Accessibility of Extracellular Space in the Rhabdome of the Living Isolated Retina of the Crayfish

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Diffusion of the tracer enzyme horseradish peroxidase (HRP) into the extracellular spaces of living isolated crayfish retinas was monitored by light- and electronmicroscopic identification of the reaction product obtained after immobilisation of the enzyme by fixation. During the incubation in enzyme containing saline (up to 3 hours) the extracellularly recorded electroretinogram (ERG) stayed normal and showed no substantial change. The reaction product of the enzyme HRP was found only extracellularly in the space between the microvilli in the rhabdome.

The results indicate an exchange of substances via extracellular pathways between the extracellular side of the photosensory membrane and the bathing fluid in the living *isolated* crayfish retina.

Introduction

In a number of investigations we studied the influence of changing the ion composition of the bathing solution on the isolated retina of the crayfish (*e.g.* Stieve and Wirth [1], Stieve and Hanani [2], Stieve, Bruns and Gaube [3]). This type of studies raises the question of the accessibility of the various extracellular compartments. According to the investigations of Fioravanti and Fuortes [4] there is strong evidence that the primary light-induced conductance increase in the visual cell membrane occurs in the photosensory membrane of the microvilli. Therefore the question of accessibility of the extracellular space and the extracellular exchange of the microvillar region is of special interest for the interpretation of the experimental results on changed ion composition of the external bathing fluid.

The question of the extracellular accessibility of the microvillar area has been studied by various authors using colloid lanthanum as a marker diffusing into chemically fixed preparations (Perrelet and Baumann [5], White and Walther [6]). They found that lanthanum could enter the microvillar area via extracellular pathways. However, we have indications to believe that fixation may change the properties of the preparation in such a way as to make certain extracellular spaces accessible which are not accessible in living cells (Weihe *et al.* [7], own unpublished results).

In contrast to the situation in the *Limulus* lateral (Fahrenbach [8]) and ventral eye (Clark *et al.* [9]) the visual cells in the *Astacus* eye are according to Krebs [10] not surrounded by a sheeth (or layer) of glial cells. The photosensory membrane in the isolated retina seems to be morphologically in direct connection, at least distally, with the bathing solution. (In the whole intact eye the situation may be different, and it is not apparent which extracellular spaces can directly exchange solution among each other.)

The extracellular spaces in the microvillar area are especially small:

Electron-microscopic studies of the crayfish eye show (Krebs [11]; Uebags, Diplomarbeit [12]) that the extracellular space is maximally 10% of the total rhabdome volume, and it consists of spaces approximately of triangular cross-section between the round microvilli. Here we studied the accessibility of this extracellular space by incubating the live isolated retina in physiological saline containing horseradish peroxidase (HRP) as a marker, while monitoring the physiological state of the preparation by measuring the electroretinogram (ERG) during the entire incubation period.

Methods and Results

The dark adapted retinas were dissected from eyes of *Astacus leptodactylus* as previously described (Stieve and Wirth [1]) and mounted in an experimental chamber containing van Harreveld solution (van Harreveld [13]). The cornea, the crystalline cones and most of the optic ganglia were removed from the retina in the dissection procedure. The

electroretinogram (ERG) was measured by means of extracellular silver/silver chloride electrodes at 15 °C. The distal part of the retina was superfused by streaming Van Harreveld saline. The retina was stimulated every 5 min by a 10 ms white light stimulus of 95,000 lx (corresponding to 1.5×10^{17} photons cm^{-2} sec^{-1} of 550 nm). The ERG evoked by each stimulus was recorded. During an initial preperiod of 20 min the amplitude of the ERG reached a stable value. Now the horseradish peroxidase (Sigma type IV) was added to the van Harreveld solution (in contact with the distal part of the retina) to a concentration of 20 mg ml^{-1}.

In order to allow for small quantities of HRP to be used, the circulation of the incubation mixture was stopped at this point and the enzyme added to the volume within the experimental chamber only. The illumination program was continued without change.

