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Endothelial cells in the oral mucosa of *Bufo marinus*

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INTRODUCTION

The oral mucosa of amphibia is unusual in that it is lined by a stratified epithelium with a ciliated border, and has an intraepithelial network of capillaries within it (Patt & Patt, 1969; Andrew & Hickman, 1974). A light and electron microscopic study was made of these capillaries to determine the structure of the endothelial cells and their relationship with the epithelial cells in *Bufo marinus* as such an arrangement of capillaries would imply transport of material between the blood and the surface of the epithelium.

MATERIALS AND METHODS

Adult cane toads (*Bufo marinus*) were anaesthetized by applying cotton wool soaked in anaesthetic ether (diethyl ether) on the abdomen. Pieces of oral mucosa were then removed and processed according to the following techniques:

(1) Fixed in 10 % formol saline processed routinely for paraffin sections and stained with haematoxylin and eosin.

(2) Freeze-dried in a Dynavac freeze-dryer at -50 °C in a vacuum of 10^{-3} Torr overnight fixed with formaldehyde vapour at 60 °C for 3 hours and then blocked in Paraplast. Sections 10 μm thick were examined for catecholamine fluorescence (Falck, Hillarp, Thieme & Torp, 1962). Control sections were not exposed to paraformaldehyde vapour.

(3) Freeze-dried, formaldehyde vapour-fixed sections were also processed for the Schmorl reaction (Bancroft, 1975), the argentaffin reaction (Sevier & Munger, 1965), lead haematoxylin reaction (Solcia, Capella & Vassallo, 1969) and the Luxol fast blue reaction (Drury & Wallington, 1967).

(4) Frozen sections 10 μm thick were processed for the Sudan black reaction (Bancroft, 1975) and the acid phosphatase reaction (Gomori lead method, Bancroft, 1975).

(5) Tissues for electron microscopy were immersed in 3 % glutaraldehyde in phosphate buffer at pH 7·4, cut into small blocks of approximately 1 mm cubes, processed routinely for electron microscopy and blocked in Spurr's low viscosity resin (Spurr, 1969). Ultrathin sections were stained with uranyl acetate and Reynold's lead citrate.

For the acid phosphatase reaction, tissue blocks were fixed as for above and processed according to the method of Barka & Anderson (1962), as described by Lewis and Knight (1977) using sodium β glycerophosphate as substrate. The blocks were then post-fixed in osmium tetroxide and ultrathin sections were stained with uranyl acetate only. The reaction was carried out at pH 5·2 using tris maleate buffer.

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Control tissue blocks were incubated in (*a*) sodium β glycerophosphate without lead nitrate, (*b*) lead nitrate without sodium β glycerophosphate and (*c*) the whole incubation medium with 5 mM sodium fluoride added as an inhibitor of enzyme activity (Lin & Fishman, 1972). Ultrathin sections were examined with a Siemens Elmiskop 1A.

OBSERVATIONS

The oral mucosa was ridged and lined by a pseudostratified columnar epithelium with a ciliated surface. Patches of non-ciliated cells were present and numerous goblet cells were seen, especially towards the posterior portion of the oral cavity (Fig. 1).

Ciliated cells bore both cilia and microvilli on their apical surfaces. Cilia had basal bodies with prominent striated rootlets which were directed downwards into the apical cytoplasm of the cells (Fig. 2). Nuclei of ciliated cells were irregular in outline and had a rim of chromatin at the periphery. Usually a prominent nucleolus was present. Desmosomes were seen between ciliated cells near their apical borders and between ciliated cells and the cuboidal cells underlying them.

The epithelium was invaded by capillaries from the lamina propria and in some places these reached the base of the surface layer of cells (Fig. 3). These capillaries were lined by a single layer of two to three endothelial cells lying on a well-defined basal lamina. Outside the basal lamina, a connective tissue sheath (about 1 μm thick) containing some collagen fibrils, was always present so that the basal lamina of the endothelium never came into contact with that of the epithelial cells. The endothelial cells overlapped each other slightly at their margins and were held together by tight junctions (Fig. 4). Nuclei of endothelial cells were usually flattened and irregular in outline and had heavy chromatin deposits. The cytoplasm was extremely attenuated and fenestrated except in the region of the nucleus (Fig. 4). Numerous pinocytotic vesicles were seen throughout the cytoplasm and lined the cell membrane on both the luminal and basal surfaces. Microvillus-like or sheet-like processes projected into the lumen and joined together to form pinocytotic vesicles on the luminal border of the endothelium.

