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Research Article

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Alterations in Pulmonary Surface Active Lipids during Exposure to Increased Oxygen Tension *

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Extracts of normal lung have been shown to contain a surface active agent that by decreasing alveolar surface tension serves to stabilize the alveoli during respiration (1–3). This surfactant has been shown to contain lipid probably complexed with protein (4, 5). Several investigators have suggested that the lipid is primarily dipalmitoyl lecithin [L-α-(dipalmitoyl) glycerophosphoryl choline], a highly surface active phospholipid (5–7).

Increased surface tension in lung extracts occurs in certain disease states associated with respiratory distress (2). In man these states include the respiratory distress syndrome of infancy and oxygen toxicity. A similar respiratory distress syndrome can be produced experimentally in dogs and rabbits by exposure to increased oxygen tension. In both clinical and experimental situations respiratory distress, pulmonary edema, and atelectasis are associated with increased pulmonary surface tension (8–10).

Our purpose in the present experiment was to induce changes in pulmonary surface tension in dogs by exposure to increased oxygen tension, to remove pulmonary surfactant by endobronchial lavage, and to measure lipid composition and surface activity. Endobronchial lavage rather than lung mincing was utilized to obtain alveolar surface lipids free of whole lung lipids, since previous studies had shown that whole lung lipids differ markedly from those of the alveolar surface (11).

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The experiment was designed so that progressive changes induced in the lungs by oxygen exposure could be studied. Thus, exposure to oxygen tensions (Po_2) greater than 550 mm Hg was stopped before definite respiratory symptoms appeared or, alternatively, exposure was continued until symptoms of severe respiratory distress and pulmonary edema were clearly evident.

Methods

Mongrel dogs weighing 10 to 20 kg were placed in a 3- × 2- × 3-foot stainless steel box sealed with a transparent Lucite lid. They were fed through a hole in the lid that could be sealed. The box was flushed and then continuously perfused with pure oxygen at 1 L per minute to maintain a positive pressure inside the box at 1 to 2 cm water. The gas inside the box was continuously recirculated through a barium hydroxide cannister. At intervals throughout the procedure samples of gas were taken from the box, and Po_2 and Pco_2 were monitored with O_2 and CO_2 electrodes. Gas tensions in the box were maintained at a Po_2 greater than 550 mm Hg and a Pco_2 less than 10 mm Hg. As controls, two dogs were exposed to a Po_2 of 150 mm Hg in the same apparatus.

The oxygen breathing was continued for periods varying from 44.5 to 52 hours in eight dogs. In two of these dogs (Table I, no. 6 and 13) the procedure was terminated at 52 and 47 hours, when tachypnea and the coughing up of pale watery fluid developed. At the completion of the exposure period, the animals were taken from the box and anesthetized with intravenous Nembutal (30 mg per kg). A modified Carlens tracheal divider was inserted through a tracheostomy and the dog ventilated on pure oxygen with a Harvard pump. The left lung was washed with saline, and the wash fluid (volume approximately 400 ml) was centrifuged, decanted, and lyophilized as previously described (11). Both chest cavities were opened and the lungs visualized. Biopsies of both lungs were taken and fixed in OsO_4 for electron microscopy (12). The animals were then exsanguinated. The lungs were removed and fixed with intratracheal buffered formalin in the inflated position for light microscopy.

As an estimate of the reliability of endobronchial washing as a sampling procedure, four sequential saline washes of 100 ml each were carried out on the left lungs of two normal dogs by the procedure described above.

TABLE I

Alterations in surface tension after exposure to increased oxygen tension

Dog no.	Exposure time	Minimal surface tension: Lung wash	Minimal surface tension: Lecithin fraction	Comments
	hours	*dynes/cm*	*dynes/cm*	
		Exposed to air atmosphere		
9	52	13	3	Control
10	52	20	0	Control
		Exposed to oxygen atmosphere		
4	44.5	6	2	Dyspnea, restlessness
8	48	7	0	Tachypnea
1	49.5	25	0	Irregular respirations
14	49.5	22	11	Tachypnea
7	52	16	6	Cyclic respirations
12	47	23	16	Pulmonary edema
13	52	22	13	Pulmonary edema
6	52	30	25	Pulmonary edema