The ERG is not recognizably altered due to the addition of the enzyme peroxidase during the complete incubation period of up to three hours. Fig. 1 shows the amplitude of the ERG of two experi-

ments. At the indicated times, two to three hours after the addition of HRP, the retina was fixed within the experimental chamber in 2% formaldehyde and 2% glutaraldehyde for two hours at room temperature and then transferred to 4 °C for overnight fixation.

The aldehyde-fixed specimens were incubated overnight at room temperature for peroxidase reaction in freshly prepared 0.5 mg/ml diaminobenzidine (DAB) in phosphate buffer (Graham and Karnovsky [14]), and 0.01% H_2O_2 which was usually added after a delay period of several hours to allow good penetration of DAB. Small amounts of additional H_2O_2 were added 3 − 5 times after several hours of incubation. After osmium fixation the samples were dehydrated and plastic-embedded (Luft [15]). In order to detect the peroxidase reaction product most clearly, no additional contrasting agents were used. Sections were cut on a Reichert-Ultracut microtome. Thick sections (some 4 μm) were examined with a Leitz Orthoplan microscope, thin sections (ca. 60 nm) were examined with a Jeol 100 C electron microscope.
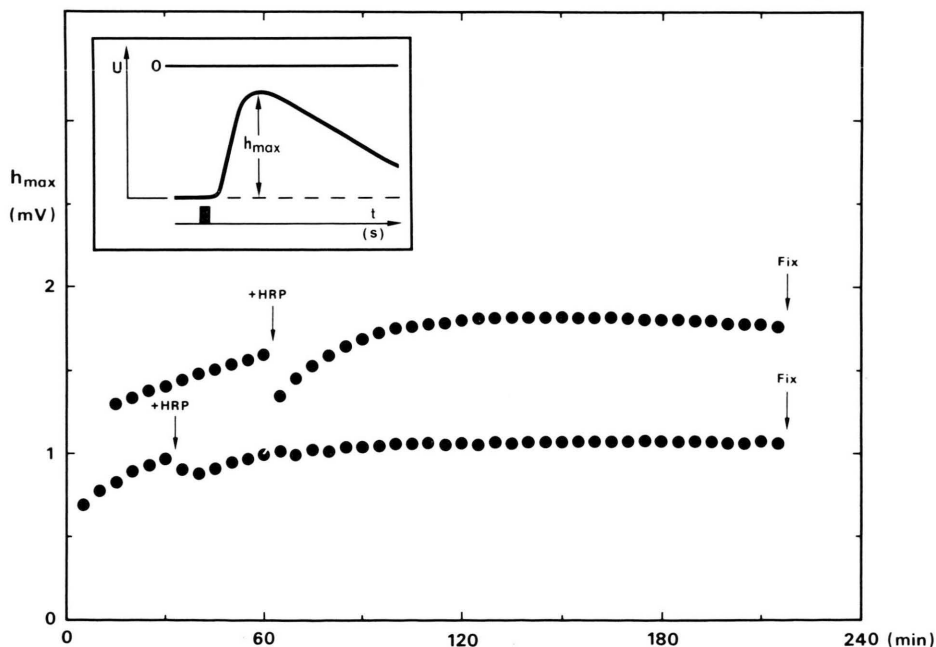

Fig. 1. Electroretinograms of two isolated crayfish retinas as described by Classen-Linke [19]. The retinas were mounted in an experimental chamber, the distal part being incubated in physiological saline, and illuminated by 10 ms pulses of white light. h_{max} (see insert) was plotted against the corresponding time elapsed after mounting the retina. At the indicated times (+HRP) horseradish peroxidase was added and the illumination program continued. Fixation (Fix) by addition of aldehydes was started while the ERG was still stable. The difference in h_{max} between the two experiments is within the normal range observed in this type of experiments.

Plate 1 a.

Plate 1 b.

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Plate 1: Light micrographs of longitudinal sections of Astacus rhabdomes. HRP can be detected in 1 a by the dark (originally red) stain visible in several bands of the rhabdomes. Plate 1 b shows the absence of the stain in an control retina treated exactly the same way except for exposure to HRP. The banding pattern was enhanced in this light micrograph by Nomarsky optics.
← d: heavily stained bands;
← 1: lightly stained bands.

