Patterns of cell death, apoptosis and necrosis and the question of recovery in light induced retinal degeneration in the rat.

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1. Abstract.

The purpose of this study was to investigate the early structural changes and the pattern of recovery in the rat retina after light damage. Methods: Albino rats were dark adapted for 36 hours. The control animals were sacrificed at the end of the dark adaptation period. The experimental animals were exposed for 2 hours to 1000 lux of white light, divided into 5 groups and killed at the following intervals: at the end of light exposure (0), 24, 48, 72 and 144 hours later. The right eyes were fixed in glutaraldehyde and processed for light and electron microscopy. The left eyes were processed for agarose gel electrophoresis of extracted retinal DNA to examine the pattern of DNA fragmentation occurring in various types of cell death. Results: Irreversible photoreceptor damage, observed after two hours of light exposure was observed in the lower temporal retina. It was characterized by condensation of cytoplasm, densification of outer segments and inner segments, including synapses, and pyknosis of the photoreceptor nuclei. Mitochondria, cilia and intracellular organelles and the arrangement of rod outer segment disks were, however, well preserved. Extensive auto and heterophagy (Muller cells) was observed but there was no inflammation. These changes were consistent with programmed cell death (apoptosis), which was confirmed by agarose gel electrophoresis showing a ladder formation characteristic for apoptosis up to 48 hours after light damage. Within 24 hours numerous photoreceptors showed, however, swelling and lysis of the inner segments, changes consistent with necrosis. Within a week chorioretinal adhesion was formed. Cell death in the retinal pigment epithelium was observed 24 hours after light exposure. The loss of the retinal pigment epithelium was followed by a breakdown of the blood retinal barrier resulting in serous retinal detachments and microcystic retinal oedema at its maximum 48 hours after exposure. Proliferation of cells in the inner nuclear layer was noted and an influx of macrophages, but no other inflammatory cells, was most pronounced between 48 and 72 hours after light damage. Lower nasal retina which primarily showed vesicular alterations of the rod outer segments as an acute response recovered normal morphology within one week. Conclusion: Diffuse white light can trigger regional retinal degeneration in a rat. Apoptosis of photoreceptors is an immediate retinal response to toxic light levels in albino rat retinae undergoing degeneration. Necrosis which was also recorded may be secondary in nature. In contrast, morphological recovery is observed in the regions in which vesicular alterations of disk membranes of photoreceptor outer segments were predominant. Such changes may signify disfunction of outer segment membranes and are enhanced by glutaraldehyde fixation.

2. Introduction.

2.1. The types of cell death.

The purpose of this chapter is to introduce the two types of cell death, necrosis and apoptosis, and briefly characterize them.

Cell death can be defined as an irreversible loss of integrated cellular function (Tonner et al. 1992). Various types of cell death occurring in different circumstances can be identified and compared using morphology as a criterion. The structure of dying cells changes in a restricted number of ways and two principal sets of changes have been described: necrosis and apoptosis. In addition a rare mode of dying is characterized by the formation of "very dark cells" (Harmon 1987).

2.1.1 Necrosis.

Necrosis is a mode of cell death induced by conditions which depart in an extreme fashion from the physiological situation. There are several descriptions of the morphological changes occurring in necrosis (Buckley 1972; Evan and Dali 1974; Finlay-Jones and Papadimitriou 1973; Ganote

et al. 1975; Jennings et al. 1975; Kloner et al. 1974; McDowell 1972a, 1972b; McLean et al. 1965; Trump and Ginn 1969; Trump et al. 1973). The major features of necrosis include cytoplasmic oedema, dilatation of endoplasmic reticulum, mitochondrial swelling with rupture of internal cristae, dissolution of cytoplasmic organelles and rupture of plasma membranes. Necrosis usually affects sheets of cells and attracts neutrophil polymorphonuclear leucocytes and other inflammatory cells. It has been reported in hypoxic injury (Jennings et al. 1975; Kloner et al. 1974; Saladino and Trump 1968), induced by complement (Hawkins et al. 1972; Prieto et al. 1967), inhibition of oxidative phosphorylation, glycolysis or Krebs cycle enzyme (Laiho and Trump 1975; McDowell 1972a, 1972b), hyperthermia (Buckley 1972), or toxins (Evan and Dali 1974; McLean et al. 1965). It is also observed in cells dying due to autolysis (Finlay-Jones and Papadimitriou 1973; Trump et al. 1965). Growing tumours frequently contain areas of necrosis and apoptosis.

2.1.2 Apoptosis.

Apoptosis is a mode of cell death in which single cells are deleted from amongst the living cells. It is a programmed cell death, as opposed to toxic necrosis, and it is a normal and essential process observed in

multicellular organisms (Duvall and Wyllie 1986; Walker et al. 1988; Wyllie 1980, 1987). The morphology of cells dying due to the process of apoptosis is very different from that of necrosis. The morphological changes occurring during apoptosis are well documented (Arends et al. 1990; Kerr et al. 1987; Kerr et al. 1972, Wyllie et al. 1980). Apoptotic cells shrink in volume with a concomitant rise in cell density and conservation of cytoplasmic organelles. The nuclear chromatin is condensed. Light microscopy of apoptotic cells reveals single cells or groups of shrunken cells usually with smooth contours and intensely stained cytoplasm. Pyknosis is one of the features of apoptosis but it can be difficult to distinguish from nuclear hyperchromatism which sometimes occurs during necrosis. Apoptosis attracts macrophages but no inflammatory reaction is observed even if apoptotic cells are present in large numbers. They are the target for immediate phagocytosis either by macrophages already present or by adjacent viable cells. Electron microscopy of apoptotic cells reveals condensation of cytoplasm and margination of nuclear chromatin. The cell adopts a convoluted outline. Intracellular organelles are densely packed. Large cells can produce multiple apoptotic bodies containing fragments of cytoplasm and cytoplasmic organelles, small cells remain as single bodies. Apoptosis is a potent stimulus for rapid phagocytosis by macrophages and adjacent

viable cells. Apoptotic cells undergo phagocytosis by nearby cells, frequently by cells of the mononuclear - phagocyte system and eventually become secondary lysosomes.

Important characteristics of apoptosis are its very short time course, less than 24 hours, and the lack of inflammatory response provoked by apoptotic cell death. Apoptosis is observed in normal tissue turnover (Kerr and Searle 1973; Wyllie, Kerr, and Currie 1973, 1980; Wyllie, Kerr, Macaskill, and Currie 1973), embryogenesis (Bellairs 1961; Farbman 1968; Hammar and Mottet 1971; Manasek 1969; O'Connor and Wyttenbach 1974; Saunders 1966; Saunders and Fallon 1966; Schweichel 1972), metamorphosis (Decker 1976; Goldsmith 1966), endocrine dependent tissue atrophy (Kerr and Searle 1973; Sandow et al. 1979; Wyllie, Kerr, and Currie 1973), growing tumours and tumour regression (Kerr et al. 1972; Searle et al. 1975). Apoptosis can be induced by a number of external stimuli including radiation (Allan et al. 1988; Hendry et al. 1982; Potten 1977; Wyllie et al. 1980; Yamada and Ohyama 1988). Various spectra of electromagnetic radiation can induce apoptosis and it has been recorded after low dose X-irradiation (Potten 1977; Potten et al. 1978; Searle et al. 1975) and UV radiation (Luokkamaki et al. 1993). Pressure induced ischemia-reperfusion injury (Buchi 1992) and photochemically induced insults (Agarwal et al. 1991) have been

recorded as provoking apoptosis.

Apart from morphological criteria for detecting apoptosis based on light and electron microscopy a biochemical criterion, the highly specific fragmentation of DNA which occurs during apoptosis, is commonly used and was applied in this study.

In 1980 Wyllie observed that during apoptosis induced by glucocorticoids in rat thymocytes extensive DNA degradation occurs at the onset of apoptotic cell death. DNA degradation, caused by activated endonuclease, occurred in a very specific pattern producing DNA fragments that were multiples of 180-200 base pairs (Wyllie 1980). Electrophoresis of isolated DNA in agarose gels reveals fragments of DNA forming a pattern resembling a ladder of regular 200 base pairs steps. This is a length of DNA wrapped around the histone octamer in a nucleosome. It indicates that the chromatin is being cleaved by endonuclease at the linker DNA between nucleosomes, producing oligonucleosomal fragments (Schwartzman and Cidlowski 1993).

These results identified a significant biochemical event, related to the activation of endonuclease during the early phases of the apoptotic process. Since the initial description of the internucleosomal DNA fragmentation in glucocorticoid treated thymocytes, it has become apparent that it is an almost universal phenomenon associated with

apoptotic cell death (for review see Schwartzman and Cidlowski 1993) and the DNA laddering associated with endonuclease activation has been used as a "marker" for apoptosis (Compton 1992).

2.1.3 Pattern of cell death in retinal degenerations.

The pattern of cell death and its significance in retinal degenerations has not been discussed until very recently (Shahinfar et al 1991, Buchi 1992, Chang et al 1993, Szczesny et al 1993c, Portera-Cailliau et al 1994). Numerous histopathological studies of either light induced or inherited retinal degenerations do not show inflammatory reaction, which is otherwise triggered by necrosis, but rather migration of macrophages (Hoppeler et al. 1988; Szczesny 1991, 1992; Tso 1989) and phagocytic activity in photoreceptors and retinal glia (Szczesny 1991).

Typical apoptotic changes in the retina were not, however, described in these conditions since in the majority of light damage studies the histology was performed some time after the insult and the change in the thickness of the outer nuclear layer was used as a criterion of damage. In the studies of retinitis pigmentosa, however, tissue was obtained and fixed hours after the death of the donor.

2.2 Light and tissue.

In this chapter a definition of light, the ways to measure it and the three major types of light induced tissue reactions are presented.

Light is a form of electromagnetic radiation and is usually defined as the part of the electromagnetic spectrum with wavelengths between 400 and 760 nm (Elkington and Frank 1984).

Light can be measured in two different sets of units. One system is a physical system called radiometric, and is used to describe and define different types of radiant energy including microwaves, X-rays and visible and invisible portions of light. The other system is a photometric system and is only applicable to optical radiation (Marshall 1984).

Light damage can be induced in biological systems in a number of ways and the following mechanisms are recognised: thermal or thermoacoustic, mechanical and phototoxic or phototochemical (Marshall 1970). Photochemical damage can be further divided into two classes.

2.3 Photochemical retinal light damage.

In this chapter the most commonly used classifications of photochemical retinal damage and the mechanism of phototoxicity are summarized.

2.3.1 Classification of photochemical light damage.

Class 1, affecting mainly photoreceptors in non-anaesthetized rodents, is associated with wavelengths resembling the visual pigment absorbtion spectrum. Class 2, affecting retinal pigment epithelium in anaesthetized primates, is caused by wavelengths peaking in the UV spectrum (Kremers and van Norren 1988; Mainster 1987). The above classifications differ from the earlier ones suggested by Noell who pioneered light damage studies in the rat model. He distinguished two classes of rhodopsin mediated damage with Class 1 characterized by damage of photoreceptors and the pigment epithelium and class 2 restricted damage of the visual cells only (Noell 1980a, 1980c). Recently it has been demonstrated, that photoreceptors are selectively damaged by threshold irradiation at wavelengths from 320 to 440nm (UV) and the retinal pigment epithelium and photoreceptors are affected by threshold irradiation with green light of 470 to 550nm wavelengths (Gorgels and van Norren 1995).

2.3.2 Mechanisms of photochemical retinal light damage.

The special nature of photochemical damage to the retina, which was

recognized by Noell (Noell et al. 1966), is still not fully understood today. In order to induce damage light has first to be absorbed by a chromophore sensitive to a particular wavelength and several pigments have been suggested: rhodopsin (Noell et al. 1966), cone pigments (Harwerth and Sperling 1975), melanin (Ham et al. 1980), cytochrome (Lisle 1982) and riboflavin (Noell 1980a). When excessive light energy is absorbed by a chromophore it becomes a photosensitizer and changes to singlet and triplet states. The singlet state is short lived and dissipates energy into a long lived triplet state which can undergo type I and type II reactions. In type I reactions activated photosensitizers react with the substrate in the tissue producing free radicals. In type II reactions photosensitizers in the triplet state react with oxygen, forming singlet oxygen (Tso 1989). Free radicals and singlet oxygen in turn may react with polyunsaturated fatty acids in photoreceptors and retinal pigment epithelium resulting in lipid peroxidation and tissue damage. It is believed that light induced free radical and singlet oxygen formation may be major components in retinal photic injury (Armstrong et al. 1989; Fliesler and Anderson 1983; Handelman and Dratz 1986; Kagan et al. 1973).

2.4 Clinical aspects of retinal light damage.

In this chapter the link between light damage and pathogenesis of some of the retinal diseases is reviewed on the basis of case reports, epidemiological data and circumstantial evidence.

2.4.1 Case studies.

An early description of solar blindness is to be found in "Phaedo" written by Plato at sometime between 427-347 BC (Verhoeff et al. 1916). The earliest description of scotoma caused by a solar burn is attributed to Bonetus (1620-1689) who practised in Geneva (Green 1985).

Solar retinitis, known also as solar eclipse retinopathy, caused by gazing at the sun and characterized by decreased visual acuity and visible retinal lesion, is a clinical example of photochemically induced retinal damage since unaided sun observation is known to increase the temperature in the retina by less than 4 degrees C (White et al. 1971) which is far below the 10 to 20 degrees C rise needed for photocoagulation. Photic maculopathy resulting from direct gazing at the sun or solar eclipse has been documented in a number of studies (Cordes 1948; Ewald and Ritchey 1970; Penner and McNair 1966).

2.4.2 Epidemiological data.

Possible cumulative phototoxic effects manifested as decreased visual acuity and macular pigmentary changes were observed in personnel working outdoors for 4 months and longer in a military base on a tropical island in the Pacific Ocean (Smith 1944). Functional studies demonstrated temporarily increased night vision thresholds in individuals exposed to bright sunlight returning to normal after eyes were protected from sunlight (Clark et al. 1946).

Evaluation of patients after cataract extraction revealed that pseudoaphakic eyes containing non-UV absorbing intraocular lenses showed a greater loss of short wave cones when compared with fellow pseudoaphakic eyes with UV absorbing implants (Werner et al. 1989). Another survey has suggested that the apparent increase of age related macular degeneration in aphakic eyes may be due to light induced damage (Liu et al. 1989). The relationship between the cumulative long term effects of visible light and the epidemiology of senile cataract, age related macular degeneration, pterygium and climatic droplet keratopathy was the subject of a recent study which concluded that high levels of exposure to blue and visible light may cause ocular damage, especially later in life and may be causally related to the development of age

related macular degeneration (Taylor et al. 1992).

2.4.3 Circumstantial evidence.

The evidence on phototoxic light effects initiated by solar radiation have been reviewed and it is considered to be a source of molecular damage in the pathophysiology of age related macular degeneration by Young (Young 1988).

2.5 Morphological studies of light damage.

Photochemical retinal damage was first demonstrated in rats, followed by studies in primates and humans (Ham et al. 1976; Lisle 1982; Noell et al. 1966; Sperling et al. 1980; Tso and LaPiana 1975).

2.5.1 Early experimental work on light damage.

The early experimental work done on light damage to the retina was conducted by Czerny in 1867, Deutschmann in 1882, both of whom used focused sunlight to induce retinal lesions in a rabbit as opposed to Widmark who in 1893 used a carbon arc source (Green 1985).

Deutschman recorded the formation of chorioretinal scar following intense exposure. Photocoagulation was used to produce thermal lesions in the retina of experimental animals in 1916 in which it was noted that near threshold doses cause alterations in the morphology of the retinal pigment epithelium (Verhoeff et al. 1916). The thermal effects of light, causing retinal photocoagulation, were subsequently studied in the human retina in eyes prior enucleation for malignancies (Maggiore 1933). A therapeutic use of photocoagulation to cause retinal burns was attempted in 1950 (Meyer-Schwickerath 1950; Moron-Salas 1950).

In 1966 it was observed that retinal exposure to light in doses far lower then those that cause thermal lesions nevertheless results in retinal degeneration. In a pioneering study Noell and his team postulated that such degeneration occurs due to the direct phototoxic effect of light on photoreceptors (Noell et al. 1966).

2.5.2 Experimental studies on photochemical light damage in animals.

Retinal degeneration in vertebrates can be induced by visible and UV radiation (Cicerone 1976; Kuwabara 1970; Noell et al. 1966; Shear et al. 1973) and has been the subject of recent reviews (Kremers and van

Norren 1988; Miller 1987; Tso 1989). Damage can be induced either by brief exposure to high intensity light (Ham et al. 1976; Kuwabara 1970; Noell et al. 1966) or prolonged exposure to relatively low intensity light (LaVail 1976; Shear et al. 1973) and is more readily produced in albino than pigmented animals (Noell and Albert 1971; Wyse and Hollenberg 1977). The degree of morphological changes in retinae damaged by light is related to several factors including the intensity of the incident light, the wavelength, the duration of exposure and the ambient temperature of the animal (Ham et al. 1976; Kuwabara 1970; Noell et al. 1966; Shear et al. 1973). Susceptibility to light damage in albino rats is also influenced by the previous light history of the animals (Penn et al. 1985; Penn and Williams 1986); the duration of preceding dark adaptation (Noell 1980b); dehydration (O'Steen et al. 1990); acute metabolic stress which protects the retina (Duncan and O'Steen 1983); the time period of light application within a 24 hours cycle (Duncan and O'Steen 1985; White and Fisher 1987) and diet which can influence the rod outer segments phospholipid membrane composition (Bush et al. 1991).

Kuwabara and Gorn described the initial abnormalities affecting the tips of rod outer segments after 3 hours of light exposure (Kuwabara and Gron 1968). Similar observations were also made for much lower light intensities (Moriya et al. 1986; Shear et al. 1973). Similar photoreceptor

abnormalities were recorded in human retinae in solar retinopathy (Hope-Ross et al. 1993). Subsequent animal studies have demonstrated that in light damaged photoreceptors the early ultrastructural changes occur in the inner segments and in the synaptic region almost simultaneously (Kuwabara and Funahashi 1976b; Moriya et al. 1986). The morphology of early light induced changes was also studied extensively in the albino rat retina exposed to threshold light levels (Reme et al. 1991; Reme et al. 1988). Alterations were reported in rod outer segments in an albino rat retina and consisted of dilatation and vesiculation of disk membranes after exposure to white light of 1000 lux for 30 minutes (Bush et al. 1991; Reme et al. 1988). The degree of vesicular alterations of the rod outer segments was used as a criterion to assess the effects of lithium and light effects on the retina (Reme et al. 1988). This criterion was used when methods of protection from light damage using a radioprotective agent WR-77913 (Reme et al. 1991), a PAF antagonist BN 52021 (Jung et al. 1993; Reme et al. 1992) and diet (Bush et al. 1991) were investigated.

2.5.3 Experimental studies on photochemical light damage in humans.

Macular swelling 3 hours after and fluorescein leakage 24 hours after exposure was observed in all 4 patients with choroidal melanoma who agreed to gaze at the sun before enucleation. Histology of these eyes revealed depigmentation and margination of nuclear chromatin in the retinal pigment epithelium, densification of the cytoplasm in some of the cells and an increased number of phagosomes. Pericytes of the choriocapillaris showed migration into the Bruch's membrane, shallow serous retinal detachment was observed and the outer segments showed tubular and vesicular alterations of the membranes. In an eye which was enucleated 12 days after sun-gazing, activation of macrophages and focal loss of photoreceptors was reported (Tso 1988; Tso and LaPiana 1975). Pathology characterized by vesiculation and fragmentation of the membranes of the photoreceptor outer segment, mitochondrial swelling, nuclear pyknosis and degenerative changes in the retinal pigment epithelium was observed in a patient 6 days after sun-gazing in an eye enucleated for malignant melanoma (Hope-Ross et al. 1993).

2.5.4 The pattern of photochemically induced retinal degeneration.

On the basis of experimental work on photic retinopathy in primates, three distinct phases of tissue response have been identified (Tso 1987, 1989). In the first, acute phase, occurring during the first 48 hours after light injury, vesicular alterations of photoreceptor outer segments, vacuolation of the mitochondria in the inner segments and swelling of the retinal pigment epithelium were noted. The second phase is characterized by the invasion of the subretinal space by macrophages from the systemic circulation and the clearing of cellular debris. A third phase of retinal photic injury is characterized by chronic degeneration with a gradual dropout of photoreceptor nuclei, a shortening of the photoreceptor outer segments and alterations in the retinal pigment epithelium which appears to be thin and flat.

2.5.5 Remarks on conventional chemical fixation and cryofixaton.

Morphological studies of retinal light damage have been almost exclusively based on chemically fixed tissue. Glutaraldehyde has been routinely used for the assessment of photoreceptor and retinal pigment epithelium ultrastructure in light damage experiments. The early

morphological changes induced by toxic light levels in photoreceptor cells have been examined after conventional tissue preparation in a number of studies (Bush et al 1991, Grignolo et al. 1969; Kuwabara 1970; Kuwabara and Gron 1968; Moriya et al. 1986, Reme et al 1988, Shear et al. 1973). It is important to consider, however, that glutaraldehyde may not be the optimal fixative to study the acute ultrastructural changes in lipid rich membranes. In recent years an alternative cryo fixation method for thicker specimens has become available. It is known as a high pressure freezing (Moor and Riehle 1968). It is based on freezing the specimen under a pressure of 2100 bar. This pressure suppresses the formation of ice crystals without the need for cryo protectants (Moor 1987). In practical work, the method allows adequate cryo immobilization of fresh cell and tissue samples up to a thickness of several hundreds of μm without visible ice crystal formation (Studer et al. 1989). The tissue is then subjected to freeze substitution procedure and postfixed with osmium tetroxide (chapter 3.7.2). In this study a comparison is made of the effects of glutaraldehyde fixation and cryofixation on the early ultrastructural changes in photoreceptors damaged by diffuse white light. The topic of possible effects of fixation on the morphology of light damaged photoreceptors is discussed in more detail in chapter 5.5. in the Discussion.

2.6 Aim of this study.

The aim of this study was to investigate tissue responses to threshold levels of photochemically induced light damage leading to retinal degeneration in the rat, including the following points:

1. To investigate the type of cell death in photoreceptors and retinal pigment epithelium triggered by light.

2. To define and differentiate between acute reversible and irreversible photoreceptor and retinal pigment epithelium alterations in response to light and to study the time course of such changes.

3. To investigate the pattern of early tissue response leading to either the degeneration or the repair of the retina including inflammatory reaction and the possibility of cell proliferation and subretinal neovascularization.

4. To investigate the influence of fixation on the morphology of rod outer segments, by comparing conventional chemical fixation with glutaraldehyde and cryofixation.

3. Materials and methods.

3.1 Statement.

All experiments were conducted in accordance with the ARVO Resolution on the Use of Animals in Research.

3.2 Animals.

Male Sprang-Dawley albino rats (Tif:RAI, Ciba Geigy AG Breeding Institute, Sisseln / Basel, Switzerland), were kept from birth under a 12 hours light / 12 hours dark cycle with lights on at 0700, a room temperature of + 24 degrees C, a relative humidity of 54% and water and food ad libitum. The illuminance level measured within the cages was 10-25 lux. In total 64 animals were used in two experiments. Thirty six and twenty eight rats were sacrificed for the first and second experiments respectively.

3.3 Light damage protocol.

Animals of 250g weight were dark adapted for 36 hours and exposed to no light (N=6 controls in the 1st experiment and N=3 controls in the 2nd experiment) or to 1000 lux diffuse light (Philips Cool White 36 W in the first experiment and Sylvania Cool White 40 W fluorescent tubes in the second experiment) for 2 hours. The lights were applied from above, the animals were free to move in steel containers. The illuminance level was controlled with a UDT S370 single channel photometer equipped with a UDT 263 photometric sensor head by adjusting the height of the neon lamps above the containers for light exposure. Readings were taken at 5 cm above the floor of the exposure containers, i.e. roughly at the eye level of the rats in 9 different locations in each of 4 containers (fig. 1). The power output of the lamps producing 1000 lux illumination was also recorded with a UDT 247 sensor head. The readings of illumination and power output of lamps, recorded in 9 standard locations in containers, are given in tables 3.3.1, 3.3.2 and 3.3.3

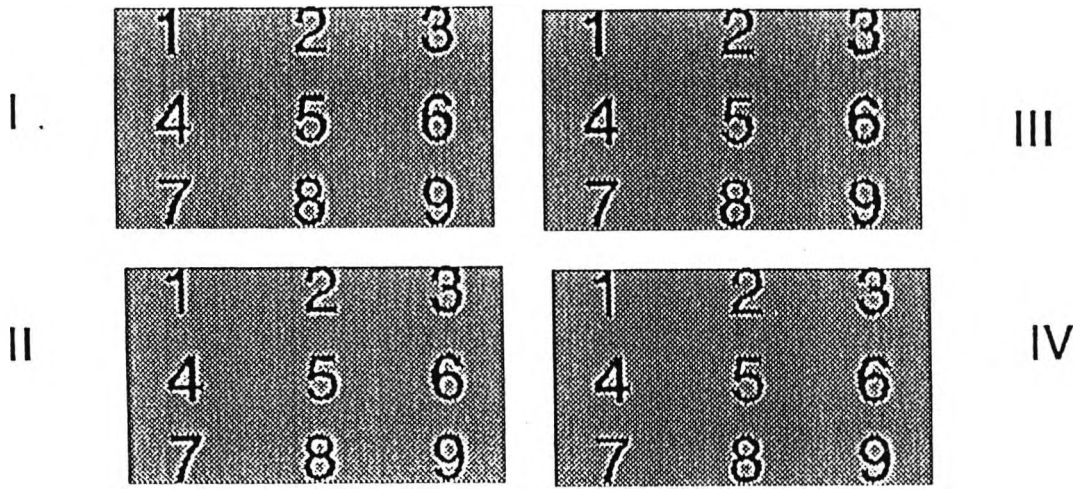


Fig. 1. Schematic drawing illustrating the arrangement of containers used for rats light exposures. Numbers indicate the points where the probes were placed to measure illumination and power of the light source.

Table 3.3.1

Philips TLD 36W/96
Comparison of the illuminance of the lamps at 15, 30, 120 minutes.

22/7 15'	Box I	Box II	Box III	Box IV	avg row
1	1234	973	1086	831	1031
2	1195	897	1197	928	1054.25
3	1018	831	1183	1055	1021.75
4	1020	1067	976	951	1003.5
5	990	1021	1053	1038	1025.5
6	826	868	1096	1064	963.5
7	1047	1224	999	1016	1071.5
8	960	1236	988	1246	1107.5
9	810	1090	1140	1230	1067.5
avg box	1011.111	1023	1079.778	1039.889	
22/7 30'	Box I	Box II	Box III	Box IV	avg row
1	1121	912	1013	887	983.25
2	1109	879	1125	908	1005.25
3	952	817	1154	1036	989.75
4	1020	1025	927	926	974.5
5	964	991	1021	1022	999.5
6	867	869	1057	1037	957.5
7	1068	1202	943	1011	1056
8	920	1104	1061	1264	1087.25
9	872	1042	971	1146	1007.75
avg box	988.1111	982.3333	1030.222	1026.333	
22/7 120'	Box I	Box II	Box III	Box IV	avg row
1	1110	955	1001	804	967.5
2	1088	900	1100	872	990
3	998	829	1171	883	970.25
4	1008	1032	905	916	965.25
5	935	963	996	985	969.75
6	839	858	1041	1012	937.5
7	1037	1172	940	1028	1044.25
8	942	1096	915	1145	1024.5
9	851	1013	1043	1183	1022.5
avg box	978.6667	979.7778	1012.444	980.8889	

Table 3.3.2

Philips TLD 36W/96
Power output of the lamps at 1000 lux measured
at 350 nm, 650 nm and 1100 nm wavelength

loc	350nm 1	350nm 2	350nm 3	350nm 4	350 avg
1	582	612	686	636	629
2	691	638	667	594	647.5
3	672	594	609	585	615
4	583	590	661	653	621.75
5	661	664	632	626	645.75
6	613	616	613	574	604
7	580	672	678	713	660.75
8	601	726	649	737	678.25
9	657	666	627	634	646
avg box	626.6667	642	646.8889	639.1111	638.6667
loc	650nm 1	650nm 2	650nm 3	650nm 4	650 avg
1	311	327	373	339	337.5
2	369	340	356	317	345.5
3	359	317	325	312	328.25
4	320	315	353	349	334.25
5	353	355	337	334	344.75
6	327	329	327	306	322.25
7	310	359	362	380	352.75
8	321	388	346	393	362
9	351	355	335	339	345
avg box	335.6667	342.7778	346	341	341.3611
loc	1100nm 1	1100nm 2	1100nm 3	1100nm 4	1100nm av
1	1029	1082	1213	1122	1111.5
2	1222	1129	1181	1051	1145.75
3	1189	1050	1076	1035	1087.5
4	1031	1044	1170	1155	1100
5	1169	1175	1118	1108	1142.5
6	1085	1090	1085	1015	1068.75
7	1026	1188	1200	1260	1168.5
8	1064	1284	1147	1303	1199.5
9	1163	1177	1108	1123	1142.75
avg box	1108.667	1135.444	1144.222	1130.222	1129.639

Table 3.3.3

Sylvania F40W/CW
Power output of the lamps at 1000 lux measured
at 350 nm, 650 nm and 1100 nm wavelengths
and the illumination levels (target 1000 lux) in exposure boxes

	350.1	350.2	350.3	350.4	avg
1	432	454	477	482	461.25
2	474	494	505	505	494.5
3	469	510	447	460	476.5
4	447	445	477	445	453.5
5	465	481	505	511	490.5
6	459	471	458	450	459.5
7	447	425	475	452	449.75
8	461	456	507	483	476.75
9	444	481	460	425	452.5
avg	457.5556	468.5556	479	468.1111	468.3056
	650.1	650.2	650.3	650.4	
1	231	248	261	262	250
2	258	263	263	278	265.5
3	253	267	239	249	252
4	232	236	256	250	243.5
5	252	249	269	270	260
6	247	247	243	239	244
7	241	223	258	248	242.5
8	257	249	255	251	253
9	248	244	244	226	240.5
avg	246.5556	247.1111	254.2222	252.5556	250.1111
	1100.1	1100.2	1100.3	1100.4	
1	761	811	820	880	818
2	850	860	883	867	865
3	839	883	800	815	834.25
4	794	783	822	796	798.75
5	833	833	884	900	862.5
6	867	808	796	790	815.25
7	802	750	880	825	814.25
8	796	774	922	860	838
9	770	800	785	752	776.75
avg	812.4444	811.3333	843.5556	831.6667	824.75
	lux.1	lux.2	lux.3	lux.4	
1	1091	1062	945	910	1002
2	1080	1016	1061	1028	1046.25
3	1052	930	1022	1024	1007
4	1094	1028	924	880	981.5
5	1065	1033	1028	1008	1033.5
6	956	940	1083	1026	1001.25
7	1039	970	966	942	979.25
8	940	1032	948	1011	982.75
9	872	965	1004	1012	963.25
avg	1021	997.3333	997.8889	982.3333	999.6389

3.3.1 Comparison of two types of lamps used in the experiments.

Compared with Sylvania F40W/CW lamps Philips TLD 36W/96 lamps produce more homogenous illumination. At the level of 1000 lux illumination Philips lamps also emitted 10% more power. The difference between the two lamps in terms of power output did not, however, exceed 10%. The comparison of illumination and power output of the lamps is illustrated in figures 1 and 2.

3.4 Time points of sampling after light damage.

The control, dark adapted animals were killed prior to light exposure. The experimental animals were exposed to 1000 lux illumination for 2 hours, divided into 5 groups (N=6 per group in the Ist experiment and N=5 per group in the second experiment) and killed by decapitation at 0 hrs, 24, 48, 72 and 144 hours after exposure. The level of illumination was set to a 1000 lux since it is believed to be roughly a threshold value for photoreceptor damage for that particular strain of rats exposed to light for 30 min (Reme et al 1988). Such intensity of light was produced by lamps being placed 160 cm above the container with animals. The time was increased, however, to be sure that the animals were irradiated

above the threshold level. The increase of light intensity to 3000 or 4000 lux was also possible but not practical, since the neon lamps had to be lowered down to 30 - 40 cm above the container with animals. Lamps positioned so close produced, however, an unacceptable rise of the temperature in the container.

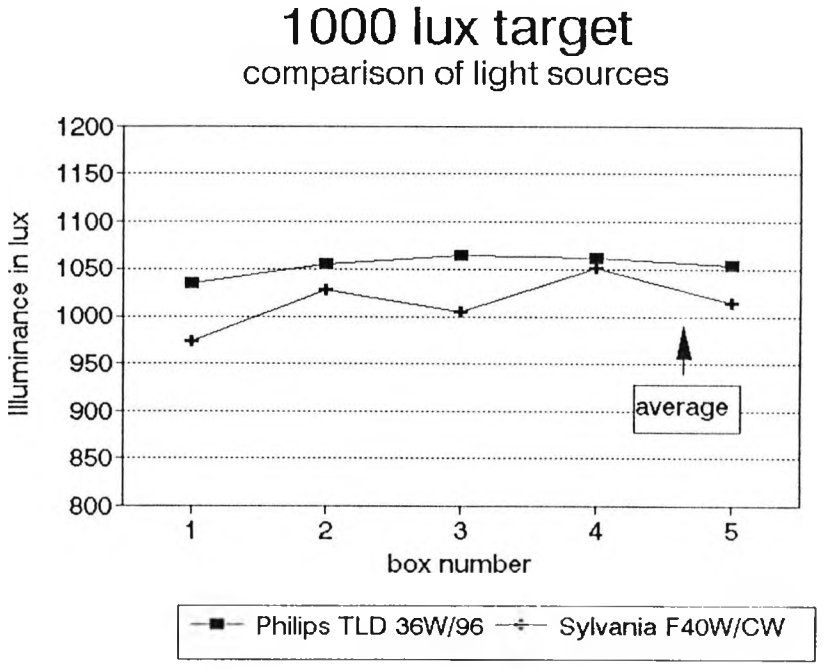


Fig. 2. Comparison of illuminance levels (target 1000 lux) of the different light sources used.

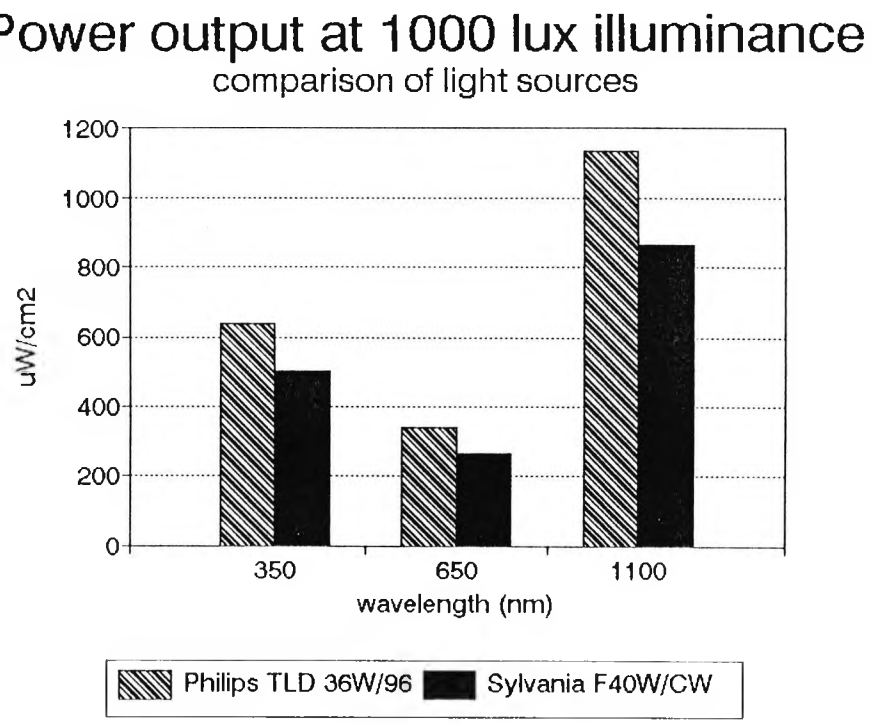


Fig. 3. Comparison of power output at 350 nm, 650 nm and 1100 nm of the different light sources used.

3.5 Tissue processing.

3.5.1 Preparation of the samples.

The retinae from the left eyes were removed in dim red light by slit corneal incision (Delmelle et al 1975) and stored in liquid nitrogen for biochemical analysis. The right eyes were processed for morphological examination. The eyes were rapidly enucleated, the anterior halves including the lens removed under a dissecting microscope equipped with a red filter transmitting 700 nm wavelength. The petri dish in which dissection was performed contained 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.3. After the tissue had remained in this fixative at 4 degrees C for at least 2 hours, it was trimmed for light and electron microscopic observation. The inferior central retina adjacent to the optic nerve head of each eye was trimmed and remained in fixative overnight. After fixation in osmium tetroxide for 1 hour and graded dehydration the tissue was embedded in Epon 812 and orientated so that the optic nerve head was always situated at the front of the block. The presence of ciliary arteries in the lower central retina was verified for each group of sections from each block as shown in fig. 4. Semithin sections were cut on a diamond knife on a Reichert microtome and routinely stained with

toluidine blue. On a few occasions acridine orange stain for condensed chromatin, and oil red O and sudan black stains for lipids were used. Microscopy was performed as described in section 3.8.

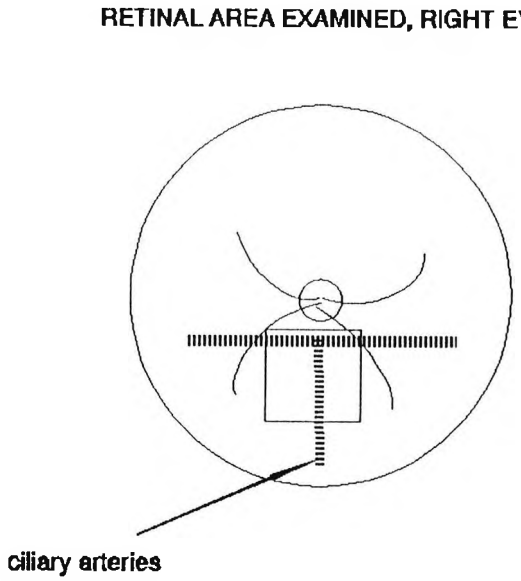


Fig. 4. Schematic drawing of the retinal area processed for histology. The optic nerve head and the ciliary arteries were the topographic landmarks used during eyecup dissection to ensure that the samples were always from the same retinal region.

3.6 Detection of DNA fragmentation in agarose gels.

DNA isolation for DNA gel electrophoresis of fragmented DNA was performed according to Ishida (Ishida et al 1992). The samples of the whole isolated retina without retinal pigment epithelium were collected as described in the literature (Delmelle et al 1975) at the same time points as the samples for the morphological examination. Samples were immediately frozen by immersion and stored in liquid nitrogen. Samples from each time point were combined. As a positive control for apoptosis the OVA-7 T helper murine cell line, strictly IL-2 dependent was used (Weller et al 1994). For DNA isolation samples in Eppendorf tubes were put on ice. 800 µl of ice cold lysis buffer containing 10 mM TrisHCl (pH=7.5), 10 mM EDTA and 0.2% Triton X-100 was added. Retinal samples were pipetted and agitated for 2 minutes. The lysates were spun for 10 minutes at 13000 rpm at 4 degrees C. The supernatant was then transferred to new Eppendorf tubes and the pellets were discarded. To each sample 800 µl of Tris-saturated phenol was added, the samples were vortexed twice, spun for 5 min at 13000 rpm at 4 degrees C. In the next step 700 µl of supernatant was transferred to a new Eppendorf tube containing 700 µl Tris saturated phenol/dichloromethane (1:1), pH=8, vortexed twice and spun for 5 min at 13000 rpm at 4 degrees C.

