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<td>Author(s)</td>
<td>Hanai, Tetsuya; Kajiyama, Masatada; Morita, Shigeru; Koizumi, Naokazu</td>
</tr>
<tr>
<td>Citation</td>
<td>Bulletin of the Institute for Chemical Research, Kyoto University (1969), 47(4): 327-339</td>
</tr>
<tr>
<td>Issue Date</td>
<td>1969-12-16</td>
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<td>URL</td>
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<tr>
<td>Type</td>
<td>Departmental Bulletin Paper</td>
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Kyoto University
Some Properties of Black Lipid Membranes Formed from Lipids Extracted from Egg Yolk

Tetsuya HANAI*, Masatada KAJYAMA**, Shigeru MORITA* and Naokazu KOIZUMI*

Received July 4, 1969

1) Six kinds of lipid fractions, such as neutral lipid (NL), phosphatidyl inositol (PI), phosphatidyl ethanolamine (PE)-phosphatidyl inositol, phosphatidyl choline (PC), sphingomyelin-phosphatidyl choline and sphingomyelin (SM), were separated from a total lipid fraction of egg yolk by means of silicic acid column chromatography. The NL fraction consisted of cholesterol ester, triglyceride, diglyceride, and cholesterol as major components. Pure PE was separated from the PE-PI fraction by means of cellulose column chromatography. Pure SM was isolated from the SM-PC fraction through alkaline decomposition of PC.

2) The formation of underwater black films containing these lipids were investigated in detail. The PC and PE gave stable black films whose capacitances were 0.34 and 0.36 μF/cm² respectively. The membranes formed from PI were less stable, the capacitance being 0.29 μF/cm². It was unable to form the black films of SM or NL. Addition of NL fraction to PI enhanced the stability of the membranes, the capacitance being 0.38 μF/cm².

3) Analytical data of fatty acid constitution of PC and PE were presented to discuss the stability of the membranes.

I. INTRODUCTION

In recent years a number of studies have been made on black lipid membranes as a structural unit of biological cell membranes. It is necessary in this field to accumulate more pieces of information on the possibility of formation and the properties of the black lipid membranes consisted of single lipids as well as of mixed lipids.

In our previous study on the formation of black films by using lipids extracted from bovine erythrocytes, it was found that phosphatidyl ethanolamine (PE) and phosphatidyl serine (PS) gave very stable membranes. Phosphatidyl choline (PC) gave less stable membranes. Sphingomyelin (SM) and neutral lipids (NL) were incapable of forming membranes. The addition of NL fractions to PC or to SM markedly improved the stability of the membranes.

In the present work some single lipids were extracted from hen egg yolk by means of column chromatography, and were examined with respect to the stability of the black films. Fatty acid chain constitution of PE and PC was also examined by means of gas-liquid chromatography, the results being discussed with reference to the membrane stability.


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II. EXPERIMENTAL METHODS

All solvents used in this work were distilled from glass stills.

A. Extraction of Total Lipids

1) York of a hen egg (about 13 g) was homogenized in 50 ml of acetone, and was filtered in a Buchner funnel.
2) The solid residue was reextracted using further 50 ml of acetone.
3) The slightly yellowish residue was mixed with 100 ml of chloroform-methanol (C-M, 2:1 v/v) solvent and was kept overnight in the dark.
4) The mixture was filtered with a filter paper.
5) The residue was reextracted with further 100 ml of C-M (2:1) solvent.
6) The filtrates was mixed with one-fifth volume of 0.1% calcium chloride solution, and was shaken vigorously (Folch Washing).
7) After standing the mixture separated into two phases. The upper phase was discarded. The lower phase containing total lipids was taken to dryness under vacuum. Yield at this stage was approximately 1.7 g.
8) The solid extract was dissolved in absolute chloroform as approximately 2% solution ready for column chromatographic separation.

B. Separation of lipids by means of Silicic Acid Column Chromatography

The total lipid extract was fractionated into single lipids by means of Mallinckrodt silicic acid (100 mesh) column chromatography as suggested by Rhodes and Dawson. The column size was 3 cm in diameter and 24 cm in length. The adsorbent was approximately 75 g. The amount of total lipids loaded was 1.7 g. Flow rate of the elution was 3 ml/min on an average. Each fraction eluted with C-M solvents in different compositions was then subjected to thin-layer chromatographic analysis for identification of lipid components (Fig. 1).

