Glycolysis and the Pasteur Effect in Rat Reticulocytes*

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SUMMARY

Under aerobic conditions, glucose consumption by rat reticulocytes was relatively low. When cellular respiratory activity was blocked by antimycin A, both glucose uptake and lactate formation were greatly increased. Pasteur control (the ratio of the rate of glycolysis in the respiratory-inhibited state to that in the uninhibited aerobic state) was very high in these cells and varied from 9 to 12.

A crossover plot of the concentrations of glycolytic intermediates in the respiratory-inhibited state as percentages of the control values showed forward crossovers at the phosphofructokinase and pyruvate kinase steps and a reversed crossover at the phosphoglyceromutase step. A third forward crossover at the 3-phosphoglycerate kinase step was suggested by an increase in the steady state concentration of 3-phosphoglycerate in the respiratory-inhibited state.

Uncoupling of mitochondrial oxidative phosphorylation by \( p \)-trifluoromethoxy-carboxyranide phenylhydrazone (FCCP) increased the rate of glycolysis, but this was further increased after the addition of antimycin A. The ratio of the rate of glycolysis in cells treated with antimycin A to that in cells treated with FCCP varied from 1.5 to 2.0. Oxidative utilization of glucose through mitochondrial activity in these cells was very low and accounted for about 6% of the total glucose utilized in the aerobic regulated state; in the uncoupled state this value rose to only about 12%.

Rat reticulocytes utilized glycerol to support glycolysis and a high Pasteur control was maintained with this substrate. This demonstrates that there is strong regulation of glycolysis beyond the phosphofructokinase step. Glycerol 3-phosphate was found in these cells when glycerol was the added substrate but was absent when glucose was the added substrate. This demonstrates the presence of glycerokinase in rat reticulocytes.

The mammalian reticulocyte occupies a unique position in the differentiation sequence which leads to the formation of the mature erythrocyte. The reticulocyte, which contains RNA and mitochondria but no DNA (1), is a transitional cell between its nucleated precursor (the normoblast) which possesses all three substances, and the mature erythrocyte, which contains none of them. The reticulocyte is capable of synthesizing lipids (2) and hemoglobin (3), but during the maturation process these cells lose mitochondria (4), and lipid and protein synthesis cease.

Previous studies of glucose metabolism (5-7) in a rabbit reticulocyte showed that they produced lactate from glucose in the aerobic state and that the Pasteur effect was weak. Studies of the aerobic control of glucose metabolism in reticulocytes are difficult because of the presence of hemoglobin. When erythrocytes were deoxygenated with nitrogen, a large increase in glycolysis was observed by Minakami et al. (8, 9). This effect was ascribed to changes in intracellular pH and in the binding of 2,3-diphosphoglycerate to hemoglobin. Ohyama and Minakami (10) observed increased lactate production in rabbit reticulocytes after treatment with KCN or 2,4-dinitrophenol but in their experiments the total adenine nucleotide content decreased considerably. This probably indicated a loss of cellular integrity, and, therefore, their results may not provide a good measure of the control of glucose metabolism in normal reticulocytes.

In the course of our studies on the respiratory activities of rat reticulocytes, we observed that these cells had a very low respiratory rate, but that it was greatly increased by the addition of \( p \)-trifluoromethoxy-carboxyranide phenylhydrazone, an uncoupler of oxidative phosphorylation. Low concentrations of antimycin A completely inhibited the respiratory activity. Since antimycin A completely inhibited the respiratory activity, this was ascribed to changes in intracellular pH and in the binding of 2,3-diphosphoglycerate to hemoglobin. Ohyama and Minakami (10) observed increased lactate production in rabbit reticulocytes after treatment with KCN or 2,4-dinitrophenol but in their experiments the total adenine nucleotide content decreased considerably. This probably indicated a loss of cellular integrity, and, therefore, their results may not provide a good measure of the control of glucose metabolism in normal reticulocytes.

In the present study of the control of glycolysis in rat reticulocytes, we measured the changes in the concentrations of glycolytic intermediates and adenine nucleotides and the rates of lactate production and substrate utilization in a single population of cells in different respiratory states. The control and experimental values were obtained from the same suspension of cells, avoiding possible errors due to biological variations in cells obtained at different times or from different animals.

