THE ULTRASTRUCTURAL BASIS OF ALVEOLAR-CAPILLARY MEMBRANE PERMEABILITY TO PEROXIDASE USED AS A TRACER

EVELINE E. SCHNEEBERGER-KEELEY and
MORRIS J. KARNOVSKY

From the Department of Pathology, Harvard Medical School, Boston, Massachusetts 02115

ABSTRACT

The permeability of the alveolar-capillary membrane to a small molecular weight protein, horseradish peroxidase (HRP), was investigated by means of ultrastructural cytochemistry. Mice were injected intravenously with HRP and sacrificed at varying intervals. Experiments with intranasally instilled HRP were also carried out. The tissue was fixed in formaldehyde-glutaraldehyde fixative. Frozen sections were cut, incubated in Graham and Karnovsky's medium for demonstrating HRP activity, postfixed in OsO_4, and processed for electron microscopy. 90 sec after injection, HRP had passed through endothelial junctions into underlying basement membranes, but was stopped from entering the alveolar space by zonulae occludentes between epithelial cells. HRP was demonstrated in pinocytotic vesicles of both endothelial and epithelial cells, but the role of these vesicles in net protein transport appeared to be minimal. Intranasally instilled HRP was similarly prevented from permeating the underlying basement membrane by epithelial zonulae occludentes. Pulmonary endothelial intercellular clefts stained with uranyl acetate appeared to contain maculae occludentes rather than zonulae occludentes. HRP did not alter the ultrastructure of these junctions.

INTRODUCTION

The permeability of the alveolar-capillary membrane to solutes and water has been the subject of many physiological studies (4, 5, 8, 11, 27, 29). However, no attempts have been made to demonstrate morphologically the diffusion barrier to proteins inferred by physiologists, largely because of the lack of adequate electron-opaque tracers which are of small enough dimensions and can be localized sufficiently sharply to reflect physiological events. With the development of a method for the ultrastructural localization of horseradish peroxidase (HRP), such a tracer is now available (15).

The ultrastructure of the mammalian alveolar-capillary membrane was first described in 1952 by Low (22) who conclusively showed the continuous nature of the alveolar epithelium. Later studies of the mouse lung by Karrer (19) further clarified the structure of the alveolar wall. It is now well established that the alveolar-capillary membrane, in its most tenuous portions, is composed of a continuous layer of squamous alveolar cells (type I), interspersed by great alveolar cells (type II), and separated from the underlying capillaries by a basement membrane. The endothelium of the capillaries is continuous and nonfenestrated. In addition, there is indirect evidence that a lipoprotein layer coats the surface of the epithelium, although this layer is usually not seen in routine electron micrographs. In thicker portions of the

alveolar wall a few fibroblasts, elastic, and collagen fibers may be seen (28).

The permeability properties of the alveolar-capillary membrane are clearly of importance in understanding the mechanism of formation and resolution of edema fluid. Drinker et al. (11), from their studies with dye-labeled proteins, suspected that the chief barrier to absorption of proteins from the alveolus lies in the alveolar epithelium, rather than in the capillary endothelium. Indeed, recent physiological studies by Taylor et al. (29) show that the over-all permeability properties of the alveolar-capillary membrane more closely resemble those of a cell membrane than those of a capillary endothelium. Thus, these workers also suggest that the alveolar epithelium represents the major barrier to diffusion of water-soluble solutes across the alveolar membrane.

The present study attempts to define morphologically the permeability barrier to the passage of a low molecular weight protein, such as HRP, across the alveolar-capillary membrane, and suggests that this barrier resides in the zonulae occludentes (12, 13) between alveolar epithelial cells.

MATERIALS AND METHODS

Animals

EXPERIMENTS WITH INTRAVENOUS HRP: Twelve unanesthetized female mice, weighing about 25 g each, were injected intravenously via the tail vein with 5–6 mg HRP (type II, Sigma Chemical Co., St. Louis) dissolved in 0.5 ml of 0.15 M saline. The mice were sacrificed by dislocating the neck at intervals from 1½ to 60 min. Two mice were given two 5 mg doses of HRP at 15-min intervals and sacrificed 15 min after the last injection.

Since HRP has been observed to cause increased vascular permeability in rats, owing to histamine and serotonin release, but not in mice (7), a histamine and a serotonin antagonist were given before the HRP. This was done by the injection of 1 mg/kg body weight pyrilamine maleate and 1 mg/kg body weight BOL 148 (2-brom-D-lysergic-acid diethylamide, Sandoz) dissolved in 0.5 ml of 0.15 M saline 5 min before the injection of 5 mg of HRP intravenously. The mice were sacrificed 3 min after HRP administration. Preliminary experiments on mice had been done with intravenously administered Evans Blue dye as a marker to demonstrate increased vascular permeability produced by intradermal injections of histamine (1:10,000) and serotonin (1:10,000). Half the animals received pyrilamine maleate and BOL 148 intravenously in the above doses before receiving histamine and serotonin. In all instances, these dosages were effective in completely inhibiting the increased vascular permeability due to exogenous histamine and serotonin.

EXPERIMENTS WITH INTRANASAL HRP: For study of the uptake of HRP from the alveolar lumen, 6 mice were anesthetized with 3 mg/100 g body weight pentobarbital sodium given intraperitoneally. 2.5 mg of HRP and 1 mg of Evans Blue dye were dissolved in 1 ml of rat serum. 0.05 ml of this solution was given intranasally over 1 hr. The animals were allowed to recover from the anesthesia and sacrificed 2–6 hr later. Preliminary experiments on 10 mice had shown that doses of HRP greater than 10 mg/ml caused the death of alveolar epithelial cells with extensive permeation by HRP of the underlying basement membrane. Evans Blue dye was added to aid in the gross localization of inhaled HRP.

CONTROL EXPERIMENTS: For a test of whether HRP changed the permeability of endothelial junctions to allow the passage of ferritin, mice were injected intravenously with 50 mg of cadmium-free ferritin (kindly supplied by Dr. G. I. Schoefl) in 0.5 ml of 0.15 M saline and sacrificed after 1 hr. In addition, mice were given 50 mg of cadmium-free ferritin in 0.5 ml of 0.15 M saline and 5 min later were given 5 mg of HRP dissolved in 0.5 ml 0.15 M saline, and were sacrificed 5 min after receiving HRP. No histochemical reaction was carried out on pulmonary tissue from these animals. Animals uninjected with HRP or ferritin served as further controls.

