

THE INFLUENCE OF INTRAVASCULAR FLUID VOLUME ON THE PERMEABILITY OF NEWBORN AND ADULT MOUSE LUNGS TO ULTRASTRUCTURAL PROTEIN TRACERS

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ABSTRACT

The permeability of the alveolar-capillary membrane of newborn and adult mice to horseradish peroxidase (HRP) and catalase was studied by means of ultrastructural cytochemistry, and the permeability to ferritin was studied by electron microscopy. The influence of varying volumes of intravenously injected fluid on the rate of leakage of the tracers from pulmonary capillaries was examined. The tracers were injected intravenously and the mice were sacrificed at timed intervals. Experiments on newborn mice with intranasally instilled HRP were also done. The tissues were fixed in formaldehyde-glutaraldehyde fixative. Chopped sections were incubated in Graham and Karnovsky's medium for peroxidase and in a modification of this medium for catalase. Tissues were postfixed in OsO_4 and processed for electron microscopy. In both newborn and adult mice, the ready passage of peroxidase through endothelial clefts was dependent on the injection of the tracer in large volumes of saline. When the tracer was injected in small volumes of saline, its passage through endothelial clefts was greatly reduced. Endothelial junctions of newborn mice were somewhat more permeable to HRP than those of adult mice. In all animals, alveolar epithelial junctions were impermeable to HRP. Catalase and ferritin did not pass through endothelial junctions. Intranasally instilled HRP in newborn mice was taken up by pinocytotic vesicles and tubules of flat alveolar cells.

INTRODUCTION

Recently Pietra et al. (31), using hemoglobin (mol wt 64,500) as a tracer, have shown that in isolated, perfused dog lung, very high pressures (above 50 mm Hg) were necessary before this tracer could be demonstrated histochemically in endothelial junctions. These authors interpreted their findings as being consistent with the concept of a labile pore size, a notion which had been postulated earlier by Shirley et al. (37).

The physiological studies of Taylor et al. (42) have indicated that the alveolar epithelium rather

than the capillary endothelium represents the major barrier to diffusion of water-soluble solutes across the alveolar capillary membrane. In our previous study on the permeability of adult mouse alveolar-capillary membranes to intravenously injected horseradish peroxidase (HRP) (mol wt 40,000), we found that the enzyme permeated endothelial junctions, diffused throughout the region of the basement membrane, but did not pass through the tight junctions of alveolar epithelium into the alveolar lumen (34). This difference in permea-

bility between pulmonary endothelial junctions and epithelial junctions has recently been reconfirmed by Taylor et al. (41) in a study in which reflection coefficients for urea, glucose, and sucrose were estimated in isolated, perfused dog lungs. From these results a pulmonary endothelial pore radius of 40–58 Å and an epithelial pore radius of 6–10 Å were calculated. However, because in our previous study we had injected HRP in relatively large volumes of saline, and in view of Pietra's findings, we carried out the present experiments to determine whether the volume in which tracers are injected plays any role in the ease with which tracer molecules pass through endothelial junctions.

We also undertook experiments to study the permeability of the alveolar-capillary membranes in newborn mice. Before birth, the pulmonary vascular bed of the mammalian fetus is a high resistance, high pressure system (3, 5). At birth dramatic hemodynamic changes take place, in that there is an immediate postnatal drop in pulmonary vascular flow. This decrease in pulmonary vascular resistance is thought to be due to the opening up of previously compressed capillaries as well as to vasodilatation caused by a fall in carbon dioxide tension and a rise in oxygen tension (13). While these events are taking place, intra-alveolar fluid, normally present and produced in the fetal lung (1, 2, 19, 20, 39), must be absorbed.

Recently Strang and his colleagues have examined the permeability of pulmonary capillaries to macromolecules in fetal and newborn lambs as well as in sheep (9). Their calculated value for mean pore radius in newborn lambs (90 Å) was significantly lower than in either fetal lambs (150 Å) or sheep (130 Å). These findings were interpreted as being compatible with the suggestion made by Shirley et al. (37) that pores may be stretched by the high capillary pressures which exist in the fetal pulmonary vascular bed, but which are much reduced in the newborn lung. In the adult lung the large pore radius was attributed to the increased vertical height of the lung in the supine sheep, portions of the lung from which pulmonary lymph is formed (9).

By using tracers of varying molecular weight, and using various volumes in which the tracers were injected, we compared the permeability of the alveolar-capillary membrane of newborn mice to that of adult mice. An attempt was also made to

elucidate the mechanism of protein uptake from the alveolar spaces in the first 2 hr after birth.

MATERIALS AND METHODS

Animals

NEWBORN ANIMALS: Female Swiss albino mice in estrus were mated overnight. The presence of a vaginal plug the following morning was considered a successful mating, and the females were thereafter kept in separate cages. The animals were allowed to deliver spontaneously and the newborn mice were used for experiments within the first 2 hr of delivery. None of the newborn mice used had been allowed to suckle.

ADULT ANIMALS: Male and female mice weighing between 25 and 30 g were used.

Experimental Procedures

INTRAVENOUS INJECTION: The various tracers (horseradish peroxidase, catalase, ferritin) dissolved in 0.01 or 0.02 ml of 0.15 M saline were injected intravenously into the orbital vein of newborn mice using the technique of Billingham and Brent (7). The skin overlying the vein was lightly swabbed with mineral oil in order to see the vessel more clearly. The solutions were injected by means of a 0.25 ml tuberculin syringe fitted with a 30 gauge hypodermic needle and with the aid of a dissecting microscope at a magnification of 25.

Horseradish peroxidase (type II, Sigma Chemical Co., St. Louis, Mo.) was administered in doses of 0.1–0.24 mg per g body weight. To estimate the osmotic effect of peroxidase injections in both adult and newborn mice, the following calculations were made. The average blood volume of adult and newborn mice is about 1.5 and 0.1 ml, respectively (17). It was assumed that the osmotic pressure of serum proteins in mice is the same as in rats, namely 26 cm H₂O (38). In newborn goats and sheep the osmotic pressure of serum proteins at birth and before suckling has been initiated is about 70% of that in adult animals (27), and a similar value for newborn mice was assumed. With the doses used and applying van't Hoff's equation for dilute solutions $\pi = cRT$, peroxidase contributed in newborn mice about 0.65 cm H₂O or 3.5%, and in adult mice 2.05 cm H₂O or 7.9%, to the osmotic pressure.

Twice-crystallized beef liver catalase (Stock No. C-100, Sigma Chemical Co., St. Louis, Mo.) was given in doses of approximately 0.50–1.0 mg per g body weight. In order to obtain a higher concentration of catalase for injection, approximately one-half of the supernatant was removed from the stock solution before sonication at 37°C as described by Venkatachalam and Fahimi (44).

Cadmium-free ferritin (kindly supplied by Dr. G. I. Schoeff) was given in a dose of 0.75 mg per g body weight. The animals which had received HRP were sacrificed 90 sec–6 min after injection, whereas those receiving catalase and ferritin were sacrificed 30–60 min after injection.

