INTESTINAL CAPILLARIES

II. Structural Effects of EDTA and Histamine

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ABSTRACT

Perfusion of the fenestrated capillaries of the intestinal mucosa of the rat with 0.05–0.1 M EDTA removes the diaphragms of the endothelial cells and detaches these cells from one another and from the basement membrane. The latter, even when completely denuded, retains effectively particles of 340 A (average) diameter. Perfusion with histamine (1 μg/ml) results in partial removal of fenestral diaphragms, occasional detachment of the endothelium from the basement membrane, and focal separation of endothelial intercellular junctions.

In a previous paper, we have shown that horseradish peroxidase and ferritin, used as probe molecules for the small and large pore system, respectively, leave the capillaries of the intestinal mucosa through the fenestrae of the endothelium (1). Since these fenestrae, usually provided with an aperture or diaphragm (2–4), appear to be important pathways for plasma-interstitial fluid exchanges, we decided to investigate their response to agents known to affect capillary permeability such as EDTA[1] (5, 6) and histamine (7, 8).

METHODS

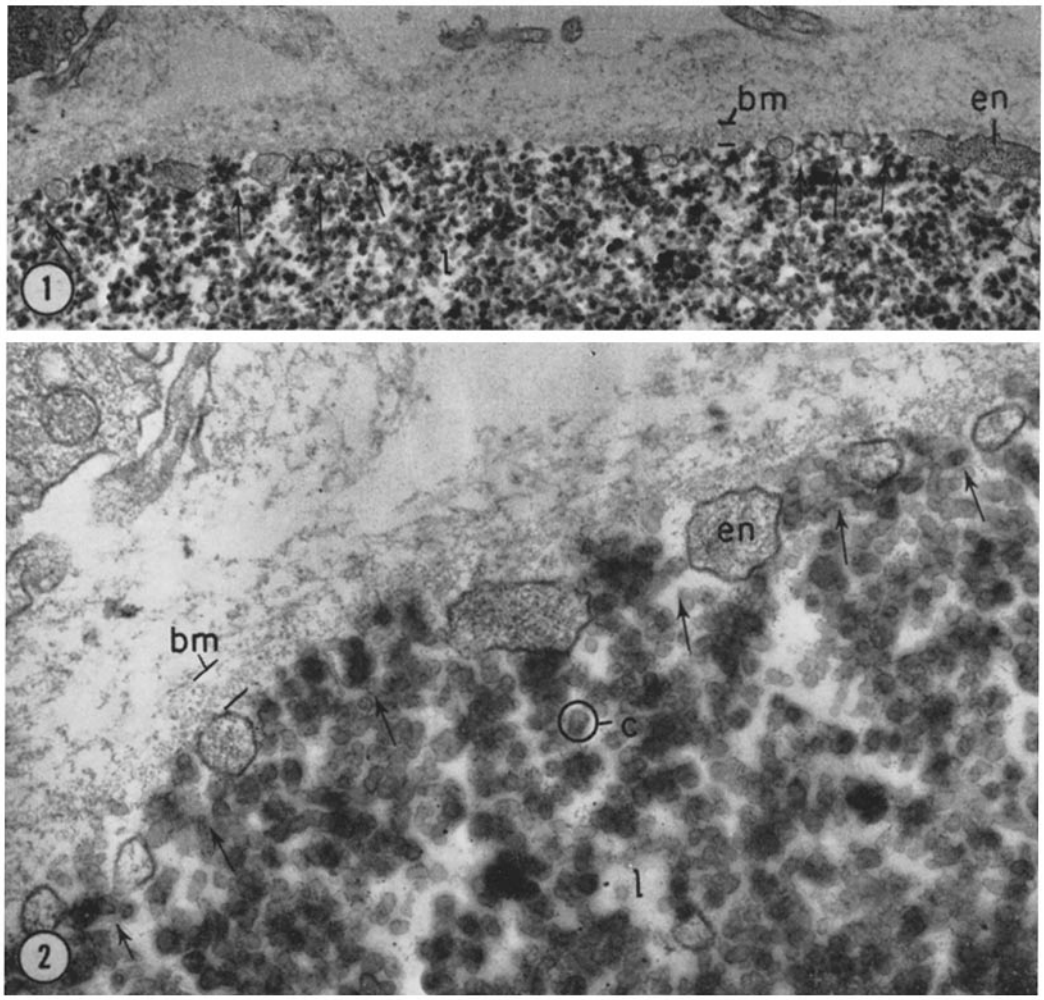
The experiments were carried out on male Sprague Dawley rats of 250–300 g weight, heparinized and anesthetized with chloral hydrate (35 mg/100 g body weight, given intraperitoneally). To obtain a better control of the composition of the intravascular medium, the drugs were administered by perfusion instead of being given systemically. The aorta was cannulated below the diaphragm with polyester tubing (0.062-in. O.D. PE 160 Intramedic) which was connected through a 37° thermostatic bath with reservoirs suspended at 150 cm above the animal. EDTA was perfused at 0.025, 0.05, or 0.1 M concentration, dissolved in Ca^{2+}-and Mg^{2+}-free carbonated saline (9); before use, the pH of the solution was adjusted to 7.2 with 0.2 N KOH. Histamine at 0.1, 0.2, and 1 μg/ml was dissolved in Earle's solution (10) containing 3% bovine serum albumin. Two to four rats were perfused with each drug concentration. Control rats were perfused with the same media without drugs. All perfusates contained 0.95% Evans blue, to facilitate the recognition of satisfactorily perfused regions, and carbon black[2] (11), or colloidal gold[3] (12) as tracers for electron microscopy. The size range of the particles was 230–480 A for carbon black,[4] and 30–300 A for colloidal gold. 10 min after beginning the perfusion, pieces of well perfused intestinal loops were fixed in 1% OsO_4 in 0.1