MATERIALS AND METHODS

Preparation and Incubation of Cell Suspension—Male albino rats (150 to 200 g) were injected subcutaneously with acetylphenylhydrazine (0.1 ml of 25% solution per 100 g body weight) for 6 consecutive days, and on the 8th day blood was collected from the heart under light anesthesia with chloroform. The

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1 A. K. Ghosh and H. A. Sloviter, unpublished observations.
blood was centrifuged at 0° for 15 min and the plasma discarded. The cells were washed three times with cold buffered NaCl solution (pH 7.4), removing the buffy coat and upper cells each time. The washed cells were filtered through absorbent cotton to remove residual leukocytes. Smears of the cell suspensions used were stained with new methylene blue and showed only an occasional leukocyte, more than 90% reticulocytes and some mature erythrocytes. The cell suspensions were always used soon after preparation.

An amount of the concentrated suspension of washed cells was added to 20 ml of oxygenated Krebs-Ringer phosphate buffer (10 mM, pH 7.4) to give a hematocrit of about 10%. The mixture was incubated at 37° with stirring in an atmosphere of 100% oxygen. At intervals, both before and after the addition of substrate, inhibitor or uncoupling agent, 2-ml samples were removed for analysis.

**Extraction for Assay of Metabolites**—The 2-ml sample removed from the incubation mixture was added to 1 ml of cold 12% perchloric acid, mixed vigorously and centrifuged at 25,000 × g for 15 min. To 2.5 ml of the supernatant were added 2.5 ml of triethanolamine-KOH mixture (0.5 ml of 1 N triethanolamine buffer, pH 7.4, + 0.35 ml of 2 N KOH per ml). The pH of the mixture was adjusted to 7.4 with a drop of 2 N KOH, if necessary, and the neutralized suspension was centrifuged at 15,000 × g for 15 min. The clear supernatant was used for the assay.

**Assay Procedures**—When glucose was the substrate, its concentration was measured with a commercial glucose oxidase reagent (Glucostat, Worthington Biochemical Corporation). When glucose was not the substrate, the change in the endogenous glucose content of the cells was determined by fluorometric measurement of the NADPH generated after addition of hexokinase to a mixture containing the sample, ATP, NADP and glucose 6-phosphate dehydrogenase.

The concentrations of glycolytic intermediates, adenine nucleotides, and NAD were estimated enzymatically by measuring the oxidation or reduction of pyridine nucleotides fluorometrically as described by Maitra and Estabrook (11), Williamson and Corkey (12) and Ghosh et al. (13). The assay of 2,3-diphosphoglycerate was done by a fluorometric adaptation of the method of Rose (14). All enzymes used were purchased from Boehringer Mannheim Corporation, and the nucleotides were obtained from Sigma Chemical Company.

**RESULTS**

**Effect of Antimycin A on Glycolysis**—The production of lactate and the changes in the concentrations of ATP, ADP, and AMP before and after the addition of antimycin A (10 μg per ml suspension) are given in Fig. 1. The lactate production in the aerobic state was quite low (3.00 μmoles per ml of cells per hour) as compared to that in the respiratory-inhibited state (33.2 μmoles per ml of cells per hour). The ratio of the rate of lactate production in the respiratory-inhibited state to that of the normal aerobic state, defined here as the “Pasteur control,” was 10.4. So high a value for the Pasteur control has not been reported before in any cellular system. The Pasteur control values obtained in our experiments with reticulocytes varied from 9 to 12.

Fig. 1 also shows that although the increase in ADP and the decrease in ATP content were quite sharp, there was no significant change in the total adenine nucleotides (ATP + ADP + AMP) of the cells after treatment with antimycin A.

Changes in the concentrations of glycolytic intermediates before and after the addition of antimycin A to the incubated suspension of reticulocytes are given in Fig. 2. It is evident that the concentrations of glucose 6-phosphate, fructose 6-phosphate, 2-phosphoglycerate, and phosphoenolpyruvate decreased and those of fructose 1,6-diphosphate, triose phosphates, 3-phosphoglycerate, and pyruvate increased quite significantly after addition of antimycin A to the reticulocytes.

**Crossover Plot of Glycolytic Intermediates**—The concentrations of glycolytic intermediates and nucleotides in reticulocytes before and after the addition of antimycin A are given in Table I. From these values we constructed a crossover plot showing the concentrations of glycolytic intermediates in cells treated with antimycin A as percentages of those in control cells (Fig. 3). Forward crossovers at the phosphofructokinase and pyruvate kinase steps and a reversed crossover at the phosphoglycerate mutase step are clearly seen in the figure. The concentration of 3-phosphoglycerate increased sharply after addition of antimycin A to the cells, suggesting a forward crossover at the phosphoglycerate kinase step; however this is uncertain because of the absence of reliable data on the concentration of 1,3-diphosphoglycerate.

