Metabolism of Phospholipids in Ehrlich Ascites Tumor

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SUMMARY

When Ehrlich ascites cells were incubated in a suitable medium containing one of a number of ¹⁴C-labeled phospholipid precursors, radioactivity was recovered from the lipid fraction. For the same concentration and specific radioactivity of precursor, the incorporation was in the following order: choline-¹⁴C > ethanolamine-¹⁴C > L-serine-¹⁴C > glycerol-¹⁴C > formate-¹⁴C > acetate-¹⁴C.

Radioactivity from choline-¹⁴C was incorporated into the 3 choline-containing phospholipids—lecithin, choline plasmalogen, and sphingomyelin. Radioactivity from ethanolamine-¹⁴C was incorporated into phosphatidyl ethanolamine, ethanolamine plasmalogen, choline plasmalogen, and lecithin. Radioactivity from L-serine-¹⁴C was incorporated into phosphatidyl serine, serine plasmalogen, and sphingomyelin, and also into phosphatidic acid and other glycerophosphatides. Radioactivity from glycerol-¹⁴C was incorporated into phosphatidic acid and the glycerophosphatides, and also into sphingomyelin. Radioactivity from formate-¹⁴C was incorporated into phosphatidyl serine, serine plasmalogen and sphingomyelin, with lesser amounts into other phosphatides. With acetate-¹⁴C, radioactivity was poorly incorporated into the phosphatides of this tissue.

These results are discussed in relation to current knowledge concerning the biosynthesis of phosphatides. Evidence is given to support the following generalizations concerning the metabolism of phospholipids in the Ehrlich ascites tumor: (a) The cytosine nucleotide pathway for the biosynthesis of glycerophosphatides is operative. (b) Lipid ethanolamine is methylated to form lipid choline. (c) Phosphatidyl serine is not decarboxylated to yield phosphatidyl ethanolamine.

There are a number of indications that the invasiveness of cancer cells may be related to the characteristics of the cell surface membranes (1, 7, 43). For example, Coman and his associates have drawn attention to the importance of such properties of the cell surface as adhesiveness (6, 31) and stickiness (8). Since it is now known that lipids, and in particular phospholipids, are essential components of all cell membranes, including both the plasma membrane and the highly specialized membranes of the mitochondria and the endoplasmic reticulum, it seemed of interest to study the metabolism of lipids in cancer cells.

Early work on the role of lipids in cancer tissue formed the subject of an extensive review by Haven and Bloor (21) in 1956. Wallach et al. (42) reported on the distribution of phosphatides in the Ehrlich ascites tumor, and Gray (20) published similar data for Landschutz ascites carcinoma cells and BP8/C3H ascites sarcoma cells.

The lipid metabolism of Ehrlich ascites cells was studied by Lee et al. (27), Figard and Greenberg (15), and Marini et al. (30). More recently, David and Rossiter (10) described the incorporation of inorganic ³²P into the individual phosphatides of Ehrlich ascites tumor in vitro. This work has now been extended to include the study of a series of ¹⁴C-labeled precursors of phospholipids.

A preliminary report of some of these experiments has appeared (38).

MATERIALS AND METHODS

The Ehrlich ascites tumor was maintained in female albino mice of the Swiss-Webster strain. Carcinoma cells (8–10 days after inoculation) were centrifuged at 700 × g for 5 min and washed twice by gentle stirring with 10 volumes of Krebs-Ringer-Tris(hydroxymethyl)amino-methane buffer containing 122 mM NaCl, 4.88 mM KCl, 1.22 mM KH₂PO₄, and 1.22 mM MgSO₄ buffered at pH 7.4 with 50 mM 2-amino-2-hydroxymethylpropane-1,3-diol. The washed cells were resuspended in 6 volumes of Krebs-Ringer-Tris buffer.

