

THE PERMEABILITY OF MUSCLE CAPILLARIES TO HORSERADISH PEROXIDASE

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ABSTRACT

The main objective of this study was to determine the pathways by which horseradish peroxidase (HRP) can cross the endothelium of muscle capillaries. Specimens of mouse diaphragm were fixed for cytochemical analysis at various intervals after intravenous injection of 0.5 mg HRP, at 4 min after intravenous injection of varied amounts of HRP, and at 4 min after intravenous injection of HRP in various volumes of isotonic NaCl. Our findings indicate that endothelial junctions serve as a barrier which may allow passage of very limited amounts of HRP. They also suggest that endothelial vesicles transfer HRP from the capillary lumen to the pericapillary interstitium as well as in the reverse direction. Increasing the volume of solution injected to ~30% of total blood volume did not increase the amount of HRP that left the capillary lumen. Our results with HRP do not provide clearcut evidence that endothelial junctions are the site of the small pore.

Physiologic studies have established that the capillaries of muscle are permeable to plasma proteins (1-4), and in this regard the capillary wall behaves as though it possesses a set of large (5-8) and a set of small pores (4, 9). Two components of the capillary endothelium are candidates for the morphologic counterparts of the pores: the abundant small cytoplasmic vesicles and the junctions between endothelial cells.¹ Ferritin² (12, 13) and colloidal particles (14-16) have been used as probes for the large pore because of their large size. After being injected intravenously these tracers were observed in sequential stages of transfer across the endothelium within vesicles; they were not observed passing through endothelial junctions. The vesicles have been identified as the

counterpart of the large pore on the basis of these observations.

Investigators have used smaller protein tracers to determine whether endothelial junctions are the counterpart of the small pore, and the conclusions from these studies are a matter of controversy. Karnovsky (17, 18) examined the localization of horseradish peroxidase (HRP)³ in junctions of muscle capillaries following intravenous injection of this tracer. He observed a continuous band of tracer extending throughout the length of the junction when there was abundant tracer in both the capillary lumen and interstitium. He also observed a similar band after shorter intervals, before a significant amount of HRP had accumulated in the interstitium. He concluded that the endothelial junctions were permeable to HRP on the basis of these observations. In a preliminary

¹ We define the endothelial junction as the region of apposition between neighboring endothelial cells.

² ~600,000 mol wt, 100-Å mol diam (10, 11).

³ 40,000 mol wt, 50-Å mol diam (17, 19).

report, Williams and Wissig (20) questioned this conclusion because in similar experiments they observed that, before a substantial amount of tracer had appeared in the interstitium, the penetration of HRP was halted abruptly at some point within the junction. On the basis of findings similar to those of Williams and Wissig with the smaller tracers, myoglobin⁴ and microperoxidase,⁵ Simionescu et al. (24, 25) also questioned Karnovsky's conclusion.

In this paper we report the results of our study of the passage of HRP across the wall of the capillaries of the mouse diaphragm. We assessed the accessibility of vesicles and junctions as routes of passage across the wall on the basis of the following criteria. After intravenous injection of tracer, sequential stages of vesicular transfer of the tracer across the endothelial cell should be observed, i.e., vesicles filling with tracer at the luminal surface of the endothelial cell, filled with tracer while free in the cytoplasm of the cell, and emptying tracer at the interstitial surface of the cell. During the filling and emptying stages, the limiting membrane of the vesicle should be continuous with the plasma membrane of the endothelial cell. During the emptying stage, the concentration of tracer within the emptying vesicle should be higher than in the interstitium, thus ruling out the possibility that the vesicle is filling rather than emptying. With regard to junctions, when there is little tracer in the interstitium and a high concentration of tracer in the capillary lumen, we should observe a continuous band of tracer extending through the junction and an aggregation of tracer in the pericapillary interstitium outside of the endothelial junction. Observation of a continuous band of tracer extending through the junction when there is a substantial concentration of tracer in both the lumen and interstitium is ambiguous evidence of passage. This appearance would also be observed if the junction is sealed at one point and tracer fills the cleft up to the point of the seal by having diffused from both the lumen and the interstitium.

We applied these criteria in the following experimental situations. We studied the localization of HRP in capillaries of the mouse diaphragm at a series of intervals after mice were injected intrave-

⁴ 17,800 mol wt, 30–35-Å mol diam (21, 22).

⁵ 1,900 mol wt (23). Simionescu et al. (24) also obtained similar results with a smaller version of microperoxidase, i.e., heme-octapeptide (~1,500 mol wt).

nously with an amount of the tracer sufficient to opacify the capillary lumens during the early stages after injection. We studied the localization of HRP in capillaries of the diaphragm 4 min after we injected mice intravenously with various amounts of HRP. Earlier investigations (26, 27) had shown that, in the pulmonary capillary bed, increasing the volume in which a given amount of tracer was administered would increase the amount of tracer that escaped through the capillary wall. This suggested that the relatively rapid expansion of plasma volume resulting from an intravenous injection of tracer could cause an artifactual increase in the size and/or number of pores in the capillary wall, a possibility also raised by earlier physiologic findings (28–32). To determine whether we might be increasing the size and number of capillary pores in our experiments, we investigated the effect of increasing the volume of injection medium on the rate of passage of HRP across the capillary wall.

MATERIALS AND METHODS

Tracers

We used chromatographically purified HRP, type HPOFF from Worthington Biochemical Corp., Freehold, N. J. and Type VI from Sigma Chemical Co., St. Louis, Mo., in this study.⁶

Injection of Tracer and Fixation of Tissue

Adult Cal/A and C₃H/HeJ mice weighing 20–24 g were anesthetized with an intraperitoneal injection of pentobarbital sodium at a dosage of 9 mg/100 g body

⁶ Nearly all of the mice were injected with Worthington Type HPOFF HRP. We assayed this enzyme preparation and found that it contained 360 purpurogallin U/mg. According to the Sigma Chemical Company, Type VI HRP has a potency of 250–330 purpurogallin U/mg. We assayed one batch of this enzyme and found that its potency was 320 purpurogallin U/mg. Because the histochemical findings obtained with the two enzyme preparations were essentially the same, we have pooled the findings obtained with them.

In an early phase of our study we employed Sigma Type II HRP as tracer. According to the manufacturer, this enzyme preparation has a potency of 100–150 purpurogallin U/mg. We assayed one batch and determined that it contained 197 purpurogallin U/mg. We obtained perplexing results with Sigma Type II HRP. Some mice had respiratory difficulties and died a few min after they were injected. We never observed similar signs of toxicity with either of the more purified enzyme preparations even when we injected much larger

weight. After a small incision was made in the right ventral cervical region, the external jugular vein was exposed by blunt dissection. HRP dissolved in isotonic saline was injected into the vein. The length of time required for injection ranged from 20–30 s for 0.1 ml to 2–3 min for 0.5 ml. The peritoneal cavity was opened with a midventral incision in the abdominal wall, and the liver and the stomach were retracted to expose the peritoneal surface of the diaphragm. The diaphragm was then fixed *in situ* by flooding its surface with a modification of Karnovsky's fixative (34) which contained 1½–2% redistilled glutaraldehyde, 1% freshly dissolved paraformaldehyde, 1% sucrose, and 0.02% CaCl₂ in 0.067 M sodium cacodylate buffer at pH 7.4. The osmolarity of the fixative was approximately 600 mosM. After 5 min fixation *in situ*, the diaphragm was removed and placed in fresh fixative at 4°C for approximately 4 h.

In the first set of experiments, we studied the localization of HRP at various time intervals after a standard amount of the tracer was injected intravenously (Table I).

In a second set of experiments, we varied the amount of HRP and the volume in which the tracer was injected. The diaphragms were fixed 4 min after injection of the tracer. The amount of HRP was either 0.5 or 5 mg per mouse; the volume injected ranged from 0.05 to 0.5 ml per mouse (Table II).

In a third set of experiments, we varied the amount of HRP injected from 0.25 to 16 mg. The HRP was injected in 0.1 ml, and specimens of diaphragm were fixed after an interval of 4 min (Table III).

Histochemical and Embedding Procedures

After fixation the tissues were washed in several changes of Tris-HCl (0.05 M, pH 7.6) or sodium cacodylate (0.1 M, pH 7.4) buffer. Sections 35–70 µm thick were cut on a Sorvall TC-2 Tissue-Sectioner (Ivan Sorvall, Inc., Newtown, Conn.), stored overnight at 4°C in one of the above buffers, and then incubated in the histochemical medium (35) the next day. We used two different incubation media and schedules of incubation: (a) 10 ml Tris buffer (as above) containing 5 mg 3,3'-diaminobenzidine tetrahydrochloride (DAB) and 0.1%

amounts. When we compared specimens from mice injected with either Sigma Type VI or Worthington Type HPOFF HRP with those from mice injected with comparable enzymatic amounts of Sigma Type II HRP, we noted, in the latter, that more reaction product appeared in the pericapillary interstitium and reaction product appeared more frequently in the cytoplasmic matrix of endothelial cells. In an earlier study Olsson and Reese (33) reported that intravenous injection of a large dose of Sigma Type II HRP could cause a fatal anaphylactic reaction in mice. To avoid complications that might arise from toxicity of the HRP, we have not included findings with Sigma Type II HRP in this report.

TABLE I
Number of Mice and Time Intervals Used in Experiments to Localize HRP* After Intravenous Injection

Time interval, min†	2	4	8	16	30	60	120
Number of mice	2	3	2	2	3	1	1

* Each mouse was injected with 0.5 mg HRP in 0.1 ml.

† The interval in this and all other experiments was measured from the time the injection was started to the time when the peritoneal surface of the diaphragm was flooded with fixative.

TABLE II
Number of Mice and Dosages Used in Experiments to Determine the Effect of Varying the Volume of Injected Solution on the Distribution of HRP

Amount of HRP injected, mg	0.5	0.5	5	5	
Volume injected, ml		0.2	0.5	0.05	0.2
Number of mice		1	2	2	2

