Tracer Studies on the Metabolism of the Gardner Lymphosarcoma

IV. The Conversion of Lactate-2-C\(^4\) to Alanine, Glutamate, and Aspartate by Tumor and Spleen Cells*

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INTRODUCTION

Tumor metabolism is characterized by a high rate of lactic acid production under both aerobic and anaerobic conditions. Lactic acid probably represents a metabolic cul de sac. Since this substance and its precursor, pyruvic acid, are normally metabolized by way of the Krebs cycle, considerable interest has been focused in recent years upon the reactions of the citric acid cycle in tumor tissues.

Early observations by Potter and Le Page (7) suggested that "oxalacetic acid oxidase" activity might be very low in tumor tissues. Recently, however, Weinhouse et al. (11) have shown that C\(^4\)-labeled fatty acids are oxidized to C\(^4\)O\(_2\) in three mouse tumors and that quinidine citrate-C\(^4\) could be isolated from the reaction mixture. It was also shown by Weinhouse and co-workers (12) that the condensing enzyme, cis-aconitase, and isocitric dehydrogenase were active in those tumors. Kit and Greenberg (4) obtained evidence indicating that the Krebs cycle was operating in the Gardner lymphosarcoma. The most recent work from the Wisconsin laboratories is in agreement with these findings (8).

Radioactive carbon-labeled lactic acid provides an excellent tool with which to study this problem further. Experiments were therefore carried out which were designed to throw additional light upon the pathways by which lactic acid is metabolized in the lymphosarcoma cells and normal mouse spleen cells. Interesting differences between these tissues in the conversions of the labeled carbon of lactic acid to alanine, glutamate, and aspartate were observed and are presented here.

METHODS

Female C3H mice, weighing 22–25 gm., were used. Food was withdrawn 3 hours prior to the time that the mice were sacrificed. The general methods used in the preparation of the cell suspensions from pooled tumors or spleens have been reported previously (3). In this study, cell suspensions were centrifuged for 5 minutes at 2,000 r.p.m. in an International refrigerated centrifuge. The cells were resuspended and twice recentrifuged, and in each case the bottom-most 0.2 ml of packed cells were discarded. In this way, many of the erythrocytes which contaminated the spleen cell suspensions were eliminated, although it was recognized that the two washes were partially removing soluble components of metabolic significance.

Incubations were conducted in Warburg flasks with 0.2 ml of 20 per cent KOH in the center wells and 0.2 ml of 20 per cent trichloroacetic acid in the sidearms. The main compartment contained 0.5 ml of the cell suspension (100–150×10\(^6\) cells) and 0.1 ml of 0.000 m lactate (84,000 counts/min) or 0.1 ml of a modified Krebs-Ringer phosphate solution. After 150 minutes at 37.5\(^\circ\)C. with air as the gas phase, the incubation was terminated by tipping in acid from the sidearm. The further procedures in the washing of the tissues and the
preparation of the protein and respiratory CO₂ for radioactive assay have been described (3, 4).

The alcohol, ether-alcohol, and ether washes were pooled, concentrated on a steam bath, transferred to shallow aluminum cups, evaporated to dryness under an infrared lamp, and counted with a flow-gas counter.

The trichloroacetic acid extracts and washes were pooled, extracted 4 times with ether, and then concentrated in vacuo to 5–10 ml. The concentrate from each incubation was chromatographed on 120 cm. x 12 mm. columns containing Dowex-50; solvent: 1.5 N HCl. Data plotted are of experiment 2 for the tumor and 4 for the spleen (see Table 1). The total counts in each of the peaks are shown in Table 1. It may be noted that the pattern of radioactivity is strikingly different in the tumor incubations than in those of the spleen cells. In the tumor, radioactivity due to alanine was double that due to glutamic acid, while that due to aspartate was comparatively low. In the spleen cell incubations the radioactivity due to the aspartate was almost as great as that due to glutamate, while the alanine peak showed the least radioactivity.

