OPEN JUNCTIONS IN THE ENDOTHELium OF THE
POSTCAPILLARY VENULES OF THE DIAPHRAGM

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ABSTRACT

We have previously established that ~30% of the endothelial junctions in the pericytic venules of the mouse diaphragm are open to a gap of ~30-60 Å, and are fully permeated by hemeundecapeptide (H11P) (mol diam ~20 Å). To estimate the size limit for molecules that can permeate these junctions, we have administered graded tracers intravenously and studied their behavior at the level of pericytic venules in bipolar microvascular fields (BMFs) in the mouse diaphragm. Horseradish peroxidase (HRP) (mol diam ~50 Å) permeated only ~50% of the open junctions of the venular endothelium. Outflow through venular junctions appeared to be modest since the tracer remained restricted to the perivenular spaces. Hemoglobin (Hb, mol diam 64 x 55 x 50 Å) permeated only a few (<5%), and ferritin (mol diam 110 Å), practically none, of the endothelial junctions of the pericytic venules. The findings suggest that under normal conditions the size limit for permeant molecules for open venular junctions is ~60 Å. Replicas of freeze-fracture preparations from appropriate regions in BMF showed that the intercellular junctions of the venular endothelium have the same organization as previously described for the corresponding segments of the microvasculature in the omentum and mesentery: discontinuous creases or grooves either free of or marked by few intramembrane particles only. Administration of histamine (topically or systemically) and 5-hydroxytryptamine (5-HT) (topically) resulted in typical focal separations of the endothelial junctions and intramural deposits of large tracer particles (carbon black) in the postcapillary venules.

KEY WORDS endothelial junctions . freeze-fracture . hemeundecapeptide . hemoglobin . histamine . horseradish peroxidase . microvasculature . microperoxidase . postcapillary venules

In previous work, we have introduced a new procedure for sampling reliably identified segments of the microvasculature of skeletal muscle. This procedure relies on the existence in the mouse diaphragm of bipolar microvascular fields (BMFs) in which the arteriole supplies and the venules drain the capillary network from opposite ends (25). Using this precise sampling, we were able to detect within the microvasculature characteristic segmental variations in: (a) the thickness of the endothelium, (b) the density of the plas-
malemal vesicle population, (c) the frequency of apparent transendothelial channels, and (d) the state of the intercellular junctions. The observations showed that, under conditions which can be considered physiological, ~25-30% of the endothelial junctions of the pericytic (postcapillary) venules are open to a gap of 30-60 Å, and are rapidly and fully permeated by probe molecules of ~20 Å diam such as the hemovendecapeptide (H11P) (26).

To get further insight into the morphological and functional characteristics of the intercellular junctions of the venular endothelium, experiments were carried out to determine: (a) the size limit of permeant molecules; (b) the intramembranous organization of these venular junctions as compared to that observed in other vascular beds (22); and (c) their sensitivity to histamine and 5-hydroxytryptamine (5-HT). In other locations (cheek pouch [29, 30]) these and other mediators were known to cause focal separations of the endothelial cells in the postcapillary venules.

The results obtained along these lines of investigation are reported in this paper.

MATERIALS AND METHODS

Materials

Animals: As in our previous work (25, 26), the inquiry was carried out on the mouse diaphragm. Male Swiss mice, weighing 20–30 g, were kept under similar standardized conditions of feeding and housing for 5–8 days before being used in the experiments to be reported.

Reagents: Reagents were obtained as follows:
- Microperoxidase (sodium salt from horse heart cytochrome c), horseradish peroxidase (HRP) (type II), hemoglobin (Hb) (type III, from sheep erythrocytes), histamine diaphosphate, and 5-HT creatinine sulfate complex (serotonin) from Sigma Chemical Co. (St. Louis, Mo.);
- ferritin (equine), 2x crystallized cadmium solution (batch no. C111/1413a) from Guenther Wagner Pelikan-Werke (Hannover, Germany);
- tannic acid, analytical reagent, cat. no. 1764, from Mallinckrodt, Inc. (St. Louis, Mo.).