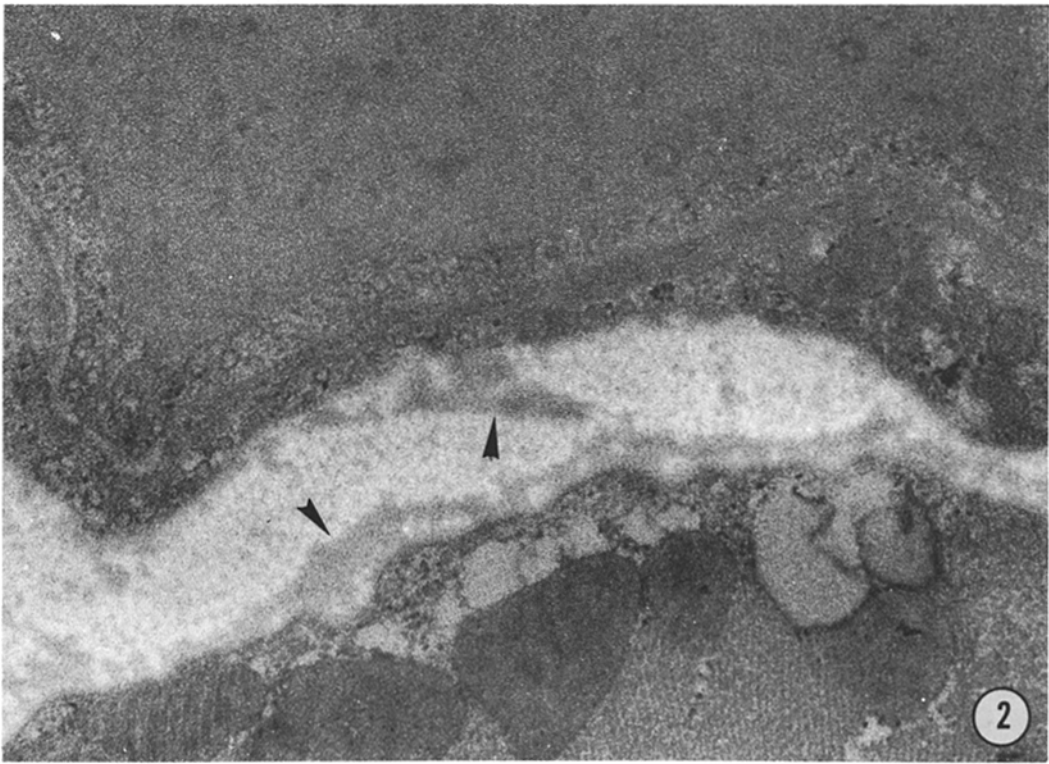
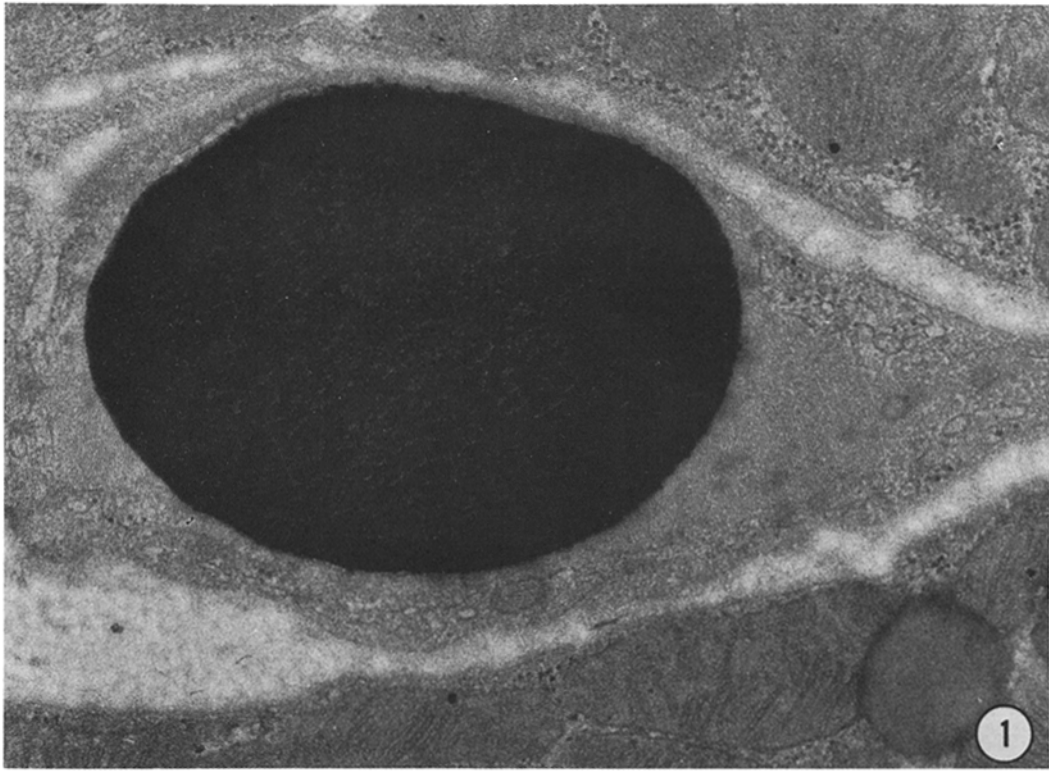
TABLE III
Number of Mice and Dosages Used in Experiments to Determine the Effect of Varying the Amount of HRP Injected on the Distribution of the Tracer

Amount injected, mg	0.25	0.5	1	1.5	2	3	4	8	16
Number of mice	1	2	1	2	4	3	4	3	1

H₂O₂ for 30–120 min, or (b) 10 ml Tris buffer containing 5 mg DAB, 0.25 mg glucose oxidase (Calbiochem, San Diego, Calif.) and 2 mM glucose (36) for 180 min. The incubations were carried out at room temperature in a Dubnoff metabolic shaker. Both procedures gave similar results. The sections were rinsed in several changes of buffer or distilled water, postfixed in 2% OsO₄ in 0.1 M sodium cacodylate buffer for 60 min, dehydrated rapidly in cold, graded acetone solutions, and embedded in low viscosity resin (37) or Epon 812 (Shell Chemical Co., N.Y.) (38). Because staining of tissue blocks in uranyl acetate solutions removed or masked the reaction product, this step was not used in the embedding procedure. Silver or silver-gray sections of selected areas were cut, stained lightly with alkaline lead citrate (39), and were examined in a Siemens Elmiskop 1.

Controls

Careful analysis of a large variety of control preparations in an earlier study (17) has shown that the



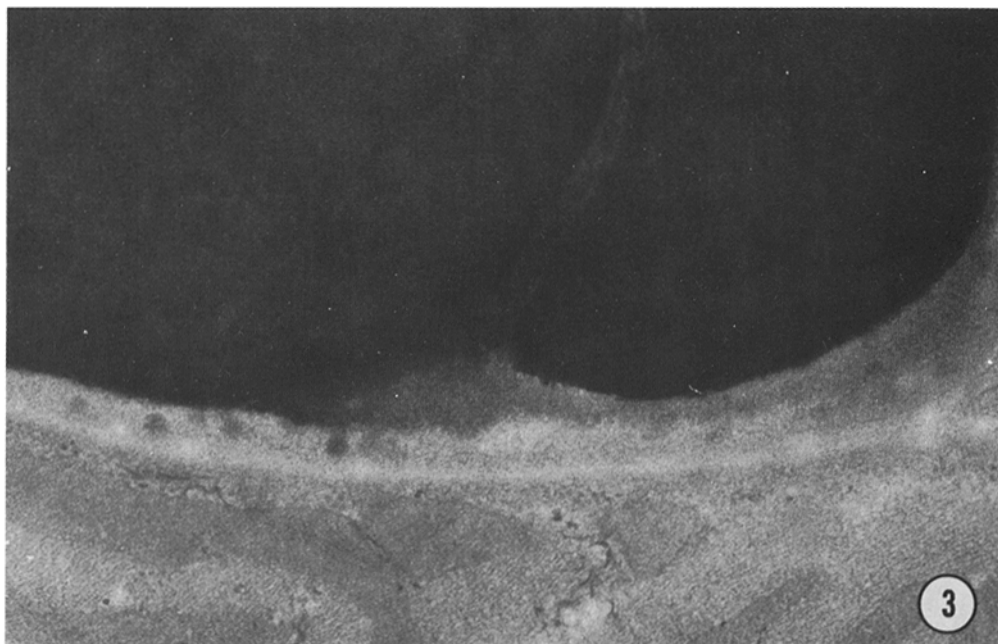


FIGURE 3 Specimens of diaphragm from control (uninjected) mice. The specimens were incubated in the histochemical medium used for localizing HRP. There are two highly reactive erythrocytes in the upper portion of the figure. The erythrocytes are separated from one another and from the endothelium by a thin layer of plasma. The plasma, particularly in the region between the erythrocytes, is considerably denser than normal. Vesicles in the endothelial cell are filled with material resembling the dense plasma. $\times 30,000$.

histochemical reaction is specific for molecules capable of reducing H_2O_2 , produces reaction product which is localized at the site of the enzyme, and does not form reaction product-like complexes which are adsorbed onto tissue. Our control specimens were diaphragms taken from uninjected mice and processed either with or without incubation in the histochemical medium.

RESULTS

Control Specimens

In specimens of diaphragm obtained from uninjected mice and incubated in the histochemical

medium, erythrocytes (Fig. 1) and specific granules of neutrophils and eosinophils were strongly positive and appeared electron opaque, i.e., deep black. The plasma within the lumen of capillaries was moderately denser than that of unincubated specimens (Figs. 1 and 2). In specimens incubated for long periods, i.e. 3 h, plasma located between an erythrocyte and an adjacent endothelial cell, and the content of endothelial vesicles in the area of apposition occasionally were denser than elsewhere (Fig. 3). This observation was probably due to leakage of hemoglobin from erythrocytes as

FIGURES 1 and 2 Specimens of diaphragm from control (uninjected) mice. The specimens were incubated in the histochemical medium used for localizing HRP.

FIGURE 1 The erythrocyte in the capillary lumen displays an intensely positive reaction. The surrounding blood plasma is slightly denser than that seen in control unincubated specimens. The small, moderately dense bodies dispersed in the plasma are presumed to be lipoprotein particles. In the lower right corner of the figure a lipid droplet in the cytoplasm of a muscle cell displays a positive reaction. $\times 28,000$.

FIGURE 2 The capillary in the upper part of the figure contains plasma that is slightly denser than that of unincubated specimens and in its lumen are small round bodies presumed to be lipoprotein particles. A pale band of connective tissue extends across the field below the capillary. The band consists principally of unstained collagenous fibers cut in cross section and seen in negative image. There are irregular patches of moderately dense material (arrowheads) among the collagenous fibers. $\times 25,000$.

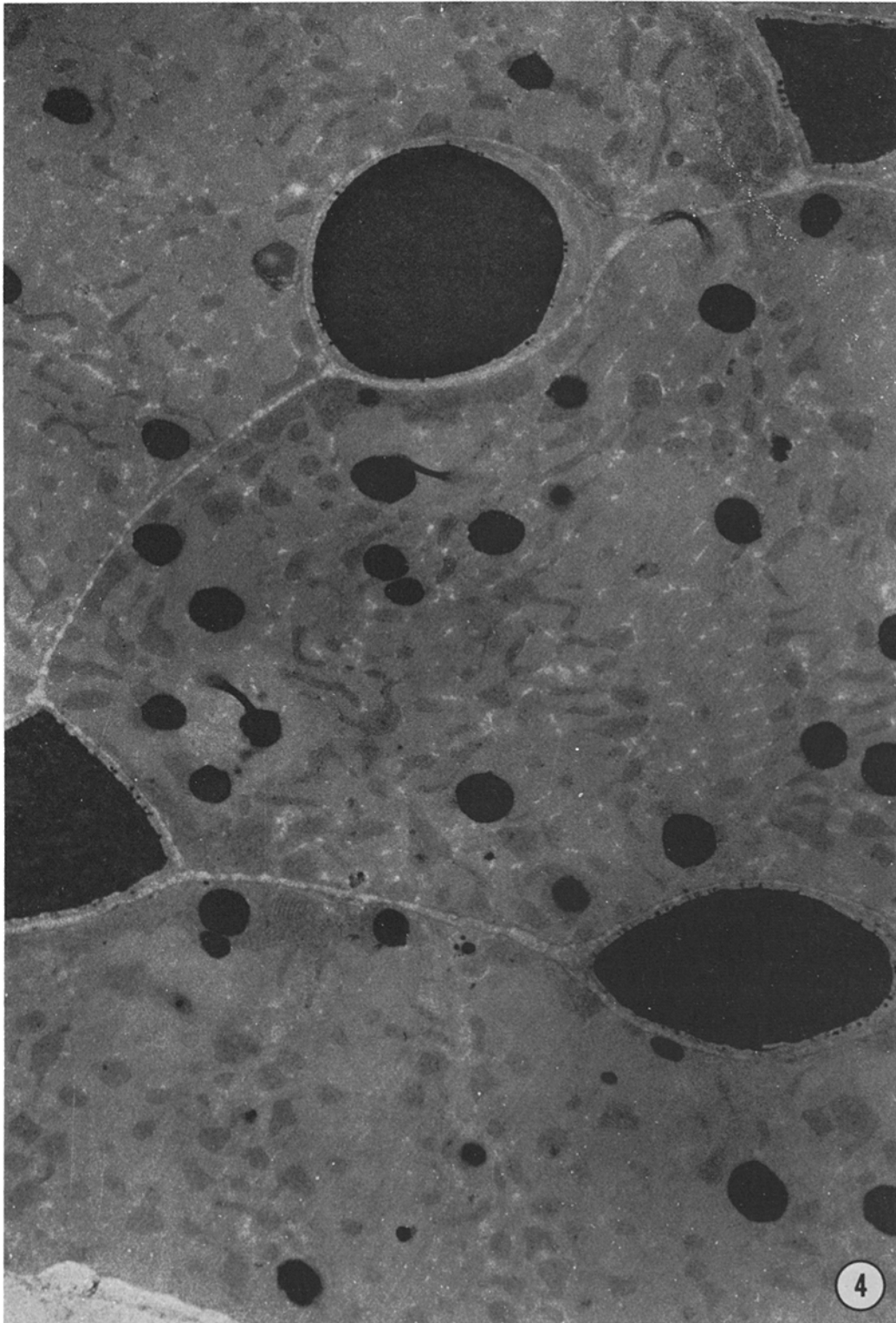


FIGURE 4 Electron micrograph at relatively low magnification of a specimen of diaphragm from a mouse that was injected with 0.5 mg HRP in 0.1 ml saline 16 min before sacrifice. The plane of section is perpendicular to the long axes of the capillaries and muscle cells. The capillaries are normally distended with blood, and the luminal reaction product is of equivalent density in each capillary. Dense lipid droplets are prominent in the cytoplasm of the muscle cells. $\times 6,000$.

reported by Karnovsky (17). Small dense bodies, circular in outline, appeared sometimes in the plasma and were presumed to be circulating lipoprotein particles (Figs. 1 and 2). The pericapillary interstitium was pale and generally free of material resembling the reaction product of HRP. In interstitial areas containing bundles of collagenous fibers, we sometimes observed a homogeneous substance of moderate density which was distributed in irregular patches among the fibers and outlined them in negative image (Fig. 2). We do not know the identity of this substance. Lipid droplets within the cytoplasm of skeletal muscle cells appeared dense black (Fig. 1). Heterophagic vacuoles of macrophages and skeletal muscle cells frequently contained dense material which could represent endogenous peroxidase or peroxidatic activity of ingested heme proteins. The sarcolemma of skeletal muscle cells often was denser than that of muscle cells in unincubated specimens, and the intracristal space of mitochondria of skeletal muscle cells frequently contained varied amounts of reaction product.

