Gluconeogenesis in the Kidney Cortex

QUANTITATIVE ESTIMATION OF CARBON FLOW*

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SUMMARY

Using analytical data and the results of degradation of glucose, lactate, and glutamate produced from [2-14C]pyruvate or lactate, we have estimated the pathways of carbon flow during active gluconeogenesis in the kidney cortex. This approach involves a steady state model and solution with the aid of a digital computer. By the isotopic method one can estimate the rates of pyruvate carboxylase, pyruvate dehydrogenase, pyruvate kinase, and fumarase exchange and these are compared with rates of gluconeogenesis and the tricarboxylic acid cycle. The cycle involving variations on the scheme [pyruvate → oxalacetate → phosphoenolpyruvate → pyruvate] was measured and found to be similar to the rate of glucose synthesis, indicating that net flux through pyruvate carboxylase and phosphoenolpyruvate carboxykinase may be nearly twice the net rate of glucose formation. The rate of fumarase exchange was about four times the net rate of the tricarboxylic acid cycle.

The major outlines of the gluconeogenic pathway have been fairly well established. Energy and in some cases reducing equivalents derived from mitochondrial oxidations are used to drive this biosynthetic pathway. In order to examine more quantitatively the relationship between the synthetic and the oxidative pathway we have used a steady state model involving an interacting system of gluconeogenesis, the tricarboxylic acid cycle, and the so-called "futile" or "useless" cycle. Analysis of the degradation patterns produced in glucose, lactate, and glutamate from 2-14C-labeled substrates permits evaluation of relative carbon flow. A rather surprisingly large rate of recycling of phosphoenolpyruvate to pyruvate is suggested by our results. Preliminary accounts of this work have been presented (1, 2).

METHODS

[2-14C]Pyruvate, [3-14C]pyruvate, and [6-14C]glucose were obtained from Amersham-Searle (Des Plaines, Ill.). L-[2-14C]-Lactate and [3-14C]lactate were synthesized by methods described in the literature. The medium from the flasks was washed out and made to a final volume of 3 ml with NaOH (CO2 free) into the hanging well. The flasks were shaken for another 3 hours to collect CO2. The flasks were then removed, immersed in 4.7 ml of CO2 free water, and aliquots were taken for determination of radioactive 14CO2 in a scintillation counter and of total CO2 by manometry. The original gas phase was 100% oxygen. At the end of the incubation 0.5 ml of n H2SO4 was injected into the medium and the mixture was put on a column of Dowex 50 to remove the substrate. The mixture was eluted with 3 ml of 4 N NaOH (CO2 free) into the hanging well. The flasks were shaken for another 3 hours to collect CO2. The medium from the flasks was washed out and made to a volume of 10 ml. Lactate, pyruvate, and glucose were assayed enzymically (4). Of the diluted medium, 8 ml were put on a column of Dowex 50 (11+ mesh, 100 to 200 mesh), columns (1 cm x 10 cm) of Dowex 50 (400 mesh, acetate form) and glutamate was eluted with 0.5 ml of 1 N NaOH (CO2 free) into the hanging well. The columns were then put on a column of Dowex 1 (acetate, 100 to 200 mesh), columns (1 cm x 10 cm) which were washed with water to 30 ml. This was taken to dryness and the glucose was purified by chromatography using Whatman No. 3MM paper and 1-butanol-acetic acid-water (4:1:2) as solvent. The Dowex 50 column was eluted with 30 ml of 2 N NH4OH and this was taken to dryness. The amino acids were then put on a column (1 x 20 cm) of Amberlite CG-4B (200 to 400 mesh, acetate form) and glutamate was eluted with 0.5 N acetic acid. Lactate was eluted from the column (1 x 11 cm) of Dowex 1 with 1 N formic acid. It was taken just to dryness in a stream of air at 37° and chromatographed on Whatman No. 3MM using 95% ethanol-concentrated NH4OH-water (160:10:30). Radioactivity on chromatograms was located with x-ray film.

The early glucose degradations (Table I) were carried out by the method of Rognstad and Woronsberg (5) in which glucose was first converted to lactate. The glucose degradations of Table III were carried out by the method of Schmidt et al. (6) involving dismutation of the glucose to α-glycerol-P and 3 P-glycerate, with subsequent degradation of glyceraldehyde. Carbon 1 of glutamate was obtained by use of Chloramine T (7) and carbon 5 by the Schmidt reaction (8). Lactate was degraded by the method of Katz et al. (9).
TABLE I
Distribution of radioactivity in glucose formed from 14C-labeled lactate and pyruvate in kidney cortex slices. Kidney cortex slices were incubated for 2 hours in 2 ml of a phosphate-salts buffer at 37° under 100% oxygen. Glucose was purified and converted to lactate which was degraded.