Methods

Tracer Experiments: The probe molecules chosen ranged from ~20 Å (H11P) to 110 Å (ferritin) diam, and comprised HRP (50 Å) and Hb (64 × 55 × 50 Å). Data regarding the tracer solutions are given in Table 1. All tracer solutions were prepared in 0.154 M NaCl, adjusted to pH 7.0 with 0.1 N NaOH, prewarmed to 38°C, and injected under light ether anesthesia into the femoral vein at a rate of 0.1 ml/s.

At selected time intervals ranging from 5 s to 10 min after tracer administration (Table II), the diaphragms were fixed in situ by injecting buffered aldehyde solution (5% formaldehyde and 3% glutaraldehyde in 0.1 M HCl-Na arsenate buffer, pH 7.2) simultaneously into the peritoneal and pleural cavities (25). After 20-min fixation in situ, the diaphragms were excised and BMFs or areas containing only venular segments of capillaries and venules were identified in the postero-lateral part of the diaphragm, under a dissecting microscope. Satisfactorily identified specimens (Fig. 1) were removed and immersed in the same aldehyde fixative for 60 min at 22°C. Specimens collected from animals injected with H11P, HRP, and Hb were incubated for 60 min at 37°C in 3-3' diamino-benzidine-H2O2 medium at pH 8.6–9.0 for H11P (21), or at pH 7.0 for HRP and Hb (3). Subsequently, all specimens including those collected from animals injected with ferritin were treated with 2% OsO4 in 0.1 M HCl-Na arsenate buffer, pH 7.2, followed by mordanting with galloylglucoses (tannic acid) (23), then dehydrated and flat embedded. Thin sections (~500–700 Å thick) were cut on a Porter-Blum MT2B ultramicrotome (DuPont Instruments-Sorvall, DuPont Co. (Wilmington, Del.), and stained for 3 min with a 0.4% aqueous solution of lead citrate. Controls for possible diffusion of tracers used or their reaction product into the tissue were carried out by adding H11P, HRP, or Hb to the fixative solution or to the incubation medium (21).

Freeze-Fracture Preparations: Diaphragms were fixed in situ by injecting intraperitoneally 2 ml/100 g body weight of a solution of 2% glutaraldehyde in 0.1 M HCl-Na cacodylate or HCl-Na arsenate buffer, pH 7.2–7.4, prewarmed to 38°C. After 15–20 min, the diaphragms were removed and BMFs identified and excised as indicated in Reference 25. From each BMF, separate specimens were collected from areas corresponding to the following vascular segments: capillaries, capillaries and venules, pericytic venules, and muscular venules (Fig. 1). After an additional 15- to 20-min immersion in the same fixative, the specimens were treated for 2 h at 4°C with 25% glycerol in 0.1 M HCl-Na cacodylate buffer, pH 7.2–7.4. Tissue fragments were then placed on metal carriers and further processed for freeze-fracturing as previously described (22, 10). 12 mice were used for this study; from the BMFs chosen, 34 replicas were prepared on which 86 endothelial junctions were examined.

Histamine and 5-HT Experiments: For these investigations we used primarily histamine which was applied either intravenously or locally. 5-HT (serotonin) was only topically employed, since it is known to be degraded in the pulmonary circulation (7, 28). The experimental protocol was as follows:

(a) Intravenous injection of either: (1) ferritin solu-
Figure 1: Bipolar microvascular field excised from the diaphragm of a mouse injected with HRP. The specimen was histochemically reacted for peroxidative activity and photographed before treatment with OsO₄. The subfields examined in tracer experiments were: middle segments of capillaries (Cₘ), venular segments of capillaries (Cᵥ), pericytic venules (Vₚ), and muscular venules (Vₘ) (as indicated on the right side of the specimen). Subfields limited to capillaries and pericytic venules collected from adjacent areas were also used. Similar BMFs were processed for freeze-fracture preparations; in this case, the subfields examined were: Cᵥ, T (vessels at the transition from capillaries to pericytic venules), Vₚ, and Vₘ (as indicated on the left side of the specimen). A marks the arteriole of the BMF, and VA, the venous arcade. × 175.

