

PERMEABILITY OF MUSCLE CAPILLARIES TO MICROPEROXIDASE

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ABSTRACT

In this study we attempted to identify a morphologic counterpart of the small pore of muscle capillaries. The existence of such a pore has been postulated by physiologists to explain the permeability of muscle capillaries to small macromolecules. We injected mice intravenously with microperoxidase (MP) and fixed specimens of diaphragm at intervals of 0–250 s after the injection to localize the tracer by electron microscopy. The small size of MP (1,900 mol wt and 20 Å molecular diameter [MD]) ensures its ready passage through the small pore since the latter is thought to be either a cylindrical channel 90 Å in diameter or a slit 55 Å wide.

MP appears in the pericapillary interstitium within 30 s of initiation of its intravenous injection. The patterns of localization of MP observed within clefts between adjacent capillary endothelial cells indicate that some endothelial junctions are permeable to this tracer. Although small vesicles transfer MP across the endothelium, we do not believe that the vesicles transfer substantial amounts of MP into the pericapillary interstitium. We did not obtain evidence that MP crosses the endothelium of capillaries through channels formed either by a single vesicle or by a series of linked vesicles opening simultaneously at both surfaces of the endothelial cell.

From our observations we conclude that some endothelial junctions of capillaries are permeable to MP, and that these permeable junctions are a plausible morphologic counterpart of the small pore.

KEY WORDS capillary permeability · capillary ultrastructure · microperoxidase · capillary small pore

Morphologic studies with tracers have shown that, of the three layers which comprise the wall of muscle capillaries, i.e., endothelium, basal lamina with enclosed pericytes, and adventitia (2), the endothelium regulates the passage of proteins and colloidal particles across the capillary wall (3, 14,

16, 27, 32, 33, 36, 43). The tracers used in these studies are of a wide range of size; the smallest is heme-octapeptide (~1,550 mol wt and ~20 Å molecular diameter [MD])¹ and the largest is

¹ In an early study, Feder (10) reported that microperoxidase (heme-undecapeptide), a tracer similar to heme-octapeptide but slightly larger, binds to protein and forms aggregates in solution. If this tracer does bind to plasma protein when injected into the circulation, its

colloidal gold (particles up to 250 Å in diameter). Until recently, two pathways have been considered as possible routes of passage across the endothelium for proteins and colloidal particles: the abundant small cytoplasmic vesicles acting as shuttles, and the cleft between adjacent endothelial cells. Morphologic studies with tracers have substantiated the hypothesis that vesicles transfer material across the endothelium and that the vesicles appear to be the only means by which large proteins and colloidal particles cross the endothelium (3, 27). Therefore, on the basis of these morphologic observations as well as recently obtained physiologic data, vesicles are considered to be one, but perhaps not the only, morphologic counterpart of the large pore postulated by physiologists to explain the permeability of muscle capillaries to large macromolecules (12, 26). Whether endothelial clefts serve as a pathway for smaller proteins and are a morphologic counterpart of the small pore postulated by physiologists (19, 28, 29) to explain the permeability of muscle capillaries to smaller macromolecules has been a subject of controversy. Karnovsky (14–16) and Karnovsky et al. (18) reported that the clefts were readily permeable to horseradish peroxidase (HRP) (40,000 mol wt and 50 Å MD [14, 39]) and cytochrome *c* (12,400 mol wt [25] and 35 Å MD [6, 20]). In contrast to these findings, Themann et al. (36) and Williams and Wissig (43) have found that permeability of the clefts to HRP is difficult to substantiate. In the latter study, limited evidence of passage of HRP through clefts was obtained only when large doses of the tracer were injected. Moreover, Simionescu et al. (32, 33) concluded that the clefts are impermeable to smaller tracers such as myoglobin (17,800 mol wt [8] and 35 Å [1]) and microperoxidase (MP) (1,900 mol wt and 20 Å MD [9, 10, 33]).

In their recent study with MP, Simionescu et al. (33) introduced a new concept concerning the role of vesicles in capillary permeability. They reported that vesicles form channels through endothelial cells of capillaries of the rat diaphragm. Each channel consists of a single vesicle or chain of linked vesicles which is open simultaneously at

dimensions would be increased. However, Simionescu et al. (33) have shown, by chromatographic analysis, that heme-octapeptide circulates in plasma in monomeric form and that only 16% of injected heme-undecapeptide is bound to plasma protein. We would not expect binding of such a small amount to affect our findings.

the luminal and interstitial surfaces of the endothelial cell. Constrictions 100 Å in diameter in the channel are located at sites where adjacent vesicles have joined and where vesicles open at the endothelial surface. After MP was introduced into the circulation, it was observed filling the length of the channels. On the basis of the physical dimensions of the channels, their frequency of occurrence, and their accessibility for the passage of MP, the authors proposed that the channels represent the counterpart of the small pore. In a subsequent paper (35), these authors reported that transendothelial channels occur more frequently in venules than in capillaries of the rat diaphragm.

In this paper we report findings from a similar study with MP. The study was carried out on capillaries of the mouse diaphragm, and our findings with regard to these microvessels do not substantiate those of Simionescu et al. (33, 35). We could not confirm their observation that vesicular channels penetrate the endothelium of muscle capillaries, and we obtained evidence indicating that MP can pass through some endothelial clefts. On the basis of these findings, we deduce that the clefts are the most likely site of the small pore of these vessels. We have reported these findings earlier in preliminary form (44).

MATERIALS AND METHODS

Tracer

Some of the MP used in this study was generously provided by Dr. Ned Feder from material he had prepared (9). The rest (sodium salt from horse heart cytochrome *c*) was obtained from Sigma Chemical Co. (St. Louis, Mo.).

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 sodium pentobarbital (9 mg/100 g body weight). In most instances, the right external jugular vein was exposed through an incision in the cervical region, and MP dissolved in isotonic saline was injected into this vein. To obtain tissue specimens for study, the diaphragm was fixed by one of three procedures. With the first procedure the peritoneal cavity was opened through a midventral incision, and the liver and stomach were retracted from the peritoneal surface of the diaphragm. The surface of the diaphragm was then flooded with a modification of Karnovsky's fixative (13). With the second procedure the diaphragm was fixed by injection of 3 ml fixative into the unopened peritoneal cavity. The

third procedure was used when the interval between injection of tracer and fixation of the diaphragm was short. The peritoneal surface of the diaphragm was exposed first, and the tracer solution was injected into the inferior vena cava. The peritoneal surface of the diaphragm was then flooded with fixative.

The modified Karnovsky's fixative contained 2.0% glutaraldehyde (Ladd Research Industries, Inc., Burlington, Vt.), 1% freshly dissolved paraformaldehyde (Matheson Scientific, Chicago, Ill.), 7.3 mM KCl, and 0.12% CaCl₂ in 0.1 M sodium cacodylate buffer, pH 7.4. Final adjustment of the pH was carried out after all components of the fixative were mixed. The fixative solution was used at a temperature of 37°-40°C. Details concerning experimental protocols are given in Table 1.

After fixation *in situ* for 10 min, the diaphragm was excised and placed overnight in fixative at 4°C. The next day the diaphragm was cut into small strips. The strips were cut from the muscular portion of the diaphragm midway between the attachment of the muscle to the central tendon and to the thoracic wall. The long axis of each strip was oriented perpendicular to the long axis of the muscle fibers.

Histochemical and Embedding Procedures

Tissue slices, 60 μm thick, were cut from the strips of muscle with a Sorvall TC-2 Tissue-Sectioner (DuPont Instruments, Sorvall Operations, Newton, Conn.). The strips were oriented so that the plane of section in the Tissue-Sectioner was parallel to the long axis of the muscle fibers. The slices were rinsed five times in 0.05 M Tris buffer, pH 8.6, and were preincubated in incubation medium without H₂O₂ for 10 min at 37°C. They were then placed in complete incubation medium at 37°C for 3 h. The histochemical incubation medium (11) contained 0.1% 3,3'-diaminobenzidine (Sigma Chemical Co.) and 0.01% H₂O₂ in 0.05 M Tris buffer (Sigma Chemical Co.), pH 8.6. The incubations were carried out in a Dubnoff metabolic shaker. After incubation the slices were rinsed five times in 0.05 M Tris buffer, pH 8.6, and were placed in a reduced osmium solution containing 1% osmium tetroxide and 1.5% potassium ferrocyanide (17) for 30-90 min at room temperature. They were then dehydrated in acetone (70, 80, 95, and 100% changes at 4°C) and embedded in Epon (21). During embedding the tissue slices were oriented so that the plane of section, during ultramicrotomy, would be perpendicular to the long axis of the muscle fibers. Tissue sections, 0.5 μm thick, were lightly stained with alkaline toluidine blue (37) and were examined by light microscopy. Silver or silver-gray sections, lightly stained with alkaline lead citrate (40), were examined by electron microscopy.