<table>
<thead>
<tr>
<th>Labeled substrate</th>
<th>Substrate concentration</th>
<th>Acetate concentration</th>
<th>Relative specific activity in glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>C-1,2</td>
</tr>
<tr>
<td>Experiment 4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-[2-14C]Lactate</td>
<td>5</td>
<td>5</td>
<td>89</td>
</tr>
<tr>
<td>L-[3-14C]Lactate</td>
<td>5</td>
<td>5</td>
<td>100</td>
</tr>
<tr>
<td>Experiment 6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-[2-14C]Lactate</td>
<td>5</td>
<td>5</td>
<td>77</td>
</tr>
<tr>
<td>L-[3-14C]Lactate</td>
<td>5</td>
<td>5</td>
<td>100</td>
</tr>
<tr>
<td>[2-14C]Pyruvate</td>
<td>5</td>
<td>5</td>
<td>74</td>
</tr>
<tr>
<td>[3-14C]Pyruvate</td>
<td>5</td>
<td>5</td>
<td>100</td>
</tr>
<tr>
<td>Experiment 8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-[2-14C]Lactate</td>
<td>10</td>
<td>10</td>
<td>79</td>
</tr>
<tr>
<td>L-[3-14C]Lactate</td>
<td>10</td>
<td>10</td>
<td>100</td>
</tr>
<tr>
<td>[2-14C]Pyruvate</td>
<td>10</td>
<td>10</td>
<td>86</td>
</tr>
<tr>
<td>[3-14C]Pyruvate</td>
<td>10</td>
<td>10</td>
<td>100</td>
</tr>
</tbody>
</table>

THEORETICAL

The approach used is based on the assumption of metabolic and isotopic steady state, i.e. that the concentrations and specific activities of the intermediates involved remain constant. This of course is an approximation, but for long time experiments (i.e. greater than 1 hour) should be reasonably valid. The model to be tested is set up, and for each carbon atom of unique specific activity, an equation is written stating that inflow of radioactivity into this carbon atom equals outflow of radioactivity. The system of equations can then be algebraically solved so that the specific activity of each carbon atom is expressed in terms of the rates. This approach of using simultaneous equations in the solution of steady state metabolic systems was developed by Katz, Wood, Landau, and Bartsch (10, 11). However, in complex systems algebraic solution is difficult. In this case data from the rates which can be directly measured together with a series of assumed values of other rates were fed to the computer program of the system of equations, and the resultant numerical values of rates adjusted by trial and error (varying the unknown values) to correspond to experiment.

The model used in this paper is shown in Fig. 1. The figure as shown represents gluconeogenesis from pyruvate. An identical model will hold for gluconeogenesis from L-lactate, except the directions of VpyR (pyruvate utilization or formation) and Vlac (lactate formation or utilization) will be reversed. Vtca is the net rate of the tricarboxylic acid cycle in the forward direction, except for the malic dehydrogenase reaction, which is diminished by the term (Vglu + Vpk). Vglu is the rate of glucose formation, while Vpk represents the rate of the pyruvate kinase reaction. Vpdh is the rate of the pyruvate dehydrogenase reaction, while Vao represents the rate of formation of acetyl-CoA either from added acetate or from endogenous substances. Vmdh represents the back rate of the reversible mitochondrial malate dehydrogenase, while Vfum represents the back rate of mitochondrial fumarase. The letters below the carbon atoms denote molar specific activities of these carbon atoms. The inflow into phosphoenolpyruvate in Fig. 1, denoted by Vglu and Vpk, actually would represent outflow from mitochondrial malate followed by cytosolic malate dehydrogenase when pyruvate is the substrate, and outflow from mitochondrial oxalacetate when lactate is the substrate. However, the data yet available are not sufficient for independent estimation of Vmdh and Vfum, and a single pool of oxalacetate and malate is used at present. Vmdh is thus arbitrarily set to a very high value for the purposes of this paper. Complete degradation of the entire carbon skeleton of glutamic acid may permit an independent estimation of Vfum.

