THE ULTRASTRUCTURAL BASIS OF CAPILLARY PERMEABILITY STUDIED WITH PEROXIDASE AS A TRACER

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ABSTRACT

The transendothelial passage of horseradish peroxidase, injected intravenously into mice, was studied at the ultrastructural level in capillaries of cardiac and skeletal muscle. Peroxidase appeared to permeate endothelial intercellular clefts and cell junctions. Abnormal peroxidase-induced vascular leakage was excluded. Neutral lanthanum tracer gave similar results. The endothelial cell junctions were considered to be maculae occludentes, with gaps of about 40 A in width between the maculae, rather than zonulae occludentes. Some observations in favor of concurrent vesicular transport of peroxidase were also made. It is concluded that the endothelial cell junctions are most likely to be the morphological equivalent of the small pore system proposed by physiologists for the passage of small, lipid-insoluble molecules across the endothelium.

INTRODUCTION

Some 60 yr ago Starling (1, 2) formulated the hypothesis that the transfer of fluid and crystalloids across the capillary endothelium was a passive process, the rate and direction being dependent on a balance between hydrostatic and osmotic forces acting across the endothelial barrier. The endothelial barrier could be considered as a filter permeable to small, lipid-insoluble molecules, but impermeable to larger ones. Landis' (3–6) work lent experimental and quantitative support to this concept.

The studies of Pappenheimer (6–9) and his colleagues established that for small, lipid-insoluble molecules diffusion is the basis for the rapid exchange which occurs between plasma and tissue. They developed the pore theory of capillary permeability, and proposed that the passage of small lipid-insoluble molecules across the capillary wall takes place through uniform, water-filled channels occupying a minute proportion of the endothelial wall. Uniform cylindrical pores of effective pore radius 40–50 A, which occupy less than 0.1% of the capillary surface would theoretically fit the data, but other geometrical configurations are also possible. While these small pores restrict the penetration of large molecules, the observed (10, 11) slow passage of molecules larger than the small pores may be accounted for either by passage through a sparser but bigger set of pores, about 250 A in radius (10, 11) or by a vesicular transport system (11, 12).

To date ultrastructural observations of capillaries have not been entirely reconcilable with the physiological data (13–16, 21). In particular, an ultrastructural entity which might be related to the small pore system has not been positively identified, even though structures of the given dimensions can be resolved by electron microscopy. Starling (1, 2), Pappenheimer (6, 9), and especially Chambers and Zweifach (17, 18) suggested the intercellular clefts between adjacent endothelial cells as likely candidates for the site of passive

passage of fluid and small molecules across the endothelium. Electron microscopic studies using colloidal and other tracers have not brought forth convincing evidence for passage of substances through the clefts (13, 16, 21, 27, 68). Furthermore, it was claimed that the intercellular clefts were sealed by tight junctions (zonulae occludentes), and they were therefore believed to be impermeable (22–24, 67). Luft (14, 15) accepts the presence of zonulae occludentes but advances reasons for regarding them as permeable.

On the other hand there has been much suggestive evidence that the micropinocytotic vesicles are a mode of transport across the endothelium. This mechanism was originally proposed by Palade (25); ultrastructural observations (21, 26–31) made by using electron-opaque tracers such as ferritin have all lent some credence to this idea, in that vesicular labeling was observed at various intervals after intravenous injections of the tracers, followed by labeling of the basal lamina.[1] Except in rare instances (16, 21), no labeling of the intercellular junctions occurred. Vesicular transport as the basis for the rapid transcapillary exchange of small molecules and fluid has been questioned, on both morphological (21, 32, 33) and physiological (12) grounds, as being insufficient in magnitude. The selective nature of capillary permeability is also hard to explain on the basis of vesicular transport: this property has been ascribed to the basal lamina (29). However, it has been suggested that vesicular transport may be responsible for the transport of large molecules (11, 12) and may represent the large pore system postulated by Grotte (10) and Mayerson et al. (11).

We have recently (34, 35) developed a new ultrastructural cytochemical tracer technique which utilizes horseradish peroxidase, a protein of relatively low molecular weight (40,000) as the tracer. Sensitivity is gained because enzymatic activity has an amplifying effect; thus the presence of the enzyme at a particular site can be detected by allowing the enzyme to act on a suitable substrate, to yield an electron-opaque reaction product in sufficient amount to be easily visualized.

The purpose of this paper is to describe the results obtained in applying this technique to the problem of localizing the sites of transcapillary passage of substances at the ultrastructural level. Evidence will be presented that the intercellular clefts, and perhaps the vesicular system, are pathways for the passage of substances across the endothelium, and it will be proposed that the former contain the ultrastructural equivalent of the "small pore system" of the physiologists (6, 9).

[1] The term basal lamina is used in this paper in preference to basement membrane.

MATERIALS AND METHODS

General

Unanesthetized white mice weighing 25–30 g were injected intravenously *via* the tail veins with horseradish peroxidase, in the dosages given below. Control mice were injected with isotonic saline only. At sequential time intervals thereafter, ranging from 1 to 60 min, the animals were sacrificed by stunning and/or neck dislocation. Cardiac and skeletal muscle (diaphragm, gastrocnemius) were fixed for 3–5 hr at room temperature in a formaldehyde–glutaraldehyde fixative (36) (see below). The fixative was injected into the pleural and abdominal cavities for fixation of the diaphragm *in situ*. The tissue blocks were then washed overnight in 0.1 M cacodylate buffer, pH 7.4, at 4°C, and then were washed briefly in cold, distilled water. They were then frozen on the stage of a Leitz freezing microtome equipped with a thermoelectric cooling device, and sections 40 μ in thickness were cut. In some experiments unfrozen sections were cut on a Smith-Farquhar tissue chopper (37). The sections were then incubated in the medium, usually for 15 min. In some experiments the incubation time was varied between 5 and 60 min. After incubation the sections were thoroughly washed in four changes of distilled water over a period of about 10 min. They were then postfixed for 1 hr in 1.3% osmium tetroxide buffered to pH 7.4 with *s*-collidine (38), were dehydrated in ethyl alcohol, were treated with propylene oxide (39) and were embedded in Epon 812 (39) or Araldite (Durcupan, Fluka AG, Switzerland). In some experiments, after the osmium-tetroxide postfixation, but before dehydration and embedding, the sections were treated with buffered uranyl acetate (40, 41) as described below. Thin sections were counterstained in alkaline solutions of lead (43, 44) and were examined either in an RCA-3G electron microscope equipped with a 50 μ objective aperture, at accelerating voltage of 50 kv, or in an AEI EM6B electron microscope equipped with a 40 μ objective aperture at 60 or 80 kv accelerating voltages. Initial magnifications were from 1500 to 100,000, and the photographic enlargement was 2-15X. Calibration was with a replica grating, 2,160 lines per millimeter.

Formaldehyde–Glutaraldehyde Fixation

This fixative was prepared as previously described (35, 36). In most experiments, however, the con-

centrated solution was diluted 1:1 with 0.1 M cacodylate buffer, pH 7.4. The final concentrations then were as follows: formaldehyde, 2%; glutaraldehyde, 2.5%; $CaCl_2$, 0.025%; cacodylate buffer, 0.09 M. Glutaraldehyde fixation was also used. Preliminary work, however, showed that preservation of structure was better with the combination aldehyde fixative although localization of the peroxidase was the same with both fixations. Aspects of the effects of different fixatives on the localization of peroxidase have previously been discussed (35).

INCUBATION MEDIUM: 5 mg of 3,3′-diaminobenzidine tetrahydrochloride[2] (Sigma Chemical Co., St. Louis, Mo.) was dissolved in 10 ml 0.05 M Tris buffer, pH 7.6. 0.1 ml of 1% H_2O_2 (freshly prepared from 30% H_2O_2) was added to the medium to give a final concentration of 0.01% H_2O_2. The medium was stable for at least 1 hr.

Peroxidase Preparations

Sigma type II peroxidase (RZ value 1.0–1.5) was mostly used in these experiments, at a dosage level of 2–5 mg per 25–30 g mouse. The peroxidase was administered dissolved in 0.5 ml isotonic saline. In some experiments either electrophoretically purified peroxidase (Worthington Corporation, Harrison, N.J.), RZ value 2.9–3.0, or Sigma type VI, RZ value 3.0, was used.

Controls

TO EXCLUDE ARTIFACTITIOUS ADSORPTION OF PEROXIDASE: (*a*) Fixed-frozen sections of tissue from uninjected normal mice were soaked in solutions of Tris-HCL buffer, pH 7.4, containing peroxidase at concentrations of 0.1–0.3 mg per ml. The sections were then transferred to the complete medium, incubated for 15 min, postfixed, and embedded as above.

(*b*) Blocks of tissue from uninjected mice were fixed in fixative containing peroxidase at a concentration of 0.01 mg per ml. Peroxidase was also present in the overnight wash in buffer. Frozen sections were cut and incubated in the complete medium and were processed for electron microscopy as above.

CONTROLS TO EXCLUDE ARTIFACTITIOUS ADSORPTION OF 3,3′-DIAMINOBENZIDINE OR ITS OXIDATION PRODUCT: Fixed-frozen sections from tissues of uninjected mice were incubated in 5 mg of diaminobenzidine in 10 ml of Tris-HCL buffer with and without subsequent washing in buffer. The sections were then incubated in Tris-HCL buffer containing peroxidase plus hydrogen peroxide, or in potassium ferricyanide, which readily oxidizes diaminobenzidine.

CONTROLS FOR DIFFUSION: (*a*) To control diffusion of reaction product, fixed-frozen sections of tissues from peroxidase-injected mice were processed for cytochemistry as given above, but in this instance were incubated in the medium for 1 hr, and then processed as usual for electron microscopy.

(*b*) To control diffusion of hemoglobin which has peroxidase-like activity and diffusion of which might give false localizations, fixed-frozen sections from tissues of normal control mice or from mice injected with saline only, were incubated in the cytochemical medium for 15–60 min, and then were processed for electron microscopy.

SUBSTRATE CONTROLS: Fixed-frozen sections of tissue from mice injected with peroxidase were incubated in media which omitted either the hydrogen peroxide, or the diaminobenzidine, from the otherwise complete incubation media.

CONTROL INJECTED MICE: Mice were injected with 0.5 ml of isotonic saline alone, and tissues were fixed and processed either for cytochemical reactions as above or for uranyl staining as given below.

CONTROLS FOR ASSESSMENT OF HISTAMINE- AND SEROTONIN-INDUCED VASCULAR LEAKAGE:[3] (*a*) For electron microscopy, mice were injected intravenously with 5 mg of horseradish peroxidase in 0.5 ml saline, and at time intervals of 10 sec and 10 min thereafter the mice were injected intravenously with 0.03 ml of filtered colloidal carbon (Pelikan). At time intervals ranging from 11 to 20 min after the initial injection of horseradish peroxidase the mice were sacrificed, and blocks of cardiac muscle were fixed and processed for electron microscopy. Leakage of carbon across the capillaries was sought as an indicator of induced vascular leakage (31, 42). In a similar experiment, 0.25 ml of 40% saccharated iron oxide was injected intravenously 1 min after the peroxidase, and the mice were sacrificed 10 min thereafter. Blocks of heart and skeletal muscle (gastrocnemius and diaphragm) were similarly fixed and processed for electron microscopy.

In other experiments mice were injected intravenously with 50 mg of cadmium-free ferritin[4] in 0.5 ml saline, and after 5 min 5 mg of horseradish peroxidase was similarly injected. The mice were sacrificed at intervals of 5–30 min after the peroxidase injection.

(*b*) For light microscopy, mice were injected in-

[2] 3,3′-diaminobenzidine (free base) may also be used (35).

[3] The author is greatly indebted to Dr. Ramzi S. Cotran for performing most of these controls. Further details, and additional observations on the rat, will be published elsewhere. (Cotran, R. S. and M. J. Karnovsky. Manuscript in preparation.)

[4] Kindly supplied by Lord Florey, and also obtained from Nutritional Biochemicals Corporation, Cleveland, O.

travenously with Evans blue, and were then injected intradermally with 0.05 ml of solutions of peroxidase at concentrations of 0.05–5 mg/ml. All preparations of peroxidase used were thus tested. The area of bluing was measured and compared to that produced in controls treated intradermally with Krebs-Ringer solution and with histamine, 5–50 μg.

In another experiment a male mouse was pretreated intravenously with carbon, and then injected intraperitoneally with solutions of peroxidase. Vascular labeling (31, 42) as an indicator of vascular leakage was sought in the cremasteric muscle and in the peritoneum.

