The Role of Phospholipides in the Uptake of Amino Acids by Ehrlich Ascites Carcinoma Cells*

W. L. GABY, H. L. WOLIN,† and I. ZAJAC

(Department of Microbiology, Hahnemann Medical College and Hospital, Philadelphia, Pa.)

SUMMARY

The phospholipides of Ehrlich ascites carcinoma of the mouse are capable of rapidly incorporating amino acids following in vivo or in vitro incubation of the cells with the labeled compounds. Glucose (0.005 M) stimulated the in vitro uptake of leucine and alanine by the phospholipides of the ascites cells. The amino acids appeared to be readily liberated by the phospholipides into the amino acid pool or protein constituents of the cell but could not be separated from the purified phospholipides electrophoretically. The presence of the amino acids could be demonstrated by paper chromatography following acid hydrolysis of the phospholipide complex.

Ehrlich ascites tumor cells are capable of rapidly accumulating glycine as well as other amino acids from the surrounding medium (1, 3, 8). Heinz and Walsh (9) have shown that preloading the ascites cells with glycine accelerated the uptake of glycine by these cells. The authors concluded from their experiments that glycine entered the cell by an active, metabolically linked transport mechanism and not by simple diffusion. Riggs et al. (14) suggest that potassium or various substitute cations are necessary for optimal amino acid concentration by Ehrlich ascites cells. Pal and Christensen (12) concluded from their experiments that either pyridoxal or manganese or both possibly participate in amino acid transport. Johnstone and Scholefield (11) indicated that, under the conditions of their experiments, the only mechanism involved in the efflux of glycine-1-C14 from ascites cells was passive diffusion. The inhibitory effects of antimetabolites which they observed were thought to be due to primary effects on the process of uptake. It is evident that many factors may influence the amino acid metabolism of Ehrlich ascites cells. It was found in previous studies that the phospholipide fractions of the mold Penicillium chrysogenum (5) and of rabbit liver (6) function in a manner compatible with a concept of amino acid transport. It was decided, therefore, to investigate the possible role of the phospholipides in the metabolism of Ehrlich ascites carcinoma cells.

MATERIALS AND METHODS

The Lettré hyperdiploid line of the Ehrlich ascites carcinoma of the mouse was used in the experiments to be described. Tumors were implanted in random-bred albino mice obtained from a local dealer, weighing between 20 and 25 gm., by the intraperitoneal inoculation of 0.2 ml. (approximately 4 × 10^7 cells) of a freshly harvested 7-day-old tumor. For in vivo experiments, mice bearing 6- or 7-day-old tumors were re-inoculated intraperitoneally with 0.5 ml. of an aqueous solution of the amino acid to be studied. Each mouse received 5 mg. of either D,L-leucine, D,L-glycine, or D,L-lysine (1.5 mg. of 1-C14-labeled amino acid + 3.5 mg. unlabeled carrier). The activity of the injected fluid varied from 5.0 × 10^7 to 8.4 × 10^7 counts/min/0.5 ml. At various designated time intervals, four or five of these mice were sacrificed and their ascites cells harvested and pooled in pre-cooled centrifuge tubes. The cells were washed several times with ice-cold saline, dehydrated with methanol, and the phospholipide fraction extracted, purified, and hydrolyzed by the method previously described (4). In vitro experiments were carried out on 6- or 7-day-old tumor cells, which had been aspirated

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† Present address: Department of Microbiology, Seton Hall College of Medicine and Dentistry, Jersey City, N.J.

