Selective reduction of phosphatidylglycerol and phosphatidylcholine in pulmonary surfactant by 4-aminopyrazolo(3,4d)pyrimidine in the rat

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Abstract Effects of 4-aminopyrazolo(3,4d)pyrimidine on pulmonary surface-active material and its surface activity were investigated in the rat. A rapid decrease in serum cholesterol was observed in rats treated with this drug and these effects continued during the entire period of treatment. Phosphatidylglycerol content in surface-active material and in the residual lung decreased significantly during three days of treatment and phosphtidylcholine content in surface-active material also decreased on the third day of the treatment. There were no changes in the contents of other phospholipids and cholesterol. The surface-active material from treated rats showed a larger surface compressibility, an elevated minimum surface tension, and a low stability index, as compared to control rats. These results show that alteration of lipid constituents significantly affects the surface properties of pulmonary surface-active material and that 4-aminopyrazolopyrimidine is a drug which can be effectively utilized to investigate lipid metabolism of the lung.—**Suzuki, Y., and R. Tabata.** Selective reduction of phosphatidylglycerol and phosphatidylcholine in pulmonary surfactant by 4-aminopyrazolo(3,4d)pyrimidine in the rat. *J. Lipid Res.* 1980. **21:** 1090–1096.

Supplementary key words cholesterol · surface compressibility · minimum surface tension

The pulmonary surfactant contains various lipids and proteins (1–4) and reduces surface tension at the air–water interface of alveoli. The major constituent of this lipoprotein is dipalmitoylphosphatidylcholine (DPPC) (1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine) (5), which is considered to be essential in reducing surface tension when the alveolar surface area is compressed. The functional significance of other constituents, such as cholesterol, minor phospholipids (PLs), or proteins is not well understood. A few attempts, however, have been made to correlate surfactant composition with surface activity by altering lipid composition in vivo (6, 7) or by comparing different kinds of natural surfactant (8, 9). Such an approach is required for the clarification of the significance of the constituents to surface activity of surfactant in situ.

In the present experiment, we observed the change in constituents and surface activity of pulmonary surfactant with the administration of 4-aminopyrazolo(3,4d)pyrimidine (4-APP) (10), a potent inhibitor of lipoprotein secretion from liver (11, 12), and our findings are reported herein.

MATERIALS AND METHODS

Animals

Female HLA Wistar rats (Japan Animal Co., Saitama, Japan) weighing 200 to 250 g were housed in an air-conditioned room and given commercial stock food (CE-2, Japan Clea Co., Osaka, Japan) and tap water ad libitum before treatment.

Drug treatment

4-APP purchased from Sigma Chemical Co. (St. Louis, MO) was dissolved in 10 mM NaH_2PO_4, adjusted to pH 3.0 with HCl (4 mg/ml). This compound was administered intraperitoneally in a dosage of 20 mg/kg once daily at 9:30–10:00 AM for 3 days. Control rats were given the vehicle. Animals were fasted from the initiation of treatment and killed at 0, 1, 2, and 3 days after treatment.

Abbreviations: 4-APP, 4-aminopyrazolo(3,4d)pyrimidine; TLC, thin-layer chromatography; GLC, gas–liquid chromatography; MS, mass spectrometry; DPPC, dipalmitoylphosphatidylcholine; PL, phospholipid; PG, phosphatidylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; SPH, sphingomyelin; FAME, fatty acid methyl ester.

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Isolation of pulmonary surface active material

Rats were anesthetized with sodium pentobarbital (50 mg/kg) (Pitman-Moore Inc., Washington Crossing, NJ) and the lungs were perfused with 20 ml of saline via the portal vein with simultaneous exsanguination from the aorta. About 2 ml of blood was withdrawn from the aorta just prior to perfusion and the serum was separated by centrifugation and stored in a deep-freezer until analysis of the cholesterol content. Lung tissues were scraped off the main bronchi and vessels, weighed and homogenized in 0.145 M NaCl buffered with 10 mM Tris-HCl (pH 7.4) containing 1 mM EDTA (called buffered NaCl, hereafter) with a Teflon-pestled Potter–Elvehjem homogenizer. The homogenates were centrifuged at 1500 rpm for 5 min and the supernatants were further processed to isolate surface active materials by sucrose density gradient ultracentrifugation, according to the method of Frosolono et al. (1) (the first centrifugation using 0.75 M sucrose was omitted and this modification did not change the relative PL content, while yielding 140% recovery of PC compared to the original method). Tissues from which total surface-active material was removed were considered as the residual lung.