**Effect of FCCP on Glycolysis**—Addition of FCCP (an uncoupler of oxidative phosphorylation) produced qualitatively similar effects to those of antimycin A on glycolysis of the reticulocytes. However, the enhancement of lactate production and the changes

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**Fig. 1.** Concentrations of ATP, ADP, AMP, and lactate in rat reticulocytes before and after treatment with antimycin A. A suspension of rat reticulocytes (0.12 ml cells per ml) in oxygenated Krebs-Ringer phosphate solution containing glucose (10 mM) at 37° was treated with antimycin A (10 μg per ml) at the point marked. Samples were withdrawn at specified intervals and analyzed. Concentrations of nucleotides are expressed as micromoles per ml of cells and the lactate values as the amount (micromoles) present in that volume of suspension which contained 1 ml of cells. The values on the curve for lactate are the calculated rates of production in micromoles per ml of cells per hour.

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2 The abbreviation used is: FCCP, p-trifluoromethoxy-carboxycyanide phenylhydrozene.
in the concentrations of some intermediary metabolites were quantitatively smaller. The ratio of the rate of lactate production in cells treated with FCCP to that in normal cells, defined here as the "aerobic ADP control" ratio, varied between 4.5 and 7.5.

**Effect of Sequential Additions of FCCP and Antimycin A on Glycolysis**—A suspension of reticulocytes in oxygenated Krebs-Ringer phosphate solution containing glucose (2.5 mM) was treated sequentially with FCCP (3.8 µM) and antimycin A (10 µg per ml); at specified intervals samples were withdrawn for determinations of the concentrations of glucose, lactate, glycolytic intermediates, and adenine nucleotides. The results obtained for glucose, lactate, and the adenine nucleotides are shown in Fig. 4. The rates of glucose utilization and lactate formation were increased by FCCP but these rates were further enhanced by antimycin A. The increase in the rate of glucose uptake after the addition of FCCP was 4.7 times and after antimycin A was 8.6 times the normal rate. The rates of the ratios of lactate formation after FCCP and antimycin A to that of the normal rate were 4.5 and 9.0, respectively. From the values for the rates of glucose uptake and lactate production in cells treated with FCCP, it was calculated that of 8.05 µmoles of glucose utilized, 1.0 µmole was oxidized; i.e. 12% of the glucose metabolized was utilized through mitochondrial activity under no ADP limitation. The ratio of the rates of lactate formation after antimycin A to that after FCCP was 2.6; this ratio varied from 1.5 to 2.0 in different experiments.

The concentrations of metabolites found in reticulocytes after sequential additions of FCCP and antimycin A are given in Table II. The total adenine nucleotide concentration (ATP + ADP + AMP) decreased after addition of both antimycin A and FCCP, but no such change was observed when either of these substances was added alone. The concentrations of hexose monophosphates decreased and those of fructose 1,6-diphosphate, triose phosphates, and 3-phosphoglycerate increased after addition of FCCP; subsequent addition of antimycin A further enhanced these changes. The concentration of 2-phosphoglycerate decreased significantly after addition of either FCCP or antimycin A, however, its concentration increased after addition of both FCCP and antimycin A. The concentration of ATP, which was lowered after addition of FCCP, decreased further after antimycin A. The concentrations of ADP and

<table>
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<tr>
<th>Metabolites</th>
<th>Before antimycin A</th>
<th>After antimycin A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose 6-phosphate</td>
<td>460</td>
<td>124</td>
</tr>
<tr>
<td>Fructose 6-phosphate</td>
<td>150</td>
<td>30</td>
</tr>
<tr>
<td>Fructose 1,6-diphosphate</td>
<td>8</td>
<td>33</td>
</tr>
<tr>
<td>Triose phosphate</td>
<td>18</td>
<td>59</td>
</tr>
<tr>
<td>3-Phosphoglycerate</td>
<td>45</td>
<td>106</td>
</tr>
<tr>
<td>2-Phosphoglycerate</td>
<td>26</td>
<td>19</td>
</tr>
<tr>
<td>Phosphoenolpyruvate</td>
<td>46</td>
<td>34</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>126</td>
<td>215</td>
</tr>
<tr>
<td>Lactate</td>
<td>1125</td>
<td>8750</td>
</tr>
<tr>
<td>2,3-Diphosphoglycerate</td>
<td>3000</td>
<td>3300</td>
</tr>
<tr>
<td>ATP</td>
<td>2500</td>
<td>1720</td>
</tr>
<tr>
<td>ADP</td>
<td>280</td>
<td>855</td>
</tr>
<tr>
<td>AMP</td>
<td>36</td>
<td>206</td>
</tr>
<tr>
<td>ATP + ADP + AMP</td>
<td>2816</td>
<td>2781</td>
</tr>
<tr>
<td>ATP:ADP</td>
<td>9</td>
<td>2</td>
</tr>
</tbody>
</table>