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from mice and washed thoroughly with cold saline. In all experiments, the total wet weight, total and viable (7) cell counts were determined. From 3 to 4.4 gm. of cells (75-90 per cent viable) were suspended in 10 ml. of Krebs-Ringer phosphate buffer, pH 7.4, containing 10 mg. of either DL-leucine or DL-alanine (1 mg. of the 1-C14-labeled amino acid + 9 mg. unlabeled carrier). The total radioactivity of these suspensions was $6.8 \times 10^6$ counts/min/ml and $6.0 \times 10^7$ counts/min/ml, respectively. The in vitro effect of 0.005 M glucose on the uptake of amino acids by these cells was also investigated, since it had been shown in preliminary experiments that this concentration of glucose stimulated respiration of the tumor cells. All the in vitro experiments were carried out for 30 minutes at 37 °C. on a rotary shaking machine which described a circle of 1-inch diameter at 120 r.p.m. Following incubation, the cells were thoroughly washed with ice-cold saline until the radioactivity of the wash saline was negligible. The extraction procedure was identical with that for the cells incubated in vivo. The total volume of fluid obtained from five mice varied from 15.1 ml. containing 5.0 gm. (wet weight) of cells to 10.6 ml. containing 6.8 gm. of cells.

Duplicate paper chromatograms were run on the aqueous fraction of the phospholipide hydrolysates with n-butanol-acetic acid-water (50:12:50 v/v/v), pyridine-acetic acid-water (50:35:15 v/v/v), and phenol-water 80:20 v/v, at 30 °C. for 12-18 hours. After the sheets were dried at room temperature, the nitrogenous spots were detected by spraying one chromatogram with 0.3 per cent ninhydrin in n-butanol and heating at 80 °C for 5-10 minutes. The distribution of radioactivity in the aqueous hydrolysates was determined by cutting out the areas on the duplicate chromatogram (not exposed to ninhydrin) which corresponded to the nitrogenous spots detected on the original ninhydrin-treated chromatogram. The radioactivity was corrected for self-absorption of the paper.

Electrophoretic studies were carried out on the purifed phospholipide fraction with Whatman #3 MM filter paper strips at 17 v/cm in a barbiturate buffer, pH 8.6, as well as a phthalate buffer, pH 4.8, at 4 °C. for 2, 4, and 16 hours. Following their removal from the chamber, the strips were dried, one sprayed with ninhydrin and heated to develop the color, and the duplicate cut in squares to determine the radioactivity in a windowless flow counter.

**RESULTS**

The lipide composition of the tumor cells is shown in Table 1. These results represent typical recoveries obtained and indicate that the phospholipide fraction comprised at least 40 per cent of the total lipides of the cell. The weight of phospholipide recorded in the table represents approximately a 65 per cent recovery. The per cent of nitrogen in the phospholipides remained constant in the control as well as in the cells incubated with amino acids.

Preliminary experiments indicated that Ehrlich ascites cells incubated in vivo with DL-leucine readily incorporated the amino acid and that the phospholipide extracted from these cells contained a substantial amount of the amino acid. The results depicted in Table 2 illustrate a typical experiment in which the uptake of DL-leucine-1-C14 was followed at 30, 60, and 120 minutes after intraperitoneal injection of the amino acid into the mouse. It is evident that fairly rapid uptake of the labeled compound by the phospholipide fraction of the ascites cell was obtained. Interestingly, the specific activity of the whole cell increased from $2.10 \times 10^6$ counts/min/gm to $3.2 \times 10^6$ counts/min/gm during the 120-minute incubation period, whereas the specific activity of the extracted phospholipide fraction decreased over the same time period. Coincident to the increase in activity of the whole cells, a corresponding decrease in the radioactivity of the supernatant tumor fluid was noted.

