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# The Formation and Properties of Bilayers Containing Lipids Extracted from Bovine Erythrocytes

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

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The formation and the properties of bimolecular membranes separating two aqueous phases have been studied using some single and mixed lipids extracted from bovine erythrocytes by means of column chromatography. Phosphatidyl ethanolamine and phosphatidyl serine were found to give very stable membranes, whose capacitances were 0.39 and 0.33 $\mu$F/cm$^2$ respectively. The membranes formed from phosphatidyl choline were less stable, the capacitance being 0.33 $\mu$F/cm$^2$. Using sphingomyelin or non-phosphorus-containing neutral lipids composed of cholesterol ester, triglyceride, diglyceride and cholesterol it was almost impossible to form thin membranes. The addition of the neutral lipid fractions to the phosphatidyl choline or to the sphingomyelin markedly improved the stability of bimolecular membranes, giving values of 0.29 to 0.34 $\mu$F/cm$^2$ for membrane capacitances. This fact implies that the neutral lipids when mixed with phospholipids play a part in the mechanical stability of cell membranes. Distinct differences in membrane stability were observed between phosphatidyl choline extracted from egg and that from bovine erythrocytes. Analytical data of fatty acid carbon chain constitution were also presented for phosphatidyl choline and phosphatidyl ethanolamine.

## I. INTRODUCTION

In the last few years, a considerable number of studies have been carried out on the formation and the physico-chemical properties of phospholipid bilayers in aqueous solutions. The "black films" provide a system which can be investigated, under controlled conditions, as a model resembling in its structure the basic unit in living cell membranes. In order to extend this work it seemed useful to obtain further information on bilayers formed from different lipids and mixtures of lipids.

Not all the lipids extracted from various sources have so far been examined systematically. Among the various kinds of naturally occuring phospholipids, phosphatidyl choline (PC) is one of the most easily obtainable, and has been used by several investigators in bilayer experiments. Phosphatidyl choline extracted from hen egg yolk was among the preparations found to form very stable black films. This has been used by Huang, Wheeldon and Thompson[1] for experiments on film stability and for electrical and optical measurements, by Huang and Thompson[2] for optical measurements of the film thickness, by Huang and Thompson[3,4] for permeability measurements, by Miyamoto and Thompson[5] for study of electrical properties, by Hanai, Haydon and Taylor[6-8] for electrical measurements, by van

* 花井哲也, 森田 滋, 小泉直一: Laboratory of Dielectrics, Institute for Chemical Research, Kyoto University, Uji, Kyoto.
** 梶山方忠: Physical Chemistry Laboratory, Faculty of Pharmaceutical Sciences, Kyoto University, Kyoto.

den Berg[9] who formed black films with the remarkably wide area of 50 $mm^2$, by Läuger, Lesslauer, Marti and Richter[10] who examined the electrical properties of films separating electrolyte solutions of varying composition, by van Zutphen, van Deenen and Kinsky[11] for studies on the effects of polyene antibiotics on the stability of bilayers, and by Tien[12] for more exact measurements of the optical thickness. The extent of these investigations reflects the fact that purified egg PC forms quite stable black films. According to observations by Huang *et al.*,[1] yeast PC also gave stable films.

In contrast to the results mentioned above, PC extracted from bovine brain and from bovine heart was reported by Bean *et al.*,[13] and by Huang *et al.*[1] respectively to give unstable black films with which further experiments were unsuccessful.

Phosphatidyl ethanolamine (PE) is also a phospholipid easily obtainable from natural sources. Phosphatidyl ethanolamine isolated from *Escherichia coli B* was used by Henn, Decker, Greenawalt and Thompson[14] for electron microscopic study of the black films, and was found to form stable films. Recently the same PE was used by Henn and Thompson[15] for composition study of the black films. Bean *et al.*,[13] however, reported that black films of PE extracted from bovine brain were not very stable.

Phosphatidyl serine (PS) extracted from bovine brain was also reported by Bean *et al.*[13] not to give stable black films.

Sphingomyelin (SM) from bovine brain was found by Bean *et al.*[13] to produce more stable black films than PE does, whereas Läuger *et al.*[10] reported that black films of bovine brain SM were stable for only a few minutes.

