Carbon Dioxide Fixation in the JA-1 Sarcoma in Vitro

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SUMMARY

Carbon dioxide fixation studies were conducted with rapidly growing JA-1 sarcoma cells in vitro. Isotope was isolated in asparagine, aspartic acid, glutamic acid, and glutamine. Chemical degradation of these compounds suggested three sites of fixation. They were carbon dioxide condensation with (a) pyruvic acid, (b) propionyl CoA, and (c) a three-carbon fragment such as the isopropyl carbons of leucine. The first reaction was found to be only slightly reversible.

Carbon dioxide was shown to be essential for growth of the JA-1 sarcoma in vitro (8). When the tumor was grown in the presence of Na₂C¹⁴O₃, isotope was isolated in incorporated purines, pyrimidines, aspartic acid, glutamic acid, and proline. Since the authors were unable to find any extensive reaction mechanism studies on carbon dioxide fixation by tumors, this area was investigated. The present report describes anabolic pathways of aspartic acid, glutamic acid, and their respective amides from carbon dioxide (bicarbonate) during rapid growth of the tumor in vitro.

MATERIALS AND METHODS

All cell cultures were conducted with the freshly excised tumor by techniques previously described (9). Medium 4a minus asparagine was used (8), and the cells were cultivated in Blake bottles (initial inoculum 2 × 10⁶ cells/ml). When the cells were in the log phase of growth, two bottles were harvested and viable cells counted to determine “cell take.” The remaining bottles received the isotope dissolved in fresh Medium 4a (minus asparagine). After an additional incubation period of 24 hours, the cells were harvested for analysis. Growth during exposure to isotope was expressed as the ratio of final cell count to “cell take.”

The cells were allowed to autolyze for 18 hours. Afterward, asparagine, aspartic acid, glutamine, and glutamic acid were isolated, and asparagine and aspartic acid were degraded by methods developed in this laboratory (11). Glutamine and glutamic acid were degraded by the method of Mosbach, Phares, and Carson (10) and Phares (12). All radioassays were conducted in a windowless gas-flow (Q-gas) counter.

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RESULTS AND DISCUSSION

The distribution of label in aspartic acid, asparagine, glutamic acid, and glutamine when the tumor was grown in the presence of Na₂C¹⁴O₃ is shown in Table 1. It can be seen that carbons 1 and 4 of aspartic acid and asparagine were equally labeled and that no isotope was found in carbons 2 and 3. These results were in accord with those reported on intact animals (1, 14). If carbon dioxide were condensed with pyruvate, the result would be oxalacetate-4-C¹⁴, which when aminated directly would yield aspartic acid-4-C¹⁴ and asparagine-4-C¹⁴. On the other hand, if oxalacetate-4-C¹⁴ were converted to fumarate or succinate via malate, the resulting compound would be a mixture of fumarate-1-C¹⁴ and fumarate-4-C¹⁴, or essentially a 1,4-C¹⁴-dicarboxylic acid (radioisotopic randomization). Subsequent conversion of this compound to aspartic acid via oxalacetate would yield equal label in carbons 1 and 4.

Another possibility was that carbon dioxide fixation occurred with propionyl CoA, resulting in a mixture of succinate-1-C¹⁴ and -4-C¹⁴, or essentially succinate-1,4-C¹⁴. (Conversion of this compound to aspartic acid via oxalacetate would also yield equal label in carbons 1 and 4.)

The labeling pattern observed in glutamic acid and glutamine showed 90 per cent of the isotope in carbon 1 and 10 per cent in carbon 5. This experiment was performed 3 times, and in each study isotope was detected in carbon 5. These results were similar to those reported for minces of hen oviduct (4) and for the intact rat (6). If the conventional tricarboxylic acid cycle were operating...
ing between an oxalacetate-1,4-C\textsuperscript{14} and a-ketoglutarate (with no other carbon dioxide fixation or other side reactions occurring), one would expect all the label in glutamic acid to be in carbon 1. Therefore, some other possibilities should be considered. These are:

a) If the citrate formed from oxalacetate-1,4-C\textsuperscript{14} and acetyl CoA would assume a symmetrical nature, the resultant mixture of citrates would essentially be citrate-labeled in all three carboxyl groups. This, in turn, could account for label in carbon 5 of glutamic acid.

b) If succinate-1,4-C\textsuperscript{14} were cleaved with the rupture of the carbon 2-carbon 3 bond (reported previously in rabbit liver slices [15]), the acetate resulting from the reaction would be labeled in carbon 1, and this would label carbon 5 of glutamic acid.

c) An additional carbon dioxide fixation could occur with a three-carbon fragment such as the isopropyl carbons of leucine. In this case the presumed reaction would involve fixation with \( \beta \)-methylcrotonyl CoA to yield \( \beta \)-methylglutaconyl CoA. This in turn would yield acetoacetate-1-C\textsuperscript{14}, which would form acetate-1-C\textsuperscript{14} (2, 18). This could account for label in carbon 5 of glutamic acid. Therefore, experiments were designed to determine which, if any, of the possibilities were occurring during rapid growth of the tumors.

