A STUDY OF STRUCTURE AND FUNCTION IN

POSTMORTEM HUMAN RETINA

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To my dear wife Yvonne &
   our beloved son Tim

ABSTRACT

The aim of the present study was to investigate structure and function in postmortem human retina and to evaluate systems for prolonging survival <u>in vitro</u>.

The main factors affecting survival were total time postmortem and the time between death and enucleation of the eye. Limited sample size precluded analysis of potential changes with donor age. Histological studies on 31 human eyes indicated that, in the periphery, photoreceptor and inner retinal structure were reasonably preserved for at least 2 to 3 days postmortem, although some vacuolation was present. In contrast to the periphery, the fovea and perifovea were always poorly preserved.

Function of human retinae was assessed under three categories: photochemistry, electrophysiology, and biochemistry.

To optimise conditions for restoring photo-transduction, pilot experiments were undertaken on rat tissue. Freshly isolated, bleached rat retinae were incubated in the dark at $37^{o}C$ for 60 minutes in Earle's medium containing 500 uM of a mixture of retinal isomers encapsulated in liposomes. This procedure induced recovery of rhodopsin to 91%, cyclic GMP to 75% and PIII amplitude to 68% of the values obtained for dark-adapted retina.

Human retinae 5 to 58 hours postmortem were incubated in fortified Eagle's medium. Dark-adapted values could not be obtained for human retina. Rhodopsin was regenerated from zero to 0.1 - 0.41 nmol/mg protein, and the regeneration rate was about twice as fast as that in the rat. Cyclic GMP increased from 15.5 - 18.8 to 23.5 - 49.2 pmol/mg protein. Photoresponses were obtained in nine out of thirteen retinae: PIII maximum amplitudes ranged from 20 - 398 uV, and thresholds from 8.8 - 1340 quanta/um^2. In three cases, b-waves were also seen.

Studies on high-affinity uptake of taurine, glycine and GABA by five isolated human retinae 5 to 36 hours postmortem showed that taurine was accumulated predominantly by photoreceptor and possibly bipolar cells, glycine by cells located in the inner nuclear layer, and GABA by Muller cells.

Protein synthesis has been investigated in seven human retinae 5 to 48 hours postmortem. Incorporation of ^{3}H-leucine into proteins and specifically into opsin was followed. Incubation in the fortified medium resulted in an increase in opsin and total protein synthesis, and a concurrent reduction of retinal vacuolation. Autoradiography revealed that in the fresh retina general protein synthesis was occurring in both neurones and glia, whereas, at longer postmortem times it was predominantly in Muller cells.

The studies have shown that human retinae and, in particular, rod photoreceptor cells survive reasonably well postmortem. Some improvements in structure and metabolism have been obtained in short-term explant culture, and it is likely that more could be achieved.

ACKNOWLEDGEMENTS

PUBLICATIONS

1. Huang,J.C., Voaden,M.J. and Marshall,J. (1990) Survival of structure and function in postmortem rat and human retinas: rhodopsin, cGMP and the ERG. Curr. Eye Res., 9: 151-162.

2. Huang,J.C., Voaden,M.J., Shallal,A. and Marshall,J. (1990) Phototransduction and opsin synthesis in the human retina postmortem and in explant culture. Invest. Ophthalmol. Vis. Sci. Suppl., 31: 77.

3. Huang,J.C., Voaden,M.J., Marshall,J. and Kemp,C.M. (1990) Electrophysiologic characteristics of human and rat retinas _in vitro_. Doc. Ophthalmol. (in press).

4. Voaden,M.J., Huang.J.C., Willmott,N.J. and Hussain, A.A. (1989) Functional recovery in human and rodent retinas _in vitro_. Biochem. Soc. Trans., 17: 741-742.

CONTENTS

PART 2: STUDIES UNDERTAKEN

LIST OF FIGURES

1. Why should we investigate postmortem human retina?

In terms of the allocation of "brain space", it could be argued that vision is man's primary and most important sense. The human retina contains more than 120 million photoreceptor cells (Østerberg, 1935), far more transducers per unit area than any other sense organ, and about 1 million optic nerve fibres (Kuffler et al., 1984). Thus the eye not only contains the most sensitive tissue in the body, but also as a whole provides the finely tuned microenvironment needed for the retina to function optimally.

The salient properties of the visual system are its extreme sensitivity, the breadth of its operating range which spans light conditions varying by more than a millionfold, its ability to monitor rapidly changing events (up to about 50 cycles per second), its high spatial acuity, and its extremely fine colour discrimination. All of these properties result from neuronal activity induced by the absorption of light.

However, although the visual system has been studied in depth for more than a century, our understanding of the mechanisms involved is still far from complete, especially in man. Physiological properties of retinal cells have been elucidated largely from studies on tissue from lower-order vertebrates, but wide ranging species differences exist as regards the detail of metabolic organization, neurotransmitter systems and processing of visual information.

Furthermore, the ultimate goal of studying vision is not only to understand its physiological mechanisms, but also to resolve visual abnormalities in man. It should also be emphasized that animal models with diseases that appear similar to those in man can only provide limited information. Therefore, there can be no substitute for studies on the human retina itself.

However, in contrast to studies on lower-order animals, ideal experimental conditions for the investigation of human tissue can hardly be achieved, and

such a limitation has greatly restricted our understanding of visual mechanisms in both normal and abnormal human retinae.

Therefore, it is necessary to investigate the human retina _in vitro_ using postmortem tissue, and a prerequisite is to establish postmortem survival and the potential for recovery of function.

2. The aims of the present study

Principal:

> To investigate survival and the potential for recovery of structure and function in postmortem human retina.

Subsidiary:
1)   To establish an optimal incubation system for structural and functional recovery in the human retina.
2)   To evaluate the feasibility of studying function in both normal and abnormal human retinae _in vitro_.

3. What can be gained from the current research?

Since almost all postmortem human retinae, available for studies in laboratories, will have been bleached and be progressively deteriorating, only a few types of studies can be expected to be productive. However, if the retinae are well preserved and functions such as transduction and metabolism are active, there is no doubt that many kinds of investigation will be possible. Further, under such condition, we may be able to perform _in vitro_ comparative studies on diseased human retinae.

For example, in patients with one form of autosomal dominant retinitis pigmentosa (AD RP), the rhodopsin complement in parts of the retina approaches 80% of the normal level and its bleaching and regeneration follow a normal time course. However, the electrical activity of the rods is very reduced and rod-mediated detection of light impaired (Kemp et al., 1988; Fitzke and Kemp, 1989). Obviously, there appears to be a defect in the

transduction mechanisms which transmit the visual signal between the rhodopsin molecules and the rod plasma membrane. By contrast, in patients with another form of AD RP, there appears to be an abnormal rod dark-adaptation curve, and the prolonged rod dark-adaptation has been reported to last for several hours (Alexander and Fishman, 1984) or days (Moore et al., 1989), instead of the norm of 40 minutes. This defect may lie in pigment regeneration and/or outer segment renewal. Hence, in order to under-stand and discriminate between the different mechanisms leading to, for example, RP, it is necessary to study the "functioning" retina <u>in vitro</u> utilizing electrophysiology, biochemistry, pharmacology and morphology.

Obviously, similar approaches may apply to various allied or unknown retinal disorders, as well as to basic studies of the human retina.

It will be shown below, that the current results have demonstrated that some aspects of the human retina can survive 2 to 3 days postmortem, and that functions such as transduction and metabolism can be partially recovered <u>in vitro</u>. Thus, the present research establishes the possibility of the above studies.

4. Structure of the thesis

This section, STRUCTURE AND CONCEPT OF THE THESIS, introduces the purpose and significance of studying postmortem human retina.

Part 1 provides a review of the necessary background material relating to the present study. It is divided into seven sections:

1.1 Morphology, in which photoreceptors are highlighted.

1.2 Rhodopsin, concerning its bleaching and regeneration.

1.3 The cGMP cascade, describing most of the factors involved, and the current concepts and controversy.

1.4 Electrophysiology, which again highlights the photoreceptors, and also concentrates on the origins of the ERG components.

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1.5 High-affinity uptake of amino acids, including taurine, glycine and GABA.

1.6 Protein synthesis, discussing general concepts of protein synthesis, and specifying opsin.

1.7 Some aspects of postmortem changes in the central nervous system.

Part 2 presents the studies undertaken. As in Part 1, Part 2 is also sub-divided into seven sections: six of them further have four subdivisions - Introduction, Methods, Results and Discussion; the last section gives concluding remarks.

Morphological studies are in section 2.1. Studies of function are considered under three categories: photochemistry (2.2 and 2.3), electrophysiology (2.4), and biochemistry (2.5 and 2.6):

2.1 Structural survival of postmortem human retina, and the affecting factors.

2.2 Regeneration of rhodopsin in isolated retinae of man and rat. Studies on rat tissue provide a data base that could not be obtained in human material. This section discusses the features of rhodopsin regeneration _in vitro_, a co-ordinate event - the phosphorylation/dephosphorylation of opsin, and the possibility of application of IPM or IRBP.

2.3 Recovery of cGMP, in both human and rat retinae. This section details the light/dark changes of cGMP content and explores the possible mechanisms leading to the cGMP recovery in previously-bleached retina.

2.4 Recovery of the ERG. This section demonstrates recovery of photoresponses in human and rat retinae following the regeneration of visual pigments and recovery of cGMP, and compares the relationship among these "key" components in retinal phototransduction. It also shows the effects of low temperature and fortified medium on survival of the photoresponse. An attempt has been made to preserve the ERG b-wave.

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2.5 Amino acid uptake. This section reveals survival in postmortem human retina of high-affinity mechanisms for uptake of taurine, glycine and GABA, and identifies sites of uptake.

2.6 Protein synthesis. This section shows improvements in protein synthesis, including opsin, and concurrent reduction of vacuolation in postmortem human retinae, after incubation in the fortified medium.

2.7 Concluding discussion and final assessment of the above studies.

PART 1: CURRENT CONCEPTS OF THE VERTEBRATE RETINA

1.1 FUNCTIONAL MORPHOLOGY

1.1.1 GENERAL STRUCTURE

All known vertebrate eyes conform to the general basic structure shown in Fig.1.1.1.
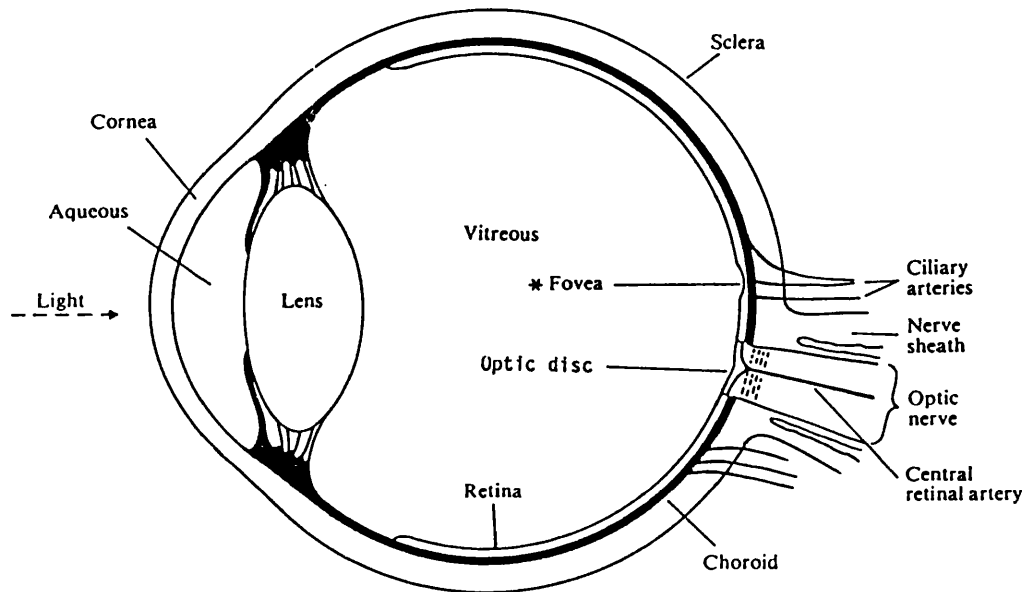
Fig.1.1.1 Schematic diagram of a horizontal section of a vertebrate eye. (After Voaden, 1979).

The outer coat, the cornea and sclera, is thick and tough and protects the delicate inner structure. The middle vascular coat, the choroid, provides the nutrients to the ocular tissue. The retina lines the inside of the back of the eye.

* The fovea, as a morphological specialization containing mainly cones, can be seen in some diurnal animals including man, but is not found in nocturnal animals such as rat.

The retina, lining the inside of the back of the eye, is embryologically and anatomically subdivided into two layers: a single layer of cuboidal neuro-epithelial cells, termed the retinal pigment epithelium (RPE), and the major part of the tissue, the neural or sensory retina. In this thesis, the term "retina" will refer only to the neural retina.

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1.1.2 TOPOGRAPHIC ANATOMY OF THE RETINA

Retinal topography can be subdivided into two general portions: the central and peripheral. The central part of the retina is 5 to 6 mm in diameter in man and contains the macula, including the foveola, fovea, parafovea and perifovea (Hogan et al., 1971; Fig.1.1.2).

The thickness of the retina varies with different areas: the thinnest portion is in the foveola, the thickest is in the parafovea, and then there is gradual thinning from the posterior to the peripheral retina (Fig.1.1.2).
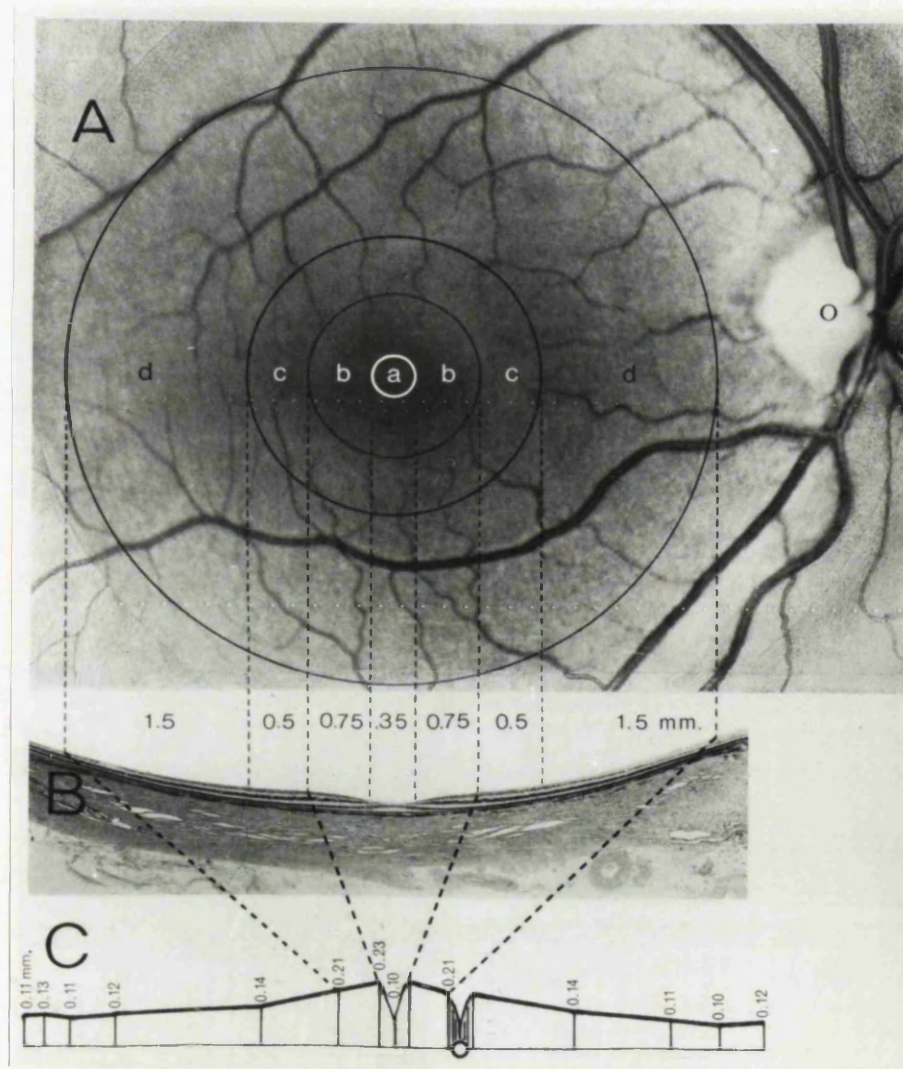
Fig.1.1.2 A typical human fundus photograph (A), matched with a meridional micrograph of the macular region (B), and profile of the thickness (C) of the human retina.
(a) the foveola; (b) the fovea; (c) the parafovea; (d) the perifovea; (O) optic disc. (Modified from Straatsma et al., 1969; Hogan et al., 1971).

1.1.3 CELLULAR ORGANIZATION OF THE RETINA

The retina is composed of 6 types of neurones that receive and process visual information before passing it on via the optic nerve fibres to the brain. They are the photoreceptors (rods and cones), and the bipolar, horizontal, amacrine, interplexiform and ganglion cells. The cell nuclei are arranged in 3 layers, separated by 2 plexiform regions where fine processes from the neurones synaptically interact. The predominant glia of the retina are termed Muller cells. In a typical picture, seen in light micrographs of vertebrate retinae, 11 sub-layers are discerned and come about by the anatomical juxtaposition of similar parts of similar cells (Fig.1.1.3; cf. Hogan et al., 1971). These are:-

1 - the RPE,

2 - photoreceptor outer segments (OS),

3 - photoreceptor inner segments (IS),

4 - the outer limiting membrane (OLM) - lying midway along the IS. This is not a real membrane, but an alignment of junctional complexes between Muller and photoreceptor cells.

5 - the outer nuclear layer (ONL), containing the nuclei of photoreceptor cells,

6 - the outer plexiform (synaptic) layer (OPL), mainly containing the synaptic contacts between photoreceptor, bipolar and horizontal cells,

7 - the inner nuclear layer (INL), containing the cell bodies of bipolar, horizontal, amacrine, interplexiform, displaced ganglion, and Muller cells,

8 - the inner plexiform (synaptic) layer (IPL): a region of complex synaptic interactions between bipolar, amacrine, interplexiform and ganglion cells,

9 - the ganglion cell layer (GC), containing the bodies of ganglion and displaced amacrine cells,

10 - the nerve fibre layer (NF), formed by ganglion cell axons converging towards the optic nerve,

11 - the inner limiting membrane (ILM), in contrast to the OLM, is formed by the basement membranes of Muller cells and into which collagen fibres from the vitreous cortex insert.
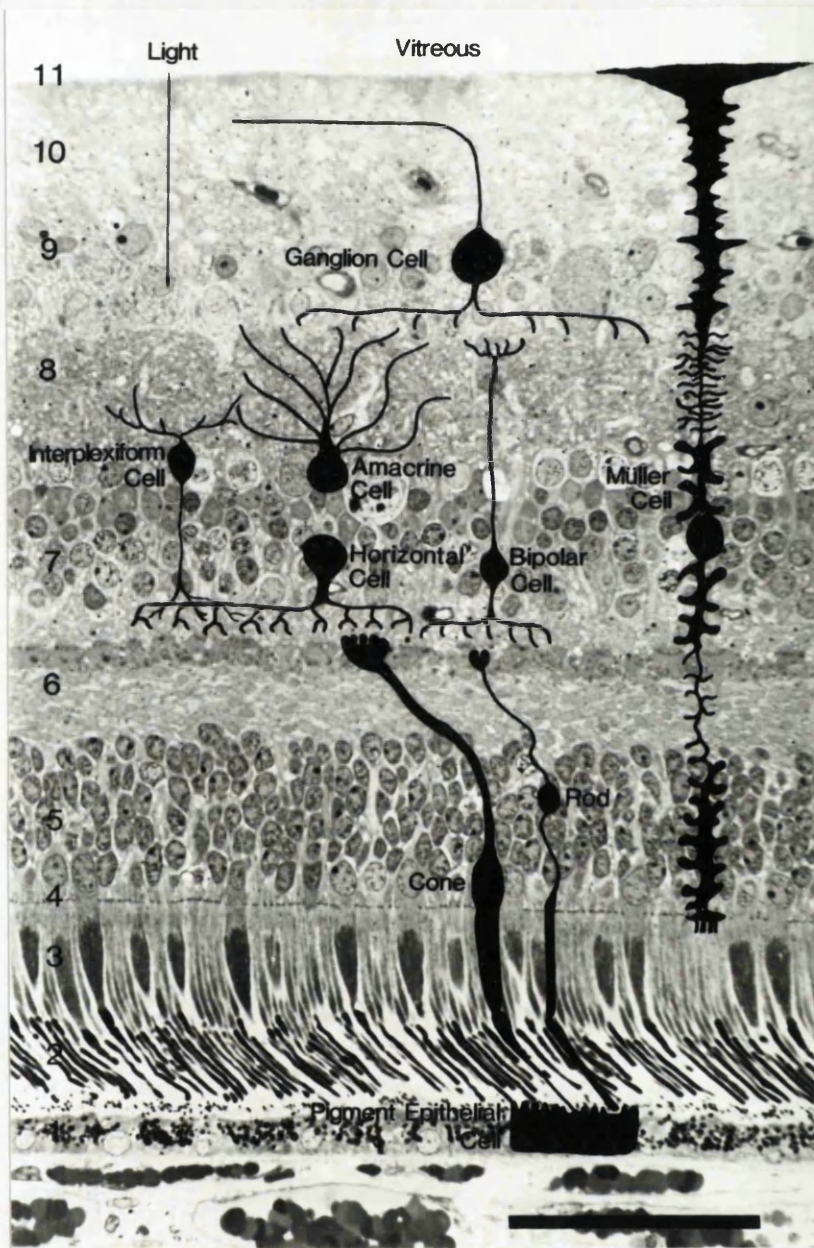
Fig.1.1.3 Photomicrograph of a transverse section of the perifoveal region of a human retina showing the 11 sublayers. (Courtesy of Prof. John Marshall).

1) RPE, 2) OS, 3) IS, 4) OLM, 5) ONL, 6) OPL, 7) INL, 8) IPL, 9) GC, 10) NF, 11) ILM. See the text for details.

Light enters the retina at the ganglion cell layer and reaches the photoreceptors where it is absorbed, starting excitation in the OS. The photoreceptor cells are connected to the bipolar cells, which in turn connect to the ganglion cells and so to the nerve fibres. Apart from this through-line, other neurones (i.e., horizontal, amacrine and interplexiform cells) make predominantly lateral and/or feedback connections.

The bar marker: 50 um.

1.1.4 THE BLOOD SUPPLY OF THE RETINA

The retina receives its blood supply from two sources. The outer half of the retina, from the OPL to the RPE, is nourished by the choriocapillaries of the choroid. The inner half is supplied by the branches of the central retinal artery (Vaughan et al., 1983).

In the central retina, there is a much denser capillary network from the central blood vessels than in the periphery. This capillary network is absent in the central part in an area measuring 0.5 mm in diameter, and the choriocapillaries is thus the only blood supply to the foveola (Hogan et al., 1971).

1.1.5 THE PHOTORECEPTOR CELLS

There are two types of photoreceptors present in the vertebrate retina: rods and cones. The schematic drawing in Fig.1.1.4 demonstrates the major morphological features of typical rods and cones. Typical rods have cylindrical OS and IS, whereas, with the exception of the fovea, the cones have a tapered OS and a bulbous IS.

Rods are able to respond in very dim light and subserve scotopic vision. The types of cones vary with species. Man has 3, which respond predominantly to red, green or blue light. Cones are the photoreceptors subserving high acuity and colour vision. They are considerably less sensitive to light than rods, and are densely packed in the macular region.

Diurnal animals such as man have duplex retinae with well differentiated rods and cones. For example, the human retina contains 90 - 120 million rods and 4.6 - 6 million cones (Østerberg, 1935; Curcio et al., 1990), with a foveola consisting of only cones. By contrast, the retinae of nocturnal animals have principally rods. In rat retina only about 1.2% of the photoreceptor cells are cones which, though collectively present in a specific area, are not so densely packed to form a foveola or fovea as in man (Cicerone et al., 1979).
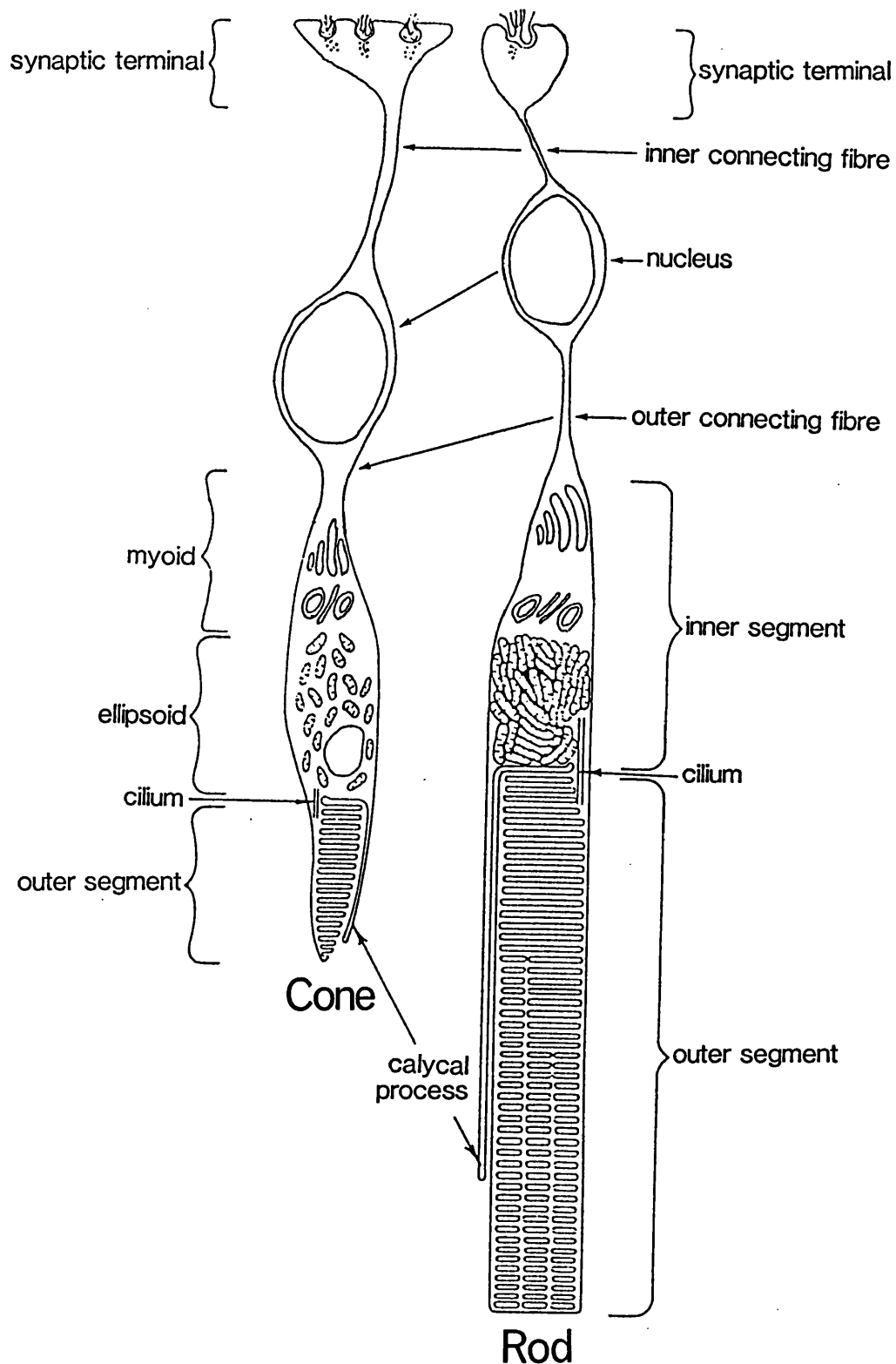
Fig.1.1.4 Schematic representation of typical rods and cones, showing major subcellular structures. (Modified from Fein and Szuts, 1982).

See text below (1.1.5.1 to 1.1.5.5) for a full description.

1.1.5.1 The outer segments

Rod OS contain a large number of parallel hollow membrane stacks or discs, rather like a stack of coins (Fig.1.1.5). New membranes are formed near the cilium of the OS by evagination of the plasma membrane (Steinberg et al., 1980). Not far from this region, the membranes become pinched off and form closed, flattened discs, totally isolated from the plasma membrane (Dowling, 1987).
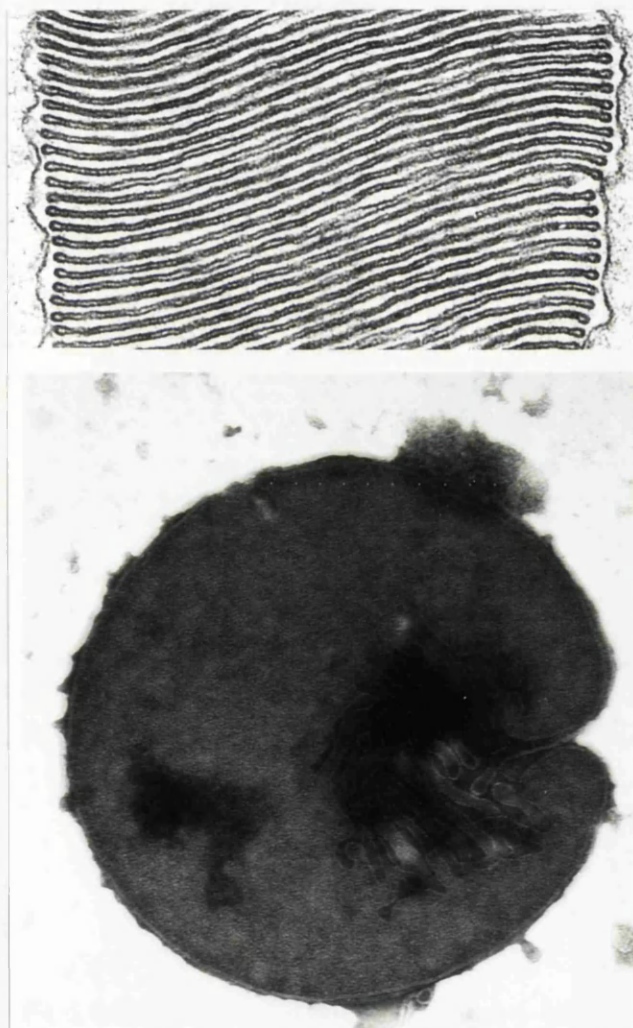
Fig.1.1.5 Electron micrographs of rod OS. (Courtesy of Prof. John Marshall).
    Upper: a cross section of a small portion of a human rod, showing the anatomical separation of the disc and plasma membrane.
    Lower: surface view of an isolated rat disc, showing the single deep incisure and fimbriate characteristic of this species. The bar marker: 1 um.

In cone OS, the arrangement of the membranes is different and the lamellae are formed from a continuously folded single membrane, so there is only a single intracellular compartment and the spaces between the lamellae are extracellular (Dowling, 1987).

Parallel to and in close proximity with each OS are long, slender (about 100 nm diameter), fingerlike structures called calycal processes (Cohen, 1972). They are outgrowths of the IS and their physiological function is unknown.

1.1.5.1.1 Composition of the disc membrane

Each rod OS contains approximately 1000 discs (Spalton and Marshall, 1984). The disc membrane is a bilayer structure which in man is composed of about 50% protein and 50% lipid (Anderson, 1983). More than 90% of the protein is the visual pigment, rhodopsin (see 1.2 for details, p.36; Vuong et at., 1987). More than 95% of the lipid is phospholipids, and the phospholipid composition is similar across various species (Daemen, 1973; Anderson, 1983). Compared with other cell membranes, it has a low cholesterol content and a higher proportion of polyunsaturated fatty acids, giving a stable (in term of gross morphology) and highly fluid (in term of molecular composition) membrane (Cone, 1972; Anderson, 1983).

1.1.5.1.2 Disc turnover in the OS

Renewal of the OS continues throughout life, with the formation of new membranes at the base, the ciliary region, being balanced by loss of "old" ones at the interface with the RPE (Young and Droz, 1968). The latter, which are shed in the form of small packages, are then phagocytosed by the adjacent RPE and degraded within phagosomes (Young and Bok, 1969; Young, 1971).

In the rod OS, disc membranes and the visual pigments they contain thus progress from the cilium towards the RPE, the total transit time being between 9 and 60 days, depending on species. For example, a single rod disc takes 9 to 13 days to migrate the length of the rod OS in

monkey, 10 days in rodents, and 7 weeks in frogs (Young, 1967,1971,1976). This would give a disc production rate of approximate 4 discs an hour in the monkey and rodents, and 1 disc an hour in the frog.

The renewal of cone OS has not received as much attention as that of the rods, mainly due to the difficulty in interpretation of autoradiographs in a fluid membrane system. Nevertheless, the process of disc shedding in cones, and phagocytosis by the RPE, appears to be similar to that in rods but slower (Hogan et al., 1974; Anderson and Fisher, 1975; Young, 1976).

1.1.5.2 The inner segments

The IS is connected to the OS by a narrow cilium. This cilium is thought to be involved in a number of cellular processes, including the transfer of metabolites and the growth and maintenance of the OS (Besharse, 1986).

From consideration of different structural elements and functions, the IS is divided into two regions: an outer portion adjacent to the OS, called the ellipsoid, and an inner portion, called the myoid (cf. Fig.1.1.4, p.25; Hogan et al., 1971).

1.1.5.2.1 The ellipsoid

The ellipsoid is densely packed with mitochondria. In mammals, these mitochodria are long and slender, and are arranged in rows approximately parallel to the IS axis, running the full length of the ellipsoid. These powerhouses for ATP production are thus pivotally situated in order to provide energy for both IS and OS needs. The ellipsoid also contains vesicles bearing opsin destined for transport to the OS (Besharse, 1986).

1.1.5.2.2 The myoid

The myoid contains rough and smooth endoplasmic reticula and the Golgi apparatus. The rough endoplasmic reticulum and the Golgi are the sites of protein synthesis and posttranslational modification, respectively

(Bok, 1985; Besharse, 1986). The smooth endoplasmic reticulum is responsible for synthesis of phospholipids and cholesterol. The distal region of the myoid contains abundant glycogen granules and intracellular vacuoles (Fein and Szuts, 1982).

1.1.5.3   The nucleus

The cell bodies of the rods and cones with their nuclei and cytoplasm are located in the ONL. The rod nuclei are round or slightly oval. Their chromatin is dispersed and a nucleolus is frequently seen. The cone nuclei are also slightly oval in shape and larger than those of the rods. They contain less heterochromatin than the rod nuclei, which accounts for their fainter staining seen with the light microscope. The structure of the cytoplasm of rod and cone cell bodies is essentially the same (Hogan et al., 1971).

1.1.5.4   The outer and inner connecting fibres

The cytoplasm of photoreceptors between the IS and the nucleus is called the outer connecting fibre. It contains some elongated mitochondria and vesicles of endoplasmic reticulum.

The narrow axon extending from the nucleus to the synaptic terminal is often called the inner connecting fibre. Inner fibres of the rods are about one fourth the diameter of those of the cones (Hogan et al., 1971).

1.1.5.5   The synaptic terminal

The synaptic terminal is the site where light-triggered signals are transmitted to the second order neurones. The terminal of rods is oval and known as a "spherule", whilst cones end in a broad, conical swelling, the "pedicle".

Both rods and cones make two types of synaptic contact with bipolar cells and only one type with horizontal cells. The main one, to both bipolar and horizontal interneurones, is via ribbon synapses, characterized by an electron-dense "ribbon" in the

presynaptic process (Ehinger and Dowling, 1987). The ribbon is orientated perpendicularly to the cell membrane and is surrounded by small synaptic vesicles that are thought to contain neurotransmitter. The photoreceptor synaptic surfaces exhibit invaginated connections, usually with two lateral processes from horizontal cells and a central dendrite from a bipolar cell. Because these invaginated synapses contain three processes, they are called "triads"; rods usually have only one triad, whilst cones may have up to twenty (Spalton and Marshall, 1984).

The other type of synaptic contact, made by photo-receptors with certain bipolar cells, is a superficial or flat one, and this is less common (Ehinger and Dowling, 1987). Freeze-fracture micrographs have shown the presence of intramembraneous particles similar to the ones observed at excitatory synapses elsewhere (Raviola and Gilula, 1975).

Various lines of evidence have suggested that glutamate is most likely to be a neurotransmitter for rods and cones, but it may not be the only one (for reviews: Ehinger and Dowling, 1987; Massey and Redburn, 1987; Daw et al., 1989).

1.1.6 HIGHER-ORDER NEURONES

There are five types of higher-order neurones in the retina (cf. Fig.1.1.3, p.23). Four of them, bipolar, horizontal, amacrine and interplexiform cells, are involved in the processing of visual signals generated by the photoreceptor cells. The fifth type, ganglion cells, is responsible for collecting the processed signals and transmitting them to the brain.

1.1.6.1 Bipolar cells

Bipolar cells are located in the INL. Their dendritic processes ramify in the outer plexiform layer, where they make contact with photoreceptor and horizontal cells via the synaptic triad, and their axonal processes terminate in the IPL, where they make contact with ganglion and/or

amacrine cells (Kolb and Nelson, 1984). In the IPL, bipolar cell synapses are identified by the presence of synaptic ribbons (Ehinger and Dowling, 1987).

Bipolar cells are designated as "ON" or "OFF" cells. Those, which are kept hyperpolarized in the dark by a neurotransmitter so that they depolarize when transmitter release is diminished or stopped by light, are called ON-bipolar cells; those, which are kept depolarized by the transmitter and hyperpolarize when the release of transmitter is diminished or stopped, are called OFF-bipolar cells.

1.1.6.2 Horizontal cells

Horizontal cells are located in the outer aspect of the INL and are laterally extensive neurones. In some species some horizontal cells have a short axon. Generally, all processes terminate in the triads of the OPL, although the axon terminal in the teleost fish has been found to contact amacrine cells in the IPL (Dowling, 1987).