Lipid extraction and analysis. Lyophilized endobronchial wash fluid obtained by the procedure described above was dissolved in approximately 45 ml water. A sample was first removed for protein determination by the method of Lowry, Rosebrough, Farr, and Randall (13); then methanol (60 ml) and chloroform (120 ml) were added with stirring. The upper and lower solvent layers were separated and re-extracted once more after the scheme previously described (11). This extraction method was found to remove more than 95% of organic phosphorus and cholesterol present. The chloroform extracts were combined, dried by rotary evaporation at 30° C, and the residue dissolved in 10 ml chloroform. After removing a sample for lipid phosphorus determination the entire sample was applied to a silicic acid-Hyflo Super-Cel column. A loading factor of 1 to 2 mg lipid phosphorus per g silicic acid Hyflo Super-Cel was used. Lipids were eluted from this column according to the schedule shown in Table II.

Identification of lipids eluted from the column was made on the basis of thin-layer chromatography in a solvent system of chloroform-methanol-water (95:35:4, vol/vol) or ethanol-chloroform-water (5:2:2, vol/vol) with detection by Ninhydrin reactivity or charring with sulfuric acid. Phosphorus was determined on each eluent fraction (11). In fractions 2 to 4 more than one component was present. To estimate the per cent distribution of each component phospholipids were separated by thin-layer chromatography, and phosphorus was determined directly by the method of Doizaki and Zieve (14). Since fraction 4 was found to be more than 90% lecithin, samples of this fraction were deacylated by alkaline methanolysis (15) and fatty acid methyl esters determined by gas-liquid chromatography. Cholesterol, triglyceride, and total weight determinations were carried out on the neutral lipid fraction (fraction 1) by methods previously described (11).

The lipids of 25 ml of citrated dog plasma were extracted and chromatographed on silicic acid in the manner described above except that the solvent volumes were increased fivefold. The lecithin-containing fraction was identified by thin-layer chromatography and a sample deacylated for determination of fatty acid methyl esters by gas-liquid chromatography.

Measurement of surface activity. Surface tension of lung extracts and lipid fractions was measured by means of a modified Wilhelmy balance (16). This consisted of a Teflon trough lined by a continuous strip of polyethylene film. The maximal surface area of the trough (60 cm^2) could be reduced to a minimal area of 10 cm^2 by a movable barrier driven linearly by a Harvard withdrawal and infusion pump. The compression and expansion cycle was 140 seconds in duration. Surface tension of a film on the surface of clean saline was measured by a platinum float suspended from a transducer and surface area by a potentiometer attached to the drive mechanism. Both suface tension and area were recorded simultaneously and continuously by an x-y recorder. Various lipid components were applied as a chloroform solution (1 μmole in 50 μl) on freshly cleaned saline in a nitrogen atmosphere.

Results

Mongrel dogs were exposed individually to an ambient Po_2 of more than 550 mm Hg for 52 hours or until definite symptoms of pulmonary distress were observed (Table I). These symptoms—restlessness, salivation, labored respirations, and lethargy—appeared within 44.5 to 48 hours, and ex-

TABLE II

Scheme for fractionation of lung wash lipids by silicic acid column chromatography

Fraction	Solvent	Volume*	Major component†
1	Chloroform	8	Neutral lipids
2	Chloroform: methanol (6:1)	6	PE, PDME
3	Ethyl acetate: methanol (3:2)	6	PDME, PS, PI
4	Chloroform: methanol (1:1)	12	PC
5	Chloroform: methanol (1:9)	12	LPC, PC, Sph

* Silicic acid column bed volumes.

† PE = phosphatidyl ethanolamine; PDME = phosphatidyl dimethylethanolamine; PS = phosphatidyl serine; PI = phosphatidyl inositol; PC = phosphatidyl choline; LPC = lysophosphatidyl choline; Sph = sphingomyelin.

