STUDIES ON FACTORS INFLUENCING STABLE BUBBLE FORMATION FROM LUNG HOMOGENATE

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It is assumed that the active secretion of surfactant from alveolar cells and the alignment of surfactant in pulmonary fluid may take an important part in forming the alveolar lining layer at the first breath of the fetus, though it has not been proved yet. As for the pulmonary fluid, it has been suggested by Scarpelli[1], that the composition of the fluid may be important in establishing and maintaining the lining layer during the immediate postnatal period. Contamination of the pulmonary fluid with interfering materials may conceivably inhibit effective layer formation.

The study on interfering materials, however, has been limited because the true nature of the alveolar lining layer and the surfactant has not been clearly elucidated. Recently, Frosolono *et al.*[2] isolated the surface-active material, which they postulated to be in its natural *in situ* form, through a refined analytical method. This has made possible the further investigation of the interrelation between surfactant and interfering materials under simulated biological conditions.

It is known that bubbles prepared by mechanical agitation of bulk liquid extracts from the lung are not stable in spite of containing a sufficient amount of surface-active material in the extract, while the bubbles, which are obtained directly by squeezing lungs and are thought to originate from well-established surface film in alveoles, are stable[3]. Many investigations have been done on the *in vivo* effect of various substances on the latter.[4] In this paper a simple *in vitro* way to evaluate an effective surface film formation using isolated surface-active material from lung and some analysis of interfering factors in forming stable bubbles in lung homogenate were reported. It would be of great importance to discover the factors which interfere with effective surface film formation in this biological materials so as to better understand the alveolar lining layer formation and maintenance in postnatal period.

MATERIALS AND METHODS

Animals: Wistar rats were obtained from the Animal Center Laboratory of Kyoto University and were housed in an air-conditioned room (temperature: 23±2°C, humidity: about 65%) and given commercial stock chow diet and tap water to drink *ad libitum*. Rat fetuses were obtained by cesarean section. Their ages were calculated from the day of copulation, which was

determined by observing vaginal smears every morning, and the day when sperms were found was considered as the 0-day of pregnancy. All rats were killed by cervical dislocation and organs were taken out immediately after sacrifice and were cooled in an ice bath. Fresh pig lung was purchased from a slaughterhouse and stored in a deep freezer until used.

Methods: *Preparation of liquid extracts*——Rat lung washings were obtained by pumping trachea with 1.0 ml of physiological saline solution several times until no more foamy fluid could be obtained and were made up to 15 ml/rat with saline, without centrifugation. In order to remove blood from the lung, perfusion was done by infusion of saline from inferior vena cava with simultaneous exsanguination from aorta under Nembutal anesthesia.

Lung tissues were scraped off from bronchi and vasculatures with pincettes and weighed. Lung and other organs were homogenized in 0.145 M NaCl in 0.01 M tris buffer containing 0.001 M EDTA (pH. 7.4) (homogenizing medium) with a glass homogenizer (2 ml/g wet tissue). An aliquot of this homogenate was diluted to suitable concentration with saline (this was labeled as Homogenate, with or without perfusion). The initial homogenate was centrifuged at 200 *g* for 5 min and the precipitate was re-homogenized with the above-mentioned medium (2 ml/g of original weight). This homogenate was centrifuged as above and the two supernatants were combined (when an aliquot was diluted to suitable concentration, it was labeled as 200 G Supernatant). Isolation of surface-active material from the supernatant was performed according to the method of Frosolono *et al.*[2] In the first ultracentrifugation step, supernatant, interface I and precipitate were obtained. The latter two were added with the homogenizing medium and re-precipitated by centrifuging at 48,000 *g* for 20 min. The precipitates were suspended in saline. These three fractions were labeled as 48,000G Sup, Interface I and 48,000G Ppt, respectively. In pig lung, fraction IB (which was most surface-active according to Frosolono *et al.*) was obtained by the second sucrose gradient ultracentrifugation and washing. For comparison, Pattle's method[4] was also used to obtain bubbles from lung directly in some experiments.

