

Contractile proteins in retinal endothelium and other non-muscle tissues of the eye

AMJAD RAHI AND NORMAN ASHTON

From the Department of Pathology, Institute of Ophthalmology, University of London

SUMMARY The present study is the first investigation to demonstrate, by employing the combined approach of immunological and electron microscopic methods, the presence of actin-like contractile proteins in the mammalian retina, the corneal epithelium and endothelium, the iris, and the ciliary body, and to confirm their presence in lens epithelium. This is also the first report to demonstrate by these methods the presence of microfilaments and intermediate filaments in retinal vascular endothelium. Since we have shown that actin filaments are especially abundant in immature retinal endothelial cells, the question of their function arises, and we have discussed their possible relevance to the closure of immature retinal vessels when exposed to hyperoxia.

The cause of the peculiar sensitivity of immature mammalian retinal vessels to hyperoxia, which react by severe constriction leading eventually to their complete and irreversible closure (Ashton *et al.*, 1954), remains unknown. One obvious explanation would be that for some reason immature vessels are more actively contractile than those of the adult, but this would be surprising since the latter possess a fully developed musculature, whereas the simple endothelial tubes of growing vessels still lack this differentiation. Curiously, the less mature the developing vasculature the greater the sensitivity to oxygen, and in seeking an explanation in the past it has been difficult to imagine upon what cellular or subcellular component any vasoconstrictor might act.

Indeed, the question whether endothelial cells are alone capable of contraction had been a matter of controversy for over a century. In an extensive review of the subject Wiedeman (1963) concluded that the accumulated evidence was overwhelmingly in favour of capillaries being non-contractile. Filaments have, however, been repeatedly observed in the cytoplasm of endothelial cells of various mammalian tissues, and it had been suggested that these might be contractile, but Rhodin (1967), although regarding the endothelial filaments as indistinguishable from myofilaments of smooth muscle, felt unable in the absence of experimental proof to conclude that this necessarily implied endothelial contractibility.

Moreover, Cecio (1967), in an ultrastructural study of capillary endothelium in the rat, believed that the lack of periodicity in the cytofilaments and the absence of nervous connections actually excluded the possible active contractility of the cells and that the filaments had only a supportive and elastic function. Becker and Murphy (1969) have since demonstrated by immunofluorescence the presence of the contractile protein actomyosin in human cardiac endothelial cells, a finding in harmony with the contractile hypothesis. In the same year Majno *et al.* (1969) published a full account of their work showing the contractile power of endothelial cells in response to histamine-type mediators.

In view of this evidence we thought it would be of interest to examine immature and mature retinal endothelium for the presence of contractile protein antigens. For the demonstration of actin filaments *in situ* 2 techniques are currently in vogue. One method relies on the capacity of actin molecules to form 'arrow head' complexes, as seen electron microscopically, when incubated with myosin subfragment-1 or heavy meromyosin (Goldman, 1975). The other, which is simpler, more versatile, and able to localise not only actin but other intracellular contractile proteins, depends on the property of smooth muscle antibody (SMA), contained either in pathological human sera or, as pure actin antibody, in the sera of immunised animals, to react with cells and tissues where the antibody-combining sites correspond to those in which microfilaments have been demonstrated ultrastructurally (Holborow *et al.*, 1975; Trenchev *et al.*, 1976). The present communication is concerned with the latter

Address for reprints: Dr Amjad Rahi, Department of Pathology, Institute of Ophthalmology, Judd Street, London WC1H 9QS

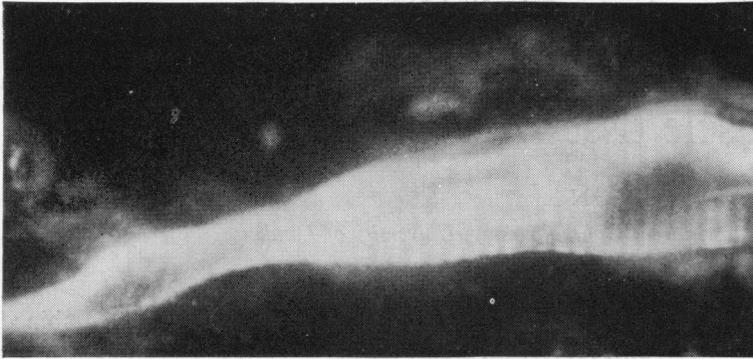


Fig. 1 Indirect immunofluorescence test on rat skeletal muscle. The smooth muscle antibody reacts with actin in the muscle fibre to produce bright fluorescence of the 'I' band. Unfixed cryostat section ($\times 1600$)

Fig. 2 Cryostat section of rat stomach treated with smooth muscle antibody and fluorescein-labelled anti-immunoglobulin. The muscularis mucosa, the vessel wall, and the muscle fibres between the gastric glands (arrows) show brilliant fluorescence ($\times 960$)

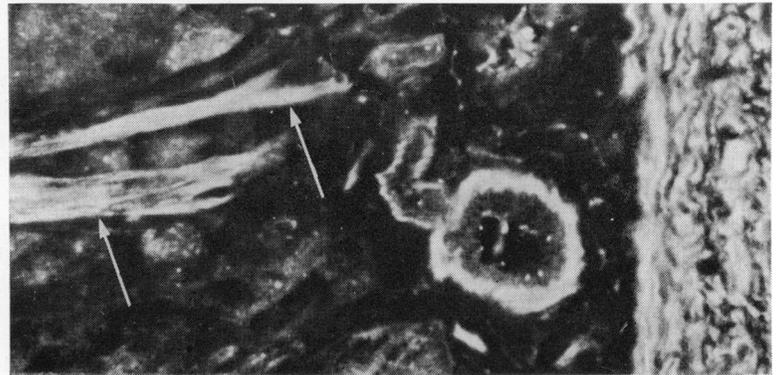


Fig. 3 Cryostat section of a rat liver. Polygonal staining of the cell periphery is due to the presence of actin-like proteins. The section was consecutively treated with smooth muscle antibody and fluorescein-labelled immunoglobulin. The nuclear fluorescence is due to the presence of antinuclear antibody in the patient's serum ($\times 960$)

technique for the immunological identification of actin and other contractile proteins in non-muscle tissues, and we report not only our observations on retinal endothelium but also our incidental findings in other ocular tissues.

Materials and methods

Blood from patients with chronic uveitis or intra-ocular melanoma which was found to contain high titres (that is, $> 1:160$) of smooth muscle antibodies

(SMA) (Rahi *et al.*, 1976), particularly against actin as judged by the standard criteria (Trenchev and Holborow, 1976), was used as the main source of antiserum for the localisation of actin-like proteins. However, the reactivity and the specificity of this serum were standardised against pure antiactin serum raised in rabbits (Holborow *et al.*, 1975; Trenchev and Holborow, 1976). Myosin was first extracted from smooth muscle homogenate of human uterus with various concentrations of KCl (pH 6.8). The remaining tissue was swollen in

Fig. 4 (a) *Indirect immunofluorescence test on frozen section of a rat kidney. The patient's serum reacts with 2 glomeruli and an artery to produce bright fluorescence of the contractile elements ($\times 640$)*