Endogenous lactic acid.—It was of interest to determine the extent to which endogenous lactic acid was diluting the radioactive lactic acid added to each flask. Analyses (4) showed that at the start of an incubation, the spleen cells contained approximately 16 μg. of lactic acid, while the tumor cells contained about 60 μg. Since 80 μg. of lactate-C¹⁴ was added to each flask, it was clear that this radioactivity was not significantly diluted by lactic acid from the spleen cells but that the dilution due to the lactic acid of the tumor cells was a factor of importance. It is also to be emphasized that, although the incubations were conducted aerobically, the glycolytic formation of lactic acid by the tumor probably exceeded the formation of lactate by the spleen cells.

RESULTS

Chromatograms.—The peaks of radioactivity obtained after chromatographing with Dowex-50 are illustrated in Chart 1. The first 40–50 fractions contained a broad, high peak of radioactivity, caused by the unreacted lactic acid or by other acidic substances such as pyruvic and acetic acids. These cups were counted in only one experiment. Three amino acids were further verified by paper chromatography (Whatman No. 1) with butanol-water-acetic acid (100:50:22.5) and phenol-water (50 cm.:19 ml.) as the solvents. However, paper chromatograms from the aspartic and glutamic acid cups, run with the second solvent, each showed an additional weak spot containing radioactivity. These two spots were close to the origin and may have been due to acidic peptides.

In order to throw further light on the results, simultaneous observations were made on the oxygen consumption, C¹⁴O₂ pro-
duction, and radioactivity of protein and lipid in the cells. In the four experiments in which oxygen uptake was measured, the average uptake per experimental flask was 137 and 170 μl. for the spleen cells and 159 and 186 μl. for the tumor cells. The presence of lactic acid raised the oxygen consumption of the spleen cells 20 per cent but did not alter the respiration of the tumor cells. This may perhaps be attributed to the fact that the tumor cells already contained considerable lactic acid, as indicated above.

Radioactivity in lipids and protein.—Lactic acid may be converted to lipids at an appreciable rate in liver tissue. Under the conditions of these experiments, however, the total radioactivity due to lipids was negligible in both the lymphosarcoma and spleen cells. It would therefore appear that this represents a minor pathway for disposing of the lactic acid by these cells.

The radioactivity to be found in the protein is a function of the rate at which lactate is converted to amino acids. It was found that the labeling of the proteins increased throughout the incubation. The specific activity of the protein from the tumor cells was somewhat lower than that of the spleen cells. However, if one corrected for the dilution caused by the endogenous lactic acid produced by the tumor cell, one found that the incorporation into the proteins of the two cell types was approximately equal. About equal rates of incorporation of radioactive alanine and glycine into the proteins of these cells were reported in a previous publication (5).

Respiratory C14O2.—In the experiments with the spleen cells, 15 and 18 per cent of the added radioactivity was found in the respiratory CO2. The figures for the lymphosarcoma were 10 and 19 per cent. Thus, citric acid was being metabolized as rapidly as it was formed. On the other hand, when the tumor cells were incubated with acetic and oxalacetate acids, citrate accumulated unless fluoroacetate was added. Thus, the tumor was apparently capable of forming citric acid more rapidly than it was able to metabolize it.

Table 1 shows that 5 times as much radioactivity was found in the glutamate of the tumor as in the spleen. An accumulation of both citrate and glutamate would be expected if the oxidation of a-ketoglutarate were the rate-limiting step in the Krebs cycle oxidations of the tumor cell. Additional data on the quantity of a-ketoglutaric acid formed and the a-ketoglutaric acid oxidase activity are required to ascertain whether this is correct. It is to be noted that the observed pattern of radioactivity in the lymphosarcoma amino acids would also be found as a consequence of several other factors: (a) a high glutamate content

### Table 1

<table>
<thead>
<tr>
<th>Exp.</th>
<th>Tissue</th>
<th>Glutamate counts/min*</th>
<th>Aspartate counts/min*</th>
<th>Alanine counts/min*</th>
<th>Ratio Glut.:Asp.:Alan.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Tumor</td>
<td>20,900</td>
<td>3,660</td>
<td>35,960</td>
<td>1:0.18:1.72</td>
</tr>
<tr>
<td>2</td>
<td>Tumor</td>
<td>24,500</td>
<td>4,440</td>
<td>55,100</td>
<td>1:0.18:2.25</td>
</tr>
<tr>
<td>3</td>
<td>Spleen</td>
<td>18,750</td>
<td>10,480</td>
<td>9,480</td>
<td>1:0.76:0.69</td>
</tr>
<tr>
<td>4</td>
<td>Spleen</td>
<td>18,070</td>
<td>11,100</td>
<td>7,660</td>
<td>1:0.85:0.59</td>
</tr>
<tr>
<td>5†</td>
<td>Spleen</td>
<td>7,440</td>
<td>7,180</td>
<td>4,640</td>
<td>1:0.97:0.62</td>
</tr>
</tbody>
</table>

