The local deletion of a microvillar cytoskeleton from photoreceptors of tipulid flies during membrane turnover

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The distal regions of the photoreceptor microvilli of tipulid flies are shed to extracellular space during membrane turnover. Before abscission, the microvillar tips undergo a transformation: they become deformed, and after conventional fixation for electron microscopy are relatively electron-lucent compared to the stable, basal microvillar segments. We now show that the electron-lucent segment is an empty bag of membrane whose P-face after freeze-etch preparation appears as densely particulate as the remainder of the microvillus. Transformation is achieved by the local deletion of a microvillar cytoskeleton which consists of a single, axial filament linked to the plasma membrane by side-arms. The filament may be partially preserved by the chelation of Ca$^{2+}$; the provision of a divalent cation (Mg$^{2+}$ or Ba$^{2+}$) stabilizes the side-arms during subsequent fixation, as has been shown previously for the rhabdomeral cytoskeleton of blowflies. Incubation of the isolated retina in the presence of 0.25 mM Ca$^{2+}$ at room temperature for 10–20 min causes proteolysis of the cytoskeleton which is blocked by as little as 0.5 mM of the thiol protease inhibitors Ep-475 and Ep-459. Loss of the cytoskeleton is accompanied by deformation of all regions of the microvilli. Local deletion of the cytoskeleton from the transformed zone of the normal rhabdom is sufficient to explain deformation of the microvillar tips, but not their subsequent abscission. The intimate association between a Ca$^{2+}$-activated thiol protease and the cytoskeleton implied by the great rapidity of proteolysis calls for a reassessment of published studies of membrane turnover by radioautography, and of the nature of light-induced damage to arthropod photoreceptor membranes.

INTRODUCTION

Photoreceptors of the compound eyes of tipulid flies shed rhabdomeral membrane to extracellular space during the catabolic phase of turnover. In each rhabdom of an ommatidium, the tips of the microvilli are transformed so as to appear relatively electron lucent in thin sections. At a later stage the microvillar segments forming the transformed zone become swollen and distorted (figures 1 and 2, plate 1); they are later shed by abscission. Shed membrane is rapidly removed from the extracellular space within the ommatidium and returned by phagocytosis to the receptors, in which it accumulates as large secondary lysosomes. In Ptilogyna,
sheding occurs during the period after dawn and is followed by a slow, renewed transformation of the distal regions of the microvilli which defines the zone that will be shed some 24 h later (Williams & Blest 1980). Williams & Blest (1980) and Blest (1980) considered that the loss of electron density by the transformed region might represent the withdrawal of a stabilizing cytoskeleton from the microvillus tips.

Blest et al. (1982) have shown that the rhabdomeral microvilli of blowflies contain a labile, Ca\textsuperscript{2+}-dependent cytoskeleton which is largely destroyed by conventional methods of fixation. A single, axial filament is linked to the plasma membrane by side-arms. In addition, microvilli are bonded together by external bridges which were demonstrated both in thin sections and in freeze-etch replicas. The mechanical importance of the cytoskeleton is not yet understood, but it is reasonable to suppose that it plays some part in generating the remarkable regularity of the microvillar architecture (Horridge & Blest 1980; Stowe 1980).

In the present paper we show that the microvillus tip is transformed by the removal of its contents, and that the deleted components exhibit many of the features of the cytoskeleton found in blowfly rhabdoms. The organization of the integral membrane proteins of the microvillus, as far as it can be resolved by the freeze-etch technique, remains the same in both the transformed and non-transformed regions.

**MATERIALS AND METHODS**

The large tipulid fly *Ptilogyna (Plusiomyia) spectabilis* (Skuse) and various species of a genus of smaller tipulids, *Leptotarsus*, were collected at sites around Canberra. Several species of *Leptotarsus* commonly coexist in a given Australian habitat, and it was impracticable to identify the subjects of experiments to species. Flies were stored for 1–4 days under a natural environmental light cycle as described by Williams (1980b) and Williams & Blest (1980), or for some experiments in continuous darkness.

