

Evidence for a Pathway of Distal Screening Pigment Granules across the Basement Membrane of the Crayfish Photoreceptor

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The distal pigment cells of *Orconectes limosus* contain two layers of large electron lucent vacuoles that are separated by layers of small right-angled platelets adjacent to the crystalline cones. The crystalline cones of the dioptric apparatus of this species have evaginations into the distal pigment cell cytoplasm. In photoreceptors of *Orconectes limosus* and *Procambarus clarkii* a dark pigment accumulation site was detected just distal to the basement membrane at the edges of each retina. These pigment accumulations occurred independent of the state of light adaptation. Ultrastructurally the pigment granules at this accumulation site resemble distal screening pigment granules according to their size (up to 1.2 µm in diameter) and fibrous structure. Distal screening pigment granules were also found in tube-like cell processes or extracellularly within and proximal to the retinal basement membrane, indicating pigment transport to and across the basement membrane. Proximal to the basement membrane screening pigment granules were also observed disintegrated to a gravel-like electron dense material in widely branched cells. Evidence was found that an electron dense material, probably resulting from disintegrating screening pigment granules, was incorporated in the integument of the eyestalk. Four hours after injection of gold particles into the eye stalk distal to the retina they were detected inside and proximal to the retinal basement membrane.

Introduction

Compound eyes are composed of structural units called ommatidia. Each ommatidium, as shown in Fig. 1, has on its distal end the dioptric apparatus composed of corneal lens and crystalline cone which is surrounded by distal, or primary pigment cells. These distal pigment cells serve to optically isolate the ommatidia in dependence of the state of adaptation (for review see [1–4]). In the superposition-type eyes of crayfish the sensory cells are separated from the cone by crystalline tracts [5]. Proximal to the cones are reticular cells that are visual sense cells containing the machinery for phototransduction in microvillar rhabdomeres. Reticular cells are pigmented too and sometimes called secondary pigment cells.

The present study was intended to yield ultrastructural information on a specific large pigment accumulation close to the retinal basement membrane of the crayfish photoreceptor. This accumu-

lation had already been observed in a previous study [6] but had not yet been described in detail.

Materials and Methods

Light- and electron microscopy

Eye stalks from ten adult crayfish (*Orconectes limosus*) kept in outdoor aquaria under a natural light cycle at 15–18 °C were removed at midday (light-adapted) or midnight (dark-adapted). The size of the animals differed only little (body weights 22–25 g). The retinæ together with the optic gangliae were excised and fixed in 4% glutaraldehyde in 0.1 M cacodylate buffer of pH 7.6 containing 2% sucrose and 2 mM CaCl₂ for several hours at 4 °C. Retinæ were postfixated with 1% OsO₄ at room temperature in 0.1 M cacodylate buffer for 3 h, bloc-stained in 5% uranyl acetate in 70% alcohol for 1 h and embedded in Spurr's medium following standard techniques. The distal part of the remaining eye stalks, after removal of the retinæ, containing corneae, crystalline cones and distal pigment cells was dissected, fixed and embedded as described above.

Additionally 5 complete eyestalks from 5 light-adapted juvenile *Procambarus clarkii* (rostrum-

telson length 1 cm) were hemisected with a razor blade and fixed as described above.

Procambarus clarkii were bred in our laboratory and kept in aquariae illuminated with fluorescent light under a 12 h light-dark rhythm.

After staining with toluidin blue semithin sections (1 μm) were examined and photographed under a Leitz Orthoplan light microscope. Ultrathin sections were stained with uranyl acetate and lead citrate and observed with a Philips EM 300.

Glycogen staining according to de Bruijn

The presence of glycogen was determined by the method of de Bruijn [7] in which specimen were kept in 0.05 M $\text{K}_3[\text{Fe}(\text{CN})_6]$ added to the OsO_4 fixative for 24 h at room temperature and afterwards embedded in Spurr's resin. Staining with uranyl acetate could not be applied in this case to prevent contrasting of ribosomes. The contrast of ultrathin sections with and without glycogen staining was compared. In both cases sections were used without poststaining.

*Injection of colloidal gold particles into the photoreceptor of *Orconectes limosus**

A small hole for pressure balance was pricked with a glass microelectrode into the cornea. Then a glass microelectrode (tip diameter approx. 30 μm) was inserted elsewhere into the cornea, and 0.5 μl colloidal gold particles (17 nm), coupled to bovine serum albumin according to the method of Horisberger [8], was injected into the space surrounding the crystalline cones distal to the retina. The position of the microelectrode is indicated in Fig. 1. The gold particles were prepared according to Frens [9]. Care was taken that the tip of the microelectrode was directed towards the distal end of the cornea to avoid damage of the basement membrane (Fig. 1). After injection the crayfish were put back into the aquariae for 4 h after which time their retinæ were isolated and embedded for electron microscopy as described above.

Results

Macroscopic observation

The optic neuropil, removed together with the retina from the eyestalk of *Orconectes*, showed several black spots.

Light microscopic observations

In semithin sections of the isolated retina of *Orconectes* accumulations of screening pigment granules were found at the margin of each retina between photoreceptors and basement membrane. Such an accumulation is schematically shown in Fig. 1. This pigment granule accumulation was observed independent of the state of adaptation.

In each eye of *Procambarus* this pigment granule accumulation was also observed (Fig. 2). One crayfish was fixed during a premolting stage indicated by the existence of two cuticulae (Fig. 2).