As described above, or (2) 0.1 ml/100 g body weight of carbon black solution.

(b) After 2 min, administration of histamine by either (1) local application, on the abdominal aspect of the diaphragm, of a thin cotton pad soaked in 0.2% histamine diphosphate solution in 0.154 M NaCl; after 5-10 min, the abdominal wall was sutured and the animal allowed to awake; or (2) i.v. injection of a
histamine diphosphate solution (in 0.154 M NaCl) in concentrations ranging from 7.0 to 200 μg (histamine base) per 100 g body weight; the injected volume was 0.1 ml/100 g body weight.

(c) After 30 min, the diaphragms were fixed in situ for 15–20 min with a buffered aldehyde solution (as described in "Tracer Experiments"), then the muscle was excised.

(d) Under the dissecting microscope, the diaphragms were screened for black vascular "tattoo." The labeled regions were photographed and further processed for electron microscopy as indicated in the tracer experiments.

In the serotonin experiments a solution of 0.25% of 5-HT in 0.154 M NaCl prewarmed to 38°C was topically applied on the abdominal aspect of the diaphragm (on a cotton pad); the specimens were fixed in situ and subsequently collected and processed as for the histamine experiments. 34 mice were used for the studies with these vasoactive mediators.

Controls for possible nonspecific effects induced by the surgical procedure or by vehicle solvents (NaCl) included: (a) opening of the abdominal cavity and the exposure of diaphragm to air; (b) the topical application of 0.154 M NaCl solution immediately before or after i.v. injection of carbon solution, and (c) intravenous administration of 0.1 ml/100 g body weight of a 0.154 M NaCl solution after the i.v. injection of a carbon black solution.

All electron microscope preparations were examined with a Philips-301 electron microscope operated at 80 kV and provided with apertures in the condenser (300 μm) and the objective (50 μm).

RESULTS

Tracer Experiments: Size Limit of Permeant Molecules

The range of molecular diameters covered by the probe molecules with which we have worked extends from ~20 Å for H11P used in our previous experiments (26) to ~110 Å for the experiments now reported (HRP, Hb, and ferritin) (Table I). The results indicate that the percentage of junctions permeated by H11P varied from ~20–30% from 0-s to 3-min interval but did not appear to increase over the rest of the time covered (10 min).

The percentage of junctions permeated by HRP (mol diam ~50 Å) increased from 6% at 30 s to 15–20% between 45 and 60 s, and appeared to remain stable thereafter up to 10 min (Table II).

Hb (mol diam 64 × 55 × 50 Å) permeated only a small fraction of endothelial junctions (2–4.5%), and concentrations high enough to be detected at the level of the venular junctions were encountered only after 30–45 s (Table II).

A single junction (out of 314 endothelial junctions examined) was detected to be permeated when ferritin (mol diam ~110 Å) was used as tracer.

Taken together, these data indicate that the percentage of open junctions in the venular endothelium is not >30% for the smaller tracer used (H11P) and decreases progressively as the diameter of the probes increases, to reach a percentage ~10 times lower for Hb, and to exclude—in practically all cases—ferritin molecules. For all tracers used, the number of junctions penetrated seemed to remain unchanged past 30–45 s; hence, there was no recruitment of newly opened junctions as a function of time after the administration of the tracer.

In the HRP experiments, in all permeated junctions the intercellular spaces were fully marked by reaction product, and small amounts of the latter were also detected in the subendothelial spaces (Figs. 2–4). In Figs. 2–4, a weak concentration gradient of reaction product can be seen in the interstitia between the endothelial cells.

