Glyconeogenesis in *Tetrahymena pyriformis*

RELATIONSHIP OF ENZYME ADAPTATION TO THE CARBON PATHWAY*

(Received for publication, April 14, 1967)

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SUMMARY

Phosphoenolpyruvate carboxykinase in the cytosol of *Tetrahymena pyriformis* is closely related to the high rate of glyconeogenesis in that organism, while the enzyme in the mitochondria does not show such a correlation. This finding, together with the observation of depression of phosphoenolpyruvate carboxykinase and malate dehydrogenase activities by glucose in the cytosol, indicates an extramitochondrial pathway for phosphoenolpyruvate synthesis from dicarboxylic acids which is similar to that postulated for rat liver and may, therefore, be of general metabolic significance. A change of oxygen tension which influences glyconeogenesis in *Tetrahymena* also has an effect on phosphoenolpyruvate carboxykinase and possibly other glyconeogenic enzymes. One factor in the repression and derepression of phosphoenolpyruvate carboxykinase was found to be related to oxygen tension and to occur independently of any carbohydrate in the growth medium.

A phosphoenolpyruvate carboxylase has been identified in this organism. Although its exact metabolic significance is somewhat unclear, the enzyme may function as the primary CO₂ fixation mechanism in *Tetrahymena*.

The ciliated protozoan, *Tetrahymena pyriformis*, is capable of synthesizing up to 23% of its dry weight in glycogen during normal growth on carbohydrate-free media (1). The predominant glycogenic precursors are most likely amino acids, although the organism can utilize added acetate, or even endogenous lipid, by way of an active glyoxylate cycle (2-4).

The strong glyconeogenic potential of *Tetrahymena*, as well as its nutritional requirements which are similar to those of mammals (5), provides a good model system for studying the regulation of carbohydrate synthesis. One important regulatory mechanism in the carbon pathway for glyconeogenesis appears to be the control of synthesis of phosphoenolpyruvate from pyruvate or di- and tricarboxylic acids. Evidence obtained from rat liver indicates that 4-carbon acids formed in the mitochondria must be transported to the cytosol as malate and aspartate, where they are converted to phosphoenolpyruvate via oxalacetate (6-8). The necessity for such a pathway is based in part upon the different subcellular distribution of two important glyconeogenic enzymes, pyruvate carboxylase, located in the mitochondria, and P-enolpyruvate carboxykinase, found in the cytosol of rat liver (9, 10). The generality of this concept might be considered likely if it could be shown that, even in organisms where enzymes exist in sufficient concentration in the mitochondria for the direct synthesis of phosphoenolpyruvate, the preferred pathway would still appear to be in the cytosol. Recent relatively pertinent experiments have indicated a high concentration of P-enolpyruvate carboxykinase in both the mitochondria and cytosol of *Tetrahymena*, and it was observed that only the soluble enzyme was glucose-repressible (11). The present communication indicates that P-enolpyruvate linked to glycogen synthesis in *Tetrahymena* is most likely formed in the cytosol, as it is in rat liver, although with a different enzyme distribution pattern, and that this glyconeogenic pathway might be of general metabolic significance. In addition, a number of other control mechanisms have been studied which may or may not be unique to this organism.

MATERIALS AND METHODS

Proteose peptone was purchased from Difco Laboratories. P-Enolpyruvate, pyruvate, fructose diphosphate, phosphohexose isomerase, glucose-6-P dehydrogenase, and p-hydroxymercuribenzoate were obtained from Sigma. Glutamate-oxalacetate transaminase was a product of Boehringer. ATP, ADP, DPNH, and TPN were purchased from P-L Biochemicals. Malic acid and "enzyme grade" ammonium sulfate were obtained through Mann. Radioactive pyruvate-2-¹⁴C and bicarbonate-¹⁴C were purchased from New England Nuclear and Calbiochem, respectively. Anthrone was a product of Eastman, and the Amberlite resin was obtained from Bio-Rad. All reagents were made up in deionized water which was prepared by passage of distilled water through a demineralizer supplied by Continental Water Company.