*Ultrastructure of the distal pigment cells of *Orconectes limosus**

The cytoplasm of distal pigment cells was densely filled with screening pigment granules (Fig. 3) but rarely contained cell organelles like endoplasmic reticulum or mitochondria. The topographic position at which the electron micrograph was taken is indicated in Fig. 1, area 1. However, Golgi bodies forming large electron lucent vesicles occurred regularly. Distal pigmentary organelles reach diameters of up to 1.2 μm and occasionally show a fibrous substructure. The distal pigment cells contain two layers of large electron lucent vacuoles, adjacent to the crystalline cones, that are

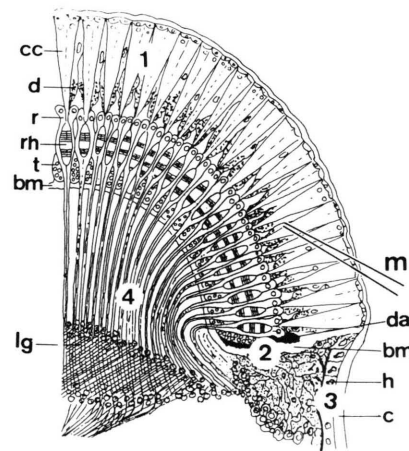


Fig. 1. Schematic drawing of a longitudinal section through one half of a crayfish eye. The numbered areas mark the positions of which micrographs were taken (bm = basement membrane, c = cuticle, cc = crystalline cone, d = distal pigment cell, da = distal pigment granule accumulation, h = hypodermis, lg = lamina ganglionaris, m = microelectrode, r = retinular cell, rh = rhabdom, t = tapetum cell).

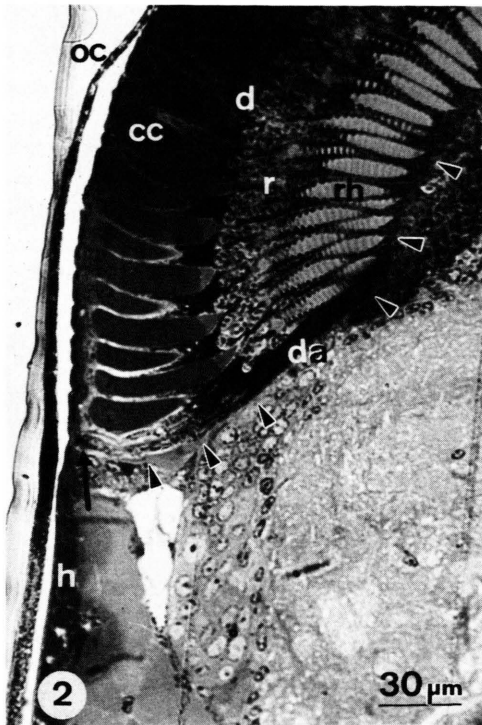


Fig. 2. Light micrograph of a semithin section of an eyestalk of *Procambarus clarkii* that had been fixed just before molting. The old cuticle (oc) is loosely fitting the eye. A large accumulation of distal pigment granules (da) is located between the retinular cells and the basement membrane the position of which is indicated by arrowheads. The arrow marks hypodermis cells (h) containing pigment granules that are ultrastructurally shown in Fig. 11 (cc = crystalline cone, d = distal pigment cell, h = hypodermis, r = retinular cell, rh = rhabdom).

separated by one layer of small rectangular platelets (Fig. 3). The crystalline cones periodically had evaginations into the distal pigment cell cytoplasm (Fig. 3). These evaginations consist of a material that is separated by membranes from the crystalline cone (Fig. 4) and forms a small border around it. Distal screening pigment granules were also found in the acellular space between the crystalline cones.

Ultrastructure of the pigment accumulation close to the basement membrane in the isolated retina of Orconectes limosus

For description of the ultrastructural morphology of the crayfish retina see [10]. The position of the pigment granules dependent on the state of

adaptation was shown previously [6]. Here only ultrastructural findings related to distal screening pigment granules are described. The topographic position of this site within the cell layer is indicated as area 2 in Fig. 1. In longitudinal sections the pigment accumulation close to the basement membrane is restricted to few narrow, longitudinal cell processes which are located at the proximal side of the basement membrane (Fig. 1, 2, 5). These cell processes are limited proximally by the basement membrane and distally by tapetum cells or retinular cells (Fig. 1, 5) and could be clearly distinguished from retinula cells by the morphology of their pigmentary organelles and cytoplasm. These pigmentary organelles are identical in morphology and size with distal pigment granules (Fig. 3) and reach diameters of up to $1.2\ \mu\text{m}$ (Fig. 5) whereas those of photoreceptor cells are maximally $0.8\ \mu\text{m}$ in diameter. Some of the distal granules are not completely electron dense, which shows that they consist of fibrous material. The cytoplasm of these cell processes often appears electron optically empty and contains only occasionally cell organelles like lucent vesicles or mitochondria (Fig. 5). This emptiness of the cytoplasm could not have been artificially induced by mechanical damaging or fixation because it was restricted to this type of cell and was never observed in adjacent retinular or tapetum cells. Moreover, such "empty cellular projections" containing distal screening pigment granules were regularly observed in the centre of the retina close to the basement membrane. However, the cellular nature of these projections is indicated because they are surrounded by their own cell membranes and contain microtubules.

Proximal to the accumulation of distal pigment granules these distal pigmentary organelles were detected within and proximal to the basement membrane (Fig. 6). Screening pigment granules and residual bodies inside the basement membrane could be either enclosed by cell membranes or not (Fig. 7). Proximal to the pigment granule accumulation single cells were located inside or just below the basement membrane and were interwoven with it by numerous cellular projections. These cells showed poor electron contrast, contained a lot of electron lucent vesicles and cell organelles and morphologically resembled retinal macrophages. Proximal to the basement membrane a widely branched system of cell processes sometimes con-