### Table I

| Tracer                        | mol wt | mol diam | conc | Osmolality | Amount injected |
|-------------------------------|--------|----------|------|------------|----------------|
| Hemeundecapeptide             | 1,900  | 20Å      | 0.5  | 315        | 5              |
| Horseradish peroxidase        | 40,000 | 50Å      | 1.0  | 313        | 10             |
| Hemoglobin                   | 68,000 | 64 × 55 × 50 | 1.0 | 302        | 10             |
| Ferritin                     | 450,000| 110Å     | 5.0  | 325        | 50             |

* 1 ml/100 g body weight.
† Estimated.
TABLE II

| Tracer experiments                  | 0-30 s | 30-45 s | 45-60 s | 1-3 min | 5-10 min |
|-------------------------------------|--------|---------|---------|---------|----------|
| Hemeundecapeptide (48)‡             | 26 ± 3.3‡ | 29 ± 4.2 | 19 ± 2.3 | 25 ± 4.1 |          |
| Horseradish peroxidase (24)         | 6 ± 1.2 | 15 ± 2.1 | 12 ± 1.6 | 11 ± 1.3 | 13 ± 2.0 |
| Hemoglobin (25)                     | 0      | 3 ± 0.4  | 2.5 ± 0.5 | 4.5 ± 0.7 | 2 ± 0.3  |
| Ferritin (22)                       | 0      | 1       | 0       | 0       | 0        |

For each diaphragm, one to four bipolar microvascular fields were collected and processed for electron microscopy (see Materials and Methods).

Intervals were counted as indicated in reference 25 (starting at 20 s after the beginning of fixation in situ).

Nos. in parentheses indicate no. of animals used. The minimum number of animals used for each time interval was four.

* In postcapillary (pericytic) venules.

‡ For H11P the figures come from previous work (25) as well as from new experiments.

§ Mean values and SD calculated for each tracer in four to eight experiments. For each experimental condition used and each time point, 60-80 junctions were examined in profiles of pericytic venules, collected from pericytic venule subfields of BMFs (see Fig. 1) or adjacent equivalent subfields (see Methods).

and the adjacent pericyte; the gradient is centered on the abluminal end of the intercellular space. At the time when the tracer diffusing through the intercellular junction and space had already reached the subendothelial spaces, the plasmalemmal vesicles were marked only on the blood front of the endothelial cells. Hence, in these specimens the tracer must have reached the venular and perivenular interstitia exclusively through open junctions.

In the Hb experiments, the reaction product marked most of the adluminal part of the intercellular spaces but the abluminal part of the latter was free (Fig. 5), suggesting that the majority of junctions were not detectably penetrated by this tracer. Fig. 6 shows an endothelial junction with two points of close contact. The reaction product marks heavily the intercellular space past the first point and only lightly the same space past the second point, suggesting that this junction was permeated at a slow rate by the tracer.

In the ferritin experiments, the tracer did not penetrate the junctions even when the latter appeared to be open to a measurable gap. Control experiments carried out as indicated under Methods gave negative results.

The penetration of the junctions of the venular endothelium by H11P was documented and illustrated in a previous paper (26).

**Organization of the Intercellular Junctions of the Venular Endothelium**

In sectioned specimens, a certain percentage of junctions (25-30%) appears to be open to a gap that measures from 20 to 60 Å (25, 26). The rest of the venular junctions show membranes in close contact or display points of membrane fusion. The measurements mentioned have to be interpreted with caution for the following reasons: (a) contrast conditions and geometry of the junctions in sections make precise measurements difficult; (b) there is no insurance that membrane components do not protrude beyond the dense outer leaflet of the plasmalemma at the level of the junctions (in fact, interacting protruding molecules must exist along the areas of cell to cell contact); and (c) changes in spacing between membranes are known to occur during preparation procedures for electron microscopy. Accordingly, we decided to rely primarily on the dimensions of permeating probe molecules, rather than measurements made on micrographs, to assess the width of the gap to which the junctions are open.