TABLE III

Composition of dog lung wash after exposure to increased oxygen tension

	Normal lung wash*	Oxygen exposure									
		Control dogs		Dogs without pulmonary edema					Dogs with pulmonary edema		
		9	10	1	4	7	8	14	6	12	13
Protein, *mg*		47	14		11	75	40	44	360	96	29
Lipid, *mg*	145	36.3	37.1	40.8	19.5	42.2	39.2	34.0	220	218	56.1
Neutral lipid, % *total lipid*	23.5	16.9	19.1	29.5	24.2	22.3	27.8	31.8	30.8	38.4	29.2
Cholesterol, % *total lipid*	9.3	8.0	9.0	20.9	18.5	17.1	13.8	6.1	15.9	19.3	12.8
Triglyceride, % *total lipid*	9.7	8.9	10.3	8.6	5.7	5.2	14.0	25.6	14.9	19.1	16.4
Phospholipid, % *total lipid*	74.1	83.1	80.9	70.6	75.8	77.7	72.2	68.2	69.2	61.6	70.8
Fraction 2, % *total lipid*	11.9	6.1	5.4	3.8	9.3	3.9	4.2	4.2	2.8	0.6	3.2
Fraction 3, % *total lipid*	2.1	20.9	12.5	10.9	15.7	12.6	18.0	9.5	1.7	9.4	14.5
Fraction 4, % *total lipid*	54.8	54.4	60.3	59.0	48.2	57.4	49.4	49.2	54.6	49.0	51.8
Fraction 5, % *total lipid*	2.1	1.4	1.5	0	2.4	3.7	0.7	5.0	10.0	2.7	1.5
Time of exposure, *hours*		52	52	49.5	44.5	52	48	49.5	52	47	52

* See (11).

posure was terminated as soon as symptoms appeared in five of the eight dogs studied. Six of the eight survived both the period of exposure and the subsequent 10-minute period of endobronchial washing. Two dogs, no. 6 and 13, developed acute respiratory distress including production of pale foamy spittle by the end of 52 hours and died during the procedure of endobronchial washing. The lungs of most but not all dogs exposed to high oxygen pressures were grossly hyperemic as compared with the lungs of the control dogs.

The lipid analyses of lung wash lipids after silicic acid chromatography (Table II) are shown in Table III. Fraction 1 contained neutral lipids that were not further fractionated by column chromatography. Cholesterol and triglyceride determinations were made on this fraction and are given in Table III. In seven of eight dogs, a small increase in the percentage of cholesterol and, thus, neutral lipids appeared on exposure to increased Po_2. There was an accompanying relative decrease in phospholipid but, since it was not possible to measure the completeness of removal of surfactant from the lungs by the washing procedure, no statement can be made as to whether there was an absolute change in any component.

Thin-layer chromatography of phospholipid fractions obtained by silicic acid column chromatography (Figure 1) made possible identification of the components and estimation of percentage composition of each fraction. Fraction 1, eluted with chloroform, contained less than 0.2% of total lipid phosphorus. Thin-layer chromatography showed a small amount of material (presumably polyglycerophospholipid) to be present in this fraction. Fraction 2 contained phosphatidyl ethanolamine and phosphatidyl dimethylethanolamine, and fraction 3, phosphatidyl dimethylethanolamine, phosphatidyl inositol, and phosphatidyl serine. The total amount of phosphatidyl dimethylethanolamine was estimated in four dogs after elution from thin-layer chromatograms. It comprised 12.3% and 8.9% of total lipid phosphorus in the control dogs (no. 9 and 10) and 7.3% and 7.5% in two dogs exposed to oxygen. Fraction 4 was phosphatidyl choline (lecithin), and fraction 5 contained lysophosphatidyl choline and sphingomyelin in small amounts. Recovery of lipids applied to the columns ranged from 96 to 107% with a mean of 101%.

The per cent composition of phospholipids (as determined by the elution scheme employed) was not affected by exposure to oxygen (Table III). Specifically, there was no decrease in the percentage of lecithin. Since fatty acyl composition of the lecithin fraction appeared to be related to surface activity (6, 11), each lecithin fraction (eluent fraction 4) was deacylated and fatty acid methyl ester distribution studied by gas chromatography. Several changes (Table IV) are apparent in the group exposed to oxygen as compared to pooled lung wash and normal dogs. First, there is a decrease in palmitic acid (16:0) from 60 to 66% in normals to 47 to 52% in animals ex-

posed to oxygen but without pulmonary edema and a further decrease to 15 to 22% in those animals with pulmonary edema. Concomitantly there is a decrease in total saturated fatty acids that is due almost entirely to the observed decreases in palmitic acid. Secondly, the development of pulmonary edema (dogs 6, 12, and 13) is accompanied by a further decrease in palmitic acid and an increase in arachidonic acid (20:4) together with a rise in total lipid and protein content of the endobronchial wash fluid (Table III).

