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ACTION OF HISTAMINE ON ENDOTHELIAL CELLS OF GUINEA-PIG ISOLATED HEPATIC PORTAL VEIN AND ITS MODIFICATION BY INDOMETHACIN OR REMOVAL OF CALCIUM

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Summary.—Methods are described for preparing guinea-pig hepatic portal vein endothelium for light microscopy and electron microscopy. Histamine (100 μg/ml) caused damage to the endothelium which was visible with both the light and electron microscopes. The damage was reduced in the absence of calcium. The reduction was more apparent with the electron microscope than with the light microscope. Indomethacin (100 μg/ml) protected the endothelial cells against the damaging effects of histamine. Possible modes of action of histamine and indomethacin are discussed.

HISTAMINE probably acts as a mediator of certain acute inflammatory responses. Several workers have shown that histamine increases the permeability of the walls of small blood vessels to particles such as colloidal carbon (Hurley, 1963; Hurley and Ryan, 1967; Cotran, Suter and Majno, 1967; Majno, Gilmore and Leventhal, 1967). Northover and Northover (1969) showed that the deposition of colloidal carbon in the walls of small venules of the rat mesentery *in vivo* after application of histamine, 5-hydroxytryptamine or bradykinin was due, in part at least, to a direct action of these substances on the endothelial cells. They also showed that indomethacin, an anti-inflammatory compound widely used in the treatment of rheumatic joint disease, reduced the amount of carbon deposited.

The present work was undertaken to ascertain whether histamine causes morphological changes in endothelial cells, and to determine whether the presence of indomethacin or the absence of calcium ions affects these changes. The guinea-pig hepatic portal vein was chosen to provide a large sheet of endothelial cells which could be exposed with a minimum of surgical trauma.

MATERIALS AND METHODS

Male guinea-pigs weighing approximately 350 g were used. The animals were killed by a blow on the back of the head. The abdomen was opened and a segment of the hepatic portal vein was dissected out and placed immediately in ice-cold Tyrode–Ringer solution (TRS) containing (mmol/l): NaCl 138; KCl 2·74; $NaHCO_3$ 10·1; $MgCl_2$ 1·06; $CaCl_2$ 2·0; NaH_2PO_4 0·416; glucose 5·68; adjusted to pH 7·5. For experiments performed in the absence of calcium, the $CaCl_2$ was omitted from the TRS and 1,2-bis-2- aminoethoxyethane-NNN′N′-tetraacetic acid (EGTA) was added in a final concentration of 0·1 mmol/l. The segment of vein was rinsed free of blood with ice-cold TRS, and then stretched to its *in vivo* length by pinning to a shallow polyethylene tray. Adventitial fat was removed and the vessel opened longitudinally, taking care that surgical instruments came in contact only with the margins of the resulting sheet of endothelium. The sheet was then pinned out with its endothelial surface uppermost and immersed in TRS gassed with 95% O_2 and 5% CO_2 at 39° for 30 min, during which time histamine and/or indomethacin were added as required.

Preparation of tissues for light microscopy.—The tissues were fixed and stained using a method similar to that of Florey, Poole and

Meek (1959). The tissue was immersed in TRS at 39° containing heparin 10 u/ml for 1 min. Exposure to heparin promoted uniform staining. Florey *et al.* also noted that heparin promoted staining of endothelial cell outlines. The tissue was then suspended in warm moist osmium tetroxide (OsO_4) vapour for 20 min, immersed in 0·5% silver nitrate solution for 1 min, rinsed briefly in distilled water and fixed for 30 min at 0° with 2% OsO_4 in barbitone-acetate buffer at pH 7·4. The tissue was then rinsed well with distilled water and dehydrated in graded concentrations (30–100%) of ethanol in water for several h, during which time it was exposed to undiffused daylight. Exposure to light was necessary to give good outlining of the endothelial cells with silver grains (Garbarsch and Christensen, 1970). The tissue was then cleared in cedar wood oil (British Drug Houses) for 3 h. It was then removed from the polyethylene tray, trimmed and mounted on a glass microscope slide in DPX mountant (British Drug Houses). In some experiments histamine acid phosphate was added to the bathing fluid in a concentration of 100 μg/ml for the final 10 min before tissue fixation. Where appropriate, indomethacin was present in the bathing fluid throughout the 30-min incubation period in a final concentration of 100 μg/ml. The indomethacin was first dissolved in a small volume of sodium carbonate solution and quickly adjusted to pH 7 with acetic acid.