Since it has been established that in the omentum the intercellular junctions of the venular endothelium have a characteristic organization detectable on freeze-cleaved specimens, we considered it of interest to investigate the organization of the corresponding junctions in the microvasculature of the diaphragm, using specimens selected for high frequency of postcapillary venules. In such preparations, endothelial junctions in postcapillary venules, capillaries, and muscular venules could be comparatively examined in the same tissue specimen. In the endothelium of the postcapillary venules, the intercellular junctions are
characterized by discontinuous and low profile junctional lines, which generally appear as separate elements provided with free ends. When parallel or nearly parallel to one another (Fig. 11), these elements are, on the average, ~200 nm apart. In contradistinction with the situation en-

**Figure 2** Pericytic venule in a mouse diaphragm, 30 s after an i.v. injection of HRP. This large field illustrates two junctions (j₁ and j₂) and the corresponding intercellular spaces marked by reaction product; j₁ is penetrated almost to its abluminal end, while j₂ is fully permeated, an associated gradient of reaction product (arrowhead) appearing in the interstitium between the endothelial cells (e) and a pericyte (p). At this time point, all plasmalemmal vesicles associated with the blood front (vb) and some vesicles apparently located in the cytoplasm (vi) are marked by reaction product. The vesicles along the tissue front (vt) are not labeled. **Inset:** Intercellular junction (j) fully marked by reaction product; the latter is also detectable in low concentration in the interstitium (arrowhead) between the endothelial cells (e) and a pericyte (p). ps, Perivascular space; l, lumen. × 60,000; Inset: × 78,000.
FIGURES 3 and 4  Pericytic venules in a mouse diaphragm, 45 s and 60 s after an i.v. injection of HRP, respectively.

**FIGURE 3**  Reaction product marks the lumen (l), the open junction, and the intercellular space (j) of the endothelium, and (in lower concentrations [arrow]) the space separating the endothelial cells (e) from a pericyte (p). At this time point, the vesicles along the blood front (vb) are marked by reaction product, whereas those associated with the tissue front (vt) are not labeled. ps, Perivenular space. × 87,000.

**FIGURE 4**  An open junction (j) of the venular endothelium (e) is marked along its entire length by reaction product. A concentration gradient of reaction product (arrow) centered on the abluminal exit of the intercellular space appears in the narrow interstitium between the endothelial cells and a pericyte process (p). Reaction product in low concentration is present in plasmalemmal vesicles open on the tissue front (vt) of the endothelium at the level of this gradient. c, Collagen fibrils; l, lumen; ps, perivenular space. × 80,000.

countered in the blood capillaries, they do not have the tendency of forming a network. These elements appear on the P faces as creases marked only in a few places by particles (Figs. 7 and 8), and on the E faces as shallow grooves free of particles. As in the omentum and mesentery (22), the endothelial junctions of the muscular venules are characterized by the presence of discontinuous rows of particles on the P face and by the occurrence of small communicating junctions adjacent to, or surrounded by, junctional ridges (Fig. 9).

As in other locations, the intercellular junctions of the capillary endothelium are characterized by a series of interconnected though generally parallel grooves (E face) or ridges (P face). The bottom of the grooves is occupied by rows of particles, occasionally fused in rods or ridges (Fig. 9).

The transition from the capillary to the venular type of junction seems to be effected abruptly without intermediary appearances. This is illus-
FIGURES 5 and 6 Pericytic venules in a mouse diaphragm 30 s and 5 min, respectively, after an i.v. injection of Hb.

**FIGURE 5** The reaction product marks the adluminal infundibulum (i) leading to the intercellular junction (j). The short abluminal segment of the intercellular space beyond the junction appears to be free of reaction product (arrowhead). Only vesicles on the blood front (vb) are labeled by reaction product; vesicles associated with tissue front (vf) as well as those apparently free in the cytoplasm (vi) are not labeled. l, Lumen; p, pericyte; ps, perivenular space. x 130,000.

**FIGURE 6** The reaction product marks the open junction (j) and forms a discontinuous gradient along the entire intercellular space beyond the junction (arrow). e, Endothelium; l, lumen; v, vesicle; p, pericyte; ps, perivenular space. x 160,000.

Illustrated in Fig. 10 in which the exposed E face of one cell has grooves or negative creases devoid of particles (as is characteristic for venular junctions) whereas in the lower part of the figure the same E face of the same cell membrane has grooves marked by rows of particles (as usually found in
the capillary type of junctions). The present sampling is not large enough to allow us to conclude that the transition is always as abrupt as indicated in Fig. 10, but in all cases so far recorded, this appears to be the case.