In another experiment, lung tissues were minced, roughly into 1 × 1 mm blocks, and suspended in buffered NaCl. Air in the lung was removed by suction under negative pressure several times and the suspension was stirred at 4°C for 90 min with care not to destroy tissues by too vigorous stirring. Then the suspension was centrifuged at 1500 rpm for 5 min. The precipitated tissue fragments were homogenized as above and centrifuged. The two supernatants were treated to isolate surface active material, as described above. The former fraction (the saline-extractable) was termed fraction E and the latter (the saline-inextractable) fraction I.

Lipid analysis

Crude lipids were extracted from surface-active materials and the residual lung according to the method of Folch, Lees, and Sloane Stanley (13). For the further separation of PLs, neutral and acidic PLs were separated by DEAE-cellulose acetate column according to the method of Rooney, Canavan, and Motoyama (14) and both neutral and acidic PLs were further separated by two-dimensional TLC of Kieselgel H (Merck, Darmstadt) with a solvent system of chloroform–methanol–water 65:25:4 for the first dimension and of chloroform–methanol–28% NH_4OH 65:35:5 for the second dimension. Qualitative analyses of PLs were performed by comparing mobilities in TLC with PL standards or by specific reagents (ninhydrin, Dragendorff's reagent, α-naphthol reagent, and PAS reaction). Other analytical procedures used for identification of phosphatidylglycerol (PG) were two-dimensional TLC by Poorthuis, Yazaki, and Hostetler (15), mild alkaline hydrolysis (16), and hydrolysis by phospholipase C from *Bacillus cereus* (grade II, Boehringer Mannheim, West Germany). Quantitation of fatty acid, glycerol, and phosphorus was performed according to the methods of Morrison and Smith (17), Renkonen (18), and Bartlett (19), respectively. Phosphatidylcholine (PC), PG, phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), and sphingomyelin (SPH) were quantitated according to the method of Kahovkova and Odavic (20).

Total cholesterol in serum was determined using O-phthalaldehyde and perchloric acid (21, 22), according to modification by Wako Chemical Co. (Kyoto, Japan). Free cholesterol in the surface-active materials was measured by GLC using 5α-cholestane as an internal standard (8).

Diglycerides obtained by hydrolysis of PC with phospholipase C (*Clostridium perfringens,* P-L Biochemicals, Inc., Milwaukee, WI) were quantitated for the relative content of various molecular species by GLC–MS, as previously reported (23).

Operating conditions of GLC-MS

A JMS-D300 mass spectrometer (JEOL Ltd., Akishima, Japan) equipped with a JGC-20K gas chromatograph and JMA-2000 computer system was used. For analysis of fatty acid methyl ester (FAME), 5% Silar-5CP coated on Celite 545A (80–100 mesh, Shimadzu, Japan) was used in a 2 × 2000 mm glass column and operated from 170°C with temperature programming of 3°C/min. For analysis of diglycerides, glycerophosphate, and glycerophosphorylglycerol, 2% OV-1 coated on Chromosorb W (AW-DMCS, 80–100 mesh) was used in a 2 × 500 mm glass column and operated at 290°C, 160°C, and 210°C, respectively. Carrier helium flow was 20 ml/min. Separator and ionizing chamber were kept at 290°C and 200°C, respectively. Electron energy for ionization was 30 or 70eV. Samples were silylated with N,O-bis(trimethylsilyl)acetamide and trimethylchlorosilane at 60°C for 15 min.

Measurement of surface activity

Various amounts of fraction E suspended in buffered NaCl or lipid extracts of fraction E dissolved in chloroform (1 μmol of PC/ml) were applied onto the surface of 50 ml of saline in the trough (maximum and minimum surface area, 36 and 9 cm^2)

