

# Membrane-associated Actin in the Rhabdomeral Microvilli of Crayfish Photoreceptors

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**ABSTRACT** Infiltration of compound eyes of crayfish, *Cherax destructor*, with the thiol protease inhibitor Ep-475 or with trifluoperazine prior to fixation for electron microscopy was found to stabilize an axial filament of 6–12 nm diam within each rhabdomeral microvillus of the photoreceptors. Rhabdoms isolated from retinal homogenates by sucrose gradient centrifugation under conditions that stabilize cytoskeletal material contained large amounts of a 42-kd polypeptide that co-migrated with insect flight muscle actin in one- and two-dimensional PAGE, inhibited pancreatic DNase I, and bound to vertebrate myosin. Vertebrate skeletal muscle actin added to retinal homogenates did not co-purify with rhabdoms, implying that actin was not a contaminant from nonmembranous structures. DNase I inhibition assays of detergent-lysed rhabdoms indicated the presence of large amounts of filamentous actin provided ATP was present. Monomeric actin in such preparations was completely polymerizable only after 90 min incubation with equimolar phalloidin. More than half of the actin present could be liberated from the membrane by sonication, indicating a loose association with the membrane. However, a large proportion of the actin was tightly bound to the rhabdomeral membrane, and washing sonicated membrane fractions with solutions of a range of ionic strengths and nonionic detergents failed to remove it. Antibodies to scallop actin only bound to frozen sections of rhabdoms after gentle permeabilization and very long incubation periods, probably because of steric hindrance and the hydrophobicity of the structure. The F-actin probe nitrobenzoxadiazol phalloidin bound to rhabdoms and labeled F-actin aggregates in other retinal components, but rhabdom fluorescence was not abolished by preincubation with phalloidin. The biochemical data indicate the existence of two distinct actin-based cytoskeletal systems, one being closely membrane associated. The other may possibly constitute the axial filament, although the evidence for this is equivocal.

The rhabdomeres of arthropod and cephalopod photoreceptors are composed of numerous microvilli which in a given cell-type are remarkably uniform in diameter and ordered in arrays of almost crystalline regularity. Successive authors have implied or suggested that a cytoskeleton is likely to underlie this organization (6, 31, 37, 59, 60, 65), and some have speculated that it will prove to be actin based. Actin is a component of rhabdoms of squid (59, 60), and the organization of other microvillar types provides well known precedents (50, 54, 68).

Conventional techniques of electron microscopy have not revealed a cytoskeletal organization within rhabdomeral microvilli with any consistency, and an early demonstration of an axial filament in microvilli of bee photoreceptors (70) has

been largely overlooked in the literature. Recently, three studies have demonstrated an axial filament linked to the plasma membrane by side-arms in photoreceptor microvilli of flies (7, 8) and squid (60). Although these filaments might, quite reasonably, be supposed to consist of actin, we have argued (7, 8) that a number of factors militate against this interpretation: the cytoskeleton is extremely labile, and treatment with tannic acid before secondary fixation with OsO<sub>4</sub> (51, 62) fails to preserve it in the photoreceptors of blowflies unless other measures are also adopted. These require either that Ca<sup>2+</sup> be chelated, or that the rhabdoms be pretreated with trifluoperazine (probably acting nonspecifically) or with E-64 derivatives. The latter reagents are highly specific inhibitors of thiol proteases (38, 66). Preservation of the side-arms

requires a divalent cation, preferably  $Mg^{2+}$ .

In this paper we show that a microvillar cytoskeleton, similar to that described in Diptera, can be preserved in rhabdoms of crayfish by infiltrating the eyes *in vivo* with either trifluoperazine or Ep-475 before fixation. Both rhabdoms and cytoskeleton survive separation from other retinal components and purification by sucrose gradient centrifugation provided that they are stabilized with Ep-475 and EGTA, and indeed some additional cytoskeletal elements can be resolved. Such preparations, like isolated squid rhabdoms (60), contain a high complement of actin. We show that a significant proportion of this actin is tightly membrane associated. The remainder is more loosely bound.

We discuss (a) the tightly membrane-associated actin fraction in terms of a postulated association between actin and integral membrane components, as has been shown to exist in the brush border microvillus (19), and in plasma membranes from a variety of cell lines (16, 40, 52) and (b) the possibility that there is an actin-based subplasmalemmal cytoskeleton distinct from the axial filament.

## MATERIALS AND METHODS

**Reagents:** Unless otherwise noted, chemicals of reagent grade were obtained from Sigma Chemical Co., (St. Louis, MO). Ultrapur urea was from Schwarz-Mann (NY), and reagents for electrophoresis from Bio-Rad Laboratories (Richmond, CA).

**Experimental Animals:** Australian crayfish (*Cherax* species, known locally as "yabbies") were either trapped from ponds and lakes near Canberra, or purchased from commercial breeders in New South Wales. They were kept in the laboratory under a 12:12 h light/dark cycle.

**Electron Microscopy:** For conventional electron microscopy, retinæ taken from light-adapted crayfish in the early afternoon were dissected quickly under a fixative solution composed of 2% glutaraldehyde (Polysciences, Inc., Warrington, PA) in 0.08 M cacodylate buffer with 0.11 M sucrose and 10 mM EGTA at pH 7.3.

To infiltrate retinæ with the thiol protease inhibitor Ep-475 (Taisho Pharmaceutical Co. Ltd., Tokyo) or with trifluoperazine dihydrochloride (Smith, Kline and French Ltd., New South Wales, Australia), we immobilized the eyes of living crayfish with dental wax, cut a small hole in each cornea, and applied a drop of either 100 mM Ep-475 dissolved in equimolar  $NaHCO_3$  or 50 mM trifluoperazine to it. Materials applied to compound eyes in this manner diffuse rapidly throughout the retinæ (39). Infiltration of the compound eyes of flies with 100 mM Ep-459 (an analogue of Ep-475) does not prejudice either the radial movements of screening pigment induced by light and darkness or receptor and resting potentials over a subsequent 12 h period, so that transduction by the receptors can be inferred to be unimpaired (11). After 15–30 min of infiltration, the retinæ were dissected into small pieces under the same fixative with 20 mM  $MgCl_2$  and kept at 4°C for 2 h. The fragments were transferred to fresh fixative containing 0.5% low molecular weight tannic acid (Mallinckrodt Inc. St. Louis, MO; reference 62) and left at room temperature overnight. They were washed for 2 h in cacodylate buffer, postfixed in cold 1%  $OsO_4$  for 30–45 min washed for 30 min in distilled water, dehydrated through an ethanol series and propylene oxide, and embedded in Araldite (Ciba-Geigy, Australia). Thin sections were cut on glass knives, mounted on Pioloform-coated or uncoated 400-mesh grids, and stained with uranyl acetate and lead citrate. They were viewed in an Hitachi H600 electron microscope operated at 75 kV, calibrated at low magnifications with a grafting replica (Polaron Instruments Inc., Hatfield, PA) and at high magnifications with a catalase crystal. Samples of isolated rhabdoms and of sonicated membrane pellets were fixed in the same solution without  $MgCl_2$  and processed with or without tannic acid.

**Purification of Intact Rhabdoms from Crayfish Retinæ:** Our method was modified from Hamacher (35). Crayfish kept under the laboratory light/dark cycle for at least 4 wk before an experiment were taken during "day" and held in darkness for 2 h. Subsequent steps were carried out under dim red light, and dissections were illuminated by red light (Schott RG 645) through a heat filter. Eyestalks were removed from the animals and opened rapidly from the basal end to expose the retina. Each optic nerve was transected, and the retina was seized with forceps, pulled out of the cup formed by the cornea, and immediately suspended in 100  $\mu$ l/retina of 10 mM PIPES buffer with 0.2 M NaCl, 10 mM  $MgCl_2$ , 1 mM EGTA, 0.5 mM ATP, 0.2 mM dithioerythritol, 2

mg/ml BSA, 0.1 mM phenylmethylsulphonyl fluoride (PMSF), and 0.1 mM Ep-475, pH 7.2. In some experiments 5 mM ascorbic acid was used as an antioxidant, but this interferes with spectrophotometric assays in the UV range.

The retinal components were disrupted with a rotating stainless steel spiral fitted to a dental drill operated at ~5,000 rpm for ~5 s, and the homogenate was layered onto a linear sucrose gradient made from 2 ml each of 5 and 40% sucrose in 10 mM PIPES buffer with 10 mM NaCl, 1 mM EGTA, 0.5 mM ATP, 0.2 mM dithioerythritol, 2 mg/ml BSA, 0.1 mM PMSF, 0.1 mM Ep-475, pH 7.2. The gradient rested on top of 0.75 ml of a cushion of 45% sucrose in the same buffer without BSA. Homogenate from as many as 10 retinæ was loaded in each tube, and centrifuged (MSE Superspeed 75 centrifuge, titanium swinging bucket rotor, 3 × 6.5 ml,  $g_{max} = 150,000$ ) for 70 min. The flocculent layer just above the cushion was carefully removed with a syringe fitted with a long needle under red light, resuspended, and stored in darkness at -80°C until use.