Fixation

The thorax was opened, and the lungs were allowed to collapse spontaneously. The trachea was exposed, a 22-gauge needle inserted, and 0.5 ml of cold formaldehyde-glutaraldehyde (FG) diluted (1:4.5) with 0.1 M cacodylate buffer, pH 7.6, (17) was infused slowly into the lung while the fixative was simultaneously dripped onto the pleural surface. The lungs were fixed in situ for 5 min. The right lung was removed and the lobes were partially cut into slices, 1–2 mm thick. Each lobe was grasped at one tip by a hemostat and fixed by submersion in cold, diluted (1:4.5) FG fixative for 3 hr. The tissue was washed overnight in cold 0.1 M cacodylate buffer, pH 7.6.

Incubation

Frozen sections, 45 μ thick, were cut on a Leitz freezing microtome equipped with a Pel-Cool thermoelectric freezing stage. The sections were incubated at room temperature for 10 min in 5 mg of 3,3′-diaminobenzidine tetrahydrochloride (Sigma Chemical Co.) dissolved in 10 ml of Tris-HCl buffer, pH 7.6. 0.1 ml of 1% H_2O_2 was then added, the

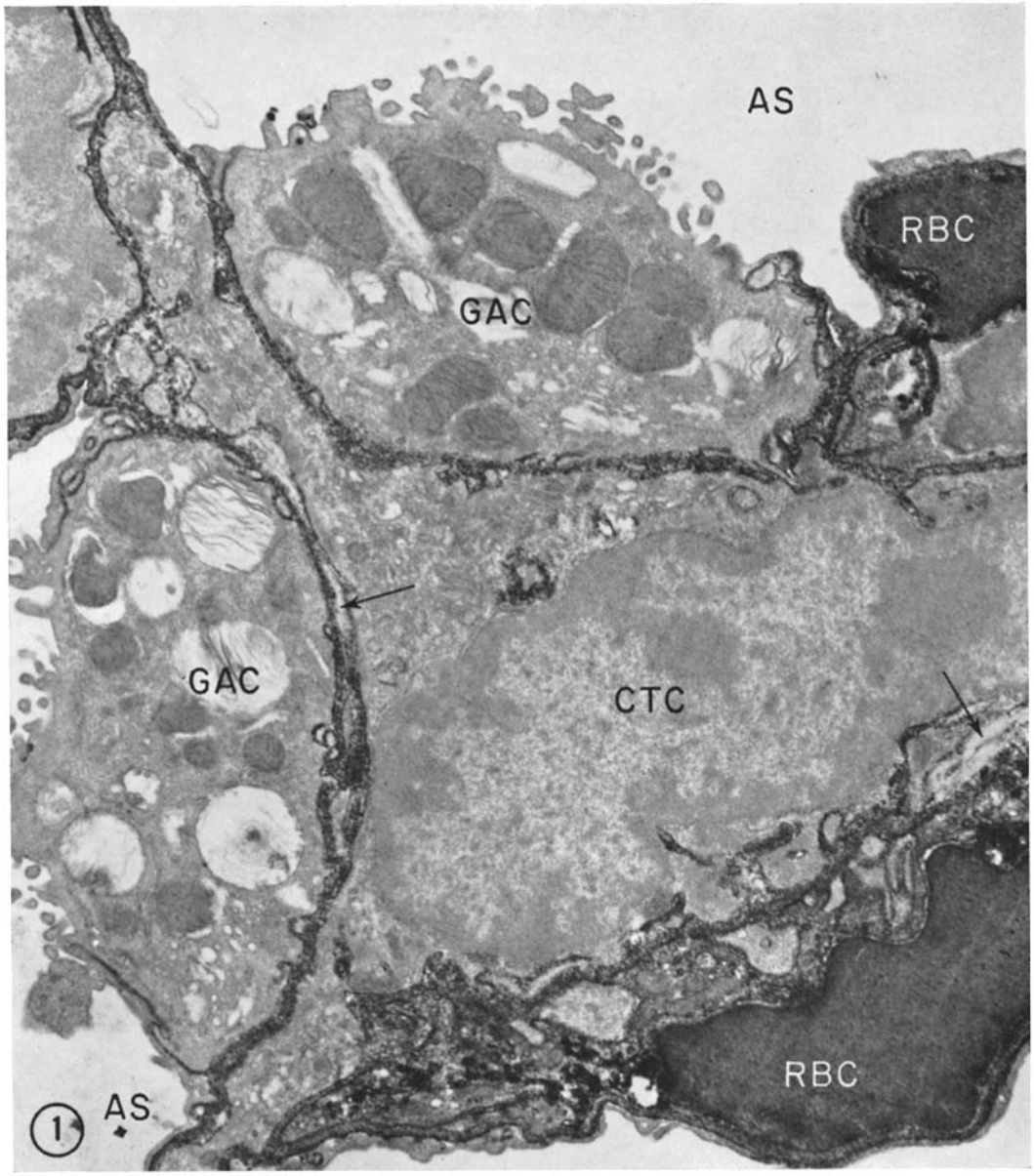

FIGURE 1 Lung from mouse sacrificed 90 sec after HRP injection. Two great alveolar cells (*GAC*) project into the alveolar space (*AS*). A connective tissue cell (*CTC*) is present between two capillaries, each of which contains a red blood cell (*RBC*). Reaction product is present in basement membranes; however, it does not permeate elastic tissue fibers (arrows). × 13,500.

solution gently stirred, and incubation continued for 15 min longer.

Postfixation

Following incubation, the sections were washed three times in distilled water and postfixed for 80 min in 1.3% OsO_4 in *s*-collidine buffer, pH 7.2 (1). Tissues were stained en bloc with uranyl acetate by a modification (18) of Farquhar and Palade's procedure (12). They were dehydrated in ethanol and embedded in Araldite (24). Thin sections were cut on an LKB ultrotome and stained with lead citrate