In experiments where nigrosin staining was used, the tissue was processed as described above until the incubation in heparinized TRS was complete. The tissue was then immersed in TRS containing nigrosin 0·5 mg/ml for 10 min at 39°. It was then rinsed with TRS and dehydrated in graded concentrations of ethanol (70–100%) in water and finally cleared by immersing in xylene for 5 min and mounted in DPX mountant.

Preparation of tissues for electron microscopy.—Hepatic portal veins of guinea-pigs were prepared and treated in the same manner as described for light microscopy up to the point of fixation in 2% OsO_4 solution. They were then rinsed briefly with distilled water, dehydrated in graded concentrations (30–100%) of ethanol in water and embedded in Durcupan ACM (Fluka) according to the manufacturers' instructions. The tissue remained pinned out until the penultimate stage of embedding. At this point the pins were removed and the tissues were trimmed and transferred for final embedding to fresh polyethylene trays (Meek, 1970). The Durcupan ACM discs thus produced were viewed with the light microscope and representative areas of endothelium chosen for electron microscopy. Sections with interference colours of pale straw or silver were cut in a plane perpendicular to the endothelial surface with glass knives on a Cambridge-Huxley Ultramicrotome Mk II, and viewed in an AEI EM6 electron microscope. Sections were cut from at least 3 sites on each specimen. Electron micrographs were taken of approximately 30 endothelial cells from each treatment group.

Assessment of morphological changes in nigrosin stained preparations.—Nigrosin stained specimens were viewed under the light microscope using a × 40 objective lens. A point-count of stained nuclei was performed on 100 consecutive fields for each specimen.

Assessment of morphological changes in silver stained preparations.—Specimens viewed under the light microscope and electron micrographs were assessed independently on at least 2 occasions by 2 observers who did not know the treatment each tissue had received. Using the light microscope, scoring was on a scale 0–4 for both the extent of damage and the amount of silver staining, where damage was scored in terms of the discontinuity of the sheet of endothelium. Electron micrographs were scored on a scale 0–5 for the amount of damage and on a scale 0–4 for the number of silver grains present. Damage in this case was scored on the degree of cytoplasmic vacuolation, the extent of convolution of the nuclear membrane, the extent of the coarse aggregation of nuclear chromatin and the extent of nuclear swelling. In addition, the longest width and the shortest width of each cell were measured in a direction perpendicular to the plane of the vessel wall. The ratio of these 2 dimensions is a convenient numerical indication of the shape of the cell.

RESULTS

Light microscopy

Silver stained preparations.—The outlines of the endothelial cells were blackened with silver (Fig. 1) as has been described by McGovern, 1956; Poole, Sanders and Florey, 1958; Florey *et al.*, 1959; Robertson, Moore and Mersereau, 1959; Gottlob and Hoff, 1967. Indomethacin increased the intensity of silver staining in the interendothelial junctions (Table I) and also on the luminal surface of the cells. This latter phenomenon varied from cell to cell, giving the endothelium a patchwork appearance which was more noticeable than in the controls (Fig. 2). Histamine caused certain characteristic changes in the appearance of the endothelium. Silver staining was more variable

TABLE I.—*Light Microscopic Changes to Endothelium Produced by Histamine, Indomethacin and Lack of Calcium*

Treatment				
Name	Concentration μg/ml	No. of observations	Damage§ scale 0–4	Silver staining§ scale 0–4
Control	—	8	1·50 ± 0·36	1·88 ± 0·33**
Indomethacin	100	6	0·88 ± 0·30	2·76 ± 0·31*
Histamine	100	18	2·03 ± 0·22	1·70 ± 0·20‡‡
Histamine + Indomethacin	100 + 100	7	1·86 ± 0·45	3·29 ± 0·20‡
Calcium free	—	6	0·96 ± 0·34†	2·50 ± 0·28***
Calcium free + Histamine	100	6	1·83 ± 0·24††	2·33 ± 0·22‡‡‡

§ indicates mean ± s.e.