All animals were stunned, and their lungs were removed and fixed by immersion.

Adult mice were injected intravenously via a tail vein with 5.0 mg HRP dissolved in 0.05, 0.2, or 0.5 ml of 0.15 M saline. The injection was given over a 3–15 sec interval. 90-sec or 6 min after injection, the animal was stunned by a blow on the head. The chest was opened and fixative was gently instilled into the trachea as well as dripped continuously onto the pleural surface for 5 min as previously described (34).

INTRANASAL INSTILLATIONS: Tiny drops of a solution composed of 0.25 mg HRP and 1.0 mg trypan blue dissolved in 1.0 ml 0.15 M saline were dropped on the unanesthetized newborn animal's nostrils and allowed to be aspirated. Between 0.01 and 0.02 ml were administered over 20–30 min. The animals were sacrificed 20–60 min later, and the lungs were removed and fixed by immersion. Trypan blue was used as a marker to localize the aspirated material grossly.

CONTROL EXPERIMENTS: To localize endogenous peroxidatic activity, lungs from both newborn and adult mice, which had not been injected with any tracers, were incubated in the complete reaction mixture at room temperature for 30 min.

To determine whether any morphologic changes could be detected in endothelial junctions following injection of large volumes of tracers, lungs from newborn mice which had been injected with the maximum volume of HRP were processed without prior incubation in reaction medium.

Fixation

All lungs were fixed in formaldehyde-glutaraldehyde (F.G.) fixative (21) diluted (1:4.5) with 0.1 M cacodylate buffer, pH 7.6, as described previously (34). The tissue was washed overnight in cold 0.1 M cacodylate buffer, pH 7.6.

Incubation

For all histochemical reactions, 40- μ thick sections were cut from fixed, washed tissue on a Smith-Farquhar TC2 tissue chopper (Ivan Sorvall, Inc., Norwalk, Conn.).

HRP: The sections were incubated at room temperature for 10 min in 5 mg of 3,3'-diaminobenzidine tetrahydrochloride (Sigma Chemical Co., St. Louis, Mo.) dissolved in 10 ml of Tris-HCl buffer, pH 7.6. 0.1 ml of 1% H₂O₂ was then added,

the solution was gently stirred, and incubation was continued for 20 min longer (16).

CATALASE: The sections were incubated at 37°C for 2 hr with intermittent agitation in 10 mg of 3,3'-diaminobenzidine tetrahydrochloride, in 10 ml of 0.05 M Tris-HCl buffer, pH 8.5, with 25 mg BaO₂ in 1 ml of 0.05 M Tris-HCl buffer, pH 8.5, sealed in a dialysis bag (44).

Postfixation

Following incubation, all sections were washed three times in distilled water and postfixed for 75 min in 1.3% OsO₄ in *s*-collidine buffer, pH 7.2 (6). Lungs from animals injected with ferritin were cut into 1 mm³ blocks before postfixation in OsO₄. Tissues were stained en bloc with uranyl acetate as previously described (22). They were dehydrated in ethanol and embedded in Araldite (Durcupan, Fluka AG, Basel, Switzerland). Thin sections were cut on an MT-2 Porter-Blum ultramicrotome (Ivan Sorvall, Inc., Norwalk, Conn.). Unstained sections, or sections lightly stained with lead citrate (43), were examined in a Philips-300 electron microscope operated at 60 kv and equipped with an anticontamination device and a 30 μ objective aperture.

MEASUREMENTS: Measurements of the width of endothelial junctions in electron micrographs were made with a dissecting microscope equipped with an ocular micrometer graduated in units of 0.1 mm units at a magnification of 25.

RESULTS

Newborn Animals

GENERAL

The lungs of newborn mice less than 2 hr old were made up of primitive air sacs, bounded by thick septa. Although the capillaries within the septa were separated from each other by abundant interstitial tissue, the tissue barrier separating the blood from the air space was quite thin. While some capillaries were filled with erythrocytes and leukocytes, many others appeared to be partially collapsed. Although the endothelium of some capillaries was quite tenuous, in most of them the endothelial cells were plump and contained many free ribosomes, polysomes, as well as moderate numbers of pinocytotic and coated vesicles. Interstitial cells containing lipid droplets were characteristic of the connective tissue between capillaries. The alveoli were lined by flat alveolar cells (type I) between which relatively few great alveolar cells (type II) were intercalated. The alveolar spaces were filled or partially filled with

amorphous material containing osmiophilic myelin figures. Some free macrophages were present in the alveolar lumen. Dilated lymphatic channels were prominent in peribronchial areas.

HORSERADISH PEROXIDASE

INTRAVENOUS EXPERIMENTS: 90 sec after the injection of peroxidase dissolved in 0.01 ml saline, or 10% of the animal's blood volume (17), reaction product in the form of black amorphous or granular deposits was limited to the pulmonary vascular lumens (Fig. 1). In most areas, the reaction product partially filled endothelial clefts, but did not extend to the basement membrane which appeared to be free of peroxidase. Some endothelial vesicles contained reaction product but these were confined to the vascular side of the endothelium. In a few isolated areas a faint staining of endothelial clefts and basement membranes with peroxidase was observed. However, we were unable to ascertain whether these areas corresponded to the more dependent portions of the lung.

6 min after injection of peroxidase in 0.01 ml saline, reaction product was present in the vascular lumen and extended through most of the endothelial clefts into the region of the basement membrane (Fig. 2). The concentration of peroxidase was higher in the capillary lumen than in extravascular sites. A few clefts were observed which contained peroxidase only at the luminal end while the distal end appeared unstained. With the outlining of endothelial clefts by reaction product, the long, tortuous appearance of some of them was accentuated (Fig. 2). In this respect they differed from the shorter clefts of adult pulmonary capillaries. However, we did not observe more clefts per capillary in the newborn mouse lung than in

adult lung. Vesicles throughout the endothelium contained peroxidase. Many of them appeared as invaginations of the cell membrane on the luminal side of the endothelium with reaction product extending into them, while others were apparently free within the cytoplasm. Similarly, reaction product in the basement membrane extended into invaginations on the alveolar side of the endothelium. Vesicles within flat alveolar epithelial cells contained peroxidase, although none appeared to open into the alveolar space. Reaction product partially filled the clefts between alveolar epithelial cells but—similar to the adult lung—it never extended the entire length of the cleft.