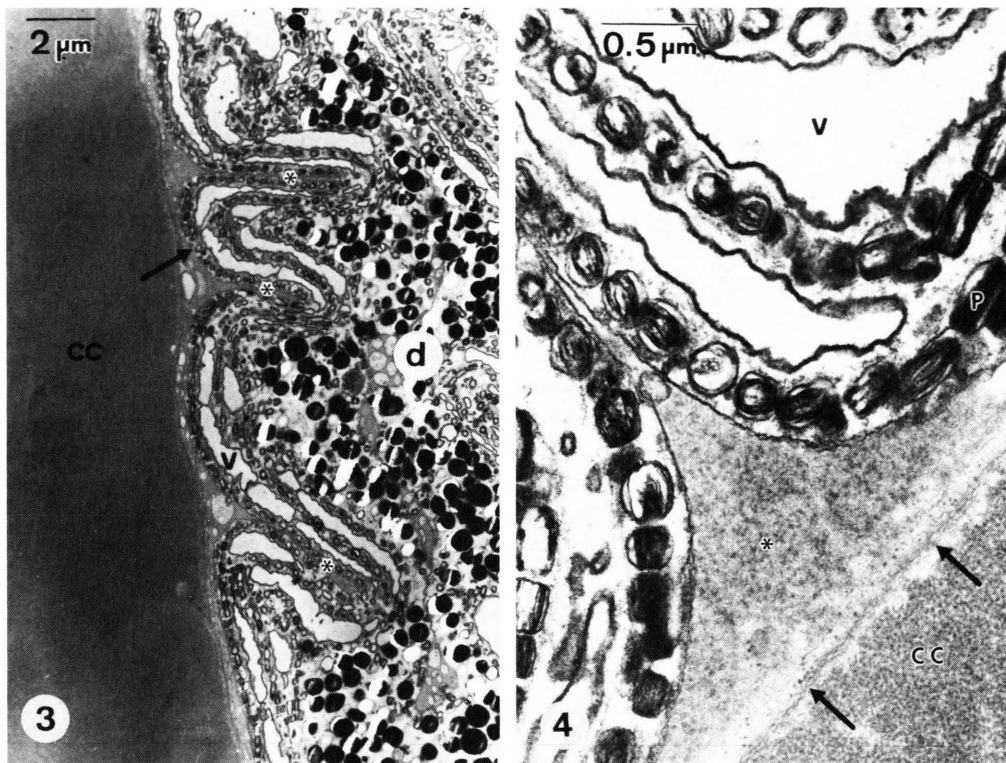


Fig. 3. This electron micrograph was taken from area 1 marked in Fig. 1. Crystalline cone (cc) and cytoplasm of distal pigment cell (d) containing numerous pigment granules from *Orconectes limosus* are shown. Extensions of the crystalline cone (marked by asterisks) project into the distal pigment cells. Pigment granules are separated from the cones by a double layer of electron lucent vacuoles (v) that are surrounded by small right-angled platelets (arrow). This leads to the formation of three layers of platelets.

Fig. 4. The material (asterisk) which forms the evaginations, shown in Fig. 3, is separated by membranes (arrows) from the crystalline cone (cc). The vacuoles (v) and platelets (p) of the pigment cells are also shown.

taining these granules was visible (Fig. 6). The cytoplasm of these cells was nearly completely filled with an electron dense gravel-like material (Fig. 6, 8) and contained almost no cell organelles. Occasionally debris of screening pigment granules occurred in these cells (Fig. 8), probably in a state of dissolving into small electron dense particles. The entire neuronal optic tract was surrounded by cells of this kind although the amount of gravel-like material differed between individual animals.

Ultrastructure of crystalline cones, distal pigment cells and pigment granule accumulation close to the basement membrane of juvenile Procambarus clarkii

The morphology of distal pigment cells, retina and crystalline cones resembled that of *Orconectes*

with the exception that the evaginations of crystalline cones towards the pigment cells were absent in *Procambarus*. The morphology of pigment granule accumulation above the basement membrane (Fig. 2) was identical to that described for *Orconectes*.

Ultrastructure of the layer between optic neuropil and cuticula of Procambarus clarkii

The topographic position of this site within the photoreceptor is indicated in area 3 of Fig. 1. Cell types like the largely branched cells below the basement membrane shown in Fig. 6, 8 for *Orconectes* are also found between the tissue of the optic neuropil and the hypodermis. These cells, the hypodermis and adjacent hemolymph lacunae contain electron dense particles (Fig. 9). Particularly small hemolymph lacunae and the basement

Plate 1. Ultrastructural observations of the distal pigment accumulation close to the retinal basement membrane of *Orconectes limosus*. The topographic position within the eye from which the figures were taken is marked as area 2 of Fig. 1.