* Counts per minute. The magnitude of the counts with the flow-gas counter is about 5 times as great as with the mica end-window tube reported in the previous papers of this series.
† Incubated at 50°C.

DISCUSSION

Experiments already reported (4) indicated that acetate-1-C14 was oxidized to C14O2 by the cells of the spleen and Gardner lymphosarcoma. The O2 utilization of the tumor cells was stimulated by succinic acid, and leucine-2-C14 was also shown to be partially oxidized to C14O2. The demonstration that lactate-2-C14 is oxidized to C14O2 and that part of the radioactivity may be found in alanine, aspartic, and glutamic acids provides further evidence that the oxidations of the Krebs cycle proceed to an appreciable extent in both the spleen and lymphosarcoma cells.

Alanine contained less radioactivity than the dicarboxylic amino acids in the incubations with the spleen cells. This, coupled with the fact that about the same amount of radioactivity was found in the latter two amino acids, suggests that the enzymes which metabolized lactic acid to CO2 were "harmoniously geared" in the spleen. This is further supported by our earlier experiments (4), since it was found that when spleen cells were incubated with oxalacetic and acetic acids, no citrate accumulated unless fluoroacetate was added. Thus, citric acid was being metabolized as rapidly as it was formed. On the other hand, when the tumor cells were incubated with acetic and oxalacetate acids, citrate accumulated in the absence of fluoroacetate, and the addition of fluoroacetate induced only a small increase in citric acid. Thus, the tumor was apparently capable of forming citric acid more rapidly than it was able to metabolize it.

Table 1 shows that 5 times as much radioactivity was found in the glutamate of the tumor as in the spleen. An accumulation of both citrate and glutamate would be expected if the oxidation of a-ketoglutarate were the rate-limiting step in the Krebs cycle oxidations of the tumor cell. Additional data on the quantity of a-ketoglutaric acid formed and the a-ketoglutaric acid oxidase activity are required to ascertain whether this is correct. It is to be noted that the observed pattern of radioactivity in the lymphosarcoma amino acids would also be found as a consequence of several other factors: (a) a high glutamate content...
which would serve as a trap when radioglutamate entered the glutamate pool, (b) a high level of glutamate-pyruvate transaminase activity in the tumor, (c) a low level of malic dehydrogenase in the tumor.

Unfortunately, only limited data are as yet available concerning these points. Semiquantitative determinations of the free amino acid content of mouse lymph nodes and lymphosarcoma tissue were reported by Roberts and Frankel (10). The paper chromatograms prepared by these investigators indicated that the lymphosarcoma cells had much less aspartate, about the same amount of glutamate, and higher levels of alanine than the lymph nodes. As for transaminase activity, the reaction between alanine and a-ketoglutarate is apparently quite slow in lymph nodes and rat or guinea pig spleen (2). The reaction between glutamate and oxalacetate is slowest in the spleen, among eight rat tissues (2). However, Redfield and Barron (9) have reported activity for the rabbit appendix which compares favorably with that of the rabbit kidney.

**SUMMARY**

When mouse lymphosarcoma or spleen cells were incubated with lactate-2-\(^{14}C\), significant radioactivity was found in the respiratory CO\(_2\). The only amino acids strongly labeled were alanine, glutamate, and aspartate; and the protein was also significantly labeled. Negligible radioactivity was found in the fats. In the spleen cells, the glutamate and aspartate contained almost equal radioactivity, while the alanine was less radioactive. On the other hand, in the lymphosarcoma cells the alanine had twice as much radioactivity and the aspartate one-fifth as much as the glutamate.

**REFERENCES**

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