[1] EDTA = ethylenediaminetetraacetate, disodium salt.

[2] Batch CII/1431a. The ink was diluted 50 times in the perfusate to give a final concentration of 2 mg/ml.

[3] Aurocoloid TM-198 obtained after complete radioactivity decay from Abbott Laboratory, Teterboro, N.J. The original concentration, 2.5 mg/ml, was raised to ~10 mg/ml in a flash evaporator.

[4] The average diameter of these particles was 340 A; the suspension contained, in addition, small clusters of particles up to 2100 A in diameter.



General Abbreviations: *l*, lumen; *en*, endothelium; *bm*, basement membrane.

Figs. 1–6 show blood capillaries in the lamina propria of the intestinal mucosa (rat) after 10-min perfusion with 0.1 M EDTA in Ca^{2+}-, Mg^{2+}-free saline. Carbon particles (India ink) were added to the perfusate as tracers.

FIGURES 1 and 2 The endothelial fenestrae (arrows) have lost their diaphragms and appear greatly enlarged. Through the patent fenestrae, the carbon particles (*c*) of the perfusate reach the basement membrane (*bm*), but do not penetrate beyond it. In the center of Fig. 1, part of the capillary wall consists only of the basement membrane. Note the pronounced fibrillar texture of the latter in Fig. 2. Fig. 1, × 17,000; Fig. 2, × 54,000.

M phosphate buffer (pH 7.4) for 2 hr, at ~0°. The blocks were subsequently stained in uranyl acetate for 30 min at 4° (13), dehydrated in graded ethanols, and finally embedded in Epon (14). Sections prepared with a Reichert OM U2 or a Servall MT2 microtome, and stained with uranyl acetate and lead citrate (15), were examined in a Hitachi HU 11C or a Siemens Elmiskop I electron microscope.

OBSERVATIONS

Intestinal capillaries, perfused with Ca^{2+}- and Mg^{2+}-free saline for up to 10 min or with Earle's