We attribute the positive reaction of erythrocytes to the peroxidatic activity of hemoglobin (40). Owing to its peroxidatic activity, hemoglobin has been used as a tracer for studies of capillary permeability (41). It is likely that the increased density of plasma seen in proximity to erythrocytes is due to leakage of hemoglobin from these cells occurring during the fixation or incubation procedures. The increase in general density of the plasma in incubated specimens may also be due to leakage of a small amount of hemoglobin from erythrocytes, but in this case, since the increased density is uniformly distributed throughout the plasma, leakage would have to occur earlier, before the time when the plasma is gelled by fixation. It would be difficult to establish conclusively whether or not injection of a tracer dissolved in saline actually causes some hemolysis because Simionescu et al. (25) detected, in chromatograms of plasma from *uninjected* rats, a low OD_{415} peak presumably due to heme protein. Vassar et al. (42) determined that hemoglobin leaked from isolated erythrocytes during fixation with formaldehyde but not during fixation with glutaraldehyde. We cannot deduce from the latter study whether or not the mixture of the two aldehydes that we used would prevent such leakage. The positive reaction of mitochondrial cristae of skeletal muscle cells is presumed to be due to the peroxidatic activity of cytochrome enzymes located in the cristae (43-45).

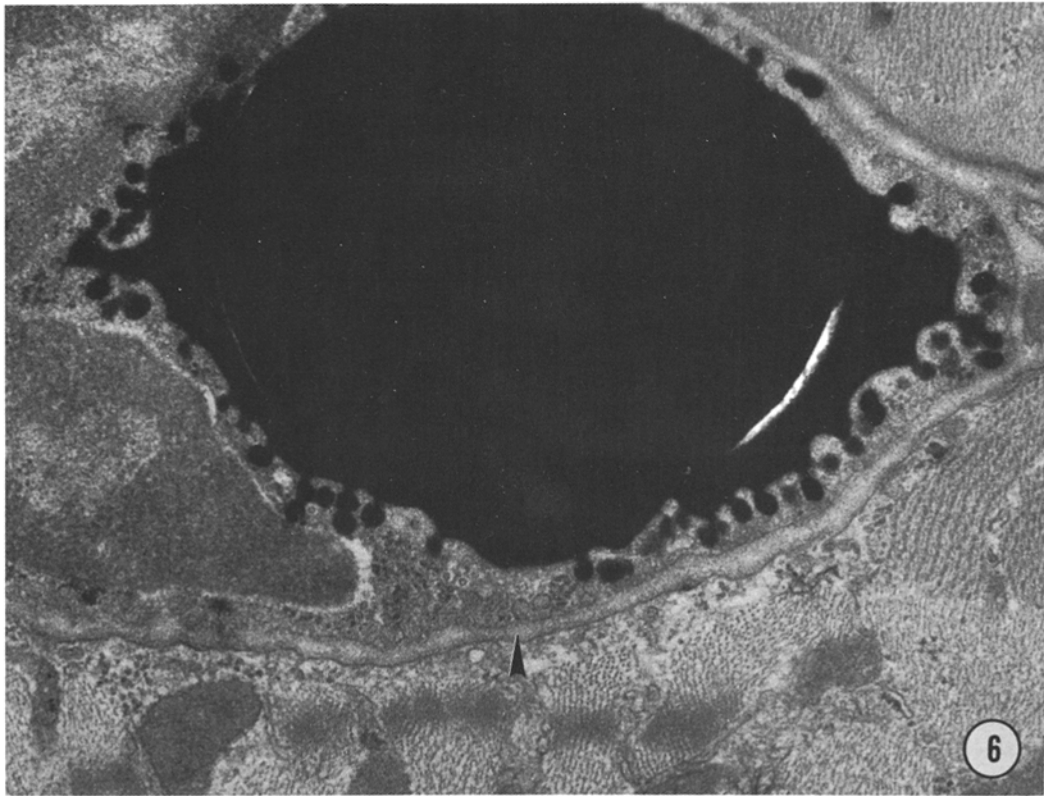
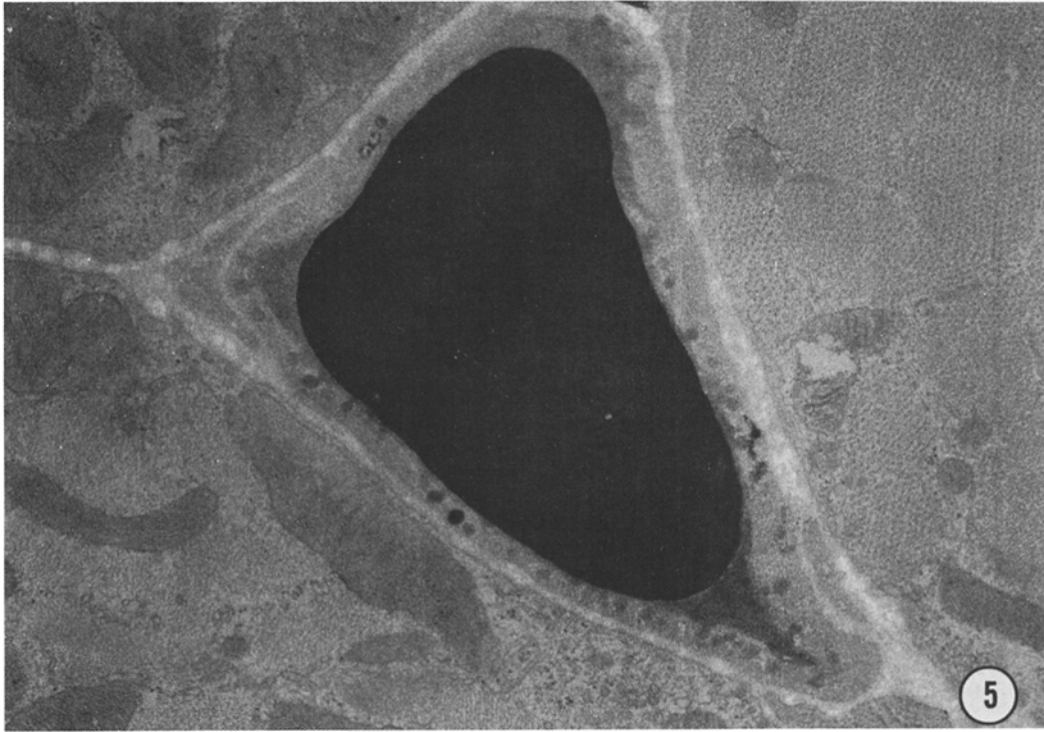
Like hemoglobin, cytochrome *c* has been employed as a tracer on the basis of its peroxidatic properties (46). We are unable to suggest an explanation for the positive reaction of the sarcolemma and lipid inclusions of skeletal muscle cells or for that of plasma lipoprotein particles. The increased density of the sarcolemma in control specimens was also noted by Karnovsky (17) and also lacks explanation.

Localization of HRP at Various Intervals after Its Intravenous Injection

HRP IN THE LUMEN: From 2 to 60 min after intravenous injection of 0.5 mg HRP, all capillaries contained electron-opaque reaction product (Fig. 4). We would expect to find uniform intravascular distribution of the tracer by 2 min because, in mice, mixing of a solute in circulating plasma is complete by 30 s after termination of its injection (47). Reaction product within the lumen was directly contiguous with the luminal surface of the endothelial plasma membrane, and any layer of polysaccharide (48, 49) or fibrinogen or fibrin (50) which coats this membrane was, evidently, readily penetrated by the tracer. By 2 h, the concentration of tracer in the plasma had decreased appreciably because the plasma density was only slightly greater than that of control specimens (Fig. 5). This suggests that a low concentration of heme protein (25) or HRP is still present in the circulation. There is little likelihood that the slight density present represents HRP bound to plasma protein because gel filtration and chromatographic analyses of HRP-containing plasma have shown that the tracer does not bind to plasma protein (19, 51). An alternative possibility that has yet to be disproved is that the initial injection of HRP caused some hemolysis, and the observed density is due to the presence of free hemoglobin in the circulation.

HRP IN ENDOTHELIAL VESICLES: The localization of HRP within endothelial cells was essentially unchanged from 2 to 60 min after its injection. Throughout this period all of the vesicles open to the capillary lumen, i.e., luminal vesicles, contained tracer equal in density to that in the capillary lumen (Figs. 6-8).⁷ Approximately 50%

⁷ Simionescu et al. (25) subdivided the total population of small endothelial vesicles into three categories: vesicles associated with the blood front of the endothelial cell, vesicles located in the interior of the endothelial cell, and



of the cytoplasmic vesicles contained tracer of approximately the same density as that of luminal vesicles (Fig. 6). We rarely saw interstitial vesicles discharging tracer into the pericapillary interstitium (Fig. 7). Wherever detectable quantities of HRP appeared in the interstitium, a similar concentration of HRP appeared concomitantly in interstitial vesicles (see later).

At 2 h, luminal and some cytoplasmic vesicles contained tracer similar in density to that in the capillary lumen (Fig. 5). A few luminal and cytoplasmic vesicles contained tracer that was markedly denser than that present in the capillary lumen. This observation is difficult to explain. It could indicate either that a small proportion of vesicles are immobilized for some time after they

vesicles associated with the tissue front of the endothelial cell. In the classification scheme of these authors, "association" with either endothelial front denotes that the vesicle is either open at that surface or lies within 800 Å of it. In our study we also subdivide the total population of vesicles into three categories, i.e., luminal, cytoplasmic, and interstitial. However, we include in the category of luminal and interstitial vesicles only those vesicles seen to be open at these surfaces of the endothelial cell. All other vesicles are classified as cytoplasmic vesicles. Our identification of cytoplasmic vesicles, although adequate for the purposes of the current study, may be somewhat naive. Karnovsky (17, 18) and Westergaard and Brightman (52) observed that endothelial vesicles, which otherwise appeared to be free in the cytoplasm, were labeled with tracer when the tissue was first fixed and then exposed to the tracer. The implication of these observations is that there is a connection, outside of the plane of section, between the interior of many cytoplasmic vesicles and the surface of the endothelial cell. Westergaard and Brightman (52) observed that a considerable number of the endothelial vesicles were linked with one another to form a chain. We did not observe with any frequency such chains of endothelial vesicles in capillaries of the mouse diaphragm. However, Bruns and Palade (53) did detect chains of two or

take up tracer or that vesicles can concentrate macromolecules that are taken up at the luminal surface of the endothelial cell.

HRP IN THE INTERSTITIUM: From 2 to 8 min after the injection of HRP, we did not detect, with certainty, tracer in the pericapillary interstitium (Figs. 6, 7, 9 *a* and *b*).⁸ However, beginning as early as 2 min after injection of HRP, we observed localized densities of finely granular texture among collagenous fibers of the interstitium. This material probably represents the earliest appearance of tracer in the interstitium.