Subsequently 600 µl of supernatant was transferred to a new tube containing 36 µl 5 M NaCl and well mixed. 1200 µl of pure ethanol was added, the solutions were well mixed and left for 60 min at -20 degrees C. Samples were spun for 20 min at 13000 rpm at 4 degrees C, the supernatant was then discarded. 1 ml of 75% ice cold ethanol was added, vortexed twice and spun for 5 min at 13000 rpm at 4 degrees C. The supernatant was discarded and pellets dried in an exsiccator. 15 µl of TE buffer (10mM TrisHCl, pH=8, and 1 mM EDTA) and 1 µl of RNAse (stock 10 mg/ml) were added and the solution was incubated for 30 min at 37 degrees C. 1.5% agarose gel containing 100 µg/ml of ethidium bromide (1 µl stock per 100 ml agarose) was prepared by dissolving agarose in TEA buffer (2 mM EDTA and 0.04 Tris-Acetate) and heating in a microwave oven. A tenth of the volume of DNA loading dye was added to the total DNA loaded. A prestained DNA size marker (DNA Marker Ladder VI, Boehringer Mannheim, Germany) was added. The gels were run at 120 V for 60 min and were examined in a UV illuminator and photographed with a Polaroid instant camera.

3.7 The influence of glutaraldehyde and cryofixation on light induced vesicular alterations of rod outer segments.

For this set of experiments the exposure times (30 min) and light levels (1000 lux and 1500 lux) were set just above the threshold levels for retinal damage. Such damage, according to the literature, is manifested as vesicular alterations and disruptions of the rod outer segment membranes and was investigated in a number of experiments (Bush et al 1991, Reme et al 1988, Reme et al 1991, Reme et al 1994). For the experiments animals of 250 g were used. Prior to light exposure the animals were dark adapted for 36 hours. Two rats were killed at the end of 24 and two at the end of a 36 hours dark adaptation period serving as dark adapted controls. The 8 remaining rats received a light exposure from diffuse, cool, white, fluorescent light for 30 minutes. Two rats were exposed to 1000 lux and 6 rats were exposed to 1500 lux. All exposures lasted for 30 min. Light exposure was followed by 60 min dark adaptation before sacrifice. Control and experimental animals were decapitated under a dim red light illumination. After rapid enucleation in a dim red light one eye from each animal was fixed by high pressure freezing and the other in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.3 overnight. After postfixation in osmium tetroxide for 1

hour and dehydration in graded alcohols the tissue was embedded in Epon 812 resin.

3.7.1 High Pressure freezing cryofixation.

High pressure freezing was performed as described earlier (Studer et al. 1989). Retinal samples were placed in 1-hexadecene (Fluka) and a disk of 2 mm diameter was punched out using an ophthalmological punch (Grieshaber, Schaffhausen, CH). The disks were mounted in aluminum support platelets (Studer et al. 1989) which were high pressure frozen in HPF 010 (BAL-TEC, Balzers, FL) Hexadecene is not miscible with water and therefore does not act as a cryoprotectant; its purpose is to exclude any gas bubbles that would collapse when subjected to high pressure (Studer et al. 1989). Hexadecene may cause allergic reactions (Tobler and Freiburghaus 1991) therefore skin contact was avoided. The frozen samples were then transported to the freeze substitution device in liquid nitrogen.

3.7.2 Freeze substitution.

Freeze substitution was carried out as described earlier (Muller et al. 1980). The freeze substitution medium consisted of water free acetone containing 2% osmium tetroxide. The samples were freeze substituted in a FSU 010 (BAL-TEC) for 8 hours at 183 K, 8 hours at 213 K, 5 hours at 243 K and 1 hour at 273 K, washed with water free acetone and embedded stepwise in Epon 812 resin. Tissue was placed 2 hours in 30% Epon in acetone, for 5 hours in 60% Epon in acetone and for 5 hours in 100% Epon, then the temperature was raised to 333 K for polymerization.

3.8 Microscopy.

Semithin sections of plastic embedded samples were cut with a diamond knife Diatome AG Biel, CH) on a Reichert ultramicrotome (Leica), stained with toluidine blue and examined in a Zeiss Axiophot light microscope. Ultrathin sections were placed on copper grids covered with parlodium support film stained with uranyl acetate and lead citrate (Reynolds 1963) and examined in a Hitachi H-7000 and Hitachi H-600 transmission electron microscopes. The apertures and accelerating voltage

were set as for the standard examination of biological samples. The photography was made using Kodak EM films. The freezing quality was estimated by looking for ice crystal segregation patterns. Areas with visible ice crystal segregation patterns were not used for interpretation.

3.9 Semiquantitative assessment.

A semi-quantitative characterization of morphological changes was performed on light microscopic sections stained with toluidine blue with a magnification of x 1000 under oil immersion. The structural alterations of photoreceptor inner and outer segments, retinal pigment epithelium, Bruch's membrane and choriocapillaris were later confirmed by electron microscopy. The degree of morphological change was graded on an arbitrary scale 0 to 3 (0 = no change, 1 = minor, 2 = moderate and 3 = severe) by comparison with control sections. Grades were applied overall. The rating of these changes was performed by an observer unaware of the experimental histories of the animals on masked sections, 4 sections for each animal. Vesicular alterations of rod outer segments *(ROS vesiculations)* were defined as membrane dilations and vesiculations seen as light bands across the width of the rod outer segments. Photoreceptor loss *(PR loss)* was defined as overall damage to

photoreceptors manifested as nuclear pyknosis or karyolysis, condensation of cytoplasm or loss of photoreceptor nuclei and thinning of the outer nuclear layer at later stages. The loss of retinal pigment epithelium *(RPE loss)* was defined as either pyknosis or karyolysis of the rpe nuclei, cell lysis or cell loss at more advanced stages of degeneration. Serous retinal detachment *(SRD)* was defined as separation and shallow lifting of the sensory retina from the retinal pigment epithelium with accumulation of the subretinal fluid. Microcystic retinal oedema *(CRE)* was defined as the presence of cystic spaces within the outer nuclear layer. *Macrophages* were identified as large monocytes with oval nuclei and cytoplasm containing numerous phagosomes. *Mitoses* were identified by characteristic mitotic figures, usually prometaphase, metaphase or anaphase. *Scar* was defined as adhesion of the remaining retinal layers and retinal glia to the Bruch's membrane deprived of retinal pigment epithelium with occasional breaks in the Bruch's membrane and invasion of glia into the choroidal space. Pyknosis of photoreceptor nuclei *(PR pyknosis)* was defined as uniformly condensed dark stained nuclear chromatin in the shrunken nuclei of photoreceptor cells. Inner segment densification *(IS densification)* was a major criterion of apoptotosis of photoreceptors. It was characterized by the presence of shrunken, condensed, dark stained outer and inner segments of photoreceptor cells

which were distinctly different from the swollen, fragmented weakly stained photoreceptor inner segments and photoreceptor debris resulting from necrosis. Retinal pigment epithelium nuclear margination *(RPE nuclear marg)* was characterized by condensation and shrinkage of the nuclear chromatin which formed a ring along the periphery of the nuclear envelope and created a poorly stained centre of the nucleus. Distinct cupping of condensed chromatin along the edges of the nucleus was clearly observed after toluidine and acridine orange staining of semithin sections. Such changes were a criterion for apoptosis in the retinal pigment epithelium. Photoreceptor nuclear margination *(PR nuclear marg)* was characterized by condensation and shrinkage of the nuclear chromatin along the wall of the nuclear envelope and formation of a dark stained ring with poorly stained centre of the nucleus. Such changes were observed at higher magnification in light microscopy preparations stained with toluidine blue and acridine orange staining of semithin sections. Photoreceptor necrosis *(PR necrosis)* was defined as occurrence of swollen cells undergoing lytic changes. Retinal pigment epithelium necrosis *(RPE necrosis)* was characterized as the presence of swollen, fragmented poorly stained cells undergoing lysis. The numerical data obtained from the semiquantitative assessment was collected in a spreadsheet program Quattro Pro which was used to prepare the graphs.

3.10 Statistics.

The results of semiquantitative analysis of morphological evaluation was analysed statistically. Differences between groups were determined using Student's t-Test. P values less than 0.05 were considered significant. Since there was some difference in power output between Sylvania and Philips lamps (tables 3.3.2, 3.3.3 and fig. 3) statistical evaluation was performed on homogenious groups of animals exposed to Philips TLD 36W/96 lamps. Each of the experimental groups evaluated histologically and then statistically included 6 animals with the exception of the 8 control animals which were not exposed to light. The following experimental groups were studied: animals killed immediately after 120 minutes of light exposure (group 0) and subsequently 24 hours, 48 hours, 72 hours and 144 hours later.

4. Results.

4.1 Macroscopic.

Macroscopic examination of the eye cups of dark adapted control animals showed normal retinal appearance. In light exposed animals the most striking changes were observed 144 hours after light exposure. A pale retinal scar was observed in all the samples on the temporal side. It varied in size and location in different animals but generally it could be described as crescent or oval shaped and extended from the inferior towards the superior retina (fig. 5) and corresponded to retinal degeneration and the formation of the chorioretinal scar.

4.2 Microscopic.

Microscopic examination of the blocks from the central part of the inferior retina revealed normal retinal anatomy in the control animals. Occasionally dark stained photoreceptor cell processes or a pyknotic photoreceptor nuclei were present (fig. 6). In all the light exposed animals retinal abnormalities were seen immediately after exposure and showed distinct regional differences and a consistent pattern of changes.

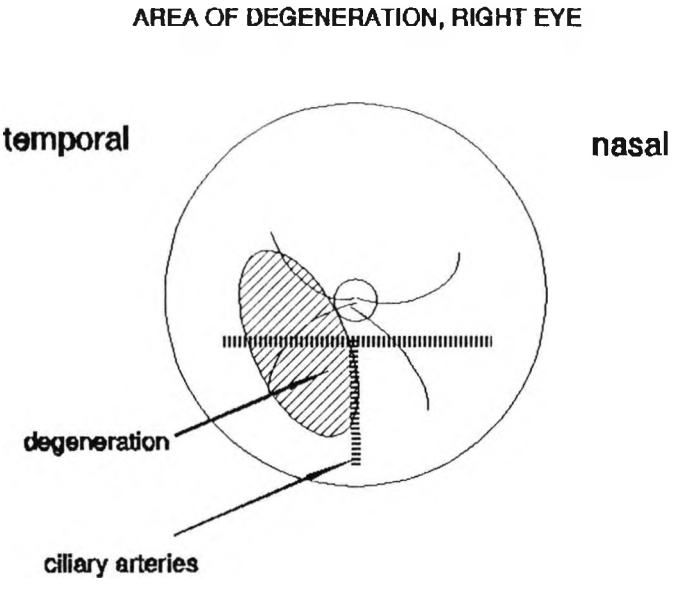


Fig. 5. Schematic drawing of the area of retinal degeneration seen as white chorioretinal scar on gross examination 72 and 144 hours after light exposure.

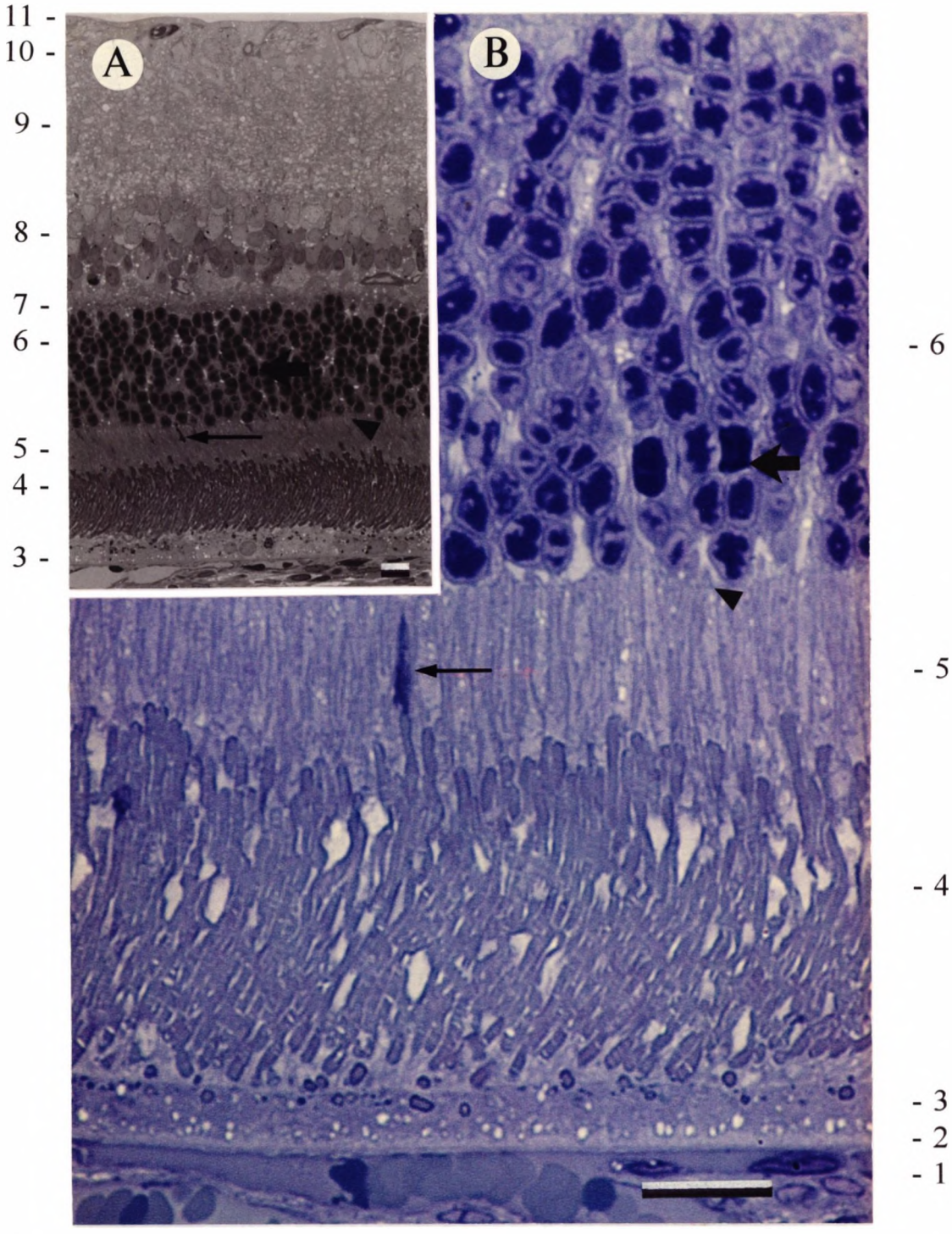


Fig. 6. Normal anatomy of the rat retina. 1 - choriocapillaris. 2 - Bruch's membrane, 3 - retinal pigment epithelium, 4 - photoreceptor outer segments, 5 - photoreceptor inner segments, 6 - outer nuclear layer, 7 - outer plexiform layer, 8 - inner nuclear layer, 9 - inner plexiform layer, 10 - ganglion cell layer, 11 - inner limiting membrane. Arrowhead indicates outer limiting membrane. In normal retina pyknotic nuclei (thick arrow) and densified inner segment (long arrow) are occasionally observed.
Bars = 10 μm.

The inferior nasal retinae revealed the well-known picture of vesicular alterations of photoreceptor outer segments and recovered relatively normal morphology within 7 days. By contrast the inferior temporal region possessed scattered but numerous photoreceptors the most striking feature of which was the densification, condensation and shrinkage of cell bodies and nuclear pyknosis seen immediately after light exposure as well as margination of the nuclei 24 hours later. Such areas showed extensive and irreversible damage resulting in loss of the outer retina within 72 hours after exposure progressing to chorioretinal scarring in 7 days (fig. 8). The inferior ciliary artery formed an approximate border between the two regions (fig. 7). A comparison of the semiquantitative evaluation of photoreceptor densification, photoreceptor nuclei pyknosis and margination and outer segments vesicular alterations in the recovery and degeneration areas is made in figures 9 and 10.

NASAL TEMPORAL

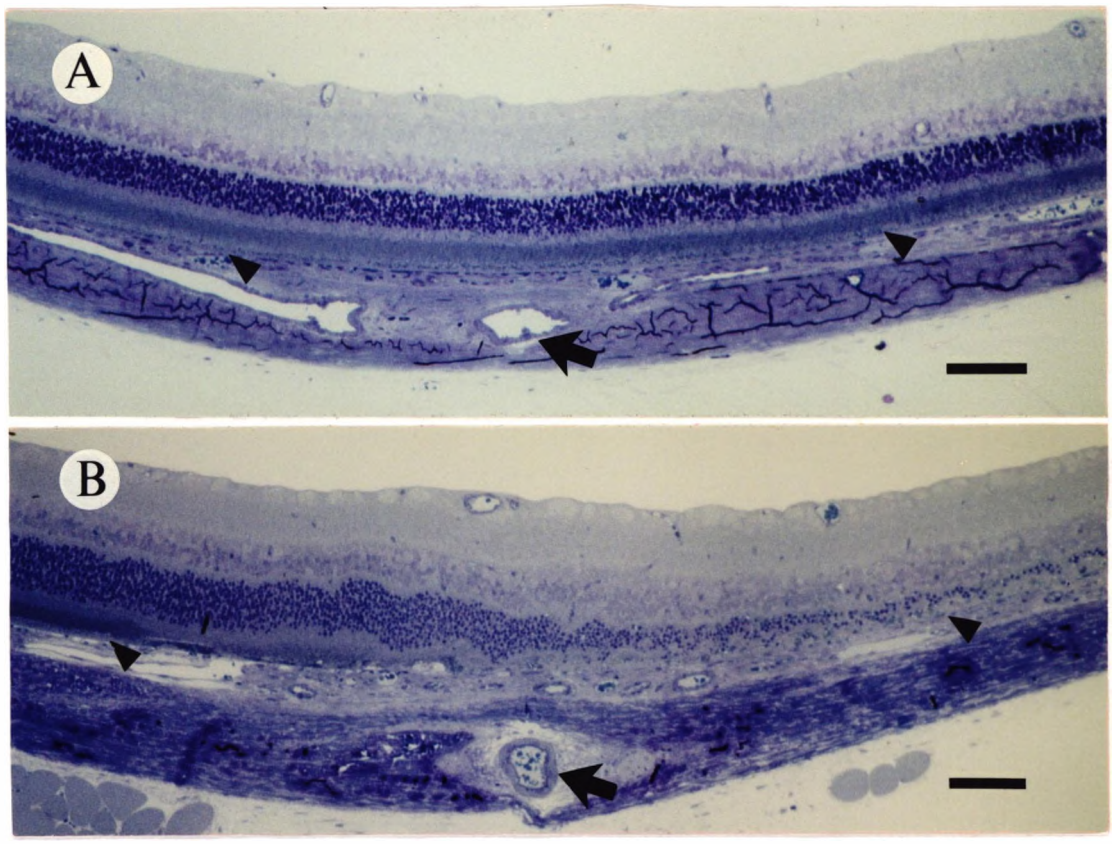


Fig. 7. Central lower part of light damaged rat retina: (A) Immediately after light exposure, (B) 144 hours after light exposure: reduction of thickness and loss of the outer retinal layers and adhesion of the remaining layers to the Bruch's membrane (arrowheads) on the temporal side. Arrow indicates inferior ciliary artery, which is a rough border between degeneration (temporal side) and recovery (nasal side) areas. Bars = 100 μm.

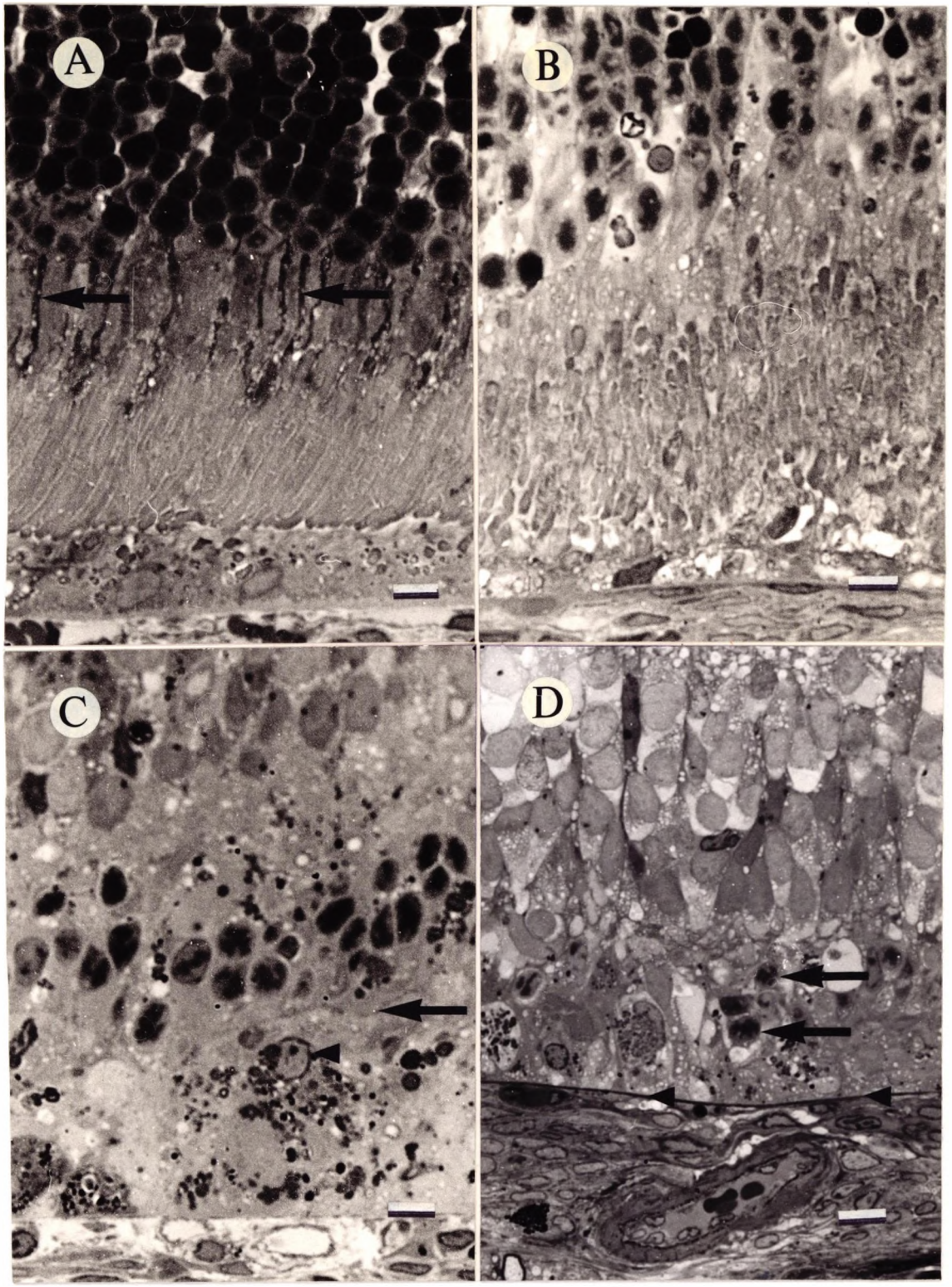
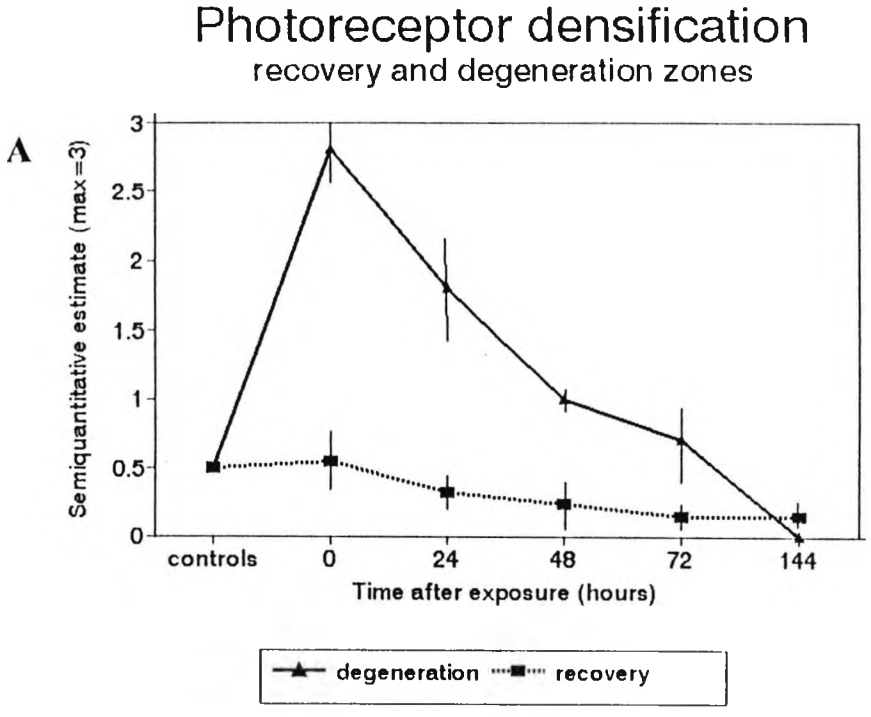


Fig. 8. Light microscopy of the degeneration zone showing the progression of changes. Photoreceptors and retinal pigment epithelium immediately after the exposure, with many photoreceptors showing pyknosis and condensation of the inner segments (arrows) (A). Outer retina 24 hours after light damage showing dropout of photoreceptor nuclei and distorted retinal pigment epithelium monolayer (B). Outer retina 72 hours after the injury with the outer nuclear layer reduced to the thickness of 2-3 nuclei. A macrophage (arrowhead) is present under the outer limiting membrane (arrow) (C). Chorioretinal adhesion 144 hours after light exposure. Single photoreceptor nuclei are identifiable (arrows). Bruch's membrane is prominent (arrowheads) and dropout of choriocapillaris is evident (D). Bars = 10 μm.



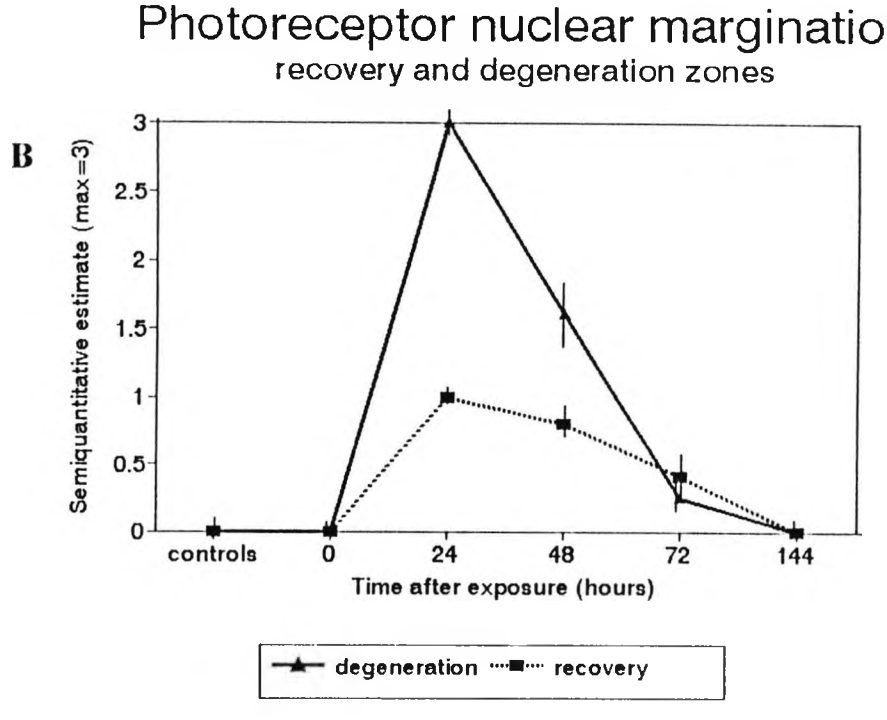
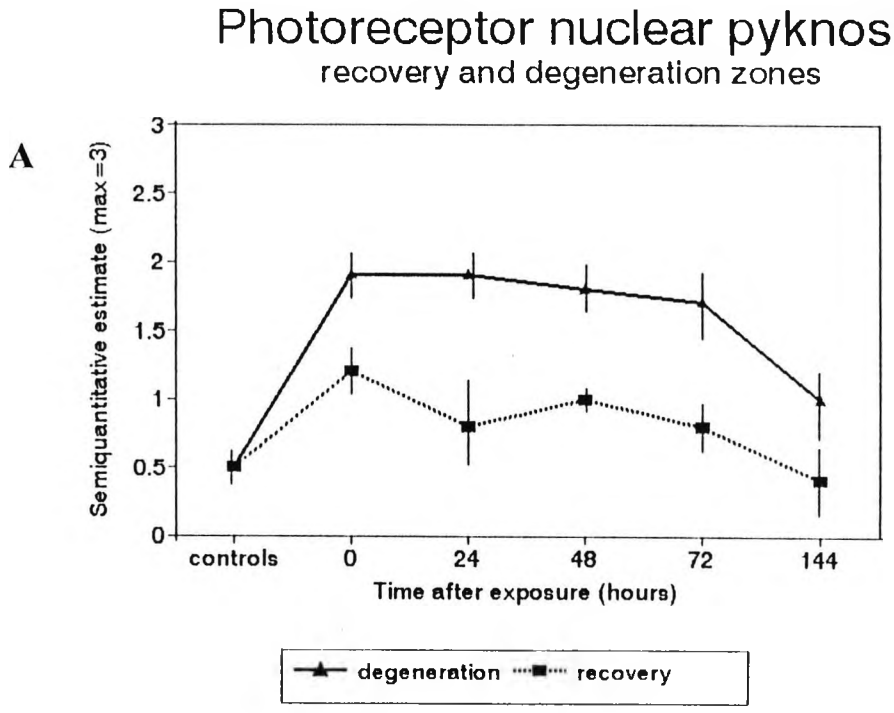


Fig. 9. A comparison of photoreceptor densification (A) and photoreceptor nuclear margination (B) in degeneration and recovery zones.



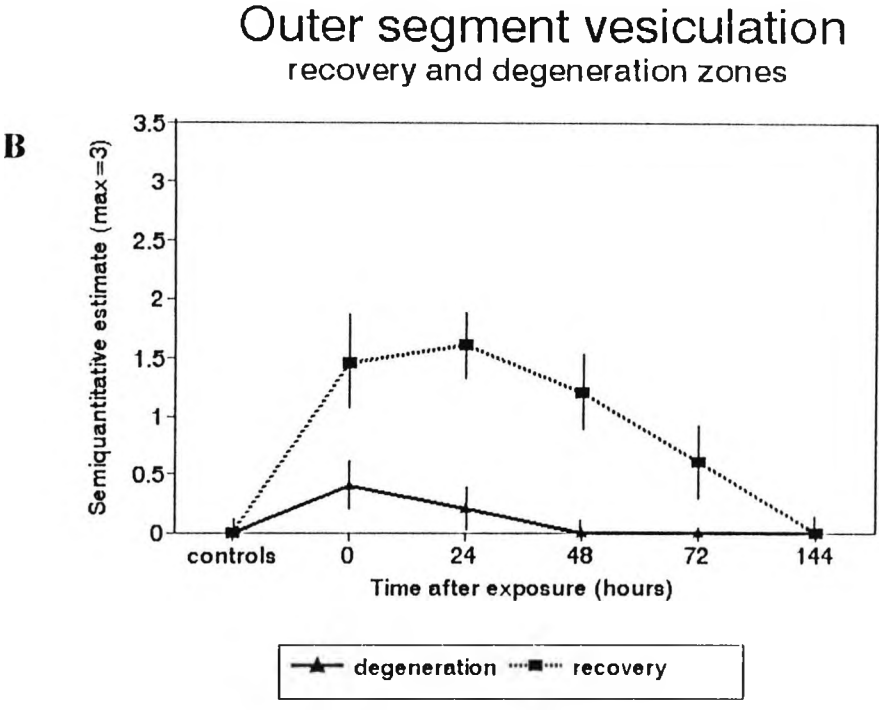


Fig. 10. A comparison of photoreceptor nuclear pyknosis (A) and rod outer segment vesiculations (B) in degeneration and recovery zones.

4.3. Light microscopy of the degeneration area.

4.3.1 Photoreceptors.

In the animals killed immediately after light exposure the most striking retinal changes were predominantly restricted to photoreceptors and consisted of densification of photoreceptor cell bodies including outer and inner segments and extensive pyknosis in the outer nuclear layer (fig. 8 A, 9 A, 10 A, 11 A). Gradual photoreceptor nuclei drop-out could already be seen in the first 24 hours and was especially pronounced in the inner aspects of the outer nuclear layer (fig. 11 B). The outer segments of photoreceptors were compact and did not show vesicular alterations. Within 24 hours apoptosis of photoreceptors was followed by extensive necrotic changes observed more centrally in the lesion (fig. 12 and 13), whereas towards the periphery photoreceptor preservation was more common. Scattered pyknotic and partially degradated nuclei showing margination of karyoplasm (fig.13, 14 A) were present. Photoreceptor and retinal pigment epithelium loss was progressive over the observation period with most of the cells being lost within 72 hours after light exposure. The chorioretinal scar was formed 144 hours after light damage (fig. 15, 17).

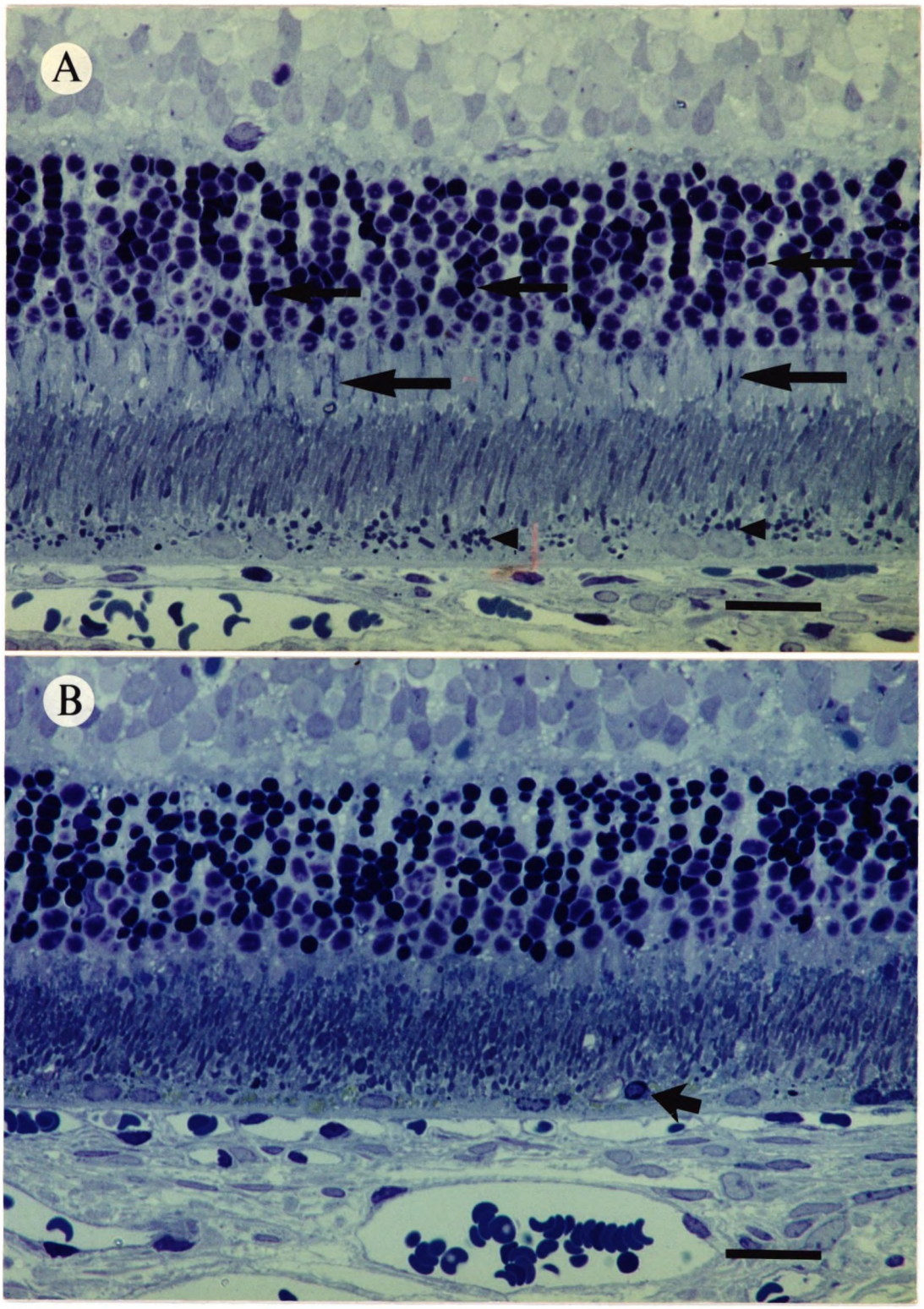


Fig. 11. Outer retina of the degeneration (temporal) area immediately after light damage (A) shows extensive pyknosis (smaller arrows) and densification of photoreceptor inner and outer segments (bigger arrows) consistent with apoptosis. The retinal pigment epithelium contains numerous phagosomes (arrowheads) but otherwise appears normal. The outer retina, degeneration area 24 hours after light damage (B), shows dropout of photoreceptor nuclei, pyknosis and viable nuclei in the outermost layer. The nuclei of the retinal pigment epithelium show various stages of condensation of karyoplasm typical of apoptosis (thick arrow). Bars = 20 μm. To compare with control please refer to fig. 6.

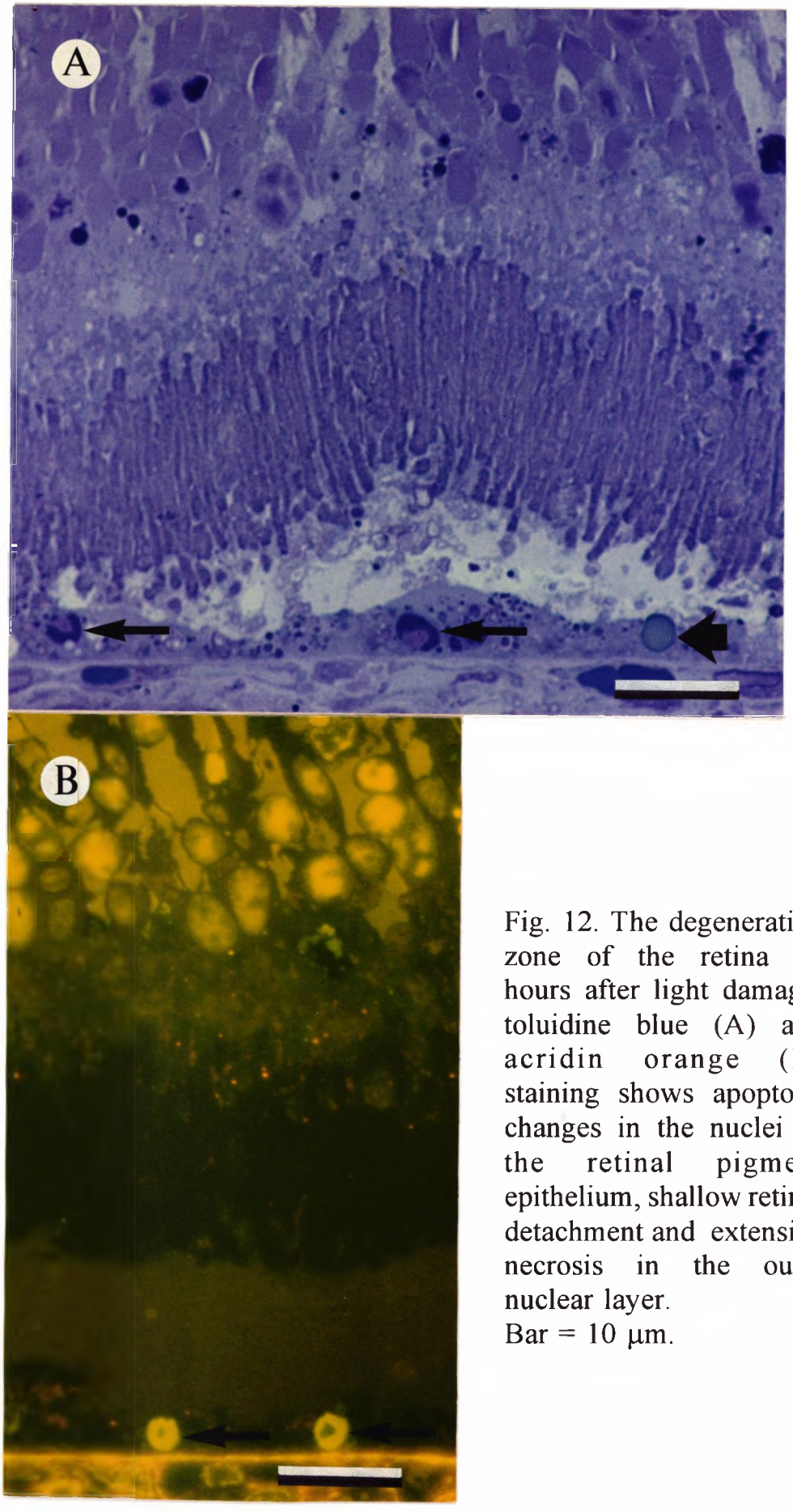


Fig. 12. The degeneration zone of the retina 24 hours after light damage: toluidine blue (A) and acridin orange (B) staining shows apoptotic changes in the nuclei of the retinal pigment epithelium, shallow retinal detachment and extensive necrosis in the outer nuclear layer.
Bar = 10 μm.

