The Ultrastructural Basis of Transcapillary Exchanges

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ABSTRACT A brief survey is given of current views correlating the ultrastructural and permeability characteristics of capillaries. Observations based on the use of peroxidase (mol wt 40,000), as an in vivo, and colloidal lanthanum, as an in vitro, ultrastructural tracer, are presented. In capillaries with "continuous" endothelium, the endothelial intercellular junctions are thought to be permeable to the tracers, and are regarded as maculae occludentes rather than zonulae occludentes, with a gap of about 40 A in width between the maculae. Some evidence for vesicular transport is also presented. It is inferred that the cell junctions are the morphological equivalent of the small-pore system, and the vesicles the equivalent of the large-pore system. Peroxidase does not apparently cross brain capillaries: the endothelial cell junctions are regarded as zonulae occludentes, and vesicles do not appear to transport across the endothelium. This is regarded as the morphological equivalent of the blood-brain barrier for relatively large molecules. The tracers appear to permeate the fenestrae of fenestrated capillaries, and the high permeability of these capillaries to large molecules is attributed to the fenestrae. Capillaries with discontinuous endothelium readily allow passage of the tracers through the intercellular gaps. A continuous basement membrane may act as a relatively coarse filter for large molecules. In general, the morphology of capillaries correlates well with physiological observations.

Since the introduction of the electron microscope and the elucidation of the ultrastructure of cells, the physiologist, biochemist, and morphologist have been able to make considerable advances in the correlation of structure and function, at least at the macromolecular level. In regard to the structural basis of transcapillary exchanges, much effort has been devoted to uncovering the morphological entities and mechanisms involved, with considerable success, particularly for large, lipid-insoluble molecules. The structural pathways for small molecules, however, can only be inferred, because of limitations in technique. The electron microscopist has been necessarily restricted to utilizing relatively large, electron-opaque tracer substances, such as ferritin, saccharated iron oxide, and so on, for detecting pathways of passage. Many

problems and questions of interpretation therefore remain unresolved, and clear and unequivocal definition of the morphological pathways whereby water, electrolytes, and proteins cross the endothelium is as yet unattained. The morphological approach suffers as well, in that volumes and rates of exchanges can only be inferred. Localization of the sites of exchange is the attainable objective. Despite these strictures, there is some degree of correlation between physiological and morphological observations.

It is the purpose of this paper to review briefly the status of the field, and to present some of our own observations based on the use of new ultrastructural tracer techniques. These techniques are based on the use of peroxidases as tracers. Mostly horseradish peroxidase (mol wt 40,000) and, to a lesser extent, human myeloperoxidase (mol wt 160,000) have been used in these studies. The use of an enzyme as a tracer has the advantage of sensitivity, in that the 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 insoluble, electron-opaque reaction product.

The details of the method have been given elsewhere (1, 2). In brief, mice were injected intravenously with peroxidase and at sequential time intervals were sacrificed. After fixation in an aldehyde-containing fixative, frozen sections or chopped sections were incubated in a medium designed for the demonstration of peroxidase activity at the ultrastructural level. The medium contains 3,3′-diaminobenzidine and hydrogen peroxide in Tris buffer. The diaminobenzidine acts as an electron donor and is oxidized at the site of enzyme activity to a highly insoluble, brown, polymeric compound. On postfixation with osmium tetroxide, this reaction product reacts avidly with the osmium, yielding a black, insoluble, noncrystalline deposit which is highly electron-opaque and gives fine and sharp localizations at the ultrastructural level. The various controls for the specificity of localization have previously been given in detail (1, 2). It was also established that in the mouse the doses of peroxidases used did not cause untoward effects, including abnormal vascular leakage due to histamine and serotonin release (2, 3).

The second tracer method we have used is the lanthanum tracer method (4), which is based on the formation of a colloidal suspension of lanthanum when it is alkalinized. After aldehyde fixation, tissues were postfixed in osmium tetroxide fixative containing the colloidal lanthanum. The lanthanum permeates though and delineates the extracellular space, and also passes into intracellular vesicles which open to the extracellular space. As the tissue is prefixed before exposure to the lanthanum, active uptake by the cells is excluded.

The capillaries studied in particular have been those of cardiac muscle, skeletal muscle, brain, lung, and kidney.

FINE STRUCTURE OF CAPILLARIES

It is unnecessary to cover here in detail the fine structure of capillaries and of the endothelium, as several papers and reviews have appeared in recent years (5–14). In particular, the review by Majno (5) is unusually comprehensive. All of these works also deal with aspects of the ultrastructural basis of transcapillary exchanges.

It is apparent that there are many differences in capillary structure between different tissues and organs, and even within the same tissue or organ the capillaries may be heterogenous in structure (5).

The general types (5) of capillaries so far described are the following.

1. Capillaries with "*continuous*" *endothelium* and continuous basement membrane; for example, those in heart, muscle, lung, skin, and nervous system.

2. Capillaries with a *fenestrated endothelium* and with a continuous basement membrane; for example, those in glomeruli, endocrine glands, and gastrointestinal tract. The fenestrae of the glomerular capillaries may, or may not be, closed by a thin diaphragm (5). At other sites, the diaphragm is usually present.

3. Capillaries (sinusoids) with an open, *discontinuous endothelium* (i.e. there are large gaps in the endothelium) and a discontinuous or absent basement membrane; for example, the sinusoids of the liver, spleen, and bone marrow.

In addition, the endothelium may be low and thin, and contain few organelles, other than micropinocytotic vesicles, or it may be high, containing varied organelles, filaments, and inclusions. The structure of the lymphatic capillary has also been recently reviewed (15).

CAPILLARIES WITH "CONTINUOUS" ENDOTHELIUM

In this paper we shall direct our attention mainly to capillaries of type 1, i.e. those of heart, skeletal muscle, lung, and nervous system, as it is with these that most of the problems in correlating structure and function have arisen. Such a capillary is shown in Fig. 1. These capillaries consist of the lumen, the endothelial cells, the basement membrane, and the pericytes.

The endothelial cells are mainly flattened and are 0.1–0.5 μ in thickness, except at the nucleus, where most of the usual cell organelles are found, and where the cell bulges to a thickness of 1–5 μ.

Most of the cell cytoplasm, especially the thinner parts, is occupied by the micropinocytotic vesicles. These vesicles, which appear to be invaginations of the plasma membrane at the luminal and basal surfaces of the cell, and also to lie free in the cytoplasm, are fairly uniform in size, being 500–800 μ in diameter, and occupy about one-half of the cell volume. They are frequently seen aligned at the luminal and basal cell surfaces, attached to the surfaces by

stalks up to about 200 A in length. Coalescence of vesicles to form a continuous channel from lumen to basal surface has not been observed (7). They are abundant in cardiac and skeletal muscle capillaries, but sparse in the capillaries of the nervous system.

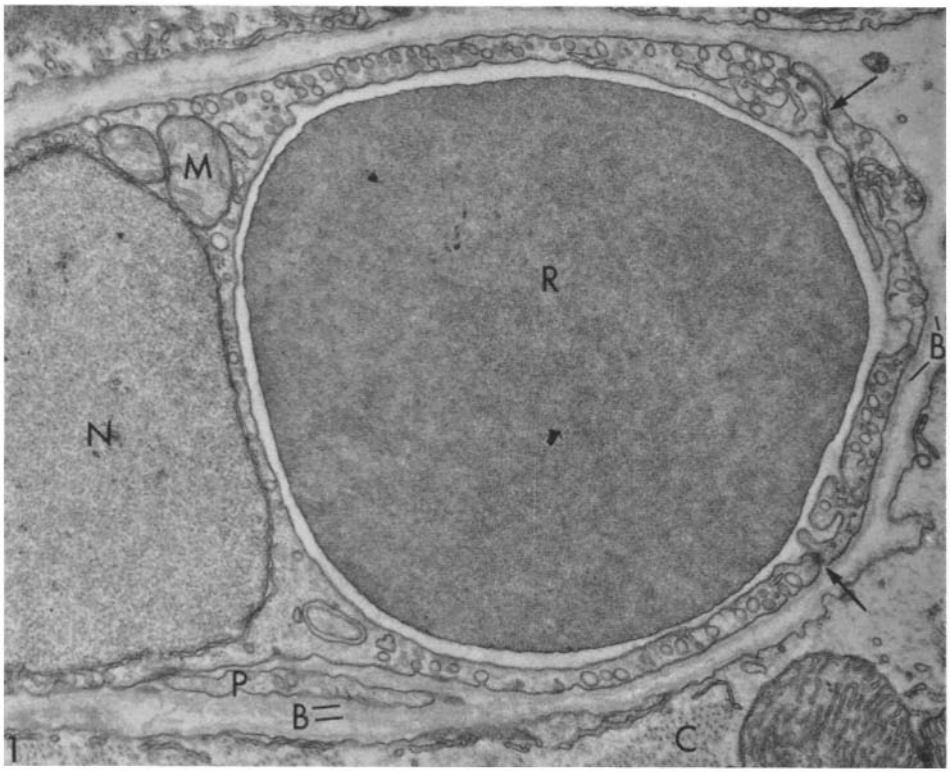

FIGURE 1. Capillary, mouse heart. Adjacent to the nucleus (*N*), there are two mitochondria (*M*). In the thinner part of the endothelial cytoplasm there are numerous vesicles, most of which are lined up at, and are apparently connected by short stalks opening to, either the luminal or basal surface membranes. Two intercellular clefts are shown at the arrows. The denser areas near the lumen represent the region of the cell junctions. The process of a pericyte, *P*, makes close contact, on the left, with the endothelial cell surface. The gray material applied to the basal surface of the endothelial cell is the basement membrane (*B*), in which the process of the pericyte is also embedded. *R* = red blood cell in the lumen. *C* = cardiac muscle. Formaldehyde–glutaraldehyde–osmium tetroxide fixation. × 24,000.

Palade, who first described these vesicles (16), suggested that they might be involved in transendothelial transport, and has (7), with others (8–11), produced experimental evidence in favor of this viewpoint.

Although the endothelium of these capillaries is referred to as "continuous," in point of fact there is an intercellular space or intercellular cleft of width 100–200 A between the lateral surfaces of adjacent endothelial cells. At at

least one point, usually close to the lumen, the adjacent plasma membranes approximate, obliterating or almost obliterating, the extracellular space. These areas, the cell junctions, will be described in detail later. As will be seen, their precise structure is of great importance in terms of their possible role in transendothelial exchange.