**Sonicated Membranes:** Small volumes of rhabdom suspensions were sonicated with an MSE sonicator (2 s at 4°C at a setting of 2  $\mu$ m) at approximate concentrations of 5–10 mg/ml protein.

Incubations in solutions of various ionic strengths were carried out in darkness at 4°C for 30 min. Digitonin (Sigma Chemical Co.) was used without further purification from a 5% solution prepared with gentle heat and subsequently clarified through a Millipore filter (Millipore Corp., Bedford, MA). Aliquots of membrane suspension were centrifuged ( $g_{max} = 70,000$ ) for 30 min and supernatants were removed carefully with a drawn-out Pasteur pipette. Analysis of washed membranes was carried out by adding SDS PAGE buffer directly to the centrifuge tubes and heating it for 5 min to 96°C. Aliquots of this mixture were analyzed by SDS PAGE.

**Membrane Cytoskeletons:** Membrane cytoskeletons were prepared by suspending an aliquot of washed rhabdomeral pellet in 0.5 ml homogenization buffer containing either 1.5% (vol/vol) Triton-100 or digitonin (wt/vol) for 30 min at 4°C, followed by centrifugation ( $g_{max} = 150,000$  for 2 h). Pellets were dissolved directly in SDS sample buffer.

**One- and Two-dimensional SDS Gel Electrophoresis:** Samples were analyzed on slab gels with a 7.5–15% acrylamide gradient using the method of Laemmli (43). Two-dimensional gel electrophoresis was carried out essentially as described by O'Farrell (56), the second dimension also employing 7.5–15% gradient gels. Gels were stained for proteins either with Coomassie Blue or with an ultrasensitive silver staining technique (72). Cross-linked BSA, myosin, actin, ovalbumin, trypsinogen, lactalbumin, and pepsinogen were used as molecular weight markers.

**Fluorescence Microscopy:** We froze crayfish eyestalks immediately after amputation by plunging them into liquid nitrogen. 5- $\mu$ m sections were cut on a cryomicrotome, air-dried on slides, and stored at -17°C.

Sections were fixed by immersion in dry acetone at -17°C for 10 min, after which they were air-dried. F-actin was visualized with the fluorescent probe nitrobenzoxadiazol phalloidin (NBD-phalloidin,<sup>1</sup> Molecular Probes Inc., Plano, TX) (3, 55), by dissolving 150 U of NBD-phalloidin in 3 ml methanol, and treating the sections with a 1:20 dilution in Van Harrevel's saline (69) for 20 min. After a subsequent wash in saline, sections were mounted in 70% glycerol in Tris-glycine buffer, pH 9.0. Controls were prepared by prelabeling sections with 1 mM phalloidin (Boehringer-Mannheim Biochemicals, Indianapolis, IN) in saline for 30 min to 10 h before treatment with NBD-phalloidin.

Binding of antibodies was performed on both acetone- and formaldehyde-fixed material after extraction with 2% saponin (Sigma Chemical Co.) in 0.2 M NaCl, 5 mM  $MgCl_2$ , 1 mM EGTA, 0.2 mM ATP, 0.05 M Tris HCl, pH 7.4, for 10 min. Following fixation, the sections were thoroughly washed in Tris-buffered saline and incubated in 2% BSA, 10% horse serum in Tris-buffered saline to block nonspecific binding sites. Antibodies against scallop actin were raised in rabbits (21) and used at a concentration of 50  $\mu$ g/ml IgG in blocking medium. After 14 h incubation at 4°C, the slides were thoroughly washed in saline (3 × 30 min) and incubated in fluorescein isothiocyanate-labeled goat-anti-rabbit IgG (Miles Biochemicals, UK). Controls were incubated with rabbit nonimmune sera, diluted 1:10 in blocking buffer, or in a scallop actin antiserum preabsorbed with insect flight muscle myofibrils for 12 h at 4°C. The slides were washed in PBS (2 × 30 min), mounted in 70% glycerol in glycine buffer, and examined by epifluorescence under a Zeiss Photoscope III. Photographs were taken on Kodak Tri-X Professional film and developed in Kodak D-76.

**DNase I Inhibition Assay:** DNase (Sigma Chemical Co., Type I, from bovine pancreas) was dissolved in 0.15 M NaCl to give an optical activity of approximately 0.02 absorbance units per minute per milliliter reaction medium under the specified conditions. DNA (Sigma Chemical Co., Type III) was used at 40  $\mu$ g/ml in 0.1 M Tris buffer, pH 7.5, with 4 mM  $MgSO_4$ , 1.8 mM  $CaCl_2$ , 0.01 mM  $NaN_3$ .

<sup>1</sup> Abbreviation used in this paper: NBD, nitrobenzoxadiazol.

Initially we performed the DNase I inhibition assay in the absence of calcium which, however, makes DNase I more susceptible to activity changes induced by detergents or chaotropic agents. Freshly isolated rhabdoms were lysed by the addition of homogenization buffer containing 1% Triton-100 to give a final protein concentration of ~5–10 mg/ml, and were vortexed. 3  $\mu$ l of this suspension was mixed with either the same volume of homogenization buffer or 1.5 M guanidinium hydrochloride in 0.5 M sodium acetate, pH 6.5. Depolymerization was allowed to take place for 30 min at 4°C, before 5  $\mu$ l of a DNase I stock solution was added, followed by addition of 1 ml of substrate solution. The time elapsing between mixing of the test sample with the DNase stock solution and start of measurements was usually <10 s. The formation of free nucleotides from high molecular weight DNA was measured as the increase of optical density at 260 nm, using a Gilford spectrophotometer with plotter (Gilford Instrument Laboratories Inc., Oberlin, OH). The equivalent in rhabdomeral membranes of one-half of a retina yielded sufficient inhibitory activity for a single assay. Estimates of actin concentrations are based on published values (12).

**Protein Determinations:** Protein concentrations were estimated according to Lowry et al (47). Actin concentrations were determined spectrophotometrically at an extinction coefficient  $E_{280\text{ nm}}^{1\%_{1\text{ cm}}} = 6.3$ .

**Myosin Binding Assay:** Chicken breast muscle myosin stored at -17°C in 50% glycerol until use was precipitated from solution by diluting it with 10 vol of 10 mM Tris-HCl with 1 mM CaCl<sub>2</sub>, 0.1 mM dithiothreitol, pH 7.5 at 4°C. After 1 h myosin aggregates were spun down for 10 min at 10,000 g, resuspended in the same buffer, and spun down again. Finally, the precipitate was suspended in the same buffer with 1% Nonidet P-40 to give a concentration of 1 mg/ml.

Either total crayfish retinae or rhabdomeral membrane fractions were dissolved in 0.5 ml of the same buffer with 0.1 mM PMSF at 4°C. The mixture was spun at 10,000 g for 5 min and resuspended in 1 ml of buffer with detergent. This washing step was repeated twice and the final pellet was dissolved in SDS electrophoresis buffer and heated to 95°C for 5 min. To presaturate myosin with actin filaments, we added 50  $\mu$ g of chicken breast muscle before blending with the rhabdomeral homogenate.