Many of these rates can be estimated directly from analytical data. Vglu is simply the difference between final amount of glucose minus the initial glucose in the tissue, which is very small in kidney cortex slices from fasted rats. Since our experiments are carried out in bicarbonate-free buffer with pure O2 in the gas phase, measurement of the CO2 formed permits estimation of the tricarboxylic acid cycle. The pyruvate dehydrogenase reaction also produces CO2 so this must be estimated and subtracted from the total CO2 produced to estimate the Krebs cycle. A preliminary estimation of Vpdk is made by measuring the difference between total disappearance of
substrate and that which is converted to glucose and lactate (or pyruvate). Other products are assumed negligible and the difference thus is assumed to represent the amount of substrate oxidized to acetyl-CoA. Determination of other rates requires isotopic data. Isotopic data also provides additional estimation of pyruvate dehydrogenase.

The algebraic representation of the model is shown in the "Appendix." An IBM 360 digital computer is used to calculate numerically the specific activities of all the carbon atoms involved by feeding in values of the known rates and ranges of values of the unknown rates.

It has been found that certain rates are more accurately estimated from the data available with the use of substrates labeled in the position 2 rather than in position 3. If [2-14C]pyruvate is used as substrate, the unknown rates can be estimated if the relative specific activities of the carbon atoms of glucose, lactate, and at least carbon atoms C-1 and C-5 of glutamate are determined.

To determine the rate of the fumarase back reaction (V_FUM), we plot the calculated values of the ratio M_3/M_2 against a range of assumed value of V_FUM, using the known experimental values of V_GLU and V_TCA. (For an example, see Fig. 2A.) This ratio is very insensitive to lipnn and lipK and these can either be set to 0, or given approximate values. From the experimental M_3/M_2 value given by the ratio of specific activities of C-6/C-5 or C-1,6/C-2,5 in glucose, one can estimate V_FUM.

To determine the rate of the pyruvate kinase reaction, the calculated values of P_3/P_2 are plotted against V_PK, using the known values of V_TCA, V_GLU, V_FUM, and V_PDH. A typical plot is shown in Fig. 2B. This curve is also quite insensitive to variation in V_PDH. The experimental value of P_3/P_2 is found by degradation of the lactate. The use of the randomization pattern in lactate to estimate the rate of recycling between pyruvate and the dicarboxylic acids has been proposed previously (12). Two methods are now available to determine V_PDH, which was previously estimated from the analytical data. The first method uses a plot of A_1/O_2 versus V_PDH for a range of values of V_PDH computed using the above determined values of V_TCA, V_GLU, V_FUM, and V_PK. Such a curve is shown in Fig. 2C. The experimental value of A_1/O_2 is given by the ratio of specific activities of C-1 to C-5 of glutamic acid, as determined by degradation. A second method uses a plot of M_3/M_2 versus V_PDH. M_3/M_2 experimentally is determined by degradation of glucose using the ratio of specific activities of C-4/C-5 or C-3,4/C-2,5. A typical curve is shown in Fig. 2D. Other model studies of mammalian gluconeogenesis have been made by Exton and Park (13), Heath (14), and Regen and Terrell (15). The mathematical approach used was that of converging series. Connett and Blum (16) have carried out model studies of gluconeogenesis in Tetrahymena using the method of simultaneous equations. Friedmann et al. (17) have also estimated recycling between pyruvate and the dicarboxylic acids in the perfused rat liver using the randomization produced in lactate with [2-14C]pyruvate as substrate. A computer model of gluconeogenesis (using a nonisotopic approach) has been formulated by Aches et al. (18).

RESULTS

Kidney cortex slices from fasted rats were incubated with L-lactate or pyruvate labeled with 14C in carbon 2 or 3 and the glucose formed was purified and degraded. The results of some preliminary experiments are shown in Table I. It is apparent that, as found by numerous other workers, extensive randomization via fumarate occurs when either lactate or pyruvate is the substrate.

To establish a more complete network of metabolic flow during gluconeogenesis, more data was required. Degradation of lactate and glutamate in addition to glucose permits evaluation of the major pathways of carbon.