Earlier studies with the light microscope had suggested the presence of a layer of "cement" substance or adsorbed protein (the endocapillary layer) on the luminal surface of the endothelial cell (17–19). This was denied in earlier electron microscope studies, but with better fixation and the use of ultrastructural cytochemical methods, supposedly specific for polysaccharides, such a layer has been observed (12, 13, 20). Similar material has been claimed to occupy the intercellular clefts, as an "intercellular cement" (17), which may play an important role in the permeability characteristics of the endothelium

The basement membrane (or basal lamina) is an acellular layer, 200–600 A in thickness, closely applied to the basal surface of the endothelium. In some sections it may have the appearance of a network of fine fibrils embedded in an amorphous matrix. By analogy with the basement membrane of the glomerulus (23), it may have a collagenous component. Histochemically there is also a polysaccharide component (24). It has been suggested that the basement membrane, especially that in the glomerulus (25), may act as a primary filtration barrier in transendothelial exchanges, but others believe it to play a secondary role as a coarse filter, being permeable to relatively large molecules (2, 5, 7, 9, 10, 14, 26).

It is not necessary to discuss the pericytes here, except to note the possibility that they may be contractile (27, 28) and that under special circumstances they can be phagocytic (29). Either of these features might modify transendothelial exchanges under certain circumstances.

PHYSIOLOGICAL CONSIDERATIONS

Extensive physiological studies have established that the capillary wall can be considered as a semipermeable membrane, and that the process of transcapillary exchange is passive in nature, dependent on diffusion, filtration, and hydrostatic and osmotic forces. Pappenheimer and his colleagues (30–32) established, in capillaries of the hind limb of the cat, that for small, lipid-insoluble molecules diffusion is the basis for the rapid exchanges which occur between the blood and the tissues. Their observations indicate that the vascular barrier 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 pores would occupy a minute fraction (less than 0.1%) of the capillary surface. [More recently, it has been claimed that the area subserving water exchanges is greater than that for small molecules (33).] Uniform, water-filled, cylindrical channels of 40–45 A radius and a population density

of 1–2 × 10^9 per cm^2 of capillary wall are compatible with the data, but the channels need not be cylindrical; slits of width 50–55 A and other geometrical configurations are also possible. The concept of a pore system was perhaps more important, as indicated by Pappenheimer, than the exact configuration and size of the channels. Moderate restriction to diffusion by this small-pore system occurs in the molecular weight range 1–10,000, 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. Thus, for plasma proteins, a high degree of molecular sieving occurs.

Utilizing dextrans of varied molecular weights, Grotte (34) and Mayerson et al. (35) were able to confirm the observations suggestive of a small-pore system, and were also able to study the slow passage of high molecular weight substances across the capillary wall. To account for the latter observations, a second system of large pores, 120–350 A in radius, and much sparser in number than the small pores, was postulated (34, 35). Alternatively, because little restriction to diffusion was noted with the molecular sizes used, a vesicular transport system was also suggested to be compatible with the data (35, 36).

It has been observed in the frog that certain dyes, after intravenous injection, first appeared extravascularly at the venous end of the capillaries (37-40). The dyes exuded through discrete foci located in the venous capillaries and venules (40). It was concluded (37–40) that there was a permeability gradient in the capillary, with higher permeability at the venous end. The foci of exudation were termed fenestellae (40), and are possibly analogous to the large pores mentioned above (34, 35). These fenestellae have not been identified in the frog at the ultrastructural level, nor is it known if the phenomenon exists in other species. Capillaries in the frog have unusual structural features (41), which may allow for unusual permeability properties.

MORPHOLOGICAL TRANSENDOTHELIAL PATHWAYS:

Intercellular Clefts

Starling, Pappenheimer (30, 32), and especially Chambers and Zweifach (17) suggested that the intercellular spaces (i.e. interendothelial spaces or clefts) would be the logical site at which passive diffusion and filtration occur, at least for relatively small molecules. The latter authors (17) suggested that the intercellular spaces were filled with a cement substance ("intercellular cement"), which might be of importance in the permeability characteristics of the capillary wall. This concept was disputed (42), and early electron microscopy did not reveal such a substance (5). More recently, the concept has been revived by Luft (12, 13), as mentioned above.

Until recently, observations on the ultrastructure of capillaries have not

been entirely reconcilable with the physiological concepts. Structures of the theoretical size of the small-pore system, which should be resolvable by electron microscopy, have not been positively identified as representing that system. The intercellular clefts, which are possible candidates, have been considered to be sealed by tight junctions, namely zonulae occludentes (43–45, 41, 12, 13). This type of cell junction (45) is formed by apposition or fusion of the external leaflets of the plasma membranes of adjoining cells to give a quintuple-layered unit, with complete obliteration of the intercellular space. This fusion of membranes extends all the way around the cells, as a continuous band or zonule, and acts as a gasket to separate the lumen effectively from the extracellular spaces basal to the zonule. At other sites (45) it has been con-

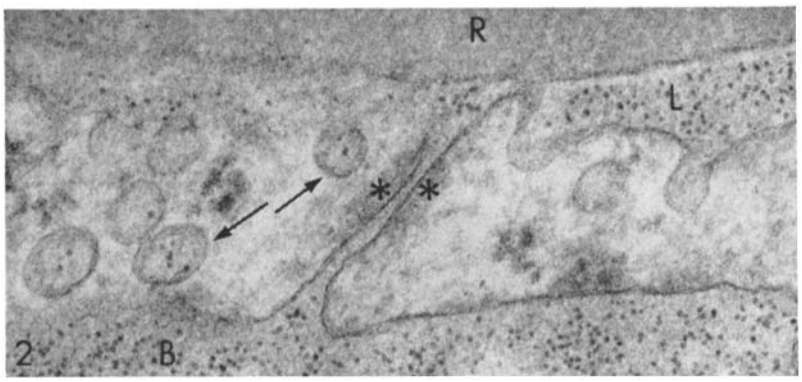

FIGURE 2. Capillary, mouse heart, 45 min after intravenous injection of ferritin. There is ferritin in the lumen (*L*), in vesicles (arrows), and in the basement membrane (*B*), but none in the intercellular cleft, or in the region of the cell junction (between the asterisks). *R* = red blood cell in lumen. × 108,000.

sidered that these structures thus form an impermeable and continuous seal, sealing the lumen from the tissue spaces. There is correlation with physiological evidence to support this concept (46, 47). Thus, if present along the intercellular clefts of the endothelium, these structures would render clefts impermeable, and some electron microscopists have therefore not considered the clefts as a likely pathway for transendothelial exchange. In support of this viewpoint, electron-opaque tracers, such as ferritin (100 A diameter), colloidal gold (30–250 A diameter), Thorotrast (100 A diameter), mercuric sulfide (70–350 A diameter), and saccharated iron oxide (40–100 A diameter), have not been found after intravenous injection, within the intercellular clefts (except rarely with saccharated iron oxide), but rather within the micropinocytotic vesicles (Fig. 2) (7–11, 48–50). On perfusion of the heart in vitro with solutions containing saccharated iron oxide, labeling of the intercellular clefts was achieved (8–10), but it was not entirely clear whether the particles had

passed through the cell junctions, and it was not shown convincingly by high-resolution microscopy that the junctions were still intact and not abnormally widened by the perfusion.

Other attempts to demonstrate the existence of an intercellular pathway by tracer techniques have been made. Two solutions of two different electrolytes were each applied to one or the other side of the endothelium: one by topical dripping, and one by intravenous injections. One substance was a cation, one an anion, and on meeting a precipitate formed, indicating diffusion pathways. Thus sodium ferricyanide and ferrous sulfate gave ferroferricyanide; sodium chromate and barium chloride, barium chromate. At both the light (51) and electron microscope (52) levels, staining of the intercellular clefts occurred, but evidence of endothelial damage was also apparent, e.g. thrombosis, permeability of endothelial nuclei to trypan blue, and ultrastructural evidence of cellular damage.

Luft (12, 13) believes that the endothelial cell junctions are indeed zonulae occludentes, but postulates that the external leaflets of the adjacent unit membranes are glycoprotein in nature, which renders the apposed external leaflets permeable. Similarly, he believes that the diaphragms closing the fenestrae of fenestrated capillaries (see below) are also derived from external leaflet material, and are also permeable. He thus draws an analogy between the structure and permeability of zonulae occludentes and the diaphragms. This ingenious synthesis has little evidence to support it, however. We disagree that the endothelial cell junctions are zonulae occludentes (see below), and consider it odd that, if zonulae occludentes are present in the endothelium, they should differ so markedly in permeability from those found elsewhere.

Micropinocytotic Vesicles

As mentioned above, labeling of the micropinocytotic vesicles has been achieved with several electron-opaque tracers, such as colloidal gold (30–250 A diameter) (48), mercuric sulfide (70–350 A diameter) (49), Thorotrast (100 A diameter) (50), ferritin (100 A diameter) (7–11), and saccharated iron oxide (40–100 A diameter) (8, 11), suggesting their possible role in transendothelial transport, a function first ascribed to them by Palade (16). In regard to ferritin, time sequence studies were particularly suggestive, in that progressive labeling of the lumen, vesicles, and basement membrane was observed over a period of about 30 min (Fig. 2) (7). No labeling of the intercellular clefts was noted. In experiments in vivo, the proportion of vesicles labeled and the number of particles found in each labeled vesicle were small. Considerable vesicular uptake of ferritin and saccharated iron was found when the heart was perfused in vitro (10) with solutions of saline containing the tracer. When whole blood was employed as the vehicle, there was little significant vesicular uptake of the tracers (10).

Vesicular transport does not appear to be unidirectional, because it appears that marker particles can also apparently pass from the extracellular spaces, through the basement membrane, label the vesicles (53), and sometimes appear in the lumen (54). The interpretation and significance of the experiments are not clear: it is possible that the lumen was labeled via the lymph, and, as shown below with the lanthanum tracer, most of the vesicles open to one or the other surface, and are thus readily labeled from such surfaces.