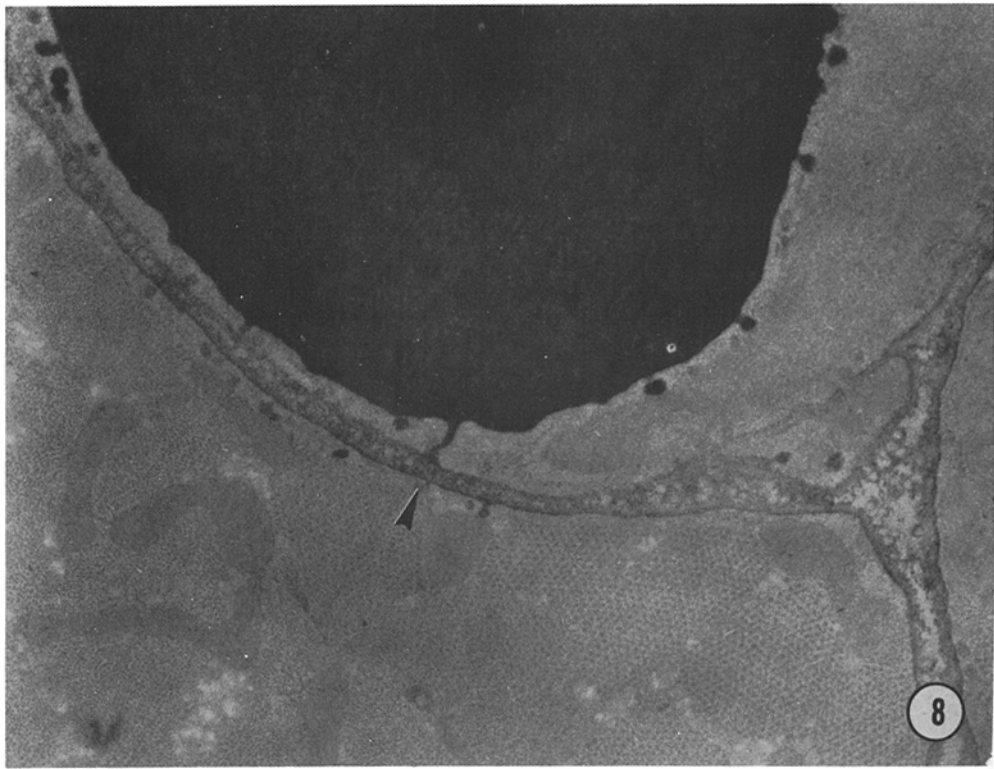
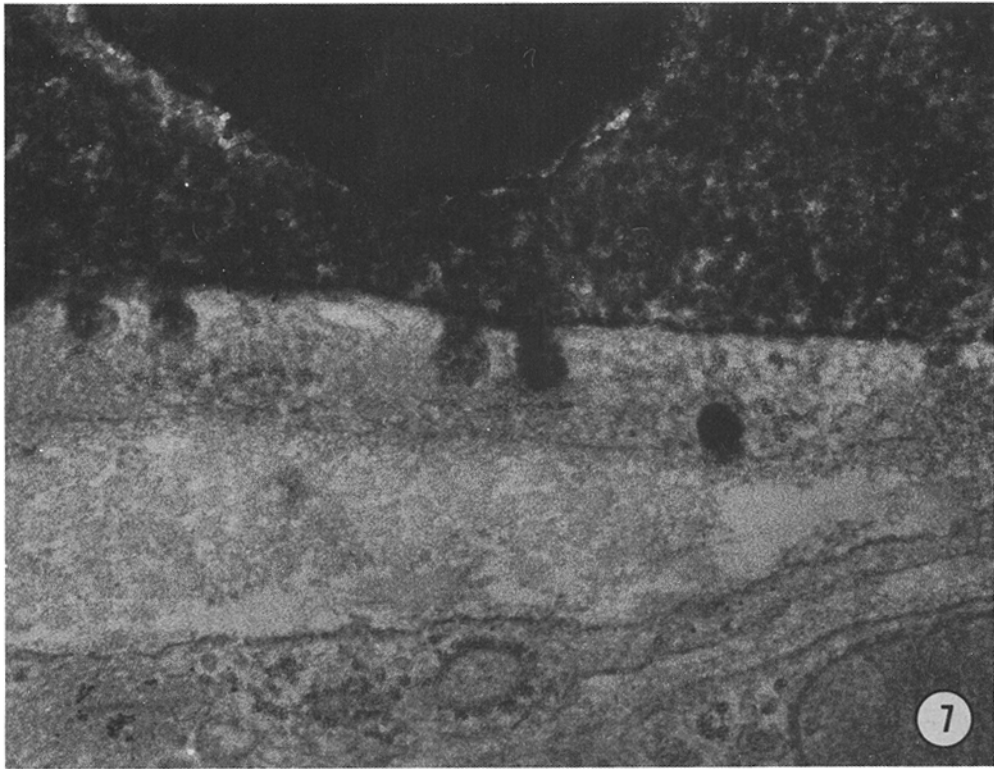
Beginning at 16 min, obvious tracer began to appear outside of capillaries in many regions (Fig. 8), especially in localized enlargements of the connective tissue space. In these areas unstained collagenous fibers stood out in negative image against the diffuse, low concentration of reaction product in the surrounding interstitial space. A high concentration of tracer frequently pooled around large bundles of collagenous fibers located

three endothelial vesicles in muscle capillaries of the rat, hamster, and guinea pig. The chains sometimes opened at one, but not at both, surfaces of the endothelial cell.

⁸ In the course of this study we noted that, after the injection of HRP, the tracer appeared first in the interstitium which bordered vessels that were broader in diameter than capillaries. We tentatively identified these vessels as venules. When tracer was clearly detectable in the interstitium, its concentration around venules appeared higher than elsewhere, and the higher concentration of tracer often extended into the interstitium bordering neighboring capillaries. In an attempt to assess the permeability of capillaries per se to HRP and to minimize confusion resulting from the escape of tracer from venules, we studied in thin sections only those capillaries located some distance from venules. Small regional variations in concentrations of tracer in the interstitium might be due to nearby venules which were not in the plane of section.

FIGURE 5 Capillary in the diaphragm of a mouse injected intravenously with 0.5 mg HRP in 0.1 ml saline 2 h before sacrifice. The plasma surrounding the highly reactive erythrocyte is denser than that seen in control specimens. Luminal and cytoplasmic vesicles of the capillary endothelium contain material comparable in density to the plasma. The content of one vesicle is markedly denser than the plasma. There is no detectable reaction product in the pericapillary interstitium or within muscle cells. $\times 16,000$.

FIGURE 6 Capillary in the diaphragm of a mouse injected intravenously with 0.5 mg HRP in 0.1 ml saline 4 min before sacrifice. All luminal and some cytoplasmic endothelial vesicles are labeled with reaction product equivalent in density to that in the capillary lumen. In the region indicated by the arrowhead are seen cytoplasmic and interstitial vesicles which do not contain reaction product. Reaction product is not detected in the pericapillary interstitium or in muscle cells. $\times 17,500$.



in the connective tissue underlying the mesothelium (Fig. 10). In thin sections examined by electron microscopy, we were unable to determine whether a mesothelium belonged to the pleural or peritoneal surface of the diaphragm. At 16 min, tracer began to appear within many inclusions in macrophages, within a small number of pinocytotic vesicles of pericytes, and within subsarcolemmal vesicles of skeletal muscle cells, i.e., small vesicles located just beneath the sarcolemma (Fig. 8), and within structures of skeletal muscle cells resembling lysosomes.

From 30 to 60 min after injection, the distribution of reaction product in the interstitium was similar to that seen at 16 min. Finely granular reaction product was detectable and was distributed diffusely throughout the interstitium in many areas, especially within the basement membrane of endothelial and skeletal muscle cells and the submesothelial connective tissue. The interstitial vesicles of endothelial cells, subsarcolemmal vesicles, and transverse tubules of skeletal muscle cells contained tracer equal in concentration to the tracer in the adjacent interstitium. Macrophages contained abundant tracer in cytoplasmic inclusions, and pericytes contained a limited amount of tracer in small inclusions.

At 2 h, tracer could no longer be detected in the pericapillary interstitium (Fig. 5), and it was no longer present within vesicles, transverse tubules or inclusions of skeletal muscle cells.

ENDOTHELIAL JUNCTIONS: From 2 to 60 min after injection of HRP, there was a steep concentration gradient of tracer across the endothelium with dense tracer in the capillary lumen and little if any tracer in the interstitium. We exam-

ined carefully the interstitial regions immediately outside of the endothelial junctions of capillaries to determine whether the tracer first appeared in these regions before its appearance elsewhere in the interstitium and whether, once recognizable tracer was present in the interstitium, its concentration in these regions was higher than elsewhere. In nearly all cases, the concentration of tracer in the interstitium outside of endothelial junctions was the same as elsewhere in the interstitium (Figs. 8 and 9 *a-d*). In only rare instances (Figs. 11 and 12) did we observe what appeared to be higher concentrations of tracer in the interstitium at or near a junction.

The abrupt change in concentration of HRP between the capillary lumen and the pericapillary interstitium was located within the endothelial junction (Figs. 9 *a-d*), usually at the first point of fusion of the apposed plasma membranes.

Effect of Varying the Volume of the Injected Solution

Despite an increase of two- and fivefold in the volume in which 0.5 mg HRP was injected (see Table II), the amount of tracer that appeared in the pericapillary interstitium of the three specimens was essentially similar to that seen in mice injected with 0.1 ml. Most of the interstitium was devoid of tracer, although some areas of interstitium and basement membranes contained granular material which could represent a barely detectable amount of tracer (Fig. 13). In none of the specimens did we detect localized concentrations of HRP in the interstitium adjoining endothelial junctions.

In the second part of this experiment a larger

FIGURE 7 Capillary in the diaphragm of a mouse injected with 0.5 mg HRP in 0.1 ml saline 8 min before sacrifice. An endothelial cell extends from right to left across the middle of the figure. The lumen of the capillary contains dense reaction product, and all luminal vesicles of the endothelial cell contain reaction product equivalent in density to that in the lumen. A solitary interstitial vesicle is seen emptying reaction product into the pericapillary space. A portion of a fibroblast appears along the lower margin of the figure, and collagenous fibers separate it from the endothelium. Reaction product is not detectable in the pericapillary interstitium. $\times 53,000$.

FIGURE 8 Capillary in the diaphragm of a mouse that was injected intravenously with 0.5 mg HRP in 0.1 ml saline 16 min before sacrifice. Dense reaction product fills the capillary lumen, and luminal vesicles of the endothelium contain reaction product of equivalent density. Reaction product of moderate density is uniformly dispersed throughout the interstitium and outlines collagenous fibers. The concentration of reaction product outside of the endothelial junction indicated by the arrowhead is not higher than elsewhere in the interstitium. A muscle cell occupies the lower portion of the field. Along its border to the left of the endothelial junction are seen several subsarcolemmal vesicles which contain reaction product. $\times 31,000$.