Since similar findings were made on a large number of specimens, we can conclude that the endothelial junctions in the microvasculature of
FIGURE 9 Mouse diaphragm: endothelial junction in a venular segment of a capillary. The outer membrane leaflet fractured along a junctional ridge (arrowheads) which on the P face (P) is marked by a few particles single or in short rows (r). The junctional network is better seen on the E face (E), where it appears as a system of predominantly parallel but interconnected grooves marked by discontinuous rows of spherical or rodlike particles (pt). This network has few free spurs (*). v, Vesicle opening. × 105,000.

FIGURE 10 Mouse diaphragm: endothelial junction from the transitional field (T) in Fig. 1. On the E face (E) of the plasmalemma of the same endothelial cell, the organization of the junction is of venular type in the upper two-thirds of the field (discontinuous grooves, sg, most of which appear free of particles) whereas in the lower third some of the junctional elements are of the type seen in capillaries (shallow grooves, g, marked by rows of particles). P, P face; v, vesicle opening. × 90,000.
the diaphragm have the same type of segmental differentiation as already described in the omen-
tum and mesentery (22). The exclusive use of specimens collected from well-identified BMFs or
from fields with high concentration of postcapillary venules makes the identification of each vessel
and of the type of junction encountered reasonably certain. Besides confirming the existence of
segmental junctional differentiations in the micro-
vasculature of the diaphragm, the new findings
demonstrate the abrupt character of the transition from capillary to venular junctions which can be
detected on the same cell (Fig. 10).

Response of the Intercellular Junctions
of the Venular Endothelium to
Histamine and 5-HT

The existence of BMFs in the diaphragm and
the high concentration of postcapillary venules
along the myotendinous junction of the phrenic
center gave us the possibility of examining the
response to histamine and 5-HT of vessels already
identified as postcapillary (pericytic) venules. We
have shown above that the junctions of the endo-
thelium of these vessels are normally permeated
by probe molecules up to 50-60 Å and have a
typical venular morphology in freeze-cleaved
preparations.

As indicated under Methods, the vasoactive
amines were applied topically (on the peritoneal
aspect of the diaphragm) or administered system-
atically (intravenous injection). Carbon black in-
jected into the circulation either before or at the
same time as the vasoactive amine was used as a
detector of histamine and serotonin effects, as
done by Majno and Palade (13) and Majno et al.
(14, 15). Intramural deposits of carbon particles
were found to be clearly limited to vessels of 12-
25 μm diam which, on account of their location,
can be easily recognized as being postcapillary
(pericytic) venules (Figs. 11–13). Smaller and less
regular deposits were found on larger muscular
venules and seemed to mark preferentially their
confluence with the larger veins which form the
phrenic arcades (Fig. 13). Similar deposits were
not encountered in arterioles or capillaries (Fig.
12).

In whole mount preparations of the injected
diaphragms, it can be easily seen that, with the
exception of BMFs, postcapillary venules marked
by intramural deposits occur throughout the mus-
cle (Fig. 11). This distribution pattern indicates
except in areas in which bipolar vascular fields
have been identified beforehand (Figs. 12 and
13).

At the electron microscope level the affected
venules showed characteristic "lesions" of the type
already described in other vascular beds (1, 4–6,
13, 15, 31). These modifications consisted of focal
separations of venular endothelial cells to a gap
ranging from 0.1 to 3 μm through which the
plasma loaded with carbon particles (used as
detector) gained access to the subendothelial
spaces to form intramural deposits (Fig. 14). The
latter had the tendency of dissecting the layers of
the vascular wall while being retained by the most
peripheral basement membrane and its associated
adventitial elements.

When used as a detector, ferritin molecules
permeated the basement membrane and reached
the perivenular spaces where they could be de-
tected around the profiles of collagen fibers (Fig.
15). Intramural deposits were of minimal size
presumably because the ferritin, unlike carbon,
was not retained by the denuded basement mem-
branes. The very dense material that fills the gap
in Fig. 15 probably represents concentrated
plasma proteins heavily stained as a result of
mordanting with galloylglucoses.