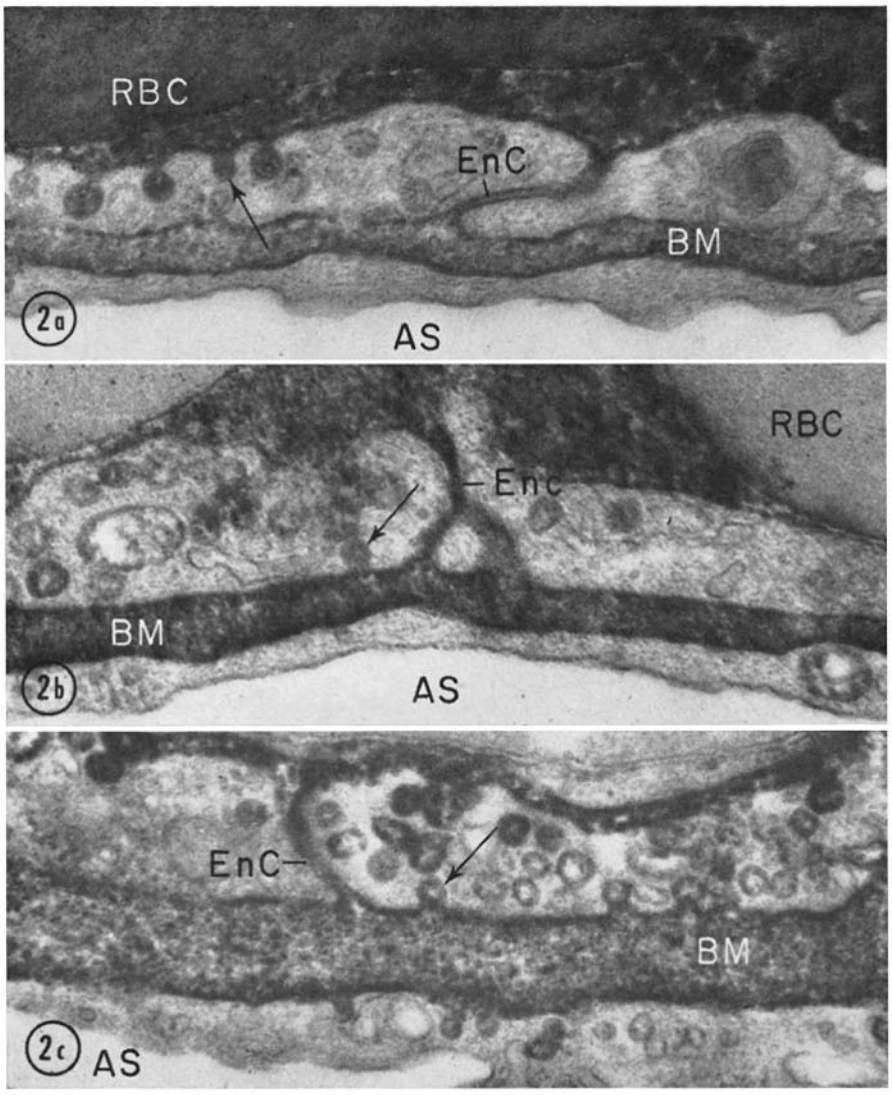

FIGURE 2 Lung from mouse sacrificed 90 sec after HRP injection. Reaction product in the capillary lumen extends through the endothelial intercellular cleft (*EnC*) into the adjacent basement membrane (*BM*). In Fig. 2 *a*, the staining of HRP is quite light. Reaction product is present in endothelial invaginations on both the capillary side (Fig. 2 *a*) and alveolar side (Fig. 2 *b*, *c*) of the cell (arrows). Several apparently unattached vesicles also contain reaction product (Fig. 2 *b*, *c*). × 46,000.

(31) and examined in an RCA EMU-3F or an AEI EM6B electron microscope.

OBSERVATIONS

Light Microscopy

Although most of the observations were made on tissue obtained from the right lower lobe of the lungs, no difference could be detected in tissue taken from other lobes. Brown reaction product was clearly visible in the capillary lumen and basement membrane 90 sec after injection of HRP. However, it became progressively less prominent in tissue obtained later than 15 min after injection. 1 hr after injection, the reaction product was patchy in distribution and absent in many areas. Rare platelet thrombi were observed in a few

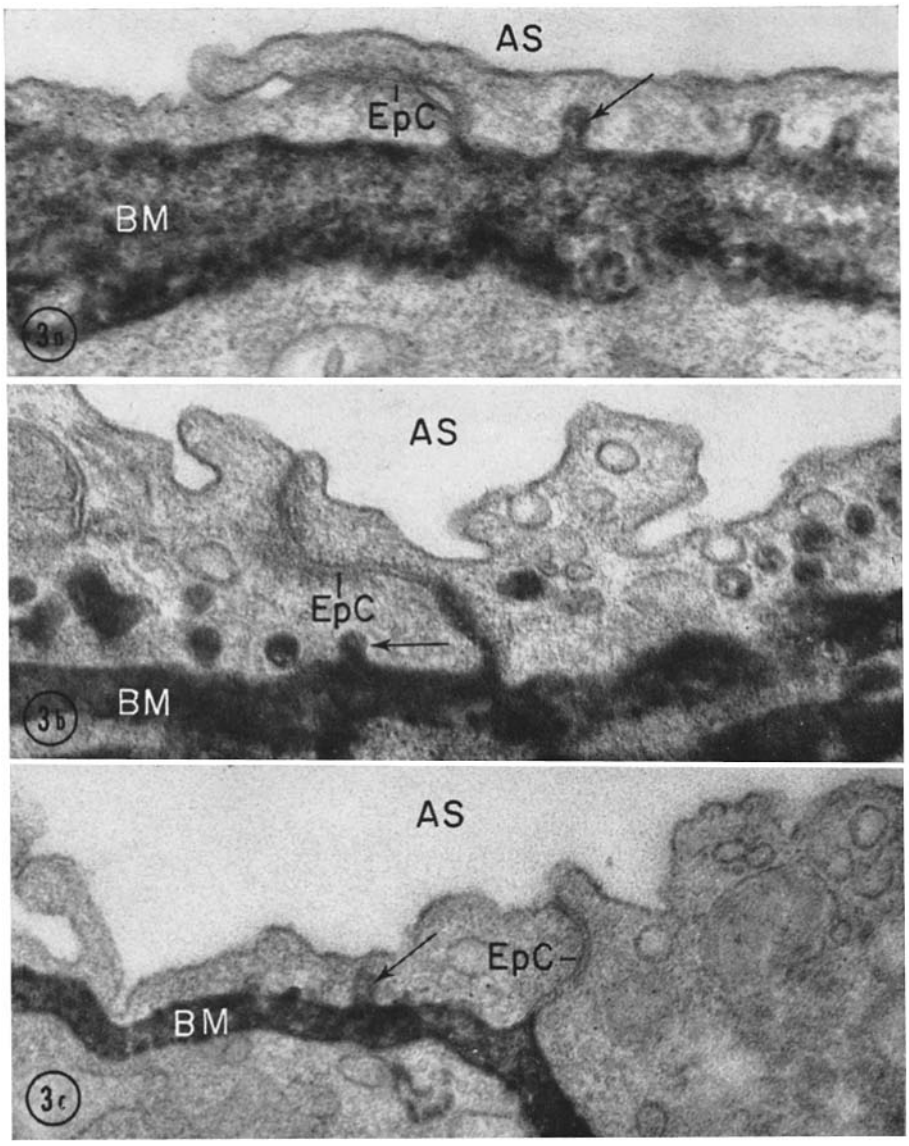

FIGURE 3 Lung from mouse sacrificed 90 sec after HRP injection. Epithelial intercellular clefts (*EpC*) are free of reaction product (Fig. 3 *a*, *c*) except in a few instances where it extends a short distance into the cleft (Fig. 3 *b*). Reaction product in the basement membrane (*BM*) extends into invaginations (arrows) on the capillary side of the epithelial cell (Fig. 3 *a–c*). A few apparently unattached vesicles containing reaction product are present in epithelial cells (Fig. 3 *b*), but none are seen to discharge their contents into the alveolar space (*AS*). *a*, × 70,000. *b*, *c*, × 67,000.

capillaries, but these were absent in specimens obtained later than 15 min after HRP injection.