Control Experiments

One mouse received no injection and served as the onset control. Its diaphragm was fixed, and specimens

TABLE I
Numbers of Mice, Dosages, and Time Intervals
Used in MP Experiments

Time interval	No. of mice	Amount of MP injected
s*		mg
-30	1	5 (0.1 ml)‡§
0	1	10 (0.2 ")
2.5	1	10 (0.2 ")
7	2	10 (0.2 ")
10	1	5 (0.1 ")
25	1	5 (0.1 ")
38	1	5 (0.1 ")
45	1	10 (0.2 ")
60	1	5 (0.1 ")
90	2	10 (0.2 ")
120	1	6 (0.2 ")
190	1	5 (0.2 ")
250	1	6 (0.2 ")

* Interval was measured as the seconds that elapsed between initiation of injection of the tracer and initiation of exposure of the diaphragm to fixative.

‡ Quantity in parenthesis is the volume of solution injected.

§ Simionescu et al. (33) injected rats intravenously with ~5 mg MP in 1 ml 154 mM NaCl and collected samples of blood for determination of plasma osmolarity. From 15 to 40 s after injection, the osmolarity of the plasma was slightly increased (~1.2%). We assume, therefore, that the injections of tracer in their study and ours did not produce changes in plasma osmolarity large enough to cause major shifts of fluid between the capillary lumen and the pericapillary space. The maximum volume of tracer solution we injected intravenously (0.2 ml) is equivalent to 12% of the blood volume of a 20-g mouse (31). In an earlier study (43), we showed that expanding the blood volume by this amount did not increase the leakage of HRP from muscle capillaries.

were collected as described above. A second mouse was injected intravenously with 0.2 ml saline, the vehicle for injection of MP. 30 s later its diaphragm was fixed, and specimens were collected in the usual manner. The specimens from each mouse were divided into two groups. One group was rinsed in Tris buffer and then embedded, and the second group was carried through the histochemical procedure for localizing MP before being embedded.

We also determined whether mice would exhibit toxic effects from an intravenous injection of MP. Two mice were anesthetized and injected with 10 mg MP in 0.2 ml saline as described above. The cervical incision made for the injection was sutured, and the mice were allowed to recover from the anesthetic. They were observed carefully for 24 h.

A second experiment was performed to determine

whether MP induces histamine release and thereby causes an increase in permeability of small blood vessels. The technique used was similar to that of Cotran and Karnovsky (5). Two mice were anesthetized with an intraperitoneal injection of sodium pentobarbital. They were immobilized in a supine position, and their ventral body surface was shaved. They each received an intravenous injection of Evans blue (0.6 mg in 0.1 ml saline), and then each mouse received a series of four intradermal injections on its abdominal surface. Both mice were injected with 0.05 ml saline and 50 μ g histamine in 0.05 ml saline. The histamine was obtained as a 1 ml ampul containing 1 mg histamine phosphate from Eli Lilly & Co. (Indianapolis, Ind.). The first mouse also received injections of 0.5 and 0.05 mg MP in 0.05 ml saline; the second received injections of 0.005 mg and 0.0005 mg MP in 0.05 ml saline. 30 min later, the abdominal skin of each mouse was excised, and the underside of the skin was examined to detect whether Evans blue dye had escaped from the circulation at the sites of injection.

RESULTS

Control Specimens

In toluidine blue-stained sections of unincubated diaphragm from uninjected and saline-injected mice, erythrocytes appeared deep blue and blood plasma appeared pale blue. In similar sections of specimens incubated in the histochemical medium, erythrocytes appeared deep brown and the plasma appeared pale brown. Under electron microscope examination, erythrocytes of incubated specimens were, in most instances, noticeably more electron-dense than those of unincubated specimens (Figs. 1 and 2). We were unable, however, to detect a consistent significant difference in the density of plasma between the two

types of specimens. The positive reaction of erythrocytes in control incubated specimens is attributed to the ability of hemoglobin to function as a peroxidase (7). The basis for the positive reaction of plasma seen in 0.5- μ m sections of specimens incubated in the histochemical medium is not clear. Vassar et al. (38) reported that fixation of erythrocytes with formaldehyde causes leakage of hemoglobin whereas fixation with glutaraldehyde does not. Our tissue specimens were fixed with a mixture of the two aldehydes. Formaldehyde, the smaller of the two molecules, might have diffused into the tissue more rapidly and caused hemolysis, thereby accounting for the positive reaction of plasma seen by light microscopy. Simionescu et al. (32) detected a low peak of absorption at OD₄₁₅, the absorption maximum for ferrihemoproteins, in plasma samples from uninjected rats. In capillaries of the diaphragm from uninjected rats, they also detected a positive reaction for peroxidatic activity in plasma close to erythrocytes. They concluded that hemoglobin from erythrocytes traumatized by experimental procedures was the most likely explanation for peroxidatic activity in the plasma.

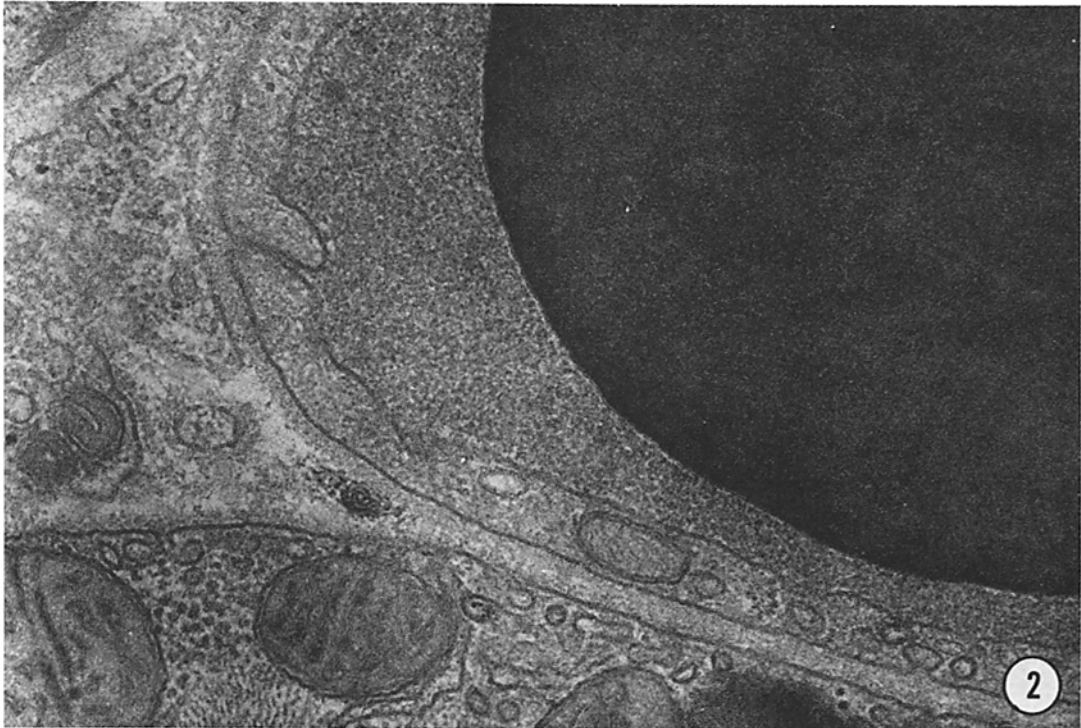
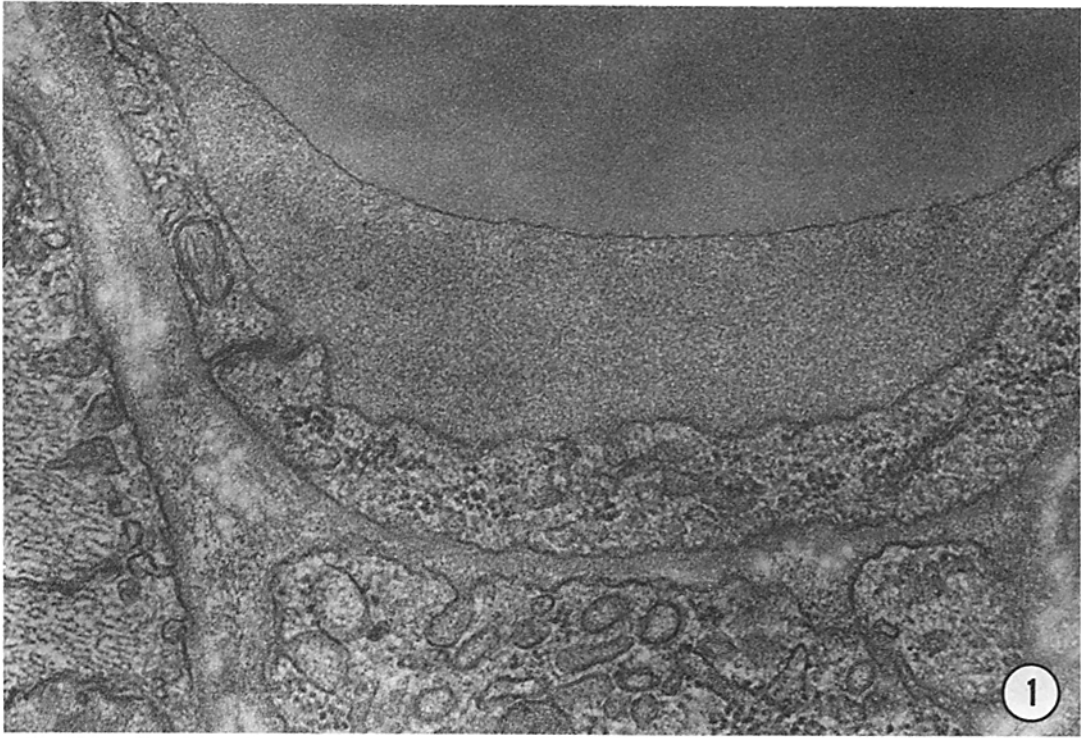
The mice which received an intravenous injection of 10 mg MP in 0.2 ml saline continued to respire at a normal rate, without difficulty (see Discussion), while tracer was being injected and for the period of time that they were under anesthesia. They recovered from anesthesia and were normally active for the remainder of the period of observation.