Paper chromatograms run on the aqueous phospholipide hydrolysate resulted in the appearance
TABLE 2

**DISTRIBUTION OF C$^{14}$ IN EHRlich ASCITES TUMOR CELLS FOLLOWING INTRAPERITONEAL INJECTION OF DL-LEUCINE-1-C$^{14}$ INTO MICE BEARING 6-DAY-OLD TUMORS**

($5 \times 10^7$ counts/min total per mouse)

<table>
<thead>
<tr>
<th>Incubation time (min.)</th>
<th>Whole tumor cells before extraction</th>
<th>Supernatant fluid</th>
<th>Amino acid pool of tumor cells</th>
<th>Phospholipides of tumor cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total wet wt. (gm.)</td>
<td>Total counts/min</td>
<td>Total volume (ml.)</td>
<td>Total dry wt. (mg.)</td>
</tr>
<tr>
<td>------------------------</td>
<td>---------------------</td>
<td>-----------------</td>
<td>---------------------</td>
<td>-------------------</td>
</tr>
<tr>
<td>30</td>
<td>3.3</td>
<td>$8.2 \times 10^6$</td>
<td>5.3</td>
<td>$1.4 \times 10^7$</td>
</tr>
<tr>
<td>60</td>
<td>3.7</td>
<td>$9.5 \times 10^6$</td>
<td>7.3</td>
<td>$1.0 \times 10^7$</td>
</tr>
<tr>
<td>120</td>
<td>3.4</td>
<td>$1.1 \times 10^7$</td>
<td>6.0</td>
<td>$5.3 \times 10^6$</td>
</tr>
</tbody>
</table>

TABLE 3

**DISTRIBUTION OF LYSINE-1-C$^{14}$ AND GLYCINE-1-C$^{14}$ IN EHRlich ASCITES TUMOR CELLS FOLLOWING INTRAPERITONEAL INJECTION INTO MICE BEARING 7-DAY-OLD TUMORS**

<table>
<thead>
<tr>
<th>C$^{14}$-labeled amino acid</th>
<th>Total amount each mouse received (counts/min)</th>
<th>Whole tumor cells before extraction*</th>
<th>Supernatant fluid</th>
<th>Amino acid pool of tumor cells</th>
<th>Phospholipides of tumor cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total wet wt. (gm.)</td>
<td>Total Radioactivity (total counts/min)</td>
<td>Total volume (ml.)</td>
<td>Total Radioactivity (total counts/min)</td>
<td>Total Radioactivity (total counts/min)</td>
</tr>
<tr>
<td>Lysine</td>
<td>9.28$\times 10^7$</td>
<td>5.0</td>
<td>5.1$\times 10^7$</td>
<td>10.6</td>
<td>3.42$\times 10^7$</td>
</tr>
<tr>
<td>Glycine</td>
<td>8.34$\times 10^7$</td>
<td>4.2</td>
<td>4.0$\times 10^7$</td>
<td>8.7</td>
<td>2.5$\times 10^7$</td>
</tr>
</tbody>
</table>

* Cells harvested after 45-minute exposure to the amino acids.

**CHART 1.**—Single dimensional chromatograms: A. Lysine control. B. Glycine control. C. Water-soluble hydrolysate of phospholipides from Ehrlich ascites tumor cells following in vivo incubation for 45 minutes with lysine-1-C$^{14}$ (radioactivity of original spot was 658 counts/min). D. Water-soluble hydrolysate of phospholipides of Ehrlich ascites tumor cells following in vivo incubation for 45 minutes with glycine-1-C$^{14}$ (radioactivity of original spot was 1500 counts/min).

**Solvent:** n-Butanol:acetic acid:H$_2$O (50:14:50 v/v/v) (15)

**Chart 1.** Single dimensional chromatograms: A. Lysine control, B. Glycine control. C. Water-soluble hydrolysate of phospholipides from Ehrlich ascites tumor cells following in vivo incubation for 45 minutes with lysine-1-C$^{14}$ (radioactivity of original spot was 658 counts/min). D. Water-soluble hydrolysate of phospholipides of Ehrlich ascites tumor cells following in vivo incubation for 45 minutes with glycine-1-C$^{14}$ (radioactivity of original spot was 1500 counts/min).

**Solvent:** Pyridine:acetic acid:H$_2$O (50:35:15 v/v/v) (2)
of a spot corresponding to the $R_f$ value of a known sample of leucine. All the radioactivity was confined to the leucine spot. Leucine was not found in the hydrolysates of phospholipides extracted from control cells.