The evidence to date thus suggests that the micropinocytic vesicles constitute a mode of transport across the endothelium, but it is doubtful that they are the main pathway by which small molecules are transferred. From what is known of vesicular transport, it appears to be too slow and of insufficient magnitude to account for the rapid transcapillary exchanges of substances of molecular weight below 10,000 (36). The luminal opening of the vesicles is about 400 A in diameter, and it is difficult to explain the selective nature of capillary permeability and physiological phenomena, such as restriction to diffusion (30–32) and molecular sieving of even small molecules, in terms of our current knowledge of vesicular structure and behavior. The vesicular system does not appear to be selective, at least to molecules smaller than carbon (250–500 A diameter), which does not label the vesicles. It is possible that other morphological or physiological phenomena, such as focal constrictions, or a diaphragm closing the luminal opening, are incorporated in the vesicular system and could allow for restriction to diffusion and molecular sieving, but there is not much evidence for this.

Large molecules, however, do slowly cross the endothelium, and appear in the lymph (34–36). These molecules are too large to pass through the small-pore system. It has been suggested that there are occasional large pores (34, 35) (approximately 250 A radius), or that vesicular transport (35, 36) could be responsible. The dimensions calculated for the large pores are in accord with the dimensions of the vesicles, and the slow passage of large molecules fits with the apparent slow net transfer of tracer particles by vesicles.

From the data for transport of large molecules, the net rates of vesicular turnover have been calculated, and indicate that vesicular transport could be largely sufficient to account for the slow transcapillary exchange of substances of high molecular weight (36). The average lifetime of a vesicle was calculated to be of the order of magnitude of 5 min; this is consistent with the electron microscopic observations using electron-opaque tracers (7), which also indicate that vesicular transport is slow.

Transcapillary exchanges are generally considered to be passive in nature, at least in regard to water and small molecules (36). On a priori reasoning, it would appear that vesicular transport would necessarily be an energy-dependent process. In heart perfusion experiments (8, 11), hypoxia, metabolic inhibitors, and cooling did not overtly affect the number and morphology of

the vesicles. It has been suggested (55) that the vesicles are moved randomly about the cell by the thermodynamic bombardment of the surrounding molecules. Theoretical calulations (56) indicate that Brownian motion seems to be largely sufficient to account for the actual migration of the vesicles across the endothelial cell cytoplasm. It was postulated that energy might be required for vesicular formation, and for an active initial intrusion of the vesicles a finite distance into the cytoplasm from one surface. This latter postulate is necessary to avoid the conclusion that the vesicles do not necessarily return an infinite number of times to the cell surface at which they originate, and thus transport their contents abortively. Perhaps this active intrusion is morphologically represented by the stalks of the vesicles, which are about 200 A in length. Thus, for an "intrusion" of 200 A, the probability of successful transport is 10% for a cell 2600 A wide and for vesicles 600 A in diameter.

In making these calculations of transit time on a random basis, many assumptions had to be made, including the viscosity of the cytoplasm of the endothelial cell. Nevertheless a transit time of the order of a few seconds seems feasible. This is at variance with the vesicular "turnover time" of a "few minutes" calculated from the transport rate of large molecules (36). These latter observations are based partly on the assumption that all observed vesicles are simultaneously available for transport in one direction, which neglects the factors of concentration gradient (55) and bidirectional transport (53–55). This would lead to roughly a 3-fold error. But the disparity should probably be attributed principally to delays at the cell surfaces, e.g. the "intrusion" process, during which the vesicles may be filling but are not transporting, and to the possible abortive nature of much of the transport process.

Ultrastructural observations indicate that most of vesicles seen are attached to the plasmalemma (57), and few are apparently free in the cytoplasm. Our own observations with the lanthanum tracer (see later) indicate that even most of those apparently free are connected to a cell surface in another plane of section. It has been inferred from their morphology (57) that vesicles, once formed, remain attached to the cell surface for appreciable lengths of time. The morphology may thus also support the hypothesis of active intrusion of the vesicles into the cytoplasm before successful transport can occur. The fact that few free vesicles are seen supports a rapid transit phase. The over-all rates of transport for large molecules (36) can thus possibly be accounted for by a few vesicles successfully transporting with a rapid transit time, in contrast to all vesicles successfully transporting slowly (36). Net transport in one direction would occur if the extracellular space were considered as a "sump" into which the transported molecules are discharged and diluted. Vesicles transporting quanta to the lumen would thus return with less of the transported material, until equilibrium was reached.

Experimental verification and quantitative data to confirm these hypotheses are currently lacking.

OBSERVATIONS WITH PEROXIDASE: CONTINUOUS CAPILLARIES

Horseradish peroxidase has a molecular weight of about 40,000 and an estimated hydrodynamic radius of about 25 A (2). Therefore, on theoretical grounds, it could be expected to permeate slowly through the endothelial pores,

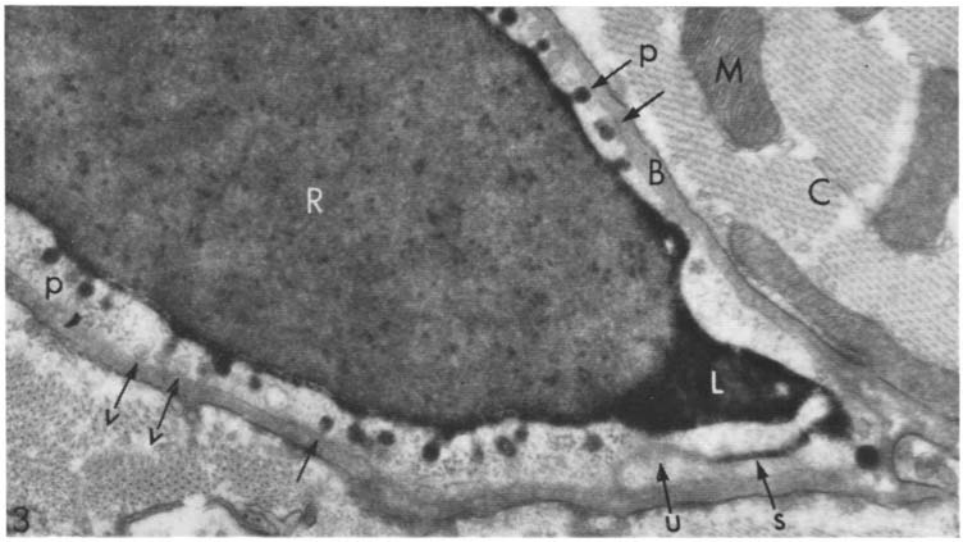

FIGURE 3. Capillary, mouse heart, 5 min after the intravenous injection of horseradish peroxidase. There is peroxidase in the lumen (*L*), around the red blood cell (*R*). Peroxidase staining (*s*) extends about halfway down the intercellular cleft, but the distal half of the cleft is unstained (*u*). Vesicles connected to the luminal surface have filled with peroxidase (*p*). A few vesicles apparently connected to the basal surface are also stained (unlabelled arrows), whereas adjacent basal vesicles are unstained (*v*). The basement itself (*B*) is virtually unstained. Such appearances are suggestive of vesicular transport. *C* = cardiac muscle; *M* = mitochondrion. × 35,000. *Figure reprinted by permission from The Journal of Cell Biology, 1967, 35:213.*

and also be transported by vesicles, if such transport were operative. The observations have been presented in detail elsewhere (2, 58).

3–5 min after the injection of horseradish peroxidase, it was noted in cardiac capillaries that many vesicles opening to the luminal surface contained horseradish peroxidase, and a few at the basal surfaces also stained (Figs. 3 and 4). In addition, the intercellular clefts were stained a considerable distance along their lengths (Fig. 3). From 5 to 15 min after the injection, nearly all the vesicles throughout the cytoplasm stained (Figs. 5 and 6). The intercellular clefts

were stained throughout their lengths (Figs. 5, 7–9), and the basement membrane and extracellular spaces contained considerable amounts of peroxidase (Figs. 5–9). Peroxidase also enters the T system of the cardiac muscle (Fig. 6).

It is possible from these observations to infer that the peroxidase has crossed the endothelium via both the clefts and the vesicles, or by one or the other. If the cleft was impermeable at the level of the cell junction, the peroxidase

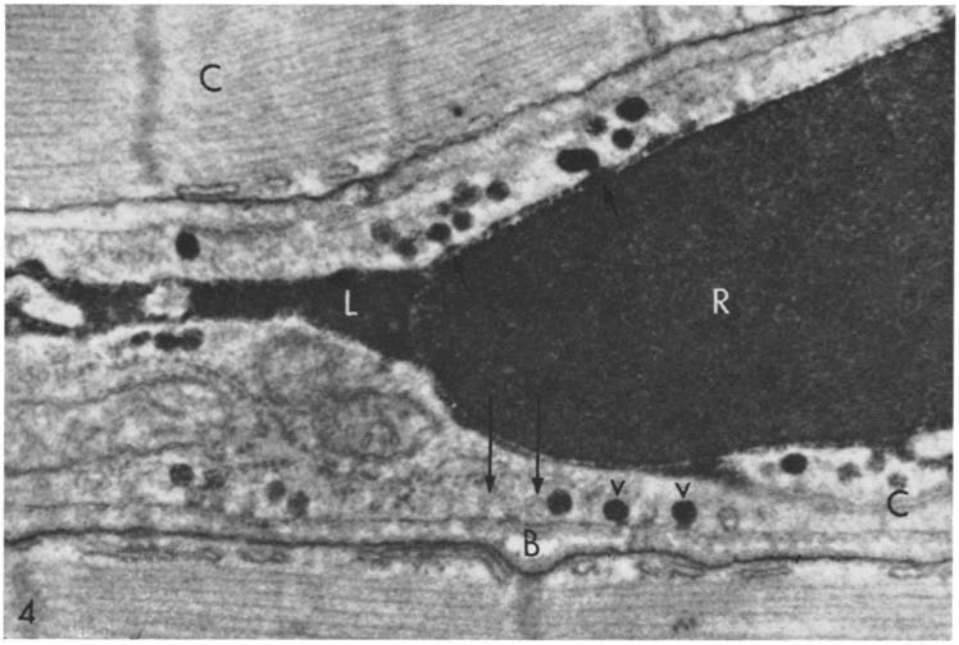

FIGURE 4. Capillary, mouse heart, 5 min after the injection of horseradish peroxidase. The red cell in the lumen (*R*) stains because of the peroxidatic activity of hemoglobin. Peroxidase is present in the lumen (*L*) and fills vesicles opening to the luminal surface (short arrows). A few vesicles opening at, or situated near, the basal surface also stain (*v*), whereas adjacent basal vesicles (long arrows) are unstained. The basement membrane is virtually unstained. The intercellular cleft (lower *C*) is stained only at its luminal end. Such images are taken as suggestive evidence for vesicular transport. Upper *C* = cardiac muscle. × 40,000. *Figure reprinted by permission from The Journal of Cell Biology, 1967, 35:213.*

could have crossed in the vesicles to the basement membrane and thereafter backed up the cleft to the cell junction. Thus the cell junction could have had peroxidase approaching from both sides, and, if it were impermeable, an unstained area should have been seen in the cleft at the level of the cell junction. Such was not observed (Figs. 5, 7–9). However, reaction product may have obscured details. Alternatively, the peroxidase could have left the lumen only via the intercellular cleft, reached the basement membrane, and filled the

basal vesicles in a retrograde fashion, giving the appearance of vesicular transport because all the vesicles would appear to be filled. Most of the vesicles seen in section do appear to open to a surface in or out of the plane of section (see later). To exclude these possibilities, instances were also found (e.g. Figs. 3 and 4) in which stained vesicles were present at the basement membrane adjacent to unstained vesicles, and the basement membrane itself was unstained. This was taken to suggest that vesicular transport had occurred. Conversely, instances were also found (e.g. Fig. 5 of reference 2) in which the intercellular cleft was stained throughout its length, whereas the basal vesi-