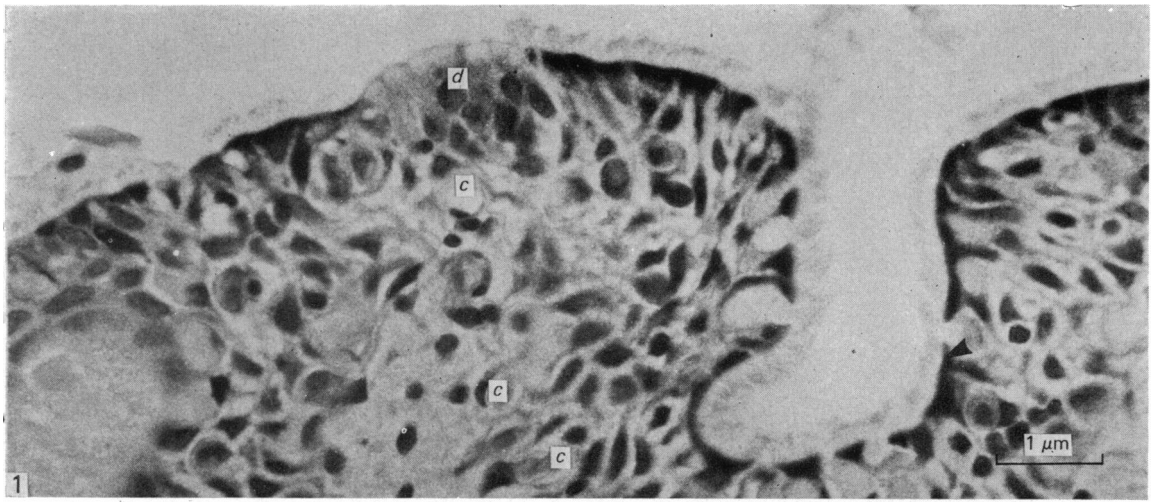
Bundles of filaments oriented in the direction of the long axis of the cell occurred, distributed throughout the cytoplasm, and apparently completely free of attachments (Fig. 5).

Electron-dense granules, oval or spherical in shape, were present in large numbers in the cytoplasm (Figs. 4, 5). They were approximately 250 nm to 400 nm in diameter and were membrane-bound. Pictures taken at higher magnification showed that these granules had a granular internal structure (Fig. 6). Endothelial cells showed a negative reaction for formaldehyde-induced fluorescence (Fig. 7); the lead haematoxylin reaction (Fig. 8), the Schmorl reaction (Fig. 9) and the argentaffin reaction (Fig. 10)

Fig. 1. Oral mucosa of cane toad showing pseudostratified columnar epithelium lining, with a ciliated border. A patch of non-cilated cells is labelled (*d*). Blood capillaries (*c*) show that endothelial cells are not stained by Luxol fast blue, while the apical portions of the ciliated cells show a positive reaction. Freeze-dried paraffin section stained with Luxol fast blue. $\times$1300.

Fig. 2. Electron micrograph of distal portion of ciliated cell showing prominent striated rootlets (*r*) and desmosomes between adjacent cells (arrows). $\times$22000.

