Differentiation of photoreceptors in cultured optic vesicles from embryos of *Rana esculenta*

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SUMMARY

Optic vesicles from embryos of *Rana esculenta* at a stage corresponding to stage 19 in the development of *Rana pipiens* were cultured for 15 days. The eyes which differentiated in vitro were much smaller than controls of the same age, due partly to the absence of the vitreous body. In many specimens a well-stratified retina surrounded by a layer of pigmented cells was found. The features of all the components of the retinal layers are described.

The complete differentiation of photoreceptors, including their outer segments, is the most interesting result obtained in these organ cultures, and is in contrast with the observations previously reported for retinal tissue cultures. The authors suggest that the source of the material (amphibia instead of birds or mammals) is the main reason for this discrepancy. Frog embryo cells are practically self-sufficient since they are endowed with nutritional substances, amongst which Vit. A and antioxidative agents (Vit. E and ascorbic acid) have been indicated as the most important factors for the structural organization and integrity of the outer segments. The role played by the pigment epithelium is also discussed.

INTRODUCTION

In a previous paper (Stefanelli et al. 1967a) we discussed factors influencing the morphogenesis of the outer segment of photoreceptors. In retinal cell aggregates of chick embryo cultivated in vitro, photoreceptors were obtained which appeared to be well differentiated, with the exception of the outer segment. Similar results have since been reported by other authors (Hild & Callas, 1967; Barr-Nea & Barishak, 1970; Sheffield & Moscona, 1970; Hansson, 1971; La Vail & Hild, 1971) who attribute the lack of formation of the outer segment to loss of contact between the embryonic photoreceptors and the pigment epithelium from which they should receive Vit. A in a form utilizable for the synthesis of visual pigments.

Sidman (1961), however, demonstrated complete differentiation of photoreceptors in an intact mouse eye in organ culture only following the addition of 11-cis retinene to the culture medium. In this case the mere presence of the pigment epithelium was not sufficient to allow a complete differentiation.

Since further research is necessary for a better understanding of the exact role of the pigment epithelium in inducing the formation of the outer segment,
we repeated the organ culture studies in which the relationship between the layers of the retina remains undisturbed. For these studies amphibian optic vesicles were chosen as the experimental material since differentiation is completed more rapidly (8 days) (Nilsson, 1964). Moreover, as early as 1934, Perri from this Institute removed optic cups from embryos of *Rana esculenta*, and after keeping them *in vitro* for only 10 days, obtained retinas, which under the light microscope appeared to be perfectly differentiated.

**MATERIALS AND METHODS**

Optic cups from embryos of *Rana esculenta* at a stage corresponding to stage 19 (Shumway, 1940) in the development of *Rana pipiens*, were isolated and cultivated in Petri dishes (4 cups to each 6 cm dish) in a medium composed of M199 diluted 50% with distilled water. The medium was changed every 2–3 days. The cups were fixed after 14–15 days of culture for light- or electron-microscopic studies.

Eyes from larvae of corresponding age and from the same batch as the donor embryos were used as controls. The fixative giving the best results for electron microscopical observations was composed of 3% glutaraldehyde, 2% formaldehyde dissolved in 0.1 M cacodylate buffer at pH 7.2–7.4. Specimens were fixed for 2 h at room temperature and for a further 48 h at 0 °C, then dehydrated in alcohol and embedded in araldite (Fluka). Ultrathin sections were stained with lead citrate according to Venable & Coggeshall (1965) prior to observation under an AEI EM 801 microscope.

The optic cups fixed and embedded for electron microscopic studies were first observed under the light microscope. Sections approximately 1 µm in thickness were stained with toluidine blue according to Rüdeberg (1967).

**RESULTS**

The eyes which differentiated *in vitro* are always much smaller than controls of the same age, due partly to the absence of the vitreous body. Sometimes only part of the retina is differentiated or the pigment epithelium may be incomplete.

Some eyes have a small lens and all the retinal layers are identifiable (Fig. 1); in the more peripheral parts, the photoreceptor outer segments are lacking and mitotic nuclei are frequently found (Fig. 2).

