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Lung Surfactants. II.¹⁾ Effects of Fatty Acids, Triacylglycerols and Protein on the Activity of Lung Surfactant

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The relations between the kinds of fatty acids and triacylglycerols present and the properties of lung surfactants were examined with modified lung surfactants. Palmitic acid–tripalmitoylglycerol, stearic acid–tristearoylglycerol and mixtures of fatty acids–triacylglycerols gave good surface activities with the lung surfactant, but oleic acid–trioleoylglycerol did not give good surface activity *in vitro*. Lung surfactants modified with palmitic acid–tripalmitoylglycerol, stearic acid–tristearoylglycerols and the mixtures restored the initial lung pressure–volume characteristics to the excised lung after these characteristics had been lost as a result of lavage *in situ*.

Fatty acids gave better surface activities with the lung surfactant than triacylglycerols. The lung surfactants modified with palmitic acid, stearic acid and a mixture of fatty acids showed better surface activities *in vitro* and gave better lung pressure–volume characteristics *in situ* than those with oleic acid and triacylglycerols.

The protein contained in the bovine lung surfactant was a lipoprotein in which the molar ratio of phospholipids to protein was about 100:1. The molecular weight of the protein was 35000 and was reduced to 10000 by pretreatment with 2-mercaptoethanol after the removal of the phospholipids from the lipoprotein. This protein contained a large proportion of nonpolar amino acids. The lipoprotein showed spontaneous spreading with 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), and with mixtures of DPPC and palmitic acid or tripalmitoylglycerol. The lipoprotein also enlarged the areas of the hysteresis loops of DPPC and the mixtures.

Keywords—lung surfactant; modified lung surfactant; fatty acid; triacylglycerol; protein; surface activity; spreading; lung pressure–volume characteristic

Although many attempts to prepare lung surfactant from various mammals have been reported,^{2–7)} there is neither a commonly accepted definition of a lung surfactant,³⁾ nor a unique marker for it, except 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC).²⁾

We recently reported a lung surfactant that showed good surface properties.⁸⁾ We examined the relations between the chemical components and the surface properties of lung surfactants modified by adding various components. We found that fatty acids, triacylglycerols and protein were indispensable components for such surface properties in addition to DPPC.^{9,10)} The lung surfactant contained palmitic acid, stearic acid and oleic acid as major fatty acids and the triacylglycerols contained palmitic, stearic or oleic acids as major acyl components.⁸⁾ We used palmitic acid as a fatty acid and tripalmitoylglycerol as a triacylglycerol in experiments on modified lung surfactant,^{10,11)} because they are the most abundant among these components in the lung surfactant.⁸⁾ Then, we examined the relations between surface properties and the kinds and amounts of fatty acids and triacylglycerols present.

Although we treated the surfactant materials with organic solvent to remove protein at one stage of the preparation, a small amount of lipoprotein still remained in it.^{1,8)} The lipoprotein had an effect on surface properties.^{9,10)} Thus, we separated the lipoprotein from the lipids in the surfactant by column chromatography, and examined its physicochemical and surface properties.

Materials and Methods

Materials—DPPC was purchased from Fluka (Buchs, Switzerland). Fatty acids and triacylglycerols were purchased from Tokyo Kasei Co. (Tokyo). All other chemicals were of reagent grade.

Chemical Analyses—The contents of chemical components were determined by the procedures described previously.¹⁾

Modification of Lung Surfactant—A lung surfactant obtained from minced bovine lung by the method described previously⁸⁾ was used. The lung surfactant was retreated with 200 volumes each (v/w) of ethyl acetate and acetone at room temperature to remove neutral lipids and fatty acids. After separation by filtration, the insoluble material was dried by evaporation and dissolved in chloroform. The chemical composition of the retreated lung surfactant is shown in Table I. Chloroform solutions of DPPC and some kinds of fatty acids and triacylglycerols were added to an aliquot of the dissolved surfactant to obtain the desired levels. The content of disaturated phosphatidylcholine (DSPC) in the surfactant was fixed at 47% and the contents of fatty acids and triacylglycerols were each varied from 1 to 25%. Palmitic acid, stearic acid, oleic acid and a mixture of fatty acids (palmitic acid : stearic acid : oleic acid = 4 : 3 : 1) were used as fatty acids. Tripalmitoylglycerol, tristearoylglycerol, trioleoylglycerol and a mixture of triacylglycerols (tripalmitoylglycerols : tristearoylglycerol : trioleoylglycerol = 2 : 2 : 1) were used as triacylglycerols. The mixture ratio used was determined by reference to that of the original lung surfactant.⁸⁾ After these adjustments, the sample was suspended in 0.9% NaCl by the procedure described previously.¹⁾

