

LIPOFUSCIN AUTOFLUORESCENCE: EVIDENCE FOR VITAMIN A INVOLVEMENT IN THE RETINA

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SUMMARY

A lipofuscin-like autofluorescence develops in the degenerating photoreceptor cells of the RCS rat, a strain with inherited retinal dystrophy. In animals with normal retinas, age-related lipofuscin accumulation in the eye is restricted to the retinal pigment epithelium (RPE). Previous investigations have established that RPE lipofuscin accumulation in the normal rat retina can be reduced by dietary vitamin A deficiency. In order to determine whether the photoreceptor-derived fluorescence in the RCS rat retina is related to RPE lipofuscin fluorescence, the influence of dietary vitamin A on the fluorophore content of the RCS rat retina was studied. Vitamin A deficiency substantially reduced the autofluorescence associated with degenerating photoreceptor cells of the RCS rat retina. A specific vitamin A-dependent fluorophore was isolated from these retinas using thin-layer chromatography (TLC). The mobility of this fluorophore on TLC differs from that of the major age-dependent fluorophore isolated from the RPE of normal rats. Thus, if the vitamin A-dependent fluorophores of the photoreceptors and RPE are related, it appears that the fluorophore generated in the photoreceptor cells must undergo chemical modification once it has been taken up by the RPE. The fact that both the RPE- and photoreceptor-associated fluorophores are vitamin A-dependent suggests that such a relationship between them is likely. These experiments indicate that the RPE is somewhat different from other lipofuscin-accumulating tissues in that a major precursor of RPE lipofuscin fluorophores originates in another cell type and enters the RPE via phagocytosis.

Key words: Lipofuscin; Retinol; Retina; Aging; Fluorescence; Thin-layer chromatography

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INTRODUCTION

Lipofuscin granule accumulation in post-mitotic cells appears to be a universal feature of the aging process in animals [1]. During senescence, lipofuscin accumulation has been observed in human neurons, cardiac and skeletal muscle, and in cells of a variety of other tissues [2,3], including the retinal pigment epithelium (RPE) [4,5]. While the occurrence of age-related lipofuscin deposition was first recognized a century ago [6], substantial uncertainty remains regarding the mechanisms underlying pigment granule formation. Determination of these mechanisms may ultimately make it possible to elucidate the role of lipofuscin in the aging process.

The RPE is an ideal tissue in which to study lipofuscin accumulation; the cells of the RPE form a single layer in the eye, which greatly facilitates collection, isolation, and quantitation of age-pigment. Other lipofuscin-accumulating cell types, on the other hand, are widely dispersed among cells not containing lipofuscin. A number of experiments have produced some insight into the mechanisms of RPE lipofuscin deposition. Autoxidation appears to be involved in age-pigment formation, since dietary deficiency in antioxidant nutrients greatly accelerates RPE lipofuscin accumulation [7,8]. Experimental evidence also indicates that the photoreceptor cells participate in the formation of age-pigment that accumulates in the RPE. In rats lacking photoreceptor cells due either to a genetic defect, or as a result of light damage, RPE lipofuscin deposition is greatly reduced [9,10]. Recent experiments have suggested that components of photoreceptor outer segments, which are normally phagocytosed by the RPE, may be major precursors of RPE lipofuscin fluorophores. In RCS rats, phagocytosis by the RPE is severely impaired, so that photoreceptor outer segment components accumulate above the RPE cells. Elements of the degenerating photoreceptor cells develop an autofluorescence similar to that of RPE lipofuscin [10]. Together, these findings suggest that components of the photoreceptor cells may be converted directly into RPE lipofuscin fluorophores via processes involving autoxidation. While the phagocytosis of photoreceptor outer segments appears to be a major contributor to RPE lipofuscin formation, the age-related accumulation of lipofuscin continues at a reduced rate in the absence of phagocytosis [10]. Thus, whereas lipofuscin appears to arise in most tissues via autophagy, both autophagy and heterophagy are involved in RPE lipofuscin deposition.

