STUDIES ON BLOOD CAPILLARIES

II. Transport of Ferritin Molecules across the Wall of Muscle Capillaries

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ABSTRACT

The pathway by which intravenously injected ferritin molecules move from the blood plasma across the capillary wall has been investigated in the muscle of the rat diaphragm. At 2 min after administration, the ferritin molecules are evenly distributed in high concentration in the blood plasma of capillaries and occur within vesicles along the blood front of the endothelium. At the 10-min time point, a small number of molecules appear in the adventitia, and by 60 min they are relatively numerous in the adventitia and in phagocytic vesicles and vacuoles of adventitial macrophages. Thereafter, the amount of ferritin in the adventitia and pericapillary regions gradually increases so that at 1 day the concentration in the extracellular spaces approaches that in the blood plasma. Macrophages and, to a lesser extent, fibroblasts contain large amounts of ferritin. 4 days after administration, ferritin appears to be cleared from the blood and from the capillary walls, but it still persists in the adventitial macrophages and fibroblasts. At all time points examined, ferritin molecules within the endothelial tunic were restricted to vesicles or to occasional multivesicular or dense bodies; they were not found in intercellular junctions or within the cytoplasmic matrix. Ferritin molecules did not accumulate within or against the basement membranes. Over the time period studied, the concentration of ferritin in the blood decreased, first rapidly, then slowly, in two apparently exponential phases. Liver and spleen removed large amounts of ferritin from the blood. Diaphragms fixed at time points from 10 min to 1 day, stained for iron by the Prussian Blue method, and prepared as cleared whole mounts, showed a progressive and even accumulation of ferritin in adventitial macrophages along the entire capillary network. These findings indicate: (1) that endothelial cell vesicles are the structural equivalent of the large pore system postulated in the pore theory of capillary permeability; (2) that the basement membrane is not a structural restraint in the movement of ferritin molecules across the capillary wall; (3) that transport of ferritin occurs uniformly along the entire length of the capillary; and (4) that the adventitial macrophages monitor the capillary filtrate and partially clear it of the tracer.

INTRODUCTION

In a preceding paper (1), we have described in detail the organization of blood capillaries in mammalian muscle and have discussed the bearing of our findings on the pore theory of capillary permeability. This theory explains the permeability characteristics of all blood capillaries by assuming the existence of two pore systems (2, 3) of which one consists of small (diam $\simeq$90 A), and the

other of large (diam $\simeq$250–700 A) waterfilled channels. The latter system is envisaged as the only passageway for molecules with a diameter larger than $\sim$80 A (2–5). The fractional luminal area presumably occupied by such pores is so small ($\lesssim$3 x 10^{-5}) in muscle capillaries that their detection and identification by direct electron microscopy become impractical and unreliable, notwithstanding the assumed large diameter of the channels (cf. 1).

Electron microscope observations have already indicated, however, that molecules and colloidal particles up to 300 A in diameter are transported across the continuous endothelium of muscle capillaries in plasmalemmal vesicles (6–9).

In the present study, we have reinvestigated this problem by following the pathway taken by ferritin molecules across the capillary wall in their movement from vascular lumina to pericapillary spaces. These molecules are of appropriate size to serve as a reliable probe for the large pore system.

The results indicate[1] that ferritin is restricted to plasmalemmal vesicles while in transit through the endothelium and support the view that these vesicles function in the transport of macromolecules across the capillary wall. They perform, therefore, in muscle capillaries the function ascribed to the large pore system by the pore theory.

MATERIALS AND METHODS

Materials

All experiments were carried out on young adult male rats of the Sprague-Dawley strain.

We selected the diaphragm as a study object because of its rich capillary network and ease with which it can be fixed in situ (1). This type of fixation insures the retention of plasma and tracer molecules in the vascular lumina which is a prerequisite for the study envisaged. In addition, in young animals weighing 100 g or less, the flat muscle is thin enough to give whole-mount preparations on which topographical studies of the vascular system can be carried out by light microscopy.

We chose ferritin as a particulate tracer for the following reasons: (a) it has the size required for a probe molecule of the large pore system: the diameter of the molecule is $\sim$110 A (11, 12); (b) it is easily detected and identified by electron microscopy on account of the electron-opaque ferric hydroxide

phosphate "micelles" disposed in the center of the molecule (13, 14); (c) it represents a particle population of remarkably uniform size; (d) it is a biological substance, a protein, similar in nature and comparable in size to most plasma proteins[2]; (e) it is well tolerated by the experimental animals so that high concentrations can be achieved in the plasma without ill effects; (f) finally, it remains in high concentration in the circulating blood for at least 24 hr.

Colloidal tracers (carbon, gold, mercuric sulfide) are less desirable because of their nonbiological nature and of the wide range of particle sizes. The range is 200–500 A for carbon (18), 30–250 A for gold (7), and 70–350 A for mercuric sulfide (19) particles. In addition, it is difficult to achieve and maintain a high concentration of such tracers in blood plasma.

PREPARATION OF FERRITIN STOCK SOLUTIONS: Horse spleen ferritin obtained from Nutritional Biochemicals (Cleveland, Ohio), Gallard-Schlesinger (Carle Place, New York), or Pentex (Kankakee, Michigan) as a 5 or 10% solution was dialyzed against 0.1 M ethylenediaminetetraacetate for the purpose of removing the Cd used for the crystallization of the protein (20). After this step, the dialysis sack was sealed closed to the ferritin solution (for the prevention of subsequent dilution), and further dialysis carried on for 24–48 hr at $\sim$4°C against several changes of 0.1 M phosphate buffer, pH 7.2. Before Cd-removal, the ferritin solution is toxic and, depending on batches, it causes shock or death in a variable number of animals; after Cd-removal, it is perfectly tolerated at all dosages so far used.[3]

The concentration of ferritin in the dialyzed stock solution was determined spectrophotometrically at 350 mμ and found to be $\sim$100 mg/ml (range: 79 to 108 mg/ml).

Experimental Procedure

Under ether anesthesia, 1 ml of stock solution/100 g body weight was slowly (1–2 min) injected into the saphenous vein of young rats weighing 100–150 g. The volume injected was estimated at $\sim$12% of

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[1] In preliminary form our findings were presented in reference 10.

[2] The diameter of albumin has been estimated at 63 A by small-angle scattering of X-rays (15) and measured at 65 X 90 A in electron micrographs (16). The dimensions of γ-globulin, measured on electron micrographs, are given as 200 X $\sim$40 A (17). The diameters of γG-globulin, α_2-macroglobulin, and 19 S γM-globulin are 60 X 150 A, 200 A, and 200 X 300 A, respectively, as determined by electron microscopy in metal-shadowed specimens (16).

[3] Because of the diluent used, the injection is expected to result in a temporary increase of blood phosphate, which appears to be perfectly tolerated by the animals.

the total blood volume (21). When van't Hoff's equation for dilute solutions (π = cRT is used), the calculated contribution of ferritin to the colloid osmotic pressure of the plasma is $\sim$1.4 cm H_2O, or $\sim$4% of the normal value (see Fig. 2). Hence, the effect of ferritin injection, on water distribution between plasma and interstitial fluid, is considered negligible.

At the time points indicated in Table I, the diaphragms were fixed in situ by injecting a 6.5% glutaraldehyde solution (in 0.1 M phosphate buffer, pH 7.4) into the peritoneal and pleural cavities. The tissue was subsequently prepared for electron microscopy as indicated in the preceding paper (1).

For intervals up to 10 min, the rats were kept under anesthesia until the injection of fixative; for longer intervals, the animals were allowed to recover from the anesthetic after the ferritin injection and reanesthetized immediately prior to the injection of the fixative.

To estimate the extent of ferritin uptake by the liver and spleen, we fixed samples of these organs and processed them for electron microscopy at several time points (Table I).

TOPOLOGICAL STUDY OF THE VASCULATURE: To study the distribution of transport sites along the vascular bed, we prepared whole mounts of the diaphragm after the intravenous injection of ferritin by fixing the muscle in situ with glutaraldehyde and staining it thereafter in toto for iron by Perl's method (22), as follows. Specimens were rinsed briefly in distilled water and then placed for 60 min in a fresh mixture of equal parts of 2% aqueous K-ferrocyanide and 2% HCl. After staining, they were rinsed briefly in distilled water, pinned out flat on Teflon discs, dehydrated in acetone, cleared in monomeric styrene for 24 hr, impregnated with Vestopal (Martin Jaeger, Geneve, Switzerland) without catalyst, and finally mounted on glass microscope slides under cover slips. The Vestopal was polymerized by ultraviolet light at room temperature.

EXAMINATION OF FERRITIN BY NEGATIVE STAINING: Negative staining with K-phosphotungstate (23) was used for the comparative examination of ferritin in the stock solution and ferritin in the plasma, after 1 hr in circulation. The object was to find out whether in the blood ferritin aggregates or binds to other proteins. No difference was found in the size and shape of the molecules. In both cases, $\sim$15% of the particles were empty shells, presumably apoferritin lacking the core of ferric hydroxide phosphate micelles. Rothen (24) has reported that purified ferritin contains $\sim$25% apoferritin.

OBSERVATIONS

Our electron microscopical observations concern mainly blood capillaries with a diameter ot $\sim$7 μ. Since their location within the capillary bed is unknown, we assume that the sections examined represent random samples from the arterial to the venous ends of the capillaries. The organization and fine structure of tissues from ferritin-injected animals were, in all instances, similar to those of the corresponding tissues of noninjected animals (controls), except for the presence of ferritin and the cellular changes associated with ferritin uptake.

The Blood

Ferritin was present in high concentration in the blood plasma that filled the capillary lumina at the earliest time point examined, 2 min (Figs. 1, 3). Thereafter, the concentration decreased, first rapidly then slowly, until sometime between 1 and 4 days when the tracer was completely cleared from the blood (Figs. 2, 11). The distribution of ferritin in the plasma remained uniform at all time points up to 1 day; the molecules did not cluster together to form large aggregates. Additional evidence on the state of ferritin molecules in the circulation was obtained by negative staining of plasma samples which showed lack of ferritin-ferritin as well as ferritin-protein aggregation. Hence, our observations refer to individual ferritin molecules, not to larger particles. The tracer did not attach to the membranes of leukocytes, blood platelets, or erythrocytes. Circulating leukocytes incorporated and concentrated ferritin into large dense bodies, and blood platelets incorporated small amounts into vesicles. The tracer did not enter erythrocytes. Leukocytes were not found penetrating the capillary walls in our specimens. In general, the amount of tracer taken up by blood cells was negligible; at all time points examined, the bulk of the tracer remained in solution in the plasma.