Examination of bubble stability——Various extracts and fractions were suspended in 10 ml of saline and stirred with a magnetic stirrer to obtain foams. The materials of the extracts and fractions forming this 10 ml of saline suspension were all derived from an original 0.6 g wet tissue. After 1 hr agitation, part of the foam was taken out and immersed into a drop of air-saturated saline. Examination of bubble stability was done according to the method of Pattle and Burgess.[4] The stability ratio (s.r.) of the bubbles was calculated by dividing surface area after 20 min (A_{20}) by initial surface area (A_0), i.e., s.r.$=A_{20}/A_0$.

Histochemical observation of bubbles——After completion of bubble stability examination, the foams on the slide glass were dried and stained with Sudan Black B, Alcian blue at pH. 2.5 and P.A.S.

Analysis of lipids——Lipids of suspensions and foams were separately extracted according to the method of Godish and Rhoades[5] and extracts dissolved in chloroform: methanol (2: 1) mixture were analyzed for the qualitative difference in lipid composition by thin layer chromatography (TLC). Development was done by solvent mixture of chloroform : methanol : water (65 : 25 : 4) in a chamber saturated or half-saturated with the solvent at room temperature. Visualisation of spots was done by heating the plates after spraying them with H_2SO_4.

Dragendorff's reagent was also used for the detection of lecithin.

RESULTS

Relationship between the sizes and the stability ratio of bubbles formed by agitation of

Fig. 1 Relationship between the initial diameters and the stability ratios of bubbles formed by agitation of homogenate of various organs and lung washings.

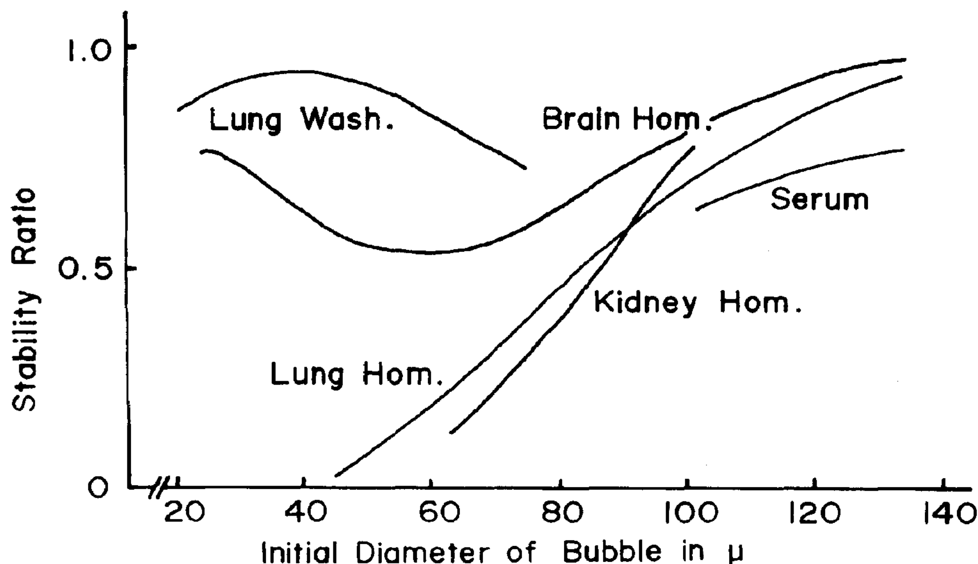


Table. 1 Stability ratio of bubbles (with initial diameters of 37 μ to 75 μ) from various extracts and fractions of organs prepared by mechanical agitation.