Phosphatidyl inositol (PI) extracted from bovine brain was used by Lesslauer, Richter and Läuger,[16] who succeeded in making black films sufficiently stable for electrical measurements.

There is no successful example in the literature of the formation of black films from cardiolipin (CL). Huang, Wheeldon and Thompson[1] reported the failure of their attempts to form black films using bovine heart CL.

Total lipid fractions from natural sources and certain lipid mixtures form films of sufficient stability for carrying out experiments. Total lipids extracted from bovine brain were used for the first time by Mueller, Rudin, Tien and Wescott[17-19] for film thickness and capacitance measurements and the demonstration of phenomena which are associated with excitability in living cell membranes. This preparation was further used by Seufert[20] for investigations of the effects of detergents on bilayers, by Babakov, Ermishkin, Liberman and Tsofina[21,22] for the study of the effects of electric fields and protein on the films, by Del Castillo, Rodriguez, Romero and Sanchez[23] who observed antigen-antibody and enzyme-substrate reactions occuring in bilayer systems, and by Cass and Finkelstein[24] for studies on the permeability of bilayers to water. Bean, Shepherd and Chan[25] measured permeability to organic solutes by using bilayers formed from mixtures of bovine brain phospholipids, $\alpha$-tocopherol and cholesterol. Total lipids extracted from bovine erythrocytes were used by Reman and van Deenen[26] who studied the lytic action of some synthetic lysolecithins and lecithins on bilayers. Vreeman[27] carried out diffusion permeability experiments by using stable

black films fromed from mixtures of PC, PS, PE and SM. Recently Andreoli, Bangham and Tosteson[28-32] carried out capacitance, resistance and voltage measurements on black films formed from total lipids extracted from sheep erythrocytes.

From the various results mentioned above, it is apparent that the stability of black films should be considered in association with the nature of the lipid together with the source from which the lipid is isolated. Further it appears that total lipid extracts or mixtures of lipids form more stable films than single lipids do. Moreover information which enables us to predict the composition of lipid solution most likely to give stable films is valuable in extending experimental studies on bilayers.

In the present study, we examined the stability of black films composed of single and mixed lipids extracted from bovine erythrocytes with particular reference to the nature of the lipids and the improvement of the stability by mixing.

## II. EXPERIMENTAL METHODS

### A. Extraction of Lipids from Bovine Erythrocytes

Bovine erythrocyte lipids were fractionated into single lipids by means of column chromatography after extraction by chloroform-methanol solvent fractionation.

Total lipid extraction was carried out according to the method of Folch, Lees and Stanley.[33]

(1) Bovine blood of 5 *l* were centrifuged at 3000 rev./min for 30 min.

(2) The sediment was washed three times with 0.9% saline solution to remove blood plasma completely.

(3) The precipitated erythrocyte pack was shaken vigorously after adding 6 l of a chloroform-methanol (C-M) mixture (2:1 by volume), and was kept overnight in the dark at 7°C.

(4) The suspension was filtered through gauze.

(5) In order to raise the efficiency of the extraction the residue obtained in Step (4) was ground, mixed with 3 *l* of a C-M (2:1) mixture, shaken vigorously and filtered.

(6) The filtrates obtained from Steps (4) and (5) were subjected to Folch's washing : A 0.9% saline solution equal in volume to one-fifth that of the filtrate was added. The mixture was shaken vigorously at 10°C, and centrifuged at 4000 rev./min for 20 min. The lower phase (about 5 *l*) was evaporated to dryness using a vacuum pump, the residue being the total lipid fraction. This extraction yielded about 5 g.

(7) The total lipid was dissolved in chloroform to give the solution for column chromatography.