Incubation.—To each of a series of 10-ml beakers was added 1.8 ml of buffer containing glucose (6.75 mg/ml) and heparin (0.112 mg/ml), 1 ml of cell suspension, and 0.2 ml of solution containing 3 μC (5μmoles) of one of a number of precursors. The beakers and contents were incubated at 37°C under an atmosphere of oxygen for 135
Radioactivity was determined in a Packard Tn-Carb perchioric acid. Another portion of the extract was added by the method of Ernster et al. (14), after digestion with liquid scintillation spectrometer (Packard Instrument Company, LaGrange, Illinois) with a 10- to 75-volt window and high voltage tap 3. The material from several beakers was pooled and divided to give 2 duplicate samples, from which the phospholipids were extracted with chloroform–methanol (2:1, v/v) and washed with ether. The washed lipid extract was reduced in volume under nitrogen, and a suitable portion was applied to filter paper impregnated with silicic acid for the chromatographic separation of individual phosphatides by the method of Marinetti (29). The labeled phospholipids were deacylated and the water-soluble products were separated by the 2-dimensional chromatographic method of Dawson et al. (11, 12). The method was modified in 2 respects: (a) the alkali-stable lysoplasmalogen fraction was hydrolyzed in 0.5 N HCl in 90% acetic acid containing HgCl₂ in order to minimize the formation of cyclic acetal compounds (9, 35); and (b) the water-soluble hydrolysis products were separated from the lipids by the biphasic system of Foleh et al. (17), as suggested by Magee and Manz (28). The labeled hydrolysis products were located on the chromatogram by staining with ninhydrin in ethanol containing 1% lutidine–collidine, 3:1, or by radioautography, and they were identified by their staining characteristics and by their Rf values relative to those of authentic markers.

Determination of specific radioactivity.—A portion of the purified lipid extract was taken for phosphorus estimation by the method of Ernster et al. (14), after digestion with perchloric acid. Another portion of the extract was added to counting vials containing 10 ml of solvent-scintillator composed of 4 gm of 2,5-diphenyloxazole and 100 mg of 1,4-bis-2-(5-phenoxazolyl)benzene per liter of toluene. Radioactivity was determined in a Packard Tri-Carb liquid scintillation spectrometer (Packard Instrument Company, LaGrange, Illinois) with a 10- to 75-volt window and high voltage tap 3.

The water-soluble hydrolysis products were eluted from the chromatograms with distilled water. A portion of the eluate was used for phosphorus determination, and another portion was taken to dryness, dissolved in 1.0 ml of hydroxide of Hyamine [1 M p-(disobutylresesoxyethoxyethyl)-dimethyl benzyl ammonium hydroxide] in dry methanol, and added to 10 ml of solvent-scintillator. Radioactivity was determined with a 10- to 75-volt window and high voltage tap 4. Quenching due to the presence of Hyamine was corrected for by the use of internal standards. The results are expressed in terms of specific radioactivity (cpm/μmole). Materials. — Glycerolphosphorylethanolamine, glycerolphosphorylserine, and glycerolphosphorylinsitol, used as markers, were prepared by deacylation of the appropriate phosphatide (11). Choline-1,2-¹⁴C, formate-¹⁴C, and acetate-¹⁴C were obtained from Tracerlab, Waltham, Massachusetts. Ethanolamine-1,2-¹⁴C was obtained from Volk Radiochemical Company, Skokie, Illinois. Glycerol-1-¹⁴C and L-serine-¹⁴C were obtained from The Radiochemical Centre, Amersham, Bucks, England.

**RESULTS**

Radioactivity from each of the precursors was incorporated into the lipids of Ehrlich ascites tumor (Table 1). At the same concentration (5 μmoles/3 ml) and same specific radioactivity (0.6 μc/μmole), choline-1,2-¹⁴C and ethanolamine-1,2-¹⁴C were the best precursors of lipids. Radioactivity from L-serine-¹⁴C, glycerol-1-¹⁴C, and formate-¹⁴C was less well incorporated, whereas radioactivity from acetate-¹⁴C was poorly incorporated.