**Fixation for transmission electron microscopy**

Flies were decapitated, the heads were immediately divided into two halves, and dissection was continued beneath various modifications of a basic stabilizing buffer (20 mM imidazole, 150 mM NaCl, 4 mM KCl, 15 mM MgCl\textsubscript{2}, 80 mM glucose, 10 mM ethyleneglycol-bis(\(\beta\)-aminoethyl)ether)-\(N\),\(\beta\)-tetraacetic acid (EGTA), 1 mM phenylmethanesulphonylfluoride (PMSF), 0.5% (by volume) dimethylsulphoxide (DMSO), pH 6.8–7.0). Head structures, including air sacs, were trimmed away until only the laminae of the optic lobes remained attached to the retinæ. After treatment with stabilizing buffer for 10–20 min at room temperature, retinæ were fixed by means of the following sequence of treatments.

(i) Place in 20 g/l paraformaldehyde, 25 g/l glutaraldehyde, 15 mM MgCl\textsubscript{2}, 10 mM EGTA, 0.09 M sodium cacodylate, 0.12 M sucrose, pH 6.8, at room temperature for 30 min. Cut each retina into two or three pieces.

(ii) Wash once, and then fix for a further 60–90 min in the same cacodylate-buffered aldehyde solution with MgCl\textsubscript{2} and EGTA replaced by 10 mM ethylenediaminetetraacetic acid disodium salt (EDTA).
(iii) Transfer to 4 g/l low molecular mass tannic acid (LMWT: Simionescu & Simionescu 1976) in cacodylate buffer with 4 mM EDTA either for 2–4 h at room temperature and then overnight at 4 °C or for 10–12 h at room temperature.

(iv) Wash in cacodylate buffer with 10 mM EDTA at room temperature for 1–3 h.

(v) Post-fix in 10 g/l OsO₄ in cacodylate buffer for 1 h.

(vi) Wash in distilled water for 30 min, dehydrate through an ethanol series and propylene oxide and embed in Araldite.

With the exceptions noted below, Ca²⁺ must be chelated from all media used to prepare rhabdoms for electron microscopy if the cytoskeleton is to survive (Blest et al. 1982). In addition, divalent cations, preferably Mg²⁺, must be present to conserve the side-arms. However, Williams (1980a) demonstrated that the conversion of microvillar membranes to myelin figures during post-fixation in OsO₄ solution is caused by the presence of Ca²⁺ and White & Michaud (1981) found that other divalent cations, including Mg²⁺, produce the same effects. The schedule of fixation given above floods the tissues with Mg²⁺ for a period sufficient to ensure the cross-linking of side-arm proteins by glutaraldehyde, and then removes it with EDTA before the stage of osmication during which myelin figures are produced.

A number of modifications to this basic protocol are described in the results section. In outline: (a) hypotonic stabilization buffers were employed in which the concentrations of NaCl and glucose were lowered to 50 mM and 40 mM respectively; (b) the protease inhibitor N-α-p-tosyl-L-lysinechloromethylketone (TLCK) was used in combination with PMSF, both at concentrations of 1 mM; (c) the powerful and specific inhibitor of thiol proteases Ep-475 (E-64c) and its analogue Ep-459 (Hanada et al. 1978; Tamai et al. 1981) were used at 0.5–1 mM in modifications of the stabilization buffer; these reagents irreversibly inhibit the Ca²⁺-activated neutral thiol proteases associated with vertebrate neurofilaments (Kamakura et al. 1981; (d) in a few experiments, BaCl₂ was substituted for MgCl₂ in the buffer.

Observations on thin sections and replicas

Grey sections were stained with uranyl acetate and lead citrate after mounting on Formvar-coated 400-mesh grids (aperture width ca. 40 μm) and viewed in a Hitachi H600 electron microscope. The relation between section thickness and microvillus diameter, which are of the same order of magnitude, produces some problems of interpretation. These, and the difficulties of matching features of freeze-etch replicas to those seen in sections of rhabdoms are discussed by Blest et al. (1982).

