Phosphoenolpyruvate Carboxykinase and the Synthesis of Glyceride-Glycerol from Pyruvate in Adipose Tissue*

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SUMMARY

In the absence of glucose, significant amounts of labeled pyruvate were converted to glyceride-glycerol by rat epididymal fat pads in vitro. Using specifically labeled pyruvate-14C we found twice as much C-2 incorporated into glyceride-glycerol as C-1. Although this ratio is not affected by changes in dietary conditions, the amount of pyruvate incorporated into glyceride-glycerol is decreased 4-fold in fasted rats as compared to fasted-refed animals. The addition of glucose to the incubation medium depressed the appearance of label in glycerol and stimulated the formation of fatty acids from pyruvate. Under all dietary conditions, over 90% of pyruvate C-1 and 43 to 49% of pyruvate C-2 incorporated into glycerol were in the α carbon atoms. These results indicate the occurrence of the dicarboxylic acid shuttle in adipose tissue and are supported by the following findings: phosphoenolpyruvate-carboxykinase activity is present in soluble and mitochondrial fractions of adipose tissue; its activity increases with fasting when compared to the fasted-refed animal; and it is sufficiently high to account for the observed rates of conversion of pyruvate to glycerol. This pathway, under conditions of glucose deprivation, may supply α-glycerophosphate for the re-esterification of fatty acids.

METHODS

Chemicals—Pyruvate-1-14C and pyruvate-2-14C were purchased from Nuclear-Chicago; NaH14CO3, from New England Nuclear; ADP, IDP, and NADH, from P-L Biochemicals; and P-enolpyruvate, malate dehydrogenase (EC 1.1.1.37), and collagenase (EC 3.4.4.19), from Sigma.

Diets—Male albino rats from Carworth Farms, weighing between 350 and 400 g, were used. They were fed Purina laboratory chow ad libitum, or fasted for 96 hours and refed for 96 hours. To minimize diurnal effects, all rats were killed at the same time of day.

Subcellular Fractionation of Adipose Tissue—In most experiments, 2.5 g of tissue from epididymal fat pads were homogenized in 6 ml of 0.25 M sucrose with a coaxial homogenizer with Teflon pestle. The homogenate was centrifuged for 30 min at 100,000 × g, and the pellet was suspended in 0.25 M sucrose, freeze-dried, and taken up in water. This preparation was used for the assay of particulate P-enolpyruvate carboxykinase activity. For the isolation of nuclei, mitochondria, microsomes, and soluble fraction, epididymal fat pads were incubated for 90 min with collagenase in Krebs-Ringer-bicarbonate buffer, pH 7.4, containing 3% bovine serum albumin (Fraction V). The cells were harvested by centrifugation, washed several times with buffer, and suspended in 0.25 M sucrose. The fat cells were disrupted by mechanical agitation on a Vortex mixer, and the bulk of the lipid was removed by centrifugation at 500 × g for 15 sec at 2°C. Nuclei were sedimented from the lipid-free homogenate by centrifugation at 100,000 × g for 15 min; mitochondria by centrifugation at 10,000 × g for 15 min; and microsomes, by centrifugation at 100,000 × g for 15 min; mitochondrial and microsomal fractions were subsequently solubilized with 1% sodium dodecyl sulfate (SDS) and dialyzed against water for 3 h, and the dialyzed fractions were then used in the assay of phosphoenolpyruvate carboxykinase activity.
eluted with a concentration gradient of 0 to 6 N formic acid by
with unlabeled malate on a column of Dowex 1-formate and
Another portion of the reaction product was chromatographed
the radioactivity was determined directly on these papers.
reaction product with aniline-citrate (13). The CO₂ evolved
ykinase reaction was determined by treating a portion of the
activity in the oxalacetate formed in the I'-enolpyruvate carbox-

liver and measured the fixation of r4C-labeled NaHC0₃ in the
the column eluent was measured as described by Hohorst (15).
the method of Busch, Hurlbert, and Potter (14). Malate in
at 38° in an atmosphere of 95% O₂-5% CO₂. The tissue was

1.25 Hmoles of ADP.

Products of P-enolpyruvate Carboxykinase Reaction—Radio-
activity in the oxalacetate formed in the P-enolpyruvate carbox-
ykinase reaction was determined by treating a portion of the
reaction product with aniline-citrate (13). The CO₂ evolved
was collected on filter paper saturated with NaOH, and
the radioactivity was determined directly on these papers.
Another portion of the reaction product was chromatographed
with unlabeled malate on a column of Dowex 1-formate and
eluted with a concentration gradient of 0 to 6 N formic acid by
the method of Busch, Hurlbert, and Potter (14). Malate in
the column eluent was measured as described by Hohorst (15).