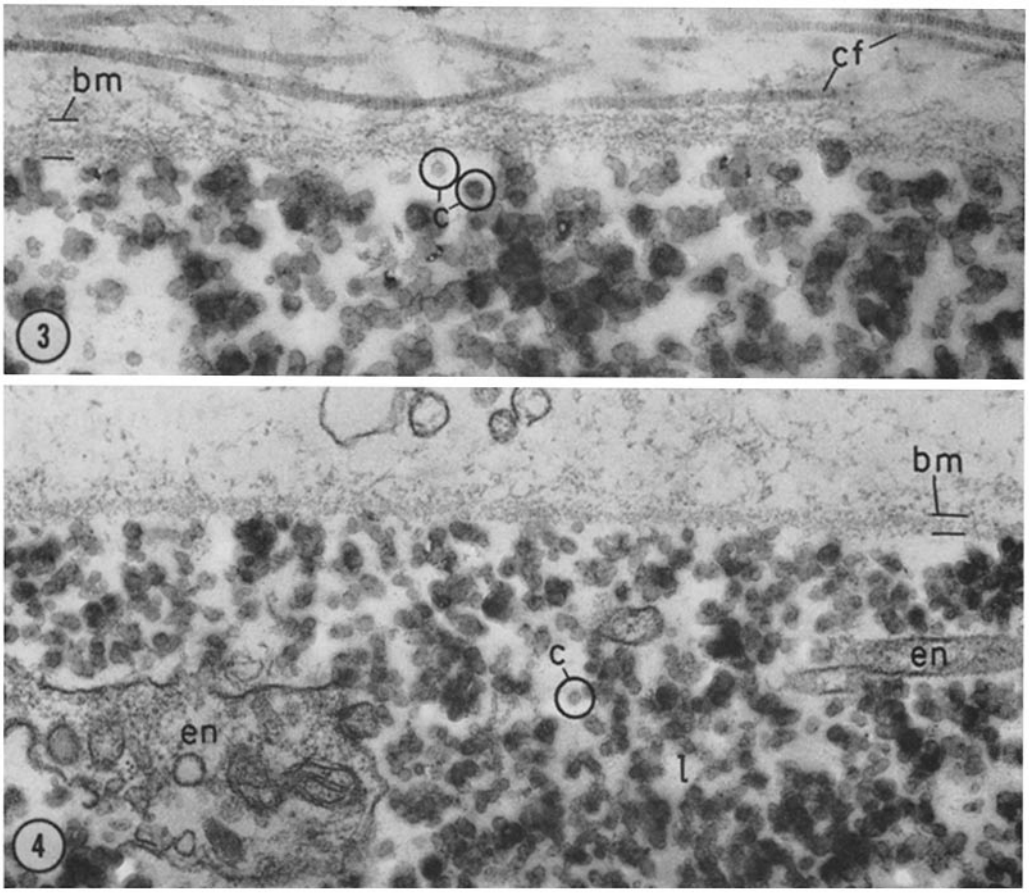


FIGURE 3 No endothelial remnants are visible in this field. The basement membrane (*bm*), which is impermeable to the tracer, shows clearly its fibrillar texture. Collagen fibrils in the lamina propria are marked *cf*. × 48,000.

FIGURE 4 Parts of the completely detached endothelium (*en*) are still visible in the capillary lumen (*l*). The carbon particles (*c*) of the perfusate reach the basement membrane (*bm*) which retains them effectively. × 40,000.

solution containing albumin, retained their normal appearance. Beyond 10–15 min, there was a pronounced increase in the number of flaps and filiform pseudopodia protruding from the endothelium into the capillary lumen, presumably on account of anoxia (see 16); otherwise, the endothelial cells seemed normal. After long perfusion with Ca^{2+}- and Mg^{2+}-free saline, a slight edema developed in the lamina propria in between the capillaries and the epithelium. Irrespective of the duration of perfusion, carbon particles were always retained within capillary lumina.

Perfusion for 10 min with 0.025 M EDTA did not cause any visible modification of capillary structure. However, when the concentration was increased to 0.05 M, the endothelial cells began to detach from the basement membrane, leaving behind a relatively wide and irregular subendothelial space. In addition, they thickened in places and acquired a highly irregular profile which suggested an increase in the number of pseudopodia and vacuoles. When the concentration of EDTA was raised to 0.1 M, damage to the capillary wall was more pronounced. The fenestrae of the endothelium lost their diaphragms, enlarged, and were frequently replaced by wide, patent discon-