Preparation of freeze-etch replicas

Retinae were fixed either without pre-treatment or after dissection beneath the basic stabilization buffer. After the second stage of fixation, glycerol was added to the EDTA-containing fixative to a final concentration of 25–30 % (by volume). Retinae mounted on gold holders were frozen in Freon 22 cooled by liquid nitrogen, fractured at −110 to −118 °C and 10⁻⁵ to 10⁻⁶ Pa in a Balzers BAF 300 apparatus equipped with a quartz-crystal thin-film monitor, and coated immediately with platinum–carbon and carbon films. The replicas were cleaned with chromic acid and/or chromosulphuric acid, mounted on 100-mesh grids (aperture width ca. 150 μm) coated with Formvar, and viewed in a Hitachi H500 electron microscope.
When necessary, replicas were removed from their grids and cleaned again, by the procedure described by Stowe (1981).

Results

Anatomy

The retinal anatomy of *Leptotarsus* resembles that of *Ptilogyna*, described in detail by Williams (1980b, 1981). Each ommatidium consists of six peripheral receptors (R<sub>1-6</sub>) arranged in a circle, and two central, tiered receptors (R<sub>7,8</sub>). Distally, the rhabdoms of R<sub>1-6</sub> and R<sub>7</sub> are contiguous; in the middle of the ommatidium all rhabdomeres are separated by a shared extracellular space; proximally, R<sub>1-6</sub> and R<sub>8</sub> are each invaginated to form a pocket which encloses the rhabdom and the extracellular space surrounding it (see for example, figure 1, plate 1). Although each receptor contains screening pigment, most abundantly in the proximal, pocketed region, it does not act as a longitudinal pupil (Kirschfeld & Franceschini 1969). The primary pigment cells just beneath the crystalline cone act as an annular field stop which opens to admit light to R<sub>1-6</sub> in the dark-adapted state at night and constricts during the day largely to confine incoming light to the two central, tiered rhabdoms. The pre-fixation treatments used in the present experiments chelate Ca<sup>2+</sup>, and thus effectively force the receptors into the dark-adapted state (Kirschfeld & Vogt 1980), irrespective of the illumination under which dissection is done. The rhabdoms of tipulids are only some 30–50 μm in length (Williams 1980). Microvillar diameters may vary from 50 to over 60 nm, occasionally within a single rhabdom.

The shedding zone

The distal microvillar regions destined for shedding form a well defined zone in sections of rhabdoms that have been conventionally fixed for electron microscopy (figure 2, plate 1). The shedding zone is produced by the gradual transformation of the tips of the microvilli throughout the day. In *Ptilogyna*, membrane is shed at some time between dawn and noon, to leave microvilli that either lack electron-lucent tips or possess short transformed regions (Williams & Blest 1980). Flies left in the dark for up to 72 h continue to assemble microvillar membrane and the return of such flies to the light provokes massive shedding. A small number of observations of *Leptotarsus* suggest that the control of turnover may be different: flies held in the dark for long periods do not assemble an excess of

Description of Plate 1

Figure 1. Transverse section through a rhabdom of *Leptotarsus* lying in the proximal pocket formed from its own photoreceptor, after pretreatment with the basic stabilization buffer, and post-fixation mordanting in LMWT. The shedding zone (Sh.Z) is clearly delimited (arrows) from the rest of the rhabdom (Rh) by its lesser electron density. (Magn. × 16 200.)

Figure 2. Longitudinal section through rhabdomeral microvilli of *Leptotarsus* after conventional fixation. The microvillar lumen shows little evidence of organized structures, and the shedding zone at the right of the figure is composed of deformed microvillar tips of lesser electron density than the stable region to the left. (Magn. × 54 000.)
Figures 1 and 2. For description see opposite.
Figures 3–5. For description see opposite.
DESCRIPTION OF PLATE 2

Figures 3–5. Transitions between the stable regions of rhabdoms of *Leptotarsus* and their transformed shedding zones, after treatment with the basic stabilization buffer and post-fixation mordanting in LMWT. There is a sharp demarcation between the shedding zones, whose microvillar segments are empty, and stable regions of the rhabdoms where the microvilli contain an imperfectly preserved cytoskeleton. (Figures 3 and 4 magn. ×120000; figure 5 magn. ×97000.)