An initial culture of *T. pyriformis* was obtained through the courtesy of Dr. W. Plaut, Department of Zoology, University of Wisconsin. The organism was maintained at 23° on a 2%
Glucose or sodium acetate was added at a concentration of 0.2%.

The reaction mixture which gave a maximum rate contained, in
from those described for the mammalian liver enzyme (7, 10).
pyruvate carboxykinase from Tetrahymena were slightly different
particulate fraction was removed by centrifugation at 32,000 ×
g for 15 min prior to the spectrophotometric studies.
were frozen and thawed three times prior to assay, and the
the i4C was counted three times for 10 min each in a Packard

The oxalacetate formed from P-enolpyruvate was coupled to
conversion of oxalacetate to malate or aspartate gave good
Comparison of the various radioactive assays with respect to
conversion of oxalacetate to malate or aspartate gave good
correlation.
Glycogen was isolated by the procedure of Good, Kramer,
and Somogyi (22) and was determined by the anthrone method
(23). Protein was assayed by the biuret method (24).

RESULTS

Adaptive Enzyme Studies—The concentration of P-enolpyruvate
Table I

Effect of growth medium on adaptation of phosphoenolpyruvate
<table>
<thead>
<tr>
<th>Growth medium</th>
<th>Cytosol</th>
<th>Mitochondria</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Specific activity</td>
<td>Total activity</td>
</tr>
<tr>
<td>Peptone</td>
<td>µmoles P-enolpyruvate/min/mg P</td>
<td></td>
</tr>
<tr>
<td>Proteose peptone</td>
<td>1.50</td>
<td>64</td>
</tr>
<tr>
<td>+Glucose</td>
<td>0.08</td>
<td>3</td>
</tr>
<tr>
<td>+Acetate</td>
<td>1.30</td>
<td>40</td>
</tr>
</tbody>
</table>

The standard assay system for 14CO2 fixation contained, in
2.0 ml, 200 µmoles of Tris-HCl buffer (pH 7.5), 30 µmoles of
NaHCO3, 5 µC of radioactive NaH14CO3, 5 µmoles of GSH, 5.0
µmoles of DPNH, and enzyme which had sufficient endogenous
malate dehydrogenase to reduce all the oxalacetate formed to
malate. For P-enolpyruvate carboxykinase, 2.0 µmoles of
P-enolpyruvate, 5 µmoles of ADP, and 5 µmoles of MnCl2 were
added. P-Enolpyruvate carboxylase was assayed under similar
conditions, except that the ADP was omitted and 15 µmoles of
MgCl2 were substituted for MnCl2. Additions for pyruvate
carboxylase were 4 µmoles of sodium pyruvate, 4 µmoles of
ATP, 0.5 µ mole of acetyl-CoA, and 15 µmoles of MgCl2. The
reaction was allowed to continue for 15 min at 30°, and was
stopped with 1.0 ml of 10% trichloroacetic acid. After
centrifugation, 2.0 ml of the supernatant solution which had been freed of
14CO2 were evaporated and redissolved in 1.0 ml of H2O.
Scintillation mixture (20) in the amount of 10.0 ml was added, and the
14C was counted three times for 10 min each in a Packard
Tri-Carb liquid scintillation spectrometer with 30% efficiency
and corrected for quenching. Pyruvate carboxylase was also
assayed by the pyruvate-2-14C-oxalacetate exchange reaction
(21). As suggested, the radioactive oxalacetate was converted
to aspartate by the addition of glutamate-oxalacetate trans-
saminase and glutamate, and was eluted from an Amberlite
column (Bio-Rad H650W-X8, 20 to 50 mesh) with 0.3 M NH4OH.
Counting of a suitable aliquot was done as previously described.
Comparison of the various radioactive assays with respect to
conversion of oxalacetate to malate or aspartate gave good
correlation.

