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Endothelial Contraction *in vivo*: a Study of the Rat Mesentery

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Summary. Electron microscopic studies have previously shown that histamine-type mediators of vascular leakage induce: (1) the appearance of gaps between venular endothelial cells; and (2) deformation of endothelial nuclei, with multiple deep infoldings. It has been postulated that these changes (and the leakage) are due to contraction of endothelial cells. The present paper has investigated this hypothesis by examining the effects of bradykinin and histamine on living rat mesenteric venules. Within 2–5 minutes after application of the mediator, some endothelial cells began to bulge; obvious bulging lasted for about 5 minutes and then subsided slowly; by 20–30 minutes after application of the mediator, the cells were again flattened. This time-course of bulging and flattening corresponded with the rise and fall in vascular permeability, as shown by carbon labeling. Electron microscopy confirmed the presence of deformed nuclei in such vessels. These observations support the concept that vascular leakage, caused by histamine-type mediators, is dependent upon endothelial contraction.

The notion of endothelial contractility has gone through several phases. It was first discussed as a mechanism of vasoconstriction in very small vessels which do not have a muscular coat. An early view had been that such vessels are passive channels, and that any increase or decrease in calibre simply reflected the state of contraction or dilatation of the corresponding arterioles. But by the time Krogh wrote his classic monograph fifty years ago (see Krogh, 1959) it was accepted that such vessels are themselves capable of independent contraction, although he believed that Rouget cells were the elements responsible—he saw "no definite evidence that the endothelium itself is contractile". Not long thereafter came the studies of the microcirculation in living connective tissues by Clark and Clark (1925, 1935) who showed that vascular narrowing in amphibia is not due to constriction by adventitial cells but can be directly attributed to active contraction of endothelial cells. Then Zweifach (1934) noticed that frog endothelial cells bulged after being prodded with a microneedle, and agreed that "endothelium is definitely contractile". On the other hand, neither the Clarks (1931, 1935) nor Sandison (1932) were able to detect contraction of endothelial cells *in mammals* (using the rabbit ear chamber technique). Thus it seemed that, although amphibian endothelium shows contractility, mammalian endothelium had somehow lost this property. This point of view was widely accepted and maintained (e.g. Zweifach, 1961a) until quite recently.

Endothelial contraction was brought back onto the stage once again by electron microscopy, but this time not in relation to vascular calibre. In the course

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of investigations into the mechanisms of vascular permeability, it was found that leakage of fluid occurs through gaps that appear between endothelial cells (Majno and Palade, 1961); the vessels affected were venules rather than capillaries (Majno *et al.*, 1961). It was then shown that, during the first minutes after the injection of histamine-type mediators into rats, many endothelial cells in the leaking venules acquired wrinkled nuclei, a characteristic of contracted cells (Majno *et al.*, 1969). On this basis, it was suggested that mammalian endothelium, in some vessels at least, can be made to contract if appropriate stimuli are applied, and that such contraction results in the appearance of gaps (and the outward passage of fluid) between endothelial cells. (Under these circumstances, the reduction of the vascular lumen was minimal—being reduced to the slight hump caused by the endothelial cell bulging into the lumen of the venule).

Although the electron microscopic evidence is highly suggestive of contraction, it cannot be taken as definitive proof, any more than a single frame of a cinematographic film can be taken as a proof of motion. Another limitation of the work mentioned above (Majno *et al.*, 1969) is that it concerned only one tissue, rat striated muscle. We therefore undertook the present study of the rat mesentery with the dual purpose of finding out: (1) whether endothelial contraction can be seen *in vivo*; and (2) whether endothelial contraction can be demonstrated electron microscopically in another tissue.

Material and Methods

We used male Wistar rats weighing 200–350 g, obtained from the Ivanovas Farm, Kisslegg/Allgäu, Germany.