Experiments with Lanthanum Tracer

Neutral lanthanum suspensions (45, 46) were also used as tracers. The technique is published in detail elsewhere (47). Blocks of myocardium were fixed in formaldehyde–glutaraldehyde, washed in buffer, and postfixed, at room temperature for 2 hr, in OsO_4–collidine containing a 1–2% suspension of neutralized lanthanum. The neutralized lanthanum was prepared by dissolving lanthanum nitrate in water and adding NaOH slowly until the solution was faintly opalescent (pH 7.6–7.8). This solution was then added to OsO_4–collidine. The final pH was about 7.0, and the final solution was also opalescent. After fixation the blocks were dehydrated and embedded as usual.

Studies on Cell Junctions

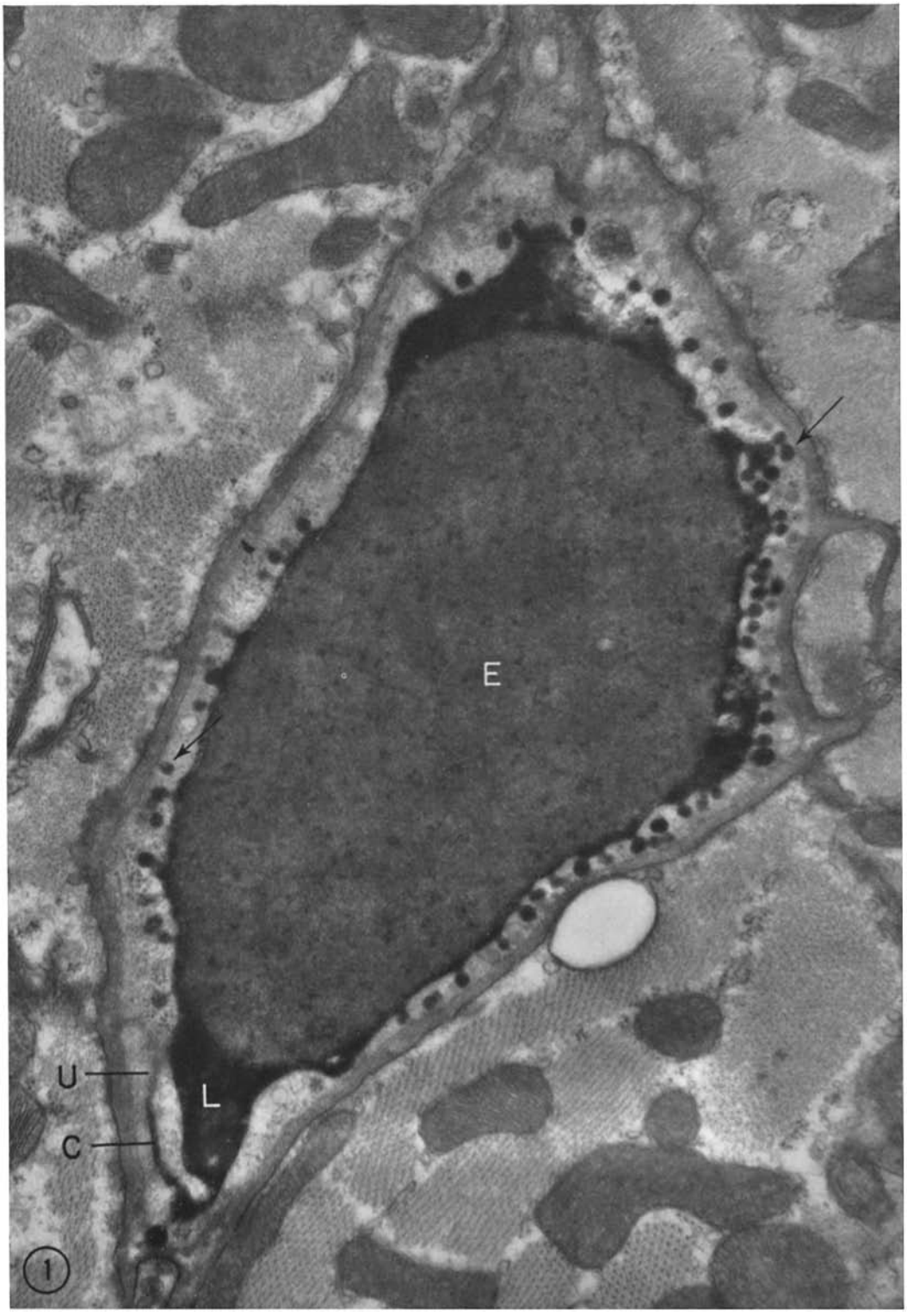
The question arose during this study as to whether the intercellular cleft between adjacent endothelial cells was sealed by a tight junction (zonula occludens) as had been proposed by others (22, 23). To investigate this point it was considered essential to resolve the unit membranes of the cell junctions as clearly as possible. In preliminary experiments comparison of fixations of myocardial capillaries was made between $KMnO_4$ (buffered and nonbuffered), OsO_4 buffered with phosphate, cacodylate or *s*-collidine, and prefixation in glutaraldehyde or formaldehyde–glutaraldehyde followed by OsO_4–collidine. OsO_4- and aldehyde-OsO_4-fixed materials were stained (*a*) by $KMnO_4$ *en bloc* (48); (*b*) by $KMnO_4$ on the grids (49, 50); (*c*) by alkaline lead (43, 44) with and without prestaining in uranyl acetate (23); and (*d*) *en bloc* by uranyl acetate, (40, 41), slightly modified, followed by alkaline lead-staining of the grids.

The most satisfactory results in terms of reproducibility, clarity, and lack of granularity were obtained with uranyl staining *en bloc*, which was adopted routinely. The method was modified from that of Farquhar and Palade (40) as follows: myocardium, diaphragm, and gastrocnemius from normal untreated mice, mice injected with saline, mice injected with peroxidase, and a normal, untreated cat were fixed in osmium tetroxide buffered with *s*-collidine (38) or phosphate (51), with 5–7.5% sucrose added (52), or in formaldehyde–glutaraldehyde (36), diluted 1:1 with 0.1 M cacocylate buffer. The aldehyde-fixed blocks were washed overnight in cold cacocylate buffer, and were postfixed in OsO_4–collidine. After the OsO_4 fixations the blocks were washed thoroughly in cold sodium hydrogen maleate–NaOH buffer, 0.05 M, pH 5.2 (53), and were then placed in cold 0.5 or 2.0% uranyl acetate dissolved in sodium hydrogen maleate–NaOH buffer, 0.05 M, pH 6.0. The final pH of the 0.5% uranyl solution was 5.2, and that of the 2% solution was 4.2. After 2 hr in the cold (4°C) and dark, the blocks were washed (three changes) in the maleate buffer, pH 5.2, and then were dehydrated and embedded as usual. The thin sections were stained with alkaline lead (43, 44). The maleate buffer buffers the uranyl solution better than Veronal-acetate buffer (40, 41) and may form a weak complex with the uranyl ions, mitigating against precipitation.

Perfusion Experiments

It was technically difficult to satisfactorily perfuse the mouse heart. However, rat hearts were perfused *via* the coronary arteries, by the method of Langendorff, with buffered formaldehyde (1%)–glutaraldehyde (1.25%) fixative, at room temperatures, at a head of 120 cm of water. After perfusion, blocks were

FIGURE 1 Capillary in heart of mouse sacrificed 5 min after peroxidase was injected. Black reaction product, at sites of peroxidase, is present in the lumen (*L*) around the erythrocyte (*E*), and in most of the micropinocytotic vesicles opening at, or close to, the luminal surface. A few such vesicles are unstained (upper right). A few vesicles within the cytoplasm and at or near the basal laminal surface are stained (arrows). Note narrow necks connecting the vesicles to the luminal surface. Most vesicles at the basal lamina, and the basal lamina itself, are unstained. The intercellular cleft (*C*) contains peroxidase for about one half of its length, from the luminal side; that portion of the cleft directed toward the basal lamina is unstained (*U*). × 35,000.


E
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fixed in buffered formaldehyde (2%)–glutaraldehyde (2.5%), and further processed as described above.

Measurements

Measurements on the width of the unit membranes and on the cell junctions were made on the photographic prints with a Leitz dissecting microscope, at a magnification of 10, and with an ocular micrometer graduated in 0.1 mm units.

RESULTS

General

It is not intended to present a detailed time-sequence study of the events occurring in the endothelium after the injection of horseradish peroxidase. To do this with any accuracy would be difficult because there was some variation not only from animal to animal but also from capillary to capillary in closely adjacent areas. Rather, the purpose here is to describe the events which occurred and the pathways whereby the peroxidase passed across the endothelium. The given time intervals after injection are those which were most commonly found to be related to the observations and are somewhat approximate.

Most of the observations in this paper were made on cardiac muscle capillaries, but were also confirmed on skeletal muscle capillaries. Because of the larger number of capillaries in the heart per unit area, it was naturally much easier to work with the heart. Furthermore, not all capillaries in skeletal muscle contained peroxidase. This was possibly due to the fact that a considerable proportion of skeletal muscle capillaries are closed under conditions of rest or mild exercise (54). In some cases efforts were made to increase the proportion of capillaries showing reaction by exercising mice on a treadmill, before or after injection

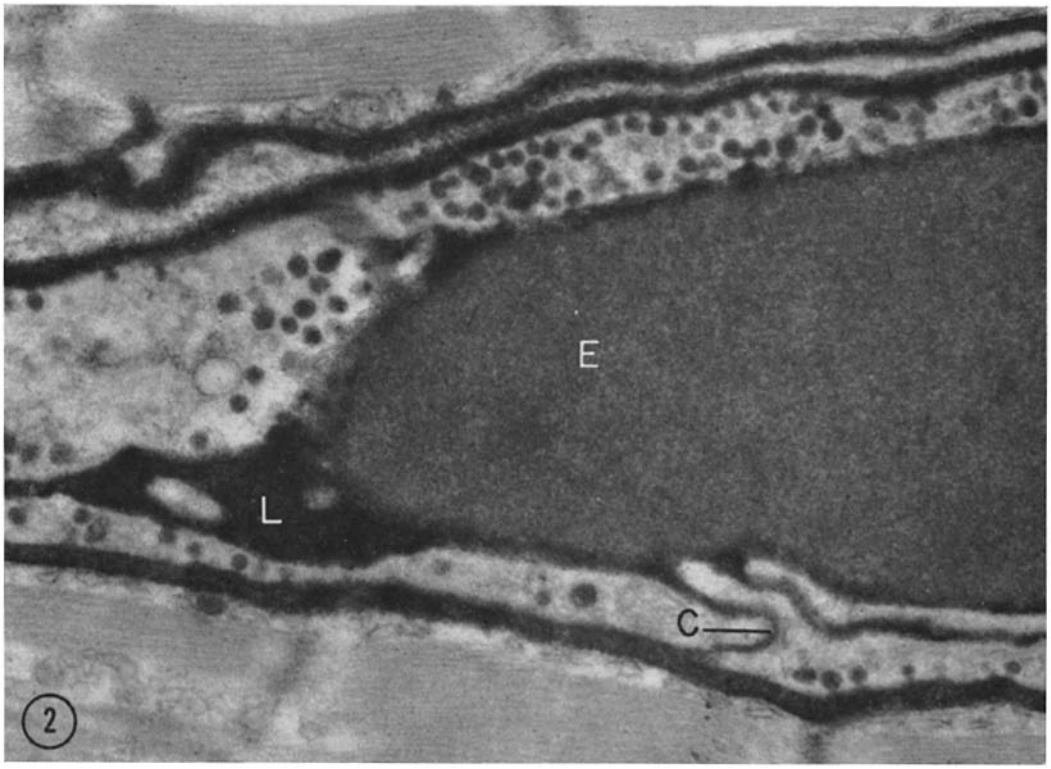


FIGURE 2 Capillary in heart of mouse sacrificed 10 min after injection of peroxidase. Peroxidase is present in the lumen (*L*) around erythrocyte (*E*). Micropinocytotic vesicles at both the luminal and basal surfaces, as well as those apparently lying free in the cytoplasm, are stained. The intercellular cleft (*C*) is stained throughout its length, from the lumen to the basal lamina. The basal lamina is also stained. × 32,000.