For choline-1,2-¹⁴C, ethanolamine-1,2-¹⁴C, L-serine-¹⁴C, and glycerol-1-¹⁴C, qualitative data are available for lipids separated on silicic acid-impregnated paper by the method of Marinetti (29). The degree of incorporation into the 6 lipid fractions recognized by David and Rossiter (10), together with comparable data for inorganic ³²P, is recorded in Table 2. In general, choline-1,2-¹⁴C and ethanolamine-1,2-¹⁴C were incorporated into the choline-containing and ethanolamine-containing lipids, respectively, whereas the radioactivity from L-serine-¹⁴C and glycerol-1-¹⁴C was found to be more widely distributed throughout the lipid fractions.

Several of the areas separated on the silicic acid-impregnated paper contained more than one lipid component.

### Table 1

<table>
<thead>
<tr>
<th>Precursor</th>
<th>Specific radioactivity (cpm/μmole)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Experiment 1</td>
</tr>
<tr>
<td>Choline-1,2-¹⁴C</td>
<td>9,700</td>
</tr>
<tr>
<td>Ethanolamine-1,2-¹⁴C</td>
<td>6,700</td>
</tr>
<tr>
<td>L-Serine-¹⁴C</td>
<td>3,600</td>
</tr>
<tr>
<td>Glycerol-1-¹⁴C</td>
<td>1,200</td>
</tr>
<tr>
<td>Formate-¹⁴C</td>
<td>1,100</td>
</tr>
<tr>
<td>Acetate-¹⁴C</td>
<td>300</td>
</tr>
</tbody>
</table>

### Table 2

<table>
<thead>
<tr>
<th>Lipid</th>
<th>Inorganic ³²P (μc/mole)</th>
<th>Choline-1,2-¹⁴C</th>
<th>Ethanolamine-1,2-¹⁴C</th>
<th>L-Serine-¹⁴C</th>
<th>Glycerol-1-¹⁴C</th>
<th>Principal components</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (+ + +)</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+ +</td>
<td>±</td>
<td>Phosphatidic acid</td>
</tr>
<tr>
<td>B (+ + +)</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>− ±</td>
<td>−</td>
<td>Phosphatidyl ethanolamine, ethanolamine plasmalogen, serine-containing lipids</td>
</tr>
<tr>
<td>C (+)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>− ±</td>
<td>−</td>
<td>Lecithin choline plasmalogen</td>
</tr>
<tr>
<td>D (+ + +)</td>
<td>±</td>
<td>±</td>
<td>±</td>
<td>−</td>
<td>± +</td>
<td>Lysophosphatidyl ethanolamine sphingomyelin</td>
</tr>
<tr>
<td>E (+)</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>− ±</td>
<td>Phosphatidyl ethanolamine sphingomyelin</td>
</tr>
<tr>
<td>F (+ +)</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>±</td>
<td>Phosphatidyl inositol</td>
</tr>
</tbody>
</table>

The lipids were separated by the method of Marinetti (33).
The phosphatides were separated by the method of Dawson et al. (11, 12).

With choline-1,2-14C as the source of radioactivity, the lecithin fraction had the greatest incorporation; it was followed by choline plasmalogen and sphingomyelin (Table 3). No radioactivity was detected in phospholipids other than those containing choline. With ethanolamine-1,2-14C, however, radioactivity was incorporated not only into phosphatidyl ethanolamine and ethanolamine plasmalogen, but also into choline plasmalogen and lecithin (Table 3).

With ethanolamine-1,2-14C, considerable radioactivity was detected in the phospholipid fraction that was stable to both alkaline and acid hydrolysis. The radioactivity was present in a compound with the properties of the cyclic acetal of glycerolphosphorylethanolamine described by Davenport and Dawson (9). This compound, which occurred in very low concentration, had a specific radioactivity similar to that of ethanolamine plasmalogen. It is assumed that a small amount of the cyclic acetal was formed from ethanolamine lysoplasmalogen during the hydrolysis procedure despite the precautions taken (9, 34). No evidence was obtained for the presence of a similar choline-containing lipid in the Ehrlich ascites tumor. With choline-1,2-14C, radioactivity was incorporated into the phospholipid fraction that was stable to alkaline and acid hydrolysis, but as described above, the radioactivity was confined to sphingomyelin.