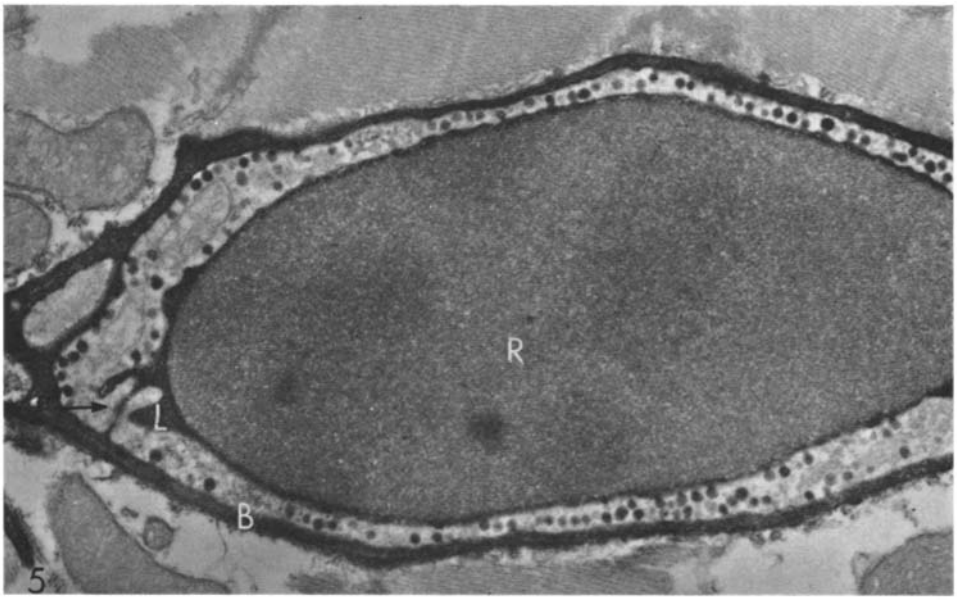

FIGURE 5. Capillary, mouse heart, 10 min after intravenous injection of peroxidase. Peroxidase is present in the lumen (*L*) around the red blood cell (*R*), throughout the intercellular cleft (arrow), and in the basement membrane (*B*). Vesicles at both luminal and basal surfaces and within the cytoplasm are also stained. × 19,000.

cles and the basement membrane were unstained. This was taken to suggest that the intercellular cleft and the cell junction are permeable to peroxidase.

Irrespective of which way the peroxidase is going in the cleft, it appears that the cleft is permeable to the tracer. Because of claims (41, 43–45) that the cell junction in the peripheral capillaries was in the nature of a zonula occludens, it became necessary to examine the cell junctions at high magnification. Such studies showed that the cell junctions were not zonulae occludentes, but that in many instances the intercellular space existed as a small gap about 40 A wide between the adjacent cell membranes (Figs. 10 and 11). In other

instances, the cell membranes appeared to fuse and obliterate the extracellular spaces (e.g. as in Figs. 20–22 of reference 2). It was therefore concluded that the cell junctions consisted of maculae occludentes, that is, discrete areas of cell membrane fusion with gaps in between, rather than continuous areas or zonulae.

To exclude further the possibility that vesicular transport was responsible

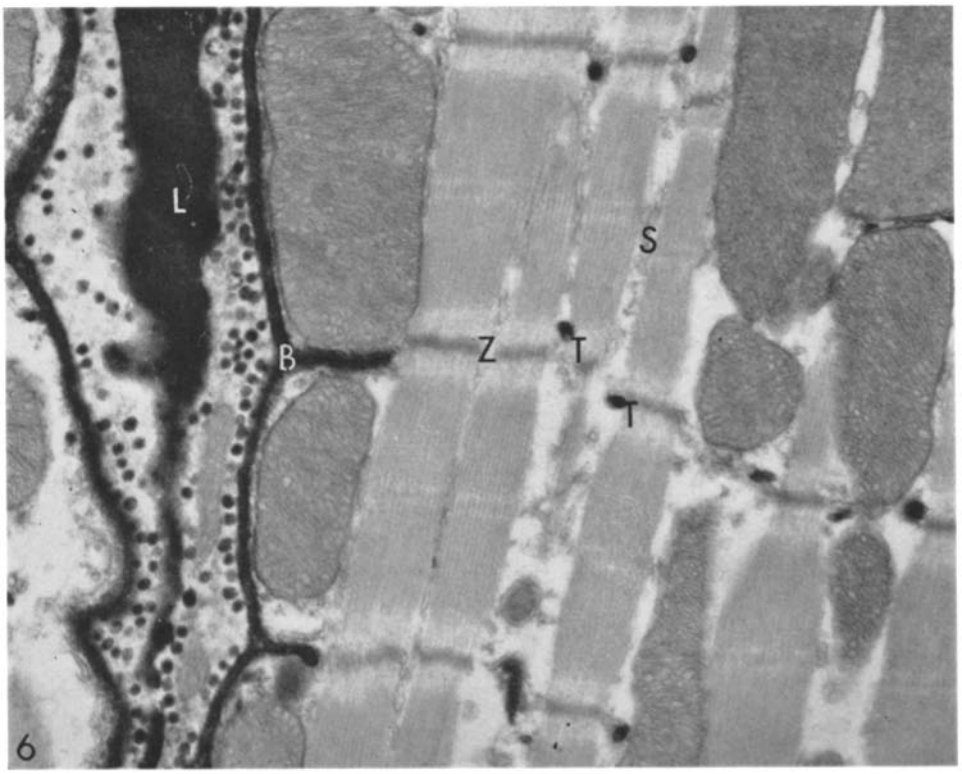

FIGURE 6. Mouse heart, 10 min after intravenous injection of peroxidase. The lumen (*L*) of the capillary, the vesicles, and the basement membranes (*B*) of the capillary and the cardiac muscle contain peroxidase. Peroxidase is also present in the invaginations of the basement membrane at the level of the Z lines (*Z*), and in profiles of the T system (*T*), but not in the sarcoplasmic reticulum (*S*). × 19,000.

in an indirect way for the staining of the intercellular clefts, the lanthanum technique was utilized on prefixed tissues in which vesicular transport was no longer possible. It was found that the lanthanum permeates the basement membrane and runs up the intercellular cleft into the lumen (Figs. 12–15) through a narrowing of the extracellular space, which represents the cell junction (Figs. 13–15). It is noteworthy that many of the vesicles fill with lanthanum (Figs. 12–15), indicating that most of those seen in a section are opening

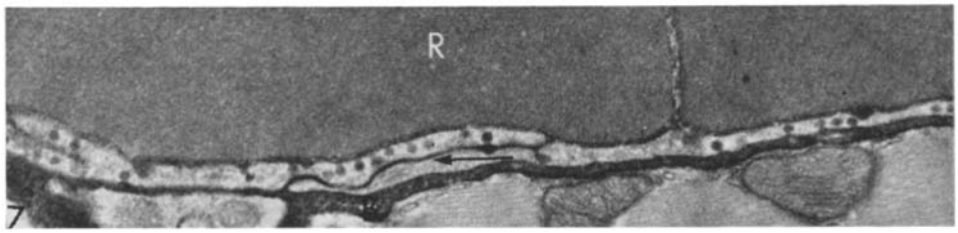

FIGURE 7. Capillary, mouse heart, 8 min after intravenous injection of peroxidase. Peroxidase is present throughout the intercellular cleft (arrow) from the lumen to the basement membrane (*B*). *R* = red blood cell in lumen. × 22,000.

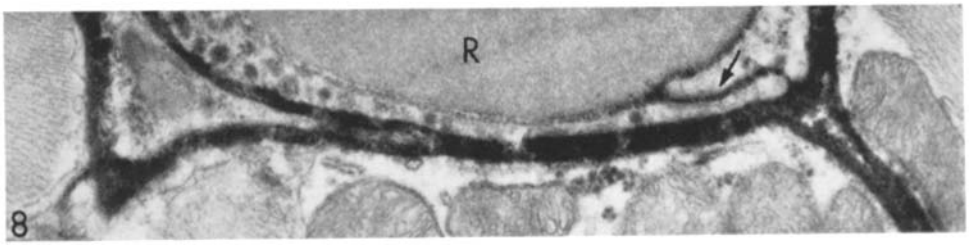

FIGURE 8. Capillary, mouse heart, 15 min after intravenous injection of peroxidase. Staining for peroxidase extends throughout the intercellular cleft (arrow), from the lumen to the basement membrane. *R* = red blood cell in lumen. × 27,000. *Figure reprinted by permission from The Journal of Cell Biology, 1967, 35:213.*

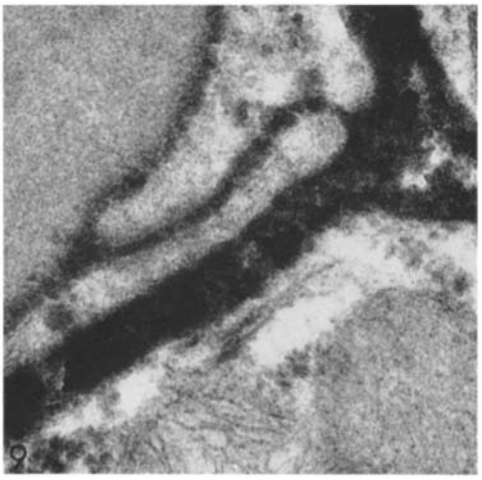

FIGURE 9. Same as Fig. 8, but higher magnification. Note that the staining in the intercellular cleft is continuous from lumen through the region of the cell junction (near the lumen). × 60,000. *Figure reprinted by permission from The Journal of Cell Biology, 1967, 35:213.*

to either the luminal or the basal surface within and without the plane of section. This could also explain much of the vesicular staining seen with the peroxidase technique.