The satisfactory retention of plasma within capillary lumina and the even distribution of ferritin therein made possible an estimate of the concentration of the tracer in the plasma as a function of time. To this intent, we counted ferritin molecules in sections in the vascular lumina at different time points and normalized the counts per unit area. Assuming an average 500-A thickness for the sections, we calculated ferritin concentration per unit volume for each time point. The data, given in Fig. 2, show that the tracer disappears from the blood plasma in two exponential,

apparently distinct phases. The first gives a half-life of ~2 hr, and the second one of ~16 hr. Since we found (v.i.) that during the first hours there is massive uptake of ferritin by both liver and spleen, we assume that the rapid clearance of the first period reflects in part this uptake. The slower clearance after 3 hr probably approximates more closely the rate of ferritin transport from the plasma to the interstitial fluid in other parts of the body.

Endothelial Tunic

At all time points investigated from 2 min to 1 day, ferritin was found in the cells, but not in the intercellular junctions of the endothelial tunic (Figs. 3, 4). By the 4th day, when the tracer was cleared from the plasma, it appeared also cleared from the endothelium.

Within endothelial cells, practically all ferritin molecules were initially found in plasmalemmal vesicles (Figs. 1, 4, 5). After 1 hr, some tracer molecules appeared and began to accumulate within multivesicular and dense bodies, but at any time point during the first 24 hr the amount of ferritin in plasmalemmal vesicles exceeded by far that found in such bodies. Ferritin did not attach to the cell membrane and was not found in any other cellular compartment such as the endoplasmic reticulum, the nucleus, or mitochondria. Occasionally, the location of a tracer molecule could not be unquestionably established, especially in sections including only a small part of an apparently labeled vesicle. In such situations, the

micrographs could not distinguish between ferritin within the grazed vesicle, and ferritin in the overlying (or underlying) cytoplasmic matrix (Fig. 8). But, since such cases were rare, and since clear-cut instances of free ferritin in the matrix were absent, we assume that in the endothelium ferritin remains restricted to the plasmalemmal vesicles and compartments involved in endocytosis and does not reach the cytoplasmic matrix, or reaches it only by accident.

Within the vesicle population, the extent of labeling was greater among vesicles on the blood front than among those on the tissue front at all time points investigated (Figs. 4, 5). It was difficult to assess frequency of labeling among vesicles which appeared located deeper in the cytoplasm because their exact relationship to the cell surface could not be determined in single sections. Such limitations do not apply to our tridimensional model (1) which represents a sector of capillary endothelium from a rat given ferritin 60 min before fixation. In this admittedly small sample, ~10% of the total vesicle population is labeled. The percentage of labeled vesicles is ~25% on the blood front, and ~20% within the cytoplasm. In this particular case, no labeled vesicles occurred on the tissue front of the endothelium. In other cases, however, ferritin-containing vesicles were found on this front (Figs. 6, 7) and many of them appeared fully or partially opened on the corresponding cell surface (Figs. 6 and 8).

Within vesicles, ferritin molecules did not adsorb or attach to the vesicle membrane, but ap-

List of Abbreviations

bm, Basement membrane
cf, Collagen fibril
db, Dense body
e, Endothelial cell
g, Glycogen
j, Intercellular junction
l, Lumen
mf, Muscle fiber

mt, Mitochondrion
n, Nucleus
pc, Pericyte
pl, Blood plasma
r, Ribosome
rc, Erythrocyte
rer, Rough endoplasmic reticulum

FIGURE 1 Blood capillary of the diaphragm of a rat 2 min after an intravenous injection of ferritin. The tracer is present in high concentration and even dispersion in the plasma (f_1). It has penetrated a number of plasmalemmal vesicles of the endothelium (f_2), especially along the blood front of the cell. There are no tracer molecules in the basement membrane and in the adventitia. rc_1 and rc_2 mark recesses of the lumen. rc_2 leads to an intercellular junction (arrow) which appears to be an occluding zonule. Fixation: glutaraldehyde-OsO_4. Section stained with lead hydroxide. × 85,000.
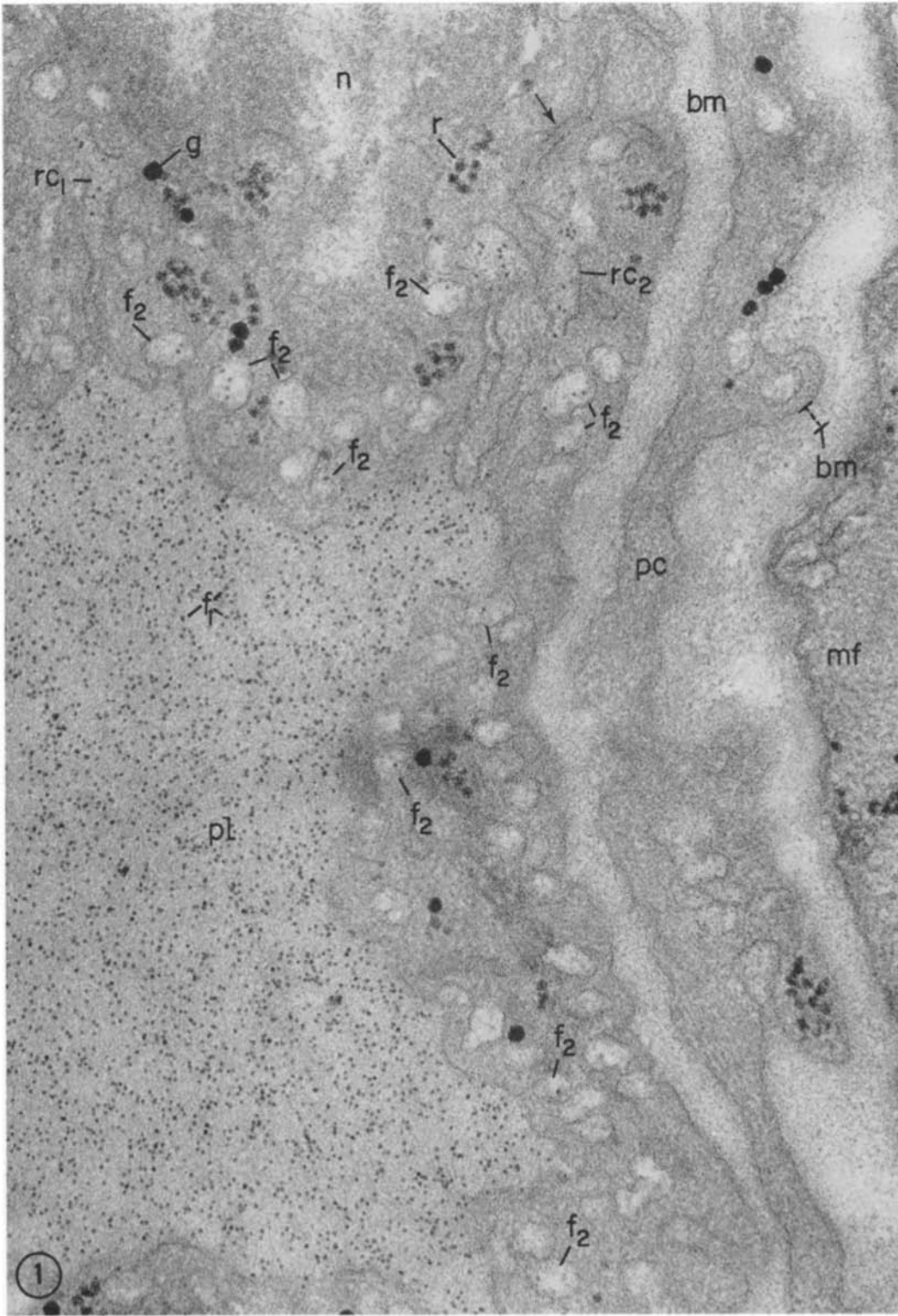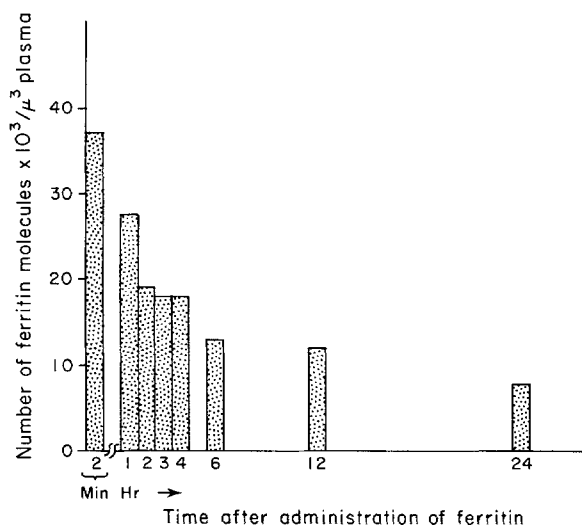
FIGURE 2 Concentration of ferritin in blood plasma established by counting ferritin molecules in sections of capillary lumina. Concentration/μ^3 was calculated by assuming an average section thickness of 500 A.

peared free in the vesicular cavity. The number of tracer molecules per vesicle profile varied from none to six, most labeled profiles containing only one to two molecules.

As already mentioned, ferritin was found extensively concentrated in dense bodies (Figs. 9, 10), and to a lesser extent in multivesicular bodies, 1–2 hr after injection. Because of the low frequency of such bodies in the endothelium, the amount of information so far obtained on them is limited. We assume that these bodies are lysosomes and that they undergo the cycle of uptake, digestion, and discharge worked out for other cell types (25), but the details and timetable of this cycle in endothelial cells remain unknown. It should be clear, however, that of all the ferritin moving through the endothelium only a small, probably negligible amount is retained in these bodies. Accumulation of i.v. injected ferritin within dense and multivesicular bodies has been observed already in the capillary endothelia of the heart and other organs (26).

The intercellular junctions of the endothelium have been described in detail in the preceding paper (1). In the capillaries of the diaphragm they appear to be practically all occluding zonules. As already mentioned, no ferritin was found in transit through these junctions (Figs. 1, 3, 4, 9).

At all time points investigated up to 24 hr, the concentration of the tracer was considerably higher in the lumina than in the endothelial tunic of the capillaries. It appears, therefore, that the main barrier to the movement of ferritin from the plasma

to the pericapillary spaces is represented by the endothelium.

No endogenous ferritin was found in any compartment of endothelial cells in control animals.

Middle Tunic

There was no accumulation of ferritin against or within the basement membrane of the capillaries at any time point; the concentration of the tracer in this layer seemed to parallel that in the adventitia and pericapillary spaces (Figs. 5, 15).