1.1.6.3 Amacrine cells

Most amacrine cell bodies are located in the inner aspect of the INL, but, in some species, a proportion are located in the ganglion cell layer. The latter are known as displaced amacrines (Masland and Tauchi, 1986). All processes terminate in the IPL.

1.1.6.4 Interplexiform cells

Interplexiform cells have been found in some species and are relatively few in number (Gallego, 1971; Boycott et al., 1975). Their cell bodies are located on the inner aspect of the INL, among the amacrine cells. In the IPL, they receive input from the amacrine cells and form a feedback pathway to the OPL where they synapse on bipolar or horizontal cells (Dowling, 1979; Massey and Redburn, 1987).

1.1.6.5 Ganglion cells

Ganglion cell bodies are situated in the innermost cell layer of the retina. A very small number, called displaced ganglion cells, are in the INL. The cells are multipolar, with dendrites extending laterally into the inner plexiform layer. Their axons run internally and then become parallel to the inner surface of the retina. Here they form the nerve fibre layer and run to exit the globe collectively in the optic nerve. In man approximately 50% of the ganglion cells are subservient to the macula.

1.1.7 GLIA: MULLER CELLS

Muller cells are giant glia that extend through the thickness of the retina from the ILM to the OLM. Their villous processes extend beyond the outer limiting "membrane". Perikarya are located in the inner nuclear layer. Muller cell cytoplasm constitutes about 35% of the retinal tissue (Rasmussen, 1972) and encapsulates and separates neurones from themselves and, when present, from retinal blood vessels.

These cells are not only structural elements. Increasing evidence shows that they play a vital role in maintaining homeostatic balance in the retina (Ripps and Witkovsky, 1985; Farber and Adler, 1986). Evidence has been obtained for the existence in the rabbit Muller cell plasma membrane of a K^+-dependent, electrogenic pump, which leads to an active K^+ accumulation (Reichenbach and Eberhardt, 1986). This appears consistent with a significant role of the Muller cells in contribution to the electroretinogram (cf. 1.4.2, p.67).

In addition to Muller cells, sparse populations of astrocytes and microglia also exist in the retina (Hogan et al., 1971; Farber and Adler, 1986).

Compared to neurones, however, glial cells remain the least well understood and the least studied cell type in the retina (Farber and Adler, 1986).

1.1.8 RETINAL PIGMENT EPITHELIUM (RPE)

The RPE consists of a single layer of hexagonal cuboidal cells (Spalton and Marshall, 1984). It lies between the neural retina and the highly vascularized choroid and is the site of the outer blood-retinal barrier. Protoplasmic processes, extending from the apical surface of the cells, surround the OS (e.g., Clark, 1986). This arrangement substantially increases the apical surface area and thus the exchange border between the RPE and photoreceptors. In addition, the RPE processes phagocytose the oldest OS material.

The RPE is the site of storage of retinoids in the eye. Here are found the activities of retinyl ester synthase and hydrolase, 11-cis retinol dehydrogenase (Bridges, 1976) and retinoid isomerase (Bridges and Alvarez, 1987; Bernstein et al., 1987).

1.1.9 INTERPHOTORECEPTOR MATRIX (IPM) AND INTERPHOTO-RECEPTOR RETINOID-BINDING PROTEIN (IRBP)

(1) IPM

The IPM is the extracellular material that occupies the space between the photoreceptor cells and the RPE. This mucoid matrix consists of a mixture of proteoglycans and glycoprotein. It has been implicated in a variety of functions such as retinal adhesion to the RPE, transport of retinoids, and transfer of nutrients to the photoreceptor cells from the choroidal vascular supply (e.g., Gonzalez-Fernandez et al., 1985; Porrello and LaVail, 1986). Recently, Hewitt et al. (1990) have reported that some factors derived from the IPM promote photoreceptor survival in embryonic chick cell culture.

It has been demonstrated histochemically that a regional heterogeneity exists in the composition of the IPM. For example, in the rat and mouse the IPM, concentrated at the apical surface of the RPE, is composed in large part of sialoglycoconjugates, whereas the IPM in the junction of the OS and IS is composed predominantly of chondroitin SO_4 A and C (Porrello and LaVail, 1986).

Components of the IPM are thought to be produced by the photoreceptor IS, Muller cells and the RPE (Edwards, 1982; Gonzalez-Fernandez et al., 1985; Hollyfield et al., 1985).

It has been recently found that cone OS are surrounded by a cylindrically shaped, extracellular domain of proteoglycan, called the cone matrix sheath. These structures have been suggested to isolate a unique extracellular environment around cone OS. In addition, they have been considered as physically linking the retina with the RPE (e.g., Hollyfield et al., 1989).

(2)   IRBP

Within the IPM, a sialic acid-containing glycoprotein that binds retinoids has been identified and designated as interphotoreceptor retinoid-binding protein, IRBP (Bridges et al., 1984; Chader and Wiggert, 1984). In contrast to cellular retinoid-binding proteins which reveal strict binding specificity, IRBP has been reported to bind retinol, retinal, retinoic acid, and retinyl acetate and palmitate, as well as several nonretinoids, e.g., palmitate and vitamin E (for a review: Saari and Bunt-Milam, 1986).

Endogenously, light-dark difference in the binding of retinol has been detected (Bridges et al., 1984; Chader and Wiggert, 1984). For example, in fully-bleached bovine eyes, up to 30% of the total molecules capacity of IRBP for all-trans retinol binding is taken up; whereas in half-bleached eyes, it is only 5-10% replete. In the latter situation the proportion of 11-cis retinol is higher (Bridges et al., 1984). Because of the differential light-dark binding and the unique localization of the protein, it has been suggested that IRBP functions as a physiological, visual-cycle retinoid carrier between the photoreceptor cells and RPE. However, this transport function should be considered as provisional until more evidence is available (for a review: Saari and Bunt-Milam, 1986). Recently, by incubation of toad RPE eye-cup with ^{3}H-retinol and IRBP, Okajima et al. (1989) found that IRBP

delivers the retinol into the RPE. Jones et al. (1989a,b) have also reported that IRBP carrying 11-cis retinal induces recovery of salamander photoreceptor sensitivity. In contrast, Ho et al. (1989) present evidence suggesting that IRBP may not be essential for retinoid transport but merely serves as a buffer protein in high light levels, allowing gradual exchange of retinoids between RPE and photoreceptor membranes.

IRBP is synthesized in the photoreceptor IS and is then transferred extracellularly to the region of the RPE apical surface (Bunt-Milam and Saari, 1983; Fong, et al., 1984a,b; Gonzalez-Fernandez, et al., 1985). Immunocyto- chemical studies have localized IRBP among the photoreceptor OS, and in greatest concentration along the apical surface of the RPE in the rat, bovine, monkey, and human (Bunt-Milam and Saari, 1983; Eisenfeld et al., 1984; Fong et al., 1984a,b; Gonzalez-Fernandez et al., 1985).

The native IRBP molecule from bovine retina has a molecular weight of about 225 to 260 kDa (Chader and Wiggert, 1984; Fong, et al., 1984b; Adler and Evans, 1985). In sodium dodecyl sulphate-gel electrophoresis, IRBP is seen at 140 kDa (e.g., Adler and Evans, 1985).

1.2 ROD VISUAL PIGMENTS: RHODOPSIN

1.2.1 STRUCTURE AND PROPERTIES OF RHODOPSIN

Rhodopsin is the visual pigment of the mammalian rod photoreceptor cell. In situ the molecule may be considered to be a multicomponent entity, consisting of an apoprotein (opsin) to which a chromophore (11-cis retinal) is covalently bound. Rhodopsin has a molecular weight of between 34-40 kDa, varying with species and/or methodology (e.g., Robinson et al., 1972; Molday and Molday, 1979; Hargrave et al., 1983). The bovine molecule consists of 350 amino acids (Hargrave et al., 1983) and is arranged in 7 alpha helices, spanning the disc membrane with the carboxyl terminal on the cytoplasmic side and the amino terminal on the intra-discal side (Fig.1.2.1). Some 7 (Wilden and Kuhn, 1982) or 9 (Stryer and Bourne, 1986) serine and threonine phosphorylation sites lie close to the C-terminal. Two oligosaccharides, consisting of mannose and N-acetylglucosamine, are covalently bound to asparagine residues close to the N-terminal (Fukuda et al., 1979).
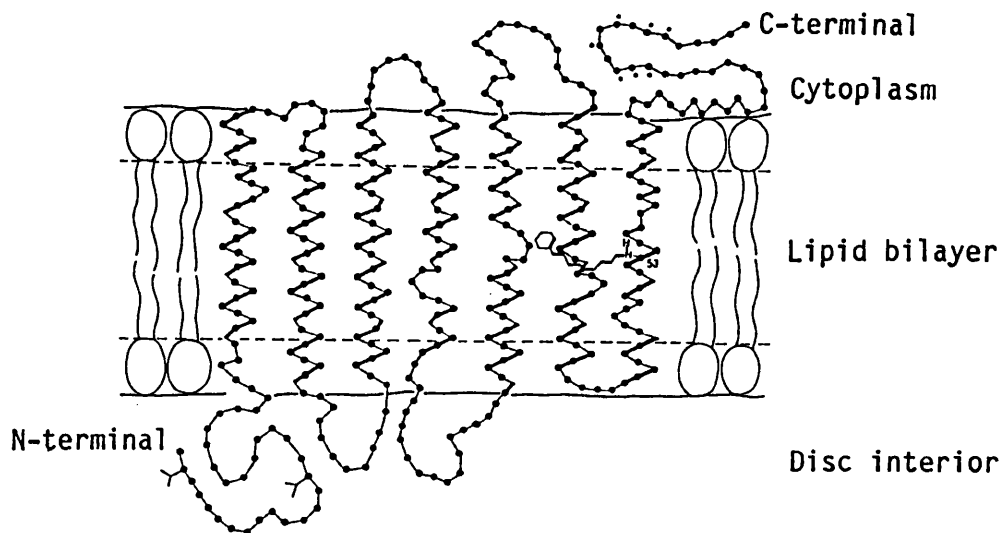
Fig.1.2.1 A model for the rhodopsin molecule in the disc membrane. (After Kemp, 1984).

Seven alpha helices spans the disc membrane with the C-terminal on the cytoplasmic side and the N-terminal on the intra-discal side. Seven phosphorylation sites (*) lie close to the C-terminal. Two oligosaccharides (Y) are bound to the N-terminal. The 11-cis retinal is attached to a lysine, the 53rd amino acid counting from the C-terminal.

There are about 10^9 rhodopsin molecules in a human rod (Dowling, 1987), and 99% of them are likely to be located in the disc membranes (Kamps et al., 1982). The surface density of rhodopsin in the disc membrane is about $30,000/um^2$, among the highest of any known integral membrane protein (Findlay, 1986). The rest of the pigment is in the plasma membrane of the rod OS and IS (Kamps et al., 1982). In the OS, the visual pigment represents more than 60% of the total protein mass and more than 95% of the disc membrane's intrinsic protein content (Vuong et al., 1987).

Since disc membranes are of a highly fluid nature, it appears that rhodopsin rotates around an axis perpendicular to the plane of the membrane and undergoes translational Brownian motion (Cone, 1972; Liebmann and Entine, 1974). The molecule is aligned in such a way that the dipole of 11-cis retinal lies parallel to the disc surface, i.e., transverse to the long axis of the photoreceptors (cf. Fig.1.2.1), thus optimising light absorption (Wald, 1968).

The absorption of light by rhodopsin alters the structure of the molecule, initiating visual excitation. In most species, the absorption maximum lies at about 500 nm: e.g., 497 nm in man (Crescitelli and Dartnall, 1953), 498 nm in rat (Dowling and Sidman, 1962), 499 nm in ox (DeGrip et al., 1972), and 502 nm in toad (Dartnall, 1954). The light-absorbing part of the molecule is 11-cis retinal, i.e., the visual pigment chromophore. Retinal is the aldyhyde derivative of vitamin A, a beta-ionone ring linked to a five double-bonded polyene chain that terminates in an alcohol group (Fein and Szuts, 1982). The 11-cis retinal is attached via an aldimine bond (-CH=N-) to a lysine residue, the 53rd amino acid counting from the carboxyl terminal of the opsin (cf. Fig.1.2.1). The covalent bond between retinal and opsin occurs via a spontaneous Schiff base reaction between the aldehyde and amino groups (Bownds, 1967).

Rhodopsin is unstable in the presence of light. When it absorbs a photon, the molecule spontaneously and rapidly progresses through a sequence of transient states

- the bleaching sequence - without the need of enzymatic
or metabolic energy (see below). In contrast, the
regenerative reactions are energy-consuming.

1.2.2 BLEACHING OF RHODOPSIN

Absorption of a photon of light by a rhodopsin
molecule involves the isomerization of retinal from the
11-cis to the all-trans form (Hubbard and Kropf, 1958).
A series of spectroscopically distinguishable, metastable
photoproducts are formed, including metarhodopsin II or
"activated rhodopsin" which is responsible for triggering
the biochemical steps transducing the light stimulus into
an electrical response. The quantum yield is about 0.65
to 0.7 (Dartnall, 1968), i.e., 65-70% of the rhodopsin
molecules that absorb a photon are activated.

The bleaching sequence of rhodopsin is shown in
Fig.1.2.2.

Rhodopsin   (505nm)
                \
                / <-- Photon
                \
6 psec   / ....photochemical reaction
                \
                ↓
          Bathorhodopsin   (545nm)

170 nsec   │ ....thermal reaction
                  ↓

          Lumirhodopsin   (515nm)

35 usec   │
                  ↓

          Metarhodopsin I   (480nm)

700 usec   │↑│   H⁺
                  │ │
                  ↓

          Metarhodopsin II (380nm) ....Rh*
                  /   \
                 /   \
50 sec   /   \   90 sec
               /   \
              ↓   490 sec ↓
Metarhodopsin III -------> Retinal-Opsin (380nm)
                                /
   (470nm)   /
                              /
               270 sec   /
                            /
                           ↓

          Retinol + Opsin

          (330nm)   (280nm)
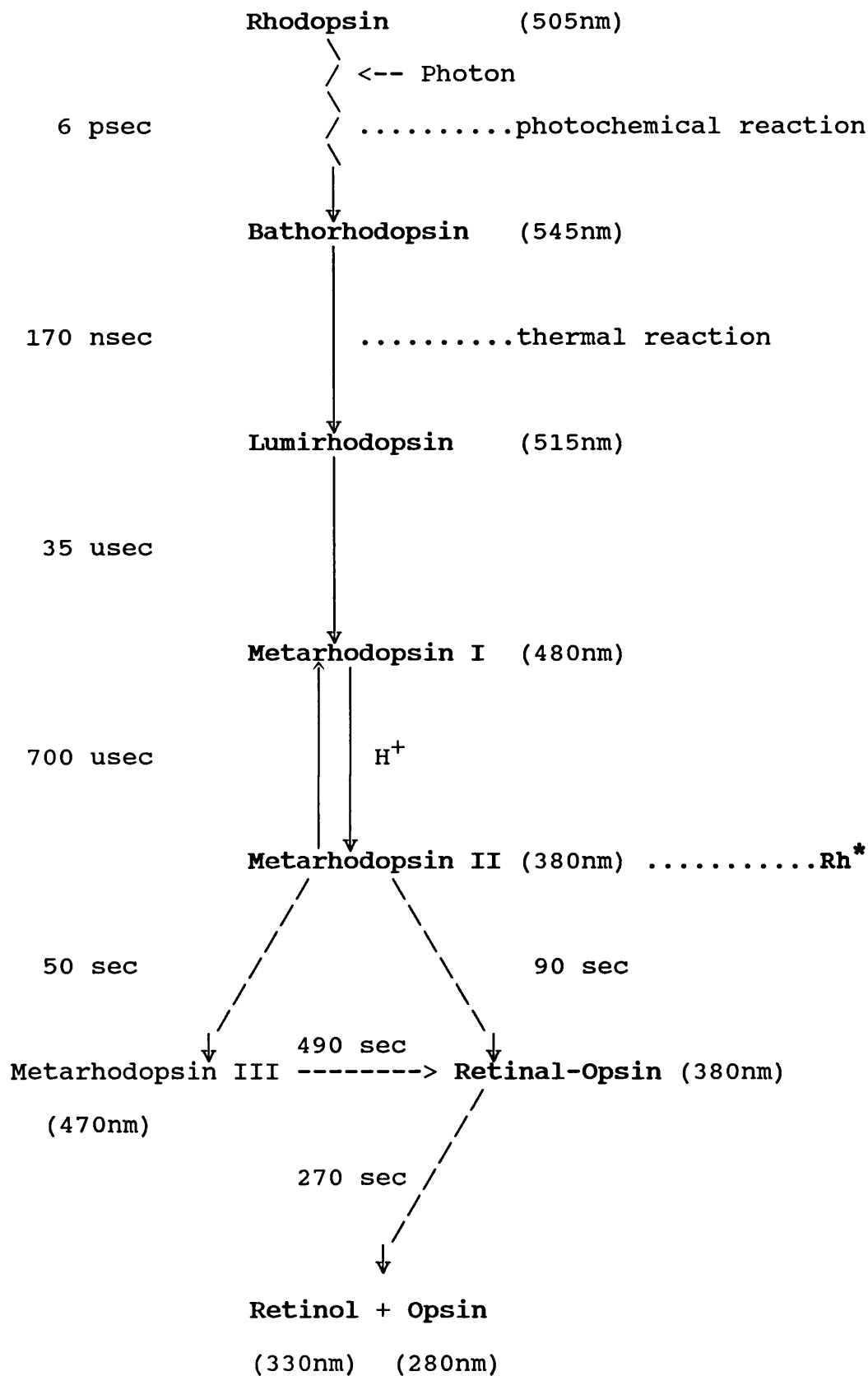
Fig.1.2.2 The bleaching sequence of rhodopsin in the
intact isolated retina at room temperature.
     The wavelength of the absorption maximum varies
slightly between species; those shown are for the frog.
The reaction half lives are approximate.   Rh* = activated
rhodopsin. (Modified from Kemp, 1984).

In the reactions shown in Fig.1.2.2, only the initial one is photochemical; all subsequent reactions are thermal. The transient intermediates produced have different spectral properties. When the reactions occur at or near to physiological temperatures, they may be broken into two groups: those fast enough to lead to the primary triggering of the rod response, and those much too slow to play any direct role in transduction (cf. Fig.1.2.2). The break occurs at the formation of metarhodopsin II (meta II).

(1) The primary reaction: formation of bathorhodopsin

The initial reaction is extremely rapid, with formation of the photoproduct, bathorhodopsin, occurring within less than 3 to 6 psec of absorption of the photon (Green et al., 1977). The isomerization of the chromophore takes place in this process (Birge, 1981).

(2) The rapid thermal reactions

As shown in Fig.1.2.2, there are three thermal reactions which are fast enough to play a role in transduction.

a) The first is the conversion of bathorhodopsin to lumirhodopsin. This transition is very rapid, with a half life of about 170 nsec in the intact rod (Ostroy, 1977).

b) The second is the transition from lumirhodopsin to metarhodopsin I (meta I), which occurs in less than about 100 usec (Ostroy, 1977). There is no major alteration in the configuration of the protein (Ostroy, 1977), but a charge displacement occurs, the latter giving rise to a component of the fast photovoltage, or early receptor potential (ERP) (see p.69), which is the first electrical activity displayed by rods following light absorption (Arden, 1969). Present evidence suggests that the ERP has no functional significance, but it has proved a useful probe of reconstituted membrane systems containing rhodopsin (Montal et al., 1981).

The reactions up to and including the formation of meta I are not affected by the surrounding medium which

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may be fully dried (Kimbel et al., 1970) or contain abnormal lipid components (Applebury et al., 1974).

c) The third is the formation of meta II. This reaction involves intermolecular processes, requires the presence of water and is sensitive to pH (Baumann and Zeppenfeld, 1981) and lipid environment (Applebury et al., 1974).

Major conformational changes occur in the protein at this stage:

i) The chromophore of meta II can be readily attacked by hydroxylamine (NH_2OH) (Johnson, 1970) and sodium borohydride ($NaBH_4$) (Bownds, 1967), indicating that it becomes more exposed.

ii) The rate, at which the C-terminal of the molecule is cleaved by thermolysin, increases with formation of meta II, suggesting that this region of the protein also becomes more exposed (Kuhn et al., 1982).

iii) The number of reactive sulphydryl groups increases, again indicating that there is an unfolding of the protein (Regan et al., 1978).

iv) Fourier transform infrared difference spectroscopy (FTIR) studies indicate that a small part of the protein back-bone undergoes a conversion from alpha-helical to beta-type structure, and, of the known photo-intermediates, the protein structure of meta II is the most distorted (Rothschild et al., 1987).

v) The blue-shifted maximum absorption of meta II (cf. Fig.1.2.2) suggests that at least a single proton per rhodopsin should have been released during the reaction, and deprotonation of the Schiff's base has been suggested to cause such a spectral shift (Fein and Szuts, 1982).

Based on the above and the timing of its appearance and degradation in the rhodopsin bleaching sequence, meta II is considered as most likely to be the "activated rhodopsin", Rh^*, which triggers the cGMP cascade (see p.47).

Much evidence suggests that meta II binds to and activates transducin (p.48) (Kuhn, 1980; Hofmann, 1986). Furthermore, experiments in measuring light-activated PDE

activity (p.49) in the presence of GTP also suggests that meta II is likely to be Rh[*] in accord with the following observations (for a review: Applebury et al., 1986):

  i) PDE activation via transducin is tightly coupled to the thermal rate dependence of meta II formation.

 ii) The pH-dependence of meta II formation matches that of PDE activation and does not implicate meta I.

iii) In weak bleaches, PDE activation persists only as long as the lifetime of meta II.

 iv) NH_2OH destroys meta II and PDE activation at the same rate.

(3)   The slow thermal reactions

As shown in Fig.1.2.2, none of the spectrally observable reactions following the formation of meta II occurs fast enough to play any direct role in triggering the transduction process. However,it has been suggested that they may reflect some regulatory role of rhodopsin in controlling the sensitivity of the photoreceptor cell, i.e., its light and dark adaptation (Ernst and Kemp, 1972; Donner and Hemila, 1979).

The overall decay of meta II terminates in its hydrolysis to retinal and opsin. Kinetic models based on spectroscopic observations describe parallel pathways: one leading directly to the final products and the other going via metarhodopsin III (meta III) (cf. Fig.1.2.2; Ernst and Kemp, 1972; Blazynski and Ostroy, 1981). It is noteworthy that, when light photolyses less then 5% of rhodopsin in the intact frog retina, no meta III can be detected but, when more than 15% is photolysed, meta III is detectable (Donner and Hemila, 1979). This supports the idea of a "storage side pathway", favoured under very high rates of bleaching (Chabre and Breton, 1979).

Rothschild et al. (1987), using FTIR difference spectroscopy, obtained the first direct evidence that the decay of meta II involves refolding of the protein from its distorted form back to its original state, i.e., opsin. They suggested that the refolding during meta II decay might restore the binding pocket of rhodopsin that

allows direct access of 11-cis retinal, but not all-trans or 13-cis retinal, to its binding site. Protonation of the Schiff base would not require further protein alteration, as indicated by the evidence that the binding is determined mainly by steric constraints (Liu et al., 1984) and by the fact that 11-cis retinal can bind spontaneously to opsin without any intermediate conformational steps (Daemen and DeGrip, 1984).

In the intact retina, free all-trans retinal, produced as a result of meta II and/or meta III decay, is enzymatically reduced to retinol via the action of retinal reductase (Cone and Cobbs, 1969).

1.2.3 REGENERATION OF RHODOPSIN

1.2.3.1 Rhodopsin regeneration in vivo

In comparison with the bleaching sequence, the regeneration of rhodopsin is poorly understood. The isomerization of all-trans retinoids to the 11-cis configuration, either by enzymatic or by photochemical means, is essential for rhodopsin regeneration in the eye (Fein and Szuts, 1982).

(1) Photoregeneration

Photoregeneration of rhodopsin from photo-intermediates (mainly meta II and meta III) is known to occur during flash-irradiation (Baumann and Ernst, 1970) and also, e.g. in the frog, during continuous illumination (Baumann, 1970). However, given their lifetime, extinction coefficients and the quantum efficiencies, the calculated rate of photoregeneration from the intermediates is less than 6% of the rate of rhodopsin bleaching (Fein and Szuts, 1982). Thus over 94% of bleached rhodopsin does not undergo photoregeneration.

(2) Enzymatic regeneration

It has been suggested that vertebrate retinae rely almost solely on enzymatic regeneration, and that this

regeneration does not occur before the visual pigment is hydrolyzed to opsin and the free chromophore (Fein and Szuts, 1982). To regenerate 11-cis retinal from all-trans retinol, two reactions must happen: (1) all-trans retinol must be isomerized to an 11-cis isomer via an isomerase reaction, and (2) it must be oxidized to the aldehyde via an oxidoreductase reaction.

It is now known that the retinal pigment epithelium (RPE) plays a major role in the regeneration of 11-cis retinal. For example, isolated retinae, stripped of their pigment layer, are unable to restore their visual pigment, even when perfused under conditions in which the photoreceptor cells maintain their light sensitivity for many hours (Weinstein et al., 1967).

A more pertinent observation in support of the role of RPE comes from the studies of Reuter et al. (1971) on the bullfrog. The rods of this retina contain either rhodopsin (502 nm) or porphyropsin (522 nm, having 11-cis 3-dehydroretinal as the chromophore). The two types of rods are normally segregated into separate geographic locations in the retina. The corresponding regions of the RPE store either retinol or 3-dehydroretinol. When the two retinal regions are excised, bleached and deliberately placed back onto each other's epithelial region, the pigment contents of the rods are exchanged: rhodopsin for porphyropsin and vice versa.

In addition, retinol isomerizing activity has been detected in the RPE of frog, rat and ox (Bernstein et al., 1987; Bridges and Alvarez, 1987). However, it is possible that a small amount of isomerizing activity also exists in the photoreceptor OS, perhaps with species variation (Bridges, 1976).

It is well established that retinoids are esterified in the RPE, and it may be that the esterification is essential for the isomerization to occur (for a review: Bridges, 1976).

To reiterate, the regeneration of rhodopsin _in vivo_ is not well understood, but a general concept may be summarized as below (for a review: Bok, 1990):

i) The all'-trans retinol, released from bleached
rhodopsin, is transported from the photoreceptor OS
to the RPE. IRBP plays a role in this process, but it
is not clear if IRBP is essential for its transport
(cf. p.34).

ii) In the RPE, all-trans retinol is esterified and, if
necessary, stored. It is then converted to the 11-cis
form. The energy for isomerization is derived, at
least in part, from hydrolysis of the ester bond.
11-Cis retinol is the product and this is then
oxidized to 11-cis retinal before released, by an as
yet unidentified process, into the IPM. It is
probably that transfer to photoreceptors is mediated
by IRBP. With species variation, small amounts of
all-trans retinol may be isomerized and oxidized to
11-cis retinal within the photoreceptor OS, without
involvement of the RPE (Bridges, 1976).

iii) 11-cis retinal binds spontaneously to opsin to form
rhodopsin (Daemen and DeGrip, 1984).

The rate of regeneration varies among visual pigments
and among photoreceptor cells. For example, human cone
pigments regenerate about four times faster than human
rhodopsin (Rushton, 1972); the rate of rhodopsin
regeneration in rat is slower than that in man (Dowling,
1963).

1.2.3.2 Rhodopsin regeneration in vitro

Regenerating rhodopsin in vitro is of direct interest
both as regards understanding the chemistry of rhodopsin
and in the generation of model systems for dynamic
investigation.

Regeneration of rhodopsin in vitro from opsin and
exogenous 11-cis retinal has be observed in rod OS (Amer
and Akhtar, 1972), rod disk membranes (Matsumoto et al.,
1978) and in some detergent-solubilized forms of rhodopsin
(Crouch, 1976), as well as in intact retina (Pepperberg,
1982; Yoshikami and Noll, 1982).

9-Cis retinal can also bind to opsin to form iso-
rhodopsin. With the exception of a slightly blue-shifted

absorption maximum (by 10 to 15 nm, cf. Table 1.2.1),
isorhodopsin is similar to rhodopsin in most respects:
(1) thermally stable in the dark, (2) photoactivated to
yield the same sequence of intermediates (Pratt, 1968),
and (3) able to initiate photoresponses (Pepperberg,
1982).

Table 1.2.1 Maximum absorbance of rhodopsin and
isorhodopsin in the rat and ox.

| Species | Maximum absorbance (nm) | | Sources |
	Rhodopsin	Isorhodopsin	
Rat	495	485	Crouch, 1976
	498		Dowling & Sidman, 1962
Ox	499		DeGrip et al., 1972
	498	483	Crouch, 1976
		487	Hubbard, 1956

Because retinal and its derivatives are hydrophobic,
they are only poorly soluble in aqueous media. To provide
rod OS with exogenous chromophores, many workers apply a
method in which retinal is suspended in media containing
1-20% ethanol (e.g., Blatz et al., 1968; Pepperberg,
1982). In addition, Yoshikami and Noll (1982) introduced
the use of phospholipid vesicles as a vehicle for
delivering chromophores, which they claim to be more
efficient than using ethanol. These lipid vesicles
neither disrupt the plasma membrane nor affect the rod
dark current even after many hours of exposure (Yoshikami
and Noll, 1982).

1.3 CYCLIC GMP (cGMP) CASCADE

1.3.1 INTERNAL TRANSMITTER HYPOTHESIS

Phototransduction comprises the sequence of events, initiated by photon absorption by the visual pigments, that leads to local modulation of photoreceptor OS membrane dark current (see 1.4.1, p.64).

Investigations of rod phototransduction have been focussed by the insight that the process requires an internal transmitter to act between a disc and the plasma membrane. This premise was deduced from the fact that rhodopsin molecules that trigger the light response are integrally embedded in the bilayer of the disc membranes, and these are not continuous with the OS plasma membrane, except in the ciliary region (cf. p.26). Moreover, since a single absorbed photon triggers an electrical response with about 10^5-fold energy amplification, many hundreds to thousands of transmitter molecules must be "produced" per chromophore isomerization (Baylor et al., 1979b).

From the 1970s, the two principal candidates for internal transmitter in rods have been calcium and cGMP (for reviews: Lewin 1985; Pugh and Cobbs, 1986).

(1) The "calcium hypothesis" suggested that Ca^{2+} is normally stored inside rod discs and that the activation of rhodopsin by light leads to its release into the cytoplasm, thus allowing it to diffuse to the plasma membrane and interact with the Na^+ channels, lowering the membrane conductance and generating the electrical response. This hypothesis dominated the field of rod phototransduction for over a decade. However, from around 1985, evidence has accumulated which refutes this concept.

(2) The "cGMP hypothesis" suggested that activated rhodopsin is capable of initiating an enzyme cascade that includes cGMP PDE and degrades endogenous cGMP (the transmitter). Since the plasma membrane Na^+ channels are normally maintained open by cGMP, an electrical response ensues. Considerable evidence has

now accumulated to show that this system functions and that the enzyme reactions lead to rapid hydrolysis of cGMP with a very large built-in amplification (see below).

1.3.2 THE MAIN PROTEINS INVOLVED IN THE cGMP CASCADE

1.3.2.1 Rhodopsin

Rhodopsin is an integral membrane protein, whose characteristics of light absorption and bleaching have been described in section 1.2 (p.36). Formation of meta II (Rh^*) during rhodopsin bleaching activates a G-protein (transducin), triggering a series of amplifying, biochemical events which are now described (see below).

1.3.2.2 Transducin

This peripheral membrane protein, originally detected and described by its GTPase ability (Wheeler and Bitensky, 1977), is a member of the GTP-binding protein (or G-protein) family, and is now given the specific name of transducin (T) (Fung et al., 1981; Stryer and Bourne, 1986). The family of G-proteins play key roles in many hormonal and sensory transduction processes in eukaryotes - carrying signals from activated membrane receptors to effector enzymes and channels (Stryer and Bourne, 1986). Transducin plays an intermediary role in phototransduction in rods and cones - carrying signals from Rh^* to the PDE (see below), leading to the hydrolysis of cGMP (Fung et al., 1981; Stryer and Bourne, 1986).

Transducin, as other G-proteins, is a trimer consisting of α (39 kDa), β (36 kDa) and γ (8 kDa) subunits (Kuhn, 1980; Fung et al., 1981). It is present in the OS in molar amounts of about 1/10th the concentration of rhodopsin.

Transducin cycles between an inactive GDP state and an active GTP one (some authors refer solely to the latter as transducin; cf. Stryer, 1986). When GTP exchanges for GDP, the T_α dissociates from $T_{\beta\gamma}$. Rh^* catalyzes this

GTP-GDP exchange. GTP.Tα, the activated form, can then stimulate the PDE. Transducin is the only protein known to be acted upon by rhodopsin.

It has been suggested that one Rh[*] molecule can catalyze the formation of GTP.Tα at the rate of about 1 per msec. The Rh[*] is then free to activate another transducin molecule until it is inhibited (Pfister et al., 1985; Wilden et al., 1986; Chabre and Deterre, 1989). Fung and Stryer (1980) have shown that the bleaching of a single rhodopsin leads to the release of up to 500 GDP, suggesting that up to 500 transducin are activated by a single Rh[*].

GTP.Tα is hydrolysed to GDP.Tα by a GTPase activity inherent in the α-subunit. It can then rejoin T$_{\beta\gamma}$ to form GDP.T. It has been shown that hydrolysis of GTP requires 0.25 to 1.5 seconds and is therefore fast enough to account for the recovery rate of the light-stimulated signal of the photoreceptor cells (Dratz et al., 1987).

1.3.2.3 cGMP Phosphodiesterase (PDE)

This second peripheral membrane protein is also loosely bound to the cytosolic face of disc membranes and is readily dissociated by reduction in ionic strength and removal of Mg^{2+} ions from the bathing medium (Baehr et al., 1979). The relative molar abundance of rhodopsin, transducin and PDE in rod OS is about 100 : 10 : 1 (Baehr et al., 1979; Stryer and Bourne, 1986).

The isolated PDE system consists of two large subunits (α and β), with molecular weights of about 88 kDa and 84 kDa, and a third small polypeptide with molecular weight of about 11 kDa (Baehr et al., 1979; Hurley and Stryer, 1982). The latter provides an inhibitory constraint (I), so that the enzyme is activated to PDE[*] when I is removed by limited proteolysis (Hurley and Stryer, 1982) or by 1-1 binding of PDE and GTP.Tα (Yamazaki et al., 1983). However, there is still some dispute about the activation process. Most recently, Kroll et al. (1989) have suggested that I does not completely dissociate from the PDE$_{\alpha\beta}$; and Deterre et al. (1988) have proposed that the composition of the native

enzyme is $PDE_{\alpha\beta}$-I_2, i.e., each PDE molecule contains two inhibitory subunits, and two GTP.T_α are required to fully activate one PDE.

The photoactivation of a single rhodopsin molecule can, via the activation of 500 transducin molecules, lead to the activation of 500 PDE molecules. Each PDE[*] can in turn hydrolyse some thousands of cGMP per second (e.g., 1,000 cGMP/PDE/sec, Fesenko and Krapivinsky, 1986; 2,000-4,000, Deterre et al., 1987; 4,200, Stryer, 1987; 1,000,000, Liebman and Pugh, 1981). Thus, through these two amplification processes, a single Rh[*] leads to hydrolysis of up to about 10^5-10^6 cGMP in, e.g., 0.5 sec, a time well within the 1 to 2 sec rise time of the photocurrent (Stryer, 1986).

The light-activated PDE is strongly regulated by ATP, to the extent that micromolar concentrations of the latter will suppress PDE activation (Liebman and Pugh, 1981; Kawamura, 1983). It has been hypothesized that ATP mediates the quenching of PDE through phosphorylation of bleached rhodopsin (Sitaramayya and Liebman, 1983; see below).

PDE activity is also affected by Ca^{2+} but there is controversy as to which way; although it has been reported that activity is reduced when the Ca^{2+} level is increased (Chader et al., 1974; Yee and Liebman, 1978; Cook et al., 1986), the opposite effect has also been suggested (Kawamura and Bownds, 1981; Stryer, 1986; Wagner et al., 1989).

The mechanisms governing deactivation of the PDE[*] after transduction are poorly understood and currently form an area of intense investigation. Various models, based on available evidence, have been proposed (cf. Pugh and Cobbs, 1986; Zuckerman and Cheasty, 1988; Kroll et al., 1989), and these are summarized in section 1.3.3.2 (p.57).

1.3.2.4 Guanylate cyclase

This enzyme is concentrated along the cilium of rod OS's, and its activity in the OS is much higher than in

other parts of the retina (Fleischman and Denisevich, 1979). The enzyme catalyzes the production of cGMP from GTP. Pannbacker (1973) has compared the activities of guanylate cyclase with the PDE in both human and bovine photoreceptor preparations in the light, and has found that the activity of the PDE is about 100 times higher than that of the cyclase.

Concentrations of ATP around 1 mM inhibit the enzyme, whilst lower levels (0.1 mM) stimulate it by about 40% (Krishnan et al., 1978).

Ca^{2+} inhibits the enzyme activity and lowering the extracellular concentration from about 10^{-6} M to 10^{-9} M stimulates the cyclase activity in a dark-adapted retina by more than 50% (e.g., Lolley and Racz, 1982). The mechanism may be complex as Pepe et al. (1986) found that the lowering of Ca^{2+} in complete darkness did not affect the cyclase, but in the presence of light, with as little as 0.07% of rhodopsin bleached, the activity increased by a factor of 30 when the Ca^{2+} concentration was reduced to 10^{-8} M. The mechanism by which light stimulates the cyclase in low Ca^{2+} remains unknown.