Measurement of Surface Properties—Surface activity was measured on a modified Wilhelmy surface balance at 37°C. The sample concentration used was 1.5 µg phospholipids/cm² surface area. One minute after carefully applying the sample, the surface area was compressed from 54.0 to 21.6 cm² at 2.4 min/cycle. The spreading rate was measured with a sample of 1.5 µg phospholipids/cm² on the surface of 0.9% NaCl in a round Teflon dish (2 cm deep and 5 cm in diameter) at 37°C. Details of these measurements were described in the previous paper.¹⁾

Measurement of Lung Pressure-Volume Characteristics *in Situ*—Lung pressure-volume characteristics were measured by the procedure of Ikegami *et al.*¹²⁾ with several modifications. The lungs were removed from Wistar rats weighing 200–250 g that had been anesthetized by the injection of sodium pentobarbital and killed by exsanguination from the abdominal aorta. A tube was inserted into the trachea, and the lung was degassed with an aspirator and placed in 0.9% NaCl at 37°C. The pressure was gradually raised with air to 30 cm H₂O with a syringe pump and then lowered to 0 cm H₂O in a like manner. The volume at a pressure of 5 cm H₂O on the deflation limb of the characteristics was represented as a percentage of the total lung capacity (TLC₃₀), that is, the volume at 30 cm H₂O pressure. Then the lung was lavaged about 20 times to remove surfactant with 10–15 ml of 0.9% NaCl each time. The measurement was performed again with the lavaged lung in the same way. After the measurement, a samples of 2.4 mg phospholipids/ml TLC₃₀ was instilled into the lung and the lung was ventilated with air to fully disperse the solution in the alveoli. After the lung had been degassed, the measurement was performed in the same way.

Separation of Protein

(1) **Sephadex LH20 Column Chromatography**—A lung surfactant obtained from minced bovine lungs¹⁾ (1.4 g of phospholipids/4 ml of chloroform-methanol, 2 : 1, v/v) was loaded on a column of Sephadex LH20 (2.8 × 105 cm) packed in the same solvent system. The column was eluted with the same solvent at a flow rate of 0.3 ml/min. Fractions of 3.6 ml/tube were collected and the contents of chemical components were measured. The lipoprotein fraction was collected and the organic solvent was evaporated off.

(2) **Sephadex G75 Column Chromatography**—After lyophilization, the lipoprotein was dissolved in 0.02 M Tris-HCl buffer (pH 8.4) containing 0.02 M EDTA-2Na and 1% sodium dodecyl sulfate (SDS), and applied to a column of Sephadex G75 (1.2 × 120 cm) equilibrated with the same buffer. The column was eluted with the same buffer at a flow rate of 0.5 ml/min. Fractions of 2.1 ml were collected and the contents of protein and phospholipids were measured.

SDS Polyacrylamide Gel Electrophoresis—SDS polyacrylamide gel electrophoresis was performed by the procedure of Fairbanks *et al.*¹³⁾ with a slight modification. About 1 mg of protein was dissolved in 1 ml of 0.01 M Tris-

TABLE I. Chemical Composition of Retreated Lung Surfactant

Composition	(%)
Phospholipids	95.7
Triacylglycerols	0.6
Diacylglycerols	0.2
Fatty acids	0.5
Cholesterol	0.0
Protein	2.2
Carbohydrate	0.8

HCl buffer (pH 8.0) containing 1% SDS and the solution was incubated at 37°C for 2 h. In some experiments, 10 μ g of 2-mercaptoethanol was added to the solution before the incubation. Electrophoresis was performed in 7.5% polyacrylamide gels (pH 7.4). Bovine serum albumin (Armour, MW=67000), egg albumin (Sigma, MW=45000), chymotrypsinogen (Sigma, MW=25000) and cytochrome c (Sigma, MW=12500) were used as standard proteins for the determination of molecular weight.

Amino Acid Analysis—Protein specimens were hydrolyzed with 6N HCl in sealed and evacuated tubes at 110°C for 24 and 72 h. Amino acids in the hydrolyzate were analyzed with a Hitachi KLA-5 automatic amino acid analyzer. Tryptophan has determined spectrophotometrically according to the method of Goodwin and Morton.¹⁴⁾

Preparation of Mixtures of Lipoprotein and Lipids—Chloroform solutions of the lipoprotein, DPPC, palmitic acid and tripalmitoylglycerol were suitably mixed and the contents of these components were adjusted to the ratio of DPPC : palmitic acid or tripalmitoylglycerol : lipoprotein=100:10:0.5 (w/w). After removal of the solvent by evaporation, the mixture was suspended in 0.9% NaCl by sonication.

