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Localization of smooth muscle-like contractile proteins in kidney by immunoelectron microscopy

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INTRODUCTION

Our recent immunofluorescent study of the tissue reactivity of rabbit antisera raised against smooth muscle actin, myosin and heavy meromyosin (Trenchev, Sneyd & Holborow, 1974) showed that these proteins have different distributions in rat kidney. Of particular interest were the immunofluorescent appearances of the glomeruli, which stained diffusely with anti-actin, diffusely but less intensely with anti-heavy meromyosin, and not at all with anti-myosin. Anti-actin also stained structures at the base of some convoluted tubule cells (Trenchev, unpublished). To determine more precisely the localization of these reaction sites we have used the indirect peroxidase-labelled antibody technique and examined the results by electron microscopy (Webb & Dorling, 1972).

MATERIALS AND METHODS

Smooth muscle actin, myosin, and heavy meromyosin were obtained from fresh and frozen human pregnant and non-pregnant uteri. Uterine tissue was homogenized and extracted three times with 0·6 M-KCl (pH 6·8) followed by one extraction with 0·3 M-KCl and one extraction with 0·1 M-KCl. The residual tissue was swollen in 0·01 M-$NaHCO_3$, with several changes. The swollen material was then dehydrated in 4 volumes of acetone with five changes at 10 minute intervals, and dried overnight at room temperature.

Actin was extracted from the acetone-dried powder with pH 8·1 buffer containing 2 mM-tris-HCl, 0·2 mM ATP, 0·5 mM mercaptoethanol and 0·2 mM $CaCl_2$. This crude actin was purified by three different methods.

(*a*) Actin was separated by column chromatography on Sephadex G-200 (Adelstein, Godfrey & Kielley, 1963) equilibrated with the above buffer. The actin emerged as the second peak after a smaller first peak, believed to contain mainly tropomyosin–troponin complex and denatured actin.

(*b*) Actin was obtained as a side-product in the procedure for purifying actinin (Goll, Suzuki, Temple & Holmes, 1972).

(*c*) Actin was purified by repeating aggregation and disaggregation. The salt concentration of the crude actin extract was adjusted to 0·1 M-KCl and 0·1 mM-$MgCl_2$. After two days the concentration of KCl was raised to 0·6 M and the mixture

was centrifuged at 164000 *g* for 2 hours. The actin pellet was rinsed, re-suspended in the original buffer and dialysed against a large volume of the same pH 8·1 buffer. This procedure was repeated twice.

The purified actin extracts gave single bands in 7·5% SDS polyacrylamide gel electrophoresis in the region of 44000 daltons. They increased about threefold the ATPase activity of myosin.

Myosin was extracted from homogenized uterine tissue with a solution containing 0·3 M-KCl, 0·1 M-KH_2PO_2 and 0·05 M-K_2HPO_2 and precipitated by 14-fold dilution with distilled water, pH 6·8. The precipitate was dissolved in salt solution containing 0·5 M-KCl, 32·5 mM-K_2HPO_4, 17·5 mM-KH_2PO_4 and 1 mM EDTA, and myosin was then precipitated between 33% and 40% ammonium sulphate saturation. The precipitate was re-dissolved in salt solution containing 0·135 M-K_2HPO_4, 0·0153 M-KH_2PO_4 and 10 mM EDTA and dialysed against the same buffer. Further purification was carried out by gradient chromatography on a column of DEAE-Sephadex A-50 equilibrated with the phosphate buffer. The eluant was the phosphate buffer containing KCl increasing to 0·5 M. The myosin emerged as a trailing peak and was concentrated by precipitation at pH 6·4. The myosin was re-dissolved in phosphate buffer and spun at 150000 *g* for 2 hours.

The purified myosin was digested with trypsin at a 1:200 weight ratio of trypsin to myosin (Hamoir, 1973) at 25 °C for 40 minutes. Heavy meromyosin was recovered by ammonium sulphate precipitation between 47% and 52% saturation. The washed precipitate was dissolved in PBS (pH 7·2), dialysed against PBS and clarified by centrifugation at 100000 *g* for 1 hour.