C. Separation of PE from PI by means of Cellulose Column Chromatography

Cellulose column chromatography proposed by Shimojo, Orii, Yamaguchi and Ohno was used for separation of PE from PI. The adsorbent was 35 g cellulose powder (product of Toyo Roshi Kaisha Ltd., Grade C, finer than 300 mesh). The column size was 2.2 cm in diameter and 35 cm in length. The amount of phospholipids applied was 150 mg. Flow rate of elution was 1.5 ml/min. The result of elution with C-M solvents in different compositions is shown in Fig. 3.

D. Isolation of SM by means of Alkaline Decomposition

Phosphatidyl choline is unstable to alkali in contrast with the alkali-stable SM. This characteristic can be utilized to remove PC from a mixture of SM and PC.
1) About 1 g of a mixture of SM and PC was dissolved in 300 ml of C-M (2:1) solvent.
2) To this solution was added 30 ml of 1 M sodium hydroxide solution in absolute methanol.
3) The mixture was allowed to stand at room temperature for 1 hour.
4) Ethyl formate (45 ml) was added and, after mixing, the solution was set aside for further 10 minutes at room temperature. This procedure neutralized excess of alkali by the hydrolysis: ethyl formate + sodium hydroxide \( \rightarrow \) sodium formate + ethanol.

5) Water (100 ml) was mixed with the solution to wash alkali.

6) The upper phase rich in water was discarded. The lower phase was taken to dryness under vacuum.

7) The dry residue, being supposed to be a mixture of SM and decomposed products of PC, was dissolved in absolute chloroform.

8) In order to separate pure SM from other ingredients the chloroform solution was applied to silicic acid column which was described in the preceding section.

E. Thin-Layer Chromatographic Analysis

Identification of the individual components and test of the purity were carried out with thin-layer chromatography plates of Kieselgel G nach Stahl. The developing solvent was a solution of chloroform-methanol-water (65:25:4 or 60:40:5, v/v/v) for identification of phospholipids, and petroleum ether-diethyl ether-acetic acid (90:10:1) for neutral lipids. Spray reagents for detecting the spots were 0.1% ninhydrin solution in butanol and 5% phosphomolybdic acid solution in ethanol.

F. Preparation of Spreading Solution and Membrane Formation

As a standard recipe of a spreading solution to generate lipid membranes, 1 ml of n-decane was added to 5.7 mg of the lipids, the values for individual cases being given in Table 1.

The n-decane was purified with effect by passing through an alumina column. The interfacial tension of the purified n-decane against water was 52.0 dyn/cm at 20°C. Adsorbent for the column was aluminium oxide standardized for chromatographic adsorption analysis accepted to Brockmann, activity grade 2 or 3, product of Merck, and was activated in the following way: The powder was mixed with 1% aqueous solution of nitric acid, and shaken vigorously. Washing of the powder with distilled water was then repeated until neutrality was confirmed with a pH test-paper. The powder was taken to dryness and kept at 150°C overnight.

With a small sable brush a small quantity of the solution was transferred to a hole of about 1.5 mm in diameter in the wall of a teflon pot which was partly immersed in an aqueous solution. The hole was illuminated with a microscope projection lamp, and was observed with a low-power (\( \times 20 \)) microscope. The spreading solution first blocked the hole in a thin liquid lamella, which spontaneously became thinner and ultimately generated the so-called secondary black film. The area of the black film was measured through crossed graticules in the microscope eyepiece.

The electrodes for a.c. measurements consisted of spirally wound platinum foil coated with platinum black to reduce electrode polarization. The film experiment was carried out at 20°C in a 0.1 M sodium chloride solution.
G. Electrical Measurements

Capacitance and conductance were measured over a frequency range of 20 Hz to 5 MHz with a conductance-capacitance bridge of the transformer ratio-arm type made by Ando Electric Co., Ltd. Film capacitance was then evaluated from a limiting capacitance at lower frequencies for a combined system in series of the film and the aqueous solution as described in an earlier paper.\(^8\)

H. Methylation of Phospholipids

Phospholipids were methylated under the presence of hydrochloric acid as the esterifying catalyst.\(^9\)

1) Dried gaseous hydrochloric acid was bubbled to be absorbed in absolute methanol until the concentration of hydrochloric acid went up to 5% by weight.