Vitamin A is among the molecular components of the photoreceptor cells that are likely precursors of RPE lipofuscin fluorophores. As an integral component of the visual pigments, vitamin A is present in the photoreceptor cells in relatively high concentration. The ease with which vitamin A is destroyed by oxidation suggests that it may be involved in the accelerated deposition of RPE lipofuscin in antioxidant-deficient animals. Evidence that photoreceptor vitamin A may participate in RPE lipofuscin deposition is provided by the finding that dietary vitamin A deficiency reduces RPE lipofuscin deposition in rats with normal retinas [11,12]. To determine

whether photoreceptor cell vitamin A could be converted directly into lipofuscin-related fluorophores *in vivo*, experiments were conducted to determine the influence of dietary vitamin A on the autofluorescence which develops in the degenerating photoreceptor cells of RCS rats.

MATERIALS AND METHODS

Experimental animals

RCS rats were obtained at weaning (21 days of age) from the foundation colony of the National Institutes of Health Veterinary Resources Branch. Animals were maintained in hanging wire cages under 12 h/12 h cyclic illumination. Maximum illumination at the cage bottoms was 100 lux. The rats were fed *ad libitum* diets containing vitamin A either in the form of retinyl palmitate (+A) or retinoic acid (−A) [12]. Metabolic derivatives of retinyl palmitate can be used in the visual process, whereas retinoic acid cannot be converted through normal metabolic pathways into forms that participate in visual transduction. Thus, in animals fed the diet containing retinoic acid, the retinas were deficient in most forms of vitamin A normally present in this tissue.

Tissue vitamin A levels

When the rats had attained 65–67 days of age, blood and eye tissue samples were taken from each animal. Plasma and eye retinol levels were measured in order to determine the effectiveness of the −A diet in reducing tissue vitamin A contents. Plasma retinol concentrations were determined essentially as described by Bieri and colleagues [13]. Eye retinol contents were measured in animals that had been light adapted under conditions that bleach at least 95% of the visual pigment in animals with normal retinas. After the rats were light adapted, their eyes were enucleated under CO₂ anesthesia, and the corneas, irides, and lenses were dissected from the eyes. The remaining eye tissues were sonicated, and retinol concentrations in the sonicates were determined using the same methods as those used for plasma [13].

Fluorescence microscopy

One eye each from several animals in each dietary group was prepared for examination by fluorescence microscopy in order to assess the effect of dietary vitamin A on the total fluorescence intensity of the degenerating photoreceptor cells. The eyes were fixed for 1 h in a glutaraldehyde/paraformaldehyde fixative [14], and were then bisected along the superior-inferior meridian. One-half of each eye was embedded in Tissue Tek II (Miles Laboratories), and 10- μ m thick frozen sections were cut along the superior-inferior meridian using an International Equipment Company model CT1 cryostat. Sections were examined and photographed using a Leitz orthoplan fluorescence microscope equipped for epi-illumination. Autofluorescence from all tissue samples was photographed with Kodak ED-135 film using a 390–490 nm

band-pass filter in conjunction with a 200 W high-pressure mercury vapor lamp for excitation and a 510 nm barrier filter in the emission path.

Thin-layer chromatography

Analysis of the fluorophore composition of eyes from both dietary groups was carried out in order to determine whether specific vitamin A-dependent fluorophores could be identified. Animals were anesthetized with CO₂, enucleated, and the corneas, irides, lenses, and vitreous were dissected away from the remaining eyecups, which were frozen immediately for subsequent analysis. Eyecups from pairs of animals were pooled for analysis. The samples were thawed and extracted in 2 : 1 chloroform:methanol [15]. The chloroform phases were evaporated to dryness under nitrogen, and the residues were resuspended in 2 : 1 chloroform/methanol containing 0.1 mg/ml metanil yellow as an internal standard. Fluorescent components of the resulting samples were separated by thin-layer chromatography (TLC) [10]. After development, the chromatograms were examined and photographed under ultraviolet illumination [10]. In order to determine the relative abundances of the various fluorescent components separated by TLC, the chromatograms were scanned with a Zeineh soft laser scanning densitometer operating in the absorbance configuration at 420 nm. Peak areas of the fluorophores were determined relative to the peak areas of the internal standard.

Statistical analysis

The statistical significance of the influences of dietary vitamin A on the various measured parameters was determined using analysis of variance.