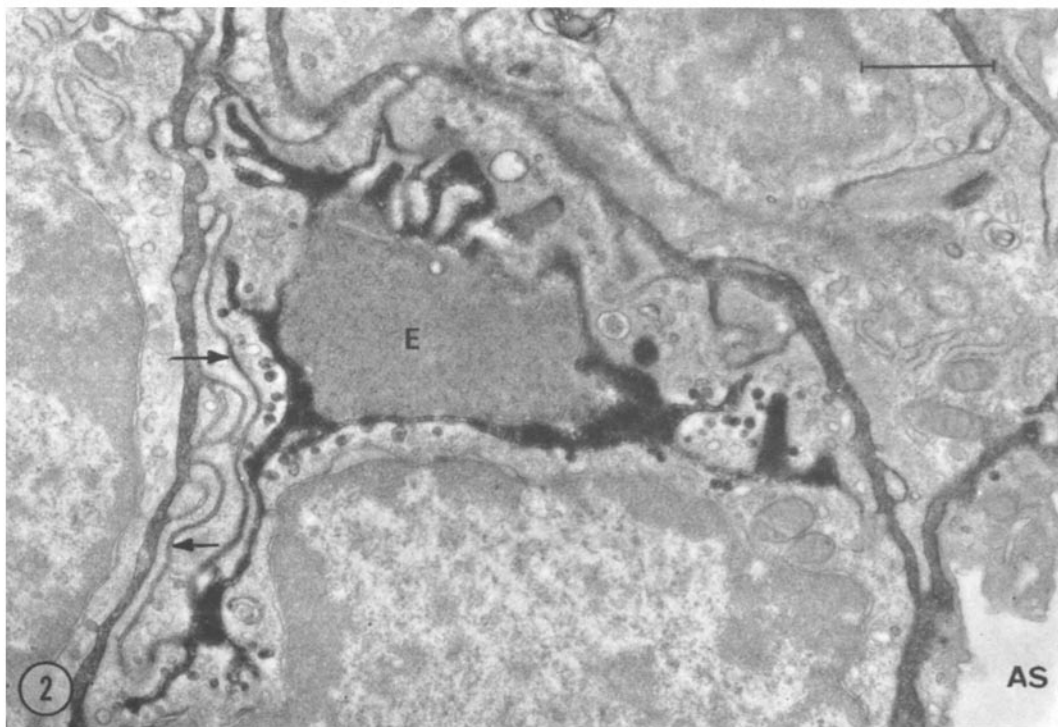
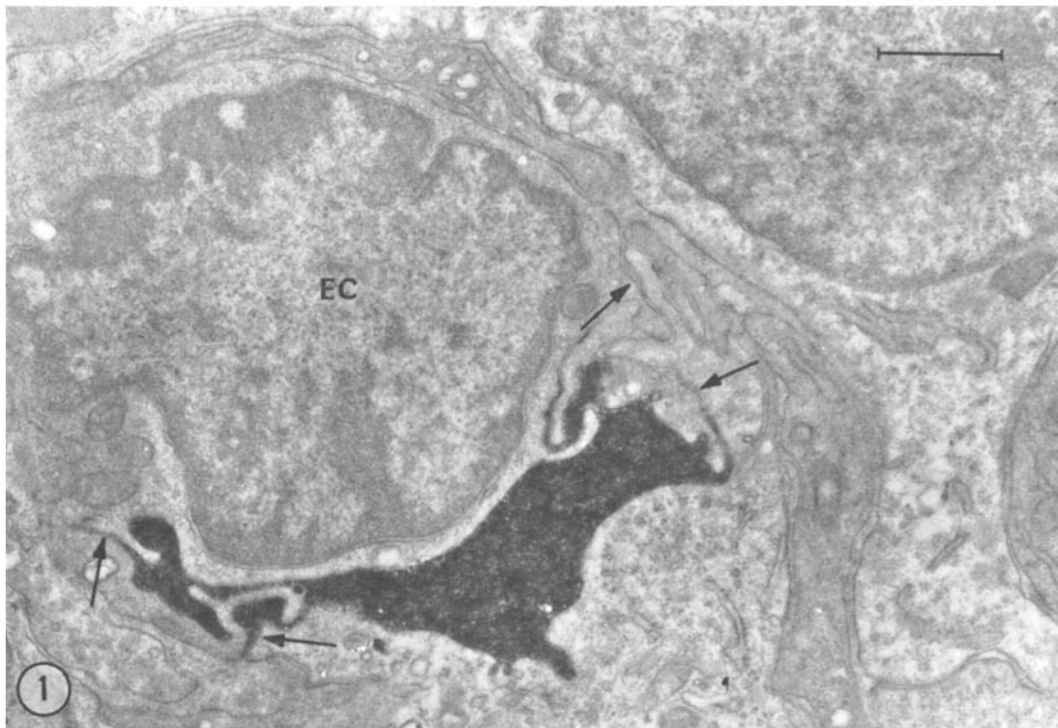
In newborn mice injected with peroxidase dissolved in the largest volume of saline (0.02 ml), the rate of leakage of peroxidase from the capillary lumen through endothelial clefts was strikingly more rapid. Within 90 sec of injection, peroxidase was found in high concentrations in the capillary lumen, endothelial clefts, and basement membrane (Fig. 3). The endothelial clefts were uniformly stained with peroxidase, and a concentration gradient could not be detected between capillary lumen and basement membrane. Vesicles throughout the endothelium as well as some alveolar epithelial vesicles were stained. The epithelial clefts between flat alveolar cells (Fig. 3) and between flat alveolar cells and great alveolar cells (Fig. 4) contained peroxidase only in their abluminal end, and none was seen in the alveolar lumen (Figs. 2-4).

INTRANASAL EXPERIMENTS: Experiments with intranasal peroxidase were complicated by the fact that, with higher concentrations of the enzyme, there was diffuse staining of some epithelial cells. This was accompanied by the appearance of low concentrations of peroxidase in the

The length of the scale line in all figures is 1 μ , Except for Fig. 7 where it is 0.8 μ .

FIGURE 1 Pulmonary capillary of a newborn mouse, sacrificed 90 sec after injection of peroxidase in 0.01 ml saline. Peroxidase within the capillary lumen partially extends into several endothelial clefts (arrows), but the basement membrane is free of the tracer. The plump endothelial cells (*EC*) contain numerous free ribosomes and polysomes in the cytoplasm. Uranyl acetate. $\times 16,000$.

FIGURE 2 Pulmonary capillary from newborn mouse sacrificed 5 min after injection of peroxidase in 0.01 ml saline. An erythrocyte (*E*) occupies the central portion of the lumen. Two very tortuous endothelial clefts (arrows) are seen on the left. The concentration of peroxidase in the basement membrane is less than in the lumen. Vesicles throughout the endothelium contain peroxidase. A flat alveolar cell abutting on the air space (*AS*) contains two stained vesicles. Uranyl acetate. $\times 17,000$.



underlying basement membrane and vascular lumen, and was interpreted as cellular damage with leakage of enzyme into the subjacent tissues. When low concentrations of the enzyme were used irregular deposits of reaction product coated the alveolar surface and extended into vesicular and tubular invaginations of the flat alveolar cells (Fig. 5 *a*). Vesicles and elongated tubules within the epithelial cells also contained peroxidase. A rare vesicle opened onto the basement membrane side of the cell (Fig. 5 *b*), but there was no detectable staining of the basement membrane by peroxidase even after 60 min. The great alveolar cells did not appear to take up any tracer, and the clefts between epithelial cells were consistently free of reaction product (Fig. 5 *b*).

CONTROL EXPERIMENTS

When lungs from uninjected newborn animals were incubated in the complete reaction mixture, peroxidatic activity was seen only in erythrocytes, in granules of eosinophils and in vacuoles of alveolar macrophages. The particulate matter in the alveolar fluid showed no peroxidatic activity.