*Pigment epithelium*

A single layer of pigmented cells was found on the outer surface of all the retinas examined. Their morphological characteristics however differ considerably according to the degree of differentiation revealed by the receptors below. Where receptors are not yet distinguishable, or where only a row of undifferentiated cells are linked to each other forming the outer limiting membrane, the
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All the figures illustrate optic vesicles removed from embryos of *Rana esculenta* at stage 19 and cultivated for 14 days.

Figs. 1 and 2. Light microscopical micrographs of semi-thick sections stained with toluidine blue. In Fig. 1 all the retinal layers are clearly recognizable. A primordium of the lens (l) can be seen. × 30. Fig. 2 illustrates a marginal part of an ‘eye’ where cell proliferation takes place, as demonstrated by the presence of mitoses (one is in the black square). Intensely stained inclusions can be seen at the bipolar cell level. *pe*, pigment epithelium, the granules do not appear to have migrated towards photoreceptors. *ros*, receptor outer segments. × 52.
cells of the pigment epithelium do not have any consistent shape. Their cytoplasm is completely taken up by roundish pigment granules already in a mature form (Fig. 3). On the apical surface only short processes are present which do not contain pigment. A finely fibrillar substance which is only slightly electron-dense fills the space between this surface and the layer of receptors. The nucleus
Figs. 4 and 5. Pigment cell layer of a cultured optic vesicle (Fig. 5) and of a control eye from a same age tadpole (Fig. 4). The comparison between the two pictures illustrates the different shape and distribution of the pigment granules within the cells. The endoplasmic reticulum is remarkably developed in both cases. In the cultured material the majority of the pigment granules seem to undergo lytic processes. \( G \), Golgi apparatus; \( \text{ros} \), receptor outer segments. \( \times 14000 \).
is found at the basal part of the cells together with a few mitochondria. In the perinuclear zone there may be a few elements of endoplasmic reticulum with ribosomes. Lipid droplets and yolk platelets are also present.

However, as soon as receptors, which have formed an inner and outer segment, are found in zones even adjacent to those mentioned above, the cells of the pigment epithelium display a cuboidal shape and have a more complex cytoplasm. In fact, the cytoplasm is mainly taken up by the smooth endoplasmic reticulum which is in the tubular form, characteristic of this type of cell (Figs. 4–5). This feature is particularly evident in the perinuclear area. The so-called myeloid bodies were not observed. The pigment granules, which are roundish or oval in shape, form a population which is not homogeneous; some have a high, uniform electron-density, but several show lighter zones, where coarse and very fine granulations occur, and occasionally membranes can be distinguished (Fig. 5). Their appearance is suggestive of lytic processes. The pigment is fairly uniformly distributed throughout the cytoplasm. The cells have an almost smooth basal surface resting on a basement membrane and an apical surface with infoldings, some of which are very long and intermingled with the protruding segments of the receptors (Fig. 7). The processes occasionally contain granules of pigment which may be slightly elongated, or more often may be roundish in shape. Yolk platelets or other electron-dense inclusions may also be present within the cells; lamellar bodies which appear to be phagocytized pieces of outer segments are occasionally observed.

The characteristics of these cells are almost identical to those observed in the control eye, except for the appearance of the pigment granules and their distribution within the cell. In fact, in the cells of the pigment epithelium in vivo the granules are all intensely opaque and migrate toward the apical portion; furthermore, while the granules of the cell body are mostly roundish, those in the finger-like projections have a rod-like shape and are more numerous compared with those in vitro (Fig. 5).

Layer of outer segments

The outer segment of the photoreceptors, as in vivo, appears to be made up of numerous overlapping flat saccules; however their configuration is often irregular. In fact, whilst the most basal saccules appear to be distended, the distal ones are often folded over or simply distorted (Figs. 6 and 7). Stacks of these discs appear to be detached and localized in the spaces between the outer segments and in amongst the villous-like processes of the pigment epithelium cells and of the Müller cells.