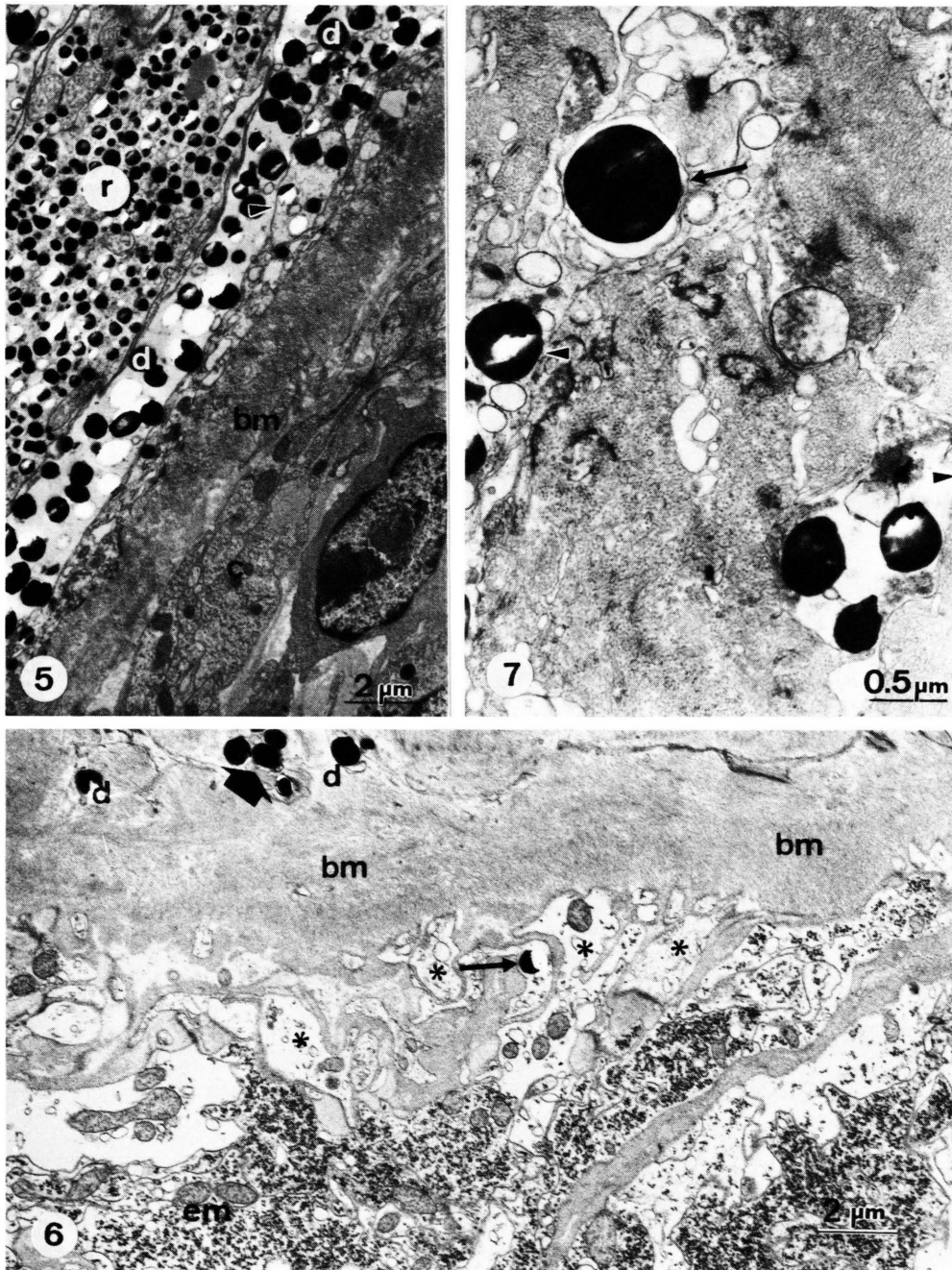


Fig. 5. Distal screening pigment granules (d) were located immediately distal to the basement membrane (bm) within cellular rays whose cytoplasm appeared electron optical empty. Cell membranes (arrowhead) however indicate the cellular nature of these projections. The screening pigment granules of the reticular cells (r) are smaller than the distal screening pigment granules. Proximal to the basement membrane cell projections (c) are interwoven with it.

Fig. 6. Some distal screening pigment granules (d) are situated within the basement membrane (large arrow), one granule is located proximal to the basement membrane (small arrow). Proximal to the basement membrane widely branched cells whose extensions are interwoven with the basement membrane (asterisks) contain large amounts of electron dense material (em).

Fig. 7. Distal screening pigment granules are located within the basement membrane. Some granules are surrounded by membranes (arrow) that are absent (left arrowhead) or incomplete (right arrowhead) in others.

Plate 2. Ultrastructural observations of hypodermis and glial cells of *Procambarus clarkii*. The topographic position within the eye from which the figures were taken is marked in area 3 of Fig. 1.