CATALASE

Up to 60 min after intravenous injection of catalase, reaction product was limited to the capillary lumen (Fig. 6). Endothelial clefts were stained only in their luminal half, and relatively few vesicles in the endothelium contained catalase. No reaction product was seen in the basement membrane or alveolar epithelial vesicles.

FERRITIN

1 hr after injection, ferritin particles were numerous in the vascular lumen, but were never found in endothelial clefts. A few particles were seen in pinocytotic vesicles, but more commonly they were found in aggregates within multi-

vesicular bodies and dense bodies in endothelial cells (Fig. 7). A rare ferritin particle was observed in the basement membrane 1 hr after injection, and the number of ferritin particles in the basement membrane did not appear to be influenced by the volume of saline in which the tracer was injected.

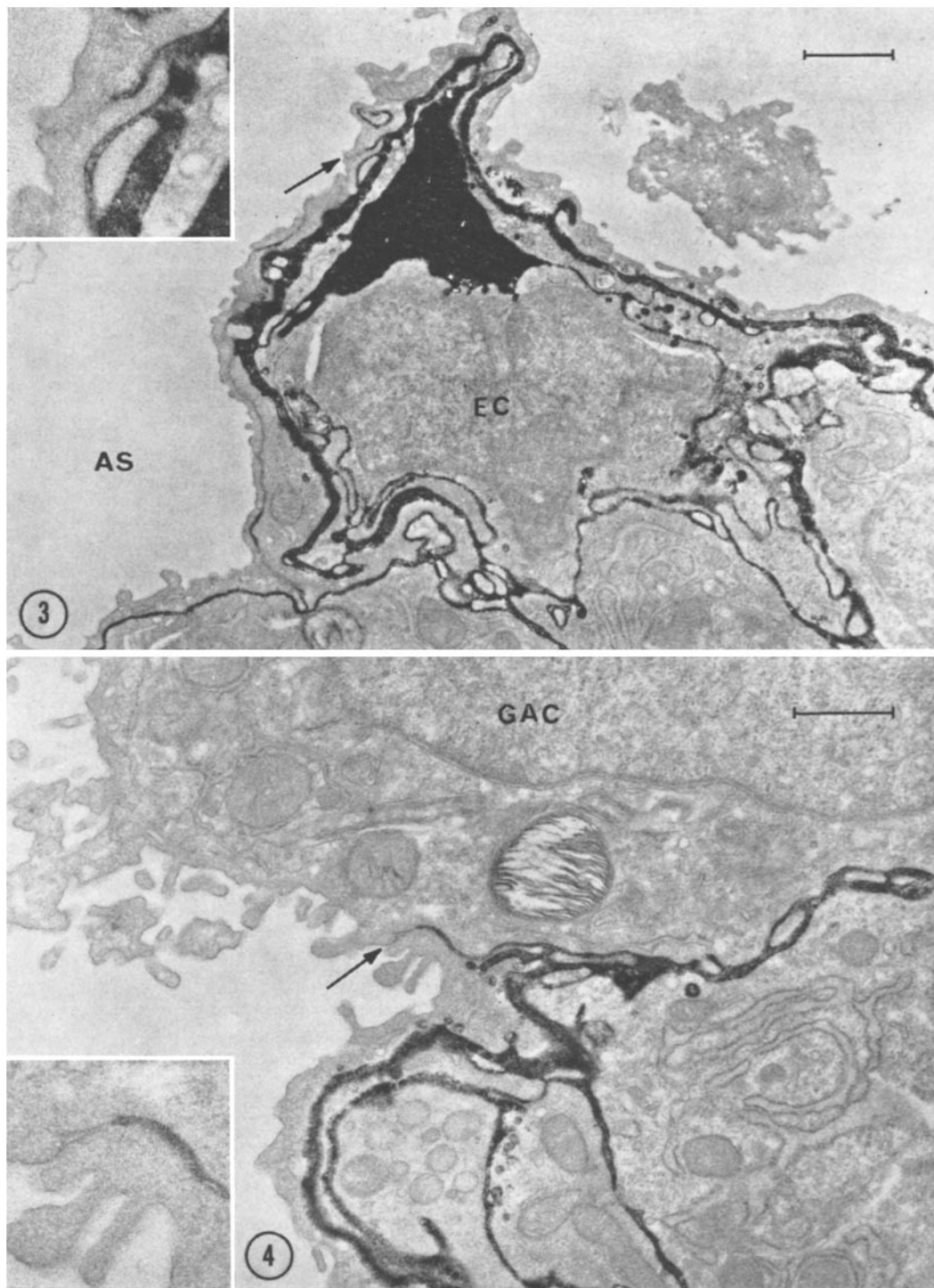
ULTRASTRUCTURE AND MEASUREMENTS OF ENDOTHELIAL JUNCTIONS

All observations were made on tissue fixed in F. G. fixative, postfixed in OsO₄, and stained en bloc with uranyl acetate. All endothelial clefts had at least one point at which adjacent plasma membranes were closely approximated, forming a cell junction. In a few endothelial junctions the gap was obliterated by fusion of the outer leaflets of the unit membrane. Some of the endothelial clefts of newborn mouse pulmonary capillaries were longer and more tortuous than those of adult pulmonary capillaries, and in the more tortuous clefts up to four junctions could be seen.

In the newborn mice there was a difference in the rapidity with which peroxidase passed through endothelial junctions, depending on the volume in which it was injected. In order to ascertain whether the width of endothelial junctions of control newborn mice differed appreciably from those injected with large volumes of tracer, the following measurements were made. The widths of the junctional gap (the area in which the adjacent unit membranes were most closely approximated) in photographic prints of 15 endothelial clefts from control, uninjected newborn mice, and of 15 endothelial clefts from newborn mice injected with peroxidase in 0.02 ml saline but not subjected to histochemical reaction, were measured and averaged, and the two groups were compared. These results were also compared to measurements which we had previously obtained in adult mice (34). The mean width and the standard error of

FIGURE 3 Pulmonary capillary from a newborn mouse sacrificed 90 sec after injecting peroxidase in 0.02 ml saline. Large amounts of peroxidase are present both in endothelial clefts and in the basement membrane, and no concentration gradient is evident. Junctions between flat alveolar cells (arrow and *inset*) are impermeable to peroxidase and the air space (*AS*) is free of the tracer. Both endothelial cells (*EC*) and epithelial vesicles are stained. Uranyl acetate. $\times 11,000$; *Inset*, $\times 36,000$.

FIGURE 4 Another area of the lung from the newborn animal shown in Fig. 3. The junction between the great alveolar cell (*GAC*) and flat alveolar cell is impermeable to peroxidase (arrow and *inset*). Uranyl acetate. $\times 14,000$; *Inset*, $\times 41,000$.



