Phosphatides in Mouse Epidermis Undergoing Normal and Abnormal Growth Changes*

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SUMMARY

Silicic acid chromatography was employed to determine the distribution of the phosphatides in mouse epidermis undergoing normal growth changes induced by the hair growth cycle. These included epidermis removed 4, 12, and 22 days after plucking of the hair in both sexes of mice. Furthermore, the distribution of phosphatides was determined in mouse epidermis made hyperplastic by three, eighteen, and 24 applications of methylcholanthrene on alternate days for 1, 6, and 8 weeks, respectively, and in carcinogen-induced squamous-cell carcinomas. There were no significant differences in the per cent distribution of the various classes of phosphatides eluted from silicic acid between epidermis undergoing normal and that with cancerous growth changes, in spite of the fact that the per cent of total phosphatides in the various examined carcinomas and epidermis varied considerably. Chromatography of the various fractions on silicic acid-impregnated paper indicated the presence of the following phosphatides in normal and treated epidermis in about the same proportions, cardiolipin-like, phosphatidyl serine, phosphatidyl ethanolamine, inositol phosphatide, lecithin, and sphingomyelin. Other phosphatides were present in all the peaks.

Previous studies on the lipide composition of mouse epidermis undergoing normal growth changes induced by the hair growth cycle and of this tissue undergoing malignant transformation produced by the topical application of methylcholanthrene have indicated that these growth changes are associated with alterations in some of the lipide constituents (3-5). In their review Haven and Bloor refer to many studies which indicate that phosphatides might be involved in the malignant transformation of cells (8), and more recent studies confirm this opinion. For example, Veerkamp et al. reported that azo dye-induced hepatomas of rats and normal rat liver differ in the content but not in the types of phosphatides present (14). Differences in the fatty acid composition of the phosphatides of normal rat liver and hepatoma were found. Also, Kögli et al. reported that certain animal tumors had a low phosphatide content but that these compounds incorporated radioactive phosphorus at a high rate (10). Again, Figard et al. found that transplantable mouse tumors had a low phosphatide content, but the fatty acid composition of the phosphatides and the proportion of different phosphatides were similar to those found in liver and red blood cells (6). In this study the distribution of phosphatides in normal epidermis, early and late hyperplastic epidermis, and squamous-cell carcinomas is reported.

MATERIALS AND METHODS

Procedures for the preparation of early and late hyperplastic epidermis, squamous-cell carcinomas, and epidermis following the plucking of hair of mice have been given (2, 4). Epidermis from 100 to 200 mice, depending upon the phosphorus content, and twenty to 30 carcinomas were pooled for each analysis. Epidermis was separated from the dermis by the procedure of Baumberger et al. (1). The total lipide of the various types of epidermis and the carcinomas was extracted by the method of Hanahan (7), as previously described (3). The total lipide fractions obtained were dried over N₂ and stored at —25°C. Chromatography of the total lipide was carried out in a mixture of 15 gm. of Mallinckrodt’s silicic acid and 7.5 gm. of SuperCel No. 545 (7). The neutral lipides were eluted with chloroform, after which the phosphatides were eluted with chloroform-methanol mixtures of increasing methanol concentration. Nitrogen was kept over the solvents and was used to increase the flow rate. The phosphatide fractions were collected automatically in volumes of 6.6 ml.; and, after removal of a portion for phosphorus determination until the elution patterns were standardized, the remainder or all for each peak was combined and the solvents were removed in vacuo under N₂ at 40°–50°C. Drying was completed under N₂.
and after being weighed the fractions were stored at \(-25^\circ C\). Under \(N_2\) for an average period of 6 months before use. The neutral lipide fraction was chromatographed on Bio Rad silicic acid by the procedure of Hirsch et al. (9). The fractions containing the sterol and wax esters, triglycerides, cholesterol, and mono- and diglycerides were collected in bulk, the solvents removed in vacuo under \(N_2\) at 40°-50° C, and the fractions stored under \(N_2\) at \(-25^\circ C\). Storage was from 1 to several months prior to interesterification and fatty acid analysis of the sterol-wax ester and ether eluate fractions.