With regard to the mice which received an intravenous injection of Evans blue and intradermal injections of saline, histamine and MP, ex-

FIGURES 1-4 and 6-10 Capillaries from control mice are shown in Figs. 1 and 2. Figs. 3, 4, and 6-10 show capillaries in the diaphragm of mice that received an intravenous injection of MP in 0.2 ml saline. The time interval stated at the beginning of the legends of Figs. 6-10 are the intervals between initiation of the injection of MP and initiation of fixation of the diaphragm *in situ*.

FIGURE 1 Capillary in the diaphragm of an uninjected mouse. This specimen of the diaphragm was not incubated in the cytochemical medium used for detecting microperoxidase. The content of the erythrocyte in the capillary is homogeneous and moderately dense. The surrounding plasma is finely granular and of similar density. The interstitium is also finely granular in texture, and its density is comparable with that of the capillary basal lamina. Pale areas in the interstitium are unstained collagenous fibers cut in cross or oblique section. $\times 48,000$.

FIGURE 2 Capillary in the diaphragm of the same mouse described in the legend of Fig. 1. This specimen of the diaphragm was incubated in the cytochemical medium used for detecting MP. The appearance of this capillary is similar to that shown in Fig. 1 except that the content of the erythrocyte in its lumen is extremely dense. $\times 61,000$.



amination of the underside of the abdominal skin revealed extravasated dye only at the sites where histamine was injected. The blue region at the site of injection was ~15 mm in diameter, the size of the bleb raised by the intradermal injection of the test substance.

General Comments

For each mouse injected with MP, we examined by electron microscopy thin sections of at least three slices of diaphragm cut with the Sorvall TC-2 Tissue-Sectioner. Each slice contained cross sections of from 15 to 60 capillaries depending upon the cross-sectional area of the slice (Fig. 3). Thus we examined approximately from 45 to 180 capillaries from each mouse. We classified vessels as capillaries when the diameter of their lumen was wide enough to accommodate only a single erythrocyte. In the region of the diaphragm from which we collected our specimens, cross sections of arterioles and venules are encountered infrequently, and we did not include observations of such vessels in this report. To observe the distri-

bution of MP in the capillary lumen, endothelial vesicles and clefts, and pericapillary interstitium, we took electron micrographs of thin regions of the capillary wall, i.e., of regions that did not include the endothelial nucleus and the area immediately adjacent to it. In the case of the interendothelial clefts, we took micrographs of all those in which the membranes of the adjacent endothelial cells appeared as distinct unit membranes throughout the length of the cleft. This allowed us to look for gradients of MP concentration in the extracellular space of the cleft and in the immediately adjacent pericapillary interstitium. Because our tissue specimens were treated with the reduced osmium procedure (17), plasma and organellar membranes appeared as distinct unit membranes when sectioned perpendicularly, and we could easily recognize sites of membrane apposition or fusion between neighboring endothelial cells.

MP in the Pericapillary Interstitium

A high concentration of MP appeared in the

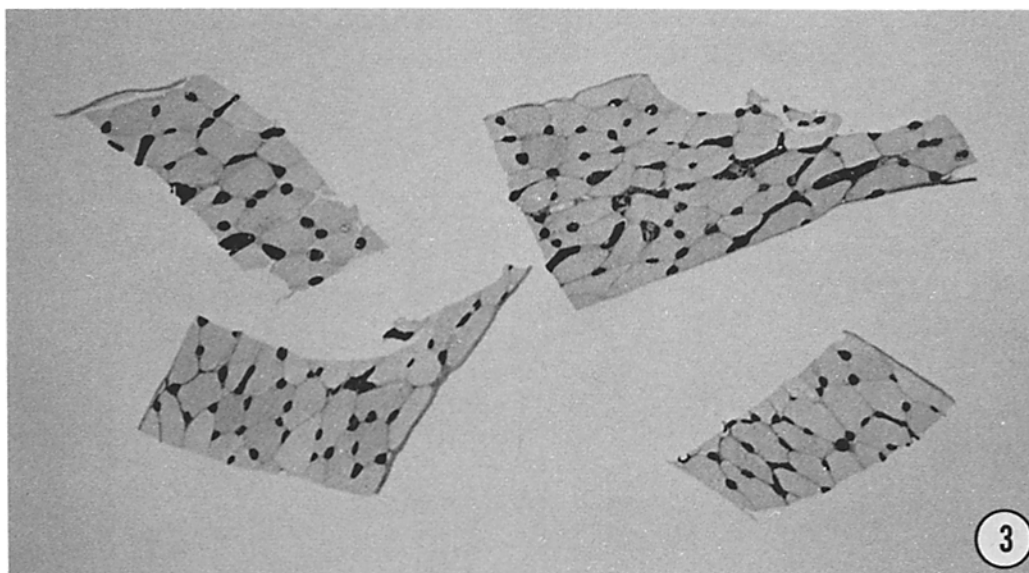


FIGURE 3 Sections of four 60- μ m thick slices of mouse diaphragm. The slices were oriented so that the plane of section is perpendicular to the longitudinal axis of the muscle fibers. Most of the capillaries are cut in cross section because they run predominantly parallel with the muscle fibers. Although the section is unstained, the capillaries appear dense because of the presence of reaction product in plasma and erythrocytes. Reaction product is also present in the interstitium where it is visible as fine lines outlining each muscle fiber. These specimens were collected from the lateral region of the diaphragm, midway between its central tendon and costal margin. Practically all of the blood vessels in the slices are identified as capillaries because they are thin-walled and their diameter approximates that of a single erythrocyte. The number of capillaries in the slices ranges from 25 (upper left slice) to 61 (upper right slice). $\times 1,100$.

interstitium bordering capillaries a short time after its intravenous injection (Table II). Even when fixation of the diaphragm and injection of the tracer were initiated simultaneously, the density of tracer in the interstitium was roughly equivalent to one-half that in the capillary lumens. The appearance at time 0, of a high concentration of tracer in the interstitium throughout the diaphragm suggests that fixation of the diaphragm by this method, i.e., bathing the peritoneal surface with fixative solution, is not rapid. To gain a more accurate idea of the rate of fixation, we injected one mouse with MP 30 s after we exposed the peritoneal surface of the diaphragm to fixative. In this specimen, we observed reaction product in the lumen of capillaries across the width of the diaphragm. We observed reaction product in the interstitium only in the middle of and near the pleural surface of the diaphragm (Fig. 4a); little tracer had escaped from capillaries near the peritoneal surface (Fig. 4b). Thus, by 30 s after exposure of the peritoneal surface of the diaphragm to fixative, fixation had proceeded to the extent that escape of tracer from subperitoneal capillaries was blocked, but blood continued to circulate in their lumens. Simionescu et al. (32) observed that capillary blood flow of the rat cremaster muscle was arrested 30 s after the inner and outer surfaces of this muscle were exposed to

fixative. Therefore, as far as the data in Table II are concerned, a correction factor of ~30 s should be added to the time intervals shown in the third column because of the slowness of the rate of fixation.