*Values expressed as the amount (nanomoles) present in that volume of suspension which contained 1 ml of cells.*

![Table I](http://www.jbc.org/)

**Fig. 3. Crossover plot of the concentrations of glycolytic intermediates in rat reticulocytes treated with antimycin A as percentages of control values.** Data from Table I were used to construct this curve. G6P, glucose-6-P; F6P, fructose-6-P; FDP, fructose 1,6-diphosphate; TP, triose phosphate; SPGA, 3-phosphoglycerate; ZPGA, 2-phosphoglycerate; PEP, phosphoenolpyruvate; PYR, pyruvate; LACT, lactate.
AMP were raised after addition of FCCP and increased further after antimycin A.

**Metabolism of Glycerol and Pasteur Effect—Lactate production in rat reticulocytes in the presence of 12 mM glycerol in normal aerobic and respiratory-inhibited states is shown in Fig. 5. Also shown are the changes in endogenous glucose concentration of the cells in these states. The Pasteur control ratio calculated from the rates of glucose disappearance was 2.4 and from the rates of lactate formation was 6.7. In the respiratory-inhibited state the calculated lactate production from glucose was 7.2 \( \mu \text{mols/ml of cells per hour} \) and 6.8 \( \mu \text{mols of lactate per ml of cells per hour} \) appear to have been produced from glycerol.

The changes in the concentrations of metabolic intermediates in the cells incubated with glycerol after the addition of antimycin A (Table III) were mostly similar to those observed with glucose as substrate. However, the concentrations of pyruvate and lactate were lower with glycerol in both the aerobic and the respiratory-inhibited states. The pyruvate to phosphoenolpyruvate ratios in both the aerobic and respiratory-inhibited states were lower with glycerol than with glucose as added substrate. The glycerol 3-phosphate contents of reticulocytes incubated with glycerol were 30 and 51 nanomoles per ml of cells in the aerobic and respiratory-inhibited states, respectively. It is interesting to note that there was no detectable amount of glycerol 3-phosphate in the reticulocytes which were incubated with glucose as substrate.

**DISCUSSION**

Our results on glucose utilization and lactate production in different respiratory states show that aerobic glycolysis in rat reticulocytes is strongly controlled, exhibiting a higher Pasteur control ratio than hitherto reported in any system. The sharp increase in glycolysis after uncoupling or inhibition of mitochondrial respiration with a subsequent decrease in ATP and increase in ADP concentrations, indicates the importance of the ATP:ADP ratio in this regulatory process in reticulocytes. Fructose 1,6-diphosphate has an important role as an allosteric activator of pyruvate kinase (16) and phosphofructokinase (17), while the increased ADP concentration, in its role as a substrate, may have further enhanced the pyruvate kinase activity. The rise in 3-phosphoglycerate concentration might also be a reflection of the role of ADP in increasing the glycolytic flux, but in the absence of reliable data on the concentration of 1,3-diphosphoglycerate a crossover at the 3-phosphoglycerate kinase step could not be shown.

The reversed crossover, i.e. the inhibition of phosphoglycerate
Fig. 5. Endogenous glucose content and lactate production of rat reticulocytes incubated at 37° with glycerol (12 mM) as the substrate before and after the addition of antimycin A (10 µg per ml). The experimental conditions were the same as given in Table III. Results are expressed as micromoles per ml of cells and the values on the curves are the calculated rates of endogenous glucose disappearance and of lactate production in micromoles per ml of cells per hour.

Table III

Effect of antimycin A on concentrations of metabolites in rat reticulocytes incubated with glycerol as the substrate

Rat reticulocytes in Krebs-Ringer phosphate solution (0.09 ml of cells per ml) containing 12 mM glycerol were incubated at 37° under oxygen. After 20 min, antimycin A (10 µg per ml) was added to the suspension. Samples drawn before and 20 min after the addition of antimycin A were analyzed as described in Table I.

