Differentiated Microdomains on the Luminal Surface of the Capillary Endothelium
II. Partial Characterization of Their Anionic Sites

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ABSTRACT To investigate the chemical nature of the cationic ferritin (CF)-binding sites of the differentiated microdomains of the capilary endothelium, the vasculature of the mouse pancreas and intestinal mucosa was perfused *in situ* with neuraminidase, hyaluronidase, chondroitinase ABC, heparinase, and three proteases: trypsin, papain, and pronase. Proteases of broad specificity removed all anionic sites, suggesting that the latter are contributed by acid glycoproteins or proteoglycans. Neuraminidase, hyaluronidase, and chondroitinase ABC reduced the density of CF-binding sites on the plasmalemma proper, but had no effect on either coated pits or fenestral diaphragms. Heparinase removed CF-binding sites from fenestral diaphragms and had no effect on coated pits. Taken together, these results indicate that the anionic sites of the fenestral diaphragms are contributed primarily by heparan sulfate and/or heparin, whereas those of the plasmalemma proper are of mixed chemical nature. The membranes and diaphragms of plasmalemmal vesicles and transendothelial channels do not bind CF in control specimens; this condition is not affected by the enzymic treatments mentioned above.

In preceding studies (20–23), we have used cationized ferritin (CF) to map the distribution of anionic sites on the blood front of the fenestrated endothelium of murine visceral capillaries. These sites were found to have a preferential distribution ranging from near absence on diaphragms and membranes of plasmalemmal vesicles and transendothelial channels to a characteristically high density on fenestral diaphragms (20, 21, 23). To characterize the chemical nature of these anionic sites, we used a series of enzymic digestions performed *in situ* (by perfusion) before exposure of the endothelial surface to the cationic probe. In selecting test enzymes, we were guided by data already recorded in the literature that indicate that the acidic groups found on the surface of eukaryotic cells are contributed by the sialyl residues of glycoproteins and glycolipids, carboxylic groups of proteins, sulfated groups of glycoproteins, and glycosaminoglycans (proteoglycans), and possibly acidic groups of phosholipids (1–4, 11, 16, 24–26). Our findings show that the anionic sites of the fenestral diaphragms of the visceral capillary endothelium are contributed primarily by sulfated glycosaminoglycans, most probably heparan sulfate; the chemical nature of the anionic sites on the rest of the endothelial surface appears to be more diverse.

MATERIALS AND METHODS

Animals

For these studies we have used 64 male Swiss albino mice weighing 25–30 g.

Chemicals

Neuraminidase, hyaluronidase, chondroitinase ABC, trypsin, papain, and Pronase P were obtained from Sigma Chemical Co., St. Louis, Mo. For each enzyme, the type and the biological source are given in Table I. Neuraminidase (Clostridium perfringens NEVA) came also from Worthington Biochemical Co., Freehold, N. J., and chondroitin ABC-lyase from Miles Laboratories, Inc., Elkhart, Ind. Heparinase (crude) was prepared in the laboratory from Flavobacter heparinum by the method of Linker and Hovingh (13, 14), but without dialysis. The partially purified heparinase thus obtained was extensively tested and found to be free of protease activities (see reference 8 for further information). Hepar-

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TABLE I

Enzymes Used to Remove the Anionic Sites from the Luminal Surface of the Capillary Endothelium (Mouse Pancreas and Jejunum)

Enzyme	Source	Concentration*‡	pH*	Temperature*	Time*	Reference
				°C	min	
Neuraminidase	Cl. perfringens (type VIII, Sigma)	0.5–2 U/ml	5.5	37	30–60	5, 7, 8, 10
Hyaluronidase	Bovine testes (type IV, Sigma)	6,000 U/ml	5.4	37	20–30	3, 9, 24
Chondroitinase ABC	Proteus vulgaris	1–2 U/ml	7.4	37	15–30	7–9, 11, 15, 24
Heparinase, crude	Fl. heparinum	0.5–1 mg/ml	7.0	30–35	20–40	2, 3, 7, 8, 13, 15
Trypsin	Porcine pancreas (type IX, Sigma)	100–400 μg/ml§	7.4	37	10–30	3, 8, 24
Papain	Papaya latex (type IV, Sigma)	3–10 mg/ml	7.0	37	10–60	15
Pronase P	Str. griseus (type VI, Sigma)	4–6 U/ml	7.4	30–35	10–20	3, 9

* These conditions have been adapted from references given in the last column.
‡ In 0.1 M PBS or sodium acetate buffer.
§ Ca^{++} and Mg^{++} free.

inase activity was assayed according to Gill et al. (8) and expressed as μmol of unsaturated double bond appearing per hour from a heparin substrate.

Cationized ferritin (CF), pI 8.4, obtained from Miles Laboratories, was dialyzed before use for 24–48 h at 4°C against 0.15 M NaCl. Dulbecco's phosphate-buffered saline (DPBS) and Minimal Essential Medium (MEM) amino acids (× 50 concentrate) were purchased from Gibco Laboratories, Grand Island Biological Co., Grand Island, N. Y. The composition of the perfusion solution, routinely used and referred to hereafter as supplemented DPBS (DPBSs), was that indicated in references 19 and 23.