DESCRIPTION OF PLATE 3

Figure 6. Longitudinal section of microvillus tips after treatment with hypotonic stabilization buffer containing 10 mM EGTA. The terminal segment of an axial filament is preserved in the central microvillus and ends abruptly (black arrow) as the tip of the microvillus dilates. Lateral densities representing presumptive side-arms, or regions of close relationship between axial filament and plasma membrane, are indicated by white arrows. (Magn. ×170000.)

Figures 7–9. Longitudinal sections of microvilli after treatment with stabilization buffer containing 0.25 mM Ca²⁺ and 1 mM Ep-459. Figure 7 shows a continuous axial structure with ill defined contacts with the plasma membrane; figure 8 shows irregular side-arms in the region bracketed between the two arrows; figure 9 shows small, circular densities (arrowed) which are often seen near the midline of the microvillus in longitudinal section, and which are assumed to be side-arms seen end on. (Magn. ×182000.)

Figure 10. Approximately transverse sections of microvilli after treatment with stabilization buffer containing 0.25 mM Ca²⁺ and 1 mM Ep-459. The transition to the shedding zone is at the far right, where the individual profiles are empty. Proximal regions of the microvilli have central densities which are fairly consistently present, linked by presumptive side-arms to the plasma membrane. (Magn. ×170000.)

DESCRIPTION OF PLATE 4

Figures 11 and 12. Longitudinal sections of microvilli after treatment with buffer containing 0.25 mM Ca²⁺ and 15 mM Mg²⁺, but without either EGTA or Ep-459. Microvilli are distorted and devoid of organized contents, and in figure 11 degraded material has dispersed into the shedding zone. The plasma membrane is poorly preserved, despite tannic acid mordanting. (Figure 11 magn. ×37000; figure 12 magn. ×182000.)

Figure 13. Transverse section of a rhabdom treated with stabilization buffer containing 10 mM EGTA and 15 mM Mg²⁺, but without the use of EDTA in subsequent processing stages to chelate Mg²⁺ after stabilization of the side-arms of the cytoskeleton. The shedding zone (large arrow) has collapsed completely during osmiumation. Small arrows indicate radial filaments of the extrarhabdomeral cytoskeleton. (Magn. ×22000.)
Figures 6–10. For description see reverse.
Figures 11–13. For description see facing plate 2.
Figure 14. Transverse section of a rhabdom from R$_{1-6}$ after injury to its axon during dissection into stabilization buffer containing 0.25 mM Ca$^{2+}$ and 1 mM Ep-459. Microvilli are grossly swollen compared to those of the stabilized rhabdom of figures 13, and the organization of the rhabdom has begun to disintegrate at the proximal ends of the microvilli, which are devoid of structured contents. (Magn. $\times$ 22000.)

Figure 15. Microvilli from a damaged rhabdom in longitudinal section. A microvesicle of internalized membrane (arrowhead) lies within a microvillus, remote from the shedding zone. In one microvillus a small length of axial filament has survived (small arrows). (Magn. $\times$ 120000.)
Figures 16-18. Freeze-etch replicas of shedding zones in three states. In figure 16, only the extreme tip of each microvillus is transformed; in figure 17, transformed segments are substantial; in figure 18, the shedding zone is vesiculating. Points at which transitions from stable to shedding zones are presumed to occur are indicated by arrowheads. (Figures 16 and 17 magn. $\times 92000$; figure 18 magn. $\times 78000$.)
Figure 19. For description see opposite.
rhabdomeral membrane, and fail to exhibit exaggerated shedding when returned to the light. A comparative study of flies identified to species is needed. For the present purposes, both genera were sampled either after being held in darkness overnight until dissection during the morning, or some 3–4 h after dawn under a normal daily cycle of illumination, in order to obtain microvilli in a range of states.