Finally, the blood-brain barrier was studied in regard to the passage of

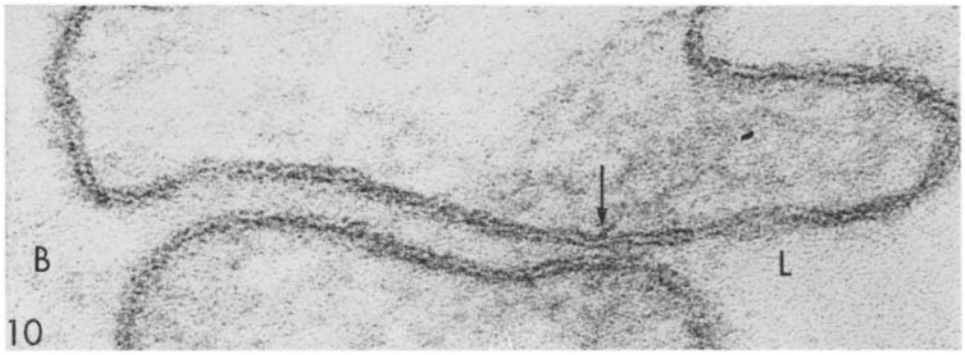

FIGURE 10. Capillary, mouse heart, intercellular cleft. *L* = lumen; *B* = basement membrane. At the cell junction (arrow) situated near the lumen, the adjacent plasma membranes approximate, but do not fuse; i.e. there is an intercellular gap about 40 A in width between the adjacent external leaflets of the unit membranes. This gap frequently contains amorphous, electron-opaque material, as shown here. Fixation and staining as in reference 2. × 200,000. *Figure reprinted by permission from The Journal of Cell Biology, 1967, 35:213.*

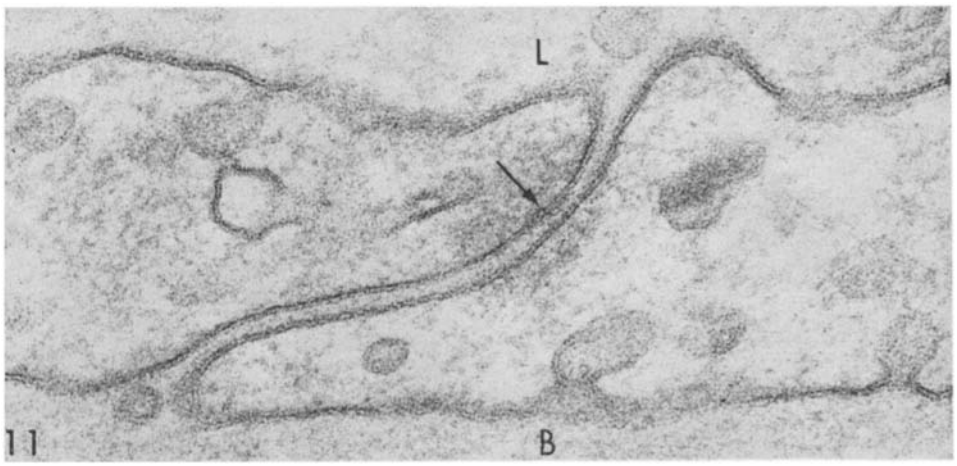

FIGURE 11. Similar to Fig. 10. × 150,000.

peroxidase. It has long been known that a barrier situated somewhere between the blood vessel lumen and the brain parenchyma appears to exclude intravascular injected substances such as protein and dyes bound to protein. Intravenous injection of peroxidase at high dosage, followed for various lengths of time, failed to show any passage of peroxidase into the brain parenchyma (58). Examination of the intercellular clefts of endothelium showed that they were sealed by extensive areas of membrane apposition or fusion,

and in no instances were gaps found (Fig. 16). These cell junctions could be considered to be true zonulae occludentes and are considered by us to be the morphological equivalent of the blood-brain barrier, at least for relatively large molecules. In addition, the relatively few vesicles appear to transport only into the endothelial cell, and not across it. These findings on the mouse

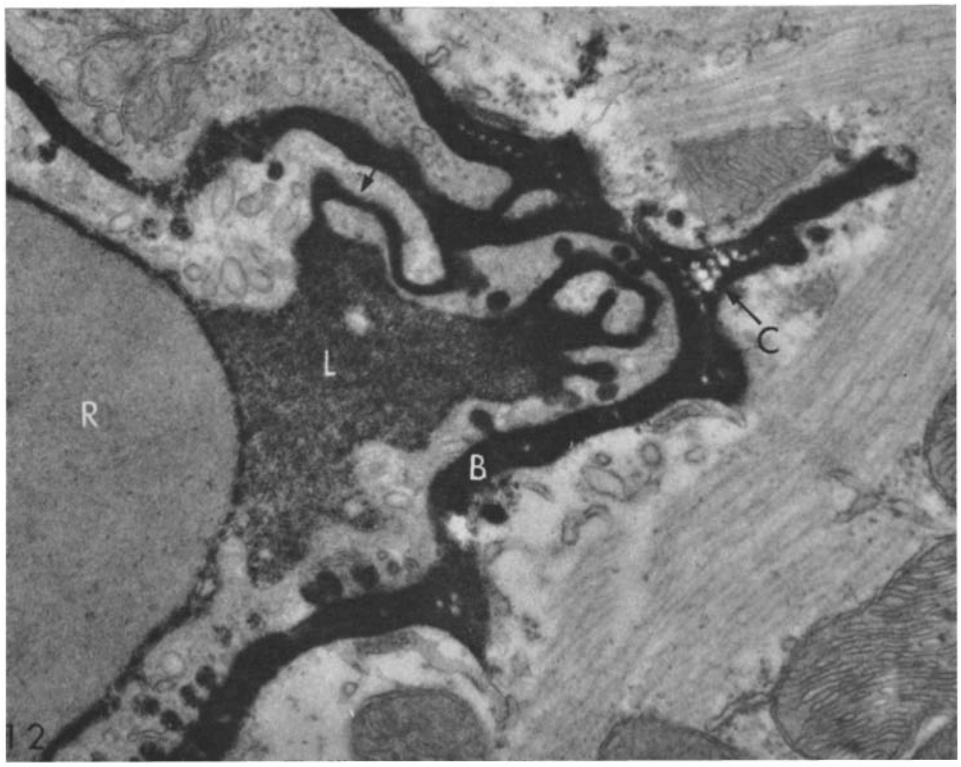

FIGURE 12. Mouse heart, colloidal lanthanum tracer applied to tissue prefixed in formaldehyde-glutaraldehyde. The lanthanum tracer has permeated the basement membranes and extracellular spaces (*B*). The basal vesicles of the endothelium which open to the basement membrane have filled with lanthanum. Collagen fibers in the extracellular space appear as clear areas (*C*), negatively stained by the surrounding lanthanum. Lanthanum fills the intercellular cleft (arrow) from the basement membrane to the lumen (*L*), and surrounds the red blood cell (*R*). Some vesicles opening to the lumen have also filled with tracer; others, apparently lying free in the cytoplasm, are unstained. × 45,000.

brain have been confirmed for the urodele brain (59), and similar cell junctions have been reported in retinal capillaries (60).

For technical reasons (2), most of the observations on "continuous" capillaries were made in the heart. It should be noted that the permeability characteristics of the cardiac capillaries have been shown to be similar to those of skeletal muscle (61).

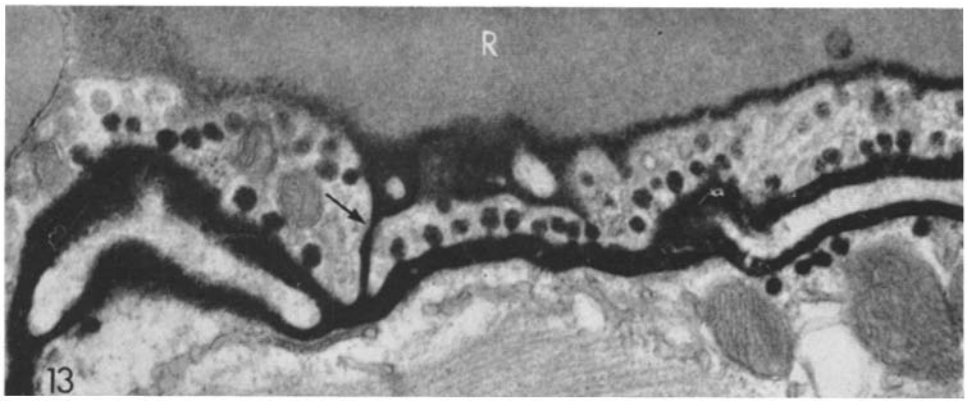

FIGURE 13. Mouse heart, lanthanum tracer applied to tissue after prefixation in formaldehyde-glutaraldehyde. The tracer has permeated the basement membranes and extracellular spaces. Collagen fibers are outlined by negative staining and appear as light lines (to the left). Endothelial vesicles opening to the basement membrane have filled with tracer. Note how some stained vesicles are situated apparently free in the cytoplasm, at a distance from the luminal and basal surfaces. They probably connect with a surface in a different plane of section. The lanthanum has apparently passed through the intercellular cleft into the lumen. The narrow waist in the lanthanum line (arrow) represents passage through the cell junction. The lumen shows less intense lanthanum staining, as do vesicles opening to the luminal surface. *R* = red blood cell. × 29,000. *Figure reprinted by permission from The Journal of Cell Biology, 1967, 35:213.*