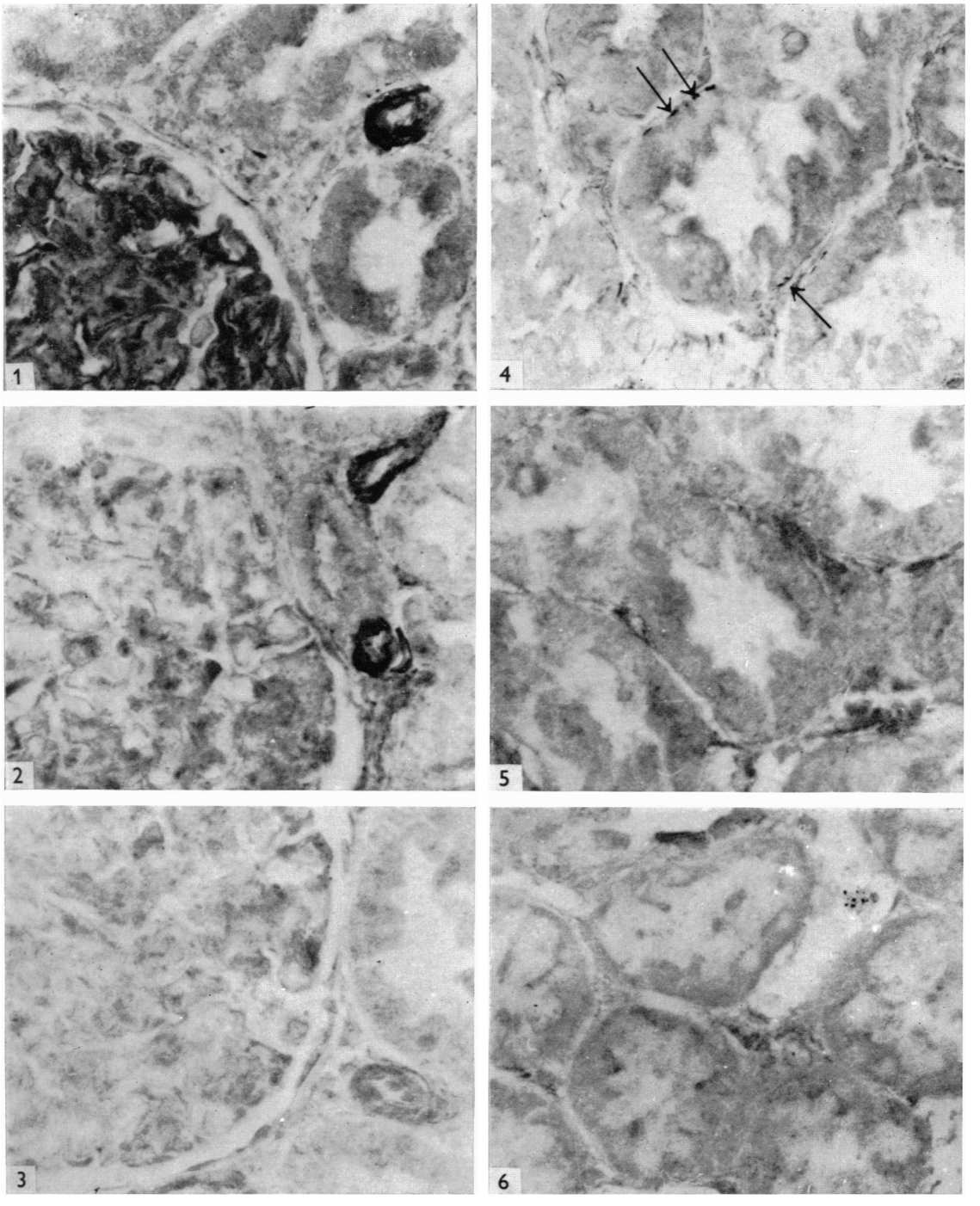
Both myosin and heavy meromyosin initially showed ATPase activity of about 500 ngPi/mg/min at 25 °C but this decreased with time. The ATPase activity was increased 3 to 4 fold by addition of actin.

Antisera against these proteins were raised in rabbits by giving each rabbit a single injection of 1 mg protein, emulsified with incomplete Freund's adjuvant. The rabbits were bled 21 days later and the sera were tested by immunofluorescence, as previously described (Trenchev *et al.* 1974). On double diffusion in agar against myosin, anti-heavy meromyosin and anti-myosin sera showed cross reactivity, but actin did not react with either.

Addition of anti-actin serum to actin preparations inhibited completely their ability to enhance the ATPase activity of myosin. Anti-actin serum alone, in the absence of actin, did not affect the ATPase activity of myosin.

The anti-actin sera precipitated in solution with crude actin. The precipitate was separated by centrifugation and the supernatant was then applied to a Sephadex G-200 column eluted with buffer as before. The second peak previously seen and identified as actin was now missing, but not the first and the third ones. The precipitate was run on SDS electrophoresis and showed a band in the actin position, but none of the other bands which appeared on electrophoresis of crude actin extract.

Immunoelectron microscopy was performed as modified by Webb & Dorling (1972). Pieces of rat kidney with one dimension not greater than 2 mm were fixed for 30 minutes at 0–4 °C in 1% paraformaldehyde in pH 7·4, 0·1 M phosphate buffer. After freezing in isopentane cooled by liquid nitrogen 10 μm cryostat sections were prepared, attached to microscope slides and treated with a 1/10 dilution of antiserum



Figs. 1–6. Photomicrographs of 6 μm cryostat sections of rat kidney treated with rabbit anti-contractile proteins followed by peroxidase conjugated goat anti-rabbit globulin. × 540.

Figs. 1–3. Show vessels and a glomerulus after (1) anti-actin, (2) anti-myosin and (3) anti-heavy meromyosin.

Figs. 4–6. Show convoluted tubules after (4) anti-actin, (5) anti-myosin and (6) anti-heavy meromyosin. Note staining at the base of tubules (arrows).

followed by peroxidase-conjugated (Avrameas, 1969) goat anti-rabbit globulin diluted 1/20. The sections were then fixed in glutaraldehyde, peroxidase activity was localized with diamino-benzidine hydrogen peroxide, and after post-osmication they were embedded in Araldite and sectioned at 600–900 nm for electron microscopy as previously described (Webb & Dorling, 1972; Holborow, Trenchev, Dorling & Webb, 1975). Most preparations were examined without further heavy metal staining but some were stained for 1–2 minutes in lead citrate (Reynolds, 1963) to increase the contrast of membranes. For light microscopy 6 μm sections were treated as above but after demonstration of peroxidase activity they were dehydrated, cleared in xylene and mounted in a resinous mounting medium. Control tests were performed using normal rabbit serum in place of anti-contractile protein serum.

RESULTS

Light microscopy (*Figs.* 1–6)

The anti-actin and anti-myosin sera gave the same patterns of staining with peroxidase conjugate as with fluorescein labelled anti-globulin. The former stained glomeruli as well as the smooth muscle in vessel walls, the latter staining only smooth muscle. In addition, anti-actin serum clearly stained dots at the bases of proximal convoluted tubule cells. The glomeruli were unstained with anti-heavy meromyosin serum by the peroxidase method, although with fluorescence this serum gave weak glomerular staining. This presumably reflects a difference in the sensitivity of the two methods.