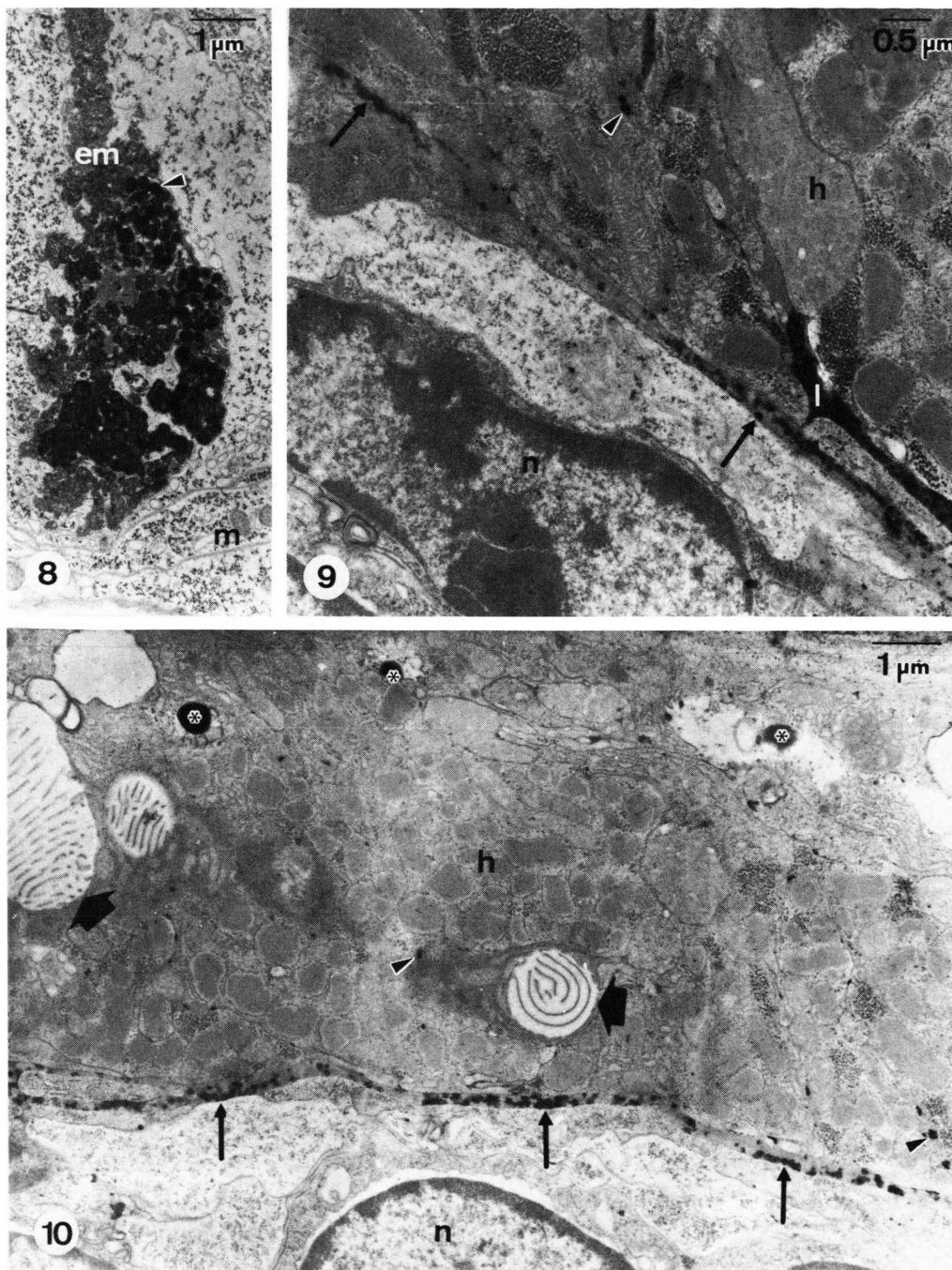


Fig. 8. Screening pigment granules (arrowhead) are shown accumulating within a cell that is probably a glial cell. This type of cell is also shown in Fig. 6 and contains only few cell organelles, but regularly mitochondria (m). Screening pigment granules seem to be fixed in a state of disintegration into an electron dense gravel-like material (em).

Fig. 9 and 10. The basement membrane of hypodermis cells (h) is marked by small arrows and contains electron dense material which is also present in a hemolymph lacuna (l). Such an electron dense material (arrowheads) or dense granules (asterisks) can also be found within the cytoplasm of the hypodermis cells. Nuclei (n) of the widely branched cells that contain electron dense material can be seen proximal to the hypodermis. In hypodermis cells electron lucent vacuoles (large arrows) contain concentric whorls or parallel arrays, depending on the plane of section.

membrane of the hypodermis that separates the hypodermis from the adjacent tissue contain large amounts of electron dense material (Fig. 9, 10). The hypodermis also shows electron dense material having the same structure as that inside the hypodermal basement membrane (Fig. 9, 10). In the crayfish that was fixed shortly before molting the content of gravel-like material in the tissue proximal to the basement membrane is smaller compared to animals in the intermolt phase. The following observations were made from this premolting crayfish. A new cuticle had been formed below the old one (Fig. 2, 11). The old cuticle, in contrast to the new, contains electron dense particles (Fig. 11). Some hypodermis cells immediately below the border between cornea and tegument contain granules resembling screening pigment granules (Fig. 12) with irregular outlines. These screening pigment granules are located within the cytoplasm or inside membrane delimited vacuoles (Fig. 12) similar to those inside the retinal basement membrane (Fig. 7). The hypodermis cells containing screening pigment granules are situated close to the distal pigment accumulation proximal to the basement membrane and appear pigmented under the light microscope (Fig. 2). Single electron dense particles (Fig. 10, 13), clusters similar to those shown in Fig. 8 or/and gravel-like material as shown in Fig. 6, 8 were detected in the hypodermis of the eyestalks of each *Procambarus*. The same was true for electron lucent vacuoles which appear as concentric whorls or parallel arrays (Fig. 10, 13) probably depending on the plane of section. These structures were associated with rough endoplasmic reticulum. In some hypodermis cells these structures were the predominant organelles. Mitochondria containing electron dense spots were found within hypodermis cells (Fig. 13) and occasionally in the branched cells below the hypodermis.

Glycogen staining according to de Bruijn

After the modified fixation according to de Bruijn the electron dense material (Fig. 6, 8) and electron dense particles within hemolymph lacunae or glial cells [7] were visible, but the contrast was not enhanced compared to tissue sections without glycogen staining according to de Bruijn and without staining with lead citrate or uranyl acetate. The material showed the same electron

contrast as screening pigment granules. Ribosomes from rough endoplasmic reticulum were not detectable.

Detection of colloidal gold particles

The ultrastructure of the retina was well preserved after injection and was not altered compared with preparations without injection. Gold particles were found at the site of distal pigment granules accumulating above the basement membrane (Fig. 14). Inside the basement membrane gold particles were detected extracellularly alone (Fig. 14) or together with distal screening pigment granules (Fig. 16).

Further gold particles were found in endosomes of macrophages (Fig. 17), glial cells (Fig. 19) and hemolymph lacunae (Fig. 18, 19) close to retinal cell axons of the optic neuropil (area 4 in Fig. 1).

Discussion

The morphology of the crystalline cones of *Orconectes* shows numerous evaginations into the distal pigment cells. Such evaginations were observed neither in young *Procambarus clarkii* in the present study, nor by Vogt [5] who investigated the internal reflection of the crystalline cones in photoreceptors of *Astacus leptodactylus*, or by Doughtie and Rao [11] in the eyes of a grass shrimp.

The double layer of large electron lucent vesicles formed by the distal pigment cells has not yet been shown ultrastructurally. The material that forms the evaginations is separated by a membrane from the crystalline cone (Fig. 4) and therefore may have light-conducting properties differing from those of the cone, so that the image quality will not be deteriorated by these evaginations. Both electron lucent vesicles and projections of crystalline cones in concert with the small platelets may be involved in internal light reflection [5]. It is unlikely that the evaginations or electron lucent vesicles originate from fixation artifacts, because the crystalline cones do not show any sign of shrinkage or mechanical alteration (Fig. 2). Moreover the evaginations are completely and evenly surrounded by alternating layers of platelets and vacuoles.