1.3.2.5 Rhodopsin kinase and phosphatase

(1) Rhodopsin kinase

It has been shown that rhodopsin has 7 to 9 phosphorylation sites situated closed to its C-terminal (p.36), and that phosphorylation via rhodopsin kinase occurs during bleaching (Kuhn and Dreyer, 1972). This 68 kDa enzyme is loosely bound to the disc membranes in the dark and tightly bound upon illumination (Kuhn, 1981). Shichi and Somers (1978) estimate the kinase content of rods at 1 per 500 rhodopsin molecules. The enzyme transfers the phosphate of either ATP or GTP to rhodopsin, which deactivates Rh[*] (Miller and Paulson, 1975; Weller et al., 1976). Sitaramayya and Liebman (1983) have shown that ATP-dependent multiple phosphorylation of rhodopsin at very weak bleaches is complete in less than 2 seconds, which is compatible with the PDE quench time of 4 seconds

measured under identical conditions. Rhodopsin phosphory-
lation and PDE quench rates are likely to be faster under
physiological conditions.

The kinase is inactive in the dark but is activated
after interaction with Rh* (Fowles et al., 1988). It is
also reported that it undergoes auto-phosphorylation and
that the unphosphorylated kinase has different enzyme or
binding characteristics from the phosphorylated one, which
may represent one of the mechanisms for the control of
rhodopsin phosphorylation (Lee et al., 1982).

There is controversy as to whether both bleached and
unbleached rhodopsin molecules are subject to
phosphorylation, since, although some studies implied that
only those molecules which had been bleached could act as
the substrate (Chader et al., 1976; Stryer, 1986), others
suggested that when small fractions of rhodopsin were
bleached, unbleached molecules were also phosphorylated
(e.g., Aton, 1986).

Phosphorylation of rhodopsin plays an important
role in the termination of phototransduction, since the
phosphorylated molecule only poorly activates transducin.
Moreover, in the presence of arrestin (see below),
phosphorylated rhodopsin is completely deactivated (e.g.,
Bennett and Sitaramayya, 1988).

Ca^{2+} may regulate rhodopsin kinase activity. Wagner
et al. (1989) have shown that, when the Ca^{2+} concentration
is decreased, the rate of transducin deactivation is
increased. Using hydroxylamine as an artificial quencher
of rhodopsin, they demonstrated that Ca^{2+} acts upon
rhodopsin kinase but not upon the rate of the GTPase.
Their results indicate that lowering of the Ca^{2+} level
increases the activity of rhodopsin kinase - increasing
the phosphorylation and accelerating transducin
deactivation. This may also explain why PDE activity is
reduced when the Ca^{2+} concentration is lowered, as
suggested by, e.g., Kawamura and Bownds (1981) (cf. p.50).

(2) Rhodopsin phosphatase

It has been shown that, in the dark, dephosphoryla-
tion of rhodopsin accompanies regeneration of the visual

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pigment _in vivo_ (Kuhn, 1974). Palczewski et al. (1989) have recently suggested that protein phosphatase 2A is likely to be the rhodopsin phosphatase which is responsible for dephosphorylation.

In preparations of isolated rod OS, the phosphatase may be lost, since dephosphorylation is usually absent (Weller et al., 1975). Its absence, however, does not prevent the bleached pigment from regenerating when exogenous 11-cis retinal is added (Weller et al., 1975; Miller et al., 1977). This suggests that the phosphorylation or dephosphorylation has no direct effect on the chromophoric binding side of rhodopsin.

1.3.2.6 Arrestin

Arrestin, or 48 kDa protein (Zuckerman and Cheasty, 1986) or retinal S-antigen (Pfister et al., 1985), is present in rods at the remarkably high ratio of at least 1/10 with rhodopsin (Pfister et al., 1985); one investigation puts the arrestin/rhodopsin ratio as 1/1 (Broekhuyse et al., 1985). Arrestin plays a significant role in the quenching of the phototransduction processes (Zuckerman and Cheasty, 1986,1988).

It has been suggested that arrestin binds to phosphorylated Rh^* but not to unphosphorylated Rh^* nor to inactive rhodopsin. It competes with transducin for binding to Rh^* (Wilden et al., 1986). Arrestin is thus suggested to serve as an inhibitory cap on phosphorylated Rh^* and to block its capacity to catalyse the activation of transducin. In the amino acid sequence of arrestin, limited but significant analogy with transducin has been detected near the C-terminal, suggesting that both proteins may recognize a common site on Rh^* (Yamaki et al., 1988).

Alternatively, Zuckerman and Cheasty (1988) have proposed another model of arrestin action during quench. They demonstrated that arrestin directly inhibits PDE^*, as indicated by its ability to inhibit activation of PDE by T_α-GMP-PNP (GMP-PNP, guanylyl-imidodiphosphate, is a non-hydrolysable GTP analog). Furthermore, in the presence

of ATP, arrestin can markedly enhance turnoff of the PDE activation velocity whilst not affecting the initial velocity (Zuckerman and Cheasty, 1986). Thereafter, by applying a cross-linking reagent to identify the target proteins for arrestin, they found that, in the presence of ATP, arrestin cross-links to both PDE and rhodopsin. The absence of ATP abolishes the cross-link with PDE but significantly increases the cross-link with rhodopsin (Zuckerman and Cheasty, 1988). They therefore hypothesized that during quench, arrestin interacts with Rh*, and ATP dissociates arrestin from Rh*. This forms activated arrestin, which subsequently binds to and deactivates PDE*.

Thus, there are possibly two mechanisms by which arrestin quenches the cGMP cascade: (1) arrestin competes with transducin for binding to phosphorylated Rh*, and (2) the arrestin, activated by Rh*, binds and deactivates the PDE*.

Activation and termination of the cGMP cascade, discussed above, is summarized below (cf. Fig.1.3.1).
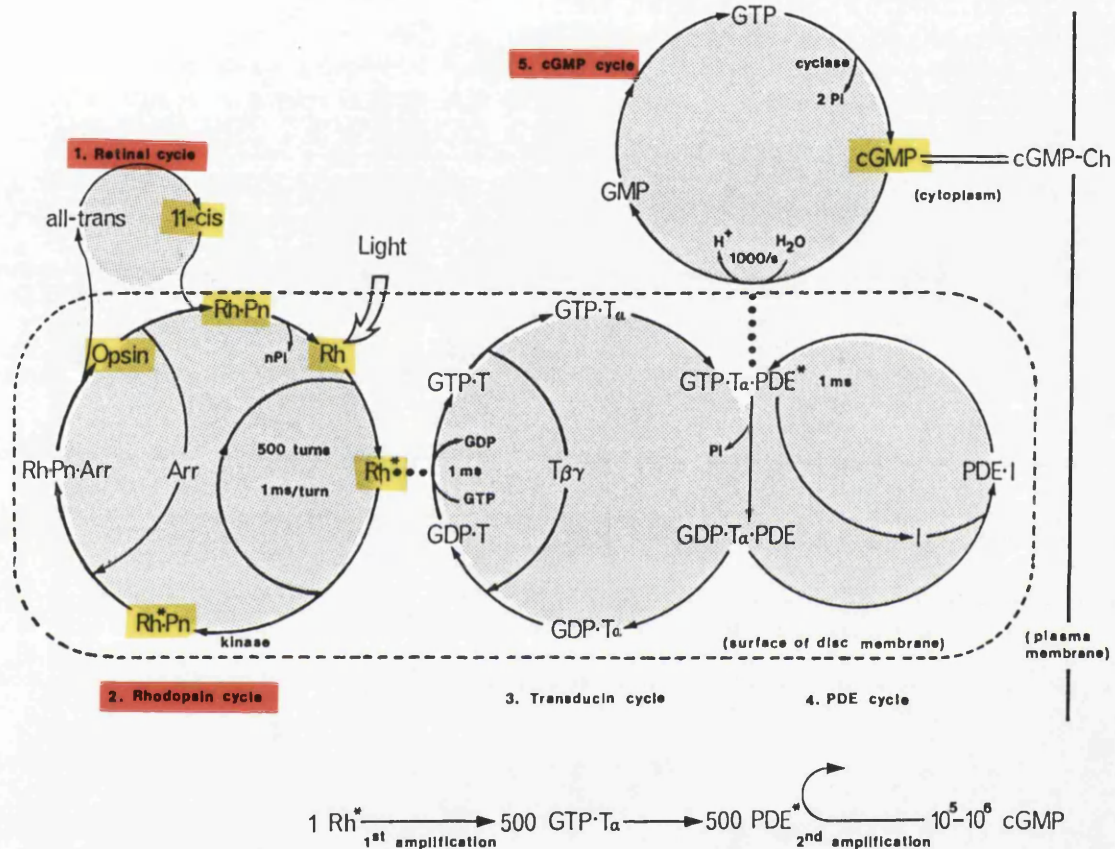
Fig.1.3.1 A summary of the cGMP cascade. (Modified from Lamb, 1986).
The five main biochemical cycles involved in photo-transduction are shown. Colour indicates the processes which have been investigated in the present studies.
Abbreviations (in order of the cycles):-
1) 11-cis and all-trans, isomers of retinal.
2) Rh, rhodopsin, with its activated form Rh*;
Rh*.P$_n$, phosphorylated Rh*;
Arr, arrestin.
ms, millisecond.
3) T, transducin, with its alpha, beta and gamma subunits.
4) PDE, phosphodiesterase, with its activated form PDE*, and the inhibitory subunit I.
5) cGMP, cyclic GMP;
cGMP-Ch, cGMP-gated channel.

1.3.3.1 Activation of the cGMP cascade:

(1) Activation of rhodopsin (Rh)

Absorption of light by Rh leads to formation of Rh*, which takes approximately 1 msec.

(2) Activation of transducin (T)

The Rh* triggers the exchange of the bound GDP for GTP, and then the GTP.T_α dissociates from $T_{\beta\gamma}$, which takes about 1 msec. Each Rh* can switch on 500 Ts within 0.5 sec. This is the first amplification of the cascade.

(3) Activation of PDE

The GTP.T_α activates the PDE, forming PDE*, which takes about 1 msec. It is generally agreed that one GTP.T_α activates one PDE, although two GTP.T_α may be required to fully activate one PDE if there are two inhibitory subunits in one PDE molecule.

(4) Hydrolysis of cGMP

PDE* hydrolyses cGMP at a rate of more than 1000/s. This is the second amplification in the cascade. Therefore, within 0.5 sec, a single Rh* can induce the hydrolysis of 10^5-10^6 cGMP molecules.

(5) Modification of the cGMP-gated channels

The cGMP-gated channels in the OS plasma membrane are kept open in the dark by a high level of cGMP; lowering of the cGMP concentration by PDE* closes the channels, modifying the dark current.

Although there is substantial evidence corroborating the above events, there have been some observations suggesting that it is the accelerated cGMP flux, rather than the lowering of the cGMP level, that subserves the modification of the cGMP-gated channels (see p.62).

1.3.3.2 Termination of the cGMP cascade

(1) Deactivation of Rh^*

Rh* is first phosphorylated by rhodopsin kinase, which is complete in less than 2 sec. Subsequently, arrestin binds to $Rh^*.P_n$, competing with transducin for binding to the phosphorylated rhodopsin, which fully blocks its capacity to activate transducin.

(2) Deactivation of $GTP.T_\alpha$

$GTP.T_\alpha$ is hydrolysed to $GDP.T_\alpha$ by the GTPase activity, which requires 0.25 to 1.5 sec. It can then rejoin $T_{\beta\gamma}$ to form GDP.T.

(3) Deactivation of PDE^*

  i. The PDE^* is deactivated by rebinding of the inhibitory subunit(s), after the $GTP.T_\alpha$ has been hydrolysed to $GDP.T_\alpha$, and/or

 ii. The deactivation of PDE^* results from a direct interaction between the $GDP.T_\alpha$ and the PDE^*, and/or

iii. The arrestin, activated by Rh^*, binds and deactivates the PDE^*.

(4) Recovery of cGMP

Consequently, the original cGMP level is restored due to the activity of guanylate cyclase.

(5) Reopening of the cGMP-gated channels

As the internal cGMP level is recovered, the cGMP-gated channels are re-opened and the dark current is restored.

It should be noted that the mechanisms that terminate the cGMP cascade, as described above, are for the rapid quenching of the response to a weak flash. They may not necessarily apply to the processes generated by a strong or continuous episode of illumination that would fully bleach the retina. Under these conditions, the gain and kinetics of the response may be modified by phenomena such as adaptation. The latter is poorly understood as yet at a molecular level (cf. Dowling, 1987). In this study, with the exception of control tissue, all experiments were designed to elicit fully-bleached conditions.

1.3.4.1 The concentration and distribution of cGMP in the vertebrate retina

The concentration of retinal cGMP varies with both species and state of light-adaptation (Table 1.3.1).

Table 1.3.1 cGMP Concentrations in retinae of various species.

Species	Dark adaptation	Light adaptation	Reduction in light (%)	*Sources
	(pmol/mg protein)			
Rat	50	15	70%	(1)
Rat	59	--		(2)
Mouse	63	--		(2)
Mouse	69	39	43%	(3)
Mouse	64	36	44%	(4)
Toad	30	--		(2)
Toad	55	25	50%	(5)
Toad	77	26	66%	(6)
Frog	25	--		(7)
Frog	44	--		(8)
Monkey	--	10-15		(9)
Man	--	10		(9)
Man	36	14	61%	(10)

*Sources:
(1) Winkler et al., 1984.   (2) Farber et al., 1981.
(3) Cohen et al., 1978.   (4) DeVries et al., 1978.
(5) Dawis et al., 1988.   (6) Meyertholen et al., 1980.
(7) Goridis et al., 1977.   (8) Kilbride and Ebrey, 1979.
(9) Newsome et al., 1980.   (10) Voaden et al., 1989.

It has been shown that, in the light-adapted rabbit retina, the photoreceptor cell layers contain about 30 pmol/mg dry wt tissue (Orr et al., 1976a) - a concentration that is at least 10-fold higher than that of any of the remaining layers; and the highest level of 95 pmol/mg dry wt is in the OS. Moreover, dark adaptation elevates cGMP about 2-fold but only in the layers containing photoreceptor cells. Similar results were found in the normal mouse, whereas congenic rodless (rd/rd) retinae had less then 5% of control values of cGMP (Cohen et al., 1978).

It follows that the values of cGMP measured in the whole retina, as shown in Table 1.3.1, although lower than in photoreceptors alone, reflect the photoreceptor pool and changes that occur in it. The unusually high level of cGMP in the OS in the dark and the reduction in light can be explained by the reactions of the cGMP cascade.

In the dark, the concentration of cGMP in rod OS is about 60 uM (Goridis et al., 1974; Cote et al., 1984). As approximately 50% of the volume of rod OS is occupied by discs (Fein and Szuts, 1982), 60 uM would be equivalent to an interdiscal cytoplasmic concentration of 120 uM. This would give approximately 10^7 cGMP molecules per rod OS, or about 1 cGMP per 100 rhodopsins.

In contrast to our detailed knowledge of cGMP homeostasis in rods, the situation in cones is more controversial. It has been proposed that, in contrast to rod-dominated retinae which contain predominantly cGMP, cone-dominant ones contain more cAMP (Farber and Lolley, 1978; Farber et al., 1981). For example, the ground squirrel retina, with 96% of all photoreceptors being cones (West and Dowling, 1975), had a dark-adapted cGMP concentration of 11 pmol/mg protein, whilst the cAMP concentration was 91 pmol/mg protein (Farber and Lolley, 1978). Most of the cGMP and about 50% of the cAMP were present in the cones, and the rest in the inner retinal layers. Furthermore, light reduced the cAMP by 30 to 50% (Farber et al., 1981). In line with these results, Newsome et al. (1980) showed that cGMP was present in lower concentrations in the macular (cone-rich) region of

human and monkey retinae than in the periphery. They demonstrated a ratio of about 3:10 in cGMP concentration, and this differential complements the differential in cone photoreceptor distribution.

However, DeVries et al. (1979) presented contradictory results. They showed that the cGMP and cAMP concentrations in dark-adapted retina of ground squirrel were 9 and 6.6 pmol/mg dry wt, respectively. Furthermore, they showed that cAMP was evenly distributed in the retinae, whereas the concentration of cGMP in the layers containing the photoreceptor cells was 10 to 40 times that found in the inner portions of the retina, and the level of cGMP in the OS layer was 10 times that of cAMP.

Despite the above contradictions, there is strong electrophysiological evidence suggesting that cGMP is the internal transmitter in both rods and cones (e.g., Haynes and Yau, 1985; Cobbs et al., 1985).

No differences have been reported in the cGMP content of dark-adapted mouse retinae isolated under either dim red light or infrared radiation (DeVries et al., 1978; Cohen, 1981). This is somewhat surprising as the red light was considered to be of sufficient magnitude to bleach a small percentage of rhodopsin, which might affect the cGMP results (Pepe et al., 1986).

Retinal cGMP appears to be relatively stable in vitro. For example, Kilbride and Ebrey (1979) found only an 11% reduction in the cGMP content of dark-adapted frog retinae kept in Ringer's solution in the dark for 30 minutes. Newsome et al. (1980) also demonstrated that the cGMP level, in light-adapted monkey retinae left in situ, was relatively stable through about 24 hours, although it declined thereafter as autolysis of the retina progressed.

1.3.4.2 Free versus bound cGMP

It must be realized that the pool of photoreceptor cGMP includes both free and bound forms. Based on single cell recording, the free cGMP concentration has been estimated to be between 2.2 and 6 uM (Matthews et al., 1985; Yau and Nakatani, 1985b; Zimmerman and Baylor, 1986), i.e., only about 4-10% of the total. Only this fraction is free to interact with the channels.

It has been suggested that the concentration of free cGMP in the cytoplasm may not only be controlled by cGMP hydrolysis by the PDE, but also by binding. Several different cGMP-binding sites exist in rod OS. Principal ones are the cGMP-gated channel protein (Matthews et al., 1985; Caretta and Saibil, 1989) and noncatalytic binding sites on the alpha and beta subunits of PDE (Yamazaki et al., 1982; Yuen et al., 1986). Fesenko and Krapivinsky (1986) have suggested that there is one binding site per 25 to 40 molecules of rhodopsin, i.e., about 10^8 sites in a rod.

It has also been proposed that ATP and GTP produce opposite effects on the cGMP-binding site pool: ATP activates, whilst GTP inhibits; both processes are light-dependent (Yamazaki et al., 1982; Fesenko and Krapivinsky, 1986). Fesenko and Krapivinsky (1986) also suggest that, under physiological conditions, the ATP-dependent, light-induced cGMP-binding time is less than 10 msec, and that the GTP-dependent, light-induced cGMP-dissociation is equally fast. Thus, the processes of association and dissociation of cGMP may occur within the time period of the change in potential of the plasma membrane.

1.3.4.3 cGMP Metabolic flux

When cGMP is hydrolysed in the presence of radio-labelled water ($H_2^{18}O$), the ^{18}O is incorporated into the alpha-phosphoryl of the guanine nucleotide. Thus the rate of hydrolysis can be determined by measuring the rate of alpha labelling (Goldberg et al., 1983). If the total level of cGMP is determined at any given time and compared with simultaneous measurements of labelled breakdown products, the cGMP metabolic flux or turnover can be estimated.

With this technique, it has been found that a consistent response of the retina to light is an accelerated rate of cGMP turnover. The response results from an equal acceleration of both cGMP hydrolysis and synthesis because the steady state level of the nucleotide remains unchanged (Goldberg et al., 1983; Ames and Barad, 1988; Dawis et al., 1988).

Dawis et al. (1988) showed that, under a low intensity illumination of 0.6 to 3 log photons/um^2/s, the metabolic flux of cGMP increased up to 8-fold within 200 sec without a change in the total concentration. At a higher light intensity of 3 to 5.4 log photons/um^2/s, the concentration of cGMP gradually declined - by 25% at 20 sec and 50% at 200 sec. The reductions were proportional to the suppression in light-enhanced cGMP turnover. Ames and co-workers also showed that the the light-enhanced cGMP flux correlates with the photoreceptor electrical responses (Ames and Barad, 1988). Therefore, the light-accelerated cGMP flux is thought to represent a biological process integral to phototransduction, and Dawis et al. (1988) have put forward an alternative model for the transduction cascade (cf. 1.3.3, p.56). In this model, it is proposed that the cGMP flux, rather than the concentration per se, is directly coupled to visual excitation, and continuous illumination activates the entire cGMP metabolic cycle.

The mechanism linking the increase in cGMP flux to the changes in membrane conductance is still unclear.

1.3.5 THE ROLE OF CALCIUM IN THE cGMP CASCADE

It is known that in the dark Ca^{2+}, as well as Na^+, enters rod OS through the cGMP-gated channels and is continually removed from the cell by an independent vectorial Na/Ca exchange mechanism that also exists in the plasma membrane (see 1.4.1.1, p.64). Thus, when the cGMP-gated channels are closed in light, the entry of Ca^{2+} into the OS from the extracellular medium is blocked but the Na/Ca exchanger continues to pump Ca^{2+} out of the OS until the intracellular Ca^{2+} level is very low (Yau and Nakatani, 1985a). A transient decrease in the cytosolic level of Ca^{2+} serves to stimulate guanylate cyclase activity (p.51), thus promoting the increase in cGMP that occurs as PDE activity is quenched, and helping to restore the dark-adapted state. The increase in the endogenous level of free cGMP opens the sodium channels again. In addition, it has also been suggested that low Ca^{2+} increases the affinity of cGMP binding to the channel protein, pushing back the equilibrium of the channels toward the open state (Caretta et al., 1988).

The re-opening of the Na^+ channels raises the cytosolic Ca^{2+} level. Conversely, a high level of Ca^{2+} depresses the cGMP level by inhibiting the cyclase and also possibly stimulating the PDE. This leads to closure of some of the cGMP-gated channels, and in turn limits the influx of Ca^{2+}.

Thus there is a feedback relationship between cytosolic Ca^{2+} and cGMP, and there is now substantial evidence to suggest that Ca^{2+} plays a modulatory role in both rods and cones and most probably mediates photoreceptor light adaptation.

1.4 ELECTROPHYSIOLOGY

1.4.1 ELECTROPHYSIOLOGY OF PHOTORECEPTOR CELLS

1.4.1.1 The dark membrane potential and dark current

The difference in electrical potential between the inside and outside of a neuronal membrane depends on the ionic concentration gradients across the membrane and the relative permeability of the membrane to the ions.

In most neurones, the transmembrane potential in the resting state depends mainly on a high internal concentration of K^+ and a relatively high permeability to K^+. This resting potential is generally about -60 to -65 mV inside, i.e., the membrane is hyperpolarized (Kuffler et al., 1984).

In photoreceptor cells, however, a fundamental physiological feature in the dark is a significant permeability of OS membrane to Na^+, which allows Na^+ to enter the cell rapidly. In addition, Ca^{2+} is permitted to enter and K^+ to leave the cell along their concentration gradients. This leads to a nett movement of positive charge from outside to inside of the OS. Here, Na^+ is the major current carrier across the membrane, and Ca^{2+} is responsible for 10-15% of the current (Yau and Nakatani, 1985a; Yau et al., 1987). To maintain electrical neutrality, there is an outward current carried by K^+ across the IS membrane. In the extracellular space, the region adjacent to the IS is thus more positive than that around the OS.

As a result of the fluxes of various ions across the photoreceptor plasma membrane, the transmembrane potential in the dark is about -30 to -40 mV inside and the cell is thus relatively depolarized (Baylor et al., 1979a; Baylor and Nunn, 1986).

Inner segment

Outer segment
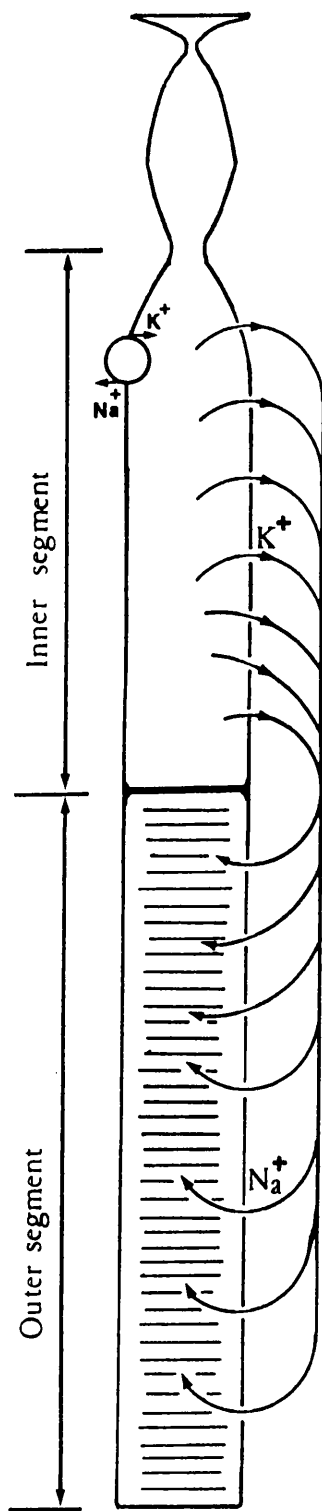
Hence, in the dark there is a flow of current which forms a loop around the IS and OS of the photoreceptor cell (Fig.1.4.1). This current is called the dark current. It flows externally around the cell from the IS to the OS and internally within the cell from the OS to the IS (Fig1.4.1). The dark current is normally about 20 to 50 pA, a level generated when only about 1-5% of the total conductance channels are open (Hagins et al., 1970; Baylor et al., 1984; Matthews et al., 1985; Yau et al., 1987).

The concentration gradient across the membrane for Na^+ and K^+ is maintained by a Na/K pump located in the IS plasma membrane (Hagins et al., 1970). The pump is electrogenic and its activity contributes to the membrane potential. The concentration gradient for Ca^{2+} is maintained by the activity of the Na/Ca exchange mechanism which may take in 3 Na^+ for every Ca^{2+} extruded and is thus also electrogenic, deriving its energy from the inward Na^+ gradient (Yau and Nakatani, 1985a). Recent evidence has suggested that K^+ is co-transported with Ca^{2+}, in the exchange stoichiometry of 4Na:1Ca,1K (Cervetto et al., 1989).

Fig.1.4.1 The dark-current of the photoreceptor cell.

1.4.1.2 Modulation of the dark current by light

As described earlier, absorption of light by the visual pigments leads to a reduction of the dark current by means of closure of the cGMP-gated channels in the OS membrane (p.56). This results in a change in the membrane potential, from a relative depolarization (p.64) to hyperpolarization (Hagins et al., 1970).

Light works in a graded manner. A very dim flash which leads to no more than one photon absorbed per rod will close about 3-5% of the total number of channels. In the case of cones the percentage is smaller. The number of the channels closed and the magnitude of suppression of the dark current increase progressively with increasing light intensity. However, the relationship between response amplitude and light intensity has to break down as the number of channels that can be closed starts to run out and eventually saturation is reached (Hagins et al., 1970; Baylor et al., 1984). Below saturation, the relationship between peak response amplitude (V) and light intensity (I) can be described by the Michaelis-Menten or Naka-Rushton equation (cf. Naka and Rushton, 1966; Aylward, 1989):

$$V = V_{max} \frac{I^n}{I^n + K^n}$$

Where V_{max} is the peak response amplitude of a saturated response, K is the intensity that elicits a half-saturated response and n represents the slope of the curve. K and n are constant for a given cell or retina, with n having a value between 0.7 and 1 (cf. Boynton and Whitten, 1970; Dowling and Ripps, 1971; Ernst and Kemp, 1972). This equation has been used extensively to estimate the parameters of photoresponses obtained from either single cell or transretinal recordings (e.g., Ernst and Kemp, 1972; Baylor et al., 1984; Aylward, 1989).

The effect of a brief flash of light is a transient current, the photocurrent, whose profile matches that of the dark current but has everywhere the opposite sign (cf. Fig.1.4.1; Penn and Hagins, 1969). That is, the photocurrent flows externally around the photoreceptor from the OS to the IS and internally within the cell from

the IS to the OS. The photocurrent thus results from suppression of the dark current (Baylor et al., 1979a). The OS membrane is the site of photocurrent initiation: local OS illumination causes local photocurrent (Hagins et al., 1970; Lamb et al., 1981). Since they are indirectly sensitive to light, the dark current and cGMP-gated channels are sometimes called the light-sensitive current and light-sensitive channels, respectively. However, use of this nomenclature is declining.

1.4.2 THE ELECTRORETINOGRAM (ERG)

The ERG is a complex waveform which represents the voltage changes that can be recorded from an eye, eye-cup or isolated retina when it is stimulated by a flash of light. Because the recording tends to be relatively stable for a long period of time, ERG potentials can be studied quantitatively under a wide variety of conditions.

1.4.2.1 Light-evoked potassium movements in the retina

When the retina is stimulated by a flash of light, there are three principal changes in K^+ arising at different intraretinal loci (e.g., Oakley and Green, 1976). These are the distal decrease, distal increase and proximal increase outlined below.

(1) The distal decrease
A large decrease in the concentration of extra-cellular K^+ occurs in the IS region. It is considered to be caused by a decrease of the passive efflux of K^+ from the light-stimulated cell (e.g., Oakley et al., 1979). This decrease in K^+ levels around the photoreceptors is believed to give rise to the vitreous-positive c-wave or the vitreous-negative slow PIII (see below).

(2) The distal increase
An increase in extracellular K^+ concentration occurs in the region of the OPL. It appears to result from the activity of ON-bipolar cells (Dowling, 1987; Stockton and Slaughter, 1989).

(3) The proximal increase

An increase in extracellular K^+ concentration occurs in the region of the IPL. There is doubt about its origin since, although it has been suggested for a decade that it derives primarily from the activity of amacrine and ganglion cells (Shimazaki et al., 1984; Dick and Miller, 1985; Karwoski et al., 1985, Dowling, 1987), Stockton and Slaughter (1989) have recently pointed out that it probably arises from the terminal region of ON-bipolar cells.

The distal and proximal increases in K^+ result in localized flows of K^+ into the Muller cells. Consequently, K^+ flows out from the Muller cell end-foot and there is thus an extracellular current flow around the Muller cells, from the end-feet to the IPL and OPL. It has been proposed that these give rise to the vitreous-positive b-wave (see below; Miller and Dowling, 1970).

1.4.2.2 The components of the ERG and their origins

The ERG consists mainly of a-, b- and c-waves (Fig.1.4.2, solid line). The first component analysis of an ERG was carried out by Granit (1933) who identified 3 basic components (PI, PII, and PIII) that disappeared successively from the ERG of a cat during deepening ether narcosis (Fig.1.4.2, dotted lines). The term "P" stands for Process, since at that time the cellular origins were unknown. The c-wave was found to disappear first, then the b-wave, whilst the a-wave disappeared last (Granit, 1933). Thus the analysis from Fig.1.4.2 indicates that the c-wave results from PI, the b-wave is due to a peak of PII, and the a-wave is accounted for by the onset of PIII.

Subsequent studies have suggested the following cells as the main contributors to the vertebrate ERG (e.g., Granit, 1947; Oakley, 1976; Dick and Miller, 1985; Dowling, 1987).

1) Photoreceptor cells. They produce a component called fast PIII (see below) as a result of the generation of the photocurrent.

2)   Muller cells. They generate two components, called
     slow PIII (see below), and the initial peak of PII,
     in response to the distal K^+ decrease and the distal
     and proximal K^+ increases, respectively.

3)   RPE cells. They produce the PI in response to the
     distal K^+ decrease.
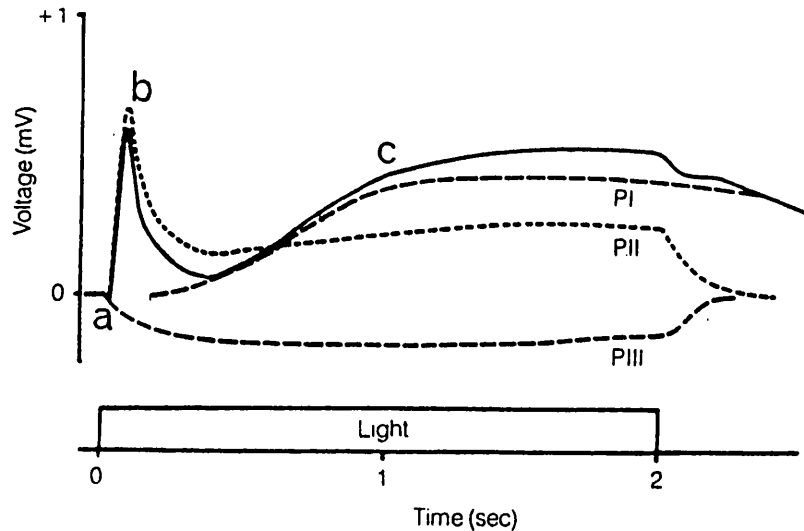
Fig.1.4.2 The ERG recorded from a cat. (After Granit, 1933).
     The solid line represents the a-, b- and c-waves, and
the dotted lines the three basic components, Granit's PI,
PII and PIII (see the text for details).

Further details of the a-, b- and c-waves and PIII
are outlined below.

(1) The a-wave and PIII

     As previously described, the a-wave is in fact the
onset of PIII (Fig.1.4.2) which consists of three
different subcomponents (Dowling, 1987). Two arise from
photoreceptor cells and have been called the early
receptor potential (ERP) and late receptor potential (or
fast PIII), respectively. The third is the slow PIII.

     The ERP, being separated into R1 and R2 and lasting
for 2 to 3 msec, is observed only when the retina is

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illuminated with a very intense, 1 msec flash (Cone, 1964; Brown and Murakami, 1964). It appears to arise from the charge displacement that follows changes in visual pigment conformation (cf. p.40).

Fast and slow PIII, on the other hand, are evoked by light flashes of ordinary intensity. The PIII complex can be isolated from the b- and c-waves by suppressing the activity of second order neurones with glutamate or aspartate and removing the retina from the RPE, respectively (Fig.1.4.3).
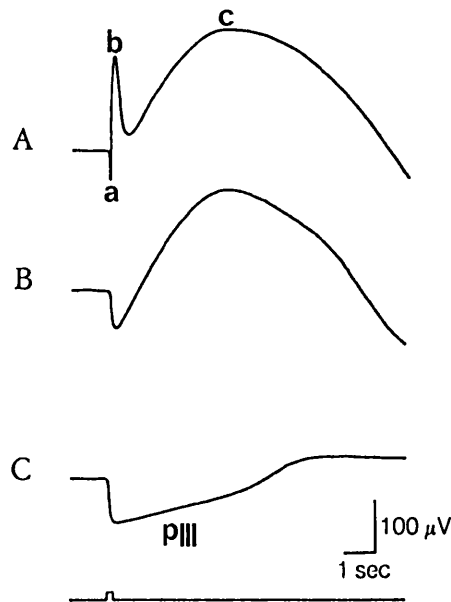
Fig.1.4.3 Isolation of PIII in the skate retina. (After Dowling, 1987).

A.   The a-, b-, and c-waves of the normal ERG recorded from an untreated eye-cup preparation.

B.   The b-wave is suppressed by immersion of the eye-cup in a medium containing 100 uM aspartate for a few minutes, but the a- and c-waves are essentially unaltered.

C.   The c-wave is eliminated by removing the retina from the RPE, and only a vitreous-negative potential, PIII, remains.

Fast and slow PIII can be differentiated with microelectrodes placed across the receptors (Fig.1.4.4). However, when recording across the whole of a retina treated with glutamate or aspartate, the leading edge of fast PIII can be distinguished from the slower component by virtue of its faster onset (Fig.1.4.4).
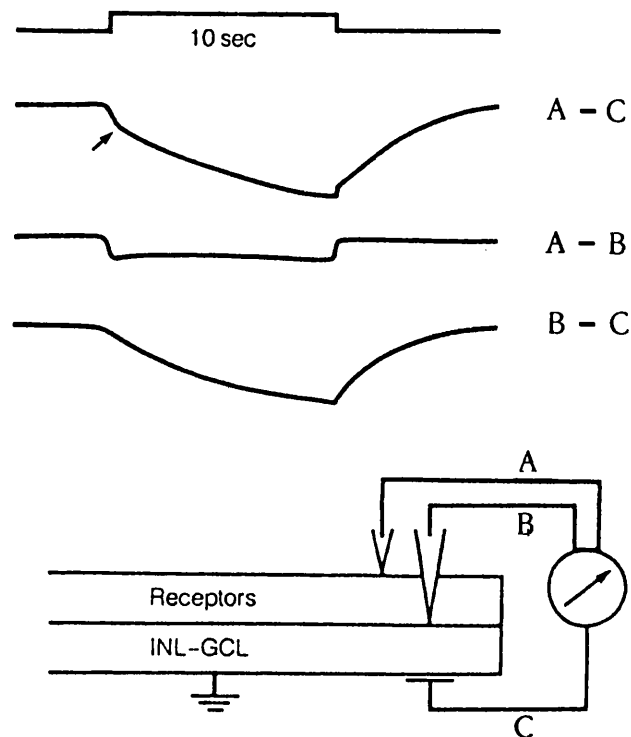
Fig.1.4.4 Schematic drawing showing the separation of fast and slow PIII in an isolated carp retina. (After Dowling, 1987).

Note: the b-wave has been suppressed by aspartate (cf. Fig.1.4.3).
A-C: The PIII complex, recorded across the whole retina.
A-B: Fast PIII, recorded across the photoreceptors only.
B-C: Slow PIII, recorded over the INL to ganglion cell span.

Note that the leading edge of fast PIII can be distinguished from slow PIII (see A-C, arrowed).

Since fast PIII arises from photoreceptor activity, it has an identical voltage-intensity function to that obtained from single photoreceptor recordings (Hagins et al., 1970). However, even though slow PIII is produced by Muller cells, Witkovsky et al. (1975) have shown that in

the carp its voltage-intensity function is identical to that derived from the rod responses. Furthermore, the spectral sensitivity function of slow PIII conforms closely to the absorption spectrum of the visual pigment. These findings indicate that slow PIII can also accurately reflect the activity of photoreceptor cells.

(2) The b-wave

As previously described (p.68), the b-wave is generated by Muller cells in response to the distal and proximal K^+ increases. This has been confirmed by the similarity of the b-wave and the intracellularly-recorded Muller cell response in the mudpuppy (Miller and Dowling, 1970). Since the distal and proximal K^+ increases result from the activities of the higher-order neurones, particularly ON-bipolar cells (cf. p.67), the b-wave reflects the activities of the neurones and of neurotransmission between photoreceptors and ON-bipolar cells.