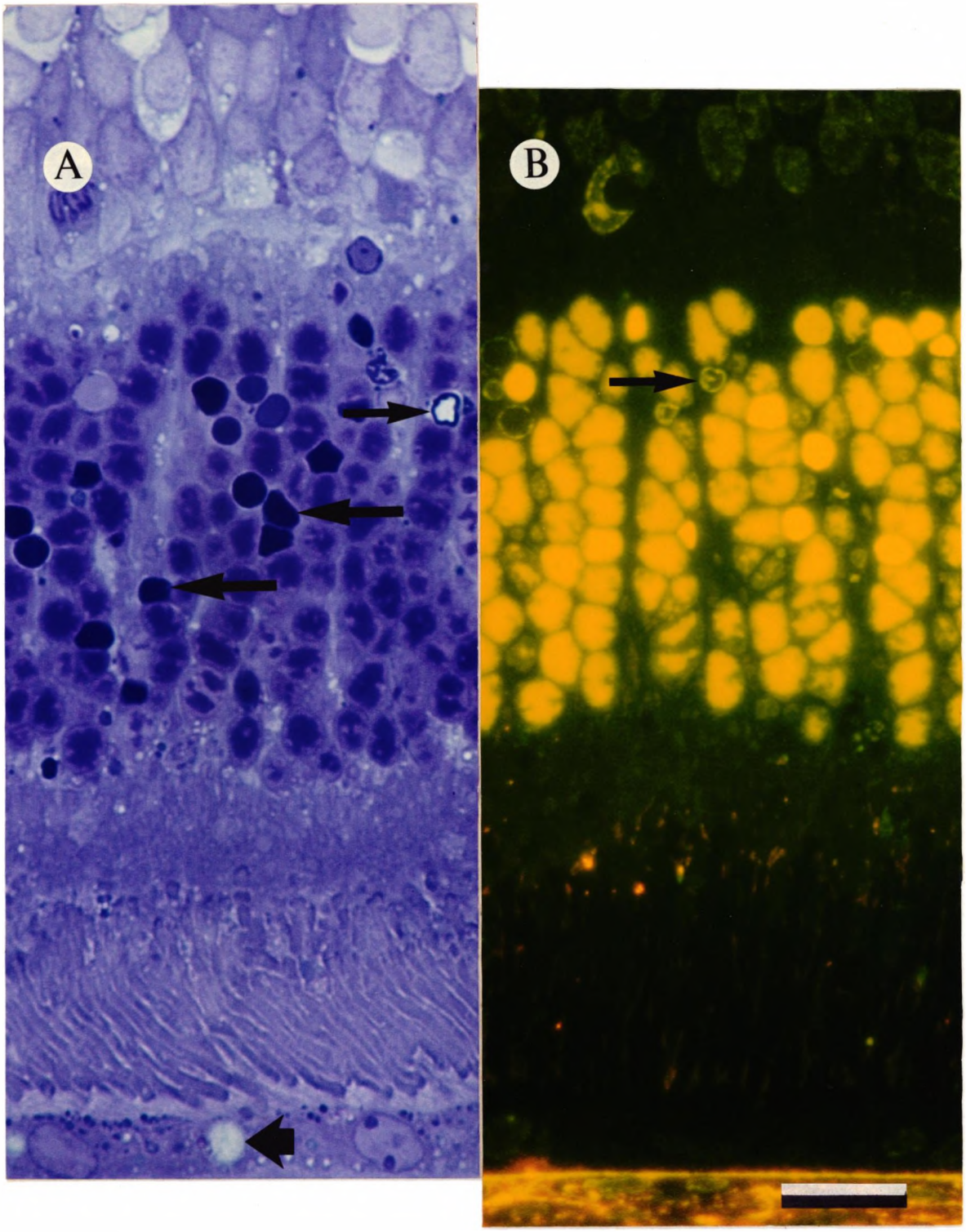
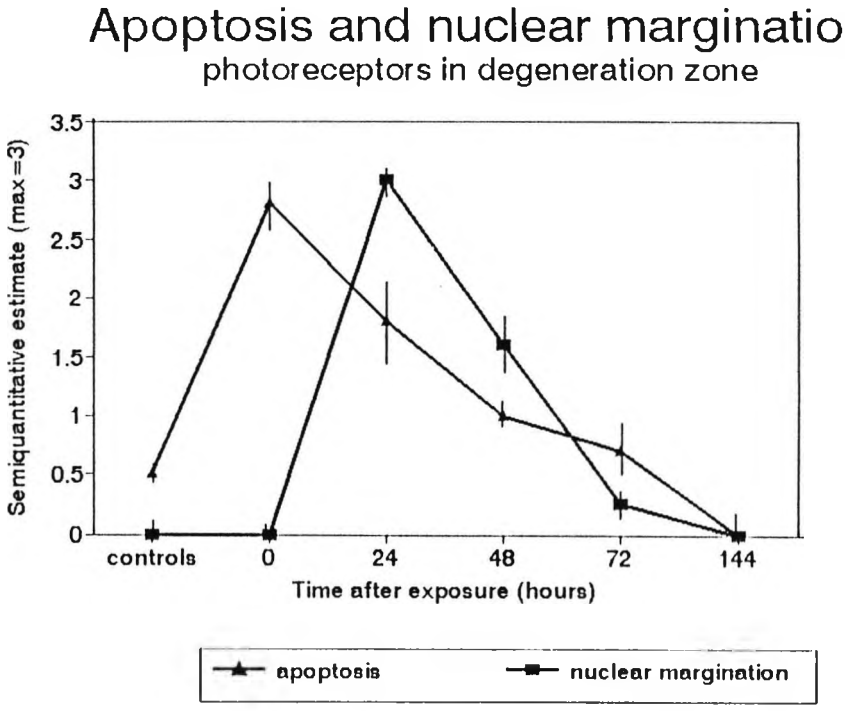


Fig. 13. The border zone between degeneration and recovery in the retina 24 hours after light damage: toluidine blue (A) and acridin orange (B) staining show drop out of photoreceptor nuclei with condensation of those remaining (arrow), some of which are degraded, only a narrow rim of karyoplasm being left (thin arrow). Other nuclei are normal. The retinal pigment epithelium shows large droplets of what are presumably lipids (thick arrow). Bar = 10 μm.

A



B

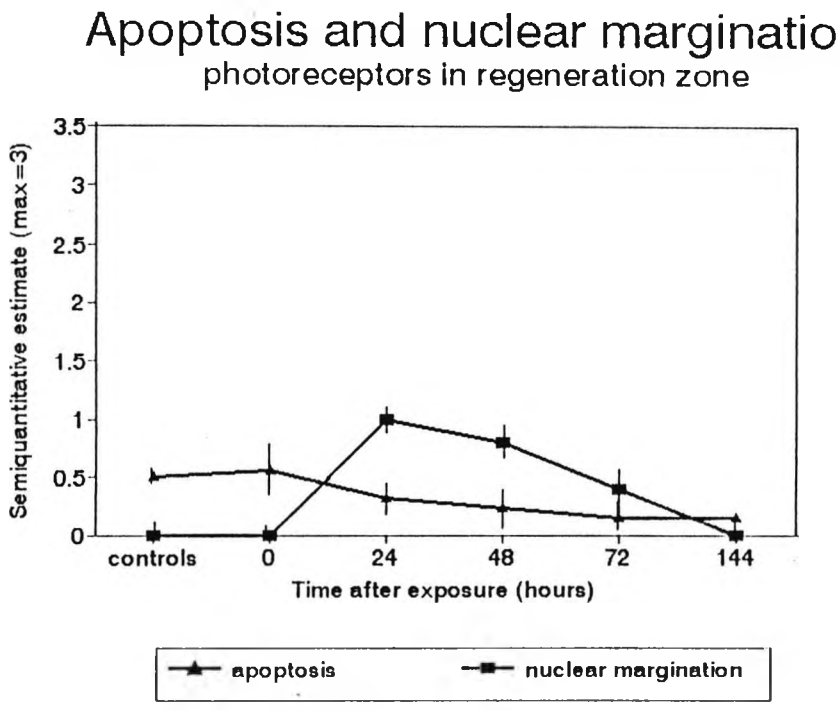


Fig. 14. Apoptosis (defined as shrinkage and densification of cells) and nuclear margination in degeneration (A) and regeneration (B) zones.

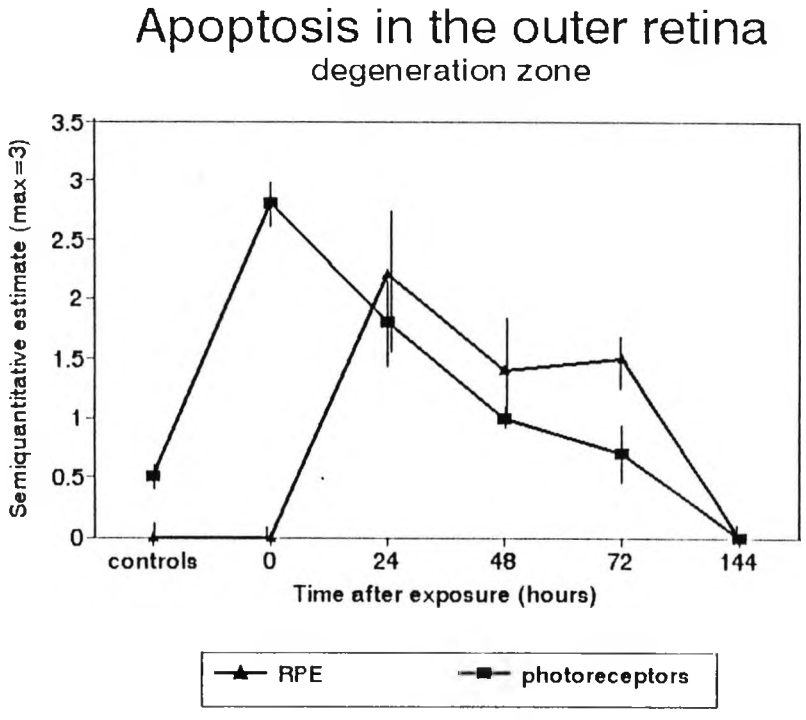


Fig. 15. Apoptosis in the outer retina in the degeneration zone.

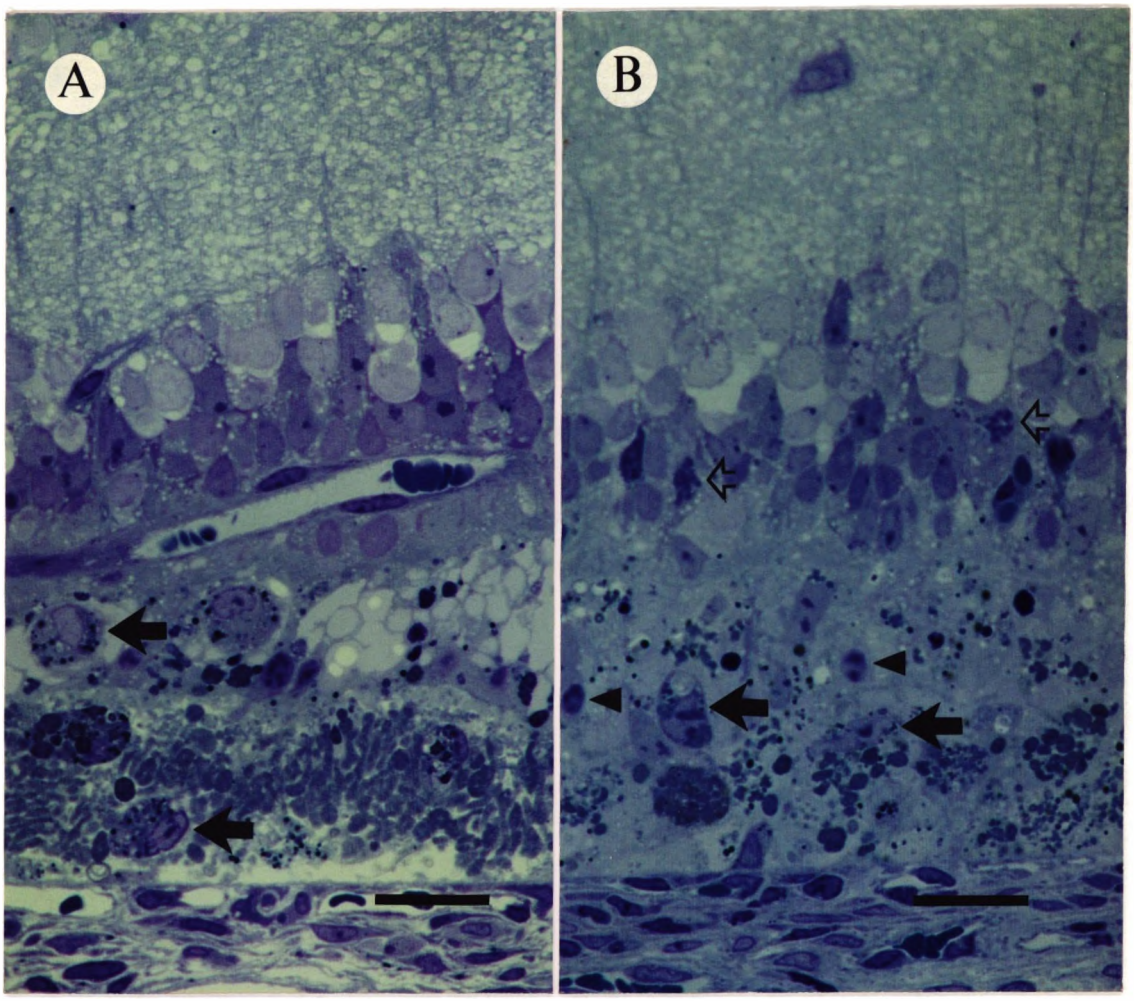


Fig. 16. Degeneration (temporal) area 48 hours after light damage (A) shows loss of photoreceptor nuclei and the retinal pigment epithelium, remains of the inner segments, microcystic retinal degeneration, infiltration of macrophages (arrows) and increased cellularity of choroid. Degeneration (temporal) area 72 hours after light damage (B) shows adhesion of the inner retina to the Bruch's membrane, debris originating from the photoreceptors, macrophages (arrows), occasional viable photoreceptor nuclei (arrowheads) and mitotic figures in the inner nuclear layer (empty arrows). The choroid is also infiltrated by monocytes. Bars = 20 μm.

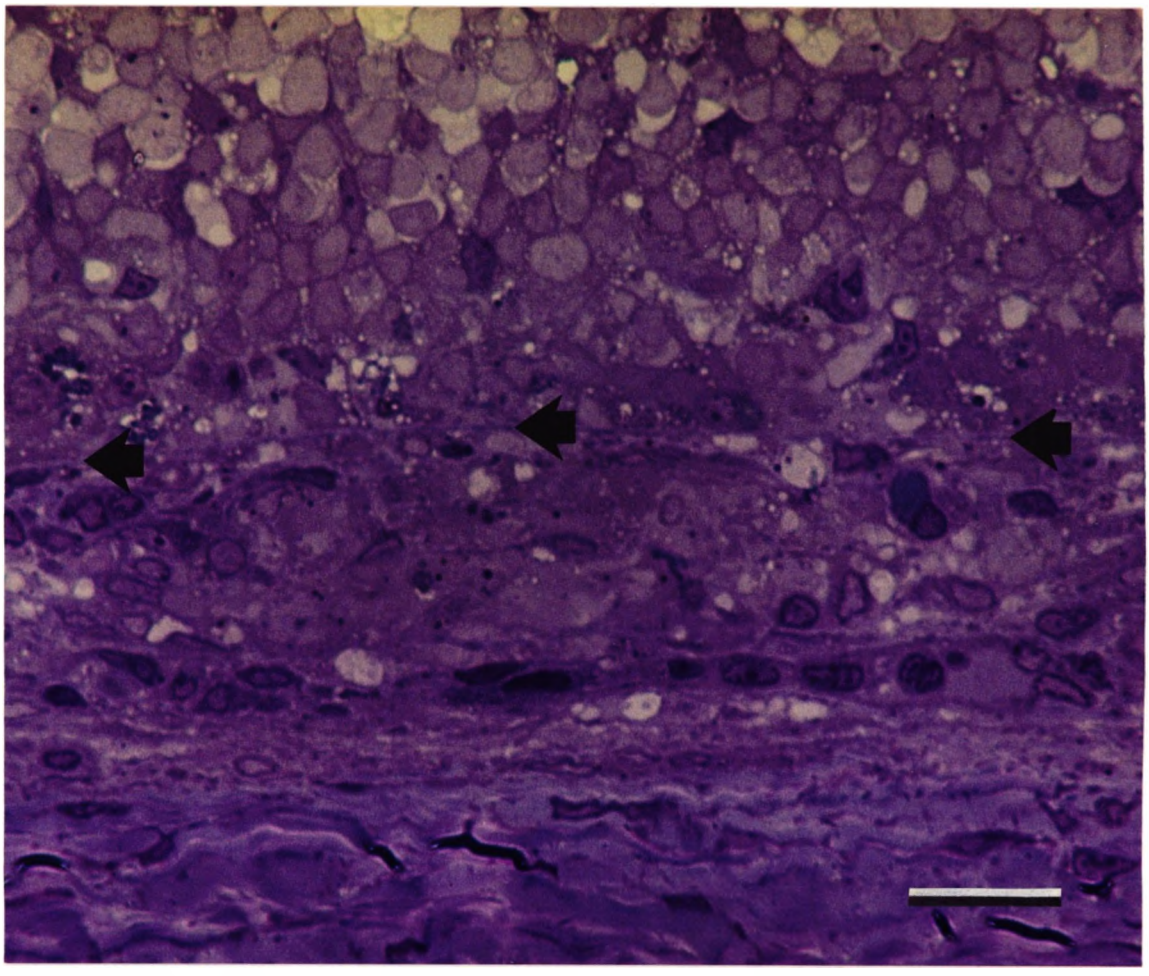


Fig. 17. Degeneration area, 72 hours after light damage shows loss of outer retinal layers (photoreceptors and retinal pigment epithelium) and adhesion of the inner retinal layers to the Bruch's membrane (arrow). Bar = 10 μm.

4.3.2 Retinal pigment epithelium, Bruch's membrane and choroid.

Immediately after light damage the retinal pigment epithelium did not show any significant pathology but an increased number of phagosomes was noted (fig. 11 A). Within 24 hours after light exposure numerous changes within the retinal pigment epithelium were observed. In the central part of the lesion shrinkage and loss of polarity of cells was observed with striking nuclear changes comprising of shrinkage of the nucleus with margination and densification of the karyoplasm around the nucleus perimeter (fig. 11 B, 12). In numerous cells large lipid droplets were present (fig. 12, 13). Towards the periphery of the lesion the general architecture of retinal pigment epithelium was better preserved but large lipid droplets were still observed (fig. 12 A). A delay between photoreceptor and retinal pigment epithelium cell death was seen (fig. 15). Within 48 hours most of the retinal pigment epithelium was lost in the central part of the lesion exposing the bare Bruch's membrane (fig. 16). This was associated with shallow serous retinal detachments and cystoid retinal degeneration (fig. 16) consistent with the loss of the blood retinal barrier function. It was followed within 72 hours after light damage by adhesion of the remaining retinal layers to the Bruch's membrane and the restoration of the blood retinal barrier function. At

this stage cystoid retinal changes were no longer observed (fig. 16 B). Retinal pigment epithelium was lost over large areas of the central part of the retinal scar at 72 hours after light damage (fig. 17). It should be noticed that retinal pigment epithelium cell death occurred within 24 hours after the onset of cell death in the photoreceptor cell layer (fig. 15) and was associated with serous retinal detachments (fig. 12, 18).

4.3.3 Other cell and tissue responses.

A reparative process in the retina was already observed 24 hours after light damage and consisted of hypertrophy of Muller cells, infiltration of macrophages which were very numerous at 48 hours (fig. 20) and prompt removal of photoreceptor debris. The influx of macrophages coincided with the loss of the retinal pigment epithelium (fig. 21) and the breaking down of the blood retinal barrier. At 72 hours after light exposure proliferation of macrophages within the retina was also observed (fig. 20). Mitotic activity was also present 48 and 72 hours after light exposure in the inner nuclear layer with some, presumably Muller cells, undergoing cell divisions (fig. 16 B). The relationship between macrophage influx and the mitotic activity within the retina is illustrated in fig. 22.

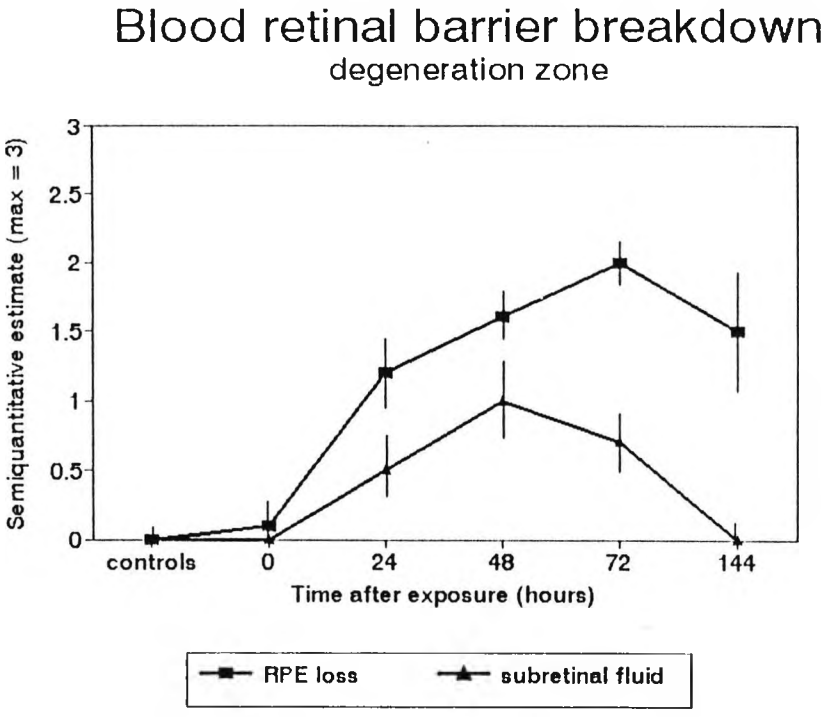


Fig. 18. Loss of retinal pigment epithelium (RPE) and dysfunction of blood retinal barrier manifested as serous retinal detachment caused by accumulation of subretinal fluid.

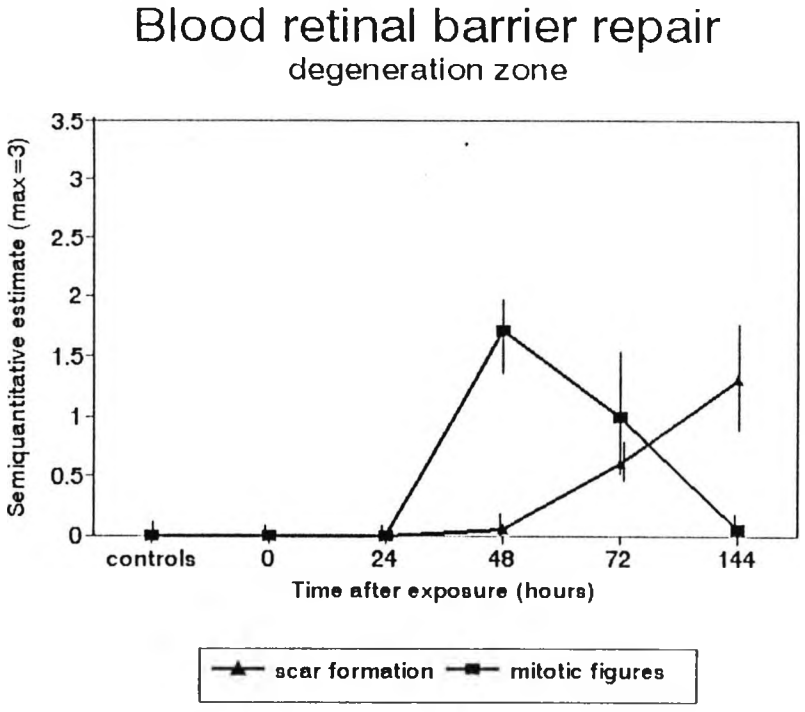


Fig. 19. Repair of the blood retinal barrier by formation of chorioretinal scar and mitotic activity in the outer retina.

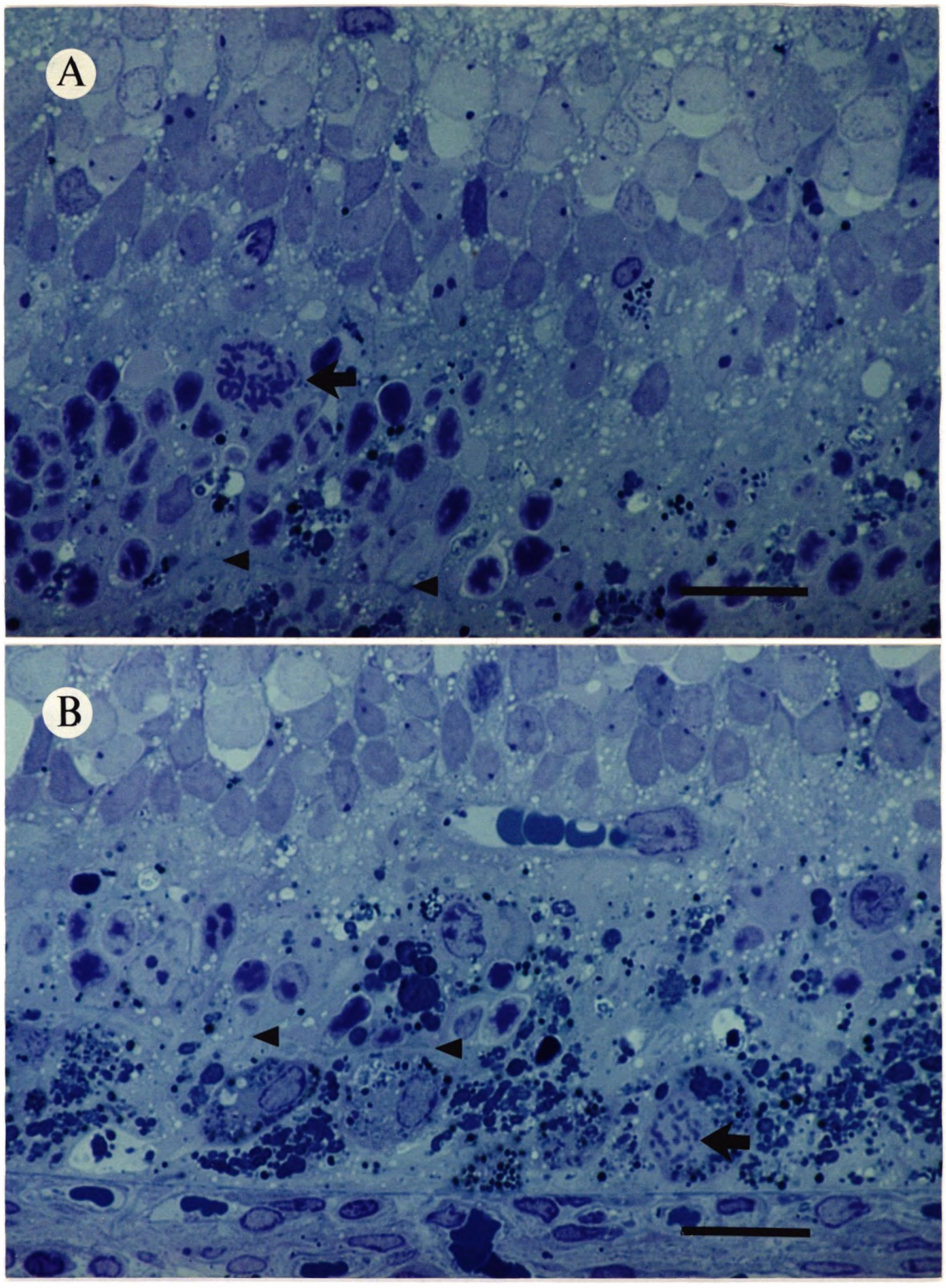


Fig. 20. Degeneration area, 72 hours after light damage showing dividing macrophages in the retina. Macrophages were undergoing mitosis (arrows) within the outer nuclear layer (A) and in the sub retinal space (B). Arrowheads indicate the outer limiting membrane. Bar = 20 μm.

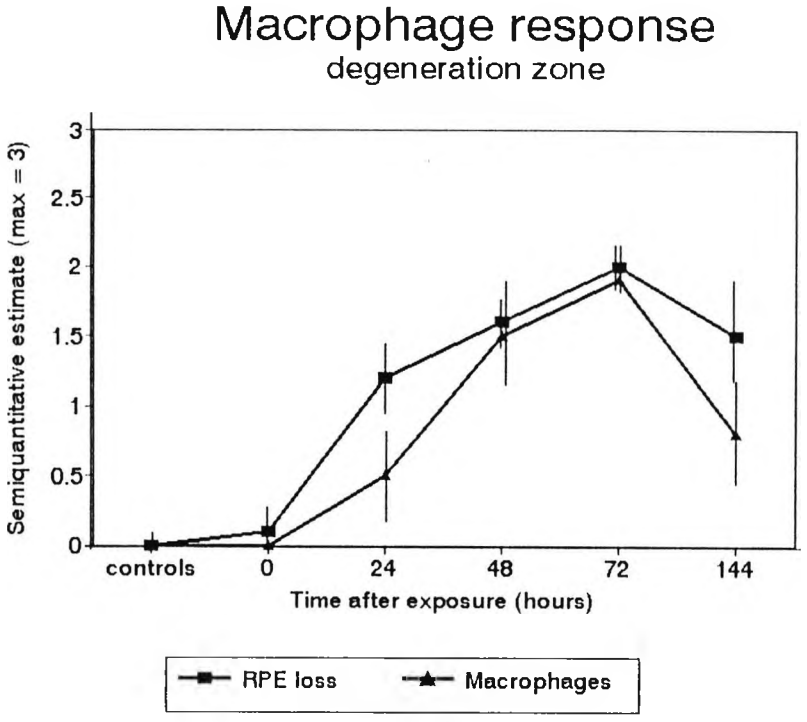


Fig. 21. Loss of the retinal pigment epithelium and accumulation of the macrophages.

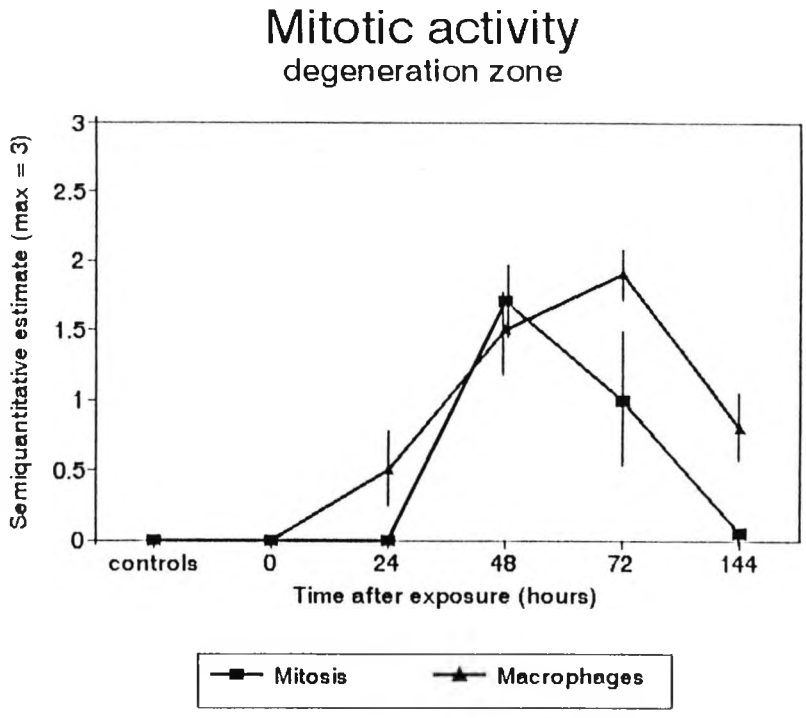


Fig. 22. Accumulation of macrophages and mitotic activity in the retina.

4.4 Electron microscopy of the degeneration area.

4.4.1 Photoreceptors.

The control retinae revealed normal anatomy (fig. 23 A, 28 A, 30 A). In light damaged samples immediately after light exposure scattered photoreceptor nuclei showed both pyknosis and karyolysis (fig. 23). Only one type of cell death was, however, predominantly observed in the outer nuclear layer. It involved scattered, single cells dying in an synchronous fashion. The earliest noticeable nuclear changes were associated with condensation and shrinkage of the nucleoplasm and the cytoplasm, including the synaptic region (fig. 24). In some cells the nuclear chromatin appeared to be divided into several smaller portions (fig. 24 A). Other nuclei contained much more condensed chromatin forming a compact, polygonal, pyknotic, shrunken nucleus (fig.23 B,D). Within 24 hours there was dissolution of karyoplasm in the central part of some of the nuclei with preservation of the thin rim along the nuclear envelope giving an impression of nuclei margination (fig. 25). The chromatin of other nuclei was very condensed and formed dense and fragmented apoptotic bodies (fig. 25). Some scattered cells, especially in the area which was the border zone between degeneration and recovery, showed

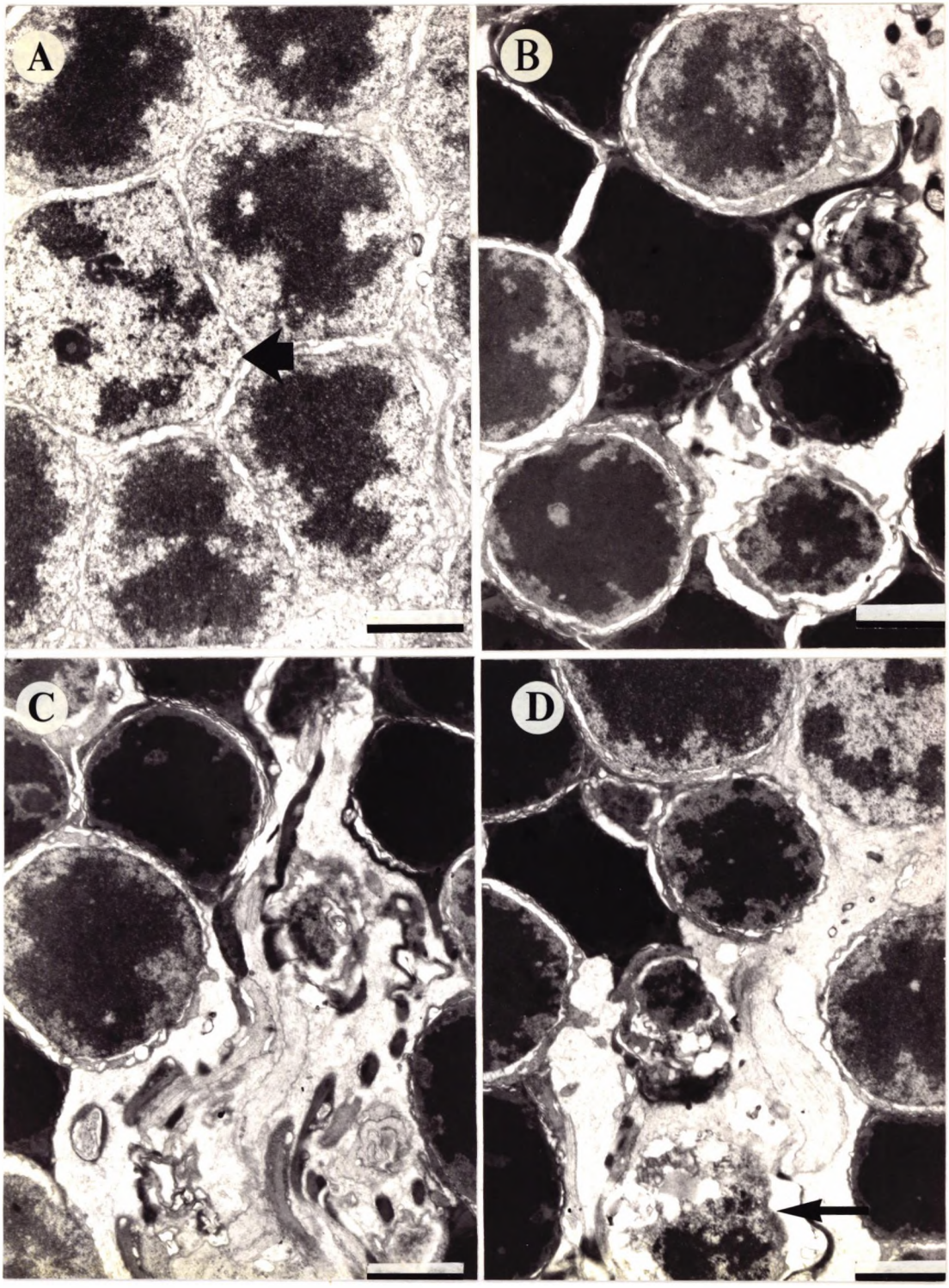


Fig. 23. Electron micrographs of the outer nuclear layer: degeneration area immediately after light exposure. Control (A), retina with normal looking photoreceptor nuclei. Thick arrow indicates cone nucleus. Light damaged retina (B,C,D): photoreceptor nuclei are shrunken and frequently pyknotic. Some nuclei are undergoing fragmentation and phagocytosis consistent with apoptosis. (D) Karyolysis which is consistent with necrosis (arrow). Bars = 2 μm.

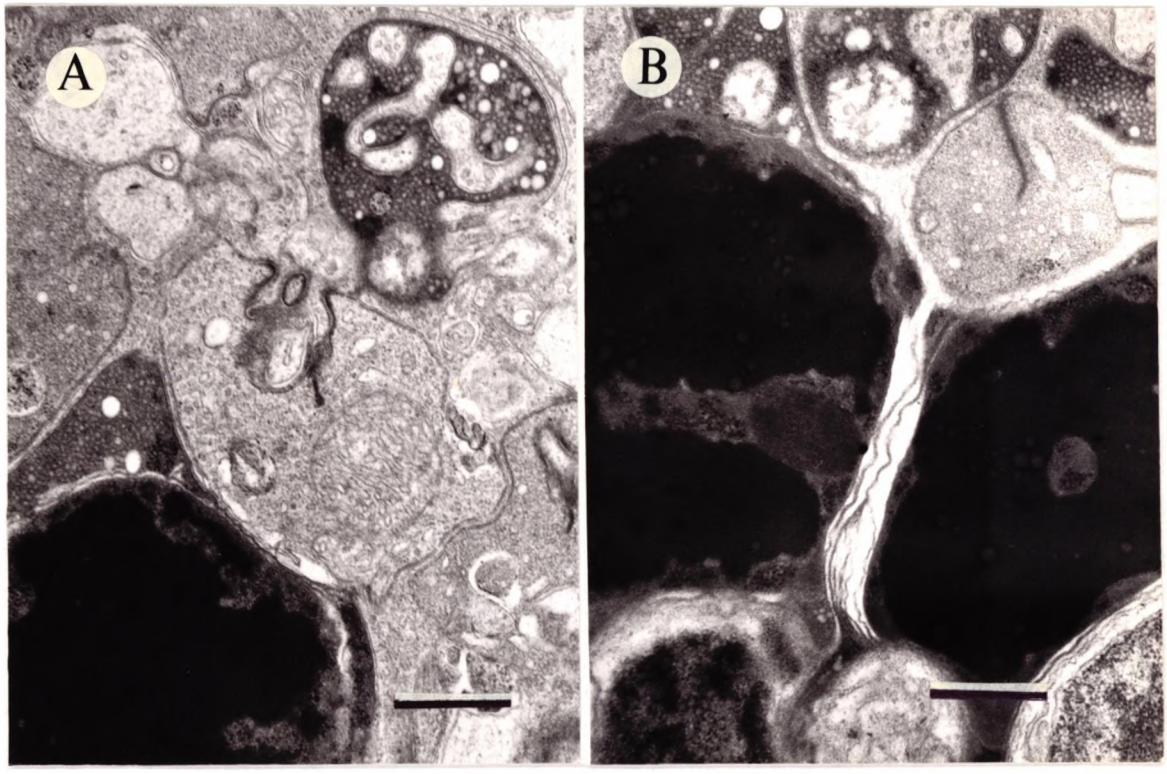


Fig. 24. Outer nuclear layer: degeneration area immediately after light exposure. Pattern of nuclear chromatin condensation (A) uniform, (B) not uniform. Bars = 1 μm.

