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AN ULTRASTRUCTURAL STUDY OF ENDOTOXIN INDUCED DAMAGE IN RABBIT MESENTERIC ARTERIES

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SUMMARY.—Mesenteric arteries from control and endotoxin-treated rabbits were studied by electron microscopy. Animals were injected intracardially with an LD_{50} dose of *Escherichia coli* endotoxin or saline 3 and 24 hr before being killed. Vessels were perfused with buffered glutaraldehyde before removal.

In control arteries endothelial cells were thin except in the nuclear region. Vesicles and caveolae were abundant in the cytoplasm. Few mitochondria, scanty endoplasmic reticulum and Golgi apparatus, free ribosomes, and glycogen granules were scattered in the cytoplasm. The endothelial cells rested on a basement membrane which was closely apposed to the internal elastic lamina. The medial smooth muscle layer contained many collagen fibres.

Endothelial cells from endotoxin-treated animals revealed extensive degeneration of subcellular organelles with only the vesicles and caveolae remaining intact. Ruptured mitochondria, areas of cytolysis and large vacuoles were prominent. The nucleus was often spindle shaped or tortuous in outline and sometimes exhibited vacuolization. Swelling and protrusion of endothelial cells into the lumen, the detachment of these cells from the internal elastic lamina, and the marked thickening of the internal elastic lamina were also prominent. Vascular endothelium reacts to histamine, serotonin, epinephrine and norepinephrine in a similar manner, suggesting that the effects observed after the administration of endotoxin may be due to the release of such chemical mediators. This study cannot differentiate between direct and indirect damage; however, it shows that bacterial endotoxin induces severe damage to continuous endothelium and internal elastic lamina.

SMALL blood vessels in different organs are lined by endothelium which differs somewhat in ultrastructural and functional aspects (Majno, 1965), with the ultrastructural variations seeming to reflect adaptations to special functions of the organ. These differences suggested that different types of vascular endothelium might respond differently to endotoxin. Particularly, the Kupffer cells which are actively phagocytic and the continuous and discontinuous endothelial cells which are weakly, if at all, phagocytic might differ in response to material which is largely particulate (Spielvogel, 1967; Stewart, unpublished). Studies by McKay and his associates indicated that this was the case.

In rats prepared for the generalized Shwartzman reaction by pregnancy, a single dose of endotoxin injected i.v. caused damage to the Kupffer cells after 1 hr, while kidney and pulmonary endothelium remained unchanged (McKay, Margaretten and Csavossy, 1966). In rhesus monkeys given a single large dose of endotoxin, the Kupffer cells were also damaged (swollen), while endothelium in the heart, lung, kidney and jejunum was undamaged for 4 hr (McKay, Margaretten

and Csavossy, 1967). However, studies by McGrath and Stewart (1969), and Coalson, Hinshaw and Guenter (1970) have shown endotoxin-induced damage in continuous endothelium in mesenteric arteries, and pulmonary capillaries.

The extent of damage following the introduction of endotoxin into the blood stream increases for at least 24 hr as indicated by our studies (McGrath and Stewart, 1969; Stewart, unpublished). Since most studies have been limited to some 4–6 hr of observation, it is possible that the full extent of tissue damage in endotoxemia has not been appreciated. This damage, whether caused by ischemia, intravascular clotting, or substances released from blood cells, is a serious factor to consider in the pathogenesis and prognosis of the disease.

The purpose of this investigation was to study the ultrastructural changes in continuous endothelium 24 hr after the introduction of endotoxin into the blood stream.

MATERIALS AND METHODS

Young adult white rabbits weighing about 2 kg. each were lightly anaesthetized with ether while either saline of 7·5 mg. of *Escherichia coli* endotoxin (Bacto-Lipopolysaccharide, Difco Laboratories, Detroit, Mich.) was injected intracardially. One rabbit was injected 3 hr and 6 were injected 24 hr before being killed. Five controls were injected with saline. Surgical procedures and perfusion were carried out as described by McGrath and Stewart (1969).