### B. Separation of Lipids

The lipids in the solution obtained from the aforementioned treatment were separated by means of Mallinckrodt Silicic Acid (100 mesh) column chromatography as suggested by Hanahan, Dittmer and Warashina[34] and by Shimojo and Ohno.[35] The column size was 3 cm in diameter and 24 cm in length. Non-phosphorus containing (non-P) neutral lipid, PS, PE, PC and SM fractions were eluted with

chloroform-methanol mixtures; the proportions of chloroform to methanol being 100:0, 90:10, 80:20, 60:40 and 40:60 (v/v) respectively. Among many fractions eluted from the column, only the fractions showing a single component were used for further black film experiments. Thin layer chromatographic analysis was carried out with Kieselgel G nach Stahl, the developing solvent being a solution of chloroform-methanol-water (60:40:5, by volume).[36]

The non-phosphorus neutral lipid fraction was further separated into four major components by means of Silica Gel (product of Kanto Chemical Co., Inc., 100–200 mesh) column chromatography.[37,38] Four major neutral lipid fractions, cholesterol ester, triglyceride, diglyceride and cholesterol, were eluted with hexane–diethyl ether mixtures; the proportions of hexane to diethyl ether being 98:2, 90:10, 80:20 and 50:50 (v/v) respectively. The fractions were identified and tested for purity using thin layer chromatograms prepared with Kieselgel G nach Stahl and developed by a solution of light petroleum-diethyl ether-acetic acid (90:10:1).[39] The pure lipids were stored at −20°C as a dried state in nitrogen atmosphere or as 0.5% solution in chloroform.

All the solvents used for extraction and separation of lipids were distilled before use.

### C. Preparation of Spreading Solution and Membrane Formation.

Unless otherwise stated, 1 ml of *n*-decane was added to 5.7 mg of the lipid, and used as a spreading solution. The *n*-decane was purified by passing through alumina columns before use. 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 immersed in an aqueous solution. The spreading solution first blocked the hole in a thin liquid lamella, which spontaneously became thinner and ultimately generated black film.

A d.c. voltage supply (0–800 mV) could be used to facilitate the drainage of the liquid lamella and to initiate formation of black film. It also provided us with a means of dispersing used solution from the hole. The electrodes for a.c. measurements consisted of spirally wound platinum foil coated with platinum black to reduce electrode polarization. The formation of the black films and the measurements were carried out at 20°C in a 0.1 M sodium chloride solution. The aqueous solutions were used unbuffered in order to avoid subsidiary effect of buffering agent on the films formed. The pH value of the aqueous solutions was 5.6–5.8 in the unbuffered state.

### D. Electrical Measurements

Both 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.

### E. Methylation of Lipids

The method for methylation suggested by Stoffel *et al.*[40] was adopted for

obtaining methyl esters of the phospholipid specimens. A 10 mg aliquot of the phospholipid specimen was mixed with 1 ml of benzene and 8 ml of hydrochloric acid-methanol solution containing absolute hydrochloric acid by 5% wt. The mixture was refluxed with a Dimroth cooler on a boiling water bath for 8 h. Until this stage, the mixture had to be kept free from water to increase reaction efficiency. Water of 4 ml was then added to the mixture. The esterified lipid was extracted from the mixture with three successive 20 ml portions of light petroleum. The extract was then dehydrated with anhydrous sodium sulfate and sodium carbonate (4:1, w/w). The final product of methyl ester of the lipid was obtained by evaporating the light petroleum under reduced pressure.

### F. Gas-Liquid Chromatography of Methyl Esters of Lipids

Gas-liquid chromatographic analysis of fatty acid constitution of methylated specimens was made by 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 standard specimens of several esters.

## III. RESULTS

### A. Formation of Black Films by Single Lipids.

i) *Phosphatidyl ethanolamine*

Among the lipids tested here, PE gave the most stable films; these were perfectly uniform black in appearance. Very brilliant patterns of interference colors were observed prior to blackening. Drainage of the films took place evenly over a period of a few minutes. The black films were stable for several hours.

ii) *Phosphatidyl serine*

Phosphatidyl serine appeared to dissolve less readily in *n*-decane. When the standard recipe was adopted, a large proportion of the material became swollen and remained at the bottom of a specimen tube. The supernatant solution was used to make black films. The PS was also capable of forming stable films which were perfectly uniform black in appearance.