At all of the time intervals shown in Table II the concentration of MP in the pericapillary interstitium is significantly less than in the capillary lumens. Thus, the capillary endothelium can restrict the diffusion of a molecule of 1,900 mol wt from the capillary lumen, and the rate of diffusion of MP through the interstitium toward lymphatics is rapid enough to prevent its accumulation in the interstitium.

Localization of MP within Endothelial Clefts

In this aspect of the study we were primarily interested in searching for evidence which would reveal whether or not MP passed from the capillary lumen to the pericapillary interstitium via endothelial clefts. We examined principally specimens in which the concentration of MP in the capillary lumen significantly exceeded that in the interstitium. We could, therefore, observe how far MP had penetrated through the cleft in one direction, i.e., from the capillary lumen towards the pericapillary interstitium.

As we stated in the preceding section, we limited our survey to clefts in which the endothelial plasma membranes of adjacent endothelial cells were recognizable as distinct unit membranes throughout the clefts. We observed three different patterns of localization of MP within such clefts (Fig. 5). In the first pattern (Fig. 5A), there was only one site of close apposition² between the membranes of the junction, and the concentration of tracer on either side of the site of apposition was the same as in the capillary lumen and pericapillary interstitium respectively (Fig. 6a and b). In the second pattern (Fig. 5B), there were one or more sites of membrane apposition in the

² Within the great majority of endothelial clefts in capillaries of the mouse diaphragm, the outer dense layers of the apposed endothelial plasma membranes come into contact with one another at one or more points. The structural nature of the contact, i.e., whether it is a close apposition, fusion, etc., is not known. In this paper we refer to it as a site of close membrane apposition simply because the intercellular space appears to be obliterated at that point. We chose this term for reasons of convenience and not to indicate that we have reached any conclusions about the nature of the structural link between the membranes.

TABLE II
Relative Concentration of MP in the Pericapillary Interstitium at Intervals after its Intravenous Injection

No. of mice	Amount of MP injected		Concentration of MP in interstitium [‡]
	mg	Time interval s*	
1	10	0	++
1	10	2.5	++ to +++
2	10	7	++
1	5	10	++
1	5	25	++
1	5	38	+ to ++
1	10	45	++ to +++
1	5	60	++ to +++
2	10	90	+++
1	6	120	++
1	5	190	++
1	6	250	++

* Measured as the seconds between initiation of intravenous injection of the tracer and initiation of fixation of the diaphragm.

‡ Estimated by assigning ++++ to the concentration of tracer in the capillary lumens.

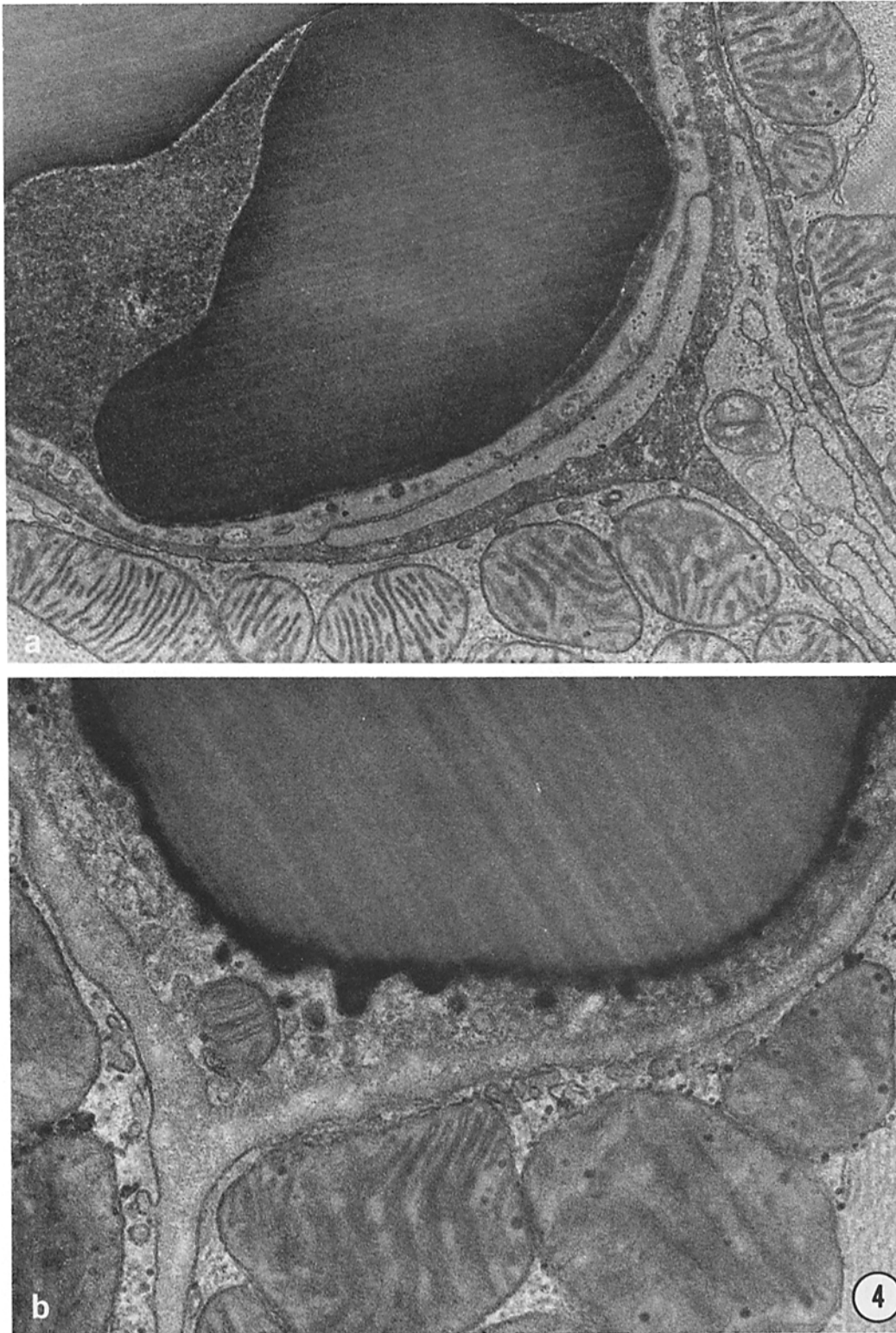


FIGURE 4 The capillaries shown in Fig. 4a and b are from the diaphragm of a mouse that was first injected intraperitoneally with fixative and then received an intravenous injection of MP 30 s later. The capillary shown in Fig. 4a was located midway between the pleural and peritoneal surface of the diaphragm. Its lumen contains dense reaction product. The pericapillary interstitium also contains reaction product but of lesser density. The capillary shown in Fig. 4b was located just beneath the peritoneal serosa. Its lumen contains dense reaction product. The pericapillary interstitium appears free of reaction product. (Note the similarity of the appearance of the interstitium in Figs. 4b and 2.) (a) $\times 32,000$; (b) $\times 56,000$.

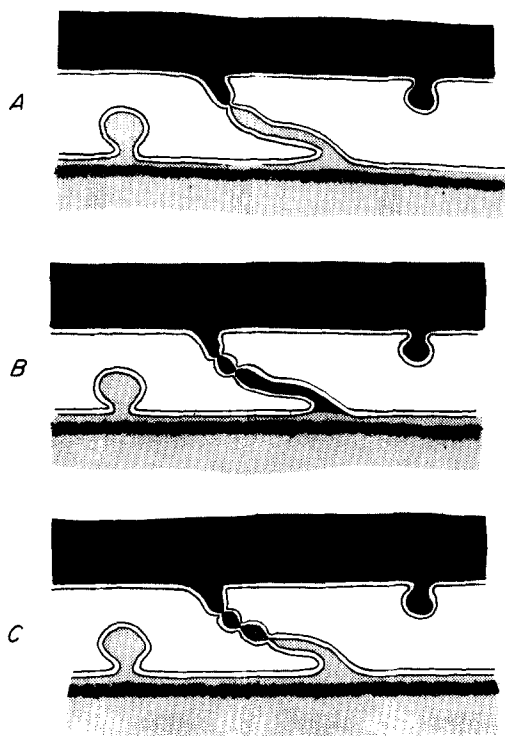


FIGURE 5 This figure illustrates the three patterns of localization of MP within endothelial clefts at short intervals after the tracer is injected intravenously, i.e., where there is dense reaction product in the capillary lumen and substantially less reaction product in the pericapillary interstitium. A detailed description of each pattern of localization is given in the text.

clefts. The concentration of MP throughout the length of the clefts was the same as that in the capillary lumen and exceeded the concentration of MP in the pericapillary interstitium (Figs. 7a, b, c, and d). At the point where the clefts opened into the pericapillary interstitium, the concentration of tracer fell abruptly from a level similar to that in the lumen to a level similar to that in the interstitium. The third pattern of localization (Fig. 5C) was observed in clefts with two or more sites of apposition of the adjacent endothelial plasma membranes. These clefts contained one or more small "compartments" between the successive sites of apposition. The density of reaction product within the compartments was intermediate between that present in the capillary lumen and that present in the pericapillary interstitium (Figs. 8a, b, c, and d). In junctions with three or more sites of membrane apposition, the concentration of tracer decreased stepwise in successive compart-

ments extending from the lumen towards the interstitium.