When the outer segments have a normal membrane arrangement, they are fairly long but never reach the same length as those in vivo. The cones are distinguishable from the rods by the structural characteristics reported by Nilsson (1964). The outer segment of the cones often appears considerably reduced. The saccules of the cones, but not of the rods, were found to be in
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Fig. 6. Nuclear zone (N), inner segments (is) and outer segments (os) of a cone. Mc, Müller cell. Arrows, outer limiting membrane. In the right lower corner of the picture apical portions of outer segments reveal a distorted array of the discs. ×15700.
Fig. 7. Inner (is) and outer segments (os) of a rod. A cone is probably present at its right side, as suggested by the big oil droplet. Irregular stacks of discs are visible among the villous projections from the tapetum cells devoid of pigment granules. em, extra-cellular matrix. × 15700.
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Fig. 8. Synaptic pole of photoreceptors just under the nucleus. Only a few fibres from the plexiform layer are engulfed within the receptor terminals to form contacts in the characteristic dyadic or tryadic configuration. The synaptic vesicles are in great number. × 35000.
communication with the extracellular space as has been shown by several authors, and confirmed using tracers (Cohen, 1968). In the outer segment of the rods, on the other hand, the characteristic lobes of the rod saccules, may occasionally appear as vertical incisures in longitudinal sections.

Layer containing inner segments and nuclei of photoreceptors

The inner segments and the cellular bodies of the photoreceptors are not unlike those observed in the eye in vivo at the same age and already described by Nilsson & Crescitelli (1970).

At the outer limiting membrane level, cellular extensions from the glial cells of Müller can be observed separating the photoreceptors (Fig. 6). Their cytoplasm is less electron-dense and contains only a few filaments and small mitochondria.

Outer plexiform layer

In this layer, the thickness of which does not differ from that of the retina in vivo at the same age, the synaptic expansions of the receptors show a high degree of differentiation. These are filled by a large number of synaptic vesicles. The synaptic ribbons are sometimes fairly long and are always perpendicular to the post-synaptic membrane; they do not present the arciform electron-dense areas described by Ladman (1958). In some cases, strongly opaque, amorphous material can be seen occupying the synaptic cleft, as already described by Nilsson & Crescitelli (1970) in larvae of Rana pipiens at early stages of retinal development.

The synaptic portion of the receptors is often immediately below the nuclei (Fig. 8). It is possible to distinguish (1) synaptic bases with an electron-dense matrix containing only vesicles and ribbons, and (2) bases with a lighter matrix containing other elements, such as small cisternae of the smooth reticulum, mitochondria and several glycogen granules. The terminations of this second type may be at the end of a short cytoplasmic process and some distance from the nucleus. Several neuronal processes are engulfed in the synaptic expansions and only occasionally does the contact have the dyad or tryad configuration described in the retina of the adult frog (Dowling, 1968). Thickening of the post-synaptic membrane is not always visible, whereas the formation of vesicles from the presynaptic membrane, described by other authors, has been observed. There are often no organelles in the post-synaptic elements and then it is difficult to ascertain whether they are dendrites of the bipolar cells or axons from horizontal cells. Several contain vesicles.

The nerve fibres making up the plexiform layer usually have a small diameter and contain neurotubules, mitochondria and glycogen granules. Of these some can be recognized as dendrites from the presence of ribosomes and reticulum elements. Synapses of the conventional type are occasionally observed between the nerve fibres; moreover, some of these form contacts with the lateral surface
Fig. 9. Reconstruction of a large Müller cell process characterized by a great number of lytic bodies. *opl*, outer plexiform layer; *bc*, bipolar cells. × 11300.
of the synaptic expansions of the receptors. These contacts can be recognized by thickening of the membranes and the presence of electron-dense material stacked against the membrane of the fibres. The intermembranous cleft shows higher opacity. No synaptic vesicles are seen.

Large glial processes from Müller cells are present, which are characterized by numerous electron-dense inclusions with myelinic figures of various forms (Fig. 9). They also contain filaments, elements of the ergastoplasmic and endoplasmic reticulum in a tubular form, and masses of glycogen granules. The perikaryon of these cells is sometimes localized at this retinal level.

Finally, in the lower part of the layer there are elongated cells lying perpendicular to the receptors; they have small flat nuclei, surrounded by very little cytoplasm in which well developed Golgi apparatuses can be seen. These cells may possibly be of the horizontal type.

**Inner nuclear layer**

This layer is made up of several rows of bipolar cell nuclei which appear to be slightly smaller than those of the receptors and with a more electron-dense matrix; chromat in is disposed in clumps. In retinas from control tadpoles the distribution of chromatin is more uniform in all the nuclei in this layer. The bipolar cells in vitro have little cytoplasm: it is rich in ribosomes, and some mitochondria and a few cisternae of the ergastoplasmic reticulum can be found in the perinuclear zone.