Results

Activities of Lung Surfactants Modified with Various Fatty Acids-Triacylglycerols

Typical surface activities are shown in Fig. 1. Palmitic acid-tripalmitoylglycerol, stearic acid-tristearoylglycerol and mixtures of fatty acids-triacylglycerols gave good surface activities with the lung surfactant. Oleic acid-trioleoylglycerol was less effective.

As shown in Fig. 2, all the lung surfactants modified with fatty acids-triacylglycerols exhibited spontaneous spreading (in decreasing order of spreading rate: oleic acid-trioleoylglycerol, the mixtures, palmitic acid-tripalmitoylglycerol and stearic acid-tristearoylglycerol).

Lung surfactants modified with palmitic acid-tripalmitoylglycerol, stearic acid-tristearoylglycerol and the mixtures showed good lung pressure-volume characteristics *in situ* and there were no significant differences among the three, as shown in Fig. 3. Lung surfactant modified with oleic acid-trioleoylglycerol showed inferior lung pressure-volume characteristics.

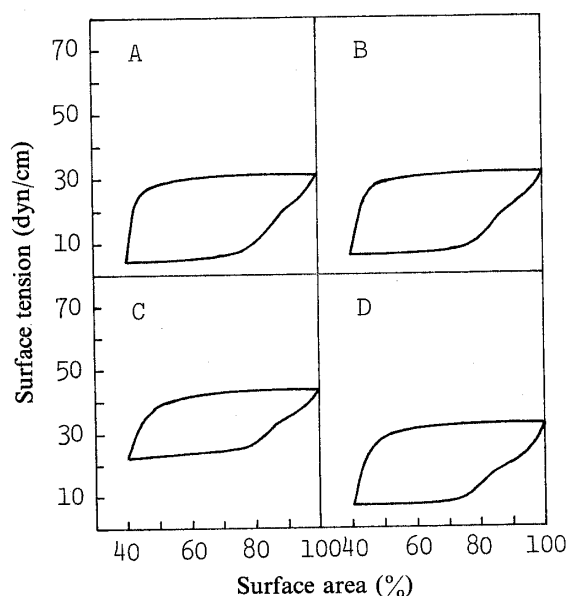


Fig. 1. Surface Activities of Lung Surfactants Modified with Various Fatty Acids-Triacylglycerols

Typical surface activities of the surfactants adjusted to 47% DSPC, 7% fatty acids and 7% triacylglycerols are shown. A, palmitic acid-tripalmitoylglycerol; B, stearic acid-tristearoylglycerol; C, oleic acid-trioleoylglycerol; D, mixtures of fatty acids-triacylglycerols.

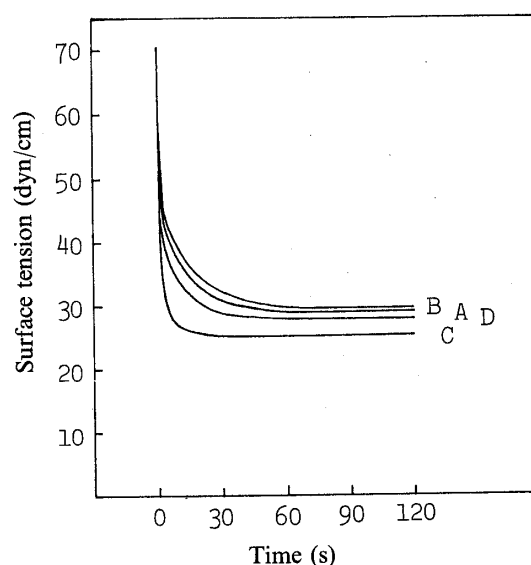


Fig. 2. Spreading Rates of Lung Surfactants Modified with Various Fatty Acids-Triacylglycerols

Typical patterns of the surfactants adjusted to 47% DSPC, 7% fatty acids and 7% triacylglycerols are shown. A, palmitic acid-tripalmitoylglycerol; B, stearic acid-tristearoylglycerol; C, oleic acid-trioleoylglycerol; D, mixtures of fatty acids-triacylglycerols.