Fig. 3. Semithin section of oral mucosa showing the relationship of the blood capillaries (*c*) to the epithelial cells. 1 μm section stained with toluidine blue. $\times$1400.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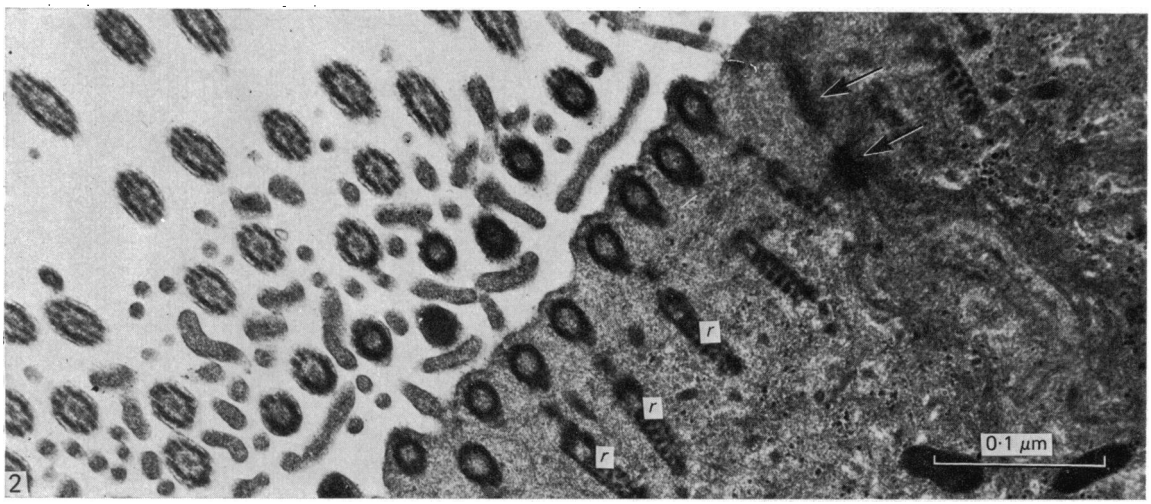