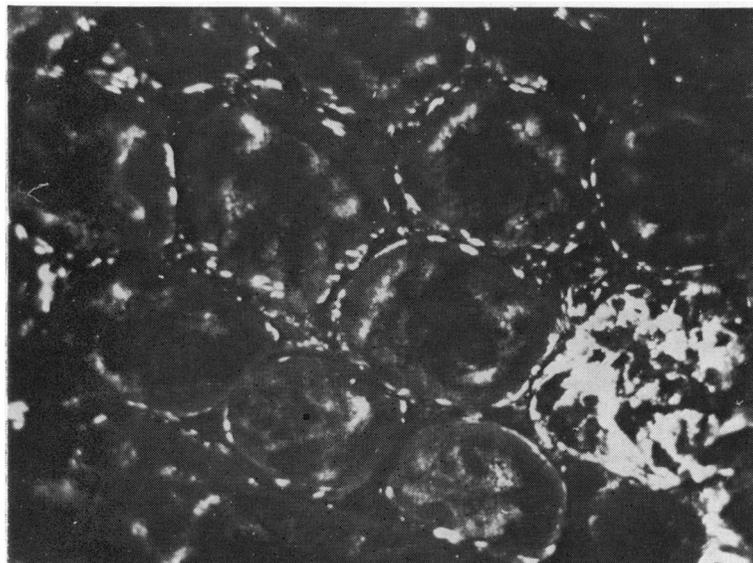
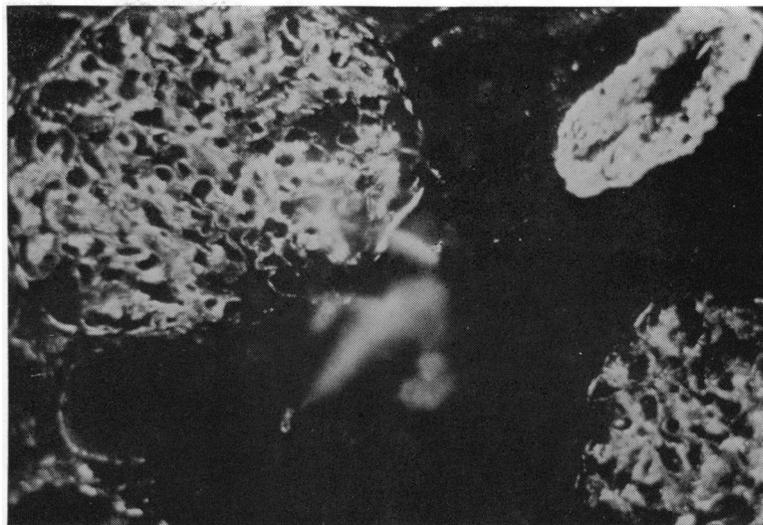


Fig. 4 (b) *Cryostat sections of a rat kidney treated in the same way with smooth muscle antibody. The peritubular fluorescence is probably due to the presence of actin-like proteins in basal processes of the tubular epithelium and muscle cells in the inter-tubular zone ($\times 320$)*

0.01 M NaHCO_3 , dried in acetone, and protein was extracted in deionised distilled water containing 2 mM-tris-HCl, 0.2 mM ATP, 0.5 mM mercaptoethanol, and 0.2 mM CaCl_2 (pH 8.2). The crude actin thus obtained was purified on Sephadex G200. Antiserum against actin was raised in rabbits by giving each rabbit a single injection of 1 mg protein emulsified with Freund's complete adjuvant. The rabbits were bled 3 weeks later and serum stored at -20°C in small aliquots.

STANDARDISATION PROCEDURE

Frozen sections of a composite block consisting of

portions of liver, kidney, skeletal muscle, stomach, and salivary gland of a rat were used to demonstrate smooth muscle antibodies (especially against actin) in patients' sera and in sera obtained from immunised rabbits.

RETINAL ENDOTHELIUM AND OTHER OCULAR STRUCTURES

For this study the following preparations were employed: (1) Frozen sections of rat eyes; (2) vascular complexes from the retinae of (a) 9-day-old rabbits, (b) adult rabbits; (3) tissue cultures of retinal vascular complexes from (a) 9-day-old

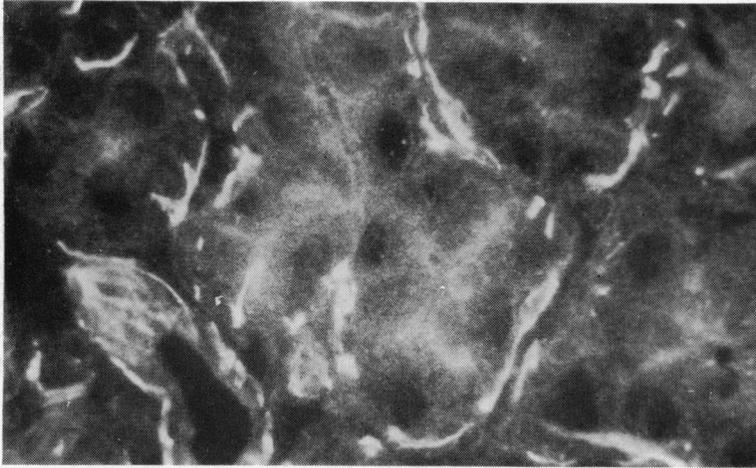


Fig. 5 Myoepithelial cells in a rat parotid showing bright periglandular fluorescence in the presence of smooth muscle antibody. Indirect immunofluorescence test ($\times 640$)

Fig. 6 Cryostat section of a rat eye showing bright fluorescence of the iris and the ciliary body. The tissue was consecutively treated with antiactin serum and fluorescein-labelled anti-immunoglobulin ($\times 320$)

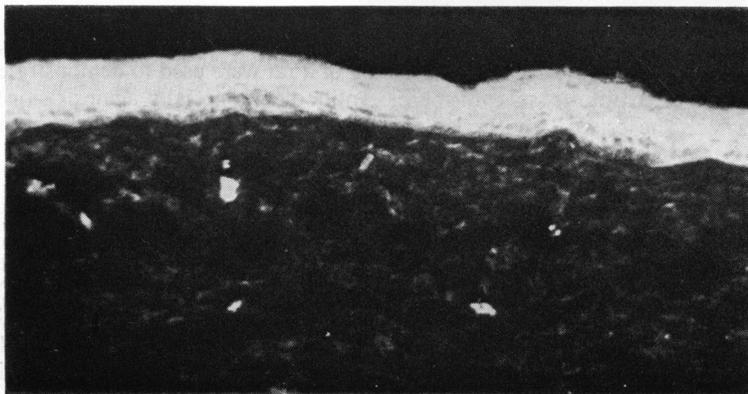
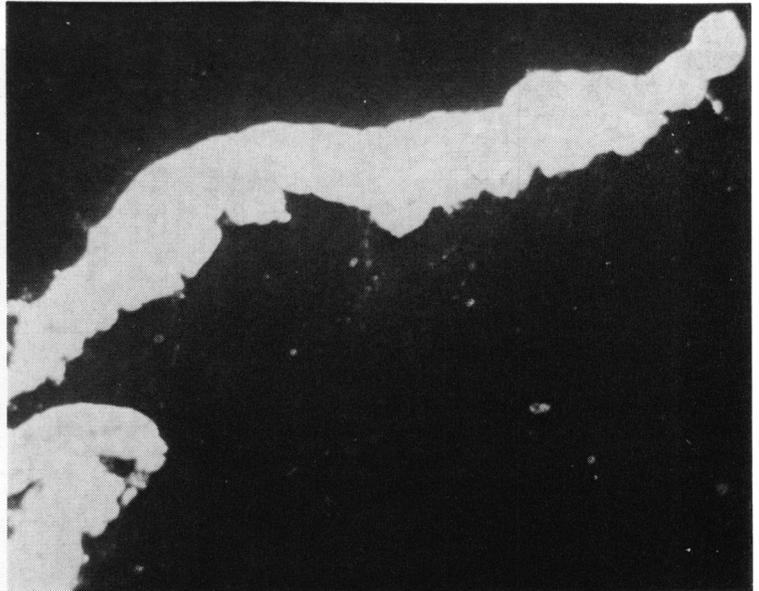


Fig. 7 Indirect immunofluorescence test on rat eye. The antiactin serum reacts with the cytoplasm of the corneal epithelium to produce bright fluorescence of the contractile elements. Unfixed cryostat section ($\times 320$)

Fig. 8 Cryostat section of a rat lens showing bright cytoplasmic fluorescence of the epithelium in the presence of anti-actin serum ($\times 320$)