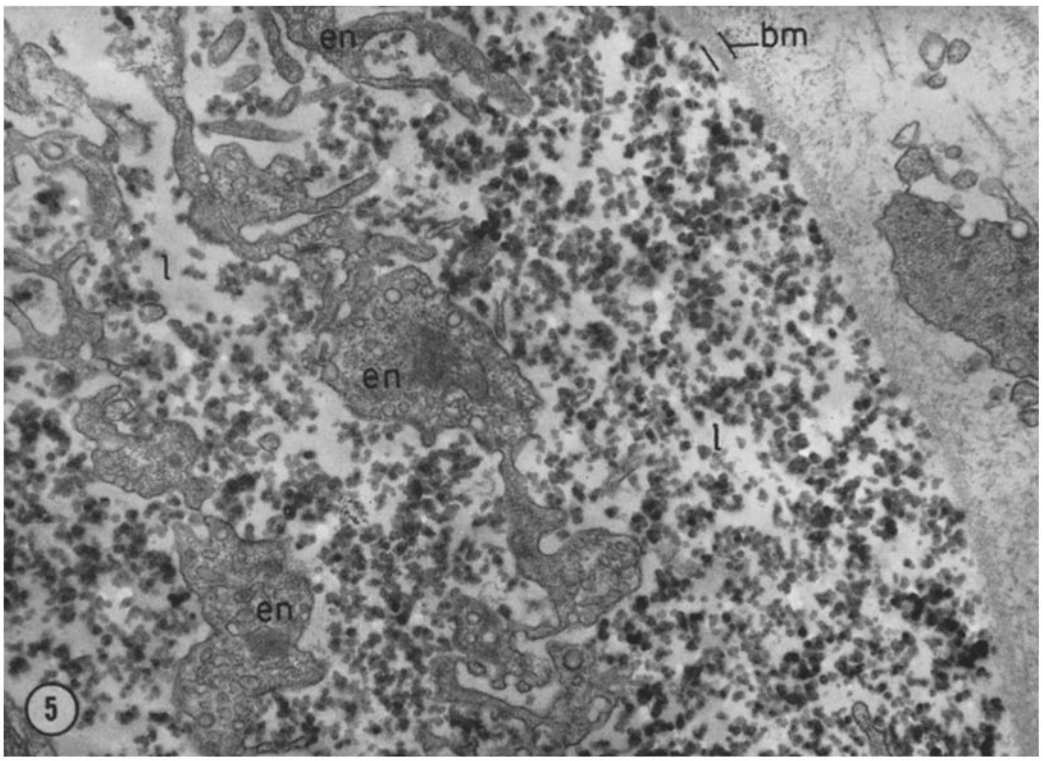


FIGURE 5 Endothelial cells (*en*) detached from the basement membrane (*bm*), which appears completely denuded, are seen "floating" in the lumen (*l*). The basement membrane retains effectively the carbon particles of the perfusate. × 21,000.

tinuities (Figs. 1, 2). The intercellular junctions were generally loosened, and the cells often appeared separated from one another by gaps or large channels. In many cases, part of the endothelium detached from the basement membrane and retracted leaving a large part of the latter directly exposed to the perfusate (Figs. 3, 4). Eventually, all the endothelial cells detached from one another and from the basement membrane to turn into freely floating cellular elements in the lumen (Fig. 5). At this point, extended regions of the capillary wall consisted only of the basement membrane (Fig. 6) which appeared slightly thickened and showed a looser, more distinctly fibrillar texture (Figs. 3, 4) than in controls. Although extensively denuded and visibly altered, the basement membrane generally retained carbon black particles; only occasionally a few of them were found embedded in the basement membrane or free in the pericapillary spaces (Fig. 6). The basement membrane also retained most colloidal gold particles larger than ~200 A. Such particles only occasionally escaped to the interstitia, while particles smaller than 200 A passed through and were often found in the pericapillary spaces. Although escaped particles ranged in size up to ~900 A, no channels of appropriate dimensions were seen in the basement membrane in their vicinity. Hence, we assume that these channels must be extremely rare and possibly transient.

Perfusion with histamine at 0.1 μg/ml had no effect on the structure of the capillary wall. When the concentration was increased to 0.2 μg/ml, edema developed under the epithelium, and in many capillaries a variable but generally small percentage of the diaphragms closing the fenestrae disappeared (Fig. 7); some of the patent fenestrae enlarged, and carbon particles began leaving the lumina through them (Figs. 8–11). However, the endothelial cells did not detach from one another or from the basement membrane and did not retract. The frequency of altered capillaries in-