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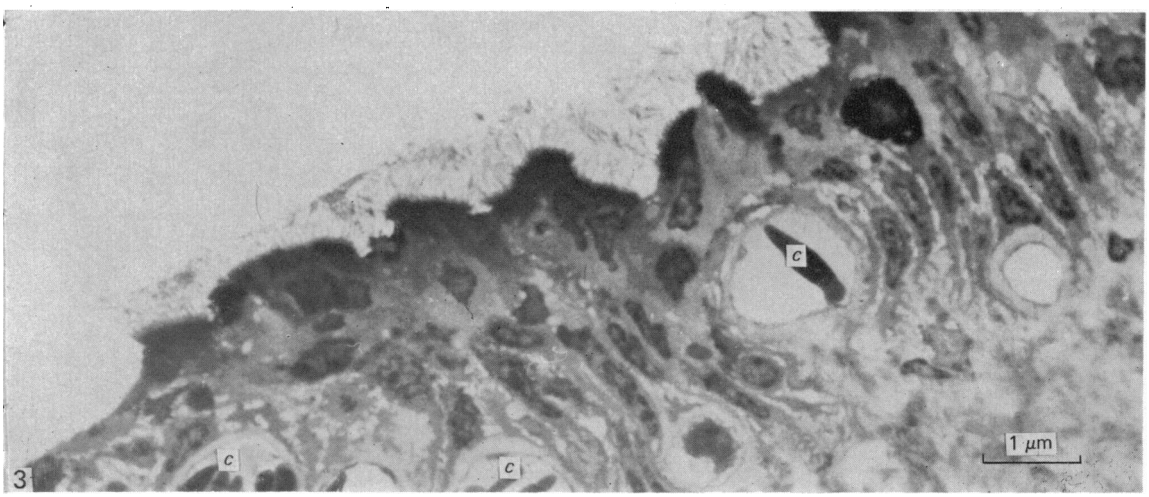
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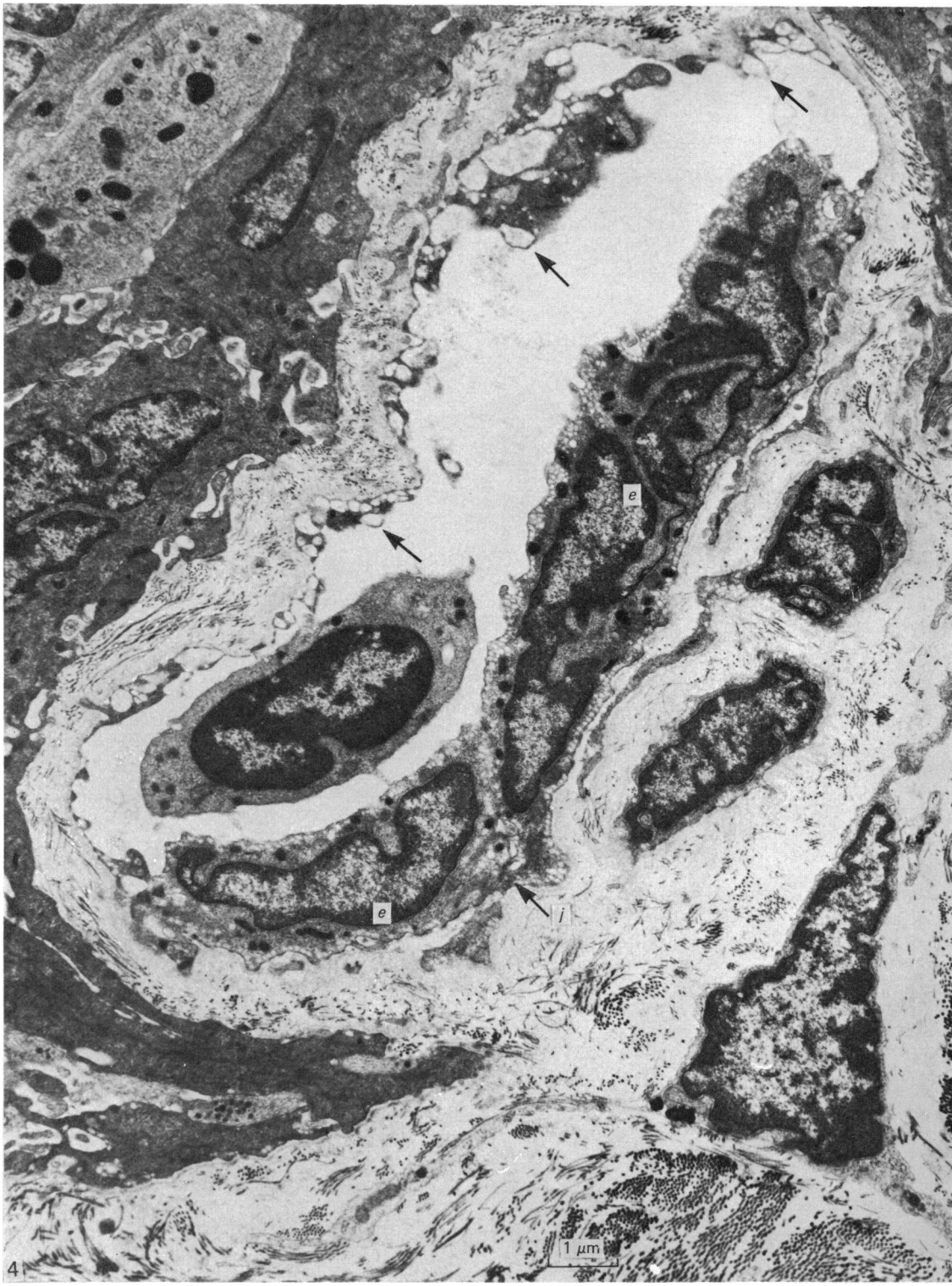
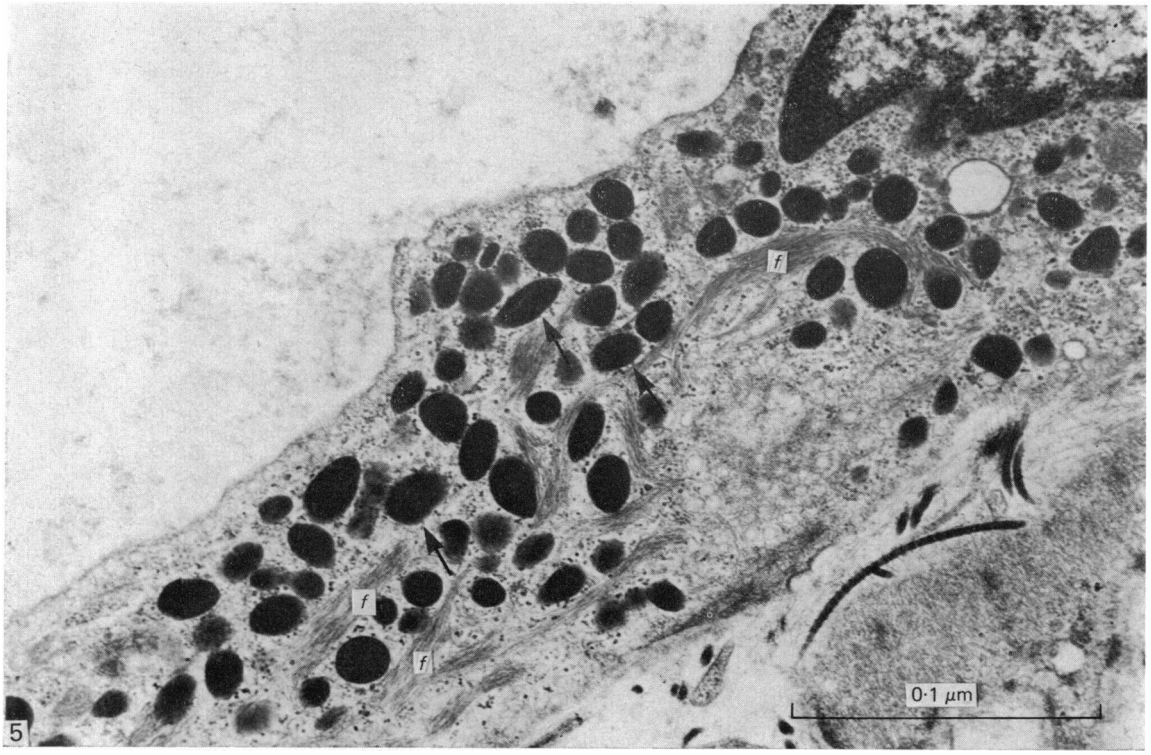