2) A 10 mg aliquot of the phospholipid specimen was mixed with 1 ml of benzene and 8 ml of the hydrochloric acid-methanol solution prepared as above.

3) The mixture was refluxed with a Dimroth cooler on a boiling water bath for 8 hours.

4) Water of 4 ml was added to the mixture at the end of the reaction period.

5) The esterified lipid was extracted from mixture with three successive 20 ml portions of petroleum ether.

6) The extract was dehydrated with anhydrous sodium sulfate and sodium carbonate (4:1, w/w).

7) The final product of methyl esters of the lipid was obtained by evaporating the petroleum ether under reduced pressure.

I. Gas-Liquid Chromatography of Methyl Esters of Phospholipids

Gas-liquid chromatographic analysis of fatty acid constitution of methylated specimens was carried out with Yanagimoto Model GCG-220 Gas Chromatograph equipped with a thermal conductivity detector. The column size was 130 cm in length and 4 mm in diameter. The column was filled with 100-120 mesh Neosorb NC coated with 20% Apiezon L. High purity hydrogen was used as the carrier gas, the flow rate being 50 ml/min. All the methyl esters examined were separated at 240°C. Identification of the retention times for esters was made by using several standards of methyl esters.

III. RESULTS AND DISCUSSION

A. Separation of Single Lipids and Confirmation of the Purity

Total lipid fraction from egg yolk was obtained by means of the extraction with C-M solvents outlined in the experimental section A. A thin-layer chromatogram of the total lipid preparation before applying to the column is given in Fig. 1 no. 1, which shows the presence of PE, PC, SM, lyso-PE, PI and NL.

The total lipid fraction was then applied to silicic acid column shown in the experimental section B for further separation into single lipid fractions.

The thin-layer chromatograms of the fractions eluted through the column
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Fig. 1. Thin-layer chromatogram of egg yolk phospholipids.
Thin-layer plate: Kieselgel G.
Developing time: 74 min. Temp.: 30°C.
Spray solution: ninhydrin and phosphomolybdic acid.
The chromatograms show typical patterns for fractions separated by means of
Mallinckrodt silicic acid column chromatography.
No. 1, total lipid fraction of egg yolk before applying to the column, after the
extraction with a chloroform-methanol (C-M, 2:1, v/v) mixture.
No. 2, neutral lipid (NL) fraction eluted from the column with an eluent C-M
(100:0, v/v).
No. 3, Phosphatidyl inositol (PI) fraction eluted at an earlier stage with an
eluent C-M (90:10).
No. 4, mixture of phosphatidyl ethanolamine (PE) and PI, eluted at later stage
with C-M (90:10).
No. 5, mixture of phosphatidyl choline (PC) and lyso-PE at an earlier stage with
C-M (60:40).
No. 6, PC fraction, at a later stage with C-M (60:40).
No. 7, mixture of PC and sphingomyelin (SM), C-M (50:50).
No. 8, SM fraction, C-M (20:80).

with C-M mixtures in 100:0, 90:10, 60:40, 50:50 and 20:80 (v/v) are given in
Fig. 1 nos. 2-8. No. 2 shows an NL fraction. No. 3 shows a PI fraction with
very slight trace of as yet unidentified component. This fraction was used as
a PI specimen in subsequent membrane experiments. No. 4 shows the presence
of PE and PI. No. 5 shows PC with a small amount of lyso-PE. No. 6 shows
a pure PC fraction, which was used as a pure PC specimen in the membrane
experiments. No. 7 shows two components of PC and SM. No. 8 shows a pure
SM fraction.

In order to confirm the constituents of the NL fraction, a fraction corres-
ponding to no. 2 was also chromatographed on silica gel G thin-layer plate, the
chromatogram being given in Fig. 2 no. 9. Concurrently spots of standard speci-
mens of cholesterol, triolein, and cholesterol palmitate are shown in Fig. 2 nos. 10, 11 and 12 respectively. The result indicates that the present NL fraction consists mainly of cholesterol ester, triglyceride, diglyceride and cholesterol.

A fraction of PE-PI mixture shown in Fig. 1 no. 4 was further applied to cellulose column which was shown in the experimental section C to obtain a pure PE fraction. In this instance care must be taken that, at the initially loading stage of the column, the effluent of PE-PI fraction is applied to the column repeatedly until the constituent PE is loaded completely on the adsorbent and no PE can be detected in the filtrate.