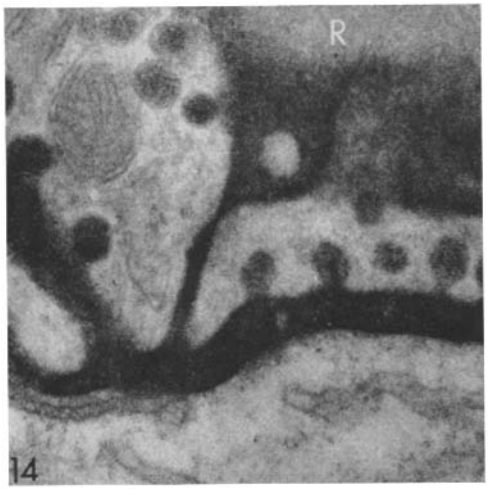

FIGURE 14. Higher magnification of the intercellular cleft shown in Fig. 13. Note lanthanum staining throughout the cleft, narrowing at the cell junction. The width of the lanthanum line at the level of the cell junction is about 65 A. It is thought (2, 4) that the lanthanum occupies both the intercellular gap at the level of the junctions (40 A) and a portion or all of both adjacent external leaflets (15 A each). *R* = red blood cell in lumen. × 87,000. *Figure reprinted by permission from The Journal of Cell Biology, 1967, 35:213.*

Observations similar to those made on cardiac capillaries were made on capillaries of skeletal muscle (2) and lung (62). In the latter, peroxidase does not enter the alveolar spaces, because the intercellular space between adjacent epithelial cells is apparently sealed by zonulae occludentes situated toward

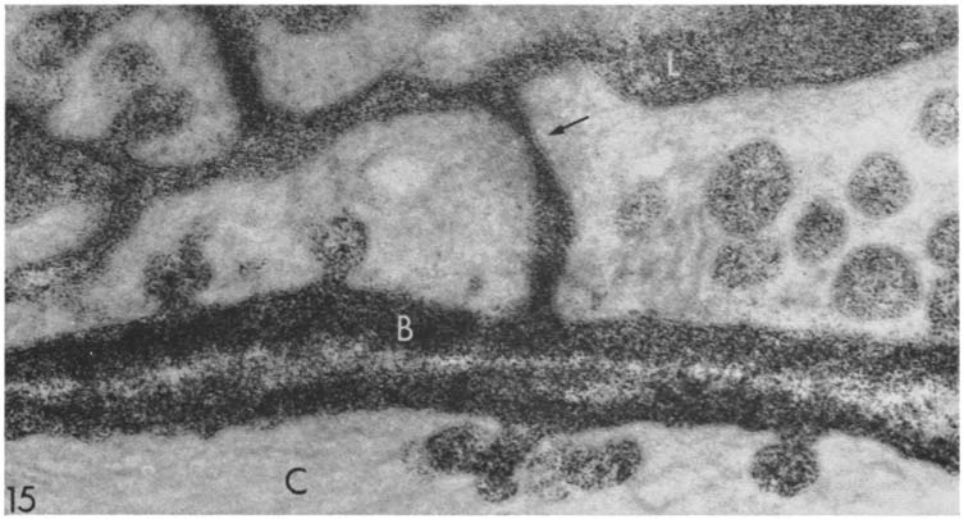

FIGURE 15. Similar to Figs. 12–14. Lanthanum fills the basement membranes (*B*), but there is little in the extracellular space between the basement membrane of the capillary and that of the cardiac muscle (*C*). The intercellular cleft is filled with lanthanum, including the narrow cell junction (arrow). The lumen (*L*) of the capillary contains lanthanum and is collapsed. × 130,000.

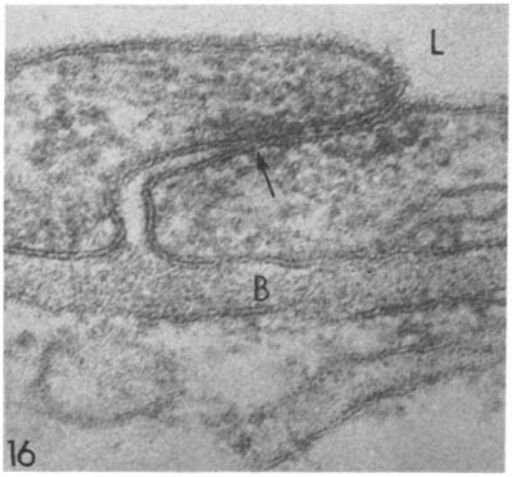

FIGURE 16. Mouse cerebrum, endothelium. The extracellular space between the adjacent endothelial cells is completely obliterated by an extensive tight junction (arrow), i.e. a five-layered structure representing close apposition or fusion of adjacent external leaflets. The width of the tight junction is less than twice the width of the nearby unit membrane, giving a ratio of about 1.7. *L* = lumen; *B* = basement membrane. × 170,000. *Figure reprinted by permission from The Journal of Cell Biology, 1967, 34:207.*

the alveolar lumen. Similar observations have been made on the choroid plexus (63).

The mesothelium has permeability characteristics quite similar to those of "continuous" endothelium (64), although recent data indicate that a component of the exchange across the mesothelium may be active (65). Indeed, Starling's original hypothesis concerning transendothelial exchanges was based on studies on the mesentery.

When the peritoneal surface of mesothelium was exposed to peroxidase, the tracer passed in a few minutes via the intercellular clefts into the basal tissue spaces (14, 66, 67). Evidence for vesicular transport in this system was minimal, if not absent.

In summary, then, it may be stated that in continuous capillaries the intercellular clefts were (*a*) permeable to peroxidase, (*b*) permeable to lanthanum tracer, and (*c*) not sealed by zonulae occludentes, and were therefore inferred to be in all probability the morphological equivalent of the small-pore system of the physiologists. Conversely, the sealing of the intercellular cleft in the endothelium of brain capillaries, and minimal vesicular transport, represents the morphological equivalent of the blood-brain barrier, at least for relatively large molecules. In addition, suggestive evidence for slow vesicular transport in peripheral "continuous" capillaries was noted, in support of the observations of others (7–11). This may represent the large-pore system in these types of capillaries (36).

FENESTRATED AND DISCONTINUOUS ENDOTHELIUM

The permeability of capillary beds varies in different areas. Physiological measurements (35) of the permeability of the cervical, intestinal, and hepatic capillary beds to dextrans showed increasing permeability to high molecular weight dextrans of the capillary beds in the above order. Each of these three capillary beds represents a mixed population of capillary types, but they are constituted primarily as follows: cervical—"continuous" type; intestinal—fenestrated type; hepatic—discontinuous (sinusoidal) type.

It is not surprising, perhaps, that the hepatic capillary bed is so permeable to large molecules, as the gaps in the endothelium are extremely large (1–10,000 A) and the basement membrane is discontinuous (5, 68, 69). Peroxidase (Fig. 17) and other tracers (68) readily gain entry to the space of Disse. Other discontinuous capillary beds, e.g. bone marrow spleen, have not been studied.

Fenestrated capillaries are widely distributed, being present in three main types of organs: (*a*) endocrine glands; (*b*) structures involved in fluid exchanges, e.g. glomerulus, ciliary body, intestine, and choroid plexus; and (*c*) retia mirabilia, e.g. renal medulla and fish swim bladder. The endothelium of

fenestrated capillaries is often attenuated (200–400 A wide) and relatively lacking in organelles and micropinocytotic vesicles (Fig. 18). The fenestrae pierce the endothelium, and are 200–1000 A in diameter. They are usually closed by a thin membrane or diaphragm, which is about 40 A in width, and which has been suggested to represent a polysaccharide or mucoprotein component of the external leaflets of the adjacent unit membrane extending

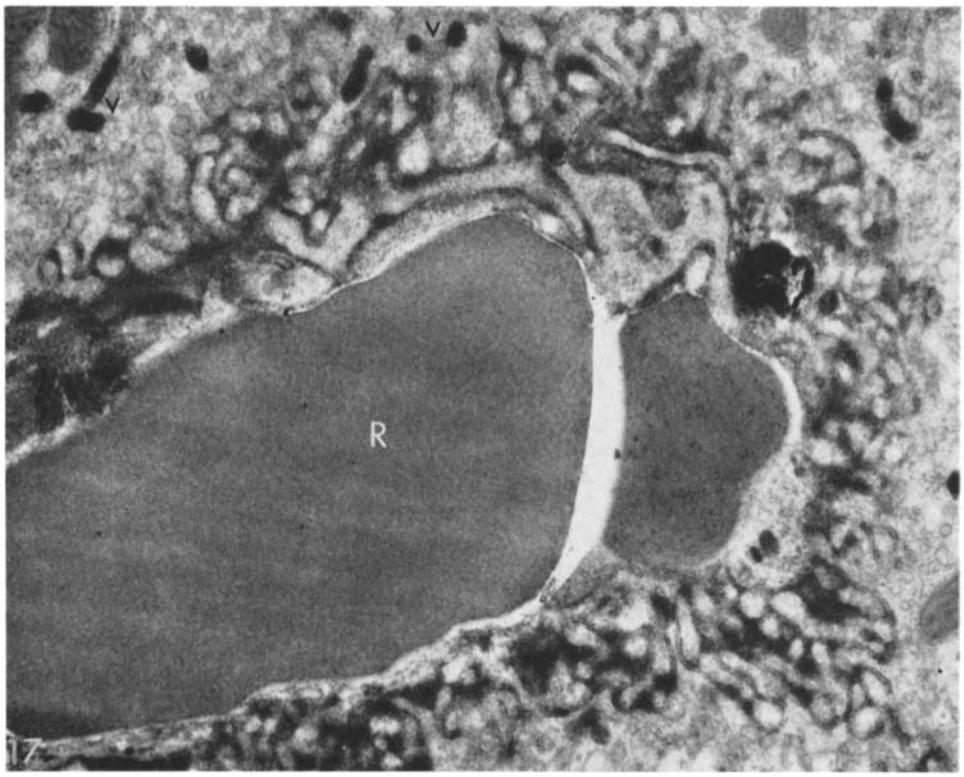

FIGURE 17. Mouse liver, sinusoidal (discontinuous) capillary, 5 min after the intravenous injection of peroxidase. The black, lacy network surrounding the capillary is the space of Disse filled with peroxidase. The reaction product outlines the microvilli on the hepatocyte surface. Within the cytoplasm of the liver cell, a few round and tubular profiles contain peroxidase (*v*). *R* = blood cell in lumen. × 18,750.

across the gap (12). In the glomerular capillaries, the diaphragm is absent, at least in the rat (5), but may be present in the mouse (70).

The fenestrae appear to occupy a considerable, but variable, proportion of the surface area of the capillaries. The proportion of the surface area of the capillaries occupied by fenestrae is known only in regard to the capillaries of the renal medulla, where it has been calculated that the fenestrae occupy 35% of the available area (70). Various interpretations have been given as to the

nature and formation of the diaphragms (5–12). The basement membrane of the fenestrated endothelium is continuous.