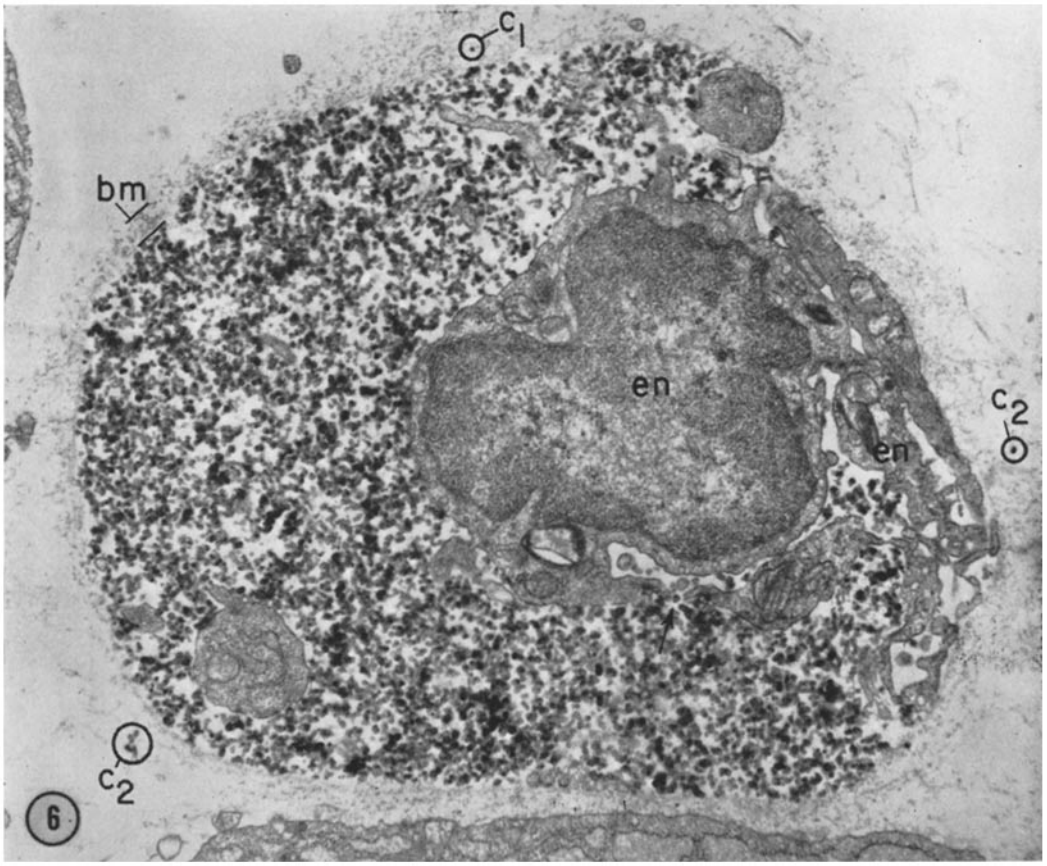


FIGURE 6 Cross-section through a blood capillary whose wall is reduced to the basement membrane (*bm*) layer on ~¾ of its perimeter. The partially detached and fully collapsed endothelium is marked *en*. A patent fenestra is indicated by an arrow. Note that a few carbon particles have escaped the lumen and are buried deeply in the basement membrane (c_1) or have reached the pericapillary spaces (c_2). × 15,000.

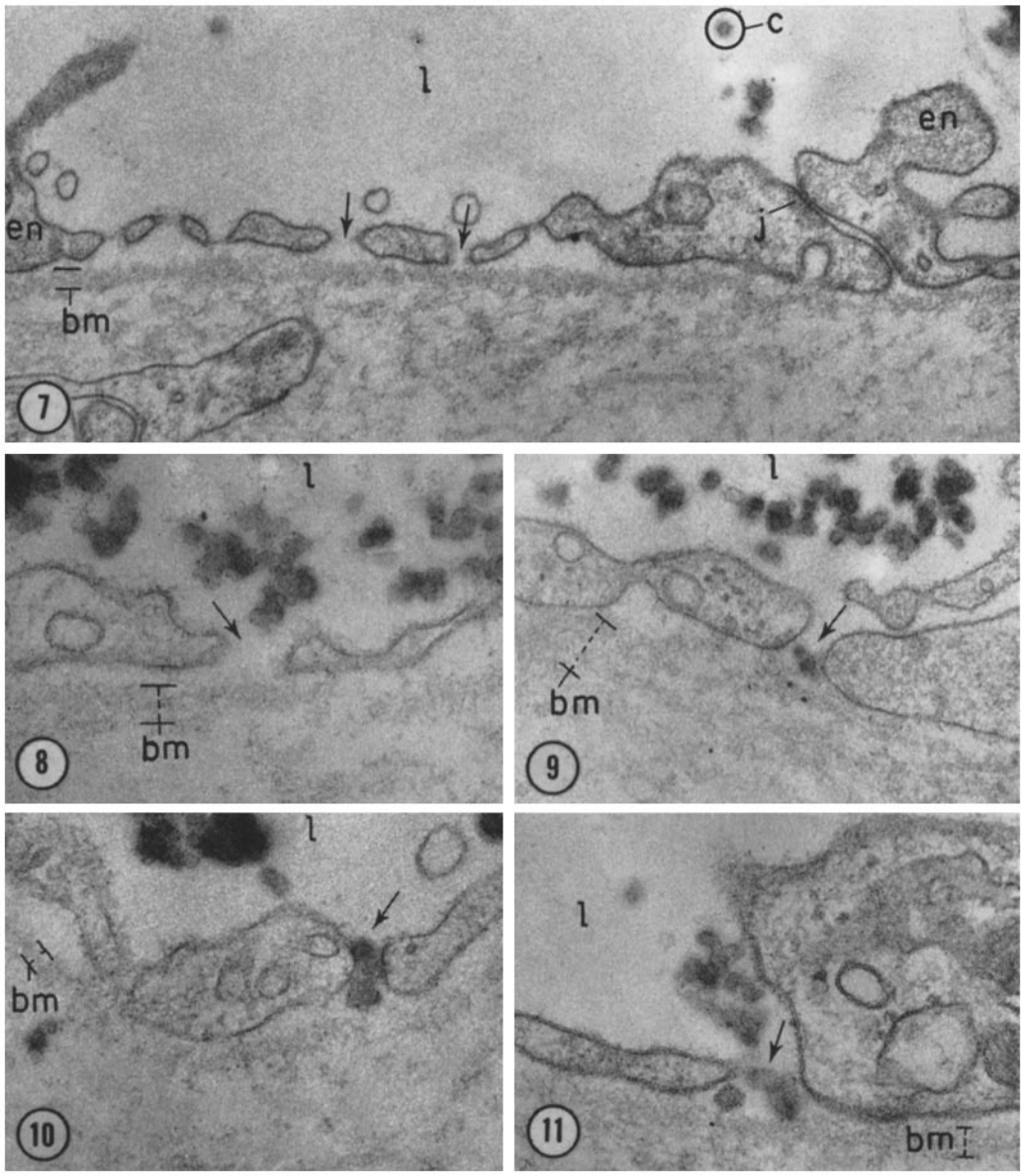
creased when the intestine was perfused with 1 μg/ml of histamine, but the modifications remained limited primarily to the loss of diaphragms by part of the fenestral population. Only occasionally we encountered focal separation of the endothelial cells from the basement membrane and from one another resulting, in the last case, in rather limited areas of exposed basement membrane. The latter successfully retained most carbon black particles, as it did in the EDTA experiments already mentioned.