The frequency dependence of the capacitance and the conductance for the systems consisting of black films of PE and of PS in 0.1 M sodium chloride solution are shown in Figs. 1 and 2. These can be explained in terms of a series combination of the film capacitance with the conductance of the aqueous phase. Complex plane plots of the capacitance and the conductance from the data in Figs. 1 and 2 are characterized by a semicircular arc as shown in Figs. 3 and 4 respectively. The characteristic was confirmed to hold for all the systems in which sufficiently stable films could be formed.

From the complex plane plots the limiting capacitance at low frequencies was

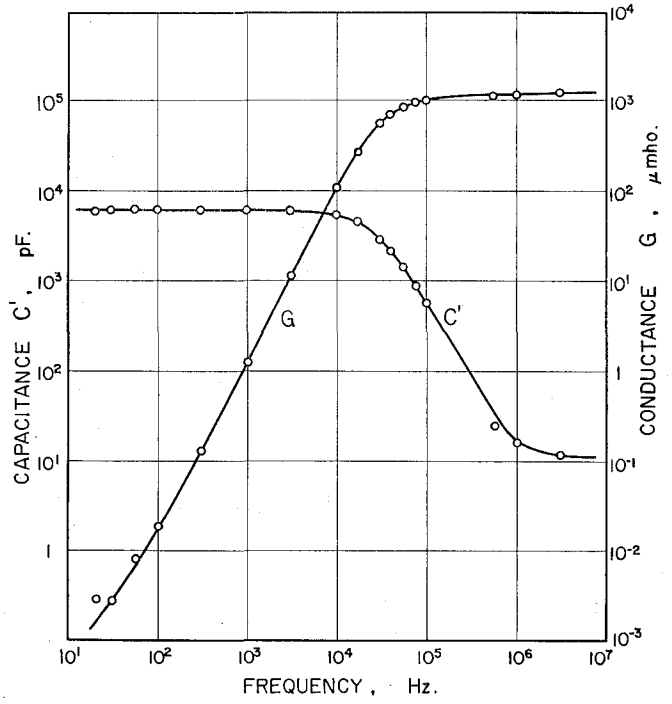


Fig. 1. Frequency dependence of capacitance and conductance of the system composed of a black film of PE and 0.1 M NaCl solution.

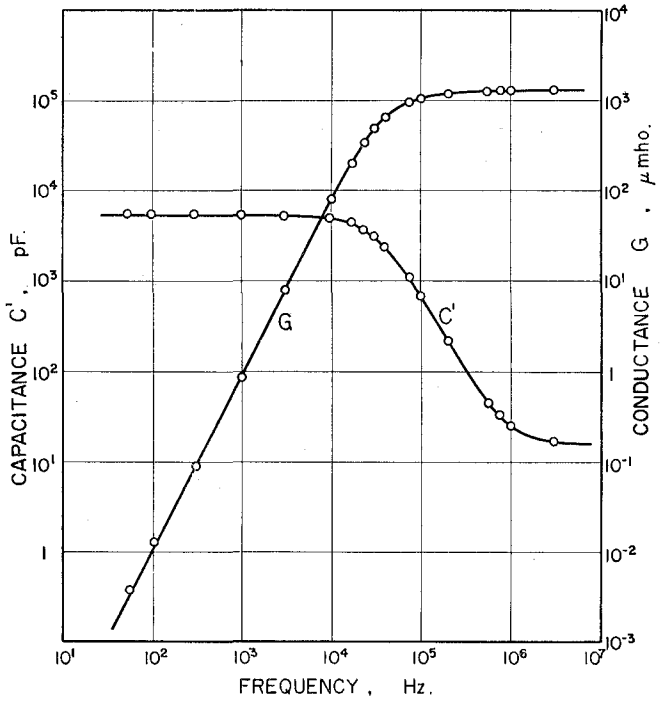


Fig. 2. Frequency dependence of capacitance and conductance of the system composed of a black film of PS and 0.1 M NaCl solution.