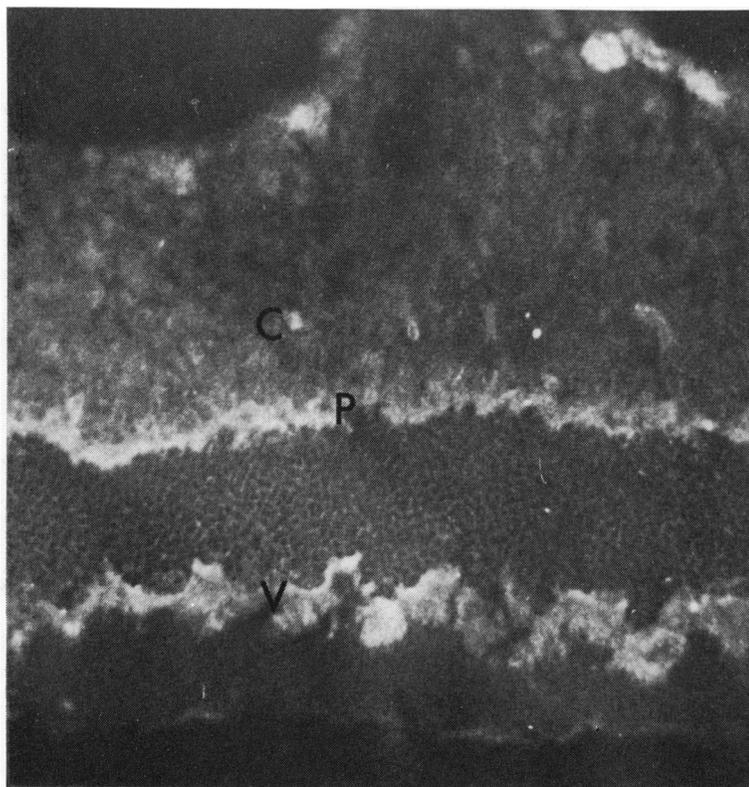
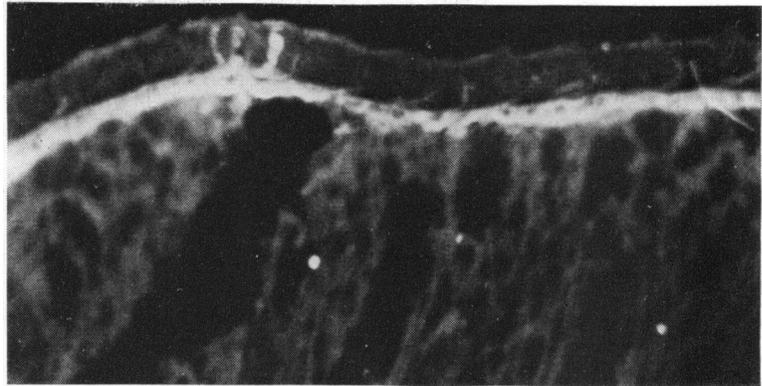


Fig. 9 Cryostat section of rat retina showing bright fluorescence of retinal capillaries (C), the outer plexiform layer (P), and the inner segments of visual cells (V). The tissue was consecutively treated with smooth muscle antibody and fluorescein-labelled anti-immunoglobulin ($\times 320$)

rabbits, (b) adult rabbits (Ashton 1961; Tripathi *et al.*, 1973).

IMMUNOFLUORESCENT TECHNIQUE

A standard method was followed using fluorescein-labelled antihuman and antirabbit immunoglobulins (Wellcome Laboratories). A Zeiss epifluorescence microscope equipped with fluorescein isothiocyanate (FITC) interference filter was used to examine the above specimens. Frozen sections of the composite block and rat eyes were air dried. The tissue-culture

preparations were washed in isotonic saline and fixed in acetone.

ELECTRON MICROSCOPY

Cultures of rabbit retinal vascular complex, human lens epithelium, and rabbit corneal endothelium were grown on Melinex discs. Fixation was carried out by floating the Melinex discs, specimen side down, on 3% glutaraldehyde in cacodylate buffer pH 7.4 for 2 hours at room temperature in covered Petri dishes. They were then washed in sucrose



Fig. 10 *Indirect immunofluorescence test. The pigment epithelium of the retina (arrow) and the photoreceptor elements in the cryostat section of a rat eye show bright fluorescence in the presence of smooth muscle antibody ($\times 960$)*

Fig. 11 *Nerve fibres in cryostat sections of an extraocular muscle showing bright fluorescence. The contractile elements in these fibres have reacted with smooth muscle antibody to produce a bright fluorescence after treatment with fluorescein-labelled antiglobulin ($\times 960$)*

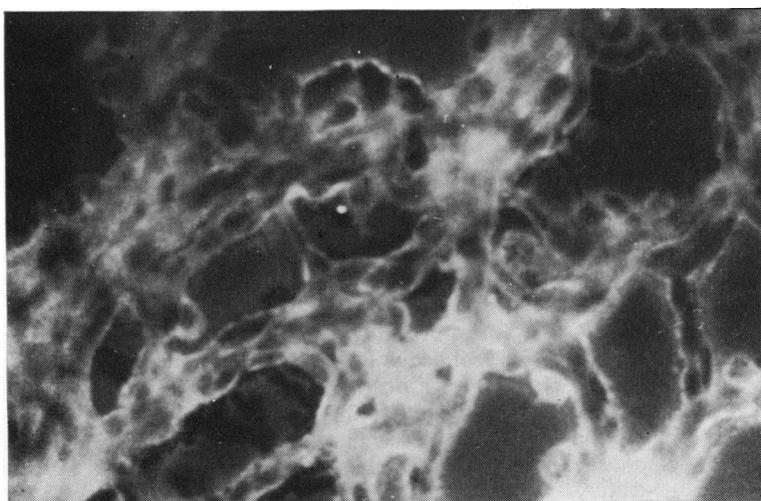
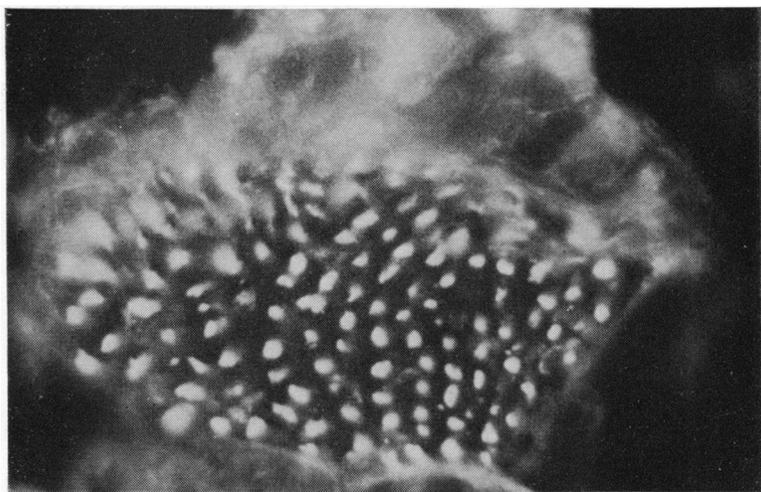


Fig. 12 (a) *Retinal vascular complex of 9-day-old rabbit stained directly after acetone fixation for the presence of smooth muscle protein. Note the mild to moderate fluorescence of the differentiating endothelial cells ($\times 213$)*

Fig. 12 (b) *Retinal vascular complex from an adult rabbit treated in the same way. The capillaries show only mild fluorescence. The large vessels which contain some smooth muscles are strongly fluorescent ($\times 213$)*