Glycogen was isolated by the procedure of Good, Kramer,
and Somogyi (22) and was determined by the anthrone method
(23). Protein was assayed by the biuret method (24).

TABLE I

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<td>1.30</td>
<td>40</td>
</tr>
</tbody>
</table>
there is an almost even distribution of the enzyme in the two subcellular compartments, which accounts for almost all of the activity when the organism is grown on proteose peptone. With the addition of glucose to the medium, there is a marked repression of the enzyme in the cytosol, whereas no effect is noted in the mitochondria. Addition of acetate has very little effect on enzyme activity, although the mitochondrial pellet is somewhat larger than under control conditions. Other organic acids besides acetate, which may have stimulated the intramitochondrial glyoxylate cycle enzymes in *Tetrahymena*, were also tested without noticeable effect on the mitochondrial P-enolpyruvate carboxykinase. Under all conditions so far studied, only the cytosol enzyme responds to repression by glucose.

Fig. 1 relates the specific activity of P-enolpyruvate carboxykinase in the cytosol and mitochondria to glycogen levels in the growing culture. Since no carbohydrate source was available, glycogen formation is due to glyconeogenesis from amino acids from the medium, and possibly from endogenous lipid of the organism. There is a sharp increase of P-enolpyruvate carboxykinase in the cytosol, which in most experiments reaches a peak after 4 days but may continue at the sustained elevated level for a more prolonged interval. Concomitantly, there is a slower rise in glycogen, which reaches a maximum slightly later than the P-enolpyruvate carboxykinase activity. By contrast, the specific activity of the mitochondrial enzyme is initially high, remains essentially unchanged during the growth of the organism, and exists in high concentration whether glyconeogenesis is formed or not. This correlates well with the fact that, when glucose is added to the growth medium, there is no re-

In Table II a series of potentially important glycogenic enzymes in the cytosol of *Tetrahymena* are compared as to their response to glucose repression. P-Enolpyruvate carboxykinase, fructose diphosphatase, and malate dehydrogenase responded identically, with a marked decrease in activity when the organism was grown on media containing glucose. Although not shown here, the mitochondrial malate dehydrogenase activity was the same whether glucose was added or omitted from the medium, and paralleled the findings for P-enolpyruvate carboxykinase. The marked adaptability of malate dehydrogenase in the cytosol is considerably greater than that noted in rat liver (7), possibly because of its close metabolic relationship to the glyoxylate cycle enzymes in *Tetrahymena*. Pyruvate carboxylase, which is known to be an extremely important glyconeogenic enzyme in higher organisms, was not found in any subcellular fraction or whole homogenate of *Tetrahymena*. The TPN-linked malic enzyme, which is related to lipogenesis in mammals (25), but could be implicated in glyconeogenesis if it were observed to be important for CO₂ fixation, was also not found in *Tetrahymena*. The observations on the absence of the latter two enzymes have been previously reported (13). The citrate cleavage enzyme, which may be a potential source of oxalacetate, was similarly not observed.

Preliminary data not presented here include a control of fructose diphosphatase in *Tetrahymena* more complex than mere glucose repression. It was necessary to add p-hydroxymercuribenzoate to the reaction mixture to obtain detectable activity under any growth conditions. EDTA was much less efficient in activating the enzyme and was inhibitory at a concentration above 0.2 mM. Neither agent shifted the pH optimum from the alkaline range to neutrality, as has been noted for other organisms (26, 27). Inhibition of the enzyme by AMP (28) was confirmed. In some respects, fructose diphosphatase from *Tetrahymena* appears similar to the enzyme isolated from the cellular slime mold (26).