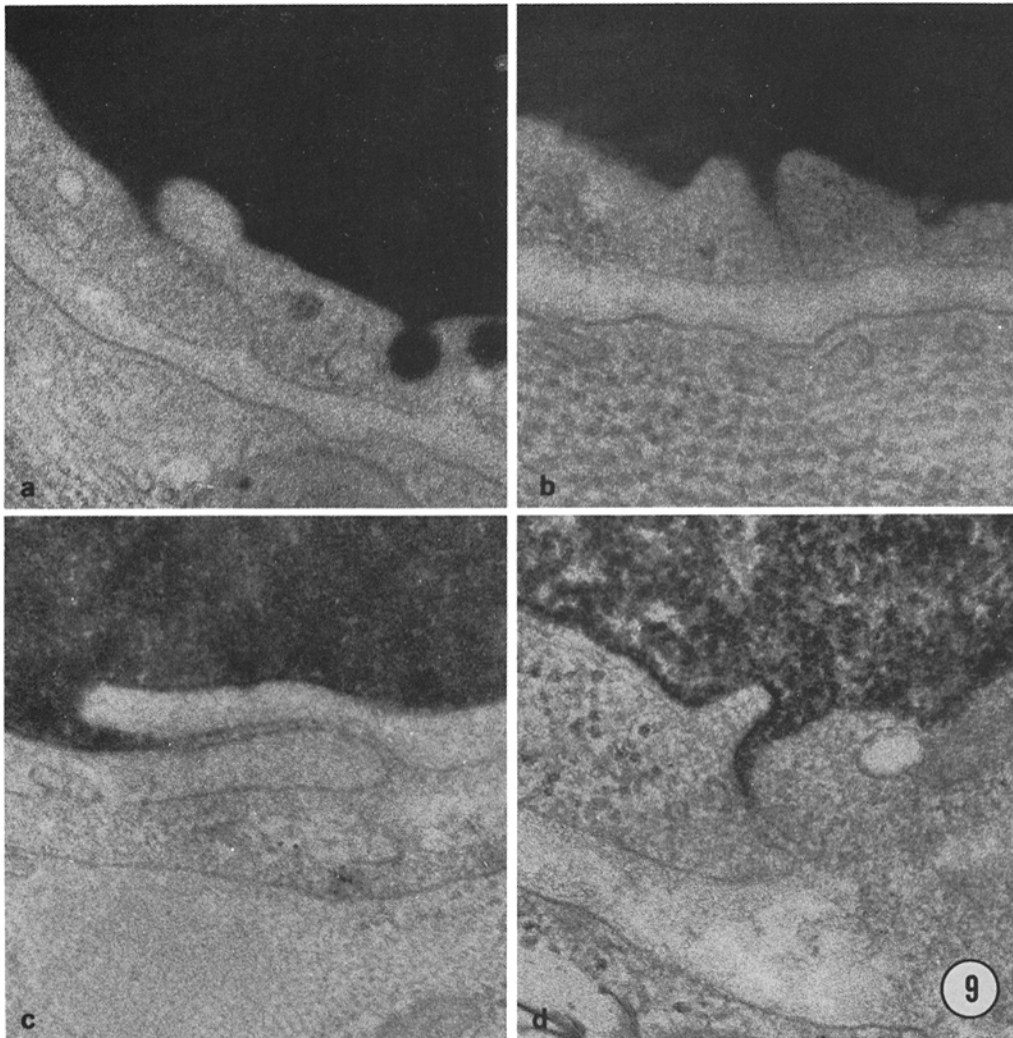


FIGURE 9 Endothelial junctions in capillaries of the diaphragm of mice injected intravenously with 0.5 mg HRP in 0.1 ml saline at 2 min Fig. 9 *a*; 4 min Fig. 9 *b*; 16 min Fig. 9 *c*; and 30 min Fig. 9 *d* before sacrifice. The luminal reaction product is extremely dense in Fig. 9 *a* and *b* and somewhat less dense in Fig. 9 *c* and *d*. Reaction product penetrates to a varied extent into each of the junctions. At some point either at the entrance to or within the junction the penetration of the reaction product terminates abruptly. There is no reaction product visible in the pericapillary interstitium in Fig. 9 *a* and *b*. The pericapillary interstitium of Fig. 9 *c* and *d* may contain barely detectable reaction product. *a*) $\times 65,000$, *b*) $\times 71,000$, *c*) $\times 60,000$, *d*) $\times 50,000$.

amount of HRP, i.e. 5 mg, was injected (see Table II). The volumes of solution injected, 0.05 ml and 0.2 ml, differed by a factor of four. The morphologic findings were similar in all animals (Fig. 14). Capillary lumens were filled with dense reaction product. A substantial amount of reaction product was present in the pericapillary interstitium, but the amount was clearly less than in the

capillary lumens. Vesicles throughout endothelial cells were labeled with tracer. Occasional endothelial cells contained varied amounts of reaction product in their cytoplasmic matrix. Within a single cell the reaction product was of uniform density. In none of the specimens were we able to detect localized concentrations of tracer in the interstitium adjacent to endothelial junctions.

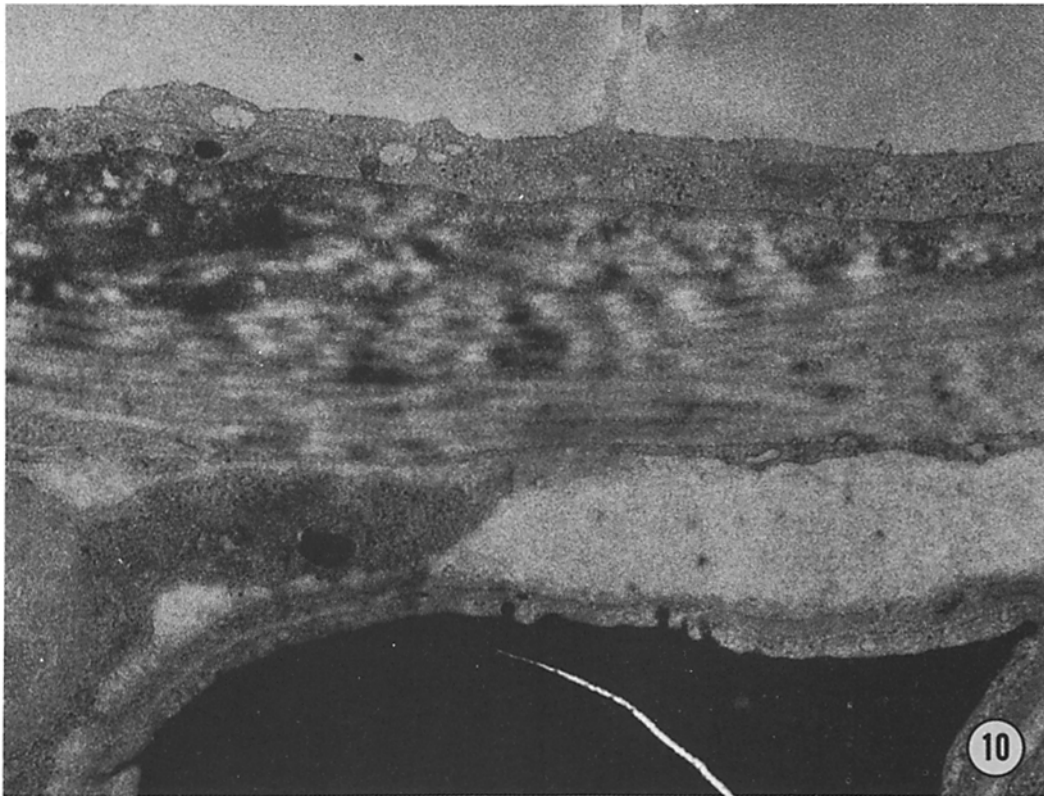


FIGURE 10 Serosal surface of the diaphragm of a mouse injected intravenously with 0.5 mg HRP in 0.1 ml saline 16 min before sacrifice. The squamous mesothelium extends across the upper portion of the field. Just beneath the mesothelium is a thick layer of closely packed collagenous fibers. A capillary is seen in the lower portion of the field. The lumen of the capillary contains dense reaction product. Dense reaction product permeates the spaces between the collagenous fibers. Interstitial vesicles of the mesothelium also contain dense reaction product. $\times 21,000$.

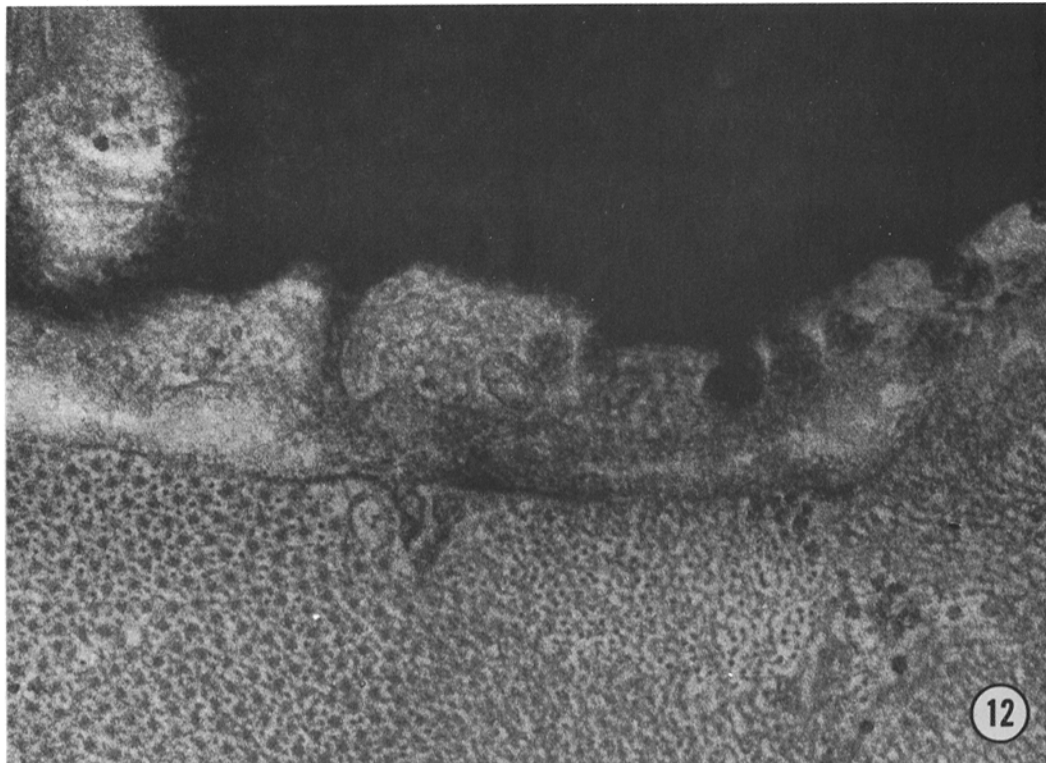
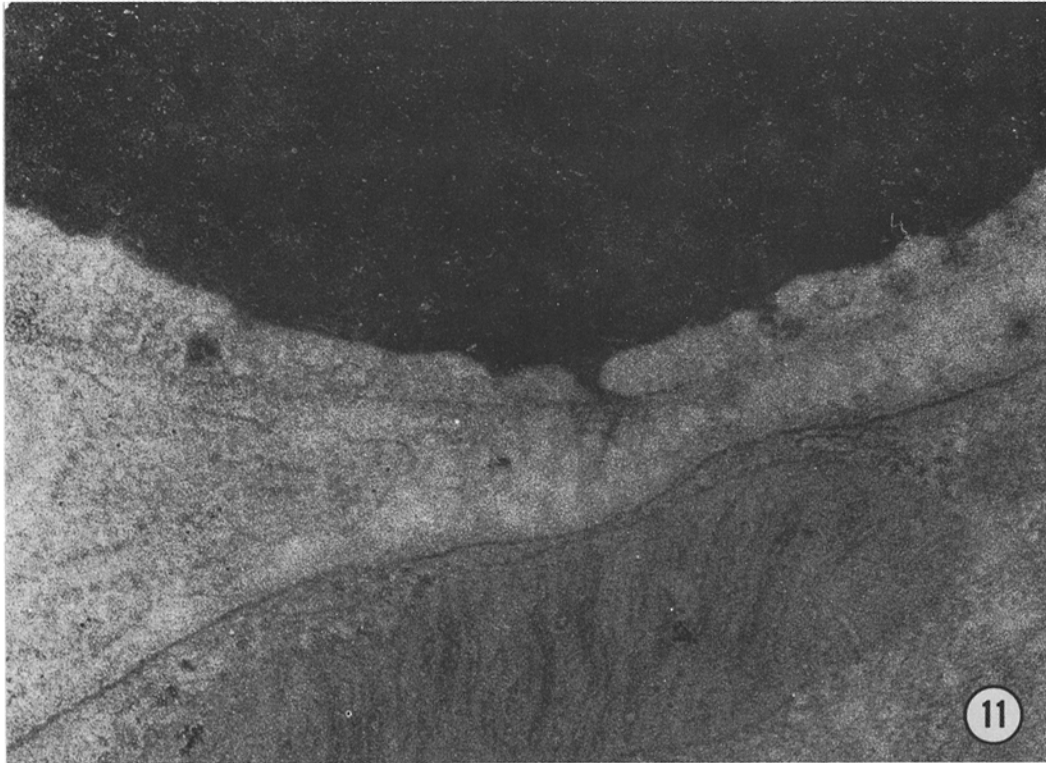
Effect of Varying the Amount of Peroxidase Injected

For this experiment, mice were injected with an amount of HRP that ranged from 0.25 to 16 mg, a 64-fold difference (see Table III). With the smallest dose injected, the capillary lumens contained a moderate amount of reaction product (Fig. 15) which was less dense than that within erythrocytes. Luminal and some cytoplasmic vesicles contained reaction product. The density of the reaction product within most labeled vesicles equalled that of the plasma, but in some vesicles the density of reaction product was greater. We were not able to detect the presence of reaction product in the interstitium.