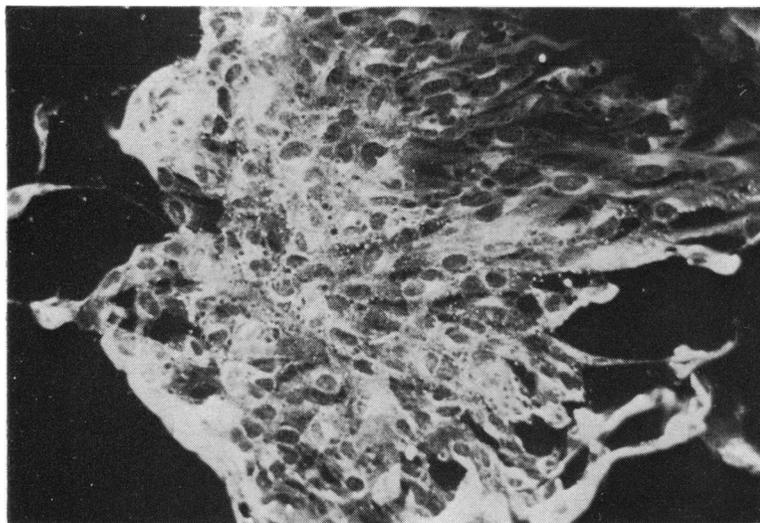
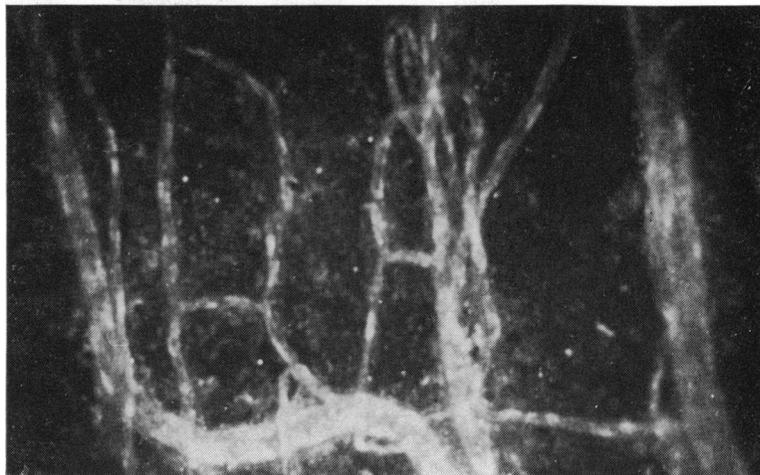


Fig. 13 *Acetone-fixed 7-day-old culture of retinal vascular complex from a 9-day-old rabbit. The endothelial cells show bright fluorescence in the presence of smooth muscle antibody ($\times 213$)*

buffer, with 2 changes (15 minutes each), followed by post-fixation in 1% isotonic OsO_4 in veronal acetate buffer for 1 hour. The cultures were washed in sucrose buffer for 15 minutes and dehydrated in ascending grades of ethanol. Excess Melinex was trimmed from the discs, and the resulting rectangles were transferred to sealed bottles for infiltration with Araldite epoxy resin. The specimens were finally embedded flat. When polymerised the Melinex was peeled off, exposing a monolayer of cells. Specimens of retinal vascular complex from adult and 9-day-old rabbits were also directly fixed in isotonic glutaraldehyde and similarly dehydrated and embedded in Araldite.

Sections were cut on a Huxley ultramicrotome using a glass knife. Semithin sections (0.5 to 1.0 μm) for light microscopy were stained with 1%

toluidine blue. Thin sections (50 to 80 nm) were stained with alcoholic uranyl acetate and aqueous lead citrate for electron microscopy. Sections were examined with a JEOL 100C electron microscope.

Findings

IMMUNOFLUORESCENT STUDIES

Standardisation

The skeletal muscles showed specific fluorescence of actin containing 'I' bands (Fig. 1). In the sections of the stomach specific bright fluorescence of the muscularis mucosa, the vessel walls, and the smooth muscle fibres between the gastric glands was observed (Fig. 2). The hepatocytes showed a polygonal staining pattern in which the contractile elements in the cell periphery showed bright fluorescence



Fig. 14 *Acetone-fixed 7-day-old culture of retinal vascular complex from an adult rabbit. The elongated endothelial cells show only weak fluorescence. Round cells in the photograph are macrophages ($\times 320$)*

Fig. 15 *Acetone-fixed 14-day-old tissue culture preparation of retinal endothelial cells from a 9-day-old rabbit showing bright fluorescence of actin filaments, which were seen to converge on the plasma membrane of these cells. The preparations were consecutively stained with anti-actin serum and fluorescein-labelled anti-immunoglobulin ($\times 960$)*



(Fig. 3). In kidney sections characteristic fluorescence of the glomeruli and the peritubular tissues was observed (Figs. 4a, b). Sections of salivary gland showed strong fluorescence of myoepithelial cells (Fig. 5). All these features showed that the serum used in the present study was rich in antibodies to actin.

Contractile proteins in ocular tissues

As expected, the iris and the ciliary body, which are known to contain a large number of smooth muscle fibres, showed strong fluorescence when treated with either the patient's serum containing smooth muscle antibody or specific antiactin serum (Fig. 6). It was of interest, however, to note a similar

Fig. 16 *Acetone-fixed 14-day-old tissue culture preparation of retinal endothelial cells from a 9-day-old rabbit showing bright fluorescence of subcortical sheath of actin filaments (also called stress fibres) which run along the length of these cells. Indirect immunofluorescent test using antiactin serum ($\times 960$)*