RESULTS

Tissue vitamin A levels

Feeding rats the $-A$ diet resulted in significant reductions in both plasma and eye retinol levels. In rats fed the $-A$ diet, the mean plasma retinol concentration was 547 ± 107 pmol/ml ($n = 7$), which was only about 46% of the retinol concentration in rats fed the $+A$ diet (1178 ± 185 pmol/ml) ($n = 6$) ($P < 0.01$). The retinol content of eyes from fully light-adapted rats was also significantly reduced as a result of feeding the animals the $-A$ diet. Animals maintained on the $+A$ diet had a mean of 104 ± 7 pmol of retinol/eye ($n = 6$), whereas those fed the $-A$ diet had only 60 ± 9 pmol/eye ($n = 7$) ($P < 0.01$).

Fluorescence microscopy

In rats fed the $+A$ diet, the layer of degenerating photoreceptor components had a bright golden-yellow emission, similar in color to the emission from lipofuscin. Deficiency in forms of vitamin A capable of participating in visual transduction resulted in a substantial reduction in the fluorescence intensity of the degenerating photoreceptor cells (Fig. 1). This reduction in fluorescence intensity did not appear to be due to a reduction in the amount of material derived from degenerating

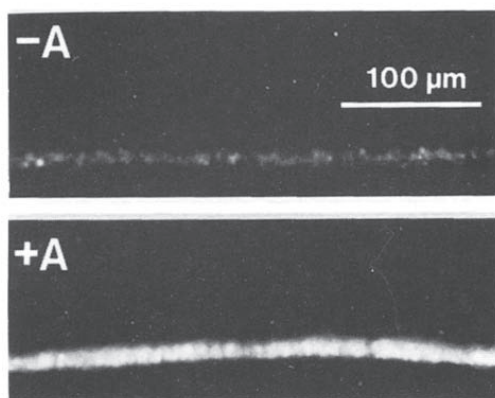


Fig. 1. Fluorescence micrographs of retinas from 66-day-old RCS rats which had been fed either the $-A$ (top) or $+A$ (bottom) diet since weaning. The total fluorescence intensity of the degenerating photoreceptor outer segment material was strongly dependent on vitamin A.

photoreceptor cells, which was similar in both dietary groups (Fig. 2), but was apparently due to reductions in the amount of fluorophores within this material.

Thin-layer chromatography

Fluorophores extracted from eyes of the experimental animals were separated using thin-layer chromatography. Feeding rats the $-A$ diet resulted in a substantial reduction in the fluorescence intensity of two components separated by TLC (Fig. 3). One component, with an R_f of 0.83 displayed a green emission under ultraviolet illumination. This component had chromatographic and fluorescence properties identical to those of an *all-trans* retinol standard [16]. Reduction in the intensity of this component by feeding rats the $-A$ diet is consistent with the finding that retinol levels were reduced in the eyes of rats fed this diet. It is unlikely that this green fluorophore contributed to the autofluorescence of the degenerating photoreceptor cells, since vitamin A fluorescence fades rapidly under ultraviolet light, whereas the fluorescence in the RCS rat retina was relatively stable.

The intensity of a stable orange fluorophore, with an R_f of 0.68, was also substantially reduced by feeding rats the $-A$ diet (Fig. 3). Expressed relative to the absorbance of the internal standard, the mean absorbance of this orange component was 63.2 ± 6.3 ($n = 5$) absorbance units in rats fed the $+A$ diet, and 20.3 ± 4.3 ($n = 5$) absorbance units in rats maintained on the $-A$ diet. The approximately 3-fold difference in the absorbance of this orange component between the two dietary groups was statistically significant ($P < 0.005$).

A variety of other fluorophores were present in the chromatograms of extracts from both dietary groups (Fig. 3). A stable orange fluorophore, with an R_f of 0.37, has been found to be associated with RPE lipofuscin [16]. A red fluorophore with relatively low mobility in the TLC system used in this study appears to be specific to the RCS rat eye. It has not been observed in extracts from human RPE or in extracts from eyes of other rat strains. Dietary vitamin A did not appear to have a consistent influence on any of these other fluorophores.