Some ferritin appeared in the plasmalemmal vesicles of pericytes (Fig. 7), but the uptake was limited and did not lead to any extensive accumulation of tracer molecules within lytic vacuoles. In this respect, the situation was markedly different from that described in histamine- or serotonin-treated small vessels in which the formation of large intramural deposits of experimental tracer particles (colloidal mercuric sulfide) in between leaflets of the basement membrane was followed by phagocytic activity of pericytes (19).

Adventitia and Pericapillary Spaces

Ferritin could be recognized in the adventitia and pericapillary spaces 5–10 min after intravenous injection (Figs. 4, 5). The concentration was exceedingly low initially, and increased slowly to approach that in the vascular lumina by ∼24 hr. Except for some accumulation within bundles of collagen fibrils, the distribution of the tracer appeared to be random. No local increases in concentration were noted in relation to any structural
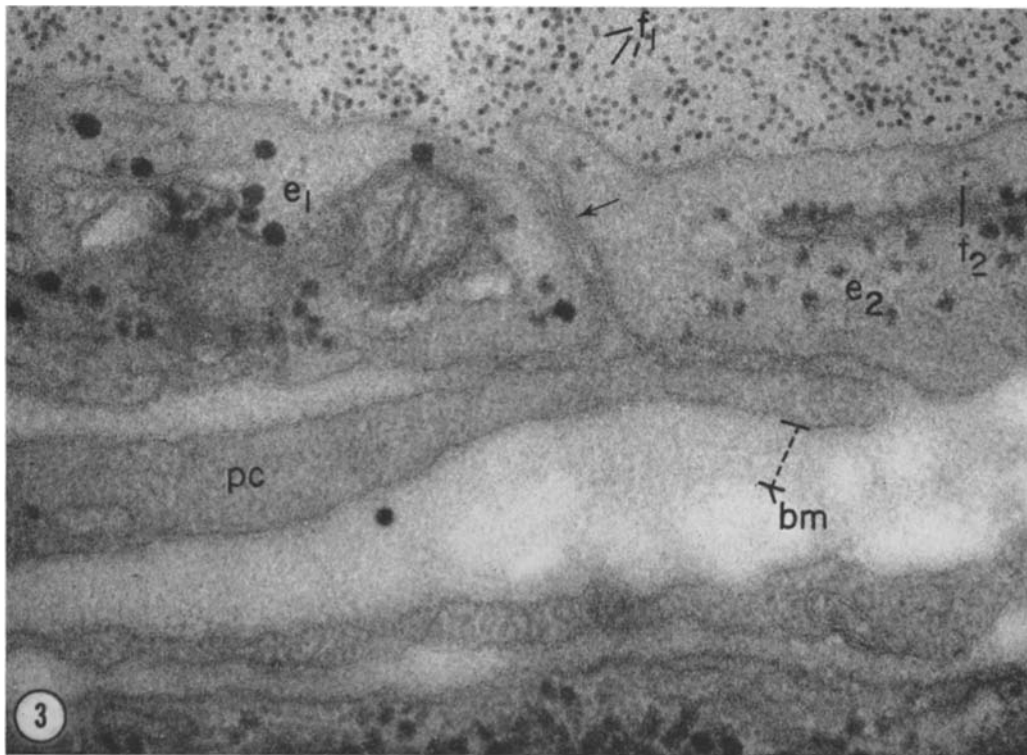
FIGURE 3 Blood capillary of a rat diaphragm 2 min after an intravenous injection of ferritin. The field includes a junction between two endothelial cells (e_1, e_2). Ferritin appears in high concentration and random distribution in the plasma (f_1). Except for a molecule in an endothelial cell vesicle (f_2), the capillary wall is free of tracer. Note that ferritin molecules have not penetrated beyond the occluding zonule (arrow) of the intercellular junction. × 170,000.

detail in the other tunics of the vessel, such as intercellular junctions or extremely attenuated regions of the endothelium. By the 4th day, the adventitial spaces, like the plasma and the endothelium, were cleared of ferritin (Fig. 11).

In contrast with its generally low concentration in the interstitia, the tracer was found in high and rapidly increasing concentration in macrophages. It already appeared—5 min after injection—in membrane infoldings, in between ruffles, and in simple or "coated" vesicles of varied sizes. Later on, there was progressive (Fig. 12), and after 1 hr extensive, even spectacular, accumulation of ferritin in large dense bodies (Figs. 13, 14) that reached up to 4 μ in diameter and literally filled the body of the macrophages. Throughout the period of observation, there was evidence of continuous uptake and progressive concentration of ingested ferritin and of fusion and enlargement of lytic vacuoles. Digestion of the tracer seemed to

proceed slowly since ferritin-filled, lytic vacuoles were still present by the 4th day of the experiments. At that time, ferritin molecules were also found in large concentrations scattered throughout the cytoplasmic matrix of the macrophages (Fig. 11). Such molecules may represent newly synthesized endogenous ferritin.

Uptake of the tracer by small vesicles, followed by its concentration in lytic vacuoles, and late appearance of ferritin molecules in the cytoplasmic matrix, were also found in the fibroblasts of the adventitia, but were carried out by these cells to a much more limited extent than by macrophages (Figs. 15, 11).

Topology of Ferritin Transport

Since ferritin iron gives an intense Prussian Blue reaction, whole mounts of diaphragm, stained by Perl's procedure, were used to study at the light microscope level certain topological aspects of
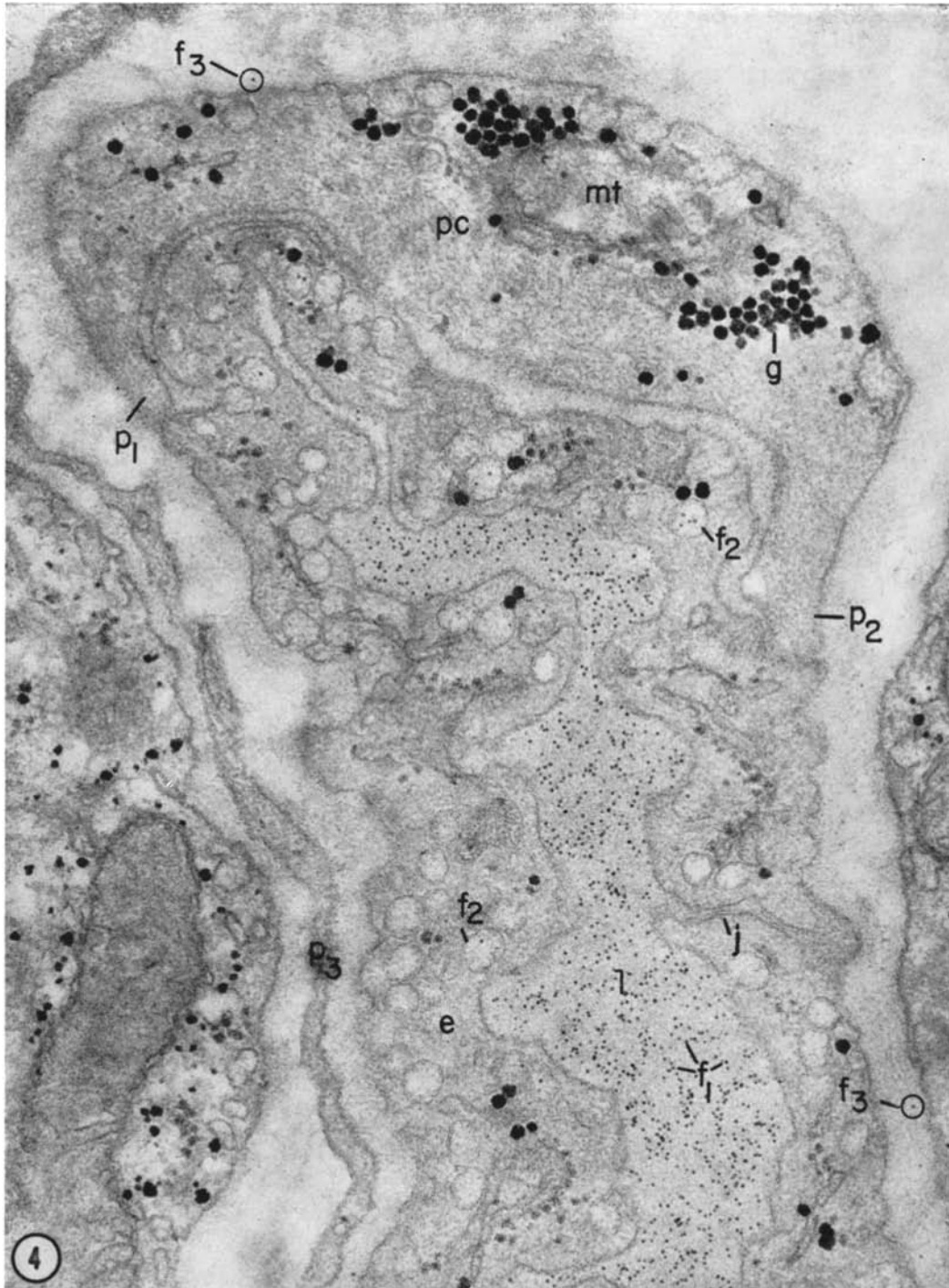
FIGURE 4   Blood capillary of a rat diaphragm 10 min after an intravenous injection of ferritin. Ferritin molecules occur in the blood plasma (f_1); in the endothelium, within plasmalemmal vesicles (f_2) most of which appear to be enclosed by the cytoplasmic matrix; and in very small numbers (f_3) in the pericapillary spaces. Note the lack of ferritin in the cell junction (j). Note also the typical pericyte at pc with its heavy load of glycogen (g) and its pseudopodia (p_1, p_2) touching the endothelium (e). p_3 marks a long, slender pseudopodium which probably belongs to a macrophage of the adventitia. × 68,000.
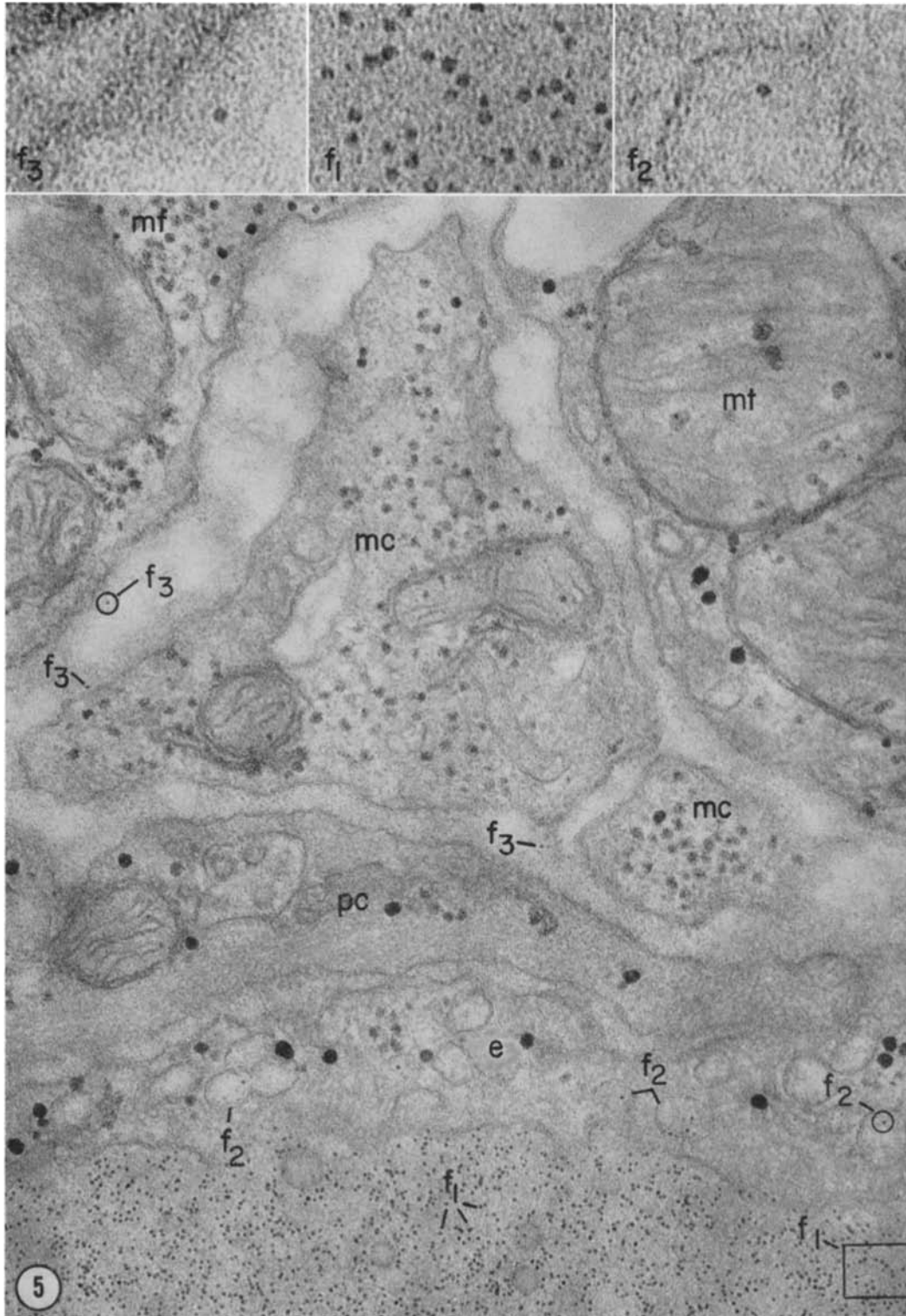
284