Fig. 4. Electron micrograph of capillary at the base of the epithelium showing endothelial cells (*e*) with fenestrated cytoplasm and electron-dense granules and numerous pinocytotic vesicles in the cytoplasm. Microvillus-like processes projecting into the lumen are arrowed. *j*, a tight junction between two endothelial cells. × 9600.



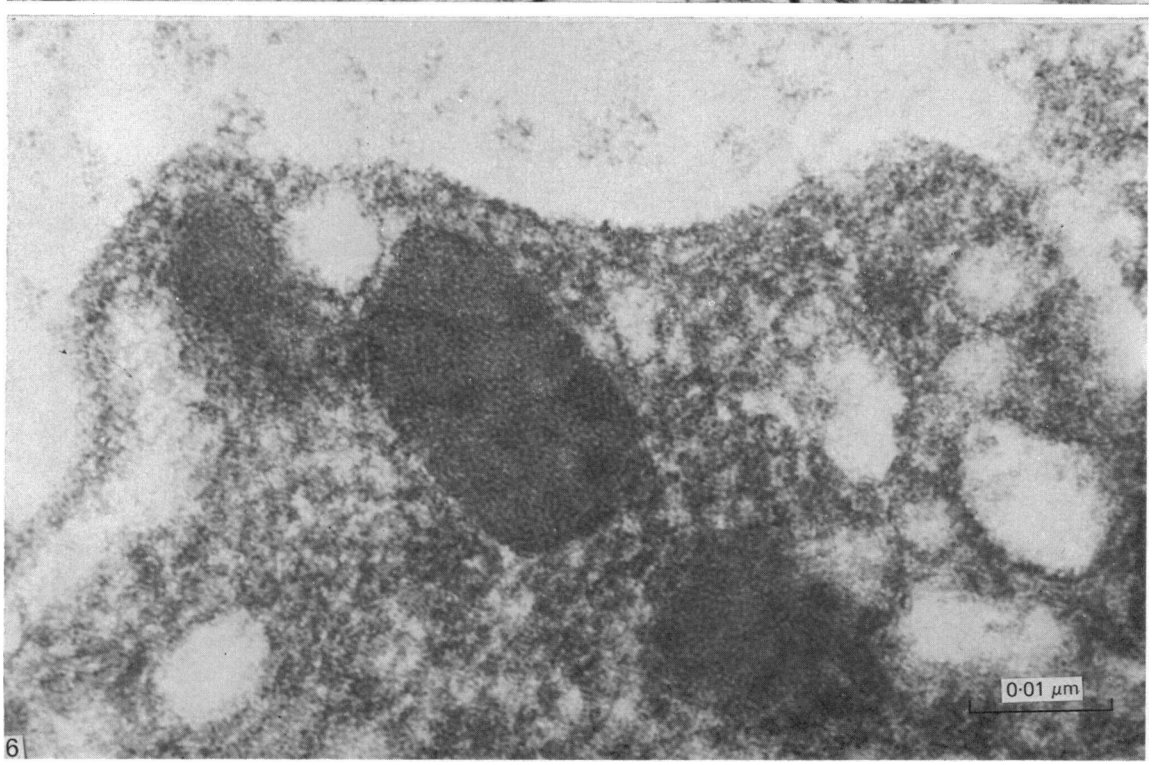
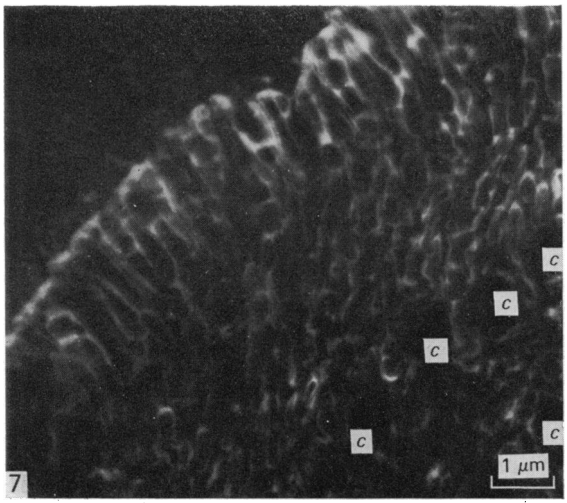


Fig. 5. Electron micrograph of portion of endothelial cell showing electron-dense granules (arrows) and bundles of filaments (*f*) in the cytoplasm. × 40 500.

Fig. 6. Higher magnification of electron-dense granules in endothelial cell cytoplasm showing their granular internal structure. × 181 000.



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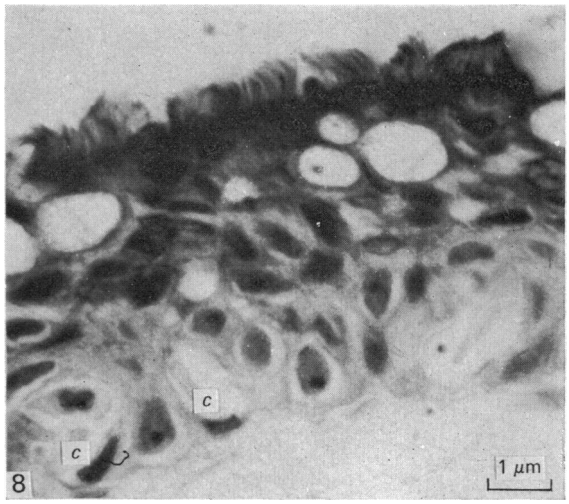

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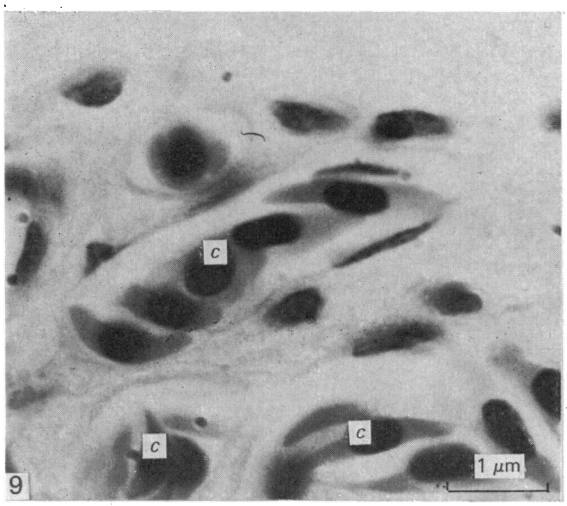

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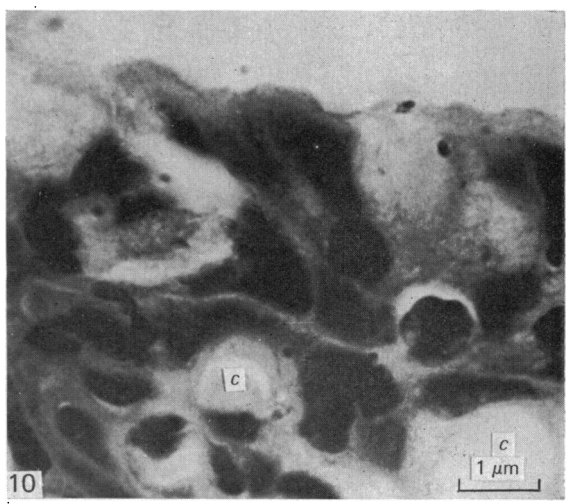