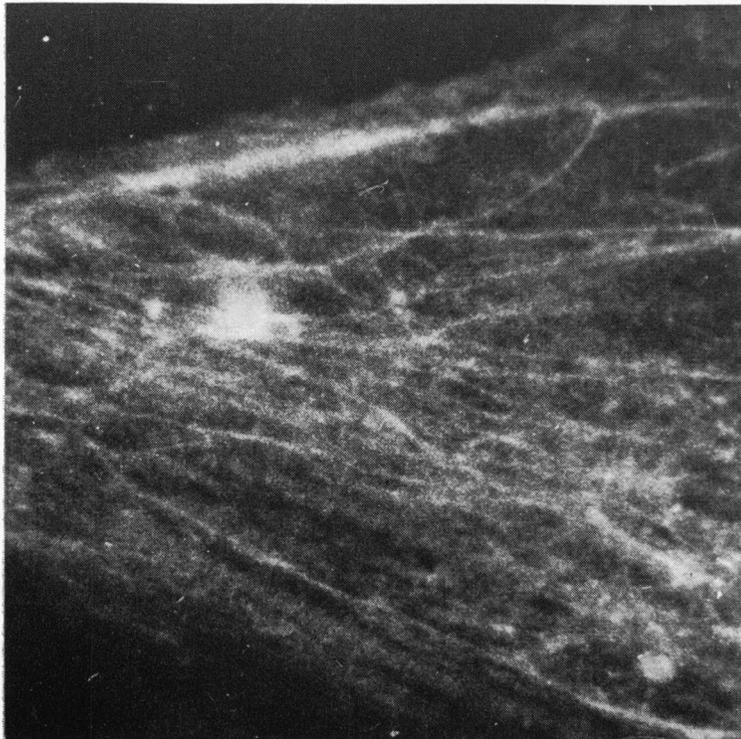
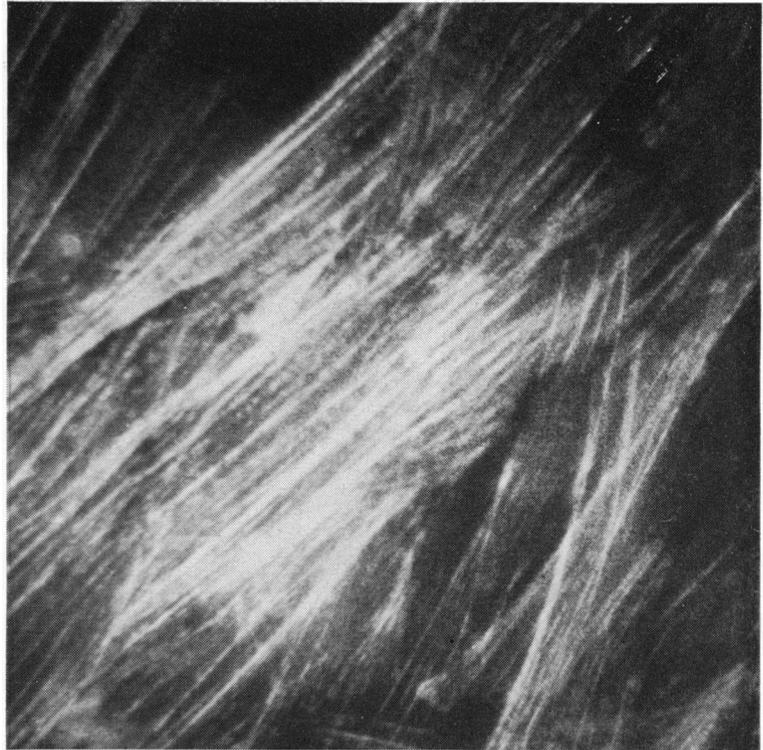
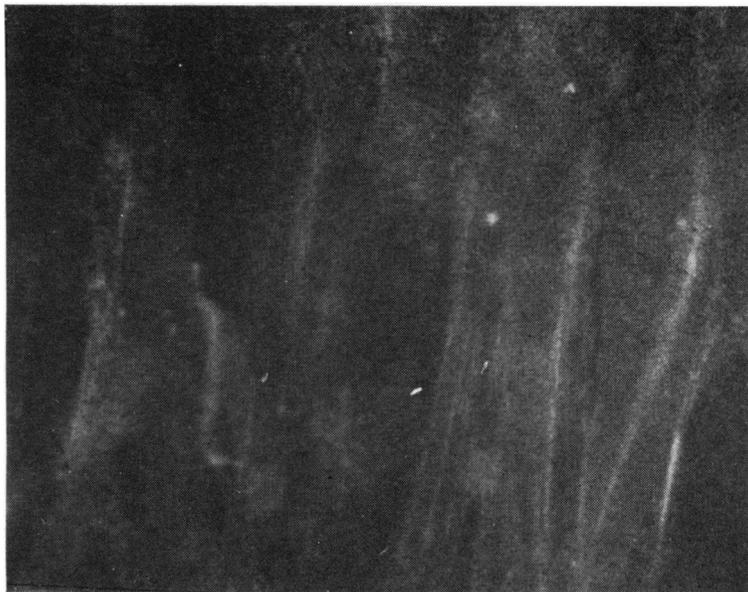


Fig. 17 *Acetone-fixed 14-day-old tissue culture preparation of retinal endothelial cell treated with patient's serum containing smooth muscle antibody. Note the loose fibrillar network in the cytoplasm of a cell indicating the presence of intermediate, 100Å (10-nm) filaments ($\times 2400$)*

Fig. 18 Acetone-fixed 14-day-old tissue culture preparation of retinal endothelial cells from an adult rabbit showing actin filaments in an immunofluorescent study. The filaments are not so numerous as in the endothelial cells from young rabbits shown in Figs. 15 and 16 ($\times 960$)



bright fluorescence in the non-muscular tissues of the eye such as the corneal endothelium and epithelium (Fig. 7), the lens epithelium (Fig. 8), the outer plexiform layer of the retina and the inner segments of the photoreceptor cells (Fig. 9), and the pigment epithelium of the retina (Fig. 10), these findings suggesting a function-related preferential accumulation of contractile proteins in these intra-ocular structures. The nerve fibres supplying the extraocular muscles also gave a positive reaction with smooth muscle antibody (Fig. 11), probably due to the presence of contractile organelles in and around the nerve fibres related to axonal transport and the activity of the Schwann cells.

Retinal vascular complex

The retinal vascular complexes obtained from 9-day-old and adult rabbits and transferred directly on glass slides for immunofluorescent studies after acetone fixation showed only mild to moderate fluorescence of the endothelial cells (Figs. 12a, b).

Tissue culture studies

Acetone-fixed, 7-day-old culture preparations of retinal vascular complexes from 9-day-old rabbits showed strong fluorescence when treated with smooth muscle antibody (Fig. 13). Cultures of similar age from adult rabbits, however, showed only a weak fluorescence of the elongated endothelial cells (Fig. 14). This may be partly due to the fact that the endothelial cells from young animals, at least in the initial stages of tissue culture, appear larger in size.

Well established 14-day-old retinal endothelial

cell cultures from young animals showed 3 types of filamentous cytoplasmic fluorescence. With anti-actin serum either the filaments appeared to be converging to a point on the plasma membrane of the endothelial cells (Fig. 15) or the fibres formed thick bundles running across the length of the cells, which have been called 'actin cables' or 'stress fibres' (Fig. 16) in various other cell lines (Pollack *et al.*, 1975; Pollack and Rifkin, 1975; Wessels *et al.*, 1971).

Culture cells stained with patients' sera which in addition to antiactin antibody contained antibodies to other contractile proteins revealed a third type of arrangement, in which the cytoplasm showed a loose fibrillar network (Fig. 17), believed to consist of intermediate, 100-Å (10-nm), filaments. These are known by a variety of names in different tissues—for example, tonofilaments, neurofilaments, endothelial filaments—and contain a different contractile protein (Kurki *et al.*, 1977).

Tissue culture preparations which were several weeks old, or had stopped growing, or had been stored for a few days in the refrigerator before staining reacted differently with antiactin sera, showing instead of a filamentous staining only diffuse cytoplasmic fluorescence. This apparent loss of filamentous material was probably due to redistribution of actin molecules into a soluble non-aggregated form, as has been reported in virus-transformed cell lines (Pollack *et al.*, 1975). It is of interest that macrophages, which are normally present among the growing retinal endothelial cells, also showed a similar diffuse cytoplasmic