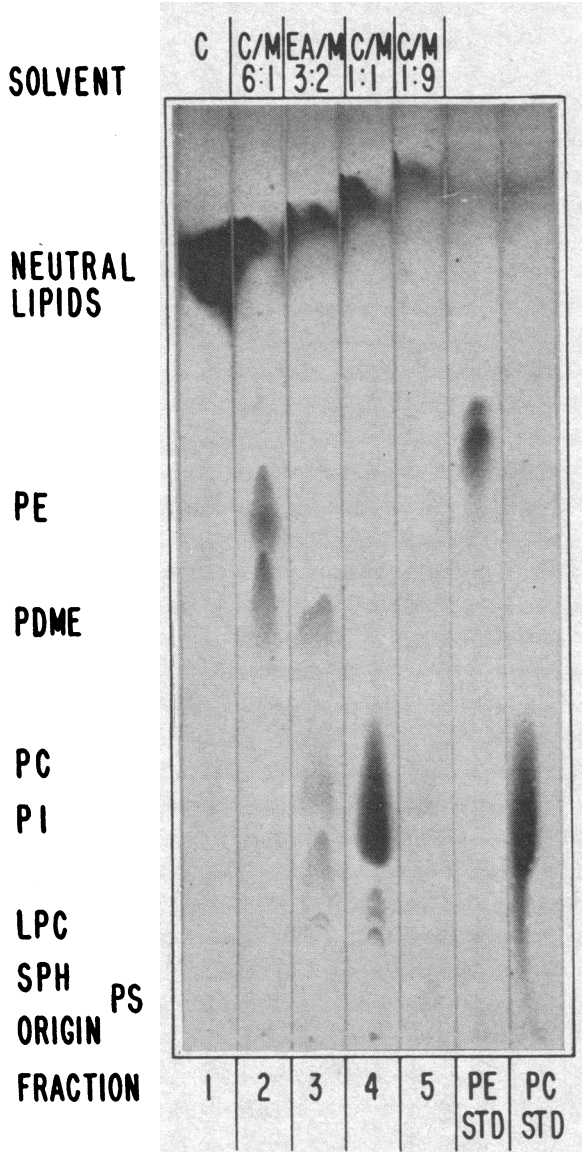

FIG. 1. THIN-LAYER CHROMATOGRAPHY OF REPRESENTATIVE DOG LUNG WASH LIPID EXTRACTS AFTER SILICIC ACID COLUMN CHROMATOGRAPHY. Eluting solvents are shown at top (C = chloroform, M = methanol, EA = ethyl acetate). Chromatography on silica gel G in chloroform-methanol-water (95:35:4, vol/vol) solvent system. Lipids identified are PE, phosphatidyl ethanolamine; PDME, phosphatidyl dimethylethanolamine; PC, phosphatidyl choline; PI, phosphatidyl inositol; LPC, lysophosphatidyl choline; SPH, sphingomyelin; and PS, phosphatidyl serine. STD = standard sample. Chromatograph shown is that obtained from dog 9.

Results of surface tension measurements are given in Table I. Surface activity was measured on the endobronchial washings immediately after collection. Normal activity (less than 21 dynes per cm) was present in both control animals and in three of the eight exposed to increased oxygen tensions. In the other animals exposed to oxygen moderate increases in surface tension were noted. Surface tension was again measured on 1 μmole of lecithin after chromatographic separation. Lecithin from normal lung decreases surface film tensions to less than 5 dynes per cm; by this criterion oxygen exposure produced no consistent changes in surface tension unless pulmonary edema was also present. In this latter situation, surface tension was consistently elevated.

Analyses made of sequential saline endobronchial washes in control dogs showed that each successive wash extracted less lipid and protein, but that the relative composition of protein, total lipid, cholesterol, total phospholipid, lecithin, and esterified fatty acids was about the same in each sample removed. Thus, sequential 100-ml washes removed 39, 24, 17, and 8 mg total lipid with protein: lipid, cholesterol: phospholipid phosphorus, and lecithin: total phospholipid values of 1.13 to 1.27, 0.75 to 0.80, and 0.64 to 0.75 (range of determinations).