Electron Microscopy

EXPERIMENTS WITH INTRAVENOUS HRP: Reaction product, in the form of black, amorphous, or sometimes granular deposits, was fairly uniformly distributed in pulmonary tissue fixed 90 sec after injection of HRP. The distribution of reaction product at this stage will be described in detail.

Reaction product was present free in the vascu-

lar lumen and extended through endothelial intercellular clefts into the adjacent basement membrane (Fig. 2 *a–c*). It was evenly distributed throughout the basement membrane, but failed to permeate elastic fibers (Fig. 1). Endothelial pinocytotic vesicles in various locations in the cell contained reaction product. Several of these vesicles were seen as invaginations of the cell membrane with reaction product extending into them from the capillary lumen (Fig. 2 *a*), while many appeared as unattached vesicles within the cell (Fig. 2 *b*, *c*). Similarly, reaction product present in the basement membrane extended into invaginations on the alveolar side of the endothelial cell (Fig. 2 *b*, *c*).

Epithelial intercellular clefts between squamous alveolar cells were free of reaction product (Fig. 3 *a*, *c*), although in a few instances reaction product extended, from the basement membrane, a short distance into the cleft (Fig. 3 *b*). Although reaction product was present in intercellular clefts between great and squamous alveolar cells, it was consistently absent from the short luminal segment of the cleft (Fig. 4). A few vesicles in the squamous alveolar cells contained reaction product; however, these vesicles were usually connected to the basal cell membrane of the epithelial cell (Fig. 3 *a–c*). Occasionally, they were observed to be apparently free within the epithelial cell cytoplasm (Fig. 3 *b*). No vesicles containing reac-

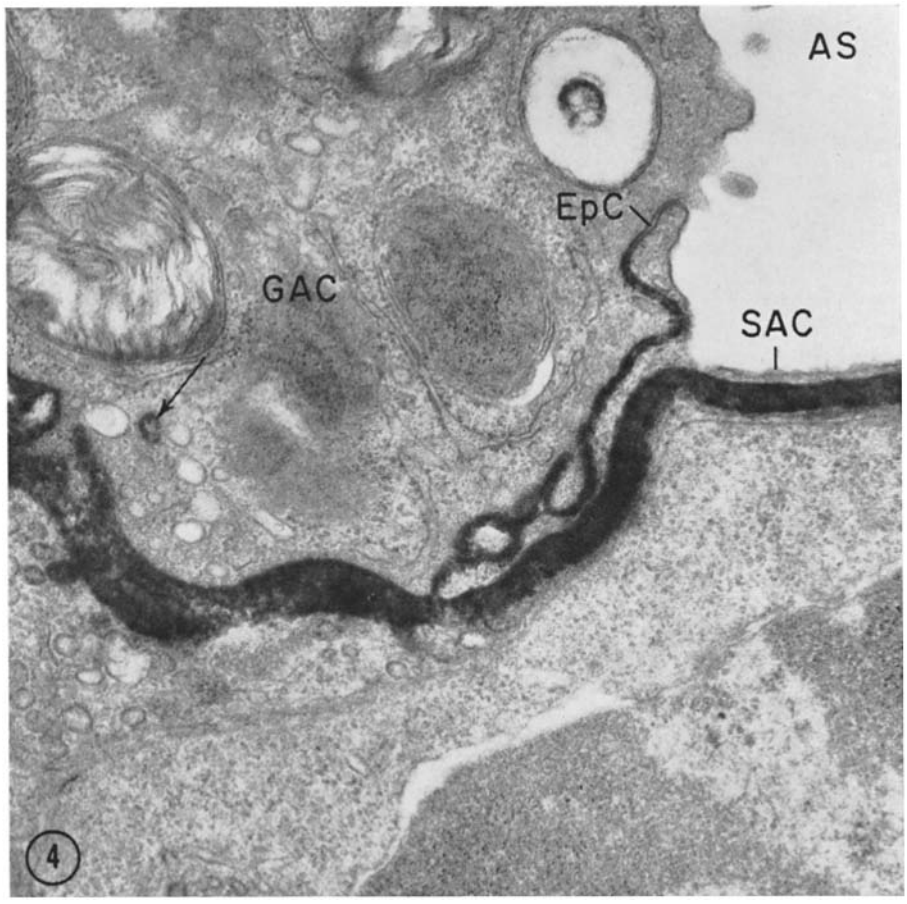

FIGURE 4 Lung from mouse sacrificed 90 sec after HRP injection. The epithelial intercellular cleft (*EpC*) between a great alveolar cell (*GAC*) and a squamous alveolar cell (*SAC*) contains reaction product except in the luminal segment of the junction where it is consistently absent. A small deposit of reaction product is seen near the base of the cell (arrow), but this is a very rare finding. × 46,000.

tion product were seen to fuse with the alveolar side of the cell membrane and to discharge their contents into the alveolar lumen. Except for a rare vesicle containing reaction product (Fig. 4), there was no pinocytotic uptake of HRP by the great alveolar cells even 60 min after injection or after giving the animal multiple doses of HRP. Reaction product was occasionally seen in connective tissue cells in the alveolar membrane (Fig. 1).

