THE PATHWAY OF HEXOSE SYNTHESIS FROM PYRUVATE IN MUSCLE*

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(Received for publication, October 3, 1957)

Although most of the reactions involved in the interconversion of glycogen and pyruvic acid are reversible, there is reason to believe that under physiological conditions the synthetic pathway may differ significantly from the degradative (1, 2). In particular, the reaction catalyzed by phosphoenolpyruvate kinase, although reversible (3), contributes little to glycogen synthesis from pyruvate in liver or liver slices. It has been estimated that at least 90 per cent of pyruvate and lactate enters the "dicarboxylic acid shuttle" before incorporation into liver glycogen (4–6). Such data are consistent with pyruvate conversion to phosphopyruvate, and ultimately to hexose in liver via the reactions described by Ochoa et al. (7) and by Utter and Kurahashi (8):

\[
\begin{align*}
(1) \quad \text{Pyruvate} + \text{CO}_2 + \text{TPNH} & \rightarrow \text{malate} + \text{TPN} + \text{H}^+ \\
(2) \quad \text{Malate} & \rightleftharpoons \text{fumarate} \\
(3) \quad \text{Malate} + \text{DPN} + \text{H}^+ & \rightarrow \text{oxalacetate} + \text{DPNH} \\
(4) \quad \text{Oxalacetate} + \text{ITP} & \rightarrow \text{phosphopyruvate} + \text{IDP} + \text{CO}_2
\end{align*}
\]

In muscle, glycogen is converted to pyruvate rapidly, while muscle glycogen formed in vivo is derived largely from blood glucose and only to a limited extent from blood lactate and pyruvate (9). Thus, in muscle the Embden-Meyerhof pathway functions primarily for the breakdown of glycogen, but in liver it is utilized as well for the synthesis of glycogen from 3-carbon precursors. It is of interest to relate these functional differences to enzymatic mechanisms. Although pyruvate incorporation into glycogen has been demonstrated in rat diaphragm in vitro (10), there is little information available concerning the pathway involved. The present studies confirm the observation that pyruvate carbon can be incorporated into muscle glycogen. However, the data are consistent with the incor-

* This investigation has been aided by grants from the Jane Coffin Childs Memorial Fund for Medical Research and the National Cancer Institute (No. C-2673), National Institutes of Health, United States Public Health Service.
poration of pyruvate carbon by way of a reversal of the phosphopyruvate
kinase reaction rather than via the less direct but energetically more favor-
able (1) dicarboxylic acid pathway.

Methods

Male Wistar rats weighing approximately 200 gm. were killed by a blow
on the head. For the studies on liver the animals were fasted for 48 hours
before use, and for the diaphragm experiments the rats were permitted
food ad libitum until the time of sacrifice. The tissues were excised im-
mmediately, plunged into ice-cold Krebs-Ringer bicarbonate solution (11),
blotted, and weighed. Approximately 300 mg. of liver slices, prepared
with the Stadie-Riggs microtome (12), or 250 mg. of diaphragm were incu-
bated in the Warburg apparatus in 2 ml. of Krebs-Ringer bicarbonate
medium at 37°, with a gas mixture of 95 per cent O₂-5 per cent CO₂. The
non-isotopic substrate was 0.022 M lactate. In the first group of experi-
ments each vessel contained 5 × 10⁶ c.p.m. (1.6 µmoles) of pyruvate-2-C¹⁴
(Nuclear Instrument and Chemical Corporation). In the second series
of experiments, similar conditions were employed except that the radio-
active pyruvate was replaced by C¹⁴O₂. One of the two side arms of each
Warburg vessel was fitted with a serum cap, through which was injected
1 ml. containing NaH¹⁴CO₃, 4.5 × 10⁶ c.p.m., after equilibration with the
gas phase was complete. The NaH¹⁴CO₃ was prepared by absorbing C¹⁴O₂
liberated from BaC¹⁴O₃ in an excess of 1 N KOH. The radioactive solution
was adjusted to the phenolphthalein end point with 0.5 N HCl. After 2
hours of incubation, the tissue was transferred to 1 ml. of boiling 30 per
cent KOH and the glycogen isolated (13). To facilitate glycogen puri-
ification, approximately 10 mg. of non-isotopic glycogen were added as
carrier in each experiment. Glycogen was hydrolyzed to glucose in 1 N
H₂SO₄ (4), and the glucose was degraded by fermentation with Leuconostoc
mesenteroides (14). It was found that cells of this organism which were
harvested during a period of vigorous CO₂ production could be lyophilized
and stored for at least 6 months, to be used as needed for the glucose deg-
gradations. The fermentation products were isolated (15) and subjected
to methods which permit the separation of each carbon atom of the glucose
molecule as BaCO₃. Ethanol was oxidized to acetic acid by heating for 2
hours at 90° with 0.5 gm. of potassium dichromate in 4 N H₂SO₄. Lactic
acid was oxidized to CO₂ and acetic acid by heating at 100° with chromium
trioxide in 1 N H₂SO₄. Acetic acid was degraded by the procedure of
Phares (16). Glucose was analyzed by the Somogyi procedure (17).

Radioactivity measurements were carried out with a "micromil" end
window counter with a counting efficiency of approximately 13 per cent.
Results

Only 0.6 per cent of pyruvate carbon was incorporated into liver glycogen, whereas 3 to 6 per cent incorporation took place in the diaphragm experiments (Table I). Thus, almost eight times as much pyruvate carbon was found in muscle glycogen as appeared in liver glycogen. On the other hand, very little bicarbonate carbon was incorporated into muscle glycogen, compared with that in liver, in which incorporation was extensive (Table I). In these experiments no attempt was made to determine whether net synthesis had occurred.