Incorporation Studies with ¹⁴C-Labeled Pyruvate—Pieces of
adipose tissue weighing 200 to 500 mg were incubated for 3
hours in calcium-free Krebs-Ringer-bicarbonate buffer, pH 7.4,
at 38° in an atmosphere of 95% O₂-5% CO₂. The tissue was
then transferred to 15 ml of chloroform-methanol (2:1), and
the lipid was extracted and saponified as described previously
between the two α carbon atoms.

TABLE I

The complete assay system, at 37°, contained 100 μmoles of
imidazole (pH 6.6), 2 μmoles of MnCl₂, 1 μmole of GSH, 1.25 μmoles
of sodium IDP, 50 μmoles of NaHCO₃ (2 μC), 2.5 μmoles of NADH,
1.5 μmoles of P-enolpyruvate, 2 units of malate dehydrogenase,
and supernatant fraction of adipose tissue in a volume of 1 ml.
The final pH was 7.2. In those experiments in which other addi-
tions were made, the amounts added were 2 μmoles of MgCl₂ and
1.25 μmoles of ADP.

<table>
<thead>
<tr>
<th>Changes in complete system</th>
<th>Bicarbonate fixed</th>
<th>CO₂ released from product by aniline-citrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>26.6</td>
<td>0.4</td>
</tr>
<tr>
<td>Omit P-enolpyruvate</td>
<td>0.2</td>
<td>0.4</td>
</tr>
<tr>
<td>Omit IDP</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>Omit MnCl₂</td>
<td>1.8</td>
<td></td>
</tr>
<tr>
<td>Omit MnCl₂; add MgCl₂</td>
<td>2.0</td>
<td></td>
</tr>
<tr>
<td>Omit IDP and MnCl₂; add ADP and MgCl₂</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>Omit NADH, malate dehydrogenase</td>
<td>14.9</td>
<td>14.2</td>
</tr>
</tbody>
</table>

Fig. 1. Identification of malate as the product of the P-enol-
pyruvate carboxykinase assay. NaH¹⁴CO₃ was incubated with
adipose tissue supernatant as described in Table I and "Methods."
A portion of the inactivated reaction mixture, after gassing with
CO₂, was chromatographed with unlabeled malate on a column
(0.8 cm x 15 cm) of Dowex 1-formate (200 to 400 mesh) and eluted
with a 0 to 6 N formic acid gradient (13). Portions of each 4-ml
fraction were taken for the determination of malate (■) and radio-
activity (○). The specific activity, in (counts per min) x 10⁻⁸
per μmole, is also indicated (■).

(16). After removal of the fatty acids by repeated extractions
with petroleum ether, the aqueous fraction, containing the
glyceride-glycerol and previously shown to be free of other
radioactive materials (3), was deionized by passage through a
column of Amberlite IR-45 (OH⁻ form) and Amberlite IR-120
(H⁺ form) resins. The eluate was evaporated to dryness under
reduced pressure and dissolved in water. One portion was
taken for total radioactivity measurements. A second was
oxidized with periodic acid, and the formaldehyde derived
from the α carbon atoms was isolated as the dimedon derivative
(17) and its radioactivity determined. It should be pointed
out that this method of glycerol degradation does not distinguish
between the two α carbon atoms.

RESULTS

The composition of the reaction mixture for the assay of
P-enolpyruvate carboxykinase, as well as the effects of additions
and deletions, is shown in Table I. In the complete assay
system, only a small fraction of the total CO₂ fixed was found in
oxalacetate as determined by treatment with aniline-citrate.
However, in similar experiments carried out with NADH and
malate dehydrogenase omitted from the reaction mixture, all
of the labeled CO₂ fixed was in C-4 of oxalacetate and thus was
detected by the aniline-citrate method. When the products of
the complete reaction system were chromatographed on Dowex
1-formate, we noted only one radioactive peak. The radio-
activity coincided with malate and had a relatively constant
specific activity (Fig. 1). These experiments indicated that
the radioactive product of the P-enolpyruvate carboxykinase
assay was malate and that oxalacetate was formed when malate
dehydrogenase and NADH were omitted. A similar result
was found if enzyme from the particulate fraction was sub-
tituated for the soluble enzyme.

As there have been reports that P-enolpyruvate carboxykinase
is not present in adipose tissue (8, 9), we have used the present
Table II

Intracellular distribution of P-enolpyruvate carboxykinase in adipose tissue

Activities are based on the assumption that yields of cells and particles were quantitative. Details of the preparation of subcellular fractions are given under "Methods," and details of the assay, in Table I.