Tissue taken from lungs fixed later than 15 min

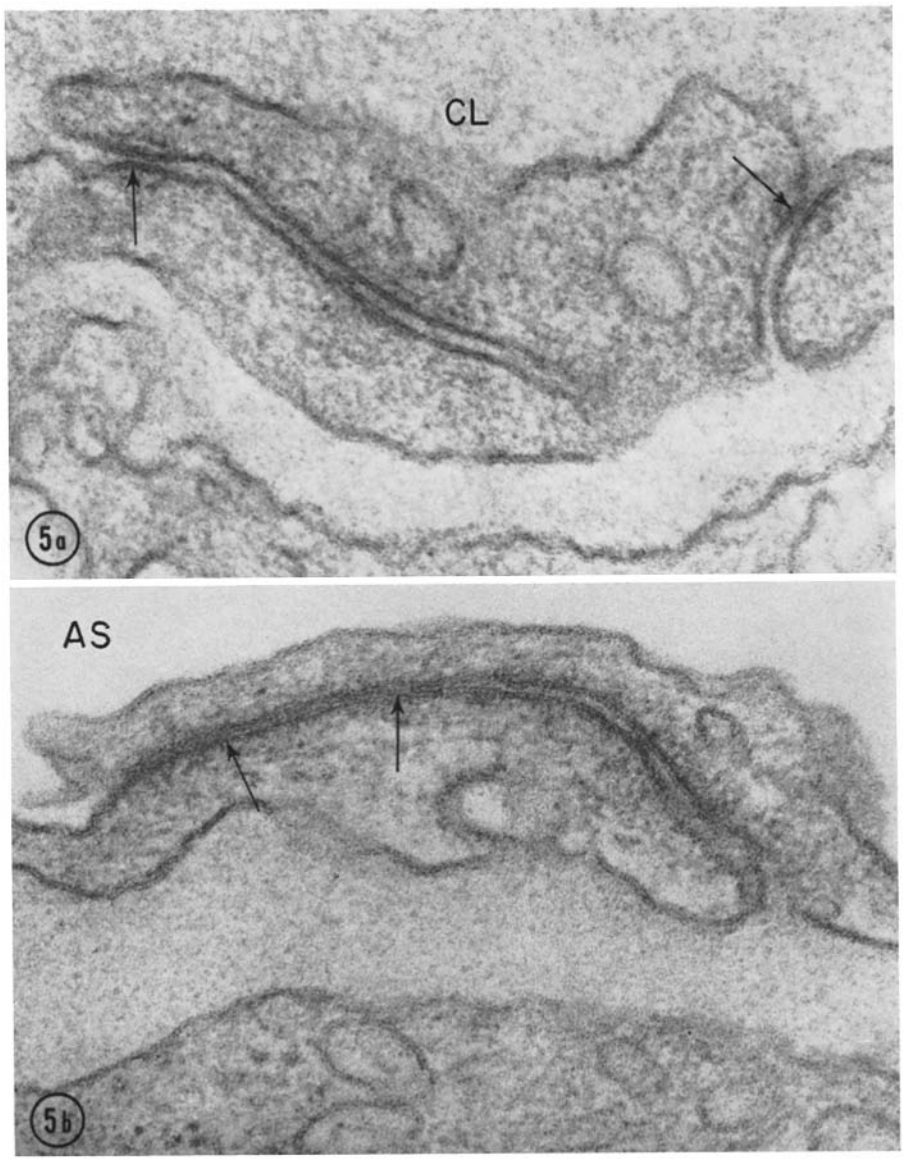

FIGURE 5 *a* Lung from an uninjected control mouse showing a portion of a capillary lumen (*CL*) and two endothelial intercellular clefts. In the junction on the left (arrow) there is a distinct gap between the bounding unit membranes, whereas in the one on the right (arrow) details are obscured. × 140,000.

FIGURE 5 *b* Lung from the same mouse as in Fig. 5 *a*. An epithelial intercellular cleft is shown. In several areas (arrows) the outer leaflets of the bounding unit membranes appear fused. This fusion usually occurs towards the alveolar end of the cleft. × 140,000.

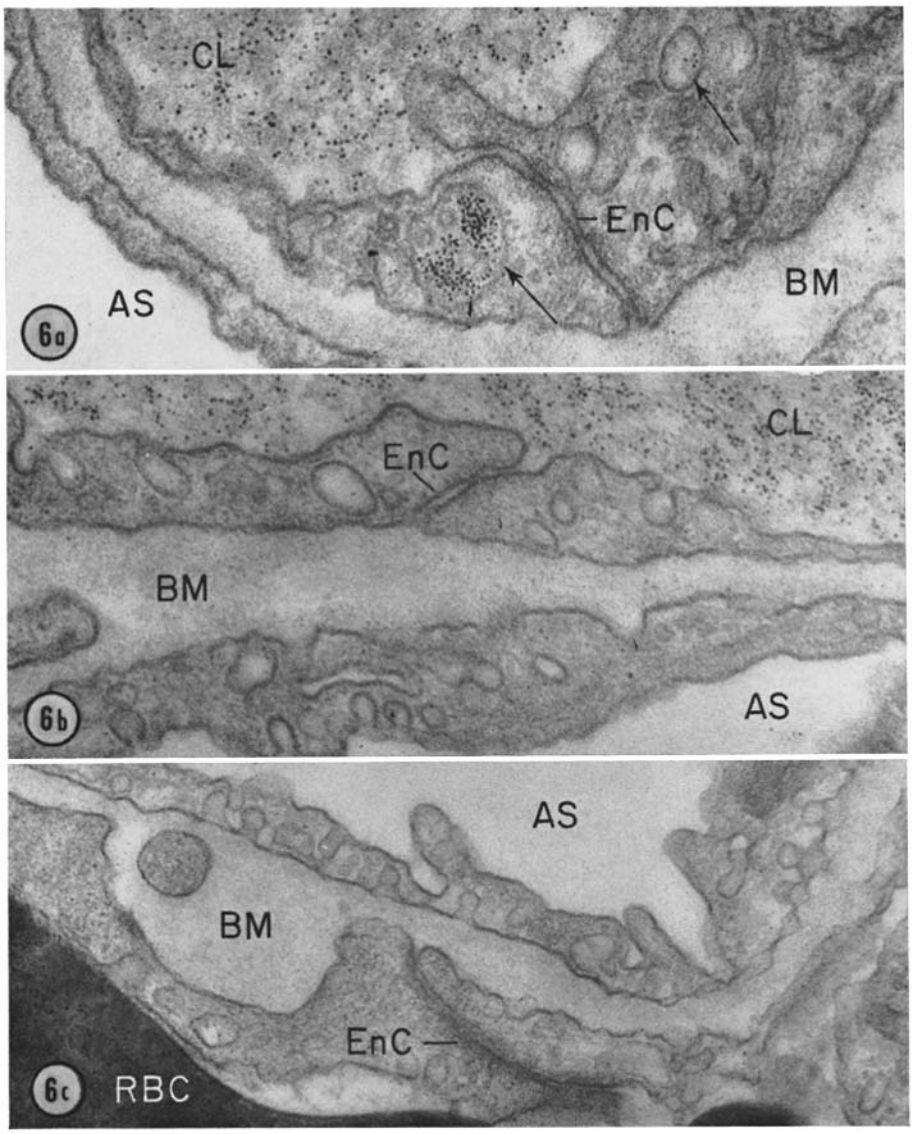

FIGURE 6 *a* Lung from mouse given 50 mg of ferritin intravenously and sacrificed 1 hr later. Note absence of ferritin from endothelial intercellular cleft (*EnC*) and basement membrane (*BM*). A few endothelial vesicles and one multivesicular body contain ferritin (arrows). × 60,000.