Examination of the phospha tid fractions for the nature and purity of components present was carried out on silicic acid-impregnated paper according to the details given by Marinetti (11). The filter paper (Whatman's No. 1) was impregnated with silicic acid according to Marinetti's procedure. Ascending chromatography of the various peak materials was carried out overnight in jars (6 inches inside diameter and 18 inches high) in 200 ml. of the solvent mixture, v/v: diisobutylketone, 40; acetic acid, 25; and water, 5. The temperature of the chromatography room was kept close to 23° C. The paper strips were then dried for 1 hour in a small hood and then subjected to the following tests (11): Rhodamine G paper strips were then dried for 1 hour in a small hood and then subjected to the following tests (11): Rhodamine G strips of the same peak, and from the RF values of the Rhodamine G spots and the various stained spots it was possible to identify many of the phosphatides.

To facilitate identification of the phosphatides in the various peaks, purified phosphatides were chromatographed and examined under the same conditions as the epidermal phosphatides. For this purpose the following phosphatides were employed: phosphatidic acid,\(^1\) L-\(\alpha\)-(dimyristoyl) lecithin, and L-\(\alpha\)-(dimyristoyl) cephalin,\(^2\) lysolecithin,\(^3\) phosphatidyl-L-serine,\(^4\) L-\(\alpha\)-lecithin dipalmitoyl,\(^4\) and L-\(\alpha\)-cephalin dipalmitoyl,\(^4\) sphingomyelin,\(^4\) and inositol phospha tide.\(^4\) Some of these standard phosphatides showed the presence of other phosphatides in varying amounts by paper chromatography.

**RESULTS**

In our previous studies the phosphatides were separated from the neutral lipides with acetone at \(-25^\circ C\). Instead of by chromatography, as was done in this report. Since some differences were found in the amounts of the neutral lipide fractions and since the recovery of these materials from the silicic acid columns averaged 93 instead of 85 percent in the work previously reported (4), the new data are given in Table 1. In general, some differences occurred in the levels of the sterol-wax esters and triglycerides and some decrease in cholesterol content and in the occurrence of measurable amounts of ether eluate. Variations in the percentage of phospholipides between post-plucking epidermis of male and female mice found currently (Table 1) and those reported earlier (4) may be due to differences in the source of mice, the diet, season of the year, or procedures. Briefly the data in Table 1 indicate that the sterol wax-ester fraction decreases in the male and female in order from 4, 12, and 22 days after plucking of hair, and that the amount of this fraction is higher in male than in female epidermis at any corresponding time of the hair cycle. The reverse situation holds for the triglycerides. The increase in the level

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\(^4\) Mann Research Laboratories, Inc., New York, N. Y.

\(^5\) General Biochemicals, Chagrin Falls, Ohio.

\(^6\) Nutritional Biochemical Corporation, Cleveland, Ohio.