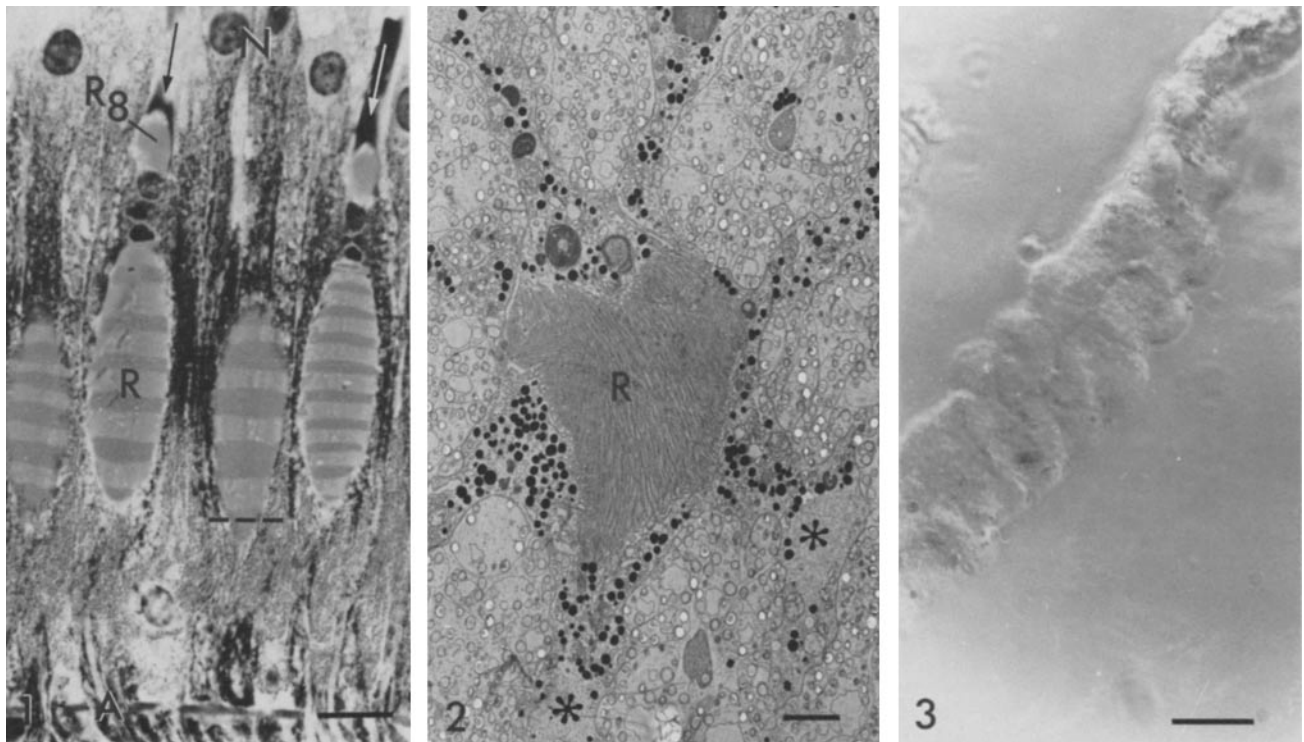
## RESULTS

### *Ultrastructure of Rhabdoms In Situ*

The disposition of rhabdoms in the crayfish retina is indicated in Figs. 1 and 2; each rhabdom consists of microvilli derived from seven photoreceptors of a single ommatidium which together form a cigar-shaped mass of rhodopsin-containing membrane.

After conventional fixation for electron microscopy, the contents of rhabdomeral microvilli usually appear homogeneous. Occasionally, microvilli in transverse section present a darker axial density which longitudinal sections very rarely display as a continuous filament. Tannic acid mordanting alone improves the resolution of the microvillar unit membranes, but does not resolve the infrastructure of their contents (7) which appear granular.

Infiltration of the retinae with Ep-475 followed by tannic acid mordanting displays a complex scaffolding within each microvillus (Fig. 4, *a* and *b*), although it is always rather irregularly preserved. A single axial filament with an apparent diameter of 6–12 nm is linked to the microvillar membranes by side-arms with diameters of 6.5–10 nm. These various diameters are probably a consequence of partial degradation; occasionally regions of axial filament as thin as 2–3 nm are seen. Similar but less satisfactory resolution of the cytoskeleton can be obtained after infiltration with trifluoperazine, and it can be visualized in fragmentary form without the use of either agent if the fixative contains 10 mM EGTA, though



FIGURES 1–3 Fig. 1: Longitudinal section through the reticular cell layer of the crayfish eye, to show the arrangement of the rhabdoms (R). Their barred appearance is due to alternating layers of orthogonally oriented microvilli. Light enters from above (arrows). Dotted line shows level of section in Fig. 2. Toluidine blue-stained Araldite section was prepared by Roland Jahnke. Bar, 20  $\mu$ m.  $\times$  500. Fig. 2: Thin section through the proximal end of a rhabdom (R) showing seven contributing retinal cells filled with dark granules of screening pigment and the beginnings of two axons (asterisks). Surrounding cells contain white reflecting pigment granules. Bar, 2  $\mu$ m.  $\times$  3,700. Fig. 3: Phase-contrast micrograph of isolated, unfixed rhabdom. "Scalloped" edges correspond to the position of bands of orthogonally arranged microvilli. Bar, 10  $\mu$ m.  $\times$  1,100.

usually as little more than an amorphous central density.

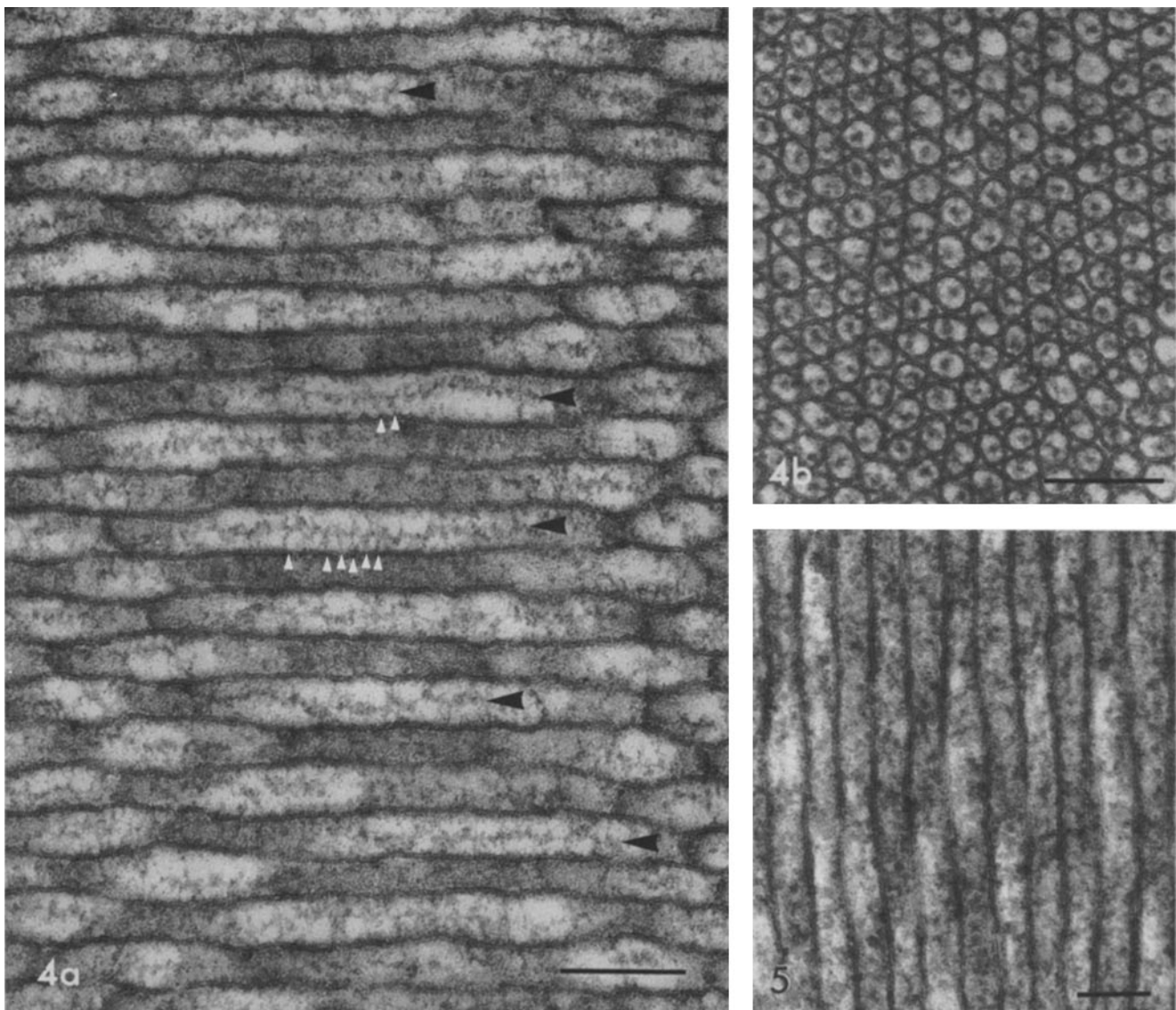
The use of tannic acid does not seem markedly to favor preservation of the cytoskeleton, but merely to improve contrast. If the trilaminar appearance of microvillar membranes is taken as an indication that penetration of tannic acid into the tissue block has been adequate, faint "ghosts" of the cytoskeleton can be seen in regions of retinae infiltrated with Ep-475 to which tannic acid has not penetrated, as, for example, in microvilli of the isolated rhabdom shown in Fig. 10. Sometimes, such variations in mordanting can be seen in different microvilli belonging to a single cell. Conversely, when the cytoskeleton has not been stabilized, tannic acid treatment merely intensifies the staining of the remaining amorphous matrix (Fig. 5).