FIGURE 6 *b* Lung from mouse given 50 mg of ferritin followed 5 min later by 5 mg of HRP, both given intravenously. The mouse was sacrificed 5 min later. Again note absence of ferritin from endothelial intercellular cleft (*EnC*) and basement membrane (*BM*). × 70,000.

FIGURE 6 *c* Lung from an uninjected mouse. Preparation was incubated in the full histochemical medium for peroxidase. Only the red blood cell (*RBC*) is stained while the endothelial intercellular cleft (*EnC*) and basement membrane (*BM*) are free of reaction product. × 46,000.

after HRP injection showed a similar but patchy distribution of the reaction product which was less dense.

The distribution of reaction product in pulmonary tissue from mice treated with pyrilamine maleate and BOL 148 before HRP injection was not morphologically different from that in pulmonary tissue of untreated animals.

EXPERIMENTS WITH INTRANASAL HRP: Pulmonary tissue from animals which had received large intranasal doses of HRP (10–25 mg/ml) showed extensive areas of cell death, involving chiefly bronchial and squamous alveolar epithelium and free alveolar macrophages. In these preparations, the necrotic cells as well as the underlying basement membrane were diffusely

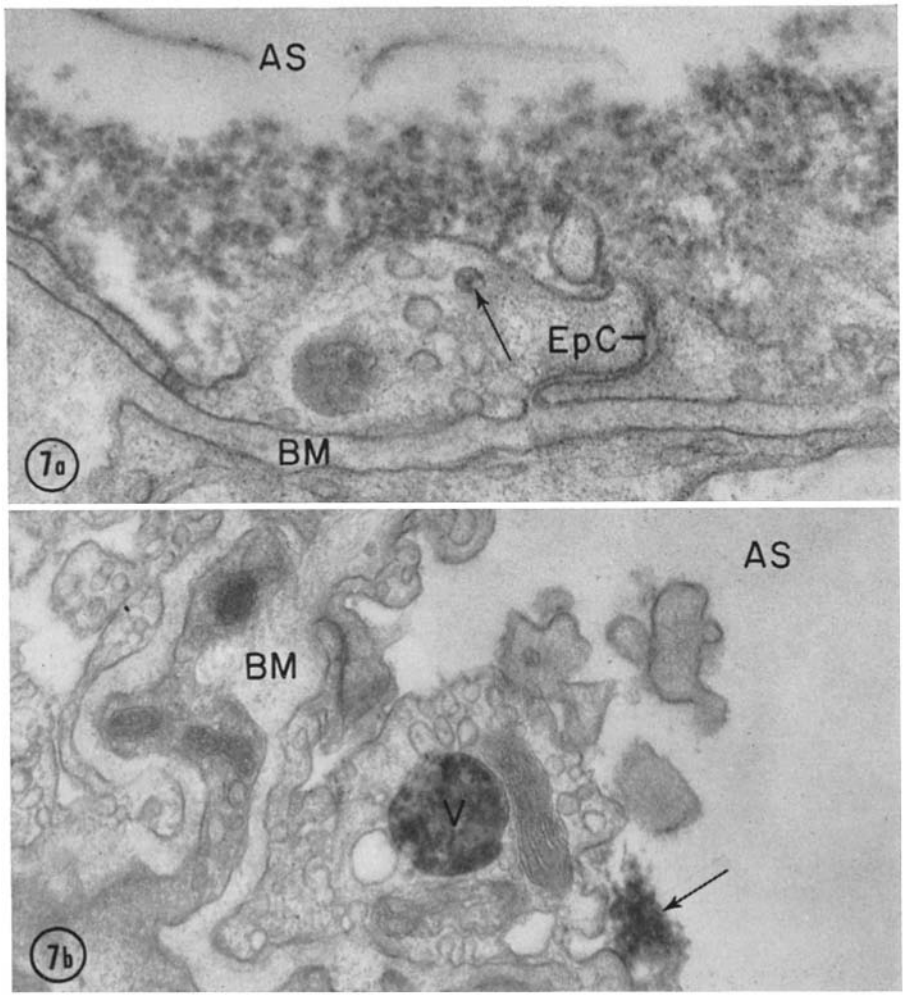

FIGURE 7 *a* Lung from mouse given intranasal instillation of 0.13 mg of HRP in 0.05 ml of serum 3 hr previously. Reaction product is present on the alveolar surface of squamous alveolar cells and possibly in a pinocytotic vesicle (arrow). However, there is no reaction product in the epithelial intercellular cleft (*EpC*) nor in the basement membrane (*BM*). × 70,000.

FIGURE 7 *b* Lung from mouse given intranasal instillation of 0.13 mg of HRP in 0.05 ml of serum 6 hr previously. Collections of reaction product (arrow) remain in the alveolar space (*AS*) as well as phagocytic vacuoles (*V*) within squamous alveolar cells. Epithelial intercellular cleft and basement membrane (*BM*) are free of reaction product. × 32,000.

stained with reaction product. With lower doses of HRP (1–5 mg/ml), only a small proportion of alveoli were labeled; in those a layer of reaction product was found on the surface of alveolar epithelial cells (Fig. 7 a). Labeled pinocytotic vesicles were only rarely demonstrated, and epithelial intercellular clefts were consistently free of reaction product. 6 hr after intranasal HRP instillation, large vacuoles containing reaction product were present in squamous alveolar cells (Fig. 7 *b*) while free alveolar macrophages contained large amounts of reaction product in vacuoles.

CONTROL EXPERIMENTS: For examination of the fine structure of endothelial and epithelial intercellular clefts, lungs from uninjected mice were processed, without prior histochemical incubation, for electron microscopy. The width of the space between the outer leaflets of the bounding unit membranes of endothelial cells was variable. In some areas, the outer leaflets appeared to be closely approximated (Fig. 5 *a*), although not apparently fused, whereas in others the intercellular space appeared to be obliterated. Although no serial sections were made, the study of a number of endothelial intercellular clefts in lung showed them to be similar in appearance to those studied in detail in skeletal and cardiac muscle (18). Thus, we consider that pulmonary capillary intercellular clefts similarly contain maculae occludentes rather than zonulae occludentes. Of those junctions examined, about half could not be categorized as definitely "open" or "closed" because tilting, or extracellular density, obscured details (Fig. 5 *a*). Of those junctions categorized, about two-thirds were considered "open." It was not possible to make accurate measurements.