In Fig. 3, no. 13 shows a PI fraction, which is the same as shown by no. 3 in Fig. 1. No. 14 is a PI-PE fraction before applying to the cellulose column. The thin-layer chromatograms of the fractions eluted through the column with C-M mixtures in 95:5, 90:10, and 50:50 (v/v) are given in Fig. 3 nos. 15, 16 and 17. From the result nos. 15 and 16 are found to show a single spot of a PE fraction, which was used as a pure PE specimen in the membrane experiments.

The amount of pure SM in the fractions corresponding to Fig. 1 no. 8 was too small to complete the subsequent membrane experiments. A pure SM specimen was further obtained from the fractions corresponding to Fig. 1 no. 7 by means of alkaline decomposition of PC, the treatment being given in the experi-
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Fig. 3. Thin-layer chromatogram of a PE-PI separation process by means of cellulose column.
Thin-layer plate: Kieselgel G.
Developing solvent: C-M-W (60:40:5).
Developing time: 70 min.
Spray solution: ninhydrin and phosphomolybdic acid.
The chromatograms show typical patterns for fractions separated by means of cellulose column chromatography.
No. 13, PI fraction, the same as shown by no. 3 in Fig. 1.
No. 14, mixture of PE and PI, the same as shown by no. 4 in Fig. 1.
The mixture was then applied to a cellulose column to separate PE from PI.
No. 15, PE fraction eluted from the cellulose column with an eluent C-M (95:5).
No. 16, PE fraction, C-M (90:10).
No. 17, mixture of PE and PI, C-M (50:50).

mental section D.

Figure 4 shows the chromatogram of the alkaline decomposition process. No. 18 is a PC-SM mixture before alkaline decomposition, the same as shown in Fig. 1 no. 7. The mixture was then subjected to the treatment of alkaline decomposition, and showed a chromatogram of no. 19, which indicates the presence of original SM and NL as decomposed product of PC. The mixture was then applied to silicic acid column prepared as before. The fractions eluted with C-M mixtures in 100:0, 40:60 and 20:80 are given in nos. 20, 21 and 22. It is seen that constituent NL was completely eluted with C-M (100:0) as shown in no. 20, and that pure SM was obtained as fractions of nos. 21 and 22, which were used as an SM specimen in the membrane experiments.

B. Formation of Black Films from Single and Mixed Lipids and the Capacitance Measurements

The PC preparation gave very stable black films. The process of drainage of the film in the hole was observed with a microscope positioned so as to catch
Fig. 4. Thin-layer chromatogram of an SM separation process by means of silicic acid column.

Thin-layer plate: Kieselgel G.
Developing solvent: C-M-W (60:40:5).
Developing time: 40 min.
Spray solution: phosphomolybdc acid.
No. 18, mixture of PC and SM, the same as shown by no. 7 in Fig. 1.
No. 19, preparation of no. 18 which was subjected to treatment of alkaline decomposition for one hour. The preparation was then applied to silicic acid column to separate SM from the rest.
No. 20, NL fraction eluted from the silicic acid column with an eluent C-M (100:0).
No. 21, SM fraction, C-M (40:60).
No. 22, SM fraction, C-M (20:80).

The light reflected from the film. Some characteristic stages of the film-blackening are shown in Plates 1 to 4. Colorful patterns of interference on the thin lamella were observed prior to blackening (Plate 1). An annular area in dark color around the colored area inside the hole is not a thin lamella but a thick layer of the spreading solution. This thick part is supposed to play a part as an excellent support of the black film generated. Drainage of the film then began spontaneously, and a dark part which is the so-called black film appeared at the lower part of the colored area (Plate 2). The drainage proceeded steadily over a period of a few minutes, resulting in the increase in the black area (Plate 3). Ultimately the colored area was changed completely to a black state (Plate 4). The borderline between the black film and the surrounding thick part of the spreading solution is shining faintly, indicating the presence of the black film. The black films of the present PC were very stable for several hours in remarkable contrast to the film of PC extracted from bovine erythrocytes. The present preparation of PE also gave very stable films similar to the
Plates 1-4. Process of the blackening of a lecithin (PC) film in 0.1M sodium chloride solution. For explanation, see the text.
case of PC.