At a more vitreal level, the cells display less electron-dense nuclei, have slightly larger dimensions and the cytoplasmic poles, turned towards the inner plexiform layer, are rich in organelles, amongst which Golgi apparatuses predominate. Sometimes vesicles with an electron-dense core are present. The same type of vesicles together with synaptic vesicles are found in some neuronal processes of the inner plexiform layer. The difference between the bipolar and the amacrine cells is less marked than in control retinas.

Another difference between in vitro and control retinas concerns the morphology and the development of the Müller cells. In fact, in vivo, these cells form a kind of net with long thin processes, which (at times) are reduced merely to the plasma membranes, separating the cells of the inner nuclear layer and the ganglion cells one from another. The Müller cell bodies are small and appear to adapt their shape to that of the interstices between the bipolar cells. In retinas differentiated in vitro the extension and branching of the glial laminae is less evident, but they are of greater calibre, and are characterized by the presence of numerous lytic bodies (Fig. 9). At the level of the more vitreal layers, the Müller processes are larger in size and have a more heterogeneous appearance. Sometimes cellular debris is clearly recognizable within them. Furthermore large drops of opaque material and yolk platelets can be found (Fig. 1).
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Fig. 10. Inner plexiform layer. *bt*, bipolar terminal; *ap*, amacrine cell process. Synaptic vesicles are present in the majority of the neural profiles. × 35000.
Fig. 11. Ganglion cell layer. In the left upper corner a portion of a cell of the lens and the basement membrane can be seen. Mf, Müller feet. The ganglion cell nuclei are surrounded by scarce cytoplasm rich in ribosomes but almost lacking the inner membranous systems. × 14000.
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**Inner plexiform layer**

The characteristics of this synaptic layer are similar in the culture material to the in vivo retina. The neuropile is made up primarily of nerve processes of low calibre, some of which contain neurotubules and of larger processes which are more irregular in shape and contain one or more mitochondria, numerous glycogen granules, synaptic vesicles and, when visible, the synaptic ribbon (Fig. 10). In vivo these elements, which can be identified as the endings of the bipolar cells, are more frequent in the deeper portion of the layer.

The majority of the nerve fibres contain synaptic vesicles. Both types of synaptic contact described in the literature at this site, are present, i.e. synapses of the conventional type (axo-axonic and axo-dendritic) and synapses characterized by the presence of ribbons. This second type however does not often appear in the dyad configuration reported in adult retinas. Furthermore, even if the ribbon is lying perpendicular to the presynaptic membrane, no particular structural peculiarities are observed on the post-synaptic side. Ribbons are not a frequent finding, but since they have very small dimensions it may be difficult to detect them on account of their position in the preparation.

In several synapses of the conventional type there is a symmetric thickening of the membranes and a migration of only two or three vesicles on the presynaptic side in spite of the fact that there are many present. The vesicles are often in both the elements making up the synapse; however, no serial-synapses were recognized.

The density of these synapses is very low also in the retina of larval stages of Rana pipiens before metamorphosis, as it was reported by Fisher (1972).

**Ganglion cell layer**

In this layer no significant differences between the tadpole retina and that of the experimental material were observed. The ganglion cells do not appear to be any larger than the amacrine cells, nor do they differ greatly in their features (Fig. 11). Groups of axons are found in between these cells close to the basement membrane. The observations refer to the cells lying immediately below the inner plexiform layer. Since the inner cavity is missing, the centre of the in vitro eye is filled by a large number of cells, the nature of which has not been clearly defined since distinctive characters are lacking. They could even be ganglion cells. In the optic cup shown in Fig. 1 a kind of lens is present but it has not completed its differentiation. It is separated from the retina by a basement membrane and connective cells.