The lungs were examined by both light and electron microscopy. By light microscopy several changes were apparent after oxygen exposure; thickening of the alveolar septum and intra-alveolar extravasation of exudate and erythrocytes were most prominent. In the electron micrographs several changes appeared after oxygen exposure. The most marked changes were swelling of the basement membrane, increased numbers of extravascular erythrocytes and phagocytes, and degenerative changes in the lamellar bodies of the type II alveolar epithelial cells. These lamellar bodies were more numerous and showed fragmentation and dissolution of their internal structure after

TABLE IV

Fatty acid composition of lecithin fraction of dog lung wash after exposure to increased oxygen tension

Dog no.	Duration of exposure	Composition of lecithin fatty acids*								Total saturation
		16:0	16:1	18:0	18:1	18:2	18:3	20:4	Other	
	hours	*moles/100 ml*								*moles/100 ml*
Normal dog lung wash†		66	5	7	9	6	0	Trace	7	78
Control dogs										
9		64	8	5	9	1	0	0	13	76
10		60	8	7	16	2	0	0	7	74
Oxygen exposed dogs										
Without pulmonary edema										
4	44.5	52	14	9	15	2	Trace	Trace	8	64
8	48	49	15	7	17	3	Trace	Trace	9	65
1	49.5	47	15	7	16	2	4	Trace	9	63
14	49.5	47	15	9	18	4	0	Trace	7	63
7	52	52	12	6	16	5	Trace	Trace	9	66
With pulmonary edema										
12	47	22	9	11	29	10	Trace	13	6	41
13	52	15	7	14	14	11	5	20	14	46
6	52	22	7	18	14	11	Trace	18	10	49
Whole dog lung		32	5	12	23	6	2	10	0	52
Plasma		16	2	22	15	18	Trace	27	Trace	39

* Trace indicates less than 1 mole per 100 ml present.
† The values for whole lung and lung wash pooled from nine normal dogs are from Morgan, Finley, and Fialkow (11).

oxygen exposure as compared with controls (Figure 2). Further histologic studies now in progress will be reported later.

Discussion

Continued exposure of dogs to increased oxygen tension ($Po_2 > 550$ mm Hg) produces progressively severe respiratory distress and death in 2 to 7 days (10). Pathologically, microscopic evidence of inflammation precedes atelectasis and pulmonary edema, which increase with the duration of exposure (17). The results obtained in the present study showed a similar progression, the alteration from the normal state becoming more marked as exposure to oxygen continued. At about 45 hours of exposure there appeared a decrease in the per cent saturation of fatty acids esterified on phosphatidyl choline, the major surface active agent. Further exposure led to pulmonary edema, possibly as a consequence of this molecular alteration and the concomitant change in surface tension. As plasma containing relatively unsaturated lecithin entered the alveolus, the degree of saturation decreased further. Surface tension of endobronchial wash increased sharply in spite of increasing amounts of lecithin within the alveolus. Clinically, respirations became labored probably due to alveolar collapse and the necessity for forcible reinflation with each inspiration. There was no discernible change in lipid distribution throughout this sequence although the total amount of lipid and protein increased as pulmonary edema occurred.

These results are at variance with those of Fujiwara, Adams, and Seto (18), who found no alteration in surface activity, no change in total lecithin content, and no change in lecithin fatty acyl composition in the lungs of guinea pigs exposed to oxygen. The lipids examined by these workers were probably derived principally from whole lung by the lung mincing procedure and not from the endobronchial wash lipids whose composition has been shown to be quite different (7, 11). Since the lipids of whole lung are relatively much more abundant as compared to endobronchial wash lipids, the latter may contribute relatively little to lipids extracted by mincing techniques. In this respect, their finding of 14.3% palmitic acid in the lecithin fraction coincides more closely with analyses of whole lung or plasma lecithin than with the 60 to 90% esterified palmitate found by most workers using endobronchial washing or foaming techniques (6, 7, 11).

It is also possible that variations in species response may account for the apparently contradictory results of Fujiwara and his associates, since the response to increased oxygen tension