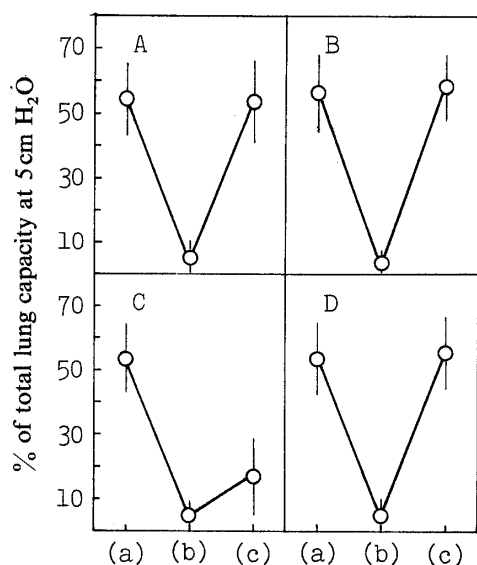


Fig. 3. Percentages of Total Lung Capacity at 5 cm H₂O of Lung Surfactants Modified with Various Fatty Acids-Triacylglycerols

The lung surfactants adjusted to 47% DSPC, 7% fatty acids and 7% triacylglycerols were used. (a), initial; (b), after lung lavage; (c), after the instillation of the lung surfactant. A, palmitic acid-tripalmitoylglycerol; B, stearic acid-tristearoylglycerol; C, oleic acid-trioleoylglycerol; D, mixtures of fatty acids and triacylglycerols. Mean \pm S.D., $n=5$.

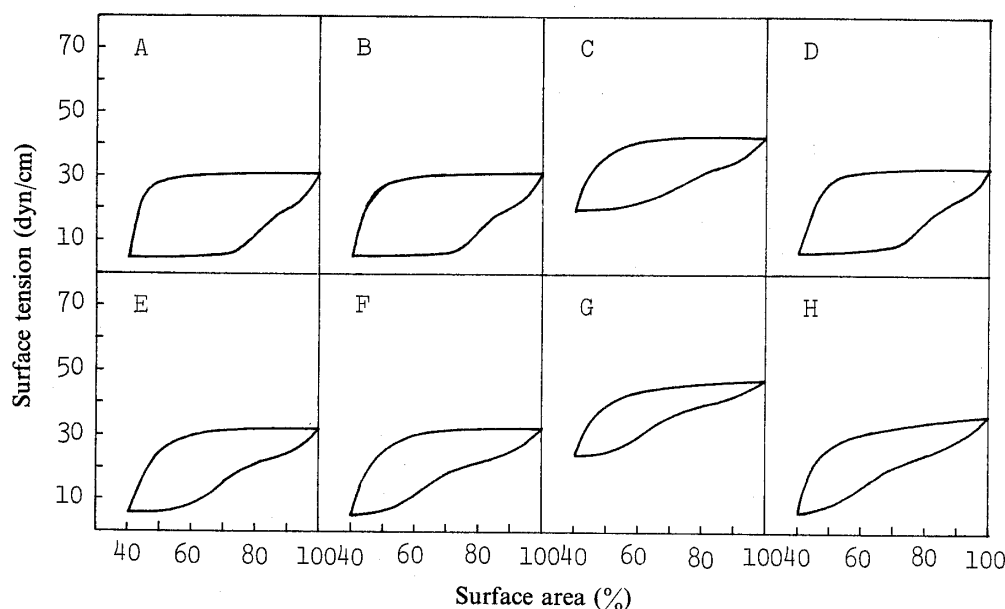


Fig. 4. Surface Activities of Lung Surfactants Modified with Various Fatty Acids or Triacylglycerols

Typical surface activities of the surfactants adjusted to 47% DSPC and 16% fatty acids or triacylglycerols are shown. A, palmitic acid; B, stearic acid; C, oleic acid; D, a mixture of fatty acids; E, tripalmitoylglycerol; F, tristearoylglycerol; G, trioleoylglycerol; H, a mixture of triacylglycerols.

Activities of Lung Surfactants Modified with Fatty Acids or Triacylglycerols

Typical surface activities are shown in Fig. 4. Fatty acids generally gave better activities with the surfactant than triacylglycerols. Palmitic acid, stearic acid and a mixture of fatty acids gave good surface activities, but oleic acid and trioleoylglycerol did not.