The microvillar cytoskeleton

Our initial attempts to stabilize the cytoskeleton followed the procedures given by Blest et al. (1982), but the amount of EGTA in the stabilization buffer was increased from 2 to 10 mM, and that of Mg$^{2+}$ from 2 to 15 mM. These treatments gave erratic and fragmentary preservation of the cytoskeleton, to reveal that the microvillar tips of the shedding zone are empty (figures 3–5, plate 2) and that some material within the microvillar lumen had, at least, been immobilized by the procedure, but the overall picture was far from satisfactory. It was only marginally improved by using hypotonic buffers which caused a proportion of microvilli to swell slightly (figure 6, plate 3). No additional improvement was yielded by combining the protease inhibitor TLCK with PMSF, and little difference could be observed between retinae supplied with 15 mM Mg$^{2+}$ as the divalent cation and those given 15 mM Ba$^{2+}$. It is likely that the very large sizes of tipulid rhabdoms compared to those of blowflies make the satisfactory removal of Ca$^{2+}$ by chelation impossible and the penetration of other reagents difficult.

Two sets of experiments were done with E-64 analogues. In one, retinae were treated during and after dissection with stabilization buffer in which EGTA was replaced by 2 mM Ca$^{2+}$ and to which was added 0.5 mM Ep-475 (E-64c). In the second series, 0.25 mM Ca$^{2+}$ was combined with 1 mM Ep-459. Both series of trials were done with and without 1 mM PMSF, and in the second series both combinations were done with normal and with hypotonic buffers. All retinae were fixed in 25 g/l glutaraldehyde solution without EGTA and with 10 mM Mg$^{2+}$, and after 30 min were transferred to fixative solution containing EDTA.

Both Ep-475 and Ep-459 are sufficient, by themselves, largely to preserve the axial filament in the presence of Ca$^{2+}$ (figures 7–10, plate 3). Ep-459 was the most

Figure 19. A diagram to summarize the events inferred to take place in the course of the daily cycle of photoreceptor membrane turnover in Ptilogyna; it combines data from the present paper with those of Williams & Blest (1980). (a) Before dawn, the shedding zone is deep and composed of empty microvillar tips that are still adherent to each other. Near them, the stable region is particularly electron-dense because it contains material transported from the shedding zone. (b) After dawn, membrane is shed from the microvilli by two routes; the microvillar segments in the shedding zone separate, vesiculate and drop off, and the membranous detritus is sequestered by pseudopodia of the receptor (PS), retrieved into phagocytic vesicles (PH.V) and concentrated into large multivesicular bodies (MVB). The multivesicular bodies also receive pinocytotic vesicles derived from the microvillar bases (PV). (c) At the completion of shedding, the microvillar tips are untransformed, and the multivesicular bodies have started to degrade to multilamellar bodies (MLB). (d) By dusk, a new shedding zone has started to form, and most secondary lysosomes are degraded to dense bodies (d.b.) in the process of lysis. The mode of addition of membrane in tipulids has not yet been analysed and is not therefore indicated in the scheme.
effective, possibly as a consequence of better penetration. Figures 11 and 12, plate 4, show the effect of incubating retinas in buffer containing 0.25 mM Ca\(^{2+}\) for 20 min without Ep-459. Without the inhibitor the microvillar contents were lysed and dispersed to become evenly distributed between the shedding zone and the basal region. In the presence of the inhibitor, the microvillar tips remained empty and the basal region contained an organized cytoskeleton (figure 10). Even in the presence of high concentrations of Mg\(^{2+}\), the side-arms were poorly preserved.

No improvement was seen as a result of adding PMSF to the medium, and it was not necessary to employ hypotonic buffers. The dissolution of the cytoskeleton in the presence of Ca\(^{2+}\), therefore, results solely from the action of a Ca\(^{2+}\)-activated neutral thiol protease. The difficulty of stabilizing the cytoskeleton by conventional fixation implies a phenomenally rapid proteolytic action, and suggests that the protease is intimately associated with the filament that it destroys.

Blest et al. (1982) believed that adding PMSF to their stabilization buffer improved the resolution of the cytoskeleton in blowfly rhabdoms, but it is now clear that the concentrations of EGTA and Mg\(^{2+}\) that they used were inadequate. The possible implication of a second, serine protease in the blowfly system requires to be re-examined in the context of experiments with E-64 analogues.