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**TABLE 1**

**Lipide Composition of Epidermis in Various Stages of Growth**

<table>
<thead>
<tr>
<th>State of Epidermis</th>
<th>Sterol and Wax-esters(^*) (per cent)</th>
<th>Triglycerides(^*) (per cent)</th>
<th>Cholesterol(^*) (per cent)</th>
<th>Ether Eluates(^*) (per cent)</th>
<th>Phospholipids(^†) (per cent)</th>
<th>Neutral Lipides(^†) (per cent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>22 days PP(^‡) sq (4)</td>
<td>12.2 ± 1.4</td>
<td>82.2 ± 3.5</td>
<td>2.7 ± 1.2</td>
<td>3.5 ± 1.3</td>
<td>6.2 ± 4.0 (4)</td>
<td>83.9 ± 3.5 (4)</td>
</tr>
<tr>
<td>12 days PP(^‡) sq (3)</td>
<td>16.8 ± 1.3</td>
<td>72.5 ± 0.8</td>
<td>2.8 ± 0.03</td>
<td>8.0 ± 2.0</td>
<td>9.7 (2)</td>
<td>91.7 ± 1.9 (3)</td>
</tr>
<tr>
<td>4 days PP(^‡) sq (4)</td>
<td>20.2 ± 3.6</td>
<td>67.4 ± 2.0</td>
<td>3.6 ± 1.0</td>
<td>8.8 ± 2.0</td>
<td>12.8 ± 2.6 (4)</td>
<td>87.2 ± 2.7 (4)</td>
</tr>
<tr>
<td>22 days PMC(^§) (4)</td>
<td>28.4 ± 1.1</td>
<td>67.6 ± 1.6</td>
<td>3.2 ± 0.7</td>
<td>6.3 ± 2.0</td>
<td>8.9 (2)</td>
<td>91.1 (2)</td>
</tr>
<tr>
<td>12 days PMC(^§) (4)</td>
<td>26.2 ± 0.9</td>
<td>61.2 ± 0.8</td>
<td>4.9 ± 0.5</td>
<td>5.9 ± 0.8</td>
<td>10.3 (2)</td>
<td>83.2 (2)</td>
</tr>
<tr>
<td>4 days PMC(^§) (4)</td>
<td>34.2 ± 3.8</td>
<td>48.4 ± 7.7</td>
<td>6.8 ± 1.5</td>
<td>10.7 ± 1.2</td>
<td>17.8 ± 11.2 (4)</td>
<td>81.9 ± 8.5 (4)</td>
</tr>
<tr>
<td>3 PMC(^§) (4)</td>
<td>5.7 ± 0.2</td>
<td>80.6 ± 4.7</td>
<td>6.5 ± 1.6</td>
<td>6.9 ± 2.8</td>
<td>19.3 ± 3.6 (3)</td>
<td>80.7 ± 3.6 (3)</td>
</tr>
<tr>
<td>18 PMC(^§) (4)</td>
<td>6.1 ± 0.8</td>
<td>79.0 ± 5.7</td>
<td>6.5 ± 1.6</td>
<td>7.3 ± 3.3</td>
<td>17.6 (1)</td>
<td>82.4 (1)</td>
</tr>
<tr>
<td>24 PMC(^§) (3)</td>
<td>11.1 ± 1.1</td>
<td>71.3 ± 4.6</td>
<td>7.7 ± 2.2</td>
<td>9.9 ± 2.0</td>
<td>24.7 ± 6.6 (5)</td>
<td>76.4 ± 7.2 (5)</td>
</tr>
<tr>
<td>Sq. ca. sq (3)</td>
<td>12.3 ± 3.2</td>
<td>57.4 ± 4.1</td>
<td>18.6 ± 4.8</td>
<td>11.7 ± 3.5</td>
<td>44.1 ± 3.8 (3)</td>
<td>55.9 ± 3.8 (3)</td>
</tr>
</tbody>
</table>

\(^*\) Expressed as % of neutral lipide fraction.

\(^†\) Percentage of total lipide following chromatography on silicic acid.

\(^‡\) PP = post-plucking of hair.

\(^§\) PMC = applications of methylcholanthrene.

\(^\#\) Sq. ca. = squamous-cell carcinoma. Number of separate analyses indicated in parentheses.
of sterol wax-ester fraction appears to be associated with the increased activity of the sebaceous glands during the initial burst of epidermal and hair follicle growth. Early and late hyperplastic epidermis (eighteen applications of methylcholanthrene, which are largely devoid of sebaceous glands, have a rather low content of sterol wax-esters, the amount of which increases in the latest hyperplastic epidermis (24 applications of methylcholanthrene) and in the carcinomas.

An elution pattern of the phosphatides from silicic acid of 4 day post-plucking female epidermis is given in Chart 1. Peaks 1, 2, and 3 are rather sharp and represent 2.5, 15.5, and 15 per cent of the phosphatides, respectively, whereas peak 4 is quite broad and has 46 per cent of the phosphatides, and peak 5 is less broad and contains 20 per cent of these components. Peak 4 could not be further resolved by further changes in the concentration of the chloroform-methanol mixtures than indicated in Chart 1 or by gradient elution. The percentage composition of the phosphatides of 4-, 12-, and 22-day male and female post-plucking epidermis, early and late hyperplastic epidermis, and the carcinomas is shown in Table 2. No appreciable differences appear to exist.

An examination of the phosphatides from each peak of ten samples (post-plucking epidermis, early and late hyperplastic epidermis, and squamous-cell carcinomas—Table 2) indicated that each fraction was a mixture of phosphatides. Approximate amounts and the types of phosphatides found in the various epidermal fractions are summarized as follows: Fraction 1—90 per cent cardiolipin-like and 10 per cent unidentified; fraction 2—80 per cent phosphatidyl ethanolamine and 20 per cent lyso-phosphatides found in the various fractions are sum-

TABLE 2
DISTRIBUTION OF LIPIDE PHOSPHORUS IN THE VARIOUS PHOSPHOLIPIDES FROM MOUSE EPIDERMIS UNDERGOING NORMAL AND ABNORMAL GROWTH CHANGES*