Although both fatty acids and triacylglycerols produced spontaneous spreading with the lung surfactant, fatty acids produced faster spreading rates at lower concentrations than triacylglycerols. As shown in Fig. 5, palmitic acid and tripalmitoylglycerol gave similar spreading rates at concentrations of 5% and over, while palmitic acid gave faster spreading rates than tripalmitoylglycerol at concentrations of 3% and less.

Lung surfactants modified with palmitic acid, stearic acid and the mixture of fatty acids

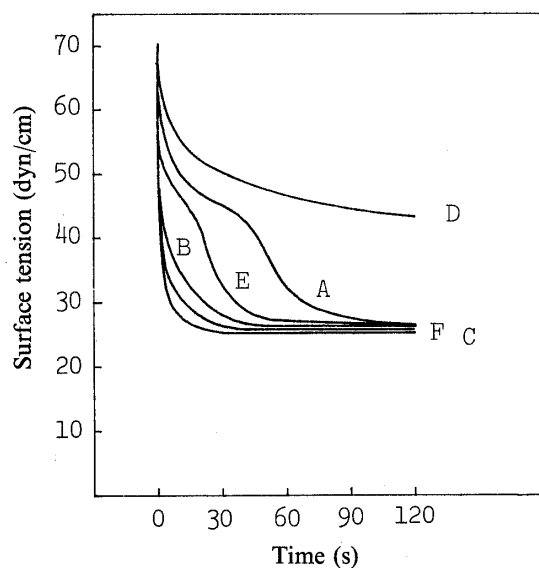


Fig. 5. Spreading Rates of Lung Surfactants Modified with Palmitic Acid and Tripalmitoylglycerol

A, palmitic acid 1%; B, palmitic acid 3%; C, palmitic acid 5%; D, tripalmitoylglycerol 1%; E, tripalmitoylglycerol 3%; F, tripalmitoylglycerol 5%.

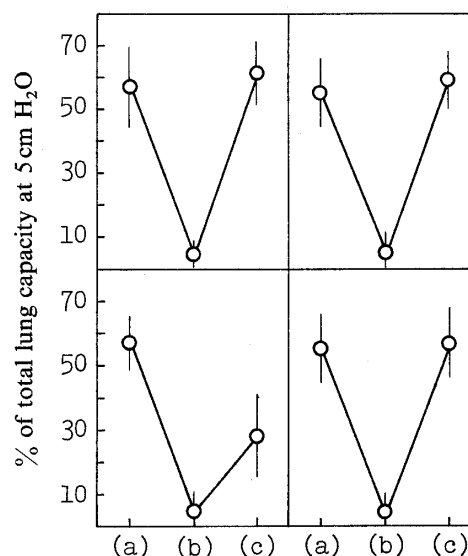


Fig. 6. Percentages of Total Lung Capacity at 5cm H₂O of Lung Surfactants Modified with Various Fatty Acids

The lung surfactants adjusted to 47% DSPC and 16% fatty acids were used. (a), initial; (b), after lung lavage; (c), after the instillation of the lung surfactant. A, palmitic acid; B, stearic acid; C, oleic acid; D, a mixture of fatty acids. Mean \pm S.D., $n=5$.

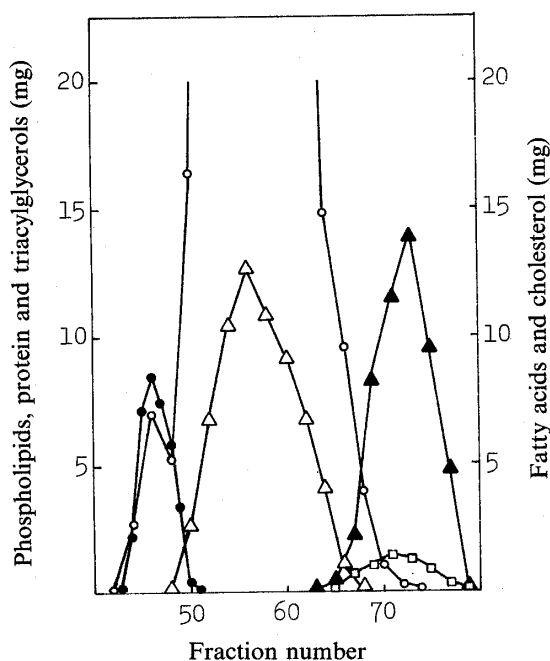


Fig. 7. Sephadex LH20 Column Chromatography of Lung Surfactant

○, phospholipids; ●, protein; △, triacylglycerols; ▲, fatty acids; □, cholesterol.