FIGURE 5 Blood capillary of a rat diaphragm 10 min after an intravenous injection of ferritin. Ferritin is present in high concentration in the plasma (f_1); at smaller concentration in the endothelium, where it is restricted to plasmalemmal vesicles (f_2); and in even smaller concentrations in the pericapillary spaces (f_3). The encircled ferritin molecules in the three positions mentioned have been enlarged in the insets, marked f_1, f_2, f_3, respectively, to show structural detail in the core of the molecules. Note the presence of a young macrophage (mc) in the adventitia. $\times$ 80,000; insets, $\times$ 290,000.
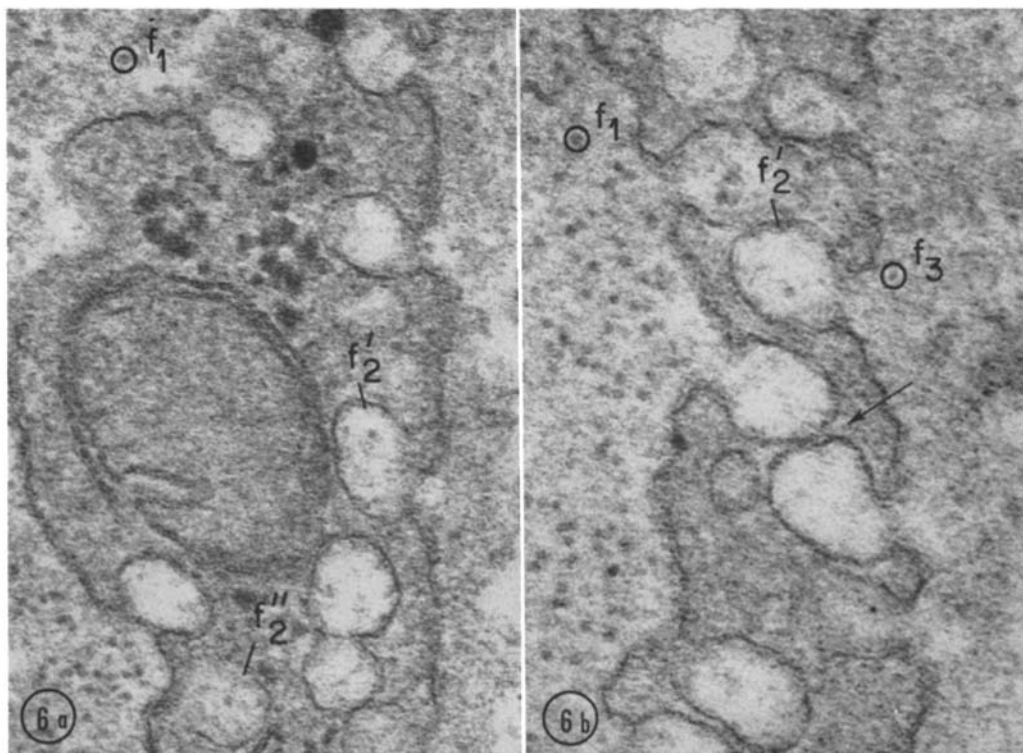
FIGURE 6   Blood capillaries in the diaphragm of a rat 60 min after an intravenous injection of ferritin. The tracer appears in the plasma (f_1), in endothelial plasmalemmal vesicles (f_2), and in the pericapillary spaces (f_3). Two of the ferritin-labeled plasmalemmal vesicles are opened on the tissue front (f_2'); and another is apparently surrounded by cytoplasmic matrix (f_2''). Note the close apposition of two vesicles (arrow), one opening on the blood front and the other on the tissue front, in Fig. 6 b. Fixation: glu-taraldehyde-OsO$_4$. Sections stained with uranyl acetate–lead hydroxyde. Fig. 6 a, $\times$ 126,000; Fig. 6 b, $\times$ 154,000.

ferritin transport. Specimens injected with carbon black were used for similar studies. We expected to find out in such preparations whether there is any evidence that the sites of exit of the tracers are uniformly distributed, or whether these tracers leave the plasma preferentially in certain segments of the vasculature, such as the small venules in which large "leaks" have been recorded in other tissues (amphibian skin (27) and mesentery (28)).

In diaphragms fixed 2 min after ferritin injection, the entire vasculature was intensely stained by the Perl reaction, the staining being restricted

FIGURES 7 and 8   Blood capillaries in the diaphragm of a rat 24 hr after an intravenous injection of ferritin. The tracer appears in the plasma (f_1), in plasmalemmal vesicles of the endothelium (f_2), and in the pericapillary spaces (f_3). Ferritin-labeled plasmalemmal vesicles of the endothelium are opened on the blood front or appear surrounded by the cytoplasmic matrix. Some of the latter are close to the tissue front of the cell (Fig. 8). At x is a ferritin molecule of uncertain location (see text). Tracer molecules appear also in plasmalemmal vesicles (long arrows) of a pericyte in Fig. 7 and in the muscle fiber in Fig. 8. Note the occluding macula (nexus) between the pericyte and endothelium in Fig. 7 (short arrow).