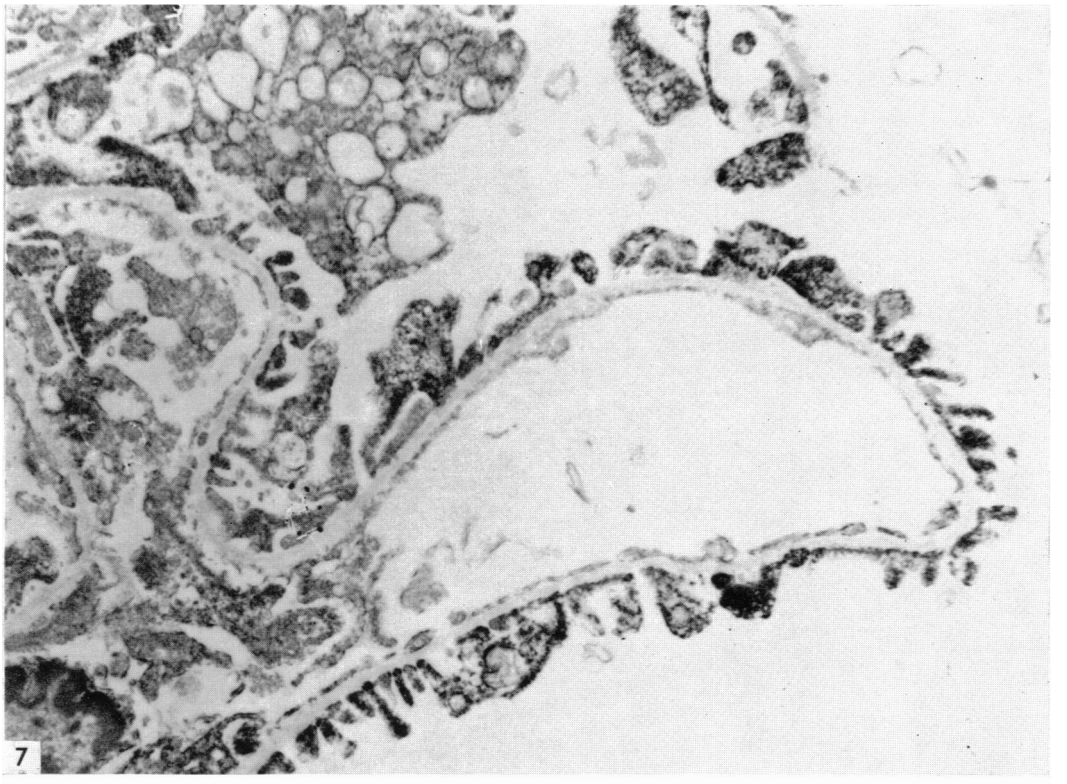
Electron microscopy

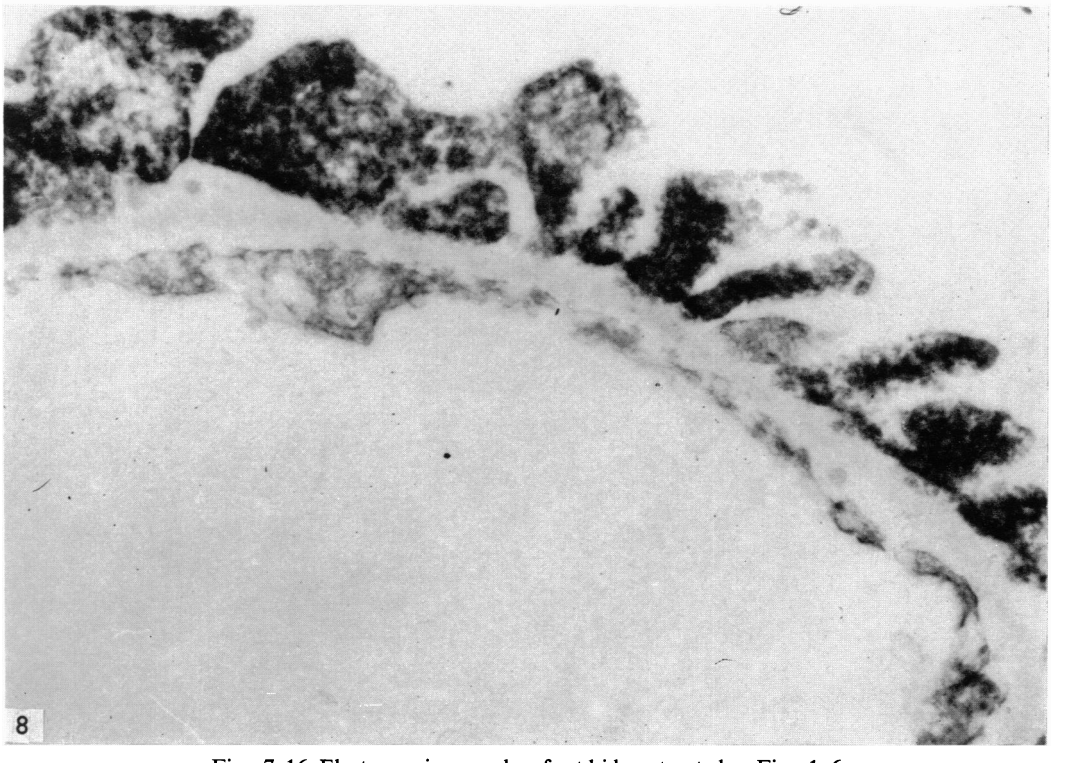
Anti-actin gave marked staining of the foot processes of glomerular epithelial cells which was strongest in the basal part of the processes abutting on the basement membranes. It appeared diffuse and no definite structure was visible (Figs. 7, 8). The foot processes of glomerular epithelial cells were also stained with anti-heavy meromyosin but this was confined to areas on the cytoplasmic membrane at each side of the foot processes near the basement membrane (Figs. 9, 10). The appearance of the glomeruli in kidney sections treated with anti-myosin serum (Fig. 12) did not differ from that in sections treated with the control serum (Fig. 11), although in the same sections the staining of small blood vessels by anti-myosin serum was very strong (Fig. 13).