Radioactivity from L-serine-14C was incorporated into most of the phospholipids studied (Table 4). As was to be expected, the highest radioactivity was observed for phosphatidyl serine. Lesser amounts of radioactivity were recovered from serine plasmalogen, phosphatidic acid, and sphingomyelin. There was a small but definite incorporation of radioactivity into both the choline-containing and the ethanolamine-containing glycerophospholipids. Glycerol-1-14C was found to contribute radioactivity to most of the glycerol-containing phosphatides; phosphatidic acid had the highest specific radioactivity and was followed by lecithin and choline phosphatides (Table 4). Next in specific radioactivity were the serine-containing phosphatides, phosphatidyl serine and serine plasmalogen, which were more radioactive than phosphatidyl ethanolamine. No radioactivity was detected, however, in ethanolamine plasmalogen, but measurable amounts were recovered from sphingomyelin.

With formate-14C, highest radioactivity was recovered from phosphatidyl serine and serine plasmalogen (Table 4). Some radioactivity also was recovered from other glycerophosphatides, but the next highest specific radioactivity was in sphingomyelin.

### DISCUSSION

The experiments reported in Table 1 indicate that choline, ethanolamine, serine, and glycerol are all good precursors of the phospholipids of the Ehrlich ascites tumor in vitro. The finding that formate-14C is also a precursor of phospholipids in this tumor confirms the report of Marinetti and Kay (30). The poor incorporation observed for acetate-1-14C agrees with the report of Gore and Popjak (19) that Ehrlich ascites tumor cells and rat ascites tumor (RD3) cannot readily form fatty acid from acetate-14C. In fact, tumors in general are unable to use acetate-14C for

### TABLE 3

<table>
<thead>
<tr>
<th>Phosphatides</th>
<th>Specific Radioactivity (cpm/µmole)</th>
<th>Choline-1,2-14C</th>
<th>Ethanolamine-1,2-14C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lecithin</td>
<td>Experiment 1: 66,200, Experiment 2: 22,900</td>
<td>Experiment 1: 2,300, Experiment 2: 1,700</td>
<td></td>
</tr>
<tr>
<td>Phosphatidyl ethanolamine</td>
<td>Experiment 1: 10,700, Experiment 2: 6,000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphatidyl serine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphatidyl inositol</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphatidic acid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Choline plasmalogen</td>
<td>Experiment 1: 13,400, Experiment 2: 8,500</td>
<td>Experiment 1: 3,300, Experiment 2: 3,400</td>
<td></td>
</tr>
<tr>
<td>Ethanolamine plasmalogen</td>
<td>Experiment 1: 6,900, Experiment 2: 4,200</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sphingomyelin</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### TABLE 4

<table>
<thead>
<tr>
<th>Phosphatides</th>
<th>Specific Radioactivity (cpm/µmole)</th>
<th>L-serine-14C</th>
<th>Glycerol-1-14C</th>
<th>Formate-14C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lecithin</td>
<td>Experiment 1: 1,700, Experiment 2: 900</td>
<td>Experiment 1: 5,900, Experiment 2: 5,000</td>
<td>Experiment 1: 1,300, Experiment 2: 1,700</td>
<td></td>
</tr>
<tr>
<td>Phosphatidyl ethanolamine</td>
<td></td>
<td>Experiment 1: 1,400, Experiment 2: 700</td>
<td>700</td>
<td>750</td>
</tr>
<tr>
<td>Phosphatidyl serine</td>
<td></td>
<td>1,400</td>
<td>1,400</td>
<td>9,200</td>
</tr>
<tr>
<td>Phosphatidyl inositol</td>
<td></td>
<td>700</td>
<td>700</td>
<td></td>
</tr>
<tr>
<td>Phosphatidic acid</td>
<td></td>
<td>4,400</td>
<td>7,600</td>
<td>5,900</td>
</tr>
<tr>
<td>Choline plasmalogen</td>
<td></td>
<td>1,900</td>
<td>710</td>
<td>960</td>
</tr>
<tr>
<td>Ethanolamine plasmalogen</td>
<td></td>
<td>2,400</td>
<td>430</td>
<td>500</td>
</tr>
<tr>
<td>Serine plasmalogen</td>
<td></td>
<td>1,700</td>
<td>5,800</td>
<td>6,900</td>
</tr>
<tr>
<td>Sphingomyelin</td>
<td></td>
<td>900</td>
<td>1,100</td>
<td>900</td>
</tr>
</tbody>
</table>