There is a significant difference (Student's t test) between values marked ** and *, and between values marked ‡‡ and ‡ ($P < 0·05$); and between values marked † and ††, values marked ** and ***, and values marked ‡‡ and ‡‡‡ ($P < 0·1$).

than in the controls and the endothelium often appeared to have split (Fig. 3). Occasionally, areas were seen where the underlying smooth muscle cells were outlined with silver. Treatment with indomethacin plus histamine resulted in less damage than with histamine alone (Table I, Fig. 4). There was little difference between the extent of damage seen in tissues bathed with TRS containing calcium and in those where calcium was absent. Similarly, the presence or absence of calcium had little influence on the extent of damage caused by histamine (Table I). However, in the absence of calcium there was a significant increase in the intensity of silver staining.

Nigrosin stained preparations.—When calcium was present, whatever other treatment was given, approximately the same low proportion of cells (0·16%) took up the stain. Thus, the amount of accidental damage caused by the dissection and by immersion in the bathing fluid was slight since damaged cells but not healthy cells take up this stain (Björkerud and Bondjers, 1972). The proportion of cells taking up nigrosin in the absence of calcium (0·02%) was significantly less ($P < 0·05$) than in the presence of calcium. Histamine caused only a slight increase in the number of stained cells both in the presence and absence of calcium.

Electron microscopy

Observations made on control preparations in the present series of experiments showed that endothelial cells were more or less flattened, with an occasional bulge in the region of the nucleus on either the luminal or the medial surface. Cell junctions were well differentiated and were sometimes of considerable length. Occasional vacuoles were seen in the cytoplasm and focal dilatations of the inter-endothelial junctions sometimes produced an appearance which resembled vacuolation (Fig. 5). Florey *et al.* (1959) observed that in aortic endothelium "silver was deposited as a continuous granular layer over the surface of the endothelium and to a variable extent in the space between the cells. Silver deposit between cells was not usually confined to the space between the cell boundary membranes, but spread to a variable distance into the adjacent cell." The silver staining of portal vein endothelium in the present experiments was in accordance with this description (Fig. 5). The nuclei of control cells had smooth outlines with only occasional indentations, which Majno, Shea and Leventhal (1969) classified as "notches" and "folds" (Fig. 5). The intimal connective tissue space was fairly narrow and regular.

Treatment of the endothelium with histamine (100 μg/ml) produced a number of changes in the appearance of the