Experimental Protocol

The usual experimental procedure consisted of the following steps: (*a*) blood removal; (*b*) enzyme perfusion; (*c*) removal of enzyme; (*d*) CF administration; (*e*) removal of unbound CF; and (*f*) fixation by perfusion *in situ*.

(*a*) Blood removal: under light ether anesthesia, the vena cava caudalis and the abdominal aorta were catheterized with no. 7405 polyethylene tubing (Clay Adams, Div. of Becton, Dickinson & Co., Parsippany, N. J.). The aortic catheter was connected to a Harvard infusion pump, and the vasculature was washed free of blood by retrograde perfusion with DPBSs, warmed up to 37°C. Using vena cava caudalis as an outlet, the perfusion was carried out in an open circuit at a flow rate of 3–4 ml/min^{-1}, for 5–6 min. In some control experiments, 0.1% Evans blue was added to the perfusate and the efficiency of the perfusion was checked by following the blue coloring of the tissue, under the dissecting microscope. In the case of the pancreas, specimens were collected from the consistently well perfused middle portion of the organ.

(*b*) Enzyme perfusion: the buffers, the pH, and the concentrations of the enzyme solutions are given in Table I. After flushing the blood with DPBSs, the solution of each enzyme to be tested was administered intermittently for 1 min, at 5-min intervals, at a rate of ~3 ml/min^{-1}. The overall duration of the perfusion (intervals included) and the temperature of the perfusate are indicated in Table I. During enzyme perfusion, the animals were kept in a thermostat at 37°C.

(*c*) Enzyme removal: after perfusion, enzyme still present in the vasculature was flushed out with DPBSs under the same conditions as in *a*.

(*d*) CF administration: CF, at a concentration of 10 mg/ml saline, was administered at a ratio of 0.7 ml/100 g of body weight. The tracer solution was maintained in the vasculature for 3–5 min.

(*e*) CF removal: unbound CF was flushed out from the vasculature with DPBSs, as under *a*.

(*f*) Fixation: the vasculature was fixed *in situ* by perfusion with either 2% glutaraldehyde or a mixture of 3% glutaraldehyde and 5% formaldehyde in 0.1 M HCl-Na arsenate or HCl-Na cacodylate buffer, pH 7.2–7.4. The fixative was perfused intermittently (following the pattern used for enzyme treatment) for a total duration of 10–15 min. In a few experiments, the enzyme solutions were perfused in a closed circuit, as described in reference 23. In a separate series of experiments, the perfusion with heparinase was either preceded or followed by neuraminidase perfusion. In these cases, a brief washing with DPBSs was interposed between the two enzyme treatments. Both enzymes were used under the same conditions as in those experiments in which each of them was the sole enzyme tested.

Control Experiments

These consisted of perfusing either DPBSs or 0.1 M NaCl-acetate buffer, pH 7.0–7.2—instead of an enzyme solution—the other conditions being the same as for enzyme digestion.

Tissue Processing for Electron Microscopy

Specimens collected from the pancreas and jejunum were processed and examined as previously described (17, 18, 23).

RESULTS

Our experiments have tested the effect of a series of proteases and glycosaminoglycan-degrading enzymes on the anionic sites previously detected by CF binding on the surface of the capillary endothelium in the pancreas and intestinal mucosa of the mouse. These results apply strictly to the enzymes and conditions used in our experiments. Different results may be obtained by varying the conditions.

As indicated in our companion paper (23), CF binding reveals the existence of differentiated microdomains connected with well-defined structural elements on the luminal front of the endothelium. These microdomains are expected to reflect local differences in the density and chemical nature of anionic sites. The results obtained in this study indicate that the nature of these sites is indeed different from one microdomain to another.

Plasmalemma Proper

The density of decorating CF, hence of anionic sites, on the plasmalemma proper is not reduced by hyaluronidase treatment, but appears to be slightly decreased after neuraminidase, chondroitinase ABC, and heparinase perfusion (Figs. 1, 2, 5). After treatment with these enzymes, CF binding acquires a patchy character, which is particularly striking in the case of heparinase (Fig. 5). Serial digestion with neuraminidase followed by heparinase is not more effective than each of the two enzymes tested alone. The converse sequence, heparinase followed by neuraminidase, results in an overall decrease in labeling by comparison with the other related treatments.

Perfusion with trypsin is only partly effective as far as removal of anionic sites from the plasmalemma proper is concerned (Fig. 6). As is well established, trypsin cleaves specifically peptide bonds on the C terminal side of positively charged residues (arginine, lysine). By contrast, Pronase P and especially papain are extremely effective in causing almost complete removal of CF-binding sites from the entire luminal aspect of the endothelial surface (Figs. 7, 8). The last two proteases have broader specificities than trypsin. Papain cleaves peptide bonds on the C terminal side of both positively charged (arginine, lysine) and (some) aromatic (phenylalanine) residues, whereas Pronase P is a mixture of bacterial proteases