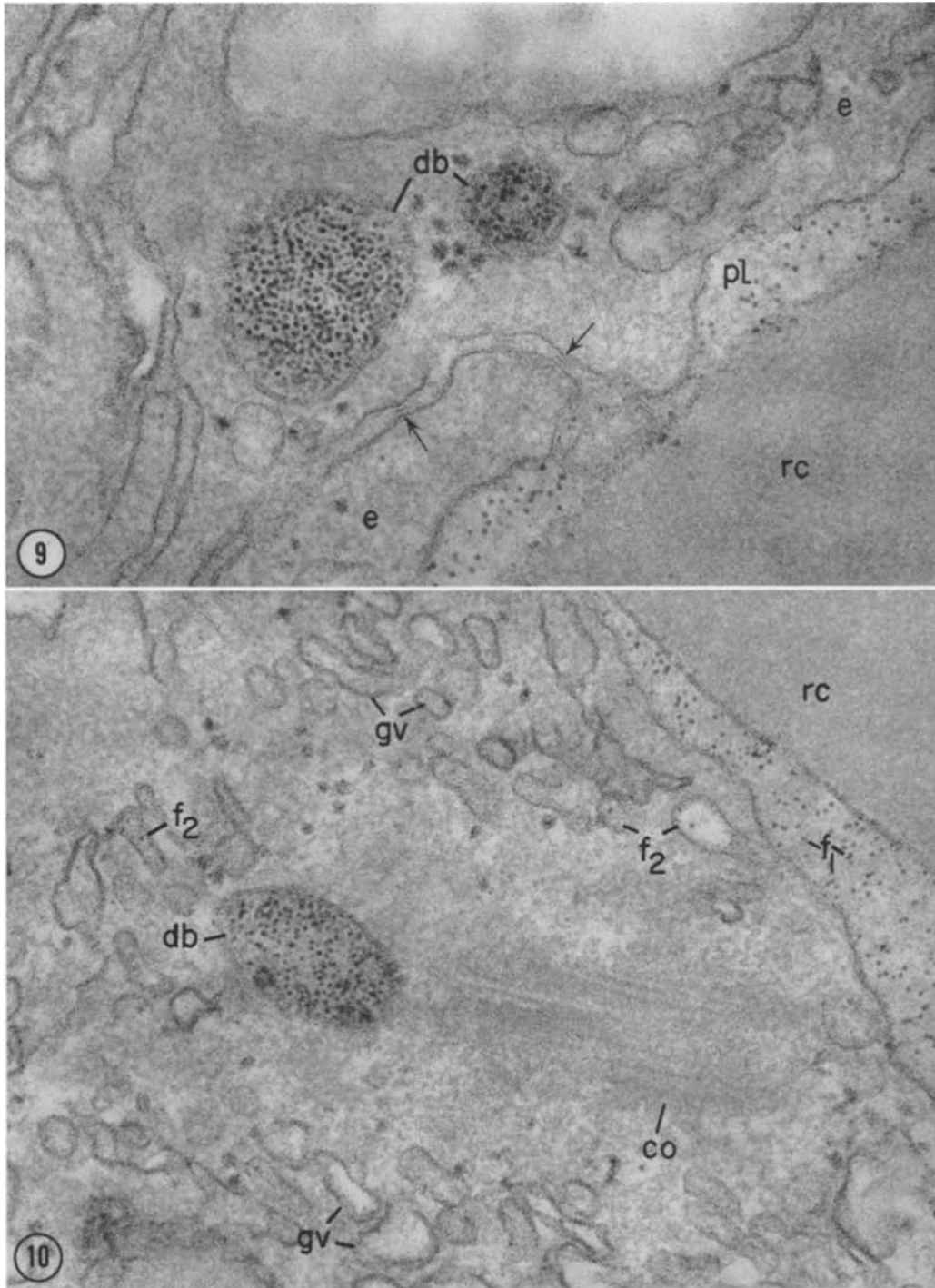Fig. 7, $\times$ 87,000. Fig. 8, $\times$ 124,000.
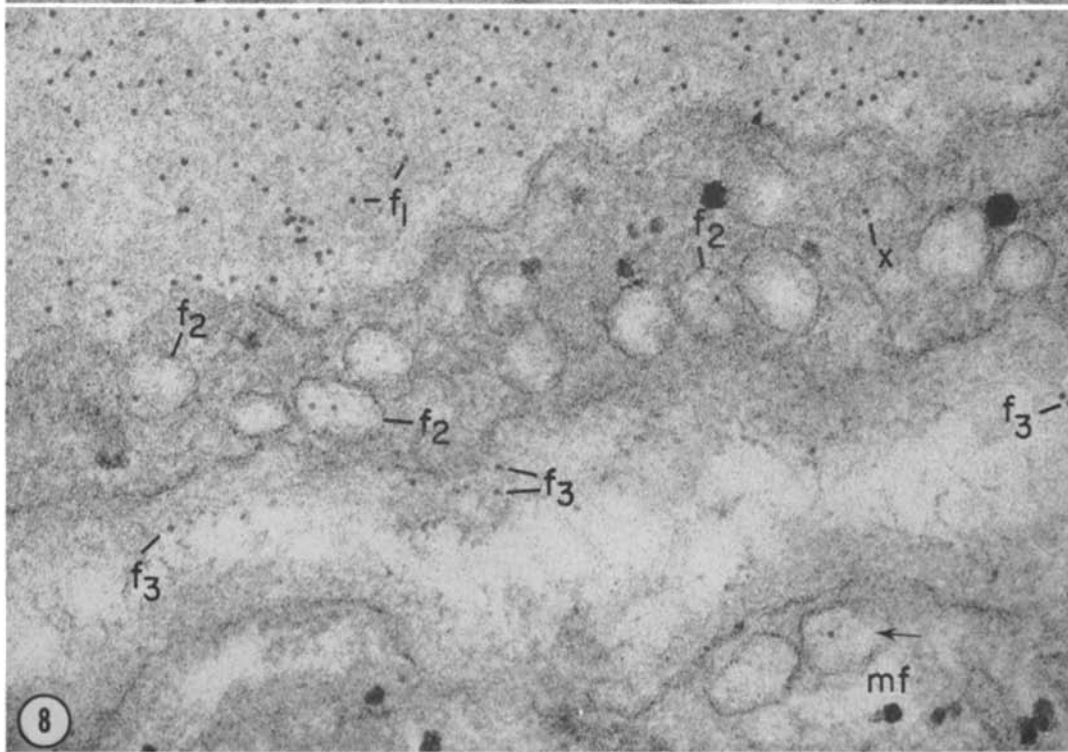
FIGURE 9   Endothelium of a small arteriole of the rat diaphragm 2 hr after the intravenous injection of ferritin. Note the two dense bodies containing ferritin in high concentration. Note also the occluding zonules (arrows) along the intercellular junction. × 60,000.

FIGURE 10   Blood capillary in the diaphragm of a rat 2 hr after an intravenous injection of ferritin. The section cuts through the centrosphere region of the cell and shows an obliquely sectioned centriole (co) characteristically surrounded by fine fibrillar material and beyond it by elements of the Golgi complex (gv). A dense body (db), a usual component of the centrosphere region, contains ferritin molecules in high concentration. Ferritin is also present in vesicles (f₂), some of which seem to be part of the Golgi complex. × 52,000.
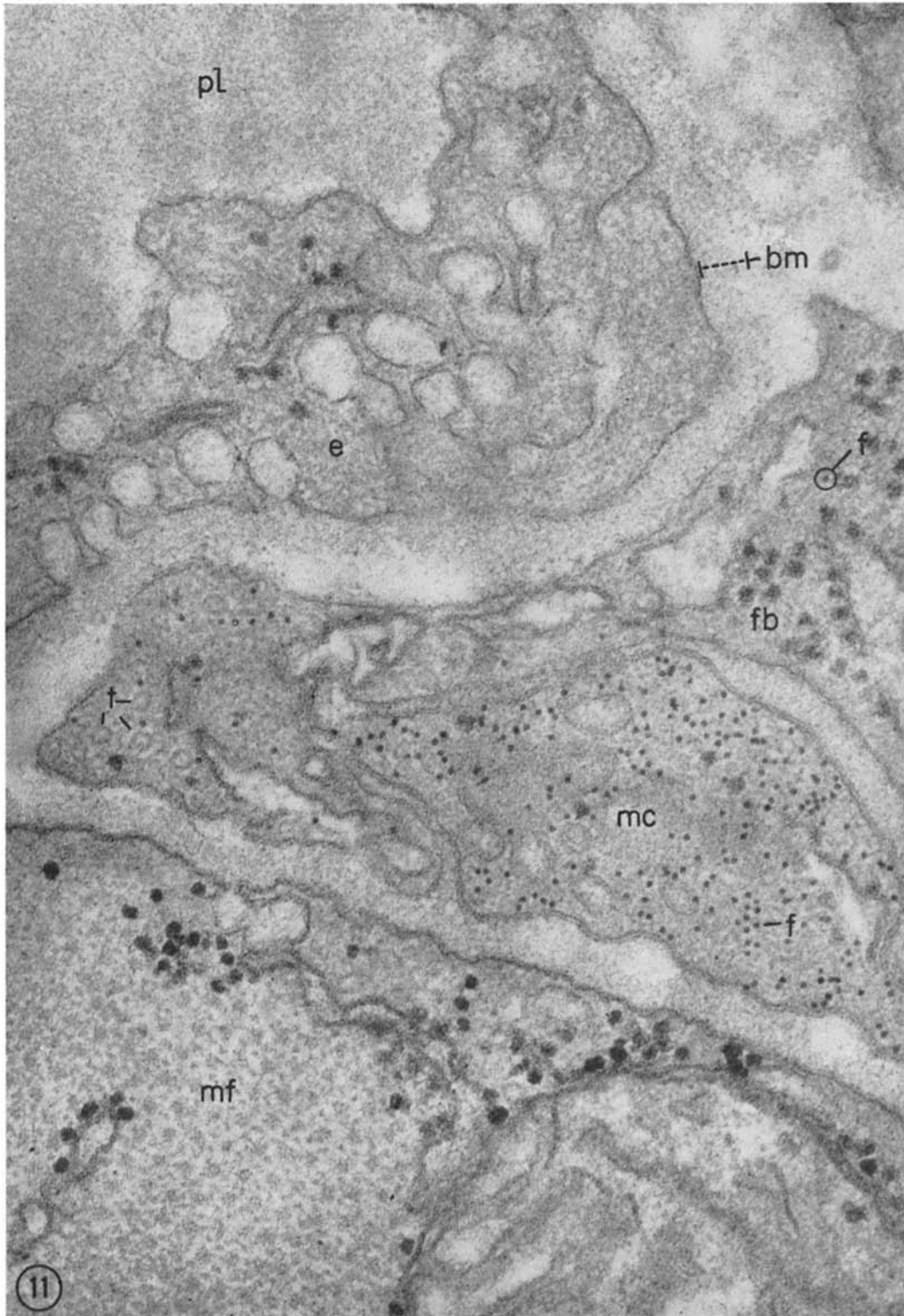
FIGURE 11 Blood capillary in the diaphragm of a rat 4 days after an intravenous injection of ferritin. The tracer has been cleared from the plasma (pl), the endothelium, the basement membrane, and the pericapillary spaces. Ferritin molecules (f) are seen only in two adventitial cells represented in the field by two relatively large pseudopodia. In the first, a macrophage (mc), it occurs in high concentration; in the second, a fibroblast (fb), it is present in small numbers. In both cases, the molecules are free in the cytoplasmic matrix and probably represent newly synthesized endogenous ferritin. Note the bundle of cross-sectioned microtubules at t. × 60,000.
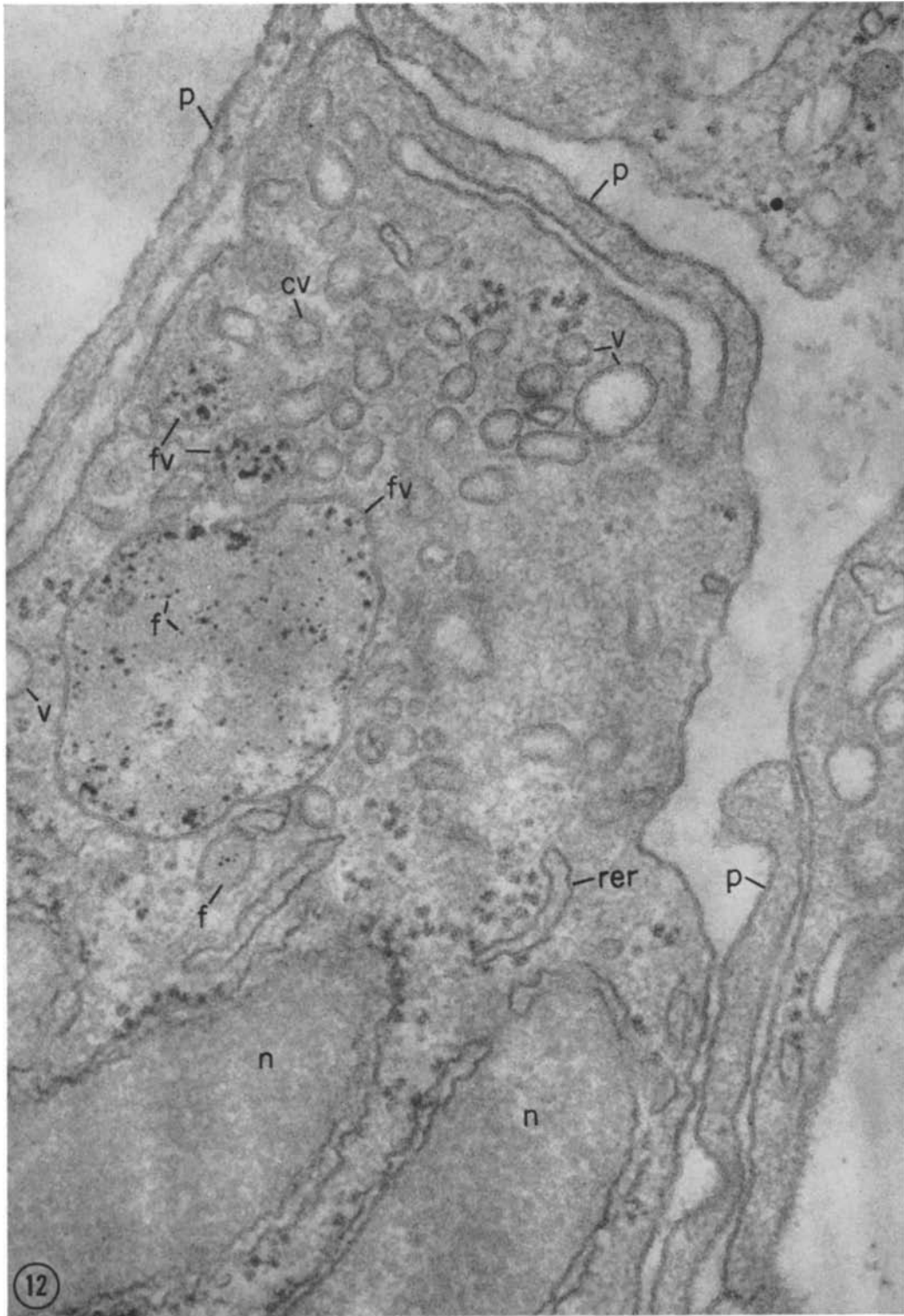
FIGURE 12   Macrophage in the adventitia of a blood capillary of a rat 30 min after an intravenous injection of ferritin. The cell characteristically shows ruffle-like pseudopodia (p), numerous vesicles (v), some of them coated (cv), and a few phagocytic vacuoles (fv). Ferritin (f) is present in a small vesicle and in the largest vacuole. × 89,000.