Following perfusion, small segments of perfused mesenteric arteries were excised and transferred to cold 3·0 per cent glutaraldehyde in Millonig's phosphate buffer with 0·005 per cent calcium chloride (Zweifach, 1965; Chambers and Zweifach, 1947; Pease, 1964). Arteries were cut into small (1–2 $mm.^3$) pieces and excess adipose and connective tissue were trimmed away under a dissecting microscope. Specimens were held for 2–26 hr at 4°. The glutaraldehyde was removed by washing in 4 changes of cold Millonig's phosphate buffer with 0·005 per cent Ca^{++} over a period of 6–20 hr. Tissues were post-fixed in 1·0 per cent osmium tetroxide in Millonig's phosphate buffer with 0·005 per cent Ca^{++} for 1–3 hr at 4°. Specimens were rinsed briefly with cold distilled water and rapidly dehydrated in cold 100 per cent ethanol (Pease, 1964; Palade, 1952, 1956; Sjostrand, 1967). While in absolute alcohol, the remaining adipose and connective tissue, made visible by the osmium fixation, were removed from the arteries, and the vessels were embedded.

EXPLANATION OF PLATES

FIG. 1.—Longitudinal section of mesenteric artery from control animal. In the endothelial cell (E) the mitochondria (M), vesicles (V), and ribosomes (R) are especially prominent. The IEL is a scalloped, electron transparent layer with a slight suggestion of denser fibres. Pleating (P) has produced an electron dense area in the IEL. Much collagen (C) borders the IEL and is dispersed between the smooth muscle cells (SM). × 20,000.

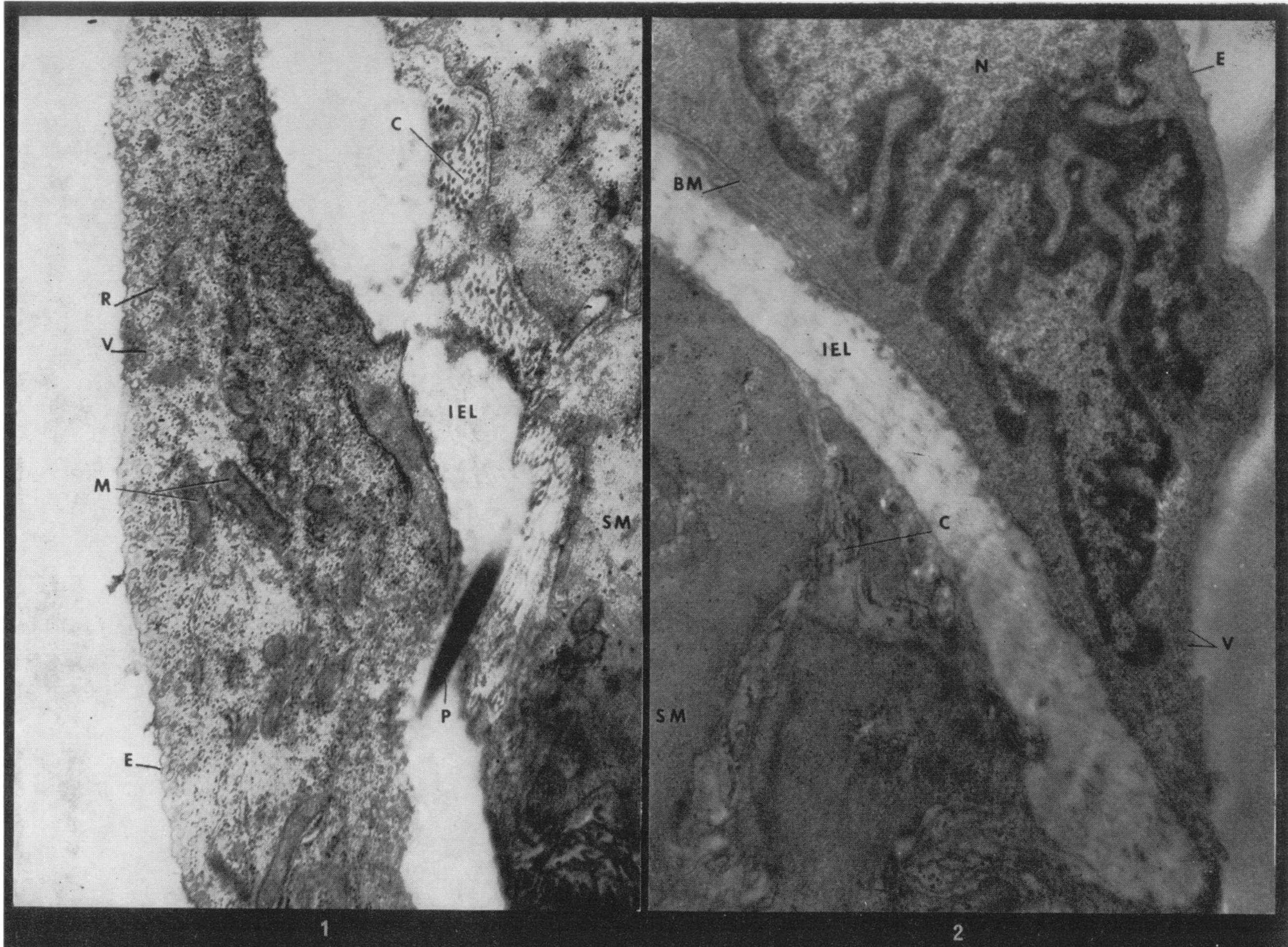
FIG. 2.—Longitudinal section of mesenteric artery from another control animal. In this section the nucleus (N) is distorted, the cytoplasm is filled with vesicles (V) and fibres of elastin are clearly visible in the IEL. × 11,000.