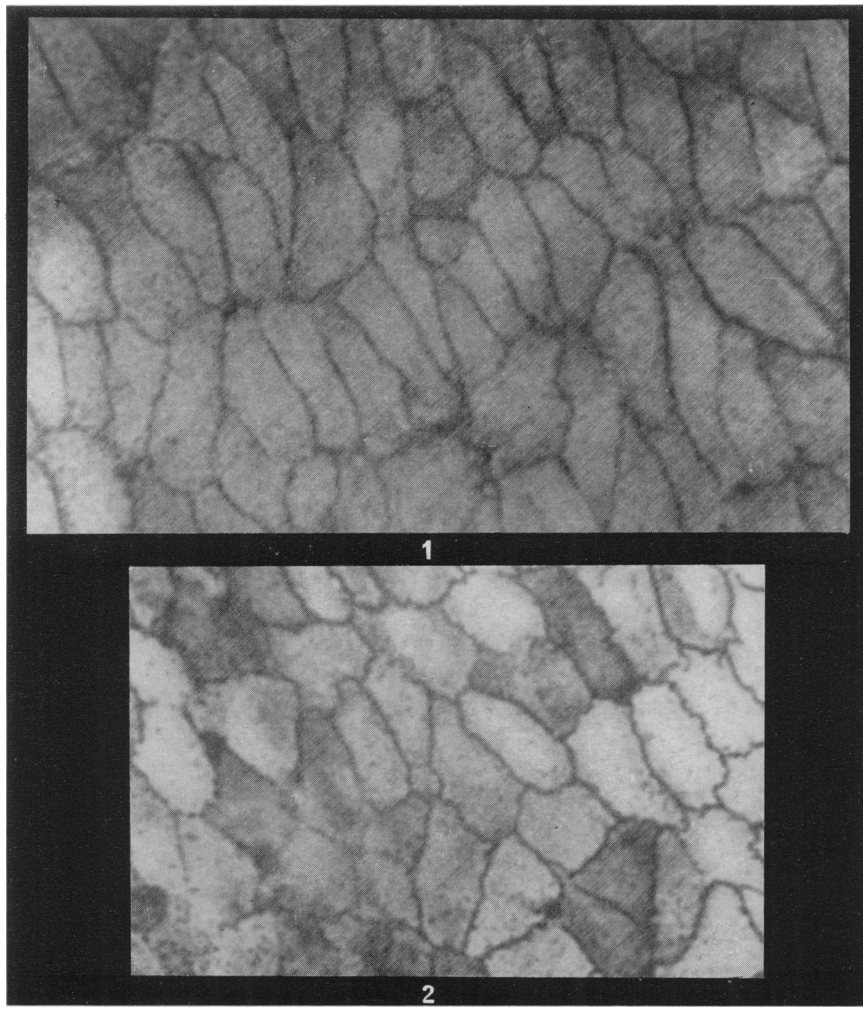


FIG. 1–4.—Photomicrographs of guinea-pig hepatic portal vein endothelium in surface view. Stained with $AgNO_3$. × 475.

FIG. 1.—Control preparation. Note the uniform blackening of endothelial cell outlines and intact appearance of endothelium.

FIG. 2.—Endothelium treated with indomethacin (100 μg/ml) for 30 min before fixation and staining. The staining of the luminal surface of some cells gives a patchwork appearance.

endothelial cells. Firstly, the amount of silver was reduced (Table II) and was present mainly on the luminal surface and rarely on the medial surface or in the cell junctions (Fig. 6). Secondly, the shape of the cells changed in one of 2 ways. In some cells the cytoplasm appeared to have moved towards the centre of the cell so that the inter-endothelial cell junctions had become extremely short. The luminal surface of the cells remained smooth but the medial surface projected focally into the intimal connective tissue space (Fig. 6) giving the appearance of "arcades" (Baumgartner, 1971). The nuclei of these cells exhibited several "notches" and "folds" and also some "closing folds" and occasional "pinches" (using the