#### *Ultrastructure and Composition of Isolated Rhabdoms*

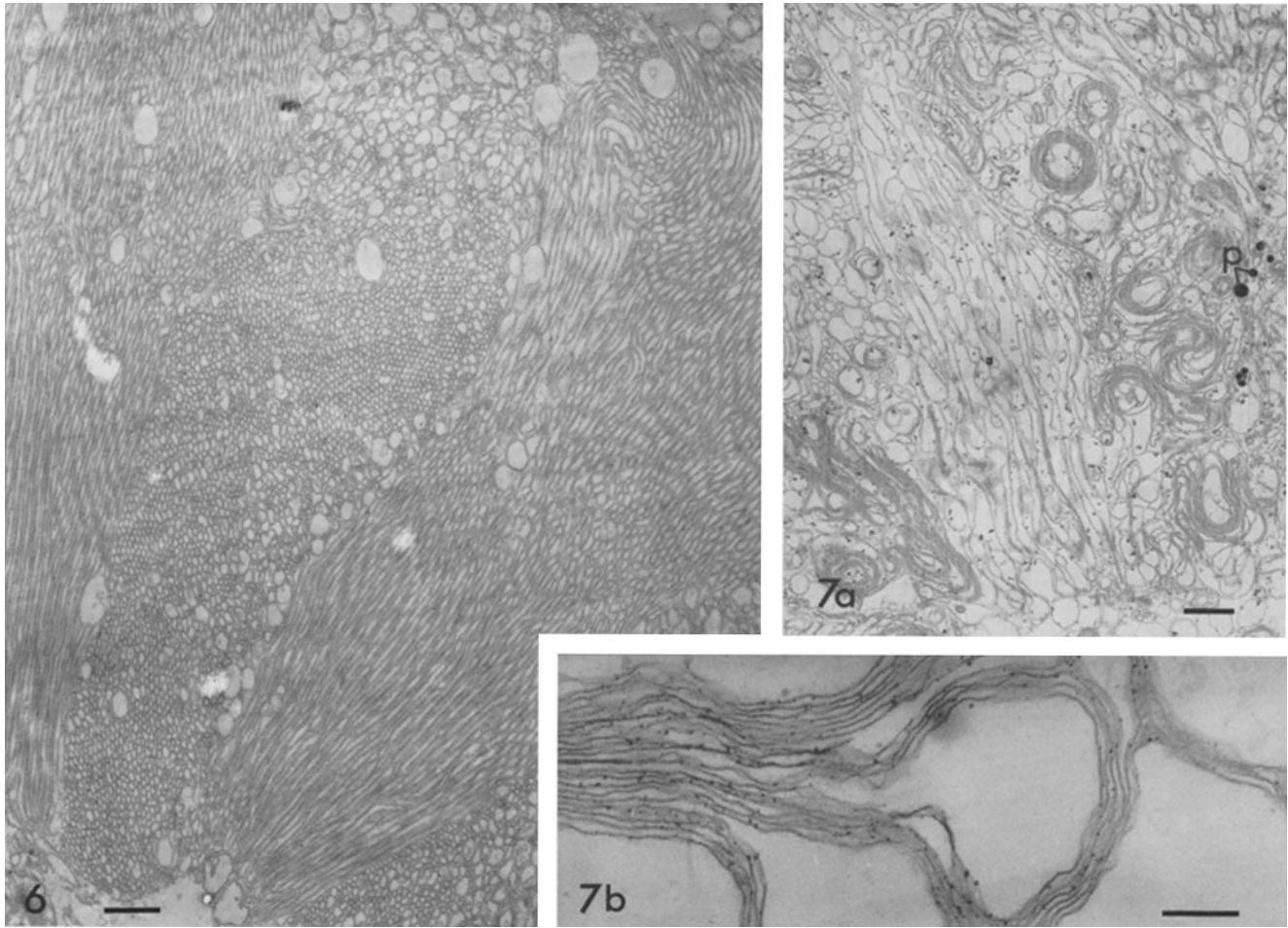
Provided that the initial homogenization is sufficiently

gentle, that the ionic composition of the homogenization medium is near to physiological values except for the omission of  $\text{Ca}^{2+}$ , and that protease inhibitors are included, the overall structure of the rhabdoms is well preserved, and cytoskeletal elements can still be seen within the microvilli (Figs. 3 and 6) despite the  $\sim 3.5$  h needed to isolate the rhabdoms from some 10 animals. While various osmolarities are known not to disrupt crayfish rhabdoms over a surprising range of concentrations in shorter-term experiments (71), our attempts to use other buffer systems that neither chelate  $\text{Ca}^{2+}$  nor inhibit proteases led to the production of disorganized membranes and myelin figures (Fig. 7). These problems have caused studies of rhodopsin photochemistry that use isolated crayfish rhabdoms to be conducted on material fixed with formaldehyde (44).

Transmission electron microscopic observations of sectioned pellets suggest that contamination of the rhabdom fraction is no more than 5% at most and probably less for



FIGURES 4 and 5 Fig. 4: Microvilli from retina infiltrated with Ep-475 before fixation and mordanted in tannic acid. (a) Longitudinal section of rhabdom showing single axial filaments (black arrowheads) with numerous side-arms (white arrowheads) within each microvillus. Bar,  $0.2 \mu\text{m}$ .  $\times 93,000$ . (b) Transverse section through microvilli. Bar,  $0.2 \mu\text{m}$ .  $\times 89,000$ . Fig. 5: Longitudinal section of microvilli mordanted in tannic acid, but without prior treatment with Ep-475 or EGTA. Microvilli contain amorphous material only. Bar,  $0.1 \mu\text{m}$ .  $\times 110,000$ .



FIGURES 6 and 7 Fig. 6: Section through a rhabdom isolated in the presence of Ep-475 and EGTA in all media. Microvilli are intact and orthogonally arranged, as in situ. Bar, 1  $\mu\text{m}$ .  $\times 8,500$ . Fig. 7: Sections through a comparatively orderly rhabdom isolated in the absence of EP-475 and EGTA. (a) Microvilli are not recognizable, but traces of their orthogonal arrangement can still be seen. There are a few adhering pigment granules (*p*). Bar, 1  $\mu\text{m}$ .  $\times 6,800$ . (b) An unstained section showing that the electron-dense deposits localized on membranes in (a) are not staining artifacts. Bar 0.2  $\mu\text{m}$ .  $\times 52,000$ .

most rhabdom preparations. Contamination is derived from plasma membranes forming sleeves round the rhabdoms, patches of unstructured cytoplasm, and pigment granules adhering to the membranes. The number of such granules is greatly reduced by the addition of BSA to the isolation media (35), but BSA cannot be subsequently removed by washing. Mitochondria or nuclei were never observed in the rhabdom fraction. When isolated rhabdoms are prepared in media lacking EGTA and inhibitors, and containing phosphate, the ordered structure largely disappears, and small accumulations of osmiophilic material are present on the membranes (Fig. 7*b*). Prepared in media containing EGTA and Ep-475, the basic organization of the rhabdom as orthogonal bands of microvilli is well preserved (Fig. 6), although the microvilli are usually rather swollen, with diameters of 90–150 nm compared with 60–70 nm for rhabdoms fixed in situ. The axial filaments after tannic acid mordanting have 6.5–12 nm diam, and are less fragmented than in rhabdoms fixed in situ, although the 2–3-nm-diam regions are seen more frequently and tend to extend for greater distances (Fig. 8). The filaments are displaced to the walls of the microvilli, sometimes lying closely against them, and are often seen to cross from one side of a microvillus to another. Intact side-arms are infre-

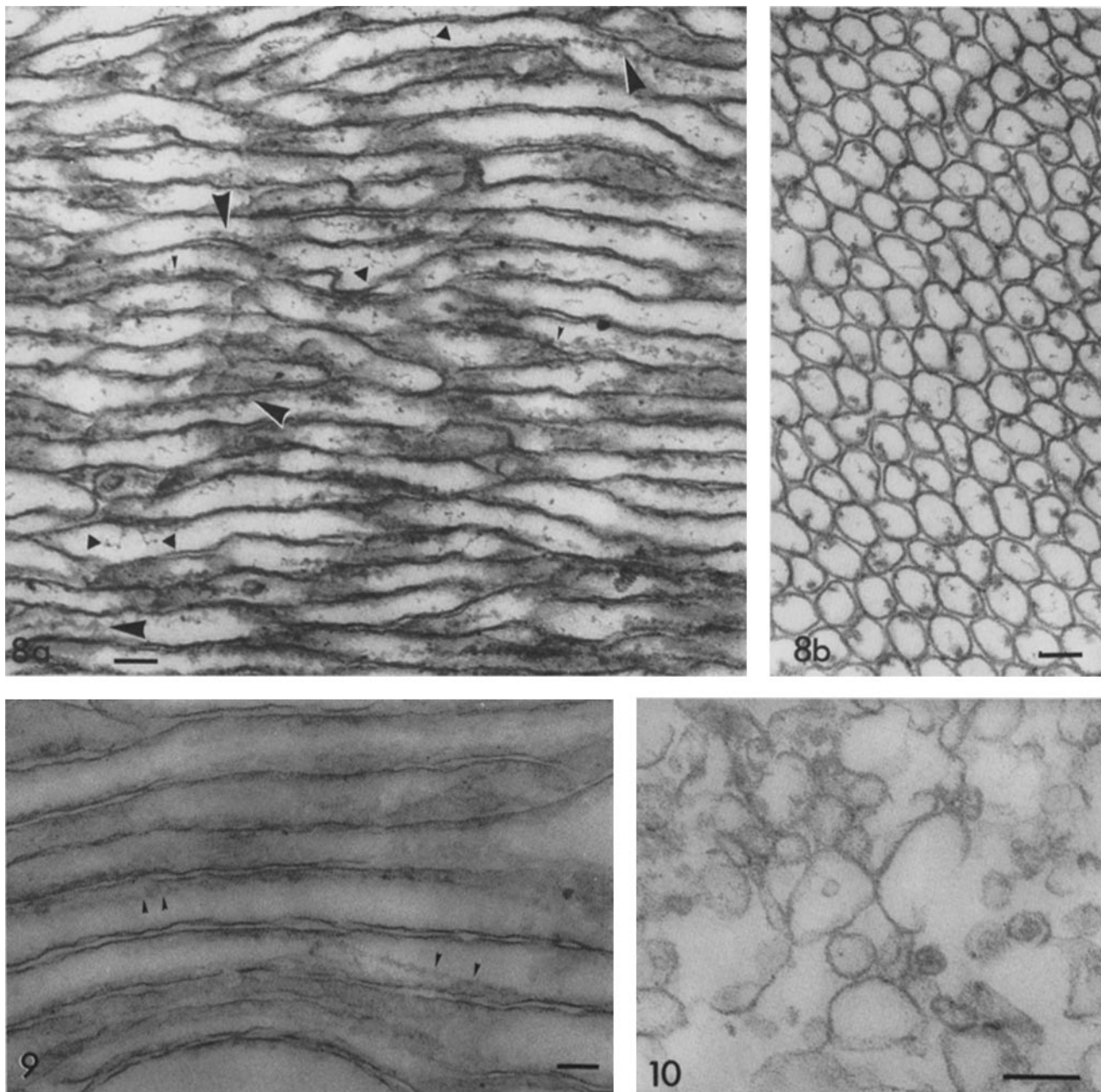
quent, but there are often globular thickenings 20–30 nm apart along each filament.