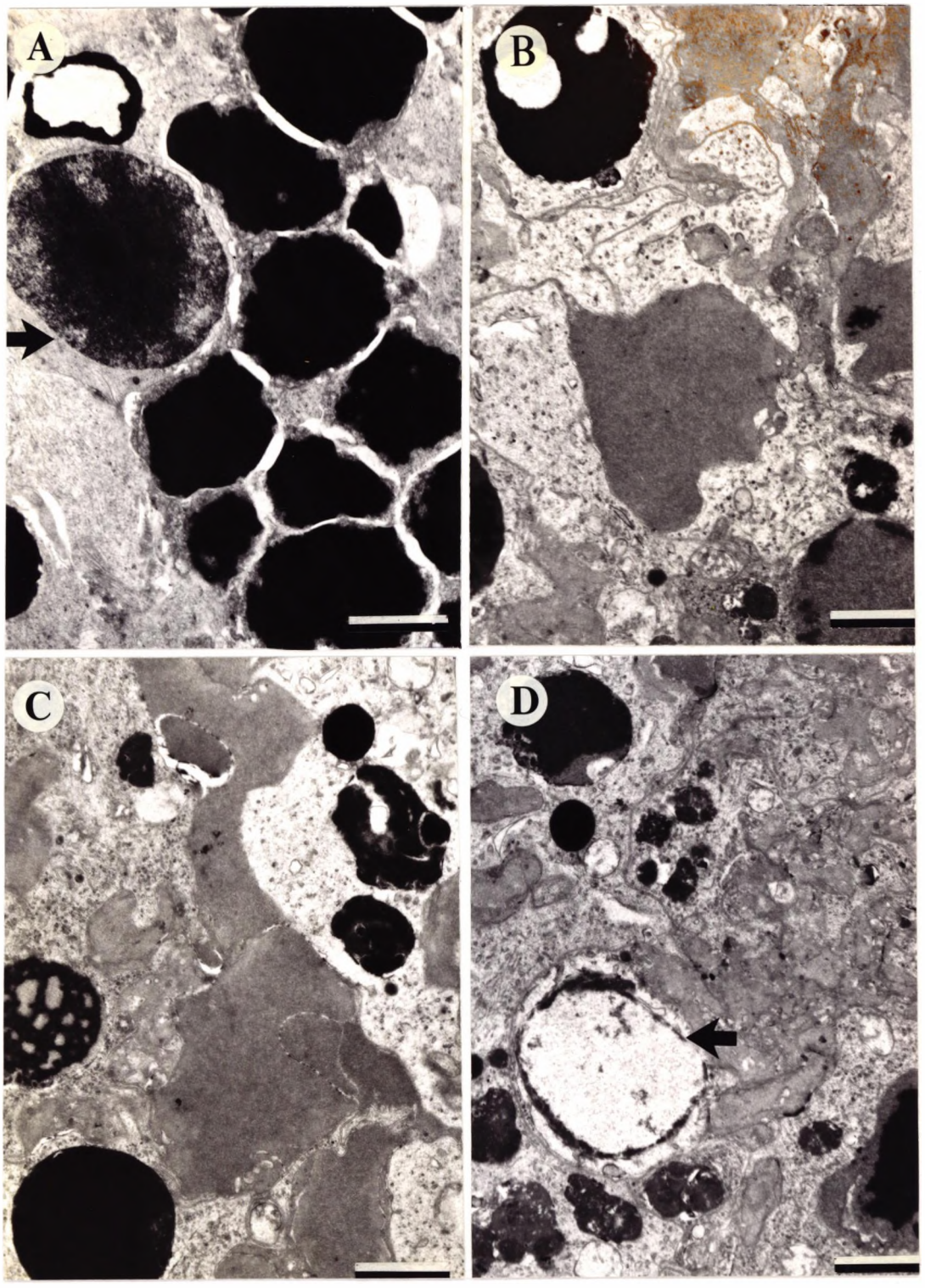


Fig. 25. Outer nuclear layer: degeneration area 24 hours after light damage. A viable nucleus (arrow) among predominantly pyknotic nuclei (A). Various stages of nuclei dissolution (B, C and D). Pseudomargination of nucleus formed by some karyoplasmic residue (arrow) (D). Bars = 2 μm.

moderate condensation of nuclear chromatin, ruptures of the nuclear envelope and chromatin leakage to the perinuclear area (fig. 26). Such cells were not observed in the central part of the degeneration zone and were not evident in the more distant areas which underwent retinal recovery. The photoreceptor cell bodies showed predominantly condensation and shrinkage immediately after light exposure. Scattered cells were affected and showed various degrees of change and varying preservation of the organelles (fig. 27). Some cells were shrunken with scanty amounts of condensed, dark stained cytoplasm (fig. 27 A, B). Mitochondria, which in some cells were relatively well preserved, (fig. 27 A, B) but in others showed various degrees of swelling and degradation (fig. 27 C, D) were also observed. In both normal looking and densified photoreceptors vesiculation of the membranes in the area proximal to the connecting cilium was frequently observed. The morphology of the connecting cilia showed normal arrangements of the ciliary microtubules (fig. 28). Within 24 hours after light exposure extensive necrotic changes were observed in the photoreceptors (fig. 29). The outer segments were very condensed in a number of cells immediately after light damage in the degeneration area. In other cells vesicular alterations of membranes were seen (fig. 30). Within 48 hours after light damage numerous outer segments were still preserved, with

very few vesicular changes seen. The inner segments were either lost or showed advanced lytic changes. The staining of disk membranes in the remaining outer segments was uneven (fig. 30 D, 34 B). At 72 and 144 hours after light exposure some photoreceptor nuclei and photoreceptor connecting cilia were identifiable (fig. 31, 39 D). Cross sections of the cilia showed normal 9+0 and symmetrical arrangement of microtubules (fig. 31). Despite the presence of cilia no outer segments were seen.

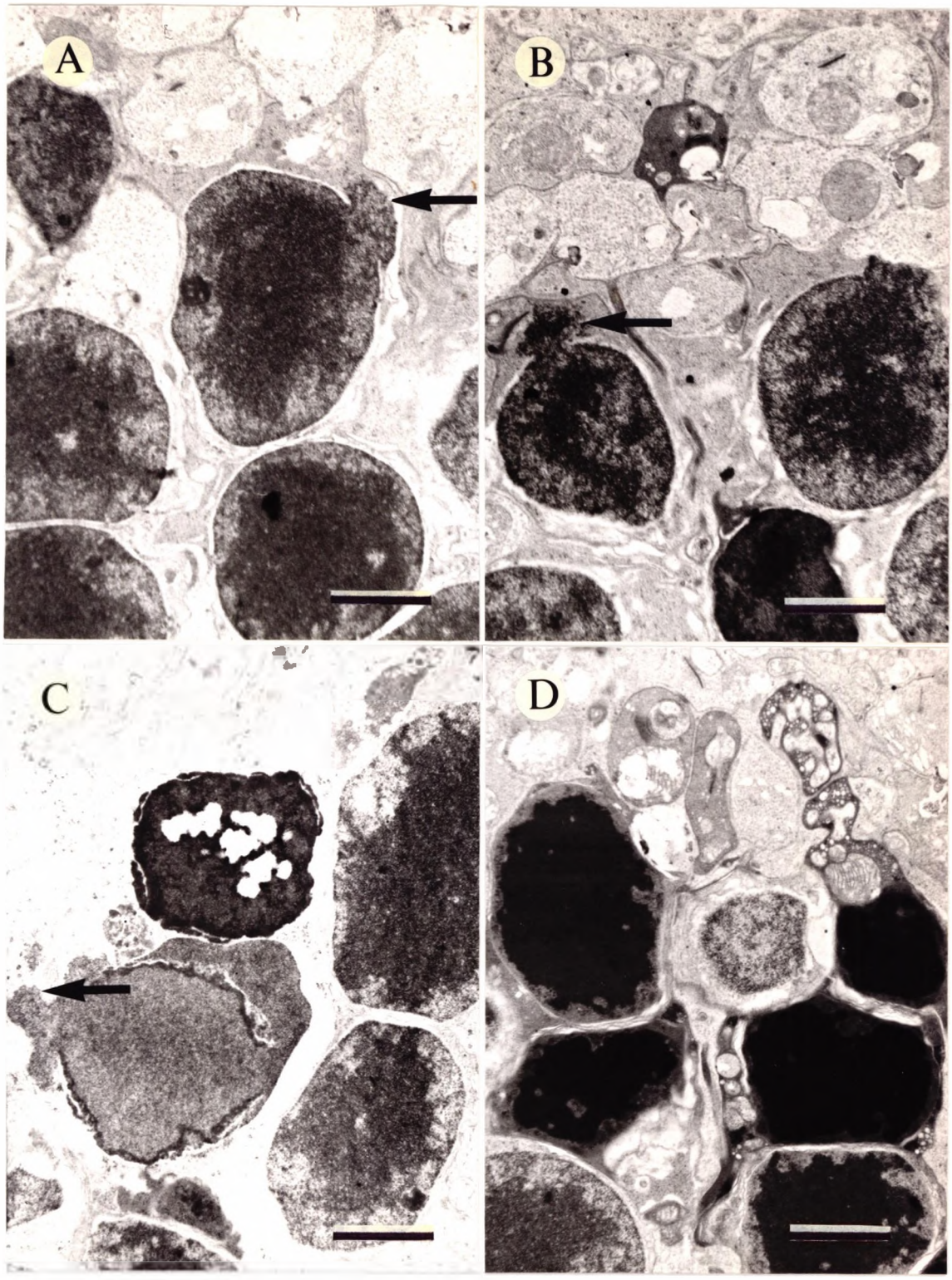


Fig. 26. Outer nuclear layer immediately (A,B,D) and 24 hours (C) after the exposure in the degeneration area. Numerous nuclei at the periphery of the lesion showed ruptures of the nuclear envelopes and ejection of karyoplasm into the cytoplasm immediately after light exposure (A, B). A similar picture was observed at 24 hours after light damage (C). Shrinkage and condensatin of nuclei was however the most common pattern of nuclear changes in the central part of the lesion (D). Bars = 2 μm.

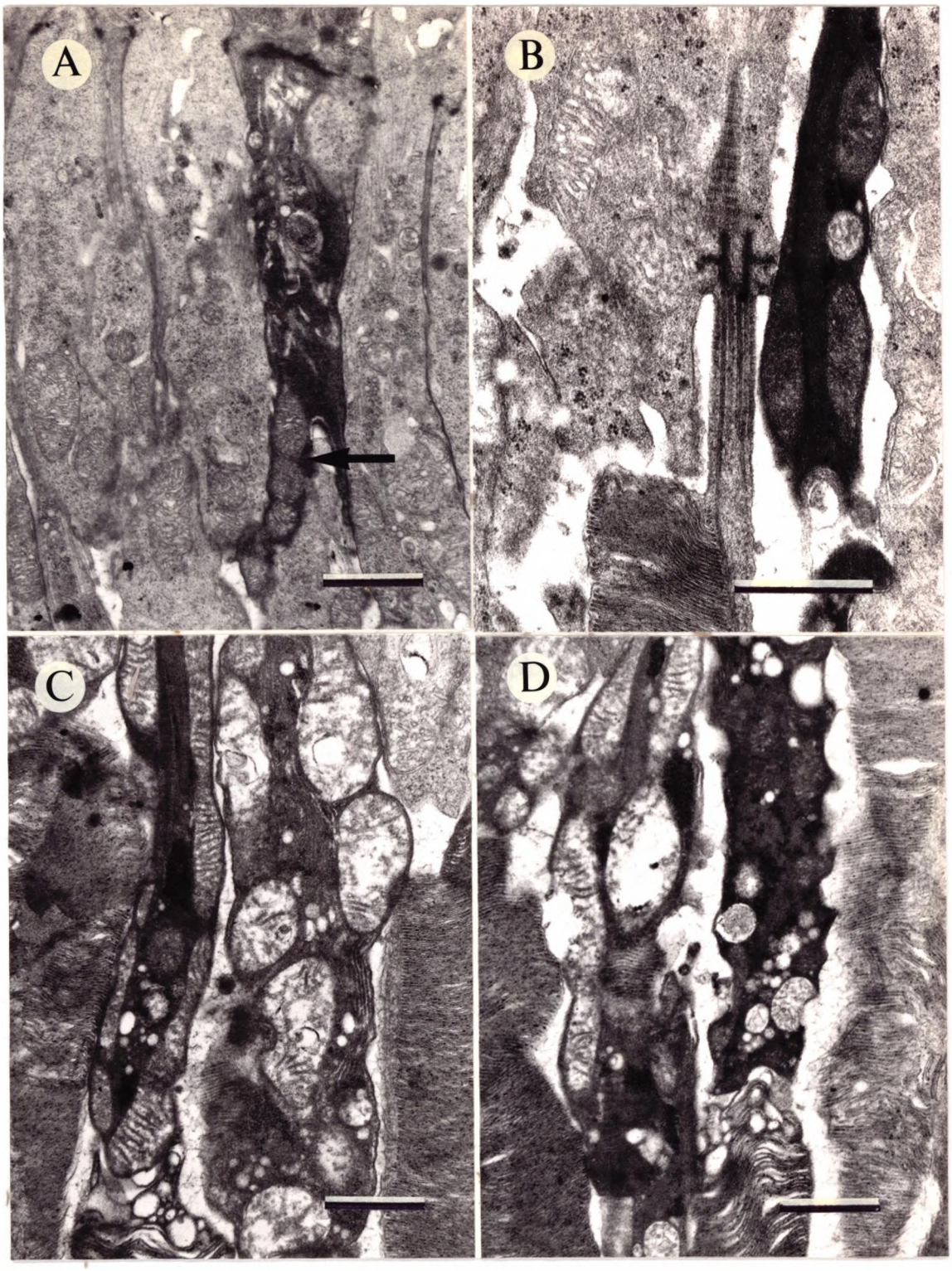


Fig. 27. Photoreceptor inner segments immmediately after light exposure in the degeneration area show a variation in the degree of shrinkage and in the condition of the intracellular organelles. Densification of the inner segment with preservation of mitochondria (arrow) (A). Very dense and shrunken inner segments (B). Condensed shrunken and condensed swollen inner segment (C). The two condensed inner segments with poorly preserved organnelles (D). Bars = 1 μm.

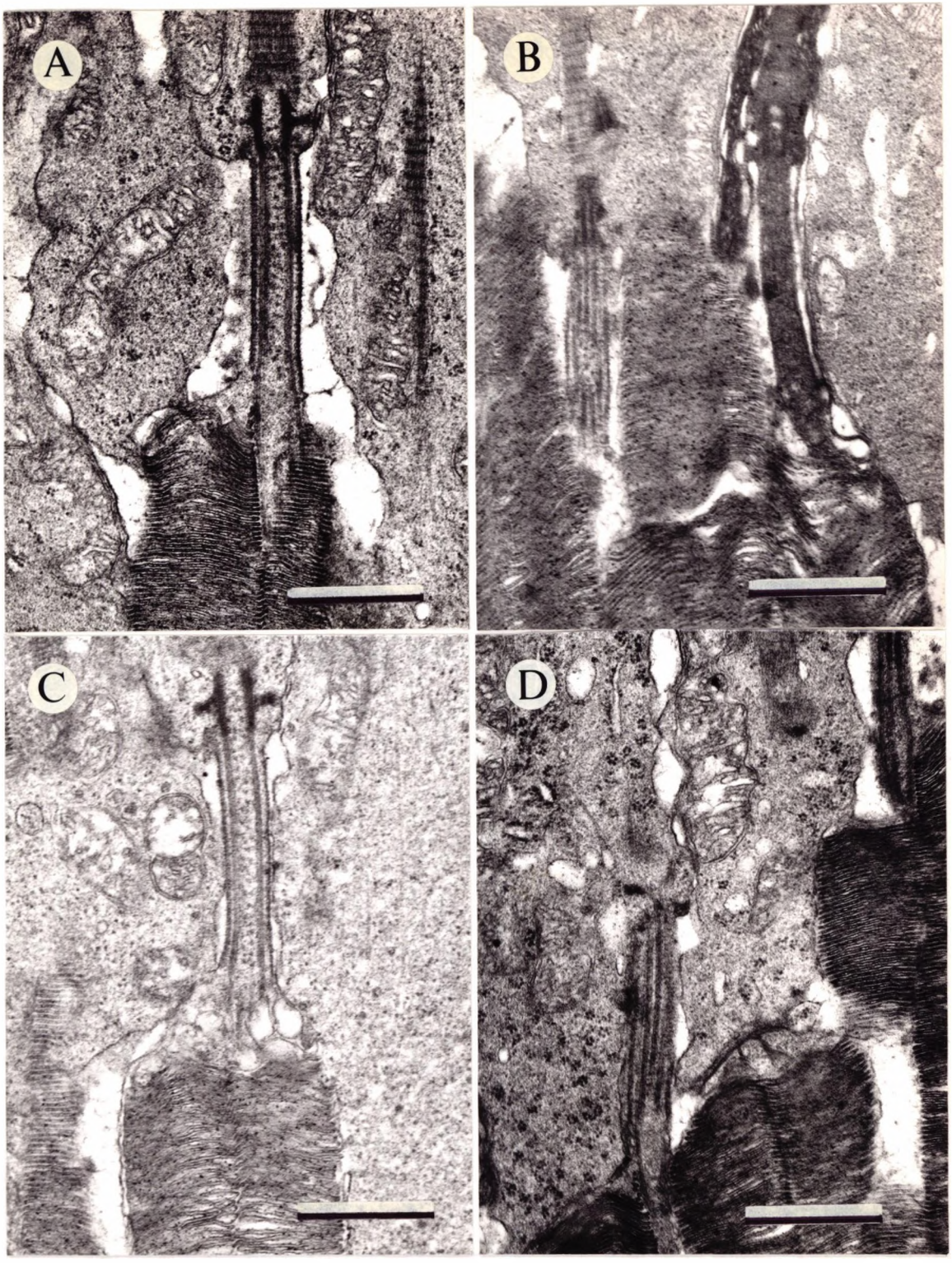


Fig. 28. Photoreceptor cilium area. Control (A) and immediately after light damage (B,C). The degeneration area (B), shows condensation and shrinkage. The recovery area (C), shows vesicular changes near the cilium. The recovery area, 144 hours after light exposure (D) shows normal morphology. Bars = 1 μm.

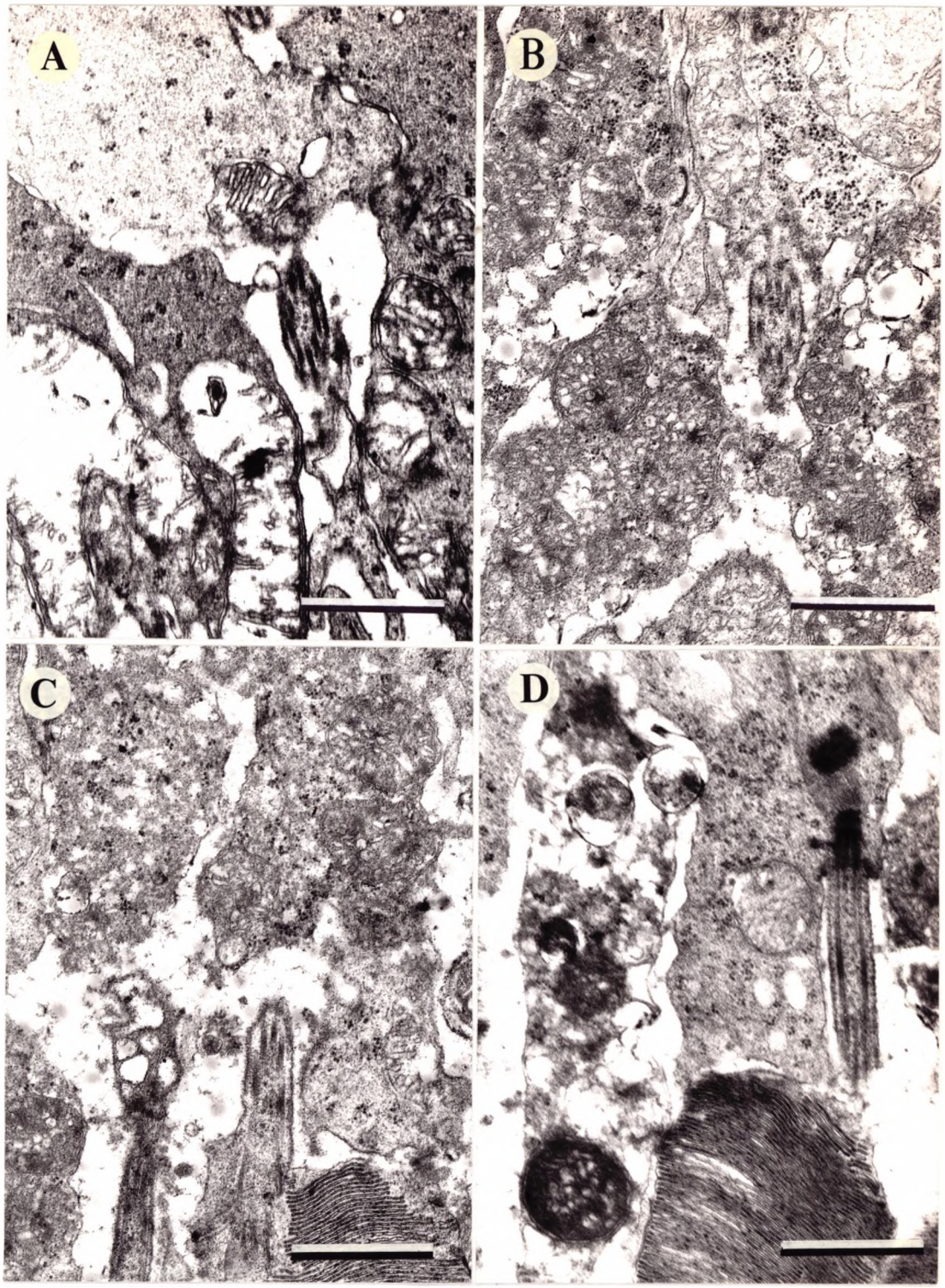


Fig. 29. Necrotic changes in photoreceptors 24 hours after light damage at the level of the connecting cilium. Distended mitochondria 24 hours after light damage (A). Vacuolization of the cytoplasm (B). Inner segment cytolytic changes (C). A better preserved inner segment next to one undergoing advanced cytolysis (D). Bars = 1 μm.

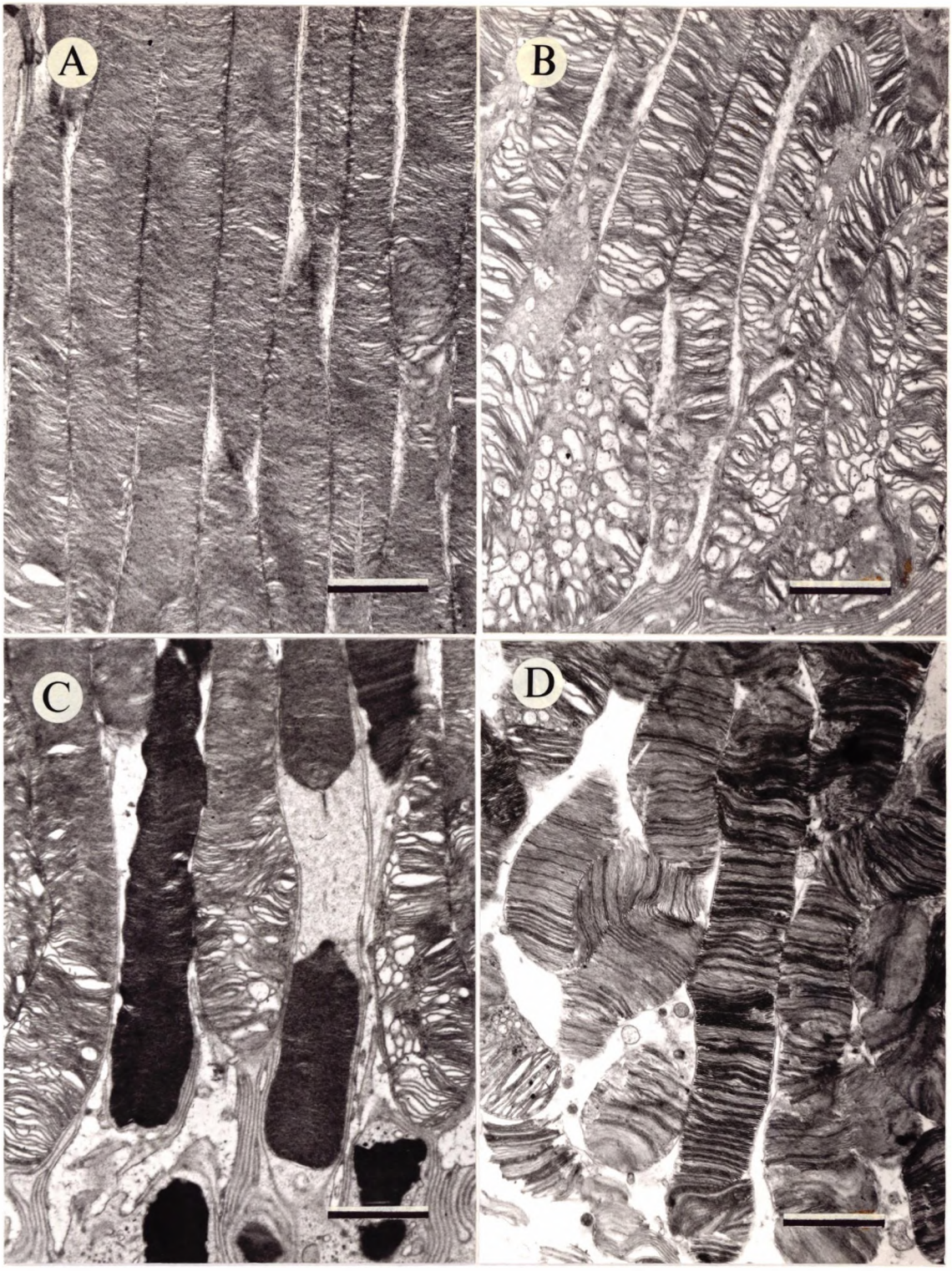


Fig. 30. Morphology of the outer segments: control (A), light damaged immediately after exposure in the recovery area showing vesicular alterations (B), in the degeneration area, showing densification of membranes in scattered apoptotic cells, while the viable cells show vesicular alterations (C), light damaged, 48 hours after exposure in the degeneration area (D), showing a regular arrangement of disk membranes, but irregilar staining of membranes (associated probably with loss of lipids and poorer osmium tetroxide staining) . Bars = 2 μm

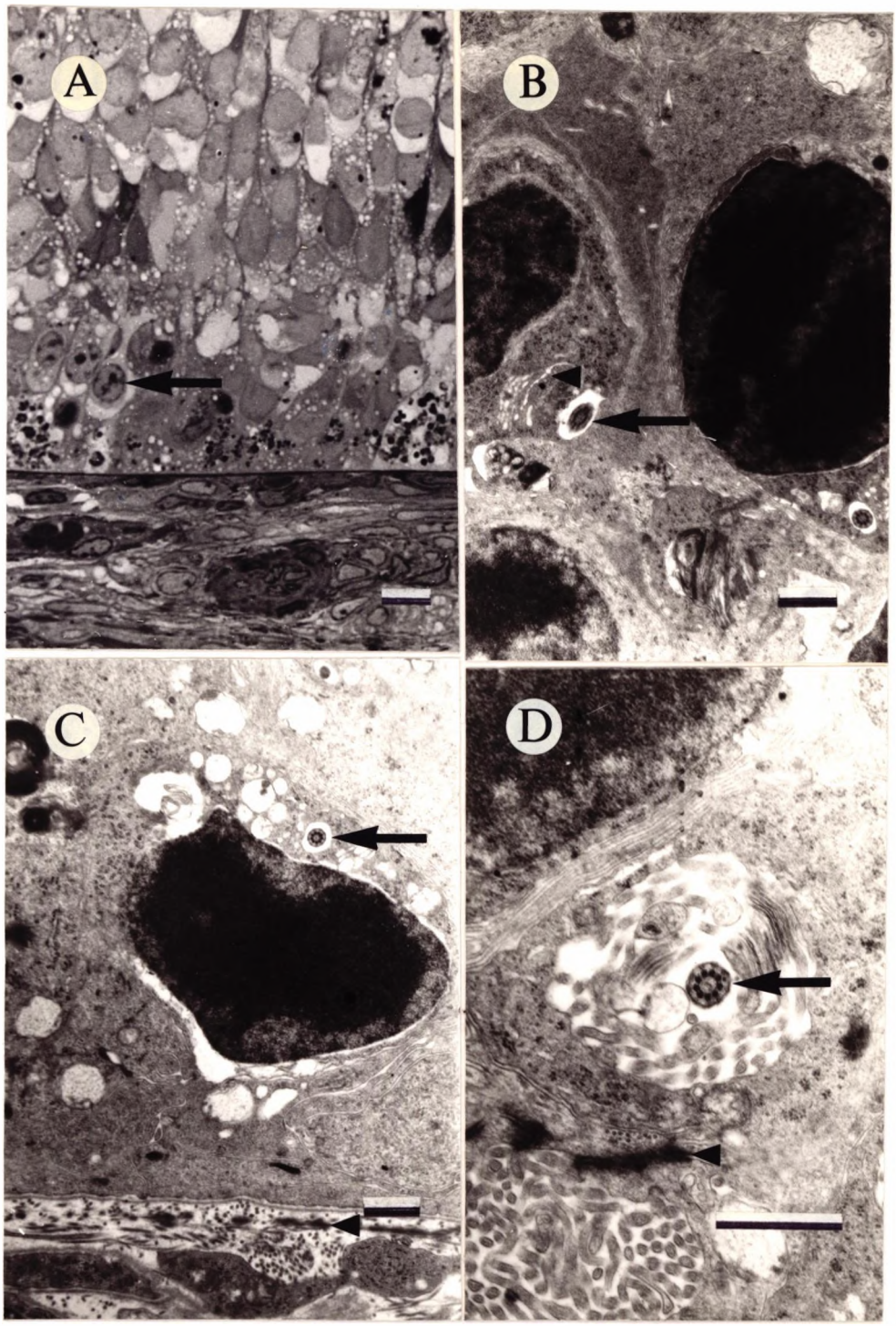


Fig. 31. Degeneration zone 144 hours after light damage. Light microscopy of the area showing a group of surviving photoreceptor nuclei (arrow) and inner segments but no outer segments. Bar = 10 μm (A). Higher magnification of such photoreceptors shows nucleus and scanty cytoplasm containing prominent Golgi cisternae (arrowhead) and the residual photoreceptor connecting cilium (arrow) (B). Such nuclei and cilia (arrow) were also found close to the Bruch's membrane.The elastic lamina of the Bruch's membrane is marked with arrowheads (C). Residual photoreceptor connecting cilium (arrow) is surrounded by villi of Muller cells. Cell junction complexes (arrowhead) forming outer limiting layer are present (D). Bars (B, C, D) = 1 μm.

4.4.2 Retinal pigment epithelium, Bruch's membrane and choroid.

The electron microscopic examination revealed some convolution or undulation of the nuclear envelope and numerous phagosomes in the retinal pigment epithelium immediately after light exposure, but no other pathology compared with the controls (fig. 32 A, B). However, a high proportion of the photoreceptors showed densification and shrinkage at the same point in time.

The most striking changes in RPE morphology occurred within the first 24 hours after exposure and included condensation of the karyoplasm along the margins of the nucleus, cell shrinkage, densification of the cytoplasm, loss of polarity of the RPE (fig. 32) and finally cell fragmentation and loss by 48 hours after the exposure (fig. 33). On rare occasions disks like membranes were observed within the lumen of the vessels of the choriocapillaris (fig. 33 C).

In the borderline area, on the margins of the degeneration area, the RPE was better preserved but contained large droplets (fig. 12, 13). These were presumably lipids, since they were stained, though weakly, with the Oil Red O and Sudan Black stains which were applied to plastic embedded, EM processed sections (not shown). Within 48 hours when most of the RPE was lost a cellular infiltration was observed (fig. 33 C,

D) within the outer layers of the Bruch's membrane, between the capillary basement membrane and the elastin layer.

Within the choroid in the degeneration area some pathology was evident 24 hours after light exposure. A diffuse choroidal oedema and accumulation of mononuclear cells were observed. Macrophages were present within the Bruch's membrane (fig. 34 A) and in the choroid. These changes coexisted with death of the retinal pigment epithelium. At 48 hours choroidal infiltration by mononuclear cells persisted (fig. 43 D). Numerous necrotic erythrocytes at various stages of degradation were present within and outside the vessels choroidal vessels (fig. 34 C, D). The Bruch's membrane was still infiltrated by monocytes (fig 34 B). At 72 hours the choroidal swelling subsided, fibrosis and focal choriocapillaris dropout were observed in some areas while in others formation of new capillaries was noted (fig 35 A). At 144 hours thinning and infiltration of Bruch's membrane by mononuclear cells (fig. 35 C) and formation of the new capillaries within choriocapillaris and choroid was also observed (fig. 35 B). In some of the immature vessels trapped erythrocytes undergoing degradation were observed (fig. 35 D).

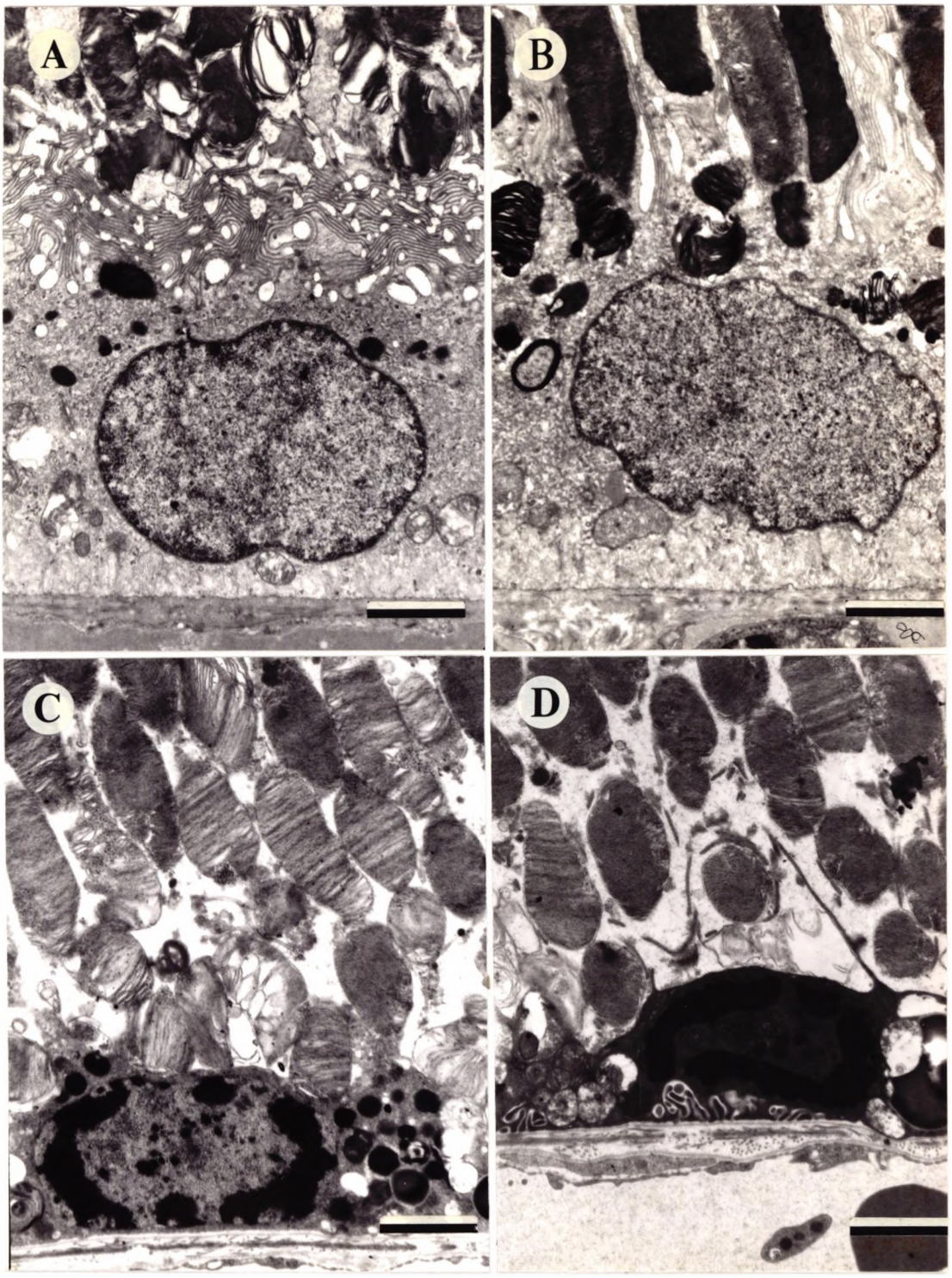


Fig. 32. Retinal pigment epithelium: Control (A), immediately after light exposure (B) showing numerous phagosomes and undulation of the nuclear envelope. 24 hours after light damage (C, D), showing margination of the nuclear karyoplasm, densification, shrinkage and loss of polarity by the cell, changes consistent with apoptosis. . Bars = 2 μm.