Exposure of the Mesentery

The experimental procedure is similar to that described by Buckley and Ryan (1969). The rat was anesthetized with sodium pentobarbitone (Nembutal, Abbott Laboratories, North Chicago, Ill., U.S.A.), 3 mg/100 g body weight, injected subcutaneously 40 minutes prior to laparotomy. The ventral abdominal skin was shaved and an incision, 3 cm long, was made in the midline; vessels in the wound edges were coagulated by electro-cautery (Electrotom 50, Martin, Tuttlingen, Germany) to prevent free blood from causing a serosal inflammatory reaction. A loop of distal ileum with a suitably vascularized mesenteric window was gently withdrawn from the peritoneal cavity and was kept moist with warm Ringer's solution containing 5% glucose. The animal was then laid on its left side and placed on a cork board shaped to fit on the microscope stage. The selected loop was carefully pinned out over a lucite disc that formed a window in the board. This disc, 3 cm in diameter and 2 mm thick, was set into the board so that its upper surface was at a level 0.5 mm above the cork. All *in vivo* observations were carried out using light transmitted from beneath the lucite window. The mesentery and its ileal loop were continually moistened with Ringer's solution; in some experiments, the network of small vessels was covered with a small piece of transparent polystyrene sheet (Polyflex, Plax Corporation, Hartford, Conn., U.S.A.), cut to an appropriate shape with scissors.

Optical and Photographic Equipment

Light microscopic observations were made with a Zeiss microscope (Standard RA, Carl Zeiss, Oberkochen, Germany) fitted with phase-contrast equipment. When the $\times 100$ oil-immersion objective was used, oil was placed directly on the mesentery (or on the polystyrene coverslip when this was being used). A heat-absorbing filter (KG1, Carl Zeiss) was interposed between the light source and the condenser. Photographs were taken at regular intervals (every minute during a test with histamine or bradykinin, or every 5 minutes during control experiments with Ringer's solution alone) using a Nikon 35 mm camera with a shutter

speed of 1/125 second and Kodak Ektachrome film EX135-20. All exposures were made with a yellow compensating filter (Kodak gelatin filter CC05Y) and an electronic flash (Ukatron UN60, Carl Zeiss) that gave a effective exposure time of 1/1000 second.

Injections and Pharmacologic Agents

To demonstrate increased vascular permeability by the carbon labeling technique (Majno *et al.*, 1961), we injected a colloidal suspension of carbon particles (Pelikan India Ink, batch C11/1431a, Gunther-Wagner, Pelikan Werke, Hannover, Germany) into the lateral tail vein, in a dose of 0.1 ml of ink/100 g body weight. Bradykinin triacetate (Sigma Chemical, St. Louis, Mo., U.S.A.) and histamine dihydrochloride (Hoffmann-La Roche, Basel, Switzerland) were used at a concentration of 0.1 mg/ml in Sørensen phosphate buffer at pH 7.4; in each case, a volume of 0.04 ml of the appropriate test solution was dripped onto the mesentery.

Processing for Light and Electron Microscopy

The animal was killed with an overdose of ether, and two intestinal loops (the loop that had been studied and an untreated control loop) were pinned out on a sheet of dental wax and fixed for light microscopy in alcohol-formol (4 parts of absolute ethanol to 1 part of 10% buffered formalin) for 18–24 hours, dehydrated and cleared in dioxane (Merck, Darmstadt, Germany), and stained with cresyl violet for the demonstration of mast cells.

For electron microscopy, the mesentery was pinned out as above, fixed for 3 hours in a 1:1 dilution of Karnovsky's (1965) fixative in cacodylate buffer (giving a final concentration of 2% paraformaldehyde and 2.5% glutaraldehyde), then left in cacodylate buffer overnight, post-fixed for $1^1/_2$ hours in 2% osmium tetroxide in s-collidine buffer, dehydrated in graded ethanols, and flat-embedded in Epon 812. After polymerization of the Epon at 60° C for 18 hours, the preparation was examined and photographed to check the topographic pattern of blood vessels against drawings and photographs that had been taken during *in vivo* observations of the same mesentery. The portion of the vascular network that had been closely studied *in vivo* was then cut out of the main block. This gave a small flat block, approximately 6 mm square and 2 mm thick, that was placed horizontally over the end of a gelatine capsule (No. 2, Parke-Davis, Detroit, Mich., U.S.A.) filled with fresh Epon. After further polymerization, the block was trimmed and sections were cut in a plane parallel to the mesenteric surface, using a Reichert OM-2 ultramicrotome. Glass knives were used for 1 μ sections and, when the mesenteric vessels were reached, ultrathin sections were then cut with a diamond knife. Some sections were mounted on 75-mesh grids coated with a Parlodion film (0.1% solution in isoamyl acetate) stabilized by a thin layer of carbon; other sections were mounted on uncoated 100-mesh grids. All sections were stained with uranyl acetate (2% in aqueous solution) and lead citrate (Reynolds, 1963). The grids were examined in Zeiss EM9 and Philips 300 electron microscopes.