FIG. 3.—Longitudinal section of mesenteric artery from yet another control animal showing a more nearly " typical " ellipsoidal nucleus. The luminal (L) edge of the endothelial cell (E) is scalloped. The basement membrane (BM) is evident between the endothelial cell and IEL. × 16,000.

FIG. 4.—Longitudinal section of mesenteric artery from animal 24 hr after endotoxin injection. A large cytoplasmic vesicle (CV) filled with amorphous material forms a bulge in the cell contour. × 18,500.

FIG. 5.—Section from another endotoxin injected animal. A giant, membrane enclosed cytoplasmic vacuole has distorted the nucleus forming a long neck. The vesicles are typical. × 30,300.

FIG. 6.—Cross section from mesenteric artery from animal 24 hr after endotoxin injection showing 2 endothelial cells, (E_1 and E_2). E_1 has 2 vacuoles (V_1 and V_2) the larger of which is mostly filled with amorphous material of intermediate density. Both vacuoles have areas of highly transparent material. E_2 bulges into the vessel lumen and contains a vacuole filled with sparsely dispersed amorphous material (V). × 15,200.



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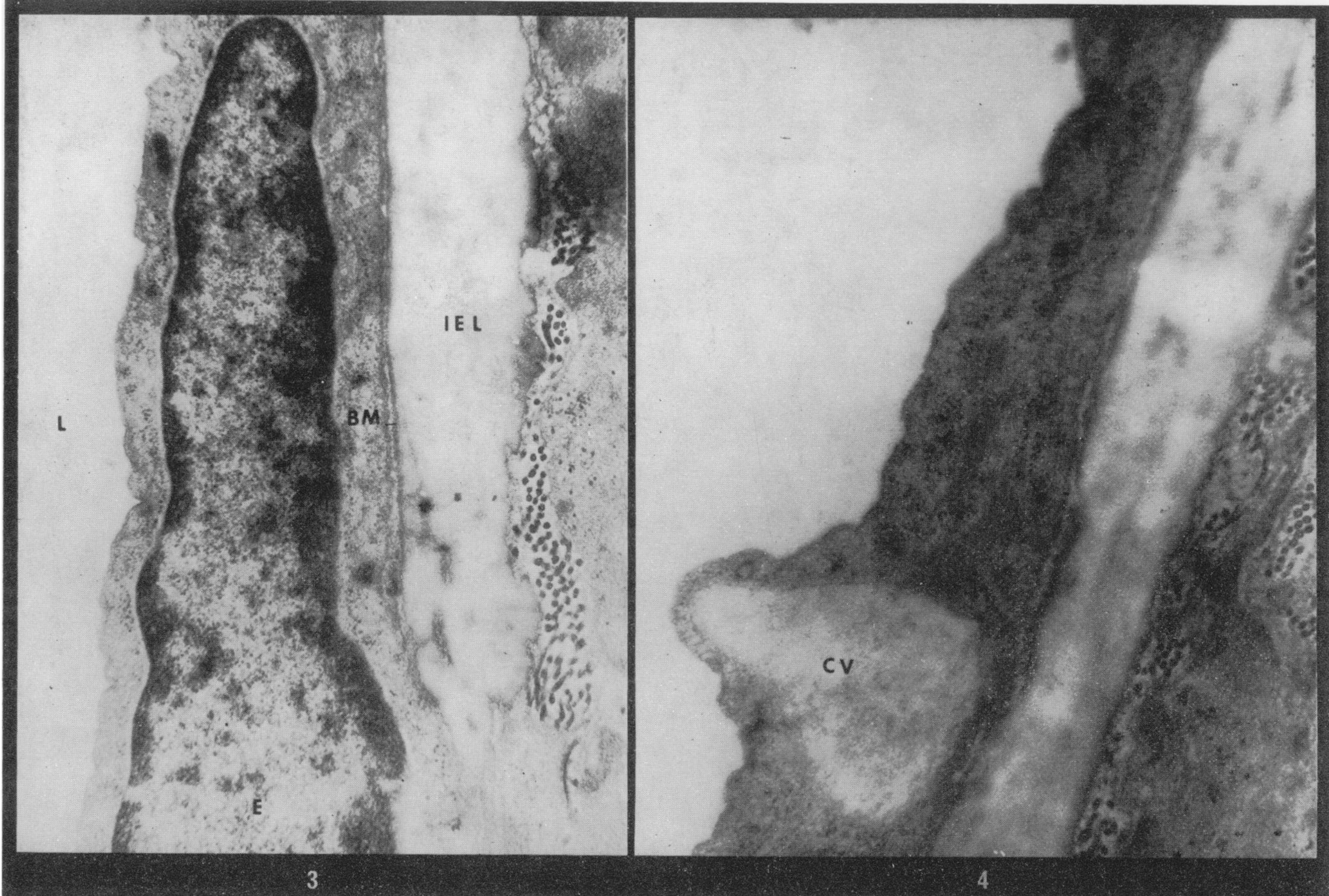