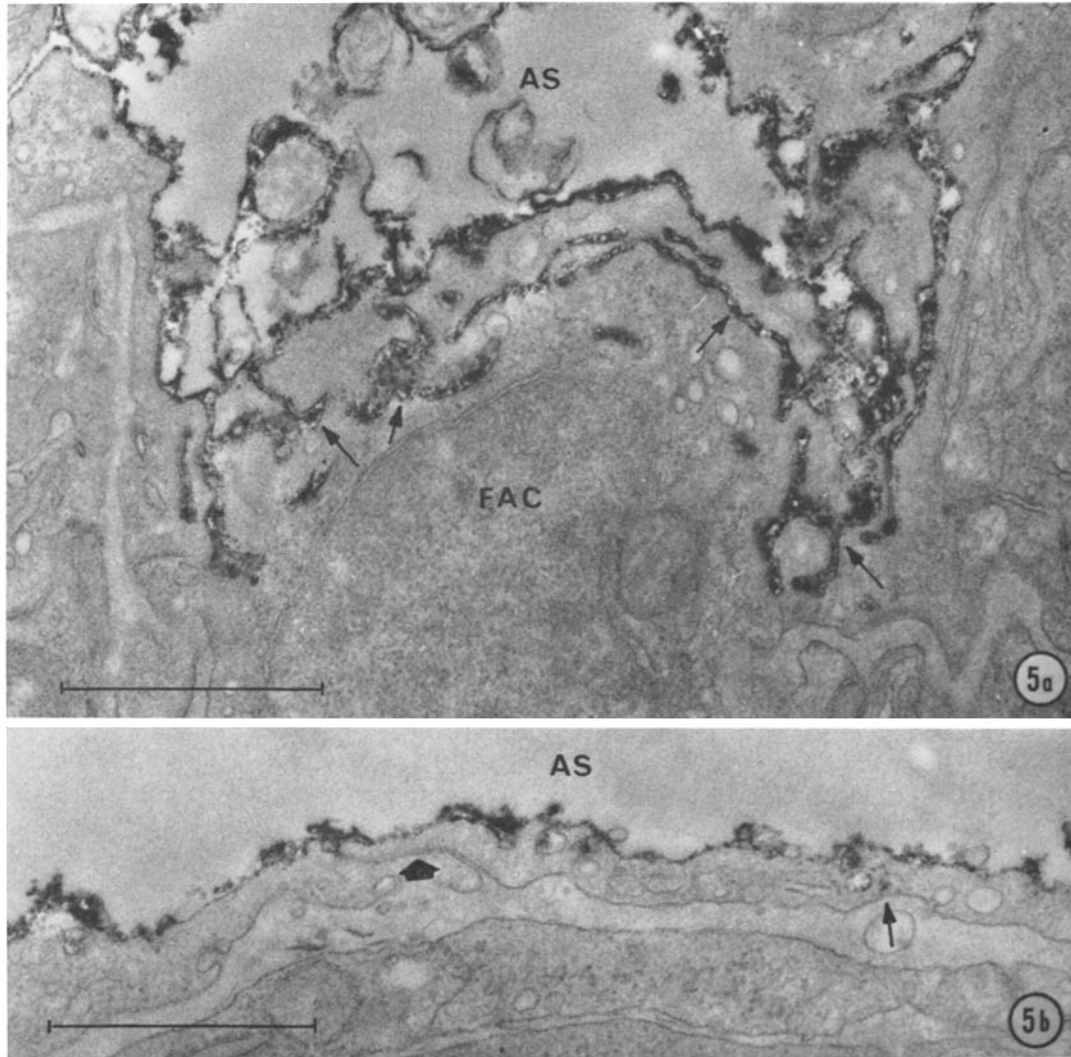


FIGURE 5 a Lung from newborn mouse, 60 min after intranasal instillation of peroxidase. The alveolar surface of flat alveolar cells (*FAC*) is covered by a film of peroxidase which extends into tortuous tubular invaginations within the cell (arrows). A few pinocytotic vesicles filled with peroxidase are also present. The basement membrane appears to be free of peroxidase. *AS*, air space. $\times 34,000$.

FIGURE 5 b Another area of the lung from the same animal as in Fig. 5 a. Peroxidase coats the surface of the flat alveolar cell but does not permeate the epithelial junction (large arrow, left). Pinocytotic vesicles contain peroxidase, one of which touches the cell membrane on the basement membrane side of the epithelial cell (small arrow, right). *AS*, air space. $\times 34,000$.

the mean of the junctional gap of uninjected control mice were 41 ± 1 A, while those of mice injected with 0.02 ml of peroxidase solution were 45 ± 2 A. However, the difference between these two values was not statistically significant ($P \sim 0.2$). Moreover, these measurements were similar

to those obtained in our previous study of adult mouse lungs (34).

Adult Animals

As in newborn mice, the rate of leakage of peroxidase from pulmonary capillary lumens

through endothelial clefts depended on the volume in which the tracer was injected. 90 sec and 6 min after injecting 5 mg peroxidase in 0.05 ml saline or 3.5% of the animal's blood volume (17), reaction product was limited to the capillary lumen. A few endothelial vesicles on both the luminal and basement membrane side of the endothelium contained peroxidase, but endothelial clefts, basement membranes, and epithelial vesicles were free of reaction product (Fig. 8).

The injection of the same amount of peroxidase in 0.2 ml saline resulted in a similar distribution of reaction product 90 sec after injection. However, after 6 min faint staining of endothelial clefts and basement membranes was evident. Endothelial as well as epithelial vesicles contained peroxidase.

Within 90 sec of injection of 5 mg of peroxidase in 0.5 ml saline or 35% of the animal's blood volume (17), reaction product was observed in the capillary lumen, throughout most endothelial clefts, in basement membranes, and in endothelial and some epithelial vesicles (Fig. 9). A few of the endothelial clefts stained heavily only in their luminal half with an abrupt loss of peroxidase staining in the distal portion of the cleft. This was interpreted as being the focal site of a tight junction. As previously described (34), all epithelial junctions were impermeable to peroxidase.

DISCUSSION

The observations reported above indicate that the rapidity with which intravenously injected peroxidase (mol wt 40,000) permeates pulmonary endothelial junctions of newborn and adult mice is dependent on the volume of fluid in which it is injected. Furthermore, high molecular weight tracers such as catalase (mol wt 240,000) and ferritin (mol wt 500,000) did not pass through endothelial clefts. Ferritin particles were present in endothelial vesicles and a few were seen in basement membranes, presumably carried there by vesicular transport.

The influence of increased intravascular fluid volumes on capillary permeability has been the subject of several physiological studies (24, 37, 45). Pappenheimer and Landis (25) have shown that capillary endothelium behaves as if it were a semipermeable membrane penetrated by water-filled cylindrical pores of 40–45 Å radius, through which small lipid-insoluble molecules equilibrate rapidly by free diffusion. For higher molecular weight substances, Pappenheimer derived, on the

basis of calculations and experiments with colloid membranes, the concept of molecular sieving (30), which suggested that during ultrafiltration through an isoporous membrane the ratio of filtrate to filtrand depends on the size of the molecule relative to the size of the membrane pore and the filtration rate. Thus, with high rates of filtration there would be greater molecular sieving due to steric hindrance at the entrance of the pore and frictional resistance within the pore, resulting in decreased filtrate-filtrand ratios. However, Shirley et al. (37), using radioactive albumin and dextran, showed that infusions producing plasma volume expansion in dogs resulted in striking increases in filtrate-filtrand ratios of these molecules. These results were interpreted as evidence for a decrease in resistance of capillaries to the passage of macromolecules or a stretching of capillary pores. They also confirmed earlier reports (24, 45) showing that infusions of saline into animals led to filtration of a protein solution which was more dilute than that filtered before infusion, but which had a greater albumin content as well as a larger volume.