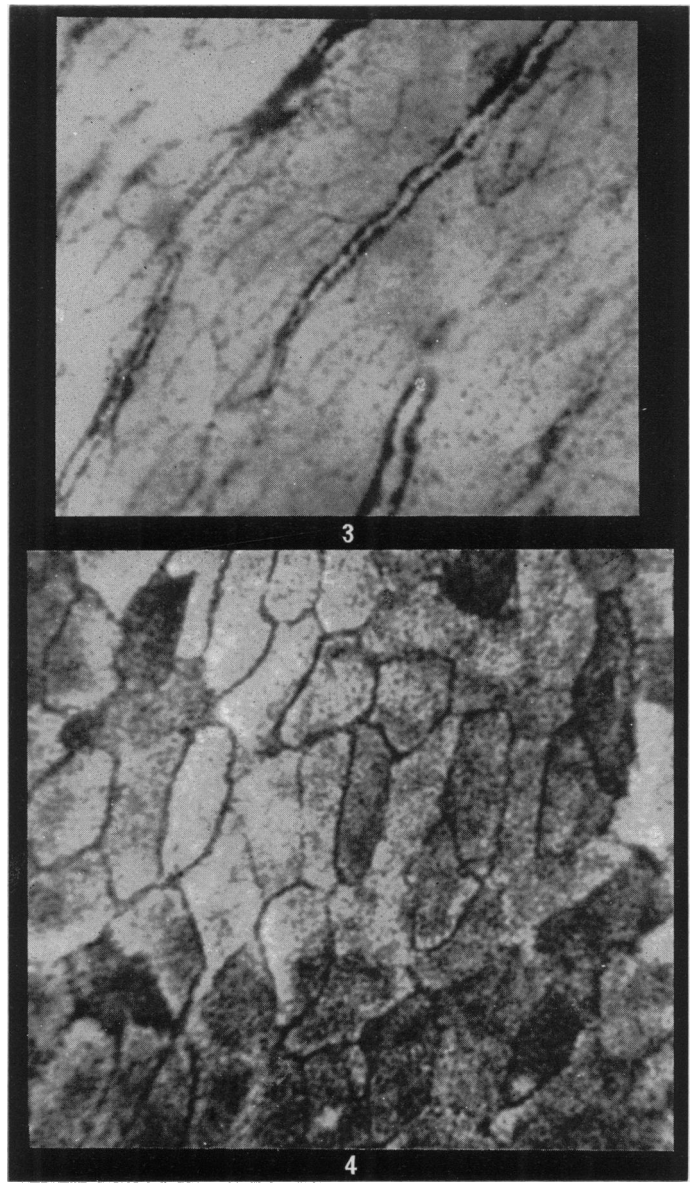


FIG. 3.—Endothelium treated with histamine (100 μg/ml) for 10 min before fixation and staining. Note the variability of staining of cell outlines, and the formation of gaps in the sheet of cells.
FIG. 4.—Endothelium treated with indomethacin (100 μg/ml) for 20 min before exposure to histamine (100 μg/ml). Cell outlines are not uniformly stained but gaps are absent.