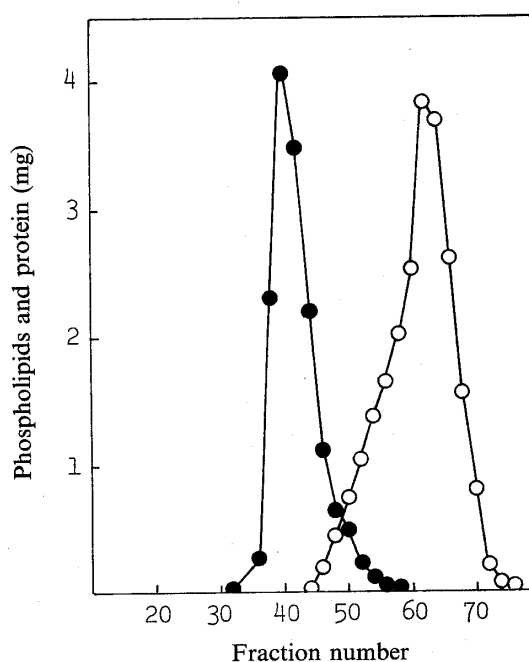


Fig. 8. Sephadex G75 Column Chromatography

○, phospholipids; ●, protein.

showed good lung pressure-volume characteristics, but that modified with oleic acid showed inferior characteristics (Fig. 6). Those modified with triacylglycerols also showed inferior characteristics.



Fig. 9. SDS Polyacrylamide Disc Gel Electrophoresis

(A), before 2-mercaptoethanol treatment.
(B), after 2-mercaptoethanol treatment.

TABLE II. Amino Acid Composition of Protein of Molecular Weight 35000

Nonpolar amino acid	Weight (%)	Residue Mole protein	Polar amino acid	Weight (%)	Residue Mole protein
Glycine	5.2	25	Aspartic acid	6.1	16
Alanine	3.8	15	Glutamic acid	3.8	9
Valine	6.5	10	Arginine	7.1	15
Leucine	20.0	55	Histidine	1.6	4
Isoleucine	7.7	21	Lysine	4.8	12
Methionine	3.4	8	Cysteine	6.7	20
Phenylalanine	2.8	6	Tyrosine	1.6	3
Tryptophan ^{a)}	1.7	3	Serine	4.8	16
Proline	10.1	32	Threonine	2.4	7
Total	61.2	175	Total	38.9	102

a) By the ultraviolet absorption method.¹⁴⁾

Column Chromatography of Surfactant on Sephadex LH20

Figure 7 shows the elution patterns of the main components of the lung surfactant. Protein was eluted first, after the void volume. Phospholipids and triacylglycerols emerged simultaneously, and fatty acids and cholesterol followed closely. Small amounts of phospholipids were also detected in the protein fractions. Fraction numbers 43—47 were collected as a lipoprotein fraction.

Gel Filtration of Lipoprotein on Sephadex G75

As shown in Fig. 8, the lipoprotein was divided into protein and phospholipids by gel filtration on Sephadex G75.

Molecular Weight of Protein

This protein gave a single band on an SDS polyacrylamide gel as shown in Fig. 9(A). The molecular weight of the protein was determined to be 35000 by comparison with the mobilities of standard proteins. When the protein was pretreated with 2-mercaptoethanol, the molecular weight was reduced to 10000 as shown in Fig. 9(B).

The molar ratio of the phospholipids to the protein of molecular weight 35000 in the

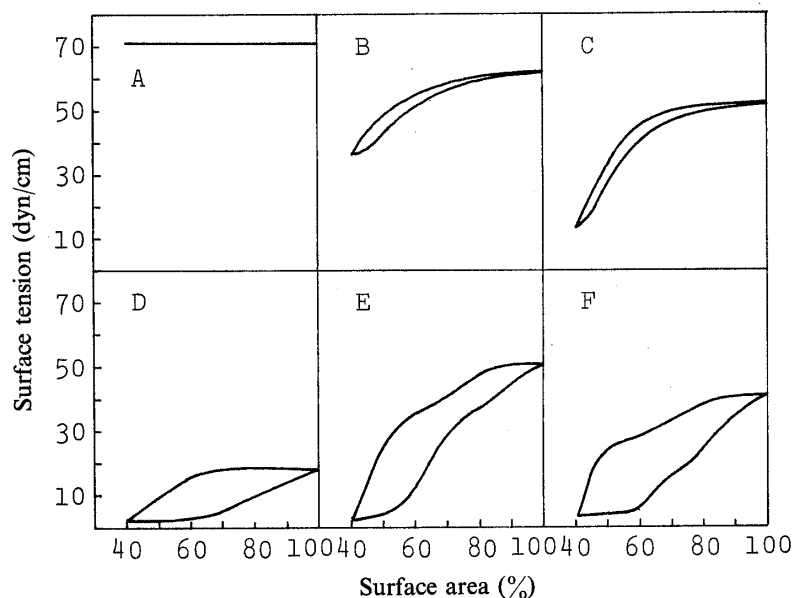


Fig. 10. Surface Activities of Lipoprotein, Mixtures of Lipids, and Mixtures of Lipoprotein and Lipids