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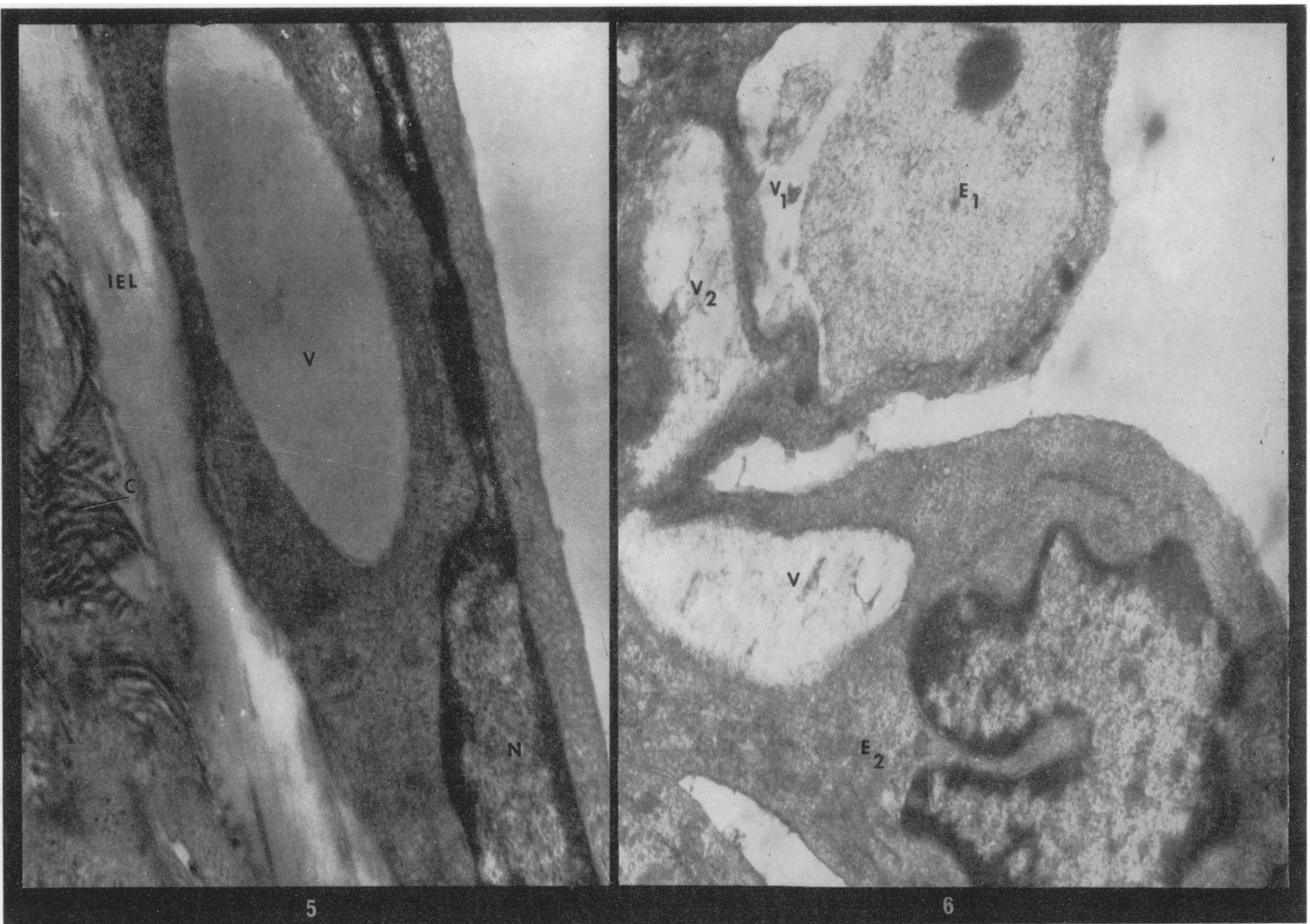