In these experiments we used either [2-14C]pyruvate or L-[2-14C]lactate in the absence and the presence of unlabeled α,β-hydroxybutyrate. Table II presents the analytical results. The molar rate of the Krebs cycle varied from about 2 to 3 times the molar rate of conversion of the three carbon substrates to glucose. β-Hydroxybutyrate stimulated the formation of glucose and inhibited the oxidation of pyruvate to acetyl-CoA. Table III shows the data from the degradation of the glucose, lactate (or alanine), and glutamate formed in these experiments. Using the model of Fig. 1 and the trial and error procedure outlined in Fig. 2, we obtained a set of calculated rates which would give isotopic patterns in the compounds degraded which are similar to those found. The calculated degradation patterns are shown in Table III beneath the experimental results. It is seen that in both experiments, the rate of the fumarase exchange reaction is from 3 to 5 times the net rate of the tricarboxylic acid cycle. The addition of β-hydroxybutyrate depressed the rate of the pyruvate dehydrogenase reaction, but has no consistent effect on the rate of pyruvate kinase.

Table IV presents the degradation patterns produced in glucose and lactate when n-[3-14C]glycerate and n-[6-14C]fructose were used as tracer substrates for kidney cortex segments in the presence of pyruvate. Since the capacity of glyceraldehyde-P dehydrogenase, P-glycerate kinase, P-glycerate mutase, and enolase in these tissues is considerable excess of net fluxes through these enzymes (19), it is to be expected that they will show considerable isotopic reversibility. n-[3-14C]Glycerate and n-[6-14C]fructose thus should introduce label on carbon 3 of phosphoenolpyruvate. Some indication of extensive isotopic reversibility between glyceraldehyde-3-P and phosphoglycerate has been shown elsewhere (1, 2). Here tracer levels of n-[U-14C]glyceraldehyde and n-[U-14C]glycerate, added to kidney cortex segments which were synthesizing glucose from lactate, produced nearly the same ratio (14C yield in glucose to 14C yield in CO_2 plus lactate), the CO_2 plus lactate yield arising from a backward or exchange pathway. The labeling pattern produced in lactate from n-[3-14C]glycerate or n-[6-14C]fructose is relevant to the problem (see "Discussion") of whether or not the randomization of 14C in carbons 1 and 3 of lactate (when [2-14C]pyruvate is the substrate) reflects recycling of phosphoenolpyruvate to pyruvate.

DISCUSSION

Estimation of the major carbon flows in a given cell can provide a great deal of useful information. It can permit construction of balances of formation and utilization of reducing power and energy, including the direction and extent of their transfer between the cytosol and the mitochondria. Quantitative or even semiquantitative estimation can help in assigning primary function to certain pathways. Thus, to assign a major role of the pentose cycle to be the formation of nucleic acid pentose is inconsistent with the fact that drainage from the pentose cycle to form these pentoses is found to be only a few percent of net cycle flux (20). Determination of fluxes in the intact cell can
Fig. 2. Procedure for estimating rates of fumarase, pyruvate, kinase, and pyruvate dehydrogenase using isotopic data. An example is shown using an assumed set of data to illustrate the procedure followed. \([2-^{14}C]\)Pyruvate (18 µmoles) were incubated with kidney cortex slices for 2 hours. Glucose (6 µmoles) was formed, 0.5 µmoles of which were from endogenous sources (estimated from an incubation without pyruvate). Lactate (2 µmoles) was formed and 1 µmole of pyruvate remained at the end of the incubation. Manometry indicated formation of 76 µmoles of CO₂. The pyruvate oxidized \((V_{FUM})\) is estimated as \(17 - [2(5.5) + 2] = 4 \) µmoles/2 hours. The tricarboxylic acid cycle \((V_{TCA})\) is \((76 - 4)/2 = 36 \) µmoles/2 hours. \((V_{TCA}/V_{GLU}) = 36/12 = 3.0\) and \((V_{FUM}/V_{GLU}) = 4/12 = 0.33\). Degradation of glucose, lactate, and glutamate gave the following patterns of relative specific activities.