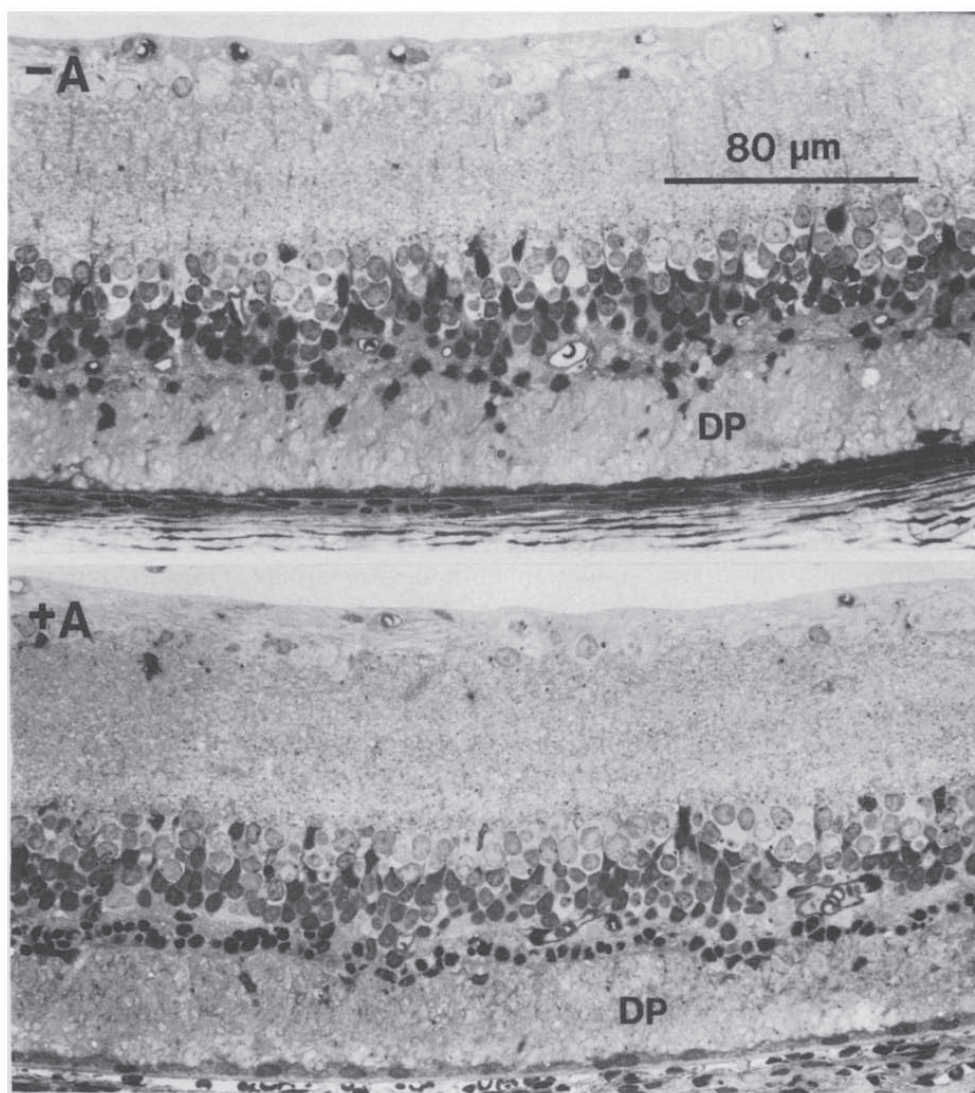


Fig. 2. Light micrographs of retinas from 66-day-old rats fed either the -A (top) or +A (bottom) diets. Dietary vitamin A had no apparent influence on the amount of material from degenerating photoreceptor cells (DP) that was present in the retina. Fewer normal-appearing photoreceptor nuclei were present in the retinas from -A animals, however.

DISCUSSION

Previous experiments have suggested that a major source of precursors for RPE lipofuscin fluorophores is the photoreceptor cell layer of the retina [9,10]. The light-sensitive pigments of the photoreceptors are contained in specialized finger-like projections of the cells called outer segments. These outer segments are turned over at a rapid rate. While new outer segment components are synthesized by the