MP within Endothelial Vesicles

The small vesicles of the endothelial cells can be divided into three classes: those that are open at the luminal face of the endothelial cell; those that are open at the interstitial face; and those that appear to be free in the cytoplasm. A certain number of the latter class have been shown to be connected with either the luminal or interstitial surface in an adjacent plane of section (14, 15, 41). Our findings indicate that whether or not a given class of vesicles contains MP depends on the distribution of the tracer in the adjacent extracellular space. For example, when there was dense tracer in both the lumen and interstitium, all luminal and interstitial vesicles as well as all but a very small number of free vesicles contained dense tracer (Fig. 9). When there was dense tracer in the lumen and a small amount of tracer in the interstitium, all luminal and some free vesicles contained dense tracer, and all interstitial and nearly all of the rest of the free vesicles contained a small amount of tracer (Figs. 10a, b, c, and d). Therefore, with regard to those vesicles open at either surface of the endothelial cell, these findings indicate that the content of MP in the lumen of the vesicle equilibrates rapidly with the content of the tracer in the bordering space. We obtained similar findings in our earlier study with HRP (43). It is important to emphasize that the presence of a high concentration of tracer in interstitial vesicles when there is an equivalent concentration in the interstitium leaves open the question of whether individual interstitial vesicles are filling or emptying. They can be classified unambiguously as emptying vesicles only when the concentration of tracer in their lumen exceeds that in the interstitium. With regard to free vesicles, our findings suggest that the concentration of MP in their lumen reflects the concentration of MP in the medium adjacent to the surface from which they originated.

Transendothelial Channels

We did not observe MP penetrating a capillary endothelial cell, from the capillary lumen to the pericapillary interstitium, via transendothelial channels.

DISCUSSION

The chief aim of our investigation was to deter-

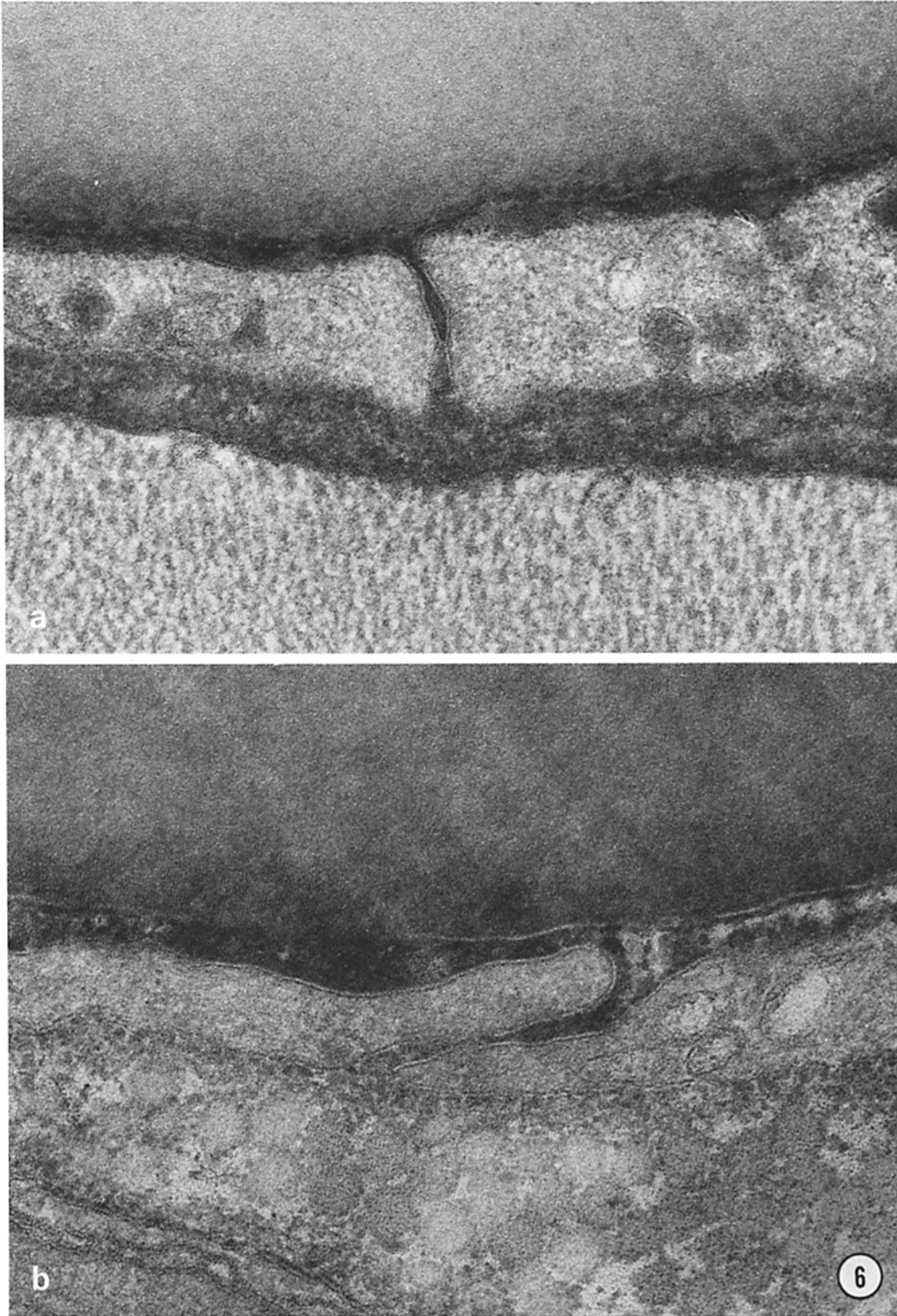


FIGURE 6 7 s (Fig. 6a), and 45 s (Fig. 6b). One site of membrane apposition appears in the endothelial clefts shown in these figures. The density of reaction product in the portion of the cleft adluminal to each site equals that in the capillary lumen, and the density of reaction product in the portion of the cleft abluminal to each site equals that in the adjacent interstitium. (a) $\times 114,000$; (b) $\times 117,000$.