Interesting studies relating glyconeogenesis in *Tetrahymena* to oxygen tension in the growth medium have been made by Levy and Scherbaum (4, 29). These workers noted that glyconeogenesis was stimulated by decreased aeration, and discussed the possible biological significance of this effect on the basis of a suggestion by Hogg (see Reference 29). It was considered that the falling oxygen tension would occur at night,

### Table II

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Glucose absent</th>
<th>Glucose present</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphoenolpyruvate carboxykinase</td>
<td>0.88</td>
<td>0.09</td>
</tr>
<tr>
<td>Fructose diphosphatase</td>
<td>0.17</td>
<td>0.06</td>
</tr>
<tr>
<td>Malate dehydrogenase</td>
<td>0.0</td>
<td>1.98</td>
</tr>
<tr>
<td>Malic enzyme</td>
<td>0.0</td>
<td>0.21</td>
</tr>
<tr>
<td>Pyruvate carboxylase</td>
<td>0.0</td>
<td>0.17</td>
</tr>
<tr>
<td>Citrate cleavage enzyme</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Fig. 1. Relationship of phosphoenolpyruvate carboxykinase activity in the cytosol and mitochondria to glycogen synthesis. Organisms were grown in 1-liter batches of 2% proteose peptone and harvested at the times indicated on the abscissa. Suitable aliquots were removed for glycogen analysis, and the remainder of the cells was fractionated into mitochondrial and cytosol components. ●—●, cytosol enzyme; ○—○, mitochondrial enzyme; ▲—▲, glycogen.
during the absence of photosynthesis by the surrounding organisms, thus initiating compensatory glyconeogenesis through the glyoxylate cycle. This would allow for the survival of *Tetrahymena* under temporary anaerobic conditions, when metabolism would depend solely on glycerogen fermentation.

Experiments relating to this important observation are shown in Table III. To Erdemeyer flasks of various size, ranging from 125 to 2000 ml in volume, 100 ml of proteose peptone medium were added, and the cultures were grown with frequent but not constant shaking at the various depths given in Column 1. Confining the work of Levy and Scherbaum (29), the proportion of glycerogen to dry weight increased with increasing depth or decreased aeration. There was also a proportionate increase in specific activity of P-enolpyruvate carboxykinase, indicating its close relationship to glyconeogenesis in this organism. Similar findings have been reported for isocitrate lyase and malate synthetase of *Tetrahymena* (3, 4). Because growth was very low in the poorly oxygenated cultures, sufficient organisms could not be obtained for fractionation of the enzyme, and the results are an expression of totally extracted enzyme by freezing and thawing. The increase in specific activity is this not as great as actually occurs, since the mitochondrial P-enolpyruvate carboxykinase is also included. When a larger culture was grown under conditions of maximum aeration and fractionated into the cytosol and mitochondrial components, depression of P-enolpyruvate carboxykinase activity in the cytosol occurred to the same extent as was found with glucose, while the mitochondrial enzyme showed no change. It should be emphasized that, in these experiments, changes in P-enolpyruvate carboxykinase activity occurred without adding glucose to the growth media. As can be seen, total dry weight of the cells and total P-enolpyruvate carboxykinase activity decreased with diminished aeration, which considerably inhibited the growth of the organism.

Table IV presents data on the effect of uncoupling and electron transfer-blocking agents on cell growth and P-enolpyruvate carboxykinase activity. It was thought that such experiments would simulate and possibly clarify the effect of decreased oxygen tension on glyconeogenesis in *Tetrahymena*. Dinitrophenol at the various concentrations noted was added to cultures in which the oxygen tension was not maximal, but in which good growth and high specific and total activity of the P-enolpyruvate carboxykinase could be obtained. An effect of dinitrophenol might be to increase oxygen consumption and decrease the energy supply to the cells. As can be seen, dinitrophenol produced an effect opposite to what was found in Table III. Increasing concentrations of dinitrophenol, besides decreasing cell growth and total activity, also lowered specific activity of P-enolpyruvate carboxykinase. Similar findings were noted with NaCN. Experiments not shown here indicated that, when dinitrophenol at 0.01 mM was added to a culture grown under maximum aerobic conditions, there was no reversal of the low P-enolpyruvate carboxykinase specific activity. It is apparent that the changes in metabolism caused by lowering the oxygen tension are much more subtle than can be produced by dinitrophenol and NaCN, particularly with regard to effective ATP generation for continued cell survival.