Stewart and Anderson.

Adequate infiltration was obtained only after prolonged incubation in Epon 812 with no catalyst. The procedure, yielding blocks of adequate sectioning quality is as follows. After the last propylene oxide change, the tissue was suspended in a mixture of monomer and propylene oxide (2 : 1) overnight. The fluid was drawn off and Epon 812 was added, evacuated in a vacuum desiccator, and held for 2 hr at 4°. The Epon 812 was drawn off and replaced with fresh plastic for 2 hr at 60°. Tissues were suspended in another change of fresh Epon 812 at 60° overnight. The Epon was drawn off and replaced with Epon plus 1·5 per cent BDMA for 2 hr at 60°. Vessels were removed from vials and placed in BEEM capsules filled with fresh Epon plus 1·5 per cent catalyst. Contents of the capsules were evacuated in the vacuum dessicator for 15 min. and polymerized at 60° for 2 days.

Sections were double stained (Frasca and Parks, 1965) with 2·0 per cent uranyl acetate and lead citrate (Reynolds, 1963). Transverse and longitudinal sections of arteries were micrographed at instrument magnifications of 3500–17,000.

RESULTS

Endothelial cells of control arteries were thin except in the nuclear region. The nuclear boundary was elliptical or irregular in shape with a densely staining border. Vesicles and caveolae were prominent in the cytoplasm, particularly along the luminal and medial edges. The cytoplasm contained a few mitochondria, some rough-surfaced endoplasmic reticulum, free ribosomes, glycogen granules and an occasional isolated vacuole (Fig. 1). The Golgi apparatus was scant. The luminal surface of the endothelium was generally flat, although scalloped edges were sometimes observed (Fig. 3). Cell junctions were not easily seen. In these preparations the basement membrane appeared as a diffuse reticulum. The intima was separated from the media by a broad zone of internal elastic lamina (IEL). Occasional dicontinuities in the IEL allowed the endothelium to protrude through and make contact with the smooth muscle cells of the media. Many radially and longitudinally oriented collagen fibres were observed in the smooth muscle layer (Fig. 1 and 2).

Injection of endotoxin produced extensive damage to the endothelium with only the vesicles and caveolae escaping damage. Mitochondria were swollen and often completely cytolysed. Giant vacuoles, taking up much of the cytoplasm and containing amorphous material, were frequent (Figs 4, 5 and 6). These vacuoles had membranes which were sometimes incomplete and differed from the occasional small vacuoles seen in endothelial cells of control animals. Many of the nuclei were spindle shaped or tortuous in outline (Fig. 5). Nuclear vacuoles were occasionally observed. In vessels of these experimental animals, the endothelium was sometimes detached from the internal elastic lamina. Once separated, it lifted away from the intima and appeared folded. In some instances the endothelium appeared to be missing entirely.

DISCUSSION

The severe damage observed in mesenteric arteries following endotoxin injection is likely representative of the real situation since it is compatible with the damage observed in thousands of cells in continuous sheets examined at the light microscope level (McGrath and Stewart, 1969). The lighter staining areas of nuclei which appeared to be vacuoles at the light microscope level apparently correspond to the long thin " necks " caused by nuclear distortion (Fig. 5). Since the density of the staining of the nuclei in these preparations depends greatly on the thickness of the structure, physically thin areas would stain much lighter, thus appearing as vacuoles.