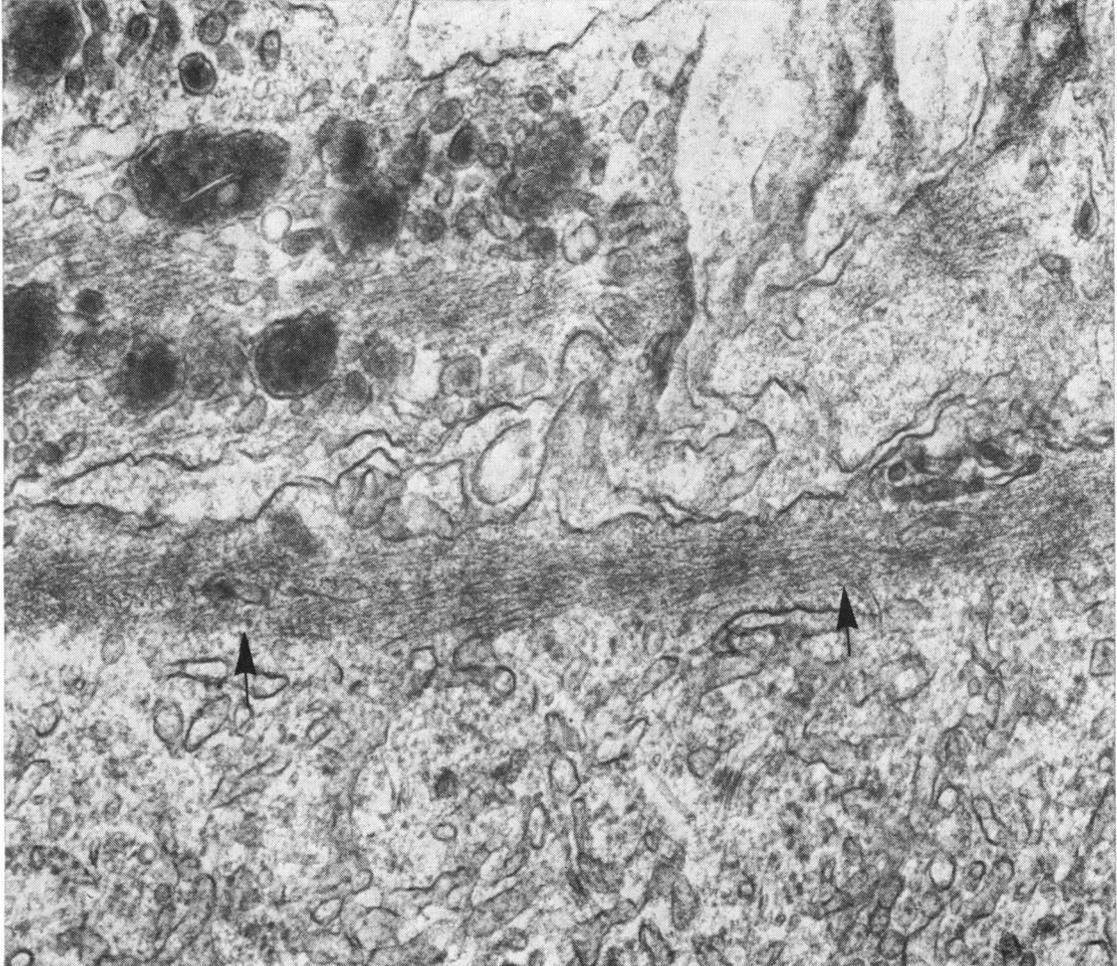


Fig. 19 Pigment epithelial cell of rat. Numerous bundles of microfilaments, 50 to 75Å (5 to 7.5 nm) in diameter, are present in the cytoplasm (arrows). Electron microscopy, $\times 52\,000$

fluorescence when treated with antiactin serum. These cells were, however, retracted and free floating.

Acetone-fixed 14-day-old tissue culture preparations of retinal endothelium from adult rabbits showed the presence of actin filaments, but their number was greatly reduced (Fig. 18). Whether this is due to a fundamental difference in the organisation of the cytoskeleton of the vascular endothelium in these 2 age groups or is simply a reflection of their *in vitro* growth potentials is uncertain, and a more detailed study is required for clarification.

ELECTRON MICROSCOPICAL STUDIES

Microfilaments in ocular tissues

Electron microscopical examination of rat retinal pigment epithelium (Fig. 19), human lens epithelium

in tissue culture (Fig. 20), and rabbit corneal endothelium grown on Descemet's membrane showed the cytoplasmic network of microfilaments (Fig. 21) which provide the structural basis for the cytoplasmic fluorescence seen in the direct immunofluorescent study.

Retinal vascular complex in tissue culture

Electron microscopical examination of the tissue culture preparations of retinal vascular complexes from 9-day-old rabbits showed the presence of a large number of actin filaments, which appeared either as the cortical network (Fig. 22a) or as subcortical sheaths, the so-called stress fibres (Fig. 22b). Electron microscopical examination of the endothelial cells in the small blood vessels of

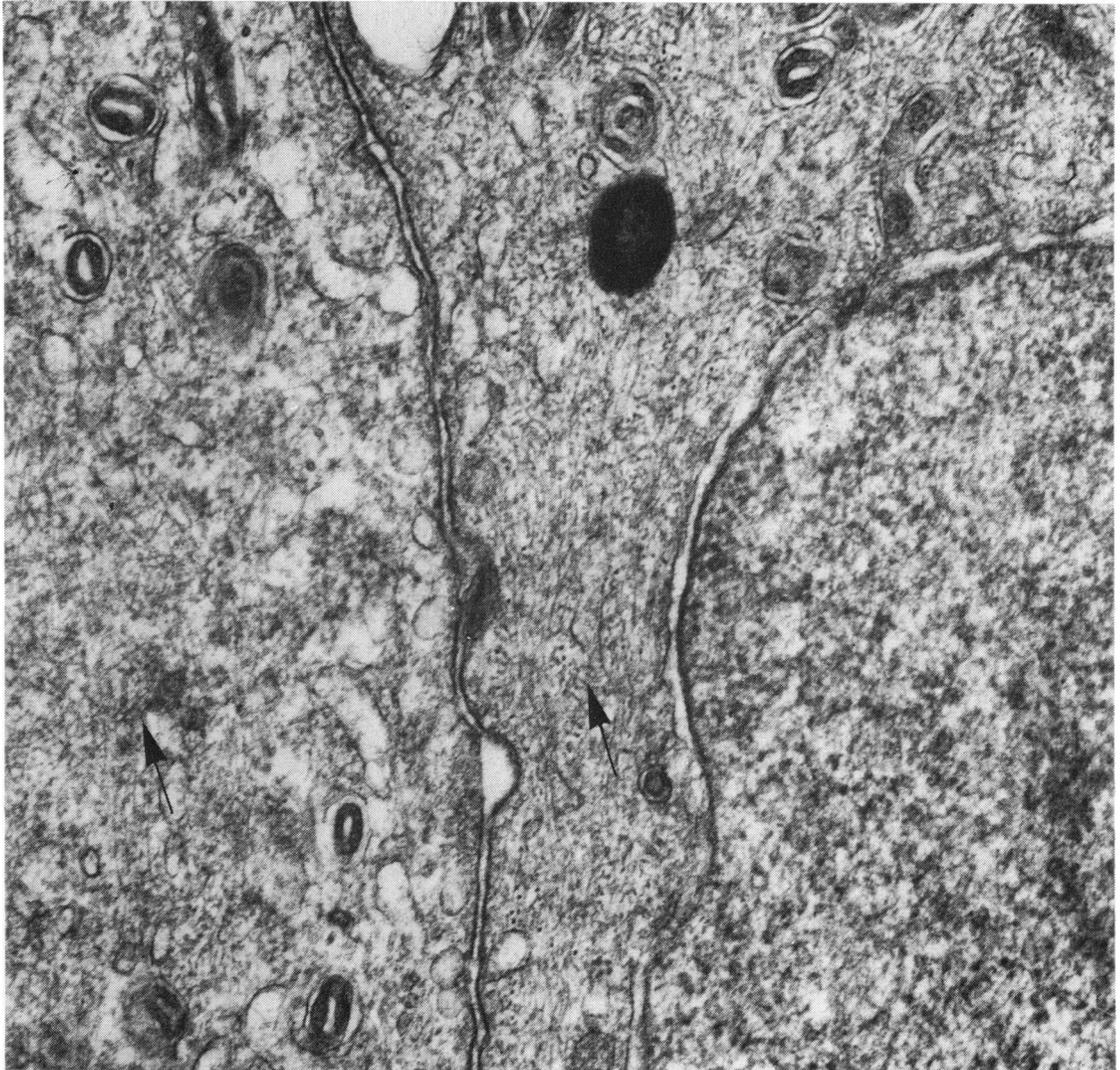


Fig. 20 Human lens epithelium in tissue culture. Numerous microfilaments, 50 to 75 Å (5 to 7.5 nm) in diameter, are present within the cytoplasm (arrows). Electron microscopy, $\times 52\,000$

the retina directly removed from the enucleated eyes of 9-day-old and adult rabbits, however, did not show such a large number of microfilaments (Fig. 23), which suggests that the microfilaments appear in large numbers only at a certain stage of physiological activity such as growth, multiplication, and cytokinesis.

Discussion

The recent interest in actin and other contractile proteins stems from the findings that many

mammalian cells contain these proteins in easily identifiable forms (Pollard and Weihing, 1974; Trenchev *et al.*, 1974). The content varies, however, in different types of normal, transformed, or neoplastic cells, and the morphological appearance of these intracellular proteins, particularly actin, depends on the physiological state of the cells and on pathological change (Pollack *et al.*, 1975; Gabbiani *et al.*, 1975).