In the case of PI, brilliant patterns of interference colors were observed prior to drainage. Blackening due to the drainage then began spontaneously and slowly. After completely blackening the films broke spontaneously, and few examples of the black films were able to be measured. The addition of NL to the PI preparation improved remarkably the stability of the black films. In this instance triolein and oleyl alcohol were also confirmed to be effective for improving the stability of the films.

In the case of SM solution in n-decane, the solution readily blocked the hole on the pot as a thin liquid lamella, which showed no tendency to become thinner. It was thus concluded that egg SM showed no possibility of forming black films. The addition of NL to the SE preparation did not facilitate drainage of the lamella to an appreciable extent. The NL preparation did not separately show the tendency to form black films.

Fig. 5. Frequency dependence of the capacitance $C'$, the conductance $G$ and the loss factor $C'' = \frac{G}{2\pi f}$ of the capacitance for the system consisting of a PC black film in 0.1M sodium chloride solution.

The film capacitance $C_b$ is approximately equal to the limiting capacitance of the composite system at low frequencies $C_l$. From the figure, $C_l=4520 \, \mu F$ and the conductance of the aqueous solution $G_a=1.24 \, \text{mmho}$ lead to a relaxation frequency $f_0=\frac{G_a}{2\pi C_b}=\frac{G_a}{2\pi C_l}=43.7 \, \text{kHz}$, which may be compared with the value $f_0=39.5 \, \text{kHz}$ obtained from the maximum point of $C''$ in Fig. 6.

Figure 5 shows an example of the frequency dependence of the capacitance, the conductance and the loss factor of the capacitance for the system consisting of a black film of PC in 0.1M sodium chloride solution. The complex plane plots of the capacitance and the loss factor from the data in Fig. 5 are characterized by a semicircular arc as shown in Fig. 6. The limiting capacitance at low frequencies was thus deduced accurately, leading to a net capacitance of the black film.
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Fig. 6. Complex plane plots of a composite system of the PC black film and 0.1M sodium chloride solution. The same data as presented in Fig. 5.

Table 1. Characteristics of Black Films of Some Lipids Isolated from Hen Egg Yolk.

<table>
<thead>
<tr>
<th>Lipid Specimen</th>
<th>Composition of spreading solution, phospholipid: neutral lipid (mg : mg) in 1ml decane</th>
<th>Number of experiments</th>
<th>Life span of black film (min.)</th>
<th>Breakdown voltage (d.c., mV)</th>
<th>Capacitance per unit area (μF/cm²)</th>
<th>General remark on stability*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphatidyl choline (PC)</td>
<td>5.7 : 0</td>
<td>13</td>
<td>120</td>
<td>100-120</td>
<td>0.342 ±0.007</td>
<td>VS</td>
</tr>
<tr>
<td>Phosphatidyl ethanolamine (PE)</td>
<td>5.6 : 0</td>
<td>9</td>
<td>&gt;120</td>
<td>110-140</td>
<td>0.362 ±0.003</td>
<td>VS</td>
</tr>
<tr>
<td>Phosphatidyl inositol (PI)</td>
<td>5.7 : 0</td>
<td>2</td>
<td>&lt; 5</td>
<td>170-210</td>
<td>0.293</td>
<td>LS</td>
</tr>
<tr>
<td>Sphingomyelin (SM)</td>
<td>5.6 : 0</td>
<td>—</td>
<td>0</td>
<td>0</td>
<td>—</td>
<td>N</td>
</tr>
<tr>
<td>Total neutral Lipid fraction (NL)</td>
<td>0 : 13.8</td>
<td>—</td>
<td>0</td>
<td>0</td>
<td>—</td>
<td>N</td>
</tr>
<tr>
<td>PI+NL fraction</td>
<td>5.6 : 14.3</td>
<td>9</td>
<td>&gt;120</td>
<td>150-220</td>
<td>0.380 ±0.011</td>
<td>VS</td>
</tr>
<tr>
<td>SM+NL fraction</td>
<td>5.7 : 5.0</td>
<td>—</td>
<td>0</td>
<td>0</td>
<td>—</td>
<td>N</td>
</tr>
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</table>

* VS, very stable.
S, stable, possible to a less extent to carry out further experiments.
LS, less stable, the life span being within ten minutes, insufficient for carrying out further experiments.
N, no possibility to generate black films.