Organ	Kind of Extracts	Stability Ratio
Lung, rat	Pattle's method	0.858 ± 0.142(25)
	Lung washings	0.957 ± 0.083(25)
	Homogenate, without perfusion	0.146 ± 0.188(16)**
	Homogenate, with perfusion	0.436 ± 0.280(15)**
	Homogenate, with perfusion, filtered through gauze	0.682 ± 0.213(15)**
	200G Supernatant	0.549 ± 0.316(29)**
	Interface I	0.972 ± 0.026(24)
	Interface I + 48,000G Sup	0.943 ± 0.096(21)
	Interface I + 48,000G Ppt	0.681 ± 0.280(30)**
	Homogenate, fetus at 20th day of pregnancy + 48,000G Ppt of adult rat	0.673 ± 0.292(11)
Lung, pig	0.1 ml of pooled Fraction, IB*	0.955 ± 0.037(17)
	0.2 ml of pooled Fraction, IB	0.973 ± 0.028(55)
Brain, rat	Homogenate	0.652(6)
	Interface I	0.0 (16)
Liver, rat	Homogenate	0.104(3)
	Interface I	0.312(6)

( ) Number of bubbles examined
* This corresponds to the isolated surface-active fraction according to Frosolono et al. 0.2 ml of this material was originated from 0.6 g wet tissue
** Statistically significant compared with rat Interface I : P<0.001

homogenates of various organs and lung washings:

As shown in Fig. 1, lung homogenate, kidney homo genate and serum formed relatively stable bubbles over 100 μ in diameter but, under this size, bubbles of these samples were unstable and their diameters diminished rapidly within 20 min. Hardly any bubbles under 60 μ in diameter were found even at the start of observation. On the other hand, the bubbles from lung washings and from brain homogenate were stable under 100 μ in diameter and especially in the case of lung washings high s.r. was obtained in bubbles with diameters under 75 μ. Bubbles from brain homogenate were relatively stable when their size was under 37 μ and over 90 μ.

Comparison of bubbles from various extracts of adult organs:

In table 1, mean s.r. of bubbles from various extracts of adult rat lung were shown. By Pattle's method, bubbles with diameters of 37 μ to 75 μ had s.r. of 0.858 and bubbles with the same range prepared by mechanical agitation in lung washings had that of 0.957. In homogenate of adult (approximately 4 months after birth) rat lung, the value was 0.146. From brain homogenate, bubbles with relatively high s.r., 0.652, were obtained but from liver homogenate, and stable bubbles of this size range were not formed.

Bubble stability of various fractions from purification steps of lung homogenate in adult rat lung and pig lung:

In perfused lung homogenate, bubbles had s.r. of 0.436. Filtration through 16 sheets of gauze or centrifugation at 200 *g* of perfused lung homogenate had no effect on s.r. In Interface I of perfused lung, s.r. was 0.972. In brain and liver, s.r. of bubbles from Interface I were 0.0 and 0.312, respectively. In Interface I of perfused lung combined with 48,000G Ppt, s.r. was 0.681 and significantly lower than that of Interface I ($P<0.001$). In Fraction IB, s.r. of bubbles from the suspension at two concentrations were 0.955 and 0.973.

Bubble stability of rat lung homogenate according to age:

In fetal rats, s.r. of bubbles from lung homogenate were 0.865 for fetuses at 20th day of pregnancy and 0.984 for fetuses at 21st day of pregnancy (Table 2). This high value was also obtained in the lung homogenate from neonates at 1 day after birth. At 5 days and 1 month after birth stable bubbles were not formed from lung homogenate. Marked decrease in s.r. was observed in bubbles from lung homogenate of fetuses at 20th day of pregnancy when combined with 48,000G Ppt of adult rat lung ($0.05<P<0.1$, compared with homogenate without addition of 48,000G Ppt) (shown in Table 1).

Table. 2 Stability ratio of bubbles (with initial diameters of 37 μ to 75 μ) from homogenate of rat lung prepared by mechanical agitation according to age.

Age at Sacrifice	Stability Ratio
Fetus, 20th day of pregnancy	0.865 ± 0.141(22)**
Fetus, 21st day of pregnancy	0.984 ± 0.020(18)**
Newborn, 1 day after birth	0.951 ± 0.040(11)**
Newborn, 5 days after birth	0.292 ± 0.226(10)
Young, 1 month after birth	0.283(6)

( ) Number of bubbles examined
** Statistically significant compared with Newborn, 5 days after birth : $P<0.001$

Fig. 2 Lipids separated by thin layer chromatography from extracts and fractions of various organs in a saturated chamber with chloroform: methanol: water (65 : 25 : 4).