The b-wave can be blocked by agents such as glutamate or aspartate that do not alter photoreceptor responses but do interfere with the synaptic transmission (cf. Fig.1.4.3; Dowling and Ripps, 1971; Cervetto and MacNichol, 1972).

(3) The c-wave

Although originating from photoreceptor activity, the c-wave arises from the RPE. Thus it cannot be recorded from an isolated retina (cf. Fig.1.4.3, p.70). The distal K^+ decrease gives rise to the generation of the c-wave as the RPE is very sensitive to changes in extracellular K^+ concentration and, like Muller cells, behaves like a K^+ electrode. Oakley et al. (1977) have shown that the intracellularly-recorded RPE response has excellent correspondence to the c-wave.

1.5 HIGH AFFINITY UPTAKE OF AMINO ACIDS

A high-affinity mechanism for uptake of neuroactive amino acids by neurones and/or glia is a characteristic property of the central nervous system including the retina. The mechanism allows rapid clearance of the amino acid neurotransmitters from the extracellular space and hence their inactivation. In the retina, the uptake systems have been characterized in detail using radiotracer techniques, including autoradiography, to locate the active cells (Morgen, 1985a). In this thesis, three of the principal neuroactive amino acids were selected to represent the retinal systems. These are taurine, glycine and GABA (gamma aminobutyric acid), and a brief survey of their properties and known/putative functions follows.

1.5.1 TAURINE

In most if not all vertebrates, including man, taurine (2-amino ethane sulphonic acid) is present in higher concentration in the retina than in any other tissue of the body (for a review: Pasantes-Morales, 1985). Within the retina, taurine is preferentially located in the photoreceptor cell layer, particularly in the ONL (Orr et al., 1976b; Voaden et al., 1977).

The involvement of taurine in photoreceptor function remains unclear, but it is now well established that the amino acid is essential for the maintenance of the structural and functional integrity of photoreceptor cells. This was first demonstrated in the cat (a species obligately dependent on dietary taurine) but appears to apply generally, including man (Pasantes-Morales, 1985). For example, dietary deficiency in the cat produces a severe disruption of photoreceptor structure, characterized by cell swelling and disorientation of the OS, and, ultimately, cell death (Hayes et al., 1975a,b). Alongside and sometimes preceding the structural changes, ERG abnormalities are also present (Schmidt et al., 1977). It is noteworthy that children and other young primates also require dietary taurine for the maintenance of normal

vision (Pasantes-Morales, 1985).

In addition to a central role in photoreceptor maintenance, taurine may also serve as a retinal neurotransmitter. This is suggested by its inhibitory effect on neuronal activity (Philips, 1978; Okamoto et al., 1983). However, a survey of taurine properties, analyzed within the scope of the criteria for establishing a transmitter role for a substance, reveals a number of inconsistencies and conflicting results (Pasantes-Morales, 1985). Thus, it is still not possible to give a definitive answer.

Nevertheless, it has been demonstrated that specific high-affinity uptake systems for taurine exist in the retinae of goldfish, frog, chick, rat, rabbit, cat, monkey and man (Ehinger, 1973; Lake et al., 1978; Starr, 1978; Salceda, 1980; Schmidt, 1981). The high-affinity uptake of taurine is sodium- and temperature-dependent (Starr and Voaden, 1972b; Schmidt, 1980).

With respect to the cellular localizations of taurine, taken up via high-affinity mechanisms, autoradiography and microdissection studies have demonstrated that photoreceptor cells are active as well as, in some species, cells in the inner retina, including bipolar cells (Lake et al., 1978; Schmidt, 1981).

Kinetic analysis of high-affinity mechanism for uptake of taurine shows quantitative similarity across species. The values of K_m range between 16 and 111 uM, and V_{max} between 330 and 580 nmol/min/g wet wt (Pasantes-Morales, 1985). If the quantity of retinal protein is estimated to be around 45 mg/g wet tissue (Yates et al., 1974; Bazan and Reddy, 1985), the above values of V_{max} are equivalent to between 7 and 13 nmol/min/mg protein.

1.5.2 GLYCINE

Glycine is present in significant concentration in the retina and is generally regarded as a major inhibitory retinal neurotransmitter (for a review: Marc, 1985).

Glycinergic retinal cells (or more precisely, glycine-accumulating cells) are present in a large population in all vertebrates examined (Marc, 1985). It

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has been suggested that there is no significant variation in high-affinity uptake between species with this amino acid (Bruun and Ehinger, 1974). Glycinergic amacrine cells have been demonstrated in all species examined (Marc, 1985) and appear to represent up to 40% of all the amacrine cells in the mammalian retina (Pourcho, 1980). Glycine is also accumulated with high-affinity by bipolar and interplexiform cells in many species (Marc, 1985). In the human retina, it is taken up by amacrine and bipolar cells (Frederick et al., 1984; Marc and Liu, 1985). Glial uptake of glycine has only been observed in the silver lamprey (Marc, 1985).

It has been shown that glycine uptake is temperature-sensitive, as well as being sodium-dependent (Voaden et al., 1974).

The kinetics of glycine high-affinity uptake has been studied in the rabbit, rat and frog (Bruun and Ehinger, 1972; Neal et al., 1973; Voaden et al., 1974). The values of K_m vary between 16.7 and 27.6 uM, and V_{max} between 5.7 and 149 nmol/min/g wet wt (equivalent to 0.13 and 3.3 nmol/min/mg protein; see p.74 for the conversion).

1.5.3. GABA

GABA is a major inhibitory neurotransmitters in the retina of several, if not all, vertebrate species. With species variation, GABAergic neurones include amacrine, horizontal and interplexiform cells (for reviews: Morgan, 1985b; Yazulla, 1986; Massey and Redburn, 1987; Ehinger and Dowling, 1987).

As with taurine and glycine, GABA uptake is both temperature-sensitive and sodium-dependent (Voaden et al., 1974).

The features of retinal GABA uptake vary not only across species but also between in vivo and in vitro conditions. Some of the variations are summarized in Table 1.5.1.

Table 1.5.1 High-affinity uptake of ^{3}H-GABA in the retina.

Species	in vivo intravitreous injection		in vitro incubation	
Mammals	Muller C.	(1)	Muller C.	(1)
	amacrine C.	(2,3)		
	interplexiform C.	(3,4)		
	ganglion C.	(5)		
Non mammals	amacrine C.	(6,7)	amacrine C.	(8,9)
	ganglion C.	(6)	bipolar C.	(8)
			horizontal C.	(8,9)
			ganglion C.	(9,10)

It can be seen that, in general, ^{3}H-GABA is taken up by Muller cells in mammalian retinae in vitro, whilst in vivo it is taken up by both Muller cells and neurones. In contrast to mammals, in non-mammalian retinae (except for the skate, cf. Yazulla, 1986) ^{3}H-GABA is taken up only by neurones, regardless of whether the studies are undertaken in vitro or in vivo.

Sources:
1. Yazulla, 1986 (a review; pig,goat,cat,baboon,rabbit).
2. Ehinger, 1977 (rabbit).
3. Freed et al., 1983 (cat).
4. Marshall and Voaden, 1975 (cat).
5. Ehinger and Falck, 1971 (rabbit).
6. Marshall and Voaden, 1974a (frog).
7. Yazulla, et al., 1984 (goldfish)
8. Voaden et al., 1974 (frog).
9. Marshall and Voaden, 1974b (chicken and pigeon).
10. Watt et al., 1984 (chicken and pigeon).

The kinetics of GABA high-affinity uptake have been studied in a range of species (Starr and voaden, 1972a; Goodchild and Neal, 1973; Neal et al., 1973; Voaden et al., 1974; Yazulla, 1986). The values of K_m range between 25 and 47 uM and V_{max} between 6.7 and 167 nmol/min/g wet wt (equivalent to 0.15 and 3.7 nmol/min/mg protein; see p.74 for the conversion).

1.6 PROTEIN SYNTHESIS

The synthesis of protein is essential for the maintenance of life. The process is a fundamental one and, for a retina to continue to function _in vitro_ as well as _in vivo_, this aspect of metabolism must be patent.

Studies of retinal protein metabolism have been concerned mainly with following the time course of metabolic processes employing labelled amino acids. Using ^{3}H-leucine _in vitro_, Steinman and Ames (1974) demonstrated:

1) High rates of protein synthesis in photoreceptor IS, perikarya of ganglion cells, and cells of the INL.

2) Low rates of synthesis in receptor cell bodies, the OPL and IPL, and Muller cells.

3) No synthesis in photoreceptor OS.

Comparable results, obtained from _in vivo_ studies, have been reported in frogs, rodents and primates (Droz, 1963; Young, 1967; Hall and Bok, 1974). Proteins with molecular weights of between 33 and 43 kDa are synthesized most rapidly (e.g., Ames et al., 1980a,b).

In photoreceptor cells, virtually all new protein molecules are synthesized in the myoid region of the IS, where rough (RER) and smooth endoplasmic reticula and the Golgi apparatus are located (p.28; Young and Droz, 1968).

As previously described, opsin is a glycoprotein that contains mannose and N-acetylglucosamine moieties (p.36). The processes of opsin synthesis and incorporation into the OS discs have been reviewed (Hargrave, 1982; Bok, 1985; Besharse, 1986) and are summarized as below.

(1) The peptide moiety of opsin is synthesized on the ribosomes of the RER, and cotranslational insertion of the peptide into the lipid bilayer membrane and core glycosylation also occur there.

(2) Processing of the oligosaccharide chain and packaging into vesicles occur in the Golgi apparatus.

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(3)   The opsin-containing vesicles migrate to and accumulate in the periciliary cytoplasm in the ellipsoid region.

(4)   The vesicular membrane is incorporated into plasma membrane near the cilium. Opsin is then transferred to newly forming discs. Disc morphogenesis occurs at the distal end of the connecting cilium by an unknown mechanism.

(5)   Once the opsin is present in the discs, 11-cis retinal is added to form rhodopsin. In addition, however, St.Jules et al. (1989) have recently reported that extracts from the RER contain a light-sensitive protein which exhibits the properties of rhodopsin. This can be labelled with exogenously-supplied ^{3}H-retinol. Thus, it appears likely that addition of the chromophore to opsin can occur as soon as the apoprotein is translated in the RER.

1.7 STUDIES ON POSTMORTEM TISSUE FROM CENTRAL NERVOUS SYSTEM (CNS)

1.7.1 POSTMORTEM CHANGES IN THE CNS

Since investigations on postmortem retinae are rare (see below), this section describes data obtained for the brain. Although the brain is thicker and denser than the retina, they both contain neurones and have high oxygen consumption. Furthermore, a similar range of neuroactive compounds, potentially serving as neurotransmitters, are found in both (cf. Bjorklund et al., 1987). Thus, information achieved from postmortem brain may help us understand some aspects of postmortem retina.

1.7.1.1 Biochemical changes in the CNS postmortem

Postmortem changes in some neurochemicals and enzymes in the human brain have been described. In the cortex concentrations of serotonin (5-HT), noradrenaline (NA), dopamine (DA), dihydroxyphenylacetic acid (DOPAC), and somatostain-like immunoreactivity (SLIR), together with the activity of phosphate-activated glutaminase (PAG), were all lower in postmortem tissue (5 to 39 hours after death) than in tissue removed antemortem. In contrast, the concentrations of glycine, GABA, aspartic acid, glutamic acid, 3-methoxy-4-hydroxyphenylacetic acid (MHPG) and homovanillic acid (HVA), were all higher in the postmortem tissue. Whilst, at the same time changes in concentration of 5-hydroxy-indoleacetic acid (5-HIAA) and the activity of choline acetyltransferase (ChAT) were not significant (Perry et al., 1981; Francis et al., 1987; Palmer et al., 1988; Lowe et al., 1988; Procter et al., 1988).

Present evidence suggests that factors influencing the amount of neurochemicals and enzymes in postmortem tissue include age, sex and agonal state of the patient, as well as postmortem delay. For example, the concentrations of HVA, 5-HIAA and MHPG were increased (Palmer et al., 1987), whilst the activities of SLIR and ChAT were reduced in aged patients (Francis et al., 1987; Procter et al., 1988). In contrast, the concentration of 5-HIAA was

higher in females than in males. The concentration of GABA and the activities of glutamic acid decarboxylase (GAD) and phosphofructokinase were reduced in patients dying after prolonged illness (Hardy and Dodd, 1983; Palmer et al., 1988).

1.7.1.2 Mechanisms of CNS neurone loss postmortem

Neurotoxicity by excitatory amino acid neurotransmitters such as glutamate has been proposed as a mechanism for neuronal cell loss in the CNS during anoxia or postmortem. This theory is based on the following evidence: both blockage of synaptic transmission and antagonism of specific postsynaptic glutamate-receptors can prevent anoxic neuronal death (e.g., Simon et al., 1984; Meldrum, 1985; Rothman and Olney, 1986). Under physiological circumstances, a very efficient uptake system in nerve terminals and glia rapidly clears the extracellular space of synaptically released glutamate (Storm-Mathisen and Iverson, 1979; Hertz, 1979). The concentration of the transmitter, therefore, never reaches toxic levels. Neurotransmitter uptake is highly energy-dependent, and under anoxic conditions the uptake systems fail to operate. Furthermore, anoxia also causes an increase of release of, e.g., glutamate into extracellular space (Bosley et al., 1983; Hauptman et al., 1984).

The mechanisms underlying anoxic neuronal death may be described as below (cf. Rothman and Olney, 1986).

Anoxia/postmortem
↓
Accumulation of excitatory amino acid neurotransmitters
↓
Sustained depolarization
↓
Influx of chloride
↓
Cations are drawn into the cell
↓
Water influx induces rapid cellular swelling
↓
Calcium accumulation causes slow necrosis
↓
Cell death
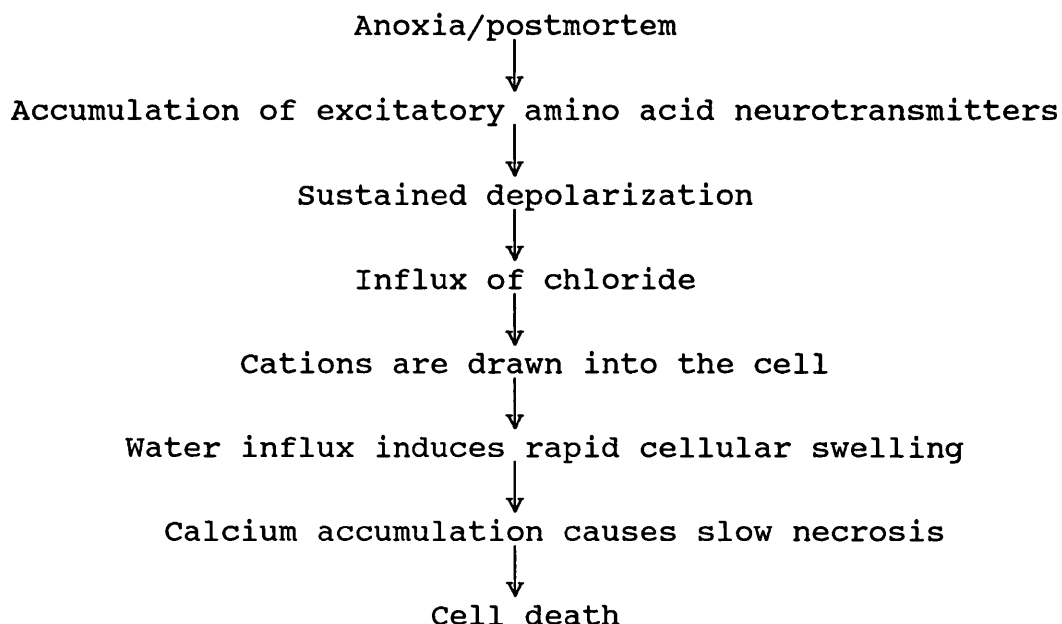
In addition to the neurotoxicity, lactic acidosis may also be an important mechanism underlying damage to CNS cells during anoxia. It has been found that excessive amounts of lactic acid - a product of anaerobic glycolysis - cause severe damage to brain cells and impair postischemic recovery, as indicated from biochemical and neurophysiological (Rehncrona et al., 1981) as well as histopathological studies (Kalimo et al., 1981). The most likely mechanism is that the consequent derangement of intracellular pH affects reactions and enzyme systems necessary for cellular viability.

1.7.2 SURVIVAL OF STRUCTURE AND FUNCTION IN THE RETINA POSTMORTEM OR UNDER ANOXIA

Previous studies have shown that interruption of both choroidal and retinal circulations in the monkey results in loss of photoresponses within 20 minutes (Fujino and Hamasaki, 1965). Interruption of the circulation to the rabbit retina, most of which in effect has only a choroidal blood supply, causes ultrastructural abnormalities in photoreceptor OS within 30 minutes and in the IS and nuclei within 2 hours (Johnson and Grierson, 1976; Johnson and Foulds, 1978). In contrast, in animals with a dual circulation, if both are suppressed then the initial change is oedema in the inner retina. Similar responses have been shown in the rat (Turnbull, 1950), cat (Reinecke et al., 1962), gerbil (Levine and Payan, 1966) and monkey (Fujino and Hamasaki, 1967).

It has been shown that the high-affinity mechanism for uptake of taurine and the capacity for rhodopsin synthesis and phosphorylation survive in human and rat retinae for at least 4 to 6 hours postmortem (Schmidt and Berson, 1980). Similar results for rhodopsin synthesis have also been demonstrated in the bovine retina (O'Brien et al., 1972).

However, our understanding of postmortem survival in the retina is still limited, especially in man. Therefore, the present study was carried out to investigate survival and the potential for recovery of structure and function in vitro in the human retina. The functional studies

include several aspects of phototransduction, amino acid uptake and protein synthesis.

In order to monitor survival of the human retina, firstly a survey of structure was undertaken using both light and electron microscopy. Secondly, to commence studies on the functional capacity of the system, it was decided to concentrate primarily on the most physiologically relevant parameter, phototransduction. Thus, electrophysiological responses to light have been monitored, and associated aspects such as visual pigment regeneration and cGMP homeostasis assessed. Although much of the thesis concentrates on these properties, studies have also been undertaken concerning survival of the retina as a whole. Thus, high-affinity uptake of three representative, neuroactive amino acids (taurine, glycine and GABA) by either neurones or glia has been investigated, and protein (including opsin) synthesis has been evaluated. In addition to the main study, the ability of some specific postsynaptic glutamate-receptor antagonists to preserve the ERG b-wave <u>ex vivo</u> has been tested using rat tissue; this was done because neurotoxicity is recognized as a major cause of neuronal loss in anoxia.

PART 2: STUDIES UNDERTAKEN

2.1   STRUCTURAL SURVIVAL OF POSTMORTEM HUMAN RETINA

2.1.1   INTRODUCTION

Histological studies were necessary, to investigate the nature of postmortem changes in human retina. Those described below deal with postmortem changes in human retinal anatomy as a function of postmortem time, enucleation time, retinal location, age, sex and the cause of death.

Morphological changes in retinae in which protein synthesis was investigated are discussed in section 2.6 (p.172).

2.1.2   MATERIALS AND METHODS

2.1.2.1   The human retina

Thirty-one normal human eyes, most of which were donated for corneal grafting, were obtained from the Eye Bank, Moorfields Eye Hospital. They were enucleated at various times after death, and were suspended in moist, sterile containers. At the hospital they were stored at $4^{o}C$, but during transport to the hospital and about 3 hours before laboratory investigation, they were at room temperature. Corneas were removed from the globes between 5 and 18 hours before retinal studies began.

2.1.2.2   Light- and electron-microscopy

For most specimens in which functional investigations were carried out, the retina was dissected (see 2.2.2.3 for details, p.101), and some portions fixed in glutaraldehyde. Only a few globes were used solely for morphology and these were fixed immediately. The concentration of glutaraldehyde was 2.5%, buffered to pH 7.4 with 0.1 M sodium cacodylate-HCl, containing 1.0 mg/ml $CaCl_2$. The specimens were then washed in 7.5% (w/v) sucrose in 0.25 M cacodylate buffer and post-fixed in 2% osmium tetroxide in 0.2 M cacodylate/HCl. The tissue was

dehydrated by means of increasing concentrations of ethanol and embedded in Araldite via epoxypropane.

For light microscopy (LM), sections, 0.8 um thick, were cut using glass knives in a Cambridge Huxley ultramicrotome. These semi-thin sections were stained with 1% toluidine blue. For transmission electron microscopy (EM), 0.08 um thick sections were cut using glass knives in a Reichert OMU4 ultramicrotome, and stained with uranyl acetate and lead citrate before being examined on an AEI 801 electron microscope.

2.1.3 RESULTS

2.1.3.1 Structural changes in postmortem human retina

Figures 2.1.1, 2.1.2 and 2.1.3 show structural changes in three postmortem human retinae. All the samples were peripheral but they vary in postmortem time and the time between death and enucleation. It can be seen that in the 5-hour-postmortem retina there was only a slight vacuolation in the nerve fibre layer (Fig.2.1.1). In contrast, tissue that was 18 hours postmortem exhibited typical vacuolation and cytoplasmic swelling, together with pyknotic nuclei (Fig.2.1.2). The pathological changes in the 34-hour-postmortem retina involved most retinal layers, except that photoreceptor IS and OS appeared surprisingly intact (Fig.2.1.3).

Histological studies of all retinal samples showed a reasonable state of anatomical preservation of photoreceptor cells. In almost all specimens there was some perinuclear vacuolation, particularly in cones, coupled with a degree of swelling of cone inner-connecting fibres. In contrast, damage to rod photoreceptors was rare. The standard of preservation of IS and OS was usually excellent, compared with the rest of the retina (e.g., Fig.2.1.3). Of particular interest was the high degree of preservation of mitochondrial membranes in the IS, and of disc membranes in rod OS (Fig.2.1.3 c,d).
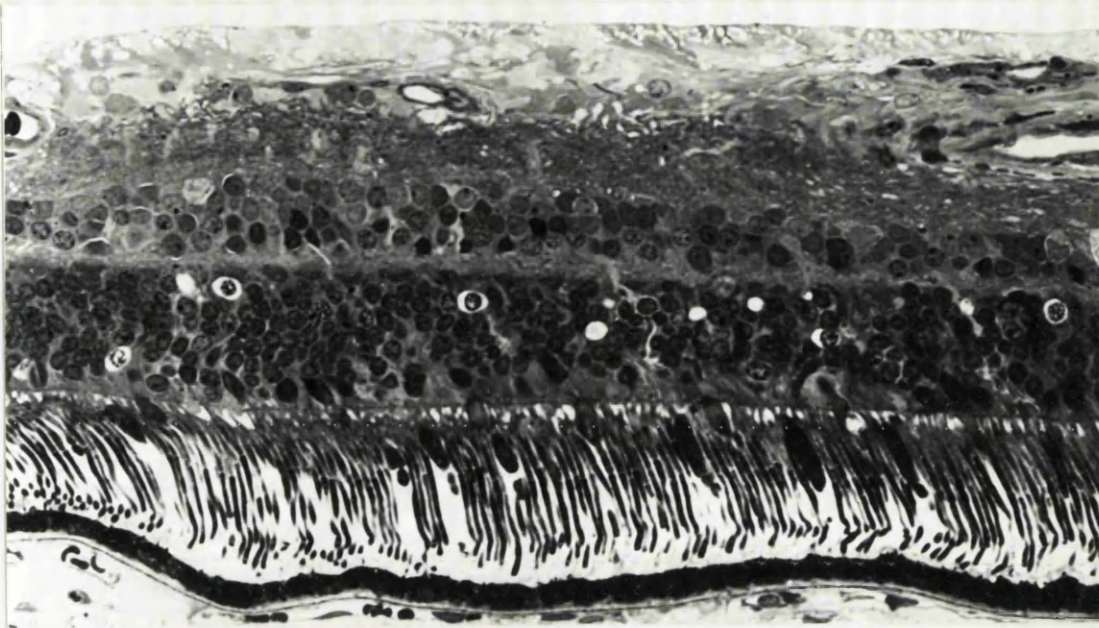
Fig.2.1.1 Light micrograph of a 40-year-old human retina, 5 hours postmortem and zero time to enucleation (Specimen 1, Table 2.1.1, p.89).

Slight vacuolation can be seen in the nerve fibre layer, but most of the other structures remained intact.

The bar marker: 100 um.

Fig.2.1.2 Light micrograph of an 18-year-old human retina, 18 hours postmortem and 6 hours to enucleation (Specimen 4, Table 2.1.1, p.89).

Vacuolation is particularly marked in ganglion cell layer but can be seen in most retinal layers.

The bar marker: 100 um.
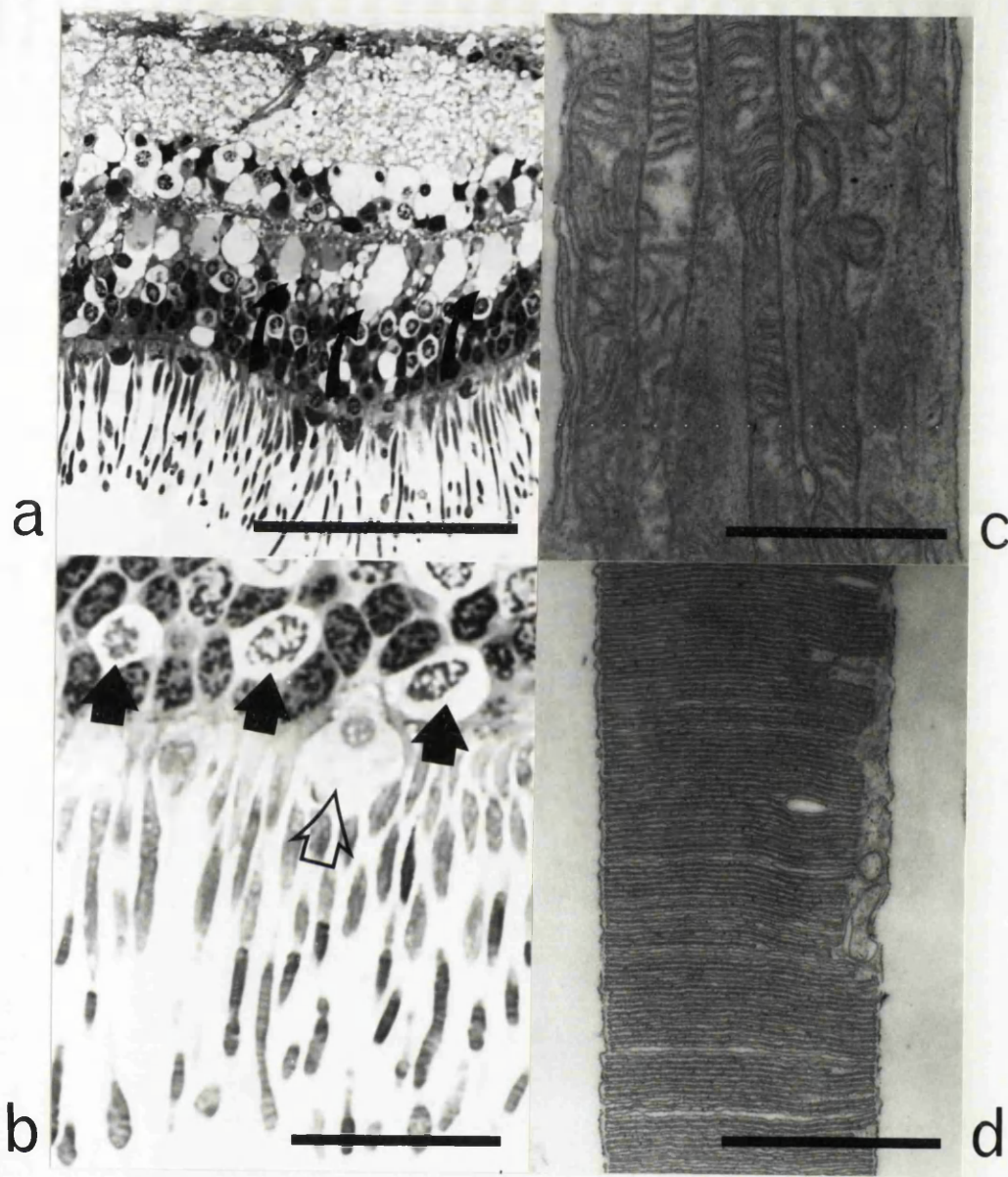
Fig.2.1.3 Light (a, b) and electron (c, d) micrographs of a 40-year-old human retina, 34 hours postmortem and 5 hours to enucleation (Specimen 15, Table 2.1.1, p.89).

a) Vacuolation can be identified in all retinal layers with the exception of photoreceptor IS and OS. Within the photoreceptor cell layer, some perinuclear vacuolation was noted together with a marked swelling of the inner connecting fibres of some cones (arrowed).

b) High power LM micrograph of the IS and OS showing a good state of anatomical preservation. In some cones, perinuclear vacuolation had occurred (solid arrows), whilst in others nuclei had migrated through the outer limiting membrane (open arrow).

c) EM micrograph of the IS of a rod cell showing a high degree of preservation of mitochondrial membranes.

d) EM micrograph showing the typical state of preservation of the disc membranes of rod OS.

The bar markers: a) 100 um; b) 25 um; c) and d) 1 um.

2.1.3.2 The factors affecting survival

In order to determine the relative contributions of the potential factors affecting survival of postmortem human retinae, specimens were analysed according to the criteria outlined below (cf. Table 2.1.1).

Structural survival was estimated by comparing the degree of anatomical preservation in both LM and EM micrographs. The loss of structural integrity was divided into 4 categories:

(++++) Best preserved, with little tissue damage (e.g., Fig.2.1.1).

 (+++) Vacuolation in some retinal layers, but other layers (INL, OPL or ONL) only slightly affected (e.g., Fig.2.1.2).

  (++) Vacuolation and cytoplasmic swelling in all retinal layers except for the IS and OS (e.g., Fig.2.1.3).

  (+) Severe damage to all retinal layers.

To provide a more detailed evaluation of retinal survival, a number of parameters of retinal function were assessed after rhodopsin regeneration (see below) and compared with the morphological appearance of individual specimens (Table 2.1.1). Functional survival was also divided into 4 categories by monitoring the threshold and amplitude of the photoresponse, and the concentrations of rhodopsin and cGMP (cf. Table 2.4.3, p.146).

Table 2.1.1 Survival of structure and function in postmortem human retinae as a function of increasing postmortem time.

No.	Total hrs post mortem	Hours to enucle- ation	Age (yr) & sex	Cause of death	Survival of structure	Survival of structure & function ERG	Rh	cG	other
1	5	0	40 M	(a)	++++	++++	++++	++++	pt aa
2	14	7	64 M	(b)	+++				pt aa
3	17	5	83 M	(c)	+++				pt aa
4	18	6	18 M	(d)	+++	+++	+++	++++	
5A	19	5	35 M	(e)	+++				
6	20	12	86 F	(f)	++	−			
7	21	5	4 M	(b)	+++				aa
8	21	10	49 M	(g)	++				
9	24	5	12 M	(e)	+++				
10	24	5	28 M	(d)	+++	+++	+++	+++	
11	24	5	85 M	(h)	+++				pt
12	29	8	44 M	(f)	++	+++			
13	30	5	66 F	(h)	++	++	+	+	
14A	31	6	42 F	(i)	++				
15	34	5	40 M	(f)	++	+++	++	++	
16	35	11	65 M	(j)	+	−			
17	36	5	58 F	(k)	++				pt aa
18	36	6	58 M	(l)	++	++	+++		
5B	36	5	35 M	(e)	++				
19	36	12	74 M	(b)	+				
20	43	5	82 M	(h)	++	+	+	++	
14B	46	6	42 F	(i)	+				
21	48	4	56 F	(d)	++				
22	48	7	17 M	(e)	+	−			
23	48	5	85 M	(h)	+				pt
24	48	7	66 M	(f)	+				pt
25	48	8	85 M	(m)	+				
26	50	21	53 M	(f)	+	−			
27	58	5	72 M	(h)	+	+			
28	65	11	63 M	(h)	+				
29	72	6	34 F	(k)	+				

Samples are tabulated in order of the total hours postmortem. In some cases where both eyes were obtained from a donor, one eye was used relatively quickly (A) and the other put aside to increase the postmortem time (B).

(++++) best structure and function, (−) no response. M = male, F = female. ERG = electroretinogram, Rh = rhodopsin, cG = cGMP, pt = protein synthesis, aa = amino acid uptake. The results of "pt" and "aa" and the details of all functional parameters will be shown in the respective sections.

Specimen (a) was from a living patient with facial tumor. For the other specimens, causes of death of the donors were: (b) Broncho-pneumonitis, (c) Kidney cancer, (d) Head injury, (e) Chest injury, (f) Cardiac arrest, (g) Colon carcinoma, (h) Cardiac failure, (i) Traffic accident, (j) Lung carcinoma, (k) Breast carcinoma, (l) Cerebral haemorrhage, (m) Pulmonary embolus.

By using the data shown in Table 2.1.1, the factors potentially affecting the survival are analysed below.

(1) Total time postmortem:

Samples are tabulated in order of the total hours postmortem. As might be expected, the samples near the top of the table generally revealed better preservation of structure and function. However, although the total time postmortem affected survival of the retinae, it was not the most significant factor.

(2) Time to enucleation:

The time between death and enucleation of the eye was found to be the most significant factor affecting survival of a retina postmortem. This can be clearly seen by comparing specimens 16 and 18, or 26 and 27. Specimens 18 and 27 showed a recovery of photo-responses whilst specimens 16 and 26 did not. Whilst it could be argued that age was a factor as 18 was marginally younger than 16, 27 was much older than 26. A striking contrast in morphology between specimens 21 and 19 (Fig.2.1.4) also confirmed that time to enucleation was very important for retinal survival, as 21 had better morphology but a longer postmortem time.

(3) Age of donor:

Age of donor may also be a factor affecting survival of tissue. However, the current results have not shown any clear relationship. For example, specimen 27 was 72 years old and 58 hours postmortem but still exhibited a photoresponse after regeneration of visual pigments. Increasing sample size and a wider assessment of functional parameters may modify this conclusion.

(4) Cause of death, and sex:

Neither the cause of death, nor sex of donor, appeared to affect retinal survival.
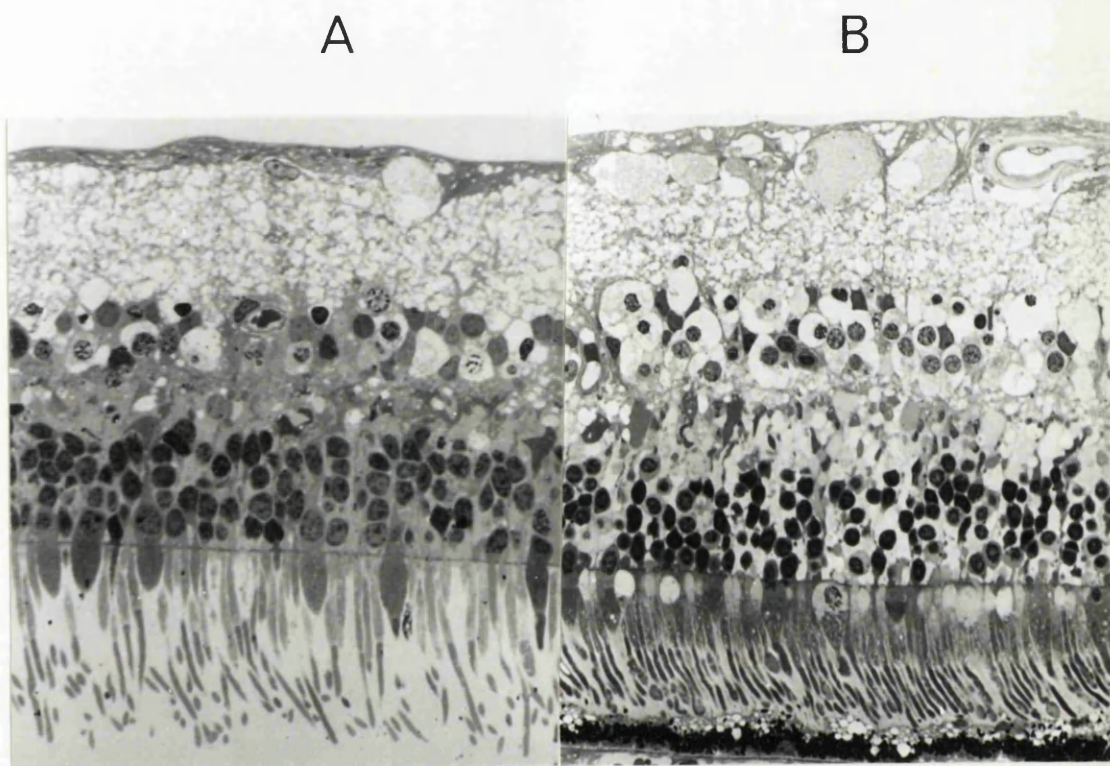
Fig.2.1.4 Effect of time to enucleation on survival of the human retina postmortem.

(A) Peripheral retina from a 56-year-old donor, 48 hours postmortem but 4 hours to enucleation (Specimen 21, Table 2.1.1).

(B) Peripheral retina (but slightly more central than in A) from a 74-year-old donor, 36 hours postmortem but 12 hours to enucleation (Specimen 19, Table 2.1.1).

Retinal structure, particularly in the photoreceptor cell layers and in the INL, was much better preserved in (A) than that in (B). The total time postmortem in the former was 12 hours longer than the latter. The excellent state of morphological preservation in the former seems to be related to the shorter time to enucleation: 4 hours, compared with 12 hours in the latter.

The bar markers: 100 um.

2.1.3.3 Variation of retinal survival in different locations in individual eyes

To determine if survival in retinal specimens in a given eye varied with geographic location, a number of specimens were isolated from a single globe. They were taken from both the peripheral and central areas. For the periphery, four samples were obtained from the superior, inferior, temporal and nasal retinae; central retina was represented by the fovea and perifovea. The studies were carried out on six human eyes (Specimens 5A, 5B, 14A, 14B, 19 and 21 in Table 2.1.1), with postmortem periods varying from 19 to 48 hours, and enucleation times from 4 to 12 hours. Of these samples 5A and B, and 14A and B were pairs, but in each case one eye was fixed first whilst the other remained unfixed at 4°C.