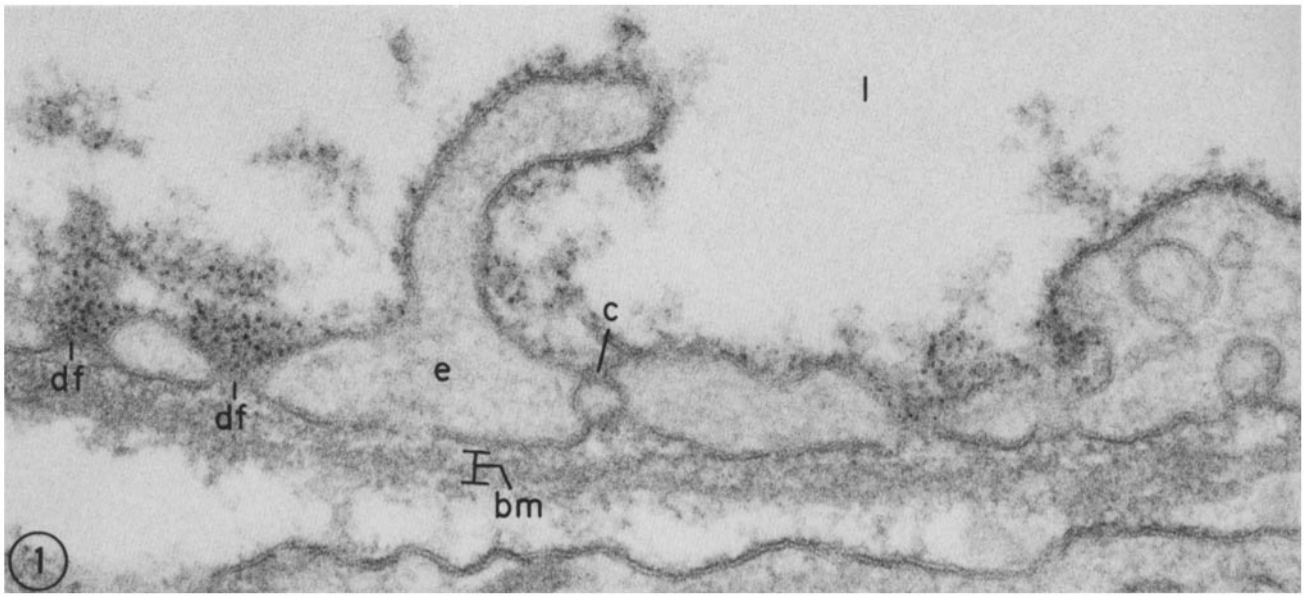


FIGURE 1 All micrographs represent small parts of endothelial cells in the microvasculature of the pancreas of the mouse, and all show cationized ferritin labeling as modified by previous perfusion with either proteases or glycosaminoglycan-degrading enzymes. *l*, lumen; *e*, endothelium; *bm*, basement membrane; *ps*, pericapillary spaces; *v*, plasmalemmal vesicle; *vd*, stomatal diaphragm of a plasmalemmal vesicle; *i*, infundibulum preceding the introit of a plasmalemmal vesicle; *cp*, coated pit; *cv*, coated vesicle; *df*, fenestral diaphragm. Fig. 1: Effect of neuraminidase perfused in closed circuit for 30 min. CF binding is partially reduced on the plasmalemma proper, but it is still present at high density on fenestral diaphragms. CF-labeled amorphous masses are also present in the lumen. The transendothelial channel at *c* is not labeled. × 125,000.