**CO₂ Fixation Studies**—Studies on CO₂ fixation by cellular fractions of *Tetrahymena* were carried out in an effort to determine which enzymes were important for this metabolic activity. As shown previously (13) and confirmed in the present report, pyruvate carboxylase and malic enzyme activity could not be demonstrated in this organism, at least under the conditions studied. P-Enolpyruvate carboxykinase was also not detected by other workers (13). In the present experiments, CO₂ fixation was measured both by the incorporation of radioactive 14C-bicarbonate into malate or aspartate and spectrophotometrically by coupling the oxalacetate formed from P-enolpyruvate to DPNH oxidation by malate dehydrogenase. The techniques used are described under "Materials and Methods." Both methods are more reliable than might be expected in crude fractions, since *Tetrahymena* contains low endogenous levels of lactate dehydrogenase which would otherwise cause interference with the assays.

The values in Table V show incorporation of CO₂ not dependent on ADP by both radioactivity and spectrophotometric measurements, indicating the probable presence of the carboxylase as well as the carboxykinase. When P-enolpyruvate was omitted from either system, activities were virtually zero, and

<table>
<thead>
<tr>
<th>Addition</th>
<th>Final concentration</th>
<th>Cell growth (optical density)</th>
<th>Specific activity</th>
<th>Total activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mm</td>
<td></td>
<td>μmol P-enolpyruvate/min/mg P</td>
<td>μmol P-enolpyruvate/min/mg P</td>
</tr>
<tr>
<td>None</td>
<td>350</td>
<td>1.06</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td>Dinitrophenol</td>
<td>0.01</td>
<td>350</td>
<td>1.27</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>0.04</td>
<td>238</td>
<td>1.00</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>0.08</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>NaCN</td>
<td>0.001</td>
<td>350</td>
<td>1.51</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>280</td>
<td>0.95</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

**Table III**

*Correlation of phosphoenolpyruvate carboxykinase activity, glyconeogenesis, and cell growth with oxygen tension*