Discrete areas at the base of proximal convoluted tubule cells were strongly stained by anti-actin (Fig. 14). In preparations counterstained with lead citrate (Fig. 15), this staining was seen to be localized within the epithelial cell adjacent to the tubular basement membrane. Anti-heavy meromyosin and normal rabbit serum gave no staining in this region of the tubule cell. Although most tubules were not stained by anti-myosin, a few showed some moderately electron-dense material at the same sites as the anti-actin-positive material (Fig. 16).

DISCUSSION

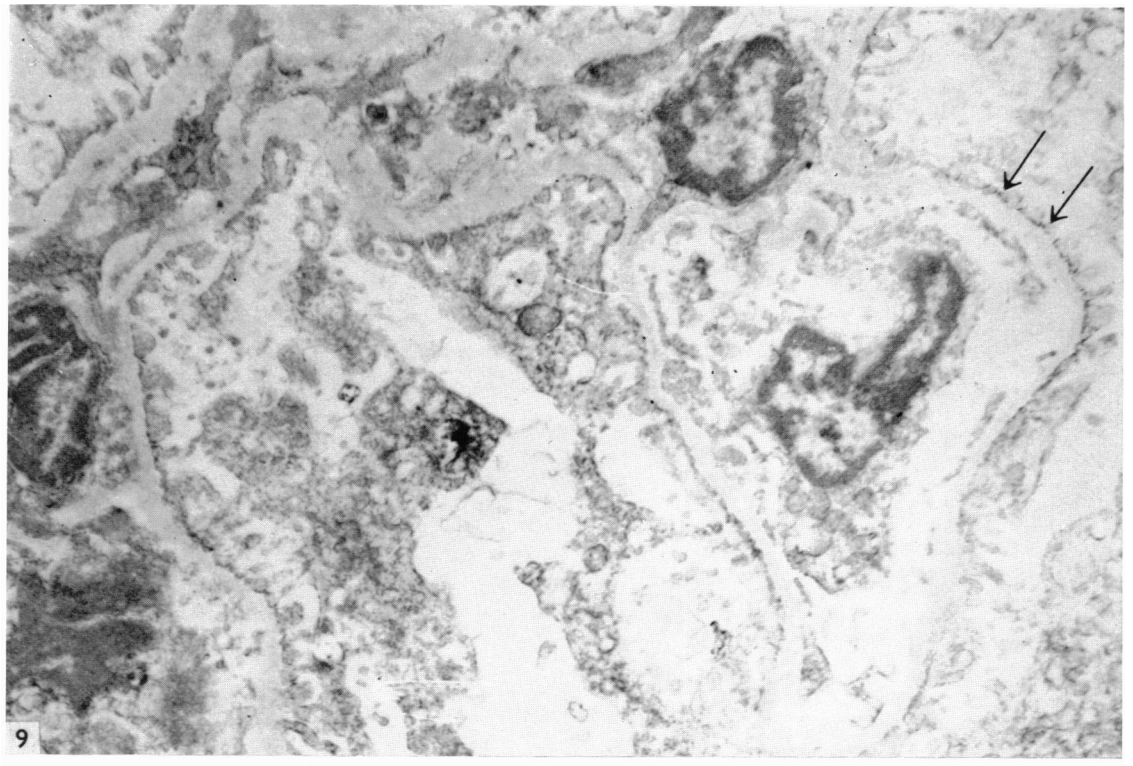
We have shown for the first time the presence of actin and heavy meromyosin within the foot processes of glomerular epithelial cells. We have also confirmed the