As mentioned above, physiological experiments have indicated (35) that capillary beds containing fenestrated capillaries are more permeable than those which have a continuous endothelium. Ultrastructural observations utilizing electron-opaque tracers have indicated that the open fenestrae of the glomerulus are freely permeable to tracers such as ferritin and colloidal gold, which appear to pass relatively unhindered through the fenestrae and, within minutes after the intravenous injection, are found within or lodged against the basement membrane (48, 71–74). In other capillaries, it has been claimed that neither ferritin (9) nor particles of Thorotrast (50) accumulate at the site of the fenestrae or pass through them, an exception being the peritubular capillaries of the mouse kidney, which were thought to be highly permeable to ferritin (Fig. 19) which was presumed to have passed through

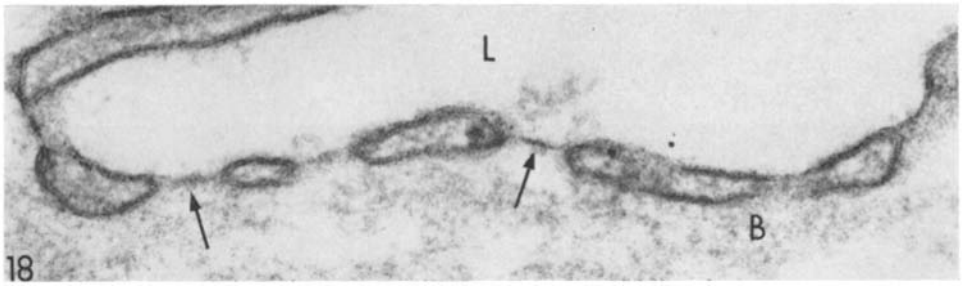

FIGURE 18. Mouse kidney, fenestrated, peritubular capillary. The fenestrae (arrows) are closed by a thin membrane. *L* = lumen; *B* = basement membrane. × 138,000.

the fenestrae (9). Similar observations were made on the fenestrated capillaries of the adrenal (8). In the same experiments, however, it was found that the fenestrated capillaries of the pancreas and the colon, for instance, did not allow the passage of ferritin. It has also been claimed that the fenestrae of the capillaries of the choroid plexus appear to be impermeable to horseradish peroxidase (75), although the vessels are permeable to the protein.

OBSERVATIONS WITH PEROXIDASE: FENESTRATED CAPILLARIES

We have confirmed the apparent permeability of the fenestrae of the peritubular capillaries in the kidney with the horseradish peroxidase technique. Minutes after the intravenous injection of the tracer, much peroxidase was found in the basement membranes of the extracellular spaces, and the fenestrae were filled with tracer (Figs. 20 and 21). The intercellular clefts were also stained, indicating their possible role as a pathway as well. The vesicles,

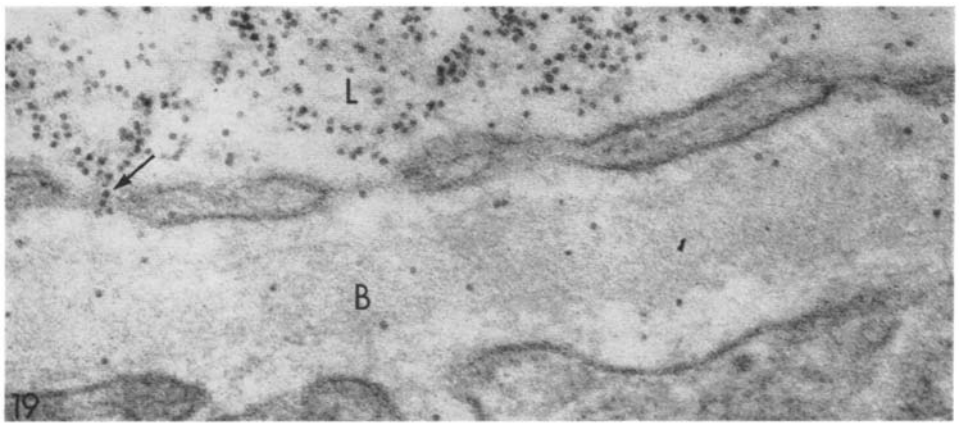

FIGURE 19. Mouse kidney, fenestrated peritubular capillary, 8 min after intravenous injection of ferritin. There is much ferritin in the lumen (*L*), which is apparently passing through the fenestrae (arrow). There are a few ferritin particles lodged in the basement membrane (*B*). × 135,000.

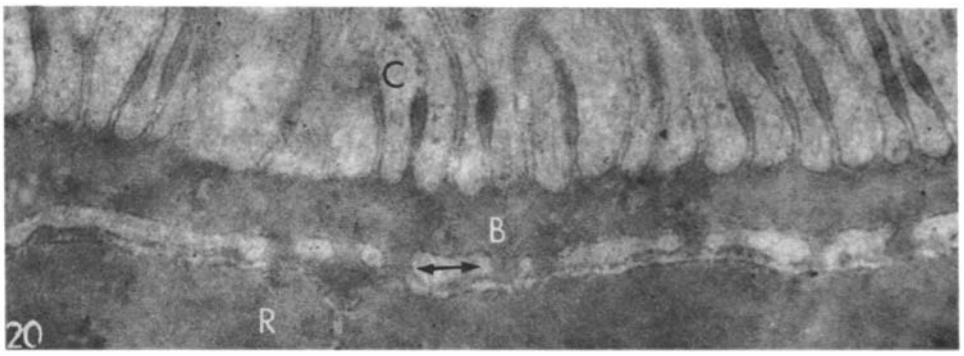

FIGURE 20. Mouse kidney, fenestrated peritubular capillary, 10 min after the intravenous injection of peroxidase. Peroxidase is present in the lumen around the red blood cells (*R*), and is apparently passing through the fenestrae (arrows), filling the basement membrane (*B*). The peroxidase also fills the extracellular spaces between the basal membranous infoldings of the renal tubular cell (*C*). × 25,000.

which were sparse in these capillaries, also contained peroxidase. Similar observations were made on the fenestrated capillaries of the sand rat (Fig. 22). Once again it was possible that the tracer had passed across the endothelium by one or more of three pathways, and that there was staining of the others by retrograde filling, giving the appearance of passage from the lumen to the basement membrane. The lanthanum tracer applied to fixed tissues excluded

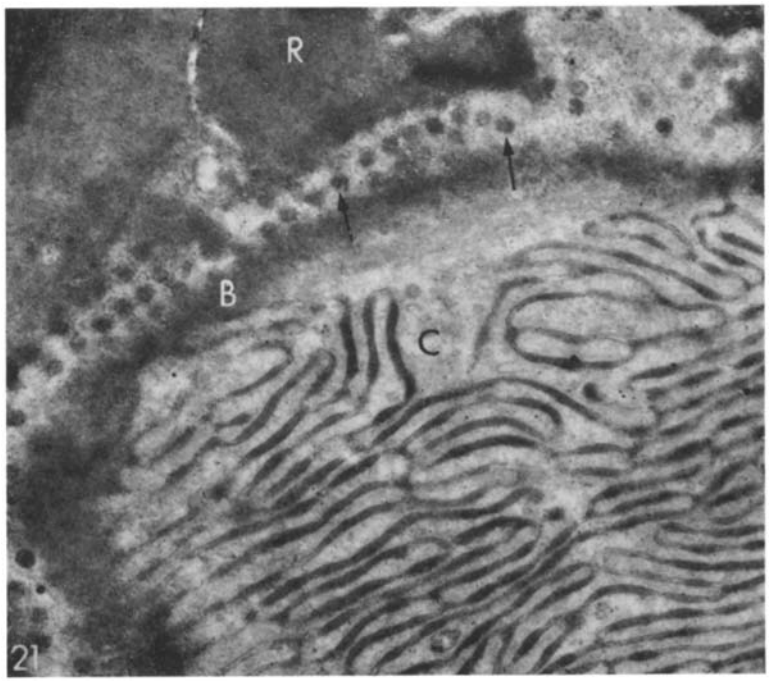

FIGURE 21. Same as Fig. 20. The endothelium is cut obliquely, and the fenestrae (arrows) are seen as round profiles penetrating the endothelium. The basement membrane (*B*) and the extracellular space between the basal membranous infoldings of the renal tubular cell (*C*) are permeated by peroxidase. *R* = red blood cell in lumen. × 25,000.

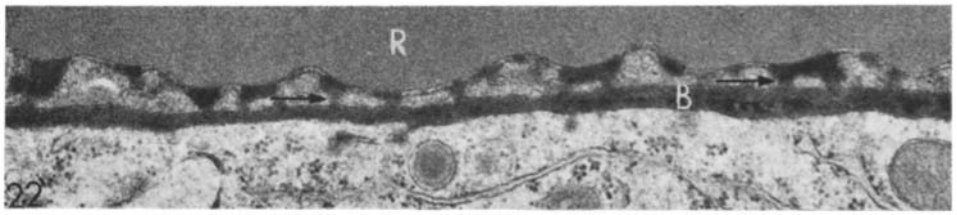

FIGURE 22. Egyptian sand rat (*Psammonys obesus*), pancreas, fenestrated capillary, 60 min after intravenous injection of peroxidase. Peroxidase is present in the lumen around the red blood cell (*R*), in the fenestrae (arrows), and the basement membrane (*B*). × 27,500. *Micrograph courtesy of Dr. Arthur T. Like.*

vesicular transport, and indicated that both the fenestrae and the intercellular clefts were permeable (Fig. 23). With neither the peroxidase nor the lanthanum technique could an unstained line, representing an impermeable diaphragm, be detected, and it was considered unlikely that staining in the

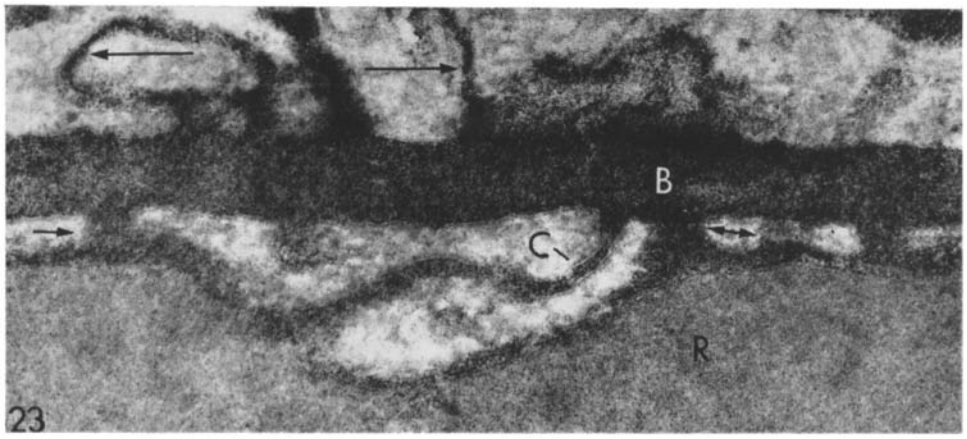

FIGURE 23. Mouse kidney, peritubular fenestrated capillary, treated with lanthanum tracer after prefixation in formaldehyde-glutaraldehyde. Lanthanum has permeated the basement membrane (*B*), the fenestrae (short arrows), the intercellular cleft (*C*), and the lumen around the red blood cell (*R*). The extracellular space between the membranous basal infoldings of the renal tubular cell also contains lanthanum (long arrows). × 174,000.

