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Adenylates, Pyridine Nucleotide Levels Not the Cause of Difference in Glycolysis between Normal and Transformed Cells

It is well established that numerous malignant tissues\(^1\), as well as virus-transformed chick embryo fibroblasts\(^2,3\), have an elevated rate of aerobic glycolysis. Although this difference in metabolism has been known for over half a century\(^4\), the reason has not been established unequivocally. Various hypotheses have been proposed over the years; frequently appearing is one which suggests that transformation induces changes in cellular energetics which cause enhanced glycolysis\(^4-8\). Examples of such a change would be a decrease in the energy charge of the adenylates\(^9\) or a decreased capacity to generate reduced pyridine nucleotides\(^10\). Both of these conditions should be reflected by differences in the steady-state pool levels of these metabolites.

Recently we have reported a method by which the steady-state metabolism of normal and transformed cells in culture can be compared under carefully controlled conditions\(^2,11\). By this method we established that the increased rate of glycolysis is reflected by elevated glycolytic intermediate pool levels. In this report we describe the comparison of the steady-state adenylate and pyridine nucleotide pools in normal and virus-transformed chick cells.

Primary cultures were prepared from 10-day old SPF chick embryos free of resistance-inducing factor essentially as described previously\(^12\). The cells were seeded in medium 199 which was supplemented with tryptose phosphate broth (2%), calf serum (1%), and heated chicken serum (1%). For studies with transformed cultures, half the cells of a single embryo were infected at the time of primary seeding with \(2 \times 10^5\) focus forming units of Schmidt-Ruppin
strain, subgroup A of Rous sarcoma virus. Secondary cultures were prepared 4 days after the primary seeding by trypsinization of primary cultures and were seeded at the desired cell concentration in 35 mm culture dishes. The concentration of glucose and calf serum was doubled (11 mM and 2% respectively) at the time of secondary seedings.

Thirty-two hours after seeding, the medium of secondary normal and transformed cell cultures was replaced by one containing 5.5 mM $[^{14}\text{C}]$-glucose (specific activity 10.2 Ci/mole and 1.25 mM inorganic $[^{32}\text{P}]$-phosphate (specific activity 1 Ci/mole). The cells were allowed to metabolize for another 16 hr under the usual growth conditions (tissue culture incubators, 5% CO$_2$ in air, 39°C). The culture plates were then transferred to the steady-state apparatus for continued metabolism and kinetic tracer studies$^2,11$. At 1 hr and again just before sampling, the medium was changed to fresh labeled medium which contained no tryptose phosphate or serum.

Plates of normal and transformed cells were killed 15, 30, and 60 min after the last medium change by rapid washing and replacement of the medium with 80% methanol (10 sec). The cells were then scraped from the dishes and sonicated. An aliquot of each sample was analyzed by two-dimensional paper chromatography and autoradiography$^{13}$. Another aliquot was analyzed for protein concentration by the method of Lowry et al.$^{14}$.

The $^{14}\text{C}$ content of glycolytic intermediate compounds and of adenylates was constant for each of the samples, showing that labeling saturation had been achieved. In previous studies, where lactate was isolated and its specific radioactivity was compared with the labeled glucose substrate after only 1 hr, we showed that there was no dilution of the radiocarbon content of glycolytic intermediate compounds$^2$. We therefore could use levels of $^{14}\text{C}$ labeling as a measure of concentrations of these compounds.
In the case of adenylates, this lack of isotopic dilution has not been demonstrated; hence absolute concentrations calculated from $^{14}$C content could be low, if dilution occurred even after 16 hr with $^{14}$C-glucose. However, at $^{14}$C saturation of these compounds it is reasonable to assume that any dilution is equivalent for the three adenylates measured; thus energy charge can be calculated accurately.

From a comparison of levels of steady-state metabolites, it is clear that the cells used in these experiments were indeed more glycolytic upon transformation (Table I). The 10-fold increase in fructose-1,6-diphosphate, as well as the increases in other glycolytic intermediates, is a distinctive feature of transformed chick cells and has been described previously in detail. In particular, the intracellular level of lactate (L), which is 5 times greater in transformed cells than in normal cells, may be taken as a measure of glycolytic flux (G). This is because most glycolytic flux is via lactate, since the rate of pyruvate metabolism via the tricarboxylic acid cycle is small compared to G in these cells. At steady state, $\frac{dL}{dt} = 0 = G - k(L)$, whence $L = G/k$, where k is the specific constant for lactate loss from the intracellular pool.

It is noteworthy that pools of metabolites of the tricarboxylic acid cycle and related amino acids are essentially the same in normal and transformed cells (Table I). A previous study showed that the rate of labeling of these pools was also similar in normal and transformed cells.

There were no changes in the steady-state pools of ATP, ADP, AMP, or consequently in the energy charge calculated from these pools, after transformation of chick embryo fibroblasts by Rous sarcoma virus (Table II).