Light microscopy of 4 μm thick sections reveal reddish-pinkish stains in the rhabdome area. This stain was not found in control experiments without peroxidase incubation (Plate 1 b).

Some 10% of the rhabdomes are more or less uniformly stained by peroxidase reaction product, the rest shows darker and lighter stained bands within the rhabdome as shown in plate 1 a. A portion of a retina where the staining can be seen in grey-tone reproduction was selected for plate 1 a. Interference contrast light microscopy shows a corresponding morphological pattern within rhabdomes of control sections without peroxidase incubation (plate 1 b). This striped appearance of the rhabdome is due to the fact that the rhabdome is constituted of packets of microvilli from the seven surrounding retinular cells in an alternating spatial arrangement (Eguchi [16]; Krebs [10]). Pictures such as shown in plate 1 a indicate that portions of the rhabdome belonging to different individual retinular cells are stained to different degrees by the product of peroxidase action, probably due to different invasion of the extracellular area by peroxidase.

Electron microscopy and light microscopy of series of alternating thin- and thick sections identify, as expected, these bands as packages of microvilli, lying at approximately right angles with respect to each other and perpendicular to the long axis of the rhabdome. An electron micrograph of a thin-section corresponding to the thick-section shown in plate 1 a reveals electron-dense material typical for the peroxidase product within the extracellular space between the microvilli (plate 2). No such material could be detected intracellularly.

Bands that appear stained darker in light microscopic pictures seem to contain more electron-dense material (plate 1 a, arrow d) while bands that appear just lightly stained (plate 1 a, arrow 1) show only a little electron-dense material typical for the peroxidase reaction product.

More darkly stained bands exhibit microvilli of larger diameter (some 0.9 μm) while less stained bands show densely packed microvilli with a smaller diameter (some 0.6 μm), typical for rhabdomes of retinas quickly fixed after dissection.

Discussion

The isolated *Astacus* retina contains at least two important extracellular compartments: the extracellular rhabdomic compartment, the space between the microvilli, and the compartment between the ommatidia. The results of the experiments described above show that one of these compartments, the extracellular rhabdomic one, can be invaded through extracellular pathways by the bathing solution, in a preparation used in electrophysiological experiments which do not show recognizable injured function. Even though in favourable views it is possible to detect peroxidase reaction product in intercellular clefts between the ommatidia it is not yet clear to us at this moment to which extent this compartment is accessible.