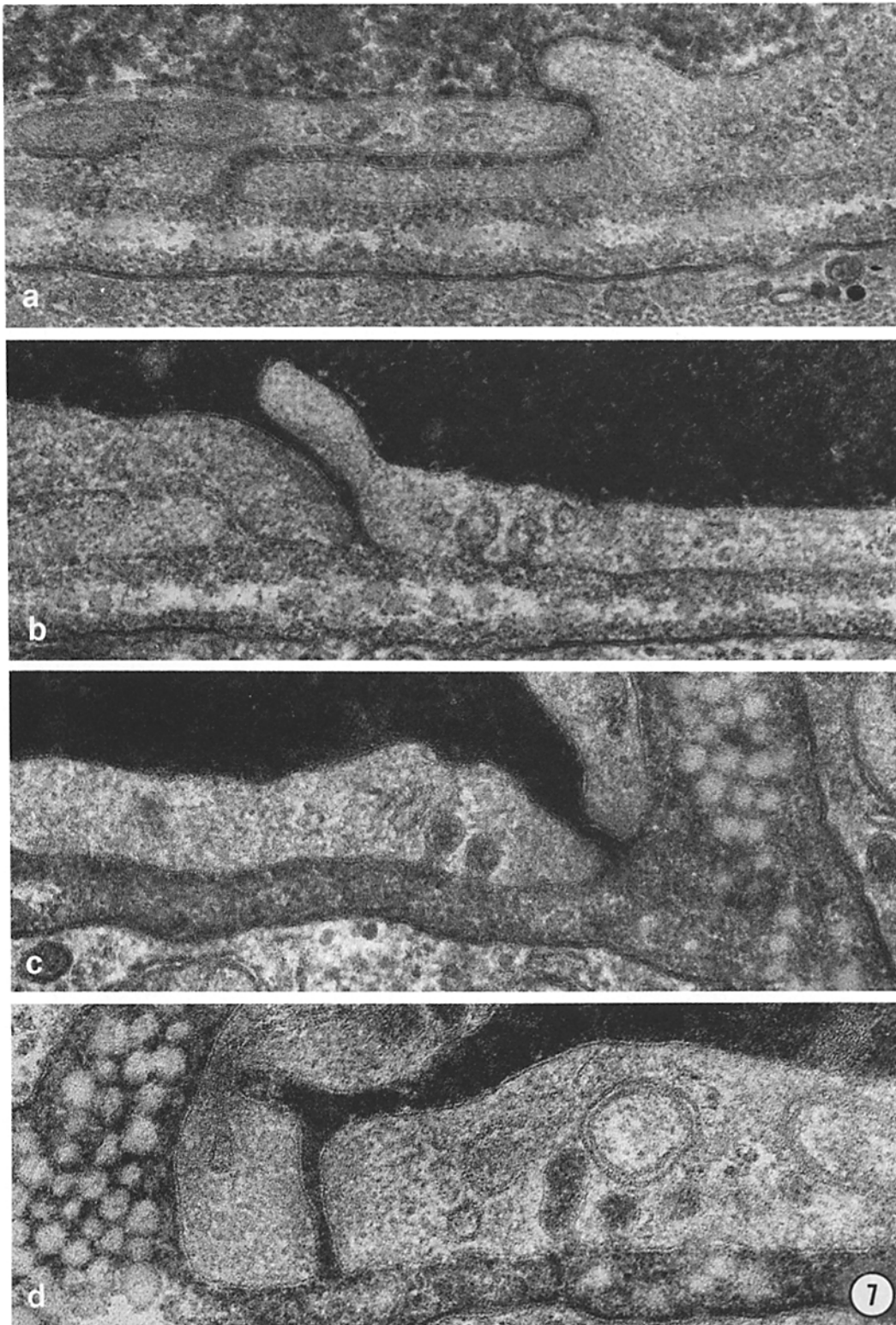


FIGURE 7 45 s (Fig. 7a); 7 s (Fig. 7b); 45 s (Fig. 7c); and 45 s (Fig. 7d). An endothelial cleft appears in each figure. The density of reaction product throughout the length of each cleft equals that in the capillary lumen. At the interstitial end of the cleft, the density of reaction product falls to the level present in the interstitium. (a) $\times 64,000$; (b) $\times 81,000$; (c) $\times 95,000$; (d) $\times 112,000$.

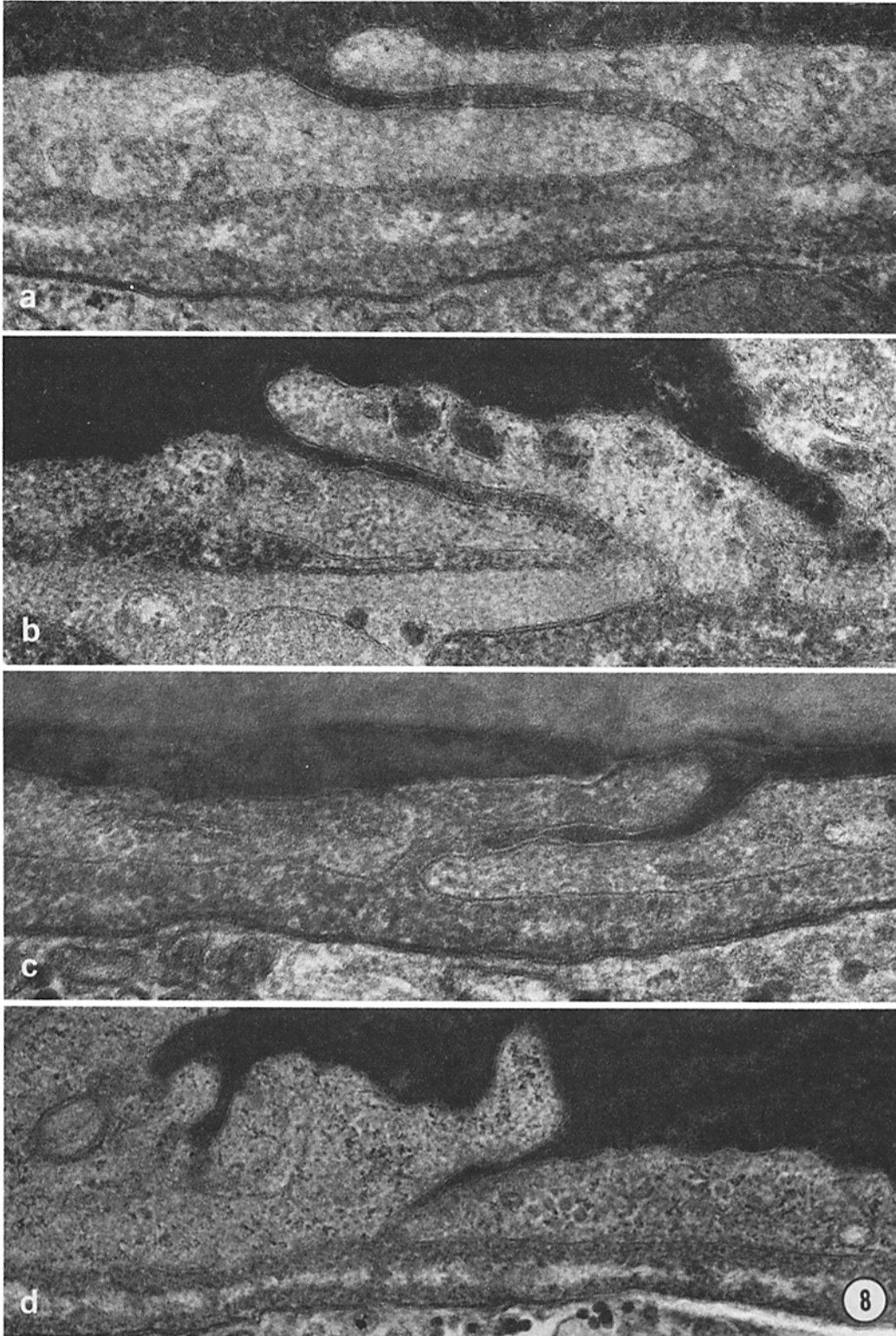


FIGURE 8 7 s (Fig. 8a); 0 s (Fig. 8b and c); and 2.5 s (Fig. 8d). An endothelial cleft appears in each figure. Two or more sites of membrane apposition appear within each cleft and subdivide the junction into a succession of "compartments." The density of reaction product in the capillary lumen exceeds that in the pericapillary interstitium. The density of reaction product decreases progressively along the length of the cleft, i.e., in successive compartments, from the luminal to the interstitial end of the cleft. (a) $\times 102,000$; (b) $\times 97,000$; (c) $\times 126,000$; (d) $\times 47,000$.