Results

Preliminary studies showed that mere exposure of the mesentery sometimes caused intramural deposition of carbon particles in the vessels of control rats, i.e. vascular leakage, probably due to ruptured mast cells releasing endogenous mediators. All animals were therefore pre-treated by degranulating their peritoneal mast cells using a modification of Fawcett's (1955) method: three intraperitoneal injections of sterile distilled water were given within the space of a week, and animals were used 10–14 days after the third dose of water; at this time, peritoneal mast cells contained no stainable granules and the local inflammatory reaction due to the injections had subsided. This procedure effectively eliminated non-specific carbon labeling due to handling of the mesentery.

In vivo Observations

In a typical experiment, the mesenteric blood vessels were directly observed and photographed before, during and after the local application of bradykinin

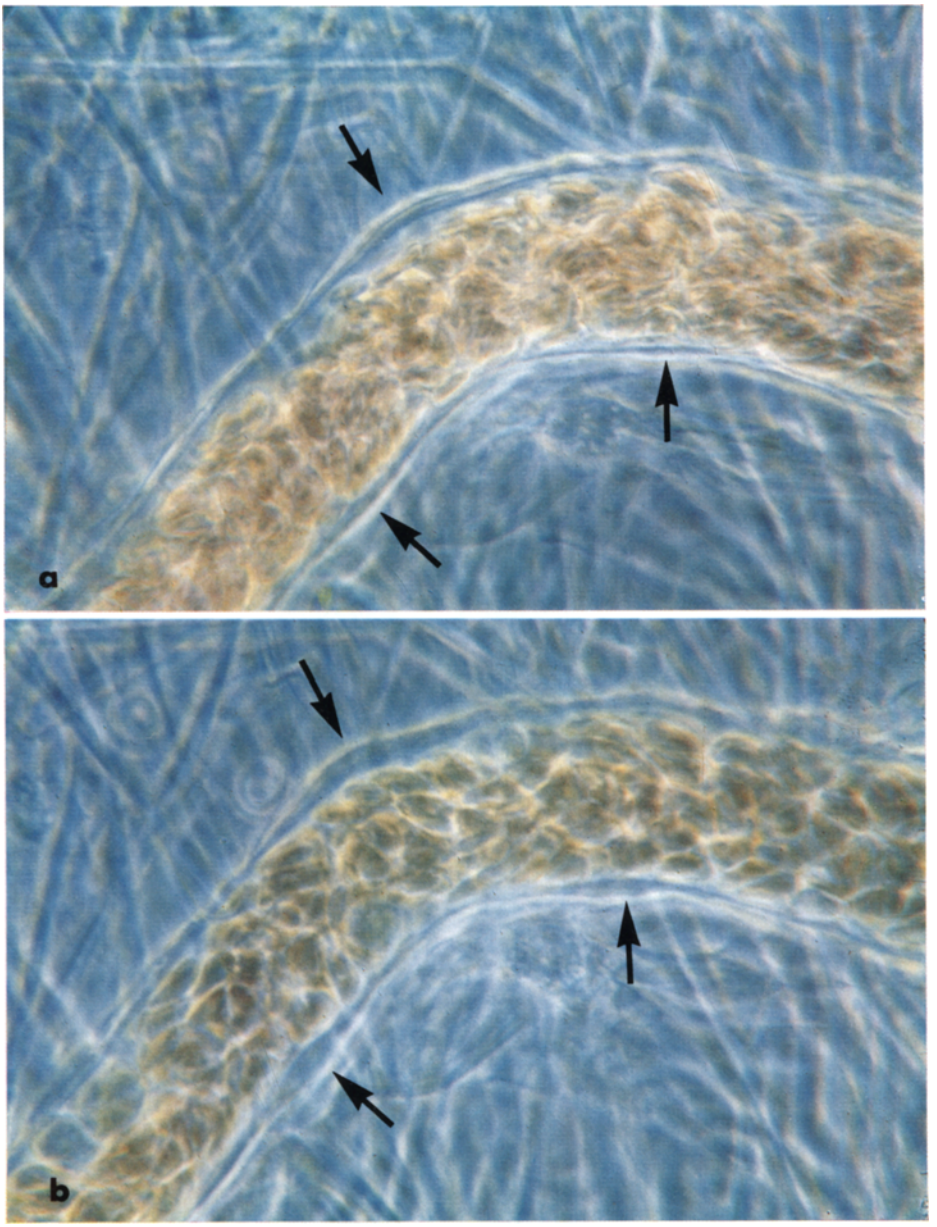


Fig. 1. a Mesenteric venule 1 minute before the application of bradykinin. Arrows point to the endothelial cells, barely visible. Phase contrast (× 1800). b The same 8 minutes after the application of bradykinin: the endothelial cells are now clearly bulging. Phase contrast (× 1800)

or histamine. In 7 rats, intravenous carbon was given 10–15 minutes before the mediator was applied, so as to confirm that non-specific carbon deposition did not occur. In 22 other rats, carbon was injected 10–20 minutes after the mediator; we had earlier found that, if the carbon was given immediately after the mediator, heavy labeling rapidly obscured endothelial cell morphology at a crucial stage of the experiment.