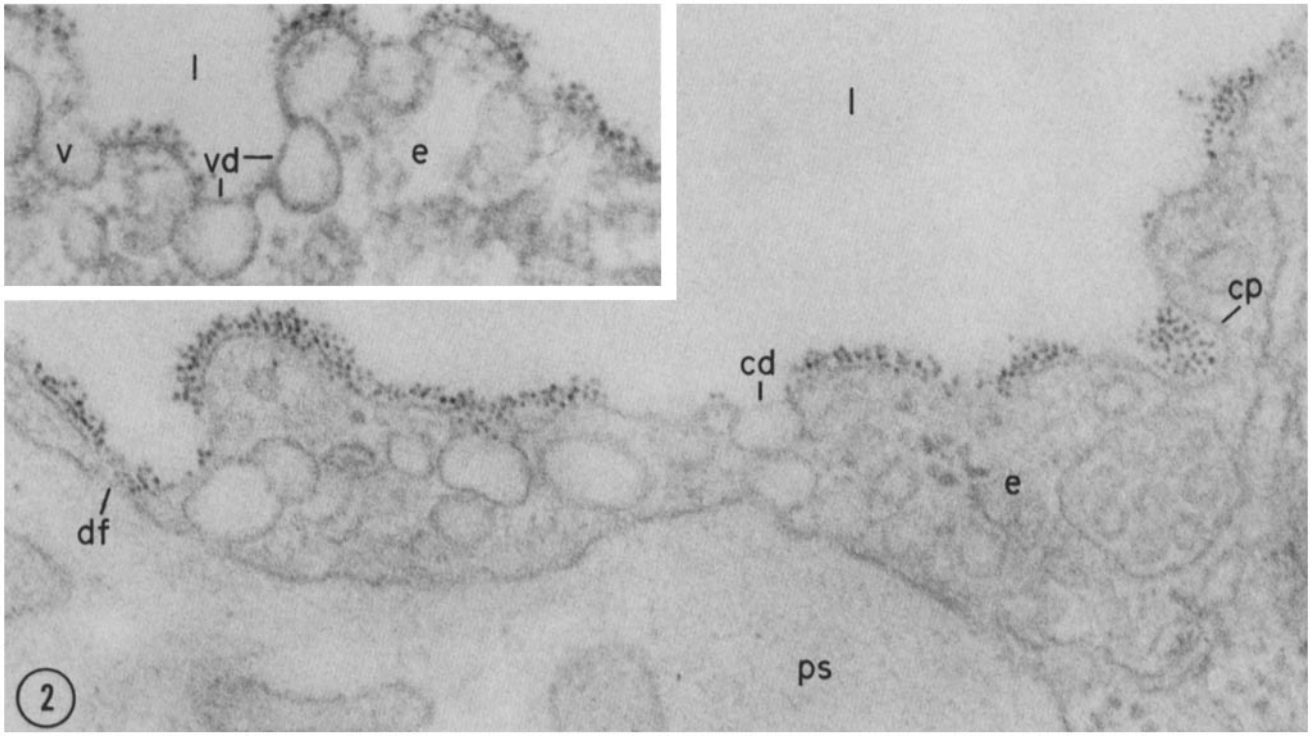


FIGURE 2 Effects of perfusion with chondroitinase ABC (30 min) on subsequent CF binding. The situation is comparable to that found in controls. *cd*, channel diaphragm. × 100,000. *Inset:* Effects of hyaluronidase perfusion (30 min). In both cases (Fig. 2 and *inset*), the luminal aspect of the plasmalemma proper is still heavily labeled. So are the coated pits and the fenestral diaphragms. Plasmalemmal vesicles and their diaphragms are not labeled. × 142,000.

capable of cleaving a wide variety of peptide bonds.

Coated pits and coated vesicles are heavily labeled by CF in control specimens. Neither neuraminidase nor any one of the glycosaminoglycan-degrading enzymes affects CF binding. The latter is reduced, however, after trypsin treatment and removed altogether by pronase and papain. The protease effects apply only to coated pits.