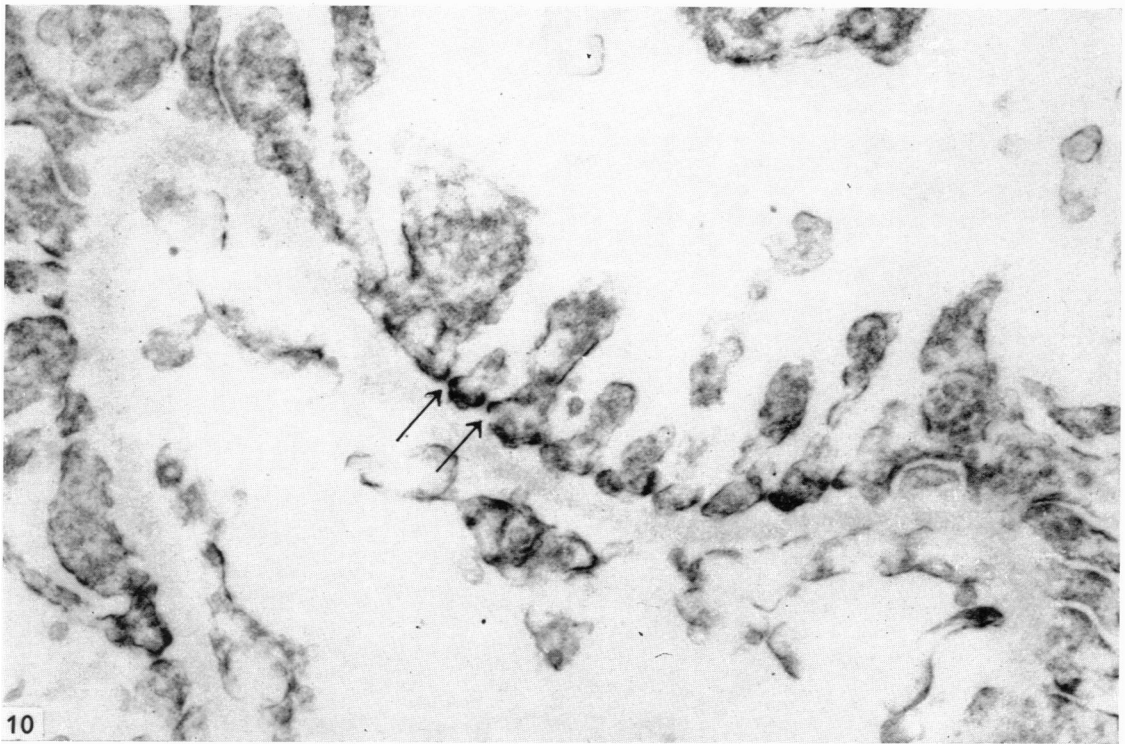




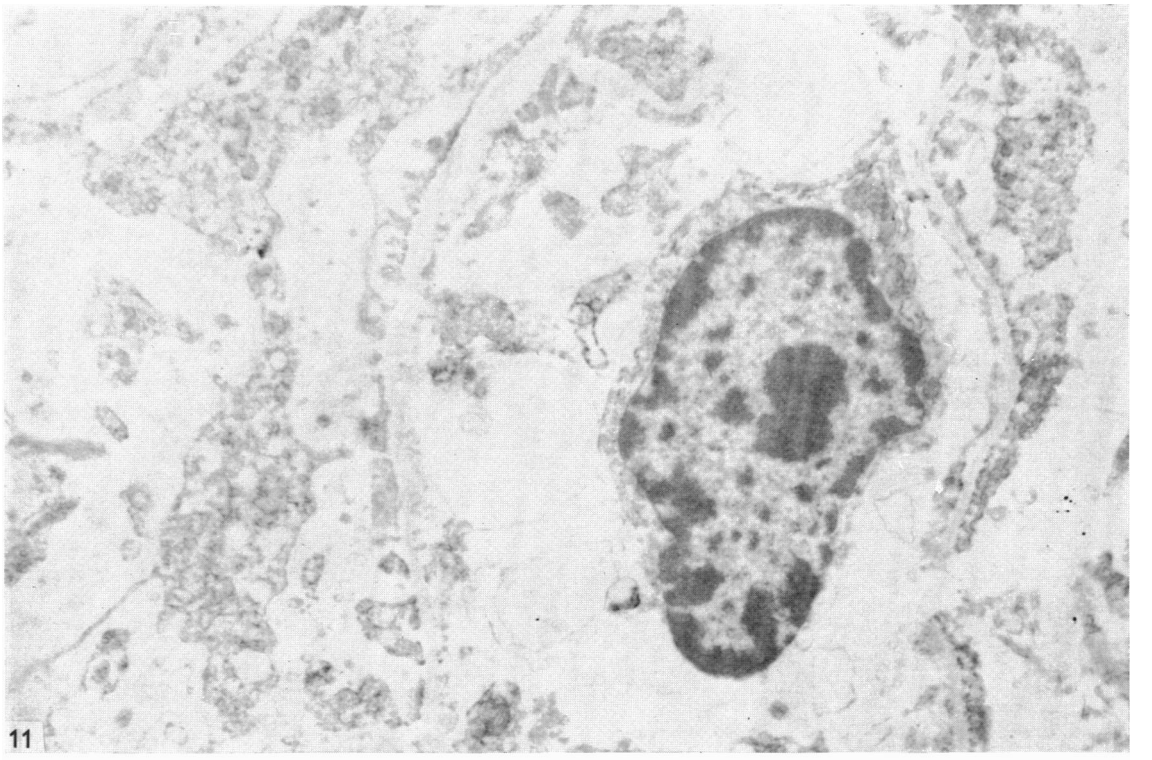
Figs. 7–16. Electron micrographs of rat kidney treated as Figs. 1–6.

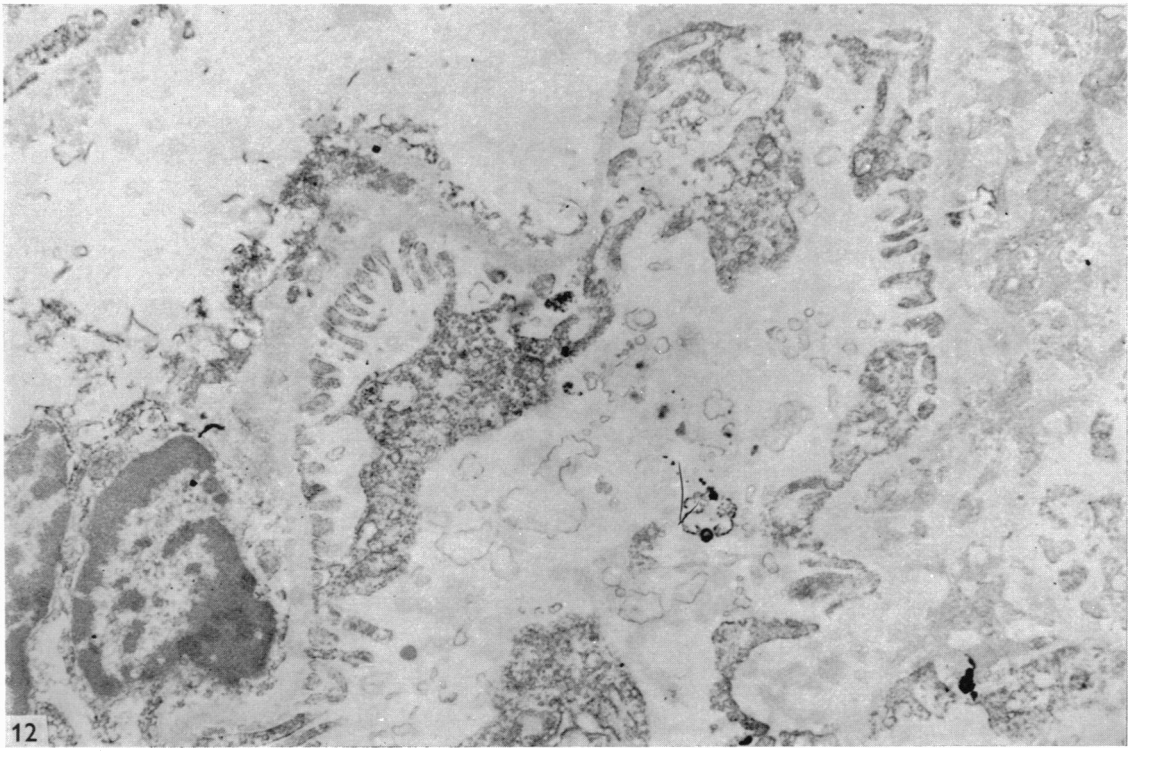
Figs. 7–8. Show the reaction of anti-actin with the foot processes of glomerular epithelial cells. Note the lack of reaction in the main part of the epithelial cells. Fig. 7, × 10000; Fig. 8, × 32000.