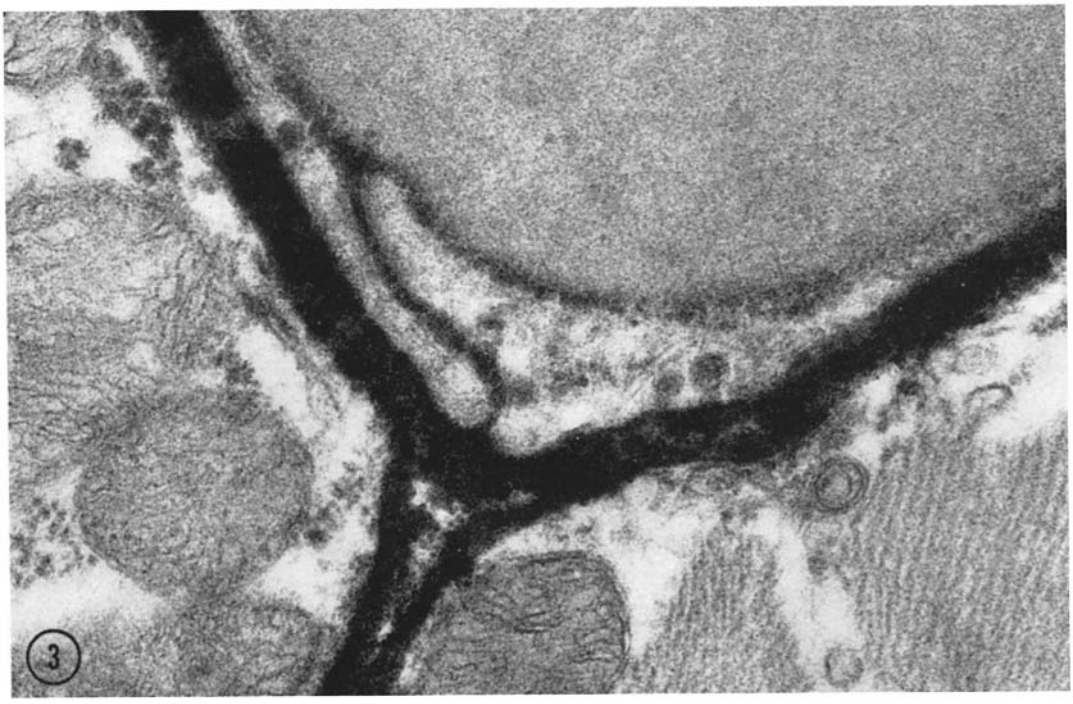


FIGURE 3 Capillary in heart of a mouse sacrificed 15 min after injection of peroxidase. Peroxidase is present in the lumen around the erythrocyte, throughout the course of the intercellular cleft, and in the basal lamina. Three vesicles opening to the basal lamina are also stained. × 60,000.

with peroxidase. This was thought to be moderately successful.

It should be mentioned here that all of the peroxidase preparations used gave similar results. In most of the experiments, Sigma Type II peroxidase was used.

In this paper the terms "peroxidase," "reaction product," and "staining" will be used interchangeably.

Peroxidase in Cardiac Capillaries

1–5 min after injection, capillaries in the heart showed marked staining in the lumen and in most of the vesicles opening to the luminal surface (Fig. 1). These vesicles appeared to have filled up with the peroxidase from the lumen. A few vesicles within the cytoplasm and at the basal surface were also stained (see also Fig. 4). A few vesicles at or near the luminal surface were unstained. Quite frequently there was staining in the intercellular cleft along a portion of its length. The staining extended from the luminal surface toward the basal lamina (Fig. 1). The basal lamina was not obviously stained at this stage.

From about 5 min onward after injection, there was staining in the lumen, in the luminal vesicles, and in addition, in vesicles throughout the cytoplasm of the endothelium cell including those apparently opening to the basal lamina (Fig. 2). The intercellular clefts now stained throughout their length (Figs. 2, 3). The basal laminae and the intercellular spaces were filled with peroxidase (Figs. 2, 3); and tubular profiles at or near the Z line in the cardiac muscle also stained: these were considered to represent the T system filling from the extracellular space (34).

The staining of the vesicles at both the luminal and tissue faces of the endothelium should not necessarily be taken to represent passage of the vesicles across the endothelium. If peroxidase had gained access to the basal lamina by some means other than by vesicular transport, for example, *via* intercellular clefts, or a vascular "leak" elsewhere, the vesicles opening to the basal lamina would have filled. Such filling of vesicles from the

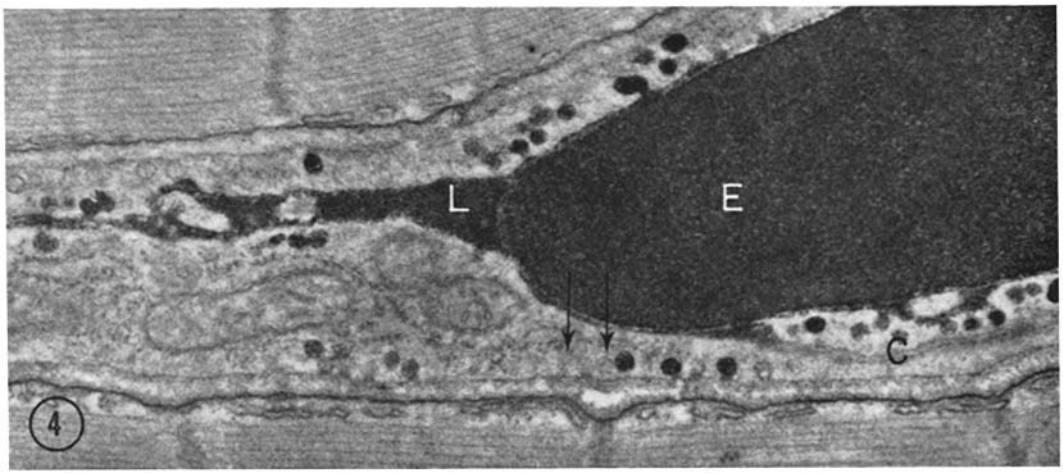


FIGURE 4 Capillary in heart of a mouse sacrificed 5 min after injection of peroxidase. Peroxidase is present in the lumen (*L*), and vesicles opening at, or close to, the luminal surface. The erythrocyte (*E*) also stains because of the peroxidatic activity of hemoglobin. Several vesicles opening at, or situated near, the basal laminal surface stain strongly whereas adjacent vesicles are essentially negative (arrows). The basal lamina shows little reaction, and the electron opacity of the sarcolemma may not represent staining for peroxidase (see text). The intercellular cleft (*C*) shows staining only at the luminal end. This type of image is taken as suggestive evidence for vesicular transport from lumen to extracellular space. × 32,000.

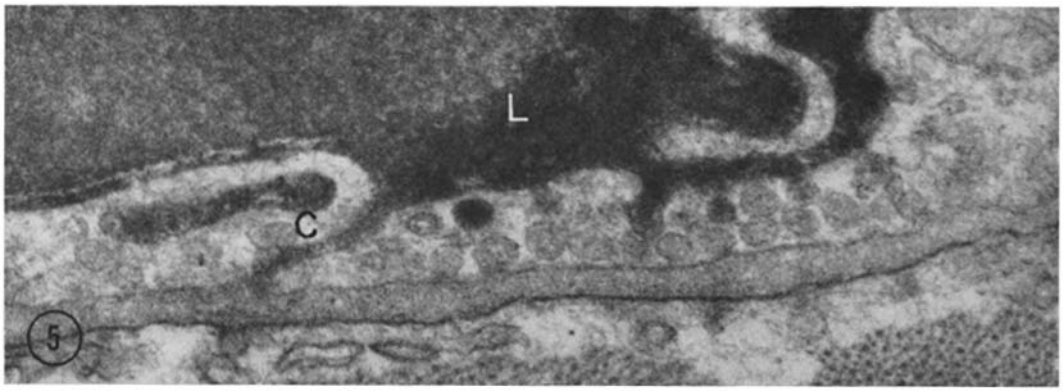


FIGURE 5 Capillary in heart of mouse sacrificed 8 min after peroxidase injection. Peroxidase is present in the lumen (*L*) and extends down the intercellular cleft (*C*). There is slightly increased density in the basal lamina near the opening of the intercellular cleft, but the rest of the basal lamina is essentially unstained. Vesicles opening at basal laminal surface are unstained. The sarcolemma appears stained (see text). × 60,000.

basal surface does occur with the lanthanum tracer, even after fixation, (Fig. 7),and also with ruthenium red (14, 15). Staining of vesicles lying apparently free in the cytoplasm may in actuality represent filling of these vesicles *via* necks which open to a surface at some other level of sectioning.

To obtain more concrete evidence for vesicular transport, instances were sought, in the early stages after peroxidase injection, where, in addition to vesicular staining on the luminal surface, some stained vesicles were present at the basal lamina, interposed amongst unstained vesicles, and where the basal lamina itself and the intercellular cleft were unstained. Figs. 1 and 4 illus-

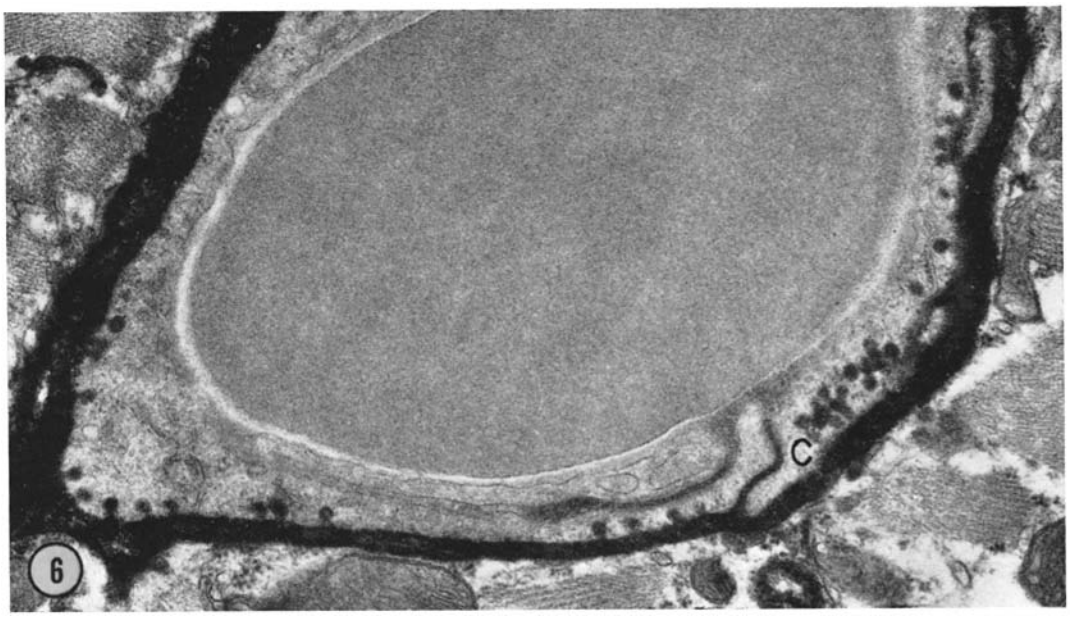


FIGURE 6 Capillary in heart of mouse sacrificed 30 min after injection of peroxidase. There are slight staining in the lumen and increasing intensity of staining down the intercellular cleft (*C*) toward the basal lamina, which is strongly stained. There are no stained vesicles at the luminal surface. Vesicles at or near the basal laminal surface are strongly stained. × 27,000.

trate such instances. This sort of evidence is taken to suggest, but not to prove, that the vesicles may indeed have crossed the endothelium. The electron density of the sarcolemma, observed in Figs. 1, 4, and 5, may or may not represent peroxidase: similar staining was found in control sections of heart taken from mice not injected with peroxidase, when these sections had been incubated in diaminobenzidine and H_2O_2.

There also were instances, in the early stages, in which the intercellular cleft stained throughout its length down to the basal lamina, but in which the basal lamina itself and the vesicles opening to the basal lamina were unstained (Fig. 5). This is taken as evidence that the peroxidase can pass down the intercellular cleft, and that the staining seen throughout the clefts at later stages is not necessarily due to reflux of peroxidase which has reached the basal lamina *via* vesicular transport. Even if such reflux were the case, the essential point is that the intercellular cleft appears to be permeable. Passage of the lanthanum tracer from the basal lamina toward the lumen *via* the intercellular cleft was shown to occur (Fig. 7) in blocks of fixed tissue. Here, hydrostatic forces in the lumen were absent.