Cells were harvested after 48 hours of growth in 2% proteose peptone medium at defined depths. Aliquots were removed for glycogen analysis and dry weights, and the remainder of the material was frozen and thawed to prepare extracts for enzyme assay as described in the text.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>cm</td>
<td>μmol P-enolpyruvate/min/mg P</td>
<td>μmol P-enolpyruvate/min/mg P</td>
<td>mg</td>
<td>mg/100 mg, dry wt</td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>1.00</td>
<td>30.0</td>
<td>83.0</td>
<td>7.1</td>
<td></td>
</tr>
<tr>
<td>1.0</td>
<td>1.10</td>
<td>26.0</td>
<td>55.0</td>
<td>8.0</td>
<td></td>
</tr>
<tr>
<td>1.5</td>
<td>1.25</td>
<td>24.0</td>
<td>41.0</td>
<td>10.0</td>
<td></td>
</tr>
<tr>
<td>2.0</td>
<td>1.80</td>
<td>21.0</td>
<td>33.0</td>
<td>10.3</td>
<td></td>
</tr>
<tr>
<td>4.5</td>
<td>2.00</td>
<td>11.0</td>
<td>19.0</td>
<td>11.7</td>
<td></td>
</tr>
</tbody>
</table>
depression of CO₂ fixation in the cytosol of glucose-grown cultures is apparent. Both assay methods were comparable and consistent for numerous experiments performed with the cytosol fraction. In the mitochondrial extract, however, whereas the ADP-dependent reaction representing P-enolpyruvate carboxykinase gave reproducible results, the values for the reaction in the absence of ADP were somewhat variable. Although the results presented are representative of at least 10 experiments, the exact concentration of P-enolpyruvate carboxylase in the mitochondria remains in some doubt. One of the problems may be the difficulty of extracting total enzyme activity from the mitochondria. Whole mitochondria gave more consistent results with the radioactivity assay; however, an accurate comparison could not then be made spectrophotometrically. Another difficulty in interpretation of the results is the decreased activity of P-enolpyruvate carboxylase in the cytosol of glucose-grown cultures. It would not be expected that an enzyme limited to CO₂ fixation would be so affected. There was no grown cultures. It would not be expected that an enzyme activity of P-enolpyruvate carboxylase in the cytosol of glucose-grown cultures. It would not be expected that an enzyme activity of P-enolpyruvate carboxylase in the cytosol of glucose-grown cultures. It would not be expected that an enzyme activity of P-enolpyruvate carboxylase in the cytosol of glucose-grown cultures. It would not be expected that an enzyme activity of P-enolpyruvate carboxylase in the cytosol of glucose-grown cultures. It would not be expected that an enzyme activity of P-enolpyruvate carboxylase in the cytosol of glucose-grown cultures. It would not be expected that an enzyme activity of P-enolpyruvate carboxylase in the cytosol of glucose-grown cultures. It would not be expected that an enzyme activity of P-enolpyruvate carboxylase in the cytosol of glucose-grown cultures. 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**Table V**

**CO₂ fixation in different cell fractions under various experimental conditions**

<table>
<thead>
<tr>
<th>Cell fraction and experimental condition</th>
<th>Specific activity</th>
<th>Specific activity</th>
<th>Specific activity</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glucose absent</td>
<td>Glucose present</td>
<td>Glucose absent</td>
<td>Glucose present</td>
</tr>
<tr>
<td></td>
<td>cpm/mg F x 10⁶</td>
<td>µmol/min/mg P</td>
<td>µmol/min/mg P</td>
<td>µmol/min/mg P</td>
</tr>
<tr>
<td>Cytosol</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Complete system</td>
<td>300</td>
<td>15</td>
<td>0.001</td>
<td>0.005</td>
</tr>
<tr>
<td>-ADP</td>
<td>53</td>
<td>7</td>
<td>0.010</td>
<td>0.002</td>
</tr>
<tr>
<td>-ADP, -Mn++, +Mg++</td>
<td>137</td>
<td>18</td>
<td>0.029</td>
<td>0.004</td>
</tr>
<tr>
<td>Mitochondrial extract</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Complete system</td>
<td>58</td>
<td>73</td>
<td>0.053</td>
<td>0.068</td>
</tr>
<tr>
<td>-ADP</td>
<td>4</td>
<td>35</td>
<td>0.015</td>
<td>0.022</td>
</tr>
<tr>
<td>-ADP, -Mn++, +Mg++</td>
<td>5</td>
<td>58</td>
<td>0.020</td>
<td>0.033</td>
</tr>
<tr>
<td>Homogenate, whole mitochondria, cytosol</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Pyruvate, ATP, Mg++, acetyl-CoA</td>
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**DISCUSSION**

Certain features in *T. pyriformis* indicate it is a satisfactory organism for studying the carbon pathway and control of glycogenesis. On a carbohydrate-free growth medium, the organism is able to synthesize large quantities of glycogen from noncarbohydrate precursors and appears to utilize a regulatory mechanism of enzyme adaptation similar to that frequently observed in higher animals (7). The strong repressive effect of glucose on the glycogenolytic enzymes fructose diphosphatase, P-enolpyruvate carboxykinase, and malate dehydrogenase when this sugar is added to the growth medium is similar to the effect of refeeding after deprivation of food, substitution of a high carbohydrate for a high protein diet, and insulin administration to diabetic rats.