Figs. 9–10. Show the reaction of anti-heavy meromyosin with the foot processes of glomerular epithelial cells. The staining is confined to the cytoplasmic membrane near the basement membrane (arrows). Fig. 9, ×10000; Fig. 10, ×32000.





Figs. 11–12. Show the appearance of foot processes after reacting with (11) normal rabbit serum and (12) anti-myosin. × 10000.

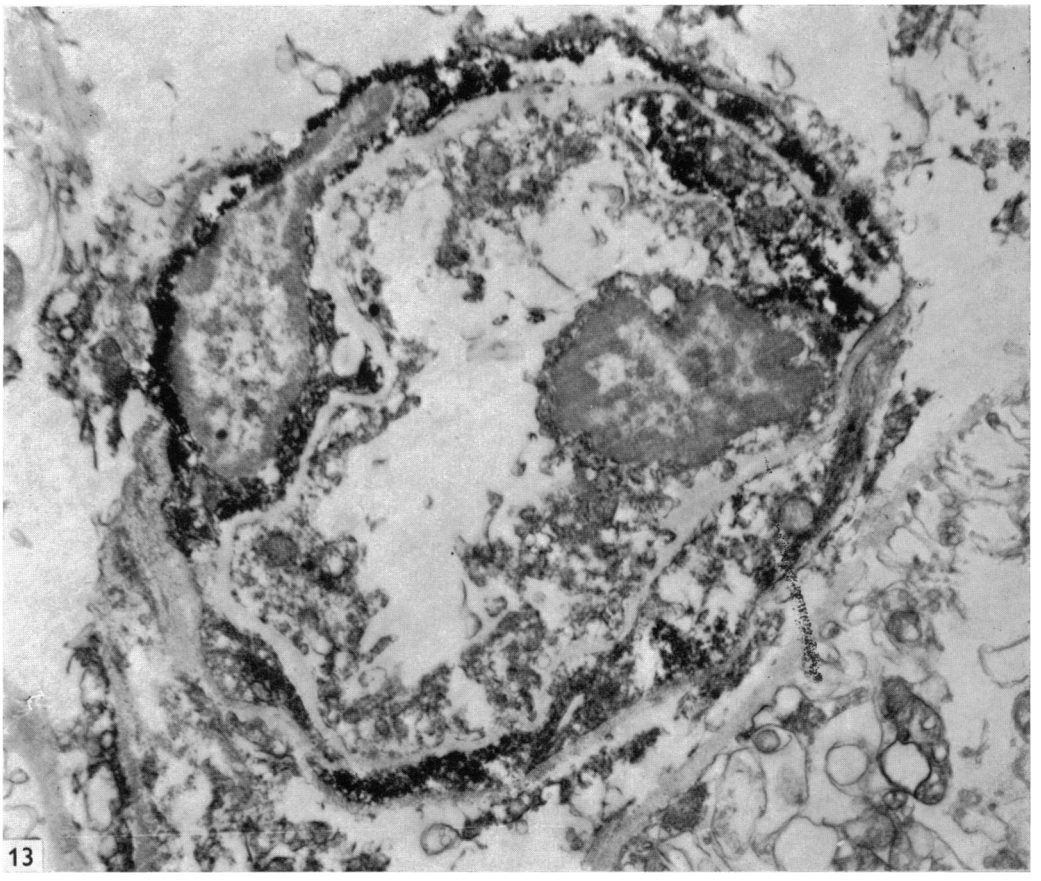


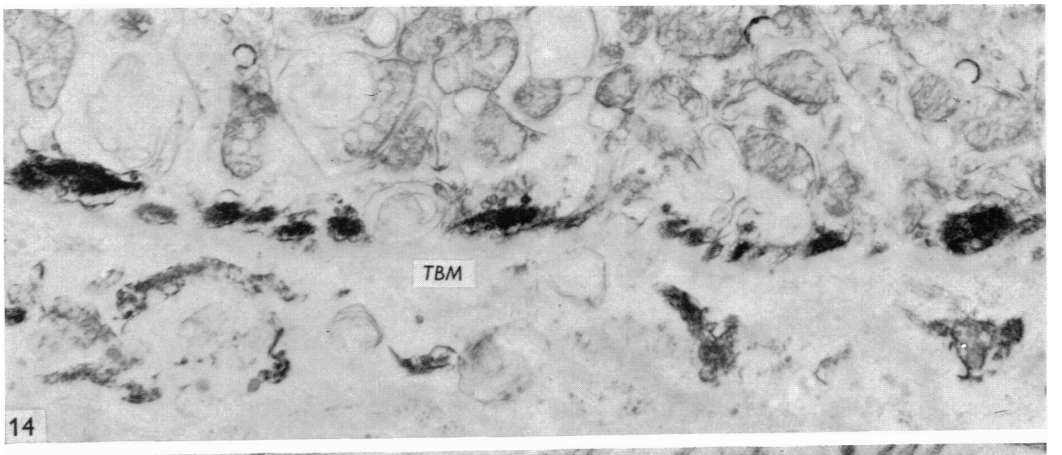
Fig. 13. The same ultrathin section as Fig. 12 but showing the reaction of anti-myosin with vascular smooth muscle. × 10000.