At late stages after the injection of peroxidase (30–60 min), capillaries were seen (Fig. 6) in which the lumen showed little staining, whereas the basal lamina and the extracellular space showed marked staining. Vesicles at or near the basal surface of the endothelium stained strongly whereas those near the luminal surface stained slightly. The intercellular clefts showed increasing intensity of reaction from the lumen toward the basal lamina. This type of image suggests progressive passage of peroxidase, both by vesicles and *via* the intercellular clefts, from the lumen to the tissue space, but the obverse interpretation is also tenable. The absence of stained vesicles within the cytoplasm could possibly be explained by a phase of rapid transit of vesicles when they are at a distance from the cell surfaces (61). Once again the intercellular cleft appears permeable, but the interpretation of the vesicular staining as indicative of vesicular transport is subject to the same difficulties as are presented above.

Lanthanum Tracer in Cardiac Capillaries

Thick (1 μ) sections from blocks of cardiac muscle which had been placed in the OsO4–collidine–lanthanum solution before embedding

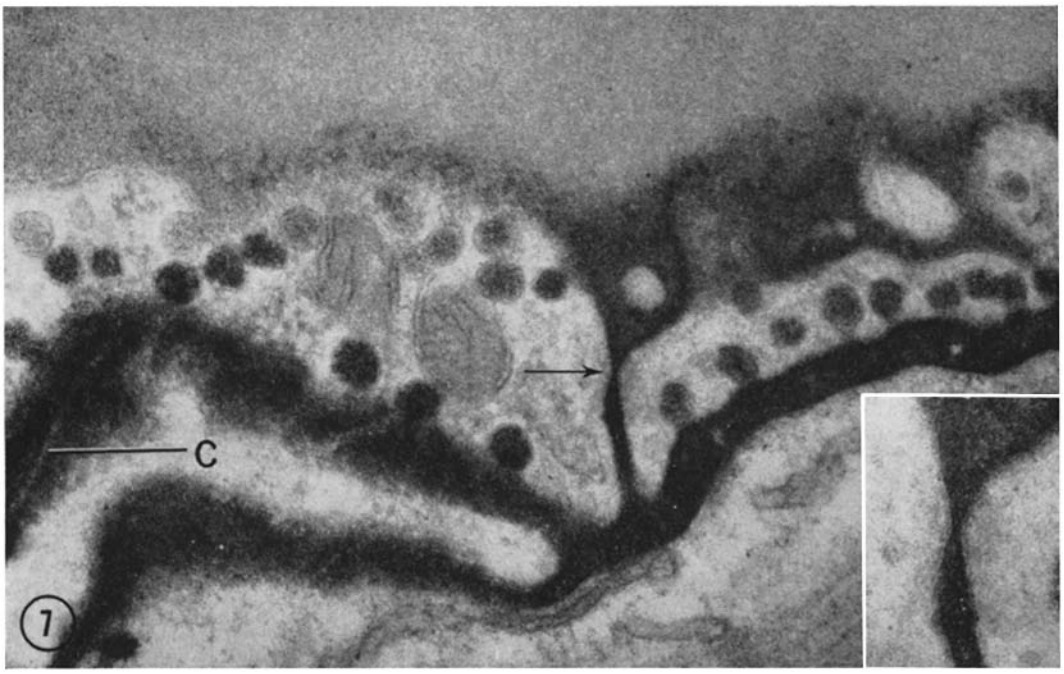


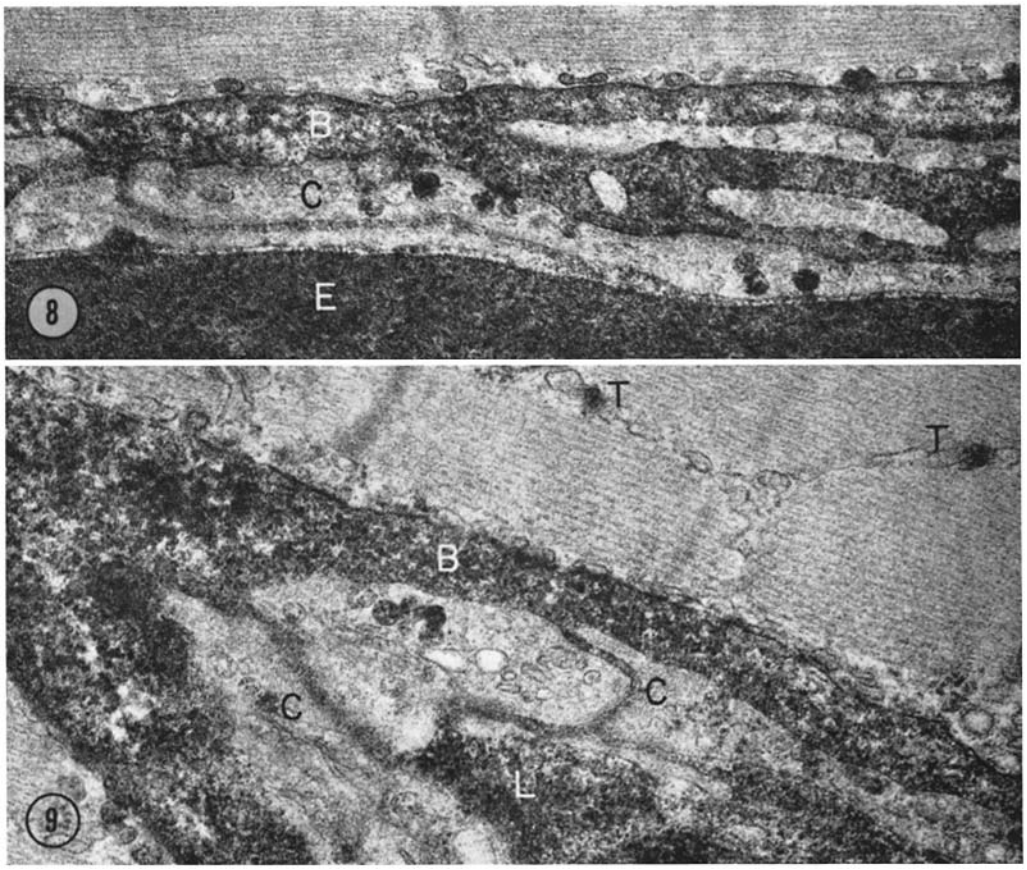
FIGURE 7 Capillary in mouse heart. Lanthanum-tracer technique applied to formaldehyde–glutaraldehyde-fixed blocks of tissue. The lanthanum has permeated the basal lamina and extracellular space. Collagen fibers are outlined by negative staining and appear as light lines (*C*). Vesicles opening to the basal lamina have filled with tracer. Note how some are situated apparently free in the cytoplasm, at a considerable distance from the basal surface. They probably connect to the basal surface in a different plane of section. The lanthanum has passed through the intercellular cleft into the lumen. The narrow waist in the lanthanum line (arrow, and inset) represents passage through the cell junction. This narrow portion is about 65 A in width (see text). With increasing distance from the opening of the intercellular cleft into the lumen, the intensity of staining in the lumen decreases, and vesicles opening to the lumen are less intensely stained than are those at the basal surface. × 87,000; inset × 180,000.

showed, by phase microscopy, permeation of lanthanum from the periphery of the blocks through the extracellular spaces. The centers of the blocks were usually unstained. These and other reasons have been presented (47) for regarding the lanthanum as a "tracer" rather than as a "stain."

By electron microscopy (Fig. 7), it was seen that an extremely electron-opaque material filled the extracellular spaces and the basal laminae and, by negative staining, delineated collagen fibers. Vesicles opening to the basal surface of the endothelium had filled up with tracer. The intercellular clefts also contained tracer which frequently filled the clefts throughout their lengths from the basal laminae to the lumina. The lumina of the capillaries contained tracer and stained less intensely at a distance from the luminal openings of the clefts. Along the course of the intercellular cleft there was frequently a narrow "waist" through which the lanthanum had apparently passed. This narrowing represents the cell junction (Fig. 7). At its narrowest point the lanthanum line filling the cell junction measured 60–70 A in width. The lanthanum, in addition to filling the extracellular space, occupied, at least in part, the external leaflets (reference 47; Revel, J. P., and M. J. Karnovsky. Manuscript in preparation) and they were therefore not distinguishable as such.

Skeletal Muscle with Peroxidase

Observations similar to those made in the heart were made in capillaries of gastrocnemius muscle and of the diaphragm. Peroxidase was present in the lumina, in vesicles, in intercellular clefts, in basal laminae, in extracellular spaces, and in the



FIGURES 8 and 9 Capillaries in diaphragm of mouse 30 min after injection of peroxidase. Erythrocyte (*E*) stains because of peroxidatic activity of hemoglobin. Peroxidase is present in lumen (*L*), throughout intercellular clefts (*C*), and in basal lamina and extracellular space (*B*). In Fig. 8, micropinocytotic vesicles at both endothelial surfaces stain, as well as subsarcolemmal vesicles. In Fig. 9, elements of the T system (*T*) contain peroxidase. Fig. 8, × 40,000; Fig. 9, × 27,000.

T system of the muscles (Figs. 8, 9). For technical reasons, such as fragility of the 40-μ sections and "cracking" of the reaction product in the lumen, presumably on cutting, which was associated with tearing of the adjacent tissues, these muscles did not yield so large a number of satisfactory images as did the heart. The time course of the transcapillary passage of peroxidase seemed to be slower in skeletal muscle than in the heart, that is, it took longer after the injection of peroxidase before strong reactions were obtained in the extracellular spaces. This does not necessarily imply differences in the rates of passage across the endothelia. There are many other possible factors which should be taken into account, such as differences in capillary population density per unit volume of tissue, relative proportions of capillaries which are open and perfused (a proportion of skeletal muscle capillaries may be closed) (54), differences in rates of lymph drainage, etc.

In their ultrastructure the cell junctions in the capillaries of skeletal muscle showed no differences from those in the cardiac capillaries (see below).

Controls for Abnormal Vascular Leakage Induced by Peroxidsae

It was important to exclude the possibility that peroxidase treatment had not caused abnormal widening of the cell junctions in the capillary

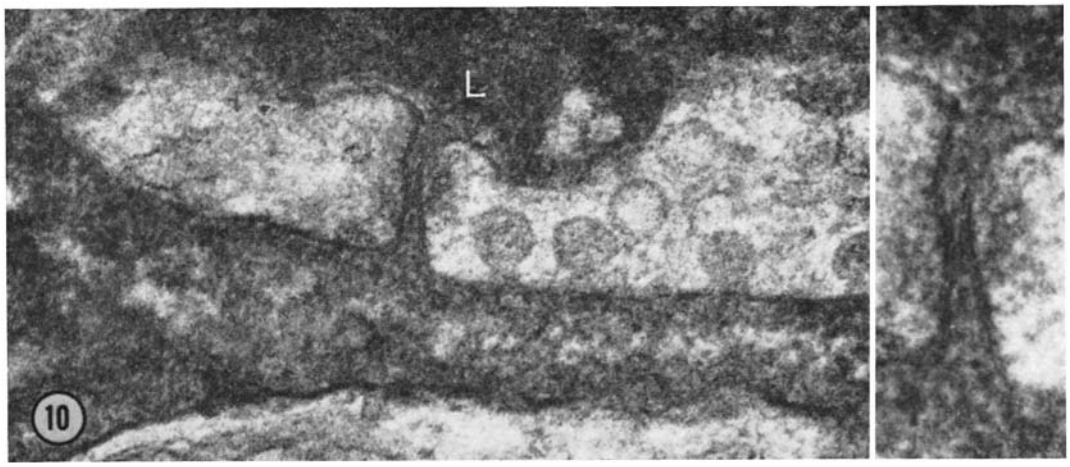


FIGURE 10 Capillary in heart of a mouse sacrificed 8 min after injection of peroxidase. Peroxidase-reacted sections were stained with uranyl acetate before dehydration. Peroxidase is present in the lumen (*L*), and extends through the length of the intercellular cleft into the basal lamina and extracellular space. In the inset, close approximation of the external leaflets of the adjacent plasma membrane is seen at the cell junction. At this level of resolution one cannot determine whether the peroxidase passes through this point of membrane apposition or around it. Vesicles abutting on the tissue face of the endothelium show a variable degree of staining. Those closer to the lumen are virtually negative. × 100,000; inset, × 190,000.

endothelium, thus allowing for excessive vascular leakage. Furthermore, the possibility of histamine and serotonin release induced by peroxidase, with consequent venular leakage (42) by way of widened intercellular clefts, had also to be considered.