For any given samples the following observations were made.

(1)   There was no apparent difference in survival between any of the four peripheral areas.

(2)   The fovea and perifovea were poorly preserved when compared to their respective peripheral samples. Central samples usually revealed detached or semidetached retinae, especially in the eyes with longer postmortem periods. They also generally showed high degrees of vacuolation, cytoplasmic swelling, and pyknotic nuclei (Fig.2.1.5).
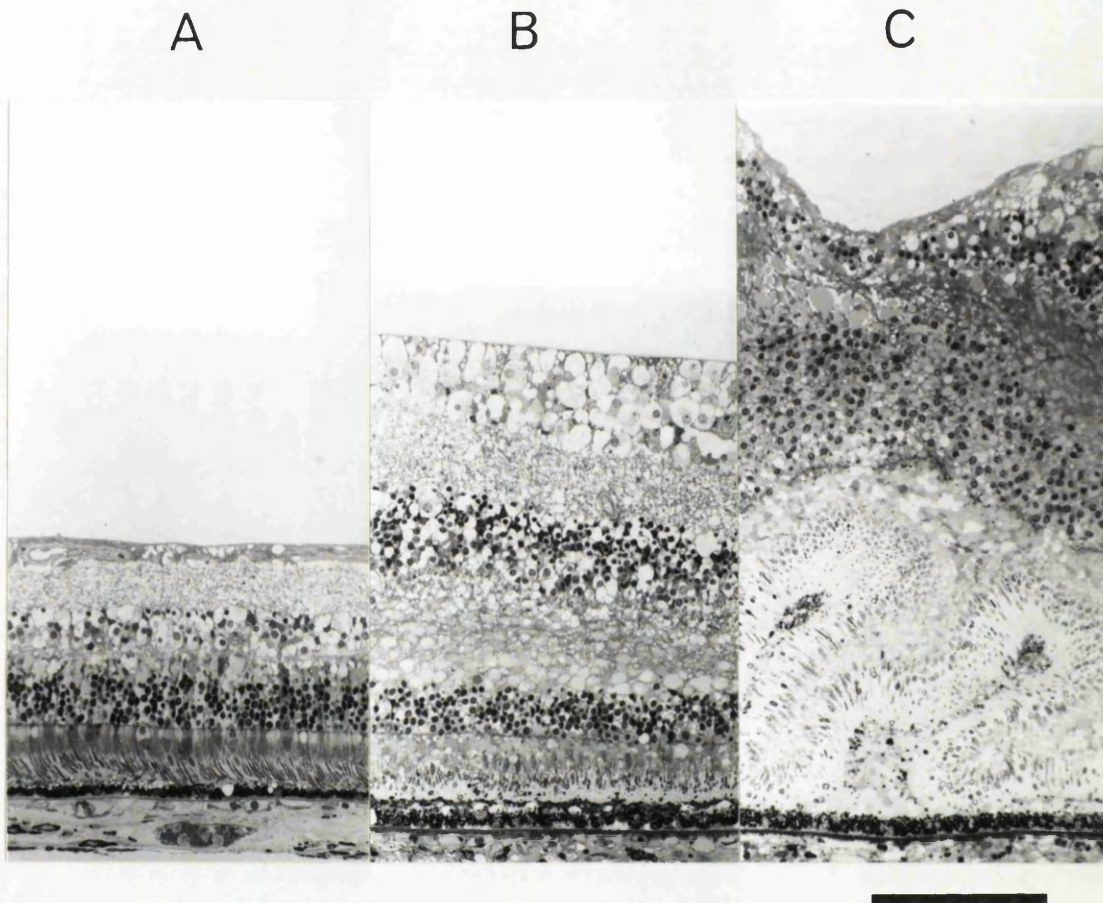
Fig.2.1.5 Comparison of structural preservation of A) periphery, B) perifovea, and C) fovea in a 36-hour-postmortem human retina (Specimen 19, Table 2.1.1).

Anatomical preservation in the peripheral retina was usually quite good, particularly in the case of photoreceptor IS and OS (A). In contrast, in the central specimens the retina was often partially detached. In (B) and (C) a high degree of vacuolation, cytoplasmic swelling, and pyknotic nuclei was apparent.

The bar marker: 100 um.

2.1.4 DISCUSSION

2.1.4.1 Structural changes in postmortem human retina

Within the time frame of the present observations, the results show that human retina and, in particular, photoreceptor cell morphology can survive reasonably well postmortem (e.g., Fig.2.1.3). The typical changes in structure are vacuolation and cytoplasmic swelling. By examining a series of specimens with varying postmortem and enucleation times, it seems that degenerative changes first appear in the nerve fibre, ganglion cell and inner plexiform layers and then spread outwards through the retina to finally involve photoreceptor IS and OS. These observations are consistent with previously-published data which show that, in animals with a dual circulation, postmortem changes occur initially in the inner retina and then extend to the outer retina (cf. p.81; Fujino and Hamasaki, 1967).

From Fig.2.1.3, it can be seen that, in the present specimens, rods survived better than cones. Since cones are densely packed in the central retina (Curcio et al., 1990), their greater vulnerability to anoxia might, at least in part, have contributed to the poorer survival of the foveal and perifoveal areas, compared with the periphery (Fig.2.1.5). Turnbull (1950) also observed that, in the rat, oedema is more pronounced in the central retina than in the periphery under experimental anoxia. With the exception of the avascular foveola, the central retina has a more extensively developed capillary system than the periphery. This indicates that the central retina requires more blood per unit volume of tissue and infers that this region has a greater nutritional need and higher metabolic rate. It would follow, therefore, that anoxia is likely to have a more profound effect in the central retina.

Lactic acidosis has been found to severely damage viability of CNS cells (Rehncrona et al., 1981; Kalimo et al., 1981; cf. p.81). The retina has a large capacity for anaerobic glycolysis, with potential rates approximately 6 times those of brain (Voaden, 1979). As the peripheral

retina has a sparser intraretinal vascular supply, metabolism may be geared to use, as well as produce, lactic acid. Whereas, in the centre, excess might normally be removed by the blood supply. It may be, therefore, that more lactic acid will accumulate in the central retina postmortem, and this merits further investigation.

In addition, neurotoxicity by endogenous excitatory amino acids, such as glutamate, is also a likely mechanism for neuronal cell loss during anoxia in the CNS (cf. p.80). Compared with the periphery, the central retina contains more neuronal elements to subserve acute vision. Thus, there is not only a denser population of photoreceptor cells, but also less pooling of the neuronal signals and, therefore, more higher-order neurones. It is recognized that glutamate and/or aspartate are major retinal neurotransmitters, not only of photoreceptors but also of inner retinal neurones (for reviews; Ehinger and Dowling, 1987; Massey and Redburn, 1987). Thus, it is possible that there is more neurotoxicity in this area postmortem.

2.1.4.2 Factors affecting preservation of the human retina postmortem

The current results indicate that the factors affecting survival of the human retina are total time postmortem and the time between death and enucleation of the eye. The latter appears to be the most significant. This conclusion is supported by the work of Kim and Takahashi (1988) who have also suggested that the time between death and enucleation is the most critical factor for the survival of retinal explants.

Enucleated eyes were routinely stored at $4^{\circ}C$. The shorter the time between death and enucleation, the earlier the refrigeration of the eye. This suggests that low temperature may reduce the rate of residual retinal metabolism and thus facilitate the survival of the retina. The positive effect of low temperature on preservation of retinal responses has been further investigated using rat tissue and this will be described below (p.148).

It should be noted that the globes used here were not optimally preserved for retinal studies, because they had been maintained and used for corneal grafting before the present investigations. If donor eyes were enucleated as quickly as possible after death and then kept at low temperature before retinal studies, the survival would undoubtedly be better.

2.2 RHODOPSIN AND ITS REGENERATION IN VITRO

2.2.1 INTRODUCTION

(1) Pigment regeneration

As previously outlined in section 1.2.3 (p.43), rhodopsin regeneration, through the binding of opsin with 11-cis retinal, is essential for restoring phototransduction. In vitro, the chromophore must be supplied. In the present experiments, mixed isomers of retinal (principally 13-cis, 11-cis, 9-cis and all-trans; see below) were employed to regenerate visual pigments. Only the 11-cis and 9-cis isomers of retinal form photoreactive complexes with opsin, yielding rhodopsin and isorhodopsin, respectively. The pigments have similar absorption characteristics and both will initiate transduction (cf. p.45): in the following discussion they will be referred to solely as rhodopsin.

Because retinal and all its derivatives are only poorly soluble in water, ethanol (1-20%) has been used by many workers to assist solubilization (p.46). Equally, Yoshikami and Noll (1978, 1982) have proposed phospholipid vesicles as vehicles for retinoid delivery to photoreceptor cells. In addition, IRBP (interphotoreceptor retinoid-binding protein) has been reported to bind retinal and its derivatives, and is widely thought to function as a visual-cycle retinoid carrier between the RPE and photoreceptor cells in vivo (cf. p.34). Its use in visual pigment regeneration in vitro has recently been reported (Jones et al., 1989b).

(2) Rhodopsin phosphorylation

The phosphorylation of rhodopsin plays an important role in the termination of phototransduction by partially deactivating Rh* (cf. p.52). The activity of the phosphorylated Rh* is then fully blocked by arrestin (cf. p.53). Subsequently, rhodopsin/opsin is dephosphorylated in the dark (cf. p.52). Because of its potential importance to recovery of function in the visual pigment, studies on phosphorylation and dephosphorylation have been included in the present programme of work.

Techniques for rhodopsin regeneration _in vitro_ were established initially with rat tissue and the following comparisons and assessments were made.

(1) The efficiency of pure 11-cis retinal versus a mixture of retinal isomers (2.2.3.1, p.108).

(2) Application of retinal in liposomes, ethanol, IPM or IRBP for rhodopsin regeneration, and exploration of the possibility of employing IPM or IRBP in delivering exogenous retinal _in vitro_ (2.2.3.2, p.111).

(3) Experiments to study the relationship between rhodopsin regeneration and phosphorylation/ dephosphorylation. This was to enhance our understanding of the factors potentially needed for recovery of phototransduction in the retina (2.2.3.3, p.113).

(4) The capability of postmortem human retina to regenerate rhodopsin (2.2.3.4, p.117).

2.2.2 MATERIALS AND METHODS

2.2.2.1 Chemicals and medium

Chemicals used were of "Analar" grade, obtained mostly from the Sigma Chemical Company (Sigma), British Drug Houses (BDH), and Fisons Scientific Apparatus Limited (FSA). Where relevant the sources of other materials will be indicated.

(1) Earle's medium: (composition in mM)

NaCl 116,   KCl 5.4,   NaHCO$_3$ 26.2,   NaH$_2$PO$_4$ 1.0,
MgSO$_4$ 0.8, CaCl$_2$ 1.8, glucose 5.5.

(2) Fortified Eagle's medium:

a) Eagle's MEM (Minimum Essential Medium) (Sigma):-

		g/L	mM
Earle's salts (as above)			
Glucose		1.0	5.5
L-Arginine.HCl		0.126	0.6
L-Cystine.2HCl		0.0313	0.1
L-Glutamine		0.292	2.0
L-Histidine.HCl.H$_2$0		0.042	0.2
L-Isoleucine		0.052	0.4
L-Leucine		0.052	0.5
L-Lysine		0.0725	0.5
L-Methionine		0.015	0.1
L-Phenylalanine		0.032	0.2
L-Threonin		0.048	0.4
L-Tryptophan		0.010	0.05
L-Tyrosine 2Na		0.0519	0.2
L-Valine		0.046	0.4
Choline chloride		0.001	
Folic acid	(vitamin Bc)	0.001	
Myo-Inositol		0.002	
Niacinamide	(vitamin PP)	0.001	
D-Pantothenic acid Ca	(vitamin B3)	0.001	
Pyridoxal HCl	(vitamin B6)	0.001	
Riboflavin	(vitamin B2)	0.0001	
Thiamine HCl	(vitamin B1)	0.001	
Phenol red Na		0.011	

b) Supplements:-
(cf. Ham and Mckeehan, 1979; Politi et al., 1988).

		g/L	mM
Taurine		0.0125	0.1
Ascorbic acid	(vitamin C)	0.352	2.0
Pyruvate		0.11	1.0
Glucose		1.0	5.5
Insulin		10 ug/ml	
Transferrin		10 ug/ml	
Foetal calf serum		10 % (v/v)	
Penicillin		100 units/ml	
Streptomycin		100 ug/ml	
Amphotericin B		250 ng/ml	

The media were pregassed with a moistened mixture of
95% O$_2$/5% CO$_2$ and had a final pH of 7.2 at room
temperature. The fortified Eagle's medium was sterilized
by ultrafiltration and used for studies on the human
retina. Earle's medium was used for studies on rat tissue.

2.2.2.2 Encapsulation of retinal in liposomes

(1) Preparation of the retinal isomers.

The techniques used for the preparation and, where relevant, purification of the retinal isomers were similar to those described by Bridges and Alvarez (1982).

An aliquot of 10 mg of all-trans retinal (Sigma) was dissolved in 10 ml of absolute ethanol in a 200 ml glass beaker on ice. The beaker was covered with a Petri dish, and the surface of the solution was gently gassed with nitrogen and irradiated with a 100 W tungsten lamp, positioned about 25 cm from the surface for 1 hour. These conditions were to photoisomerize the all-trans retinal to a photostationary mixture of isomers. The retinal in ethanol was quantified in a spectrophotometer, using the extinction coefficient of 24,935/M/cm at 369 nm.

Aliquots of the resultant photoisomerate of retinals were fractionated by high-pressure liquid chromatography (HPLC) (Bridges and Alvarez, 1982). The HPLC system was produced by Varian Associates Ltd. and consisted of a 5060 liquid chromatograph, a UV-50 detector, a 4270 integrator and a Water's u Porasil column (7.8 mm x 30 cm). The mobile phase was 13% ether in hexane (v/v), pumped at 0.6 ml/min, and detection was carried out at 310 nm. In the photoisomerate, the quantity of both 11-cis and 9-cis retinal together accounted for 27 $\pm$ 2% of the total (mean $\pm$ SEM, n=6). Some pure 11-cis retinal was collected for an initial assessment of relative efficiency but the crude photoisomerate was used for most experiments.

(2) The preparation of liposomes.

The techniques used for preparation of liposomes to encapsulate retinal were similar to those previously described (Perlman et al., 1982; Voaden, 1985).

The designated amounts of 11-cis retinal or photoisomerate of retinal, together with 100 ul of phosphatidyl choline (100 mg/ml in chloroform, Sigma) were pipetted into a small glass tube covered with aluminium foil. The mixture was immediately evaporated to dryness with nitrogen under dim red light. This was carried out to minimize further photoisomerization and oxidation of

retinal. The pellet was resuspended in 0.5 ml of Earle's medium and the container, after gassing with nitrogen, capped and vortexed for 1 minute. The mixture was then sonicated for 10 minutes at one third power in a model W-150 sonicator, equipped with a tapered microtip (MSE Ltd.). Throughout the sonication surface of the suspension was gassed with nitrogen, and the glass tube was located in a beaker filled with water at 10°C. Sonication broke the liposomes into small, unilamellar lipid vesicles containing the retinal (cf. Yoshikami and Noll, 1982). Afterwards, the preparation of liposomal retinal was kept cool in the dark, and used within 30 minutes.

2.2.2.3 Preparation of isolated rat and human retinae

(1) Rat:

Female albino Wister rats, weighing 200 to 300 grams, were killed by cervical dislocation after light ether anaesthesia. After enucleation, the eyes were placed in a semiautomatic device consisting of two plates of perspex with a rotating blade that was adjusted to bisect the eye at the ora serrata. The anterior portion together with lens and vitreous were discarded. The posterior eye-cup was transferred to a Petri dish containing Earle's buffer and then everted. The retina was then carefully teased from the pigment epithelium and underlying tissue using forceps.

In some experiments the rats were maintained in total darkness overnight and their retinae dissected under dim red light. The source was a 15 W red incandescent bulb (Philips, PF712) and the samples were never closer than 40 cm. In other experiments the rats were light adapted and their retinae isolated under normal room lighting. Exposure of the latter to ambient light was continued for a further 5 minutes after isolation and no rhodopsin was then detectable by difference spectroscopy at 500 nm (see below).

(2) Man:

Normal human eyes were obtained as described in section 2.1.2.1 (p.83). All were dissected under room light. The wall of the globe was cut circumferentially with scissors about 2 mm behind the ora serrata. The anterior portion of the globe, including the lens and vitreous, was removed and discarded. After adding about 2 ml of the fortified medium into the posterior eye-cup, the retina was carefully separated from the RPE by using a smooth stainless steel probe. When the whole retina had been detached, it was finally isolated by severing the optic nerve proximal to the lamina cribrosa.

After transference to a Petri dish containing the same medium, the retina was carefully cut into approximately 20 mm^2 portions. Samples used for this study were from the peripheral retina and did not include any specimen from the posterior pole.

2.2.2.4   Incubation of the retina

In the rat experiments, one entire retina was incubated per ml of incubation medium. In human experiments a similar volume was used per isolated sample of peripheral retina. All incubations were carried out in the dark at 37°C and samples were shaken gently. All incubation media were gassed with a moistened mixture of 95% O_2/5% CO_2 throughout.

Unless otherwise stated, retinal in liposomes was used for rhodopsin regeneration.

2.2.2.5   Quantification of rhodopsin

Whenever possible the manipulations described here were performed in darkness, dim red light being used only when essential.

After incubation, the retina was carefully transferred to a Petri dish of fresh medium in order to minimize free retinal. It was then placed in a glass homogeniser containing 0.5 ml distilled water and homogenized at low speed for 1 minute. The homogenate was

transferred to an Eppendorf tube. The homogenizing tube and pestle were washed with two 0.5 ml aliquots of water, and the washes and homogenate combined. A 75 ul aliquot was then taken for protein estimation (see below).

The remainder was centrifuged at 12,000 g in a SORVALL RC2-B centrifuge (SS-34 rotor) for 10 minutes. The supernatant was decanted and any residual liquid, adherent to the sides of tube, carefully wiped away. A 0.45 ml aliquot of 1% octyl glucoside (Sigma) in 100 mM sodium phosphate buffer, pH 7.4, was added to the precipitate. This was then vortexed for 1 minute and maintained at $4^{\circ}C$ for 2 hours to allow sufficient time for the detergent to act on the membranes and release the rhodopsin. A 50 ul aliquot of freshly-prepared 1 M hydroxylamine, neutralised to pH 7.0 with NaOH, was then added.

The suspension was centrifuged at 8,000 g in an Eppendorf microcentrifuge for 5 minutes, and the supernatant transferred to a cuvette to be scanned from 650 to 450 nm in a Perkin Elmer spectrophotometer (UV-VIS 552). It was then bleached for 5 minutes with a 100 W incandescent bulb placed 30 cm from the surface. This light exposure was sufficient to bleach all the rhodopsin. The sample was then re-scanned, after adjusting the plotter to a baseline level at 650 nm where no absorbance change was assumed to occur.

The difference in absorbance at 500 nm before and after the bleaching was measured, and the amount of rhodopsin calculated using the extinction coefficient of 40,000/M/cm (Hubbard et al., 1971). The concentration of rhodopsin was calculated on a per mg protein basis.

2.2.2.6 Protein estimation

Protein in the retina was measured by the method of Lowry et al. (1951), as modified by Miller (1959). Two reagents were used: (A) containing alkaline copper which reacts with the protein, and (B) Folin Ciocalteau which reacts with the protein-copper complex, resulting in a chromatic endpoint.

Bovine serum albumin (Sigma, A-8022) was used as standard. Reagent A was made from: 0.5 ml of 5% $CuSO_4$, 4.5 ml of 1% Na/K tartrate and 50 ml of 10% Na_2CO_3 in 0.5 M NaOH. Reagent B was 1:10 diluted Folin Ciocalteau Phenol reagent (BDH).

The standard and retinal proteins were made up to 1 ml with distilled water, and 1 ml of reagent A was then added. The mixtures were left to stand for 5 minutes. The above procedures were carried out at $4^{\circ}C$. Subsequently, 3 ml of reagent B was added and the mixture was then incubated at $50^{\circ}C$ for 10 minutes. Samples were then pipetted into cuvettes and the absorbance at 660 nm determined. Protein concentrations were estimated by construction of a standard curve.

2.2.2.7 Preparation of IPM and IRBP

The techniques used for preparation of IPM and IRBP were essentially those described by Adler and Evans (1985).

Fresh bovine eyes were obtained approximately 4 hours postmortem from a slaughter house. They were transported to the laboratory on ice. A total of 50 retinae were dissected out, and each eye-cup was subsequently rinsed twice with 2 ml of ice-cold PBS (140 mM NaCl and 5 mM NaH_2PO_4, pH 7.4). The 50 retinae and a total of 400 ml of the PBS solution were collected in a large beaker, and stirred with a magnetic stirrer at 20 rev/min for 30 minutes. The mixture was then filtered through a fine mesh. The residue on the filter was resuspended in a further 200 ml of PBS and stirred for 15 minutes before being refiltered. The two filtrates were combined and centrifuged at 20,000 g for 80 minutes. Half of the supernatant (300 ml) was removed and 10 ml aliquots were concentrated by ultrafiltration at $4^{\circ}C$, using a stirred cell (Amicon, 8010) and ultrafiltration membrane (Amicon, YM5). After the protein content had been measured, the concentrates, designated IPM, were frozen. The remaining 300 ml of the supernatant was used to isolate IRBP.

IRBP is the major glycoprotein of the IPM and the method used to isolate it was affinity adsorption onto concanavalin A-sepharose beads (Adler and Evans, ~~1983~~, 1985). Aliquots of the supernatant containing 40 mg protein were stirred with 5 ml concanavalin A-sepharose beads in 20 ml of buffer (140 mM NaCl, 10 mM Tris, pH 7.0) for 1 hour at room temperature. The supernatants were discarded and the beads washed twice with the same buffer. IRBP was liberated by stirring the beads in 20 ml of 50 mM alpha-methyl D-mannoside in the same buffer for 30 minutes. The supernatants containing IRBP were then concentrated as described for IPM, and stored frozen.

Adler and Evans (1985) have shown that IPM, prepared as described above, has no serious contamination from retinal cell components. Densitometry of SDS gels of the IPM preparation showed that protein of molecular weight at 140 kDa (potentially IRBP) accounted for about 10% of the total. In contrast, in the IRBP preparation, 140 kDa protein was about 92% of the total. These proportions were similar to those described by Adler and Evans (1985) who reported levels of 12% and 94% respectively.

2.2.2.8 Phosphorylation and dephosphorylation of rhodopsin

The method used to determine the state of phosphorylation of rhodopsin was modified from those described by Miller and Paulsen (1975) and Kamps et al. (1986). The $Na_2H^{32}PO_4$ was obtained from DuPont Inc.

Dark-adapted rat retinae were dissected under dim red light in Earle's medium. The retinae were incubated in the dark at 37^OC for 60 minutes in the same medium containing $Na_2H^{32}PO_4$ (final concentration 0.1 mM, activity 0.1 mCi/ml). It has been suggested that the endogenous pools of phosphate donor equilibrate with $^{32}PO_4$ within 45 minutes, but that a 60 minute incubation gives better reproducibility (Kamps et al., 1986). After incubation, the retinae were fully bleached by exposure to room light for 10 minutes. They were then separated into 3 groups and further incubated for various periods in Earle's medium containing no radiolabelled phosphate. One group

was incubated under normal room light, the second kept in the dark, and the third incubated in the dark with 500 uM retinal photoisomerate. At designated times, the phosphorylation or dephosphorylation reactions were stopped by emerging the samples of retina into 0.2 ml of electrophoresis "Sample" buffer containing 2% sodium dodecyl sulphate (SDS) (cf. 2.6.2.4, p.173; Sitaramayya and Liebman, 1983).

The retina was then dispersed by intermittent sonication at 50% power in the model W-150 sonicator for a total of 20 seconds. The resultant suspension was subjected to SDS-polyacrylamide gel electrophoresis (cf. 2.6.2.4, p.173). Aliquots of 25 ug protein were run in each lane. The opsin band was identified by both comparing molecular weight markers and opsin immunoblotting using rabbit antiserum (cf. 2.6.2.5, p.174). After photography, the gels were dried and direct autoradiography was performed with Hyperfilm-MP X-ray film (Amersham). Exposure was carried out at $-70^{\circ}C$ for 2 days.

The radioactivity in the opsin band on the dry gels was then released using the method described by Dion and Pomenti (1985) (cf. 2.6.2.6, p.175) and counted by scintillation spectrometry (cf. 2.6.2.3, p.173).

As regards the protein content of the retinal samples which were treated with SDS-containing "Sample" buffer, preliminary experiments showed that SDS affected protein estimation. Thus, aliquots of the retinal suspension were precipitated 3 times with 12% trichloroacetic acid (TCA) (final concentration), so as to wash out residual SDS. The final pellets were solubilized with 0.5 ml of 0.5 M NaOH at $4^{\circ}C$ overnight. The proteins in the resulting suspension were then estimated with the modified Lowry's method (cf. p.103), using bovine serum albumin, similarly treated with the sample buffer and TCA, as standard.

An effort has been made to estimate the phosphate incorporation into opsin by dividing the moles of ^{32}P by the moles of opsin present. This relationship between phosphate and (rhod)opsin is abbreviated as the P/R ratio.

A standard curve of $^{32}PO_4$ molarity was established by using a graded series of molecular concentrations of $Na_2H^{32}PO_4$ added to prepared protein suspensions. Blank samples were obtained by adding $Na_2H^{32}PO_4$ to TCA-precipitated protein and then washing as described above. The concentration of (rhod)opsin could not be determined spectrophotometrically (cf. p.102), as the proteins had been denatured by SDS. Instead, the average rhodopsin content of normally dark-adapted rat retinae (cf. Table 2.2.1, p.109) was used to estimate the P/R ratio.

It should be noted that P/R values may not represent absolute levels of phosphorylation of (rhod)opsin. In practice, they may be low because the radiolabelled phosphate donors (e.g., ^{32}P-ATP), synthesized by 60 min incubation with $Na_2H^{32}PO_4$, may account for only a portion of the endogenous phosphate donor pool. In other words, non-^{32}P-labelled phosphorylation of (rhod)opsin can not be estimated by radioactivity counting.

Given this limitation, in the following experiments phosphorylation of rhodopsin was expressed as dpm/mg protein, instead of the P/R value. This gave a simple and adequate comparison of incorporation of $^{32}PO_4$ into (rhod)opsin between individual samples.

2.2.3   RESULTS

2.2.3.1 Rhodopsin regeneration in isolated, bleached rat retinae

Table 2.2.1 and Fig.2.2.1 show rhodopsin regeneration in bleached, isolated rat retinae incubated with various concentrations of either 11-cis retinal, or retinal photoisomerate, encapsulated in liposomes. It can be seen from Table 2.2.1 and Fig.2.2.1A that full regeneration of rhodopsin was achieved with 100 uM 11-cis retinal, and 91% regeneration attained with 500 uM of the photoisomerate.

Fig.2.2.1 also compares the efficiencies of 11-cis retinal and photoisomerate in rhodopsin regeneration. Increasing the amount of the mixed isomers did not give the same proportional increase in efficiency as 11-cis retinal. Instead, there was a decrease in their efficiency as the concentration increased (Figure 2.2.1B).

Table 2.2.1 Rhodopsin in dark-adapted, bleached and "regenerated" rat retinae.

Retinal samples	Rhodopsin nmol/mg protein	(%)
Dark-adapted	* 1.16 ± 0.06 (5)	100
Bleached	zero	0
Regenerated with		
11-cis retinal, 50 uM	1.01 ± 0.03 (6)	87
100 uM	1.18 ± 0.04 (5)	102
photoisomerate, 100 uM	0.59 ± 0.03 (4)	51
200 uM	0.82 ± 0.03 (4)	71
400 uM	1.03 ± 0.03 (4)	89
** 500 uM	1.06 ± 0.05 (6)	91

Incubation time for rhodopsin regeneration was 60 minutes. Results are expressed as the mean ± SEM (n).

Note: The average rat retina was found to contain 1.12 ± 0.14 mg protein (n = 8). Thus, a rhodopsin concentration of 1.16 nmol/mg protein (*) represents approximately 1.30 nmol/retina.

The rate of rhodopsin regeneration with 500 uM photoisomerate of retinal (**) has been studied and below is compared with that obtained for the human retina (see Fig.2.2.6, p.118).
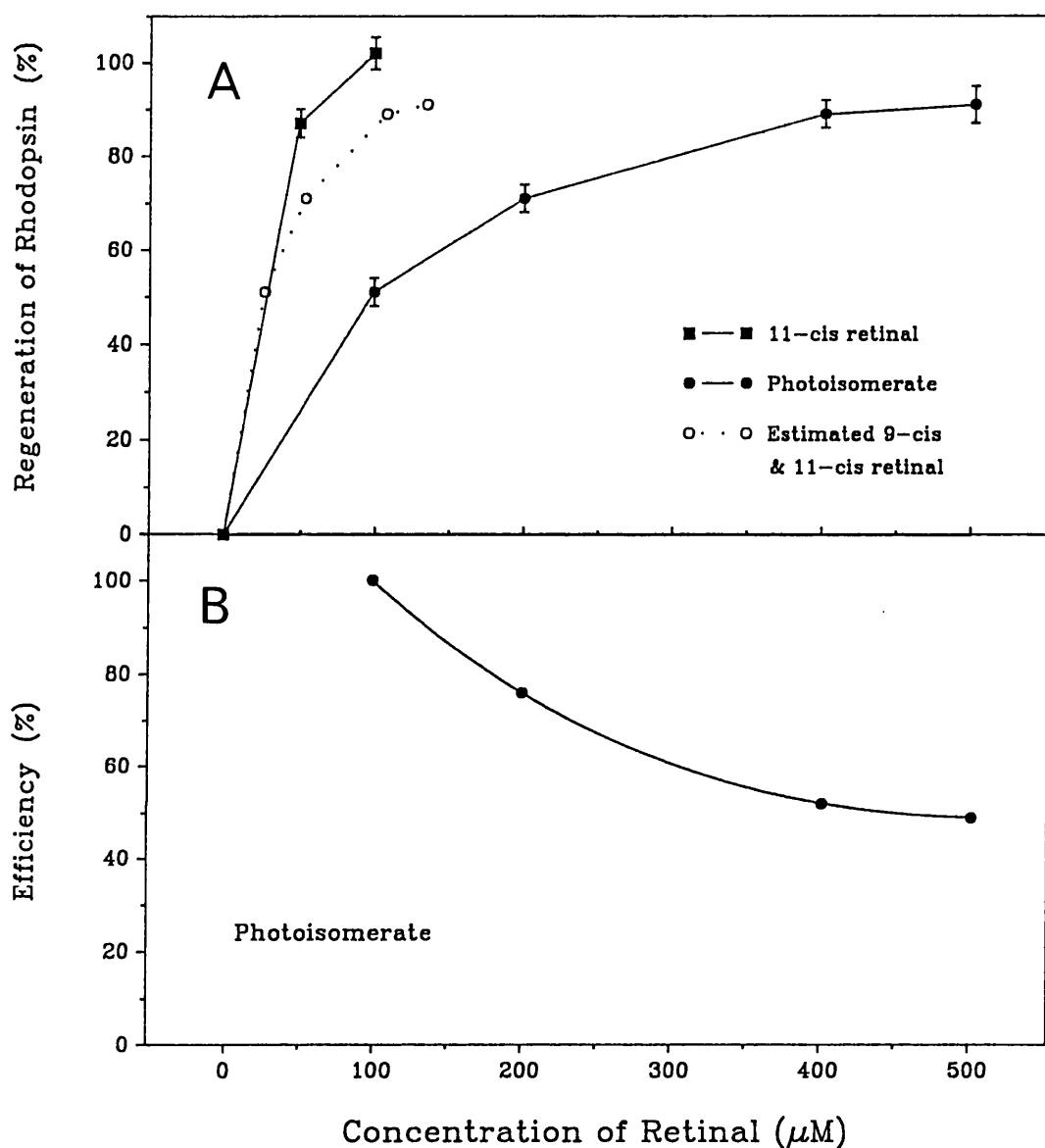
Fig.2.2.1 Rhodopsin regeneration in bleached rat retinae incubated for 60 minutes in different concentrations of 11-cis retinal or the retinal photoisomerate.

(A) Rhodopsin regeneration as a function of retinal concentration. The combined amounts of 9-cis and 11-cis retinal in the photoisomerate were estimated as 27% of the total (see p.100).

(B) Efficiency of the photoisomerate was calculated by dividing the concentration of 11-cis retinal used for regenerating a certain level of rhodopsin by the estimated concentration of 9-cis and 11-cis retinal present in the photoisomerate that regenerated the same amount of rhodopsin (cf. curves ■ ——— ■ and O .... O in A).

2.2.3.2 Carrier systems for retinal

The aim was to compare the efficiencies of retinal, solubilized in ethanol or encapsulated in liposomes, for the regeneration of rhodopsin. A second study explored the possibility of employing either IPM or IRBP in the delivery of retinal for rhodopsin regeneration in vitro.

Isolated, bleached rat retinae were individually incubated in 1 ml of incubation medium containing 500 uM photoisomerate of retinal. Retinal was encapsulated in liposomes, or dissolved in ethanol, or mixed with IPM or IRBP, as described in the Methods (2.2.2, p.98). As in the previous experiments, 10 mg phosphatidyl choline was used, or, following the procedure of Pepperberg (1982), 3% ethanol (v/v) was employed. The amount of IPM was equivalent to 0.5 mg protein and IRBP to 0.1 mg protein. The choice of these amounts of IPM and IRBP was based on pilot experiments which showed that the above concentrations of IPM and IRBP resulted in optimal regeneration of rhodopsin. Increasing the amount of IPM or IRBP did not increase the level of rhodopsin regenerated. It is known that IRBP constituted about 10% of the total protein in the IPM preparation and about 92% of the total in the IRBP preparation (cf. p.105). It is also known that the molecular weight of IRBP is 140 kDa. Thus, 0.5 mg protein in the IPM contained approximately 0.36 nmoles of IRBP (equivalent to 0.36 uM) and 0.1 mg protein in the IRBP preparation contained approximately 0.66 nmoles of IRBP (equivalent to 0.66 uM).

It should be noted that, in the samples with IPM or IRBP, 3% ethanol was necessarily present to dissolve exogenous retinal.

Fig.2.2.2 shows that the application of liposomes resulted in the highest level of rhodopsin regeneration (91% of the dark-adapted level), 3% ethanol gave a level of 61%, and IPM and IRBP, 73% and 75% respectively.
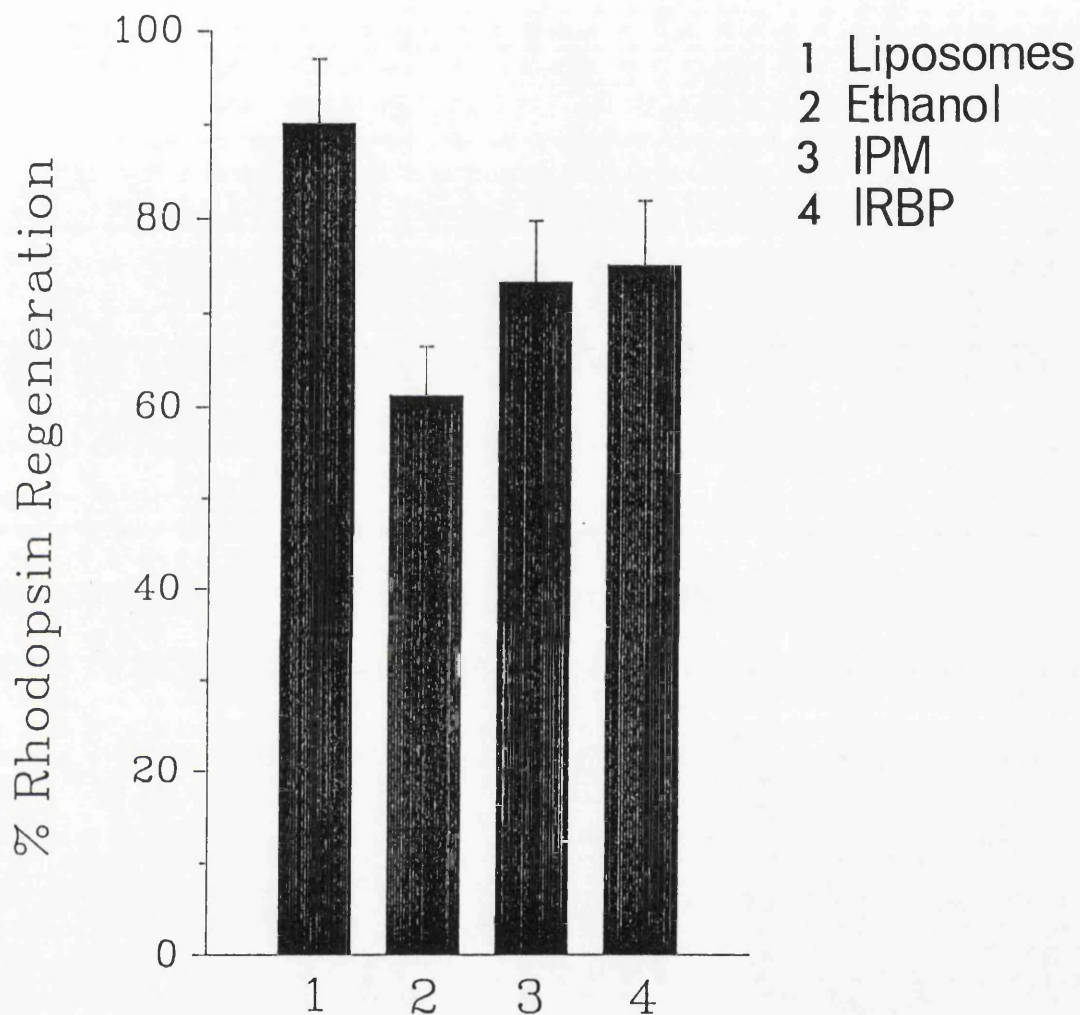
Fig.2.2.2 Application of retinal in liposomes, ethanol, IPM or IRBP to regenerate rhodopsin in isolated, bleached rat retinae.