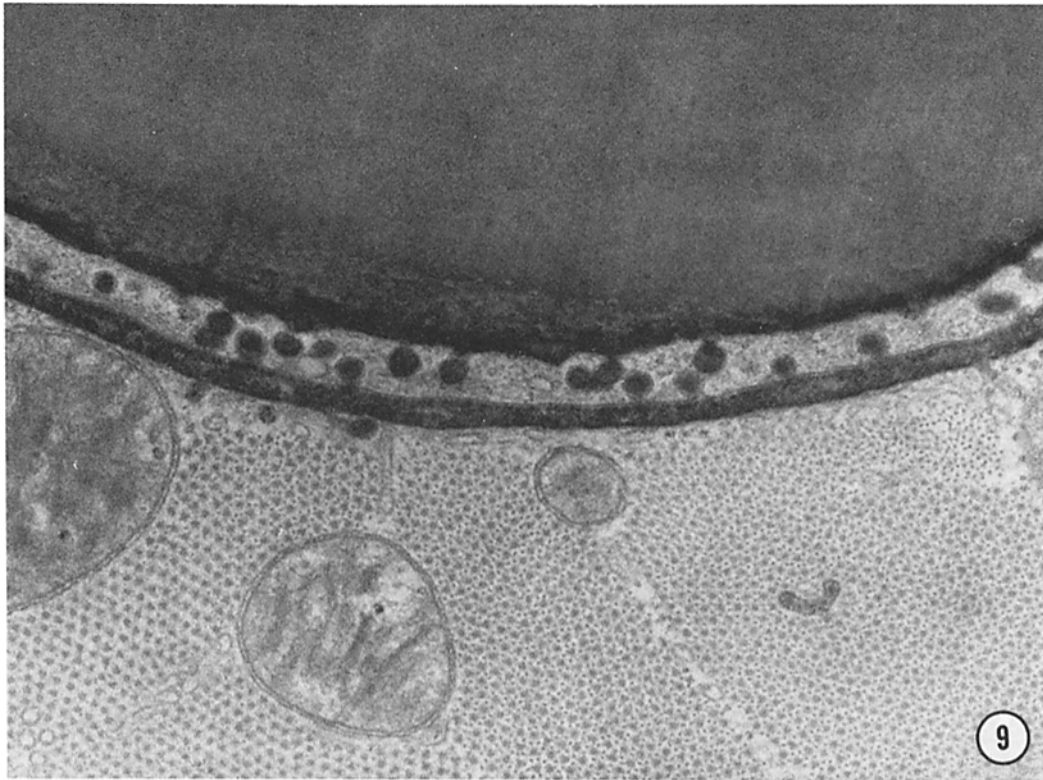


FIGURE 9 2.5 s. An erythrocyte appears in the capillary lumen in the upper portion of the figure. A narrow layer of plasma separates it from the endothelial cell located below it. A skeletal muscle cell appears in the lower portion of the figure. The density of reaction product in the plasma exceeds that within the erythrocyte. The density of the reaction product in the plasma and in the pericapillary interstitium is approximately the same. Lumenal, interstitial, and nearly all cytoplasmic vesicles contain reaction product equal in density to that in the capillary lumen and interstitial space. An occasional cytoplasmic vesicle appears empty. $\times 52,000$.

mine how MP, a small polypeptide, crosses the endothelium of muscle capillaries. On the basis of our findings, we conclude that MP crosses the endothelium principally via endothelial clefts. The main evidence for this conclusion stems from the three patterns of distribution of MP that are seen within endothelial clefts at short intervals after the tracer is injected intravenously, i.e., when the density of tracer in the pericapillary interstitium is appreciably less than in the capillary lumen. The first pattern (Fig. 5 A) lends itself to ambiguous interpretation. On the one hand, MP could be leaking through the cleft, but at a rate too slow to permit it to accumulate in the cleft distal to the site of membrane apposition before diffusing away into the surrounding pericapillary interstitium. On the other hand, the site of membrane apposition could be impermeable to MP, and the distal portion of the cleft is filled with MP which has

diffused into it from the interstitium. Since the pattern of distribution of MP within the cleft is the same whether or not the membrane apposition is permeable to MP, the first pattern of distribution does not permit us to decide unequivocally whether the cleft is permeable to MP.

The second pattern of localization (Fig. 5 B) can be interpreted as unequivocally indicating that the cleft is permeable to MP because it is unlikely that the distal portion of the cleft can accumulate and concentrate tracer from the adjacent interstitium. The third pattern of localization (Fig. 5 C) also indicates that the endothelial cleft is permeable to MP because the progressive decrease in concentration of tracer within compartments between successive sites of membrane apposition signifies that the tracer has circumvented or penetrated each site.

As yet we do not know the structural basis for

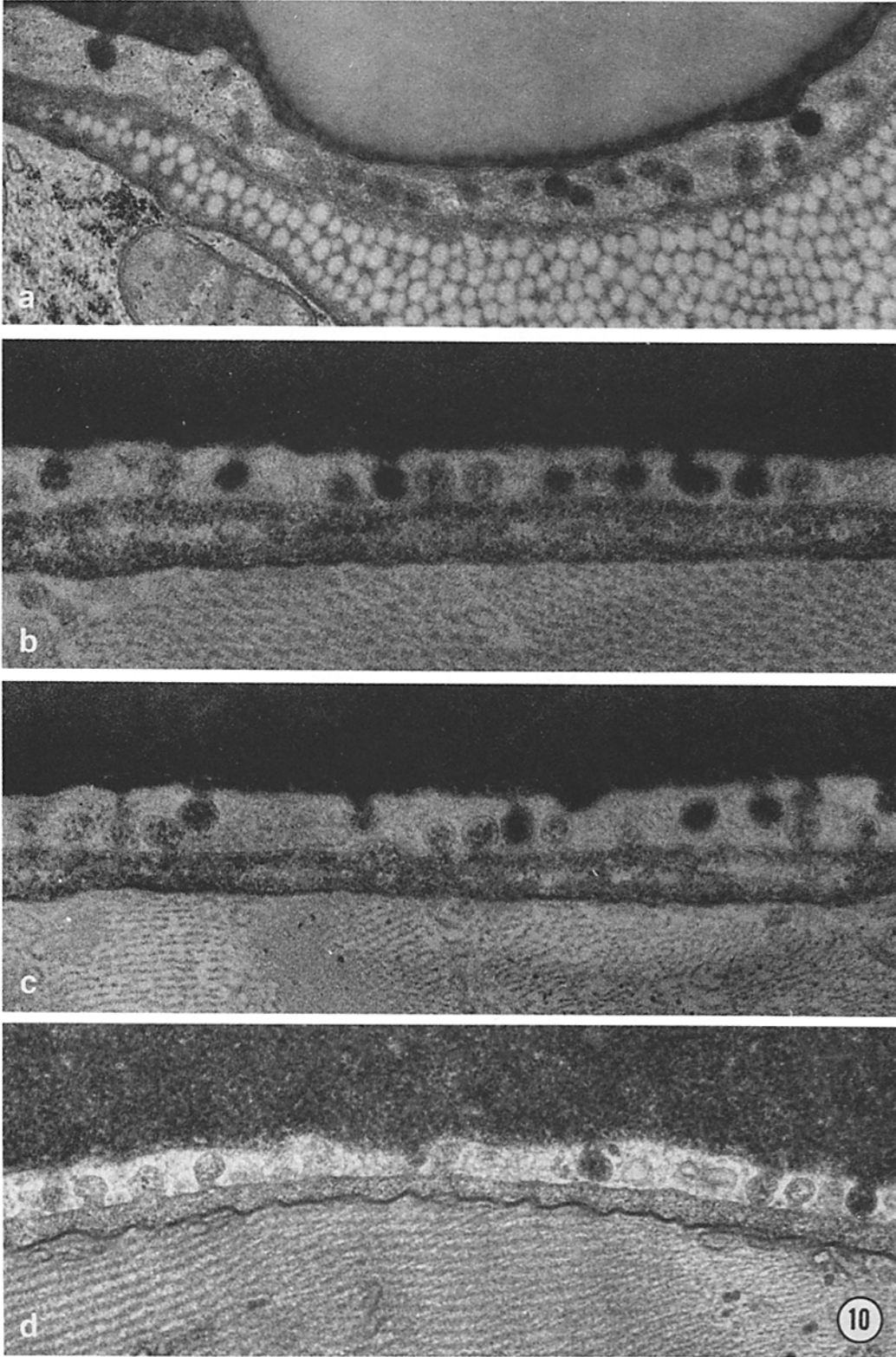


FIGURE 10 7 s (Fig. 10*a-c*), and 45 s (Fig. 10*d*). In the capillaries shown in these figures, the density of reaction product in blood plasma exceeds that in the pericapillary interstitium. Luminal and some cytoplasmic vesicles contain reaction product equal in density to that in the plasma, whereas interstitial and the rest of the cytoplasmic vesicles contain reaction product equal in density to that in the pericapillary interstitium. (*a*) $\times 58,000$; (*b*) $\times 75,000$; (*c*) $\times 73,000$; (*d*) $\times 57,000$.

the permeability of some endothelial junctions to MP. However, certain observations of thin-sectioned and freeze-fractured muscle capillaries suggest possible explanations. In sections of endothelial clefts in capillaries of the mouse heart, gastrocnemius, and diaphragm, Karnovsky (14) noted that, at the sites of close apposition, the plasma membranes of adjacent endothelial cells frequently were separated by a 40-Å gap. However, according to Williams (42), such gaps are few in number. In 57 endothelial clefts in capillaries of the mouse diaphragm, she observed only two in which the membranes were separated by a gap of 25–35 Å at the site of close apposition, and in 37 endothelial clefts of capillaries of intestinal smooth muscle of the same species, she observed only two with a gap of similar dimensions at the site. Bruns and Palade (2) examined endothelial clefts in thin sections of various striated muscles in several species of small laboratory animals and reported that, except for capillaries of the tongue, gaps between the membranes at sites of close apposition were rare. Karnovsky proposes that the gaps represent, in three dimensions, slits ~40 Å wide between adjacent endothelial cells and that they serve as the ultrastructural analogue of the small pore. In this case, it should ultimately be shown that the slits occupy <0.02% of the capillary surface area, the proportion of the total capillary surface area that is occupied by the small pore according to physiologic findings (16). At present, we cannot determine the fractional surface area occupied by such slits for two reasons. First, we do not know the dimensions of capillary endothelial cells and thus cannot calculate the total length or surface area of endothelial clefts per unit of capillary surface area. Second, data from freeze-fracture studies indicate that slitlike passages between endothelial cells, if indeed they exist, are tortuous (34). Thus it would be extremely difficult to determine the fraction of the total length of the endothelial clefts that is occupied by such openings.