In many of the routinely prepared peroxidase-stained intercellular clefts it was difficult to discern the cell junctions, probably because of tangential cuts, or because reaction product obscured details. In peroxidase-reacted material stained *en bloc* with uranyl acetate the trilaminar unit-membrane structure of the endothelial plasma membranes lining the clefts could be discerned (Fig. 10, and inset). At at least one point the external leaflets of the adjacent unit membranes were closely opposed, formed the so-called cell junction, and apparently obliterated the extracellular space. No obvious widening of the extracellular space and the cell junction was seen (Fig. 10, inset). Whether the peroxidase passes through or around the point of contact of external leaflets could not be resolved.

The fine structure of cell junctions in control and peroxidase-treated capillaries is depicted in Figs. 13–19 (controls) and in Figs. 11, 12, 20, and 21 (peroxidase-treated); as will be described below, no differences could be detected in the ultrastructure of the endothelial cell junctions of peroxidase-treated, saline-treated, and untreated mice.

Save for an occasional labeled venule in the peritoneum, gross, light microscopic and electron microscopic studies revealed no leakage of carbon particles, and no vascular labeling (42) of venules, or, for that matter, of capillaries, in peroxidase-treated mice. All the peroxidase preparations gave the same results. Saccharated iron oxide was not detected in the intercellular clefts in peroxidase-treated mice, except for occasional particles, which were also found in the control mice. Ferritin was noted in the micropinocytotic vesicles, but was never found in the endothelial cell junctions or in the intercellular clefts (Figs. 11, 12) of experimental and control mice. 30 min after it was injected ferritin was observed in the basal laminae of the capillaries (Fig. 12); presumably it arrived there by vesicular transport (26, 27). No differences in the amounts of vesicular or basal laminal labeling were detected between control and experimental mice. These observations are in accord with others (26, 27) on the passage of ferritin across the capillary.

At sites where peroxidase was injected locally (skin, peritoneum), intravenously administered Evans blue or carbon particles did not leak to a

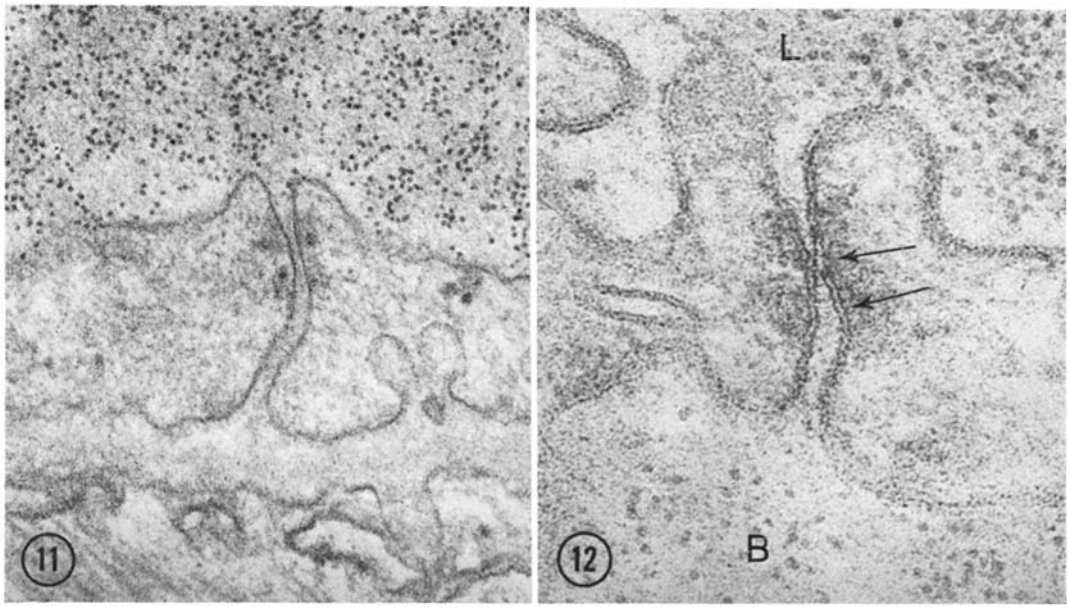


FIGURE 11 Capillary in heart of mouse injected with ferritin, 10 min, and with peroxidase, 5 min, before sacrifice. The lumen (above) shows a high concentration of ferritin, but there is none in the intercellular cleft. This preparation was not treated with uranyl *en bloc*, so that the external leaflets of the unit membranes are not seen and the cell junction appears open (see Fig. 12). × 110,000.

FIGURE 12 Capillary in heart of mouse injected with ferritin 30 min, and with peroxidase, 20 min, before sacrifice. Tissue stained with uranyl *en bloc*. There is ferritin in both the lumen (*L*) and basal lamina (*B*), but none in the intercellular cleft. Micropinocytotic vesicles were labeled (not shown). The cell junction shows touching or fusion of the external leaflets of the adjacent plasma membranes, with complete obliteration of the extracellular space. The width of (*a*) the unit membrane is ~65 A, and of (*b*) the cell junction is ~125 A. The ratio of (*b*):(*a*) is ~2.0. The arrows show the levels at which the measurements were made. The radial depth of the intermediate line is ~100 A. × 200,000.

greater extent than was seen in Krebs–Ringer-treated controls, whereas locally administered histamine readily induced leakage.

It was concluded that, in the mouse, horseradish peroxidase did not act as a significant releaser of histamine and serotonin, in the doses used in this study. It should be noted, however, that in the rat (Cotran, R. S., and M. J. Karnovsky. Manuscript in preparation.) and guinea pig (65) increased vascular permeability occurs. It has been shown that the enzyme preparations cause histamine and serotonin release in the rat (Cotran, R. S., and M. J. Karnovsky. Manuscript in preparation.), with consequent leakage of venules, but not of capillaries, as would be expected (42). These effects are readily overcome by pretreatment with antihistamine and antiserotonin agents.

Cytochemical Controls

None of the control experiments for specificity of the enzymatic reaction and for accuracy of localization gave rise to staining which could have been confused with the specific reaction and localization of the peroxidase, with one minor exception. This was an increase in electron-opacity of the cardiac sarcolemma of nonperoxidase-treated mice, (similar to that illustrated in Figs. 1, 4, and 5), sometimes found after incubation of sections in the cytochemical medium. The only other staining found was in the controls for artifactitious adsorption of ingredients of the medium, reaction product, or peroxidase. In some of these, clumps of reaction product were found at the surface of the tissues, with no particular localization. Prolonged incubation up to 1 hr

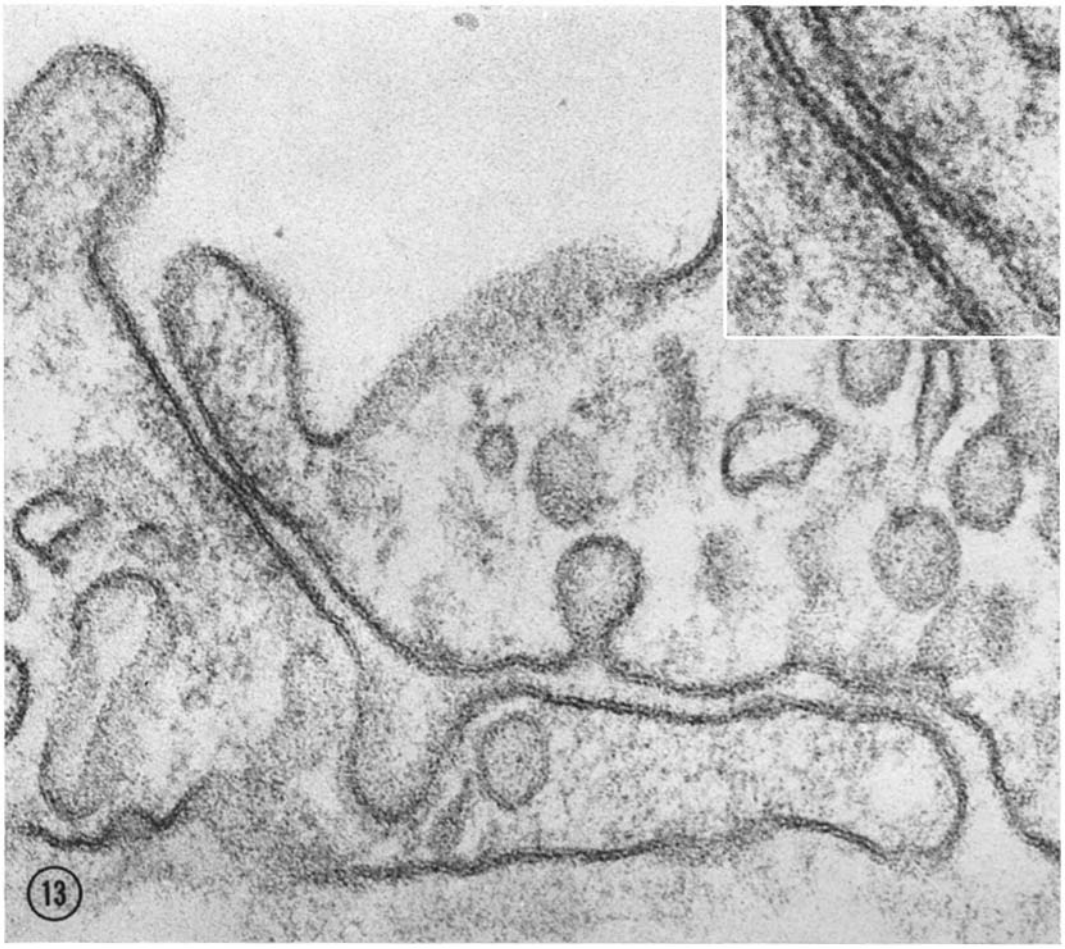


FIGURE 13 Cell junction in capillary of normal mouse heart. Lumen above. The cell junction has close approximation of the adjacent external leaflets, and the extracellular space appears to be obliterated. At higher magnification (inset) the external leaflets do not touch, and are distinctly separate. The gap between them is ~40 A in width, and contains electron-opaque material which tends to obscure detail Osmium-tetroxide fixation, uranyl staining *en bloc*. × 160,000; inset, × 350,000.

showed little diffusion of reaction product or of peroxidase, but on occasion slight staining, presumably of diffused hemoglobin, was found around red cells after 1 hr of incubation.

Ultrastructure of Endothelial Cell Junctions

In tissues treated by uranyl acetate staining *en bloc*, the individual leaflets of the endothelial plasma membranes were clearly defined. The outer leaflets were generally less electron opaque and somewhat thinner than the inner leaflets.

In capillaries of untreated, saline-injected, and peroxidase-injected mice, the width of the intercellular cleft, for most of its length, was from 100 to 200 A. At at least one point, generally close to the lumen, the adjacent plasma membranes were closely approximated, over a radial length of 100 to 400 A, to form a so-called cell junction (Figs. 13–22). More than one of these junctions was occasionally seen in one plane of section through the cleft (Figs. 19–21, 24, and 26).

At relatively low magnifications, touching or fusion of the adjacent external leaflets appeared to obliterate the extracellular space of a cell junction

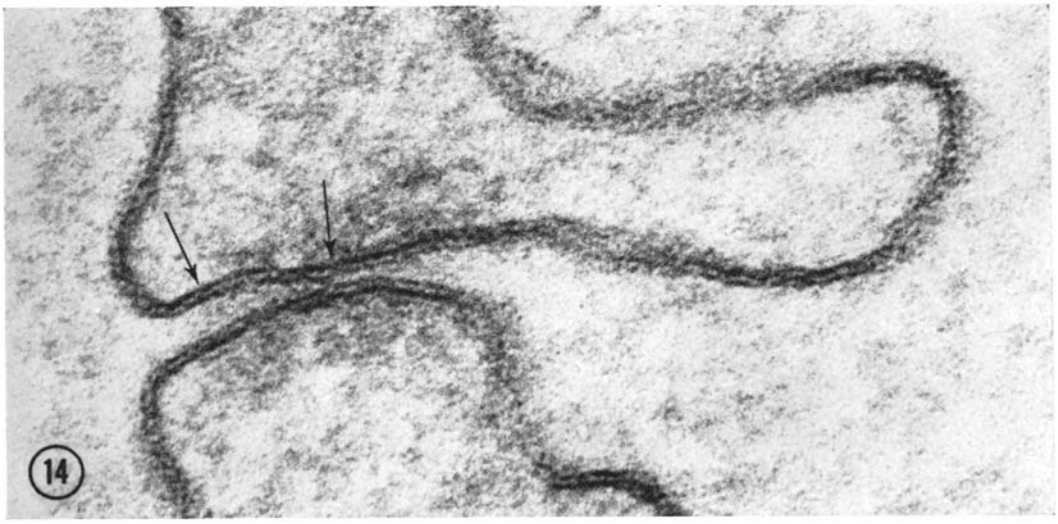


FIGURE 14 Cell junction in capillary of normal mouse heart. Lumen to the right. There is a gap between the adjacent external leaflets, which is 30–40 A in width. Electron-opaque material is present in the gap. The ratio, width of cell junction: width of unit membrane, is about 2.5, giving a calculated gap of ~35 A. Arrows as in Fig. 12. Osmium-tetroxide fixation, uranyl staining *en bloc*. × 300,000.