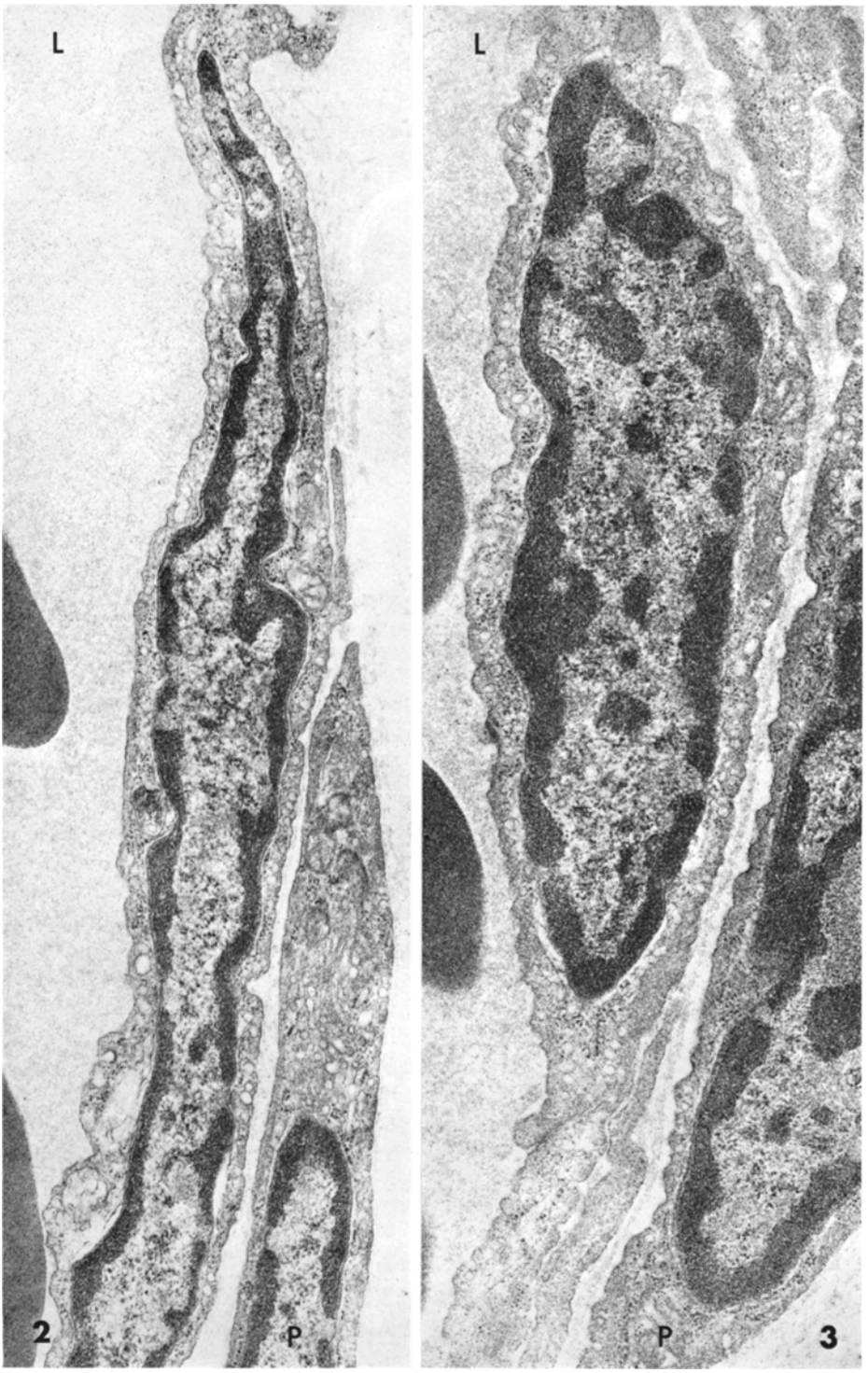
Attention was focussed on the endothelial cells of venules because these are the vessels that are predominantly affected by histamine-type mediators (Majno *et al.*, 1961). They were identified by their post-capillary position and from our experience of the calibre of mesenteric vessel that is blackened with carbon during increased permeability. Another factor that governed selection of a suitable field was the clarity with which a reasonable number (say 3 ore more) endothelial cells could be seen exactly "side-on" in the vessel wall, because it is only by observation of such cells that *in vivo* changes can be recorded—the shape of cells on the "front" or "back" of the vessel cannot of course be discerned.

Following the application of bradykinin (19 experiments) or histamine (10 experiments), no change was noted until, at various times between 2 and 5 minutes later, endothelial cells began to "bulge", i.e. the normally flattened cells became thicker and more fusiform. At the middle of this fusiform swelling, the nucleus was often seen to be more prominent and rounder than usual. It was not possible to give an overall estimate of the percentage of endothelial cells that bulged in a particular venule because, as mentioned above, only cells seen in profile could be studied; however, of those cells that could be seen clearly, most developed some degree of bulging—indeed, in some instances, it was only when such bulging occurred that the presence of a suitably situated endothelial cell was revealed. Fig. 1 shows the appearance of endothelial cells in a mesenteric venule (a) 1 minute before and (b) 8 minutes after the application of bradykinin. The cells usually remained obviously bulged for about 5 minutes, following which they slowly re-flattened over the next 10–15 minutes. By 20–30 minutes after application of the mediator, the endothelium was almost back to its original state; in 4 rats, bradykinin or histamine was re-applied to the mesentery at about 45 minutes and again produced a brisk but transient endothelial cell bulging. At no stage did bradykinin or histamine induce a detectable change in venular calibre or blood flow; leukocytic margination and sticking were absent. Carbon labeling always occurred in response to the bradykinin or histamine: black deposits could usually be seen around the ends of cells that had bulged.