Glucose

<table>
<thead>
<tr>
<th>Glucose</th>
<th>C-1</th>
<th>C-2</th>
<th>C-3</th>
<th>C-4</th>
<th>C-5</th>
<th>C-6</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-1</td>
<td>75</td>
<td>100</td>
<td>45</td>
<td>45</td>
<td>100</td>
<td>75</td>
</tr>
<tr>
<td>M₂</td>
<td>1</td>
<td>0.5</td>
<td>1</td>
<td>1</td>
<td>1.0</td>
<td>0.5</td>
</tr>
<tr>
<td>M₁</td>
<td>0.5</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>M₃</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>

The curves in A, B, C, and D are obtained by inserting ranges of values of rates in the computer program of the model. All rates in these figures are expressed relative to \(V_{GLU}\) set equal to 1. Fig. 2A shows that the curve of \(M₃/M₂\) versus \(V_{FUM}\) is rather insensitive to \(V_{FK}\) and \(V_{PK}\) and thus to estimate \(V_{FUM}\) we arbitrarily set these rates to 0. When estimates of \(V_{PK}\) and \(V_{FUM}\) are available one could return to this figure and re-determine \(V_{FUM}\). From the \(C-6/C-5\) ratio in glucose \((= M₃/M₄)\) of 0.75, \(V_{FUM}\) from Fig. 2A is estimated to be 12. From the \(P₁/P₃\) ratio, available from the degradation of lactate, of 0.09, \(V_{PK}\) is estimated from Fig. 2B to be 0.5. From the \(C-5/C-1\) ratio in glutamate \((= A₁/O₄)\) of 0.67, using \(V_{PK} = 0.5\), \(V_{FUM}\) is estimated from Fig. 2C to be 0.3. From the \(C-4/C-5\) ratio in glucose \((=M₁/M₃)\) of 0.45, \(V_{FUM}\) is also estimated from D to be 0.3.
TABLE II
Rate of formation of major metabolic products from pyruvate or lactate in kidney cortex slices. Values except where otherwise indicated are μmoles per 125 mg of tissue (wet weight) per 2 hours. Substrate concentration and dl-β-hydroxybutyrate concentration, when added, was 10 mM.

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Substrate</th>
<th>αβ-Hydroxybutyrate</th>
<th>Substrate to glucose</th>
<th>Total to glucose</th>
<th>Substrate utilized</th>
<th>Lactate or pyruvate formed</th>
<th>Substrate oxidized (VPDH)</th>
<th>CO₂ formed</th>
<th>Acetyl-CoA to CO₂</th>
<th>Vtca/Vglu</th>
<th>Vpyr/Vglu</th>
</tr>
</thead>
<tbody>
<tr>
<td>42 A</td>
<td>Pyruvate</td>
<td>-</td>
<td>8.4</td>
<td>9.2</td>
<td>17.4</td>
<td>1.3</td>
<td>7.7</td>
<td>61.8</td>
<td>27.0</td>
<td>3.00</td>
<td>0.84</td>
</tr>
<tr>
<td>42 B</td>
<td>Pyruvate</td>
<td>+</td>
<td>11.2</td>
<td>12.0</td>
<td>17.4</td>
<td>1.6</td>
<td>4.6</td>
<td>67.6</td>
<td>31.5</td>
<td>2.80</td>
<td>0.38</td>
</tr>
<tr>
<td>90 A</td>
<td>Pyruvate</td>
<td>-</td>
<td>6.5</td>
<td>7.7</td>
<td>17.5</td>
<td>1.4</td>
<td>5.6</td>
<td>50.0</td>
<td>20.2</td>
<td>2.63</td>
<td>1.25</td>
</tr>
<tr>
<td>90 B</td>
<td>Pyruvate</td>
<td>+</td>
<td>11.2</td>
<td>12.4</td>
<td>18.0</td>
<td>2.2</td>
<td>4.5</td>
<td>52.5</td>
<td>24.0</td>
<td>1.93</td>
<td>0.36</td>
</tr>
<tr>
<td>90 C</td>
<td>L-Lactate</td>
<td>-</td>
<td>4.0</td>
<td>5.8</td>
<td>12.8</td>
<td>0.3</td>
<td>7.9</td>
<td>43.4</td>
<td>17.8</td>
<td>5.07</td>
<td>1.36</td>
</tr>
<tr>
<td>90 D</td>
<td>L-Lactate</td>
<td>+</td>
<td>7.8</td>
<td>9.0</td>
<td>10.3</td>
<td>0.3</td>
<td>2.2</td>
<td>40.2</td>
<td>22.0</td>
<td>2.45</td>
<td>0.24</td>
</tr>
</tbody>
</table>

TABLE III
Isotopic yields and degradation patterns in lactate and glucose in experiments with [2-14C]pyruvate and l-[6-14C]-lactate. The experiments are those described in Table I in the same order. The “calculated” values of the degradation patterns are those which are obtained using the computer program and the values of the rates given in the last four columns. When [2-14C]lactate was used, alanine was degraded.