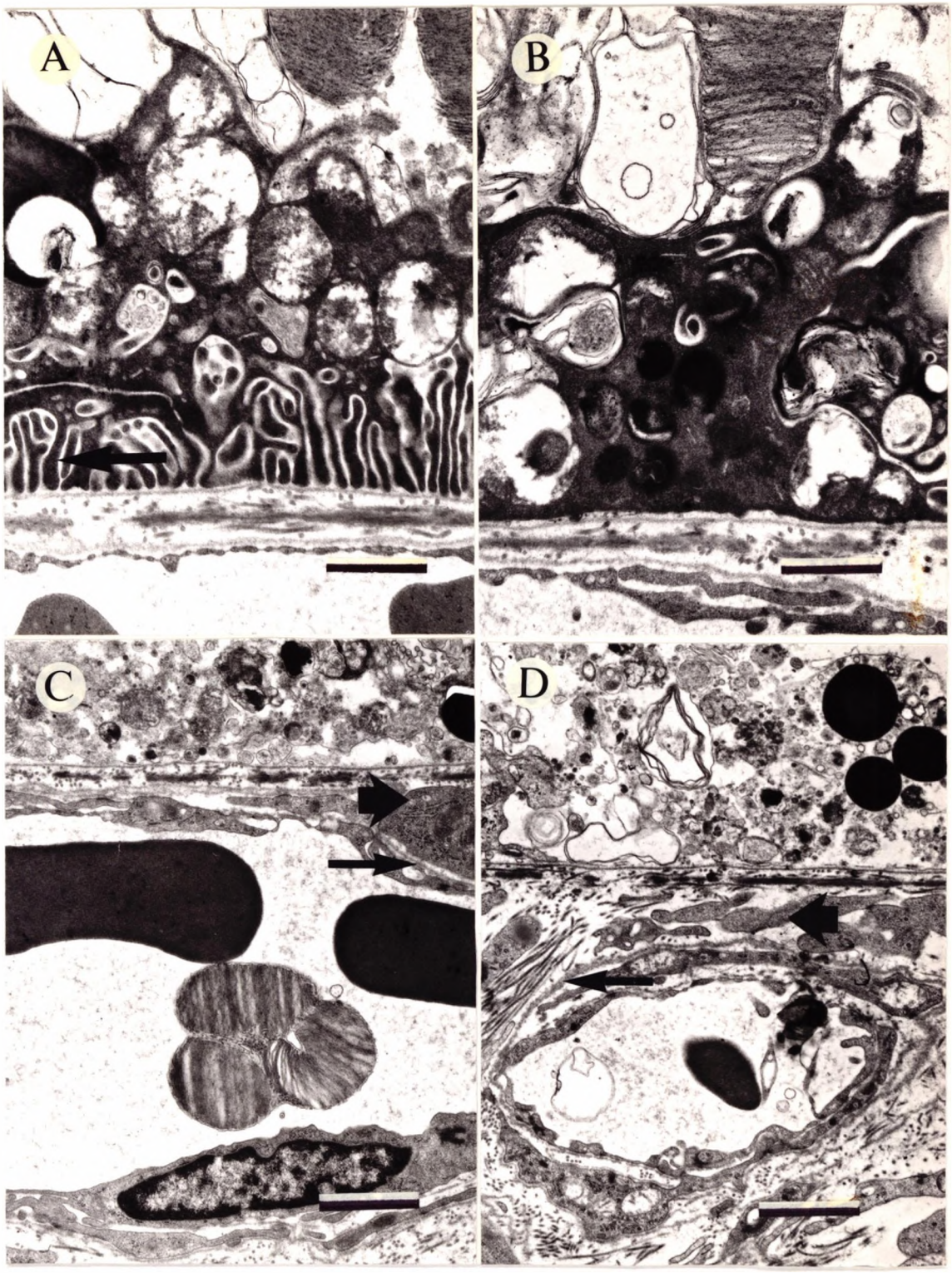


Fig. 33. Retinal pigment epithelium, degeneration area (A,B): 24 hours after light exposure, showing shrinkage and densification of cells, loss of apical infoldings but preservation of basal infoldings (arrow) (A) and a complete loss of polarity (B). Bruch's membrane, degeneration area, 48 hours after light exposure (C,D): Retinal pigment epithelium is necrotic and between the elastin layer and the endothelial basement membrane (arrow) of the Bruch's membrane a cellular infiltration can be seen (thick arrows). Bars (A,B) = 1 µm, (C,D) = 2 µm.

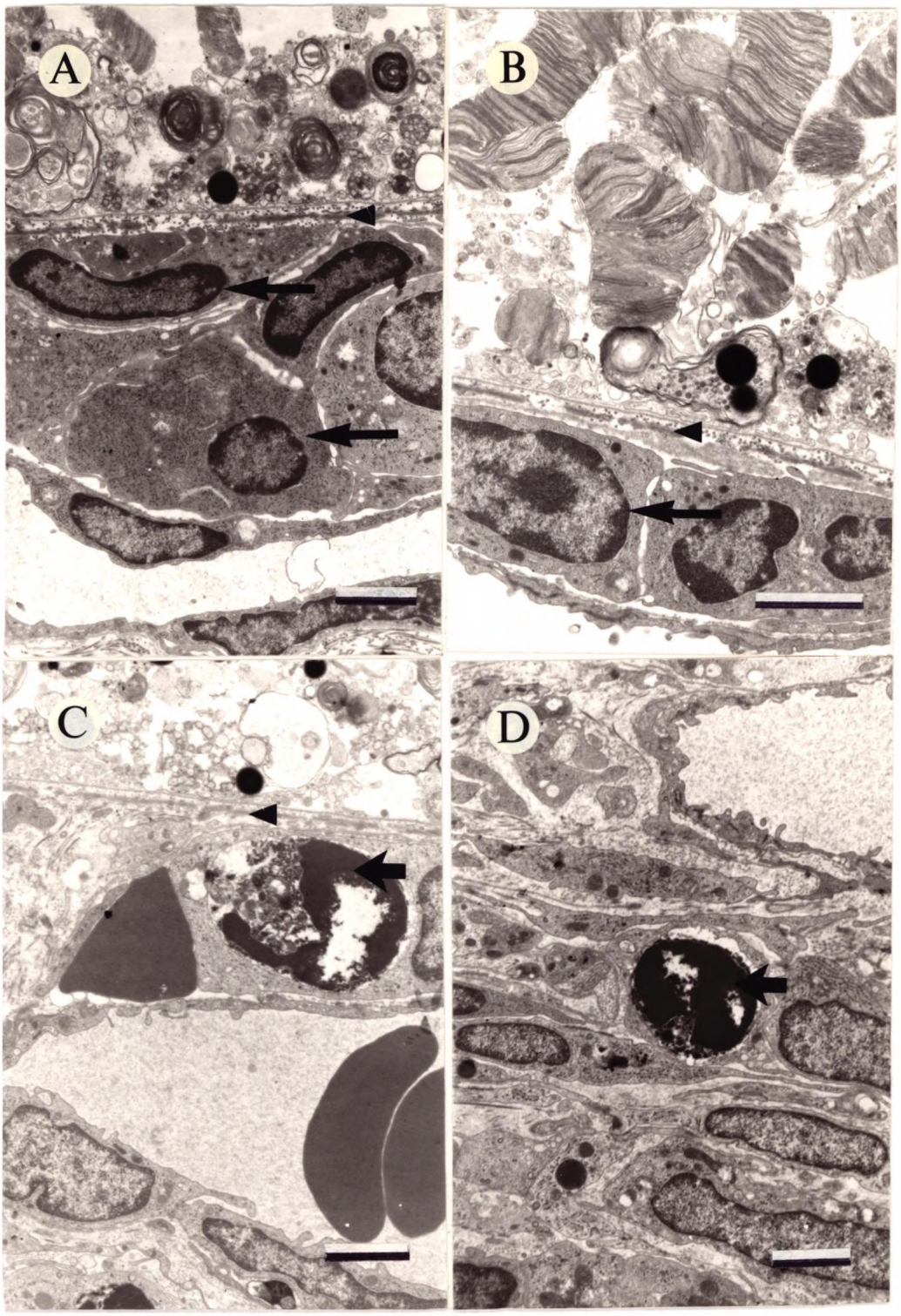


Fig. 34. Changes within the Bruch's membrane and choroid in the degeneration zone. Infiltration of the outer aspects of the Bruch's membrane (elastin layer is marked with arrowheads) by numerous mononuclear cells (arrows) is apparent within 24 hours (A) and 48 hours (B) after light damage. At 48 hours extravasation and subsequent desquamation of erythrocytes (thick arrows) is seen between choriocapillaris and the Bruch's membrane (C) and in deeper choroidal layers (D). Bars = 2 μm.

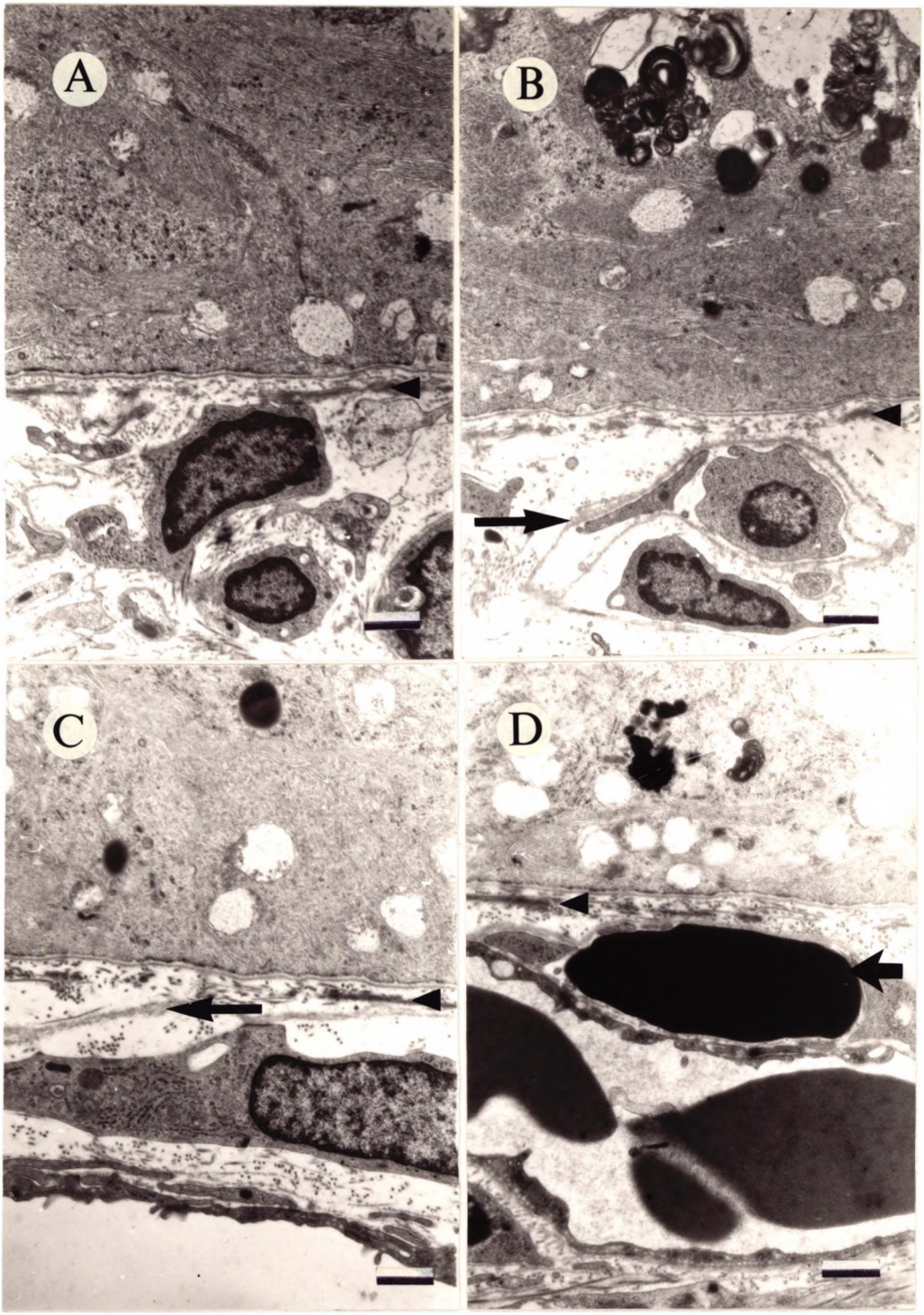


Fig. 35. Changes in the Bruch's membrane and choroid in the degeneration zone. Foci of new vessel formation were present 48 hours post exposure, but were more prominent 72 (A) and 144 hours (B) after damage. At that stage glial tissue was firmly attached to the Bruch's membrane. 144 hours after light damage mononuclear cells with prominent rough endoplasmic reticulum (C) and erythrocytes (D) trapped in immature vessels (thick arrow) were still present in the Bruch's membrane. Elastin layer of the Bruch's membrane is marked with arrowheads. Basement membrane like material is marked with arrows. Bars = 1 μm.

4.4.3 Other cell and tissue responses.

Within 24 hours and thereafter macrophages were present in the sub retinal space and in the outer retina (fig. 16, 20, 36 C, D).

At 48 and 72 hours after the light damage numerous mitotic figures were observed in the outer and inner retina (fig. 16 B, 20, 36 A, B).

Condensed cell-derived debris was shed and formed inclusion bodies in Muller cells within the first 24 hours after light damage (fig. 37). Inclusion bodies were uniformly electron dense in appearance, resembling very condensed chromatin (fig. 37 A, B; fig. 38 A, B, C) or more granular (fig. 37 D) or membranous suggesting cytoplasmic origin (fig. 37 B,C) or they were of variable density consisting of loosely packed, heterogeneous, granular electron dense nuclear and cytoplasmic material of dead cells (fig. 37 D and 38 D). The inner retinal layers showed some oedema manifested as separation between the cells at 24 and 48 hours after light exposure (fig. 39 A). These changes were not observed at 72 hours when cell debris was observed within Muller cells close to the inner limiting membrane (fig. 39 B). At 24 and 144 hours abnormal cell junction complexes were observed in that region (fig. 39 C). At the same time the outer limiting membrane showed some remodelling (fig. 39 D).

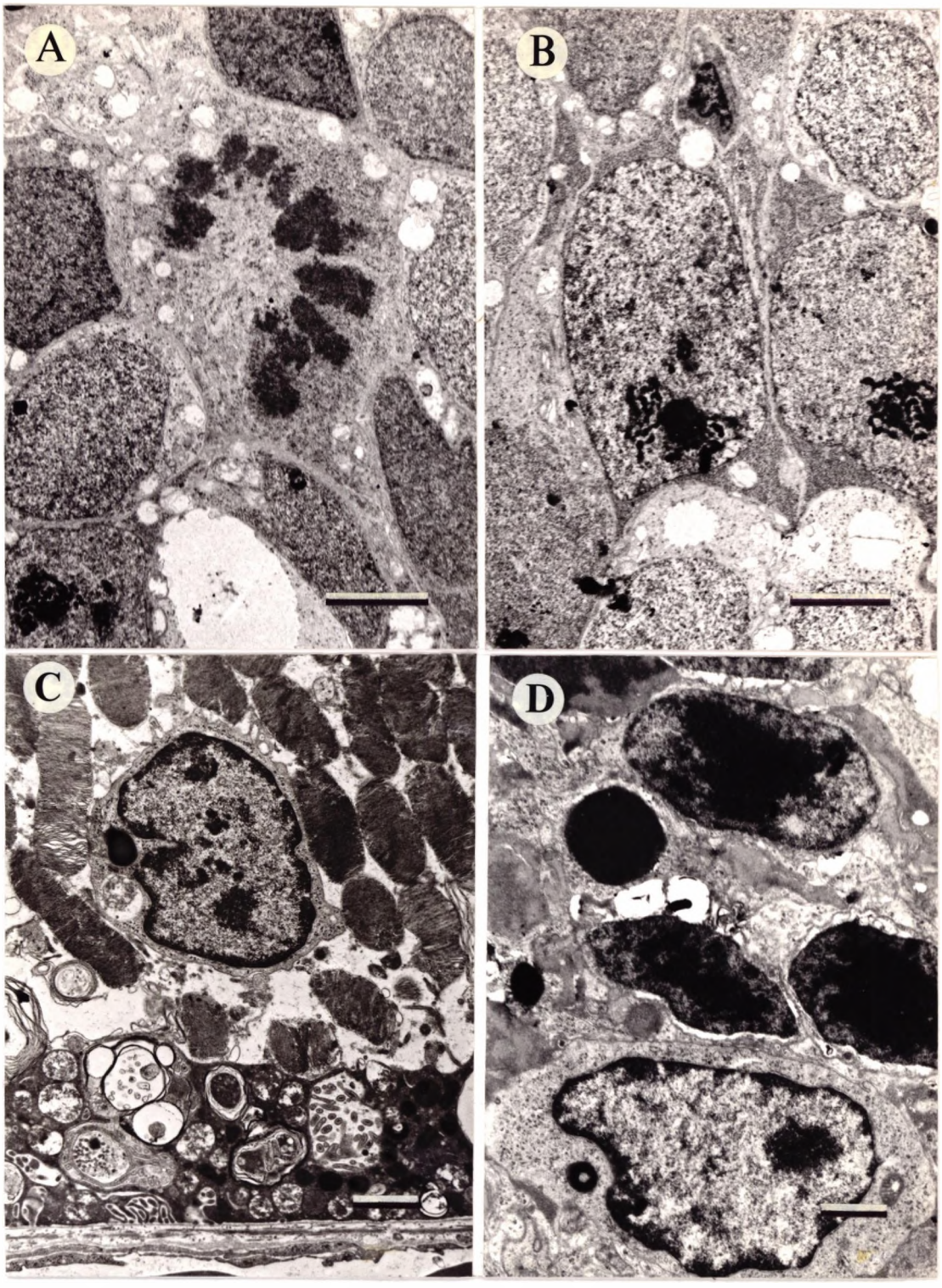


Fig. 36. Inner nuclear layer (A, B), 72 hours after light damage in the degeneration area showing proliferation of presumably Muller cells, a mitotic figure (A), chromatin clumping (B). Bar = 4 μm. The degeneration area 24 hours after light exposure infiltrated by macrophages in the sub-retinal space (C), in the outer nuclear layer (D). Bar = 2 μm.

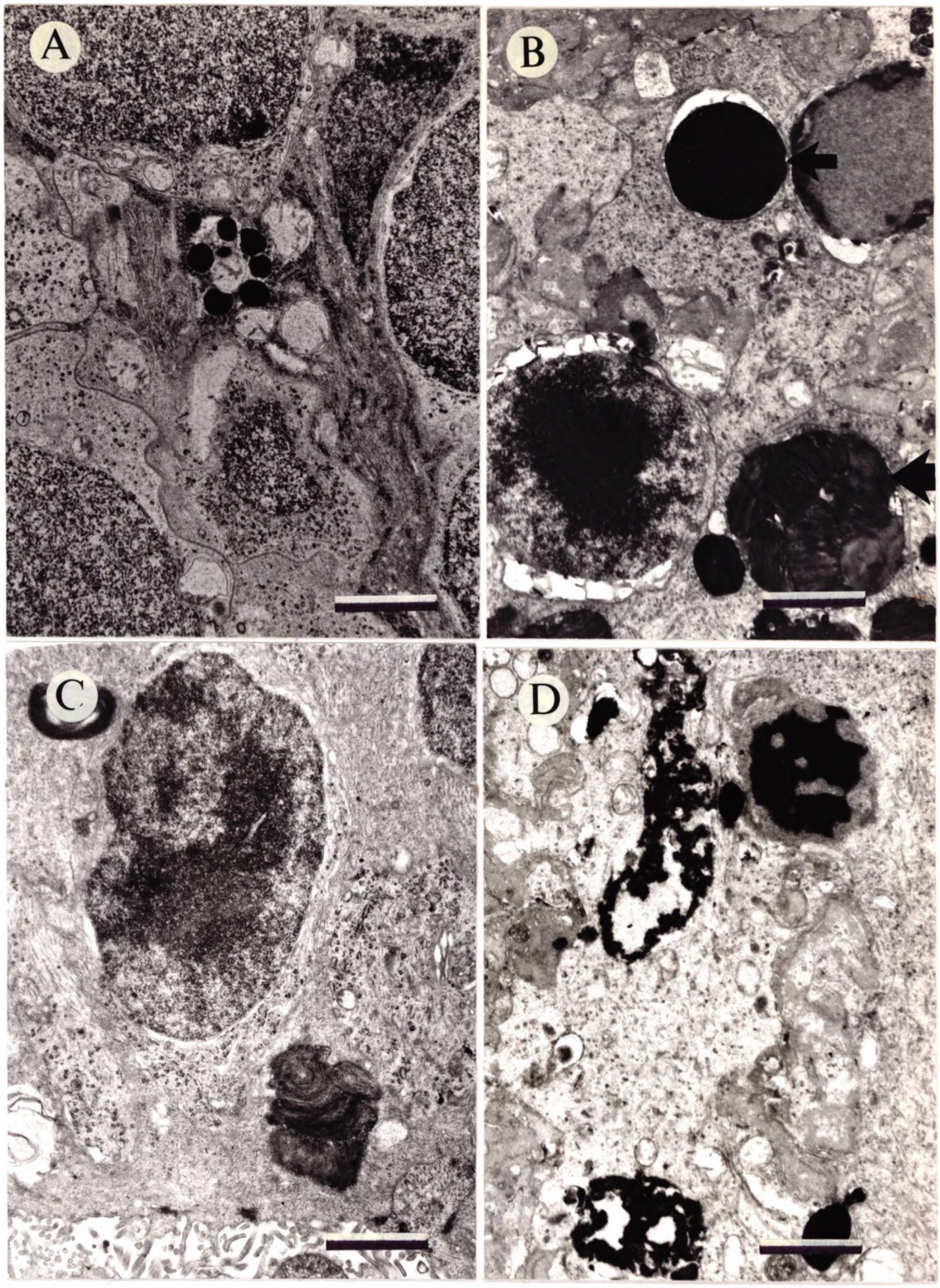


Fig. 37. Photoreceptor derived debris in the retina 24 hours after light damage in the degeneration area: densely condensed debris within Muller cell in the inner nuclear layer (A), chromatin debris and lipid rich cell-derived debris (thick arrow) forming membranes in the outer nuclear layer (B), membranous debris close to the outer limiting membrane (C), some nuclear residue forming dense granular deposits in the outer nuclear layer (D). Bars = 2 μm.

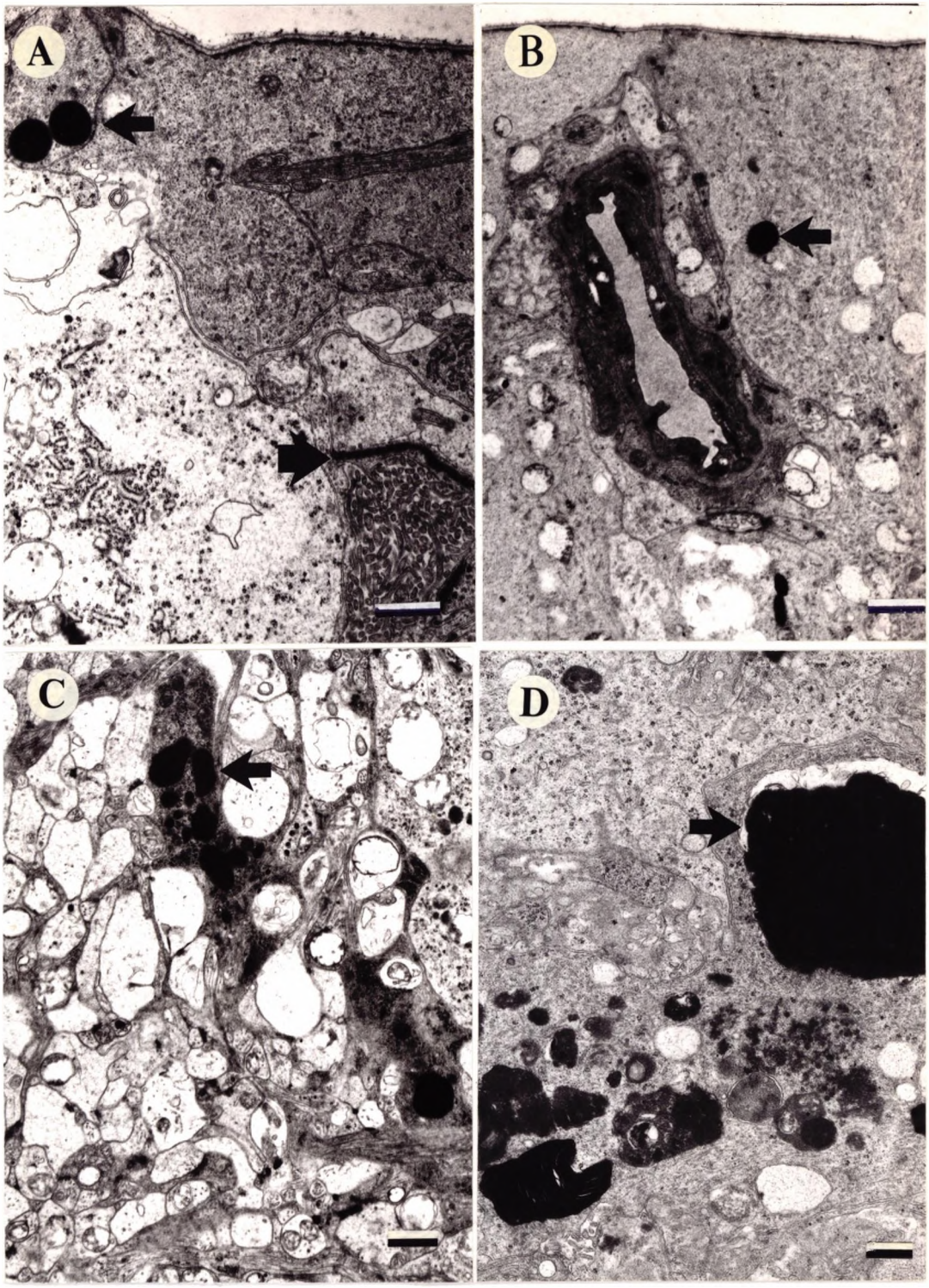


Fig. 38. Photoreceptor derived debris (arrows) within the glial cells 72 hours after light damage (A) close to the inner limiting membrane (thick arrow indicates abnormal cell junction complexes), in proximity to the retinal vessels (B), within the inner plexiform layer (C) and in the outer nuclear layer (D). Bar = 1 μm.

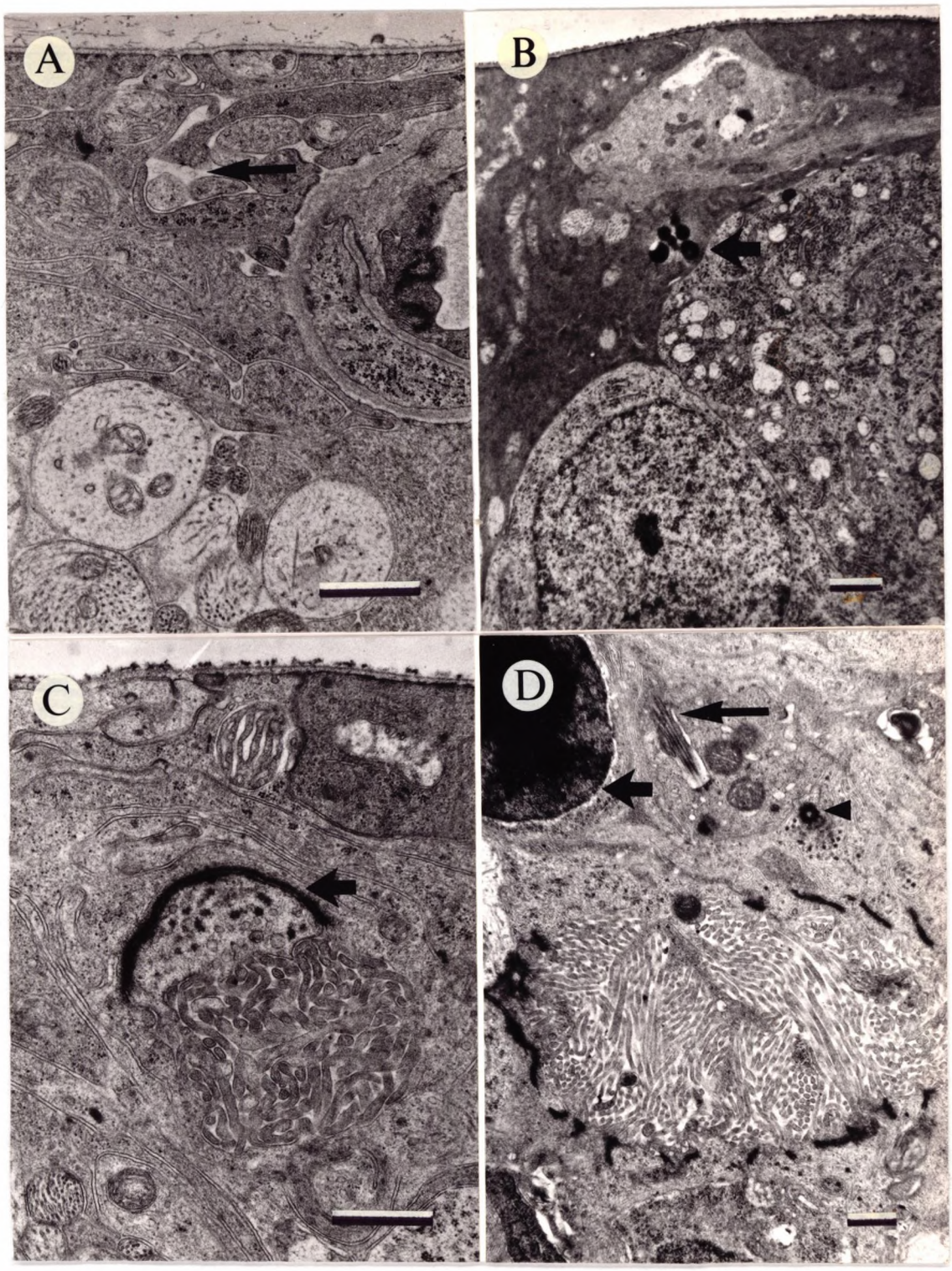


Fig. 39. Degeneration area: 24 hours after light damage oedema (arrow) is seen in the region of the inner limiting membrane (A), at 72 hours the oedema has subsided and photoreceptor-derived debris (arrow) can be seen in the Muller cell end plates (B) and abnormal cell junction complexes (arrow) can be observed between glial cells (C). The outer limiting membrane shows dysplasia and formation of cystic spaces filled with apical villi of Muller cells (D). Occasional viable photoreceptor nucleus (arrow), connecting cilium (long arrow) and centriole (arrowhead) are present.
Bars = 1 μm.

4.5 Light microscopy of the recovery area.

4.5.1 Photoreceptors.

The most striking pathology observed in the retinal region which recovered normal morphology within a week were vesicular alterations of the photoreceptor outer segments and some pyknotic nuclei in the outer nuclear layer as seen in the control animals. (fig. 40 and 41). Vesicular alterations of the outer segments were observed immediately after light exposure and persisted in varying degrees up to 72 hours after damage. At 144 hours the retina had regained its usual appearance (fig. 42).

4.5.2 Retinal pigment epithelium.

The retinal pigment epithelium did not show any abnormalities on light microscopy examination at any time during observation. In control and experimental animals some vacuolation of the retinal pigment epithelium was observed. Vacuoles in the retinal pigment epithelium were also present in all other control groups of the animals used in other experiments conducted in the laboratory. Controls from the following

experiments were examined: effects of dietary fish oil on acute light induced photoreceptor damage in the rat retina (Reme et al 1994), modification of light and/or lithium induced effects in the rat retina by the PAF antagonist, BN 52021. (Reme et al 1992, Jung et al 1993), light damage in the rat retina: effect of a radioprotective agent (WR-77913) on acute rod outer segment disk disruptions (Reme et al 1991), light damage in the rat retina: effect of dietary deprivation of N-3 fatty acids on acute structural alterations (Bush et al 1991). In the published reports, however, the photographs of the control, dark adapted animals were not included. Vacuolation of the retinal pigment epithelium in all control animals was not uniform, some scattered areas with affected cells were present next to the areas with better preserved cells.

4.5.3 Cell and tissue responses.

Light microscopy did not reveal any other abnormalities besides the outer segment vesicular alterations in photoreceptor cells. At the end of the observation period in the recovery area the outer and inner retinal layers showed normal morphology in the light damaged animals which did not differ from the histology of controls.

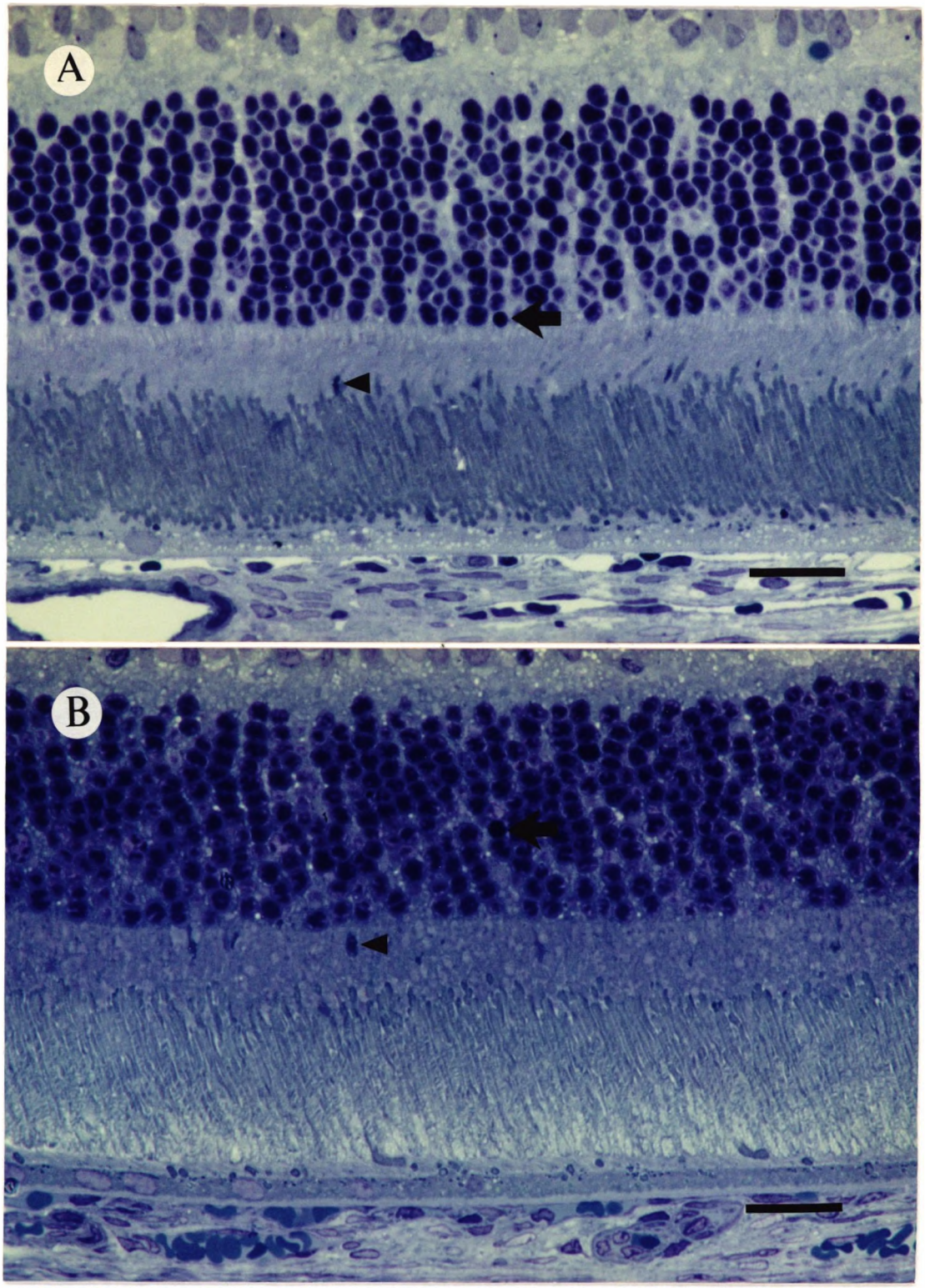
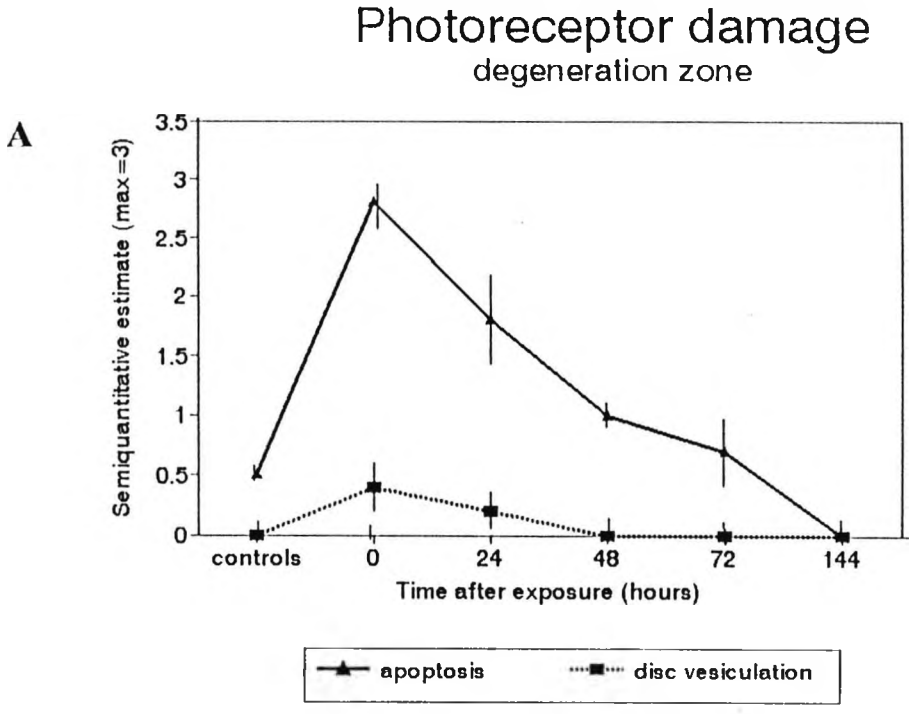


Fig. 40. Control (A): outer retina shows occasional pyknotic nuclei (arrow) and densified inner segments (arrowhead) of apoptotic photoreceptor cells. The outer retina of the recovery (nasal) side immediately after light damage (B) shows prominent vesicular changes of the outer segments, occasional pyknotic nuclei (arrow) and densified inner segments (arrowhead) as in the controls. Bars = 20 μm.



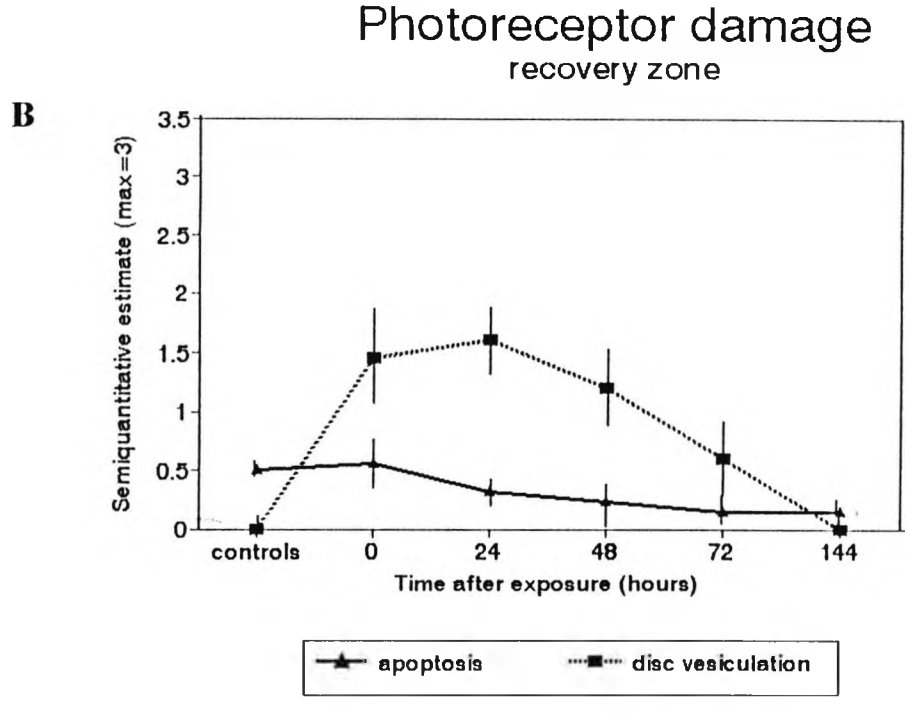


Fig. 41. Relation between apoptosis of photoreceptor cells and vesiculation of rod outer segment membranes in degeneration zone (A) and regeneration zone (B).