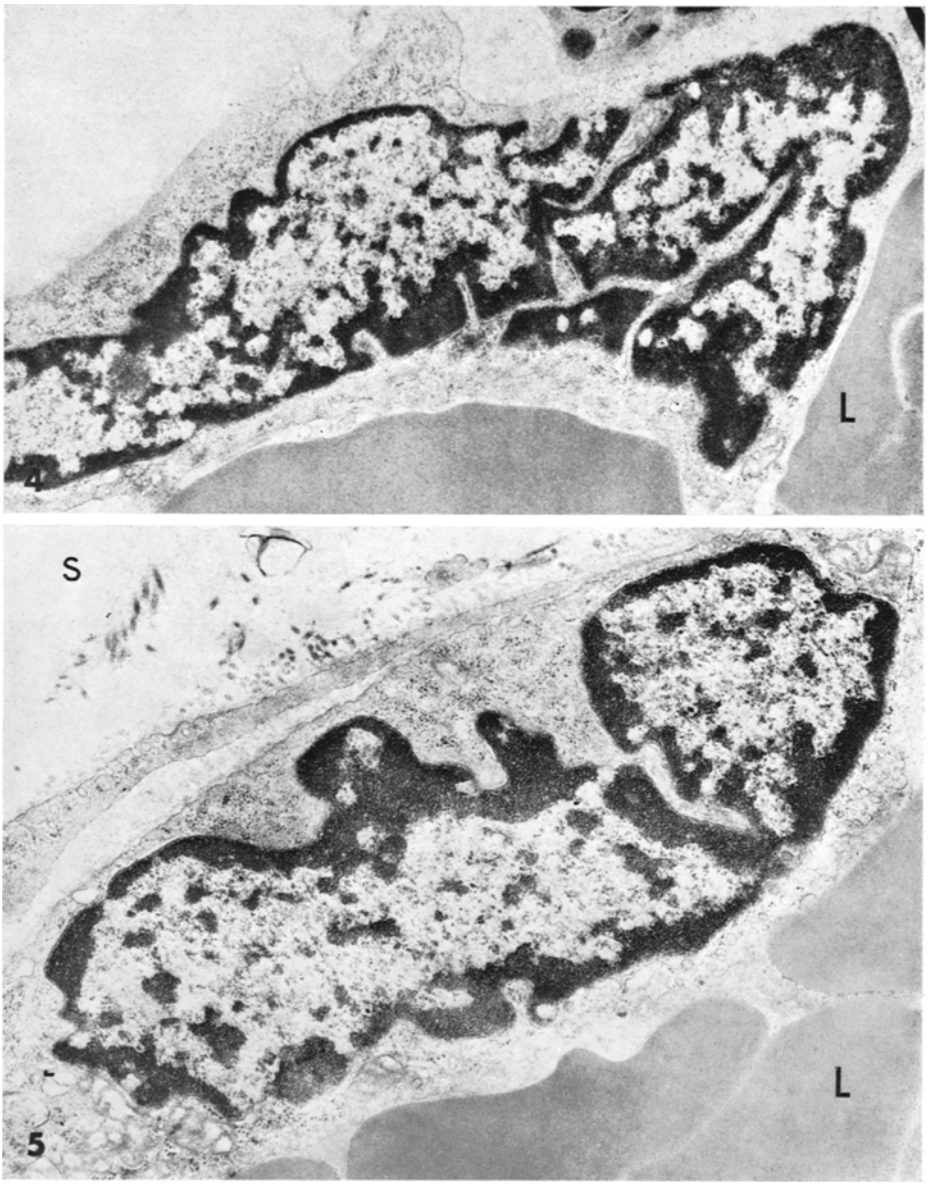
Control animals (7 experiments) were treated by exposing the mesentery as usual and keeping it moist with Ringer's solution for periods ranging from 30 to 130 minutes: no bulging of endothelial cells occurred. In 4 of these animals, bradykinin or histamine was then applied (at times ranging from 30 to 65 minutes after the initial exposure of the mesentery) and in each case the endothelial cells bulged promptly as described above.