The swollen microvilli also contain meandering, 3-nm filaments of consistent appearance (Fig. 8) whose status is uncertain. Possibly, they are protofilaments derived from the axial structure; alternatively, they might be reassembled side-arm proteins or cytoskeletal elements stripped from the inner face of the microvillar membranes.

One- and two-dimensional electrophoresis of the rhabdomeral fraction followed by silver staining of the gels reveals about a dozen major polypeptides and as many minor constituents. A major protein of most preparations has a molecular weight of 35,000, identical with that of the rhodopsin of other arthropods (60, 71). Two-dimensional isoelectric focusing reveals only one isoelectric species with a pI slightly alkaline with respect to BSA (Fig. 11). Amounts of rhodopsin varied considerably between different preparations and rarely exceeded ~30% of the total rhabdomeral proteins, but conventional two-dimensional isoelectric focusing of membrane proteins may not accurately reflect their relative amounts because of incomplete solubilities in the lysis buffer (1).

Depending on the pretreatment of samples for electrophoresis, numerous oligomers of rhodopsin can be detected. The





FIGURES 8–10 Fig. 8: Longitudinal (a) and transverse (b) sections through swollen microvilli from a single rhabdom isolated in the presence of Ep-475 and EGTA, mordanted in tannic acid. Axial filaments (large arrowheads) are of normal width, but exhibit narrow regions (small arrowheads) with globular blebs. Microvilli also contain thin, meandering filaments (triangles). Bar,  $0.1 \mu\text{m}$ .  $\times 67,000$ . Fig. 9: Longitudinal section through microvilli of a rhabdom isolated in the presence of Ep-475 and EGTA, but without tannic acid mordanting. Axial filaments are faintly visible (arrowheads). Bar,  $0.1 \mu\text{m}$ .  $\times 64,000$ . Fig. 10: Section through a pellet of sonicated membrane fraction. Bar,  $0.1 \mu\text{m}$ .  $\times 120,000$ .

molecular weight of these may be high enough to prevent them from penetrating the separating gel during electrophoresis, thus making it difficult to quantitate the amount of opsin (28). The dimer of opsin co-migrates with BSA in one-dimensional PAGE (67 kd). Serum albumin firmly adheres to the rhabdomeral membrane during the isolation procedure and therefore obscures the presence of opsin dimers. However, after isoelectric focusing we were unable to detect any oligomers of opsin (Fig. 11).

Another major component of similar isoelectric point to rhodopsin is a 142-kd protein that binds concanavalin A (Con

A) (de Couet, H. G., manuscript in preparation) and is assumed to be an integral membrane protein. The most acidic polypeptides are represented by 87.5-, 71-, and 57-kd proteins, and a cluster of 49–56-kd peptides. The latter tended to be obscured by ill-defined deposits after silver staining. A third major constituent is a 42-kd protein which we show to be actin.

Comparison of the electrophoretic profiles of isolated rhabdoms and whole retinal extracts shows that all components in the former are enriched relative to their amounts in whole retinae with the single exception of a 49-kd polypeptide which

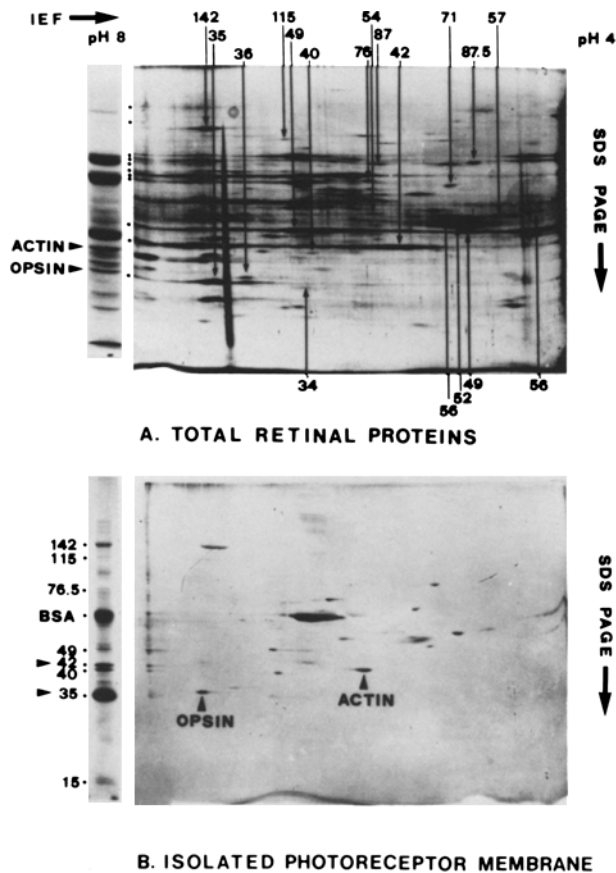


FIGURE 11 Two-dimensional PAGE of crayfish total retinal proteins (A) and of isolated rhabdoms (B). 7.5–15% gradient gels were silver stained for protein. Rhabdoms equivalent to 10 retinas were loaded on gel B; gel A is equivalent to two whole retinas. The position of actin and the assumed position of opsin are marked with arrows. The large amounts of BSA added during the isolation procedure bind nonspecifically to the rhabdoms and give an internal molecular weight marker that includes polymeric forms detected by silver staining.

survives in no more than trace amounts in the membrane fraction, and which we therefore conclude to be a cytoplasmic contaminant.

### Identification of the 42-kd Polypeptide as Actin

The 42-kd polypeptide co-migrates with actin from locust flight muscle in one- and two-dimensional electrophoresis; its isoelectric point also corresponds to that of  $\beta$ -actin from vertebrate cytoplasm and smooth muscle (23) (Fig. 12). The co-purification of BSA with the rhabdomeral fraction noted above suggests that actin might be a contaminant of the isolated rhabdoms, derived from other retinal structures. To test this, we exploited the lower pI of vertebrate  $\alpha$ -actin. The addition of chicken breast muscle actin to the rhabdomeral fraction and electrophoresis of the mixture yields two well distinguished spots at 42 kd (Fig. 12d). If, however, the  $\alpha$ -actin is added (0.5 mg/ml final concentration) to a homogenate of whole retinae whose rhabdoms are then isolated by our standard procedure without any additional washing steps, not even trace amounts of  $\alpha$ -actin can be distinguished on the subsequent two-dimensional gels (Fig. 12c). Since the myofibrillar actin was treated the same way as the membrane-associated form it cannot be argued that partial denaturation

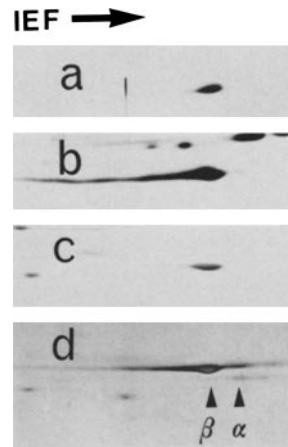


FIGURE 12 Co-migration of the 42-kd polypeptide of isolated rhabdomeral membranes with locust myofibrillar actin in two-dimensional gel electrophoresis. (a) Isolated rhabdomeral membrane. (b) Isolated rhabdomeral membrane + 50  $\mu$ g glycerinated myofibrils. No difference between the isoelectric points of the two peptide species can be detected. (c) Vertebrate skeletal muscle actin added to a total retinal homogenate does not detectably co-purify with the rhabdomeral membranes. (d) Co-electrophoresis of rhabdomeral membranes and vertebrate skeletal muscle  $\alpha$ -actin reveals the two isoelectric species, membrane actin corresponding to the  $\beta$ -form. Figures show small areas of the slab gels in the actin region. Silver stain.

leads to the exposure of hydrophobic segments that could interact nonspecifically with the membrane: rhabdomeral  $\beta$ -actin is specifically associated with the microvillar membranes.