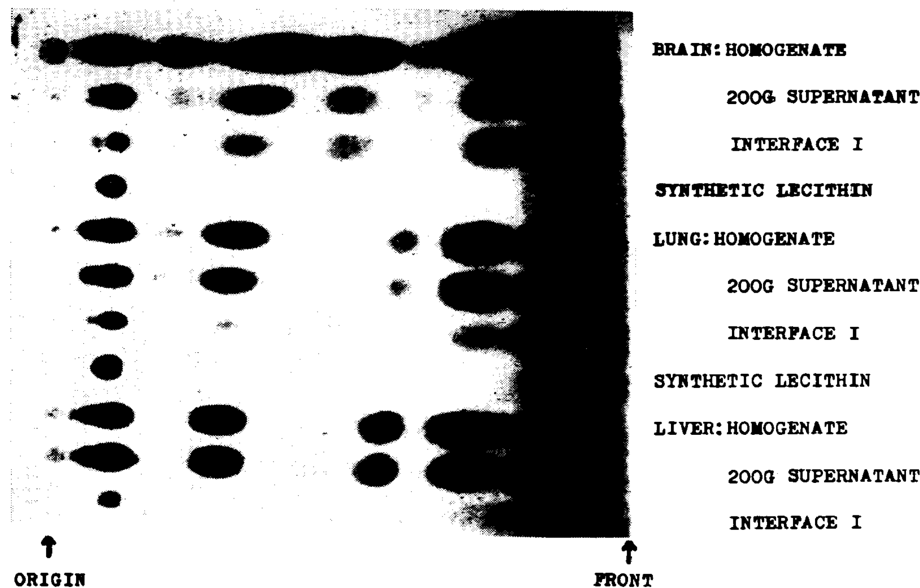
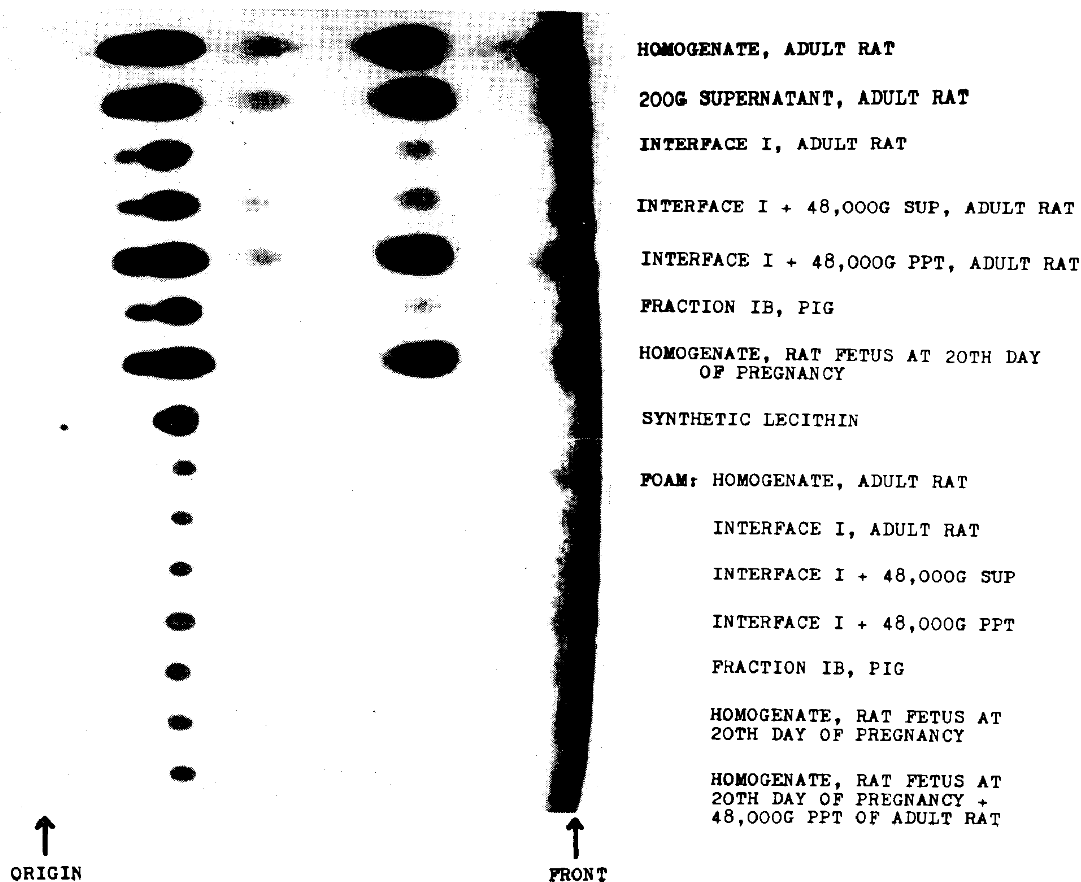