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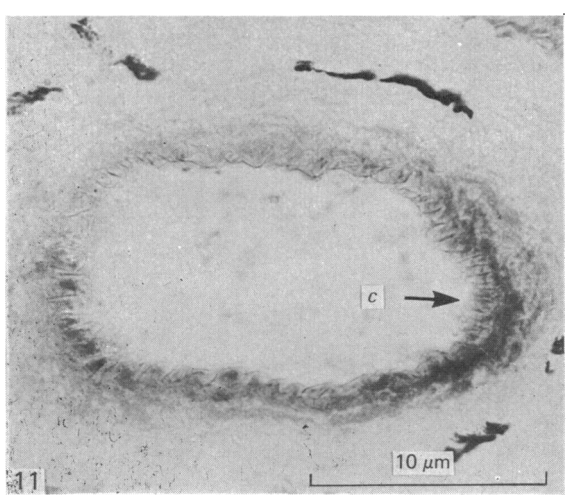

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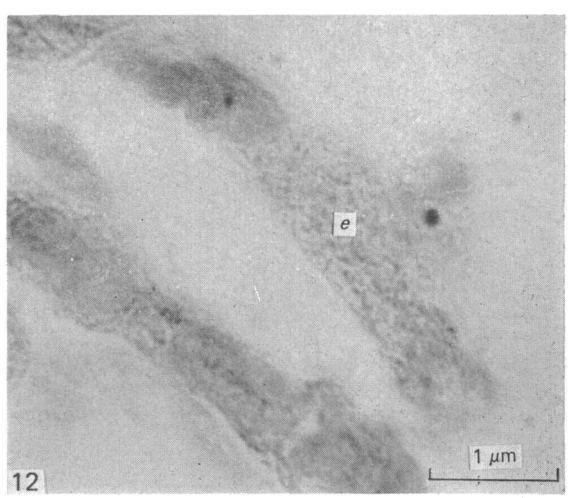

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were also negative. Both Sudan black B reaction (Fig. 11) and Luxol fast blue reaction (Fig. 1) were negative in endothelial cells, but some of the apical cytoplasm of the ciliated cells in the epithelium showed a positive reaction with Luxol fast blue. Acid phosphatase reaction in the frozen sections showed a positive result (Fig. 12), which was confirmed at electron microscopic level by a positive reaction in the granules (Fig. 13). Control sections incubated (*a*) in the buffer and sodium β glycerophosphate, without lead nitrate (Fig. 14), (*b*) in the buffer and lead nitrate without sodium β glycerophosphate (Fig. 15) and (*c*) in the full incubation medium with 0·5 mM sodium fluoride added as an inhibitor of enzyme activity (Fig. 16) all showed negative reactions in the granules.

DISCUSSION

The situation in the oral mucosa of amphibia, where the epithelium is invaded by capillaries up to the base of the distal layer of cells, is probably unique among vertebrates (Patt & Patt, 1969). These capillaries resembled lymphatic capillaries (Bloom & Fawcett, 1975) in that they were lined by fenestrated endothelium with very attenuated cytoplasm and microvilli or folds projecting into the lumen, but unlike lymphatic capillaries, they had a complete basal lamina. The endothelial cells were separated from the epithelial cells by a thin connective tissue sheath separating the basal lamina of the endothelium from that of epithelial cells. This is unlike the situation in the mammalian lung where these basal laminae come into close contact with each other or actually fuse together. The extreme attenuation of the cytoplasm of endothelial cells, the fenestrations and the abundance of micropinocytotic vesicles suggest active transport of material through the endothelium.

Endothelial cells of the toad contained bundles of filaments distributed throughout the cytoplasm. They could have a contractile function, as contractibility has been recognized in amphibian capillaries (Hama, 1961). It has also been suggested by Ludatscher (1978) that endothelial filaments in the dermal capillaries of man could have a contractile function to vary the size of the vessels for thermal regulation. This view is supported by Laweryns, Baert & de Loecker (1976) who have described two types of filaments in the endothelium of lymphatic vessels and have suggested that the thick filaments form a plastic cell skeleton while the thin filaments are actin-like and form a contractile system. However Cecio (1967) is of the opinion that these filaments are mainly supportive in function as they are not attached to any plates in the cell membrane.

Electron-dense granules described by Weibel & Palade (1964) in the pulmonary

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Fig. 7. Freeze-dried section of toad oral mucosa showing absence of fluorescence for catecholamines in capillary (*c*) endothelium. Formaldehyde-induced fluorescence. × 700.