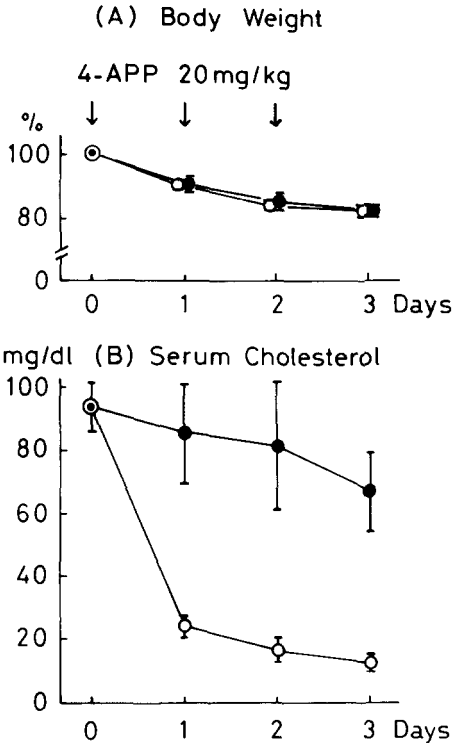

Fig. 1. Body weight (A) and serum cholesterol level (B) during treatment with 4-aminopyrazolopyrimidine (4-APP). 4-APP was administered intraperitoneally as indicated by arrows. Closed circles show control group and open circles 4-APP-treated group. Each point shows mean of values obtained from four to eight animals and vertical bars indicate standard deviations of the mean.

of a modified Wilhelmy balance (Acoma surfactometer, Osaka, Japan) with a microsyringe. After standing 15–20 min, the surface tension was measured with constant expansion and compression of the surface at 1 cycle/3 min at 23–25°C. Stability index (24) and surface compressibility at 15 dynes/cm were calculated from the recorded area-tension diagrams when two successive curves showed the same tracings.

Statistical analysis

Difference between means was statistically evaluated by Student's t test.

RESULTS

Body weight and serum cholesterol

A steady decrease in body weight was observed in control and in 4-APP-treated groups after initiation of fasting with no significant difference between these groups (**Fig. 1A**). In the 4-APP-treated group, a significant decrease in serum cholesterol level was found even after 1 day of the treatment and the level remained around 20 mg/dl during the treatment. In the control group, the cholesterol level fell to 68 mg/dl on the 3rd day of the treatment compared to 95 mg/dl of the initial value (statistically significant at $P < 0.001$) (Fig. 1B).

Lipid content in total surface active material and residual lung

In the neutral PL fraction, three main spots were found having R_f values of 0.40, 0.13, and 0.07 in the first dimension and 0.41, 0.22, and 0.12 in the second dimension, respectively. These were identified as PE, PC, and SPH. In the acidic fraction, the spot having R_f values of 0.20 in the first and 0.12 in the second dimension was PS, the spot having R_f values of 0.07

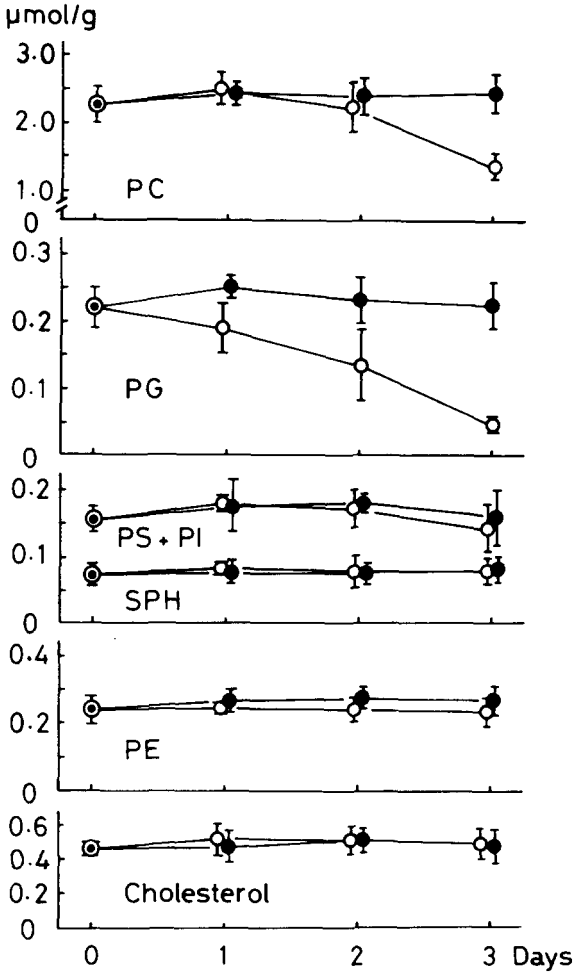

Fig. 2. Change in phospholipid and cholesterol content during 4-aminopyrazolopyrimidine (4-APP) treatment in total surface active material. Closed circles indicate control group and open circles indicate 4-APP-treated group. Treatment was the same as shown in Fig. 1. Abbreviations are PC, phosphatidylcholine; PG, phosphatidylglycerol; PS, phosphatidylserine; PI, phosphatidylinositol; SPH, sphingomyelin; PE, phosphatidylethanolamine. Each point shows the mean value from four to six animals and vertical bars indicate standard deviations of the mean.