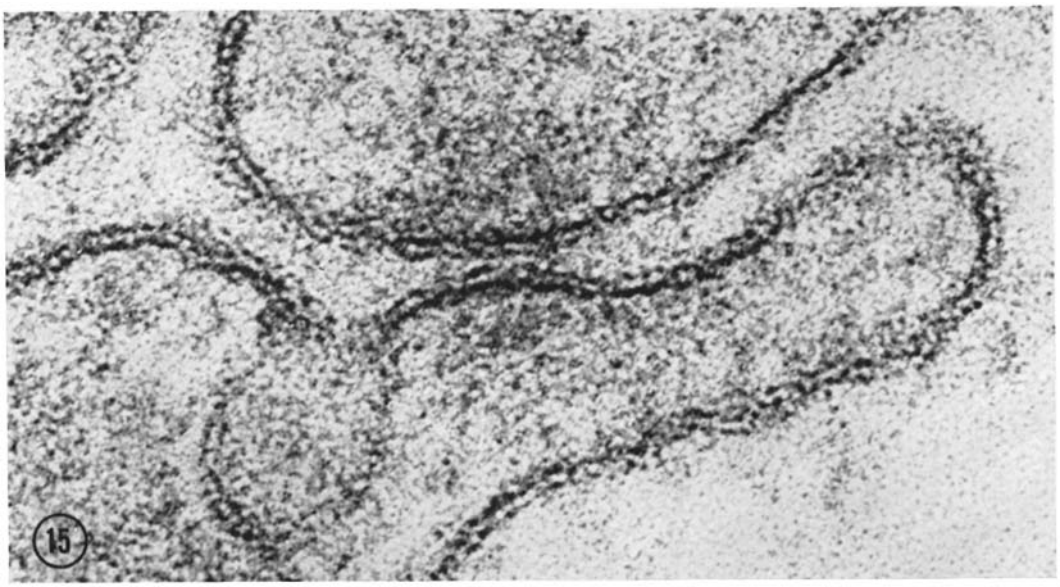
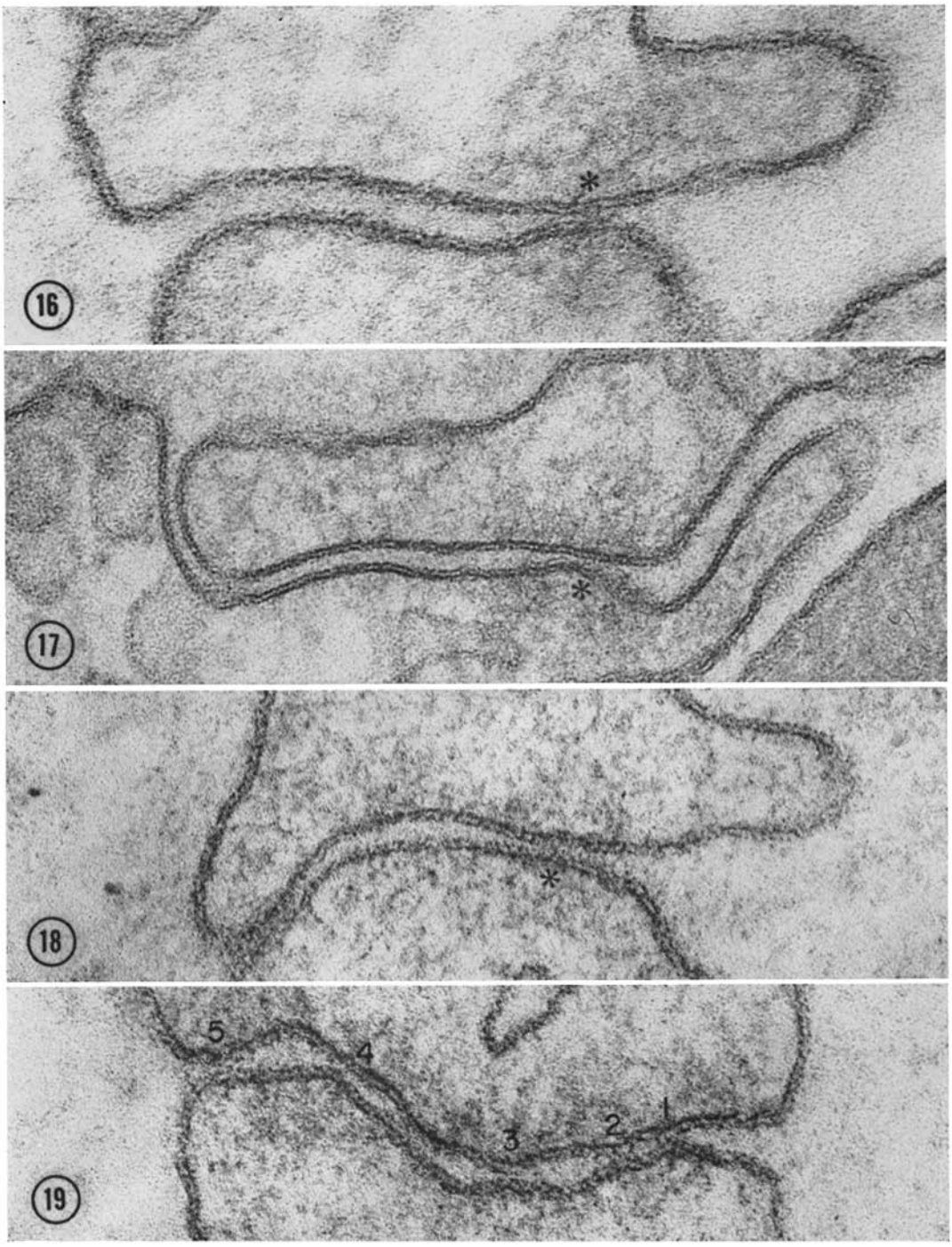


FIGURE 15 Cell junction in capillary of normal mouse heart. Lumen to the right. There is a definite gap of ~40 A between the adjacent plasma membranes. Osmium tetroxide fixation, uranyl staining *en bloc*. × 420,000.

(Fig. 13). At higher magnifications, a small gap was frequently seen between the external leaflets (Figs. 13, inset; 14–18). This gap was about 40 A in width, and frequently contained a somewhat electron-opaque material (Figs. 13, inset; 16, 18) which sometimes made measurement difficult. In rarer instances the intercellular space appeared to be truly obliterated (Figs. 22, 24, and similar to

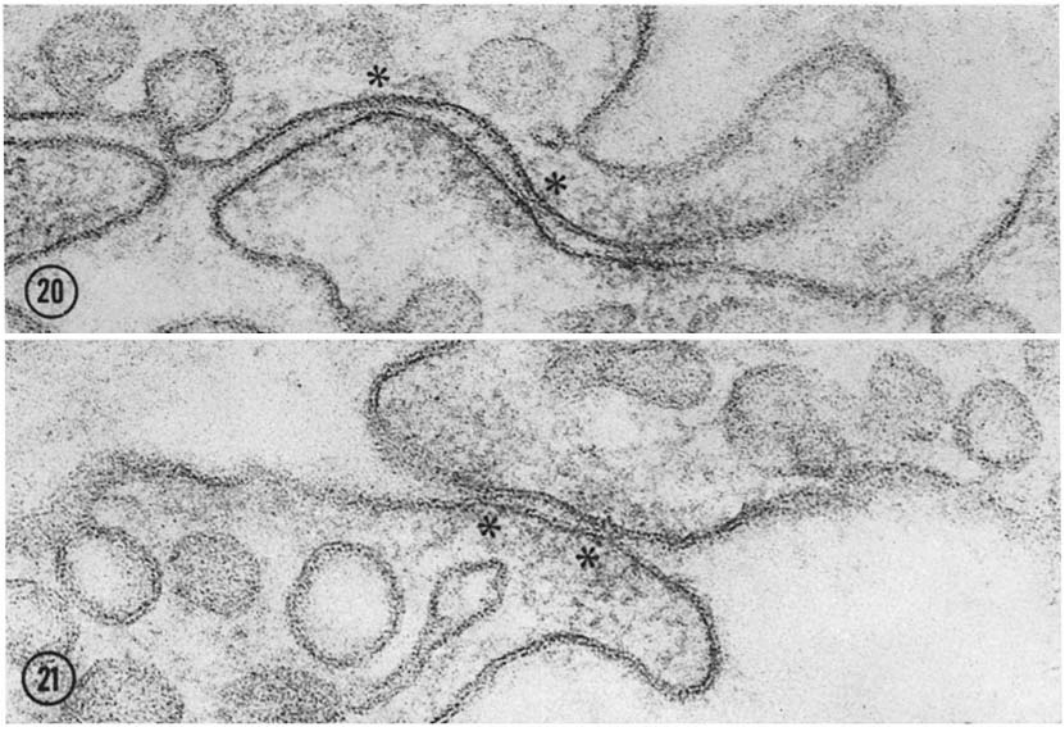


FIGURES 16 and 17 Normal mouse heart, cell junctions in capillaries. Osmium-tetroxide fixation, uranyl staining *en bloc*. Lumina to the right.

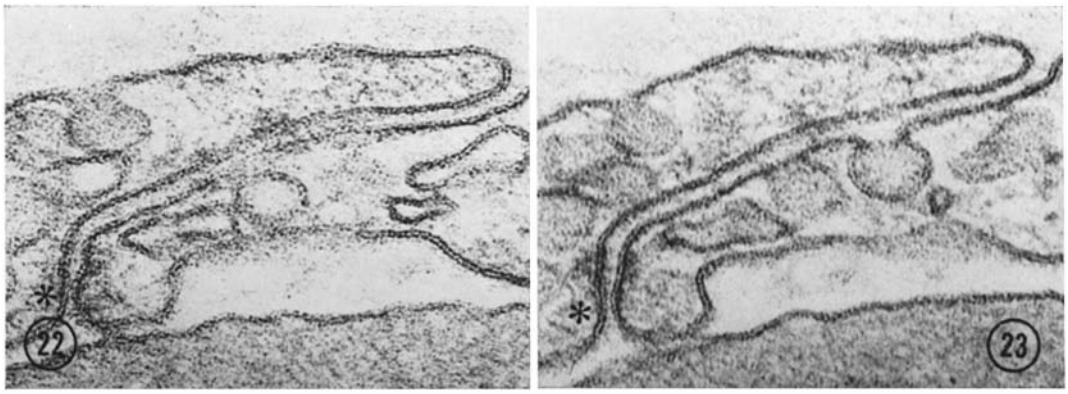
Fig. 16, there is a gap of 30-40 A width between the external leaflets at the cell junction (*). The gap contains dense material. × 300,000. Fig. 17, the lower plasma membrane at the cell junction (*) is cut tangentially, and can be seen not to fuse with the upper one. × 200,000.

FIGURES 18 and 19 Cell junctions in capillaries of diaphragm of normal mouse. Osmium tetroxide fixation, uranyl staining *en bloc*. Lumina to the right.

Fig. 18, at cell junction (*) there is a gap of ~40 A between the adjacent plasma membranes, which contains electron-opaque material. Toward the basal lamina (left) the membranes turn tangentially, and details cannot be seen. × 280,000. Fig. 19, at cell junctions *1* and *2* there is apparent obliteration of the extracellular space. At *3*, *4*, and *5* a gap ~40 A in width is present. An intercellular cleft such as this, with more than two cell junctions, is rare. × 300,000.



FIGURES 20 and 21 Cell junctions in capillaries of hearts from mice injected with peroxidase, and sacrificed after 8 and 12 min respectively. Lumina to the right. At the cell junctions (*) there is close apposition of the plasma membranes and apparent obliteration of the extracellular space. There is no abnormal widening of the junctions. Osmium-tetroxide fixation, uranyl staining *en bloc*. × 200,000.