Fig. 3 Lipids separated by thin layer chromatography from various extracts and fractions of rat lung and their foams in a half-saturated chamber with chloroform: methanol: water (65 : 25 : 4).



Thin layer chromatographic observation of lipids of various extracts and fractions and their foams formed by agitation:

Changes in chromatographic pattern of lipids in the brain, lung and liver homogenate according to the purification step were shown in Fig. 2. In the lung and liver, the only lipid which was concentrated in any significant amount by ultracentrifugation was in the lecithin fraction. This spot yielded a reddish orange color through spraying Dragendorff's reagent and had the same mobility as synthetic lecithin both in the saturated and in the half-saturated chamber. In brain, however, a significant amount of contamination by other lipids was still observed in Interface I. In Fig. 3, comparison of lipid composition among fractions of lung homogenate at various purification steps and foams from them was shown. Though addition of 48,000G Sup to Interface I did not change the pattern of lipid composition, addition of 48,000G Ppt markedly altered the pattern and it was almost the same as that of Homogenate or 200G Supernatant. Compared with these findings, little differences were found in lipid composition in foams among 7 extracts and fractions.

Histochemical observation of bubbles after drying:

By microscopic observation, no marked difference was detected in staining properties of surface of bubbles with Sudan Black B stain between lung homogenate and Interface I but significantly larger quantities of balck granules were found in interspaces of bubbles in the lung homogenate. In Alcian blue and P.A.S. stain, significantly larger amounts of stained materials were demonstrated around the bubbles in Homogenate than in Interface I, although in the latter and in pig Fraction IB, those materials were found in small amount. The grade of staining intensity of these materials were summarized in Table 3.

Table. 3 Staining intensity of materials around bubbles dried on glass plates.

Kind of Extracts	Staining Methods		
	Sudan Black B	Alcian Blue at p.H. 2.5	P.A.S.
Pattle's method	+ ~ ++	+	+ ~ ++
Lung washings	+ ~ ++	+ ~ ++	
Homogenate, without perfusion	++ ~ +++	++ ~ +++	++ ~ +++
Homogenate, with perfusion	++ ~ +++	++ ~ ++++	+ ~ +++
Homogenate, with perfusion, filtered through gauze	+ ~ +++	+ ~ ++	
200G Supernatant	+ ~ +++	+	+ ~ ++
Interface I	+	+	+
Interface I + 48,000G Sup	+	+	+
Interface I + 48,000G Ppt	+ ~ ++	+ ~ ++	+ ~ ++
Homogenate, fetus at 20th day of pregnancy	+ ~ ++	+ ~ ++	+ ~ ++
Homogenate, fetus at 20th day of pregnancy + 48,000G Ppt of adult rat	++	+++	+++
Homogenate, fetus at 21st day of pregnancy	++ ~ ++++	+ ~ ++	+++