The giant cytoplasmic vacuoles containing amorphous material were similar to those described in endothelia of vessels after anoxia, thermal injury, and perfusion with various vasoactive agents (Cotran, 1956; Hoff and Gottlob, 1967*a*, *b*; Reynolds, 1963; Sinapius, 1967). Similar changes occur in local antigen-antibody or hypersensitivity reactions (Stetson, 1964; Stewart, 1963), in lesions of the Shwartzman variety induced by bacterial extracts (Thomas, 1954), and during the reaction to local infection (Zweifach, 1959). Vacuolization, according to Altschul (1954), indicates the beginning of cell decay. A number of theories for vacuole genesis after injury have been postulated. Manuelidis (1958) describes vacuole formation as the result of mitochondrial swelling. Buckley (1962) attributes them to endoplasmic reticulum swelling. Trump and Janigan (1962) consider them to be derivatives of lysosomes. Hoff and Gottlob (1967*a*) suggest they may be pseudopodia of adjacent endothelial cells protruding into the cytoplasm where they appear as vacuoles.

The vesicles and caveolae remained structurally unaffected by the endotoxin treatment. Jennings and Florey (1967) showed that these structures were independent of various perfusion techniques, and histamine, serotonin and the inflammatory response were without visible effect on the vesicles. Some authors have stated that the number of vesicles is increased under abnormal conditions, but the evidence is not convincing (Majno, 1965).

Other lesions exhibited after endotoxin administration also seem to resemble those induced by other types of cellular injury. Cytoplasmic degeneration close to the intercellular junction has been observed after thermal injury, mechanical agitation, and exposure to chemicals (Cotran, 1965; Majno and Palade, 1961; Marchesi, 1964; French, 1963; Cotran and Majno, 1964). Such gaps in the endothelial lining may lead to the increased permeability associated with injured blood vessels (Majno, 1965; Cotran, 1965; French, 1963).

The separation of the endothelium from the internal elastic lamina likely corresponds to the sloughing and desquamation of endothelial cells after cellular injury (Altschul, 1954; Zweifach, 1964; McGrath and Stewart, 1969). Swelling of endothelial cells with their consequent protrusion into the vessels lumen (Fig. 6) has been reported in other systems (Altschul, 1954; Cotran, 1965; Mannuelidis, 1958; Zweifach, 1964; Hoff and Gottlob, 1967*a*, *b*; Majno and Palade, 1961; Florey, 1961; Marchesi, 1964; Zweifach, 1965).

The similarity in the response of vascular endothelium to diverse types of injury has led Cotran (1965) to postulate that the alterations are chemically mediated. Since the vasoactive agents histamine, serotonin, epinephrine and norepinephrine bring about the same kinds of pathological changes as observed in this study (Mannuelidis, 1958; Hoff and Gottlob, 1967*a*, *b*; Buckley, 1962; Friederici, 1965), these alterations may be due to the liberation of these substances by endotoxin or endotoxin-antibody complexes. Numerous investigators favour the theory that endotoxin exerts its vascular action *via* these vasoactive substances (Thomas, 1954; Brake, Emerson, Wittmers and Hins, 1964; Delaunay, Lebrun and Cotrereau, 1947; Delaunay, Boquet, Lebrun, Lehoutt and Delaunay, 1948; Gilbert, 1960; Hinshaw, 1964; Hinshaw, Gilbert, Kuida and Visscher, 1958; Kobold, Katz and Thal, 1963; Morris, Smith and Assali, 1965; Thomas, Sweifach and Benacerraf, 1957; Tsagaris, Koehler and Kuida, 1963; White, Ross, Barajas and Jacobsen, 1966; Zweifach and Thomas, 1957). This study cannot distinguish between direct and indirect action of endotoxin on endothelium, but it does

present physical proof that bacterial endotoxin induces severe damage to continuous endothelium as well as to Kupffer cells. It also indicates that extensive cellular damage is a significant aspect of endotoxaemia which cannot be ignored. In addition to loss of function, extensive cellular injury may result in the release of histones which cause aggregation of fibrinogen (Stewart and Niewiarowski, 1970). These aggregates may obstruct capillaries and thus contribute to collapse of the peripheral circulation.

This study indicates that animals which do not succumb due to circulatory collapse or thrombosis during the first 24 hr of endotoxaemia are still in a grave situation because of the extensive cellular damage. The extent of damage observed in the liver (Stewart, unpublished) would be expected to result in loss of function while the extensive vascular damage would seriously impair permeability thus interfering with the supply of fluid, salts, nutrients and oxygen to all cells.

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