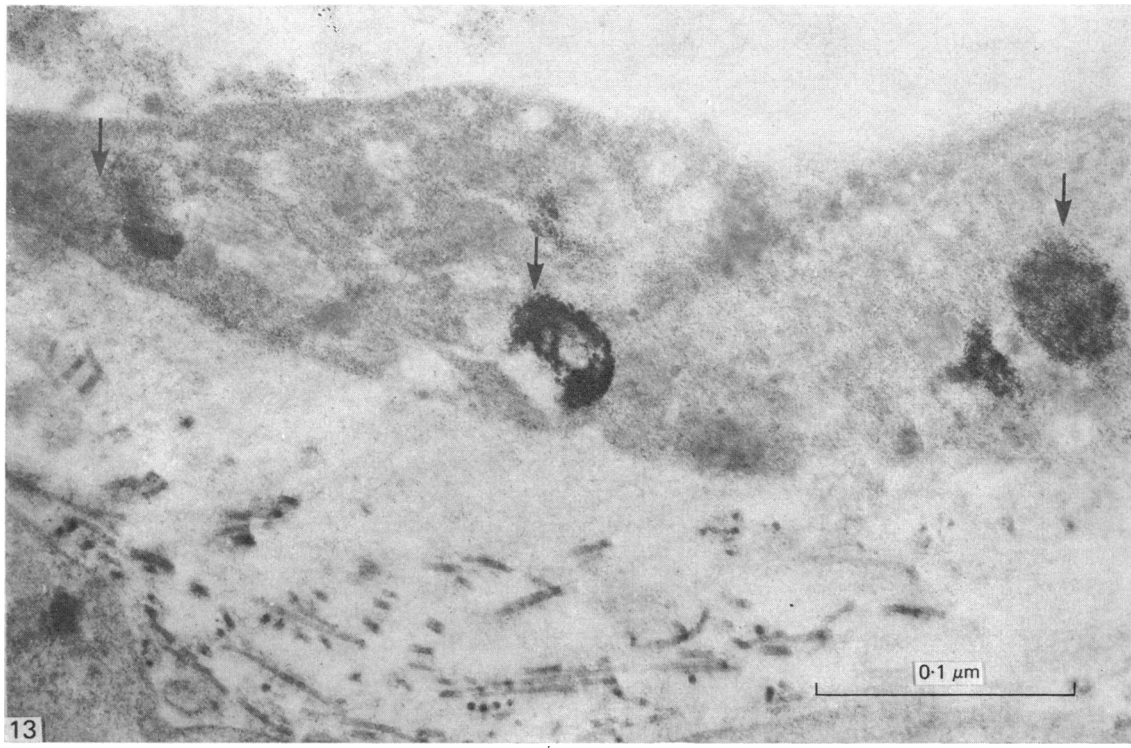
Fig. 8. Toad oral mucosa stained with lead haematoxylin. Endothelial cells of capillaries (*c*) show a negative reaction. × 700.

Fig. 9. Capillaries (*c*) in toad oral mucosa showing the lack of reaction in endothelial cell cytoplasm to the Schmorl reaction. × 1250.

Fig. 10. Capillaries (*c*) in toad oral mucosa showing the lack of stainable material in endothelial cell cytoplasm with the argentaffin reaction. × 1000.

Fig. 11. Frozen section of toad oral mucosa stained with Sudan black B. Capillary (*c*) endothelium shows no reaction. × 30.

Fig. 12. Frozen section of toad oral mucosa. Capillary endothelium (*e*) shows a positive reaction for acid phosphatase. × 1600.



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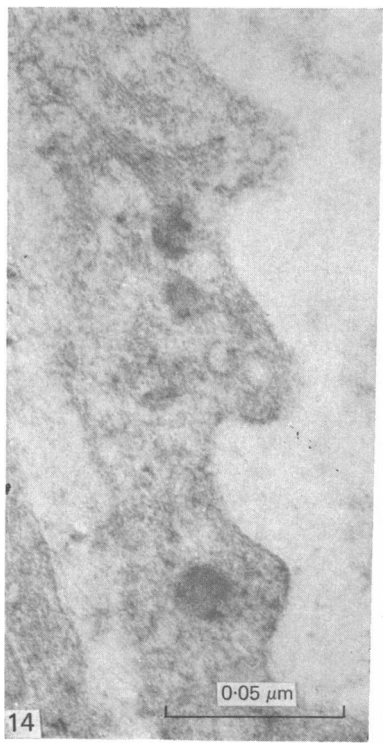

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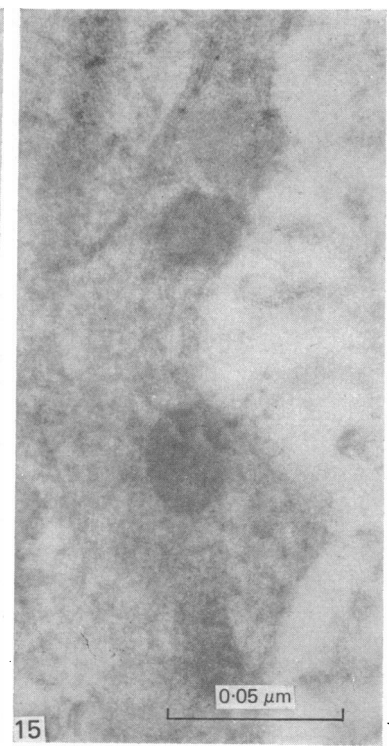