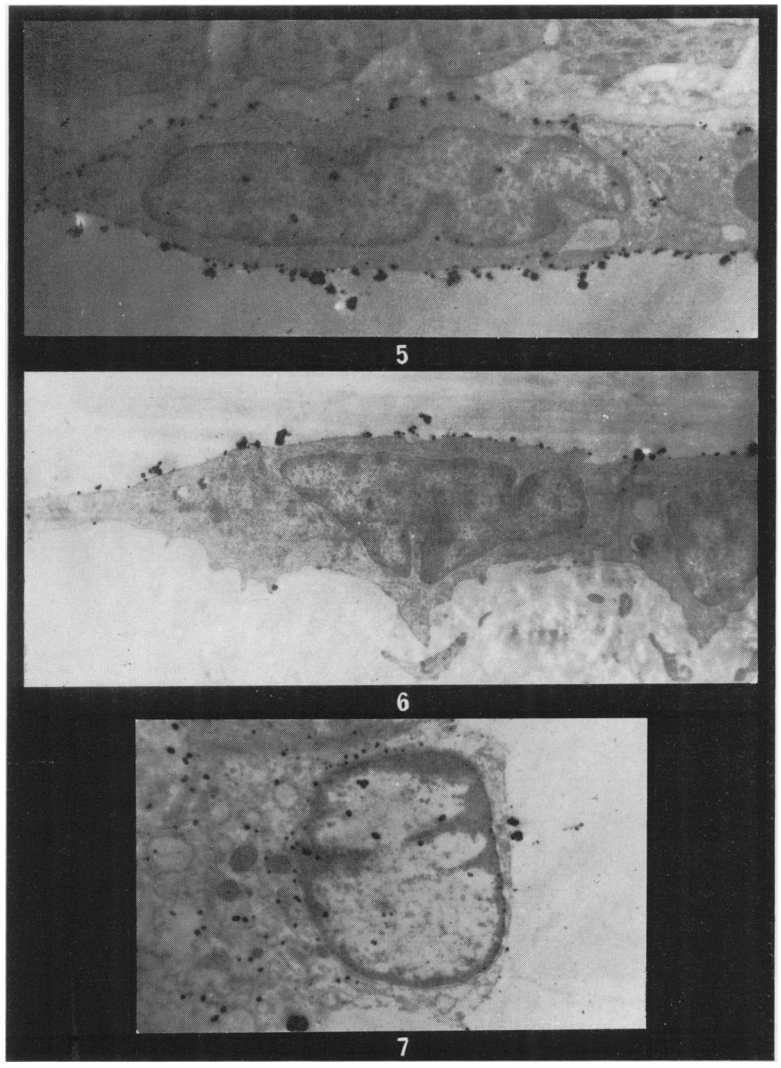
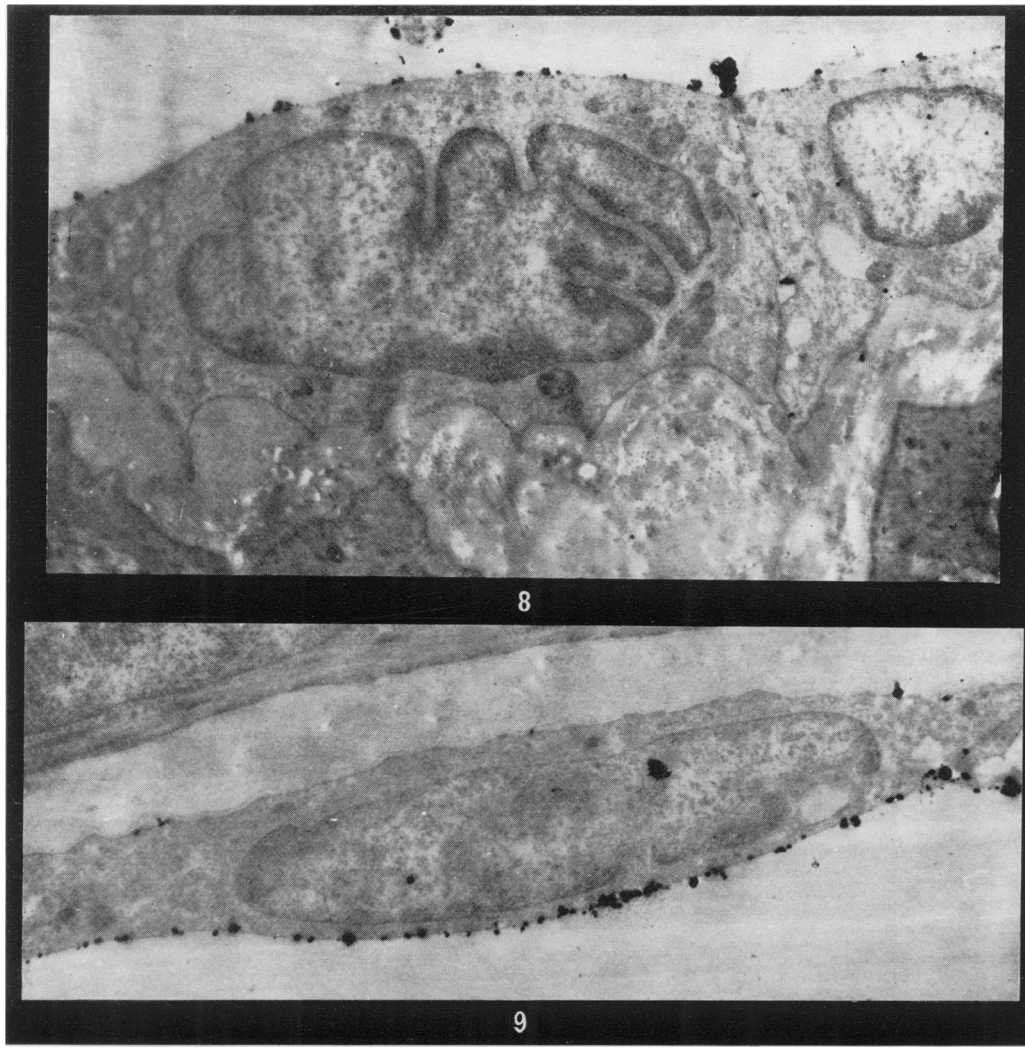


FIG. 5–7.—Electron micrographs of guinea-pig hepatic portal vein endothelium. × 5875.

FIG. 5.—Endothelial cell from control preparation. Note the fairly regular shape of the cell and the large numbers of silver grains on both luminal and basal surfaces and in the intercellular junction.

FIG. 6.—Endothelial cell after treatment with histamine (100 μg/ml), showing "arcading" on the basal surface, absence of silver grains and narrowing of the cell in the region of the intercellular junction.

FIG. 7.—Endothelial cell after treatment with histamine (100 μg/ml) showing accumulation of chromatin at the periphery of the nucleus, considerable vacuolation and discontinuity of the cell membrane.



FIGS 8 & 9.—Electron micrographs of guinea-pig hepatic portal vein endothelium. × 8250.