in the first and 0.15 in the second dimension was PI and the spot having R_f values of 0.32 in the first and 0.57 in the second dimension was PG. By phospholipase C hydrolysis of this lipid, diglycerides including dipalmitoyl, palmitoyl-palmitoleoyl, palmitoyl-oleoyl, and palmitoyl-linoleoyl diglycerides were found in the ether fraction of hydrolyzate and α-glycerophosphate in the water fraction, which had a characteristically high m/e 357 (base peak) and low m/e 243 ions compared to β-glycerophosphate in addition to m/e 445 (M-15) by GLC–MS. Mild alkaline hydrolysis yielded glycerophosphorylglycerol, which gave m/e 591 (M-15), 516 (M-90), 503 (M-103), 445, 357 (base peak) and 299 ions by GLC–MS, and FAME, whose profile was 2.8% myristic, 53.5% palmitic, 6.6% palmitoleic, 4.6% stearic, 11.5% oleic, 12.5% linoleic, 6.5% arachidonic, and 2.6% other acids (mole ratio). The observed ratio of fatty acid to phosphorus was 2.1:1 (mol/mol) and that of glycerol to phosphorus was 1.9:1.

The change in the PL content together with cholesterol content in the surface-active material is shown in **Fig. 2.** There was a marked decrease in PG even after the 1st day of treatment and the content decreased to about one-fifth of the control on the 3rd day of the treatment (the mean values were significantly smaller than those of control group at $P < 0.05$, 0.05, and 0.001 for the 1st, 2nd and 3rd day of treatment, respectively). PC content also decreased to 58% of control value on the 3rd day ($P < 0.001$). **Fig. 3** shows the change in the PL content of the residual lung and we found that the decrease in PG was less pronounced, compared to the surface active material, although the differences were statistically significant on the 1st ($P < 0.05$), 2nd ($P < 0.05$), and 3rd ($P < 0.001$) days of treatment.

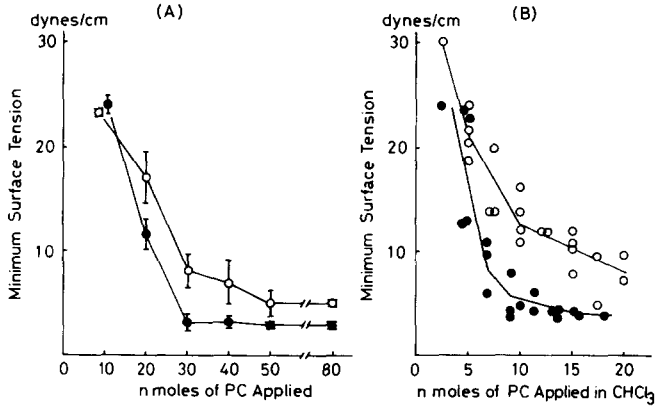

Fig. 4. Minimum surface tension of the suspension of fraction E (surface-active material isolated from saline extract of minced lung) (A) and lipid extracts of fraction E (B) in control (closed circles) and in 4-aminopyrazolopyrimidine (4-APP)-treated rats (open circles). The amount of surface active material or lipid applied to the trough of a Wilhelmy balance was expressed as the content of phosphatidylcholine (PC). In Fig. A, each point represents mean of four to six determinations and vertical bars indicate standard deviations of the mean.