With all higher doses, opaque reaction product appeared in the capillary lumen; it was not possi-

ble, therefore, to discern differences in luminal concentration of tracer. All luminal vesicles contained HRP equal in density to that in the lumen. The proportion of labeled cytoplasmic vesicles depended upon whether tracer was present in the pericapillary interstitium. When the pericapillary interstitium did not contain tracer, approximately half of the cytoplasmic vesicles contained tracer as dense as that in the lumen, and the rest were unlabeled. When there was a substantial concentration of tracer in the pericapillary interstitium, essentially all of the cytoplasmic vesicles were labeled with tracer (Fig. 16). With few exceptions (Fig. 7), the interstitial vesicles contained tracer equal in concentration to that in the interstitium (Fig. 16).

When 0.25–1.5 mg HRP was injected, little tracer appeared in the interstitium (Fig. 15).



FIGURES 11 and 12 Capillaries from the diaphragm of mice injected intravenously with 0.5 mg HRP in 0.1 ml saline at 16 min (Fig. 11) and 30 min (Fig. 12) before sacrifice. The lumen of each capillary contains dense reaction product. An endothelial junction appears near the center of each figure. Dense reaction product extends ablu-menally throughout the length of the junction. There is a localized concentration of reaction product in the pericapillary interstitium outside of each junction. In Fig. 11 the concentration is small and is centered just outside of the junction. In Fig. 12 the concentration is larger and lies to the right of the interstitial terminus of the junction. $\times 56,000$ (Fig. 11); $\times 69,000$ (Fig. 12).

Reaction product appeared in some regions of submesothelial connective tissue, in subsarcolemmal vesicles, and occasionally as enhanced granularity of muscle and endothelial basement membranes.

When 2.0–4.0 mg HRP was injected, HRP was clearly present in some areas of the interstitium and questionably present in others. When 8 or 16 mg HRP was injected, the amount of tracer observed in the interstitium varied; there was an abundance of tracer in some regions (Fig. 16) and less in others. The variations were large in scale, i.e., the amount of tracer present in the interstitium around a single capillary was always uniform but the amount varied among capillaries in different regions of a specimen. A consistent feature of these capillaries was the frequent appearance of tracer within the cytoplasmic matrix of endothelial cells (Fig. 17). The amount within the matrix varied; in some instances the density of intracellular tracer equalled that in the capillary lumen. Where the intracellular components of such endothelial cells were not obscured by the tracer, the components appeared normal.

In all specimens in this part of the study, we did not detect localized concentrations of tracer in the interstitium adjoining endothelial junctions that were higher than in surrounding areas. Within most junctions there was a sharp decrease in concentration of tracer at a point in the junction where the apposed endothelial plasmalemmae were fused. In endothelial junctions with two points of fusion, the membrane-bound space between the two points of fusion is a "compartment" within the junction. In specimens from mice injected with a large amount of HRP, some of these compartments contained tracer that was lower in concentration than that in the lumen and higher in concentration than that in the interstitium (Fig. 17).

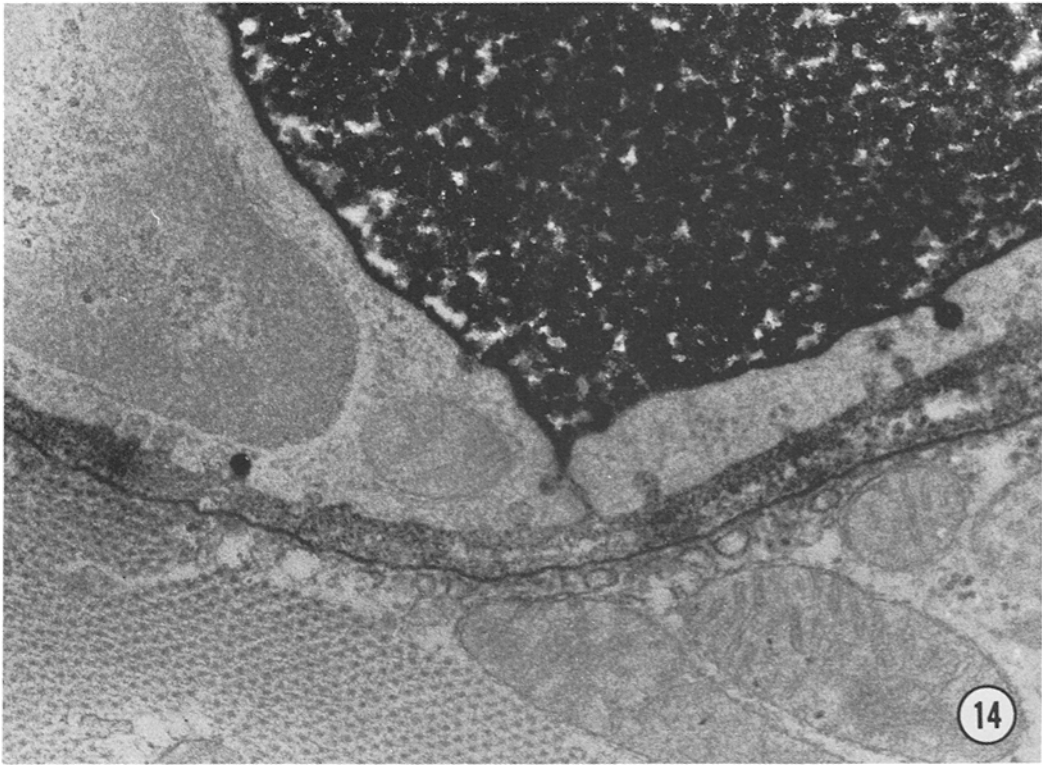
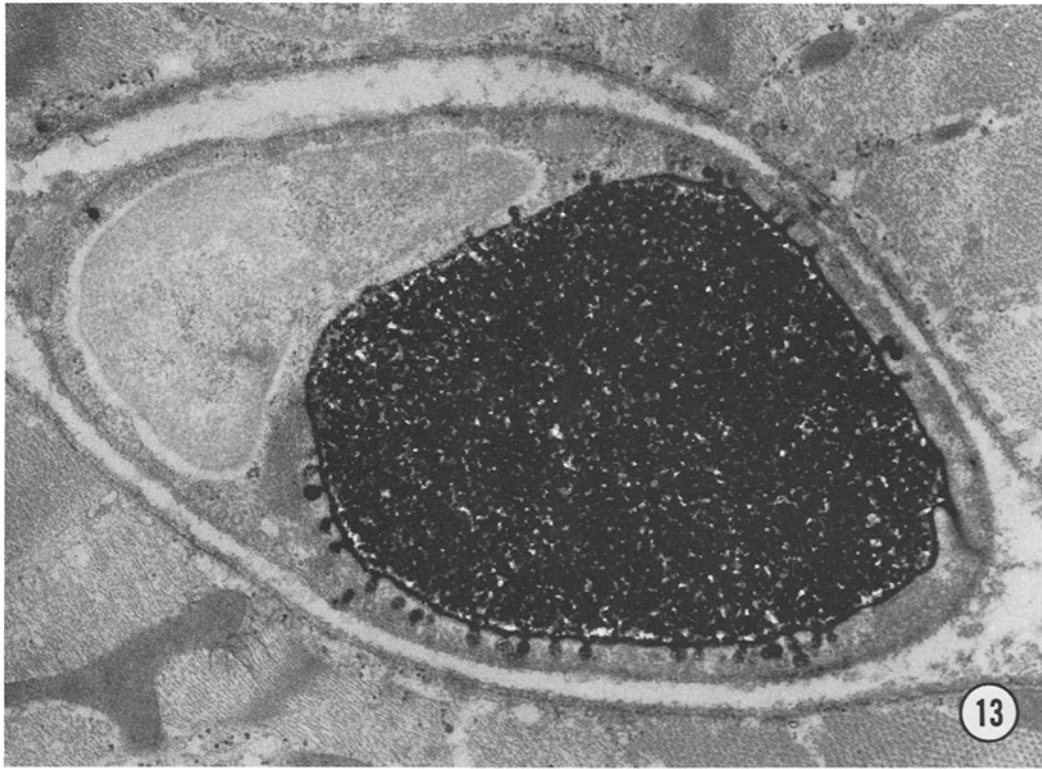
DISCUSSION

The results of these experiments show clearly that the capillary endothelium acts as a barrier to the escape of HRP from the circulation, since, at those intervals at which there was a substantial concentration of tracer in the blood plasma, there was appreciably less in the pericapillary interstitium. This difference in concentration cannot be assessed quantitatively because of inherent limitations in the cytochemical procedure for detection of HRP. First, the procedure permits direct visualization of

reaction product, not individual tracer molecules. The amount of reaction product that is deposited is dependent upon several variables, e.g., the proportion of enzymatic activity that persists after fixation, the duration of incubation in the reaction mixture, the temperature of incubation, etc. Second, once there is sufficient HRP present within an intracellular or extracellular compartment to render the compartment opaque to electrons, any additional increase in concentration of HRP within the space cannot be perceived. Nevertheless, it is clear from this and earlier work (17) that some HRP does enter the pericapillary interstitium, indicating that the capillary endothelium is not impervious to the tracer, and this observation is consistent with physiologic findings (4, 54). Our major concern is to evaluate, on the basis of our morphologic observations, whether endothelial vesicles and junctions are sites of "leaks" through the endothelial barrier.

The results of studies with tracers such as ferritin, colloidal gold, and saccharated iron oxide which can be recognized as individual molecules or particles in the electron microscope have verified the hypothesis that vesicles transfer fluid from the capillary lumen to the pericapillary space (12–16). The verification is based on the observation that, soon after these tracers are introduced into the plasma and before any tracer has appeared in the pericapillary space, tracer is detected in a small proportion of the lumenal, cytoplasmic, and interstitial vesicles of endothelial cells.

With HRP, morphologic observations substantiate less clearly vesicular transfer of tracer across the endothelium, principally because of the rarity with which discharge of tracer into the pericapillary interstitium is observed. When there was a high concentration of HRP in the capillary lumen and little or no HRP in the interstitium, we rarely observed tracer in interstitial vesicles. Thus, in contrast with the findings from studies with ferritin and various colloids, we could not readily obtain clearcut evidence that vesicles actually discharge HRP into the interstitium after transferring it across the endothelial cell. We do not interpret this as indicating that discharge does not occur because studies with ferritin clearly indicate that vesicles are capable of completing the transfer process. Instead, we believe that, once the vesicle opens at the cell surface, equilibration of HRP between the vesicular lumen and the adjacent extracellular space occurs rapidly and is not arrested by fixation with aldehydes. Additional proof of the rapid



equilibration of tracer between interstitial vesicles and the adjoining interstitium is derived from the experiments in which mice received larger amounts of HRP and a substantial amount of HRP was present in the interstitium. Under these conditions, all interstitial vesicles contained a concentration of HRP similar to that in the interstitium (see Fig. 16). Since the interstitial vesicles appeared empty when little or no HRP was detectable in the interstitium, we conclude that the interstitial vesicles now contain HRP because they have incorporated it from the interstitium. That the labeling of interstitial vesicles with HRP under these conditions indicates that the vesicles are incorporating tracer from the interstitium and not discharging it is corroborated by observations of the subsarcolemmal vesicles of skeletal muscle cells. Like the interstitial vesicles of endothelial cells, subsarcolemmal vesicles do not contain HRP if there is none in the interstitium. However, once tracer appears in the interstitium, it is also observed in subsarcolemmal vesicles, and its concentration in these vesicles parallels that in the interstitium (see Figs. 8 and 16). In the case of the subsarcolemmal vesicles, it is clear that they cannot be discharging tracer but can only be incorporating it from the interstitium.