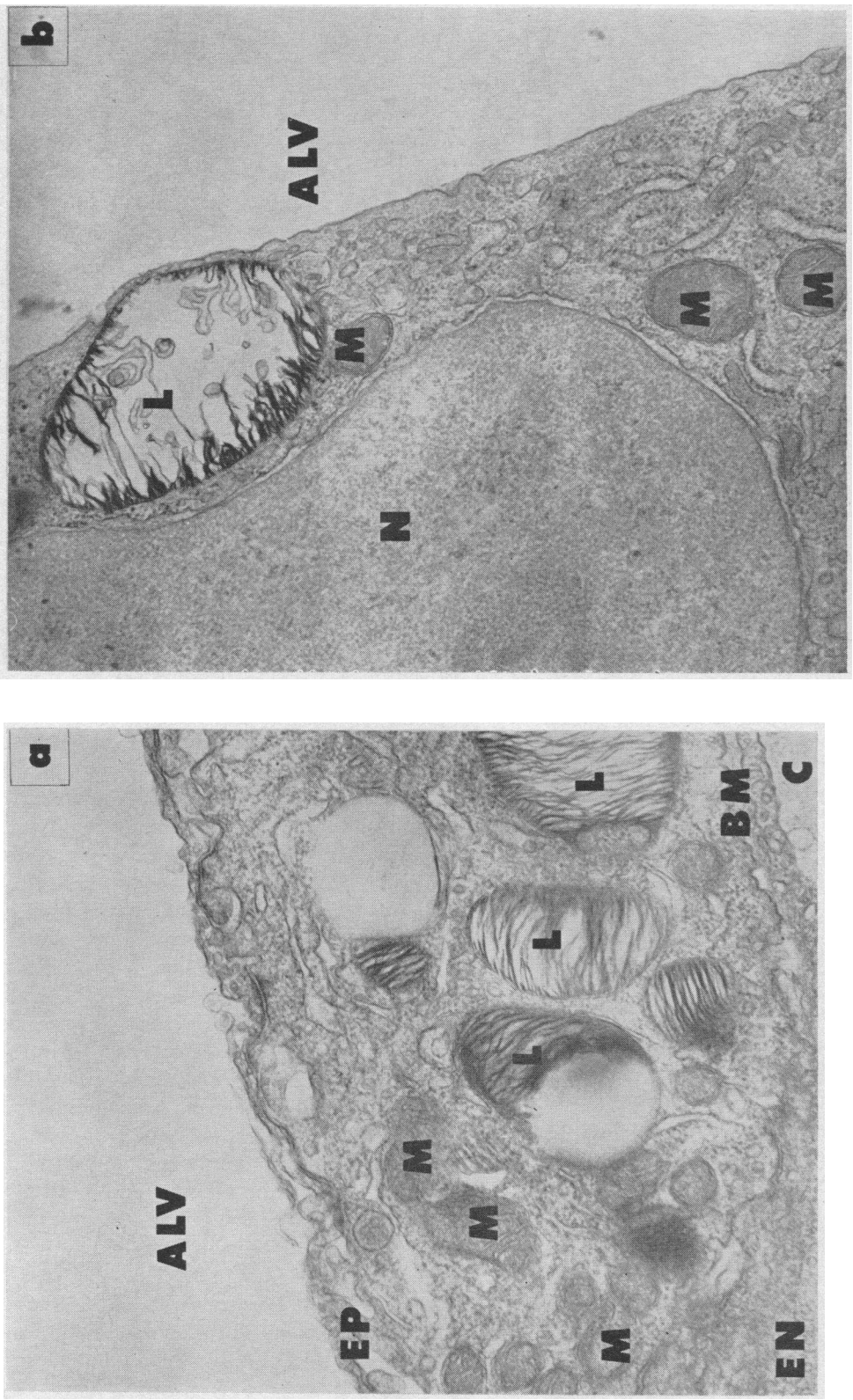

FIG. 2. ELECTRON MICROGRAPHS OF NORMAL AND OXYGEN-EXPOSED DOG LUNG. Sections from inflated right (unwashed) lungs prepared for electron microscopy illustrate alterations of structure of alveolar epithelial cells (type II). Section A from control dog (no. 9) and B from oxygen-exposed dog (no. 4, 44.5 hours). Abbreviations: ALV = alveolar space, BM = basement membrane, C = capillary, EN = endothelial cell, EP = alveolar epithelial cell (type I), L = lamellar body, M= mitochondria, N = nucleus. Magnification: × 43,350.

varies among different animal species. Thus, rats (10, 19), mice (20), and guinea pigs (18) do not manifest the same progressive symptoms of distress and increased surface tension of surfactant extracts on exposure to oxygen that are seen in rabbits (8–10), dogs (10), and man (21, 22). Also, variability in response of individual animals of the same species is apparent. For example, in the present study the degree of unsaturation or appearance of pulmonary edema was not consistently related to duration of exposure although the trend was clearly apparent. Oxygen exposure has been found to produce increased surface tension in lung wash in dogs and rabbits by Giammona, Frayser, Caldwell, and Bondurant (10) and in rabbits by Hackney, Collier, Conrad, and Coggin (8) and Collier (9), but no lipid analyses were carried out by these workers. Therefore, the question of species variation remains unanswered, but for the dog, a species that responds to oxygen exposure with consistent changes in surface activity, the relationship of oxygen exposure to biochemical alterations appears clear.