The axial filament of the blowfly microvillus terminates distally in a dense structure at the tip of the microvillus, termed the ‘cap’ by Blest et al. (1982), and the neck of the microvillus contains an electron-dense plug which surrounds the end of the filament. Neither structure is found in the tipulid microvillus. Distally, the filament ends abruptly at the boundary of the empty region of the shedding zone (figure 6); the terminal region often seems especially electron-dense and may differ from the proximal part of the filament. The longitudinal side-arm periodicity lies around 40 nm, and is variable, as it is in the blowfly.

**The effects of divalent cations during fixation**

White & Michaud (1981) noted that failure to remove divalent cations, including Mg\(^{2+}\), from media used during fixation for electron microscopy produced membrane whorls from rhabdomeral membrane. Williams (1980a) earlier ascribed such effects to failure to remove Ca\(^{2+}\), and suggested that they only occurred in regions from which membrane was about to be shed in the course of turnover. The prolonged tannic acid treatments used in the present work exacerbate these effects, and confirm Williams’s hypothesis. Without EDTA to chelate Mg\(^{2+}\), the shedding zone collapses completely (figure 13, plate 4); in some sections, the basal region of the rhabdom is also damaged to a lesser degree in a pattern that resembles the distribution of pinocytic vesicles (cf. figure 19).

**Damaged cells**

Blest et al. (1982) proposed that in the course of conventional fixation destruction of the cytoskeleton occurred because rapid depolarization of the invertebrate photoreceptor consequent upon damage generates an influx of calcium ions; it was suggested that this would switch on any Ca\(^{2+}\)-activated protease associated with the cytoskeleton. The optic lobes and lamina of *Leptotarsus* can be detached from the retina by pulling them gently: the whole basement membrane is stripped away.
neatly from the retina, breaking all the receptor axons. When this is done immediately that a half head has been immersed in buffer containing Ep-459, all rhabdoms assume the appearance shown in figures 14 and 15, plate 5. The microvilli are swollen, often distorted, empty of organized contents, and disintegrating in a characteristic manner at their bases. If the medulla is separated from the lamina by a cut severing the axons of R_{7-8} alone, only the rhabdoms of those two cells are affected. This suggests that depolarization induced by damage does, indeed, activate rapid proteolysis before the inhibitor has penetrated to the rhabdom.

Freeze-etch observations

Observations were made on replicas derived from retinae in a variety of shedding states. Satisfactory replicas of tipulid rhabdoms were difficult to obtain, because of problems associated with their large size and the rugged fracture topography generated by the large pigment granules. Shedding zones in three states are shown in figures 16–18, plate 6. In figure 16, shedding has been recently completed, and only the extreme tips of the microvilli have been retransformed. Comparisons between replicas and sections show that the shedding zone starts at approximately the levels marked for exemplary microvilli by arrowheads. In figure 17, an extensive fringe has been transformed, and the irregular microvillar profiles of the shedding zone contrast with the regularity of the untransformed segments at the right. The microvillar tips in figure 18 have started to vesiculate and detach. In all cases, the density of particles on the P-faces of transformed and untransformed regions appears to be essentially the same.

The extrarhabdomeral cytoskeleton

Radially disposed elements are often observed at the base of the rhabdom, most frequently in retinae that have been treated with E64 analogues. Some are visible in figure 13. They will not be discussed here other than to note that one type with a diameter of 8–10 nm and irregular projections appears similar to the axial filament of the microvillus, is aligned parallel to the microvilli and is sometimes so disposed as to suggest that it may be in continuity with the axial cytoskeleton. We have seen similar components in retinae of blowflies and Drosophila, and they resemble radially arranged fibrillar elements in the retinulae of toadbugs (Burton & Stockhammer 1969). Similar radial arrays have also been observed in Musca (K. Kirschfeld, personal communication). A second type consists of a loosely radial arrangement of microfibrils which have the ultrastructural appearance of actin filaments. Even after treatment with PMSF and Ep-459 or Ep-475 in combination, neither component is consistently seen outside the rhabdom, and there is no relation between successful preservation of the microvillar cytoskeleton and the presence of the extrarhabdomeral elements.