The question has been raised whether a significant amount of the plasma protein that leaks from capillaries returns to the circulation by reverse passage across the capillary wall. The prevailing belief has been that protein returns solely via the lymphatic system (55). Whether reverse passage actually occurs is disputed among physiologists (56, 57). Reverse passage could be carried out by the vesicular transfer system since this system would be capable of operating against the concentration gradient of plasma protein across the capillary wall. Morphologic observations from our

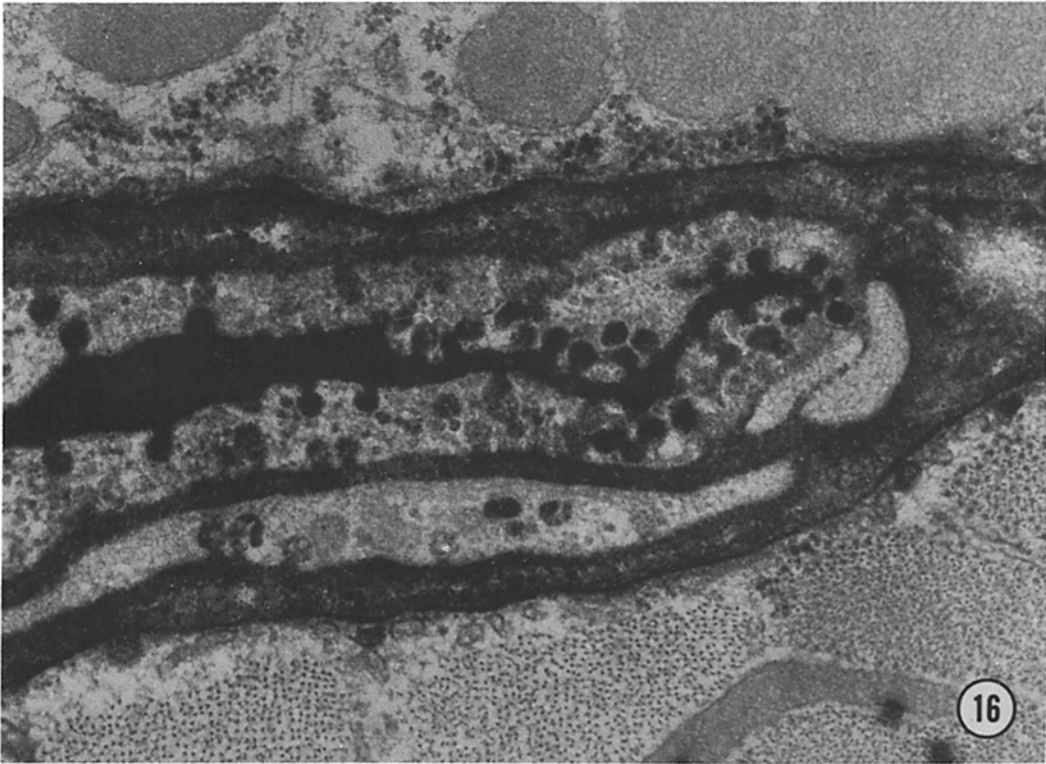
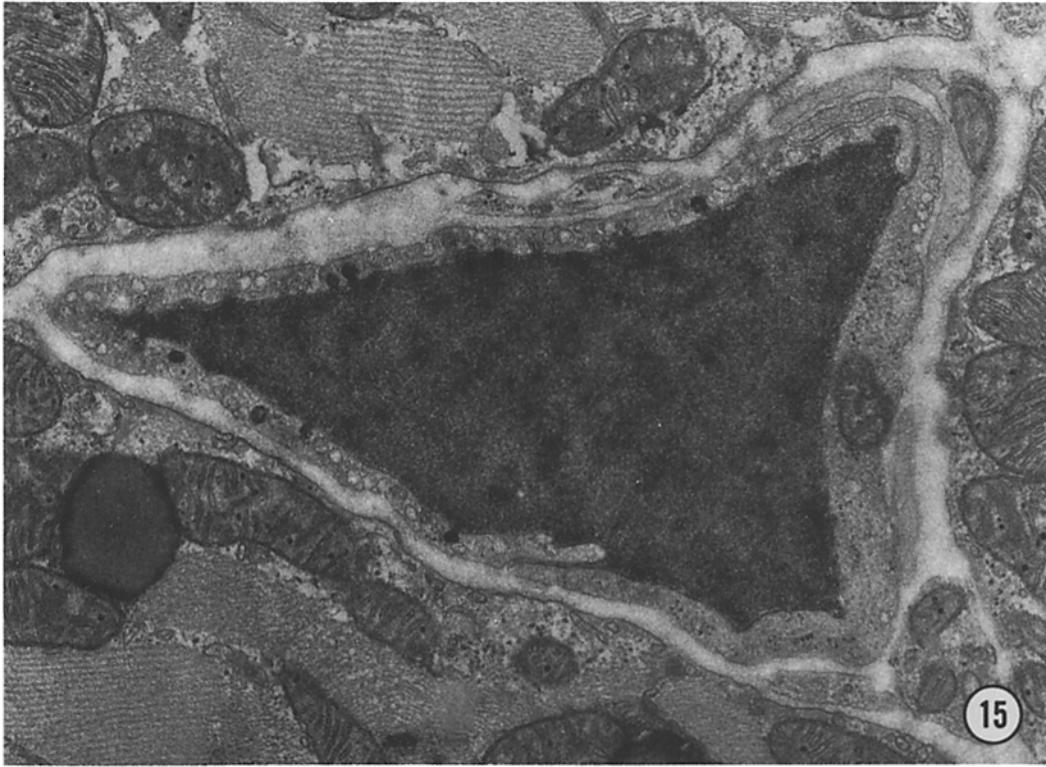
study suggest that reverse transfer does occur, but we cannot gauge its quantitative importance vis a vis lymphatic drainage. At early intervals after injection of moderate amounts of HRP, when there is little detectable tracer in the pericapillary interstitium, 50–70% of cytoplasmic vesicles are labeled with HRP. In similar experiments carried out with myoglobin as tracer, Simionescu et al. (25) also report that 65% of cytoplasmic vesicles become labeled with tracer before the appearance of significant amounts of tracer in the interstitium. In our study the proportion of labeled cytoplasmic vesicles does not vary as the interval after injection increases as long as the amount of HRP in the interstitium remains small. After larger amounts of HRP have been injected and there is a substantial amount of HRP in the pericapillary interstitium, essentially all of the cytoplasmic vesicles contain tracer. One would expect to observe these proportions of cytoplasmic vesicles labeled with tracer under the two experimental conditions cited if about half the cytoplasmic vesicles are transferring fluid from the capillary lumen to the interstitium and the other half are transferring fluid in the reverse direction.

Additional support for the hypothesis of reverse vesicular transfer stems from the results of a preliminary study by one of the authors (58) which demonstrated that ferritin, injected intraperitoneally in mice, appeared in high concentration in the pericapillary interstitium of the diaphragm whereas the concentration of ferritin in the plasma of capillary lumens was low. At this time, endothelial vesicles contained numerous ferritin molecules which were presumably being transferred across the endothelium from the pericapillary interstitium to the capillary lumen.

In this study we sought evidence to prove the hypothesis that the endothelial junctions of capil-

FIGURE 13 Capillary in the diaphragm of a mouse injected intravenously with 0.5 mg HRP in 0.5 ml saline 4 min before sacrifice. The capillary lumen contains dense reaction product, and reaction product is seen in luminal and some cytoplasmic vesicles of the endothelial cell. The pericapillary interstitium contains barely detectable reaction product visualized as increased density and coarseness of the basement membranes of the endothelial and muscle cells and as small fuzzy masses scattered in the interstitium. $\times 17,500$.

FIGURE 14 Capillary in the diaphragm of a mouse injected intravenously with 5 mg HRP in 0.1 ml saline 4 min before sacrifice. The lumen contains dense reaction product, and there is moderately dense reaction product in the pericapillary interstitium. A single luminal and a single interstitial vesicle of the endothelium contain reaction product comparable in density with that of the lumen. The rest of the interstitial vesicles of the endothelium contain reaction product approximating in density that present in the interstitium. A vesicle is seen opening into the endothelial junction in the center of the figure. $\times 47,000$.



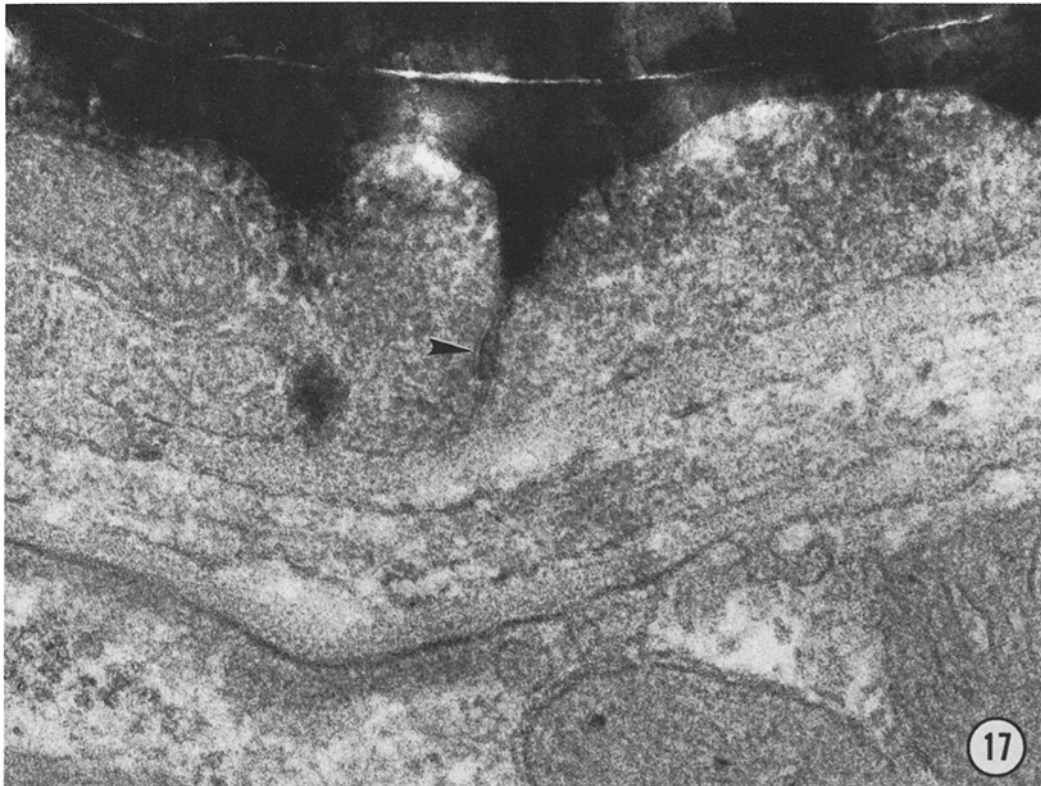


FIGURE 17 Endothelial junction in a capillary of the diaphragm of a mouse injected intravenously with 4 mg HRP in 0.1 ml saline 4 min before sacrifice. The capillary lumen contains dense reaction product, and there is barely detectable reaction product in the pericapillary interstitium. The arrowhead points to a compartment within the endothelial junction. Although the plasma membranes of the apposed endothelial cells are poorly visualized, the upper and lower limits of the junctional compartment appear to be demarcated by sites of membrane fusion. The density of the reaction product within the compartment is intermediate between that of the lumen and that of the pericapillary interstitium. $\times 81,000$.

larities of the diaphragm serve as passageways through which HRP passes from the capillary lumen to the interstitial space. In our opinion, the most unambiguous evidence should be based on the two criteria cited in the introduction.

In this study we did not detect, except in extremely rare instances, localized concentrations of HRP in the interstitium adjacent to endothelial junctions, and we observed an abrupt interruption of penetration of HRP within the junction at the

FIGURE 15 Capillary in the diaphragm of a mouse injected intravenously with 0.25 HRP in 0.1 ml saline 4 min before sacrifice. The lumen contains moderately dense reaction product (less dense than that of the erythrocyte in Fig. 1). Luminal and some cytoplasmic vesicles contain reaction product. The density of the reaction product in some of these vesicles exceeds that of the luminal reaction product. Neither the interstitium nor interstitial vesicles contain reaction product. $\times 25,000$.

FIGURE 16 Capillary from the diaphragm of a mouse injected intravenously with 16 mg HRP in 0.1 ml saline 5 min before sacrifice. The lumen of the capillary and the pericapillary interstitium contain dense reaction product. All endothelial vesicles contain reaction product. Subsarcolemmal vesicles of the muscle cell in the lower portion of the field and vesicles in a cell located below the endothelium and presumed to be a pericyte also contain reaction product. $\times 47,000$.

point of fusion of the endothelial plasma membranes. These observations are clear evidence that the junction serves as a barrier to the passage of molecules of the size of HRP. Can we determine whether the barrier is partial or complete? On the basis of the molecular size of HRP and assumptions concerning the viscosity of interstitial fluid, we have calculated the diffusion rate of HRP in the fluid (see later). The diffusion rate is high enough to allow rapid equilibration in the pericapillary interstitium of any HRP that enters the interstitium at one or more points along the perimeter of the capillary. Because of such rapid equilibration, it is unlikely that we would detect HRP emerging from junctions if the amount emerging is small. Therefore, although our observations do not furnish evidence to support the hypothesis that HRP can pass from the capillary lumen into the surrounding pericapillary space through endothelial junctions, they do not exclude the possibility that passage does occur but on an undetectable scale.