In Table 1 are summarized the results of the measurements: composition of lipids in the spreading solution, number of experiments, life span of the black films, breakdown voltage, capacitance per unit area and general remark on stability.

C. Results on Gas-Liquid Chromatographic Analysis

In our previous paper it was reported that PC extracted from bovine erythrocytes did not give stable black films whereas the PE gave stable films. In
the present investigation both egg PE and egg PC gave stable films as described above. As regards the stability of the black films, it was sometimes considered plausible that the presence of unsaturated bonds in the hydrocarbon chain might be responsible for the stability of the black films. In hopes of finding a clue to the stability of black films, the fatty acid composition was examined in the previous work. The result showed that bovine erythrocyte PC contained almost no oleyl chains, while erythrocyte PE contained no linoleyl chains. For further consideration in the present work gas-liquid chromatographic analysis was carried out for the present egg PC and PE, the result being shown in Table 2. It is seen that egg PC contains no linoleyl chains (18: 2) and egg PE contains no oleyl chains (18: 1) although both phospholipids gave stable membranes. It thus follows that no simple correlation is found between the film-stability and the presence of oleyl or linoleyl chains in the present case.

Table 2. Fatty Acid Carbon Chain Composition of PC and PE Extracted from Egg Yolk.

<table>
<thead>
<tr>
<th>Carbon number: number of unsaturated bond</th>
<th>Phosphatidyl choline</th>
<th>Phosphatidyl ethanolamine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>wt.%</td>
<td>no.%</td>
</tr>
<tr>
<td>14 : 0</td>
<td>&lt; 0.1</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>16 : 0</td>
<td>33.4</td>
<td>36.6</td>
</tr>
<tr>
<td>16 : 1</td>
<td>&lt; 0.1</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>18 : 0</td>
<td>6.2</td>
<td>6.1</td>
</tr>
<tr>
<td>18 : 1</td>
<td>37.1</td>
<td>36.8</td>
</tr>
<tr>
<td>18 : 2</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>20 : 0</td>
<td>1.3</td>
<td>1.2</td>
</tr>
<tr>
<td>20 : 3</td>
<td>1.4</td>
<td>1.3</td>
</tr>
<tr>
<td>20 : 4</td>
<td>2.3</td>
<td>2.1</td>
</tr>
<tr>
<td>22 : 0</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>22 : 5</td>
<td>8.7</td>
<td>7.4</td>
</tr>
<tr>
<td>22 : 6</td>
<td>9.8</td>
<td>13.2</td>
</tr>
</tbody>
</table>

Table 3. The Average Number of Carbon Atoms and Unsaturated Bond in the Fatty Acid Carbon Chains.

<table>
<thead>
<tr>
<th></th>
<th>Average number of carbon atom per chain</th>
<th>Average number of unsaturated bond per chain</th>
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</thead>
<tbody>
<tr>
<td>Egg</td>
<td>19.0</td>
<td>0.86</td>
</tr>
<tr>
<td>(Phosphatidyl choline)</td>
<td>(Phosphatidyl ethanolamine)</td>
<td></td>
</tr>
<tr>
<td>Bovine erythrocyte</td>
<td>18.0</td>
<td>1.54</td>
</tr>
<tr>
<td>(Phosphatidyl choline)</td>
<td>(Phosphatidyl ethanolamine)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>18.9</td>
<td>1.11</td>
</tr>
</tbody>
</table>

Unsaturated bonds in hydrocarbon chains sometimes exert remarkable influence on the properties of surface films of lipids on water. The density of the unsaturated bonds therefore might be responsible for the film-stability. Table 3 shows the average number of carbon atoms per chain and the average number of unsaturated bonds per chain for PC and PE of egg and of bovine erythrocytes, the values being calculated from the data shown in Table 2 for egg lipids
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and from the data in the previous paper\textsuperscript{1} for lipids of bovine erythrocytes. Bovine erythrocyte PC is seen not to show a value different distinctly from those for the other three kinds of phospholipids. Hence the average number of unsaturated bonds cannot explain the unstable nature of bovine erythrocyte PC-films. The instability of bovine erythrocyte PC-films remains a problem for future exploration.

ACKNOWLEDGMENTS

The authors wish to express their sincere thanks to Prof. T. Shimojo, Sapporo Medical College, for his advice on the techniques of extraction and separation of lipids. The part of the present study was financially supported by a grant from the Ministry of education.

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