to blood vessels (Fig. 16). By 30 min, discrete particles along the vessels became reactive and their number increased progressively until by 24 hr clusters of intensely reactive droplets appeared uniformly distributed at a spacing of ~50 μ along the entire vasculature (Fig. 17). On the basis of our electron microscope observations, we identify these clusters as histocytes or "fixed" tissue macrophages. There were no "clouds" of blue stain or carbon particles of the type expected for large leaks in any part of the vasculature, and there was no early or preferential accumulation in macrophages along small venules.

Ferritin Uptake By The Liver And Spleen

We examined liver and spleen specimens at the time points indicated in Table I to find out whether large amounts of ferritin were removed from the circulation of these organs. The tracer was found in the usual series of endocytic structures in von Kupffer cells, hepatocytes, and spleen macrophages. The amounts incorporated were already large at the first point investigated (10 min) and increased continuously thereafter. Early accumulation of i.v. injected ferritin in von Kupffer cells and splenocytes has been already demonstrated in the mouse by Florey (26).

DISCUSSION

Our findings indicate that ferritin molecules appear in the adventitia and pericapillary spaces of the diaphragm within minutes after their intravenous injection and accumulate slowly during the following 24 hr within these spaces and the macrophages that populate them. During this entire period, practically all the ferritin that penetrates the endothelium is restricted to plasmalemmal vesicles, and only a small negligible proportion is segregated within lytic vacuoles.

We assume that the tracer is carried across the endothelium, from the plasma to the pericapillary spaces, by plasmalemmal vesicles, and we base our assumption on the following reasons:

A. We did not find the tracer within intercellular junctions or within other compartments of the endothelial cells.

B. We found ferritin-containing vesicles in all situations expected in a transport operation across the endothelium, i.e., open on the blood front, closed within the cytoplasm, and opened on the tissue front.

C. The examination of whole-mounted dia-

phragms failed to detect large "leaks" of the type described by Rous et al. (27, 29) and Landis (28) along small venules. It demonstrated, instead, the uniform labeling of adventitial macrophages along the whole vasculature, especially along capillaries.

D. We found only small amounts of ferritin—by negative staining—in the peritoneal fluid during these experiments; this rules out the remote possibility that the tracer reached the interstitia of the diaphragm through the peritoneal mesothelium.

Since at all time points within the first 24 hr, the concentration of the tracer dropped abruptly at the plasma:endothelium boundary, and since no marked differences in tracer concentration were found among the successive tunics of the vessel, we identify the endothelium as the main barrier in the movement of ferritin across the capillary wall of muscle capillaries.

Since the ferritin molecule has the right size for a probe of the large pore system, our findings indicate that in muscle capillaries the plasmalemmal vesicles are the structural equivalent of the large pore system. This conclusion is supported by previous results obtained by us and by others with colloidal particles injected in the general circulation (6, 7) or introduced in the perfusion fluid of an isolated heart preparation (8, 9). The possibility that the large pore system of the capillary wall is represented by plasmalemmal vesicles has already been envisaged by Mayerson et al. (30).

The identification of the plasmalemmal vesicles as the means by which large molecules reach the interstitial fluid in muscle capillaries seems well established in our experiments; yet it poses a number of problems to be discussed in some detail below.

A. The pore theory considers the large pores as open, water-filled channels along which molecules move by diffusion or hydrodynamic flow. The vesicles imply a discontinuous type of transport in bulk which requires a rather complex series of operations, with diffusion involved in part of them only (loading, discharge).

B. Vesicles and large pores have about the same diameter, but not the same frequency. The vesicles are five to six orders of magnitude more numerous. Since they imply, however, a discontinuous type of transport, this difference in apparent frequency could be accounted for by the time a vesicle needs to traverse the endothelium. We should point out,

however, that calculations of large pore frequency are based on macromolecule concentration in the lymph, and generally assume that there is no retention of the molecule concerned in the interstitia of the tissues. This assumption is rendered questionable by the finding that large amounts of ferritin are accumulated and apparently degraded in the macrophages of the diaphragm in our experiments.

C. Nothing is known at present about molecular mechanisms involved in, and amount of energy required for, this type of transport. It has been assumed that the formation of plasmalemmal vesicles requires energy, and it has been pointed out that the frequency of vesicles in the endothelium is not changed by cooling, anaerobiosis, respiratory poisons (31), or suppression of blood supply (32). It has also been assumed that Brownian movement (33, 34) could account for the transfer of a vesicle from one front of the cell to the other within the time expected from its function (34, cf. 35), energy being possibly required only for membrane fission ("ejection") and fusion. With the evidence available, the problem cannot be solved since, if energy is required, neither its amount nor its source is known. The extent of metabolic inhibition obtained in reference 30 is uncertain, and the assumption that vesicle frequency should decrease with decline in available energy is questionable. Transport could be stopped without necessarily reducing the number of containers involved in the operation.

Average Duration of a Half Cycle

On the assumption that all dextran molecules larger than 200,000 mol wt are transported across the endothelium by vesicles, Renkin (35) has calculated the average lifetime of a vesicle, i.e., the time needed for loading, traversing the endothelium, and unloading, at $\sim$300 sec.

We can now recalculate this value starting from more detailed and more reliable parameters (1). For a capillary with an internal radius of 4 μ and an 0.35-μ-thick endothelial tunic, the endothelium represents only $\sim$18% of the entire vessel volume and the plasmalemmal vesicles only $\sim$6% of endothelial volume. The corresponding figures were estimated at 50% and 20%, respectively, by Renkin (35). For an entire perfused limb ($\sim$50 g of which 1 g is blood), the capacity of the plasmalemmal vesicles of the entire vascular bed can be estimated at 5.4 $\times$ 10^{-3} cm^3. Since this capacity is used to clear (presumably by whole plasma transfer) 1.6 $\times$ 10^{-4} cm^3 plasma per second of its large dextran molecules, the average lifetime of a vesicle calculates to $\sim$34 sec, which is about one order of magnitude shorter than Renkin's estimate. Assuming that only 70% of the endothelial volume (the attenuated periphery without the thick perikaryon) is functional, the figure is reduced to 24 sec. Since liftetime may imply that the vesicle is destroyed at the end of this period, the parameter in question could be called "half cycle" or "traverse time."

The Large Pore System in Other Capillaries

Studies carried out on perfused heart preparations have shown that colloidal iron (particle diameter $\simeq$20–70 A) and ferritin reach the pericapillary spaces by vesicular transport and also by "leaking" along the intercellular junctions of the endothelium (8, 9).

It seems, therefore, that in the myocardium, in contradistinction with what we found in the diaphragm, the large pore system is represented by plasmalemmal vesicles as well as open intercellular junctions. It should be pointed out, however, tha