<table>
<thead>
<tr>
<th>Subcellular fraction</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mmoles NaHCO₃ incorporated/mg of tissue</td>
<td>mmoles NaHCO₃ incorporated/mg of tissue</td>
</tr>
<tr>
<td>Supernatant</td>
<td>11.3</td>
<td>11.7</td>
</tr>
<tr>
<td>Microsomes</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>1.3</td>
<td>0.9</td>
</tr>
<tr>
<td>Nuclei</td>
<td>0.4</td>
<td>0.4</td>
</tr>
</tbody>
</table>

Table III

Effect of diet on P-enolpyruvate carboxykinase activity in rat adipose tissue

Enzyme activities are expressed per g of tissue, per mg of nitrogen, or per total fat pad, and are shown as the mean ± S.E.M. for the number of animals in parentheses. Details of the assay are given in Table I. All methods of expressing the soluble enzyme activity show significant differences at the 5% probability level between the values in animals fasted for 96 hours and those fasted for 96 hours and refed chow for 96 hours. In no case were the differences between particulate enzyme activities significant at the 5% level.

Table IV

Incorporation of specifically labeled pyruvate-¹⁴C into glyceride-glycerol and fatty acids in vitro by adipose tissue from normal, fasted, and fasted-refed rats

Pieces of adipose tissue were incubated in calcium-free Krebs-Ringer-bicarbonate buffer, pH 7.4, for 3 hours at 38°C. The buffers contained 5 mM sodium pyruvate, and, where indicated, 5 mM glucose, 0.1 unit of insulin, 0.33 pmol of pyruvate-1-¹⁴C, or 0.33 pmol of pyruvate-2-¹⁴C. Values are the means ± S.E.M. for five rats. In all experiments, the differences between incorporation in tissue from fasted rats and fasted-refed rats are significant at the 5% probability level.

Table: Conversion of Pyruvate to Glycerol in Adipose Tissue

<table>
<thead>
<tr>
<th>Label and treatment</th>
<th>Pyruvate incorporated</th>
<th>Glyceride-glycerol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µmoles/g tissue/3 hrs</td>
<td>% total</td>
</tr>
<tr>
<td>Pyruvate-¹⁴C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fasted</td>
<td>0</td>
<td>0.58 ± 0.11</td>
</tr>
<tr>
<td>Fed</td>
<td>0</td>
<td>0.39 ± 0.06</td>
</tr>
<tr>
<td>Fasted-refed</td>
<td>0</td>
<td>0.18 ± 0.03</td>
</tr>
<tr>
<td>Pyruvate-²¹⁴C</td>
<td>0.16 ± 0.04</td>
<td>1.42 ± 0.29</td>
</tr>
<tr>
<td>Fasted</td>
<td>2.00 ± 0.45</td>
<td>1.03 ± 0.11</td>
</tr>
<tr>
<td>Fasted-refed</td>
<td>17.00 ± 3.78</td>
<td>0.51 ± 0.14</td>
</tr>
<tr>
<td>Pyruvate-²¹⁴C + glu-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cose + insulin</td>
<td>3.30 ± 0.74</td>
<td>0.38 ± 0.08</td>
</tr>
<tr>
<td>Fasted</td>
<td>10.03 ± 1.68</td>
<td>0.21 ± 0.03</td>
</tr>
<tr>
<td>Fasted-refed</td>
<td>25.45 ± 4.40</td>
<td>0.06 ± 0.01</td>
</tr>
</tbody>
</table>

The use of collagenase to release adipose tissue cells, followed by rupture of the cells on a glass surface, minimizes the possible contamination of the cellular components with ruptured subcellular organelles. Previous studies with adipose tissue pyruvate carboxylase (7) have shown that this method of cellular disruption results in a negligible loss of activity from isolated mitochondria. As indicated in Table II, nearly all of the P-enolpyruvate carboxykinase activity was in the soluble fraction. Adipose tissue mitochondria contained only 6 to 10% of the total P-enolpyruvate carboxykinase activity, and even lower levels of activity were present in the nuclei and microsomes.

The results of changes in dietary conditions on the activity of soluble or particulate P-enolpyruvate carboxykinase of adipose tissue are shown in Table III. Alteration in dietary pattern had no effect on P-enolpyruvate carboxykinase in adipose tissue mitochondria. As both the weight and nitrogen content of adipose tissue vary widely under different dietary conditions, we have expressed the P-enolpyruvate carboxykinase activity in units per mg of nitrogen and units per total fat pad, in addition to units per g, wet weight. When any of these methods of expressing activities is used, adipose tissue from 96-hour fasted rats has a significantly (p < 0.05) higher P-enolpyruvate carboxykinase activity than noted in adipose tissue from fasted-refed animals.