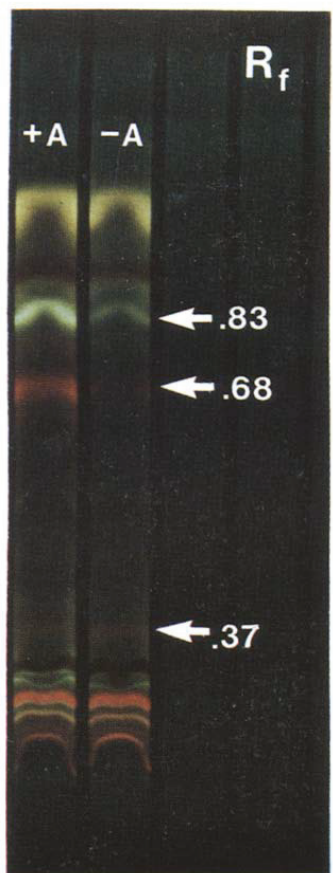


Fig. 3. Fluorescence photograph of thin-layer chromatograms of chloroform-methanol extracts from eyes of RCS rats fed the +A diet (left lane) and the -A diet (right lane). Autofluorescent emission was induced by ultraviolet illumination of the TLC plate [10]. The green bands with R_f values of 0.83 have been tentatively identified as retinol. The effect of diet on the intensity of the orange band with an R_f value of 0.68 correlated with the influence of dietary vitamin A on the fluorescence intensity of the degenerating photoreceptor cells. Other fluorescent components visible on the chromatograms were not consistently affected by diet.

photoreceptor cells themselves, old fragments of the outer segments are shed from the photoreceptors and are taken up and degraded by the RPE. When retinas lacking photoreceptors due either to light damage or to a genetic defect were studied, it was discovered that RPE lipofuscin deposition was substantially reduced [9,10]. It thus appeared likely that components of the photoreceptor outer segments were being converted into RPE lipofuscin fluorophores. Strong support for this hypothesis came from studies on the RCS rat. In this rat strain, the ability of the RPE to take up shed outer segment components is severely impaired, so that these components accumulate above the apical surface of the RPE (Fig. 2). A lipofuscin-like autofluorescence was found to develop in this material, providing the most direct evidence available that

components of the outer segments can be directly converted into RPE lipofuscin fluorophores.

The question then arose as to what the specific molecular components of the outer segments were that gave rise to the lipofuscin fluorophores. Investigations had shown that vitamin A deficiency significantly reduced RPE lipofuscin accumulation in rats with normal retinas [11,12]. Since vitamin A is present in the photoreceptor outer segments in large amounts as a component of the visual pigments, it appeared possible that the vitamin A within the outer segments was converted into lipofuscin fluorophores. On the other hand, the effect of vitamin A deficiency on RPE lipofuscin accumulation may have been the result of an indirect effect of vitamin A on RPE metabolism. In order to distinguish between these possibilities, the effect of vitamin A deficiency on the RCS rat retina was studied. Because RPE phagocytosis is impaired in the RCS rat, it was possible to study the influence of vitamin A on the development of outer segment-derived autofluorescence under conditions where the influence of the RPE is excluded. The finding that this outer segment-derived autofluorescence was substantially reduced as a result of vitamin A deficiency in the RCS rat provides strong evidence that vitamin A contributes directly to the formation of lipofuscin in the RPE.

The major stable vitamin A-dependent fluorophore extracted from the RCS rat retina corresponds to a minor lipofuscin-associated fluorophore present in the RPEs of humans and rats with normal retinas [16]. The major stable orange fluorophore associated with RPE lipofuscin has a considerably lower mobility (R_f of 0.37) in the TLC system used in this study [16]. It is possible that the R_f 0.83 orange fluorophore seen in the RCS rats is generated in the photoreceptor cells even in animals with normal retinas, but is altered chemically after being taken up by the RPE in such a manner as to alter its mobility on TLC. Hydrophobic compounds are frequently hydroxylated or otherwise made more polar during the course of normal metabolism. Such an increase in polarity would help account for a decreased chromatographic mobility on silica sorbents. In the RCS rat, such a conversion process would occur only to a minimal extent, since the ability of the RPE to engulf shed photoreceptor outer segment components is severely impaired in these animals. The observation that RPE lipofuscin fluorescence is reduced by vitamin A deficiency in rats with normal retinas is consistent with the possibility that RPE lipofuscin fluorophores and the orange fluorophore isolated from the RCS rat retina are related.