Glyconeogenesis in mammals is influenced by hormones, particularly insulin, glucagon, and adrenocortical steroids. In the main, the rat has been used as the experimental animal as it can be easily manipulated to show hormonal effects. Recent studies by Lardy et al. (6–8) and supporting evidence by Haynes (33) on the carbon pathway for gluconeogenesis in rats have suggested a hypothesis for a series of reactions which account for transport of 4-carbon acids from the intramitochondrial compartment, where oxaloacetate is synthesized from pyruvate.
by pyruvate carboxylase, to the cytosol, where it is converted to P-enolpyruvate by P-enolpyruvate carboxykinase. There is strong evidence, although indirect, that the reactions from pyruvate to P-enolpyruvate in the rat are under hormonal control (36–38). The basic requirement for this pathway in rats appears to be the separation of P-enolpyruvate carboxykinase and pyruvate carboxylase by the mitochondrial membrane barrier. In a number of other animals, P-enolpyruvate carboxykinase is located in both the mitochondrial and cytosolic compartments (9, 10). Thus there might not be a requirement for the transport of oxalacetate, since it could form P-enolpyruvate directly in the mitochondria. In fact, synthesis of P-enolpyruvate has been readily accomplished by the combination of the two partially purified mitochondrial enzymes from avian liver (9) as well as from whole mitochondria from various mammals (40, 41). The carbon pathway for gluconeogenesis in the rat may thus be specific for this species and not represent a more generalized metabolic scheme. One method of ascertaining the metabolic pathway, although indirectly, is to test the adaptability of key enzymes during gluconeogenesis and its repression in animals which have P-enolpyruvate carboxykinase in both the cytosol and mitochondria. Unfortunately, this is difficult to do experimentally. P-Enolpyruvate carboxykinase in the cytosol of fowl does not seem to adapt4 and in humans and monkeys, where enzymes have been found equally in both the cytosol and mitochondria of the liver,5 adaptive studies are almost impossible to do on a large scale. Tetrahymena, although it is much lower on the evolutionary scale, does possess certain advantages to test the generality of the carbon pathway in gluconeogenesis. The present studies have shown that, whereas this organism contains both a cytosol and mitochondrial P-enolpyruvate carboxykinase, only the former shows a close correlation with gluconeogenesis. P-Enolpyruvate carboxykinase in yeast was found to be repressible in glucose-grown cells (42), but no data were presented on the subcellular distribution of the enzyme. Of further significance is the similar response of malate dehydrogenase in the cytosol to P-enolpyruvate carboxykinase. Holzer (43) has indicated that the cytoplasmic malate dehydrogenase of yeast, which is required for gluconeogenesis from acetate via the glyoxylate cycle, is absent when the cells are grown on glucose. On the basis of studies in Tetrahymena by Hogg and Kornberg (3), malate is synthesized intramitochondrially by the glyoxylate cycle enzymes isocitrate lyase and malate synthetase, which are also closely integrated with gluconeogenesis. While the malate formed could be converted to P-enolpyruvate in the mitochondria by malate dehydrogenase and P-enolpyruvate carboxykinase, the present results would indicate that malate which is utilized for glycogen synthesis diffuses into the cytosol and is converted to P-enolpyruvate by the extramitochondrial enzymes. The definite advantage of this pathway, besides supplying carbon fragments, would be the transport of reducing equivalents from the mitochondria for reversal of the tricarboxylate dehydrogenase step in the cytosol. The importance of generating reducing power by an extramitochondrial pathway for gluconeogenesis has been discussed in a number of publications (6, 7, 8, 44, 45).