DICSUSSION

The importance of Ca^{2+} in the regulation of capillary permeability, presumably by binding the endothelial cells together with a protein-Ca "cement," has been recognized for more than 30 years (17–20). Hence it was expected that EDTA, a powerful Ca-complexing agent, will affect capillary permeability and related structural features in the capillary wall. In fact, "stasis" accompanied by an increase in permeability has already been recorded by light microscopy in the capillaries of EDTA-irrigated mesentery[5] (6).

Our findings give more precise indications, at a pertinent dimensional level, on structural alterations which are caused by EDTA, and which appear to be directly related to increased permeability in the fenestrated capillaries of the intestinal mucosa. Some of these alterations, e.g., separation

[5] In contradistinction to the capillaries of the intestinal mucosa, mesenteric capillaries have a continuous (nonfenestrated) endothelium (36).



Figs. 7–11 show blood capillaries in the lamina propria of the intestinal mucosa (rat) after 10-min perfusion with 3% serum albumin in Earle's solution containing 1 μg/ml histamine. Carbon particles were added to the perfusate as tracers.

FIGURE 7 The treatment has not affected the intercellular junction (*j*) but has led to partial detachment of the endothelium from the basement membrane and disappearance of the apertures of two (arrows) of the six fenestrae seen in this field. × 66,000.

FIGURES 8–11 These figures show individual fenestrae which have lost their diaphragms and through which carbon particles reach the basement membrane (Figs. 9–11). Fig. 8, × 85,000; Fig. 9, × 67,000; Fig. 10, × 73,000; Fig. 11, × 80,000.

of endothelial cells from one another and from the basement membrane, were to be expected in view of the known importance of Ca^{2+} in cell-to-cell and cell-to-substrate adhesion (21, 22, see also 23). Another, i.e., the removal of fenestral apertures, is entirely novel, at least as far as we are aware. It implies that Ca^{2+} is necessary either for binding together the components of the aperture, presumably proteins (24) and polysaccharides (25), into a thin film, or for anchoring the latter in the fenestral frame, or for both. It also has some interesting functional implications, since our recent work (1) has established that the apertured fenestrae represent the small pore system, and has suggested that permanently or transiently patent fenestrae correspond to the large pore system in this type of capillaries.

The effects of histamine are only in part similar to those of EDTA; the drug removes a certain percentage of fenestral apertures, but affects much less extensively and intensively than EDTA cell junctions and endothelium–basement membrane adhesion. The mechanism of histamine action cannot be explained at present, since the chemical nature of its receptors is unknown. Ca^{2+} is apparently required for histamine activity, presumably histamine-receptor interaction (26–28), and exogenous histamine administered per os is known to be concentrated in the intestinal mucosa (29), which is already richer than other tissues in endogenous histamine (30). These points, especially the high local concentration, might have some bearing on the effects observed. In any case, it should be pointed out that in the intestinal mucosa the site of action (i.e., capillaries) and the type of structural alteration observed (i.e., removal of fenestral apertures) are different from those described in the muscle vasculature where the vessels affected are small venules (50–200 μ in diameter) and possibly the venous ends of capillaries (11), and where the structural effect consists in focal separations of endothelial cells along their junctions (31).