The endothelial clefts in pulmonary tissue from mice which had been given HRP but which had not been subjected to the histochemical reaction mixture showed no detectable widening or alteration of the intercellular junction. Furthermore, the presence of HRP had no detectable effect on the distribution of ferritin in the pulmonary capillaries (Fig. 6 *a*, *b*). Ferritin particles were prominent in the vascular lumen but consistently absent from endothelial clefts. A few pinocytotic vesicles contained some ferritin, and a rare ferritin particle was observed in the basement membrane, 1 hr after ferritin was given.

Intercellular clefts between squamous alveolar cells, as compared to endothelial clefts, were longer and more tortuous. Thus, the entire length of the membranes lining the cleft was seldom perpendicular to the plane of section. Along the course of the cleft there were several areas in which the outer leaflets of the apposed unit membranes appeared fused (Fig. 5 *b*). These areas of fusion were usually observed towards the luminal end of the cleft. An area of fusion was similarly observed in the luminal segment of the cleft between great and squamous alveolar cells. Since they are consistently present in all normal (perpendicular) planes of section, we consider these areas of fusion in epithelial intercellular clefts to be zonulae occludentes.

We also subjected pulmonary tissue from uninjected animals to incubation in the complete histochemical medium to ascertain the presence of endogenous peroxidatic activity. In these preparations, reaction product was observed in erythrocytes (Fig. 6 *c*) because of the peroxidatic activity of hemoglobin, and in granules of eosinophils and neutrophils. Alveolar macrophages found in the alveolar lumen contained irregular, membrane-lined deposits of reaction product. No intercellular clefts, pinocytotic vesicles, or basement membranes contained reaction product.

DISCUSSION

Physiological studies have shown that vascular endothelium behaves essentially as a semipermeable membrane with water and small water-soluble solutes diffusing through water-filled pores (23). Restriction to diffusion of solutes is, in part, dependent on their molecular size, whereas net flow across the endothelium depends on hemodynamic forces. We have shown that nonfenestrated capillaries of the lung allow the relatively rapid passage of HRP through endothelial intercellular clefts in a manner analogous to that observed in nonfenestrated systemic capillaries (18) other than those of the brain (26). It has been previously shown that endothelial cell junctions of heart and skeletal muscle capillaries are maculae occludentes, with a gap of approximately 40 A in width between the points of membrane approximation. It was inferred that these structures are the morphological equivalent of the small pore system (18). We consider that the capillary junctions in lung are analogous to those in heart and skeletal muscle. Although we have been unable to demonstrate "tight" junctions as recently implied by Kistler et al. (21), these authors did not attempt

to distinguish between maculae occludentes and zonulae occludentes. The permeability of pulmonary capillaries to HRP is perhaps remarkable when one considers pulmonary capillary pressures. Estimates of the latter have been interpolated from pulmonary arterial diastolic pressures and mean left arterial pressure. Their values have been little more than half of those found in systemic capillaries (14), thereby apparently creating conditions which do not favor the filtration of water-soluble solutes across the endothelium. Nevertheless, it is our impression that HRP crossed pulmonary endothelium faster than cardiac endothelium. We have no ready explanation for this difference; possibly differences in the nature or number of pores could account for it. The role of pinocytosis in the net transport of protein across the pulmonary capillary endothelium remains to be established and cannot be determined from our observations. The problems in interpreting the vesicular labeling of endothelium in favor of vesicular transport have previously been discussed (18). In our experiments, ferritin showed minimal evidence of vesicular labeling 1 hr after injection.

Previous physiological work on the permeability of the alveolar-capillary membrane to urea (8) and radioactively labeled ions (4) drew attention to the unexpectedly slow rate of equilibration of urea and certain ions between blood and alveolar fluid. On the basis of this work, it was suggested that the basement membrane might function as a diffusion barrier. Moreover, Chinard (5), using the multiple indicator dilution technique, suggested that bonded water molecules are an integral part of this barrier. He divided solutes into those which enhance the normal structure of water and, therefore, enter the barrier (proteins, hydrocarbons, alcohols, gases), and those which disrupt it (ions), to explain the observed behavior of solutes in the alveolar wall. Although this hypothesis could explain the diffusion of HRP in basement membranes, it could not, when postulated, take into account the observed barrier to diffusion of HRP in the intercellular junctions of alveolar epithelial cells.

The presence of HRP in the basement membrane and its subsequent disappearance by 60 min after injection are of significance when one considers the distribution of lymphatic channels in the lung. Tobin has claimed that lymphatic vessels are absent from alveolar walls and that they make their appearance only at the level of alveolar ducts (30). Thus, any excess interstitial fluid in the alveolar wall might diffuse along the basement membrane until it reaches a lymphatic channel at the level of an alveolar duct.

Our findings that cell junctions in intercellular clefts between alveolar epithelial cells constitute the chief barrier to diffusion of HRP across the alveolar-capillary membrane are analogous to those reported by Brightman in the choroid plexus (3). We interpret these junctions to be similar to those demonstrated morphologically (12) and physiologically (9) in epithelia of a variety of hollow organs, and, therefore, consider them to be zonulae occludentes. Farquhar and Palade have tested their hypothesis that tight junctions (zonulae occludentes) serve as impermeable seals by correlating the presence of these junctions in frog epidermis (13) with the physiologically demonstrable impermeability of the epidermis to water and certain small molecules (25). However, the permeability properties of the lung are not entirely analogous to those of the frog epidermis: the alveolar-capillary membrane is relatively impermeable to small water-soluble solutes, but freely permeable to water. Thus, it has long been known that large amounts of water are readily absorbed from the lung (6), and that there is rapid equilibration of water across the alveolar-capillary membrane (29). The area of the alveolar wall through which water diffuses is not established. Recently Yudilevich and Alvarez have suggested that water may diffuse through both intercellular clefts and endothelial cells of cardiac capillaries (32); it is possible, but not as yet demonstrated, that a similar situation might pertain to alveolar epithelium.

In attempting to trace the fate of protein-containing edema fluid, physiologists have studied the absorption of protein from the alveolar space (11, 27). Both Drinker et al., using T1824-labeled protein (11), and Schultz et al., using I^{131}-labeled protein (27), point out the slow rate of absorption of protein, most of the labeled protein returning to the lymphatic circulation (11) but some of it apparently entering pulmonary capillaries (27). More recently, studies in dogs and guinea pigs (2, 10) report a relatively rapid absorption of intact, radioactively labeled serum proteins from the alveolar spaces into the circulatory system. Our studies on the fate of intranasally instilled HRP have been hampered by sampling problems,

in that many alveolar spaces were not labeled; however, our observations indicate that the epithelial junctions remain impermeable to HRP when the latter is in the alveolar lumen. A small amount of HRP may be transported across the epithelium by means of pinocytotic and later phagocytic vacuoles. These findings are similar to those described by Karrer with intranasally instilled carbon particles (20).