Hartung [17] and Stieve and Hartung [18] measured the outflow of different radioactive labelled extracellular markers, $^{35}SO_4^{-2}$, [^{14}C]-Inulin and [^{3}H]-Inulin from the isolated *Astacus* retina under similar conditions as used in the experiments described here. The loss in extracellular markers can be described as the sum of 2 exponential functions with exchange rates $t_{0.5}$ of 1.5 and 5 min. Hartung [17] interpreted this as the exchange of two extracellular compartments, the faster one between the ommatidia and the slower one from the extracellular space between the microvilli of the rhabdome. The observations described here are in agreement with this suggestion as both extracellular compartments can be reached by extracellular pathways. The striped appearance of the peroxidase staining of some rhabdomes is conspicuous. It shows that extracellular space belonging to different retinular cells constituting a rhabdome can probably be of different accessibility. This may not be a normal property of rhabdomes but may be due to the different reaction of individual retinular cells to experimental stress. Presumably the long stay in van Harreveld's solution causes a certain swelling and loosening of the packing of the microvilli constituting the rhabdome which is restricted to the domains of individual retinular cells. Even though the in-
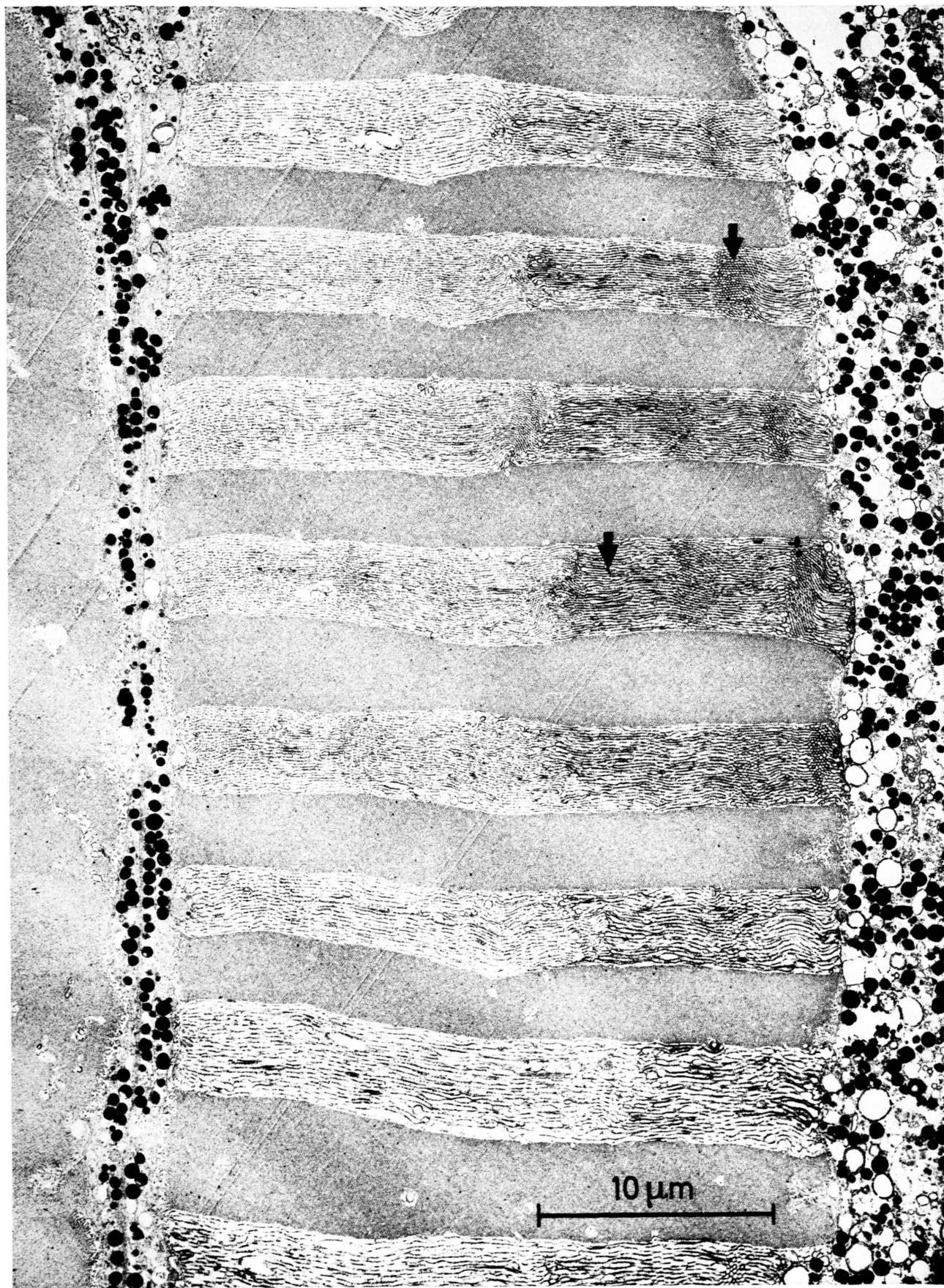
Plate 2.

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Plate 2. Low power electron micrograph of a longitudinal section of an Astacus rhabdome treated with HRP. This micrograph corresponds to the section shown in plate 1 a. Again the dark stain is due to the HRP-reaction product (arrows).

fluence of H_2O_2 during the histochemical reaction appears to be harmful to the ultrastructure the peroxidase reaction is not the cause of this swelling as it can be observed without HRP and H_2O_2 treatment as well. However, immediately after dissection, before mounting in the experimental chamber, the ultrastructure of the microvilli seems undisturbed. The accessibility of the extracellular spaces in

the rhabdome in the *intact eye* may be different since the rhabdomic area is closed laterally by tight junctions (Krebs, unpublished results) between retinular cells and distally by the crystalline cone.

Our preliminary results show that in living *Limulus* ventral nerve photoreceptors the extracellular space of the microvillar area is not accessible via extracellular pathways.

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