Adult Mice

Recently, Karnovsky, using HRP (22) and cytochrome *c* (23) (mol wt 12,000) as tracers, and serial sections, has presented evidence that the gaps in endothelial junctions of heart and skeletal muscle capillaries are the most likely ultrastructural equivalent of the small pore system postulated by physiologists. Although the exact geometry remains to be established, Karnovsky envisions the gaps as being a series of oval slits bounded at each end by a macula occludens and having a width of 40 Å. We have reported similar evidence for pulmonary capillaries in adult mice (34).

Although the present observations agree with our previous findings on the permeability of the alveolar-capillary membrane to HRP in adult mice (34), they also lend morphological support to the concept of stretched capillary pores. HRP with a molecular weight of 40,000 and an estimated radius of equivalent hydrodynamic sphere of 25–30 Å (22) would be expected to be significantly restricted, at normal filtration rates, in its passage through pores of approximately 45 Å radius. Furthermore, conditions normally present in pulmonary capillaries do not favor the filtration of water-soluble molecules across the endothelium: pulmonary capillary pressures (5–15 mm Hg) are

approximately one-half of those found in systemic capillaries and therefore well below the opposing colloid osmotic pressure of plasma proteins (15). Thus it is not surprising that peroxidase, when injected in a very small volume of saline (0.05 ml), remains confined to the pulmonary capillary lumen and that none can be demonstrated in endothelial clefts or basement membranes within the first 6 min of injection. That the contribution of injected peroxidase to the total osmotic pressure of serum proteins is negligible is indicated by the calculations discussed earlier. However, when peroxidase is injected in large volumes of saline (0.5 ml), it rapidly permeates—within 90 sec—endothelial clefts and the adjacent interstitial space. Presumably the transient increase in intravascular fluid volume stretched endothelial junctions or opened up previously closed endothelial junctions, allowing the ready passage of peroxidase through them. These results are perhaps not entirely comparable to those obtained from experiments in which steady states of increased venous pressure or increased plasma volumes were established before their effect on capillary filtration was studied (4, 24, 37, 45). However, they are consistent with the observations of Pietra et al. (31), who reported the necessity of using extremely high intravascular pressures (50 mm Hg) before hemoglobin, a larger molecule than peroxidase, could be detected passing through endothelial clefts. These authors suggested that endothelial pores may be stretched or opened up by increased hydrostatic pressures.

Newborn Mice

Relatively little is known about the permeability of fetal and neonatal capillaries. However, they are thought to resemble regenerating capillaries (26) which have shown to be permeable to such large tracer molecules as colloidal carbon (35).

Gaps between endothelial cells, allowing the escape of chylomicra, have been described in cutaneous and muscular capillaries of newborn mice (40). That pulmonary capillaries might be analogous in structure and permeability to regenerating capillaries would not be surprising, in that there is considerable growth of alveoli—and presumably capillaries—after birth. Indeed, in newborn rats Weibel, using morphometric methods, established that between the fifth and tenth days of life the growth of the surface area of the lung is proportional to the eighth power of the growth of linear dimensions of the body and the lung (46).

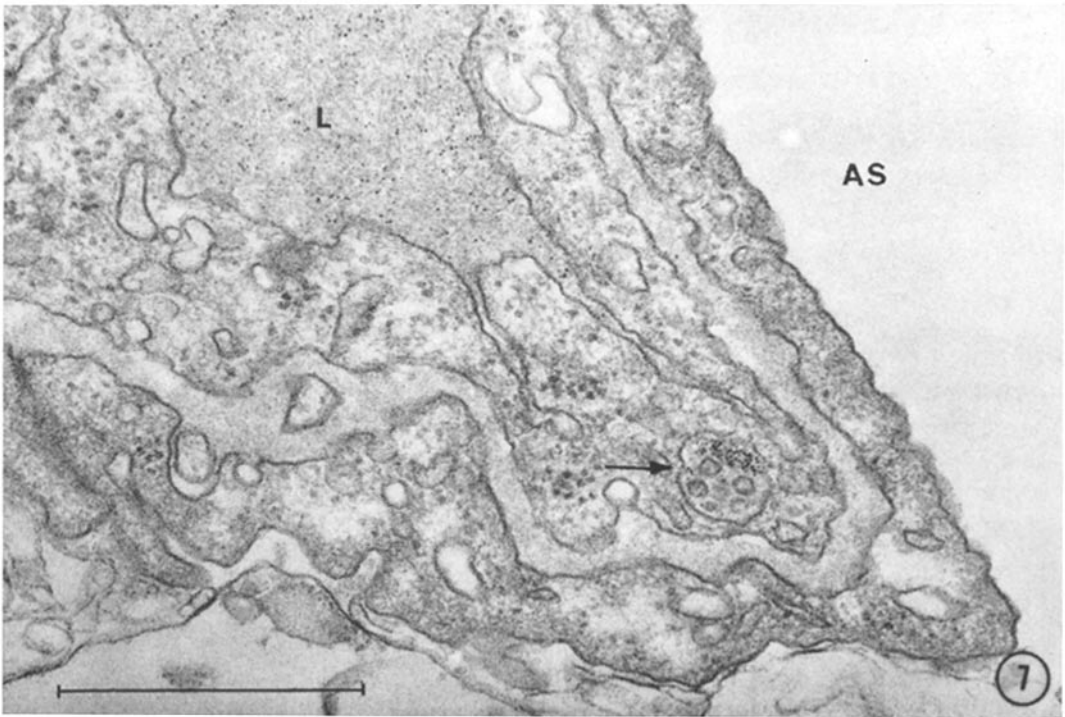
PERMEABILITY OF ENDOTHELIAL CLEFTS:

In a recent study on the permeability of the ovine alveolar-capillary membrane, Strang and his colleagues reported that in newborn lambs the mean endothelial pore radius (90 Å) was considerably smaller than that of fetal (150 Å) or adult (130 Å) sheep, while the pore area per unit path length in newborn lambs was about 5–15 times greater than in either of the latter. Furthermore, they postulated the presence of a few large pores or leaks which would allow the passage of dextran molecules of a molecular radius of 110 Å (9).