Electron Microscopy

Venular endothelial cells were examined electron microscopically in 7 mesentery preparations: 4 animals were treated with a mediator (bradykinin 1 rat, histamine 3 rats) and the mesentery was fixed when endothelial cells bulged;



Figs. 2 and 3



Figs. 4 and 5. Nuclei in wall of mesenteric venules, 9 minutes after the application of a mediator. Fig. 4: Bradykinin (× 15300). Fig. 5: Histamine (× 17000). In both cases, the contracted nuclei show surface wrinkles and deep indentations. *L* erythrocytes in vascular lumen; *S* extravascular space

Figs. 2 and 3. Normal nuclei in wall of mesenteric venules. Fig. 2: Flattened, elongated type (× 21800). Fig. 3: Ovoid type (× 21400). *L* lumen; *P* pericyte

Table 1. Effect of bradykinin and histamine on the shape of endothelial nuclei in rat mesenteric venules

	Controls (Ringer's solution)	Bradykinin	Histamine
Nuclear shape:			
flattened	18	2	8
ovoid	9	5	7
contracted	0	8	8
Total number of nuclei	27	15	23

the remaining 3 animals were exposed to Ringer's solution alone for 5, 50 or 80 minutes before fixation. None of these animals received intravenous carbon.

Particular attention was paid to the nuclei of endothelial cells, which were graded into three groups: (a) "flattened" nuclei had a narrow, elongated shape and a smooth, unindented outline (Fig. 2); (b) "ovoid" nuclei were oval in shape and had a smooth outline (Fig. 3); and (c) "contracted" nuclei were oval or rounded but their main feature was the presence of a wrinkled outline, often with multiple deep indentations (Figs. 4 and 5). As shown in Table 1, the nuclei of control preparations were mostly flattened (Fig. 2), some were ovoid (Fig. 3), but none could be called contracted. On the other hand, preparations treated with bradykinin or histamine showed contracted nuclei in many endothelial cells (Figs. 4 and 5). Gaps between endothelial cells were also seen following bradykinin or histamine, but never in the controls.

Discussion

In 1961, electron microscopic studies suggested a morphologic explanation for increased vascular permeability: histamine-type mediators induce the appearance of small gaps between endothelial cells (Majno and Palade, 1961). It was not clear whether this cellular separation was due to an active endothelial contraction or to a direct loosening effect on the junctional regions. To explain it, an hypothesis was proposed by Rowley (1964): mediators constrict veins and so raise the hydrostatic pressure in the microcirculation; and as the small vessels distend, the venules burst at their endothelial seams—Rowley attributed the relative sparing of the capillaries (Majno *et al.*, 1961) to their smaller diameter (Laplace's law says that, for a given intraluminal pressure, the wider the tube the greater is the tangential stretching force in its wall). However, it has since been demonstrated that leakage can occor in the absence of venous spasm (Majno *et al.*, 1967; Buckley and Ryan, 1969). It was then noticed in electron micrographs of leaking venules that endothelial nuclei are rounded and show multiple deep infoldings of their membranes (Majno *et al.*, 1969). This appearance is typical of that seen in contracted cells (Lane, 1965; Bloom and Cancilla, 1969; Stromberg *et al.*, 1969), and gives logical support to the concept that gaps are caused by endothelial contraction. On the other hand, it could be argued that fixation in the presence of a mediator produces

these peculiar nuclei. Therefore, more direct evidence was needed that endothelial cells become shorter and thicker at the right time, i.e. during increased permeability of the vessel. The present *in vivo* observations provide this evidence.

We studied endothelial cells that could be clearly seen in profile in rat mesenteric venules. Such cells are normally flattened and inconspicuous (Fig. 1a); control preparations remained unchanged like this for up to 2 hours. However, within 2–5 minutes after the local application of bradykinin or histamine, the endothelial cells began to bulge (Fig. 1b); these bulges often contained a prominent, rounded nucleus. Electron microscopy at this stage (Table 1, Figs. 4 and 5) confirmed the presence of nuclear deformations similar to those described by Majno *et al.* (1969) (in venular endothelium of rat skeletal muscle). Obvious bulging lasted for about 5 minutes and then subsided slowly; by 20–30 minutes after application of the mediator, the endothelium was again flat.