The results described above were obtained with
histamine, the vasoactive amine used in most of
our experiments. 5-HT was employed in a more
limited number of cases, with similar results.

In control experiments isotonic saline solution
(0.154 M NaCl) applied topically or injected
systemically did not induce any detectable changes
at the level of the venular endothelium in the
diaphragm.

DISCUSSION

The endothelium of postcapillary (pericytic) ven-
ules appears to be characterized by: (a) partially
open intercellular junctions, (b) special intrame-
membranous organization of the junctions, and (c)
specific sensitivity to vasoactive amines such as
histamine and 5-HT. In the past, such properties
have been demonstrated individually in the venu-
lar endothelium of a variety of vascular beds: e.g.
omentum and mesentery, for intramembranous
organization of the junctions (22); mesentery
(19), cremaster (13), and cheek pouch (6, 29,
30), for sensitivity to histamine and 5-HT; and
mouse diaphragm, for the existence of open junc-
tions (25, 26). In this study, we have taken
advantage of a recent procedure for precise sam-
pling of the microvasculature of the mouse dia-
Figure 11. Mouse diaphragm 30 min after an i.v. injection of carbon black followed by topical application of histamine. Large field in the postero-lateral quadrant of the diaphragm (muscular portion). Discontinuous intramural deposits of carbon mark pericytic (postcapillary) venules of ~15–30 μm outer diam (arrowheads). Note that venules of this type appear randomly distributed throughout the entire field. The same situation applies to the whole muscle (from insertions to the phrenic center) with the exception of the few BMFs (**) which are supplied by arterioles from one end and are drained by venules at the opposite end. In this region of the diaphragm, the BMFs are limited to the area indicated by brackets. At this magnification the arterioles are not visible. The pericytic venules (Vp) can be recognized on account of their intramural deposits of carbon black. VA, venous arcade; mp, musculo-phrenic vessels. Note that the muscular venule marked Vml does not have pericytic venular affluents along the BMF side (**). pa, Phrenic artery. The inset shows a whole diaphragm excised from an animal treated as for Fig. 12. The boxed area corresponds to a field similar to that shown in Fig. 12. pc, Phrenic center. × 120; inset: × 1.5.
Mouse diaphragm 30 min after an i.v. injection of carbon black and topical application of histamine. Bipolar microvascular field shown at a higher magnification. The arteriole can be detected with some difficulty in the upper part of the field (arrows). The capillaries (C) are hardly visible in this type of preparation, whereas the postcapillary venules (Vp) are clearly seen on account of their intramural deposits of carbon black. In this case, some of these venules form a small arcade (a). x 600.

The junctional system of the venular endothelium appears to be uniform in organization when examined in the cleavage plane of the correspond-

phragm (25), and have succeeded in localizing all three properties in the endothelium of the pericytic venules of this muscle.
Mouse diaphragm 30 min after an i.v. injection of carbon black and topical application of histamine. Detail of a series of postcapillary venules (Vₚ) collected by muscular venules (Vₘ) draining into the phrenic vein (PV) along the myotendinous junction of the phrenic center. The postcapillary venules are clearly marked by intramural deposits of carbon black, and most of them show a characteristic orientation perpendicular to the predominant direction of the capillaries and muscle fibers. Note the intramural deposits that mark the points of confluence (arrowheads) of some muscular venules with the venous arcade. × 300.

The structural details revealed by this procedure suggest frequent and relatively extensive discontinuities along the junctions. Yet, in sectioned specimens and especially in tracer experiments, only a fraction (~30%) of the venular junctions are found open; the rest (~70%) behave like tight junctions. It follows that the special organization of the venular junction is a necessary but not sufficient condition for the existence of open junctions. This inference points out again the difficulty in extrapolating from intramembranous structural features to conditions prevailing at the surface of the plasma-lemma at the level of intercellular contacts. This situation is further illustrated by the findings at the level of muscular venules where the intramembranous organization of the junctions is similar to that found in the endothelium of pericytic venules; yet the frequency of open junctions detected in section or by tracers is much lower.