In freeze-fractured specimens of muscle capillaries (personal observations and reference 34), the junction between adjacent endothelial plasma membranes appears as a narrow network of linear elements or strands. The strands appear as ridges on the PF face of the endothelial plasma membrane and as complementary shallow grooves on the EF face. Because the number of strands across the width of the junction ranges from 1 to 5, the junction bears some resemblance to the

occluding zonule of "leaky" epithelia (4, 30), but it differs from the epithelial junction in important respects. In the latter, a row of closely spaced particles or a continuous linear strand surmounts the ridge on the P face of the plasma membrane. In the endothelial junction, a relatively discontinuous row of intramembrane particles is found along both the ridges and grooves of the junction; the majority of the particles adhere to the groove on the EF face (34). Because complementary replicas have not yet been examined, it is not clear whether the particles on the PF and EF face together form a continuous single file. If they do not, this might mean that there are minute interruptions in the line of fusion between the membranes. In capillaries of the rat omentum and mesentery, the strands of the endothelial junction are staggered and have free ends. Occasional unobstructed channels can be traced through the network from one side to the other (34). Because capillaries of the rat omentum and mesentery and those of the mouse diaphragm resemble each other closely when examined in thin sections, it seems likely that their structure will appear similar when visualized with the freeze-fracture technique. If the strands of the network are faithful markers of lines of apposition between membranes of adjacent endothelial cells, the discontinuities in the network suggest that the endothelial junction is a fascia or macula occludens, as originally postulated by Karnovsky (14), rather than a zonula occludens as proposed by Bruns and Palade (2). Such discontinuities, restricted in distribution, could account for the rapid leakage of MP through endothelial clefts observed in this study. Because MP, on account of its small molecular size, should be able to pass through the small pore of the capillary wall, the discontinuities could be the ultrastructural counterpart of this pore.

Because the strands in the endothelial network are staggered, the terminations of the strands at the site of discontinuities usually overlap. On account of this morphologic feature, we would not expect the occurrence of interruptions in the junction to be easily recognized in sectioned specimens (Fig. 11). Therefore the true frequency of their occurrence would be difficult to assess in such specimens.

Luft (22, 23) observed that the apposed outer dense lamellae of the plasma membranes in endothelial junctions form a single layer 35–50 Å thick which stains with ruthenium red, suggesting that the layer contains acid mucopolysaccharide

or an acidic protein (24). He noted that the width of the layer, i.e., 35–50 Å, is similar to the width of the slit that Landis and Pappenheimer (19) postulated as a possible configuration of the small pore. Luft (22, 23) postulated that the layer formed by the apposed lamellae could function as the small pore provided that its content of acid mucopolysaccharides or proteins endows the layer with the properties of a gel that is porous enough to allow passage of molecules of up to 40,000 mol wt. The concept that the opposed outer dense lamellae form a permeable structure is not unique to capillary endothelium. The fused outer dense

lamellae in the tight junctions of leaky epithelia are also assumed to be porous since they permit passage of ions and water (4, 30).

At this stage, it is not possible to decide whether the discontinuities in the lines of apposition between adjacent endothelial plasma membranes or the gel-like properties of the apposed outer dense lamellae of the membranes enable MP to pass through the clefts.

It is important to consider whether vesicular transfer contributes significantly to the outflow of peroxidatic tracers from muscle capillaries. Thus far a number of tracers of this type have been used in ultrastructural studies of capillary permeability. They have been administered to laboratory animals in roughly equivalent dosages, i.e., dosages sufficient to produce dense reaction product in capillary lumens after intravenous injection of the tracer. When we surveyed the observations made with the tracers, we noted that the time, after intravenous injection, at which a significant amount of tracer first appears in the pericapillary interstitium varies inversely with the molecular weight of the tracer (Table III). This implies that the rate at which these tracers escape into the pericapillary space is roughly proportional to their rate of diffusion, i.e., tracers of smaller molecular size escape more rapidly from the circulation than do larger tracers. This is consistent with physiologic findings (19) and is what one would expect to observe if the tracer crossed the capillary wall principally by diffusion through openings or "pores." If these tracers passed across the capillary wall principally by vesicular transfer, we would expect them to appear in the interstitium at approximately the same time provided that we make

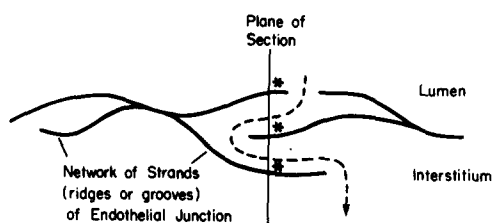


FIGURE 11 Diagram of the freeze-fracture image of the linear strands in endothelial junctions of the rat mesentery and omentum. The diagram is based on the observations of Simionescu et al. (34). A plane of section has been drawn through a region of the network where the strands are discontinuous and staggered. Examination of a thin section through the cleft coincident with this plane would show that the pathway for diffusion through the cleft is restricted by a succession of three sites of membrane apposition (asterisks). The image of the cleft in a freeze-fracture preparation would indicate that there is, however, a tortuous potential pathway for diffusion (dashed line) that passes between interruptions in the network of strands to circumvent the membrane appositions.

TABLE III
Time of Initial Appearance of Various Peroxidatic Tracers in the Pericapillary Interstitium after Their Intravenous Injection

Tracer	mol wt	Time of appearance	Reference
Heme-octapeptide	1,500	40 s	Simionescu et al. (33)
Heme-undecapeptide (MP)	1,900	40 s	Simionescu et al. (33)
Heme-undecapeptide (MP)	1,900	30 s	This study
Cytochrome c	12,000	1 min	Karnovsky (16)
Myoglobin	17,800	1 min	Simionescu et al. (32)
HRP	40,000	5 min	Karnovsky (14)*
HRP	40,000	16 min	Williams and Wissig (43)*

* In his study, Karnovsky injected mice intravenously with 2–5 mg Sigma Type II HRP. In our study with HRP, we injected mice intravenously with 0.5 mg Worthington Type HPOFF HRP (Worthington Biochemical Corp., Freehold, N. J.). The Worthington preparation has twice the enzymatic potency as the Sigma Type II preparation. We injected, in effect, one-fifth to one-half the amount of HRP injected by Karnovsky, and this presumably explains the longer interval before HRP appeared in the interstitium in our study.

the following assumptions: first, that the presence of the tracers in the plasma does not alter the rate at which the vesicular transfer system functions, and, second, that vesicles remain open at the luminal surface of the endothelial cell long enough to allow each tracer to equilibrate between the content of the vesicle and the luminal plasma. The second assumption is probably valid for peroxidatic tracers because the density of reaction product in luminal vesicles and in the capillary lumen is always the same. In other words, vesicular transfer, being a bulk transfer system, should operate in such a manner that the amount of tracer it transfers to the interstitium should depend primarily on the concentration of tracer in the capillary lumen and not on the molecular weight of the tracer. Because this is not consistent with physiologic data (19) or the data shown in Table III, it is unlikely that the vesicular transfer system is the principal means by which peroxidatic tracers of up to 40,000 mol wt cross the endothelium of muscle capillaries.

The observation by Simionescu et al. (33) that MP crosses endothelial cells of muscle capillaries via a channel formed by a single vesicle or a chain of linked vesicles has not been confirmed in this study. Therefore, we are not able to substantiate their hypothesis that transendothelial channels are the ultrastructural analogue of the small pore of muscle capillaries. This question should be examined further because the channels may be tortuous and hence difficult to discern in thin sections. Moreover, such channels are reported to occur more frequently in venous vessels (35), and we have not yet studied such vessels in detail.

Because of its small molecular size, MP should cross the wall of muscle capillaries principally via the small pore (19). Our findings from this study indicate fairly conclusively that a certain number of the clefts between endothelial cells allow passage of the tracer; whether or not the tracer can pass through the remainder of the clefts is still undecided. Our findings do not furnish evidence that significant amounts of MP cross the capillary wall either via vesicular transfer or via transendothelial channels. We conclude, therefore, that endothelial clefts represent one structural analogue of the small pore of capillaries. The structural properties of the cleft that enable it to function as a small pore are not yet resolved. The pore could be represented by tortuous discontinuities in the close appositions between the plasma membranes of adjacent endothelial cells or by the

porosity of the conjoined outer dense lamellae at the site of close membrane appositions.

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