Due to the instability of the pyridine nucleotides under usual chromatographic procedures, another method was used to quantify these
steady-state pools. Cells used for these measurements were grown in parallel as described above except that no radioactive label was included in the growth medium. One hour after the last medium change, plates of normal and transformed cells were killed by the addition of either 0.1 N NaOH or 0.1 N HCl directly after removal of the medium. The cells were collected, placed in tubes and heated for 15 min at 60°C. Acidic conditions destroy the reduced forms while basic conditions destroy the oxidized forms. A sensitive fluorescence enzyme cycling assay was used to measure the quantity of pyridine nucleotides in each extract. This method allows accurate measurement (sensitive to 20 pmoles) of each nucleotide except NADPH. Treatment of the extract with NaOH produces a compound which inhibits the measurement of NADPH. By use of internal standards, it was shown that the actual values were 40% too low and therefore must be corrected. There were no appreciable differences in pyridine nucleotide levels in normal and transformed chick cells (Table III). Thus, transformation of cells by Rous sarcoma virus appears not to have altered their ability to generate or transport reducing equivalents.

Differences in the levels of energy metabolites and (or) the enzymes which utilize them, have been reported for other systems, as well as for chick cells transformed by other viruses. In each of these cases an increased rate of glycolysis was noted and was attributed to a stimulatory effect caused by observed differences in energy-coupling metabolite levels.

An increased rate of growth after confluency, as well as an increased rate of glucose transport, are universally accepted as attributes of transformed cells. Such increased metabolism can rapidly deplete nutrients from the growth medium, leading to nutrient starvation which in turn could give
rise to differences in energy metabolite levels\textsuperscript{21}. Therefore, carefully controlled nutritional conditions are necessary for comparison of the metabolism of normal and transformed cells.

We have established that under controlled conditions of steady-state metabolism, the levels of metabolites directly related to the maintenance of cellular energetics (adenylates and pyridine nucleotides) are essentially identical in normal and transformed chick cells. This eliminates, at least in the case of chick cells, the possibility that an imbalance of energy metabolites induces increased glycolysis upon virus transformation. However, these results are consistent with the proposal that the increased rate of glycolysis in virus-transformed cells is a consequence of an increased rate of glucose uptake\textsuperscript{2,24}.

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Table I. Steady-state Metabolite Pools in Normal and Virus-transformed Chick Cells

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Pool in Normal Cells</th>
<th>Pool in Transformed Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n-gram-atoms $^{14}$C/mg protein</td>
<td>n-gram-atoms $^{14}$C/mg protein</td>
</tr>
<tr>
<td>FDP</td>
<td>4.4</td>
<td>48.3</td>
</tr>
<tr>
<td>Lac</td>
<td>8.1</td>
<td>38.4</td>
</tr>
<tr>
<td>HMP</td>
<td>4.2</td>
<td>10.7</td>
</tr>
<tr>
<td>3-PGA</td>
<td>0.9</td>
<td>2.4</td>
</tr>
<tr>
<td>Glut</td>
<td>51.4</td>
<td>55.0</td>
</tr>
<tr>
<td>Asp</td>
<td>6.9</td>
<td>6.9</td>
</tr>
<tr>
<td>Ala</td>
<td>3.7</td>
<td>4.1</td>
</tr>
<tr>
<td>Cit</td>
<td>2.8</td>
<td>2.8</td>
</tr>
</tbody>
</table>

Average of three time points taken after 15 hr of labeling of cells as described in the text. Each metabolite pool was isolated by two-dimensional paper chromatography and quantitated by its $^{14}$C content as described in the text.
Table II. Steady-state Adenylate Pools and Energy Charge of Normal and Transformed Chick Cells

<table>
<thead>
<tr>
<th>Adenylate or Pool in Normal Cells</th>
<th>Pool in Transformed Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMP</td>
<td>3.9</td>
</tr>
<tr>
<td>ADP</td>
<td>19.6</td>
</tr>
<tr>
<td>ATP</td>
<td>152.3</td>
</tr>
<tr>
<td>E.C.</td>
<td>0.92</td>
</tr>
</tbody>
</table>

These steady-state pools represent the average of the three time points (three separate plates) taken after 15 hr of labeling of cells as described in the text. Measurements were made on the same samples used to determine the pools in Table I. The energy charge was calculated by use of the formula $E.C. = \frac{[ATP] + 1/2[ADP]}{[ATP] + [ADP] + [AMP]}$ (ref. 21).
Table III. Pyridine Nucleotide Pools in Normal and Transformed Chick Cells

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>Pool in Normal Cells (nmoles/mg of protein)</th>
<th>Pool in Transformed Cells (nmoles/mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAD</td>
<td>15.5</td>
<td>16.3</td>
</tr>
<tr>
<td>NADH</td>
<td>0.5</td>
<td>0.6</td>
</tr>
<tr>
<td>NADP</td>
<td>3.0</td>
<td>2.9</td>
</tr>
<tr>
<td>NADPH</td>
<td>1.5 (2.5)</td>
<td>1.3 (2.2)</td>
</tr>
</tbody>
</table>

Each of these measurements was carried out on a combined extract from four plates of cells grown as described in the text. The pools represent an average of 3 separate experiments. NADPH values (low due to the presence of an inhibitor) were corrected (in parentheses) by use of internal standards.
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