Except in very rare instances, the absence of uptake of HRP, from the basement membrane side, by the great alveolar cells is interesting in light of the suggestion (28) that these cells may give rise to free alveolar macrophages. If indeed these cells are precursors of alveolar macrophages, they appear to lack any appreciable phagocytic ability while interpolated between flat alveolar cells. This finding confirms observations made by Karrer using intranasally instilled carbon particles (20).

This study was supported by Grant HE 9125 and Training Grant GM 1235 from the National Institutes of Health, United States Public Health Service.

Mr. Edward Gadowski provided expert help with photography.

Received for publication 26 December 1967 and in revised form 30 January 1968.

REFERENCES

1. BENNETT, H. S., and J. H. LUFT. 1959. S-collidine as a basis for buffering fixatives. *J. Biophys. Biochem. Cytol.* **6**:113.
2. BENSCH, K. G., E. DOMINGUEZ, and A. A. LIEBOW. 1967. Absorption of intact protein molecules across the pulmonary air-tissue barrier. *Science.* **157**:1204.
3. BRIGHTMAN, M. W. The intracerebral movement of proteins injected into blood and CSF of mice. *In* Progress in Brain Research. Elsevier, Amsterdam. In press.
4. CHINARD, F. P., T. ENNS, and M. F. NOLAN. 1962. The permeability characteristics of the alveolar capillary barrier. *Trans. Assoc. Am. Physicians.* **75**:253.
5. CHINARD, F. P. 1966. The permeability characteristics of the pulmonary blood-gas barrier. *In* Advances in Respiratory Physiology. C. G. Caro, editor. Edward Arnold Ltd., London.
6. COLIN, G. 1873. Traité de physiologie comparée des animaux. J-B Baillière et fils, Paris. 2nd edition. **2**:109–110.
7. COTRAN, R. S., and M. J. KARNOVSKY. 1967. Vascular leakage induced by horseradish peroxidase in the rat. *Proc. Soc. Exptl. Biol. Med.* **126**:557.
8. CROSS, C. E., P. A. RIEBEN, and P. F. SALISBURY. 1960. Urea permeability of alveolar membrane; hemodynamic effects of liquid in the alveolar spaces. *Am. J. Physiol.* **198**:1029.
9. DIAMOND, J. M., and J. McD. TORMEY. 1966. Role of long extracellular channels in fluid transport across epithelia. *Nature.* **210**:817.
10. DOMINGUEZ, E. A. M., A. A. LIEBOW, and K. G. BENSCH. 1967. Studies on the pulmonary air-tissue barrier. I. Absorption of albumin by the alveolar wall. *Lab. Invest.* **16**:905.
11. DRINKER, C. K., and E. HARDENBERGH. 1947. Absorption from the pulmonary alveoli. *J. Exptl. Med.* **86**:7.
12. FARQUHAR, M. G., and G. E. PALADE. 1963. Junctional complexes in various epithelia. *J. Cell Biol.* **17**:375.
13. FARQUHAR, M. G., and G. E. PALADE. 1965. Cell junctions in amphibian skin. *J. Cell Biol.* **26**:263.
14. FISHMAN, A. P. 1963. Dynamics of Pulmonary Circulation. Handbook of Physiology. Section 2, Circulation. **2**: chapter 48. American Physiological Society Washington, D.C.
15. GRAHAM, R. C., and M. J. KARNOVSKY. 1966. The early stages of absorption of injected horseradish peroxidase in the proximal tubules of mouse kidney: Ultrastructural cytochemistry by a new technique. *J. Histochem. Cytochem.* **14**:291.
16. GRAHAM, R. C., and M. J. KARNOVSKY. 1966. Glomerular permeability: Ultrastructural cytochemical studies using peroxidases as protein tracers. *J. Exptl. Med.* **124**:1123.
17. KARNOVSKY, M. J. 1965. Formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy. *J. Cell Biol.* **27**:137A. (Abstr.)
18. KARNOVSKY, M. J. 1967. Ultrastructural basis of capillary permeability studied with peroxidase as a tracer. *J. Cell Biol* **35**:213.
19. KARRER, H. E. 1956. The ultrastructure of the mouse lung. *J. Biophys. Biochem. Cytol.* **2**:241.
20. KARRER, H. E. 1960. Electron microscopic study of the phagocytosis process in the lung. *J. Biophys. Biochem. Cytol.* **7**:357.
21. KISTLER, G. S., P. R. B. CALDWELL, and E. R. WEIBEL. 1967. Development of fine structural damage to alveolar and capillary lining cells in oxygen-poisoned rat lungs. *J. Cell Biol.* **32**:605.

22. Low, F. N. 1952. Electronmicroscopy of the rat lung. *Anat. Record.* **113**:437.
23. PAPPENHEIMER, J. R. 1953. Passage of molecules through the capillary walls. *Physiol. Rev.* **33**:387.
24. LUFT, J. H. 1961. Improvements in epoxy resin embedding methods. *J. Biophys. Biochem. Cytol.* **9**:409.
25. MACROBBIE, E. A. C., and H. H. USSING. 1961. Osmotic behaviour of the epithelial cells of frog skin. *Acta Physiol. Scand.* **53**:348.
26. REESE, T. S., and M. J. KARNOVSKY. 1967. Fine structural localization of a blood-brain barrier to exogenous peroxidase. *J. Cell Biol.* **34**:207.
27. SCHULTZ, A. L., J. T. GRISNER, S. WADA, and F. GRANDE. 1964. Absorption of albumin from alveoli of perfused dog lungs. *Am. J. Physiol.* **207**:1300.
28. SOROKIN, S. P. 1966. Respiratory system. *In* Histology. R. O. Greep, editor. McGraw-Hill Book Co., New York. 2nd edition.
29. TAYLOR, C. E., A. C. GUYTON, and V. S. BISHOP. 1965. Permeability of the alveolar membrane to solutes. *Circulation Res.* **16**:353.
30. TOBIN, C. E. 1954. Lymphatics in the pulmonary alveoli. *Anat. Record.* **120**:625.
31. VENABLE, J. H., and R. A. COGGESHALL. 1965. A simplified lead citrate stain for use in electron microscopy. *J. Cell Biol.* **25**:407.
32. YUDILEVICH, D. L., and O. A. ALVAREZ. 1967. Water, sodium and thiourea transcapillary diffusion in the dog heart. *Am. J. Physiol.* **213**:308.