(Table 2). Wallach et al. (42), Figard and Greenberg (15), and Lee et al. (27) reported peaks with multiple components when attempts were made to separate the lipids of Ehrlich ascites tumor in silic acid columns. Because of the poor resolution on the silic acid paper and the failure of the method to separate plasmanogrames of phosphatides, the lipids were deacylated and separated by the procedure of Dawson (11, 12).
the synthesis of fatty acid (34), although long-chain fatty acids, such as palmitate-1-14C, are readily incorporated into the lipids of most tumors (33), including Ehrlich ascites cells (16).

The finding that the radioactivity of choline-1,2-14C may be recovered from the 3 choline-containing lipids but from no other phospholipid (Table 3) suggests that, once the choline-containing lipids are formed in the Ehrlich ascites tumor, they are metabolically quite stable and are not converted into other lipids. It seems likely that the choline-containing lipids are formed by way of metabolic pathways already known for other tissues. It is currently believed that in the biosynthesis of these lipids choline is first converted to cytidine diphosphate choline (2), which may transfer phosphorylcholine to diglyceride to form lecithin (25, 41), to plasmalogenic diglyceride to form choline plasmalogen (26, 32) or to ceramide to form sphingomyelin (32, 40). Unpublished experiments with a microsomal preparation of Ehrlich ascites tumor and cytidine diphosphocholine-1-14C indicate that the above pathways are operative in this tissue.

The findings for ethanolamine-1,2-14C (Table 3) are for the most part as anticipated. In other tissues ethanolamine is known to be converted to cytidine diphosphate ethanolamine (2), which may transfer phosphorylethanolamine to diglyceride to form phosphatidyl ethanolamine (25, 32) or to plasmalogenic diglyceride to form ethanolamine plasmalogen (26, 32). Again unpublished experiments with a microsomal preparation and cytidine diphosphate ethanolamine-1-14C indicate that these pathways are operative in the Ehrlich ascites tumor. The finding that radioactivity from ethanolamine-1,2-14C was incorporated into both lecithin and choline plasmalogen strongly suggests that the methylation pathway proposed by Bremer and Greenberg (5), whereby lipid ethanolamine is methylated by S-adenosyl methionine to form lipid choline, also is operative in the Ehrlich ascites tumor. The experiments of Figard and Greenberg (15) indicate that the enzyme system responsible for the methylation is considerably less active in the tumor tissue than in the mouse liver. The finding that choline plasmalogen had a higher specific radioactivity than lecithin, an observation that contrasts with the results when choline-1,2-14C was the precursor, suggests the possibility that, for this tumor, lecithin may be formed more readily than choline plasmalogen by the cytosine nucleotide pathway, but that choline plasmalogen is formed more readily than lecithin by the methylation of lipid ethanolamine. This topic is being pursued further.

As mentioned previously, some radioactivity from ethanolamine-1,2-14C was incorporated into a compound with the properties of the cyclic acetol of glycerolphosphorylethanolamine described by Davenport and Dawson (9). The compound yields a water-soluble ninhydrin-positive product on hydrolysis with 2 N hydrochloric acid for 4 hr at 105°C. Some of this water-soluble product, probable ethanolamine hydrochloride, also was formed during the hydrolysis of the vinyl ether bond of the plasmalogen. Since on chromatography with phenol this compound has an Rp not greatly different from that given by glycerolphosphorylserine, it was erroneously reported as being derived from serine plasmalogen (38).