<table>
<thead>
<tr>
<th>Relative specific activity</th>
<th>Rates used for calculation (Vglu set equal to 1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>Vtca</td>
</tr>
<tr>
<td>C-4, M1</td>
<td>65.5</td>
</tr>
<tr>
<td>C-5, M2</td>
<td>65.8</td>
</tr>
<tr>
<td>C-6, M3</td>
<td>40.3</td>
</tr>
<tr>
<td>C-7, P1</td>
<td>45.2</td>
</tr>
<tr>
<td>C-8, P2</td>
<td>60.0</td>
</tr>
<tr>
<td>C-9, P3</td>
<td>61.6</td>
</tr>
<tr>
<td>C-10, P4</td>
<td>34.3</td>
</tr>
<tr>
<td>C-11, P5</td>
<td>33.8</td>
</tr>
<tr>
<td>C-12, P6</td>
<td>58.7</td>
</tr>
<tr>
<td>C-13, P7</td>
<td>60.4</td>
</tr>
<tr>
<td>C-14, P8</td>
<td>26.6</td>
</tr>
<tr>
<td>C-15, P9</td>
<td>33.3</td>
</tr>
</tbody>
</table>

a Scheme 1.

TABLE IV
Isotopic yields and degradation patterns in lactate and glucose in experiments with d-[3-14C]glycerate and d-[6-14C]fructose. The isotopic substrates were added at tracer levels (about 0.2 μmoles) to kidney cortex slices together with 20 μmoles of unlabeled pyruvate and 20 μmoles of acetate. In flasks 2 and 4, 30 μmoles of glucose were also added.

<table>
<thead>
<tr>
<th>Flask</th>
<th>Labeled substrate</th>
<th>Isotopic yields</th>
<th>Relative specific activities</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glucose</td>
<td>Lactate</td>
<td>C-6</td>
</tr>
<tr>
<td>1</td>
<td>d-[3-14C]Glycerate</td>
<td>45.0</td>
<td>4.4</td>
</tr>
<tr>
<td>2</td>
<td>d-[3-14C]Glycerate</td>
<td>45.4</td>
<td>3.9</td>
</tr>
<tr>
<td>3</td>
<td>d-[6-14C]Fructose</td>
<td>49.7</td>
<td>3.7</td>
</tr>
<tr>
<td>4</td>
<td>d-[6-14C]Fructose</td>
<td>46.9</td>
<td>3.0</td>
</tr>
</tbody>
</table>

also be of some use in the determination of potential regulatory or rate-limiting steps. Establishment of rapid isotopic exchange of a given enzymic reaction in the cell tends to rule out major rate limitation or primary target of hormone action at this site. On the other hand, if the actual flux through a given enzymic reaction in the intact cell is the same or nearly the same as the maximal rate of the enzyme reaction in a homogenate, the reaction obviously must be considered in the potential rate-limiting class.

The model of kidney cortex metabolism which we have attempted to fit is plainly simplified in several regards. For example, a more realistic model would include separate mitochondrial and cytosolic pools of oxalacetate and malate. Much more data would be required in order to measure the rates of exchange of mitochondrial and cytosolic oxalacetate and malate and also the exchange rates of the two malate dehydrogenases. Preliminary studies (1) have indicated that the enzymic steps between phosphoenolpyruvate and glucose show varying degrees
of isotopic reversibility, and more comprehensive models should include such reversibility.

There is no absolute test for the validity of a model of a biological system. The model should be put through a number of independent and sensitive tests, if these agree reasonably well one can at least propose the model as a good working hypothesis. Thus, for example, we have measured the rates of pyruvate dehydrogenase by two independent and sensitive isotopic methods, as well as by an analytical balance of carbon, and find reasonable agreement among these three approaches.

It is evident from the relative rates of the Krebs cycle and glucose synthesis that gluconeogenesis uses only a fraction of the ATP generated in the mitochondria, most of which is presumably used in the transport processes essential to kidney function. We have estimated that the rate of "fumarase" exchange is about 4 times the forward rate of the Krebs cycle. This rate actually may be a composite of malate dehydrogenases exchange (both in the mitochondria and cytosol) and fumarase. Preliminary experiments using total degradation of glutamate (to determine the randomization of isotope in mitochondrial oxalacetate) as well as degradation of malate, suggest that the rate of mitochondrial malate dehydrogenase exchange is approximately the same as that of fumarase exchange.