For two reasons it seems not probable that the accumulation of pigment cells above the basement

Plate 3. Ultrastructural observations of hypodermis cells and cuticle of *Procambarus clarkii*. The topographic position within the eye from which the figures were taken is marked in area 3 of Fig. 1.

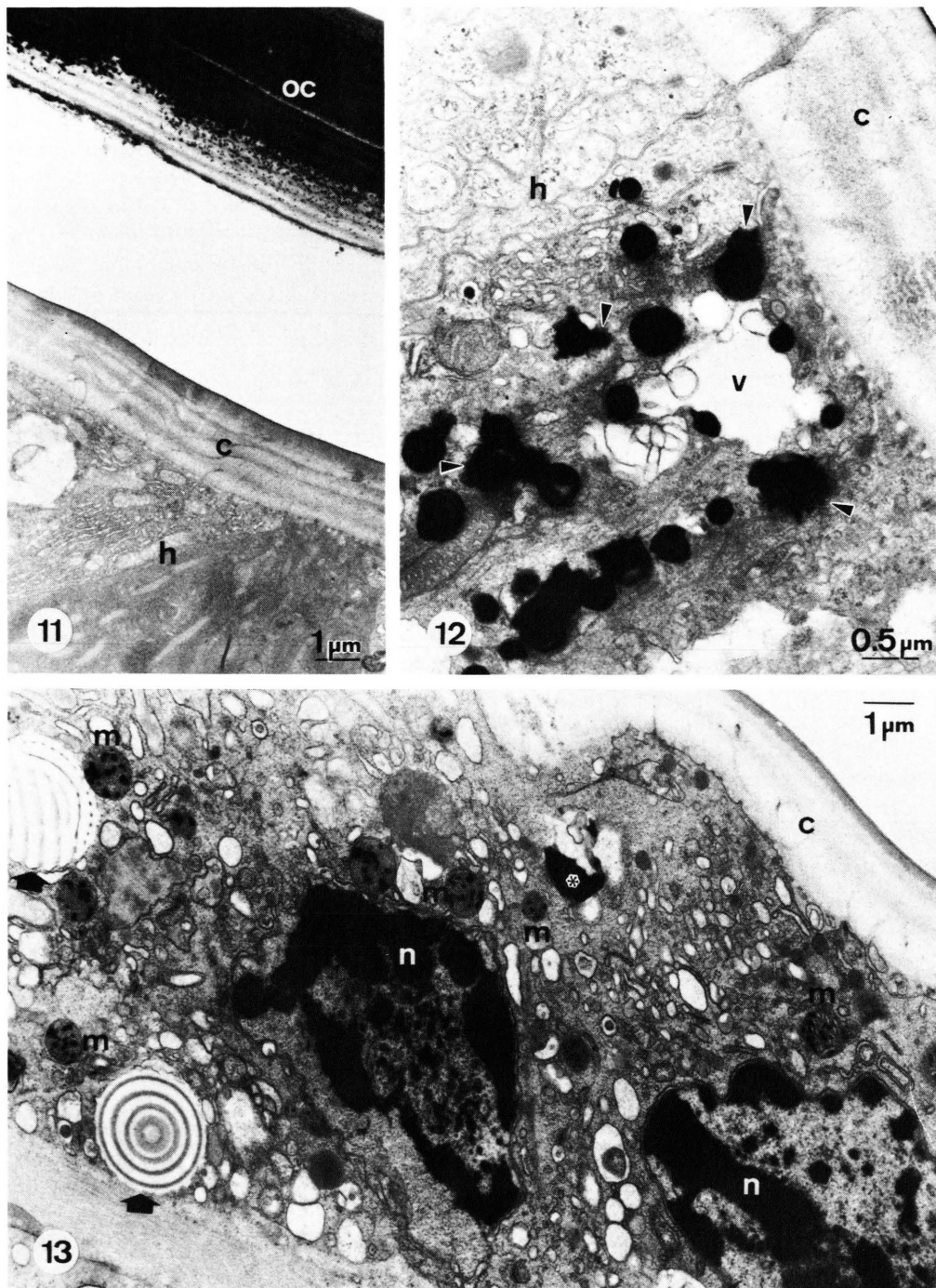


Fig. 11. The old cuticle (oc) is loosely fitting the eyestalk and more electron dense than the newly formed still incomplete cuticle (c) shortly before molting (h = hypodermis cell).

Fig. 12. Granules that are ultrastructurally identical with screening pigment granules but are smaller than the majority of distal screening pigment granules are located within the cytoplasm of a hypodermis cell (h). Some of these granules have irregular outlines and their content seems to flow into the cytoplasm (arrowheads). Other granules are located within a membrane delimited vacuole (v) (c = cuticle).

Fig. 13. The mitochondria (m) of hypodermis cells of the eyestalk are heavily pigmented. Electron dense material (asterisk) and lucent vacuoles (large arrows) that contain concentric whorls or parallel arrays are regularly present in the hypodermis cells (n = nuclei, c = cuticle).

Plate 4. Detection of gold particles inside or close to the retinal basement membrane 4 h after injection into the distal ommatidia. The topographic position within the eye from which the figures were taken is marked in area 2 of Fig. 1.