Additional groups of mice bearing 7-day-old ascites tumors were injected I.P. with either DL-lysine-$l$-$C^{14}$ or DL-glycine-$l$-$C^{14}$. The mice were sacrificed 45 minutes later and the harvested tumor cells washed thoroughly with cold saline. Following the determination of the total radioactivity of the cellular and fluid portion of the tumor, the cells were extracted first with lipide solvents and then with boiling water to extract the amino acid pool. Table 3 represents the typical results obtained from pooling the ascites cells from groups of five mice for each amino acid studied. It is evident from these results that both lysine and glycine were actively taken up by the phospholipide fraction of the tumor cells. Whereas the radioactivity of the cells was approximately equal (1 x $10^4$ counts/min/mg wet weight of cells incubated with lysine as compared with 9.5 x $10^4$ counts/min/mg cells incubated with glycine) the phospholipides extracted from the cells incubated with glycine contained more radioactivity than the phospholipides obtained from cells incubated with lysine. On the other hand, there was less radioactivity in the boiling water extract (amino acid pool) of the glycine-exposed cells than in the extract from the cells exposed to lysine. These results were confirmed by repeated experiments.

Paper chromatograms of the phospholipide hydrolysates revealed that the radioactivity was concentrated in those spots corresponding to the known injected amino acids (Chart 1).

The results of in vitro experiments comparing the influence of 0.005 m glucose on the uptake of DL-leucine-$l$-$C^{14}$ and DL-alanine-$l$-$C^{14}$ by Ehrlich ascites cells suspended in Krebs-Ringer phosphate buffer are shown in Table 4. It is evident that the phospholipides of these cells took up the labeled compounds in vitro and that the uptake was stimulated by the presence of 0.005 m glucose. It was of interest to note that, while there was increased radioactivity of the whole cells incubated with glucose, the activity of the boiling water extract of the cells did not increase.

<table>
<thead>
<tr>
<th>TABLE 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Influence of 0.005 M Glucose on Uptake of DL-Alanine-$l$-$C^{14}$ and DL-Leucine-$l$-$C^{14}$ by Ehrlich Ascites Carcinoma Cells in Vitro</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Total wet weight of cells (gm.)</th>
<th>Total amount of radioactivity added (counts/min)</th>
<th>Total radioactivity of tumor cells* (counts/min)</th>
<th>Radioactivity of amino acid pool of tumor cells (total counts/min/total mg)</th>
<th>Radioactivity of phospholipides of tumor cells (total counts/min/total mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.65</td>
<td>DL-Alanine-$l$-$C^{14}$ 6.0 x 10^7</td>
<td>1.6 x 10^6</td>
<td>3.6 x 10^7/18.75</td>
<td>8.3 x 10^7/13.5</td>
</tr>
<tr>
<td>3.55</td>
<td>DL-Alanine-$l$-$C^{14}$ 6.0 x 10^7</td>
<td>3.15 x 10^6</td>
<td>8.2 x 10^7/21.4</td>
<td>5.4 x 10^7/14.5</td>
</tr>
<tr>
<td>3.29</td>
<td>+ 0.005 M glucose DL-Leucine-$l$-$C^{14}$ 6.8 x 10^4</td>
<td>5.6 x 10^4</td>
<td>1.9 x 10^7/25</td>
<td>6.5 x 10^7/23.5</td>
</tr>
<tr>
<td>3.29</td>
<td>DL-Leucine-$l$-$C^{14}$ 6.8 x 10^4</td>
<td>8.8 x 10^4</td>
<td>1.18 x 10^7/22.5</td>
<td>1.5 x 10^7/23.5</td>
</tr>
</tbody>
</table>

* 30-minute incubation.