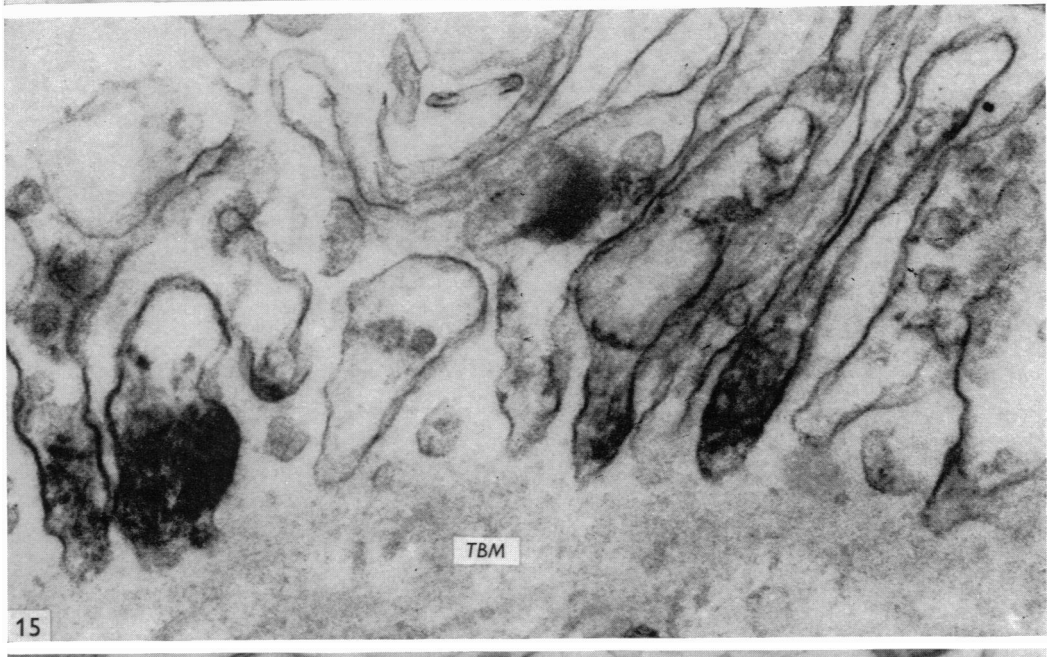
finding of Rostgaard, Kristensen & Nielsen (1972), by a different method, that actin is present in the basal regions of convoluted tubule cells.

These specific immune reactions were satisfactorily demonstrated only in unfixed or very lightly fixed tissues which, despite consequent poor preservation, retained sufficient structure to allow more precise localization of antigens than was possible by light microscopy.

Negative results by this method must be viewed with caution. Lack of reaction may be due to (1) absence of antigen, (2) presence of antigen in a form in which it is unable to combine with antibody, or (3) presence of antigen in a site which is inaccessible to antibody under the conditions of the method. However, the lack of reaction with anti-myosin in glomerular foot processes is unlikely to be due to (3) because anti-actin was able to penetrate to this site and because other intracellular sites were well stained by anti-myosin (Fig. 13).

The apparent failure of anti-myosin serum to stain sites where anti-heavy meromyosin antibody binds could be accounted for by a difference in the available





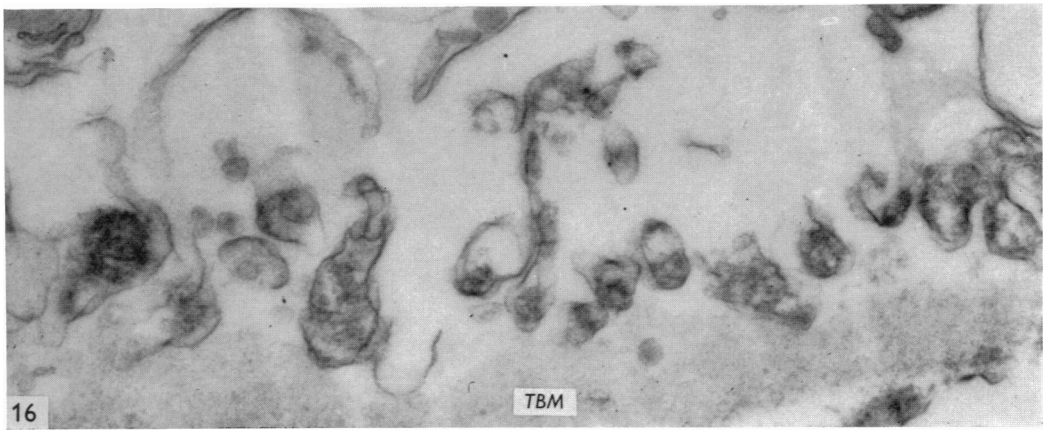


Fig. 14–15. Show the reaction of anti-actin with discrete areas at the base of proximal convoluted tubules. Fig. 14, ×10000; Fig. 15, counterstained with lead citrate to increase the contrast of membranes, ×32000. *TBM*, tubular basement membrane.