An alternative hypothesis for P-enolpyruvate synthesis has been proposed by Henning, Stumpf, and Seubert (38), who found a significant amount of pyruvate carboxylase in rat liver cytosol during the stress of gluconeogenesis. They maintained that there is no necessity for a transport mechanism of oxalacetate, since it can be formed directly in the cytosol by the combined activities of pyruvate carboxylase and P-enolpyruvate carboxykinase. While there has yet been no confirmation of their findings except when the mitochondria are prepared at room temperature (46), the inherent difficulty presented by their hypothesis would be the necessity of generating acetyl-CoA in the cytosol for the activation of pyruvate carboxylyase. As has been shown, this would require transport of acetyl-CoA from the mitochondria by the citrate pathway (47), and the citrate cleavage enzyme for the formation of acetyl-CoA in the cytosol is considerably reduced during active gluconeogenesis (7, 48, 49).

If P-enolpyruvate carboxykinase in the cytosol is primarily involved in gluconeogenesis, the possible action of the enzyme in the mitochondria is unclear. It may act predominantly for CO2 fixation to replenish the citric acid cycle with necessary oxalacetate. However, it has been shown that mutants of certain microorganisms which contain P-enolpyruvate carboxykinase, but not P-enolpyruvate carboxylase, cannot grow unless supplied with tricarboxylic acid intermediates, indicating that CO2 fixation does not occur physiologically through the carboxykinase (50, 51). Other work in microorganisms (52), as well as in mammals (7, 57), indicates that P-enolpyruvate carboxykinase acts exclusively as a gluconeogenic enzyme. This may, therefore, be the reason why Tetrahymena contains P-enolpyruvate carboxylyase, since P-enolpyruvate carboxykinase is the only other possible source for synthesis of 4-carbon fragments which could be demonstrated. Another possible function of P-enolpyruvate carboxykinase in the mitochondria could be the synthesis of P-enolpyruvate from noncarbohydrate precursors for the formation of benzoquinone intermediates and cell wall precursors. Although it is known that Tetrahymena is unable to synthesize any of the essential aromatic amino acids which would occur by way of the shikimic acid pathway, it has been demonstrated that at least a fragment of the pathway for the synthesis of ubiquinone has been retained (53).

The stimulation of gluconeogenesis by decreased aeration in culture of Tetrahymena appears to be an important factor in the metabolism of this organism. Hogg has made the intriguing suggestion that after a fall in oxygen tension there is a subsequent compensatory increase in the glyoxylate cycle to augment the synthesis of glycogen (see Reference 29). If this hypothesis is correct, one could further postulate that the increased succinate production by the enzyme isocitrate lyase might be sufficient to reverse electron transport and reduce the pyridine nucleotides necessary for gluconeogenesis. Since decreasing oxidative phosphorylation by low concentrations of dinitrophenol or NaCN does not mimic the effect of diminished oxygen tension, there must be a much more subtle effect on electron transfer and ATP production by the latter phenomena. It has been shown in the present work (Table III) that repression and depression of P-enolpyruvate carboxykinase in Tetrahymena can occur independently of any exogenous carbohydrate in the growth medium and are closely related to oxygen utilization by the organism. Whether a similar mechanism relating electron transfer and oxidative phosphorylation to the regulation of gluconeogenesis in higher organisms exists cannot be answered by the present data, but presents an intriguing possibility. Recent

1 E. Shinog, unpublished observations.

1 This suggestion was put forward by Dr. H. A. Lardy.
studies have shown that addition of fatty acids to rat liver mitochondrial preparations stimulate the accumulation of glycogen, as well as increasing CO2 fixation, while inhibiting the pyruvate oxidase complex (8).

Numerous studies in a variety of organisms indicate a similarity in the regulation of glycogen synthesis, which appears to be a physiological response in all organisms to a nutritional restriction.

By using these results, we can conclude that the control of carbohydrate utilization in higher animals is reflected by a sustained elevated rate of glycogen synthesis, which is incompatible with life unless controlled by hormonal factors exists, an inability to utilize carbohydrate.

Acknowledgment—We appreciate the helpful discussions and continued support of Dr. Henry A. Lardy.

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