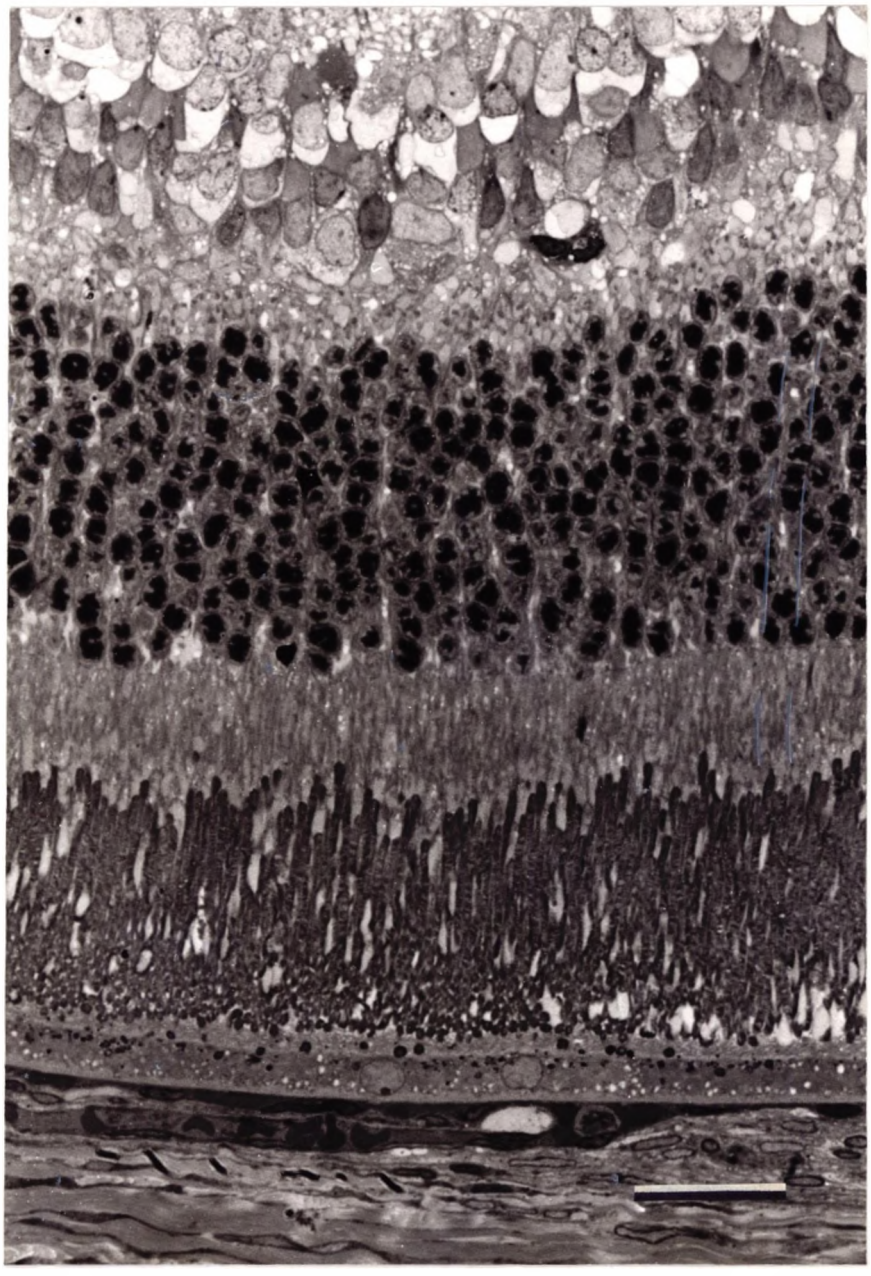


Fig. 42. The retina from the recovery area 144 hours after exposure shows apparently normal morphology and preservation of the photoreceptors and the retinal pigment epithelium. Bar = 20 μm.

4.6 Electron microscopy of the recovery area.

4.6.1 Photoreceptors.

Photoreceptor outer segments showed characteristic alterations in the distal portion immediately after exposure. The membranes were vesiculated and disks were dilated from the tip area along 1/2 or 2/3 of the length of the outer segments towards the base and photoreceptor cilium area (fig. 40 B, 45 A, 49 C). Scattered condensed and shrunken inner segments were occasionally seen as well as single pyknotic nuclei. These dark and pyknotic photoreceptors did not occur more frequently than in the control animals. Altered morphology of rod outer segment membranes was observed with some variability up to 72 hours after light damage. At 144 hours the vesicular alterations were no longer observed and the morphology of the outer segments (fig. 42, 43) did not differ from that of the control animals (fig. 40 A).

4.6.2 Retinal pigment epithelium.

The only abnormality observed within the retinal pigment epithelium was some oedema manifested as separation of the cells at their lateral borders

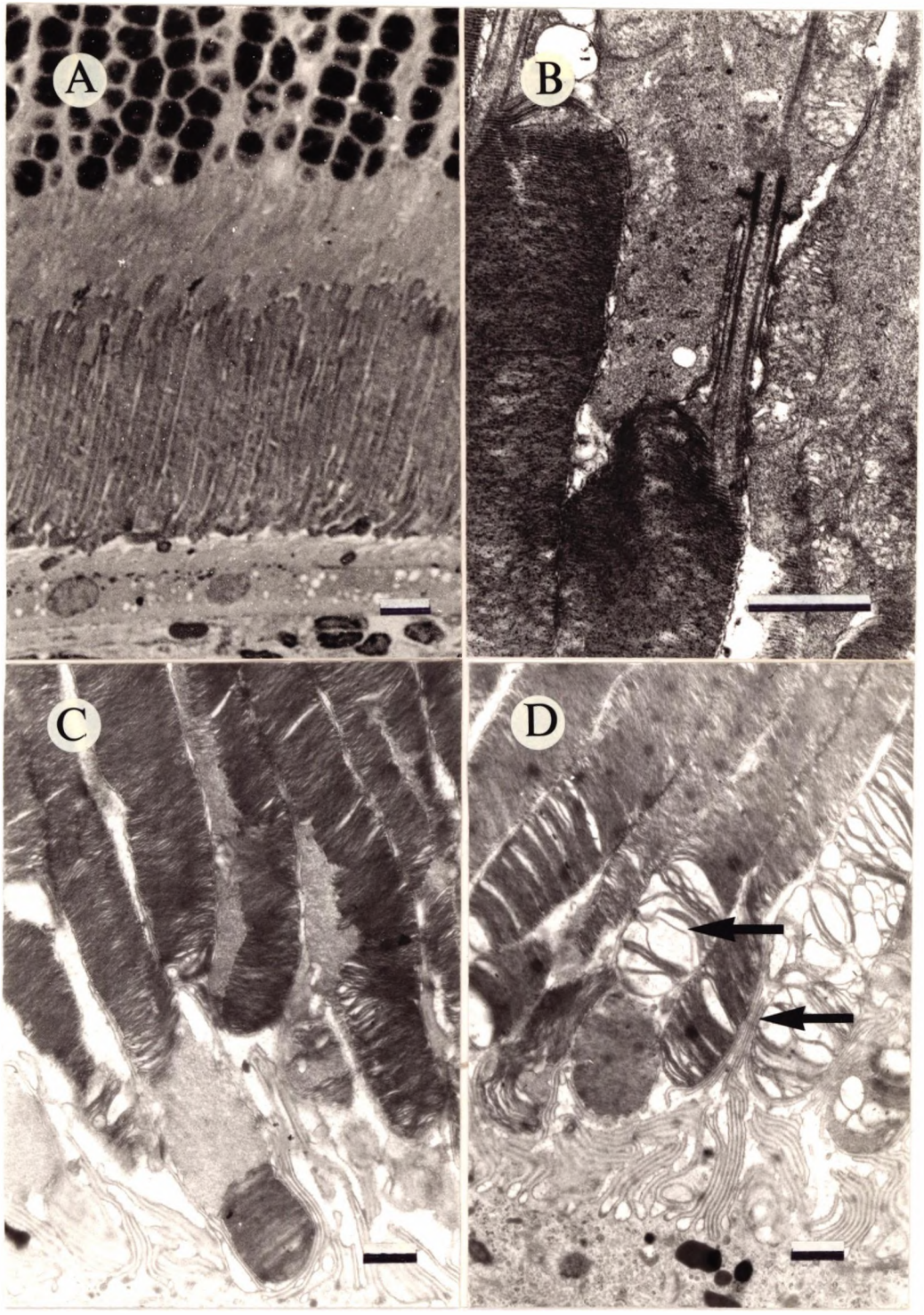


Fig. 43. Regeneration zone, 144 hours after light damage shows normal morphology of photoreceptors and pigment epithelium on light microscopic examination (A), bar = 10 μm. The inner segments, connecting cilium and proximal (B) and distal (C) parts of outer segments show normal ultrastructure, but in some cells the outer segments still show some residual vesicular alterations of the tips (arrows) (D). Bars (A,B,C) = 1 μm.

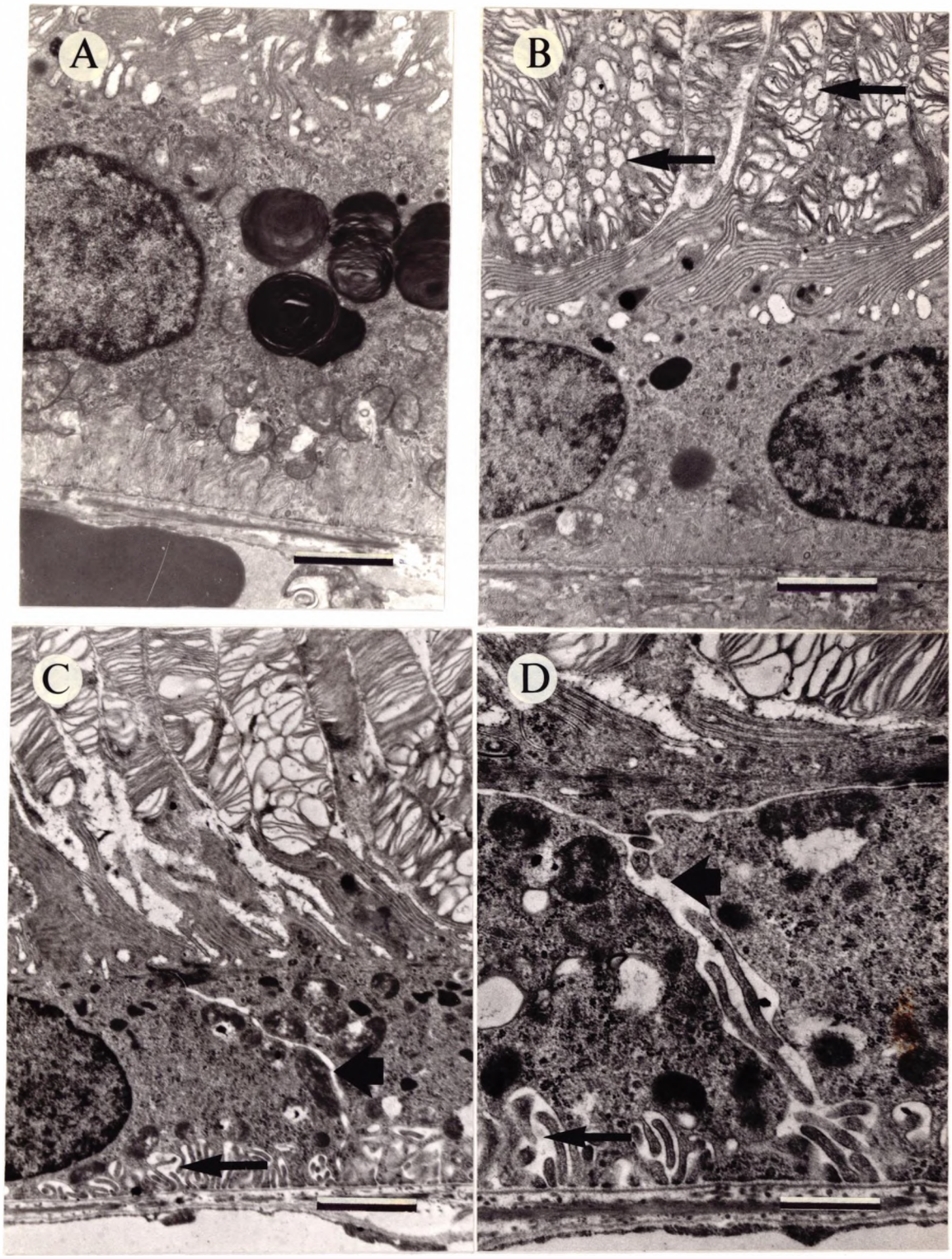


Fig. 44. Retinal pigment epithelium. Control (A), recovery area (B), immediately after light damage, showing apparently normal morphology of the retinal pigment epithelium and vesicular alterations of the rod outer segments (arrows). 24 hours hours after light exposure in the recovery area (C, D) some oedema is present. The spaces in the basal infoldings area (arrows) and in between cells (thick arrow) are increased suggesting swelling of the extracellular space also prominent in figures 31 D, 32 A, 33 C. Bars (A,B,C) = 2 μm, (D) = 1 μm.

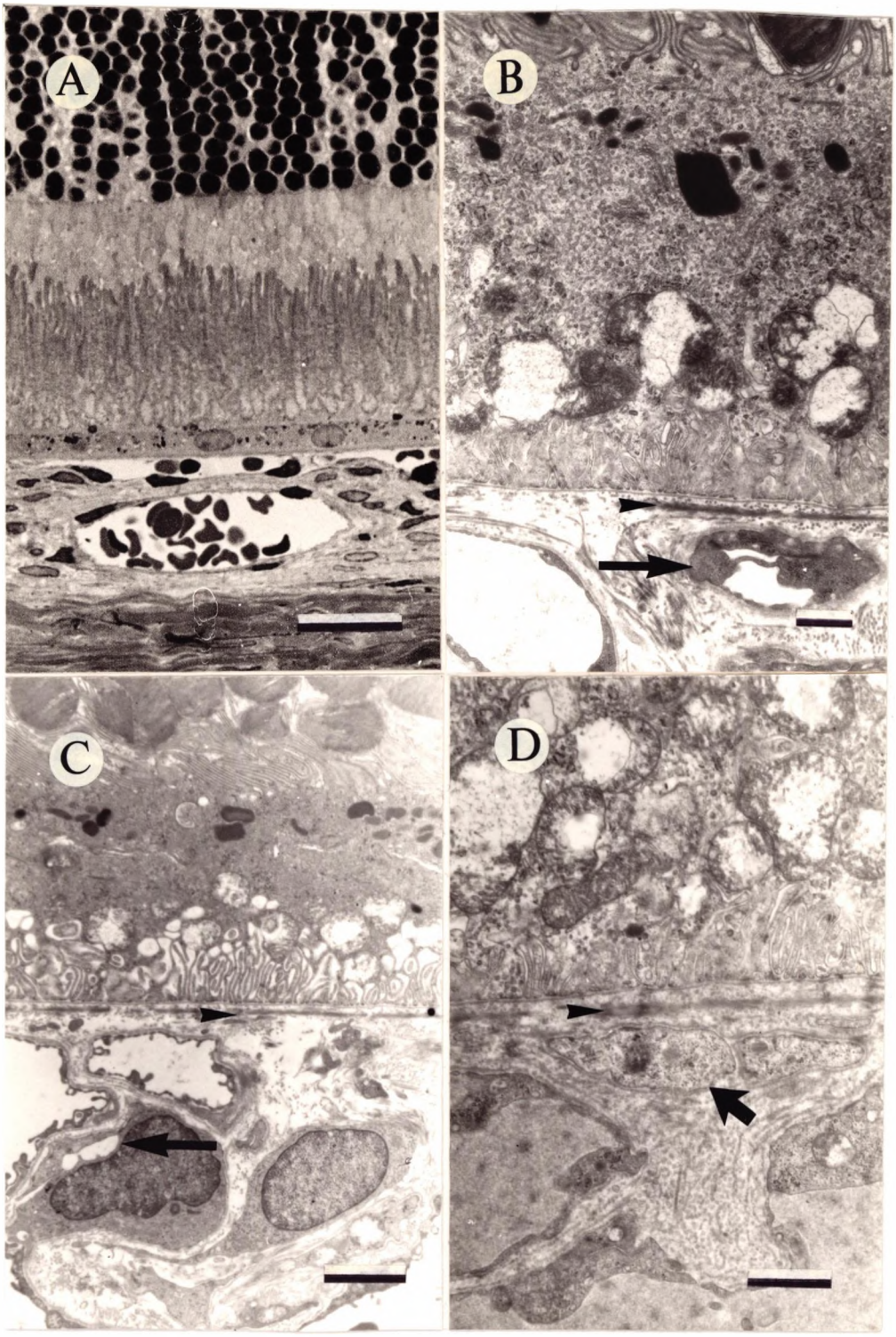


Fig. 45. Regeneration zone. Light microscopy of the outer retina showing vesicular alterations of photoreceptor outer segments and apparently normal appearance of the retinal pigment epithelium and choroid 48 hours after light damage (A). Electron microscopy revealed distended mitochondria in the retinal pigment epithelium and formation of new vessels (arrows) in the choriocapillaris in samples obtained 48 (C) and 72 (B) hours after light exposure. Some ghost vessels in the form of residual basement membranes (thick arrow) were occasionally present 144 hours after light damage (D). Bar (A) = 20 µm. Bars (B,C) = 1 µm. Bar (D) = 2 µm. Arrowheads = elastin layer of Bruch's membrane.

and swelling at the level of the basal infoldings (fig. 44). These changes were only observed 24 hours after light damage and imply a transient insufficiency of the blood retinal barrier at the level of retinal pigment epithelium. Oedema of the retinal pigment epithelium subsided, however, and was not observed at any other time points.

4.6.3 Other cell and tissue responses.

No other abnormalities in the inner and outer retina were recorded in this study. During the time of observation occasional formation of new vessels in the choriocapillaris was recorded (fig 45 B, C). Some dropout of capillaries was also present and was manifested as obliteration and fibrosis of the lumen (fig 45 D). No others changes in morphology of the Bruch's membrane, choriocapillaris and choroid were detected in the recovery area.

4.7 Agarose gel electrophoresis.

The extraction of the DNA was performed from the whole retinae which included both the recovery and the degeneration areas.

Fragmentation of the DNA into segments, which were multiplications of 180-200 base pairs of DNA consistent with apoptosis was observed immediately after light damage and was more pronounced 24 and 48 hours after damage. At the same time points random fragmentation of the DNA consistent with necrosis was also observed in the background. No DNA fragmentation, suggesting either apoptosis or necrosis was observed in controls and light damaged retinae 72 and 144 hours after light damage (fig. 46 and 47).

These results are consistent with the morphological observation of extensive cell death via apoptotic and necrotic pathways occurring immediately after light damage and lasting for 48 hours. By 72 hours the outer retinal layers are lost and the reparative processes in the remaining layers are well established.

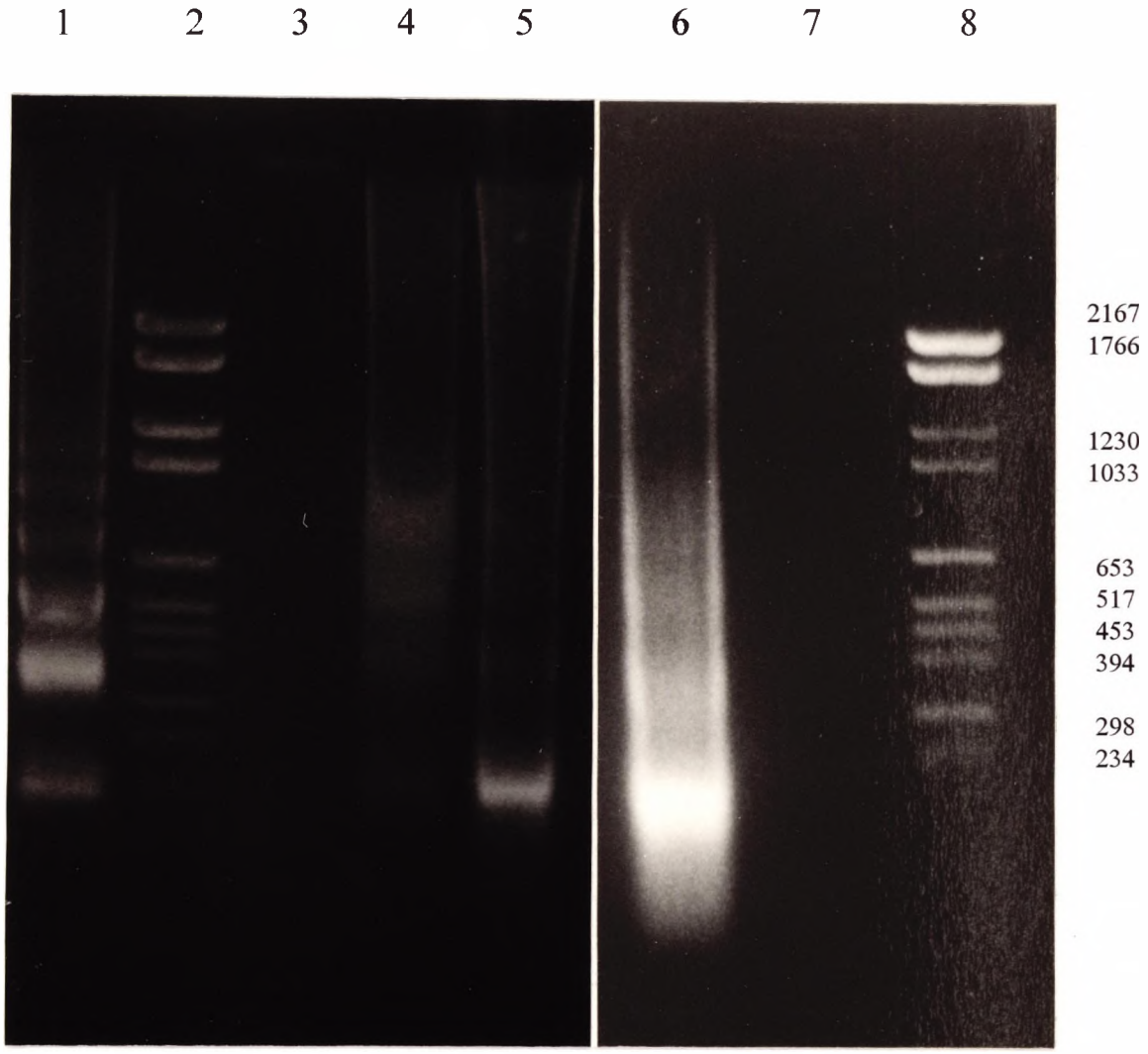


Fig 46. Agarose gel electrophoresis of fragmented DNA demonstrating a ladder pattern characteristic for apoptosis in light damaged retinae immediately after light exposure (band 4), 24 (band 5) and 48 (band 6) hours later. The gel was run for 45 min.

1; Positive apoptosis control (OVA-7 T Helper Cell Line, murine, strictly IL2-dependent, provided by Dr Michael Weller, Department of Internal Medicine, Division of Clinical Immunology, University Hospital Zurich, Switzerland), showing fragmentation of DNA resulting in the formation of a typical ladder pattern. 2 and 8; DNA Marker Ladder VI standard (DNA-Langenstandards VI, Boehringer Mannheim). 3; Dark adapted control showing no DNA fragmentation. 4; Light damaged retinae immediately after exposure show a smear of fragmented DNA (observed in necrosis) and formation of bands characteristic for apoptosis. 5; Light damaged retine 24 hours after light exposure showing bands of fragmented DNA (characteristic for apoptosis).

6; Light damaged retina 48 hours after light exposure showing bands of fragmented DNA characteristic for apoptosis and the DNA smear observed in necrosis. 7; Light damaged retina 72 hours after light exposure showing no DNA fragmentation.

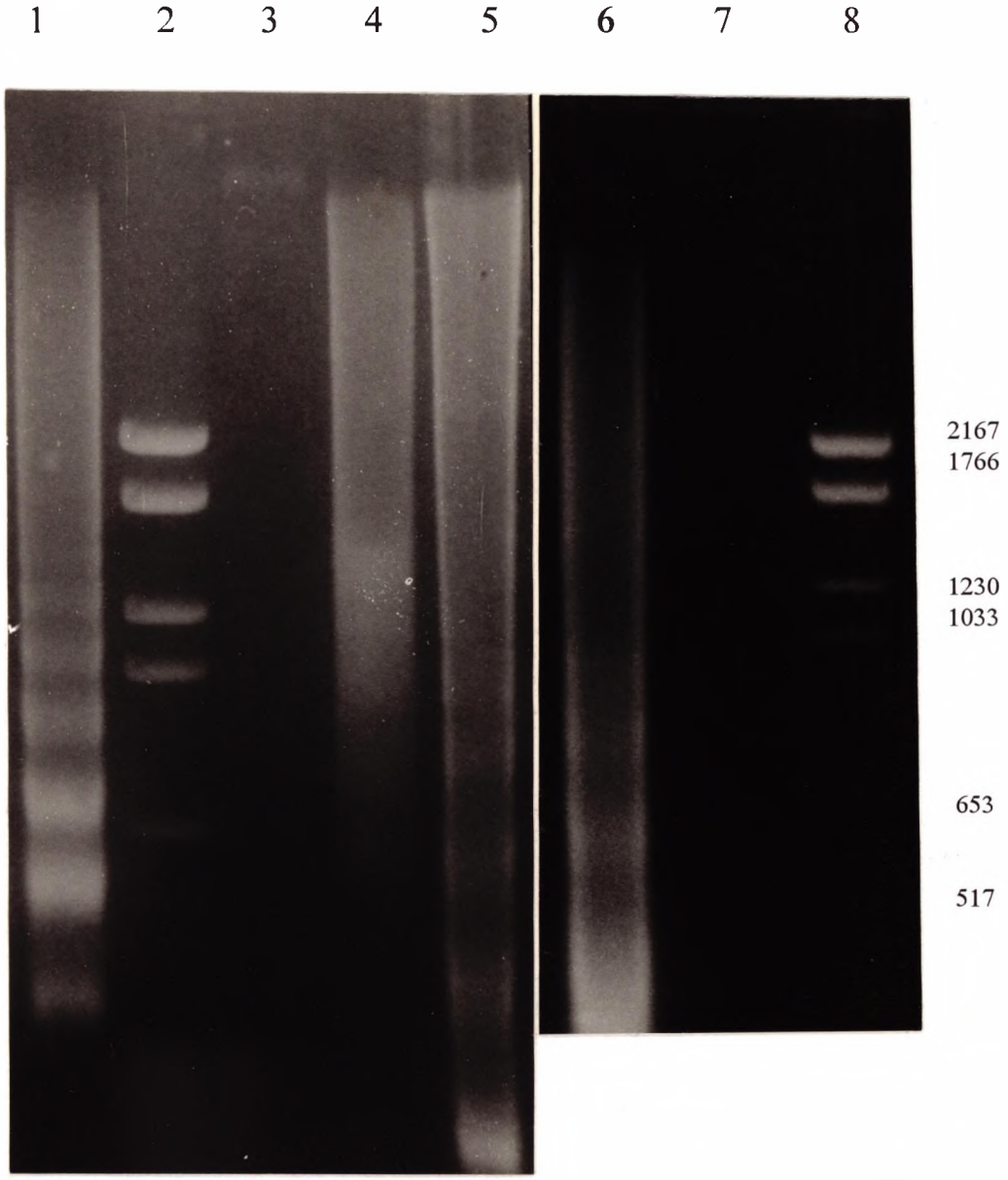


Fig 47. Agarose gel electrophoresis of fragmented DNA demonstrating a ladder pattern characteristic for apoptosis in light damaged retinae 24 (band 5) and 48 (band 6) hours after light exposure. Samples obtained immediately after light damage show a smear of fragmented DNA characteristic for necrosis. The gel was run for 90 min. 1. Positive apoptosis control (OVA-7 T Helper Cell Line, murine, strictly IL2-dependent, provided by Dr Michael Weller, Department of Internal Medicine, Division of Clinical Immunology, University Hospital Zurich, Switzerland), showing fragmentation of DNA resulting in formation of a typical ladder pattern. 2 and 8; DNA Marker Ladder VI standard (DNA-Langenstandards VI, Boehringer Mannheim). 3; Dark adapted control showing no DNA fragmentation. 4; Light damaged retinae immediately after exposure show a smear of fragmented DNA characteristic for necrosis. Bands typical of apoptosis seen in gel run for 45 minutes were not observed in a gel run for 90 minutes. 5; Light damaged retine 24 hours after light exposure showing bands of fragmented DNA characteristic for apoptosis. 6; Light damaged retina 48 hours after light exposure showing bands of fragmented DNA characteristic for apoptosis and DNA smear observed in necrosis. 7; Light damaged retina 72 hours after light exposure showing no DNA fragmentation.

4.8. Results of the comparative study of the influence of fixation with glutaraldehyde and high pressure freezing on morphology of rod outer segments.

4.8.1 Dark adapted photoreceptor cells.

Glutaraldehyde fixed rod outer segments (fig. 48 A, C) showed a regular arrangement of the stacked disk membranes. The plasma membrane was undulated and in close apposition with stacked disks. High pressure frozen rod outer segments (fig 48 B, D) also showed a very regular arrangement of the stacked disk membranes. The plasma membrane was less undulated and a space always appeared between plasma membrane and stacked disks with a width of approximately 50 nm which was filled with filamentous and fine granular material (asterisk, fig 48 D) The glutaraldehyde fixed region of the connective cilium showed a prominent calyx (arrow, fig 48 A). In high pressure frozen samples the calyx was much less pronounced and, therefore, often not in the plane of section (fig. 48 B). In the proximity of the connecting cilium of high pressure frozen samples (arrow, fig. 48 B) small disk-like structures were positioned very close to the microtubules. Membrane bound vesicles were also observed in that region. The cytoskeletal elements visible in

the glutaraldehyde fixed cilium such as microtubules and ciliary rootlets were also present in the high pressure frozen samples. In the synaptic region the morphological changes between glutaraldehyde and high pressure freezing fixation were consistent with what has been seen in the other parts of the cell. Membranes undulations, and some degree of swelling of the mitochondria were more accentuated in glutaraldehyde fixed samples (fig.50 A). In high pressure frozen material mitochondria were darker, more condensed and they contained fine dark granules. The membranes and cristae were in close contact and attached to each other (fig.50 B).

4.8.2 Light damaged photoreceptors.

Figure 49 and 50 (C, D) exemplifies reversible morphological alterations in light damaged photoreceptor cells. Rod outer segment disk membranes of glutaraldehyde fixed samples underwent extensive vesicular alterations and swelling (fig. 49 A, C) whereas the light damage in high pressure frozen rod outer segments was considerably less evident. Only some small vesicles were observed together with local irregular disruptions of membrane stacks (asterisk, fig. 49 B, D). The synaptic region of glutaraldehyde fixed light damaged cells (fig. 50 C) showed undulating

undulating membranes, mitochondria with swollen cristae and many small vacuoles in the cytoplasm. High pressure frozen light damaged synaptic regions (fig. 50 D), however showed smooth membranes. The outer and inner membrane of the mitochondria were in close contact, as well as the two membranes of the cristae giving a characteristic appearance of cryofixed mitochondria. The mitochondria contained numerous minute dark inclusions (the same features were also observed in mitochondria of high pressure frozen dark adapted samples, picture not shown). In contrast to dark adapted synapse regions, the mitochondria of light damaged synapse regions were often surrounded by Golgi cisternae (G, fig. 50 D) and exhibited various degrees of autophagy (arrow).

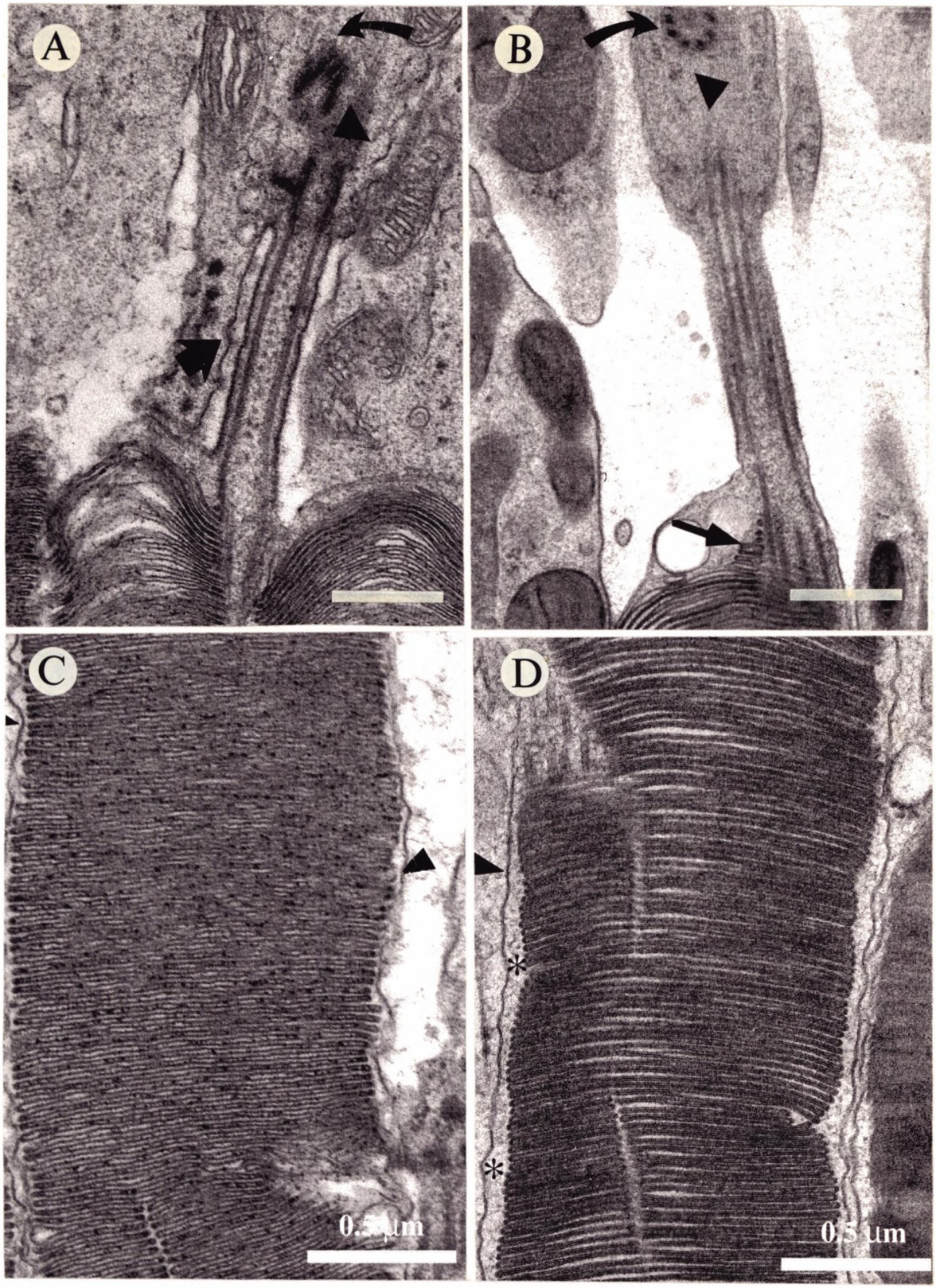


Fig. 48. Dark adapted retina. Ciliary region in glutaraldehyde fixed cell (A) and high pressure frozen fixed cell (B). The glutaraldehyde fixed sample shows a prominent extension of the inner segment in the form of calyx (thick arrow). The calyx is less pronounced after high pressure freezing and therefore is often not included in the plane of section (B). Small disks (arrow) are visible in the proximity of the ciliary microtubules in cryo-fixed cells (B) Centrioles are marked with bent arrows. Ciliary rootlets are marked with black triangles. The usual arrangement of disk membranes in the outer segment after glutaraldehyde fixation (C) and high pressure freezing fixation (D). The space between plasma membrane (marked with arrowheads) and disks is larger after high pressure freezing (black asterisk) (D). Bars = 0.5 µm.

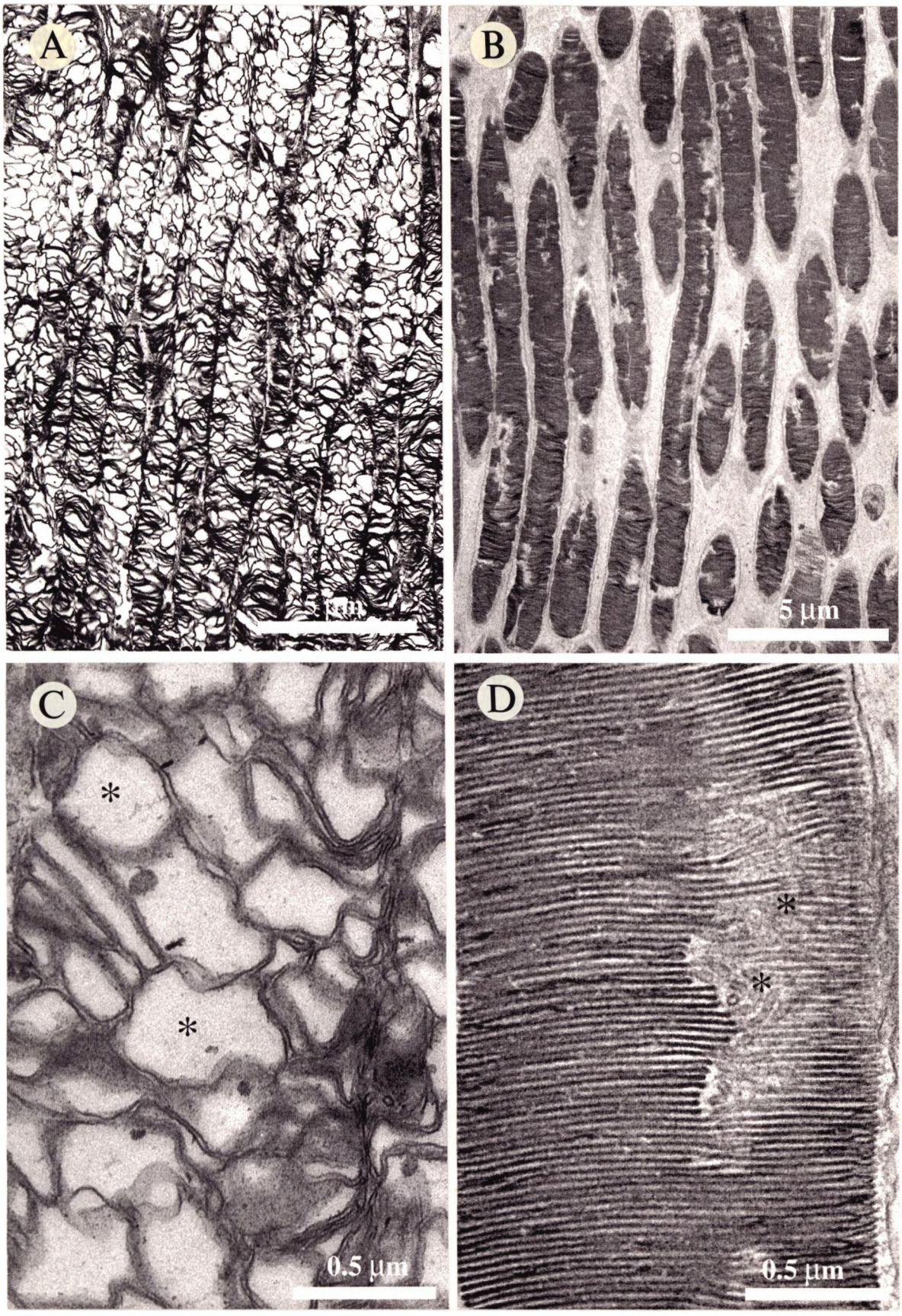


Fig. 49. Light damaged retina. Low magnification of rod outer segments, glutaraldehyde fixed sample (A) and high pressure freezing fixed sample (B). Higher magnification of glutaraldehyde fixed tissue (C) show extensive vesicular alterations of disk membranes (asterisks). High pressure frozen rod outer segments (D), however, show only minute disruptions of the disks and discrete vesicular and tubular alterations of membranes (asterisks). Bars = 0.5 µm.