The degradation data are shown in Table II. Marked isotope randomization was observed in the glycogen glucose isolated from the liver slices incubated with pyruvate-2-\(^{14}\)C. The specific activity of carbon atoms 1 and 6 was approximately equal to that of carbon atoms 2 and 5, respectively. In muscle glycogen glucose, on the other hand, the isotope was predominantly in carbons 2 and 5, and no radioactivity was found in positions 1 and 6.

### Table I

<table>
<thead>
<tr>
<th>C(^{14}) substrate</th>
<th>C.p.m. fixed in glycogen per gm. tissue</th>
<th>Ratio, liver/diaphragm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyruvate, 5 (\times 10^4) c.p.m.</td>
<td>2800</td>
<td>16,800</td>
</tr>
<tr>
<td></td>
<td>2600</td>
<td>29,000</td>
</tr>
<tr>
<td>NaHCO(_3), 4.5 (\times 10^7) c.p.m.</td>
<td>4100</td>
<td>61</td>
</tr>
<tr>
<td></td>
<td>3000</td>
<td>70</td>
</tr>
</tbody>
</table>

### Table II

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Per cent radioactivity in glycogen glucose carbon atoms</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Carbon 1</td>
</tr>
<tr>
<td>Liver</td>
<td>19</td>
</tr>
<tr>
<td>Diaphragm</td>
<td>0</td>
</tr>
<tr>
<td>“</td>
<td>0</td>
</tr>
</tbody>
</table>
The very small quantity of pyruvate carbon incorporated into liver glycogen is probably attributable to the high Na medium used in these experiments. This has been shown to be much less satisfactory for glycogen synthesis in liver than a high K medium (18). The high Na medium was selected, however, to provide conditions similar to those employed for a previous study of muscle glycogen formation (10).

The extensive isotope randomization in carbons 1, 2, 5, and 6 of liver glycogen glucose is similar to that observed by others who have used pyruvate-2-C^{14} as substrate (4, 6). The virtual equality of isotope concentration in carbons 1 and 6 with that in carbons 2 and 5, respectively, is consistent with the estimate (4, 6) that at least 90 per cent of pyruvate is converted by liver to a symmetrical dicarboxylic acid before its entry into the glycolytic pathway. Krebs has pointed out that a consideration of the energy changes involved in phosphopyruvate formation from pyruvate leads to the conclusion that those reactions involving dicarboxylic acids as intermediates would be more likely to occur under physiological conditions (1). Hence, it is not surprising to find this pathway to be the major synthetic route in liver, the tissue mainly responsible for pyruvate conversion to glycogen in vivo. In muscle, on the other hand, the data suggest that none of the pyruvate is converted to a symmetrical dicarboxylic acid. Two alternative explanations are available to account for this result. Either the direct phosphorylation of pyruvate by a reversal of the phosphopyruvate kinase reaction or the absence of equilibration of C_4 acids by fumarase (Equation 2) would result in pyruvate-2-C^{14} incorporation without spread of isotope to positions 1 and 6 of hexose. Consistent with the first of these alternatives are the observations of Dickens and Glock (19), who found very low concentrations of glucose 6-phosphate and 6-phosphogluconic dehydrogenases in muscle. Evidence is accumulating to suggest that these enzymes represent the major mechanism for the generation of reduced TPN in mammalian tissues (2). The conversion of pyruvate to malate requires TPNH (Equation 1), and the absence of the dicarboxylic acid shuttle in muscle may be related to the low activity of these TPNH-producing enzymes in this tissue.

In view of these findings concerning the mechanism of muscle glycogen formation from pyruvate, the observation of Crane and Ball that CO_2 incorporation in pyruvate by rat diaphragm and liver occurs to an approximately equal extent (20) evokes additional questions. Carbon dioxide fixation is thought to involve the formation of the same symmetrical C_4 intermediate which accounts for spread of isotope from pyruvate-2-C^{14} (Equations 1 to 4). Since in our studies with pyruvate no evidence of those reactions was found in muscle, the possibility must be considered that CO_2 is fixed in muscle tissue by some other mechanism. One possi-
bility is an exchange of the pyruvate carboxyl group with $^{14}C\text{O}_2$ in the pyruvate oxidation reaction. This exchange has been demonstrated with bacterial and mammalian enzyme systems (21, 22).

The greater activity in carbon 5 than in carbon 2 of the hexose in the diaphragm experiment recalls a similar phenomenon in rat liver glycogen (6, 23), and is consistent with incomplete equilibration of the triose phosphates before their condensation to form hexose.

**SUMMARY**

Radioactive carbon was incorporated into the glycogen of rat diaphragm and of rat liver slices incubated with pyruvate-$^{14}C$ or $^{14}CO_2$. Isotope distribution was determined in glycogen glucose isolated from liver and from diaphragm after incubation with pyruvate-$2^{14}C$. $^{14}C$ was extensively randomized throughout the liver hexose molecule, but glucose isolated from diaphragm was labeled almost solely in carbon atoms 2 and 5. The patterns of radioactivity suggest that in liver pyruvate enters the glycolytic pathway via the dicarboxylic acid shuttle, whereas in muscle entry is achieved by way of a reversal of the phosphopyruvate kinase reaction.

**BIBLIOGRAPHY**

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