In control specimens, none of these, plasmalemmal vesicles,

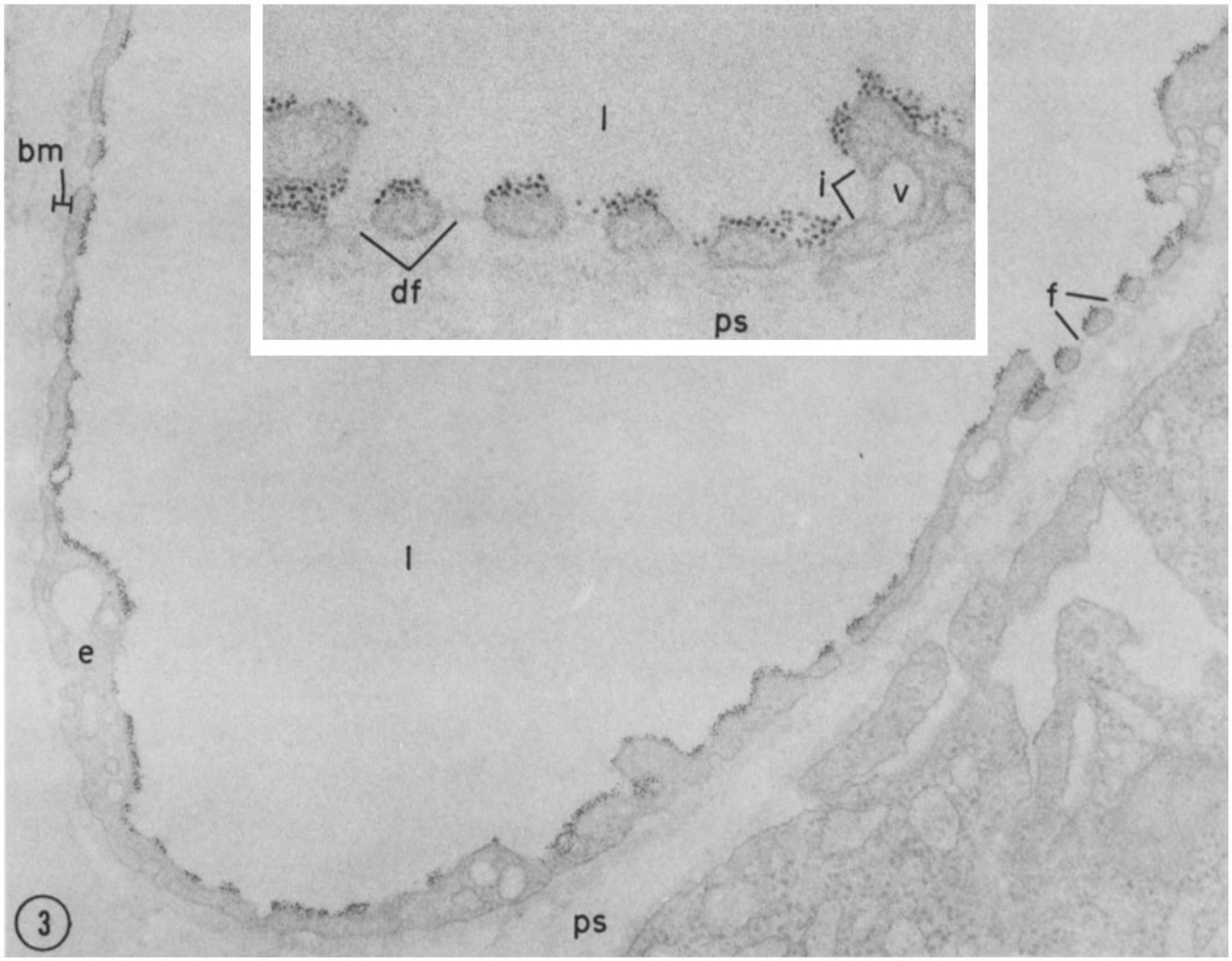


FIGURE 3 General view of a blood capillary after heparinase perfusion (30 min) followed by CF decoration. Anionic sites have been effectively removed from practically all fenestral diaphragms. The decoration of the luminal surface of the endothelial plasmalemma is patchy. The decorated domains as well as alternating CF-free areas vary considerably in size. Plasmalemmal vesicles and their associated infundibula are not labeled. × 38,000. *Inset:* A series of fenestrae at higher magnification taken from the upper right corner of Fig. 3. It illustrates the extensive (but not complete) removal of CF-binding sites from fenestral diaphragms, and the lack of CF binding to a plasmalemmal vesicle, its stomatal diaphragm, and its associated infundibulum. × 120,000.

transendothelial channels and cognate diaphragms, is marked by CF. Moreover, the infundibula leading to plasmalemmal vesicles are generally not labeled by the cationic probe. This lack of labeling is not affected by any of the enzymatic treatments tested.

In control experiments, fenestrae and fenestral diaphragms are the structures more heavily and consistently labeled by the cationic probe. CF binding is not detectably affected by either hyaluronidase or chondroitinase ABC (Fig. 2), but it is effectively and dramatically eliminated by heparinase treatment (Figs. 3, 4). As already mentioned, this enzyme also changes the labeling of the plasmalemma proper from a nearly continuous to a definitely patchy appearance.

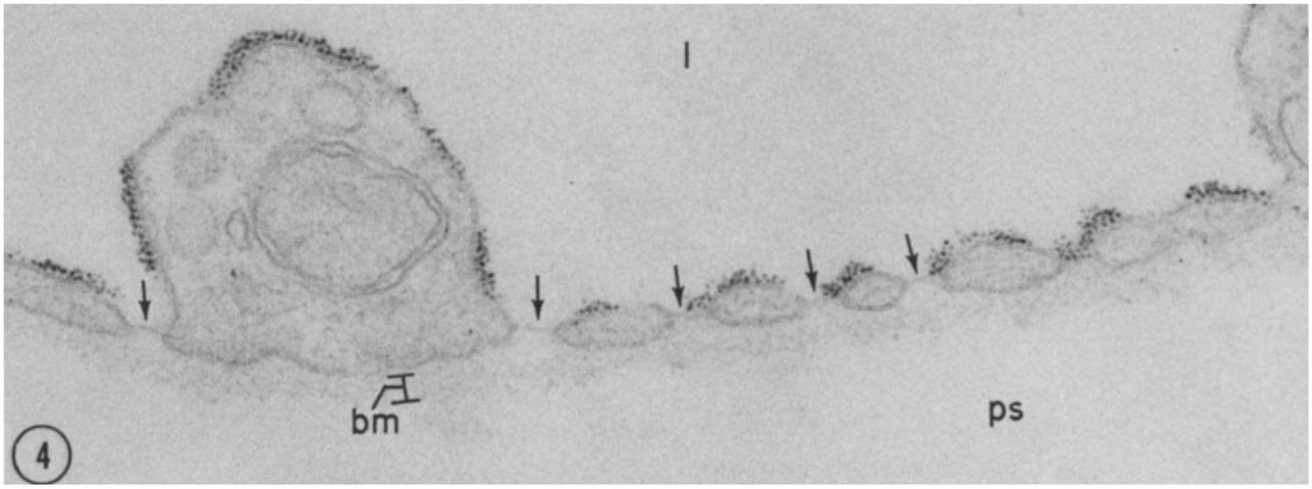
Papain and Pronase P are also extremely effective in the removal of CF-binding sites from the fenestrae and their diaphragms (Figs. 7, 8). Trypsin reduces CF binding only slightly, but occasionally appears to remove or weaken the fenestral diaphragms themselves as indicated by the passage of plugs or large clusters of CF through the affected fenestrae (Fig. 6).