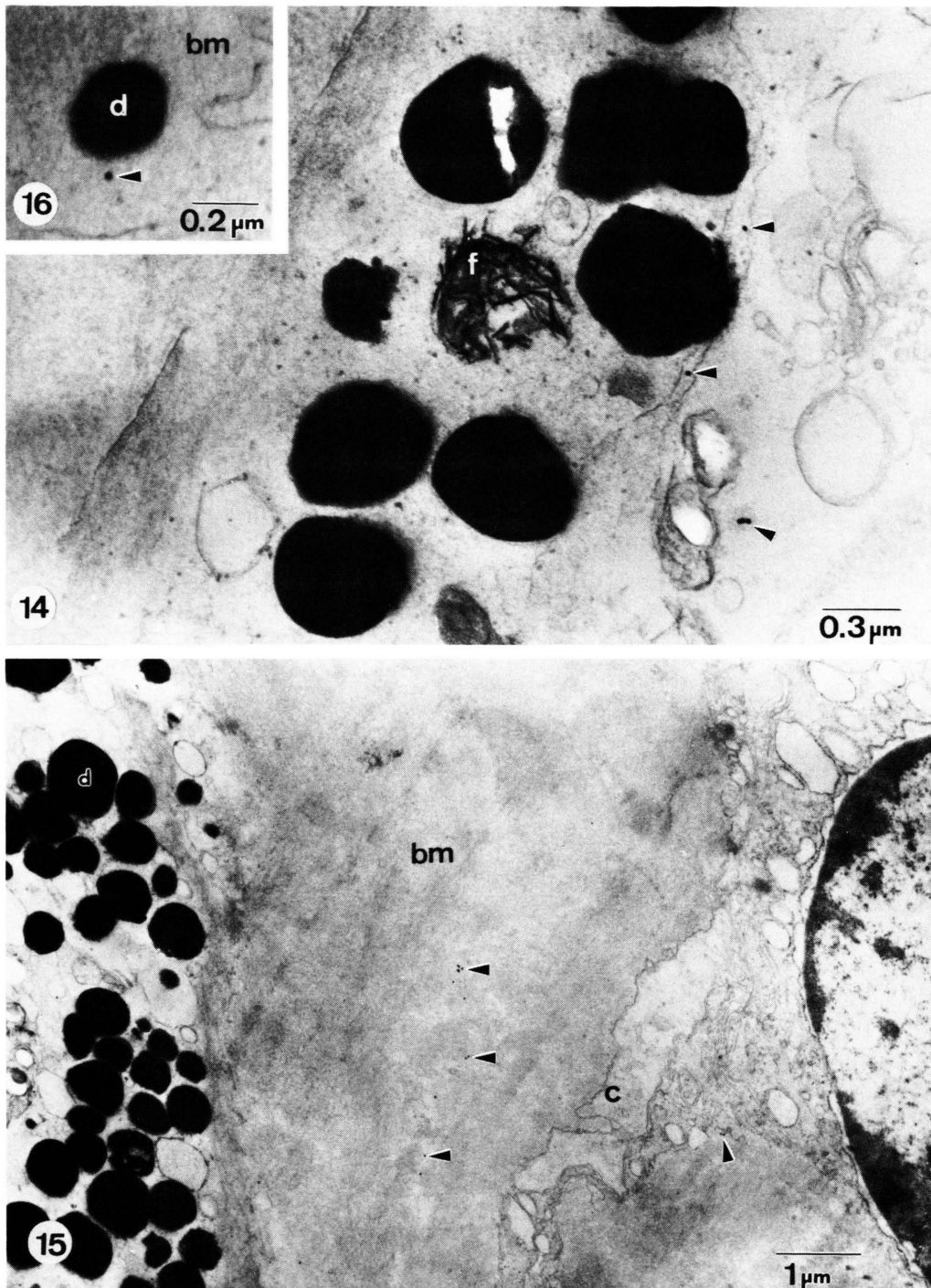


Fig. 14. Distal screening pigment granules are shown immediately distal to the retinal basement membrane. One granule consists of fibres (f). Gold particles (arrowheads) are located close to pigment granules.

Fig. 15. Distal pigment granules (d) are located proximal to the basement membrane (bm). Gold particles (arrowheads) are located inside the basement membrane or in close contact to a cell (c) interwoven with it.

Fig. 16. A distal screening pigment granule (d) is located extracellularly close to a gold particle (arrowhead) inside the retinal basement membrane (bm).

Plate 5. Detection of gold particles proximal to the retinal basement membrane 4 h after injection into the distal ommatidia. The topographic position within the eye from which the figures were taken is marked in area 4 of Fig. 1.