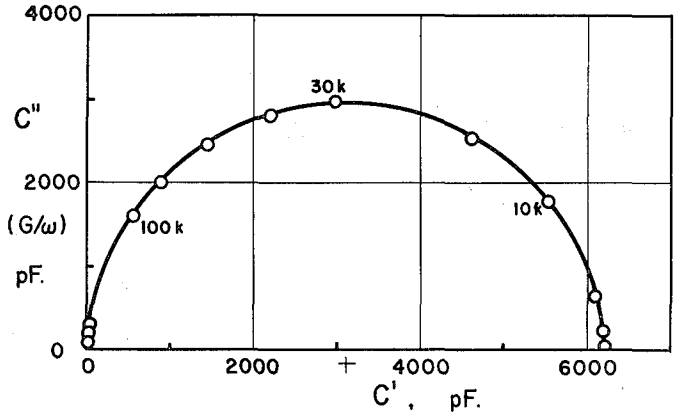


Fig. 3. The plots of the imaginary part against the real part of the complex capacitances for the data on a PE-film system given in Fig. 1. The measuring frequencies are shown on the graph.

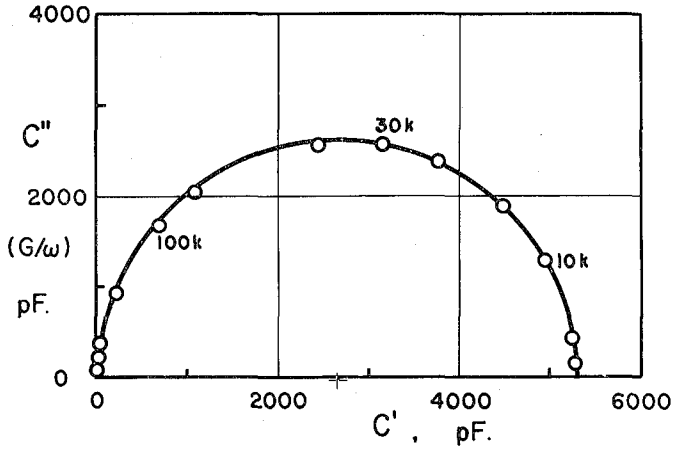


Fig. 4. The plots of the imaginary part against the real part of the complex capacitances for the data on a PS-film system given in Fig. 2.

deduced accurately by extrapolation. The net capacitance of the films was then evaluated from the low frequency capacitance using the standard methods for complex capacitance calculations described in an earlier paper.[6] The capacitance per unit area is given in Table 1, together with the life span of the black films and the breakdown voltage. The latter quantity is to a certain extent an indication of the stability of the films. It may be said from experience that the higher the breakdown voltage the better the stability of the black films.

The d.c. conductance, which was not measured directly in the present study, can be estimated roughly by extrapolating the conductance to lower frequencies using the data shown in Fig. 1. This approximate method gave values of the d.c. conductance less than $1.0\times10^{-8}$ mho/cm$^2$ for all the black films examined.

Table 1. Characteristics of Black Films of Some Lipids Isolated from Bovine Erythrocytes.

| Lipid Specimen | Composition of spreading solution phospholipid: neutral lipid (mg:mg) in 1 ml decane | Number of experiments | Life span of black film (min.) | Break down voltage (d.c. mV) | Capacitance per unit area ($\mu$F/cm$^2$) | General remark on stability* |
|---|---|---|---|---|---|---|
| Phosphatidyl ethanolamine (PE) | 5.7:0 | 23 | > 120 | 210–250 | 0.386 ±0.012 | VS |
| Phosphatidyl serine (PS) | 5.7:0** | 14 | > 120 | 110–180 | 0.328 ±0.007 | VS |
| Phosphatidyl choline (PC) | 5.7:0 | 8 | < 10 | < 60 | 0.326 ±0.022 | LS |
| Sphingomyelin (SM) | 5.7:0** | — | 0 | 0 | — | N |
| Non-phosphorus total neutral lipid fraction (NL) | 0:5.7 | — | 0 | 0 | — | N |
| PC+cholesterol ester fraction | 5.7:4.4 | 6 | > 120 | 130–200 | 0.337 ±0.008 | S |
| PC+triglyceride fraction | 5.5:4.9 | 6 | > 120 | 100–160 | 0.348 ±0.029 | S |
| PC+diglyceride fraction | 2.5:6.1 | 6 | > 120 | 100–120 | 0.289 ±0.010 | S |
| PC+cholesterol fraction | 5.7:2.0 | 6 | > 120 | 130–170 | 0.316 ±0.011 | S |
| PC+total NL fraction | 5.7:1.8 | 6 | > 120 | 200–220 | 0.332 ±0.006 | S |
| | 5.7:4.3 | 9 | > 120 | 110–190 | 0.337 ±0.005 | VS |
| | 5.6:8.7 | 12 | > 120 | 130–210 | 0.399 ±0.023 | VS |
| PC+mixture of four NL fractions | 5.7:10.0*** | 6 | > 120 | 140–160 | 0.297 ±0.059 | VS |
| SM+total NL | 5.6:2.0 | 2 | > 60 | ~ 180 | 0.384 | S |
| | 5.7:4.2 | 8 | > 120 | 350–430 | 0.363 ±0.006 | VS |
| | 5.7:8.3 | 6 | > 120 | 550–650 | 0.393 ±0.094 | VS |