Fig. 16. Shows the slight reaction of anti-myosin with areas at the base of a proximal convoluted tubule. ×32000. *TBM*, tubular basement membrane.

antigenic determinants of myosin and heavy meromyosin. Heavy meromyosin, although part of the bigger myosin molecule, presumably possesses antigenic determinants which become accessible only after trypsin cleavage and are not available in intact myosin. This explanation was supported by the results of absorption experiments. Anti-heavy meromyosin antibody was not completely absorbed by myosin and the staining with anti-myosin serum was only partly affected by absorption with heavy meromyosin or light meromyosin. These newly arising antigenic determinants on heavy meromyosin are perhaps the most immunogenic.

It is known that heavy meromyosin exhibits the enzymic activity of myosin (Malik & Martonosi, 1972; Oplatka, Werber & Danchin, 1974) and also its ability to combine with actin. It is also known that the myosins in different tissue cells may differ from one another in chemical structure (Pollard & Weihing, 1973; Burridge, 1974). It seems possible that in some non-muscle mammalian cells a heavy meromyosin-like protein takes the place of myosin and forms complexes with actin. Recently it has been shown that heavy meromyosin is capable of generating a mechano-chemical force by interacting with actin filaments in the presence of ATP (Oplatka *et al.* 1974).

We have found this combination of heavy meromyosin and actin (Trenchev *et al.* 1974; Holborow *et al.* 1975) in other non-muscle cells such as those of duodenal, bronchial and bile duct epithelium, thyroid epithelium and newly formed vascular endothelium. In other cells, such as macrophages and platelets, actin-myosin complexes were found rather than actin–heavy meromyosin complexes. It seems, therefore, that some non-muscle cells are equipped with an actin–myosin system in which both components are similar, at least antigenically, to those in smooth-muscle actin–myosin, while epithelial cells have a slightly different combination of actin with a heavy meromyosin-like protein. The foot processes of glomerular epithelial cells appear to possess the latter contractile system. The sliding theory of Huxley (1973) for non-muscle cells postulates that one contractile protein is fixed and the other dispersed in the cytoplasm. According to this it seems possible that assemblies of actin molecules may interact consecutively with fixed heavy meromyosin-like molecules, thus causing movement of the foot process cytoplasm.

SUMMARY

Contractile proteins of smooth muscle type were found in kidney cells by immunological methods. The reactions of rabbit anti-actin, anti-heavy meromyosin and anti-myosin antisera with rat kidney were investigated by immunoelectron microscopy. Anti-actin stained specifically the foot processes of epithelial cells in the glomerulus, the basal processes of tubular epithelial cells, and the cytoplasm of smooth muscle cells. Anti-heavy meromyosin stained the foot processes at their bases near the cell membrane and close to the basement membrane. Anti-myosin stained the cytoplasm of vascular smooth muscle cells. It is suggested that actin and heavy meromyosin-like proteins may act together to cause movement of foot process cytoplasm.

REFERENCES

ADELSTEIN, R. S., GODFREY, J. E. & KIELLEY, W. W. (1963). G-actin: preparation by gel filtration and evidence for a double stranded structure. *Biochemical and Biophysical Research Communications* **12**, 34–38.

AVRAMEAS, S. (1969). Coupling of enzymes to proteins with glutaraldehyde. Use of the conjugates for the detection of antigens and antibodies. *Immunochemistry* **6**, 43–52.

BURRIDGE, K. (1974). A comparison of fibroblast and smooth muscle myosins. *FEBS Letters* **45**, 14–17.

GOLL, D. E., SUZUKI, A., TEMPLE, J. & HOLMES, G. R. (1972). Studies on purified α-actinin. I. Effect of temperature and tropomyosin on the α-actinin/F-actin interaction. *Journal of Molecular Biology* **67**, 469–488.

HAMOIR, G. (1973). Extractibility and properties of the contractile proteins of vertebrate smooth muscle. *Philosophical Transactions of the Royal Society* B **265**, 169–182.

HITCHCOCK, S. E. (1971). Detection of actin filaments in homogenates of developing muscle using heavy meromyosin. *Developmental Biology* **25**, 492–501.

HOLBOROW, E. J., TRENCHEV, P. S., DORLING, J. & WEBB, J. (1975). Demonstration of smooth muscle contractile protein antigens in liver and epithelial cells. *Annals of the New York Academy of Sciences* (in the Press).

HUXLEY, H. E. (1973). Muscular contraction and cell motility. *Nature* **243**, 445–449.

ISHIKAWA, H., BISCHOFF, R. & HOLTZER, H. (1969). Formation of arrowhead complexes with heavy meromyosin in a variety of cell types. *Journal of Cell Biology* **43**, 312–328.

MALIK, M. N. & MARTONOSI, A. (1972). The regulation of the rate of ATP hydrolysis by H-meromyosin. *Archives of Biochemistry and Biophysics* **152**, 243–257.

OPLATKA, A., WERBER, M. M. & DANCHIN, A. (1974). Specific interaction of cobaltic complexes with myosin. *FEBS Letters* **47**, 7–10.

POLLARD, T. D. & WEIHING, R. R. (1973). Cytoplasmic actin and myosin and cell movement. *Critical Reviews in Biochemistry* **2**, 1–65.

REYNOLDS, E. S. (1963). The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *Journal of Cell Biology* **17**, 208–212.

ROSTGAARD, J., KRISTENSEN, B. I. & NIELSEN, L. E. (1972). Electron microscopy of filaments in the basal part of rat kidney tubule cells, and their *in situ* interaction with heavy meromyosin. *Zeitschrift für Zellforschung und mikroskopische Anatomie* **132**, 497–521.

TRENCHEV, P., SNEYD, P. & HOLBOROW, E. J. (1974). Immunofluorescent tracing of smooth muscle contractile protein antigens in tissues other than smooth muscle. *Clinical and Experimental Immunology* **16**, 125–136.

WEBB, J. A. & DORLING, J. (1972). The use of peroxidase-labelled anti-globulin for ultrastructural localization of tissue antigens reacting with serum antibodies. *Journal of Immunological Methods* **2**, 145–157.