FIG. 8.—Endothelial cell incubated with indomethacin (100 μg/ml) before treatment with histamine (100 μg/ml). Compared with Fig. 6 there is less narrowing of the cells at the junctions, although there is still some " arcading ". Note also the presence of silver grains on the luminal surface and in the intercellular junctions.

FIG. 9.—Endothelial cell after incubation with indomethacin (100 μg/ml) for 30 min. Compared with Fig. 5 the cell margins are smoother and the nucleus is less indented.

nomenclature of Majno *et al.*, 1969). Other cells showed definite swelling under the influence of histamine. The nuclei became noticeably rounded, causing the cells to bulge on the luminal surface. Chromatin accumulated at the periphery of the nuclei, giving them a hollow appearance. The cytoplasm of the cells contained many vacuoles and in some cases the plasma membrane was discontinuous (Fig. 7). Possibly, the disappearance of some cells in this manner could account for the splitting seen with the light microscope.

Treating the endothelium with indomethacin before exposure to histamine

TABLE II.—*Electron Microscopic Changes to Endothelium Produced by Histamine, Indomethacin and Lack of Calcium*

Treatment					
Name	Concentration μg/ml	No. of observations	Longest width§ divided by shortest width	Damage§ scale 0–5	Silver staining§ scale 0–4
Control	—	34	$3{\cdot}5 \pm 0{\cdot}35^{1}$	$1{\cdot}4 \pm 0{\cdot}17^{7}$	$1{\cdot}7 \pm 0{\cdot}20^{13}$
Indomethacin	100	33	$2{\cdot}7 \pm 0{\cdot}26^{2}$	$1{\cdot}0 \pm 0{\cdot}11^{8}$	$1{\cdot}1 \pm 0{\cdot}09^{14}$
Histamine	100	32	$5{\cdot}5 \pm 0{\cdot}61^{3}$	$2{\cdot}2 \pm 0{\cdot}26^{9}$	$0{\cdot}7 \pm 0{\cdot}08^{15}$
Histamine + Indomethacin	100 / 100	33	$3{\cdot}4 \pm 0{\cdot}24^{4}$	$1{\cdot}2 \pm 0{\cdot}09^{10}$	$0{\cdot}7 \pm 0{\cdot}06^{16}$
Calcium free	—	21	$3{\cdot}0 \pm 0{\cdot}30^{5}$	$0{\cdot}55 \pm 0{\cdot}06^{11}$	$2{\cdot}1 \pm 0{\cdot}10^{17}$
Calcium free + Histamine	100	42	$3{\cdot}6 \pm 0{\cdot}48^{6}$	$1{\cdot}0 \pm 0{\cdot}02^{12}$	$1{\cdot}0 \pm 0{\cdot}01^{18}$

§ indicates mean ± s.e.

There is a significant difference (Student's t test) between the values marked with the superscript 3 and those marked 1, 4 or 6, the value marked 7 and those marked 8 or 11, the value marked 9 and those marked 10 or 12, the values marked 11 and 12, the value marked 13 and those marked 14, 15, or 18, the values marked 15 and 18, the values marked 17 and 18 ($P < 0{\cdot}05$); and the values marked 1 and 2 ($P < 0{\cdot}01$).

reduced the amount of damage caused by histamine. Some cells showed arcading and others a slight tendency to bulge on the luminal surface but changes of shape were not statistically significant ($P > 0{\cdot}1$, Table II) when compared with controls. In all cases the cell junctions approximated to control lengths (cf. Fig. 5, 8). Vacuoles were present in some cells but by no means all. The nuclei of these cells only showed "notches" and "folds". Silver grains were readily detectable on the luminal surface of all cells, and cell junctions showed one or more grains (Fig. 8). Most cells had a scattering of silver on their basal surfaces.

Endothelium treated with indomethacin alone appeared similar to that of control preparations although the nuclei were smoother in outline and vacuoles, where present, were smaller than in controls (Fig. 9, Table II). There was, however, a significant decrease in the amount of silver deposited, this being manifest as an almost total absence of silver on the medial surface of the cells.