A, lipoprotein; B, a mixture of DPPC and tripalmitoylglycerol; C, a mixture of DPPC and palmitic acid; D, a mixture of lipoprotein and DPPC; E, a mixture of lipoprotein, DPPC and tripalmitoylglycerol; F, a mixture of lipoprotein, DPPC and palmitic acid.

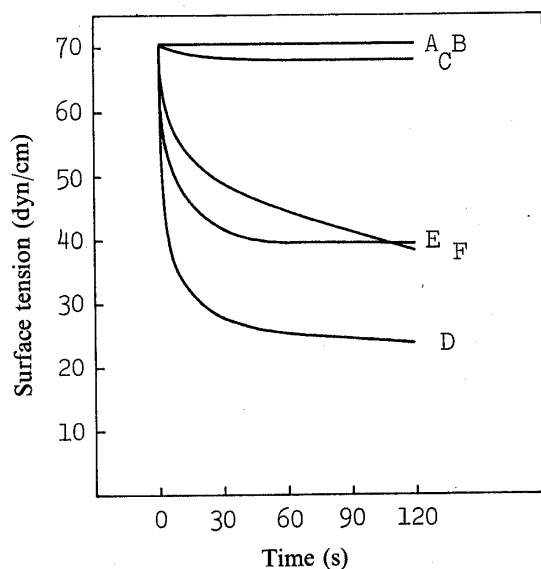


Fig. 11. Spreading Rates of Lipoprotein, Mixtures of Lipids, and Mixtures of Lipoprotein and Lipids

A, lipoprotein; B, a mixture of DPPC and tripalmitoylglycerol; C, a mixture of DPPC and palmitic acid; D, a mixture of lipoprotein and DPPC; E, a mixture of lipoprotein, DPPC and tripalmitoylglycerol; F, a mixture of lipoprotein, DPPC and palmitic acid.

lipoprotein was about 100:1.

Amino Acid Composition

The amino acid composition of the protein of molecular weight 35000 is shown in Table II. It contained a high proportion of nonpolar amino acids, corresponding to about 1.7 times the amount of polar amino acids. This protein was rich in leucine and proline, but poor in tyrosine, tryptophan and histidine.

Surface Properties of Lipoprotein

Figure 10 shows the surface activities of the lipoprotein, mixtures of lipids, and mixtures of the lipoprotein and the lipids. The lipoprotein did not show any surface activity at all. The mixtures of the lipids showed scarcely any hysteresis loops. Although the data are not shown,

DPPC, like the lipoprotein, did not show any surface activity. The lipoprotein rapidly decreased the surface tension in compression, in comparison with DPPC and the mixtures of lipids, and resulted in hysteresis loops having large areas and minimum surface tensions of under 10 dyn/cm.

The spreading rates of the lipoprotein, the mixtures of lipids, and the mixtures of the lipoprotein and the lipids are shown in Fig. 11. The lipoprotein and the mixtures of lipids showed scarcely any spreading. Although the data are not given, DPPC also failed to show spreading. The lipoprotein caused spreading of DPPC and the mixtures of lipids.

Discussion

It is well known that there are fatty acids and triacylglycerols in lung surfactant preparations. In total, they account for 1–10% of the contents of lung surfactants, but the role that they play is not clear.^{2,15–17)} They have been regarded as components derived from blood and lung tissue, and as components that inhibit the surface activity of lung surfactant phospholipids.^{15,18,19)} We found, however, that these components are indispensable as factors for the spreading of lung surfactant.^{9,10)} They are also concerned in the lowering-raising of the surface tension in compression–expansion cycles on a modified Wilhelmy surface balance. They presumably assist in the formation of the multilamella of phospholipid monomolecular films in compression and their collapse in expansion. They may be interposed among the regions of nonpolar fatty acid residues of phospholipids in a manner similar to the relation of phospholipids and cholesterol in monomolecular films.²⁰⁾

Fatty acids gave better surface activity than triacylglycerols. This result suggests that fatty acids can shift more easily among hydrophobic residues in phospholipid films than triacylglycerols, and that the ability to shift is related to the molecular shape. The cylindrical shapes of saturated fatty acids can easily adapt to the dynamic surface action in compression–expansion cycles.