### The State of Actin in the Rhabdomeral Membrane

The highly sensitive DNase I inhibition assay (13) was used to evaluate the state of actin in the rhabdomeral membrane. DNase I is only inhibited by monomeric actin, so that careful choice of experimental conditions allows its contribution to the membrane-associated pool to be assessed.

Using membranes that had been freshly lysed in 1% Triton-100 under conditions that strongly favor the formation of F-actin and stabilize the F-G equilibrium (12), we consistently found 60–70% of the total actin in the sample to be monomeric, depending on the preparation (Table I). The time elapsing between membrane lysis and DNase I inhibition measurements was kept to a minimum and was usually <10 min. Disruption of the membranes by sonication increased the pool of monomeric actin owing to the mechanical lability of polymeric actin. Since the rhabdoms had undergone severe mechanical stress due to the disruption and isolation procedures, a proportion of the monomeric actin found in freshly lysed membranes is likely to result from the depolymerization of originally filamentous actin in situ. Estimates of the actin concentration in the samples on the basis of a standard actin preparation gave values between 1.5 and 2.5 mg/ml, which is well above critical concentration. Preincubation of the samples in the presence of 2.5 mM  $\text{CaCl}_2$  increases the pool of monomeric actin (Table I), which can be attributed either to the effect of a calcium-activated depolymerization factor present in the sample or to the increase in critical concentration (13).

The depolymerization within minutes of F-actin by DNase I (49) is blocked by phalloidin, which also prevents its depolymerization by other agents and can induce polymerization under conditions that favor the formation of monomers (25, 45). Addition of 0.5 mM phalloidin to a membrane sample gradually increases the amount of filamentous actin disclosed by the DNase I inhibition assay. Total repolymerization however was only achieved after a minimum of 90 min incubation.

TABLE I  
DNase I Inhibition Measurements of Actin Pools in Freshly Isolated Photoreceptor Membranes

Treatment	% of total actin in sample
Monomeric actin	
After membrane lysis	68.9
After 5 min in 0.6 M KI	98.2
After 30-min preincubation with 2.5 mM CaCl <sub>2</sub> /1 mM EGTA	77.2
Monomeric actin associated with sedimentable membrane vesicles after sonication	60.2
Monomeric actin in supernatant after sonication and centrifugation	86.2
Total actin remaining in supernatant after sonication	56.0
Total actin in pellet	44.0
Monomeric actin in sample incubated	
14 h at 4°C without ATP	n.d. inhibition
14 h with 2 mM ATP	87.0
With 2 mM ATP and DNase I	99.0
Control at <i>t</i> = 0	80.9
0.5 mM phalloidin	
<i>t</i> = 0 m	96.4 units
<i>t</i> = 20 m	83.7 units
<i>t</i> = 60 m	45.4 units
<i>t</i> = 90 m	n.d. inhibition

Rhabdoms were washed as described, excess liquid was removed from the pellet, and washing medium was replaced by homogenization buffer containing 1% Triton X-100 to give a final concentration of ~5 mg/ml protein. Membranes were lysed by being vortexed repeatedly and measurements were begun within 10 min of membrane lysis.

Phalloidin incubation experiments are expressed as inhibitory units according to Blikstadt et al. (13), since estimates of the percentage of monomeric actin are less reliable over the wide range of enzymatic activities observed in these experiments. Membrane samples used in long term incubations were briefly sonicated and therefore contain a high complement of monomeric actin. *n.d.* inhibition, no detectable inhibition.

tion. This rather large rate constant of repolymerization under conditions that strongly favor the formation of F-actin and are far above critical concentration (25) could be explained by the presence of actin capping factors that prevent polymerization. Since phalloidin acts by stabilizing actin nuclei as they are formed, the generation of filamentous actin will take longer in the presence of such binding factors owing to the various dissociation equilibria.

A little less than two percent of the total actin pool in lysed rhabdoms as assayed in the presence of guanidinium HCl is not accessible to DNase I after depolymerization in the presence of 0.6 M KI. Although nothing is known about the dissociating effects of potassium iodide on actin-associated proteins, this effect could be caused by a tightly actin-bound factor.

To evaluate the relative contribution of peripherally and tightly membrane-associated actin within the microvilli, we sonicated a freshly prepared rhabdom suspension in a large volume of homogenization buffer and sedimented membrane material at 12,000 *g*, which leaves cytoskeletal material in the supernatant. The pellet was then lysed in a small volume of the same buffer containing 1% Triton X-100, following which monomeric and polymeric pools of actin were assayed in both fractions. The experiment revealed that more than half of the

total actin in the sample is liberated into the supernatant fraction by mechanical disruption of rhabdoms. As can be expected from the low protein concentration that results from the dilution step (~150 μg/ml), most of the actin liberated by sonication proved to be monomeric but can be assumed to have been derived from filamentous actin in the sample. In contrast, the pellet fraction exhibited a somewhat smaller amount of monomeric actin than did a total rhabdomeral lysate. We therefore conclude that this membrane-associated actin mainly exists in the form of filaments or short oligomers. SDS PAGE of supernatants revealed that few proteins other than actin are released from the membranes. Among these are a 55-kd protein, proteins of 87 and 115 kd, and small amounts of the major 142-kd glycoprotein (Fig. 3). This experiment does not preclude the possibility that a proportion of the membrane-associated actin is bound in a form that renders it inaccessible to DNase I. To evaluate this possibility, we divided a sonicated membrane suspension into two equal aliquots, one of which was incubated with DNase I for 14 h at 4°C, while the other was left on ice for the same period of time before mixing it with the enzyme. The inhibitory activities were assayed at *t* = 0 and after 14 h. The results indicated that little change in the monomeric actin pool occurred over this time period if DNase was not present. Thus, a slow disruption of filamentous or oligomeric actin by DNase I takes place even in the absence of detergent. That DNase I does not appear to co-sediment with the membrane-bound actin fraction after 30 min of incubation (1 mg/ml) and one successive washing step (Fig. 13) supports this interpretation.

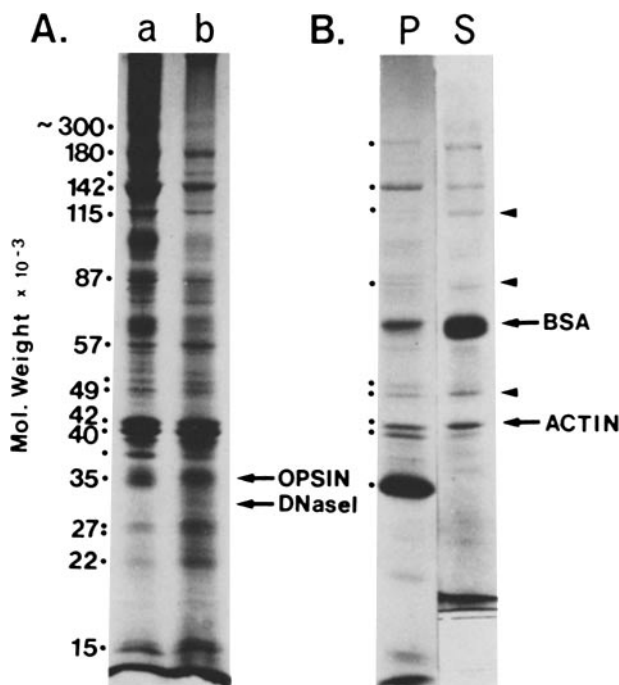


FIGURE 13 (A) Accessibility of membrane-bound actin to DNase I. Cytochrome *c* (a) or DNase I (b) were added at 1 mg/ml to aliquots of sonicated membranes and incubated for 30 min on ice. Pellets obtained after subsequent centrifugation were analyzed on SDS gels and stained with Coomassie Blue. Neither protein co-purifies with the membrane fraction. The position of DNase I is indicated (arrow). (B) Polypeptide composition of the supernatant fraction after sonication and centrifugation. Arrowheads indicate polypeptides that are preferentially lost. Substantial amounts of actin remain in the supernatant. *P*, pellet; *S*, supernatant.



A control sample was incubated with the same concentration of cytochrome *c*, a peripheral membrane protein, to assess nonspecific interactions (Fig. 13A).

We conclude that an actin fraction exists that is tightly bound to the membrane, that does not dissociate on washing, and that does not bind DNase to a significant extent and therefore does not contribute to the pool of monomeric actin in the sample. No filamentous material could be seen in sonicated membrane preparations (Fig. 10) which consist of vesicles 25–250 nm diam and upwards, many of whose diameters approximate those of the microvilli. Failure to demonstrate filaments ultrastructurally should, however, be interpreted with caution.