Our observations indicate that the pulmonary endothelial barrier of newborn mice differs from that of adult animals in several respects. In newborn mice the endothelial cells were plump, containing many free ribosomes, and resembled immature endothelium described by others (12). Many of the endothelial clefts were longer and more tortuous than in adult lungs, presumably because of a greater overlapping of adjacent endothelial cells; however, more endothelial clefts per capillary than in adult animals were not observed. Whereas all capillaries contained intraluminal peroxidase, many of them were collapsed, re-

FIGURE 6 Lung from newborn mouse sacrificed 1 hr after injection of 0.02 ml catalase. The tracer extends only partially into the endothelial cleft (arrow) and the basement membrane is free of the tracer. A few vesicles contain a small amount of catalase (double arrow). Uranyl acetate and lead citrate. *AS*, air space. $\times 25,000$.

FIGURE 7 Lung from newborn mouse sacrificed 1 hr after injection of ferritin suspended in 0.02 ml saline. Ferritin is confined to the capillary lumen (*L*) and a cluster of ferritin particles is present in a multivesicular body (arrow) within the endothelium. Elsewhere a few particles were present in pinocytotic vesicles, but most of them were in vacuoles and multivesicular bodies. No ferritin particles are present in the basement membrane. Uranyl acetate and lead citrate. *AS*, air space. $\times 50,000$.



flecting possibly the relatively low pressure in pulmonary capillaries of newborn animals as reported by Strang et al. (9). Although the degree of permeability of pulmonary capillaries to peroxidase depended, as in adult animals, on the volume of saline in which it was injected, there was some indication that capillaries of newborn mice behaved as though they were somewhat more permeable. That is, even within 90 sec of injecting peroxidase in the smallest volume of saline used, there were a few areas in the lung where leakage through endothelial clefts had occurred. This does not necessarily mean that pulmonary endothelial junctions of newborn animals are themselves wider or more permeable; they may simply be more susceptible to being stretched by slight increases in intravascular fluid volume.

It is perhaps not surprising that we were unable to measure any difference in the width of endothelial pores in animals receiving peroxidase in large volumes of saline as compared to those receiving the tracer in small volumes of fluid. This is possibly so for the following reasons. Firstly, the stretching of pores caused by the injection of large volumes of fluid is probably a transient event. Secondly, during removal of the tissue for fixation intravascular pressures fall to levels much lower than those present in vivo. In order to demonstrate widened endothelial clefts, fixation would have to be done by perfusion at those pressures which cause increased leakage of protein such as peroxidase. Nonetheless, it is unlikely that the pore radii of pulmonary endothelium of newborn mice are as large as those reported for newborn lambs (9). As pointed out by Karnovsky (22), a cylindrical pore of 45 Å radius corresponds to a slit having an equivalent width of 55 Å. Our figure of 45 Å for the average junctional width was considerably lower than the radius of 90 Å or the slit of equivalent width of

110 Å reported for newborn lambs (9). Our values, instead, resemble more closely the calculated pore radii of 40–58 Å obtained by Taylor and Gaar (41) from estimates of reflection coefficients of urea, glucose, and sucrose in isolated dog lungs.

That the injection of relatively large volumes of fluid into newborn mice did not cause anatomical disruption of endothelial junctions is evidenced by the fact that catalase (mol wt 240,000) (33) remained confined to the capillary lumen and extended only partly into endothelial clefts even 60 min after injection. Furthermore, these results also indicate that pulmonary endothelial pores of newborn mice are of such dimensions, even when transiently stretched by large fluid volumes, that they are impermeable to molecules the size of catalase.¹ They would also tend to preclude the presence of a few large pores postulated by Strang et al. (9) in newborn lambs, which might allow the passage of such large molecules as catalase, although if the large pores are extremely few relative to the frequency of small pores, the former could easily escape detection.

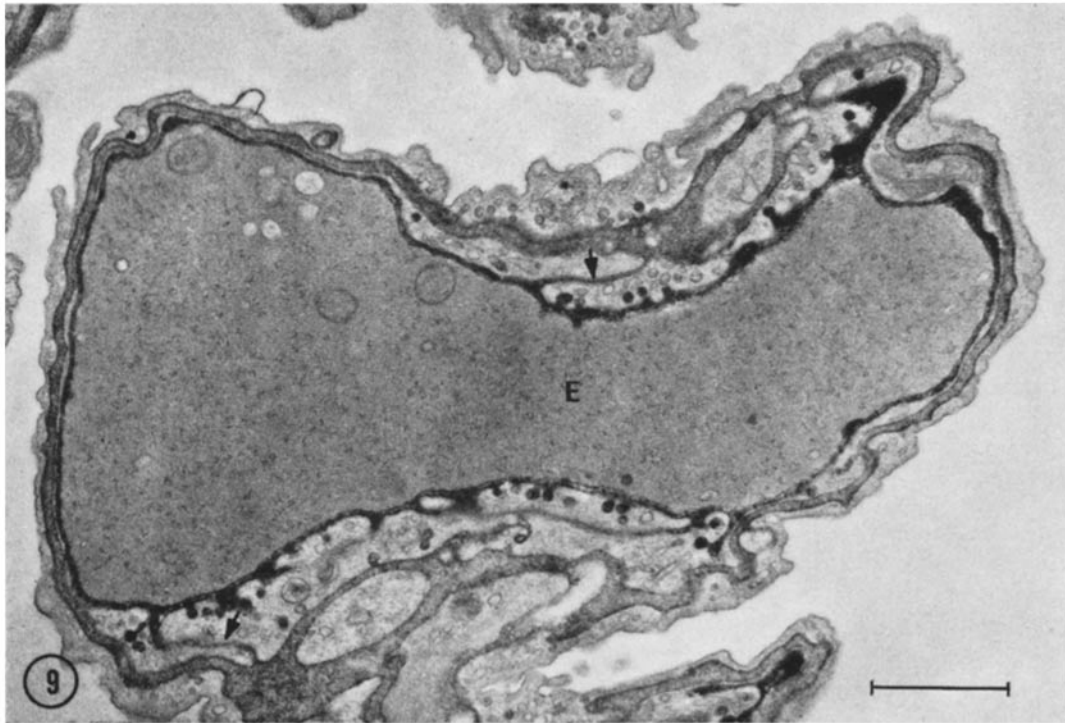
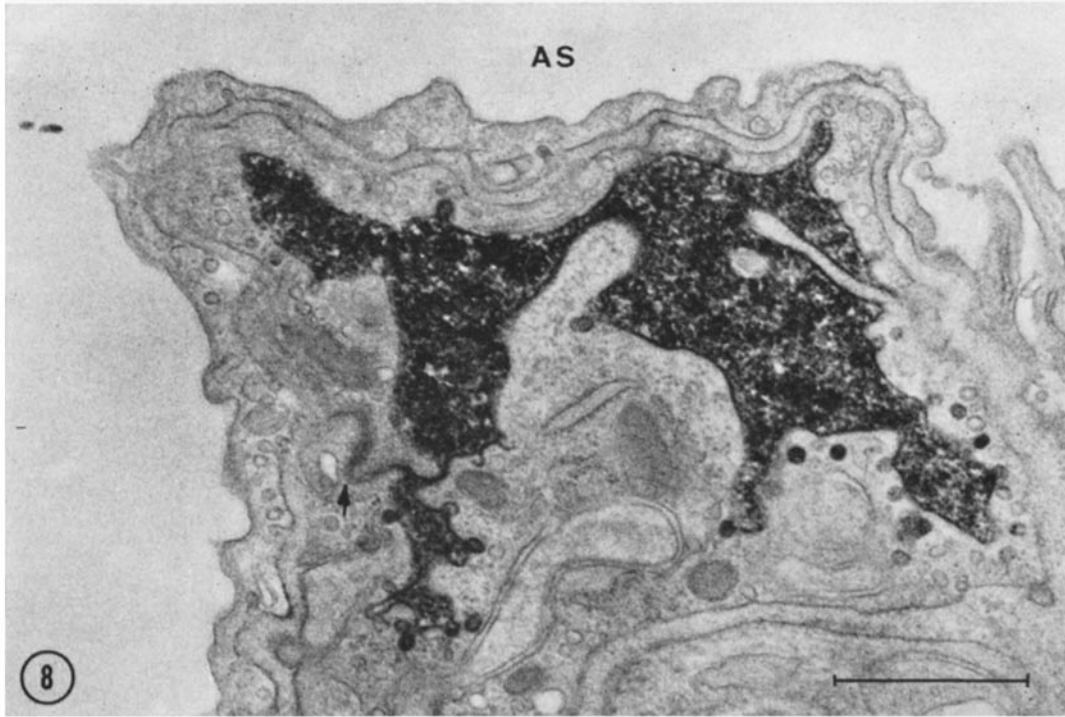
ENDOTHELIAL VESICULAR TRANSPORT:

Relatively few pinocytotic vesicles were present in the endothelium and vesicular transport appeared to be slow just as in adult mice (34). Furthermore, as indicated by Winne on theoretical grounds (47), the injection of large volumes of fluid did not appear to influence the rate of vesicular transport. The distribution of ferritin particles in vesicles, dense bodies and multivesicular bodies, was similar to that described by Bruns and Palade in

¹ Catalase has a sedimentation coefficient $S_{20,w}$ of 11.4S (33). The intrinsic viscosity of native catalase is low, implying a compact confirmation of the protein molecule. It has been estimated that the axial ratio of native catalase (as an equivalent hydrodynamic ellipsoid) is less than 5 and that the major length of the molecule is about 240 Å (33).

FIGURE 8 Pulmonary capillary from an adult mouse injected with 0.05 ml peroxidase solution and sacrificed 6 min later. Peroxidase is confined to the capillary lumen and extends down almost the entire length of an endothelial cleft (arrow). Pinocytotic vesicles in the luminal half of the endothelial cells contain peroxidase. Very little, if any, peroxidase is present in the basement membrane. AS, air space. $\times 25,000$.

FIGURE 9 Pulmonary capillary, containing an immature erythrocyte (E), from an adult mouse injected with 0.5 ml peroxidase solution and sacrificed 6 min later. Peroxidase in the lumen permeates endothelial clefts (arrows) and stains the basement membranes. Pinocytotic vesicles throughout the endothelium contain peroxidase. $\times 18,000$.



diaphragmatic capillaries (10). It is of some interest that the uptake of catalase by pinocytotic vesicles was considerably less than that of HRP. This may be related to the lower isoelectric point of catalase (pH 5.7) as compared to HRP (pH 7.2) (38), for it has been reported that pinocytotic cells take up protein with a basic charge more readily than protein with a negative charge (32).

THE EPITHELIAL BARRIER AND PROTEIN UPTAKE FROM ALVEOLUS: The epithelial junctions of newborn mouse lungs were consistently impermeable to peroxidase and thus resembled those of adult mice (34). This relative impermeability of alveolar epithelial junctions as compared to alveolar capillary junctions has recently been confirmed by Taylor and Gaar, who estimated reflection coefficients for urea, glucose, and sucrose across alveolar membranes and alveolar capillaries, respectively, and calculated a pore radius of 6–10 Å for alveolar epithelium (41). This is of interest in relation to the origin and composition of lung liquid. It is well known that a considerable quantity of fluid is present in the fetal lung at birth (1) and that its electrolyte composition is quite different from that of amniotic fluid (2). Furthermore, ligation of the trachea in rabbits (11, 20) results in overdilatation of the fetal lung, indicating that the liquid is formed *in situ* in the lung. The low concentration of protein (0.03 g/100 ml) in lung liquid (8) suggests that it may be formed by ultrafiltration from lung capillaries. However, the site through which protein gains entrance to the alveolar space in fetal animals remains to be established for, although epithelial junctions are tight and impermeable to peroxidase at the time of birth, their permeability during earlier stages of development is unknown. That alveolar epithelial cells may be important in further modifying the composition of alveolar fluid is evidenced by the fact that the total CO₂ content and the pH of lung liquid are strikingly different from those of plasma (2).

The removal of lung liquid in newborn lambs occurs within the first 5 hr after birth, and this is accompanied by a large increase in lymph flow from the lung (19). By means of intranasally installed peroxidase, an attempt was made to establish the manner in which protein is absorbed from the alveolar space during the first 2 hr after birth. The distribution of inhaled peroxidase in newborn mice differed from that reported in adult animals (34), in that the tracer was more evenly

distributed throughout the lung and that it coated alveolar surfaces more uniformly. This was probably due to mixing of peroxidase with residual lung liquid in the alveoli. Similar observations with intratracheal injections of Evans blue dye into sheep and newborn lambs have been reported (19). In newborn mice the tracer was taken up not only by pinocytotic vesicles of flat alveolar cells but also by tubular invaginations which resembled the lamellae of "micropinocytosis vermiformis" described by Orci et al. (29) in Kupffer cells and which have been correlated with the cellular uptake of colloidal material and proteins. Such tubular invaginations were not observed in adult mice (34). In no instance was peroxidase observed passing through epithelial junctions, and of the cells lining the alveoli, only flat alveolar cells took up the tracer.

The problem of protein absorption from alveolar spaces has been the subject of a number of studies (14, 28, 36). Although it is generally thought to be a slow process, it has recently been reported (28) that radioiodinated albumin leaves alveoli of dog lungs more rapidly than was previously appreciated. The present observations would favor a relatively slow pinocytotic transport across the alveolar epithelium: even 1 hr after intranasal administration of peroxidase, none could be demonstrated in basement membranes. The apparent susceptibility of alveolar epithelium to cell damage by relatively large amounts of protein such as peroxidase resulted in diffuse staining of epithelial cells with leakage across the alveolar epithelium and artefactual staining of the underlying basement membrane. This susceptibility to damage of the epithelium could be important in the interpretation of studies of protein uptake from alveoli.

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A recent paper (Clementi, F. 1970. *J. Histochem.*

Cytochem. 18:887.) has suggested that permeation of HRP through pulmonary endothelial cell junctions is dependent on high doses of HRP. The volume of saline in which HRP was injected was kept constant at 0.3 ml. We, however, find no such relationship to dosage. As we kept the dose constant, irrespective of the volume injected, we find rather a relationship to volume.

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