**DISCUSSION**

As might have been expected, following the experiments of Strangeways & Fell (1926) and Perri (1934) respectively on chick and frog embryos, culturing
optic vesicles resulted, in a large number of specimens, in the formation of a stratified and differentiated retina. However, the degree of this differentiation, which in the above mentioned studies had been ascertained by light microscopical observations, appeared to warrant analysis under the electron microscope, since recent attempts to culture mammalian or chicken retina (Hild & Callas, 1967; Sheffield & Moscona, 1970; Hansson, 1971; La Vail & Hild, 1971) had shown at the ultrastructural level that photoreceptors were unable to complete their differentiation. In rat retina in vitro stratification occurs, the different types of cells acquire their distinctive characteristics and synaptic contacts are set up between the components of the plexiform layers. However, the tissue is different from that of control embryos at the same age, and the main difference lies in the outer segments of the photoreceptors: they are either completely lacking, or do not develop beyond the very early phases of their formation. This finding is also valid for the chicken retina and in addition stratification is quite irregular in the latter (Ieradi, personal communication).

In contrast to these observations, in our cultured optic vesicles we found a better differentiation of all the tissue elements, in particular the formation of the outer segments. From the structural complexity of the receptors it was even possible to distinguish rods and cones.

Several factors may play a role in determining the different behaviour observed in culture conditions, one of which is the origin of the experimental material, i.e. amphibian rather than mammalian or bird embryos. The amphibian embryo cells are all endowed with nutritional material, of which the yolk platelets are the morphological expression. They are therefore self-sufficient and are less dependent upon the integrity of the embryo. Ever since the early experiments of Holtfreter (1931) it has been known that amphibian cells can be maintained in culture, even in a simple physiological solution, for extended periods of time. All the factors essential for the structural integrity of the photoreceptor may be present in the yolk platelets. In addition to Vit. A, Daemen (1973) stresses the importance of antioxidative agents (Vit. E and ascorbic acid) to protect the unsaturated compounds, including retinene, present in the external segments.

It should be emphasized that the procedure used in this study was organ culture and not tissue culture, which ensures that the reciprocal relationships between the cells in a complex organization are maintained. Several authors consider that the preservation of contact between pigment epithelium and neural retina is essential for the building up of the outer segments. Experimental evidence has not as yet been obtained due to the impossibility of preventing the separation of the two retinal components in tissue cultures.

Culture experiments carried out by Sidman (1961) on intact mouse eyes explanted before the formation of the outer segments, demonstrated that even in these conditions the photoreceptors were not completed, unless 11-cis retinene, and not its isomer or Vit. A, was added to the culture medium. The
In vitro differentiation of photoreceptors in Rana cells of the pigment epithelium, unlike the in vivo tissues, are therefore unable to metabolize either Vit. A or the all-trans form of its aldehyde. Furthermore, Sidman did not study whether the addition of 11-cis retinene had the same effect on the differentiation of neural retina even in the absence of the pigment epithelium. It is possible that in vitro the cells from the mammalian and bird pigment epithelium lose some of their metabolic and morphologic characteristics. The lack of tyrosinase synthesis in some cells and the decreased activity of this enzyme reported by Whittaker (1967) appear to support this hypothesis. We also observed an alteration in the metabolism of the pigment since in our material degradation of the pigment granules apparently takes place. However, this does not necessarily imply changes in the possible metabolic pathways involved in the organization of the outer segments of photoreceptors.

The close correlation both in vivo and in vitro between the degree of differentiation reached by the pigmented cells and that of the receptors in contact with them, is in our opinion significant. At the sites in which the receptors had not formed the inner segment, the cells of the tapetum did not show the complex system of tubules of the smooth endoplasmic reticulum which is characteristic of this type of cell, and which was first described by Porter & Yamada (1960). Yamada & Ishikawa (1965) in electron microscopical observations on the development of the human eye also took into consideration this particular relationship. In addition, Dowling & Sidman (1962) described in the pigment cells in the retina of mice affected by retinal dystrophy a certain degree of involution of the endoplasmic reticulum when degeneration of the outer segments was completed.

In attempts in this Institute (Ieradi, personal communication) to cultivate strips of retina still attached to the tapetum from 6-day-old chick embryos, it was found that after 13 days of culture the pigmented cells had characteristics common to 11–12 days of development in vivo, even though they showed no signs of cellular damage and pigment granules were forming. Correspondingly, the receptors appeared to have reached the same developmental stage, in zones in which a certain organization of the neural tissue had been preserved. Numerous elements of the rough endoplasmic reticulum were present in the cytoplasm of the pigmented cells, whilst the smooth reticulum was lacking. Therefore, from the findings outlined above, it might be concluded that to maintain contact between tapetum and neural retina might not be a sufficient condition to ensure the formation of the outer segments of the photoreceptors in vitro.