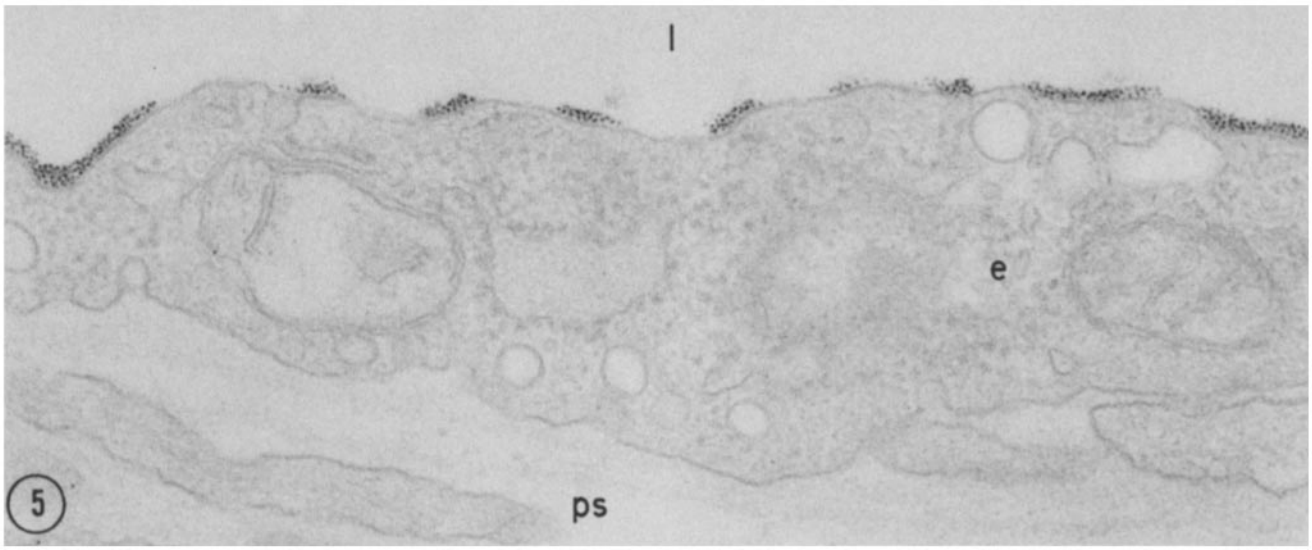
Fig. 9 gives in graphic form the effect of each enzyme tested on the CF binding on each of the endothelial structures described above. It also attempts to give a semiquantitative assessment of these effects.

DISCUSSION

Our results indicate that enzyme digestion carried out in perfusion experiments is demonstrably effective at least for some of the enzymes tested, e.g., proteases of broad specificity and heparinase. They also indicate that the differentiated microdomains of the luminal aspect of the endothelium have a distinct biochemical basis which appears to be different from one domain to another.

The results that can be interpreted now with a reasonable degree of certainty concern the chemical nature of the anionic





FIGURES 4–5 Effects of heparinase perfusion (40 min). In Fig. 4, CF-binding sites have been eliminated from practically all fenestral diaphragms (arrows), but the diaphragms themselves are apparently still present in their usual location. CF decorates most of the surface of the plasmalemma proper. × 92,000. Fig. 5 illustrates a thicker portion of an endothelial cell in a region provided with few plasmalemmal vesicles along the luminal front of the cell, and gives an example of sharply defined patchy distribution of CF binding sites detected after heparinase perfusion. × 90,000.

sites of the fenestral diaphragms. They cannot be ascribed to sialyl residues of glycoproteins or glycolipids because of the negative results obtained with neuraminidase. They appear to belong to heparan sulfate or heparin because they are effectively removed by heparinase, an enzyme preparation of relatively broad specificity: it degrades hyaluronic acid, chondroitin sulfates ABC, dermatan sulfate, as well as heparan sulfate and heparin; but it is free of protease activity on a variety of substrates (8). Accordingly, by itself heparinase cannot define the chemical nature of the polyanionic molecules it degrades; however, because the results obtained with other glycosaminoglycan-degrading enzymes, e.g., hyaluronidase and chondroitinase ABC, are negative, it can be concluded (by difference) that the anionic sites of the fenestral diaphragms are contributed by heparan sulfate or heparin. The fact that the sites can be demonstrated after the extensive washing included in our experimental protocol suggests that they are contributed primarily by heparan sulfate,[1] but their definitive identification must wait the results of further experimentation with an enzyme of strict specificity, such as heparitinase (2, 9, 11–13). The glycosaminoglycans present on the fenestral diaphragms appear to belong to proteoglycans or be closely associated with other proteins because the corresponding anionic sites are also effectively removed by proteases of broad specificity such as pronase and papain. After removing the anionic sites, these proteases still leave behind a structurally recognizable diaphragm or diaphragm remnant. It is noteworthy that the fenestral diaphragms themselves appear to be more susceptible to trypsin attack, as suggested by the presence of large CF clusters on the abluminal site of a fraction of the fenestral population, after perfusion with trypsin.

The results obtained for other microdomains are more difficult to interpret. For instance, the anionic sites of coated pits (and coated vesicles) appear to be contributed by proteoglycans and/or glycoproteins. These sites are removed by proteases of broad specificity, but the nature of their glycoproteins or

[1] After extensive perfusion-washing of other vascular beds *in situ*, we were not able to detect sulfated glycosaminoglycans in the perfusate (D. Popov, M. Simionescu, and N. Simionescu. Unpublished observations).