Another finding that deserves comment is the extensive but incomplete impermeability of the basement membrane to particles larger than ~200 A. Previous work has established that this membrane is freely permeable to horseradish peroxidase (diameter ≃50 A) and ferritin (diameter ≃100 A) in muscular (32, 33) as well as intestinal (1) capillaries. The new finding indicates that in visceral capillaries, as in the small vessels of muscle (31), skin (34, 35) and mesentery (36, 37), the basement membrane can function as an additional, coarser permeability barrier when denuded. Each layer of the capillary wall thus appears to have its own permeability characteristics. Hence, the pore theory (38, 39) should be considered with this conclusion in mind. For the small pore system, the size-limiting, structural discontinuities must be located exclusively in the endothelium, and the same apparently applies for the large pore system as far as particles smaller than ~200 A are concerned. For larger particles, however, discontinuities or channels equal to, or larger than, 700 A must be postulated in the basement membrane, in addition to the patent fenestrae in the endothelium. Our observations suggest that the endothelial and basement membrane components of the large pore system are rarely in register with one another. Hence, upon their escape through a fenestra, particles larger than ~200 A must diffuse over relatively large distances in the subendothelial space until they gain access to an appropriately sized channel through the basement membrane.

Finally, our observations indicate that in addition to its role of second line permeability barrier, the basement membrane functions as a structural support of the capillary wall, a view already advanced and discussed by Plenk (40) and by Pease (41). Even in the quasi-complete or complete absence of the endothelium, the basement membrane maintains the general form of the vessel, keeps it open as a circulation channel, and acts as an effective partition between the channel lumina and the interstitia of the tissue, at least for particles larger than ~200 A.

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REFERENCES

1. Clementi, F., and G. E. Palade. 1969. Intestinal capillaries. I. Permeability to peroxidase and ferritin. *J. Cell Biol.* **41**:33.
2. Bennett, H. S., J. H. Luft, and J. C. Hampton. 1959. Morphological classification of vertebrate blood capillaries. *Amer. J. Physiol.* **196**:381.
3. Rhodin, J. A. G. 1962. The diaphragm of capil-

lary endothelial fenestrations. *J. Ultrastruct. Res.* **6**:171.