All samples were incubated in the dark at 37°C for 60 minutes with 500 uM mixed isomers of retinal as described in the text. Each value represents the mean ± SEM of at least 4 estimations.

The significance of differences: $p < 0.01$ between (1) and (3), or (1) and (4); $p < 0.05$ between (2) and (3), or (2) and (4); and $p > 0.05$ between (3) and (4), by the Student paired t-test.

2.2.3.3 Phosphorylation and dephosphorylation of rhodopsin

Fig.2.2.3 shows protein and phosphorylation profiles of isolated rat retinae. It can be seen that:-

1) Light predominantly induced phosphorylation of opsin (b′, c′).

2) Subsequent incubation in the dark for up to 60 minutes caused some dephosphorylation of opsin or rhodopsin (d′-i′).

To compare quantitatively the levels of the phosphorylation of (rhod)opsin shown in Fig.2.2.3, the radioactivity incorporated into the opsin bands (both monomer and dimer) was released and counted. The results are plotted in Fig.2.2.4, and show that:-

1) Exposure of the dark-adapted retina to light induced phosphorylation of opsin, and most of the phosphorylation occurred in the first 10 minutes of exposure (I).

2) Subsequent incubation in the dark resulted in dephosphorylation of opsin, and 60 minutes incubation allowed approximately 44% of the molecules to be dephosphorylated (II).

3) Rhodopsin regeneration had no apparent effect on the dephosphorylation (cf. II and III).

In addition, Fig.2.2.3 shows that some unidentified proteins, e.g., 22-23, 28, 30, 46, 145, and 220-245 kDa, also exhibited light-induced phosphorylation. Light-induced phosphorylations of 46 and 245 kDa proteins have been reported by Kamps et al. (1986), and 220-240 kDa proteins by Szuts (1985). The significance of the phosphorylation of these proteins is not clear.

Fig.2.2.5 compares the degree of rhodopsin regeneration and dephosphorylation in the rat retina under identical conditions. It can be seen that rhodopsin regeneration was not parallel to its dephosphorylation. For example, after 60 minutes incubation, 91% of the dark-adapted level of rhodopsin was regenerated, whilst only 44% of the total molecules were dephosphorylated. That is, approximately half of the regenerated rhodopsin was still phosphorylated.

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Fig.2.2.3 Protein and phosphorylation profiles of the isolated rat retina.

    The separating gel was 10% acrylamide. Abbreviations: Mw, molecular weight markers; Rh, opsin bands (arrowed).

    Lanes **a-i**, protein profiles; **a'-i'**, autoradiograms.

I: **a**, Dark-adapted (DA) retina incubated with $Na_2H^{32}PO_4$ for 60 min. **b**, DA retina incubated as in (a) but subsequently exposed to room light for 10 min to bleach the rhodopsin fully. **c**, DA retina incubated and exposed as in (b) but further incubated under room lighting for 60 min. It can be seen that predominant phosphorylation of opsin (monomer and dimer) was induced by illumination (**b'**) and that a further 60 min exposure to light caused slightly more phosphorylation (**c'**).

II: Retinae were incubated and exposed as in (Ib) and then incubated in the dark for (**d**) 15, (**e**) 30 and (**f**) 60 min. It can be seen that incubation in the dark reduced the density of the radio-labelling in the opsin bands (**d'-f'**).

III: Again, retinae were first incubated and exposed as in (Ib) and then incubated in the dark but with 500 uM photoisomerate of retinal for (**g**) 15, (**h**) 30 and (**i**) 60 min, to regenerate rhodopsin. There was a similar reduction in the density of the radio-labelling in the opsin bands (**g'-i'**) to that seen in (II).

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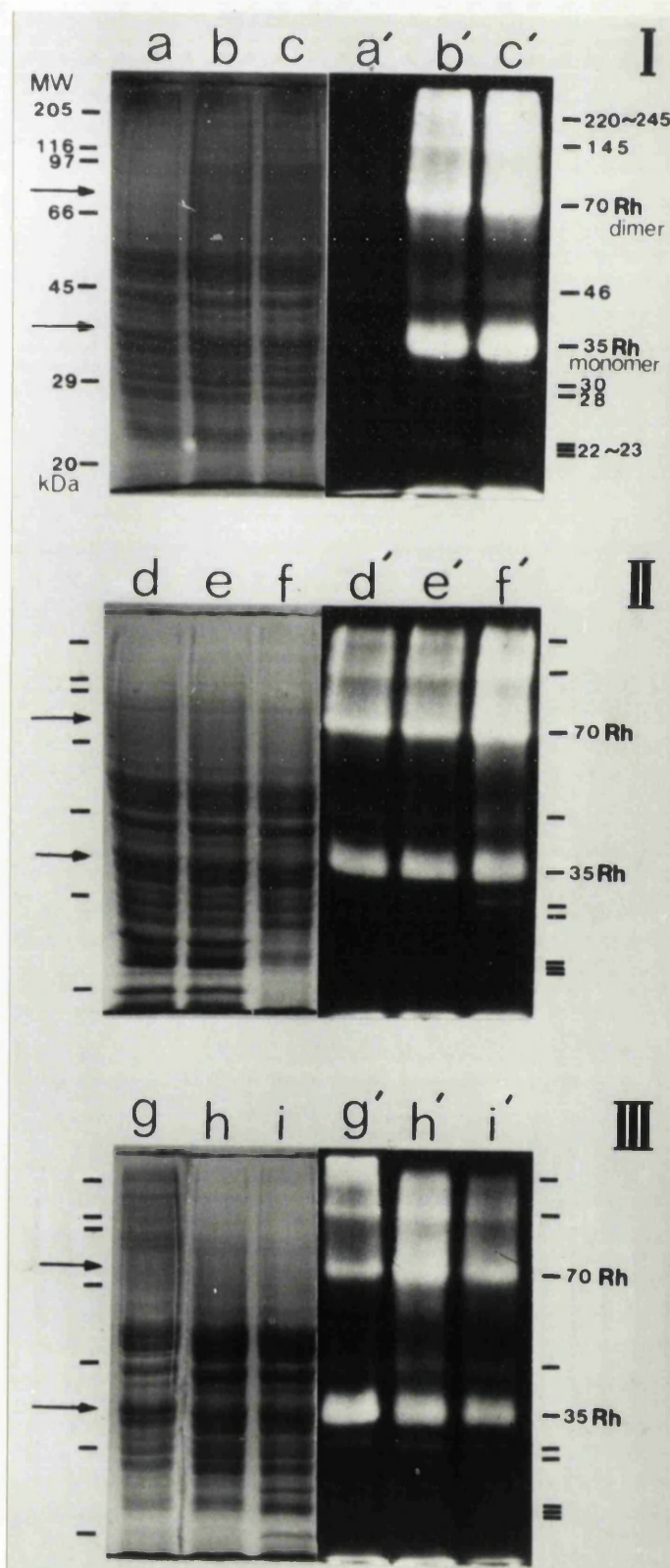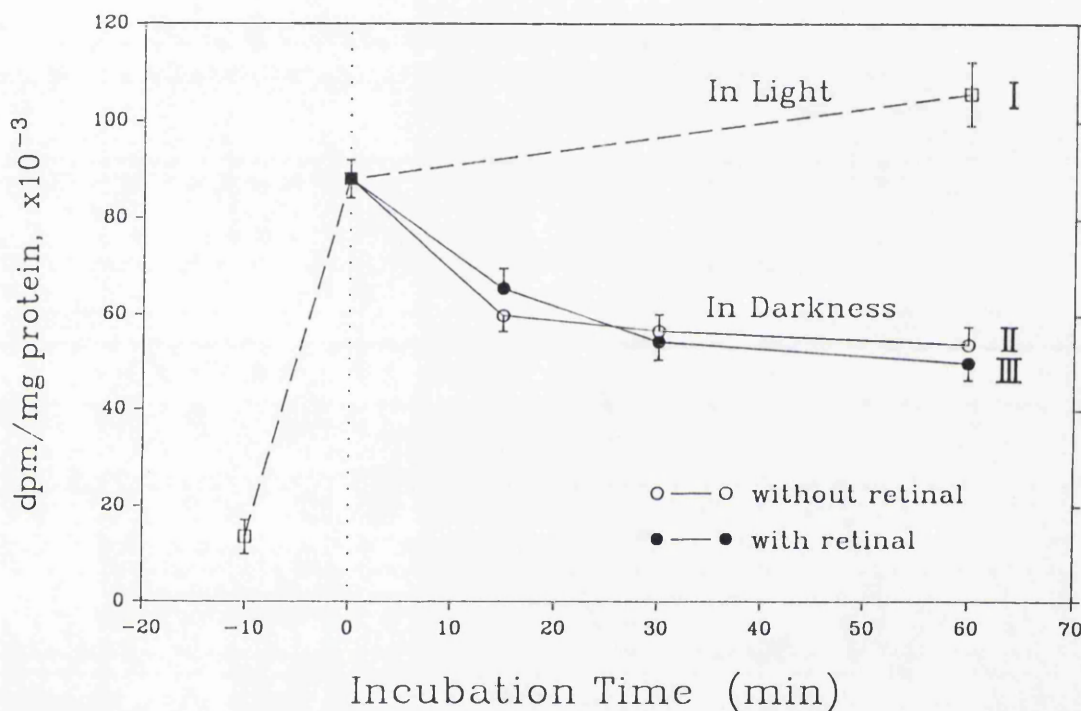
Fig.2.2.4 Incorporation of ^{32}P into rhodopsin/opsin in the rat retina.

The three curves represent, respectively, the changes in radioactivity incorporated into the opsin bands (monomer and dimer) of the three groups in Fig.2.2.3.

All retinae were prelabelled by incubation in the dark for 60 minutes with $^{32}PO_4$, and then:-

  I: The retinae were exposed to light.

 II: After exposure to light for 10 minutes, the retinae were incubated in the dark again.

III: After exposure to light for 10 minutes, the retinae were incubated in the dark but with 500 uM photoisomerate of retinal to regenerate rhodopsin.

Each point represents the means ± SEM of 3 estimations.
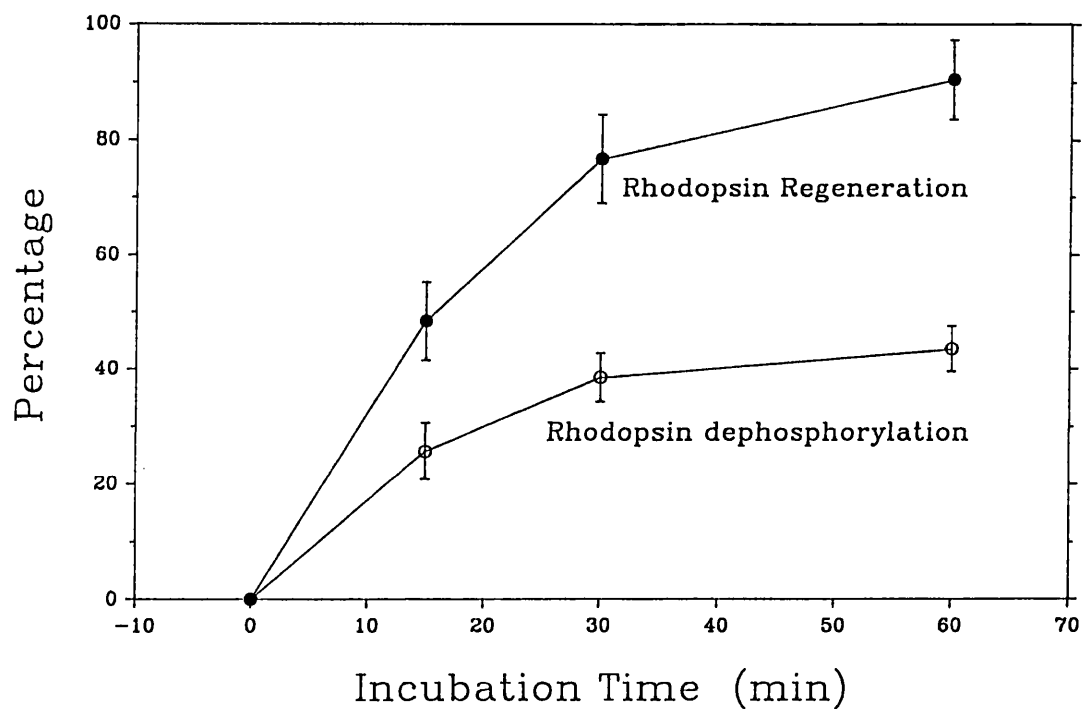
Fig.2.2.5 Comparison between rhodopsin regeneration and dephosphorylation.

Isolated, bleached rat retinae were incubated in the dark at $37^{\circ}C$ for designated periods with 500 uM photoisomerate of retinal.

The data are derived from Fig.2.2.1 and Fig.2.2.4.

2.2.3.4 Rhodopsin regeneration in the human retina

Seven bleached human retinae, 5 to 43 hours postmortem, were incubated in the dark for 60 minutes in fortified Eagle's medium with 100 uM 11-cis retinal or 500 uM photoisomerate of retinal. Rhodopsin was regenerated to between 0.10 and 0.41 nmol/mg protein (Table 2.2.2).

Table 2.2.2 Rhodopsin regeneration in the human retina postmortem.

Sample No.	Total hours postmortem	Hours to enucle- ation	Age & sex	Regenerated rhodopsin nmol/mg protein
1	5	0	40 M	0.41
4	18	6	18 M	0.34
10	24	5	28 M	0.35
13	30	5	66 F	0.15
15	34	5	40 M	0.27
18	36	6	58 M	* 0.34 ± 0.03 (3)
20	43	5	82 M	0.10

The retinae were incubated with either 100 uM 11-cis retinal (Specimen 20) or 500 uM photoisomerate of retinal (all others). Rhodopsin was undetectable in all samples before incubation. Samples are tabulated in order of total time postmortem (cf. Table 2.1.1, p.89).
(*), mean ± SEM (n).

Fig.2.2.6 compares the rate of rhodopsin regeneration in a bleached, 36-hour-postmortem human retina (Specimen 18, Tables 2.2.2) to that of freshly-isolated, bleached rat retinae. It can be seen that rhodopsin was regenerated more quickly in the postmortem human retina than in the rat. The maximum level of regenerated rhodopsin was attained after about 30 minutes for the human tissue, compared with about 60 minutes for the rat.
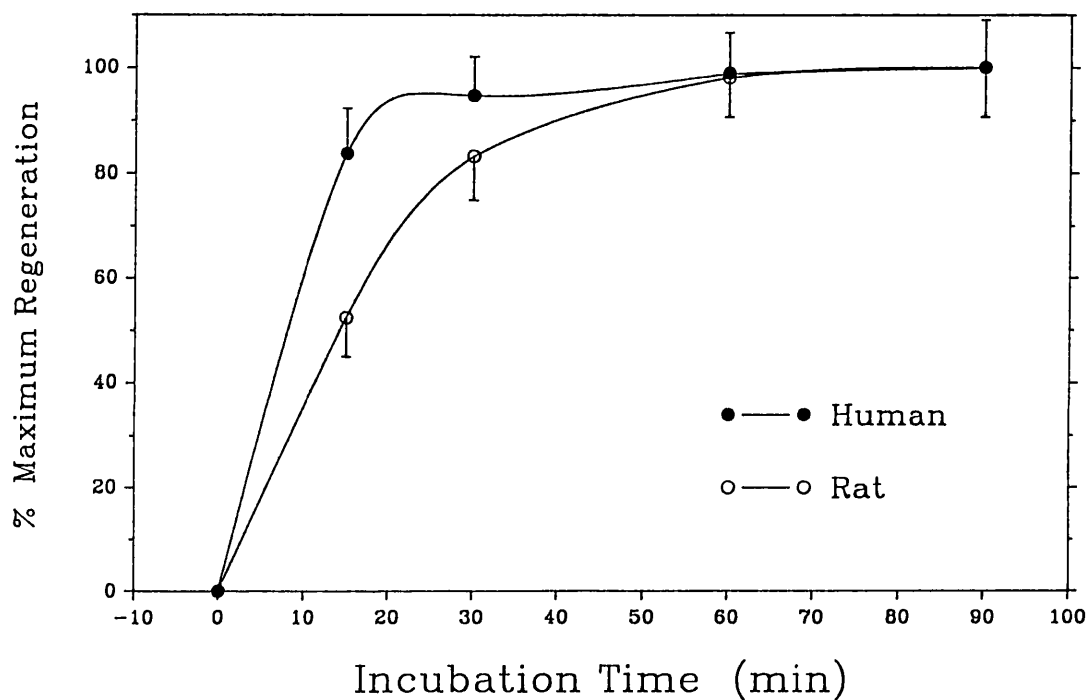
Fig.2.2.6 Comparison of the rates of rhodopsin regeneration in human and rat retinae.

Portions of a bleached, 36-hour-postmortem human retina (Specimen 18, Table 2.2.2) or freshly-isolated, bleached rat retinae were incubated with 500 uM photoisomerate of retinal for the times designated. Each point represents the mean ± SEM of at least 3 estimations.

2.2.4 DISCUSSION

2.2.4.1 Regeneration of rhodopsin in isolated rat retinae

The present results show that the rhodopsin concentration in the dark-adapted retina is 1.16 nmol/mg protein, or 1.30 nmol/retina (Table 2.2.1). These values are comparable to those previously published for the rat of between 1.23 and 1.30 nmol/retina (Dowling and Sidman, 1962; Dowling, 1963).

To regenerate rhodopsin in isolated, bleached retinae, several studies have employed either 9-cis or 11-cis retinal, formed by photoisomerization of the all-trans form and then purified by either thin layer chromatography or HPLC (Hubbard et al., 1971; Bridges and Alvarez, 1982; Pepperberg, 1982). However, 11-cis retinal is not commercially available, and its isolation is very tedious and time-consuming. Thus, as a major aim of the present study was to develop relatively simple procedures that could then be more readily employed in investigating human tissue, the substitution of 11-cis retinal by a crude photoisomerate of all-trans retinal was investigated. The current results suggest that more than 90% of the dark-adapted level of rhodopsin can be regenerated by employing 500 uM of the mixed isomers (Fig.2.2.1A). However, increasing the amount of crude photoisomerate did not give a proportional increase in pigment regeneration. That is, compared to 11-cis retinal, the efficiency decreased as the concentration of crude isomers increased (Fig.2.2.1B). It has been suggested that, under steady state rates, the delivery of retinoids to photoreceptor cells by liposomes is limited by the rate of diffusion through both the aqueous medium and the plasma membrane (Yoshikami and Noll, 1978). In a crude photoisomerate, inactive and active forms of isomers may compete for access to the chromophore binding site. Thus, since the delivery rate is limited, the competition might cause inhibition of the active forms. Competitive inhibition is likely to be more noticeable with increasing concentration - as observed here (cf. Fig.2.2.1B). Nevertheless, the results described here in this section and to be shown in 2.3.3.1 (p.128)

and 2.4.3.1 (p.141) suggest that the crude photoisomerate is adequate for investigating general photoreceptor function.

As shown in Fig.2.2.1A, after 60 minutes incubation full regeneration of visual pigment can be obtained with 100 uM 11-cis retinal, or 91% regeneration with 500 uM mixed isomers encapsulated in the liposomes. The above concentrations of retinal are less than those used in previous studies. For example, Yoshikami and Noll (1982) used liposomes containing approximately 600 uM 11-cis retinal, and Pepperberg (1982) used an ethanol suspension containing 1500 to 4500 uM 9-cis or 11-cis retinal. Because retinoids are retinotoxic, it is advantageous to keep exogenously-applied levels as low as possible.

2.2.4.2   The retinal-transport function of IRBP and the possibility of employing IPM or IRBP for rhodopsin regeneration in vitro

As previously described, IRBP can bind retinoids, and it has been suggested that it transports them between the photoreceptor cells and the RPE. However, more studies are required to verify the transport function of IRBP (cf. p.34). In relation to this concept, an aim of the present study was to provide evidence of the proposed retinoid-transport function and also to explore the possibility of employing IRBP or IPM in rhodopsin regeneration.

The current results have shown that the application of retinal, complexed with IRBP or IPM (0.36 to 0.6 uM IRBP), produces an increase in the amount of rhodopsin regenerated in the rat retina (Fig.2.2.2). This is in agreement with Okajima et al. (1989) who reported that IRBP delivered all-trans retinol into toad RPE, and with Jones et al. (1989a,b) who found that IRBP carrying 11-cis retinal induced recovery of sensitivity of salamander photoreceptors.

In those experiments where 3% ethanol was used as the sole delivery system, the suspension of retinal remained opaque, suggesting that it was not completely dissolved. By contrast, in 100% ethanol, a yellowish, transparent

solution of retinal was always obtained. Thus, it is likely that the "undissolved" retinal might not be able to diffuse into cell membranes, hence hindering rhodopsin regeneration. The increase in the regeneration by addition of IRBP or IPM may be explained as below. IRBP may facilitate the process of diffusion by binding the "undissolved" retinal and delivering it to the membranes, thus increasing pigment regeneration. However, analysis of the efficiency of retinal delivery by IRBP is complicated by the presence of ethanol in the preparation.

In addition to IRBP, other proteins in the IPM may also be able to carry retinoids. For example, bovine serum albumin (BSA) has been shown to deliver ^{3}H-retinol to the RPE but its efficiency is much lower than that of IRBP (e.g., 3 uM IRBP is more efficient than 90 uM BSA; cf. Okajima et al., 1989). Because the IPM and IRBP preparations were relatively impure with respect to the existence of other potential retinal-carrying proteins, it is difficult to make a precise comparison between these two preparations in term of the efficiencies.

Under present experimental conditions, the results indicated that phosphatidyl choline liposomes (10 mg/ml) were of the greatest efficiency in delivering retinal to facilitate rhodopsin regeneration. This supports the observations of Ho et al. (1989) that transfer of retinol from the liposomes (5 mg/ml) to rod OS membranes can be accomplished rapidly via the aqueous phase, and is about 8 times as fast as that from IRBP (2 uM). They suggest that IRBP may serve as a buffer protein for retinoids in the interphotoreceptor space, instead of an active transport protein. In contrast, Okajima et al. (1989) showed that retinol transfer into the RPE by IRBP (3 uM) is comparable to, or exceeds, that by the liposomes (0.9 mg/ml). It is possible that the above differences in the efficiencies of retinoid delivery may relate to the relative concentrations of the liposomes, IRBP, and retinoid applied, as well as other experimental conditions. It is also possible, however, that they may reflect binding or uptake differences between the two target membranes of rod OS and RPE.

The present study, together with those of Ho et al. (1989) and Okajima et al. (1989), also revealed that applying a higher concentration of IRBP did not increase the delivery rate of the retinoid. This suggests a competition for retinoids by IRBP and cell membranes, which is consistent with the view that the interaction of retinoid and IRBP is a passive, reversible binding event (Ho et al., 1989; Okajima et al., 1989). Comparison of the findings in each of the above studies indicates that, unlike IRBP, higher concentrations of the liposomes may increase the delivery rate of retinoids.

The present experiments, applying retinal together with IPM or IRBP in rhodopsin regeneration studies, are preliminary, and more investigation is necessary. However, the current results suggest that it may be desirable to employ either IPM or IRBP in rhodopsin regeneration _in vitro_. The use of these agents may be especially important in studies of retinal explant culture where longer-term exposure of photoreceptor OS to the agents will be necessary. It has also been suggested that the interaction of retinoid with IRBP protects cell membranes against the toxic effects of isolated retinoid (Perlman et al., 1982; Jones et al., 1989a,b). Furthermore, transient binding with IRBP may also prevent retinoids from degradation in an aqueous environment (Futterman and Heller, 1972, Ho et al., 1989). In addition to these considerations, IPM may contain additional factor(s) that promote OS survival (Hewitt et al., 1990). Thus, it is possible that a simple preparation of crude IPM will be more beneficial than IRBP in culture systems.

2.2.4.3 The lack of relationships between rhodopsin regeneration and either phosphorylation or dephosphorylation

The present study has demonstrated that, in the isolated retina, both opsin and regenerated rhodopsin can be phosphorylated and dephosphorylated (Figures 2.2.3 - 2.2.5), supporting the suggestion that phosphorylation has no direct effect on the 11-cis retinal binding site of rhodopsin (cf. p.53).

It was noted earlier that light-induced phosphorylation might also occur in some "light-sensitive" polypeptides with molecular weights comparable to that of opsin, e.g., the alpha and beta subunits of transducin (39 and 36 kDa, respectively; p.48). However, opsin is about 10 times more concentrated than, e.g., transducin in the OS (cf. p.48) and each opsin molecule has 7 to 9 phosphorylation sites (cf. p.36). Therefore, it is unlikely that phosphorylation of other proteins would significantly have interfered with quantification of opsin phosphorylation.

As previously discussed, phosphorylation of activated rhodopsin reduces the rate of its catalytic coupling to transducin (cf. p.52). Therefore, in the isolated retina, the regenerated but still phosphorylated rhodopsin may act less efficiently in inducing photoresponses. This may thus explain why, in the isolated previously-bleached retina, the photoresponse recovered in the dark to only about 70% of the initial dark-adapted level whilst rhodopsin was regenerated to 90% or higher of the initial level (cf. Fig.2.4.2, p.142).

Incorporation of $^{32}PO_4$ during rhodopsin phosphorylation is expressed in this study as dpm/mg protein (cf. Fig.2.2.4). If this incorporation is calculated in moles phosphate/mole rhodopsin (i.e., the P/R ratio; cf. p.106), maximum phosphate incorporation, induced by a total of 80 minutes' exposure to light (cf. Fig.2.2.4), would be equivalent to approximately 0.3 P/R. This value appears low, compared with Kuhn and Wilden's results (1982). They incubated isolated bovine rod OS under white light for 60 minutes with a high concentration of exogenous ^{32}P-ATP (3 mM), and obtained a P/R ratio of 6 to 6.5. However, in

the present study, the radiolabelled phosphate donors were synthesized by means of incubation and might account for only a portion of the endogenous phosphate donor pool. Because only the labelled phosphorylation could be counted, the low P/R ratio in the current results is predictable. By comparing the P/R of 0.3 derived from the present data with the "saturated" level of 6-6.5 obtained by Kuhn and Wilden (1982), it may be deduced that 60 minutes' dark-incubation with $Na_2H^{32}PO_4$ under the present conditions resulted in labelling of about 5% of phosphate donor pool in the retina.

It should be noted that there are several advantages in employing isolated, intact retinae and endogenously-labelled phosphate donors, instead of isolated rod OS and exogenously-supplied ^{32}P-ATP, as the regime more closely relates to the physiological state in the following ways.

1)   The levels of phosphate donors approximate those in vivo.

2)   Potentially, a more normal enzyme profile is maintained. For example, it has been shown that the phosphatase responsible for opsin dephosphorylation, as well as opsin kinase or its cofactors, are lost from preparations of isolated rod OS when plasma membranes have been made permeable to ^{32}P-ATP (Miller and Paulsen, 1975; Kamps et al., 1986).

3)   The preservation of contact between the OS and IS may allow the passage of some essential factors from one to the other.

Thus, the "intact" system is likely to allow a more meaningful comparison between endogenous events such as opsin phosphorylation/dephosphorylation and changes in cGMP homeostasis (cf. 2.3.4.3, p.134).

2.2.4.4 Regeneration of rhodopsin in the postmortem human retina

In a 5-hour-postmortem human retina (Specimen 1, Table 2.2.2), rhodopsin regenerated to a level of 0.41 nmol/mg protein. This value compares well with that of 0.43 ± 0.06 nmol/mg protein (n=7), obtained for similarly incubated baboon tissue 1 to 2 hours postmortem (Voaden,M.J. unpublished observation). No dark-adapted value for rhodopsin could be obtained for the human retina.

Furthermore, the range of concentrations of rhodopsin (0.1 to 0.41 nmol/mg protein), found in "regenerated" human retinae 5 to 43 hours postmortem (Table 2.2.2), blankets the mean value of 0.28 nmol/mg protein, determined by Barbour et al. (1985) using opsin immunoassay on 12- to 24-hour-postmortem tissue. Table 2.2.2 also shows that, in general, the fresher the retinae, the higher the level of regenerated rhodopsin.

In the present study, the choice of 60 minutes exposure to retinal in order to regenerate visual pigment in the human retina was based on results obtained for mouse (Voaden et al., 1989) and rat tissue (cf. Fig.2.4.2B, p.142). However, previous studies have suggested that rat opsin regenerates more slowly than the opsins of other species (Dowling, 1963; Crouch, 1976). This was why a comparison was made between rhodopsin regeneration rates in rat and human tissue. The results demonstrate that the regeneration rate in a 36-hour-postmortem human retina was about twice as fast as that in freshly-isolated rat tissue (Fig.2.2.6), suggesting that a shorter incubation time might suffice for the human retina postmortem.

Thus, the above results indicate that the capacity of opsin to combine with 11-cis retinal to form rhodopsin survives well in aged human tissue.

2.3 RECOVERY OF CYCLIC GMP

2.3.1 INTRODUCTION

Considerable evidence has indicated that cGMP serves as the internal transmitter required in vertebrate photo-transduction (cf. 1.3, p.47). In the dark, a high level of cGMP is found in the retina and more than 95% of it is located in photoreceptor cells, with the highest concentration in the OS. Absorption of light activates rhodopsin and triggers the cGMP cascade, leading to hydrolysis of cGMP and hence modifying the cGMP-gated channels in the plasma membrane. Termination of the cGMP cascade is accomplished via a series of deactivation reactions, which results in restoration of a higher cGMP steady state concentration and resets the retina for a photoresponse. Thus, the aim of this portion of the present study was to investigate the changes in cGMP content accompanying rhodopsin regeneration.

Since availability of human retinae was limited, some investigations were performed using rat tissue.

2.3.2 MATERIALS AND METHODS

2.3.2.1 Preparation and incubation of the retina

In general, materials and techniques were as described in section 2.2.2 (p.98).

Unless otherwise stated in the text, retinal samples were co-incubated with those for rhodopsin studies (2.2.2.4, p.102).

2.3.2.2 cGMP Measurement

Techniques used for the assay of cGMP were similar to those described by Doshi et al. (1985), as outlined below.

At the end of incubation, the retina was removed under dim red light and then homogenized for 1 minute in 0.5 ml of 0.1 N HCl. The resultant homogenate was boiled for 1 minute at $90^{\circ}C$. As this destroyed all enzyme activity the rest of the operation could be performed under room lighting. An aliquot of 25 ul of the homogenate was taken for protein estimation (cf. p.103). The remainder was centrifuged in an Eppendorf microcentrifuge at 8000 g for 5 minutes. An aliquot of 25 ul of the resulting supernatant was evaporated down under nitrogen at $50^{\circ}C$ and stored at $-20^{\circ}C$.

The cGMP content of the stored aliquots was measured using a cGMP radioimmunoassay kit supplied by Amersham International Ltd. The assay was based on the competition, between unlabelled cGMP and a fixed quantity of the tritium-labelled compound, for binding to an antiserum which had a high specificity and affinity for cGMP. The greater the concentration of unlabelled cGMP, the less the radioactive cGMP was bound to the antiserum. The content of cGMP in the samples was then determined from a linear standard curve produced with known quantities of unlabelled cGMP, and the concentration calculated on a per mg protein basis.

2.3.3 RESULTS

2.3.3.1 cGMP levels in the rat retina

Fig.2.3.1 compares the changes in concentration of cGMP in either dark-adapted or bleached rat retinae during incubation. It shows that:-

1)   The normal dark-adapted level of 48.2 pmol cGMP/mg protein gradually decreased when the retina was incubated in the dark, and had declined by 14% after 60 minutes ($P < 0.025$).

2)   The light-adapted level of 15.7 pmol cGMP/mg protein was 33% of the dark-adapted one.

3)   When bleached retina was incubated in the dark the light-adapted level of cGMP increased by 88% within the first 30 minutes, and the higher level was then maintained for at least another 60 minutes.

4)   However, when the bleached retina was incubated in the dark in the presence of retinal to regenerate rhodopsin, the above level of cGMP rose further, reaching 130% of the initial bleached level in 60 minutes.

5)   By contrast, when a bleached retina was incubated in the light, cGMP did not increase.

Thus, the dark-adapted level of cGMP was reduced in light but recovered to a considerable extent in the dark. Furthermore, the latter recovery commenced regardless of whether rhodopsin was regenerated, but regeneration of rhodopsin enhanced the recovery of cGMP (Fig.2.3.1).

In order to analyse the probable mechanisms underlying the recovery of cGMP in the bleached retina, the relationship between rhodopsin regeneration and dephosphorylation, and cGMP recovery, was studied (Fig.2.3.2). It can be seen that, during incubation in the dark, the increase in the concentration of cGMP and dephosphorylation of opsin followed a similar time course, even without regeneration of the visual pigment (Fig.2.3.2.B); whilst regeneration of rhodopsin resulted in a further increase in the concentration of cGMP, though the dephosphorylation remained unchanged (Fig.2.3.2A).
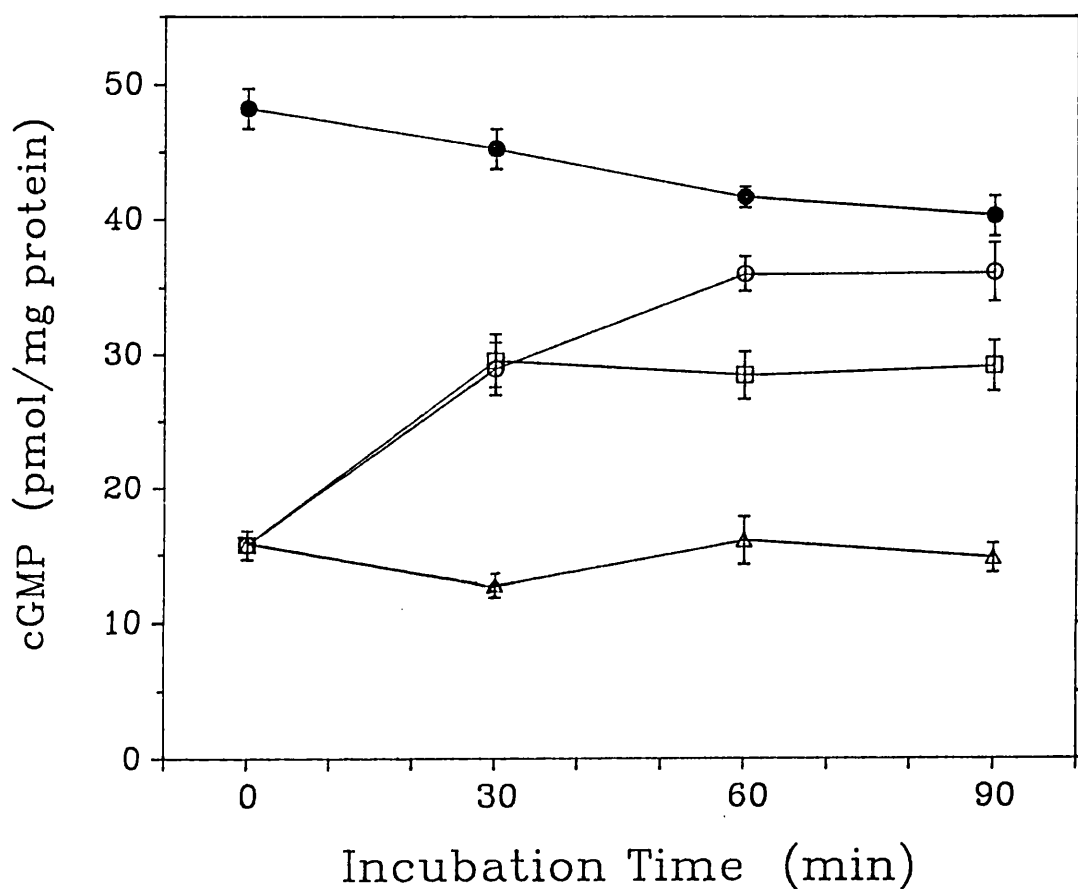
Fig.2.3.1 The concentrations of cGMP in dark-adapted, bleached and "regenerated" rat retinae.

●————● normally dark-adapted and incubated in the dark,
△————△ bleached and incubated in ambient light,
□————□ bleached and incubated in the dark,
○————○ bleached and incubated in the dark with 500 uM photoisomerate of retinal to regenerate rhodopsin.