The spreading rates of the surfactant modified with various fatty acids and triacylglycerols increased in the order oleic acid–trioleoylglycerol, palmitic acid–tripalmitoylglycerol and stearic acid–tristearoylglycerol, and this order is in accord with that of their melting points. Their abilities to lower the surface tension in compression were in the inverse order to their spreading rates. Although oleic acid and trioleoylglycerol are liquid at 37 °C, they are unsaturated lipids and have bent molecular shapes. We presume that the spreading rate of lung surfactant is related to the state of the fatty acids and triacylglycerols in the lung surfactant, and that the lowering-raising of the surface tension in compression–expansion cycles is related to the molecular shapes of the components.

The role of cholesterol in lung surfactant is not clear. Cholesterol was not contained in our surfactants (Table I), but cholesterol is contained at a level of 5–11% in lung surfactants according to some reports.^{2,15,21)} It is well known that cholesterol stabilizes phospholipid films,^{20,22–24)} but some researchers reported that cholesterol inhibited the surface activity of lung surfactant.^{15,25–27)} We previously found that cholesterol did not inhibit the surface activity at a low concentration, but did at a high concentration. Cholesterol is probably not an important component in lung surfactant.¹⁰⁾ We presume that, although cholesterol can form stable films with phospholipids in static conditions, *e.g.* liposomes, the flat shape of the cholesterol molecule impedes the formation and collapse of the multilamellar surfactant films in the dynamic surface action of compression–expansion cycles at a high concentration.

In many cases, preparations of lung surfactant were found to contain 10–20% protein.^{2,16,28,29)} The significance of the protein controversial. Although some reports suggested that the protein associated with lung surfactant was a contaminant derived from serum,³⁰⁾ most researchers have concluded that natural surfactant contains lipoprotein.^{31,32)}

Our results support the latter conclusion, but the lipoprotein content that we found was far smaller than those of established lung surfactants. The lipoprotein fully enhanced the surface properties of the lung surfactant at low concentrations of 0.5–2%.¹⁰⁾

King *et al.* obtained non-serum lipoprotein (lipoprotein peculiar to the lung) from the lung surfactant of dogs; the molecular weights of the proteins were 35000 and 10000.^{17,33)} The protein of molecular weight 35000 formed a complex with DPPC and assisted the adsorption of DPPC at the interface of a subphase. The adsorption rate depended on the ratio of lipoprotein–DPPC, but it took 60 min to change the surface pressure by only 5 dyn/cm at a ratio of 1–750.¹⁷⁾ Other researchers have also reported that surfactant lipoprotein reduced the adsorption time of DPPC.^{4,34)} Recently, Suzuki *et al.* reported on the reconstitution of surfactant lipoprotein with pig pulmonary surfactant,³⁵⁾ and described the surface activity and the effect of the lipid constituents of the reconstituted lipid–protein complex.³⁶⁾

Our lipoprotein caused the spreading and the surface activity of DPPC and mixtures of DPPC, palmitic acid and tripalmitoylglycerol. This reaction occurred fully at a concentration of 0.5%, when the molar ratio of lipoprotein–DPPC was about 1–7500 in the mixture. This reaction was not induced by bovine serum albumin or bovine serum β -lipoprotein at concentrations of 0.5–3%.

The molecular weight of the protein was 35000-daltons, similar to those of some other mammalian species.^{37,38)} It is presumed that the protein of molecular weight 10000 is a metabolic fragment of the 35000-daltons protein on the basis of the kinetics of the precursor–product reaction,³⁹⁾ but there is no direct evidence of the relation between them. Sawada *et al.* found a protein having a molecular weight of 36000-daltons in a bovine lung surfactant, but they could not detect a 10000-daltons protein.^{32,40)} Although the 10000-daltons protein was not detected in our native surfactant either, we found that the protein was derived from the protein of molecular weight 35000 by treatment with 2-mercaptoethanol after the removal of the phospholipids of the lipoprotein.

The protein of molecular weight 35000 was contained a large proportion of nonpolar amino acids as already reported for the corresponding proteins of other mammals.^{38,39)}

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