Two major questions arise:

1. Do Endothelial Bulges (and Nuclear Deformations) Occur at the Same Time as Leakage? The time-course for bulging corresponds well with the known time-course for increased vascular permeability due to the action of histamine-type mediators: "This type of response begins in the first minute or two after injury and reaches a peak in 5–7 min. It usually subsides in 10–15 min and never lasts more than 30 min" (Wilhelm, 1971). Our own carbon labeling studies confirm that this pattern of leakage is true for the rat mesentery.

2. Is Bulging Due to Contraction of the Endothelial Cells? Contraction of an elongated cell would be expected to produce a bulging cell. However, we should consider the possibility that bulging may be caused by something other than an active contraction. For example, the junctions could somehow be loosened by the mediator, this being followed by a "recoil" of the endothelial cells due to an intrinsic tone (although of course even this implies a contractile capacity). We cannot exclude such a mechanism. But there is no evidence that histamine-type mediators have any direct effect on junctions, whereas such agents do induce active cellular contraction in other systems. Another possible explanation for bulging is that the endothelial cells swell because they become filled with fluid, either due to injury or to a transcellular passage of water across the vessel wall. This is not the case: electron microscopy showed no sign of an increase in cytoplasmic fluid.

Evidence favouring an active contraction of endothelial cells in the present experimental model is as follows:

1. Our time-course of endothelial bulging-flattening corresponds to the contraction-relaxation response shown by contractile cells exposed to smooth muscle stimulants.

2. Because there is no detectable increase in venular calibre as leakage develops (Majno *et al.*, 1967; Buckley and Ryan, 1969; and the present study), intercellular gaps are almost certainly due to endothelial shortening. Such gaps were seen electron microscopically and, although cell borders could not be clearly identified in our *in vivo* preparations, we usually saw carbon deposits on either side of cells that had bulged.

3. We found wrinkled endothelial nuclei—the evidence for this being due to an active cellular contraction is discussed elsewhere (Majno *et al.*, 1969).

4. Endothelial cells contain potentially contractile material: cytoplasmic fibrils have been described (e.g. Majno *et al.*, 1969), and actomyosin-like material has been located in certain endothelial cells by immunofluorescence techniques (Becker and Murphy, 1969).

In our *in vivo* studies, it was not possible to estimate the proportion of endothelial cells that contracted, but electron microscopy confirmed the observation by Majno *et al.* (1969) that not all endothelial nuclei show signs of contraction. The reason for this is not clear but may be related to the precise time of fixation of the tissue: we noticed that bulging *in vivo* was not maximal in all cells at the same time. It would also not be surprising if an occasional endothelial cell did not contract at all, perhaps depending on its cell-cycle stage: histamine-type mediators cannot induce leakage from immature venules in newborn animals (Majno, 1964; Little, 1969) or in healing wounds (Hurley *et al.*, 1970). Such a variation of responsiveness would account for the normally patchy and focal nature (Majno, *et al.* 1961) of vascular leakage.

Endothelial contraction was observed long ago in amphibian capillaries (Clark and Clark, 1925, 1935; Zweifach, 1934). Zweifach (1957, 1961b) also noted that histamine induces endothelial swelling, but did not give experimental details. Although it was thought at first that mammalian endothelium had suffered "an evolutionary loss of contractile power" (Clark and Clark, 1935), swelling of rabbit endothelial cells was eventually seen in ear chambers by Sanders *et al.* (1940); this effect occurred spontaneously or could be provoked by sympathetic stimulation. More recently, transient bulging of endothelial cells has been recorded in sphincters of hepatic sinusoids (McCuskey, 1966) and in pancreatic capillaries (McCuskey and Chapman, 1969). However, there has been no attempt in any of this work to correlate bulging with increased vascular permeability.

In conclusion, our findings suggest (1) that the electron microscopic appearances interpreted as "endothelial contraction" correspond to a cellular shortening and bulging *in vivo*; and (2) that the phenomenon occurs also in the rat mesentery. This study, then, reinforces the view that histamine-type mediators increase vascular permeability by causing contraction, and hence separation, of endothelial cells.

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