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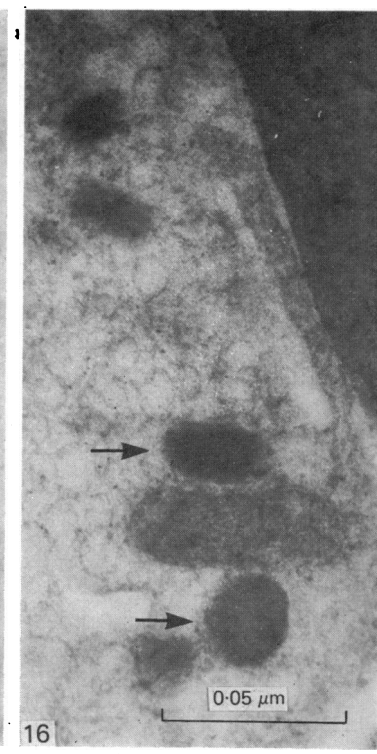

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arteries of rat and man were numerous. Their distribution and sizes are consistent with the findings of Stehbens (1965), Piezzi, Santolaya & Bertini (1969) and Santolaya & Bertini (1970) in amphibia. Histochemical tests show that these granules are not pigments, because both the Schmorl and argentaffin reactions are negative. The negative argentaffin, lead haematoxylin and formaldehyde-induced fluorescence reactions show that they do not have the characteristics of APUD cells (Pearse, 1977). These results are in agreement with the findings of Piezzi *et al.* (1969) that endothelial cell granules in toad arteries show negative activity for the chromaffin and argentaffin reactions. However, Iijima & Wasano (1978) have reported that venous endothelia in the carp contain granules which show positive reaction for catecholamines. Their finding that the specfic granules contain catcholamines would support the report by Bertini & Santolaya (1970) that aqueous extracts of granules in toad endothelium exhibit a strong hypertensive effect when injected into rats. Piezzi *et al.* (1969) investigating the endothelium of *Bufo arenarum* and Lemeunier, Burri & Weibel (1969), investigating these endothelial granules in mammals, have stated that they do not show acid phosphatase activity.

In the capillaries of the oral mucosa of the cane toad, however, we have shown acid phosphatase activity in the granules, implying that they could have some lysosomal function (De Duve, 1963). The finding of thorotrast in the dense bodies of endothelium of mammals, when this was given three to twenty four hours before killing would seem to suggest that these bodies are concerned with phagocytosis (Buck, 1958). Moreover Hruban, Vigil, Slessers & Hopkins (1972) reported the occurrence of peroxisomes in the endothelium of small vessels of mice.

These granules occur in the endothelium of all vertebrates (with the exception of birds) and are more abundant and larger in the lower vertebrates like fish, amphibia and reptiles than in mammals (Santolaya & Bertini, 1970; Bertini, Piezzi & Gutnerrez 1972). They are abundant in venous endothelium (Gorgas, Böck, Tischendorf & Curri, 1977; Iijima & Wasano, 1978) and in the endothelium lining lymphatic vessels (Tabuchi & Yamamoto, 1974). Our findings in the cane toad suggest that they could have some lysosomal function.

SUMMARY

The oral mucosa of the cane toad (*Bufo marinus*) is lined by a pseudostratified columnar ciliated epithelium containing an intraepithelial network of capillaries, which penetrates it to the bases of the distal layer of cells. The capillaries are lined by fenestrated endothelium lying on a complete basal lamina. A connective tissue sheath, approximately 1 μm thick, surrounds the capillaries and separates them from the

Fig. 13. Electron micrograph of portion of endothelial cell showing positive acid phosphatase in electron-dense granules (arrowed). × 33600.

Fig. 14. Electron micrograph of control section for acid phosphatase incubated in sodium β glycerophosphate only showing no reaction in electron-dense granules. × 46000.

Fig. 15. Electron micrograph of control section for acid phosphatase in endothelial cell incubated in lead nitrate without sodium β glycerophosphate showing no reaction in electron-dense granules. × 43900.

Fig. 16. Electron micrograph of control section incubated in substrate for acid phosphatase with 5 mM sodium fluoride in the incubation medium. Electron-dense granules (arrows) show no reaction. × 46000.

surrounding epithelial cells. Endothelial cells resemble those in lymphatic capillaries in that they show microvillus-like processes or folds projecting into the lumen and also have extremely attenuated and fenestrated cytoplasm except in the nuclear region. Numerous pinocytotic vesicles, bundles of filaments and many electron-dense granules occur in the cytoplasm. These granules are oval or round in shape and approximately 250–400 μm in diameter. Histochemical tests on the endothelial cells show that the granules do not contain pigment, as both the Schmorl and argentaffin reactions are negative. Both the Sudan black B and Luxol fast blue reactions are also negative showing the lack of stainable lipids. The formaldehyde-induced fluorescence, the argentaffin reactions and lead haematoxylin reactions are negative, indicating that they do not have the characteristics of endocrine cells. The acid phosphatase reaction gives a positive result, localized to the site of the granules by electron microscopy and suggesting that these granules in amphibian capillaries may have a lysosomal function.

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