Radioactivity from L-serine-14C was incorporated into most phosphatides. The direct incorporation of L-serine-14C into phosphatidyl serine has been reported for other tissues (3, 22), and a similar incorporation of L-serine-14C into serine plasmalogen is not surprising. The incorporation of radioactivity from L-serine-14C into sphingosine, and hence into sphingomyelin, is a well-known reaction sequence (see Brady et al. (4) for reference). On the other hand, the incorporation of radioactivity from L-serine into phosphatidic acid was unexpected and suggests the conversion of L-serine into glycerol, which is then incorporated into phosphatidic acid. The further incorporation of radioactivity into the choline-containing and ethanolamine-containing phospholipids would thus be readily explained.

To confirm this suggestion, the labeled phosphodiester derivatives were obtained by the deacylation of lecithin, phosphatidyl ethanolamine, and phosphatidyl serine were eluted from the chromatograms and hydrolyzed with 6 N hydrochloric acid for 24 hr at 110°C in a sealed tube. On rechromatography only glycerolphosphorylserine, derived from phosphatidyl serine, was found to contain radioactivity in the base moiety. The phosphodiester derivatives derived from lecithin and phosphatidyl ethanolamine contained radioactivity that was confined to the glycerol moiety. These experiments thus demonstrate that in the Ehrlich ascites tumor L-serine is converted into the glycerol moiety of phosphatidic acid and hence into the other glycerophosphatides. The conversion of L-serine to intermediates of carbohydrate metabolism in other tissues is well known (13).

The experiments also provide no evidence that the Ehrlich ascites tumor contains enzymes that catalyze the decarboxylation of phosphatidyl serine to yield phosphatidyl ethanolamine, a reaction that has been reported for other tissues (3, 24). If such enzymes were present, the ethanolamine moiety of phosphatidyl ethanolamine would have been radioactive. The absence of decarboxylation of phosphatidyl serine to yield phosphatidyl ethanolamine in Ehrlich ascites tumor was reported by Marinetti and Kay (30).

Radioactivity from glycerol-1-14C was incorporated into most of the glycerol-containing phosphatides, with the highest specific radioactivity in phosphatidic acid, followed by lecithin and choline plasmalogen. These findings are consistent with current views concerning the biosynthesis of these phosphatides from n-o, β-diglyceride by the cytosine nucleotide pathway (25, 26, 32, 41). The much lower incorporation of radioactivity from glycerol-1-14C into phosphatidyl ethanolamine is presumably also by way of the cytosine nucleotide pathway (25, 32). The finding of radioactivity from glycerol-1-14C in phosphatidyl serine confirms the observations of others for different tissues (18, 37), but it leaves unexplained the metabolic pathway whereby phosphatidyl serine is formed. If the phosphatidyl serine becomes labeled from radioactive serine by the exchange reaction postulated by Borkenhagen et al. (3) and Hübscher (22) and referred to above, glycerol-1-14C first would have to be converted to L-serine, a transformation known to occur in some tissues (23, 36, 44). Further evidence that this transformation occurs in the Ehrlich ascites tumor is the finding that radioactivity from glycerol-1-14C was recovered from sphingomyelin, a lipid known to be formed from serine (Table 4).
The finding that radioactivity from formate-¹⁴C is incorporated into phosphatidyl serine confirms the report of Marinetti and Kay (30). As is well known, formate may serve as a precursor for L-serine (39), which may then be incorporated into phosphatidyl serine (3, 22). Conversion of formate-¹⁴C to serine also explains the recovery of radioactivity from sphingomyelin. As pointed out above, L-serine may serve as a precursor of sphingosine in other tissues (4). The incorporation of radioactivity from L-serine-¹⁴C into other glycerophosphatides has been referred to above and explains the recovery of radioactivity from formate-¹⁴C in the choline-containing and ethanolamine-containing glycerophosphatides.

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