* VS very clean and stable without any shining spot on it, preceded by steady drainage.
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 cut further experiments.
N no possibility to generate black films.

** Since the supernatant was used for generating black films, the concentration of phospholipid in the spreading solution is in effect lower than this value.

*** Neutral lipid 10 mg consisting of 2.1 mg cholesterol ester fraction, 3.1 mg triglyceride fr., 1.5 mg diglyceride fr. and 3.3 mg cholesterol fr.

iii) *Phosphatidyl choline*

Black films stabilized by PC differed in appearance from those formed using PE and PS in that minute bright islands of thick film remained in the black film. Bright and curved lines were also observed. The application of a d.c. voltage of about 30 mV caused these bright regions to disappear giving a uniform black film.

The stability of films formed from this preparation of PC was not very satisfactory as compared with the results reported previously for egg PC[1-12] and yeast PC;[1] in fact, the black films lasted only about 10 min, and the films were broken by applying d.c. voltages as low as 50 mV. Consequently it was not possible to measure the capacitance and conductance over the whole frequency range. However a few points could be obtained at frequencies below 1 kHz which enabled us to extrapolate to the limiting capacitance at low frequencies and so to evaluate the net capacitance of the films.

iv) *Sphingomyelin*

Sphingomyelin (SM) appeared to dissolve less readily in *n*-decane. Using the standard recipe, undissolved sphingomyelin remained at the bottom of the specimen tube. The supernatant was used to make black films. The liquid lamella filling the hole showed interference color patterns at first, then as the film drained, it became entirely first order yellow, this being the interference color produced on reflection in a thin hydrocarbon film of about 1500 Å thick. No black area was observed. A d.c. voltage applied across the film did not cause further thinning to the black state. Pure SM was thus found not to form black films.

v) *Non-phosphorus total neutral lipid fraction*

In this instance the liquid lamella filling the hole did not thin sufficiently to show interference colors but remained in a macroscopically thick state, thus showing no tendency to generate black film. A d.c. voltage applied across the films did not cause them to drain.

It is thus concluded that bovine erythrocyte PE and PS can form stable black films, whereas PC gives less stable films. Pure SM and the non-phosphorus neutral lipid fraction did not separately form black films.

### B. Formation of Black Films by Mixed Lipids.

In contrast to the results when single lipids were used, it was found that the addition of some of the neutral lipid fraction to PC and SM improved the stability of the black films, enabling us to make further measurements. The composition of the spreading solution, the capacitance, the breakdown voltage and the life span of black films formed from such mixtures are given in Table 1.

The addition of the total neutral lipid fraction to PC greatly enhanced the stability of the films, while addition of each one of the neutral lipid fractions separately improved the stability appreciably. In particular it should be noticed that stable films were obtained with the mixture of SM and the total neutral lipid fraction, although our earlier results showed that neither of these substances tended to form black films when used alone. The capacitances of PC and SM films were found to increase as the amount of the neutral lipid fraction increased. A similar tendency was also found by Hanai *et al.*[8] using mixtures of egg PC and cholesterol.