Probably the most surprising result of these studies is the estimated high rate of recycling of phosphoenolpyruvate to pyruvate. Preliminary presentation of these results (1, 2) was met by much skepticism in this aspect of the interpretation of the model. It has been (reasonably) assumed that in an efficiently regulated system the "glycolytic" enzyme, pyruvate kinase, should be essentially totally suppressed when gluconeogenesis is taking place (for discussion, see reviews (21, 22)). Thus, it was suggested that the randomization of isotope in lactate may have been caused not by pyruvate kinase recycling but rather by isotopic exchange reactions involving either pyruvate carboxylase or malic enzyme. Indeed, Krebs and Veech (23) have postulated that pyruvate carboxylase may catalyze a "near-equilibrium" reaction in liver much as are thought to be catalyzed by the very active dehydrogenases.

Scrutton and Utter (24) and McClure et al. (25) showed that, respectively, chicken liver and rat liver pyruvate carboxylase catalyzed the reverse (oxalacetate to pyruvate) reaction at about 10% of the rate of the forward reaction. However, this required an oxalacetate concentration of nearly 1 mM which is 2 to 3 orders of magnitude higher than the estimated oxalacetate concentration in rat liver (26). McClure and Lardy (27) reported that the rat kidney pyruvate carboxylase is very similar to the rat liver enzyme in regard to apparent K_m values.

Perhaps a more serious candidate for an exchange reaction is malic enzyme. This is not a highly active enzyme in gluconeogenic tissues; in rat liver malic enzyme activity is reportedly about 1/4 that of pyruvate carboxylase (23) while malic enzyme levels in rat kidney are reported to be about 1/4 those in rat liver (28). In addition, the rate of any reversible exchange reaction will be governed by the rate in the slower direction; the rate of the pyruvate → malate reaction (even when saturated with CO_2) is about 1/3 that of the malate → pyruvate reaction. Our experiments are initiated in 100% oxygen and while CO_2 is generated during the incubation, its concentration is much lower than the rather high K_m for CO_2 of the malic enzyme.

Although these considerations suggest that these exchange reactions are not major factors in the randomization produced in lactate, more direct proof that pyruvate kinase is involved was sought. Table IV shows that ^14C from compounds which enter the gluconeogenic pathway "above" phosphoenolpyruvate appears in lactate. If one assumes that P-enolpyruvate carboxykinase is essentially irreversible in the intact cell, the labeling of lactate must occur via pyruvate kinase. On the other hand, if P-enolpyruvate carboxykinase exchange causes labeling of oxalacetate and subsequently malate, interpretations based again on malic enzyme and pyruvate carboxylase could again be invoked. However, if this were the case the ^14C in lactate should be largely randomized, and it is not.

The fact (Table IV) that the extent of labeling in lactate from n-[3-^14C]glycerate or n-[6-^14C]fructose is not greatly affected by the inclusion of 90 μmoles of glucose in the incubation medium suggests that the recycling via pyruvate kinase occurs in the gluconeogenic cells. An alternate explanation could have been formation of ^14C-labeled glucose from the substrates in the gluconeogenic cells, and subsequent glycolysis in another population of cells. Such a pathway should have been affected by the large glucose trap.

One can only speculate at this stage on possible functions of a pathway involving recycling via pyruvate kinase. One possible pathway is that which has been described as a "futile" or "useless" cycle involving the sequence: pyruvate → oxalacetate → P-enolpyruvate → pyruvate. With the use of the transaminase inhibitor, amino-oxyacetate (29), we have confirmed the proposal of Lardy et al. (30) that oxalacetate transfer out of the mitochondria requires the dual transaminase mechanism. Thus aspartate should also be included in this cycle (Fig. 3, Cycle A).