The chief contractile elements of skeletal muscle are made up of a series of myosin and actin molecules. The myosin molecule is composed of 2 anti-

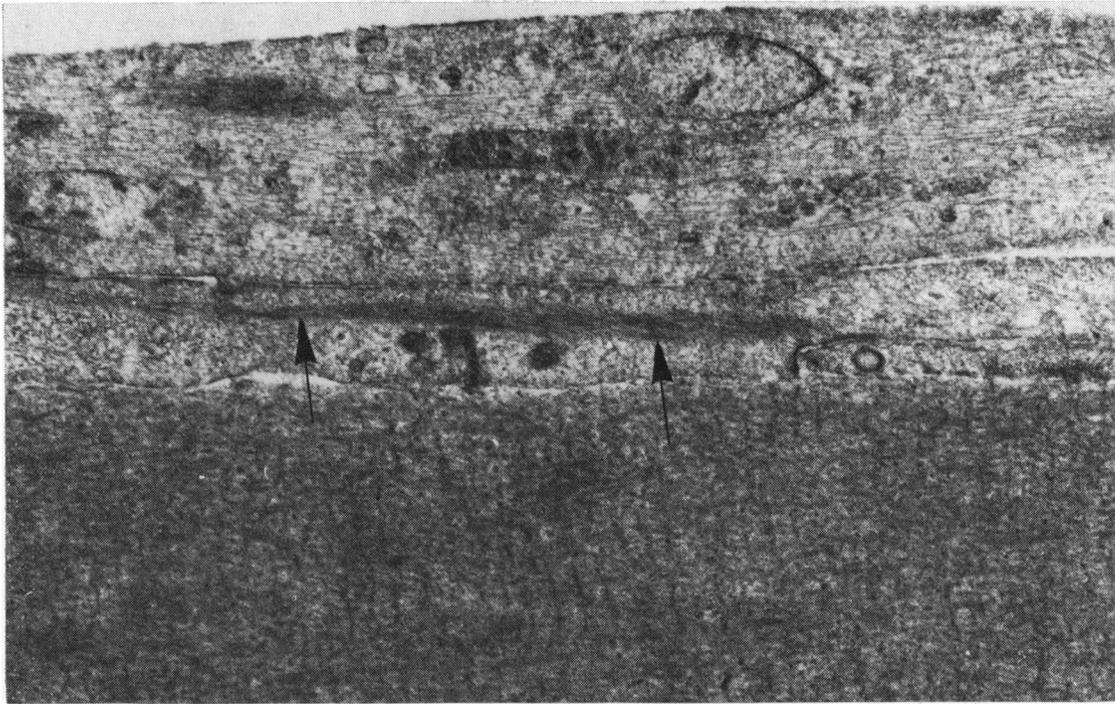


Fig. 21 Rabbit corneal endothelial cells in tissue culture on Descemet's membrane. Numerous microfilaments lie in parallel in the longitudinal axis of the cell (arrows). Electron microscopy, $\times 10\,000$

genically distinct components—light meromyosin and heavy meromyosin. The actin filaments on the other hand consist of double stranded F-actin helix, each containing several molecules of G-actin. The actin filaments also contain 2 additional antigenic proteins—tropomyosin and troponin.

In skeletal muscle cells the myosin and actin filaments interdigitate and show a banded arrangement (A and I bands), whereas in smooth muscle cells the actin and myosin filaments have no such spatial arrangement and lie at odd angles to each other, being mostly orientated in the longitudinal direction of the smooth muscle cell. Electron micrographs show typical actin filaments (50 to 75 \AA (5 to 7.5 nm) in diameter), some of which are apparently attached by their tips to the smooth muscle cell membrane, thereby providing a method for mechanically coupling the contractile fibres to the cell surface.

Large fibres, 180 to 250 \AA (18 to 25 nm) in diameter, forming microtubules and consisting of the contractile proteins α and β tubulin, are also seen in many cells, particularly in neural tissues, platelets, mast cells, and the mitotic spindle. Intermediate filaments, 100 \AA (10 nm) in diameter, distinct from actin filaments and microtubules are also to

be found in eukaryotic cells—markedly in epidermal and glial cells. They are regarded as cytoskeletal structures, and the protein of the filament has been named desmin or skeletin. Other proteins, such as filamin, spectrin, actinin, C-protein, and J-protein, have also been identified with various cellular structures.

Several activities of non-muscle cells depend on these specialised intracellular structures (i.e., actin microfilaments, microtubules, and intermediate filaments), and they are believed to be concerned with cell shape, mobility, and mitosis, with the tight junctions between cells and their adherence to glass surfaces in tissue culture, etc. (Lazarides, 1975; Feeney and Mixon, 1976).

OCULAR TISSUES IN GENERAL

The presence of the 3 major cytoplasmic filamentous structures in various tissues of the eye, such as the corneal epithelium, the lens epithelium, the various layers of the sensory retina, and the endothelium lining Schlemm's canal, has been known for some time (Kuwabara, 1965; 1968; Hogan *et al.*, 1971; Piatigorsky *et al.*, 1972; Rafferty and Esson, 1974; Grierson and Lee, 1975).