Each point represents the mean ± SEM of at least four estimations.
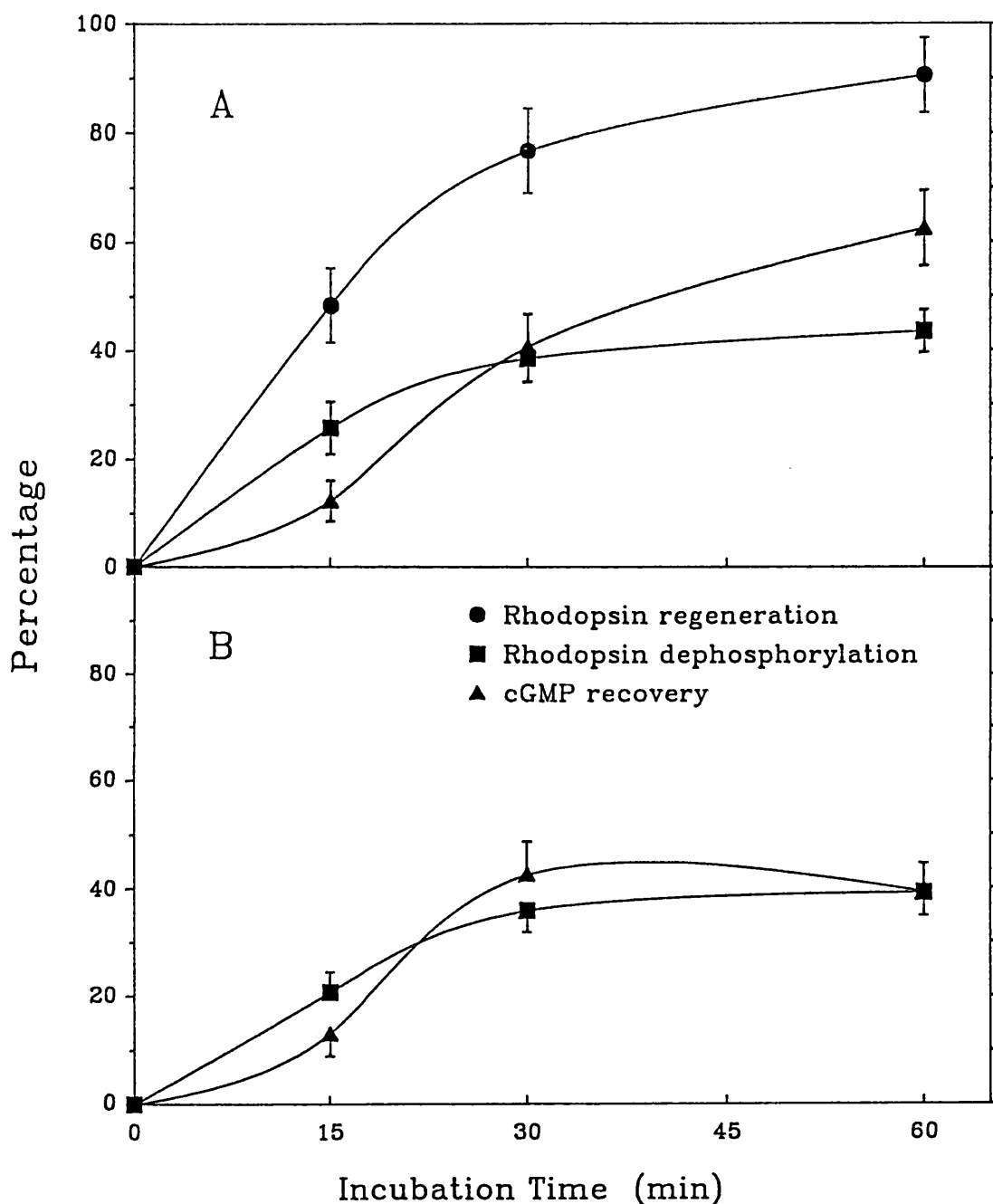
Fig.2.3.2 Comparison of rhodopsin regeneration and dephosphorylation, and cGMP recovery, in "regenerated" rat retinae.

Previously bleached rat retinae were incubated in the dark (A) with or (B) without 500 uM photoisomerate of retinal.

The data for rhodopsin regeneration and dephosphorylation are derived from Fig.2.2.1 (p.110) and Fig.2.2.4 (p.115), respectively. Each point for rhodopsin regeneration or cGMP recovery represents the mean ± SEM of at least 4 estimations. Each point for the dephosphorylation represents the mean ± SEM of 3 estimations.

2.3.3.2 cGMP Recovery in postmortem human retina

Table 2.3.1 shows cGMP levels in six postmortem human retinae before and after incubation with active isomers of retinal. It can be seen from Table 2.3.1 that all the samples studied showed some increase.

Table 2.3.1 Recovery of cGMP in postmortem human retinae.

Sample No.	Total hours postmortem	Hours to enucle- ation	Age & sex	cGMP recovery pmol/mg protein before	after
1	5	0	40 M	17.9	48.6
4	18	6	18 M	18.5	49.2
10	24	5	28 M	15.5	36.4
13	30	5	66 F	16.2	23.5
15	34	5	40 M	17.0	31.5
20	43	5	82 M	16.5	34.0

The bleached retinae were incubated in the dark at $37^{O}C$ for 60 minutes with 100 uM 11-cis retinal (Specimen 20), or 500 uM photoisomerate of retinal (all others). Samples are tabulated in order of total time postmortem (cf. Table 2.1.1, p.89). Values of cGMP represent those before and after incubation, respectively.

2.3.4 DISCUSSION

2.3.4.1 The concentration of cGMP

The dark-adapted value of 48.2 pmol/mg protein found for cGMP in the rat retina compares well with the values of 50 and 59 pmol/mg protein found by Winkler et al. (1984) and Farber et al. (1981), respectively. The light-adapted value of 15.7 pmol/mg protein was also in line with the 15 pmol/mg protein found by Winkler et al. (1984) (cf. Fig.2.3.1 and Table 1.3.1, p.58).

Consistent with the higher levels of cGMP seen in dark-adapted retinae, intracellular cGMP levels were also increased in "regenerated" tissue. However, comparison with normal values is complicated by the changes that occurred in "non-regenerated" levels on incubation. That is, the content in dark-adapted retinae incubated in the dark decreased by 6.6 pmol/mg protein, whereas the content in bleached retinae incubated similarly increased by 13.8 pmol/mg protein, thus lessening the light/dark difference (Fig.2.3.1).

Although it is well recognized that medium composition can modulate the endogenous level of cGMP in photoreceptor cells (e.g., Cohen, 1984; Meyertholen et al., 1986), this is not likely to explain the current observation on bleached retinae, as the concentration of cGMP in similar tissue, incubated in the light, was stable (Fig.2.3.1).

It is noteworthy that incubation in the dark resulted in recovery of cGMP in bleached retinae, regardless of whether rhodopsin was regenerated (Fig.2.3.1). Partial recovery of sensitivity, through a process believed to be independent of pigment regeneration, is a common observation when light-exposed, rhodopsin-containing retinae are maintained in the dark (e.g., Dowling and Ripps, 1972; Pak, 1982). It is possible that the present observation connects with this phenomenon.

It is known that dark-adaptation consists of two distinct phases: (1) a fast component of unknown mechanism, not apparently related to the level of visual pigment in the eye, and (2) a slower component related to

pigment concentration (for reviews: Dowling, 1987; Ripps and Pepperberg, 1987). Based on the current observations, it is possible that some cGMP recovery, independent of rhodopsin regeneration, governs, at least in part, the fast phase of dark-adaptation.

The mechanisms subserving cGMP recovery in isolated, bleached retinae will be discussed below (2.3.4.3, p.134).

2.3.4.2 Recovery of cGMP in postmortem human retina

The concentrations of cGMP in bleached and "regenerated" human retinae (Table 2.3.1) should be compared with the values of 14 and 36 pmol/mg protein, found by Voaden et al. (1989) for light and dark-adapted tissue, respectively, processed between one and three hours after surgical removal of the eyes for malignant melanoma, and with the 10 pmol/mg protein found by Newsome et al. (1980) in peripheral tissue from light-exposed human eyes up to 20 hours postmortem.

In the present study, light-adapted/bleached levels appeared constant for at least 40 hours postmortem. In contrast, Newsome et al. (1980) found that levels declined after only one day in tissue "obtained at autopsy". The difference may relate to the time between death and enucleation, which, as previously discussed, was the most significant factor affecting survival of postmortem human retinae (cf. p.95). The results also suggest that, potentially, cGMP metabolism survives for at least 40 hours postmortem, as increases in cGMP on incubation were seen in tissue 24, 30, 34 and 43 hours postmortem (Table 2.3.1). This finding is consistent with the observations on the long-term survival of OS morphology (Fig.2.1.3d, p.87), rhodopsin regeneration and photo-response recovery (see below, Table 2.4.3, p.146) in the same retinae.

2.3.4.3 What brings about recovery of cGMP in an isolated, bleached retina?

As previously described, light triggers the cGMP cascade, leading to hydrolysis of cGMP (see p.56). Thus for the situation in a dark-adapted retina to be regained, the sequence of events must be reversed. A turnoff mechanism has been proposed (see p.57) and is reiterated below.

1) After a flash of light, activated rhodopsin is first partially inactivated by phosphorylation, and its activity then fully blocked by binding of arrestin.

2) Subsequently, transducin is inactivated when $GTP.T_\alpha$ is hydrolysed to $GDP.T_\alpha$ by intrinsic GTPase activity.

3) Deactivation of the PDE is poorly understood but may be effected by (i) rebinding of the inhibitory subunit(s), and/or (ii) a direct interaction between the PDE and $GDP.T_\alpha$, and/or (iii) binding of arrestin.

4) The original cGMP level is restored in part because of the increased activity of guanylate cyclase.

Thus, the combination of these events results in termination of the cGMP cascade and permits <u>rapid recovery</u> from flash activation, allowing perception of repeated stimuli.

However, the above mechanisms may not apply to the fully-bleached retina, particularly postmortem tissue, because the whole system of phototransduction may have been changed. For example, in bleached tissue, Rh[*] (meta II) should have degraded into the final products, opsin and retinol, because the half life of the bleaching process is a few minutes at room temperature (cf. Fig.1.2.2, p.39). This means that there is no Rh* continuing to activate transducin. Furthermore, it has been suggested that light-induced binding of transducin to rhodopsin is transient, the gradual decline having a half time of approximately 17 minutes both in darkness and in sustained light (Mangini et al., 1986).

Little is known of the activities of transducin, PDE and guanylate cyclase in bleaching/postmortem conditions, but it is clear that cGMP is maintained at a low level.

This implies that the activity of PDE remains relatively high and/or that of cyclase is lower. The current results show that the concentration of cGMP increases when previously bleached retinae are incubated in the dark (Fig.2.3.1), indicating that the balance between the activities of PDE and cyclase is altered. What mechanisms are involved in these processes?

The rise in cGMP concentration commences in the dark and coincides with dephosphorylation of opsin. The recovery is then further enhanced when rhodopsin is regenerated (Fig.2.3.1, Fig.2.3.2). More studies are needed to see if the initial correspondence of events is fortuitous or if they are related. One approach would be to see if cGMP recovery is reduced when dephosphorylation of opsin is suppressed. Unfortunately, a method has not yet been devised that will allow inhibition of opsin dephosphorylation without affecting the overall enzymic environment of the OS and, thus, cGMP metabolism. For example, in suspension of rod OS whose membrane has been made permeable to exogenous ATP, opsin dephosphorylation can be suppressed by either supplying a high concentration of ^{32}P-ATP ($\geq$ 5 mM, cf. Miller et al., 1977) or by purification of the OS to reduce the activity of the phosphatase responsible for the dephosphorylation (cf. Miller and Paulsen, 1975). However, neither method is suitable for the current purpose.

It has been shown, using immunoelectron microscopy, that the subcellular concentration of some photoreceptor specific proteins is light-dependent. For example, in dark-adapted toads (Mangini and Pepperberg, 1987,1988) and mice (McGinnis et al., 1989), arrestin is found in the highest concentration in the myoid region of rod IS, and light-adaptation results in movement of arrestin from the IS to the OS. It has also been shown that light-induced binding of arrestin to phosphorylated rhodopsin inhibits the subsequent dephosphorylation by a phosphatase (Palczewski et al., 1989). Based on these studies, it appears likely that light stimulation results in arrestin moving from the IS to the OS, thus increasing binding to the phosphorylated rhodopsin/opsin; whilst, in the dark,

arrestin is released and returns to the IS, hence allowing the dephosphorylation to occur.

In contrast to arrestin, McGinnis et al. (1989) have also observed that, in dark-adapted mouse retinae, the alpha and beta subunits of transducin are mainly in the OS, and light induces their movement from the OS to the IS. Therefore, it may be that in the dark the subunits transfer back to the OS, to link with the gamma peptide and reconstitute transducin. Possibly, the "regeneration" of transducin in some way facilitates cGMP recovery.

In summary, several processes take place in photoreceptor cells in the dark, coinciding with recovery of cGMP. They are:-

1) rhodopsin regeneration,

2) rhodopsin/opsin dephosphorylation,

3) release of arrestin from phosphorylated rhodopsin and its removal from the OS to the IS, and

4) the opposite movement of the alpha and beta subunits of transducin and possible reconstruction of the inactive complex in the OS.

It is not known whether the reactions of arrestin and transducin depend on rhodopsin regeneration, but it is clear that dephosphorylation of opsin and initial recovery of cGMP, at least _in vitro_, do not. It may be that an unknown agent (or agents) becomes active in darkness and triggers opsin dephosphorylation and cGMP recovery. From this viewpoint alone it is of considerable interest to search for the 'dark-activated' agent(s) as they might play an important role in photoreceptor adaptation.

2.4 RECOVERY OF THE ERG

2.4.1 INTRODUCTION

(1)   The ERG

Previous sections have detailed studies of regeneration of rhodopsin and recovery of cGMP in rat and human retinae (see p.108 and p.128). The present section presents the subsequent recovery of electrical responses in such tissue, and estimates the relationship of these three aspects of phototransduction.

The electroretinogram (ERG) is a complex response representing the voltage changes across the retina when it is stimulated by a flash of light (see p.67). In an isolated retina, the a-wave (the leading edge of fast PIII) and the b-wave can be recorded, and application of glutamate (5 to 100 uM) can eliminate the b-wave, leaving PIII responses, including the fast and slow components.

It is common practice to use the potential gradient of either fast PIII (e.g., Asano, 1977), or slow PIII (e.g., Witkovsky et al., 1975), or the PIII complex (e.g., Ernst and Kemp, 1972) as a measure of the photoreceptoral activity. In the present study, the PIII complex was used to estimate the photoresponse, and, when appropriate, the b-wave also monitored.

The effects of low temperature and fortified Eagle's medium on preservation of the rat ERG was also studied.

(2)   NMDA (N-methyl-D-aspartate) receptors and the b-wave

As it was found that the b-wave declined rapidly postmortem both in rat and human retinae (see Results), the following investigations were undertaken to see if NMDA receptors were involved in synaptic transmission between photoreceptors and higher-order neurones and if NMDA antagonists would prolong b-wave survival.

Considerable evidence has indicated that generation of the b-wave is related to synaptic transmission between photoreceptors and ON-bipolar cells (see p.72), and that glutamate is most likely to be the major neurotransmitter

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(Ehinger and Dowling, 1987; Massey and Redburn, 1987).
Four types of glutamate-receptors have been found in the
vertebrate retina: NMDA, APB (2-amino-4-phosphonobutyric
acid), quisqualate and kainate (Miller and Slaughter,
1985; Colemen et al., 1986).

As previously described, anoxic damage to CNS
neurones is due, in part, to neurotoxicity by excitatory
amino acids, such as glutamate (see p.80). It has been
further demonstrated that this excitotoxicity is mediated
via NMDA receptors. Pharmacological blockage of the
receptors by NMDA antagonists such as ketamine (non-
competitive) or APH (2-amino-7-phosphono-heptanoic acid;
competitive) can significantly protect the neurones
against hypoxic injury in the brain (Simon et al., 1984;
Goldberg et al., 1987; Choi et al., 1988; Marcoux, et al.,
1988). It has also been reported that dextromethorphan (a
non-competitive antagonist of NMDA receptors) can protect
the _in vivo_ rabbit retina against ischemic damage and
preserve the b-wave (Yoon and Marmor, 1989). Furthermore,
NMDA is known to cause spreading depression (Drejer et
al., 1989), and to have neurotoxic actions in the chick
retina _in vitro_ (Zeevalk and Nicklas, 1989), and both of
these actions can be blocked by non-competitive NMDA-
receptor antagonists such as ketamine or MK-801 [(+)-5-
methyl-10,11-dihydro-5H-dibenzo(a,b)cyclohepten-5,10-imine
maleate].

Ketamine and MK-801 act at the level of the receptor-
associated ion channel rather than the transmitter
recognition site, resulting in blockage of the receptor.
In addition, these antagonists can be washed from the
binding sites, and receptor function recovered (Kemp et
al., 1987).

Therefore, it was considered possible that loss of
the b-wave from the retina postmortem might be due to
anoxic damage involving NMDA receptors. To investigate
this, it was necessary to study fresh tissue, and an
attempt was made to protect rat retinae against anoxic
postmortem damage by using the antagonists, ketamine and
MK-801.

2.4.2 MATERIALS AND METHODS

2.4.2.1 Incubation of the retina

Incubation of isolated rat and human retinae was identical to that for studies on rhodopsin regeneration (cf. 2.2.2, p.98).

To verify the effects of low temperature and fortified Eagle's medium (p.99) on survival of the ERG, dark-adapted rat retinae were maintained in the dark in the following conditions: (1) in the intact eye, (2) in 15 ml of fortified Eagle's medium (10 retinae), and (3) in 15 ml of Earle's medium (10 retinae). The medium was contained in a 50-ml lidless glass jar which was then placed in a two-litre light-proof jar with a small amount of water in the bottom. The large jar was tightly covered after being gassed with 95% O_2/5% CO_2. The retinae were then kept at 4°C. Two retinal samples from each group were taken on a specified day for measurement of the ERG, and the remainder was continuously incubated under the same conditions.

2.4.2.2 Application of ketamine and MK-801

(1) Application of ketamine in vivo:

Dark-adapted rats were injected intraperitoneally with 100 mg/kg ketamine under dim red light and, after approximately 5 minutes, they had lost their righting and other reflexes. Control animals were anaesthetized with ether. Both groups were then killed by cervical dislocation, and the enucleated eyes stored at room temperature for the times indicated in the text.

(2) Application of ketamine and MK-801 in vitro:

Either ketamine (final concentration 0.15 or 0.3 mM; cf. Marcoux et al., 1988) or MK-801 (final concentration 0.1 or 0.2 mM; cf. Anderton and Millar, 1989) was added to the perfusion medium during measurement of the ERG.

For comparison, the effect of DL-APB (2-amino-4-phosphonobutyric acid) was also studied. This selectively acts on ON-bipolar cells, and eliminates the b-wave (Porciatti et al., 1987; Powers et al., 1988).

2.4.2.3 ERG recording in isolated retinae

Isolated retinae were positioned with the ganglion cells downwards onto Whatman no. 1 filter paper, and placed in a perspex chamber. Both surfaces of the tissue/filter paper preparation were perfused at 7 ml/min with 400 ml of recirculating Earle's medium (p.99), containing 2% foetal calf serum. The time taken for the solution to reach the retina was approximately 20 seconds. The medium was gassed continuously with a moistened mixture of 95% O_2/5% CO_2 and maintained at 35°C. In order to assess b-wave survival, ERG responses in all the human and some of the rat retinae were recorded intact. For other rat retinae, PIII was isolated by the addition of 10 mM (final concentration) glutamate to the medium. The tonicity was maintained by omission of an equivalent amount of sodium chloride.

Photoresponses were recorded with cotton wick and Ag/AgCl electrodes, connected to a high input impedance preamplifier with a band pass of 0 - 200 Hz. The light stimulus was 1.5 mm in diameter and of wavelength 550 nm. This was derived from a light emitting diode (LED) and relayed by a glass fibre bundle. Stimulus duration was 0.2 second and maximum intensity 2.0 x 10^4 quanta/um^2, as measured by a radiometer (UDT, Model 40X). Interval between the flashes was at least 30 seconds. The light intensity was adjusted by using neutral density filters (NDF). The responses were displayed on an oscilloscope (Tektronix 5110) and recorded on an RML 380Z computer. A response of 3 uV was taken as threshold, and response sensitivity was defined as the inverse of the stimulus threshold value.

PIII amplitude vs. log intensity curves were fitted by the Naka-Rushton equation, $V/V_{max}=I^n/(I^n+K^n)$ (p.66). The data were analysed using purpose written software and a NAG routine (Numerical Algorithm Group) on the University of London main frame computer.

2.4.3 RESULTS

2.4.3.1 Recovery of the ERG in isolated, bleached rat retinae

Fig.2.4.1 shows typical ERG responses from a normally dark-adapted, isolated rat retina in the absence (top trace) and presence (bottom trace) of 10 mM glutamate. It can be seen that the a- and b-waves and PIII component were distinctive in the native retina, and that the b-wave was eliminated by application of glutamate, revealing fast and slow PIII.
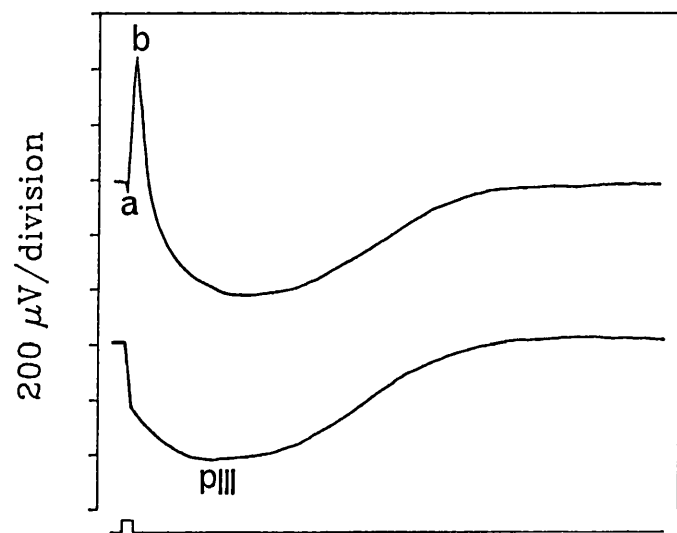
Fig.2.4.1 The ERG recorded from a normally dark-adapted, isolated rat retina.

The upper trace shows the a- and b-waves together with a PIII component. The lower trace, recorded 5 minutes after the perfusion medium was replaced by one containing 10 mM glutamate, shows the leading edge of the fast PIII combined with slow PIII to form the complex response.

The sweep duration was 10 seconds and stimulus duration 0.2 seconds, as indicated by the markers beneath the records. Stimulus intensity: $\log I = -0.9$, equivalent to 3,700 quanta/um^2. a = a-wave; b = b-wave; PIII = PIII complex.

Photoresponses were obtained following regeneration of visual pigment in isolated, previously-bleached rat retinae. Fig.2.4.2 compares levels of rhodopsin, cGMP and PIII in both dark-adapted and "regenerated" rat retinae.

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It can be seen from Fig.2.4.2A that when dark-adapted retinae were incubated in the dark at 37°C for up to 90 minutes, rhodopsin levels were unchanged but there was a significant decrease in cGMP (14%, P < 0.025, at 60 minutes) and a decline in PIII, the latter falling from an initial value of 579 ± 41 (8) to 443 ± 40 (4) uV after 60 minutes incubation.

Fig.2.4.2B shows that, when previously-bleached retinae were incubated with 500 uM photoisomerate of retinal in the dark, rhodopsin, cGMP and PIII recovered to considerable extents, with optimal recoveries at about 60 minutes of incubation.
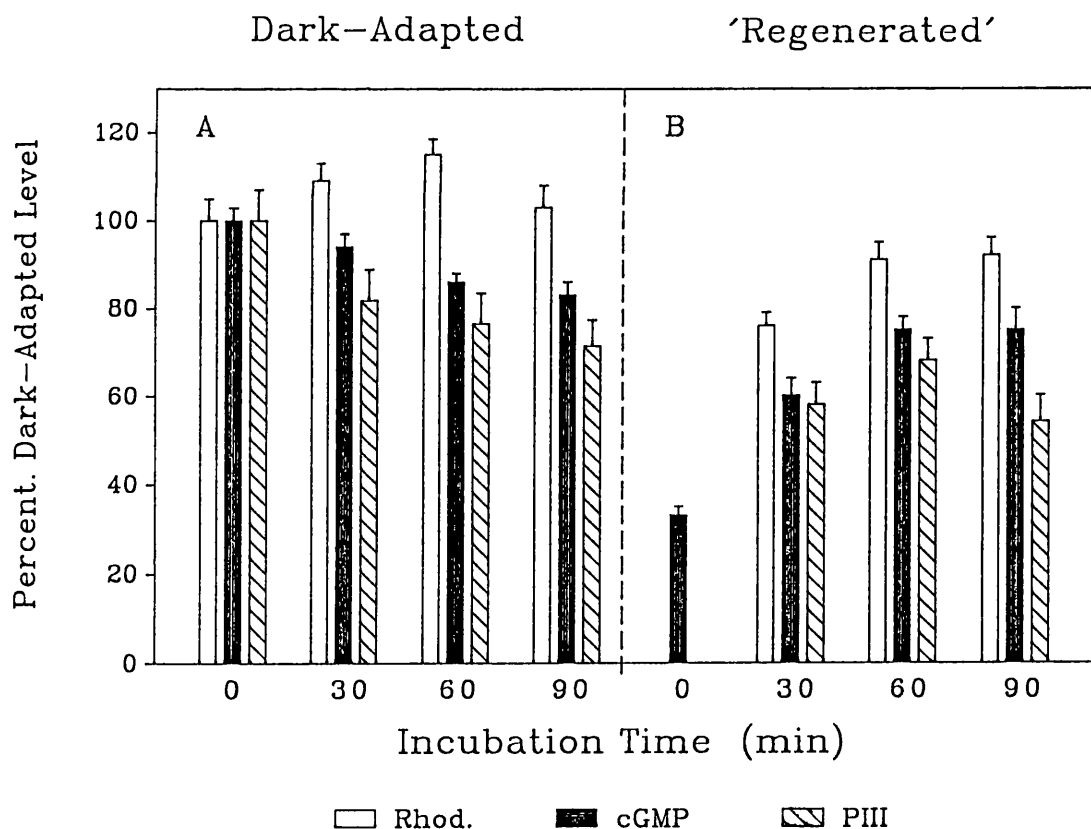
Fig.2.4.2 Rhodopsin, cGMP and the PIII in rat retinae (A) normally dark-adapted and (B) bleached and then incubated with 500 uM photoisomerate of retinal.

The tissue in A and B was incubated as described in the text and for the times indicated. PIII responses were elicited with the maximum stimulus of 2×10^4 quanta/um^2. Each point represents the mean ± SEM of at least four estimations.

The efficiency of 500 uM photoisomerate of retinal, in inducing recovery of phototransduction, was compared with that of 100 uM 11-cis retinal. The results in Table 2.4.1 show that, although 100 uM 11-cis retinal is better as regards rhodopsin regeneration, the photoisomerate is also effective and likely to be adequate for recovery of photoresponses.

Table 2.4.1 Comparison of recovery of rhodopsin, cGMP and PIII in isolated, bleached rat retinae.

Retinal samples	Rhodopsin nmol/mg protein	cGMP pmol/mg protein	PIII uV
Control: normally DA	$1.16 \pm 0.06(5)$	$48.2 \pm 1.5(10)$	$579 \pm 41(8)$
DA + 60min incubation	$1.33 \pm 0.04(4)$	$41.6 \pm 0.8(5)$	$443 \pm 38(4)$
Regenerated: with 100 uM 11-cis retinal	$1.18 \pm 0.04(5)$	$36.0 \pm 1.9(4)$	$407 \pm 26(4)$
500 uM isomers of retinal	$1.05 \pm 0.04(4)$	$36.0 \pm 1.3(5)$	$394 \pm 39(4)$

Control: Normally dark-adapted rat retinae (DA) and the levels after the retinae were incubated in the dark for 60 minutes (DA + 60 min incubation).

Regenerated: Bleached rat retinae were incubated in the dark for 60 minutes with either 100 uM 11-cis retinal or 500 uM photoisomerate of retinal.

PIII responses were elicited with the maximum stimulus of 2×10^4 quanta/um^2. Results are expressed as the mean $\pm$ SEM (n).

Recovery of the b-wave was also assessed in 4 "regenerated" retinae, incubated for 60 minutes with 500 uM photoisomerate of retinal. All samples showed recovery of the b-wave with maximum amplitudes of between 10 and 60 uV. In contrast, values of the b-wave obtained for normally dark-adapted retinae were between 100 and 220 uV

(n=6); and those for dark-adapted retinae, incubated in the dark for 60 minutes, were between 50 and 150 uV (n=5). It was found that b-wave amplitudes varied considerably among samples, even in the normally dark-adapted retinae. In contrast, the isolated PIII response was more consistent.

Table 2.4.2 compares the thresholds of PIII, and the values of K and n. It can be seen that:-

1)   The threshold for the PIII in a normally dark-adapted rat retina was approximately 2.3 quanta/um^2. If the cross-sectional area of a rat rod OS is about 1.8 um^2 (cf. Fig.1.1.5, p.26) and quantum efficiency is 0.65 to 0.70 (Dartnall, 1968), the threshold was then equivalent to the absorption of about 2.5 to 3 quanta/rod. This is in agreement with Cone (1963) who proposed that the threshold for the a-wave in the rat required the absorption of 2 to 4 quanta/rod.

2)   The intensity required for the half maximum response, i.e. K, was about 85 quanta/um^2.

3)   When the dark-adapted retina was incubated in the dark for 60 minutes, sensitivity decreased, as both the threshold and K increased. This was concurrent with a reduction of the maximum response (i.e., 579 against 443 uV, Table 2.4.1).

4)   When the bleached retina was incubated in the dark with 500 uM photoisomerate of retinal for 60 minutes, sensitivity was recovered to a large extent.

5)   n represents the slope of the curve. The values of n in normally dark-adapted retinae were between 0.7 and 0.8, whereas larger variations of between 0.6 and 1.0 were obtained for the other two conditions. This probably indicates that cellular function was more consistent in the former group of retinae.

Table 2.4.2 Thresholds and values of K and n in the normally dark-adapted rat retina (DA), dark-adapted retina incubated in the dark for 60 minutes (DA + 60 min), and the "regenerated" retina.

	Threshold quanta/um^2	K quanta/um^2	n
DA:	2.3 ± 0.5 (11)	85 ± 19 (6)	0.7-0.8
DA + 60 min:	2.7 ± 0.4 (6)	132 ± 28 (5)	0.6-1.0
Regenerated:	3.2 ± 0.6 (4)	198 ± 54 (4)	0.6-1.0

The "regenerated" retinae were previously bleached and subsequently incubated in the dark for 60 minutes with 500 uM photoisomerate of retinal. Values of K and n were given by computer, as described in the text. Results are expressed as the mean $\pm$ SEM (n).

2.4.3.2 Recovery of photoresponses in postmortem human retina

Exogenous application of "active" isomers of retinal induced increases in the concentrations of both rhodopsin and cGMP in postmortem human retinae (cf. p.117 and p.131). Photoresponses were also obtained from these samples, and the results, together with those for rhodopsin and cGMP, are summarized in Table 2.4.3. It can be seen that:-

1) Human retinae 5 to 58 hours postmortem produced photoresponses, with PIII varying from 20 to 398 uV; on three occasions a small b-wave of 5 to 10 uV was also seen.

2) In general, fresher retinae exhibited greater sensitivity and larger amplitudes, which was consistent with the higher rhodopsin and cGMP levels.

3) It was surprising that the PIII maximum amplitude in specimen 1 (5 hours postmortem) reached 398 uV, a level of the same order as the average of 394 uV for freshly-isolated, "regenerated" rat retinae (cf. Table 2.4.1). As yet, even fresher or dark-adapted human retinae have not been available for this study.

4)   It should be noted that the recovery of sensitivity, i.e., the threshold, was not always parallel to that of maximum response. For example, specimen 15 appeared most sensitive to light, but its maximum response was not correspondingly high.

Table 2.4.3 Phototransduction in postmortem human retinae.

Sample No.	1	4	10	12	13	15	18	20	27
Total hrs postmortem	5	18	24	29	30	34	36	43	58
Hours to enucleation	0	6	5	8	5	5	6	5	5
Age (yrs)	40	18	28	44	66	40	58	82	72
ERG PIII:									
threshold ($quanta/um^2$)	18	53	53	35	85	8.8	85	504	1340
K ($quanta/um^2$)	504	313	132	295	1000	132	955	2530	3700
n	0.6	0.8	1.0	0.7	0.7	0.6	0.8	NE	NE
V_{max} (uV)	398	35	45	50	25	34	35	20	20
b-wave (uV):	10	–	–	–	–	10	–	5	–
Rhodopsin (nmol/mg prot)	0.41	0.34	0.35	NE	0.15	0.27	0.34 (*n=3)	0.10	NE
cGMP (pmol/mg prot) before:	17.9	18.5	15.5	NE	16.2	17.0	NE	16.5	NE
after:	48.6	49.2	36.4	NE	23.5	31.5	NE	34.0	NE

The retinae were incubated in the dark for 60 minutes with 100 uM 11-cis retinal (Specimen 20) or 500 uM photoisomerate of retinal (all others). Maximum stimulus was 2×10^4 quanta/um^2. Samples are tabulated in order of total time postmortem (cf. Table 2.1.1, p.89). The values were obtained for individual explants of retina, except for (*). NE = not estimated. The results for rhodopsin and cGMP, previously given in Tables 2.2.2 (p.117) and 2.3.1 (p.131) respectively, are reiterated here in order to facilitate comparison with the electrophysiology.

In specimens 10 and 15 (Table 2.4.3), PIII responses were also measured after portions of the retinae were incubated at 37°C in the fortified medium for 24 hours: the amplitudes reached approximately 50% of those seen before the incubation. This might be explained by the observations that, although retinal vacuolation was generally reduced after 24 hours' incubation, photoreceptor OS became swollen (cf. Fig.2.6.4, p.181).

Fig.2.4.3 illustrates the photoresponse recovered in a 34-hour-postmortem human retina, and its PIII amplitude vs. log intensity curve (Specimen 15, Table 2.4.3). It can be seen that there was a small b-wave of 10 uV (Fig.2.4.3A), and that the PIII amplitudes were reasonably fitted by the Naka-Rushton equation (Fig.2.4.3B).
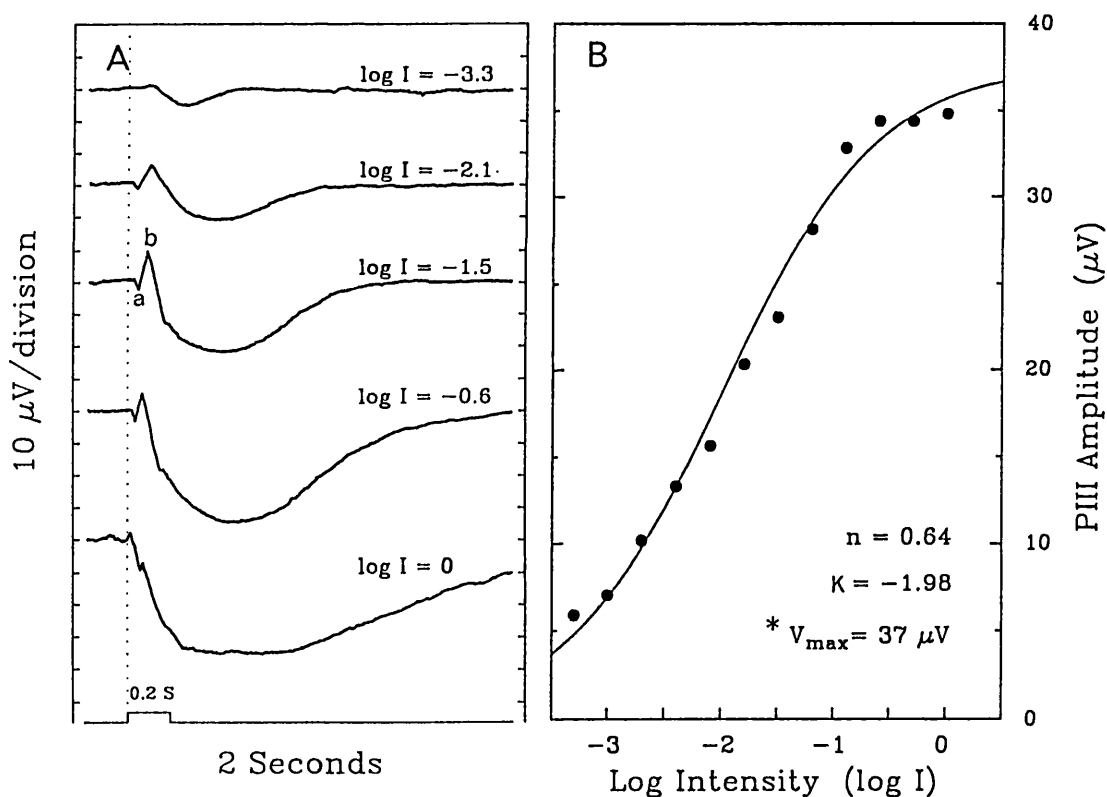
Fig.2.4.3 The photoresponse recovered in a 34-hour-postmortem human retina (Specimen 15, Table 2.4.3).

The retina was incubated with 500 uM photoisomerate of retinal for 60 minutes, as described in the text.

(A) Photoresponse amplitude vs. stimulus intensity. Intensity: log I = -3.3 is equivalent to 8.8 quanta/um^2; log I = 0 to 2 x 10^4 quanta/um^2. a = a-wave; b = b-wave.

(B) The PIII amplitude vs. log intensity curve. The solid circles represent the values of the PIII response. The continuous line is derived from the data and fitted by computer as described in the text. The values of n, K and V_{max} were given by computer, and the K, log I = -1.98, is equivalent to 132 quanta/um^2.