FIGURE 13 Macrophage in the adventitia of a blood capillary (rat diaphragm) 60 min after ferritin injection. The tracer is present in the extracellular space (f) and in one (v) of the numerous coated vesicles seen along the periphery of the cell. It already occurs in relatively high concentration in a series of dense bodies (db_1 — db_4) some of which (db_4) contain in addition remnants of other ingesta. Note the absence of the tracer in the dense body marked db_5, and its uneven concentration in the others. These differences in ferritin content describe variations in the time and frequency of fusion of newly formed, ferritin-rich, phagocytic vacuoles with old, ferritin-free, dense bodies. No such fusion has yet occurred apparently for db_5. Compare the amount and distribution of the tracer in this macrophage with those seen in macrophages 30 min (Fig. 12) and 24 hr (Fig. 14) after ferritin injection. $\times$ 61,000.
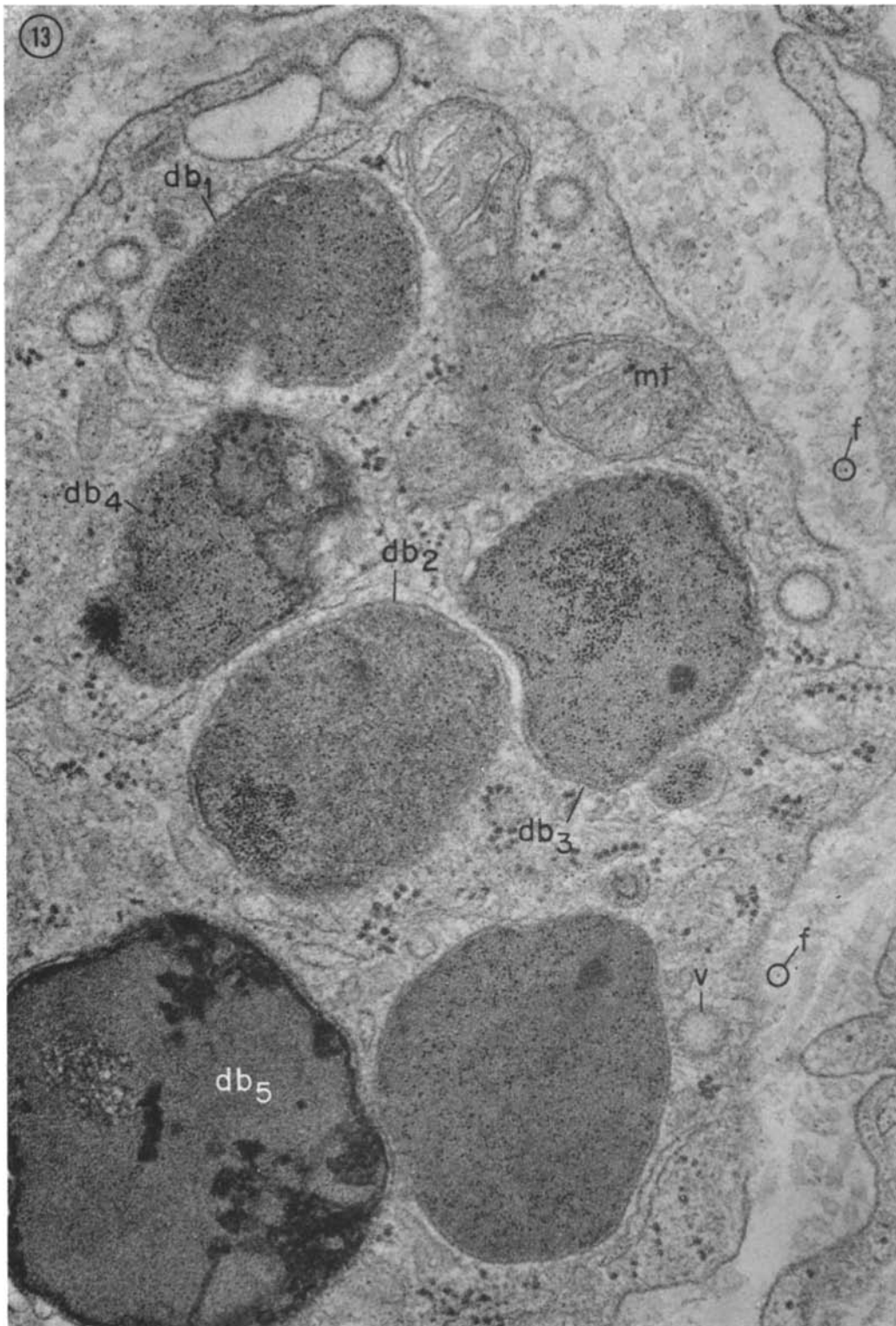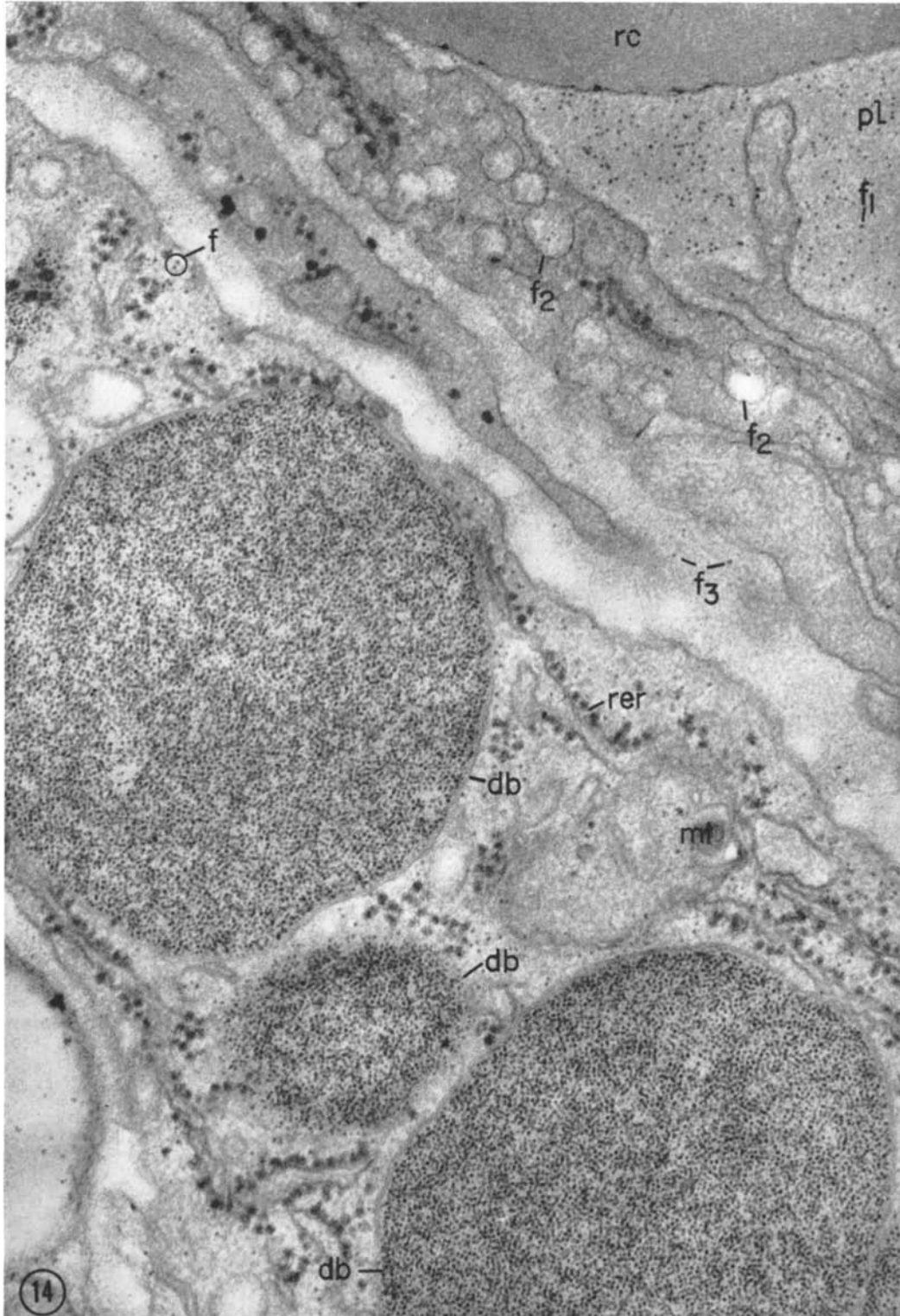
FIGURE 14   Macrophage in the adventitia of a small venule in the diaphragm of a rat 24 hr after the injection of ferritin. The tracer is present in the plasma (f_1); in the endothelium, restricted to endothelial cell vesicles (f_2); and in the spaces of the adventitia (f_3). It occurs in very high concentration in three dense bodies (db) of the macrophage. Note also that the tracer is present in low concentration in the cytoplasmic matrix of the macrophage. In this location, it probably represents endogenous, newly synthesized ferritin. $\times$ 73,000.
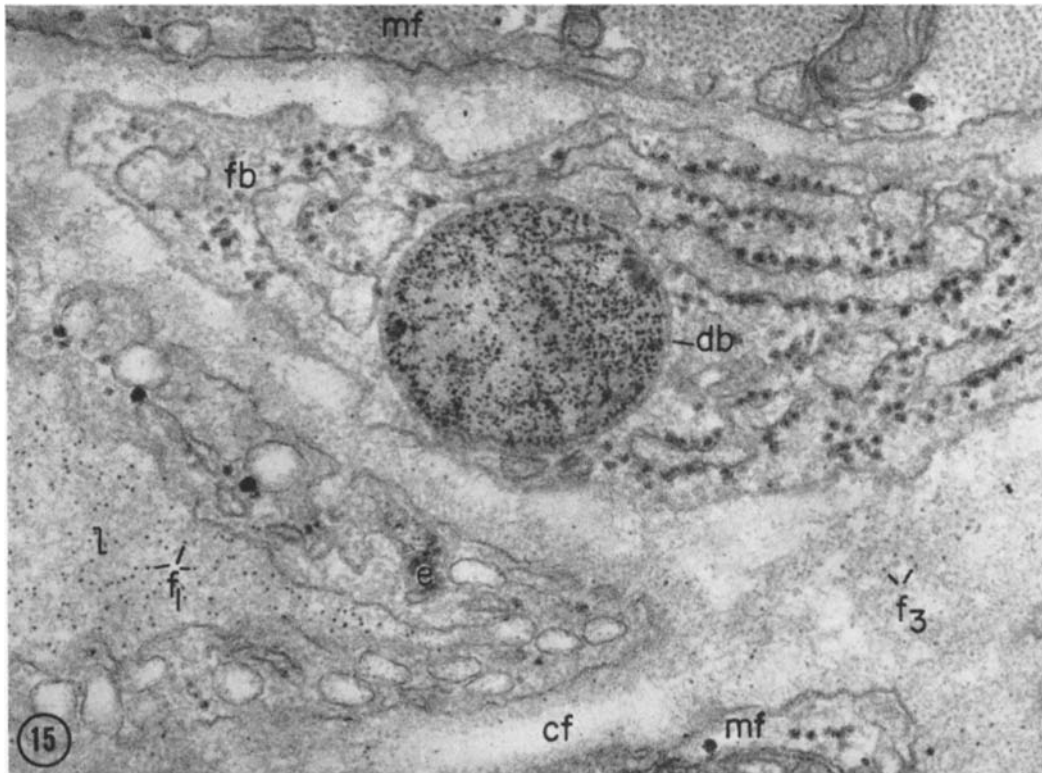
FIGURE 15 Blood capillary in the diaphragm of a rat 12 hr after the intravenous injection of ferritin. The tracer is present in decreased concentration in the plasma (f_1) and in increased concentration in the pericapillary spaces (f_3). Ferritin also occurs in high concentration mixed with amorphous and membranous residues in a dense body (db) in a fibroblast (fb) of the adventitia. X 67,000.

the permeability of the capillary bed of the perfused heart is known to be higher than that of the heart in situ (36) and that in isolated cat hind limbs permeability has been shown to be influenced by the presence and concentration of proteins in the perfusate (reference 2, p. 994). It should also be pointed out that the protein concentration of cardiac lymph is considerably higher than that of the limb-lymph (37, 38). Hence, in the case of the perfused myocardium we are dealing with a capillary permeability that is basically higher than in skeletal muscle, and can be further increased by perfusion in vitro. These aspects should be taken into consideration in interpreting findings at the level of the capillary wall.