DISCUSSION

The bubbles observed in this experiment were able to move independently when in close proximity and were not surrounded by ghosts of thickness comparable with their radii. According to Pattle,[6] these are indications that the stability of bubbles is due to the existence of surface active film. As shown in results, from brain homogenate and liver homogenate, and even from the Interface I of these organs, stable bubbles could not be formed at all, although, especially in liver Interface I, purification of lecithin seems to be more complete than in lung Interface I. This is supposed to be due to the difference in the composition of fatty acids of lecithin.[7] By choosing the bubble size from 37 μ to 75 μ, s.r. examination on bubbles formed by agitation can be specific for the determination of effective surface film formation by lung surfactant and a s.r. value of 0.97 can be considered as a standard for the presence of the effective lining film without interference, when substances to be examined in 10 ml saline are extracted from not less than 0.6 g of lung.

According to Pattle and Burgess,[4] the criteria for the presence of normal amount of surface-active material in lung is that s.r. of bubbles squeezed from lung be in the range of 0.60 to 0.87. The results of this experiment concur with that criteria. It was shown that washing-out blood from lung has a marked effect on raising s.r. in homogenate and this is consistent with the report of Scarpelli *et al.*,[8] in which lower minimum surface tension was obtained in mince of perfused lung than in mince of unperfused lung, when surface tension was measured with the modified Wilhelmy balance. It is reasonale to conclude that contamination of blood is one of the factors which decreases bubble stability.

In the present experiment, materials in 10 ml of agitated liquid were adjusted to originate from the same amount of wet lung tissue, so the same amount of surface-active material (or even greater in unpurified samples since the loss during purification steps was not considered in calculation of concentration) was contained in each suspension. The results obtained here, therefore, can be interpreted that materials which interfere with the formation of stable bubble are present in lung homogente of the adult rat even after clearing blood from the lung. Stability of pulmonary bubbles depends mainly on low surface tension of surface film and insolubility of lining film in water. It is known that neutral lipids[9] and ether extracts of tissue[10] inhibit surface activity of dipalmitoyl lecithin on lung extracts. The mechanism of inhibition of surface activity by neutral lipids is explained by substitution of surfactant with neutral lipids and then raising the surface tension of the film. The presence of a large amount of neutral lipids in unpurified extracts was suggested by TLC (although actual identification was not done, it was inferred from solubility in solvents and from mobilities in TLC). The stability of bubbles from unpurified extracts may be partly affected by contamination of neutral lipids but, from the analysis of lipid composition of foams, it was found that there was little difference in lipid composition between bubbles with low s.r. and high s.r. This result suggests that the surface films among these foams would not be so different from eachother. Since there was a great amount of Alcian blue and P.A.S. positive materials (these might be only indications for contaminating materials and the presence of other materials not stained should be also

considered) around bubbles in unpurified extracts, the possibility must be taken into consideration in the first place that these materials may play some more important part in interfering with stable bubble formation than the difference in lipid composition of surface films. Adhesion of surface-active material to another substance in subphase may possibly destroy the stability of surface film and cause it to collapse, in a similar manner to Shulman and Hughes' model[11].

Interestingly, it was shown that in fetal and early neonatal lung stable bubbles could be formed even in homogenate. Fetal rat lung contains surface-active material in late pregnancy, as has been demonstrated by Pattle[12]. Lungs of newborn animals have higher stability index than adult ones and adult excised lung loses its normal stability after numerous cycles of inflation and deflation, as has been reported by Gruenwald[13]. Although recent investigations revealed the developmental changes in lecithin synthesis and quality of lecithin[14,15] in lungs, there seems to be no report that fetal animals have more powerful surface-active material than adults. As for the present experiment, no observable qualitative difference was found in lipid composition of bubbles between adults and fetuses. So this high stability of bubbles of fetal rat lung homogenate might be due to different properties in subphase from adult one. It is noticeable that 48,000 G Ppt of adult rats still had the ability to reduce stability of bubbles with high s.r. in fetal rat lung homogenate. The results of Gruenwald's seem to indicate *in vivo* presence of factors in adult rat lung to inhibit effective surface film formation or maintenance. No definite conclusion can be drawn about the nature or mechanism at present.