When large amounts of HRP were injected, in some endothelial junctions in which there were two successive points of fusion the space enclosed between the two points behaved like a separate small compartment; it contained a lower concentration of tracer than that in the capillary lumen and a higher concentration than that in the adjacent interstitial space (see Fig. 17). Apparently the content of this small compartment did not equilibrate rapidly with that of the two large adjacent spaces. The fact that tracer appeared within this compartment suggests that the membrane fusion closest to the lumen is not serving as an impenetrable barrier to HRP. Hüttner et al. (59) reported findings similar to this in the endothelium of large arteries fixed in solutions containing colloidal lanthanum. In these vessels, lanthanum penetrated into several of the series of compartments formed by sequential fusions of the endothelial membranes.

There are three routes by which HRP might enter such junctional compartments. First, the fusion between the apposed endothelial plasma membranes could be relatively loose or porous enough to allow molecules of the size of HRP to seep through. Second, the line of fusion between the apposed membranes may be discontinuous, and HRP could circumvent the line of fusion at a nearby site outside of the plane of section. Third, HRP could be deposited in the compartment by pinocytotic vesicles.

The first possibility is not excluded by data on capillaries published up to this point or obtained in this study. In an earlier study based on observations made with ruthenium red, a cationic dye that combines with acid polysaccharides and acidic polypeptides, Luft (48) proposed that the fused outer lamellae of the apposed endothelial membranes formed a loose meshwork of hydrated acid polysaccharide and protein. On the basis of our findings, the meshwork could have a few pores large enough to allow small quantities of HRP to leak through. On the basis of studies with ferritin (13, 60), the meshwork would not have pores large enough to accommodate this larger tracer.

The second possibility seems unlikely because in studies with ferritin and particulate tracers carried out *in vivo*, such tracers have not been detected within junctional compartments.⁹ If the sites of fusion of apposed endothelial plasmalemmae seen as points in transverse section are actually lines of fusion in three dimensions which do not form continuous belts around each endothelial cell, *i.e.* they are maculae rather than zonulae occludentes (17), then larger particulate tracers would be able to enter endothelial junctions at the free ending of a line of fusion, and such tracers should appear, in sections, within the junctional compartments. Failure to observe these tracers within junctional compartments suggests that the compartments are closed in the sense that they are bounded by continuous lines of membrane fusion and each line of fusion terminates by fusing with an adjacent line. Therefore, in terms of their penetrability by ferritin and particulate tracers, the endothelial junctions behave as though they are zonulae rather than maculae occludentes. The possibility cannot be ruled out, however, that discontinuities are present which are small enough to exclude ferritin yet large enough to permit passage of HRP.¹⁰

⁹Jennings et al. (16) perfused rat hearts *in vitro* with balanced salt solutions containing saccharated iron oxide and observed numerous particles of this tracer in endothelial junctions. This observation does not resemble anything seen in studies of normal capillaries carried out *in vivo*, and assessments of the toxicity of their tracer and the normal physiologic behavior of the *in vitro* heart preparation will have to be made before the observation can be placed in proper perspective.

¹⁰Discontinuities in lines of fusion of capillary junctions should be detectable in freeze-fractured preparations. In a preliminary report of such a study, Simionescu et al. (61) describe capillary tight junctions as formed by "staggered strands and grooves." The ultrastructural significance of this terminology is not clear.

With regard to the third possibility, adjacent endothelial cells of muscle capillaries usually make blunt contact with or overlap one another to a small degree, but do not interdigitate extensively with one another (13, 17, 53). Vesicles are occasionally seen open at the surface of the endothelial cell within the small area of lateral apposition (17; see Fig. 14), and it is conceivable that such vesicles could deposit material into junctional compartments. In our studies, we did not observe this phenomenon. Adjacent endothelial cells of large arteries interdigitate more extensively along their lateral margins, and vesicles depositing material into junctional compartments have been observed (51, 59, 62) in these vessels.

On the basis of our current knowledge it is not possible to decide which of the three possible explanations for the presence of HRP in junctional compartments are valid.

As another aspect of the permeability of endothelial junctions, we determined whether an acute increase in plasma volume at the time of injection of HRP would cause a morphologically detectable increase in the rate of escape of the tracer from muscle capillaries. In an earlier morphologic study, Pietra et al. (41) had shown that capillaries of an isolated lobe of dog lung perfused with a solution of hemoglobin began to leak hemoglobin into the interstitium when the pressure in the pulmonary artery was raised from its normal value of 10–15 mm Hg to 55 mm Hg. They concluded that the site of the leak was the junction between capillary endothelial cells although they were unable to discern any ultrastructural change in junctions after the increase in capillary hydrostatic pressure. In subsequent studies of pulmonary capillaries of newborn and adult mice, other workers (26, 27) showed that, when the volume of solution in which HRP was injected was increased, greater amounts of the tracer leaked from pulmonary capillaries. This effect was pronounced, for example, when the volume of solution injected into adult mice was 35% of the total blood volume. The authors localized the site of increased leakage to the endothelial junctions, although they could not perceive any ultrastructural change in the junctions.

In our study we were unable to detect any increase in the amount of HRP appearing either generally throughout the pericapillary interstitium or in localized areas adjacent to junctions when the volume in which the tracer was injected was increased to as much as 30% of the total blood

volume, i.e., 0.5 ml injected in a 20 g mouse (47). This increase in volume was similar to that used by Schneeberger (26) and Schneeberger and Karnovsky (27) to cause an increase in escape of HRP from pulmonary capillaries. We found that, even when large volumes were injected, the site of fusion between apposed membranes remained functionally intact and continued to serve as a barrier to the escape of the tracer. Several explanations can be offered for the discrepancy between our results and those of earlier morphologic studies. In the first place, if, when we injected HRP in a large volume, the amount of HRP appearing in the pericapillary interstitium increased by only a small factor, we might not be able to detect the difference for reasons stated at the beginning of the Discussion. Second, an acute increase in blood volume produced by a single intravenous injection may have a relatively greater effect on the permeability of pulmonary capillaries than on systemic capillaries. The systemic circulation has been shown to have compartments such as the vasculature of the liver which can serve as temporary storage sites to accommodate sudden excesses of blood volume (63). The pulmonary circulation presumably lacks such storage sites. The presence of such storage sites could blunt the effect of an acute increase in blood volume on capillary hydrostatic pressure. At first glance our findings seem to disagree with the "stretched pore" phenomenon observed by physiologists (28, 29, 32). They report that the large pore postulated to exist in the capillary wall increases in size when plasma volume is expanded, and this allows plasma protein molecules to escape more readily from the circulation. However, to demonstrate the phenomenon, they infuse over a protracted period solutions of albumin or of high molecular weight dextran that are much more concentrated than the tracer solutions we used. It seems, therefore, that we did not observe the phenomenon because we did not achieve a comparable degree of plasma expansion.

Schwartz and Benditt (51) have determined that the half-life of HRP in the circulation of the rat is approximately 13 min and that, by 30 min after injection, the longest interval studied, its concentration is approximately 30% of its initial maximum at 30–60s. Similar findings were observed in mice by Clementi and Palade (64). In our experiments, we assume that the concentration of HRP in the lumen of capillaries of the mouse diaphragm decreased at a similar rate during the first 60 min after injection of 0.5 mg HRP, even though we

could not detect this decline morphologically, because the lumen was electron-opaque throughout this period. During the period after injection of HRP, tracer was first detected at scattered sites in the interstitium at 16 min. By 30 min, it was more readily detectable in the interstitium and was more widespread in distribution. Since the progressive accumulation of HRP in the interstitium from 16 to 30 min after its injection occurred while the concentration of HRP was declining in capillary plasma, it is clear that the concentration of this tracer in the interstitium does not parallel closely the concentration of tracer in plasma but lags behind it. Presumably accumulation of HRP occurs because HRP is not removed from the interstitium at a rate comparable with that at which it enters. We assume that HRP is removed from the interstitium by two principal means: uptake and destruction by cells, and uptake and removal by lymphatics. In this study we observed small amounts of tracer within vesicular elements of skeletal muscle cells and somewhat larger amounts in endocytic organelles of tissue macrophages. In neither did the amount detected seem large enough to account for the disposal of a substantial amount of tracer that leaked from small blood vessels. The bulk of the disposal was probably being carried out by lymphatics. In our examination of the muscular component of the mouse diaphragm, we have encountered only few vessels resembling lymphatics in this tissue. They are located at scattered points between the mesothelium and underlying skeletal muscle along both the pleural and peritoneal surfaces of the diaphragm. This confirms their location in the mouse as described by Yoffey and Courtice (55). The tracer, therefore, has to diffuse a substantial distance through the interstitium before it can reach the closest lymphatic. This could account for pooling of tracer in the interstitium. Another factor which could explain pooling of tracer in the interstitium would be its slow diffusion through the interstitial ground substance which is thought to have the consistency of a gel. The diffusion rate of proteins in the interstitium of skeletal muscle has not been measured. Simionescu et al. (25) have estimated the diffusion rate of myoglobin (17,800 mol wt) in the interstitium by assuming that the viscosity of interstitial fluid is similar to that of the cytoplasm of cells. We have carried out similar calculations for HRP, and its diffusion rate should fall in the range of 4.6–46 $\mu\text{m}/\text{min}$. Thus, HRP, over a period of 5 min could diffuse a distance of as

little as 23 μm . This relatively slow rate of diffusion could also account for its accumulation in the muscular portion of the diaphragm before it reached the site of the draining lymphatics.

On the basis of analyses of the rate at which lipid insoluble molecules of a range of sizes pass through the wall of capillaries in muscle, the capillary wall appears to behave like a passive membrane possessing a set of large and small pores (5, 7, 9). According to most recent findings the small pores have a diameter of 90 \AA (4) and an aggregate pore area of 0.02% of the capillary surface area (65). Alternatively, the small pore could be a slit 55 \AA wide occupying the same fractional surface area (66). The large pores have a diameter of 240–800 \AA (5, 8). They are far fewer in number, i.e., there is one large pore for every 34,000 small pores (5). Morphologists have sought to identify the structural equivalents of the two sets of pores by utilizing tracer molecules. As mentioned in the introduction of this paper, studies with ferritin and colloidal gold have identified the vesicles as the equivalent of the large pore. The identification of the equivalent of the small pore is in dispute.

In studies with HRP, a molecule which should use both large and small pores to cross the capillary wall (8), Karnovsky (17, 18, 65) identified the endothelial junction as the equivalent of the small pore. In our study of the passage of HRP across the wall of the capillaries of the diaphragm we have been unable to corroborate the findings of Karnovsky. Our results lead us to the conclusion that the endothelial junction acts as a barrier to the passage of HRP but may allow a small amount of the tracer to leave the circulation. They are not sufficient evidence to justify identification of the junction as the small pore. We are unable to explain the discrepancy between our findings and those of Karnovsky. He illustrated most of his findings with capillaries from the heart, and there are suggestions in the physiological literature that they are more permeable to large macromolecules than are other muscle capillaries (67). Their greater permeability to large molecules has been interpreted as indicating that they have more large pores. We would then expect the ultrastructural basis for this difference to be some property of the vesicular transfer system rather than of the endothelial junctions.

Findings with tracers of smaller size are also in dispute. Karnovsky (65) reported that cytochrome *c* could pass through endothelial junctions whereas Simionescu et al. (25) reported that myoglobin

could not. The latter group of workers has published a preliminary account (24) of a study with microperoxidase in which they report that endothelial junctions are impermeable to this small tracer. A tracer of this size should be an ideal probe for the small pore, since according to the data of Renkin (54), one would predict that the amount passing through the small pore would be 100 times greater than that passing through the large pore. We have also carried out a study with microperoxidase and have published a preliminary account of our findings (68). We found evidence of passage of microperoxidase through endothelial junctions, and we judged the evidence strong enough to justify identification of the junction as the site of the small pore.

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