Discussion

Although the microvillar cytoskeleton of tipulid flies has proved more difficult to preserve than that of blowfly photoreceptors, the same features have been
revealed: an axial filament can be distinguished, usually in rather fragmentary form, linked by side-arms to the plasma membrane. When side-arms do not survive fixation, periodicities are often observed, which probably represent their degradation products. In the tipulid microvillus, however, the cytoskeleton ends abruptly at a greater or lesser distance from the tip. The segment from which it is deleted corresponds to the region observed to be deformed in thin sections and freeze-etch replicas, but adjacent microvillar tips remain cemented together until shedding, presumably because the extracellular bridging material is still intact. Thus the internal cytoskeleton can be postulated to contribute to the rigidity of the microvillus and therefore to the regularity of the whole assembly. Nevertheless, substantial lengths of microvillus from which the cytoskeleton has been deleted must remain intact throughout the day; while deletion precedes shedding, and appears to define the limit of what will be shed, it cannot itself be responsible for abscission. Cytoskeletal deletion may, however, underlie the vulnerability of shedding zones to divalent cations during fixation, which the present study incidentally confirms.

The shedding of microvillar membrane is known to be a daily event (Williams & Blest 1980). It follows that, as soon as a segment of microvillus has been shed, a new shedding zone is created by the withdrawal of material from the microvillus tip that remains. Much of this material must consist of the cytoskeleton. A postulated sequence of events that combines our inferences about the behaviour of the cytoskeleton with the data of Williams & Blest (1980) is given in figure 19.

Transformation of local regions of microvilli by cytoskeletal deletion implies that the control of the microvillar infrastructure is complex. Blest et al. (1982) inferred that the cytoskeleton has an associated Ca$^{2+}$-activated protease with a calmodulin requirement as a minimal explanation of their pharmacological observations. The effectiveness of Ep-459 and Ep-475 in protecting the tipulid cytoskeleton confirms the present of a Ca$^{2+}$-activated protease, for these analogues are specific inhibitors of thiol proteases, including vertebrate neurofilament proteases, thiol cathepsins and papain, while having no activity against either non-thiol proteases (trypsin, α-chymotrypsin, pepsin, elastase, bacterial collagenase) or non-proteolytic thiol enzymes (e.g. porcine lactate dehydrogenase, bacterial glyceraldehyde-3-phosphate dehydrogenase) (Tamai et al. 1981; K. Hanada, personal communication). The vulnerability of the cytoskeleton to Ca$^{2+}$ in pretreatment solutions and fixatives implies that the protease is present throughout the length of the microvillus; either a second mode of activation exists, or deletion is mediated through a different enzymatic system. The results of preliminary experiments in which tipulid retinae were incubated in media combining the calmodulin blocking agent trifluoperazine with calcium were ambiguous, although the same media stabilize the cytoskeleton in blowfly rhabdons (Blest et al. 1982). A role for calmodulin in the control of the cytoskeleton in tipulid rhabdons is questionable, and in the blowfly microvillus it requires more direct proof.

Procedures for stabilizing the cytoskeleton of tipulid rhabdons are analogous to those effective for the blowfly, and the structures revealed are similar. Blest et al. (1982) note that the ultrastructural evidence does not allow a satisfactory identification of the nature of the axial filament. Saibil (1978) and Horridge & Blest (1980) have suggested that actin may form the basis of the cytoskeleton, following
the precedent of the vertebrate intestinal brush border (see Horridge & Blest (1980) and Blest et al. (1982) for a comparative discussion). In tipulids, as in blowflies, measurements of the axial filament are difficult to interpret because it seems likely that it is always partly degraded and there is much adherent material. The microvilli of leech photoreceptors are wide enough to accommodate bundles of longitudinal filaments that are stable to conventional fixation and probably consist of actin (illustrated without comment by Walz (1979)). During the morphogenesis of crayfish photoreceptor microvilli, a similar bundle, also stable to conventional fixation, has been observed; it vanishes later in development as the primordial microvilli narrow to the diameters characteristic of the mature rhabdom (Hafner et al. 1982). While it is possible that a bundle of actin filaments is thinned out to yield a single survivor to which a Ca$^{2+}$-activated protease is then added so that the cytoskeleton becomes unstable, it seems more likely that an actin skeleton is replaced by a neurofilament. The behaviour of the cytoskeleton to divalent cations (Blest et al. 1982) and the presence of an associated Ca$^{2+}$-activated neutral thiol protease are consonant with recent findings on invertebrate neurofilaments (Eagles et al. 1981; P. A. M. Eagles, personal communication), although the rate of proteolysis is remarkable.