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>Concentrations (nmol/ml of cells)</th>
<th>Metabolites</th>
<th>Concentrations (nmol/ml of cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before antimycin A</td>
<td>After antimycin A</td>
<td></td>
</tr>
<tr>
<td>Glucose 6-phosphate</td>
<td>372</td>
<td>100</td>
<td>Fructose 6-phosphate</td>
</tr>
<tr>
<td>Fructose 1,6-diphosphate</td>
<td>8</td>
<td>23</td>
<td>Triose phosphate</td>
</tr>
<tr>
<td>3-Phosphoglycerate</td>
<td>48</td>
<td>64</td>
<td>2-Phosphoglycerate</td>
</tr>
<tr>
<td>Phosphoenolpyruvate</td>
<td>53</td>
<td>27</td>
<td>Pyruvate</td>
</tr>
<tr>
<td>Lactate</td>
<td>700</td>
<td>5350</td>
<td>Glycol 3-phosphate</td>
</tr>
<tr>
<td>2,3-Diphosphoglycerate</td>
<td>3400</td>
<td>3200</td>
<td>ATP</td>
</tr>
<tr>
<td>ADP</td>
<td>200</td>
<td>1020</td>
<td>AMP</td>
</tr>
<tr>
<td>ATP + ADP + AMP</td>
<td>2800</td>
<td>2800</td>
<td></td>
</tr>
<tr>
<td>ATP : ADP</td>
<td>12.8</td>
<td>1.5</td>
<td></td>
</tr>
</tbody>
</table>

* Values expressed as the amount (nanomoles) present in that volume of suspension which contained 1 ml of cells.

mutase activity at low ATP:ADP values, is more difficult to explain. The steady state ratio of 3-phosphoglycerate to 2-phosphoglycerate increased from a control value of 2 to 6 in the respiratory-inhibited state. Although 2,3-diphosphoglycerate acts as a cofactor in phosphoglycerate mutase activity, the decrease in its concentration in cells treated with antimycin A or FCCP was not large enough to account for this behavior of phosphoglycerate mutase. Perhaps phosphoglycerate mutase is a rate-limiting step in reticulocyte glycolysis because of its comparatively low concentration. Therefore activation of enzymes on either side of it (phosphoglycerate kinase and pyruvate kinase) by increased ADP concentration in the respiratory-inhibited state might have produced such a change in the ratio of 3-phosphoglycerate to 2-phosphoglycerate concentrations.

The results obtained after the sequential addition of FCCP and antimycin A to reticulocytes showed that the Pasteur control is always larger than the "aerobic ADP control" of glycolysis in this system. This is also observed when the cells are treated with either of the substances singly. This difference, however, was not observed by Lynen (19) in similar experiments with yeasts. We observed a decrease in ATP and an increase in ADP and AMP concentrations after addition of antimycin A to cells previously treated with FCCP. Although this change in ATP concentration was not large, it might have a large effect if it were localized in an area where phosphofructokinase and pyruvate kinase are situated.

Our studies on glycerol metabolism in reticulocytes demonstrated that these cells contain glycerokinase and that the glycerol 3-phosphate produced from glycerol is utilized to form lactic acid. The presence of glycerokinase in reticulocytes had been suggested from previous experiments which showed the incorporation of [14C]glycerol into glycerides and glycerophosphatides (20). The enhanced lactic acid production after the addition of antimycin A and the quite high Pasteur control value with glycerol as substrate clearly demonstrated that strong regulation of glycolysis exists beyond the phosphofructokinase step. Our results, showing that the stimulation of pyruvate kinase was accompanied by a marked decrease in ATP:ADP ratio, suggest that the ATP:ADP ratio also is important in the regulatory process at these later steps.

The comparatively low glycolytic rate with glycerol as the substrate may be due to low glycerokinase activity or limitation by availability of NAD, or both. The low pyruvate to phosphoenolpyruvate ratio in aerobic metabolism may be due to a decrease in the concentration of ADP as a result of its utilization for the direct oxidation of glycerol 3-phosphate in the mitochondria.

It is not clear why there is such a high Pasteur control in rat reticulocytes. Since antimycin A has such a large effect, it is difficult to explain how these cells under ordinary conditions maintain their energy reserve (high ATP:ADP ratio) with such low mitochondrial oxidative activity. We calculated that about 6% of the total glucose utilized by the cells is oxidized through the mitochondria in the aerobic state and that even in the uncoupled state this value is only 12%.

Since the movement of ATP out of mitochondria is very slow in the controlled state (21), the regulatory effects of ATP would be enhanced if phosphofructokinase and pyruvate kinase were localized near the ATP-generating system of the mitochondrion. This might explain the very high Pasteur control in reticulocytes. This suggestion is supported by our finding that washed reticulocyte mitochondria contain phosphofructokinase and pyruvate kinase activities. Localization of glycolytic enzymes in mito-
chondrial or other membranes has been reported in many cellular systems (22-25). Lynen (19) and Hess (26) have also proposed compartmentation of ATP to explain the Pasteur effect.

It is possible that the variations in Pasteur control observed in different systems may be due to variations in the distribution of regulatory enzymes between cytosol and mitochondria and to differences in ATPase activity.

REFERENCES
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