Actin-containing microfilaments have been

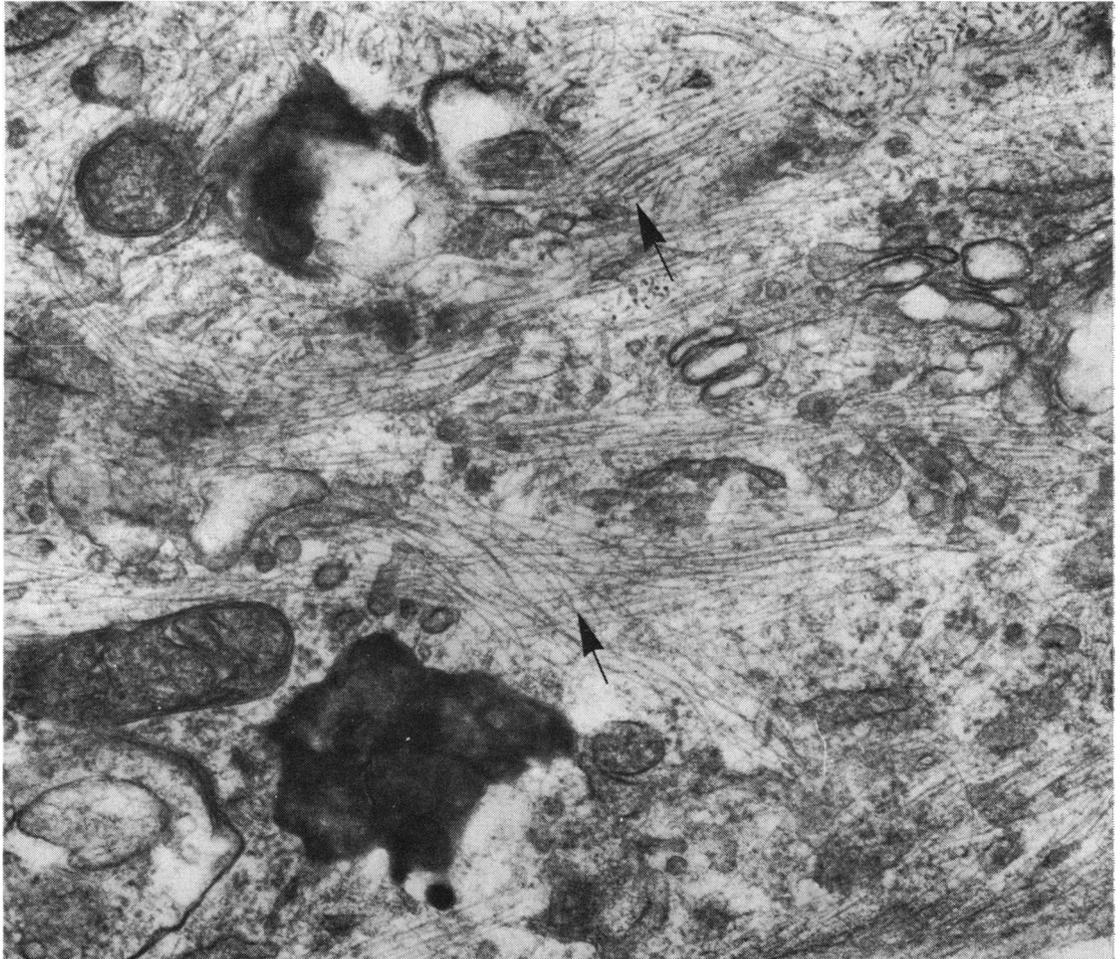


Fig. 22 (a) A 7-day-old culture of retinal endothelium from a 9-day-old rabbit. Numerous microfilaments, 50 to 75 Å (5 to 7.5 nm) in diameter, lie at odd angles to each other, but in the general longitudinal axis of the cell (arrows). Electron microscopy, $\times 52\,000$

demonstrated in the retinal pigment epithelium and the corneal epithelium by the 'arrow-heading' technique (Gipson and Anderson, 1977), but there is only 1 ophthalmological study using immunofluorescent methods (Lonchampt *et al.*, 1976). These workers demonstrated microfilaments in rabbit lens epithelium using antiactin serum, and also microtubules reacting strongly to tubulin antibody: the filaments were also visualised by conventional electron microscopy.

Intermediate filaments in the form of tonofilaments, neurofilaments, glial filaments, and endothelial filaments are as familiar in the eye as elsewhere in the body (Hogan *et al.*, 1971), and both microtubules and actin filaments have been described in the retinal pigment epithelium of man and monkey

(Burnside and Laties, 1976) and of the rat (Burnside, 1976).

The present study, however, is the first investigation to demonstrate, by employing the combined approach of immunological and electron microscopical methods, the presence of actin-like proteins in ocular tissues and to show their preferential disposition in the mammalian retina (that is, pigment epithelium, inner segments of visual cells, and the plexiform layers), the corneal epithelium and endothelium, the iris, and the ciliary body, and to confirm the previous report on the lens epithelium (Lonchampt *et al.*, 1976).

RETINAL VASCULAR COMPLEXES (RABBIT)

This is also the first report to demonstrate by these

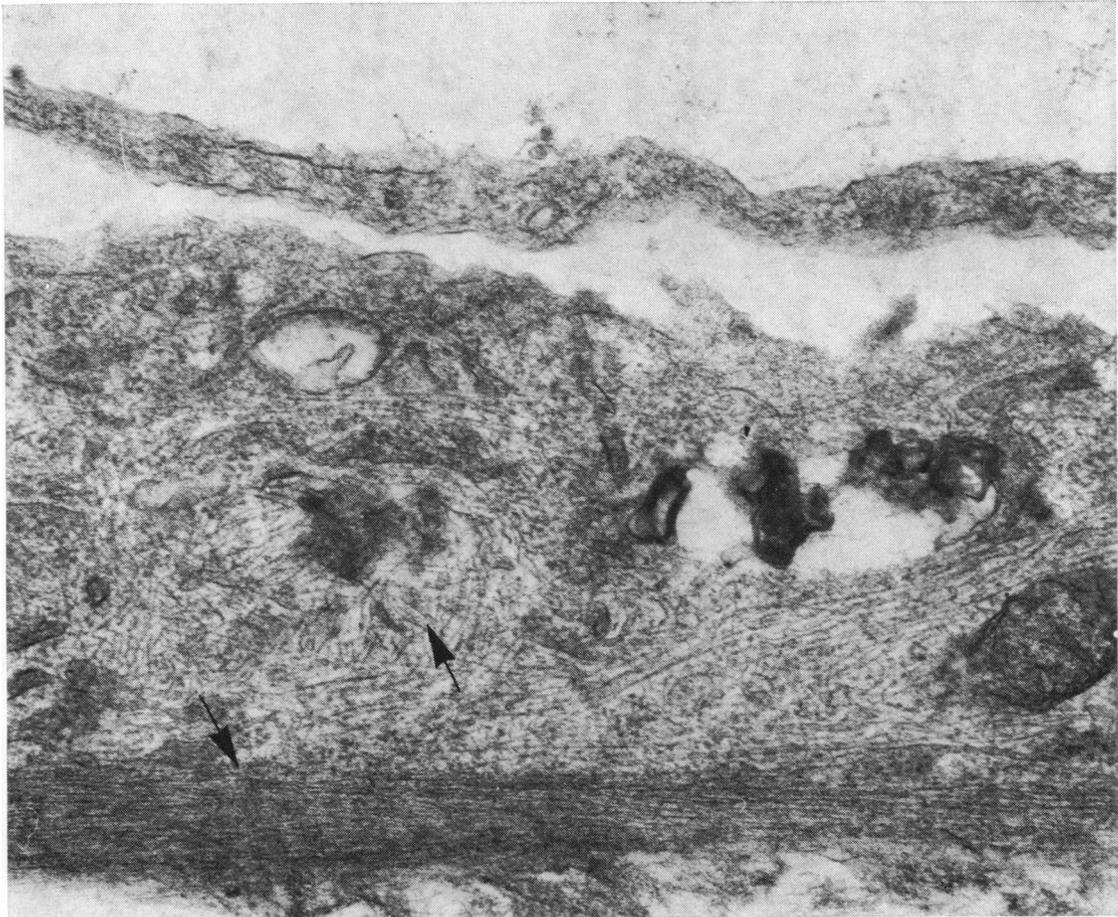


Fig. 22 (b) Another cell from the same culture showing a subcortical 'actin cable' or 'stress fibre' (arrow) and other disorientated microfilaments (arrow). Electron microscopy, $\times 52\,000$

methods the presence of microfilaments and intermediate filaments in the retinal vascular endothelium, although filaments have been previously described in pericytes (Ashton and Tripathi, 1977). Our findings that actin filaments are especially abundant in immature retinal endothelial cells raises the question of their function. Although the role of such filaments in muscles and in phagocytic cells is well documented, their precise role in vascular endothelium is unknown, but their presence at least suggests that these primitive cells are well equipped to contract when appropriately stimulated. That contraction of immature retinal vessels of the rabbit does occur as a result of oxygen exposure has been demonstrated in *in vivo* experiments (Ashton *et al.*, 1972; Tripathi *et al.*, 1974), and it would now seem reasonable to attribute at least the initial closure of the circulation to reactive contraction of these

growing vasoformative cells. Moreover, the fact that we found actin microfilaments most evident in the more primitive cells would correspond to the greater reactivity to hyperoxia to be found in the ingrowing cells of the peripheral retinal complex as compared with the more mature vessels differentiating posteriorly. Such a reactive contraction to oxygen in the immature retina was suggested many years ago (Ashton *et al.*, 1954) when a few pilot experiments to test the possible effect of antagonism by vasodilators (for example, Prisol, CO_2) were carried out, but with negative results. It might now be rewarding to examine experimentally *in vivo* or *in vitro* the possible influence of substances known to cause disruption of microfilaments—for example, cytochalasin B (Rathke *et al.*, 1975).

To explain the comparative immunity to hyperoxia found in the adult retinal circulation, which has



Fig. 23 Developing retinal vessel from a 9-day-old rabbit. Microfilaments, 50 to 100Å (5 to 10 nm) in diameter, are present within the cytoplasm (arrows) but less prominent than in the corresponding cultured cell (see Fig. 21). Electron microscopy, $\times 52\,000$

fully developed muscular coats in its large vessels, additional factors to simple contractility would need to be invoked.

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