It is clear that the microvillar segments forming the shedding zones of tipulid rhabdoms are void of contents in life. This conclusion is reinforced by a comparison between retinae incubated in Ca$^{2+}$-containing buffers with and without the addition of Ep-459. In the presence of the inhibitor, the microvillar infrastructure is stabilized, and the transformed region remains empty. Incubated with Ca$^{2+}$ alone, the cytoskeleton is lysed and the products are dispersed throughout the microvilli so that the shedding zone differs little in electron density from the basal segments. A further consequence is the implication that each microvillus possesses a system that transports mobilized materials towards its base.

An important question concerns the state of the membrane proteins and phospholipids in the transformed segments of the shedding zone. The freeze-etch technique does not distinguish between rhodopsin and metarhodopsin, nor does it reveal anything about the phospholipid components of the membrane. Thus, the present experiments do not allow us to say that the instability of the transformed region of the microvillus arises solely from the absence of the cytoskeleton; alterations to membrane components could also contribute to changes in its conformation (Kirkpatrick 1979). However, after incubation in 0.25 mM Ca$^{2+}$ alone, all regions of the microvilli tend to an irregular and distorted appearance consistent with a mechanical role for the internal cytoskeleton. This would not necessarily require the cytoskeleton to be static, and it is not impossible that it will prove to be in a state of flux in the living cell.

The demonstration of a microvillar cytoskeleton has more general significance. For example, strong illumination causes the microvillar organization of some rhabdoms to break down; light of defined wavelengths can be used selectively to destroy particular receptors in an ommatidium while sparing others, so that the spectral characteristics of the vulnerable cells can be inferred (see for example: Gribakin 1975; Welsch 1977; Langer et al. 1979). Analogous selective effects have been described when compound eyes are fixed for electron microscopy in aldehyde.
solutions of varied pH (Menzel & Blakers 1975). Authors have been tempted to ascribe these results of stress or hyperillumination to unspecified properties of the transductive membranes themselves, while admitting that a satisfactory explanation eludes them. The collapse of a supportive cytoskeleton in the face of excessive calcium fluxes mediating their effects through proteolysis is a more attractive basis for speculation, for it can be tested by simple experiments.

All rhabdoms fixed by conventional methods for light or electron microscopy are clearly left in a partly degraded state. Thus, the cumulative results of experiments in which rhabdoms are labelled by radioautography (Perrelet 1972; Hafner & Bok 1977; Krauhs et al. 1978; Stein et al. 1979) are subject to two major difficulties of interpretation. (i) If cytoskeletal components turn over and are labelled by $^3$H-leucine, then after fixation they will be broken down and topographically redistributed within each microvillus. Such a dispersal might account for the puzzling observation that, after label has been injected, rhabdoms seem rapidly to be labelled throughout their depth. (ii) If proteolysis reduces the cytoskeleton to fragments of sufficiently small size, labelled cytoskeletal proteins might elude detection on gels, and allow the conclusion of Stein et al. (1979) that the bulk of $^3$H-leucine is incorporated into rhodopsin at the end of short-term labelling experiments.

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References


Local deletion of a microvillar cytoskeleton


Saibil, H. 1978 Actin in squid retinal photoreceptors. J. Physiol., Lond. 281, 17P.


Stein, R. J., Brammer, J. D. & Ostroy, S. E. 1979 Renewal of opsin in the photoreceptor cells of the mosquito. J. gen. Physiol. 74, 565-582.


Williams, D. S. 1980b Organisation of the compound eye of a tipulid fly during the day and night. Zoomorphologie 95, 85-104.