In these experiments, the phospholipides of the Ehrlich ascites cells were demonstrated by the in vitro incubation of thoroughly washed cells in Krebs-Ringer phosphate buffer containing glycine-$l$-$C^{14}$. The experiments were run in triplicate. One set of flasks was removed from the rotary shaker after incubation for 30 minutes at 37°C. A second set of flasks was removed after 1 hour. The cells in the third set of flasks were centrifuged after 30 minutes incubation with the $C^{14}$-labeled glycine, washed thoroughly with cold saline, resuspended in phosphate buffer containing unlabeled glycine, and reincubated for an additional 30 minutes. Immedi-
ately following incubation, the cells from each flask were washed thoroughly with ice-cold saline, and the radioactivity was determined in the amino acid pool and phospholipide fractions.

The results presented in Table 5 demonstrate that the amount of radioactivity present in the phospholipides and cellular components increased with time (from 30 minutes to 1 hour). These results also indicate that the radioactivity of the phospholipides of those cells incubated with C14-labeled glycine and subsequently reincubated with unlabeled glycine decreased, whereas the radioactivity of the amino acid pool and protein fraction of the cell continued to increase. The results of the electrophoretic studies carried out on the purified phospholipide fractions showed no evidence of migration of any nitrogenous or labeled compound. Furthermore, 0.005 M concentration of glucose stimulated the in vitro uptake of alanine and leucine by the phospholipide fraction of the cell. The amino acids are tightly bound to the phospholipides and could not be separated electrophoretically from the lipid complex. It was of interest to note that, as previously reported with the use of various other types of cells, the phospholipides extracted from Ehrlich ascites cells would not take up radioactivity when shaken in vitro with C14-labeled amino acids.

These findings are in agreement with the results of previous studies carried out on the phospholipides of the mold *Penicillium chrysogenum* and of rabbit liver cells. Additional evidence for a dynamic, labile lipide-amino acid complex functioning as an intermediate stage in the incorpora-

### TABLE 5

**The Uptake and Release of Radioactivity by Phospholipides Following in Vitro Incubation of Ehrlich Ascites Cells with DL-Glycine-1-C14**

<table>
<thead>
<tr>
<th>Experimental Conditions*</th>
<th>Total dry wt. of cells (mg)</th>
<th>Total radioactivity (counts/min X 10^5)</th>
<th>Total amino acid pool (counts/min X 10^5)</th>
<th>Total protein cell fraction (counts/min X 10^5)</th>
<th>Total phospholipide fraction (counts/min X 10^5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>471.3</td>
<td>5.1</td>
<td>65.8</td>
<td>0.012</td>
<td>424.2</td>
</tr>
<tr>
<td>B</td>
<td>496.0</td>
<td>5.1</td>
<td>84.0</td>
<td>0.53</td>
<td>391.3</td>
</tr>
<tr>
<td>C</td>
<td>371.9</td>
<td>5.1</td>
<td>50.4</td>
<td>0.26</td>
<td>316.0</td>
</tr>
</tbody>
</table>


† Cellular residue following extraction of phospholipide and amino acid pool.

C14-labeled compound from the origin as detected by 0.3 per cent ninhydrin and radioactivity determination in a windowless flow counter.

**DISCUSSION**

It is evident from these results that the phospholipide fraction of the Ehrlich ascites mouse tumor cells is capable of taking up amino acids following *in vitro* or *in vivo* incubation of the metabolizing cells with the respective amino acid. Reproducibility of the results was confirmed by repeated experiments. The data presented in the first table represent typical variations observed between individual experiments. Glycine was taken up by the phospholipides more rapidly than was lysine, indicating that the lipid complex does not incorporate all amino acids at the same rate. It is also evident that the radioactive amino acids are being readily liberated by the phospholipides into an intracellular pool regardless of a constantly increasing cellular concentration of the 

**REFERENCES**

The Role of Phospholipides in the Uptake of Amino Acids by Ehrlich Ascites Carcinoma Cells

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