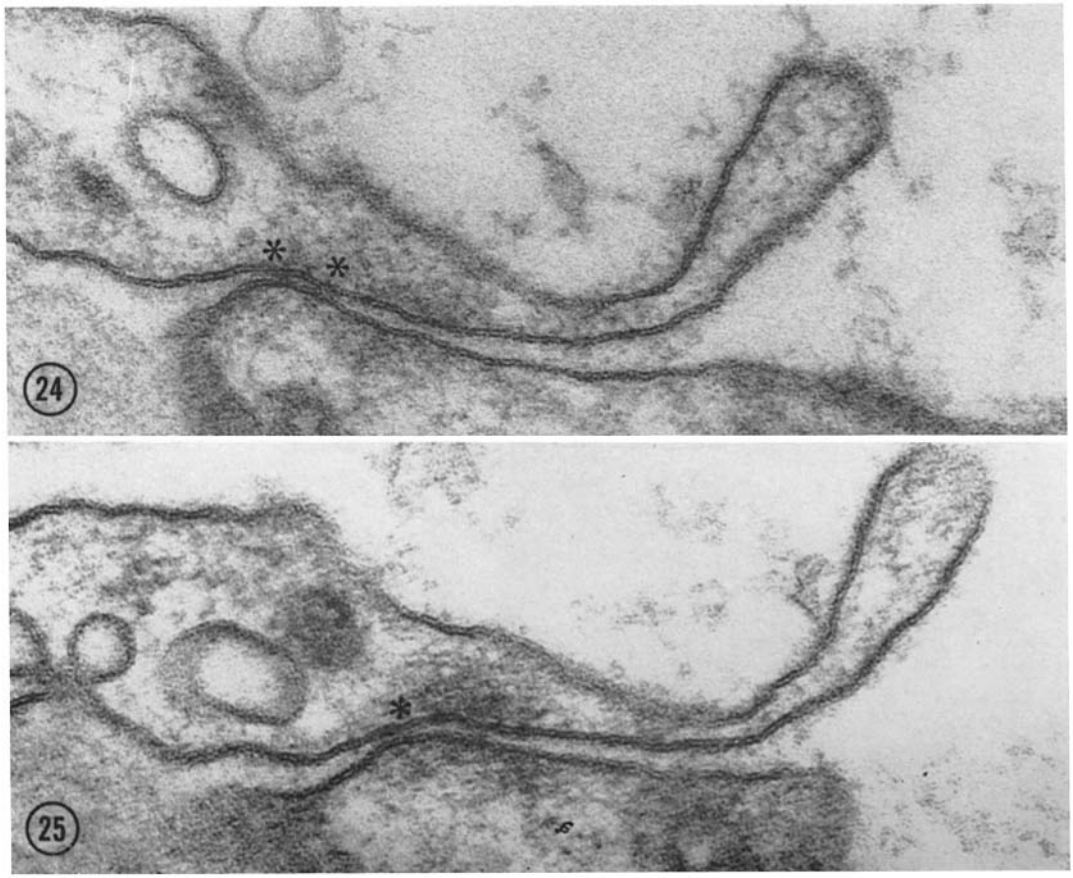


FIGURES 22 and 23 Cell junction in heart of normal mouse sectioned at two different levels. Lumina below. In Fig. 22 the cell junction (*) shows apparent obliteration of the extracellular space by fusion of the adjacent external leaflets. In Fig. 23, sectioned at a different level, the same junction has a small gap of ~30 A between the adjacent external leaflets. Osmium-tetroxide fixation, uranyl staining *en bloc*. × 100,000.

Figs. 12, 20), by touching or fusing of the adjacent external leaflets.

The endothelial cell junctions of peroxidase-treated mice (Figs. 20, 21) showed no differences from those of controls. The ratio of the width of the cell junction at its narrowest point to the width of an adjacent unit membrane (of the plasmalemma) was measured in 32 untreated mice (C), in 20 saline-treated (S), and in 20 peroxidase-treated (E). All of this material was fixed in osmium tetroxide–collidine with sucrose added. The way in which the measurements were made is indicated in Figs. 12 and 14. The ratios of widths of cell junctions: widths of unit membrane were almost always 2.0 or greater than 2.0. The means and their standard errors were $\bar{C} = 2.400 \pm 0.061$; $\bar{S} = 2.463 \pm 0.063$; and $\bar{E} = 2.522 \pm 0.056$. The differences $\bar{C} - \bar{S}$, $\bar{C} - \bar{E}$, $\bar{S} - \bar{E}$, were not significant, having P values by the t test of $0.4 < P < 0.5$, $0.1 < P < 0.2$, and $0.4 < P < 0.5$, respectively. Similar observations were made on capillaries in osmium tetroxide-fixed mouse gastrocnemius and diaphragm (Figs. 18, 19) and cat heart and gastrocnemius, in formaldehyde–glutaraldehyde-perfused rat heart, and in unperfused mouse heart fixed in formaldehyde–glutaraldehyde. There were no apparent differences in the various fixations, species, and perfused and unperfused capillaries.

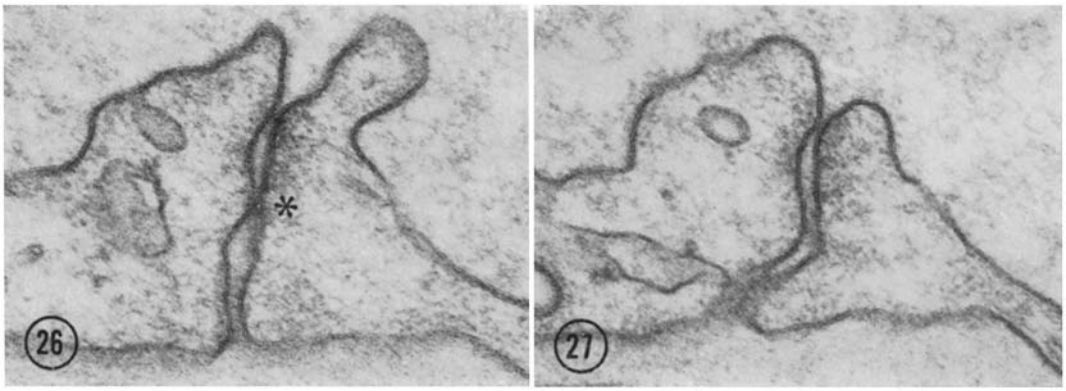
Attempts were made to obtain micrographs of serial sections of a particular cell junction. Because of tilting of the junction, tangential or oblique views were frequently obtained, which obscured the detail required. However, if a cell junction



FIGURES 24 and 25 Similar to Figs. 22 and 23. Lumina to the right. The junctions in Fig. 24 (*) have opened up in Fig. 25, which is sectioned at a different level. The gap measures ~20 A. Osmium-tetroxide fixation, uranyl staining *en bloc*. × 150,000.

was satisfactorily visualized at two different levels, it was possible to note that at one level the extracellular space appeared to be obliterated, whereas at another level the same cell junction had a small gap between the adjacent plasma membranes (Figs. 22–25).

Sometimes the intercellular cleft displayed in one plane of section more than one junction at various levels along its course from lumen to basal lamina. Some of these junctions had small gaps, whereas others appeared closed (Fig. 19). The same intercellular cleft cut at different levels sometimes showed complete disappearance of one or more junctions (Figs. 26, 27). However, complete absence of a junction along the course of an intercellular cleft from lumen to basal lamina was found only extremely rarely.



FIGURES 26 and 27 Cell junction in capillary of normal mouse heart, sectioned at two different levels. The one junction (*, Fig. 26) disappears, whereas that closest to the lumen (above) persists. Osmium-tetroxide fixation, uranyl staining *en bloc*. × 87,000.

DISCUSSION

The observations reported above indicate that horseradish peroxidase, a protein tracer of relatively low molecular weight (40,000), passes across the capillaries into the extracellular spaces *via* the intercellular clefts. An additional mode of transport may be by means of micropinocytotic vesicles, but, as pointed out above, the evidence for this latter route for peroxidase is not completely convincing, despite some suggestive observations.[5]

In this latter regard the observations made with ferritin (mol wt 500,000) as a tracer (26–28) are suggestive of a slow (26) transcapillary passage by means of vesicles. The evidence is unconvincing, however, that the vesicles are the major or sole mode of passage across the endothelium, especially for water and small lipid-insoluble molecules. It is difficult to reconcile our present knowledge of vesicular structure and transport (21, 25–28, 11–13, 32–33) with much of the physiological data, and to explain phenomena such as restriction to diffusion (6, 9), and molecular sieving (6, 9) of even relatively small molecules in these terms. On both morphological (21, 32, 33) and physiological (12) grounds, vesicular transport has been considered to be too slow and of insufficient magnitude to account for the rapid transcapillary exchange of substances of molecular weight below 10,000. The calculated net rates of vesicular turnover (12) indicate that vesicular transport is largely sufficient to account for the slow transcapillary exchange of high molecular weight substances (12). Possibly, plasma proteins, hormones, antibodies, and the like, could pass across the endothelium by this means. Physiological observations on capillary permeability to high molecular weight substances (>100,000 mol wt) are compatible with this view

[5] In an earlier publication (34), the author was under the then current belief that the intercellular clefts were sealed by tight junctions (*zonulae occludentes*) (22, 23) which were assumed to be impermeable (23). The observed transcapillary passage of peroxidase was consequently attributed to vesicular transport. The significance of staining in the clefts was not appreciated, nor had the studies on the fine structure of the cell junctions reported here been made.

(11, 12). It has been suggested on the basis of physiological data that the vesicular transport system may represent the "large pore" system, as the experimental observations are at present interpretable in terms of both types of system (10–12). On a priori reasoning, it seems that vesicular transport would necessarily be an energy-requiring process which would be at variance with the belief that transcapillary permeability, at least for water and small molecules, is a purely passive process (6, 9). It has been proposed, on theoretical grounds, that Brownian motion may underly, at least in part, the movement of vesicles (61).

According to the work of Pappenheimer and his colleagues (6–9), the capillary endothelium behaves as if it were a semipermeable membrane, penetrated by water-filled channels or pores, through which pass water and small, lipid-insoluble molecules. The total cross-sectional area of the pores would occupy less than 0.1% of the capillary surface. Uniform, water-filled, cylindrical channels of 40–45 A radius, and a population density of $1–2 \times 10^9$ per cm^2 of capillary wall would fit the experimental data. The channels need not actually be cylindrical: slits of equivalent width, 50–55 A,[6] and other geometrical configurations are also possible. Small molecules exchange rapidly between plasma and interstitial fluid by diffusion through these pores, and net volume flow of fluid is dependent on hydrodynamic forces. With increasing molecular size, restriction to diffusion becomes greater, and for large molecules, such as plasma proteins, a high degree of molecular sieving occurs. The residual transport for molecules larger than the small pores may represent either diffusion through a sparse system of large pores (radius ~250 A) (10, 11), or vesicular transport (11, 12).

Moderate restriction to diffusion by the small cylindrical pore (40–45 A radius) occurs in the molecular weight range 1000–10,000, and above this the restriction is greater. Theoretically, substances of molecular weight greater than 90,000 would be almost completely restricted, at normal rates of filtration. Horseradish peroxidase of molecular weight 40,000 , and an estimated equivalent radius of 25–30 A,[7] would be expected to pass slowly, but definitely, through the pores. As it has been observed that horseradish peroxidase passes along the intercellular clefts, this suggests that the limiting pore be located along this pathway, presumably at the cell junction, which is the narrowest portion of the cleft.

Indeed, Starling (1, 2) as well as Pappenheimer (6, 9) suggested that the intercellular cleft or space would be the logical site at which filtration occurs. Chambers and Zweifach (17–18) postulated that the intercellular cleft was filled with an intercellular cement, which could account for the permeability characteristics of the endothelium. This concept of intercellular cement fell somewhat into disrepute with the application of electron microscopy, as little, if any, intercellular material was visualized. It has recently been revived by Luft (14, 15) who obtains staining with ruthenium red in the intercellular cleft, which he attributes to the presence of a mucopolysaccharide.

Electron microscopists have hitherto discounted the intercellular cleft as a route of passage across the endothelium, for three main reasons. Firstly, it has been claimed that the intercellular clefts are sealed by tight junctions, i.e. zonulae occludentes (22, 23, 67); secondly, there has been no convincing evidence at the ultrastructural level that a tracer passes through the clefts (13, 16, 21); and thirdly, there has been a preoccupation with vesicular transport, which has been demonstrable, to some degree, by tracer experiments (21, 26–28).