The removal of calcium from the bathing fluid affected neither the shape of the cells nor the amount of silver deposited in the cell margins, but the damage score was considerably less (Table II). This was manifest as an almost complete absence of vacuoles. The addition of histamine (100 μg/ml) to the calcium-free bathing fluid caused no change in the shape of the cells but there was an increase in the amount of damage and a decrease in the amount of silver deposited (Table II). However, when compared with the effects of histamine in the presence of calcium, there were significant decreases in the amount of shape change and in the amount of damage caused (Table II). There were occasional places where the endothelial cell had disintegrated and in these areas silver grains were visible between the smooth muscle cells.

DISCUSSION

The observations made with the light microscope agree reasonably well with those using the electron microscope. Lower scores for silver staining were obtained, however, using the electron microscope in the indomethacin treated group and in the calcium-free histamine treated group as compared with their scores using the light microscope (cf Tables I and II). This may be explained by the fact that in both groups, in contrast to the controls, silver was deposited on the luminal surface of the cells but hardly at all on the medial surface. Since the scores for the electron micrographs were based upon the deposition of silver on *both* surfaces of the cell, whereas only the luminal surface was viewed with the light

microscope, it is to be expected that the scores for the former will be lower than those for the latter. The variability of the results obtained with the light microscope was greater than with the electron microscope. In view of this, the remainder of the discussion is restricted to the results obtained with the electron microscope.

Workers who have studied the effects of various forms of injury on the endothelium of blood vessels suggest that the characteristic signs of damage are vacuolation (Berdjis and Vick, 1968; Hoff, 1970; Entrican, Simpson and Stalker, 1972), changes in shape of the nucleus (Majno *et al.*, 1969; Joris, Majno and Ryan, 1972), bulging of the cell (Majno *et al.*, 1969), cytoplasmic extrusions (Hoff and Gottlob, 1967; Entrican *et al.*, 1972) partial detachment of endothelial cells from the sub-endothelial connective tissue in several foci—" arcading "—(Baumgartner, 1971), and complete separation of endothelial cells from the internal elastic lamina (Tranzer and Baumgartner, 1967; Gaynor, Bouvier and Spaet, 1970; Wright and Giacometti, 1972). Peters, Müller and De Duve (1972) have found many of the above phenomena in varying degrees in isolated suspensions of smooth muscle cells from rabbit aorta. They concluded that the most rounded cells were the most damaged.

The results of the present experiments indicate that venous endothelium of the guinea-pig reacts to histamine in several of the above ways and this substance can, therefore, be said to damage endothelial cells.

In the present experiments both indomethacin and the absence of calcium reduced the amount of damage and the shape changes caused by histamine, but whereas the absence of calcium caused a significant increase in the amount of silver deposited, indomethacin had no effect. Majno *et al.* (1969) suggested that histamine-like mediators cause vascular endothelial cells to contract and bulge. The discovery of actomyosin in endothelial cells by Becker and Murphy (1969) is evidence in favour of this hypothesis. It is well known that the entry of calcium into muscle cells is required for the shortening of their actomyosin filaments (Ashley, 1970), and this would explain why in the absence of calcium histamine causes little change in the shape of endothelial cells. Furthermore, Tapp and Trowell (1967) have shown that under anoxic conditions vacuolation occurred in acinar cells of the rat submandibular gland, but only when calcium and bicarbonate ions were present in the bathing fluid. Similarly, in the present experiments the number of vacuoles seen in endothelial cells was considerably reduced in the absence of calcium. Northover (1971, 1973) showed that indomethacin can reduce the influx of calcium into smooth muscle cells. It seems reasonable to suggest, therefore, that indomethacin protects venous endothelial cells against the damaging effects of histamine by reducing the influx of calcium.

Northover (1975) has recently shown that the histamine induced and heat induced depolarization of endothelial cells is calcium dependent and this agrees with the observations in the present experiments that the damaging effects of histamine on endothelial cells are considerably reduced in the absence of calcium.

Work is in progress to see whether endothelial cells react similarly to other mediators of the inflammatory response and whether the endothelial cells of small blood vessels, particularly the terminal venules, behave in a similar way to the endothelial cells of the hepatic portal vein.

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