The separations of the endothelial cells induced by histamine and 5-HT are discontinuous, like the opening along the junctions, but at present it is not possible to ascertain whether the histamine and 5-HT response is limited to intercellular junctions already open or can occur at any other location in the junctional system of the venular endothelium.

The findings reported in this paper extend and strengthen data already published on the importance of the precise sampling in the localization of various structural features involved in vascular permeability and especially in the localization of open junctions. The fact that the latter were
Figure 14 Mouse diaphragm, 45 min after an i.v. injection of carbon black followed by histamine. In this field, the intercellular junction appears open at three different sites (j) to gap ranging from ~50 to 250 nm. Carbon particles were accumulated in an intramural deposit (*) developed between the endothelium (e) and its basement membrane (bm). pl, Platelet. × 65,000.
Mouse diaphragm, 5 min after the i.v. administration of ferritin immediately followed by histamine. A wide separation appears between endothelial cells (e) in between the arrows. It is assumed that it results from the focal opening of a junction (which is still visible at j). The basement membrane (bm) has retained in part the plasma proteins which appear heavily concentrated between it, the endothelium and the platelet, pl. The tracer (arrowhead) is visible in the lumen (l), along the basement membrane (bm), and in the perivenular spaces (ps) where it is preferentially associated with collagen fibrils (c). p, Pericyte; rb, red blood cell. × 45,000.
encountered exclusively in pericytic (postcapillary) venules in our experiments suggests that the cases of open junctions reported in the past in the capillary endothelium were due to misidentification of the vessel examined (8, 9, 32). Mistakes in identification are difficult to avoid when the observations rely exclusively on a random sampling. For instance, with the exception of BMFs, postcapillary venules are encountered throughout the muscular portion of the diaphragm, as illustrated in Fig. 12. Hence, there is no guarantee that samples collected from the middle portion of this muscle contain exclusively blood capillaries, as recently claimed by Wissig and Williams (32).

Open junctions of the venular endothelium are the equivalent of slit pores restricted to a special region of the microvasculature. Hence, the question arises as to how these slits fit into the pore theory of capillary permeability. Our observations indicate that the open junctions are quite heterogeneous in effective width since the frequency of patent junctions decreases with increasing size of probe molecules. Under normal conditions, they do not appear to represent the large pore system, or a variant of the latter, because they are not permeated by ferritin. They do not seem to represent the small pore system either, primarily because of their restricted distribution which can be put in proper perspective by the following consideration. A BMF of the diaphragm supplied by a single arteriole has usually ~80 capillaries, collected by ~8 pericytic (postcapillary) venules draining into approx. two muscular venules. In the entire microvascular bed of such a BMF, the surface of the capillary endothelium is estimated at 0.75 mm², whereas the venular endothelium does not exceed 0.03 mm². Within this small endothelial territory, only a small fraction of the junction is open. Moreover, as already mentioned (26), diffusion of macromolecular tracers through the perivascular spaces appears to be limited to the immediate vicinity of the postcapillary venules. At present the most plausible assumption is that the open junctions of the venular endothelium constitute an additional pathway for molecules up to 50–60 Å diameter, distinct from the two pore systems postulated by the pore theory of capillary permeability (2, 11, 18). This pathway was not detected as a distinct pore in physiological investigations probably because it falls within the range of the small pore system. Earlier studies mentioned, however, the preferential escape of "slowly diffusible dyes" at the level of postcapillary venules (5, 12, 20, 27). Yet, in those cases, as well as in the Landis experiments on frog mesentery, the possibility of a concomitant, superimposed inflammatory reaction (due to the exposure to air of the organ under investigation) cannot be ruled out. In our present interpretation, the small pores are represented by the transendothelial channels which are distributed throughout the entire microvasculature, being more concentrated at the level of the venular segments of the capillaries. In a recent reference it is maintained that such channels are rare and difficult to identify (32), yet they have been detected in a reasonably large number of cases by several investigators (see reference 16, 21, and 33 for a more detailed discussion of this point).

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