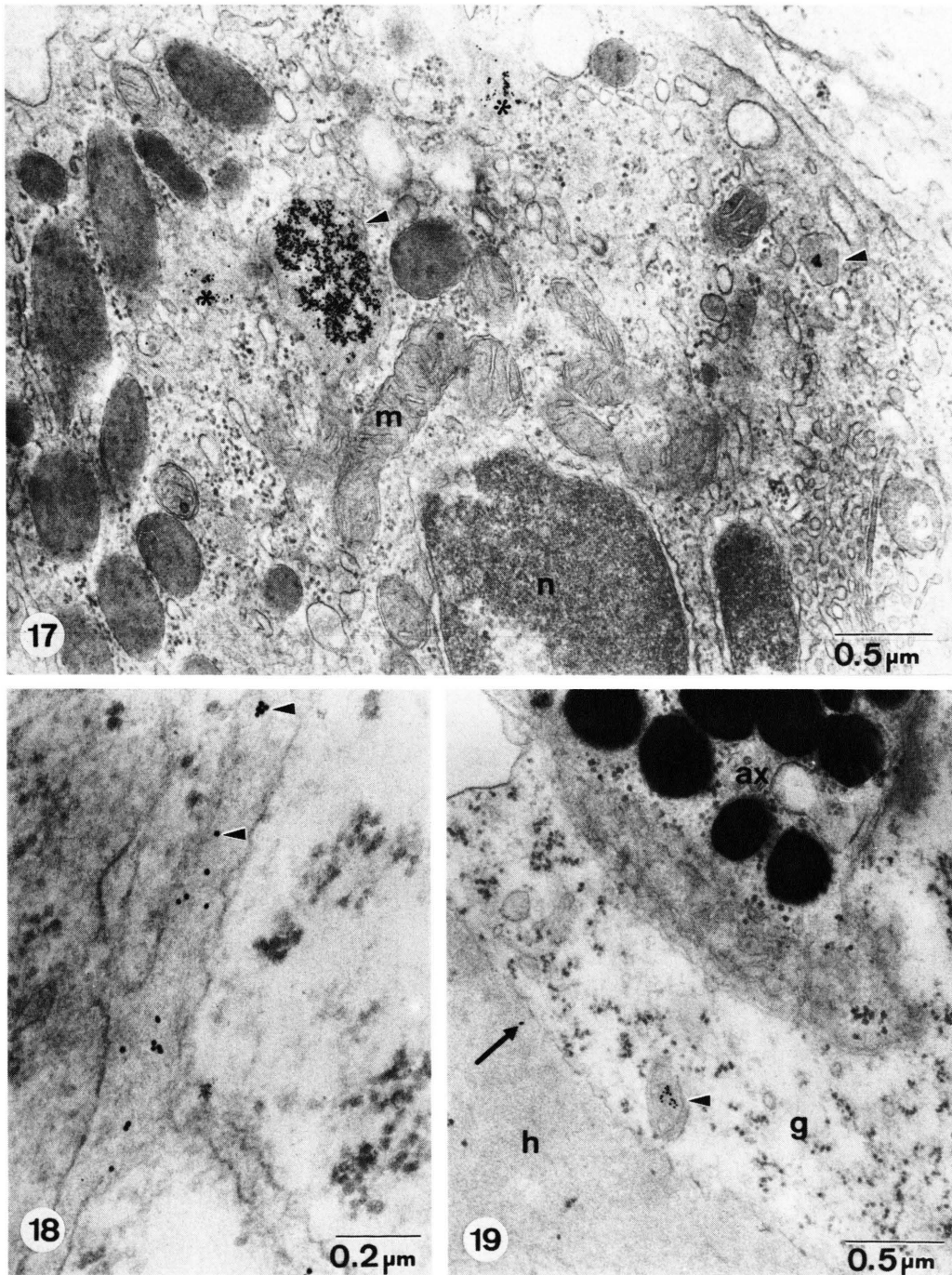


Fig. 17. Gold particles are shown inside of endosomes (arrowheads) or in the cytoplasm (asterisks) of a hemocyte. The hemocyte is located inside a hemolymph lacuna of the optic neuropil (n = nucleus, m = mitochondrion).

Fig. 18. Gold particles (arrowheads) are shown inside a hemolymph lacuna of the optic neuropil.

Fig. 19. Gold particles are located inside an endosome (arrowheads) of a glial cell (g) and inside a hemolymph (h) lacuna (arrow). A photoreceptor cell axon (ax) containing proximal screening pigment granules is also shown.

membrane is involved in light-screening to protect light-sensitive structures. The pigment granules are unable to protect light sensitive membranes because the light reaches them after passing the rhabdoms, and they do not show light-dependent migration.

A possible explanation for the findings is that processes of distal pigment cells transport aged screening pigment granules into basal direction, using a specific pathway not to disturb the light flux to the sensitive membranes. Although direct connections between distal pigment cells and the cellular rays were not found, it is probable that both are of the same cell type, judged by the similarities of cytoplasm and pigmentary organelles. Distal pigment granules in the cytoplasm interwoven with extracellular rays of the retinal basement membrane were also shown for a grass shrimp [11] and correspond to the findings of the present study. Because distal screening pigment granules were found intracellularly proximal to the basement membrane they may have been taken up by certain cells (Fig. 7) and degraded to a gravel-like material (Fig. 6 and 8). The nature of these cells is unknown but they seem to store this gravel-like material. This material might be incorporated during formation of the next cuticle and thus be removed from the animals. This is in accordance to the observation that shortly before the molt of young *Procambarus* such an electron dense material appeared within the hemolymph (Fig. 9), the basement membrane and cytoplasm of the hypodermis cells (Fig. 9 and 10).

The nature of this electron dense material remains uncertain, but unchanged contrast after staining according to de Bruijn indicates that these particles are neither glycogen nor ribosomes. Furthermore such material was found extracellularly [6].

Carotinoids and ommochromes are possible candidates. Both pigments are present in the eyes and integument of crustacea [12–16]. The carotinoid astaxanthin was found in the eyes of different crustacean species in a much greater concentration than in the remainder of the body [12]. On the oth-

er hand conjugated carotinoids, as chromoproteins both inside and outside chromatophores, play an important role in the general coloration of crustacea, especially decapods. Denaturation of this protein carotinoid complex by heat results in the marked color change from dark green or blue to bright red observed when a lobster is boiled [13].

Pigmentation of mitochondria that was not observed in the hypodermis of the body [17] may be induced by metabolites of ommochrome synthesis like kynurenine, perhaps originating from degraded screening pigment granules. Kynurenine may be oxidized to xanthommatin by an enzyme system in the mitochondria. Indeed such a pathway has already been demonstrated. Yoshi and Brown [18] found that enzymes from rat liver mitochondria were able to synthesize hydroxykynurenine from O_2 and kynurenine *in vitro*. Addition of cytochrome C resulted in the formation of xanthommatin.

A kynurenine 3-hydroxylase is also located in the outer membrane of mitochondria of insects [19].

Gold particles found inside or proximal to the basement membrane seem not to have been transported to these sites by the pressure of the injection procedure. The intraretinal pressure was balanced by the second hole in the cornea. More likely the gold particles were transported towards and across the retinal basement membrane by an active transport mechanism. Distal screening pigment granules seem also to be transported on the same route. These conclusions are indirectly supported by the existence of an active and selective transport mechanism across the retinal basement membrane of flies [20].

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