Since the labeling patterns of the amides were the same as their corresponding amino acids, it was assumed that the aspartic acid and glutamic acid were the immediate precursors of the amides when carbon dioxide fixation was involved. As a result, all subsequent degradation studies were made on hydrolysates of cell protein that represented the combination of the incorporated amino acid and its respective amide. In these experiments the cell protein was prepared as described previously (7).

To account for the label in carbon 5 of glutamic acid, fumarate-1,4-C\textsuperscript{14}, succinate-1,4-C\textsuperscript{14}, and succinate-2,3-C\textsuperscript{14} were used. The distribution of isotope found in incorporated aspartic acid and glutamic acid is shown in Table 2. Of interest was the fact that no detectable isotope was found in carbon 5 of glutamic acid when the 1,4-C\textsuperscript{14}-labeled dicarboxylic acids were administered, and no activity in carbon 4 of glutamic acid when succinate-2,3-C\textsuperscript{14} was given the tumor. As a result, the possibility of the symmetrical nature of citrate or the cleav-

### Table 1

<table>
<thead>
<tr>
<th>COMPOUND</th>
<th>ASPARTIC ACID</th>
<th>ASPARAGINE</th>
<th>GLUTAMIC ACID</th>
<th>GLUTAMINE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cpd</td>
<td>580</td>
<td>568</td>
<td>73.3</td>
<td>42.4</td>
</tr>
<tr>
<td>(counts/min/μmole)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

| Carbon 1 | 305 | 167 | 68 | 37 |
| Carbon 2 | 0   | 0   | 0  | 0  |
| Carbon 3 | 0   | 0   | 0  | 0  |
| Carbon 4 | 323 | 181 | 0  | 0  |
| Carbon 5 | 0   | 6   | 5  |    |

* Initial activity, 1,138,000,000 counts/min. Growth during exposure to isotope = 3.2 (ratio of final cell count to “cell take”).

### Table 2

<table>
<thead>
<tr>
<th>ISOTOPES</th>
<th>FUMARATE-1,4-C\textsuperscript{14}</th>
<th>SUCCINATE-1,4-C\textsuperscript{14}</th>
<th>SUCCINATE-2,3-C\textsuperscript{14}</th>
<th>DL-ALANINE-3-C\textsuperscript{14}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AA| GA</td>
<td>AA</td>
<td>GA</td>
<td>AA</td>
</tr>
<tr>
<td>Carbon 1</td>
<td>96</td>
<td>32</td>
<td>1820</td>
<td>420</td>
</tr>
<tr>
<td>Carbon 2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Carbon 3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Carbon 4</td>
<td>67</td>
<td>0</td>
<td>170</td>
<td>0</td>
</tr>
<tr>
<td>Carbon 5</td>
<td>0</td>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Growth:</td>
<td></td>
<td>2.3</td>
<td>2.2</td>
<td>1.9</td>
</tr>
</tbody>
</table>

* Initial activity, ca. 1,000,000 counts/min.
\dagger Initial activity, ca. 4,000,000 counts/min.
\ddagger Initial activity, ca. 57,000,000 counts/min.
\$ AA = aspartic acid plus asparagine; GA = glutamic acid plus glutamine.
\| Ratio of final cell count to “cell take.”
To determine whether the carbon dioxide condensation occurred with pyruvate, D,L-alanine-2-C\textsubscript{14} was administered to the tumor (Table 2). Although carbon 5 of glutamic acid contained the highest activity (indicating that the predominant pathway into the tricarboxylic acid cycle was through acetyl CoA), significant isotope was found in carbons 2 and 3 of aspartic acid. This clearly indicated that carbon dioxide fixation was occurring with pyruvate, but appreciable radioisotopic nor alanine-2-C\textsubscript{14} (Table 2) gave detectable activity in carbon 4 of glutamic acid from the JA-1 sarcoma.

The apparent discrepancy of the amount of isotope found in carbon 4 of glutamic acid between the JA-1 sarcoma in vitro system and the Murphy-Sturm in vivo system should not be interpreted as conflicting results. One system contained only tumor cells under actively growing conditions, whereas in the other system the isotope was exposed to metabolizing cells from a variety of organs as well as tumor cells, which makes interpretation difficult. Similar apparent discrepancies have been resolved with respect to the labeling of carbon 4 of brain glutamic acid in rats (3).