<table>
<thead>
<tr>
<th>TREATMENT OF EPIDERMIS</th>
<th>PER CENT PHOSPHORUS IN FRACTION NUMBER</th>
<th>RECOVERY OF PHOSPHORUS FROM COLUMN (per cent)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 4:1†</td>
<td>2 2.5:1†</td>
</tr>
<tr>
<td>2 days ♀ and ♂ post-plucking (3)</td>
<td>4.0 ± 1.1</td>
<td>18.6 ± 3.4</td>
</tr>
<tr>
<td>4 days ♀ post-plucking (3)</td>
<td>2.6 ± 0.1</td>
<td>18.4 ± 3.6</td>
</tr>
<tr>
<td>4 days ♂ post-plucking (4)</td>
<td>1.6 ± 0.4</td>
<td>20.4 ± 2.9</td>
</tr>
<tr>
<td>12 days ♀ and ♂ post-plucking (5)</td>
<td>2.4 ± 0.3</td>
<td>19.1 ± 2.5</td>
</tr>
<tr>
<td>3 applications MC‡ (4)</td>
<td>3.0 ± 0.9</td>
<td>17.2 ± 2.3</td>
</tr>
<tr>
<td>18 applications MC‡ (2)</td>
<td>2.4</td>
<td>19.6</td>
</tr>
<tr>
<td>24 applications MC‡ (3)</td>
<td>2.3 ± 0.8</td>
<td>18.5 ± 2.7</td>
</tr>
<tr>
<td>Squamous-cell carcinoma (2)</td>
<td>2.0</td>
<td>18.5</td>
</tr>
</tbody>
</table>

* No. separate samples analyzed indicated in parentheses.
† Ratio of chloroform to methanol v/v.
‡ MC = methylcholanthrene.
into the lecithins, whereas in Ehrlich ascites tumor cells this isotope was recovered in about equal amounts in lecithin and phosphatidyl ethanolamine. In a comparison of the phosphatide content of azo dye-induced hepatoma of the rat and normal liver cells, Veerkamp et al. found that the hepatoma had about half as much phosphatides as normal liver cells (14). However, the percentage of the various phosphatides—namely, sphingomyelin, lecithin, cephalin, choline phosphatides, and noncholine phosphatides—was about the same in the liver and hepatoma cells (14). The incorporation of P32 into phosphatidyl choline and ethanolamine was lower in the liver cells than in the hepatoma. The hepatoma had a high content of plasmalogens.

Figard et al. also reported that the phosphatide content of some transplantable tumors was much less than that found in normal liver cells, but the proportions of the principal phosphatides was about the same in the normal liver cells and tumor cells (6). As indicated in Table 2 no real differences appear to exist in the amounts of the various phosphatides found in normal epidermis undergoing growth changes induced by the hair growth cycle or in early and late hyperplastic epidermis or in the carcinomas. This is so in spite of the fact that the total phosphatide content of the tissues employed varies considerably (Table 1). As indicated earlier the fractions obtained from the various carcinomas and epidermis contain the same principal phosphatides. Actually the percentage of the various phosphatides and the types found in the fractions given in Table 2 agree well with those reported by Figard et al. (6) for mouse liver cells, Hepatoma 134, red blood cells, and other transplantable tumors of mice and are in the same order of magnitude with those reported by Wallach et al. for Ehrlich ascites tumor cells (15). In Ehrlich ascites carcinoma cells the phosphatides account for 58 per cent of the total lipide, and of this 13.7 per cent is found in the nuclei, 29 per cent in the mitochondria, 52.5 per cent in the microsomes, and 5.4 per cent in the supernatant fraction (15). Wallach et al. found the same phosphatides in Ehrlich ascites carcinoma cells in the fractions obtained from silicic acid as indicated above for other tissues but in addition quantitized their findings as follows: Of the nitrogen-containing phosphatides (81–93 per cent of the total phosphatides) lecithin was 39 per cent, phosphatidyl ethanolamine, 24 per cent, sphingomyelin, 14 per cent; and uncertain, 13 per cent. Of the remainder (7–19 per cent), phosphatidic acid comprised 3 per cent, inositol phosphatides 4 per cent, and acetal phosphatides 12.5 per cent. It is apparent then that much better methods for the separation of phosphatides, such as developed by Rouser et al. (12, 13), will have to be employed to determine whether any real differences exist between the phosphatides of normal and malignant cells.

REFERENCES

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