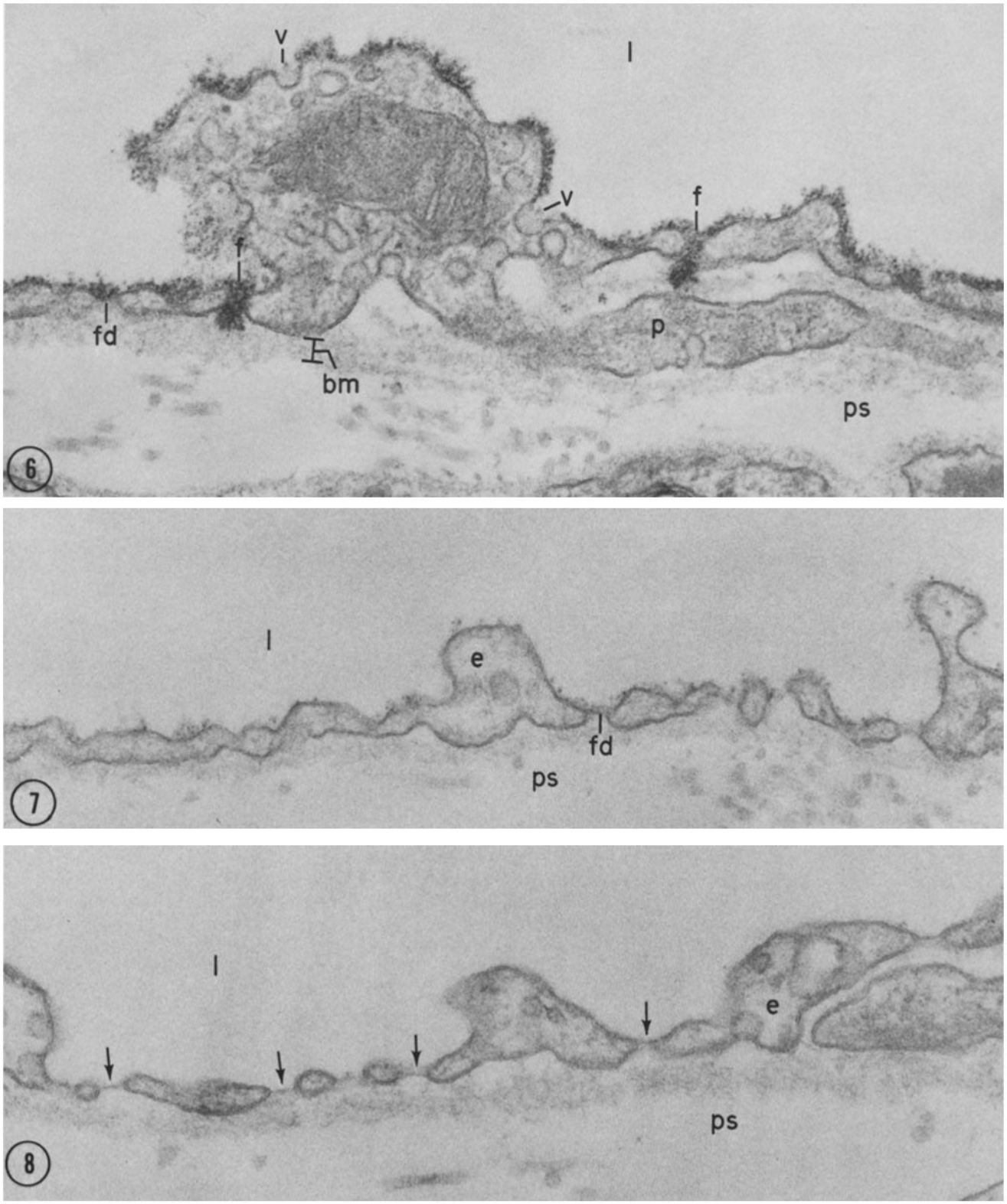
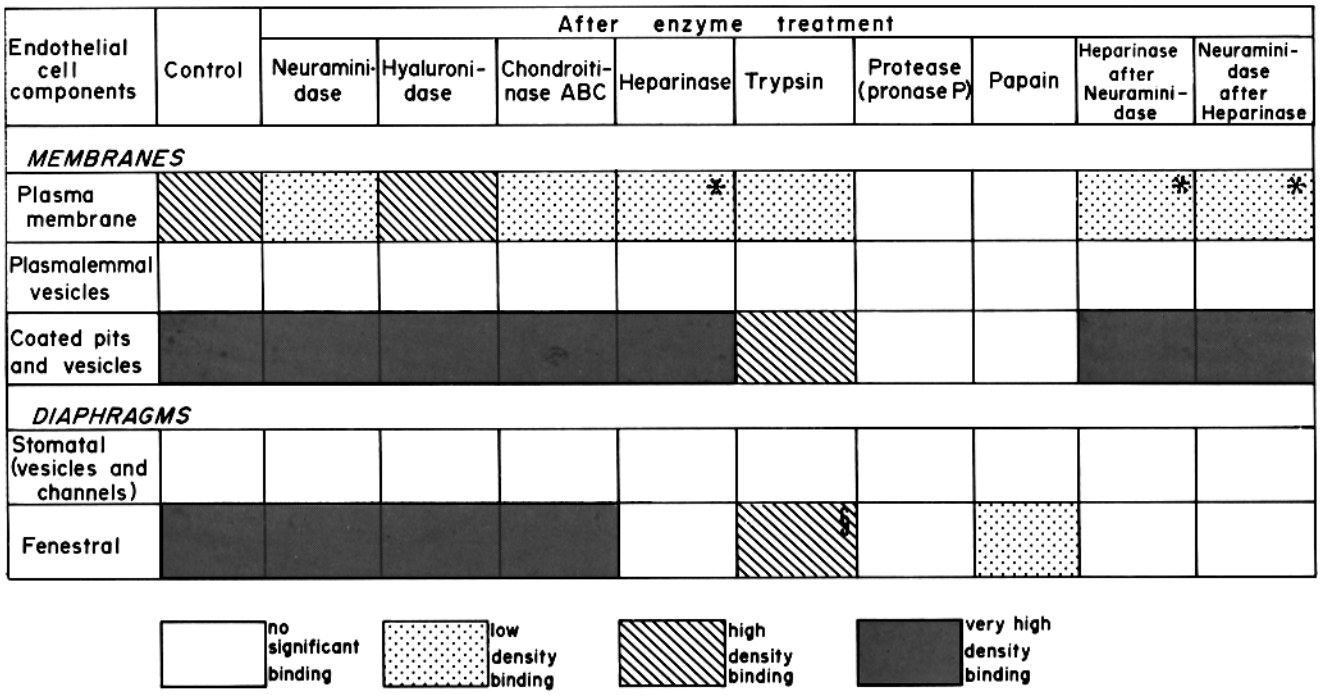