4. LUFT, J. H. 1965. The ultrastructural basis of capillary permeability. *In* The Inflammatory Process. B. Zweifach, L. Grant, and R. T. McCluskey, editors. Academic Press Inc., New York. 121.
5. KELENYI, G., and J. KASZA. 1959. The local oedema producing effect of disodium ethylenediamine tetraacetate (Na_2EDTA). *Experientia.* **15**:56.
6. ZWEIFACH, B. 1964. Microcirculatory aspects of tissue injury. *Ann. N. Y. Acad. Sci.* **116**:831.
7. ROCHA E SILVA, M. 1966. Action of histamine upon the circulatory apparatus. *In* Handbook of Experimental Pharmacology. **18/1**:238.
8. STERN, P. 1966. The relation of histamine to inflammation. *In* Handbook of Experimental Pharmacology. **18/1**:892.
9. ZWILLING, E. 1954. Dissociation of chick embryo cells by means of a chelating compound. *Science.* **120**:219.
10. EARLE, W. R. 1943. Production of malignancy in vitro. IV. The mouse fibroblast cultures and changes seen in the living cells. *J. Nat. Cancer Inst.* **4**:165.
11. MAJNO, G., G. E. PALADE, and G. SCHOEFL. 1961. Studies on inflammation. II. The site of action of histamine and serotonin along the vascular tree: a topographic study. *J. Biophys. Biochem. Cytol.* **11**:607.
12. PALADE, G. E. 1961. Blood capillaries of the heart and other organs. *Circulation.* **24** (Pt. 2): 368.
13. FARQUHAR, M. G., and G. E. PALADE. 1965. Cell junctions in amphibian skin. *J. Cell Biol.* **26**:263.
14. LUFT, J. H. 1961. Improvements in epoxy resin embedding procedures. *J. Biophys. Biochem. Cytol.* **9**:409.
15. VENABLE, J. H., and R. A. COGGESHALL. 1965. A simplified lead citrate stain for use in electron microscopy. *J. Cell Biol.* **25**: 407.
16. BRUNS, R. R., and G. E. PALADE. 1968. Studies on blood capillaries. I. General organization of muscle capillaries. *J. Cell Biol.* **37**:244..
17. CHAMBERS, R., and B. ZWEIFACH. 1934. Intercellular cement and capillary permeability. *Physiol. Rev.* **27**: 436.
18. ZWEIFACH, B. 1955. Structural make up of capillary wall. *In* Symposium on Bioflavonoids and Capillaries. *Ann. N.Y. Acad. Sci.* **61**:670.
19. LECOMPTE, J. 1964. Action des ions alcalino-terreux sur les parois vasculaires. *In* Handbuch der experimentellen Pharmakologie. **17/2**:845.
20. MAJNO, G. 1965. Ultrastructure of the vascular membrane. *In* Handbook of Physiology. Sect. II: Circulation. W. F. Hamilton and P. Dow, editors. American Physiological Society, Washington, D. C. **3**:2293.
21. HERBST, C. 1900. Über das Auseinandergehen von Furchungs- und Gewebezellen in kalkfreiem Medium. *Arch. Entwicklungsmech.* **9**:424.
22. MOSCONA, A. 1962. Cell suspensions from organ rudiments of chick embryos. *Exp. Cell Res.* **3**: 535.
23. WEISS, L. 1967. Studies on cell deformability. IV. A possible role of calcium in cell contact phenomena. *J. Cell Biol.* **35**:347.
24. PALADE, G. E., and R. R. BRUNS. 1968. Structural modulations of plasmalemmal vesicles. *J. Cell Biol.* **37**:633.
25. LUFT, J. H. 1966. Fine structure of capillary and endocapillary layer as revealed by ruthenium red. *Fed. Proc.* **25**: 1773.
26. KWAITKOWSKI, H. 1941. Observations on the relation of histamine to reactive hyperaemia. *J. Physiol.* **100**:147.
27. COHEN, Y., and G. VALETTE. 1960. Effets de la chélation du calcium sur les propriétés vasomotrices de l'histamine et de l'adrenaline *in vitro*. *C. R. Soc. Biol.* **154**:905.
28. JONES, R. G. 1966. Chemistry of histamine and analogs. Relationship between structure and pharmacological activity. Handbook of Experimental Pharmacology. **18/1**:1.
29. PARROT, G. L., and G. NICOT. 1966. Absorption de l'histamine par l'appareil digestif. Handbook of Experimental Pharmacology. **18/1**:148.
30. SMITH, A. N. 1953. Release of histamine by the histamine liberator compound 48/80 in cats. *J. Physiol.* **121**:517.
31. MAJNO, G., and G. E. PALADE. 1961. Studies on inflammation. I. The effect of histamine and serotonin on vascular permeability: an electron microscopic study. *J. Biophys. Biochem. Cytol.* **11**:571.
32. KARNOVSKY, M. 1967. The ultrastructural basis of capillary permeability studied with peroxidase as a tracer. *J. Cell Biol.* **35**:213.
33. BRUNS, R. R., and G. E. PALADE. 1968. Studies on blood capillaries. II. Transport of ferritin molecules across the wall of muscle capillaries. *J. Cell Biol.* **37**:277.
34. COTRAN, R. S. 1965. The delayed and prolonged vascular leakage in inflammation. II. An electron microscopic study of the vascular response after thermal injury. *Amer. J. Pathol.* **46**:589.
35. COTRAN, R. S. 1967. Studies on inflammation. Ultrastructure of the prolonged vascular response induced by *Clostridium oedematiensis* toxin. *Lab. Invest.* **17**:39.
36. MARCHESI, V. T. 1962. The passage of colloidal carbon through inflamed endothelium. *Proc. Roy. Soc. Biol.* **156**:550.

37. MOVAT, H. Z., and N. V. P. FERNANDO. 1963. Allergic inflammation. I. The earliest fine structural changes at the blood tissue barrier during antigen-antibody interaction. *Amer. J. Pathol.* **42**:41.

38. PAPPENHEIMER, J. R., E. M. RENKIN, and L. M. BORRERO. 1951. Filtration, diffusion and molecular sieving through peripheral capillary membranes. A contribution to the pore theory of capillary permeability. *Amer. J. Physiol.* **167**:13.

39. LANDIS, E. M., and J. R. PAPPENHEIMER. 1963. Exchange of substances through the capillary walls. *In* Handbook of Physiology. Sect. II. Circulation. W. F. Hamilton and P. Dow, editors. American Physiological Society, Washington, D. C. **2**:961.

40. PLENK, H. 1927. Über argyrophile Fasern (Gitterfasern) und ihre Bildungszellen. *Ergeb. Anat. Entwicklungsgesch.* **27**:302.

41. PEASE, D. C. 1960. The basement membrane: substratum of histological order and complexity. *In* Vierter internationaler Kongress für Elektronen-Mikroscopie, Berlin. W. Bargmann, editor. Springer-Verlag, Berlin. 139.