Our results apply strictly to muscle capillaries, i.e. capillaries with a continuous endothelium, of the type found in the diaphragm and probably other skeletal muscles. In view of the structural diversity known to exist among blood capillaries in various tissues and organs, it is unlikely that the plasmalemmal vesicles represent the structural equivalent of the large pore system in all capillaries. The pore theory assumes that large pores of comparable dimensions and structural characteristics exist in all types of capillaries and explains difference in permeability from one type to another by appropriate differences in pore frequency (3, 30). In visceral capillaries, the most likely structural equivalent of the large pore system is the population of diaphragmed fenestrae, and in the liver, if any restriction exists in the movement of large molecules from the plasma to the lymph, it should be looked for at the periphery of the lobule, in between the spaces of Disse and the lymph vessels.

Relation of Vesicles to the Small Pore System

Finally, we should indicate that, with the evidence at hand, it is difficult to estimate the role

TABLE I

Number of Rats Examined at Each Time Point after Intravenous Ferritin Injection

		Time points*																			
		Minutes												Hours					Days		Total
Tissue	Prepared for	0	1	2	4	5	10	15	20	30	45	60	90	2	3	4	6	12	1	4	No. of animals
Diaphragm	Electron microscopy	15‡	7	7	1	3	10	2	3	8	1	11	1	2	2	3	1	3	2	1	76§
Liver	"						1							1					1		3
Spleen	"						1							1						1	3
Diaphragm	Whole mount	2‡	1	1				1		1		2							2		10§

* Time elapsed between the end of intravenous injection and the initiation of fixation.

‡ Control animals, noninjected.

§ Including control animals.

played by plasmalemmal vesicles in the over-all exchanges between the plasma and the interstitial fluid, i.e., exchanges which, according to the pore theory, are effected primarily through the small pore system. It is clear that a certain amount of water and small, water-soluble molecules is moved across the endothelium by such vesicles, but the amount may be comparatively small. To account for all exchanges between plasma and interstitial fluid, the average half cycle of a plasmalemmal vesicle should last only 0.012 msec, which is extremely unlikely.

In recent studies, horseradish peroxidase has been used as a probe molecule that can probably penetrate both pore systems (mv ≃40,000; molecular diameter ≃40 A). Results obtained by Karnovsky and Cotran on mouse myocardium suggest that the probe reaches the pericapillary space through both vesicles and intercellular junctions (39, 40). This finding could indicate, like Jennings' and Florey's observations (9), that in the capillaries of the myocardium, the large pore system includes open intercellular junctions, or conversely,

that the junctions represent the small pore system of these vessels. Karnovsky and Cotran (39) and more recently Karnovsky (41) favor the second interpretation. Yet neither the width of the intercellular slits (~40 A according to reference 41), nor apparently their aggregate area (cf. 1) satisfy at present the requirements set forth by the pore theory.

The difficulties encountered in our attempts to identify the small pore system make one wonder whether current preparation procedures succeed in preserving relevant structural details that may depend on conditions in their immediate environment. For instance, the intercellular spaces could represent the small pore system, provided we assume that they are open in vivo to a gap of 70–90 A,[4] and partially or completely collapsed in

[4] The width of the intercellular gap required by the slit pore variant of the pore theory was originally calculated at 37 A (42), but no subsequent correction for osmotic reflection is available for this figure. Such a correction has increased the diameter of cylindrical pores from ~60 A (42) to ~90 A (2). From results

FIGURES 16 and 17 Diaphragms of rats after an intravenous injection of ferritin. Whole mounts stained with iron by the Prussian Blue method.

FIGURE 16 Specimen fixed 2 min after the injection. Photomicrograph shows the vascular network of the muscle stained on account of the high concentration of ferritin in the plasma. Venules are marked ve and capillaries, cp. × 256.

FIGURE 17 Specimen fixed 24 hr after injection. The vascular bed is only partially visible (cp) on account of the decreased concentration of ferritin in the circulation; instead, clusters of heavily stained granules (mc) appear regularly spaced along all capillaries. These clusters are identified as tissue macrophages (cf. Fig. 14). In the row marked by arrows, the center-to-center spacing of the macrophages is ~50 μ. × 256.
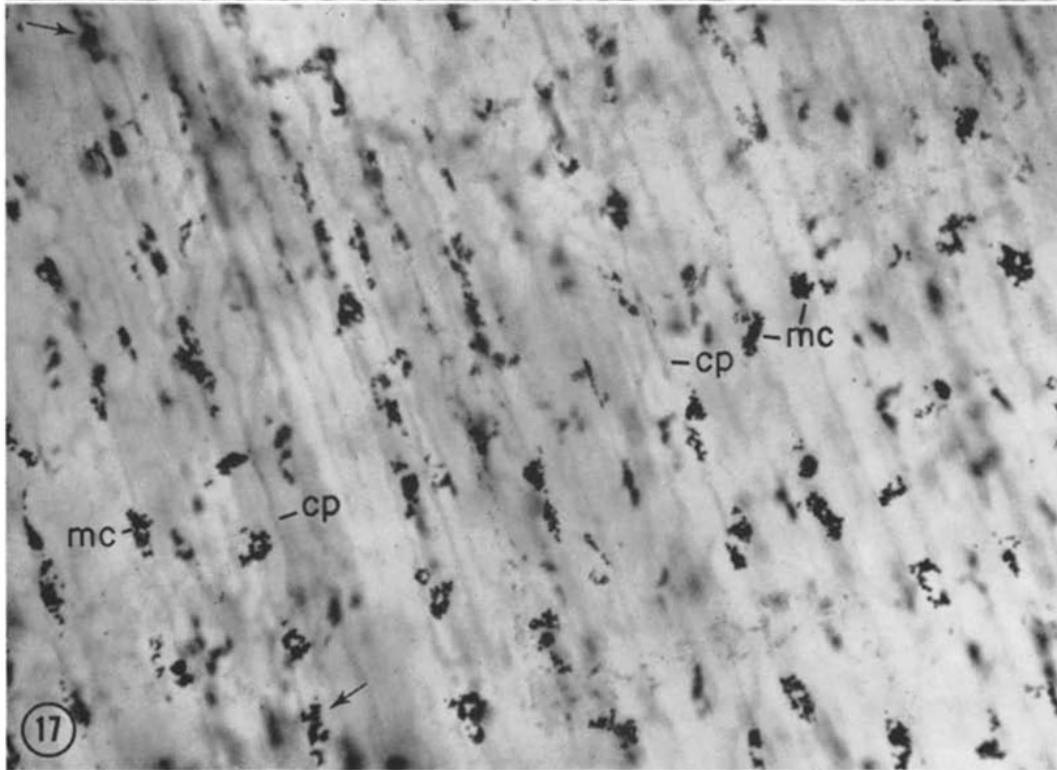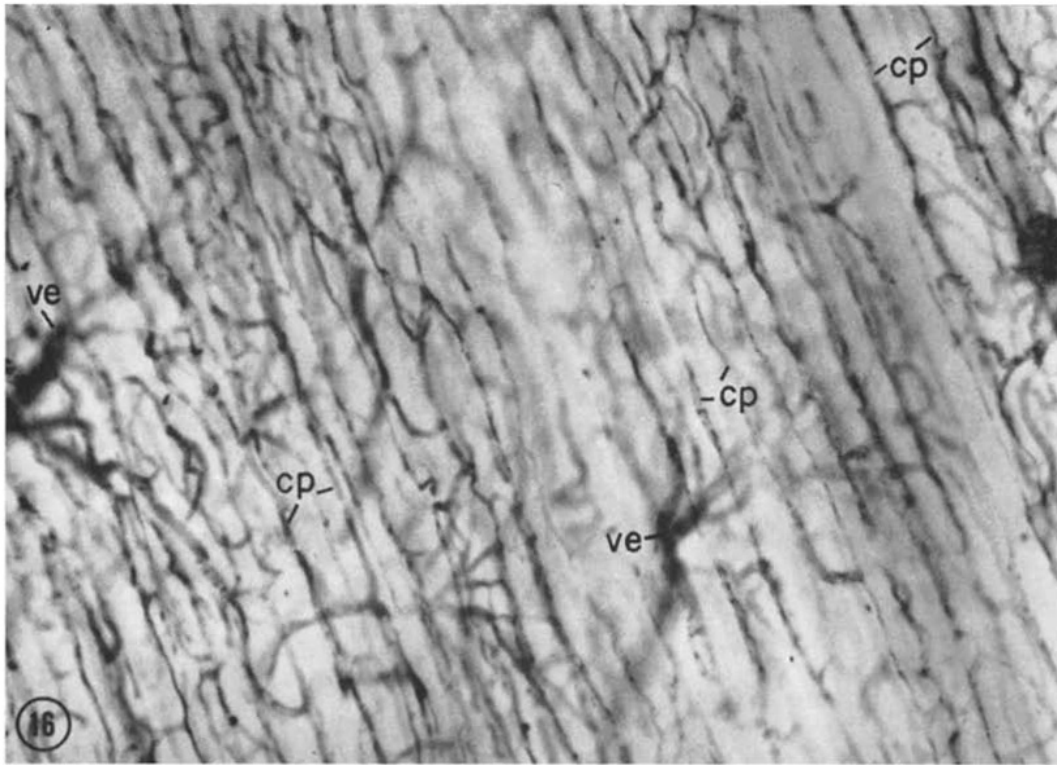
fixed specimens, because—among other factors—
the hydrostatic and osmotic pressure of the plasma
and interstitial fluid are not controlled during
fixation. Conversely, this system, like the large pore
system, could be accounted for by the plasma-
lemmal vesicles, provided that at any given time
the vesicle population establishes 15 to 20 patent
vesicular chains from one cell front to another, and
provided that each chain has a stricture of ~90 A
which could be located in an intercalated neck or

in a closing diaphragm. Such chains may exist in
the living endothelium, but may break down
during fixation, as a result of changes in the con-
formation of the proteins of the membrane.

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obtained with calibrated dextrans used as probe
molecules, Grotte (3) has calculated the diameter of
the small pores at 70–90 A. Since dextran molecules
behave as "hydrodynamic spheres" (3, 43), slit pores
should have approximately the same width.

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