FIGURE 6 Effects of trypsin perfusion (30 min). CF binding to the luminal surface of the endothelium is more uneven than in controls, and is limited to the plasmalemma proper and fenestral diaphragms. Plasmalemmal vesicles and their stomatal diaphragms are not labeled. Large plugs of aggregated cationized ferritin have penetrated through two fenestrae and appear to be retained by the basement membrane (left) and by the basement membrane and a pericyte (p) (right). × 70,000.

FIGURE 7 Effects of papain perfusion (10 min). Practically all the anionic sites have been removed from the entire luminal surface of the endothelium. × 70,000.

FIGURE 8 Pronase treatment (20 min) results in complete removal of all CF-binding sites from the luminal surface of the endothelium. Note that in this case (as well as after papain treatment), fenestral diaphragms appear to be still present in their usual location (arrows). × 70,000.



(9)

FIGURE 9 Effects of enzyme treatments on the anionic CF binding sites of the endothelial cell surface (luminal domains).

glycosaminoglycans (if present) is uncertain. It is possible that the sites are contributed by keratan sulfate or sulfated glycoproteins because they are resistant to neuraminidase and to all glycosaminoglycan-degrading enzymes so far tested; but it is possible that the conditions used in our experiments were not optimal for some of the enzymes used.

The membranes, as well as the diaphragms of the plasmalemmal vesicles and transendothelial channels, remain distinct microdomains. They are not decorated by CF in the controls, and their affinities for our cationic probe are not modified by any of the enzymes tested.

Finally, the anionic sites present on the luminal site of the plasmalemma proper appear to be contributed, like those of the other domains, by proteoglycans and acidic glycoproteins, because they are effectively removed by proteases. Partial removal by neuraminidase (especially following heparinase treatment) and by chondroitinase ABC and heparinase suggests that these sites are contributed by a variety of macromolecules, e.g., sialoglycoproteins as well as glycosaminoglycans. The most intriguing results were obtained with heparinase, which often revealed a patchy distribution of heparinase-resistant anionic sites on the plasmalemma (in addition to the effective removal of anionic sites from fenestral diaphragms). It is possible that the intercalated CF-free patches represent the infundibula usually associated with plasmalemmal vesicles. These infundibula, described in more detail in our companion paper (23), have few or no CF-binding sites; they may be preferential sites for plasmalemmal vesicle fusion to, or detachment from, the plasmalemma proper. In fact, similar intercalated CF-free patches can be seen at lower frequency in control exeriments; their increase in frequency after heparinase treatment remains to be explained; it may have physiological significance or it may be an experimental artifact: patching of other anionic sites by CF may be facilitated by the preliminary removal of heparan sulfate.

The results obtained with proteases of broad specificity suggest that all or practically all anionic sites on the capillary endothelium are provided by either acidic proteins or proteoglycans. Sites contributed by acidic glycolipids seem to be either absent or extremely sparse. Currently available preparations of glycosaminoglycan-degrading enzymes (hyaluronidase, chondroitinase ABC) may be contaminated by proteases. Except for heparinase (8), they were not tested for proteolytic activities because their effects were limited and, in addition, quite different from those obtained with proteases.

A point that deserves further attention is the apparent resistance of fenestral diaphragms to both broad specificity proteases and glycosaminoglycan-degrading enzymes. It suggests extensive cross-linkage of the constitutive molecules.

We have not studied the effect of the enzymes used on the permeability of the capillary wall because our experimental conditions were adjusted primarily for studying CF binding. Permeability modifications remain to be investigated in further work with appropriate probe molecules.

The presence of sulfated glycosaminoglycans, especially heparan sulfate, has been recorded in cultured endothelia (1, 2, 7, 26). Apparently, endothelial cells (from bovine aorta) are able to synthesize a variety of sulfated glycosaminoglycans. It

can be assumed that these properties apply also to endothelia from other parts of the vasculature, including the capillary endothelia investigated by us in this study. Enzymatic removal of anionic sites from the vasculature endothelium of bone marrow sinusoids was tried before by De Bruyn and his collaborators (5, 6). The only enzyme tested was neuraminidase. As in our case, it did not reduce CF binding to either the endothelial cell surface, or coated pits, or fenestral diaphragms at neutral pH.

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