## IV. DISCUSSION

It is concluded that all the lipid components isolated from bovine erythrocytes can form stable black films provided that they are present in suitable mixtures. Par-

ticularly addition of cholesterol, cholesterol ester, diglyceride and triglyceride is seen to reinforce the black films so that the films may change more flexible and pliable to mechanical shock. Some of proteins[8, 31, 42] were found to be effective for reinforcement of the black films. In this case, however, addition of the proteins decreased the rate of drainage of the black films, and made the films more stiff.

For experiments of the black films depleted of steroids, the use of diglyceride or triglyceride is advisable for reinforcement. Alternatively oleyl alcohol and oleic acid are also found to be effective for increasing the film stability.

As regards the electrical conductivity, all the mixed black films showed conductances of the same order of magnitude as those of films formed from single lipids, the values being lower than those for natural membranes by factors of $10^3$ to $10^7$.

According to the present results, PC extracted from bovine erythrocytes seems to give less stable films, while egg PC[1-12] and yeast PC[1] were reported to give stable films. In order to confirm the diversity of the results on PC specimens, the stability of black films of PC extracted from egg yolk was tested with the same equipment and the same degree of technical skill as those used in the erythrocyte PC. The egg PC was confirmed to give very stable films also in the present case. A chloroform solution of the egg PC was then bubbled with air for 8 hours at 25°C. After the chloroform was evaporated, decane was added to the residual egg PC specimen. The decane solution was then bubbled with air for further 8 hours at 60°C. This decane solution of egg PC still generated stable black films. The result shows that PC specimens are stable to the exposure to oxygen in air during the extraction procedure. It is therefore less probable that the less stability of the films formed from the present erythrocyte PC is due to oxidation of the PC molecules in the extraction procedure.

According to analytical results of lipid distribution reported by Nelson,[41] no PC was detected in erythrocytes of sheep, cow or goat. He suggested that appreciable

Table 2. Fatty Acid Carbon Chain Composition of PC and PE Extracted from Bovine Erythrocytes

| Carbon number : number of unsaturated bond | Phosphatidyl choline | | Phosphatidyl ethanolamine | |
|---|---|---|---|---|
| | wt.% | no.% | wt.% | no.% |
| 14 : 0 | 1.4 | 1.8 | — | — |
| 16 : 0 | 12.1 | 13.7 | 5.6 | 6.4 |
| 16 : 1 | — | — | 1.6 | 1.9 |
| 18 : 0 | 21.2 | 21.6 | 5.1 | 5.3 |
| 18 : 1 | < 0.1 | < 0.1 | 50.6 | 52.6 |
| 18 : 2 | 24.7 | 25.5 | < 0.1 | < 0.1 |
| 20 : 0 | 6.7 | 6.2 | — | — |
| 20 : 3 | 14.3 | 13.5 | 5.3 | 5.1 |
| 20 : 4 | 9.3 | 8.9 | 9.7 | 9.3 |
| 22 : 0 | 4.1 | 3.5 | 2.8 | 2.4 |
| 22 : 5 | 6.3 | 5.5 | < 0.1 | < 0.1 |
| 22 : 6 | — | — | 9.1 | 8.1 |
| unidentified | — | — | 10.1 | 8.9 |

amounts of PC which were reported in other literature on cow erythrocytes might well have arisen from failure to remove all of the serum lipoproteins from the packed-cell preparation. If this is the case in the present extraction, unstable black films generated from the present PC fraction might reflect the nature of PC of the serum lipoprotein.

The difference in film-stability between egg PC and bovine erythrocyte PC might be attributed to the variation in their fatty acid constitution. In hopes of giving a clue to the problem, gas-liquid chromatographic analysis was carried out for the present PC and PE. The analytical results of fatty acid carbon chain constitution are given in Table 2. It is noticed in Table 2 that the bovine erythrocyte PC contains almost no oleyl chains in contrast to the abundance in those for the PE. The absence of oleyl chains in PC thus might have some connection with the poor stability of the films. Strictly speaking the comparison should be made between the same kind of phospholipids obtained from different natural sources. Such a consideration will be made in a later paper including detailed investigation on egg lipids.

## ACKNOWLEDGMNTS

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