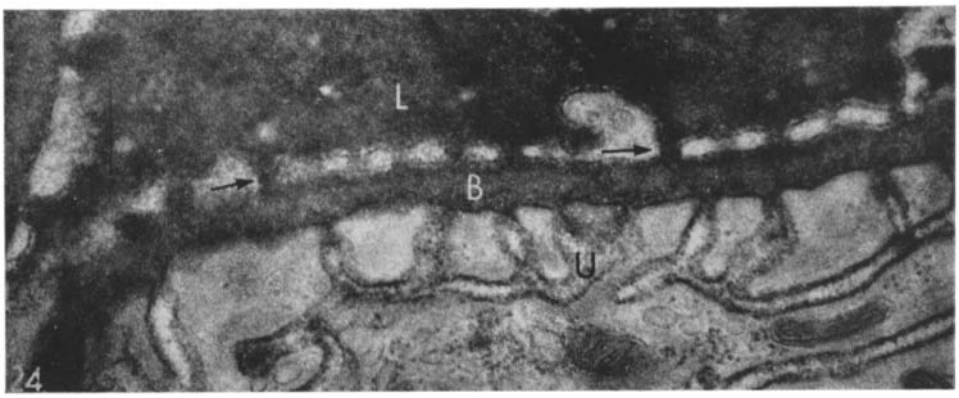

FIGURE 24. Mouse kidney, glomerular fenestrated capillary, 3 min after the intravenous injection of peroxidase. Peroxidase is present in the lumen (*L*), the fenestrae (arrows), and the basement membrane (*B*), and has entered the urinary space (*U*). Reabsorption of peroxidase from the tubular lumina into proximal tubular cells can be detected at this stage (1, 26). × 25,000. *Figure reprinted by permission from The Journal of Experimental Medicine, 1966, 124:1123.*

fenestrae merely represented filling from both sides of an impermeable diaphragm. In all probability, the fenestrae were the major pathway responsible for the relatively rapid transendothelial passage of the peroxidase, as they occupy a high proportion of the surface. The results were also in accord with those obtained with ferritin (Fig. 19) (9). Nevertheless, differences may exist

(8, 9, 50, 75) in fenestrated capillaries at other sites, despite apparent structural similarities. A systematic investigation of fenestrated capillaries in various sites and species has not been made.

In regard to the open fenestrae of the glomerular capillaries, we found that in the mouse the endothelial pores were permeable not only to horseradish peroxidase (Fig. 24) but also to myeloperoxidase, which has a molecular weight of 160,000. This is in accord with previous work with ferritin (mol wt 500,000) (74). Both the peroxidase tracers appeared to permeate the basement membrane relatively freely, but myeloperoxidase in our estimation was restricted in its passage by the epithelial slits, whereas the smaller molecule, horseradish peroxidase, freely reached the urinary spaces (26).

In summary, it appears, at least for those fenestrated capillaries studied, that the fenestrae may well account for the higher permeability of these types of capillaries to large molecules.

GENERAL SUMMARY

A brief review of the fine structure and permeability characteristics of capillaries is given. Capillary beds may vary greatly in structural and permeability characteristics at various sites, and particular capillary beds may be composed of structurally mixed populations of capillaries. Various views held to date on the ultrastructural basis of transcapillary exchanges are discussed. Observations based on the use of intravenously injected horseradish peroxidase (mol wt 40,000) as an ultrastructural tracer are presented.

In capillaries with "continuous" endothelium (e.g. those of cardiac and skeletal muscle), peroxidase appears to pass through the cell junctions and the intercellular clefts. The cell junctions are patent at intervals, and are thus interpreted to be maculae occludentes, rather than zonulae occludentes, with a gap of about 40 A in width between the maculae. It is inferred that the cell junctions are probably the morphological equivalent of the small-pore system proposed by physiologists for the passage of small, lipid-insoluble molecules. Suggestive evidence for slow vesicular transport was also obtained. Vesicular transport may thus be responsible for slow transendothelial passage of large molecules, and represent the large-pore system proposed by physiologists.

Peroxidase did not cross the endothelium of the "continuous" capillaries in the brain, either via intercellular clefts, which are thought to be sealed by zonulae occludentes, or by vesicular transport, and it is proposed that the blood-brain barrier, at least for molecules the size of peroxidase or larger, resides at the level of the endothelium.

Peroxidase appeared to pass readily through the fenestrae of the fenestrated capillaries studied, i.e. those in the glomerulus, pancreas, and peritubular capillaries of the kidney. The increased permeability to large molecules of

capillary beds consisting largely of fenestrated capillaries is related to the relatively large area occupied by the fenestrae.

The high permeability of capillaries with discontinuous endothelium (e.g. sinusoids of liver) is attributed to the presence of large gaps between adjacent cells, and, to some extent, to the discontinuous nature of the basement membrane.

The continuous basement membrane of "continuous" and fenestrated capillaries may act as a relatively coarse filter for large molecules.

It is apparent that there is great variation in the structure of capillaries, and differences in the permeabilities of capillary beds can be related to variations in structure. Such considerations should be kept in mind whem comparing the permeabilities of capillary beds. The concept of a "uniform pore" is useful in physiological thinking, but needs to be translated into morphological terms. For instance, the "large-pore" system in "continuous" capillaries may be represented by vesicular transport, whereas in other capillary beds fenestrae or large interendothelial gaps may be responsible. Moreover, in any one capillary bed, the population of capillaries may be heterogeneous.

Although there is good over-all correlation between the structure and permeability of capillaries, further work is required to establish this more firmly. Collaborative studies between physiologists and morphologists would be desirable to account for the permeability characteristics of a particular capillary bed.

It is also necessary to have new and better ultrastructural tracers, especially in a molecular size range approximating the substances used in physiological experiments. Furthermore, structural features which may correlate with the subtleties of permeability differences, such as the permeability gradient between the arterial and venous ends of single capillaries (37–40), species differences, and the problems of water exchanges (33), remain to be investigated.

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Discussion

Question from the Floor: Have you considered using cryogenic techniques with small molecules, and a radioautographic approach for use in this problem?

Dr. Karnovsky: We've considered using them but we haven't really done it. I think

we are a little scared that at the ultrastructural level, even with cryogenic techniques, some shifts of soluble small molecules might occur. Furthermore, the resolution of current electron microscopic radioautography may not be sufficient for our purposes. I think this is something that could and should be tried.

What we have tried to do is on the other side of the scale, namely, to hook the peroxidase onto various other proteins. We can possibly do this with some coupling reagents, and we can hope to make molecules of different sizes which have the enzyme attached. For instance, we can hook it onto γ-globulin by the method of Nakane and Pierce (1967. *J. Cell Biol.* **33**:307) using a difluorodinitrodiphenylsulfone coupler. The yields are small, and there are technical problems, but the method looks promising for making larger tracers. It is also possible that we could break the peroxidase up into smaller moieties which are still enzymatically active for use as smaller tracers.

Dr. Zierler: My question should be preceded by a statement of fact from either Dr. Chinard or Dr. Renkin. It has to do with the adequacy of the total diameter of clefts to account for the observed movement of water across the capillary. Can it be calculated, from observed data on movement of tritiated water across the capillary, how much surface area ought to be available for that movement, and is there then an adequate surface area in the junctions?

Dr. Chinard: Very briefly, the calculations made by Pappenheimer and his associates indicated that the net flux of water can be accounted for by passage through pores, if such exist, across the cells or at the cell junctions through clefts. With respect to the transcapillary exchange of water, I don't think a definite answer can be given. We do not know the number of times a water molecule crosses back and forth across the junction between the vascular and extravascular compartments.

I would like to put a question to Dr. Karnovsky. He has shown the way in which peroxidase may or may not get across. We have to make some guesses as to the regions and areas that are involved with respect to the passage of water and ions. There is a pressure difference between a vascular compartment and an extravascular compartment, as Dr. Wiederhielm has indicated quite clearly. Where do you think that pressure gradient is, in view of your knowledge of the electron microscope picture? Is it at the basement membrane, across all the basement membrane, or where?

Dr. Karnovsky: This is something we don't know, and I don't think we have any way of really telling from these pictures. All we can really show is a possible pathway of passage, but I don't think we can go farther than that.

In regard to the pore area, I think Dr. Pappenheimer's figure is less than 0.1 % of the capillary surface, which is compatible if you make assumptions and calculate out the area occupied by these junctions. In regard to water exchange, as I understand it as a nonphysiologist, this is rather disputed. I think Yudilevich and Alvarez (1967. *Am. J. Physiol.* **213**:308), for instance, claim that the area of capillary surface involved in water exchange is greater than can become accounted for by the pores. So in this regard the pore area is too small, if they are correct.

Dr. A. L. Copley: It was of interest to me that what Dr. Karnovsky said about the horseradish peroxidase, and it may interest this audience that several years ago we developed and described a quantitative test for capillary permeability of skin tissue using horseradish peroxidase (COPLEY, A. L., and B. CAROL. 1964. *Life Sci.* **3**:65).

Also, if I understand you correctly, Dr. Karnovsky, in regard to the work of Dr. Luft about mucopolysaccharides, you did not find any such substances surrounding the endothelial cell. I think that Luft bases his work on the precipitate formed when ruthenium red reacts with mucopolysaccharides. Dr. Scheinthal, Mr. Polanskyj, and I have found, in my laboratory, that quite a number of substances, such as proteins, proteinpolysaccharides, and protein-free carbohydrates, can react with ruthenium red. So I don't see how specific the ruthenium red reaction can be, other than its specificity for anionic groups.