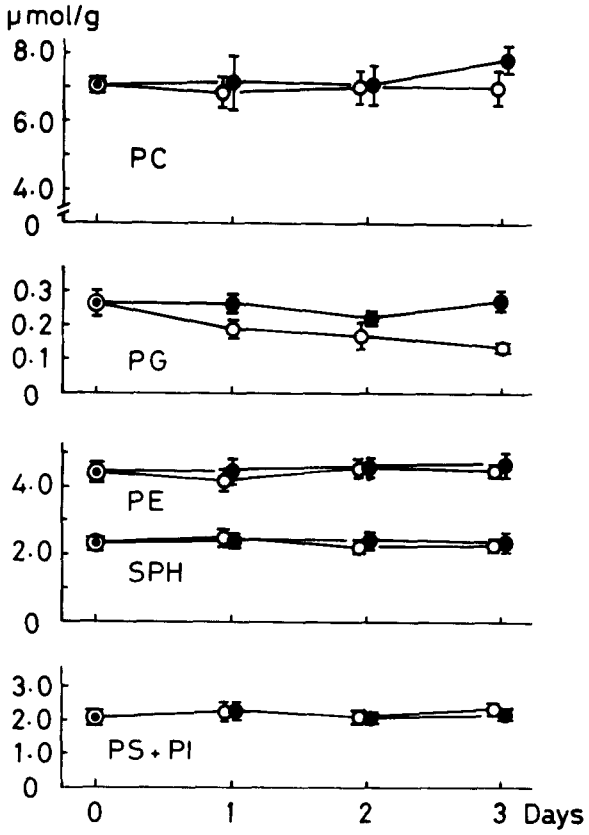

Fig. 3. Change in phospholipid content in residual lung tissue in control and 4-aminopyrazolopyrimidine (4-APP)-treated rats. Symbols and abbreviations are the same as in Fig. 2. Each point shows mean of values obtained from four to six animals and vertical bars indicate standard deviations of the mean.

In the control rats, PC and PG contents of fraction E of surface-active material on the 3rd day of the treatment were 1.10 ± 0.27 and 0.11 ± 0.03 μmol/g wet weight of lung and those of fraction I were 1.09 ± 0.14 and 0.11 ± 0.03 μmoles/g. On the other hand, in 4-APP-treated rats, these values were 0.63 ± 0.09 (statistically significant at $P < 0.01$ compared to that of control rats), 0.02 ± 0.012 ($P < 0.001$), 0.74 ± 0.07 ($P < 0.01$) and 0.03 ± 0.004 μmol/g ($P < 0.001$), respectively.

There was no difference in the relative content of major species of PC between control and 4-APP-treated groups on the 3rd day of the treatment (data not shown).

Surface activity of fraction E of surface-active material from control and 4-APP-treated rats

Saline-extractable surface-active material was obtained on the 3rd day of the treatment and, in control

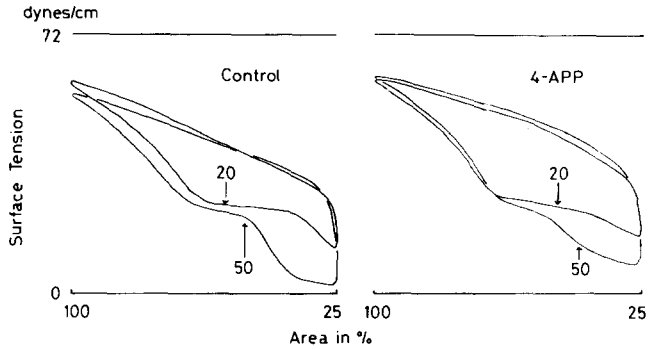

Fig. 5. Surface tension-area curves at low (20 nmoles of PC) and high (50 nmoles of PC) concentration of the suspension of fraction E from control and 4-aminopyrazolopyrimidine (4-APP)-treated rats.

rats, we found that about 22 nmol of PC was required to obtain a minimum surface tension of 10 dynes/cm with suspension of this fraction, but only 6.6 nmol of PC was required when lipid extract of this fraction was used in chloroform (**Fig. 4A** and **B**). With various amounts examined, surface-active material from 4-APP-treated rats exhibited a larger minimum surface tension than that of control rats and about 28 nmol of PC was required with suspension of fraction E and 16 nmol of PC with lipid extract in order to obtain the minimum surface tension of 10 dynes/cm. The representative tension-area curves from control and 4-APP-treated rats are shown in **Fig. 5.** Stability indices were smaller in 4-APP-treated rats than in control rats (**Fig. 6**).

Surface compressibility at 15 dynes/cm of surface tension calculated by the formula: $C = 1/A\ dA/d\gamma$ was larger in the 4-APP-treated group at all amounts examined and the difference between the control and 4-APP-treated groups decreased when the amount applied was increased (**Table 1**).

DISCUSSION

We have clearly demonstrated that the administration of 4-APP caused a significant decrease in the content of PG in the pulmonary surface-active material in the rat. PG was repeatedly identified in the pulmonary surface-active material in the dog (25), rat (14, 26), rabbit, monkey, and man (14) or in the pulmonary perfusate (27), and was established not only to be one of the components of this material but also to be the substance actively synthesized in the lung. All analytical data presented in this experiment for PG corresponded well to data reported by others (14, 25, 27).