A peculiar feature observed in the pigmented cells of the cultured optic vesicles is the different distribution and form of the pigment granules compared with those found in vivo. The granules are predominantly roundish in shape and occupy all the cytoplasm. It is interesting to note that in eyes formed following the transplant of optic vesicles under the epidermis of frog embryos, which likewise are not in a condition to promote central visual projections, we observed, as in vivo, the displacement of the pigment into the apical part and
fringes of the tapetum cells, with a differentiation in the form of the granules (Caravita and Cataldi, unpublished observations). The factors controlling the intracellular movements of pigment in relation with visual function are numerous and complex, and their role in culture condition is worthy of more detailed experimental work. In the cytoplasm of the pigmented cells phagocytized portions of the outer segments can be seen, suggesting a process of continuous degradation from the apex and proliferation from the base of the lamellae, as already demonstrated in adult frogs (Young, 1971). Nevertheless, fairly large stacks of these lamellae are visible in the extracellular spaces above the limiting membrane. This pattern is similar to that observed by Dowling & Sidman (1962) and by Bok & Hall (1971) in dystrophic retinas and which is attributed to a lack of phagocytosis of the material which has detached from the receptors. It is not possible, in the present state of our knowledge, to establish whether the rather irregular aspect of the tips of the outer segments observed in our material can be interpreted as the onset of involution due to disordered metabolism of the tissues in vitro.

As far as synaptic contacts are concerned, our studies confirm the findings of other authors on cultured retinal tissue (Stefanelli et al. 1967b; Hild & Callas, 1967; Sheffield & Moscona, 1970; La Vail & Hild, 1970), i.e. the presence of synapses both of the conventional type and of the type characterized by the presence of synaptic ribbons. Furthermore, the plexiform layers do not appear to be significantly different from those of the control retinas, either in the morphology of the components or in the number or degree of maturation of the synapses. Although ribbons and several synaptic vesicles are present in the endings, the majority of these have not yet achieved the configuration of dyads. In studies on the differentiation of mouse retina, Olney (1968) reported that the dyads form later than the conventional synapses.

Serial-synapses of the conventional type in the inner plexiform layer are particularly numerous in amphibian and avian retinas with complex receptive fields, in comparison with mammalian retinas (Dowling, 1968). This anatomical feature has been correlated with differences in the processing of visual information. Fisher (1972) measured the density of serial-synapses in Rana pipiens at several larval stages and found that it rapidly increased with the onset of metamorphosis. He suggested that this might be correlated with changes in motion sensitivity. The cultured eyes examined in the present study do not reach an age corresponding to the stages considered by Fisher, so that we cannot discuss their synaptic organization in relation to function. Interesting information about this problem could be gained by the study of retinas cultured for longer periods of time.

In contrast to observations by La Vail & Hild (1970), no decrease was found in the number of amacrine and ganglion cells (using visual evaluation only), nor was there any lack or difficulty in identifying Müller cells. On the contrary, these latter cells, in our material, had an almost hypertrophic appearance.
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Their extensions are of greater calibre, even if less-branched, between the cells of the nuclear layers, and the perinuclear cytoplasm is more abundant and richer in cytomembranes. The cell processes are characterized by the presence, at all levels, starting from the outer plexiform layer, of numerous inclusions of various types; for example, lipid droplets, yolk platelets, myelinic bodies, cytolysosomes. These cells appear to be involved in intense lytic activity, which is in keeping with their glial nature.

It can be concluded from these investigations that in amphibia, in contrast to birds and mammals, the photoreceptors are able to form their own outer segments, since they are probably provided with the metabolites required for the synthetic cycle of the visual pigments and are also able to make use of them. Thus frog retina would be ideal material for investigations aimed at establishing the role of the pigment epithelium in the morphogenesis of photoreceptors.

Supported in part by a grant of the National Research Council.

REFERENCES


(Received 11 June 1974, revised 17 September 1974)