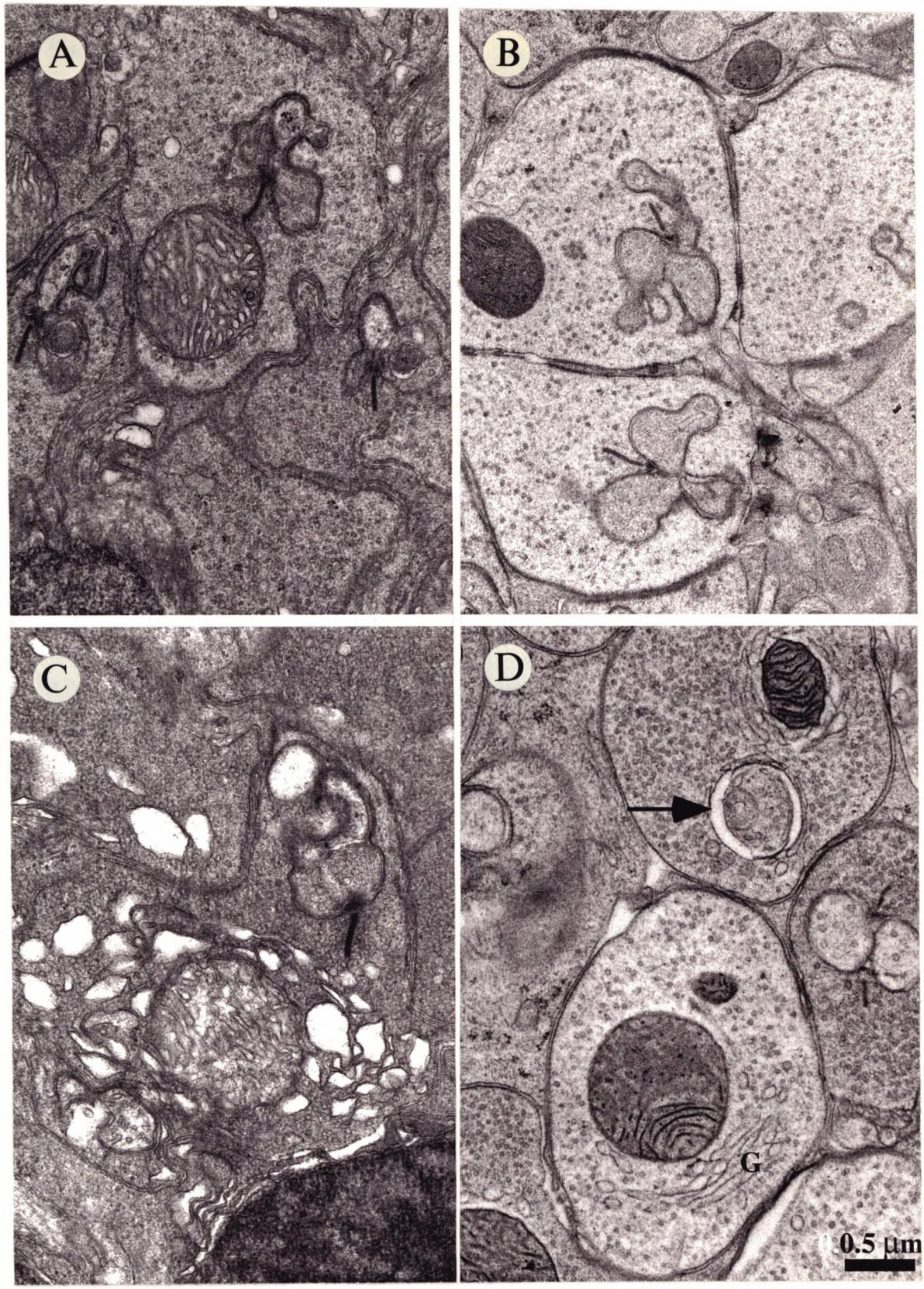


Fig. 50. Photoreceptor synaptic region of dark adapted retina after glutaraldehyde fixation (A) and after high pressure freezing (B). Photoreceptor synaptic region of light damaged retina (C, D). Vesicular changes are present in the cytoplasm surrounding the mitochondria in the photoreceptor synaptic region in the glutaraldehyde fixed sample (C). In cryo-fixed samples (D) mitochondria appear to be surrounded by Golgi compartments (marked with G) and some autophagic vacuoles are present (arrow).

4.9 Tabulated summary of the results and of the statistical evaluation.

A summary of the morphological changes in the degeneration area and their time sequence is presented schematically in figure 50.

The tabulated results of semiquantitative assessment of morphological changes in degeneration and recovery areas are presented in tables 4.8.1 A and B. Tabulated results of statistical analysis are presented in tables 4.8.2 - 4.8.10. The t-test was performed on the data obtained from the homogeneous group of animals exposed to the light from Philips TLD lamps. Each experimental group contained 6 animals.

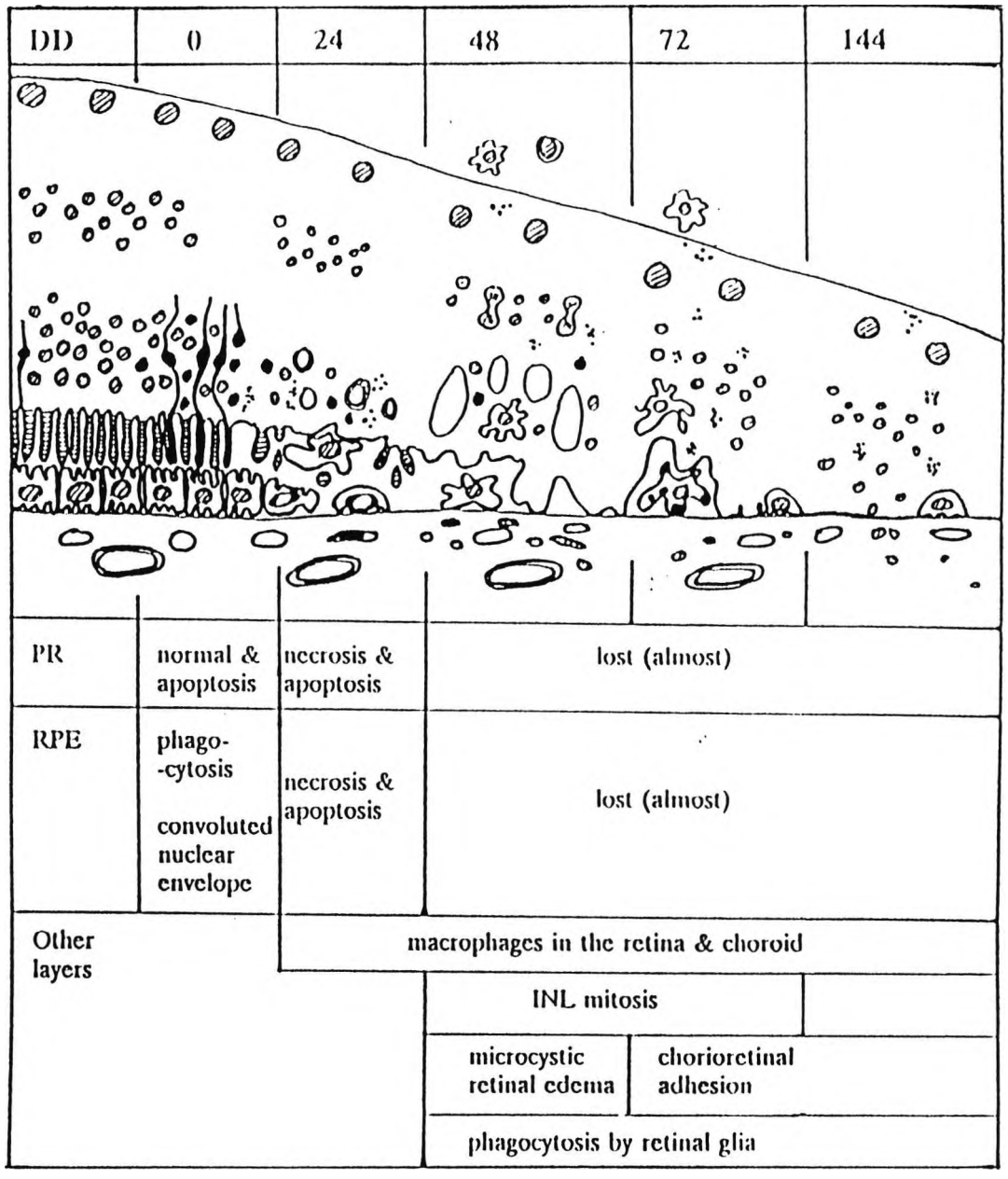


Fig. 51. Schematic drawing summarizing the time sequence of the most important morphological changes induced by light in the rat retina undergoing degeneration.
DD - control dark adapted retina.
0, 24, 48, 72 and 144 hours after light exposure.

Table 4.8.1 A

Degeneration zone. Tabulated results of a semiquantitative assessment of light microscopy sections. Table contains average values of 3 readings from each of the 11 experimental animals per each experimental group. Control group included 8 animals.

DEGENERTION	controls	0	24 hrs	48 hrs	72 hrs	144 hrs
ROS vesiculations	0	0.4	0.2	0	0	0
PR loss	0	1.9	1.6	1.9	1.9	1.5
RPE loss	0	0.1	1.2	1.6	2	1.5
SRD	0	0	0.5	1	0.7	0
CRE	0	0.05	0.09	1.1	0.2	0.5
macrophages	0	0	0.5	1.5	1.9	0.8
mitosis	0	0	0	1.7	1	0.05
scar	0	0	0	0.05	0.6	1.3
PR pyknosis	0.5	1.9	1.9	1.8	1.7	1
IS densification	0.5	2.8	1.8	1	0.7	0
RPE nuclear marg	0	0	2.2	1.4	1.5	0
PR nuclear marg	0	0	3	1.6	0.25	0
PR necrosis	0	1	3	2.6	0.5	0
RPE necrosis	0	0	0.5	2	0	0

Standard deviation	control	0std	24std	48std	72std	144std
ROS vesiculations	0	0.4	0.4	0	0	0
PR loss	0	0.3	0.6	0.4	0.3	0.7
RPE loss	0	0.3	1	0.8	0	0.9
SRD	0	0	0.6	1	0.8	0
CRE	0	0.15	0.2	0.9	0.3	0.8
macrophages	0	0	0.7	0.8	0.3	0.6
mitosis	0	0	0	0.6	1	0.14
scar	0	0	0	0.1	0.3	0.9
PR pyknosis	0	0.3	0.4	0.4	0.5	0.5
IS densification	0	0.4	0.7	0	0.5	0
RPE nuclear marg	0	0	1.2	0.8	0.5	0
PR nuclear marg	0	0	0	0.5	0.24	0
PR necrosis	0	0	0	0.5	0.5	0
RPE necrosis	0	0	0	0	0	0

ROS - rod outer segments
PR - photoreceptors
RPE - retinal pigment epithelium
SRD - shallow retinal detachment
CRE - cystoid retinal oedema
IS - photoreceptor inner segments
std - standard deviation
hrs - hours

Table 4.8.1.B
Regeneration zone. Tabulated results of a semiquantitative assessment of light microscopy sections. Table contains average values of 3 readings from each of the 11 experimental animals per each experimental group. Control group included 8 animals.

REGENERATION	controls	0	24 hrs	48 hrs	72 hrs	144 hrs
ROS vesiculations	0	1.45	1.6	1.2	0.6	0
PR loss	0	1.1	0.6	0.3	0.4	0.4
RPE loss	0	0.05	0	0	0.1	0.2
SRD	0	0	0	0	0	0
CRE	0	0.05	0	0	0	0.3
macrophages	0	0	0	0	0	0.2
mitosis	0	0	0	0	0	0
scar	0	0	0	0	0	0.4
PR pyknosis	0.5	1.2	0.8	1	0.8	0.4
IS densification	0.5	0.55	0.32	0.23	0.14	0.14
RPE nuclear marg	0	0	0	0	0	0
PR nuclear marg	0	0	1	0.8	0.4	0
PR necrosis	0	0	0	0	0	0
RPE necrosis	0	0	0	0	0	0

Standard deviation	ddstd	0std	24std	48std	72std	144std
ROS vesiculations	0	0.7	0.5	0.6	0.6	0
PR loss	0.2	0.5	0.4	0.2	0.4	0.8
RPE loss	0	0.15	0	0	0.2	0.6
SRD	0	0	0	0	0.1	0
CRE	0	0.15	0	0	0	0.6
macrophages	0	0	0	0	0	0.4
mitosis	0	0	0	0	0	0
scar	0	0	0	0	0	0.8
PR pyknosis	0	0.4	0.6	0	0.3	0.5
IS densification	0	0.42	0.24	0.33	0.22	0
RPE nuclear marg	0	0	0	0	0	0
PR nuclear marg	0	0	0	0.24	0.37	0
PR necrosis	0	0	0	0	0	0
RPE necrosis	0	0	0	0	0	0

ROS - rod outer segments
PR - photoreceptors
RPE - retinal pigment epithelium
SRD - shallow retinal detachment
CRE - cystoid retinal oedema
IS - photoreceptor inner segments
std - standard deviation
hrs - hours

Table 4.8.2

Outer Segment Vesiculations
t-Test: Paired Two Samples for Means

	Degeneration	Recovery
Mean	0.1	0.8
Variance	0.03	0.5
Observations	6	6
Pearson Correlation	0.7	
Pooled Variance	0.08	
Hypothesized Mean Difference	0	
df	5	
t	-2.8	
P(T<t) one-tail	0.02	
t Critical one-tail	2.02	
P(T<t) two-tail	0.04	
t Critical two-tail	2.57	

Table 4.8.3

Photoreceptor Loss
t-Test: Paired Two Samples for Means

	Degeneration	Recovery
Mean	1.47	0.47
Variance	0.55	0.13
Observations	6	6
Pearson Correlation	0.64	
Pooled Variance	0.17	
Hypothesized Mean Difference	0	
df	5	
t	4.25	
P(T<t) one-tail	0.004	
t Critical one-tail	2.02	
P(T<t) two-tail	0.008	
t Critical two-tail	2.57	

Table 4.8.4

Retinal Pigment Epithelium Loss
t-Test: Paired Two Samples for Means

	Degeneration	Recovery
Mean	1.07	0.06
Variance	0.7	0.006
Observations	6	6
Pearson Correlation	0.4	
Pooled Variance	0.03	
Hypothesized Mean Difference	0	
df	5	
t	3.09	
P(T<t) one-tail	0.01	
t Critical one-tail	2.02	
P(T<t) two-tail	0.03	
t Critical two-tail	2.57	

Table 4.8.5

Serous Retinal Detachment
t-Test: Paired Two Samples for Means

	Degeneration	Recovery
Mean	0.4	0
Variance	0.19	0
Observations	6	6
Pearson Correlation	#div/0	
Pooled Variance	0	
Hypothesized Mean Difference	0	
df	5	
t	2.08	
P(T<t) one-tail	0.05	
t Critical one-tail	2.02	
P(T<t) two-tail	0.09	
t Critical two-tail	2.57	

Table 4.8.6

Cystoid Macular Oedema
t-Test: Paired Two Samples for Means

	Degeneration	Recovery
Mean	0.3	0.06
Variance	0.18	0.01
Observations	6	6
Pearson Correlation	0.16	
Pooled Variance	0.008	
Hypothesized Mean Difference	0	
df	5	
t	1.55	
P(T<t) one-tail	0.09	
t Critical one-tail	2.02	
P(T<t) two-tail	0.18	
t Critical two-tail	2.57	

Table 4.8.7

Macrophage Influx
t-Test: Paired Two Samples for Means

	Degeneration	Recovery
Mean	0.78	0.03
Variance	0.61	0.007
Observations	6	6
Pearson Correlation	0.01	
Pooled Variance	0.0007	
Hypothesized Mean Difference	0	
df	5	
t	2.34	
P(T<t) one-tail	0.03	
t Critical one-tail	2.02	
P(T<t) two-tail	0.07	
t Critical two-tail	2.57	

Table 4.8.8

Mitotic Activity
t-Test: Paired Two Samples for Means

	Degeneration	Recovery
Mean	0.46	0
Variance	0.53	0
Observations	6	6
Pearson Correlation	#div/0	
Pooled Variance	0	
Hypothesized Mean Difference	0	
df	5	
t	1.54	
P(T<t) one-tail	0.09	
t Critical one-tail	2.02	
P(T<t) two-tail	0.18	
t Critical two-tail	2.57	

Table 4.8.9

Scar Formation
t-Test: Paired Two Samples for Means

	Degeneration	Recovery
Mean	0.33	0.07
Variance	0.28	0.03
Observations	6	6
Pearson Correlation	0.9	
Pooled Variance	0.08	
Hypothesized Mean Difference	0	
df	5	
t	1.61	
P(T<t) one-tail	0.08	
t Critical one-tail	2.02	
P(T<t) two-tail	0.17	
t Critical two-tail	2.57	

Table 4.8.10

Photoreceptor Pyknosis
t-Test: Paired Two Samples for Means

	Degeneration	Recovery
Mean	1.5	0.8
Variance	0.34	0.09
Observations	6	6
Pearson Correlation	0.82	
Pooled Variance	0.14	
Hypothesized Mean Difference	0	
df	5	
t	4.45	
P(T<t) one-tail	0.0034	
t Critical one-tail	2.02	
P(T<t) two-tail	0.0067	
t Critical two-tail	2.57	

5 Discussion.

This discussion of light damage in the retina is divided into a number of parts. In the first part acute morphological changes in photoreceptors are discussed with separate sections for reversible and irreversible changes including comment on the various forms of cell death observed in this study (chapters 5.1-5.3). In the second part the question of the morphological recovery of the outer retina is discussed with emphasis on the structural recovery of the outer segment membranes (chapter 5.4). Other morphological findings including inflammation, proliferation and choroidal changes are discussed in chapter 5.5. The discussion of effects of chemical and cryofixation on the morphology of light damage in photoreceptors is covered in the final chapter 5.6.

This study investigated the pattern of cell death in light damaged rat retinae. It demonstrated that a low dose of light (1000 lux) applied over a short period of time (120 minutes) induces regional degeneration of the retina in male Sprang-Dawley rats. The degeneration zone was located in the lower temporal part of the fundus. In the areas in which the outer retinal layers were irreversibly damaged apoptosis of photoreceptors was observed immediately after light exposure. Within the next 24 hours

necrosis of photoreceptors was observed. The retinal pigment epithelium, which appeared normal immediately after light exposure, showed a delayed response. The pattern of cell death in the retinal pigment epithelium was, however, the same as in the photoreceptors. Cell death occurred 24 hours after light exposure and exhibited features of apoptosis. Samples collected 48 hours after light damage showed that necrosis had followed the initial apoptotic changes. In the degeneration zone breakdown of the blood retinal barrier, activation of macrophages, cell proliferation and scar formation were found.

In the retinal area, located nasally to the degeneration zone, which did not develop retinal degeneration, structural alterations in the arrangements of rod outer segment disk membranes were observed immediately after light exposure. The vesiculation of membranes was most extensive in the area of the outer segment tips. Morphological recovery was observed within a week of light exposure. In additional experiments it was demonstrated that vesiculation and blebbing of disk membranes induced by light was predominantly associated with glutaraldehyde fixation. Such changes were not found when the retinae were cryofixed. It was concluded, therefore, that the vesicular alterations of rod outer segments in outer segment membranes reversibly damaged by light are fixation artifacts.

5.1 Regional differences in the morphology of light damage.

The fate of the retina after light irradiation, either degeneration or recovery, varied with the region of the eye. Samples from the lower temporal quadrant were characterized by extensive and immediate apoptosis of photoreceptors and developed irreversible retinal degeneration. Samples from the lower nasal quadrant, which showed pronounced vesiculation of photoreceptor outer segments immediately after the exposure recovered normal retinal morphology within one week of the injury.

The regional differences in retinal response to light injury have already been observed in the rat. However, the superior retina was reported to be more prone to light damage than the inferior part. Williams and his co-workers have done extensive studies on the "sensitive area" of the rat retina, situated in the upper temporal quadrant (Bush and Williams 1991; Penn and Williams 1986; Rapp and Williams 1980). These authors concluded that the superior temporal area was more susceptible to light damage due to anatomical (longer rod outer segments), biochemical (more rhodopsin) and neurochemical (more dopaminergic neurons) properties. Their illumination system provided equal illuminance levels within the cage of the animal, but their studies extended the light

exposure over days and / or weeks. In other light damage studies, the superior temporal retina was also most severely affected (Lam et al. 1990; Li et al. 1985) but the position of the light source was not specified. In this study an alternative and well established light damage set-up was used (Reme et al. 1988, Reme et al. 1991, Reme et al. 1992, Reme et al. 1994). The light was delivered from the ceiling and it was also reflected by the interiors of the cages. In addition exposure to light was brief. A direct comparison between these studies using different light delivery systems and various exposure times sometimes extending over days or weeks may therefore be misleading. Also different light sources may emit different spectral energies and cause different retinal irradiance despite the apparent similar photometric value given in luxes. Even in this study a 30% difference in power output was observed for different neon lamps tested despite the fact that their illuminance was the same and equal to 1000 lux. The severe and irreversible lesions in the lower temporal retina may reflect higher irradiance levels in this area. Further studies are needed to explain the regional differences in retinal responses to light. In experiments in which the light dose was increased beyond the levels applied in this study, the nasal area also showed irreversible lesions (unpublished observations). Recently it has been demonstrated in the same animal model that after increasing the

illuminance levels up to 3000 lux, photoreceptor apoptosis was induced in the entire fundus within 30 to 60 minutes after the exposure (Hafezi et al 1995).

5.2 Acute reversible photoreceptor changes.

A distinct feature of early, reversible photoreceptor changes was the vesicular alterations of the rod outer segments (fig. 40 B, 45 A, 49) in the nasal portion of the retina which recovered normal morphology within a week. Vesicular alterations of the rod outer segments, shown schematically in figure 52, have been described in early light damage studies (Grignolo et al. 1969; Kuwabara and Gron 1968). They preceded photoreceptor degeneration in some (Grignolo et al. 1969; Kuwabara and Gron 1968) but not all of these studies (Kuwabara and Funahashi 1976a; LaVail 1976; Moriya et al. 1986). Vesicular and tubular alterations of photoreceptor outer segments have also been observed across the species during development (De Robertis 1960; Yamada and Ishikawa 1965). Similar changes are seen in ageing photoreceptors as well as photoreceptors in which damage is induced by thermal and photochemical mechanisms, suggesting that vesicular degeneration of outer segment membranes is a process common to all compromised

photoreceptor cells (Marshall 1984). In a limited number of histopathological studies of chemically fixed eyes with solar retinopathy, vesicular and tubular alterations and fragmentation of photoreceptor outer segments have been consistently observed and coexisted with other photoreceptor and retinal pigment epithelium pathologies (Hope-Ross et al. 1993; Tso 1988; Tso and LaPiana 1975). Although the prognosis for patients with solar retinopathy is good, central scotomas frequently persist (MacFaul 1969) suggesting recovery in general but with some of the retina irreversibly damaged. It has recently been reported that vesicular changes in the membranes of photoreceptor outer segments observed in light damage experiments in rats are produced during glutaraldehyde fixation (Szczesny et al. 1993b, Walther et al. 1993). Further details of the influence of fixation on photoreceptor ultrastructure are discussed in section 5.6 "Effects of fixation".

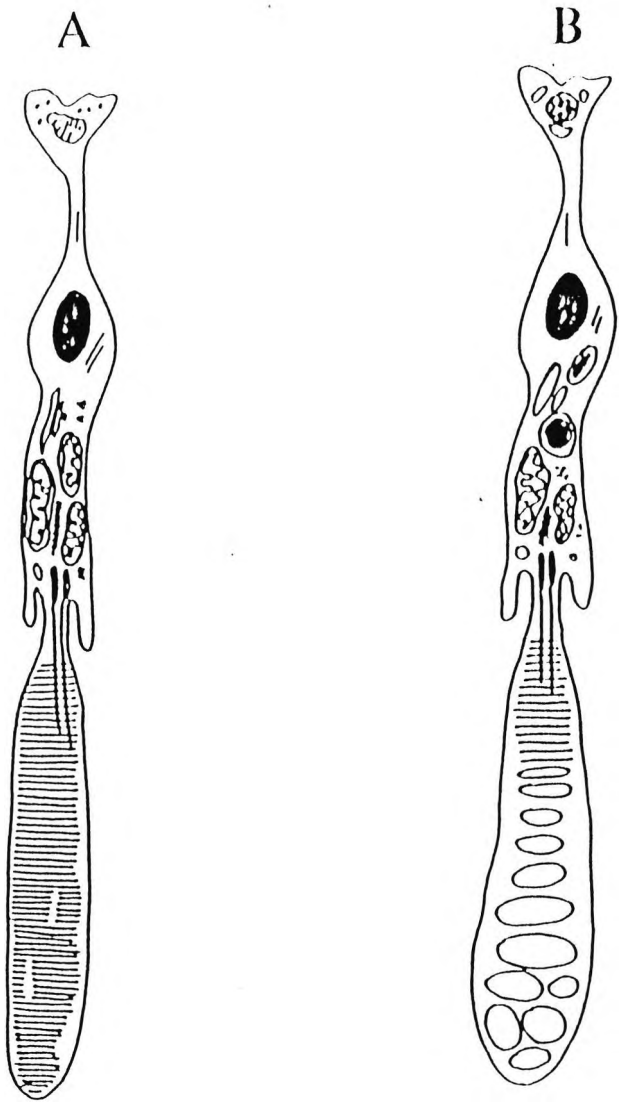


Fig. 52. Schematic drawing of normal (A) and light damaged (B) rod outer segments. Vesicular alterations of rod outer segment membranes in light damaged retinae were found to be reversible and are enhanced during fixation in glutaraldehyde.

5.3 Acute irreversible photoreceptor changes.

5.3.1 Apoptosis.

In the temporal retina, which showed irreversible damage, acute lesions consisted of dark, condensed photoreceptor cells with pyknotic nuclei which were isolated and separated by either unaffected or by necrotic cells. The cytoplasmic organelles within the condensed cells were recognizable and presented a relatively regular structure. At later stages phagocytosis of dark stained material was seen in the Muller cells. The morphology of the dying cells and the tissue response to this process were characteristic for apoptosis.

The occurrence of apoptosis in the light damaged retinal samples analyzed in this study was also confirmed by the presence of internucleosomal cleavage of DNA into fragments of approximately 200 base pairs which is a commonly used biochemical marker for apoptosis (for reviews see Bowen 1993, Compton 1992, Schwartzman and Cidlowski 1993). Agarose gel electrophoresis of the DNA extracts revealed fragmentation of DNA into larger fragments forming a ladder pattern consisting of bands which were multiplications of 200 base pair fragments. Fragmentation of DNA was present immediately after light

exposure, being more marked after 24 hours, still present after 48 hours but not observed 72 hours after light damage. DNA fragmentation was not observed in dark adapted controls. In all the samples which displayed the biochemical features of apoptosis a smear of randomly fragmented DNA consistent with necrosis was present in the background. Artificial DNA fragmentation, a result of tissue processing, was not observed in the controls and 72 hours after light damage histological examination confirmed that not only apoptosis but also necrosis were found in the outer retina.

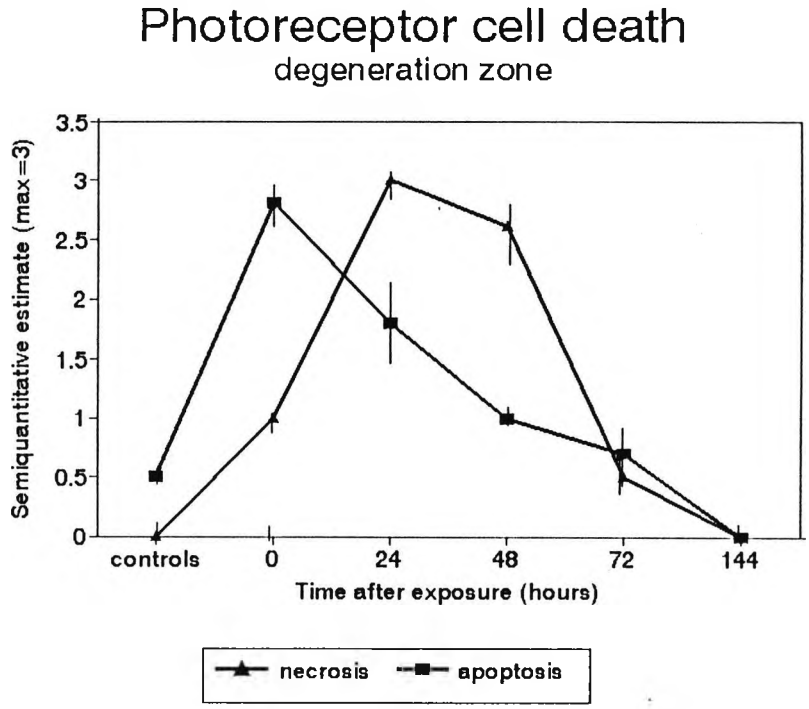
It is of interest that histological examination revealed extensive apoptosis, manifested as densification and shrinkage of photoreceptors immediately after light damage. The ladder pattern of DNA fragments was, however, most clear in samples obtained 24 hours later when apoptosis of photoreceptors was manifested histologically as nuclear margination (fig. 13, 25 D) and widespread necrosis of the inner segments was present (fig. 29). At the same time apoptotic changes in the retinal pigment epithelium were also present (fig. 12 and 32 D). The time of onset of apoptosis in photoreceptor cells and the retinal pigment epithelium was different. A 24 hours delay between the peaks for apoptosis and necrosis was constant for both groups of cells suggesting

an evolution of changes (fig. 53). The issue of coexistence of various forms of cell death is discussed in section 5.4.1.

5.3.1.1. Agarose gel electrophoresis of the DNA extracted from rat retinae.

Apoptosis can be detected in the cells by using either morphological or biochemical criteria. Cell shrinkage, condensation, nuclear chromatin densification and margination have been observed in this study. Other morphological features like fragmentation of cells and blebbing were not seen in photoreceptors but the dense apoptotic bodies, which were the final result of cell fragmentation were frequently observed in the light damaged retinae.

The most frequently applied biochemical method of detection of apoptotic cell death in the tissue is demonstration of characteristic DNA fragmentation. Such fragments have the length of approximately 200 base pairs or a multiplication of this value and are most commonly demonstrated in an agarose gel electrophoresis of the DNA extracts. Other methods are also known and are mentioned in chapter 5.3.1.3. Agarose gel electrophoresis is well established and reliable and was chosen to confirm biochemically the existence of apoptosis in this study.



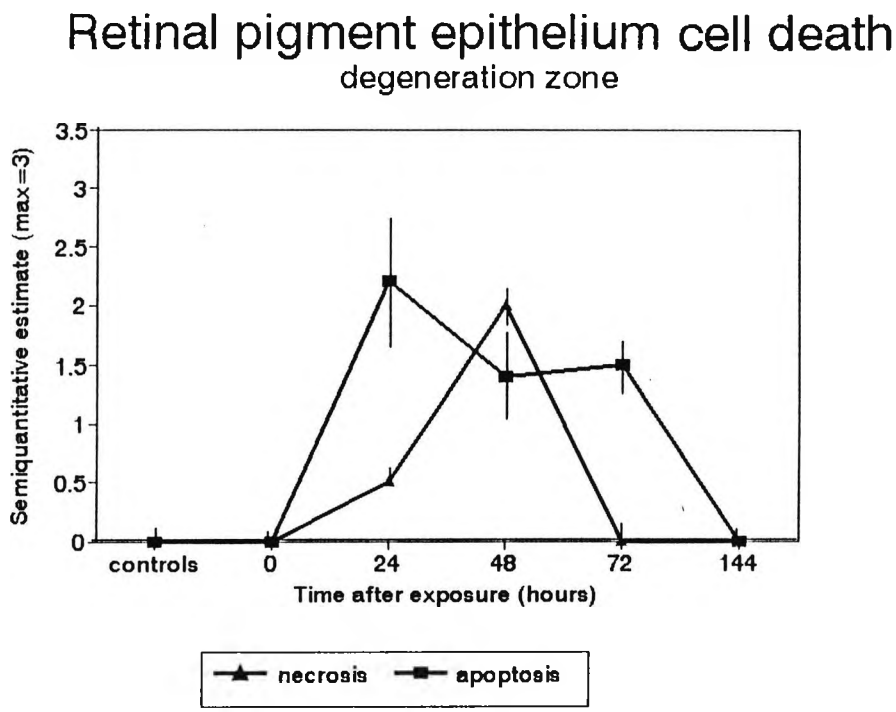


Fig. 53. Apoptosis and necrosis in photoreceptors (A) and retinal pigment epithelium (B).

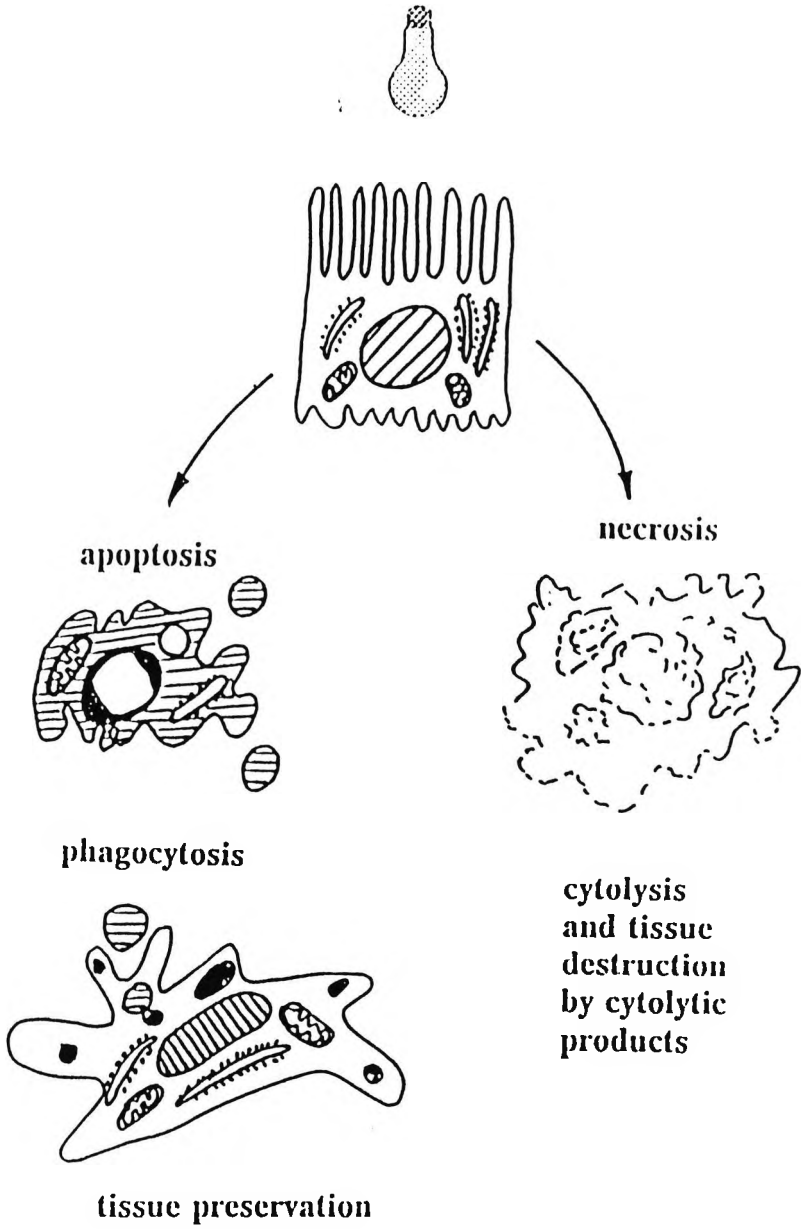


Fig. 54. Schematic drawing of the retinal pigment epithelial cell undergoing either apoptosis or necrosis.

For a single DNA sample for agarose gel electrophoresis 5 complete retinae from the given time point after light exposure were combined for the DNA extraction. Such an approach was taken in order to obtain enough DNA to give a signal during electrophoresis. Apart from the control retinae from the animals which were not exposed, each exposed retinae contained both degeneration and recovery areas. It is argued that in the recovery areas neither apoptosis nor necrosis was observed on histology in the fellow eyes and therefore no artefactual DNA fragmentation from the recovery areas was expected during agarose gel electrophoresis. Another reason for not separating the recovery and degeneration areas for the DNA extractions was the technical difficulty. It was not possible to dissect precisely and isolate only the degeneration areas after a retina was harvested with a technique commonly applied for biochemical studies in the rat retina (Delmelle et al 1975) and stored in liquid nitrogen before DNA extraction.

5.3.1.2 Apoptosis in the retina.

A review of the literature reveals pictures of photoreceptors undergoing apoptosis in a number of earlier reports on light damage, although the authors did not comment on the pattern of cell death or on the

significance of such dark condensed cells in retinal degenerations (Lam et al. 1990; Li et al. 1985; Moriya et al. 1986; Organisciak et al. 1992; Reme et al. 1988). Recently it has been suggested that such morphological changes in photoreceptors, namely shrinkage, condensation and pyknosis, may be the result of apoptosis (Shahinfar et al. 1991), a genetically controlled and programmed mechanism of cell death distinctly different from necrosis (Kerr et al. 1972; Wyllie et al. 1980). Apoptosis in the retina following injury has been recently described in ischemia - reperfusion injury (Buchi 1992) and has been known to occur during retinal development (Penfold and Provis 1986; Young 1984). It is interesting that in this study, apart from the photoreceptors which showed apoptosis immediately after light damage, the retinal pigment epithelial cells revealed features of apoptotosis within 24 hours after the observed photoreceptor alterations. The appearance of changes in the retinal pigment epithelium, shown schematically in figure 54, was as in the photoreceptors, consistent with the apoptotic cell death that has been observed in various tissues during embryogenesis and tissue remodelling, tumour regression or after exogenous induction (Drewett et al. 1993; Kerr et al. 1972; Tilly et al. 1992; Wyllie et al. 1980). It is of importance to mention that apoptosis has also been linked with inherited retinal degenerations. Degenerative changes in retinitis

pigmentosa may result from apoptotic cell death (Jones et al. 1992, Szczesny 1995). Recent studies of the pattern of cell death in inherited retinal degenerations in animal models demonstrate that apoptosis has been a common pathway of photoreceptor elimination in rd, rds and rhodopsin mutant mice (Chang et all. 1993). Apoptosis has also been demonstrated as a mode of photoreceptor cell death in inherited retinal dystrophy of RCS rat (Tso et al. 1994). The critical question to ask is, which cellular mediators regulate apoptotic cell death in the retina and at which stages do these regulatory mechanisms become a common pathway for inherited and acquired degenerations? It is of paramount importance to understand such mechanisms since if apoptosis is the initial process leading to retinal degeneration irrespective of the cause, it may be a possible target for pharmacological intervention.

5.3.1.3 Apoptosis in the eye.

Although the literature on apoptosis in the eye was rather limited when the experiments for this thesis were conducted, in the last 2 years during the Association for Research in Vision and Ophthalmology Annual Meetings (ARVO 1994 and 1995) apoptosis received a lot of attention. It is becoming clear that it is a widespread phenomenon associated with

development and disease. Programmed cell death has been linked for the first time with some of the most common ocular pathologies. Apoptosis, studied in various animal models of human ocular diseases, was found in the pathogenesis of retinal degenerations, both inherited (Jones et al. 1994, Adler et al. 1994, Papermaster et al. 1994, Wong et al 1994, Szczesny 1995) and acquired. The later included light induced degenerations (Li et al. 1994, Abler et al. 1994, S-H Li et al. 1994, Chang et al. 1994, Szczesny et al. 1994) and those resulting from lead poisoning (Fox 1994), aging (Fan et al 1995), retinal detachment (Chang et al 1995, Cook et al 1995, Wilson et al 1995). Apoptosis was also observed in corneal injury and healing (Zagorski and Szczesny 1994, Arar et al. 1994, Wise et al. 1994, Ishizaki et al 1995), has been linked with the pathogenesis of cataract formation of both the inherited (Vrensen and Graw 1994) and the acquired (Li et al. 1994, Li et al 1995) forms. Apoptotic cell death was also reported in the ganglion cells in an animal model of glaucoma and after optic nerve transection (Quigley et al 1994) and transient increase of the intraocular pressure (Li et al 1995). The most common techniques used to detect apoptosis were light and electron microscopy (Li et al. 1994, Abler et al. 1994, S-H Li et al. 1994, Chang et al. 1994, Papermaster et al. 1994, Zagorski and Szczesny 1994, Szczesny et al. 1994). The TUNEL technique, based on specific

labelling of nuclear DNA fragmentation in situ, introduced by Gavrieli (Gavrieli et al. 1992) and agarose gel electrophoresis of fragmented DNA has been frequently used to detect apoptosis (Li et al. 1994, Abler et al. 1994, S-H Li et al. 1994, Chang et al. 1994, Quigley et al. 1994). More sophisticated techniques to detect apoptosis have involved studies of apoptotic gene expression by either detecting the DNA of the TRPM-2/Clusterin gene (Wong et al. 1994) or the specific mRNA for c-fos gene (W-C Li et al. 1994) or clusterin (Jones et al. 1994). In some cases commercially available antibodies for proteins which occur in the cell during apoptosis were used (Arar et al. 1994).