### Inhibition of DNase I Decays with Time

When aliquots of freshly sonicated rhabdoms were incubated for 14 h at 4°C in the absence of ATP in homogenization buffer, all capacity to inhibit DNase I was lost, independently of the presence of nonionic detergents or antioxidants (Table I). If, however, 2 mM ATP was included in the incubation medium, no loss of inhibitory activity was observed. Similar progressive denaturation of G-actin has been observed in liver plasma membranes (67), where the hydrolysis of free nucleotides leads in turn to the loss of actin-bound nucleotide and failure to inhibit DNase I. In the photoreceptor membrane of invertebrates, a number of nucleotide processing enzymes involved in phototransduction can be assumed to be present and partly responsible for this effect. We emphasize the importance of this observation because a number of structural and functional studies (23, 31, 60) did not recognize the need for actin stabilizing conditions if the cytoskeleton is to survive. Incubation of rhabdomeral membranes in the presence of Ca<sup>2+</sup> for 4 h at room temperature gave no evidence of any

proteolytic activity present in the sample that could be responsible for the breakdown of cytoskeletal elements (not shown).

### Myosin Binding Assay

The myosin affinity technique (26, 40) was used as a second method to assess binding of actin to the microvillar membrane. The result of submitting a detergent lysate of total retinae to repeated precipitations by filamentous myosin, followed by analysis of the actomyosin complex, shows that at least part of the retinal actin binds to vertebrate myosin (Fig. 14d), as indicated by the increase of material co-migrating with actin. The same result is obtained from a detergent lysate of isolated rhabdoms, indicating that at least some of the rhabdomeral actin can bind myosin (Fig. 14).

Two further polypeptides (49 and 54 kd) co-sediment with myosin filaments in extracts of whole retinae. The 49-kd component is a major polypeptide in whole retinae; both are represented as minor components in isolated rhabdoms (Fig. 14f) and cannot be detected after the myosin affinity technique if binding is blocked by presaturating the myosin filaments with vertebrate skeletal muscle actin (Fig. 14b). Unfortunately, the position of the major high molecular weight polypeptides is obscured by the myosin heavy chains. Since none of the co-purifying peptides is a major component of the rhabdomeral membrane, we consider that either they originate from other parts of the tissue, or their binding is nonspecific.

Protein components of the membrane cytoskeleton associated with sedimentable membranes could not be detected with certainty by the myosin affinity technique; impurities in the myosin preparation precluded the use of ultrasensitive staining methods.

### Subcellular Localization of Actin

Frozen sections of the rhabdoms were used to demonstrate the presence of actin by antibodies to scallop actin (21) and by NBD-phalloidin. Our approaches to the localization of actin were determined by penetration problems and nonspecific fluorescence of the pigment granules within the photoreceptors.

Frozen sections provide a built-in control: the retina is isolated from the main haemocoel by a so-called basement membrane, a composite structure built in part from processes derived from the subcorneal cone cells and from glia. The latter contain microfilaments (9), and can be used to assess penetration by labels.

Sections labeled with NBD-phalloidin show intense fluorescence associated with basement membrane structures, and a lesser fluorescence of the rhabdoms (not shown). Myofibrils usually present at the periphery of the eyecup in sections also show intense staining of I bands. Preincubation of the sections in phalloidin for up to 16 h largely abolishes the fluorescence at both basement membrane and myofibril sites, but does not notably affect that of the rhabdoms, indicating that the fluorescence is mainly due to nonspecific binding of the probe.

No differences could be demonstrated between the binding of NBD-phalloidin to sections prepared from frozen, untreated eyes, from retinae infiltrated with Ep-475, and from retinae floated on media containing Ep-475 and EGTA, despite the ultrastructural differences between the states of the

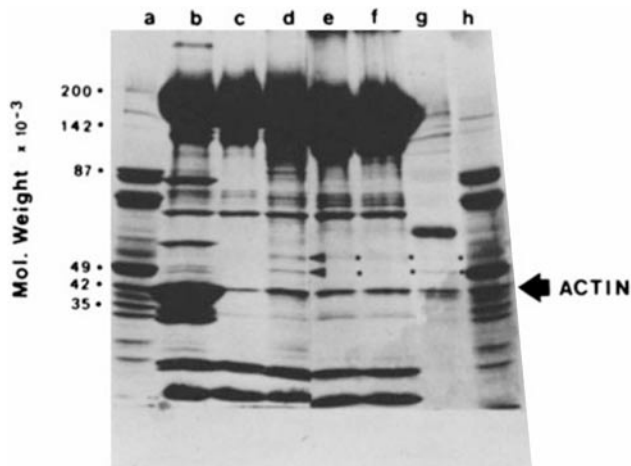


FIGURE 14 Myosin binding assay. Samples were incubated with myosin filaments in the presence of detergent and washed several times (cf., Materials and Methods). (a and h) Total retinal proteins. (b) Myosin preincubated with 50  $\mu$ g of purified vertebrate skeletal muscle actin, then added to a detergent lysate of whole retinae. (c) Purified myosin. (d) Whole retinal proteins. Two polypeptides co-purify with myosin (arrowheads) and are not present in the myosin preparation. (e) Purified rhabdoms. The slight increase in material co-migrating with actin compared with c indicates the presence of actin in the membrane fraction. (f) Same as for e, but using half the concentration of membranes. (g) Isolated rhabdoms without myosin. Coomassie Blue stain.

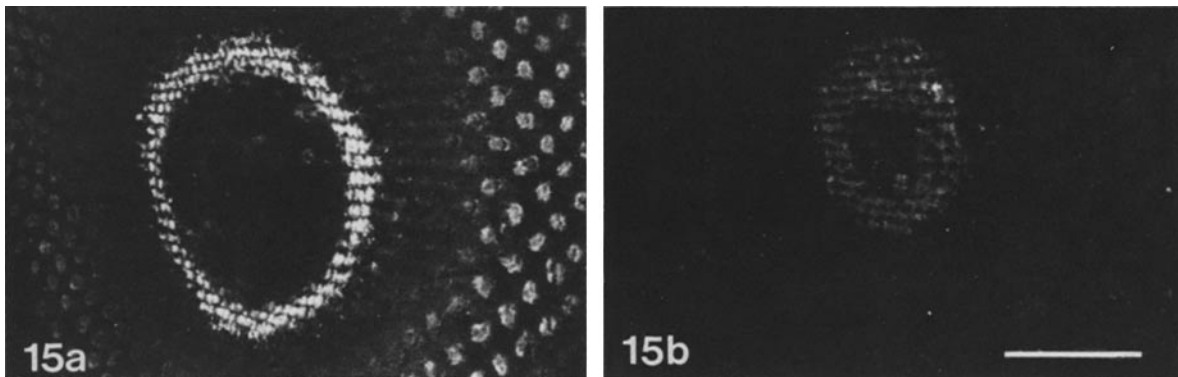


FIGURE 15 Fluorescence micrographs of frozen cross-sections of crayfish retina. (a) After incubation with anti-scallop actin followed by fluorescein isothiocyanate-labeled anti-rabbit IgG. Strongly labeled central ring is basement membrane, peripheral spots are rhabdoms. (b) Adjacent section incubated with rabbit nonimmune serum. Bar, 50  $\mu\text{m}$ .  $\times 260$ .

microvillar filaments in treated and untreated eyes. In initial experiments, antibodies left the rhabdomeral region completely unlabeled when the same penetration and fixation regimes were used. Only extraction of frozen sections with 2% saponin under actin-stabilizing conditions before fixation and prolonged incubation periods with the primary antibody yielded positive rhabdom labeling with consistency, whereas nonimmune sera gave negative results under similar conditions (Fig. 15). Preabsorption of antiserum with insect myofibrils was not sufficient to completely abolish labeling, probably due to the marked species-specific properties of the antibody (21). Nonimmune sera were occasionally found to label the basement membrane weakly even after extensive blocking procedures, probably owing to the presence of autoimmune antibodies to actin which are frequently present in human and in rabbit sera.

#### Protein Composition of the Membrane Matrix

To assess whether the membrane-associated actin is peripherally bound to the photoreceptor membrane or is an integral part of it, membranes were extracted with solutions of low and high ionic strengths (Fig. 16). Relative amounts of actin in the membranes are altered drastically only if detergents are employed. Extraction of sonicated membranes with either 0.5% digitonin or 0.5% Triton X-100 and subsequent centrifugation at 100,000  $g$  leaves behind a purplish pellet which consists of an insoluble membrane framework. SDS PAGE shows it to contain actin (42 kd), a very low molecular weight polypeptide (15 kd), and components at 40, 142, 150, and 180 kd. The material at 142 kd may represent the 145-kd protein described by Saibil (60) from squid photoreceptor membranes after Triton X-100 extraction. We also find a selective enrichment of the two other high molecular weight polypeptides during the extraction step, whereas a minor amount of 142-kd polypeptide and actin can always be detected in the supernatant after a 30-min extraction under conditions favoring polymerization of the latter.