The minimal surface tensions obtained on endobronchial wash were variable in both experimental and control animals for reasons that are not entirely clear although several tentative explanations may be offered. In an effort to remove as much surface active lipid as possible endobronchial washing with large volumes of saline was employed; consequently the concentration of surface active material in the wash was probably low. It is also possible that the protein moiety of the surface active lipoprotein complex (4–6) may have been denatured or inactivated during the washing procedure. We have not found any evidence to suggest that increased amounts of surface activity "inhibitors" are present or that alterations of protein have occurred in response to oxygen exposure although a specific search for the latter has not been made. Thus, it is possible that inhibition of pulmonary surface active lipoprotein by a mechanism such as that described by Abrams and Taylor (5) may also account for decreased surface activity after oxygen exposure, but such a mechanism would not account for alterations in the lipid components such as we have described.

It is important to consider whether the differences in esterified fatty acids observed after oxygen exposure might be due entirely to the introduction of plasma lipoprotein into the alveolar space. That such is not the case in those dogs without pulmonary edema (no. 1, 4, 7, 8, and 14) is indicated by the values for esterified arachidonate (20:4) in the lecithin fraction (Table IV). Since plasma lecithin contains 27% esterified arachidonate, the presence of amounts of plasma in the alveolus sufficient to cause the observed differences would elevate arachidonate levels above the trace amounts actually observed. On the other hand, in the dogs with pulmonary edema (no. 6, 12, 13) the observed differences could be partially due to plasma lipids.

The mechanism by which oxygen exposure induces biochemical alterations in the lecithin molecule is not clear. We have previously suggested (11) that dipalmitoyl lecithin, the principal pulmonary surface active agent, is formed in lung by conversion of phosphatidyl ethanolamine to lecithin by way of phosphatidyl dimethylethanolamine. A decrease in phosphatidyl dimethylethanolamine content was found in the two dogs exposed to oxygen, but the significance of this observation cannot be evaluated at present. There is electron microscopic evidence, largely circumstantial, to suggest that the site of production of surfactant is the lamellar body of the alveolar macrophage (granular pneumocyte) (23–25). Alteration of these lamellated structures occurs in response to increased P_{CO_2} in guinea pigs with a significant increase in pulmonary surface tension (23, 24). If, as outlined above, decreased biosynthesis of surface active lecithin is caused by oxygen exposure, changes in the lamellar bodies might be expected in the present experiment. An increase in number of these bodies was apparent after oxygen exposure as well as apparent fragmentation and dissolution of their internal structure. Similar alterations in the structure of the lamellar bodies were noted by Treciokas (26) in rats exposed to oxygen, but in neither study was it possible to obtain direct evidence for a link between lamellar bodies and the production of surfactant.

Summary

The syndrome of progressive respiratory distress, pulmonary edema, and increased pulmonary surface tension was induced in eight dogs by exposure to oxygen tensions greater than 550 mm Hg for 44.5 to 52 hours. Pulmonary surfactant

was extracted by endobronchial washing for measurement of lipid composition and surface activity. Five dogs developed respiratory distress without pulmonary edema. In these dogs endobronchial wash surface tension was normal or slightly increased, and total lipid distribution was essentially normal. Esterified fatty acids in the lecithin fraction were consistently altered with a reduction in palmitate and total saturated fatty acids.

Three dogs developed pulmonary edema with increased surface tension, increased total lipid and protein, and relatively decreased total phospholipid in the endobronchial washings. Esterified fatty acids in the lecithin fraction were markedly altered with palmitate levels about one-third normal. Esterified arachidonate was present that was attributed to intra-alveolar plasma.

Electron micrographs of the lung after oxygen exposure showed thickening of alveolar basement membrane and alterations in the structure of the lamellar bodies of the type II alveolar epithelial cells.

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