The function of PG, however, is unknown and it was speculated that it might play some role in stabilizing the surfactant lipoprotein complex (27) or that it might function as a regulator of the surface properties of PC (25). Hallman and Gluck (9) reported that a larger surface compressibility was obtained in the surfactant lacking PG rather than in the control surfactant having sufficient PG. The larger surface compressibility of surface-active material of 4-APP-treated rats is compatible with these findings. However, with the present experimental results, it cannot be concluded that only the reduction of PG content is responsible for the altered surface properties, because, in 4-APP-treated rats, the relative amount of cholesterol and other minor PLs to PC is elevated compared to the control rats. Addition of cholesterol to surface-active material (28) or to pure DPPC film (29, 30) significantly raises minimum surface tension or alters the surface tension-area characteristics. In order to draw a precise conclusion about the causative material in the altered surface properties, an analysis should be made by changing the single component to show that surface compressibility and minimum surface tension could be independently determined.

To obtain minimum surface tension of 10 dynes/cm, larger amounts of surface-active material in suspension were required, both in control and in 4-APP-treated rats, than the lipid extract. This would be due to more effective surface spreading of lipids by the

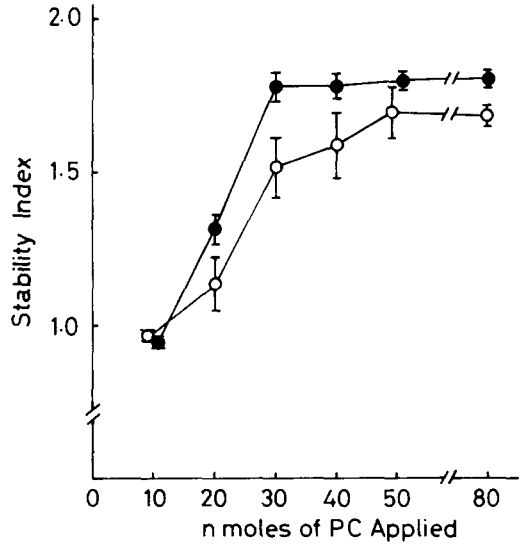

Fig. 6. Stability index of suspension of fraction E in control (closed circles) and 4-aminopyrazolopyrimidine (4-APP)-treated rats (open circles) calculated by the formula: S.I. = $2(\gamma max - \gamma min)/(\gamma max + \gamma min)$ (24). The amount of surface active material was expressed as the content of phosphatidylcholine (PC). Each point indicates the mean of four to six determinations and vertical bars indicate standard deviations of the mean.

TABLE 1. Surface compressibility of pulmonary surface-active materials[a] in control and in 4-aminopyrazolopyrimidine-treated rats

Experimental Group	PC Applied (nmoles)		
	50	40	30
	cm/dyne	*cm/dyne*	*cm/dyne*
Control	0.011 ± 0 (4)[b]	0.012 ± 0.001 (5)	0.012 ± 0.002 (6)
4-APP	0.016[c] ± 0.002 (4)	0.020[c] ± 0.005 (4)	0.023[d] ± 0.004 (4)

[a] Fraction E obtained on day 3 of treatment (See text).
[b] Mean ± S.D. at 15 dynes/cm of surface tension at 23–25°C (No. of experiments).
[c] Statistically significant difference from control at $P < 0.01$.
[d] Statistically significant difference from control at $P < 0.001$.

organic solvent. About 2.5 times more PC in the lipid extract was required in 4-APP-treated rats to obtain a minimum surface tension of 10 dynes/cm than in control rats, while only 1.3 times more suspension was required. Thus, protein(s) in the surface-active material may play some role in the control of surface activity.

Although it is difficult to assess the biochemical changes induced by 4-APP-treatment, it is certain that this change was not simply due to disturbance in the secretion of surface-active material from type II cells into alveoli. In contrast to the action of 4-APP in the liver in which accumulation of lipids was observed (11, 12, 31), reduction of PG and PC in the surface-active material was accompanied by the same degree of reduction of these lipids in both fraction E and I, the former representing mainly extracellular origin and the latter intracellular origin. According to Godinetz, Sanders, and Longmore (27), incorporation of radioactivity from labeled glucose, lactate, and acetate was larger in PG than in PC and PE. Thus, the rapid decrease of PG after treatment with 4-APP may indicate that this lipid has the most rapid turnover rate of all the known PLs in the lung, particularly in the surface-active material.

Further study is underway to clarify the mechanisms involved and important clues related to the problem of the control aspect of lipid metabolism in the lung, and an explanation of the relationship between surface activity and the constituents of surface-active material should be forthcoming.

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