No qualitative differences were found between extractions with Triton X and digitonin; rhodopsin is preferentially removed from membranes by either detergent, although digitonin has recently been shown to be less effective in solubilizing membrane components, presumably because it forms insoluble complexes with cholesterol (46).

A number of minor constituents can be visualized in extracted membranes after silver staining of gels, or by submit-

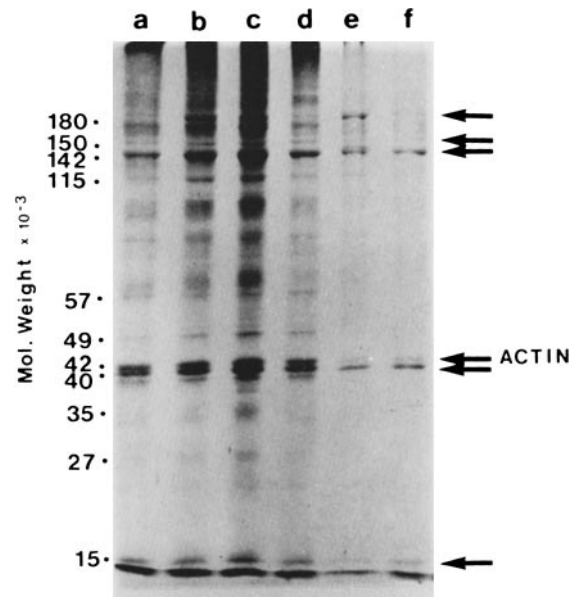


FIGURE 16 The nature of the actin-membrane interaction and of the membrane-associated cytoskeleton, assessed by washing membrane fractions with high ionic strength buffers or buffers containing detergents. Rhabdoms were sonicated and divided into aliquots, and large excesses of washing solutions were added before high-speed centrifugation. Pellets were lysed directly in PAGE buffer and applied to slab gels. Arrows indicate the positions of detergent-resistant polypeptides. Amounts of proteins applied to gel pockets varied in individual experiments, but relative amounts of actin co-sedimenting with the membranes were only altered considerably by detergent extraction. (a) Van Harreveld saline. (b) 2 mM  $\text{MgCl}_2$ . (c) 1 mM EDTA. (d) 1 M NaCl. (e) 1% digitonin. (f) 0.2% Triton X-100. Coomassie Blue stain.

ting higher concentrations of proteins to SDS PAGE, but their status is undetermined.

#### DISCUSSION

##### Membrane-associated Actin in the Crayfish Rhabdom

Association between actin and the plasma membrane of many cells is well documented (4, 5, 17, 18, 32, 41, 47, 52, 53, 58). Best understood is the role of actin in the erythrocyte submembrane cytoskeleton, where together with band 4.1

protein it participates in the spectrin network (recently reviewed by Branton et al., reference 14); the filamentous network parallels the membrane face, to which it is bound.

Recent evidence suggests that this type of membrane association is not restricted to the red cell but represents a universal building block in the architecture of plasma membranes in general (15, 30). In this situation actin behaves in a manner suggestively similar to our findings for rhabdoms. In both cases, no filaments can be visualized ultrastructurally, and actin is present in the form of oligomers. Oligomeric actin behaves similarly to F-actin in DNase inhibition assays in not inhibiting the enzyme initially and gradually disrupting the cytoskeleton during prolonged incubation (61), implying that actin is removed from the sedimentable fraction as a high affinity complex which stays in the supernatant.

The validity of the comparison, however, rests on our assumption that the membrane-bound actin in our rhabdom preparation has not been introduced as a contaminant. Failure of vertebrate  $\alpha$ -actin to co-purify with rhabdoms indicates that any contaminating, nonrhabdomeral actin must stem from the small fraction of other membranes in the final preparation. Our estimate of 5% adulteration from this source is cautiously pessimistic, and we suspect that on average it is rather less. Even at 5% it is difficult on this basis to account for the strong actin band disclosed by SDS PAGE and common to both crayfish and squid (60) rhabdoms and the large proportion of it that is membrane bound.

In other systems, 60% of membrane-bound actin has been shown to be readily solubilized by detergents (4) and removed from the membrane by depolymerizing agents (52), or by dialysis against solutions of low ionic strength (32), which is consistent with the findings presented here. Similarly, in brush border microvilli there is a considerable amount of actin associated with Triton-extracted membrane vesicles after separation from the core (20). It is implied that in the native rhabdom, membrane-associated actin is present in two forms, the larger part loosely bound and able to dissociate from the membrane, while the remainder is tightly bound to the membrane matrix with which it co-purifies after detergent extraction. The tightly bound actin fraction is presumably linked to one or more integral membrane proteins, directly, or via actin-binding, spectrin-like proteins that are in turn linked to an integral membrane component. We have not been able to prove the latter possibility directly. However the fine, meandering filaments seen in isolated rhabdoms appear morphologically similar to spectrin-like proteins (15, 29, 30). Recent demonstrations of actin-binding, spectrin-like proteins in cell types other than erythrocytes (15, 29, 30) including the vertebrate brush-border microvillus, suggest that this molecular component should be sought in the future.

Interestingly, an association of a ConA-binding 140-kd protein with actin in Triton cytoskeletons of brush border membrane vesicles has recently been reported (20) and was suggested to represent a putative anchorage site for actin in the membrane.

### *The Axial Cytoskeleton*

The biochemical results presented here strongly suggest that actin could form or constitute a large part of the axial filament, as proposed for squid microvilli (60), since at least half of the filamentous actin present within the microvilli can be liberated from the membrane by gentle mechanical disruption, and no clear alternative molecular candidate has been found.

On the other hand ultrastructural observations, both those reported here and our previous work on arthropods (7, 8), are hard to reconcile with this interpretation. The large diameter of the filament and the existence of 2–3-nm subfilaments when the central filament is partially degraded are more indicative of intermediate or neurofilament affinities than of actin. Sidearms linking the axial filament to the membrane are more frequent than are those borne by the single actin filament. Morphological preservation of the axial filament is not dependent on tannic acid but is apparently sensitive to a  $\text{Ca}^{2+}$ -activated thiol protease, as are some invertebrate neurofilaments (24). However, we have not yet been able to demonstrate significant proteolytic activity of any nature biochemically.

Hafner et al. (34) found microfilaments within developing crayfish microvilli, but in the same study were unable to demonstrate them in the adult rhabdom. A bundle of actin filaments is however clearly visible in the photoreceptor microvilli of the leech (10), and its preservation characteristics are similar to those of gut microvillar cytoskeletons rather than those of arthropod photoreceptor microvilli.

We are currently attempting to resolve the problem of the identity of the axial filament and its associated proteins by raising monoclonal antibodies to rhabdomeral proteins.

### *Possible Roles for Membrane-bound Rhabdomeral Actin*

Several previous discussions of the composition and architecture of rhabdomeral microvilli are affected by our results. It is no longer clear what proportion of proteins in rhabdomeral membrane fractions actually consist of rhodopsin, for published estimates may have been prejudiced by contamination of the integral membrane proteins by actin (for example, see references 2 and 36). Proteins labeled in autoradiographic experiments on photoreceptor membrane turnover (33, 42, 57, 64) have been assumed to be rhodopsin without positive evidence. Whether actin turns over with the membrane remains to be determined.

Another important issue concerns the effects of membrane-associated actin on the microvillar architecture, and on the properties of the integral membrane proteins to which it is bound. The rigidity and regularity of photoreceptor microvilli have been attributed to the high sterol content of the membrane (36), which has also been proposed to account for the fact that the translational mobilities of rhodopsin in invertebrate microvillar membrane are of notably lesser magnitude than those measured for rhodopsin in the disks of vertebrate rod outer segments (31). In squid microvilli, the axial filament bears too few side-arms to sustain the assumption that each rhodopsin molecule is bound to a side-arm (60). The same stricture applies to crayfish. Although we do not yet know to what integral membrane protein actin is bound, nor the stoichiometry between rhodopsin and membrane-associated actin, it seems possible that translational diffusion of rhodopsin in the microvillar membrane may be constrained, in part, by an association with a submembrane cytoskeleton, such a restrictive mechanism not necessarily requiring that all rhodopsin molecules be tethered. Precedents for such a model are to be found in the relationship between the mobility of integral membrane proteins and the cytoskeleton in erythrocytes (27), and in a recent demonstration that the mobility of Con A receptors on the surface of lymphocytes is increased by several orders of magnitude when the plasma membrane

is separated from its underlying F-actin cytoskeleton (73). Tethering of rhodopsin would be of even greater significance if it were to determine and standardize its rotational orientation in the bilayer, because such a restriction might explain the fact that electrophysiological measurements of the polarization sensitivities of some arthropod photoreceptors, including those of crayfish, are much greater than can be attributed to the form dichroism derived from freely moving rhodopsin molecules (63).

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