C-1 of pyruvate was converted to glyceride-glycerol at less than half the rate of C-2 by adipose tissue from fed, fasted, and fasted-refed rats (Table IV). From 91 to 99% of the label of pyruvate-1-¹⁴C and from 43 to 49% of that of pyruvate-2-¹⁴C incorporated into glycerol was in the α carbon atoms. In addition, the rates of incorporation into glycerol were significantly higher (p < 0.05) in epididymal fat pads from fasted rats than from fasted-refed animals, while the incorporation into fatty acids followed the opposite pattern. Addition of glucose and insulin to the incubation medium depressed glycerol formation from pyruvate and increased by several fold the rate of fatty acid synthesis from pyruvate, but did not alter the dietary effects.
DISCUSSION

In the present report, we have demonstrated that C-1 of pyruvate is incorporated in glycerol at about half the rate of C-2 of pyruvate. This finding is consistent with the formation of α-glycerophosphate via a pathway involving a symmetrical 4-carbon intermediate such as fumarate. This process would involve the loss of 1 of the labeled carbons when oxalacetate is randomized through fumarate and then decarboxylated to P-enolpyruvate (Fig. 2). Further support for this pathway is obtained from the degradation of glyceride-glycerol to determine the distribution of label. Virtually 100% of C-1 of pyruvate and approximately 50% of C-2 of pyruvate that were incorporated into glycerol were found in the α carbons. This finding eliminates the direct reversal of pyruvate-2-14C to P-enolpyruvate via pyruvate kinase, as no label would appear in the α carbons of glyceride-glycerol. However, Christophe et al. (5) have reported an equivalent incorporation of C-1 and C-3 of pyruvate into the α carbons of glyceride-glycerol in adipose tissue, suggesting a direct reversal of pyruvate to P-enolpyruvate via pyruvate kinase.

An alternative explanation of these experiments is possible if, prior to glycerol formation, pyruvate-2-14C becomes pyruvate-2,3-14C. This is possible by a series of reactions involving carboxylation to oxalacetate, randomization to fumarate-2,3-14C, and decarboxylation of malate to pyruvate (Fig. 2). Label from pyruvate-2-14C could thus be incorporated into the α carbons of glycerol via pyruvate kinase without the participation of the dicarboxylic acid shuttle. However, the appearance of approximately 50% of the label from C-2 of pyruvate into the α carbons of glycerol would require complete randomization of pyruvate-2-14C to pyruvate-2,3-14C before the pyruvate kinase step. In addition, the pyruvate kinase reaction would have to be reversible. As neither of these possibilities is likely, we have concluded that the conversion of pyruvate to glyceride-glycerol involves the dicarboxylic acid shuttle.

If the dicarboxylic acid shuttle occurs in adipose tissue, it requires P-enolpyruvate carboxykinase for the conversion of oxalacetate to P-enolpyruvate as well as pyruvate carboxylase for oxalacetate formation (7) (Fig. 2). Young, Shrago, and Lardy (8) and Weber et al. (9) were unable to detect any P-enolpyruvate carboxykinase activity in rat adipose tissue. We have demonstrated this enzyme in the cytoplasm of the fat cell separated with collagenase and homogenized under conditions which would cause a minimum disruption of cellular organelles. Although P-enolpyruvate carboxykinase is measured in the reverse direction, the assay method is sensitive, and the observed activities are sufficient to account for the rate of pyruvate converted to α-glycerophosphate (compare Tables III and IV).

A close correlation between P-enolpyruvate carboxykinase activity and the conversion of pyruvate-14C to glyceride-glycerol was noted in adipose tissue taken from rats under varying dietary conditions. Increasing lipogenesis in adipose tissue by fasting the rats for 96 hours and refedding for 96 hours reduced both the activity of soluble P-enolpyruvate carboxykinase and the incorporation of pyruvate into glycerol as compared to the levels in fasted rats. The over-all rates of pyruvate conversion to glycerol could be depressed by the addition of 5 mM glucose plus insulin to the incubation medium, but the differences between the fasted and fasted-refed animals remained. It therefore does not seem likely that this difference in the rate of pyruvate incorporation into glycerol is due to a dilution of labeled pyruvate by tissue glycogen, which is present in relatively high concentration in tissue from fasted-refed animals (18).

Unlike liver, adipose tissue does not synthesize glucose from pyruvate, as several key enzymes in gluconeogenesis are absent (9). From the results of this and several other studies, it appears that adipose tissue does carry on an active process of α-glycerophosphate synthesis from pyruvate (3-5). The physiological significance of such a pathway may lie in the constant requirement for α-glycerophosphate for triglyceride synthesis even during periods of fasting (19-21). It thus seems probable that pyruvate may supply α-glycerophosphate during periods of glucose deprivation.

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REFERENCES

18. Shafirr, E., Shapiro, B., and Wertheimer, E., in A. E.
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