The net result of this cycle is simply a loss of energy, and the

![Fig. 3. Hypothetical cycles involving recycling of phosphoenolpyruvate to pyruvate. Cycle A is the much discussed "futile" or "useless" cycle with the addition of the dual transaminase mechanism of oxalacetate transfer. Cycle B would provide an energy driven transfer of reducing equivalents from the mitochondria to the cytosol.](http://www.jbc.org/content/85/1/6052/fig/3)
function of this is certainly not evident. It is conceivable that
modulating the pyruvate kinase backflow is a means of regulat-
ing gluconeogenesis. While more efficient controls would be
considered more likely, it should be remembered that, in the
kidney, gluconeogenesis itself uses only about 20% of the ATP

Another possibility which we have suggested previously (2) is
that malate rather than aspartate (or oxalacetate) is involved,
the cycle being as shown in Fig. 3, Cycle B. The net result of
this cycle is an energy-driven transfer of reducing equivalents
from the mitochondria to the cytosol. Some kind of transfer of
reducing hydrogen (without carbon) is required when pyruvate
is the gluconeogenic substrate to furnish the reducing equivalents
for the lactate which is also formed. In the experiments of
Tables II and III, the amount of lactate formation measured at
the end of the experiment was smaller than the calculated rate
of recycling of phosphoenolpyruvate. However, kinetic experi-
ments using similar conditions show that lactate formation
reaches a maximum after about 1 hour, after which time lactate
begins to be converted to glucose. Thus, it is conceivable that
a marked export of reducing equivalents occurred in the first
half of the incubation, possibly followed by a net import of re-
ducing equivalents into the mitochondria when lactate became
the major substrate. The use of pyruvate (or alanine) as a sole
substrate, the calculated rate of recycling via pyruvate
kinase was about 30% of the rate of gluconeogenesis.

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APPENDIX

The model is shown in Fig. 1. Metabolic and isotopic steady
state is assumed. Thus the amount of radioactivity entering a
given species of carbon atom equals the amount of activity
leaving. The specific activity of carbon 2 of the 2-14C-labeled
substrate is set equal to 1. The rate of glucose synthesis (VGLU)
is also set equal to 1 for purposes of computation. The follow-
ing equations hold when [2-14C]pyruvate is the labeled sub-
strate:

\[
V_{PK}M_1 = (V_{GLU} + V_{FK} + V_{PDH} + V_{LAC})P_1
\]

C-2 of pyruvate

\[
V_{FYR} + V_{PK}M_1 = (V_{GLU} + V_{FK} + V_{PDH} + V_{LAC})P_2
\]

C-1 of pyruvate

\[
V_{PK}M_1 = (V_{GLU} + V_{FK} + V_{PDH} + V_{LAC})P_1
\]

C-2 of acetyl-CoA

\[
V_{PDH}P_2 = V_{TCA}A_2
\]

C-1 of acetyl-CoA

\[
V_{TCA}P_2 = V_{TCA}A_1
\]

C-1 and C-2 of fumarate

\[
V_{TCA}(A_1 + M_1)/2 + V_{FUM}(M_4 + M_3)/2 = (V_{TCA} + V_{FUM})F_2
\]

\[
V_{TCA}(A_1 + M_1)/2 + V_{FUM}(M_4 + M_3)/2 = (V_{TCA} + V_{FUM})F_2
\]

\[
C-4 of malate

(V_{TCA} + V_{FUM})F_0 + V_{MDH}O_4 = (V_{TCA} + V_{FUM} + V_{MDH})M_4
\]

C-3 of malate

(V_{TCA} + V_{FUM})F_4 + V_{MDH}O_3 = (V_{TCA} + V_{FUM} + V_{MDH})M_3
\]

C-2 of malate

(V_{TCA} + V_{FUM})F_3 + V_{MDH}O_2 = (V_{TCA} + V_{FUM} + V_{MDH})M_2
\]

C-1 of malate

(V_{TCA} + V_{FUM})F_2 + V_{MDH}O_1 = (V_{TCA} + V_{FUM} + V_{MDH})M_1
\]

C-4 of oxalacetate

(V_{TCA} + V_{FUM} + V_{MDH} - V_{Glu} - V_{FK})M_4
\]

C-3 of oxalacetate

(V_{TCA} + V_{MDH} - V_{Glu} - V_{FK})M_3
\]

C-2 of oxalacetate

(V_{TCA} + V_{MDH} - V_{Glu} - V_{FK})M_2
\]

C-1 of oxalacetate

(V_{TCA} + V_{MDH} - V_{Glu} - V_{FK})M_1 + (V_{Glu} + V_{PK})P_1
\]

CO_2

\[
V_{TCA}(O_1 + O_2) + (V_{Glu} + V_{FK})M_4 + V_{PDH}P_1
\]

= (2V_{TCA} + V_{PDH})C - (V_{Glu} + V_{PK})C

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