Confirmation of the hypothesis that RPE lipofuscin fluorophores are derived from the vitamin A-dependent fluorophore generated in the photoreceptor cells should be possible using cultured RPE. Normal RPE cells are capable of phagocytosis in culture [17]. By feeding such cells the autofluorescent material derived from RCS rat retinas, the ability of the RPE to convert the photoreceptor-derived fluorophore into a lipofuscin-specific fluorophore can be evaluated. In conjunction with the association now established between vitamin A and RPE lipofuscin accumulation, such experiments will greatly enhance our understanding of the mechanisms of RPE lipofuscin formation.

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REFERENCES

- 1 H. Donato, Jr., M.A. Hoselton and R.S. Sohal, Lipofuscin accumulation: effects of individual variation and selective mortality on population averages. *Exp. Gerontol.*, **14** (1979) 141-147.
- 2 E.A. Porta and W.S. Hartroft, Lipid pigments in relation to aging and dietary factors (lipofuscins). In M. Wolman (ed.), *Pigments in Pathology*, Academic Press, New York, 1969, pp. 192-236.
- 3 K.R. Brizzee and J.M. Ordy, Cellular features, regional accumulation, and prospects of modification of age pigments in mammals. In R.S. Sohal (ed.), *Age Pigments*, Elsevier/North-Holland, Amsterdam, 1981, pp. 102-156.
- 4 L. Feeney, Lipofuscin and melanin of human retinal pigment epithelium. *Invest. Ophthalmol. Vis. Sci.*, **17** (1978) 583-600.
- 5 G.L. Wing, G.C. Blanchard and J.J. Weiter, The topography and age relationship of lipofuscin concentration in the retinal pigment epithelium. *Invest. Ophthalmol. Vis. Sci.*, **17** (1978) 601-607.
- 6 H. Koneff, Beitrage zur kenntniss der Nervenzellen in den peripheren Ganglien. *Mitt. Naturforsch. Ges. Bern*, (1886-1887) 13-44.
- 7 M.L. Katz, W.L. Stone and E.A. Dratz, Fluorescent pigment accumulation in retinal pigment epithelium of antioxidant deficient rats. *Invest. Ophthalmol. Vis. Sci.*, **17** (1978) 1049-1058.
- 8 W.G. Robison, Jr., T. Kuwabara and J.G. Bieri, Vitamin E deficiency and the retina: photoreceptor and pigment epithelial changes. *Invest. Ophthalmol. Vis. Sci.*, **18** (1978) 683-690.
- 9 W.G. Robison, Jr., T. Kuwabara and J.G. Bieri, The roles of vitamin E and unsaturated fatty acids in the visual process. *Retina*, **2** (1982) 263-281.
- 10 M.L. Katz, C.M. Drea, G.E. Eldred, H.H. Hess and W.G. Robison, Jr., Influence of early photoreceptor degeneration on lipofuscin in the retinal pigment epithelium. *Exp. Eye Res.*, (1986) 561-573.
- 11 W.G. Robison, Jr., T. Kuwabara and J.G. Bieri, Deficiencies of vitamins E and A in the rat. Retinal damage and lipofuscin accumulation. *Invest. Ophthalmol. Vis. Sci.*, **19** (1980) 1030-1037.
- 12 M.L. Katz, C.M. Drea and W.G. Robison, Jr., Relationship between dietary retinol and lipofuscin in the retinal pigment epithelium. *Mech. Aging Dev.*, **35** (1986) 291-305.
- 13 J.G. Bieri, T.J. Tolliver and G.L. Catignani, Simultaneous determination of α -tocopherol and retinol in plasma or red cells by high pressure liquid chromatography. *Am. J. Clin. Nutr.*, **32** (1979) 2143-2149.
- 14 M.L. Katz and W.G. Robison, Jr., Age-related changes in the retinal pigment epithelium of pigmented rats. *Exp. Eye Res.*, **38** (1984) 137-151.
- 15 J. Folch, M. Lees and G.H. Sloane-Stanley, A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.*, **226** (1957) 497-509.
- 16 G.E. Eldred, Questioning the nature of age pigments (lipofuscin) in the human retinal pigment epithelium and their relationship to age-related macular degeneration. In J.M. Ordy, D. Armstrong and M.F. Marmor (eds.), *Environmental Design for Optimum Vision in the Elderly*, Plenum Press, New York, in press.
- 17 R.B. Edwards, The use of tissue culture techniques to study normal and diseased retinal pigment epithelium. *Prog. Retinal Res.*, **2** (1983) 51-66.