Rat lung had a disadvantage for further sub-fractionation of 48,000 G Ppt because of the small amount obtained, although it was favorable in perfusion. Further investigation on interactions between surface-active material and materials in 48,000 G Ppt is now on progress in pig lung.

SUMMARY

A simple *in vitro* way of examining materials interfering with the formation of stable bubbles from lung surfactant was presented. The method consists of agitation of liquid extracts with a magnetic stirrer for 1 hr and examination of bubble stability under a microscope. Bubble stability of perfused lung homogenate from adult rat was significantly greater than that which was not perfused. Foams from isolated surface-active material and lung homogenate of fetal rat showed high stability but those from adult rat lung homogenate showed low stability. In analysis of lipid composition by thin layer chromatography, no significant differences were found among foams from various extracts and fractions from purification steps of surfactant in spite of much difference among the extracts and fractions themselves. Contamination with materials, which were precipitated by sucrose gradient ultracentrifugation of adult rat lung homogenate at 48,000 g, inhibited stable bubble formation in half-purified surface-active material from adult rat lung and in fetal lung homogenate. From these results, it is suspected that inhibiting factor(s) are present in adult rat lung homogenate which interfere with stable bubble formation from surfactant, even if the surfactant itself is sufficient in amount.

REFERENCES

1) Scarpelli, E. M.: The surfactant system of the lung, Lea & Febiger, Philadelphia, 1968, pp. 169.
2) Frosolono, M. F. *et al.*: Isolation, characterization, and surface chemistry of a surface-active fraction from dog lung. J. Lipid Res., 11: 439-457, 1970.
3) Pattle, R. E.: Properties, function and origin of the alveolar lining layer. Nature, 175: 1125-1126, 1955.
4) Pattle, R. E. and Burgess, F.: The lung lining film in some pathological condition. J. Path. Bact., 82: 315-331, 1961.
5) Godish, D. and Rhoades, R. A.: Quantitative recovery of phospholipids from alveolar wash. Resp. Physiol., 10: 396-404, 1970.
6) Pattle, R. E.: Properties, function, and origin of the alveolar lining layer. Proc. Roy. Soc. Biol., 148: 217-240, 1958.
7) Dobiasova, M. *et al.*: Fatty acid composition in developing rats. Biochim. Biophys. Acta, 84: 538-549, 1964.
8) Scarpelli, E. M. *et al.*: Preliminary identification of the lung surfactant system. J. Appl. Physiol., 23: 880-886, 1967.
9) Fujiwara, T. *et al.*: Fetal lamb amniotic fluid: Relationship of lipid composition to surface tension. J. Pediat., 65: 824-830, 1964.
10) Tierney, D. F. and Johnson, R. P.: Factors in tension-area relationship of pulmonary surface films. Physiologist, 4: 122, 1961, cited by Fujiwara *et al.* in reference No. 9.
11) Shulman, J. H. and Hughes, A. H.: Monolayers of proteolytic enzymes and proteins. IV. Mixed unimolecular films. Biochem. J., 29: 1243-1252, 1935.
12) Pattle, R. E.: The formation of a lining film by foetal lungs. J. Path. Bact., 82: 333-343, 1961.
13) Gruenwald, P.: Effect of age on surface properties of excised lungs. Proc. Soc. Exp. Biol. Med., 122: 388-392, 1966.
14) Gluck, L. *et al.*: Biochemical development of surface activity in mammarian lung. IV. Pulmonary lecithin synthesis in human fetus and newborn and etiology of the respiratory distress syndrome. Pediat. Res., 6: 81-99, 1972.
15) Zackman, R. D.: The enzymes of lecithin biosynthesis in human newborn lungs. III. Phosphoryl choline glyceride transferase. Pediat. Res., 7: 632-637, 1973.