**TABLE 3**

**CONTRIBUTION OF ISO TOPE TO INCORPORATED ASPARTIC ACID AND GLUTAMIC ACID**

<table>
<thead>
<tr>
<th>ISOTOPE</th>
<th>PROPYCIC ACID-2-C\textsubscript{14}</th>
<th>ISOUCINE-U-C\textsubscript{14}</th>
<th>Leucine-U-C\textsubscript{14}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AA| GA</td>
<td>AA</td>
<td>GA</td>
</tr>
<tr>
<td>Carbon 1</td>
<td>857</td>
<td>253</td>
<td>67</td>
</tr>
<tr>
<td>Carbon 2</td>
<td>5100</td>
<td>1480</td>
<td>88</td>
</tr>
<tr>
<td>Carbon 3</td>
<td>5110</td>
<td>1390</td>
<td>93</td>
</tr>
<tr>
<td>Carbon 4</td>
<td>736</td>
<td>28</td>
<td>54</td>
</tr>
<tr>
<td>Carbon 5</td>
<td>24</td>
<td>24</td>
<td>24</td>
</tr>
<tr>
<td>Growth 1| 2.2</td>
<td>1.8</td>
<td>1.4</td>
<td>1.4</td>
</tr>
</tbody>
</table>

\* Initial activity, 86,000,000 counts/min.
\dag Initial activity, 25,000,000 counts/min.
\| Initial activity, 86,000,000 counts/min.
§ AA = Aspartic acid plus asparagine; GA = glutamic acid plus glutamine.
\| Ratio of final cell count to "cell take."

randomization took place presumably via fumarate or succinate before the resultant oxalacetate was aminated.

The lack of detectable activity in carbon 4 of glutamic acid (in the D,L-alanine-2-C\textsubscript{14} studies) deserves some consideration. Freedman and Graff (3) reported that when normal rats and Murphy-Sturm sarcoma-bearing rats were given intraperitoneal injections of D,L-alanine-2-C\textsubscript{14}, some activity was in carbon 4 of liver glutamic acid. In the sarcoma, however, appreciable isotope was recovered in carbon 4 of glutamic acid. They explained this on the basis of a condensation of carbon dioxide with pyruvate, and the resultant oxalacetate-2,3-C\textsubscript{14} (after radioisotopic randomization via fumarate) was doubly decarboxylated to yield uniformly labeled acetyl CoA. Subsequent condensation of the acetyl CoA with oxaloacetate would result in labeled carbons 4 and 5 in tumor glutamic acid. In the present studies, neither succinate-2,3-C\textsubscript{14} nor alanine-2-C\textsubscript{14} (Table 2) gave detectable activity in carbon 4 of glutamic acid from the JA-1 sarcoma.

Experiments were then conducted to clarify three points. They were:

a) Propionic acid-2-C\textsubscript{14} was administered to ascertain whether carbon dioxide fixation was occurring through propionyl CoA. Further, if this reaction did occur, the initial isotopic activity administered should be sufficiently high to study the reversibility of the pyruvate-carbon dioxide reaction as suggested by Freedman and Graff (3).

b) Isoleucine-U-C\textsubscript{14} was administered to the tumor to confirm the propionic acid studies.

c) Leucine-U-C\textsubscript{14} was administered to determine whether carbon dioxide fixation would occur with the isopropyl carbons of leucine (presumably via β-methylcrotonyl CoA and β-methylglutaconyl CoA). The results are given in Table 3.

From the propionic acid-2-C\textsubscript{14} study it was apparent that carbon dioxide fixation did occur, but the pyruvate-carbon dioxide condensation was only slightly reversible in the JA-1 sarcoma (see activity
in carbons 4 and 5 in glutamic acid). The labeling distribution found when isoleucine-U-C\(^{14}\) was given to the tumor suggested the formation of acetyl CoA and propionyl CoA, which presumably entered the tricarboxylic acid cycle by carbon dioxide fixation to give succinyl CoA via methylmalonyl CoA.

The isotopic leucine investigations gave results in the labeling distribution of aspartic acid and glutamic acid that supported the earlier suggestion of carbon dioxide fixation with the isopropyl carbons of leucine via \(\beta\)-methylcrotonyl CoA.

If the citric acid cycle was used solely for the purpose of supplying energy for the tumor, then the reduction of a metabolic by-product, such as carbon dioxide, should not be involved. Studies with actively growing JA-1 sarcoma cells, however, have indicated entry of carbon dioxide into the cycle at three points. These involved carbon dioxide condensation with pyruvate, propionyl CoA, and, to a lesser extent, \(\beta\)-methylcrotonyl CoA. Therefore, the cycle was utilized as a site of appreciable synthesis. For example, in one experiment JA-1 sarcoma cells ("cell take" = 3 \(\times\) 10\(^8\); final cell count = 1 \(\times\) 10\(^9\)) fixed 16 mg. of carbon dioxide during a 24-hour period.

Two other experiments were conducted to determine net synthesis of compounds from carbon dioxide and the effect of growth rate on net synthesis. In JA-1 cells (average generation time, 24 hours) 32.7 \(\times\) 10\(^{-17}\) moles carbon dioxide/cell/24 hours were utilized in net synthesis, whereas cells growing with an average generation time of 19 hours utilized 53.4 \(\times\) 10\(^{-17}\) moles carbon dioxide/cell/24 hours for net synthesis. This, and the fact that carbon dioxide is required for growth of the tumor, indicated that substrates that are produced from the carbon dioxide fixation system are necessary for growth and are not satisfied by other metabolic pathways.

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REFERENCES

Carbon Dioxide Fixation in the JA-1 Sarcoma *in Vitro*

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