[6] In the earlier calculations (7–9) the equivalent pore radius for cylindrical pores was given as 30 A, and the equivalent slit width as 37 A. The figure for cylindrical pores was later (6) revised upward to 40–45 A equivalent pore radius, which, as pointed out by Luft (15) would presumably correspond to an equivalent slit width of 50–55 A.

[7] Commercial horseradish peroxidase preparations contain multiple components (64) which can be separated by electrophoresis. They do not differ in size, molecular weight, absorption spectrum, enzymatic activity, or amino acid composition. Almost all migrate to the cathode at pH 8.6. A series was observed (64) of $S^\circ_{20w} = 3.4–3.7$. Assuming a compact globular molecule, behaving in a fashion similar to a variety of proteins, we estimate the radius of an equivalent hydrodynamic sphere to be roughly 25–30 A.

It remains for further work to show whether the peroxidase may breakdown in the bloodstream into smaller, enzymatically active fragments. Obviously, this would favor intercellular passage across the endothelium. Also, the degree of binding to plasma proteins has not as yet been established, but is not thought to be significant in view of the rapid passage of peroxidase through the glomerulus (69). Preliminary fractionation studies also indicate that binding is minimal.

Zonulae occludentes would be, by definition, continuous bands or zonules extending all around the capillary. They would be formed by fusion of the outer leaflets of the adjacent endothelial plasma membranes at the level of the cell junctions. By analogy with zonulae occludentes at other sites (23), it has been considered that these structures, if present in the capillaries, would form an impermeable[8] and continuous seal which would seal the lumen from the tissue spaces (22). The papers of Muir and Peters (22) and of Peters (67) are frequently quoted in support of zonulae occludentes sealing the intercellular clefts of capillaries. It is significant, however, that most of the illustrations in these papers are of junctions in the endothelia of capillaries in the brain and its appendages.

We have confirmed that the cell junctions in cerebral capillaries are indeed zonulae occludentes (62). In contrast to the cell junctions found in noncerebral capillaries, they occupy a much larger proportion of the radial length of the intercellular cleft and are thought to form an unbroken belt between the endothelial cells. In comparison with those of the noncerebral capillaries, the ratios of width of cell junctions to width of adjacent unit membranes was 2.0 or less, with an average of 1.7. Peroxidase did not pass through these cerebral junctions. We consider this structural feature (zonulae occludentes) of the cerebral endothelium to be part of the morphological basis of the blood–brain barrier, at least in regard to substances of molecular weight 40,000 or greater. Also, vesicular transport may be lacking (22). Cunha-Vaz et al. (66) have reached similar conclusions in regard to retinal versus iridic capillaries.

Farquhar and Palade (23) also regarded the cell junctions in capillary endothelium as being zonulae occludentes. They stated that the occluding zonule frequently takes the form of a focal stricture or "pinch." While we agree that there are focal strictures in noncerebral capillaries, we do not, from the observations presented here, regard these as being zonulae occludentes, but rather as a series of maculae occludentes, i.e. small, discrete, and spotty areas of obliteration of the intercellular space. Both the zonula and macula are characterized by fusion of the external leaflets of adjacent plasma membranes, to form quintuple-layered cell junctions, but whereas this fusion is invariably found in every plane of section of a zonula, gaps would appear as one moves away from the point of fusion in the macula. This seems to be the case in the noncerebral endothelia studied here, where sometimes the junction appears "tight," but frequently a small intercellular gap is present. The same junction may thus be truly tight and less tight at different levels. Thus one envisages a series of "buttons" which open up slightly between the tight areas. The spaces thus formed could be oval slits, but the exact geometry of these slits is at present unknown. The distance between the tight areas, i.e. the length of the slits, and the maximum width attained remains to be established. The latter, from our present data, appears to be about 40 A, but may well be wider in vivo. The depth of the junctions from luminal to tissue surfaces varies from 100 to 400 A.

Pappenheimer (6) has calculated that the pores should occupy less than 0.1% of the surface area of the capillary. This calculation is based on a path lenth of 10^{-4} cm, i.e. the whole width of the thinner portions of the endothelial wall. For slits about 55 A in width, located within the intercellular clefts of a capillary 10 μ in diameter, the percentage of slit surface area to capillary surface area is about 0.02%. However, as the cell junctional area is only a few hundred angstroms in length (=path length) and not 1 μ, the percentage of surface area occupied by the pores would be considerably less than 0.1%, and of the same order of magnitude as 0.02%, i.e., a minute fraction of the capillary surface.

Previous attempts have been made by using various tracers to demonstrate the existence of an intercellular pathway. Mende and Chambers (19) applied solutions of two different electrolytes on each side of an endothelial membrane: one by topical dripping; one by intravenous injection. One substance was an anion and one a cation, which on meeting would precipitate and thus indicate diffusion pathways. For instance, with sodium ferricyanide and ferrous sulfate, ferroferricyanide (Turnbull blue) precipitated along the intercellular clefts, as was seen when the preparations were examined by light microscopy. In their ex-

[8] Although the structure of the zonulae occludentes suggests impermeability, the evidence for this was originally based on impermeability to large tracer molecules, such as hemoglobin (23). More recently, correlation of structure with physiological observations has been more convincing (40, 63). Lanthanum (Revel, J. P., and M. J. Karnovsky. Manuscript in preparation.) and peroxidase (reference 62, Revel, J. P., and M. J. Karnovsky. Manuscript in preparation.) do not permeate true zonulae occludentes.

periments endothelial damage was indicated by the staining of endothelial nuclei by intravenously injected Trypan blue, and by the presence of thrombi. Ohori (20) and Takada (55) performed similar experiments by electron microscopy and confirmed that the precipitates were in the intercellular clefts. Their micrographs also show evidence of much cellular damage. Saccharated iron oxide (diameter 20–100 A) (68) has been found in cell junctions after perfusion of the heart (21, 26, 68), especially in the presence of metabolic inhibitors. It was not entirely clear whether the particles had passed through the junctions (21) and it was not shown by high-resolution microscopy that the junctions were still intact.

On the other hand, horseradish peroxidase appears to pass through the cell junctions after intravenous injection of peroxidase; the cell junctions appear morphologically intact, and do not apparently differ from those of controls. It is possible that peroxidase itself, or a minor impurity, could subtly affect the permeability of capillary junctional complexes. However, there was no detectable peroxidase-induced leakage of various tracers, such as carbon, ferritin, and saccharated iron oxide. Admittedly, these means of detection of abnormal leakage may be too insensitive to detect subtle changes in the junctional complexes, but, if there were self-induced leakage of peroxidase, a relatively large molecule, the changes at the junctions would probably be not inconsiderable; the changes probably would be detectable with some of the nonenzymatic tracers and also at the morphological level. Furthermore, prolonged exposure to high levels of peroxidase did not increase the permeability of brain capillary junctional complexes (62). There were also no abnormal vascular (venular) leaks from peroxidase-induced histamine and serotonin release in the mouse, as was demonstrated in the various controls performed to exclude this possibility.

The basal lamina probably plays a secondary role in capillary permeability, since horseradish peroxidase, as well as ferritin (26–28) and saccharated iron oxide (13), appear to pass across quite freely. Large particles such as colloidal carbon (diameter 250–500 A) (13, 16, 42, 56, 57) and HgS (diameter 70–300 A) (31) are largely retained, at least temporarily, following abnormal vascular leakage.

In the light of the observations presented here, and the above considerations, we believe that passage of substances from plasma to tissue does occur *via* the intercellular clefts, and that the morphological equivalent of the small pore system of the physiologists (6–9) is the cell junction. Admittedly, the width of the "slits" found here (~40 A) does not correspond exactly with the size of the theoretical pores previously proposed (6–9), but this is not necessarily in disagreement with the theoretical concept. It would be somewhat unreasonable, in our opinion, to expect to find by electron microscopy structures which correspond exactly in dimensions with the pores proposed by the physiologists. The concept of a pore system was more important than the exact configuration and size of the channels. As Pappenheimer himself states (6); "there are no reasons for supposing, however, that the channels through capillary walls are either cylindrical or perfectly uniform. Alternative models utilizing different pore geometries or a limited distribution of pore sizes could be devised to simulate the observed capillary permeability." Pappenheimer points out that the simple physical model closely approximates the morphology of the capillary wall; in view of our observations, we would agree with this. The mean pore radii be calculated are equivalent radii, i.e. they tell one nothing of the actual shape of the pore, but tell one that the capillary wall behaves as it if contained pores of such dimensions. Furthermore, the theoretical pores are regarded as water-filled channels, with smooth, rigid walls (6, 9). The cell junctions as observed here probably do not fit this description. Apart from other considerations, which have been listed by Luft (15), there is frequently electron-opaque material within the gap of the junction, the nature of which is unknown. It may be similar to the endocapillary layer and intercellular cement, which stain with ruthenium red and are considered by Luft (14, 15) to be acid mucopolysaccharide in nature.

Other rather obvious factors which should be taken into account in relating the theoretical pores to the observed structure of the cell junctions are the changes in structure and dimensions which may be produced by fixation, embedding, staining, microscopy, inaccuracy of measurement, and so on. In regard to staining, we have shown[9] that poststaining with alkaline lead of thin sections of tissue pretreated with uranyl *en bloc* may decrease the apparent width of the gap seen in some cell junctions. For example, in the intercalated disc of the heart the so-called tight junction has a gap of

[9] Revel, J. P., and M. J. Karnovsky. Manuscript in preparation.

about 20 A in width, which can almost be obliterated by heavy lead staining. Furthermore, we do not know exactly what chemical entities and what portions of the external leaflets, endocapillary layers and intercellular cements the uranyl and lead are staining.

Luft (14, 15) believes that the external leaflets of the endothelial plasma membranes are permeable to and are stained by ruthenium red. Accepting the evidence of Muir and Peters (22) for the presence of zonulae occludentes, Luft argues that that intermediate line of this quintuple-layered complex represents fusion of the external leaflets, and that thus this fusion line is composed of a common layer of mucopolysaccharide derived from each external leaflet. This fusion line would, he argues, be functionally permeable to water and small molecules, although structurally it would appear closed. By the assumption of various widths for the strata of the unit membranes, the fusion line was calculated to have a width of 35–5 A.

We are doubtful about the existence of zonulae occludentes in the endothelium, except in the brain, but support Luft's contention about the permeability of the external leaflets, to the extent that the lanthanum tracer permeates the external leaflets as well as the small intercellular gap (~20 A) at some cell junctions, for instance in the so-called tight (close) junctions of the intercalated disc of the heart (46, 47). It is of interest that the minimum width of the lanthanum line penetrating the endothelial cell junction, as in Fig. 7, is 60–70 A, which could be accounted for by permeation through adjacent external leaflets (2 × 15 A = 30 A) plus an intrajunctional gap of 30–40 A. Lanthanum does not, however, penetrate through zonulae occludentes, for instance in the intestinal epithelium, proximal tubule of kidney, frog skin, and the brain capillaries, and neither does the horseradish peroxidase.[9] These observations indicate that the endothelial cell junctions are not so impermeable as are zonulae occludentes (23, 40, 63). There is also physiological evidence for high impermeability of zonulae occludentes to small molecules (40, 63).

The mesothelium has permeability characteristics quite similar to those of the capillary endothelium (58, 59), and there are also morphological resemblances (13, 16). Indeed, Starling's hypothesis was based on studies made on the mesentery (1, 2). It is of interest that on exposure of the peritoneal surface to peroxidase, the tracer passed through the intercellular junctions into the basal tissue spaces (16, 60).[10] There was no evidence suggestive of vesicular transport in this tissue, at least up to 1 hr after exposure to peroxidase.

Much further work is required to establish the structure, geometry, and chemical composition of endothelial cell junctions. It will only be then that more exact reconciliation between the morphological and physiological data will be possible. The observations presented in this paper strongly suggest, however, that the capillary endothelium cell junctions are permeable and are the ultrastructural equivalent of the small pore system of the physiologists.

This work was supported by grant HE-09125 from the National Institutes of Health, United States Public Health Service. The author was the recipient of a Lederle Medical Faculty Award, 1963–1966.

Miss Antoinette Ebener provided skilled technical assistance. Mr. Robert Rubin provided expert help with the photography. The author appreciates the help and advice of Doctors Ramzi S. Cotran, Stephen M. Shea, David S. Papermaster, and Jean-Paul Revel.

Received for publication 20 April 1967; revision accepted 9 June 1967.

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