5.3.1.4 Therapeutic implications of programmed cell death.

Numerous mechanisms involved in apoptotic cell death have been studied. In addition to the expression of apoptotic genes and the protein synthesis mentioned above, activation of endonuclease and the role of calcium (S-H Li et al. 1994) and the protein kinase C pathway have also been investigated (Chang et al. 1994). Apoptosis has been blocked to some extent by cycloheximide which is a protein synthesis inhibitor (Shahinfar et al 1991, S-H Li et al. 1994), by aurintricarboxylic acid which is an endonuclease inhibitor (Chang et al. 1994), phorbol myristate

acetate, a protein kinase C activator (Chang et al. 1994), and flunarizine, a calcium channel blocker (S-H Li et al. 1994), which has already been shown in previous studies to reduce light damage (Edward et al 1991). These have been the first encouraging trials on animal models adding to our knowledge of the known mechanisms controlling apoptosis in retinal degenerations and give some hope for new therapeutic approaches in the future.

5.3.1.5 Evolution of the terms "Apoptosis" and "Programmed cell death".

In recent years the two terms "apoptosis" and "programmed cell death" have been used interchangeably to describe cell death as distinct from necrosis. However, there is an opinion that "apoptosis" and "programmed cell death" are two distinct processes. Martin and his collaborators argue that the term "programmed cell death" is a functional term, used to describe cell death that is a normal part of the life of a multicellular organism (Martin et al 1994). Apoptosis is a descriptive term introduced by Kerr and colleagues to characterize a type of cell death showing a distinct set of morphological features (Kerr et al 1972). The word apoptosis (αποπτοσισ) is derived from the two Greek words apo (απο)

meaning off and ptosis (πτοσισ) meaning falling. The word describes leaves falling from trees. Martin and his colleagues are of the opinion that although in many instances apoptosis may represent programmed cell deaths it can not be said that all programmed cell deaths take the form of apoptosis.

Cell death showing the morphological and biochemical features of apoptosis can be induced by wide range of stimuli and drugs and has been reviewed in the introduction. Such instances of cell death are not programmed as in the developmental sense but represent the cell's responses to lethal stimuli from the environment.

In both programmed cell death, as during embryonic development, and in apoptosis, as a response to lethal injury, a specific and clearly defined, genetically controlled programme leading to cell elimination is activated. The execution of such a programme by the cells to be eliminated depends on the energy supply, the activation of endonuclease, protein synthesis, the expression of newly formed cell membrane antigens and characteristic structural changes in the cell. It is in this sense that apoptosis can be understood as programmed cell death as opposed to necrosis.

5.3.2 Necrosis.

Necrosis can be defined as a process which involves progressive derangements in energy and substrate metabolism (Hillis and Braunwald 1977) as opposed to apoptosis which is genetically encoded.

Among the scattered pyknotic nuclei and the shrunken, condensed cell bodies of photoreceptors observed immediately after light exposure and consistent with apoptotic cell death, numerous cells showed features of necrosis, especially when the outer nuclear layer was examined. Karyolysis was also observed immediately after light exposure although only in a small number of cells. Within 24 hours, however, necrotic, lytic changes in the inner segments of photoreceptor cells with relatively well preserved outer segments dominated the picture. A similar 24 hours shift was noticed in the pattern of cell death in the retinal pigment epithelium. It was interesting to note that apoptosis of the retinal pigment epithelium was observed 24 hours after light injury and not immediately after it as in photoreceptors. Once it had occurred it was followed within the next 24 hours by necrosis in a similar pattern to the photoreceptors. Therefore the same pattern of cell death for both photoreceptors and retinal pigment epithelium and the same time scale were observed, but with the different times of onset after injury. It may be speculated that

photoreceptor cells are directly damaged during light exposure and the retinal pigment epithelium cell death is a response to an accumulation of toxic products resulting from that injury. This topic is further discussed in section 5.3.3 " Lysosomotropic cell death" below.

5.3.3 Lysosomotropic cell death.

The death of the retinal pigment epithelium in the inferior temporal retina was preceded by an increase in the number of phagosomes and the formation of abnormal large lipid droplets within the cells. There was a noticeable delay between photoreceptor cell death occurring immediately after exposure, and the death of the retinal pigment epithelium within 48 hours (fig. 15). One of the possible explanations of such a delay in retinal pigment epithelium cell death is the generation of toxic products within the pigment epithelium triggered by excessive light exposure and the subsequent increased shedding and phagocytosis of rod outer segments. Such a situation may result in an overstretching of the retinal pigment epithelium's capacity to degrade the debris. When this happens, production of lysosomotropic detergents within the cells loaded with photoreceptor derived lipid rich membranes may occur. Such substances may include retinoid derivatives accumulated within the lysosomes

which, after reaching critical micelle concentration, are able to induce membrane lysis and release lysosomal enzymes into the cell causing it to die (Firestone and Pisano 1979, Miller et al. 1983). The morphology of cell death induced by lysosomotropic detergents includes features like vacuolization, blebbing of plasma membranes, cell rounding and finally cell death (Miller et al. 1983), all observed within the retinal pigment epithelial cells between 24 and 48 hours after light damage. The release of lysosomal enzymes into the cytoplasm, which is the postulated killing event (de Duve et al. 1974; de Duve and Wattiaux 1966; Miller et al. 1983), may occur some time after the excessive burst of phagocytosis induced by toxic levels of light. Such a release of lysosomal enzymes has been demonstrated to be induced by photosensitive dyes (Allison et al. 1966), carcinogens (Allison 1969) and viruses (Allison 1967). Retinoid reaction products, forming lysosomotropic detergents have also been postulated as playing a role in the pathogenesis of age-related retinal degeneration (Eldred 1993). Exposure to light, resulting in a burst of phagocytosis (Reme et al 1985), may well increase production of such substances and be linked with the retinal pigment epithelial cell death and the retinal degeneration observed in this study.

5.3.4 The possible mechanisms of cell death.

Apoptosis and necrosis are two fundamental types of cell death. It appears that the key mechanisms of apoptosis are the activation of endonuclease and transglutaminase. Endonuclease causes the cleavage of DNA at the nucleosomal linker regions giving rise to regular oligonucleotide runs of DNA. When agarose gel is performed, such runs give rise to a characteristic "ladder". The occurrence of a "ladder" is the most commonly used biochemical marker of apoptosis. (for reviews see Bowen 1993, Fesus et al. 1991, Gerschenson and Rotello 1992). It is thought that activation of transglutaminase leads to an extensive cross linking of proteins beneath apoptotic membranes (Fesus et al. 1991).

In necrosis, on the other hand, membrane damage is the key mechanism (Searle et al. 1982, Trump et al. 1973) and has been emphasised as a major event in light-induced degenerations. Vesicular alterations induced by light in rod outer segment membranes may be the first sign of such damage. It is believed by some that such changes are the initial step leading to photoreceptor degeneration (Reme et al. 1994). During injury the initial alterations of cellular metabolism and electrolyte homeostasis may involve at least four major pathways leading to the loss of membrane integrity (Buja et all. 1993) all of which have been suggested

as occurring in retinal degenerations: membrane phospholipid degradation (Wiegand et al. 1983); the production of amphipatic lipids and lysosomotropic detergents (Eldred 1994); damage to the cytoskeleton (Szczesny 1992b) and the generation of toxic oxygen species and free radicals (Gottsch et al. 1990).

5.3.5 Cell injury and gene activation.

It has been demonstrated recently that injury to the cell can result in the expression of certain genes and that products of such activated genes can modulate the response of the injured cells. Such genes include c-fos, c-jun, c-myc, Erg-1 and jun B, some of which induce heat shock or stress proteins, members of a class of proteins known as chaperonins (Buja et al. 1993). There is evidence for interrelationships between oxidative stress, changes in Ca^{+2} and the expression of chaperonins (Maki et al. 1992). It has been speculated that chaperonins can interact with constitutive proteins so as to inhibit protein degradation (Buja et al. 1992). A number of comprehensive reviews on what has already been established in relation to the molecular mechanisms controlling apoptosis have been published recently (Martin et al. 1994, Collins and Riva 1993, Wyllie 1993). However, these mechanisms are not yet fully understood.

It is worth discussing some of the known mechanisms and exploring the relationship between apoptosis and peroxidation for the purposes of this thesis. It is known that apoptosis can be induced by the activation of genes such as c-myc. Expression of other genes, for instance bcl-2, counteracts the induction of apoptosis by c-myc (Collins and Rivas 1993). In fact bcl-2 inhibits most types of apoptotic cell death. It is of interest that bcl-2 is localized to intracellular sites of oxygen free radical generation including mitochondria, endoplasmic reticulum and nuclear membranes (Hockenbery et al. 1993). Following an apoptotic signal cells sustain progressive lipid peroxidation. Overexpression of bcl-2 appears to function as a suppressant of lipid peroxidation and thus may regulate an antioxidant pathway at sites of free radical generation (Hockenbery et al. 1993). Lipid peroxidation may be the key event leading to photoreceptor cell death in photic retinopathy (Armstrong et al. 1989, Handelman et al. 1986, Kagan et al. 1973, Wiegard et al. 1983). It has been shown that antioxidants protect the retina from light damage (review Tso 1989). The relation of bcl-2 gene to both inherited and acquired retinal degeneration and the impact of toxic stimuli like light on the expression of bcl-2 would be logical topics to explore. Constant developments in molecular cell biology and the continuous search for a better understanding of the mechanisms controlling apoptotic cell death

open new horizons in retinal research, particularly in the field of photoreceptor degenerations irrespective of the cause.

5.4 Interpretation of the conflicting data on retinal recovery from light damage.

As mentioned in the introduction there are conflicting opinions on the ability of the retina to recover from light damage. In all past studies dealing with threshold retinal lesions and the problem of recovery the question of apoptosis was not considered, attention being paid rather to the membrane changes in the outer segments. Apoptotic cells were, however, a constant feature of the micrographs of light damaged retinae although the numbers of apoptotic cells varied from one experiment to another, the protocols of the experiments being different. It is difficult to make direct comparisons, since different groups used different units (either radiometric or photometric) to characterize the light, exposure times varied from hours to weeks, different species of animals were used, animals were sacrificed at various intervals after exposure and different parts of the retina were processed for histology. Kremers and van Norren have compared the experimental methodology of different groups working on light-damage in their recent review (Kremers and Van

Norren 1988).

Considering the results presented in this thesis, it appears that the authors of the conflicting reports were in fact presenting consistent results. Those investigators who used energies below the threshold levels for lethal damage, observed vesiculations of the rod outer segments and retinal recovery within 2 weeks. This type of damage, produced by fluorescent lamps was named "mild damage" (Kuwabara 1970). When the light doses were increased, vesicular alterations of membranes were still observed, but cell death also became apparent. Some recovery was reported from such damage, which was named "advanced damage" (Kuwabara 1970). The electron micrographs of "advanced damage" (fig. 2 of already quoted Kuwabara paper) show morphological changes which were also observed in this study in the "degeneration area" (fig. 12, 13, 33 A and B).

In another paper dealing with photoreceptor recovery from light damage, vesiculation of rod outer segments, swelling of mitochondria and an increased number of autophagic vacuoles were reported in the cells which recovered (Moriya et al. 1986). However, scattered pyknotic dying cells have also been observed by these authors and an analogy to "normal cell death in the embryonic chick spinal cord" reported originally by O'Connor and Wyttenbach in 1974 was made (Moriya et

al. 1986). O'Connor and Wyttenbach were in fact reporting apoptosis in embryogenesis (O'Connor and Wyttenbach 1974) two years after the term "apoptosis" had been introduced in the literature (Kerr et al. 1972). Kerr and his co-authors discuss in their paper the earlier descriptions of non-necrotic cell death, a phenomenon which has been known for many years before under different names. Moriya, to my knowledge, made therefore the first link between retinal light damage and the process widely known now as "apoptosis" or "programmed cell death". By definition, due to the nature of apoptosis, dying cells are "deleted in the midst of living tissue" (Wyllie 1993) without any disruption of the overall architecture of the retina. The difference between "recovery" and "degeneration" areas observed in this study is principally related to the different number of cells in which programmed cell death was triggered.

In the "recovery" area only a small number of cells is undergoing apoptosis and the morphological appearance of the outer nuclear layer and inner segment layer is similar to that of the controls. Only rare scattered cells show densification and pyknosis. Such cells are eliminated without any noticeable disturbance to the overall retinal architecture. Such a situation probably occurred in the experiments with threshold light levels, as used by Moriya and his co-workers (Moriya et al 1986). The process of programmed cell death was initiated in just a small

number of cells, these cells were promptly deleted and overall retinal morphology left intact this being interpreted as retinal recovery from light damage. With higher doses of light lethal to larger numbers of cells apoptosis was more widespread. Necrosis also was recorded and the final outcome was retinal degeneration. It seems quite logical for apoptosis to be the preferential option for cell death in the nervous tissue since it is a less disruptive way of eliminating lethally injured cells than primary necrosis and the inflammation which follows.

In the "degeneration" area a large number of cells is affected and apoptosis is triggered in response to light irradiation. The final result is retinal degeneration and the elimination of the photoreceptor and retinal pigment epithelial layer within a week without much disturbance to the inner retinal layers.

The different reaction of tissue to light exposure, ie recovery or degeneration, may be related to retinal irradiance. A recent study in the same strain of rats confirmed that photoreceptor apoptosis is triggered in the inferior central retina after exposure to 1000 lux, but involves the entire retina if the exposure is increased to 3000 lux (Hafezi et al 1995). There are also a number of other factors influencing susceptibility to light damage. The degree of expression of programmed cell death in photoreceptor response to toxic light levels varies depending on the

strain of rats used in an experiment. It has been demonstrated that both Lewis and Wistar albino rats exhibit more apoptotic cells than Fisher and Bufallo strains in the same exposure conditions (Lai et al 1995). Other factors including pigmentation or lack of it, the sex of the animal, hydration, the stress levels and others have been reviewed in the introduction (section 2.5.2 "Experimental studies on photochemical light damage in animals").

5.4.1 Coexistence of various forms of cell death.

Necrosis has been observed in the retina by several investigators and has been reported as present within 24 hours after light irradiation (Moriya et al 1986, Shahinfar et al 1991). A similar observation was made in this study. Interestingly one of the features of apoptosis, nuclear margination of photoreceptor nuclei, was most frequently observed 24 hours after light damage and coincided with widespread necrosis of the photoreceptor inner segments. Apoptotic followed by a lytic appearance of the outer nuclear layer in the later stages of degeneration induced by ischaemia-reperfusion insult has been reported (Buchi 1992). The existence of transitional stages between characteristic types of cellular degeneration is known (Clarke 1990). It is believed that apoptotic cells

and bodies can undergo "secondary necrosis", where apoptotic bodies lose their capacity for volume homeostasis. This leads to less specific lytic morphological changes (Wyllie 1985) and was observed 24 hours after injury in this study. Figure 25, 26 C and D show nuclei undergoing apoptosis while the inner segments in figure 29 show lytic changes typical for necrosis. The characteristic segregation pattern of the DNA demonstrated by gel electrophoresis (fig. 47) confirms the diagnosis of apoptosis which is followed by secondary necrosis seen particularly clearly in the inner segments.

5.4.2 Vesicular alterations of outer segment membranes.

The issue of reversibility of the outer segment membranes alterations have been discussed in the literature for some time. It has been reported that lamellar disk structure undergoes a transition to a structure which shows no bilayer membrane structure in response to light stress. After the removal of the light stimulus vesicular and tubular structures appear to rearrange themselves reproducing the normal lamellar disk structure (Moriya et al 1986). It has been postulated that such transformations of the outer segments are related to the transition of membranes from a lamellar to a hexagonal phase (Corless and Costello 1981, Gruner et al

1982). It has been postulated that lamellar to hexagonal phase transition of disk lipids is triggered by the shrinkage of the distance between the external disk surfaces (Gruner et al 1982). Shrinkage of the outer segment membranes has been reported as occurring in response to photoreceptor bleaching (Chabre and Cavaggioni 1975). In the same paper the authors have also documented swelling of the disks upon hypotonic shock which was more pronounced in bleached than dark adapted retina. It suggests that in the light damaged membranes lipids are prone to phase transition and membranes to osmotic changes and that these may be enhanced by fixation. The temperature also influences the fluidity of lipid rich membranes. Disk lipids are highly fluid below 20°C, and the transition temperature further decreases for the lipids in rod outer segment membrane matrix (Lamba et al 1992). Cooling of the specimen by a cold fixative may further influence the behaviour of membranes and make them prone to structural alterations. The effects of chemical and cryo-fixation are discussed in the following section below.

In most of the studies of the retinal changes induced by light, vesicular alterations in the rod outer segments have been identified as acute light induced changes (Reme et al. 1988, Reme et al. 1991, Reme et al. 1992). It has been suggested incorrectly in the light of the results presented in this thesis that these alterations are the first step leading to more severe

retinal lesions (Reme et al. 1994). The process of apoptosis has been ignored. It is, however, of great importance and an understanding of the mechanisms of apoptotic cell death in degenerating retinae may have far reaching clinical consequences.

5.5 Effects of fixation.

A distorted morphology of the outer segment membranes manifested as distinct dilation, vesiculation and structural alteration of disk membranes was documented in several retinal light damage studies. The nature and significance of such changes has not been clearly established. The issue of reversibility of the vesicular alterations of rod outer segments has been controversial since such changes were followed by photoreceptors degeneration according to some investigators (Grignolo et al. 1969; Kuwabara and Gron 1968) while others reported photoreceptors recovery from such alterations (Kuwabara and Funahashi 1976a; LaVail 1976; Moriya et al. 1986). Recently it has been postulated that vesicular alteration of rod outer segment membranes are the initial stages photoreceptor degeneration in a rat retina (Reme et al. 1993, Reme et al 1994). The degree of vesicular alterations of the rod outer segments has also been used as a criterion to assess the effects of lithium and light

effects on the retina (Reme et al. 1988) and to investigate methods of protection from light damage using the radioprotective agent WR-77913 (Reme et al. 1991), PAF antagonist BN 52021 (Jung et al. 1993; Reme et al. 1992) and diet (Bush et al. 1991).

It is known, however, that glutaraldehyde fixation can induce structural alterations and these include enhancement of hypoxia dependent changes in mitochondria (Minnassian and Huang 1979; Rash 1974), intramembrane particle aggregation and dispersal (Landis and Reese 1981) and artifacts of lipid mobility including membrane blistering, fusion and stretching (Bretscher and Whytock 1977; Chandler and Heuser 1979; Hasty and Hay 1978). During the process of chemical fixation diffusion barriers are altered unpredictably resulting in uncertainty about the preservation of the shape and the volume of intracellular and extracellular compartments (Rash 1983). Photoreceptor outer segments represent a unique part of the cell and are composed of lipid rich membranes (Fliesler and Anderson 1983). Fluidity of such membranes is mostly unaffected by glutaraldehyde but reduced to zero during postfixation with osmium tetroxide (Hayat 1989). Postfixation with osmium tetroxide on the other hand usually takes place hours or days after the experiments and in the mean time samples are stored either in glutaraldehyde or a buffer.

Such considerations are important in the interpretation of acute structural alterations in photoreceptor outer segments. These unpredictable effects of glutaraldehyde fixation are avoided by the use of cryo-immobilization, which allows a biological sample to be immobilized under defined physiological conditions, due to its high time resolution for dynamic cellular events. It has recently been applied to study the light response in non vertebrate retina (Ischikawa et al. 1993; Tsukita et al. 1988), photoreceptor development and disk morphogenesis in mice (Obata and Usukura 1992), the fine structure of the embryonic avian eye (Cacchillo and Allenspach 1992), the surface structures of disk membranes (Roof and Heuser 1982), opsin transport (Miyaguchi and Hashimoto 1992) and distribution in the rat photoreceptors (Miyaguchi et al. 1992) and the secretion and transport function of the ciliary body epithelium in a rat (Eggli et al. 1991). Cryo-fixation is much faster than chemical fixation: it stops all physiological processes within a few milliseconds. The main problem related to cryo-fixation is distortion of the biological structure by the formation of ice crystals that occur if cooling rates are insufficient. The thermal conductivity of frozen water is relatively poor so a cooling rate high enough to prevent formation of disturbing ice crystals (at ambient pressure) is only obtained in the outermost layer of a specimen (up to a depth of about 5 um from the surface). In order to

prevent ice crystal formation in thicker samples cryoprotectants (usually combined with aldehyde fixation) were frequently used for the freeze fracture replica technique (Moor 1971; Sjostrand and Kreman 1978). It is, however, documented that this pretreatment induces numerous artefact (Plattner and Bachmann 1982). Due to improvements in high pressure freezing technique, introduction of simpler commercially available equipment it is possible to cryofix biological samples which are thicker than 200 μm (Moor 1987, Studer et al 1989).

5.5.1 Glutaraldehyde versus high pressure freezing cryofixation.

The ultrastructure of dark adapted photoreceptors after high pressure freezing and after glutaraldehyde fixation was very similar (fig.48). Light damaged photoreceptors, however, showed remarkable morphological differences depending on the fixation technique used (fig 49). Glutaraldehyde dramatically increased alterations of the rod outer segment membranes in retina which had been damaged by diffuse white light. Several physiological factors have been already postulated as contributing to alterations of rod outer segment membranes. They include ionic imbalance (Chabre and Cavaggioni 1975), photochemical membrane damage (Dayhaw-Baker et al. 1986; Girotti 1990), lipid

peroxidation (Armstrong et al. 1989; Handelman and Dratz 1986; Kagan et al. 1973; Wiegand et al. 1983) and light mediated biochemical cascades (Girotti 1990; Jung and Reme 1993). This study demonstrates the severe effects of glutaraldehyde fixation on the morphology of light damaged photoreceptor outer segments. The secondary effects of glutaraldehyde fixation are excluded by fast cryo-immobilization (Moor 1987). High pressure frozen and freeze substituted samples of light damaged photoreceptors revealed initial ultrastructural alterations such as autophagy and rearrangement of Golgi in the synaptic region and alterations in the outer segments suggesting that the whole cell is affected relatively early during light damage (Walther et al. 1993). These results agree with earlier studies based on glutaraldehyde fixed material (Kuwabara and Funahashi 1976b; Kuwabara and Gron 1968; Moriya et al. 1986), but the effects become much more clear after high pressure freezing fixation (fig. 49). It appears from this study that photoreceptors showing vesicular alterations of outer segments recover normal morphology within a week after injury. Similarly the retinal areas which predominantly show such changes recover normal architecture within one week. It remains to be seen if morphological recovery is parallel to functional recovery in such eyes. In a limited number of histopathological studies of chemically fixed eyes with solar retinopathy

in Man, vesicular and tubular alterations and fragmentation of photoreceptor outer segments have been consistently observed and coexisted with other photoreceptor and retinal pigment epithelium pathologies (Hope-Ross et al. 1993; Tso 1988; Tso and LaPiana 1975). In such eyes, despite good prognosis, central scotomas frequently persist (MacFaul 1969), suggesting overall recovery with some of the retinal area irreversibly damaged.

High pressure frozen samples revealed some structural details which have been consistently observed by other investigators using different cryofixation techniques. High pressure freezing shows filaments between disk and plasma membrane similar to those described in outer segments in a toad after cryofixation combined with freeze fracture and etching (Roof and Heuser 1982). Disk like structures close to ciliary microtubules and membrane bound vesicles in the ciliary region, significant with respect to the role of the cilium in the process of disk formation and renewal, were observed as in other studies (Miyaguchi and Hashimoto 1992; Obata and Usukura 1992). On the other hand extension of the inner segment in the form of a calix around the photoreceptor connecting cilium was infrequent in high pressure frozen samples. One of the possible explanations is that elongation of the inner segment may

be a function of post mortem time as has been demonstrated in glutaraldehyde fixed retinae (Uehara et al. 1989).

5.5.2 "Dark cells" and chemical fixation.

The importance of fixation and embedding procedures in the detection of "dark cells" with condensed cytoplasm and or nucleus has been shown (Klein-Szanto et al. 1982). It has been demonstrated that after glutaraldehyde fixation and standard tissue processing for electron microscopy twice as many dark cells are present compared with fixation with Bouin's fluid (Klein-Szanto et al. 1982). Some of the negative effects of glutaraldehyde fixation are omitted by using cryo fixation. The question of fixation induced artifacts and the morphology of light damaged photoreceptors has been addressed recently (Szczesny, Reme, et al. 1993; Walther et al. 1993). It has been demonstrated in these studies that vesicular alterations of rod outer segments are induced by glutaraldehyde during fixation of light damaged samples. However, the morphology of apoptotic cells in light damaged retinae is similar after glutaraldehyde and cryofixation methods (Szczesny, Walther, et al. 1993). The quantitative differences in numbers of "dark cells" in light

damaged retinae depending on fixation techniques being used have yet to be established.

5.6 Patterns of cell and tissue responses.

5.6.1. Activation of macrophages.

Histopathological studies of light induced and inherited retinal degenerations do not show typical inflammatory reactions but rather an increased presence of macrophages (Hoppeler et al. 1988; Noell et al. 1966; Szczesny 1991, Szczesny 1992; Tso 1989). The occurrence of macrophages has been associated with major photoreceptor loss in the early phase of photic injury when more then 25% of cells were eliminated as estimated by a nuclei count (Tso 1989). One of the aims of this study was to examine the tissue response and to look at the pattern of inflammatory reaction in retinae injured by light. It was observed that in retinae undergoing light induced degeneration distinct mononuclear cells and macrophages were present but no other inflammatory cells were to be seen. Macrophage activity has been associated with apoptotic cell death and also with tissue necrosis. Activated macrophages were the primary cytotoxic cells in inflammatory

lesions 24 hours after stimulation (Cohn 1978; North 1978; Rappolee and Werb 1989). Macrophages have also been observed in cell death and tissue remodelling in the mouse eye (Lang and Bishop 1993; Sanyal et al. 1984). Numerous phagocytic cells (Noell et al. 1966; O'Steen and Lytle 1971) and macrophages have been noticed in light damage studies (Hoppeler et al. 1988; Tso 1973). It has been hypothesized that leukotriene may be a chemotactic agent attracting macrophages to assist in the phagocytic activity of the pigment epithelium and Muller cells in light damaged retinae (Tso 1989) in a response to visual cell death. Biochemical studies have shown a light elicited release of arachidonic acid (Jung and Reme 1993). Metabolites of arachidonic acid, such as the leukotrienes, may constitute chemotactic agents for macrophages in the eye as postulated by Tso. A semiquantitative evaluation of the tissue sections shows an influx of macrophages following the loss of retinal pigment epithelium (fig. 21) and breakdown of the blood retinal barrier observed within 24 and 48 hours after light damage. The peak for mitotic figures is also followed by an increase in macrophage presence within the retina and the sub retinal space up to 72 hours after the injury (fig. 20 and 22). It may suggest that apart from the resident retinal macrophages a certain number of cells may arrive from the blood stream and also that the cells which are recruited can divide in the retina.

5.6.2 Retinal proliferation.

In normal adult mammalian retinae cell mitoses are not observed. In clinical situations pathological proliferation is observed in epiretinal membranes, massive retinal gliosis, proliferative vitroretinopathy and proliferative forms of age related macular degeneration (ARMD). In this study cell divisions were frequently observed. The inner nuclear layer revealed numerous mitotic figures in the degeneration zone. The highest incidence of mitoses was observed 48 to 72 hours after the injury (fig.20 22, 36 A, B). Some of these cells were clearly macrophages dividing in the sub retinal space and within the retina. The identity of other dividing cells within the inner nuclear layer remains to be established. Some of these cells may be derived from retinal glia and may be associated with the reparative processes and scar formation (fig 19). In experimental conditions mitosis in retinal vascular cells, presumed Muller cells, retinal pigment epithelium and possibly other cell populations has been reported in the albino rat retina after ischemia followed by reperfusion (Stefansson et al. 1988). In his study Stefansson reported mitotic activity at 48 and 96 hours after the release of central retinal vascular occlusion and he ceased to observe it by the seventh day. A similar time course was observed in this study. The occurrence of mitosis in the retina has been

also reported in presumed amacrine cells following kainic acid poisoning (Lessell et al. 1980) and in pericytes in talc retinopathy (Kaga et al. 1982).

Mitotic activity in retinal pigment epithelium, Muller cells, astroglia and migratory cells within the retina has been also reported (Burke and Smith 1981). A reference to an unpublished observation of mitotic activity in response to retinal detachment has been made (Stefansson et al. 1988). Mitogenesis has also been reported in autoradiographic studies of the rat neurosensory retina, retinal pigment epithelium, choriocapillaris and deep choroid after krypton laser photocoagulation (Zhang et al. 1993). In a classical paper on light damage (Noell et al. 1966) mitotic figures were observed within the inner retinal layers 2 to 3 days after exposure when visual cells in the same area were undergoing lysis. Mitotic figures in the inner nuclear layer of light damaged retinae can be identified in another study, in which mitotic activity was explicitly described in the retinal pigment epithelium (O'Steen and Karcioglu 1974).

5.6.3 Choroidal changes.

Although the mechanisms leading to retinal degeneration induced by light are not fully understood it is thought that they are the result of

photochemical or photo-oxidative mechanisms (Lisle 1982; Noell 1980). Recently it has been proposed, that photosensitization with haematogenous protoporphyrin IX contributes to the development of age related macular degeneration by induction of changes within Bruch's membrane and choriocapillaris (Gottsch et al 1990). In experimental light damage studies some abnormalities were recorded at the level of Bruch's membrane and the choroid. Proliferation of retinal vessels into the sub retinal space (Kuwabara 1970), choroidal neovascularization (Tso and Woodford 1983) and placoid proliferation of the retinal pigment epithelium (Friedman and Kuwabara 1968, Tso 1973) have been observed after various periods after retinal light injury. Retinal vessel abnormalities have also been observed in phototoxic retinopathy in rats (Bellhorn et al 1980, Shiraki et al 1983).

In this study an acute inflammatory reaction was observed in the choroid as a response to light induced retinal degeneration. Initial damage to the choriocapillaris was followed by some neovascular activity probably associated with tissue reparation and scarring. At later stages choriocapillaris dropout was recorded.

There are some similarities between the light induced pathology observed in this model and the choroidal reaction observed in photodynamic insult (Buchi et al 1994, Wilson et al 1991). Damage to the choriocapillaris,

the breakdown of the blood retinal barrier and the inflammatory reaction in the choroid was observed in all these studies. In photodynamically induced lesions the main feature was damage and obliteration of choriocapillaris with preservation of the larger choroidal vessels. Recanalization of the choriocapillaris was also observed with endothelial cells containing abundant rough endoplasmic reticulum. Such cells were located within the denuded basement membrane of the former vessels (Wilson et al 1991). A similar sequence of events was observed in this study.

Damage and disruption of the retinal pigment epithelial cells and subsequent breakdown of the blood retinal barrier manifested as shallow serous retinal detachment in response to photodynamic injury has been reported (Wilson et al 1991) and was also observed in this study.

Infiltration of the choroid with monocytes after light damage was a striking feature noticed in the degeneration area. A similar reaction was recorded in the case of photodynamic insult (Wilson et al 1991). One of the potent chemoattractants for leucocytes and monocytes are leukotriens, LTB_4 in particular. It has been demonstrated that intravitreal injection of LTB_4 induces polymorphonuclear and monocytes infiltration of the choroid but not the neuroretina (Krauss and Woodward 1993). Leukotriens, breakdown products of arachidonic acid which is present in

photoreceptor outer segments, have been proposed to be chemoattractants in the light damaged retina (Tso 1989). Recently a release of LTB_4 by light damaged retina has been reported (Reinboth et al 1995).

The results of this study support the concept of direct phototoxic injury to the choriocapillaris which occurs in parallel to retinal light damage (Szczesny et al 1995). In a study in which exogenous photosensitizer was used, the changes in the choroid were more pronounced (Buchi et al 1994, Wilson et al 1991). Being a nocturnal rodent the rat has a Harderian gland filling the orbit and the posterior part of the globe is adjacent to it. Harderian gland produces endogenous photosensitizers, primarily protoporphyrin IX (Tomio and Grinstein 1968, Vaughan et al 1991) which is a potent photosensitizer and may contribute to retinal and choroidal light damage in a rat. Further studies exploring the concept of photosensitization with protoporphyrin IX in light damage studies in a rat are required.

5.6.4 Blood retinal barrier.

Light induced retinopathy in the rat and other experimental animals is associated with changes in the blood retinal barrier function, something

which has been observed in this study. The pathology was related to disfunction of the barrier leading to shallow serous retinal detachments followed by cystoid retinal oedema. The changes were prominent within 48 hours after the light injury and were associated with retinal pigment epithelium cell death. Endothelial abnormalities in choriocapillaris were also present. The cells were fragmented and often detached from their basal lamina. The vessel walls, Bruch's membrane and choroid in general were infiltrated by numerous mononuclear cells. Sub-retinal fluid, retinal oedema and microcystic retinal degeneration were transient. These changes subsided after the remodelling of the outer limiting membrane and the formation of cell junction complexes at the level of and proximal to the inner limiting membrane. Within 7 days of the injury chorioretinal scar was formed and no evidence of gross leakage and fluid accumulation within the retina was to be found. The breakdown of the blood retinal barrier coincided with retinal pigment epithelial cell death (fig 18). Restoration of the blood retinal barrier function was preceded by mitotic activity in the outer retina (fig.19 and 55) and followed formation of chorioretinal scar (fig. 56). Abnormalities of permeability and subsequent remodelling of the retinal vascular bed have been reported in light damage studies (Bellhorn et al. 1980; Shiraki et al. 1983). Disfunction of the blood retinal barrier can be induced by low

intensity white light. The breakdown of the blood retinal barrier studied in the rabbit was shown to be transient, to occur at the level of the retinal pigment epithelium resulting in increased transcellular permeability of the cells and to be observed before any ultrastructural changes become evident within the cells (Putting et al. 1992). The vesicular alterations of photoreceptor outer segments observed in the recovery zone and reported in the literature by many authors (Grignolo et al. 1969; Kuwabara and Gron 1968, Moriya et al. 1986, Reme et al 1988) may be linked to such a discrete disfunction of the retinal pigment epithelium described by Putting, resulting in increased fluid permeability and swelling of outer segment membranes. In this study, however, the retinal pigment epithelium showed some transient ultrastructural changes, manifested by cells swelling, and widening of the extracellular spaces between the neighbouring cells. Such changes were observed within the first 24 hours of light damage in the recovery zone. Vesicular alterations of the outer segment membranes were, on the other hand, primarily associated with the type of fixation and were dramatically enhanced by the use of glutaraldehyde. Reparative processes in the retina manifested as proliferation of cells and scar formation associated with the restoration of barrier function may indicate the role of Muller cells and glial tissue in repair and maintenance of a barrier in light damaged

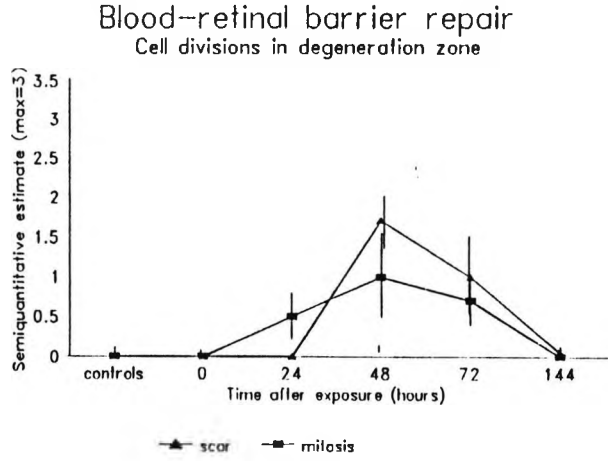


Fig. 55. Mitotic activity in the retina and blood retinal barrier dysfunction.

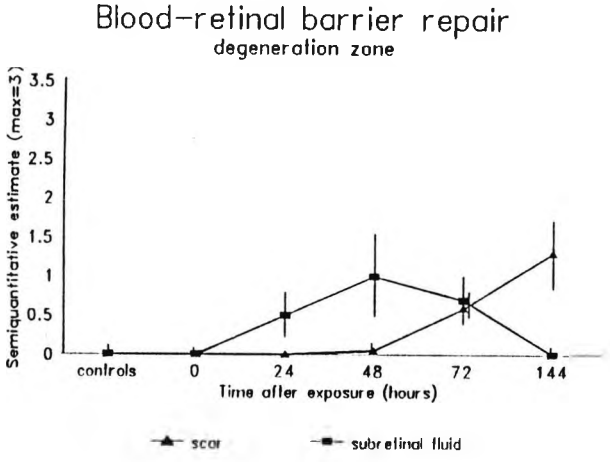


Fig.56. Association of blood retinal barrier repair and scar formation in the retina.

retinae. The role of these cells in the formation of the blood retinal barrier has been documented recently (Tout et al 1993).

6. Summary and conclusions.

1. Toxic levels of diffuse white light of 1000 lux illuminance delivered for 120 minutes induced reversible and irreversible lesions in the albino rat retina.

2. There were regional differences in the rat retina showing different patterns of response to injury. In the samples from the inferior nasal part of the retina a recovery of normal morphology was observed within a week. In the samples from the inferior temporal retina progressive degenerative changes were recorded which resulted in loss of photoreceptors and retinal pigment epithelium within 7 days after the insult. The regional differences were probably related to differences in retinal irradiance with lower temporal retina receiving more light with the illumination set up used in this study.

3. Reversible retinal lesions were characterized by vesicular alterations of the photoreceptor outer segments observed immediately after the

exposure and transient oedema of the retinal pigment epithelium present within 24 hours of light damage. The retina, however, recovered normal morphology over a period of 7 days after the exposure. Vesicular changes may be related to lipid transition from lamellar to hexagonal phase. This process may also be associated with transient increase in membranes permeability followed by reparative process and restoration of normal homeostasis. The structural membrane changes themselves are produced by glutaraldehyde fixation of light damaged membranes and are not observed in light damaged cryofixed samples.

4. Irreversible retinal changes leading to degeneration and loss of photoreceptor cells and retinal pigment epithelium were consistent with apoptosis. In photoreceptors such changes were observed immediately after the exposure. Necrosis of photoreceptors was also evident at the same time and was widely spread within the first 24 hours after exposure. The retinal pigment epithelium initially showed an increased number of phagosomes and, within 24 hours of the photic injury, also displayed features of apoptosis. Apoptosis is triggered by toxic light levels and appears to coexist with necrosis, especially at later stages. Cell death of the retinal pigment epithelium was delayed and showed a mixed pattern of apoptosis and necrosis with many cells showing lytic changes.

Cell death of the retinal pigment epithelium may be a secondary response related to an accumulation of excessive amounts of rod outer segments debris degradated to form toxic products like lysosomotropic detergents.

5. Prompt removal of photoreceptor cell debris by neighbouring cells, predominantly Muller cells and by macrophages, a lack of inflammatory response and preservation of the inner retinal architecture were consistent with the tissue response observed in apoptosis. The influx of macrophages is correlated with a breakdown of the blood retinal barrier. Additionally macrophages are undergoing rapid cell divisions in the retina with a maximum number of mitotic figures observed 48 to 72 hours after injury.

6. Mitotic activity in the retina undergoing degeneration is explained by rapid tissue repair after the insult. No pathological proliferation of cells leading to retinal neovascularization or epiretinal membrane formation was seen during the 7 days period of observation. The observation time was too short, however, to exclude the possibility that such pathologies may develop as a later sequel in retinal degeneration induced by light.

7. During the 7 days of observation retinal degeneration progressed in certain stages: initially a rapid onset of cell death was seen, followed within 24 - 48 hours by the breakdown of the blood retinal barrier, manifested as shallow retinal detachments and cystoid retinal degeneration, and prompt repair leading to chorioretinal adhesion within 72 hours of the injury. The inner retinal layers were not affected and were well preserved at the end of 7 days' observation. The mechanisms which control the tissue responses in this model of retinal degeneration are not fully understood. The well defined and clear pattern of changes and the time scale of their occurrence may be, however, useful in planning further research in the field of retinal degeneration. They may also be of use in planning therapeutic approaches targeted at the prevention of primary and secondary pathologies leading to retinal degeneration animals and humans.

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