2.4.3.3 Effects of low temperature and fortified Eagle's medium on survival of the ERG

It can be seen from Fig.2.4.4 that fortified Eagle's medium at 4°C maintained photoresponses of the rat retina longer than did standard Earle's medium, and that in turn the latter was better than maintaining the retina in situ in an enucleated eye.   An additional experiment showed that, when rat globes were left at room temperature for only 16 hours, photoresponses were undetectable (n = 2).   B-wave survival was poor, even in the fortified Eagle's medium, with less than 20% of the normal response being present after only 24 hours, and about 5% after 48 hours in vitro.
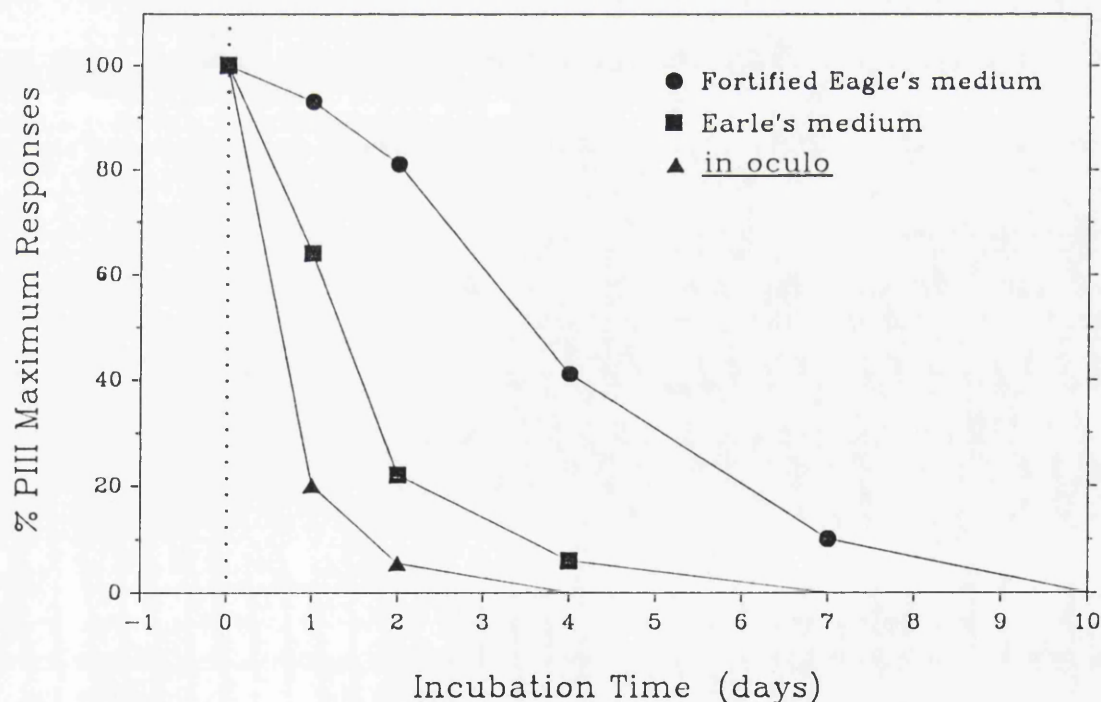
Fig.2.4.4 Survival of the rat ERG in vitro.
Dark-adapted rat retinae were maintained at 4°C in oculo or in fortified Eagle's medium or Earle's medium for the times indicated. Maximum stimulus: Log I = 0, equivalent to 2×10^4 quanta/um^2. Each point represents the mean of two estimations.

Fig.2.4.5 shows that the threshold and amplitude of neither the a- nor the b-wave of normal rat retinae were affected by the presence of up to 0.3 mM ketamine in the perfusion medium. Similarly, MK-801 (up to 0.2 mM) was without effect. In addition, intraperitoneal injection of 100 mg/kg ketamine prior to tissue isolation had no effect (data not shown).
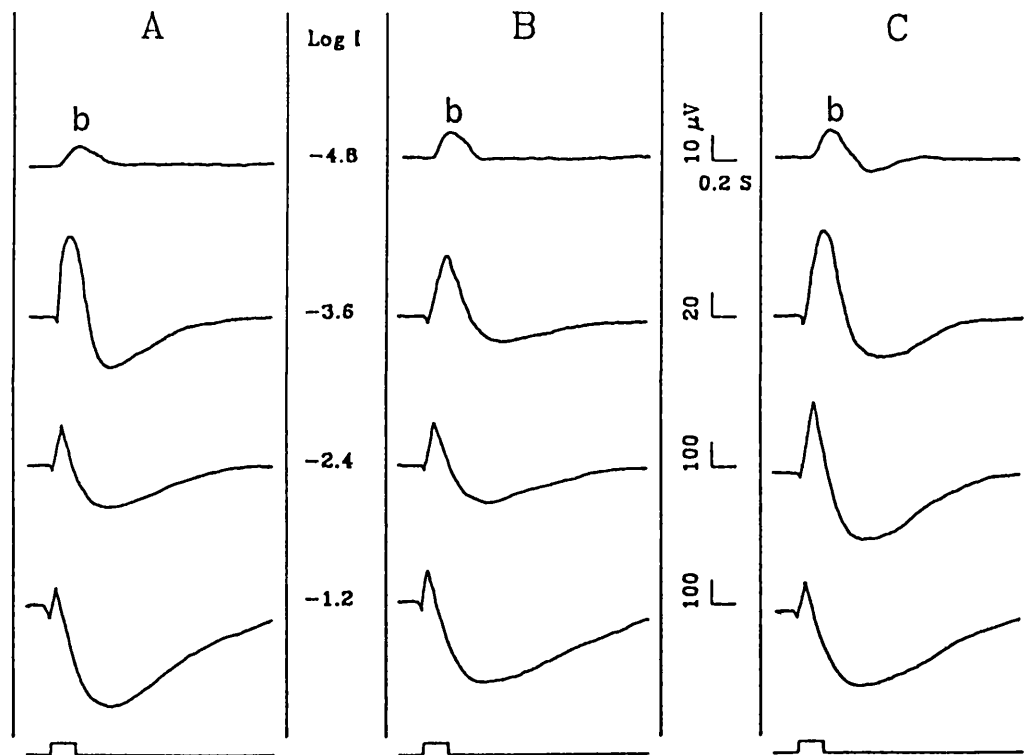
Fig.2.4.5 Effect of ketamine on the ERG.
   The ERG was measured in a dark-adapted, isolated rat retina. Ketamine was added to the perfusion medium to a final concentration of 0.15 mM. (A) before, (B) 4 minutes after, and (C) 16 minutes after the application of ketamine. Note: increasing the concentration of ketamine to 0.3 mM did not change the responses.
   The sweep duration was 2 seconds and stimulus duration 0.2 seconds. b = b-wave.

To see if ketamine could prolong survival of the b-wave during postmortem anoxia, dark-adapted eyes, taken from rats previously injected with the drug, were left in the dark at room temperature for 30 and 60 minutes, respectively, and then the retinae were isolated. For comparison, eyes from rats without ketamine pretreatment were kept for the same periods of time.

Following 30 minutes storage and subsequent perfusion with oxygenated medium, a b-wave gradually manifested, taking about 10 minutes to reach its peak of 10 uV: it then declined (Fig.2.4.6). No b-wave was seen in eyes, stored _ex vivo_ for 60 minutes. Prior administration of ketamine made no difference to the above observations.
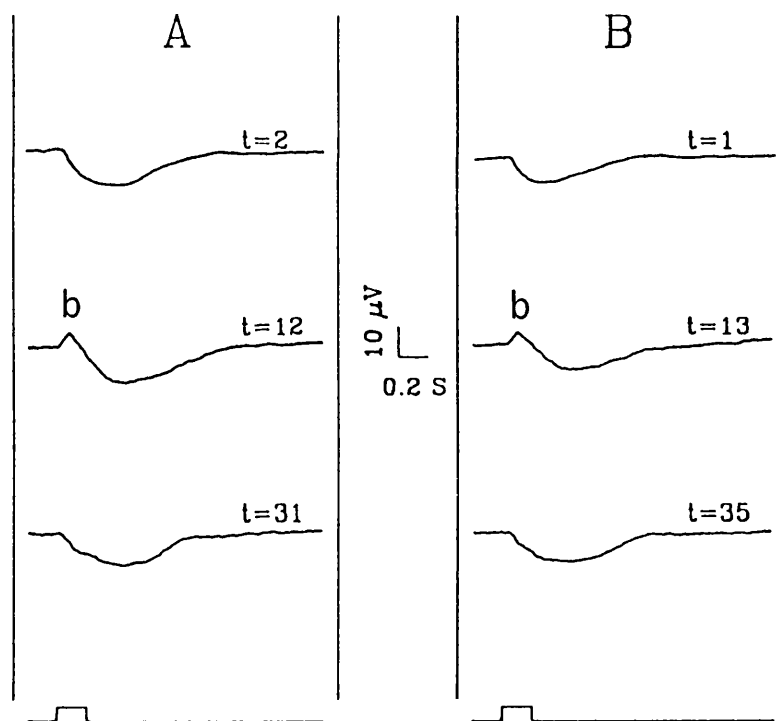
Fig.2.4.6 Pretreatment with ketamine.
    Dark-adapted eyes were kept in the dark at room temperature for 30 minutes before measurement of the ERG: (A) with and (B) without prior intraperitoneal injection of ketamine (see the text for details).
    The sweep duration was 2 seconds and stimulus duration 0.2 seconds. Stimulus intensity: $\log I = -2.7$, equivalent to 35 quanta/um^2. t = time in minutes after the start of retinal perfusion. b = b-wave.

Addition of 0.01 to 0.1 mM APB to the perfusion medium blocked the b-wave (Fig.2.4.7) - showing that the system was responsive to a drug that acts selectively on ON-bipolar cells (Porciatti et al., 1987; Powers et al., 1988).
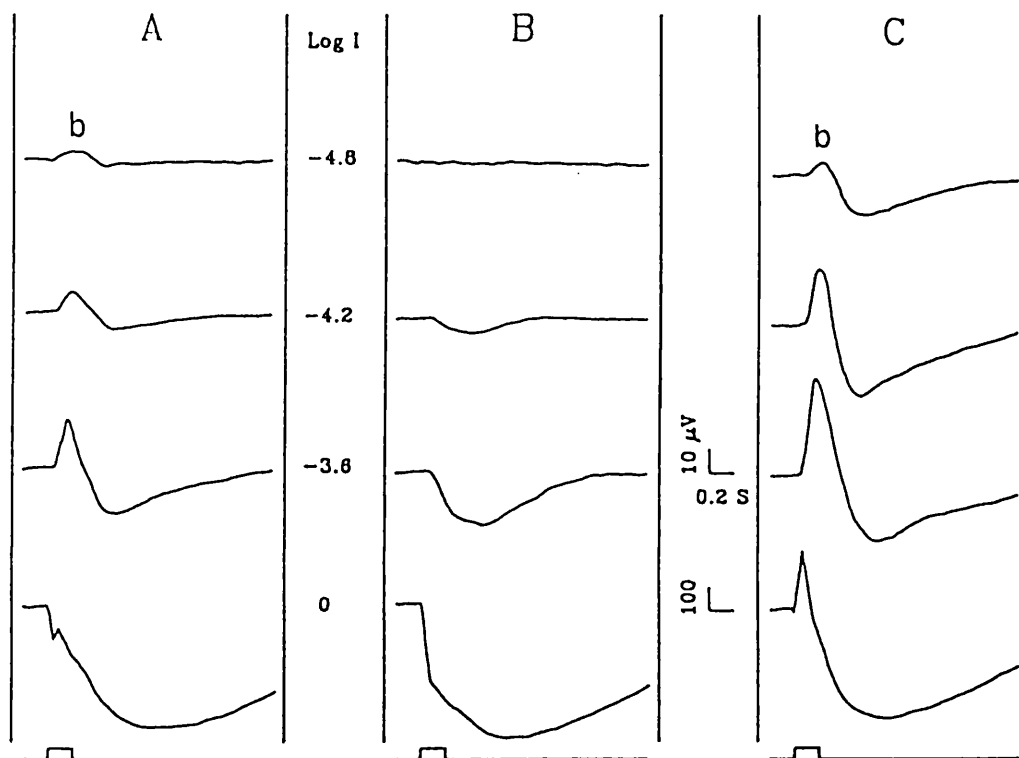
Fig.2.4.7 Effect of APB on the b-wave.

The ERG was measured in a dark-adapted, isolated rat retina. APB (final concentration 0.1 mM) was added to the perfusion medium, as described in the text.

(A) before and (B) 2 minutes after the application of APB. (C) 2 minutes after reperfusion with non-APB medium. The sweep duration was 2 seconds and stimulus duration 0.2 seconds. b = b-wave.

2.4.4 DISCUSSION

2.4.4.1 Relationship of recovery of rhodopsin, cGMP and the ERG in the isolated rat retina

Previous studies have shown that photoresponses are re-established when visual pigment is regenerated in isolated, partially-bleached retinae by application of either exogenous 9-cis or 11-cis retinal (e.g., Pepperberg, 1982; Yoshikami and Noll, 1982). However, no systematic investigations have been reported as regards the potential for recovery of phototransduction and the relationship between recovery of rhodopsin, cGMP and the ERG in the regeneration system.

The current results show that, although visual pigment was restored to almost the initial dark-adapted level, the sensitivity and amplitude of the photoresponse did not recover equally (Tables 2.4.1 and 2.4.2, Fig.2.4.2). The reasons are unknown. However, dark-adapted retinae incubated in the dark for, e.g., 60 minutes also revealed a significant reduction in the sensitivity and amplitude of the photovoltage (Tables 2.4.1 and 2.4.2 and Fig.2.4.2) and similar observations have been reported by Ernst and Kemp (1972). In addition, although 91% of rhodopsin was regenerated after 60 minutes incubation in the dark with 500 uM photoisomerate of retinal, approximately 50% of the visual pigment was still phosphorylated, as previously shown in Fig.2.2.5 (p.116); phosphorylated rhodopsin is known to act less effectively in initiating phototransduction than dephosphorylated form (cf. p.52). This might well contribute, therefore, to the reduction in the photoresponse.

It is also noteworthy that there was generally a close correspondence between the endogenous concentration of cGMP and the height of the PIII complex (Fig.2.4.2). The correspondence may be fortuitous since both are only indirect measures of their "key" components (cf. p.137). The central role of cGMP in determining the conductance of photoreceptor OS and hence the dark current is now well established (cf. p.55). It is generally recognized that the bulk of retinal cGMP is present in photoreceptors, but

it is also known that less than 10% is free and contributing to the dark current (see p.61). It is not clear what factors determine the equilibrium between free and bound cGMP. The binding constants for cGMP at specific sites on PDE and other OS proteins are likely to be important but there is also evidence to suggest that the concentration of free cGMP does not always parallel that of bound (Cote et al., 1986; Willmott et al., 1988; Voaden and Willmott, 1990). Thus, the possibility that the determinants may change, particularly in the previously-bleached and/or postmortem tissue, must be born in mind.

Although, in "regenerated" rat retinae, PIII was recovered to approximately 89% of the level obtained for dark-adapted retinae, incubated in the dark (Table 2.4.1), b-wave recovery was poor and ranged only between 10 and 60 uV, as compared with 50 and 150 uV in dark-adapted tissue (see the text, p.143). Since generation of the b-wave involves synaptic transmission between photoreceptors and higher-order neurones, it is possible that this mechanism is impaired in fully-bleached tissue.

2.4.4.2 Lack of effect of non-competitive NMDA-receptor antagonists against anoxic degeneration of the b-wave

The observation of partial recovery of the b-wave in the rat retina after 30 minutes anoxia (Fig.2.4.6) is in agreement with the findings of Winkler (1972) who reported that a b-wave could be regained in the rat if the retina was perfused, within 60 minutes of anoxia, with oxygenated medium. Thereafter, there was no recovery.

By comparing Fig.2.4.5 and Fig.2.4.7, it is unlikely that the lack of effect of ketamine and MK-801 on the b-wave is due to an inability of these drugs to penetrate the tissue, but rather that the b-wave is not mediated via NMDA receptors.

The present results indicate that ketamine and MK-801 have no effect on the b-wave in the rat retina (Fig.2.4.5 and the text, p.149). This is in agreement with Bloomfield and Dowling (1985) who found that NMDLA (N-methyl-D,L-aspartate) had no clear effect on rabbit ON-bipolar

cells, and with Coleman and Miller (1988) who showed that D-2-amino-5- and D-2-amino-7-phosphonovaleric acids (competitive NMDA antagonists) had no obvious effect on the ERG in the mudpuppy. Thus the current observation confirms that there is little or no synaptically activated NMDA-receptor component to ON-bipolar cell responses in the vertebrate retina.

As neither the b-wave nor PIII was better preserved after ketamine pre-treatment in vivo (Fig.2.4.6) and the photoresponses were not affected by NMDA antagonists in vitro as discussed above, it is unlikely that their postmortem decline involves NMDA-receptor activation. At first sight, this may appear surprising in view of the previous positive finding in vivo relating to the effects of transient ischaemia (Yoon and Marmor, 1989; cf. INTRODUCTION, p.138). However, it is possible that different mechanisms underly in vivo ischaemia and postmortem anoxic damage, and this requires further investigation using other pharmacological agents.

2.4.4.3 The potential for recovery of phototransduction in postmortem human retina

Preliminary experiments using rat tissue showed that the photoresponse could be preserved for at least 7 days in the fortified Eagle's medium at 4°C; whereas there was only 4 days survival in Earle's medium under similar conditions (Fig.2.4.4). The fortified medium was, therefore, used for studies on human tissue.

The current results show that phototransduction can be partially recovered in human retinae at least 58 hours postmortem (Table 2.4.3). However, survival in most specimens was poor. That is, the human retinae exhibited a 1 to 3 log unit increase in the threshold of PIII, a 1 to 2 log unit increase in the value of K, and, in most specimens, only a tenth of the maximum amplitude of the photoresponse obtained for the rat retina (cf. Tables 2.4.1 to 2.4.3).

However, as previously discussed with reference to structural preservation (see p.95), if all donor eyes were enucleated as quickly as possible after death and kept at

low temperature before retinal studies, it is likely that better functional survival could be achieved. This suggestion is supported by the observation that photoresponses in the rat retina _in situ_ were preserved for at least 2 days at $4^{\circ}C$, compared with less than 16 hours at room temperature (cf. Fig.2.4.4 and the text, p.148).

It is noteworthy that the recovery of response sensitivity does not always parallel that of the maximum amplitude (Table 2.4.3). For example, specimen 1 (5 hours postmortem) had a threshold of 18 quanta/um^2 and a maximum response of 398 uV. In contrast, the threshold of specimen 15 (34 hours postmortem) was 8.8 quanta/um^2, but its maximum response only 34 uV (Table 2.4.3; Fig.2.4.3). According to the intensity-time relation of fast and slow PIII voltages shown by Witkovsky et al. (1975), the threshold response obtained with a 0.2-second stimulus is almost entirely due to fast PIII. Thus, the present observations might be explained if the threshold response is triggered by the reaction of a small number of surviving photoreceptors, whilst the maximum response reflects a summation of activities of photoreceptor and Muller cells (in which slow PIII is generated). This suggestion is supported by tissue morphology, as general vacuolation and cytoplasmic swelling were present in most retinal layers of specimen 15, whereas photoreceptor IS and OS were well preserved (cf. Fig.2.1.3, p.87).

It is interesting that the b-wave was apparent in the ERGs obtained from three human specimens (Table 2.4.3, Fig.2.4.3), since this implies synaptic transmission between photoreceptors and ON-bipolar cells (cf. p.72). However, because the b-wave is only an indirect measure of function, its loss does not necessarily mean loss of neurotransmission (Masland and Ames, 1975), and in the future more direct studies of the responses of higher-order neurones should be undertaken. As a preliminary to this, the uptake of some neuroactive amino acids has been investigated and is discussed in the next section (2.5).

2.5 HIGH AFFINITY UPTAKE OF AMINO ACIDS

2.5.1 INTRODUCTION

Although photoreceptors are of prime importance with respect to the perception of light, vision is dependent on a fully functioning retina and on the processing of the information that occurs in the higher-order neurones. Although it is beyond the scope of the present thesis to deal in depth with survival of the latter, preliminary studies have been undertaken as regards survival of high-affinity mechanisms for the uptake of neuroactive amino acids into retinal cells. The carriers mediating accumulation of such amino acids are located in plasma membranes and, in general, are sodium dependent (cf. 1.5, p.73); sodium gradients are established by ATP dependent mechanisms. Thus, the uptake of amino acids serves as a good index of cell survival.

As previously described, taurine is essential for photoreceptor maintenance and is also a potential neurotransmitter in the retina. It has been shown to be taken up with high-affinity by photoreceptors and other neurones (see p.74). Glycine and GABA are inhibitory neurotransmitters in the inner retinal layers and, with species variation, are accumulated by neurones and/or Muller cells (see p.74-76). Therefore, these 3 amino acids were selected for investigating survival of various cells in postmortem human retinae.

Since fresh human retinae were not available for study, some investigations were undertaken using fresh baboon tissue.

The approaches used were autoradiography and kinetic analysis.

2.5.2 MATERIALS AND METHODS

2.5.2.1 Incubation of the retinae

Human retinae, 5 to 36 hours postmortem, were dissected in Earle's medium (p.99). Portions of tissue were individually incubated in the dark at 37°C for 20 minutes in 1 ml of Earle's medium containing a radio-labelled amino acid (obtained from Amersham International Ltd.).

For autoradiography, the activity of the isotopes was 100 uCi/ml, and concentration of ^{3}H-taurine 2.9 uM, ^{3}H-glycine 2.0 uM, and ^{3}H-GABA 2.0 uM. To establish the kinetics of the uptake systems, concentrations of 5, 10, 20 and 40 uM were used, with activity of 1, 2, 4, and 8 uCi/ml, respectively.

Freshly enucleated eyes from baboons, previously anaesthetized for from 30 minutes to 3 hours with alpha-chloralose (60 mg/kg), were stored at 4°C in moist, sterile containers for various periods of time before use. The retinae were isolated as described for human tissue (p.102), and then incubated at 37°C for 10 minutes with 2 uCi/ml taurine, glycine or GABA at a concentration of 1.0 uM.

After incubation, retinal samples were washed in fresh medium for 2 minutes. Some were then fixed for autoradiography (see below) and others were dispersed by sonication (p.106). Accumulated radioactivity was counted by scintillation spectrometry (cf. 2.6.2.3, p.173). As appropriate, the protein concentration was estimated as described in section 2.2.2.6 (p.103).

2.5.2.2 Autoradiography

Retinal samples were fixed in 2.5% glutaraldehyde, buffered in 0.1 M sodium cacodylate-HCl, and LM microscopy was performed as described in 2.1.2.2 (p.83).

For LM autoradiography, 0.8 um thick sections were mounted on glass slides, subbed with 0.05% chrome alum in 0.5% gelatine. Ilford K5 dipping emulsion was used, and the slides were coated, using the semiautomatic coating

device described by Marshall and Faulkner (1977). The slides were subsequently stored in a light-tight box with dried silicon gel. The exposure was carried out at 4°C for 6 to 8 weeks. The emulsion was then developed using KODAK D19 developer and fixed with KODAK Unifix.

2.5.3 RESULTS

2.5.3.1 Autoradiography of amino acid uptake

Fig.2.5.1 shows sites of high-affinity uptake of taurine by human retinae a) 5, b) 17 and c) 21 hours postmortem (cf. Specimens 1, 3 and 7, Table 2.1.1, p.89). It can be seen that taurine was predominantly taken up by photoreceptors and some neurones in the INL (Fig.2.5.1a,b). Muller cells were also labelled. In the 21-hour-postmortem retina, label was diffusely distributed and no cell specifity could be discerned (Fig.2.5.1c).

Fig.2.5.2 shows high-affinity uptake of glycine by retinae a) 17, b) 21 and c) 36 hours postmortem (Specimens 3, 7 and 17, Table 2.1.1, p.89). In all samples, glycine was predominantly accumulated by cell perikarya located in the INL and by synapses in the IPL and OPL. The labelling in both plexiform layers together with the number and distribution of labelled nuclei in the INL suggests that some bipolar cells are labelled and, possibly, a few amacrine and horizontal cells. In the 17-hour-postmortem specimen, some ganglion cells had also taken up glycine heavily (Fig.2.5.2a). It was interesting to note that intensive radio-labelling was also seen in some cells surrounding the walls of arteries (Fig.2.5.2a) and veins (not shown). Glycine uptake by photoreceptor cells was evident (Fig.2.5.2). It is noteworthy that glycine uptake into the photoreceptors and neurones in the INL was still occurring in the retinae 21 and 36 hours postmortem, whereas taurine uptake at these tissues had noticeably declined (cf. Figs. 2.5.1c and 2.5.2b,c).

Fig.2.5.3 shows high-affinity uptake of GABA by human retinae a) 17, b) 21 and c) 36 hours postmortem (Specimens 3, 7 and 17, Table 2.1.1, p.89). It can be seen that GABA was principally accumulated by Muller cells in all the retinal samples.
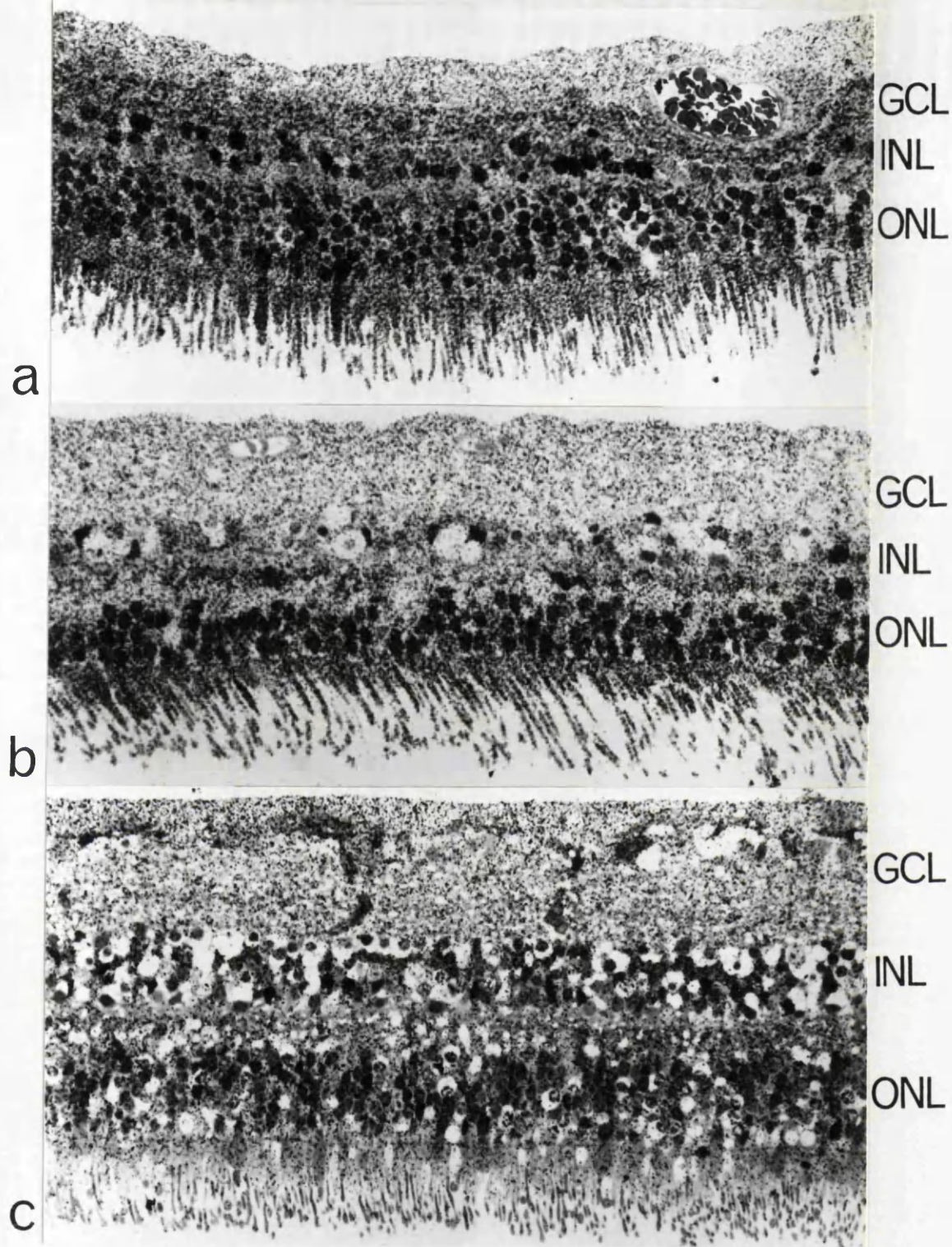
Fig.2.5.1 Autoradiographs of taurine uptake by human retinae a) 5, b) 17 and c) 21 hours postmortem (Specimens 1, 3 and 7 in Table 2.1.1, p.89).

The retinae were incubated at 37°C for 20 minutes with 2.9 uM ^{3}H-taurine (100 uCi/ml). Intensive radiolabelling can be seen in photoreceptors and some neurones in the INL (a, b). Whereas diffuse labelling is seen in c). Muller cells were also labelled. ONL = outer nuclear layer. INL = inner nuclear layer. GCL = ganglion cell layer. The bar marker: 100 um.
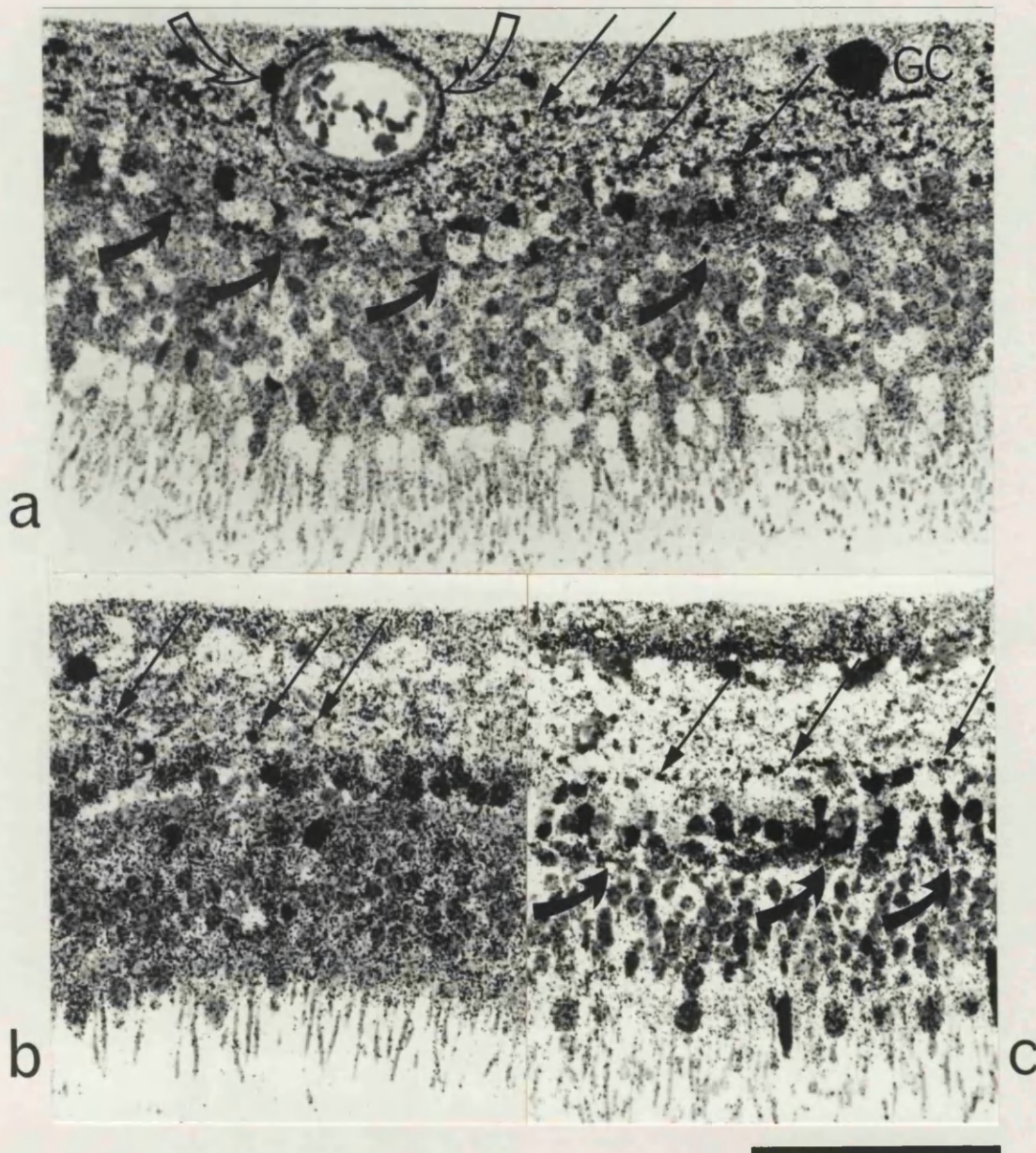
Fig.2.5.2 Autoradiographs of glycine uptake by human retinae a) 17, b) 21 and c) 36 hours postmortem (Specimens 3, 7 and 17 in Table 2.1.1, p.89).

The retinae were incubated at 37°C for 20 minutes with 2.0 uM ^{3}H-glycine (100 uCi/ml). In all samples radio-labelling occurred principally in cells located in the INL and was also present in discrete locations in the OPL (close arrows) and IPL (fine arrows). In sample a) some ganglion cells (GC) and some cells surrounding retinal arteries (open arrows) were also intensively labelled. Intense labelling can also be seen over photoreceptors, particularly their IS. The bar marker: 100 um.
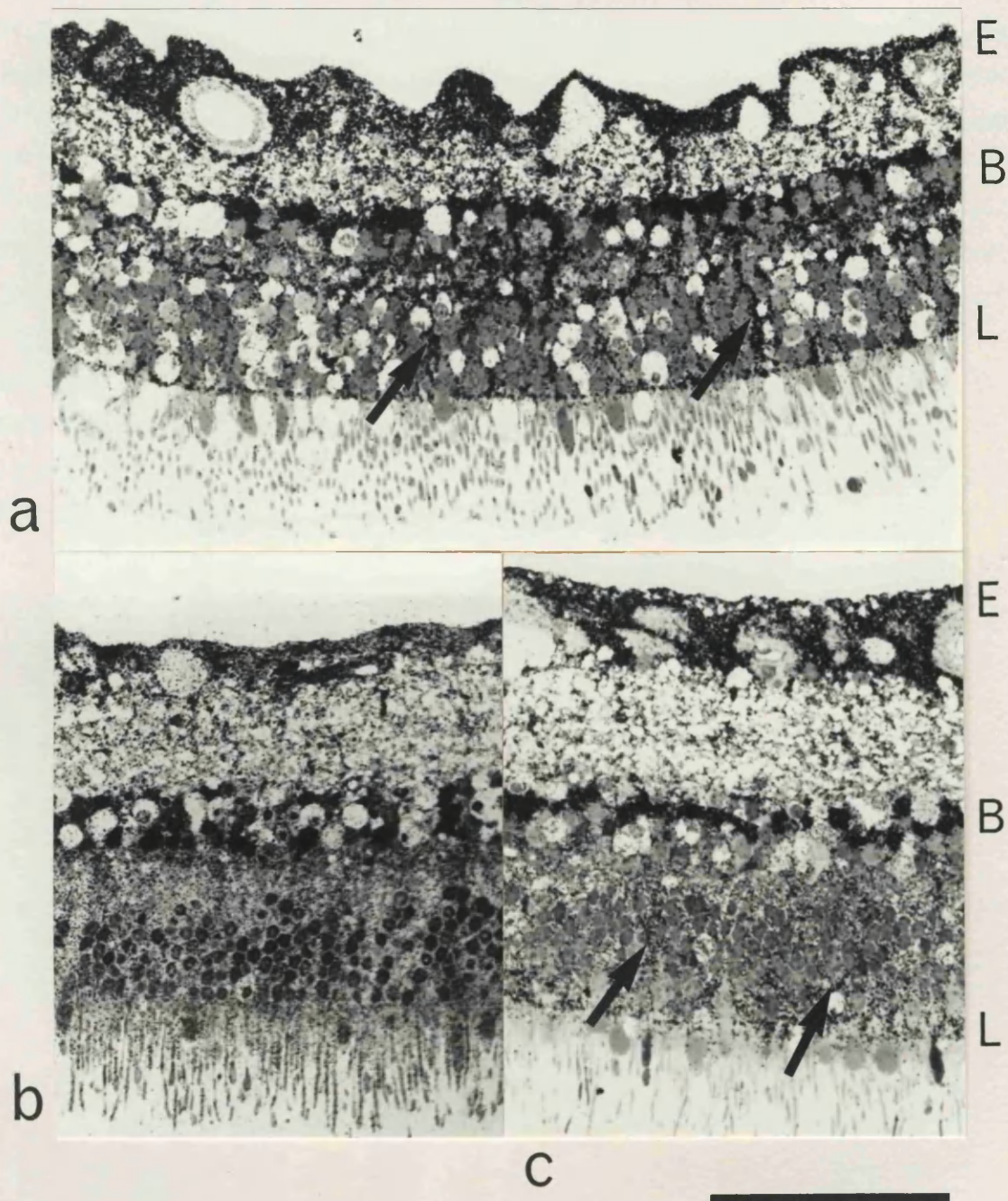
Fig.2.5.3 Autoradiographs of GABA uptake by human retinae
a) 17, b) 21 and c) 36 hours postmortem (Specimens 3, 7
and 17 in Table 2.1.1, p.89).

The retinae were incubated at 37°C for 20 minutes
with 2.0 uM ³H-GABA (100 uCi/ml). The end-feet of the
Muller cells (E), their cell bodies in the INL (B), their
passage through the ONL (arrowed) and their contribution
to the outer limiting membrane (L) were particularly
clearly labelled. The bar marker: 100 um.

2.5.3.2 The kinetics of amino acid uptake

The kinetics of high-affinity uptake of taurine, glycine and GABA were assessed in two human retinae, 14 and 21 hours postmortem (Specimens 2 and 7 in Table 2.1.1, p.89). The values of K_m and V_{max} are shown in Table 2.5.1. It can be seen that high-affinity uptake of taurine occurred in the 14-hour-postmortem specimen but not in the 21-hour-postmortem one. In contrast, high-affinity uptake of glycine and GABA remained in both retinal samples.

Further comparisons of postmortem survival of taurine, glycine and GABA uptake were undertaken using fresh baboon retinae. Fig.2.5.4 shows that accumulation of ^{3}H-taurine was maintained at a constant level for about 6 hours postmortem, whereas ^{3}H-glycine and ^{3}H-GABA uptake survived unchanged for at least 12 hours. These results are similar to those obtained for the human retina (cf. Figures 2.5.1 to 2.5.3; Table 2.5.1).

Table 2.5.1 The kinetics of high-affinity uptake of amino acids in the human retinae, 14 and 21 hours postmortem (Specimens 2 and 7 in Table 2.1.1, p.89; cf. Figures 2.5.1 to 2.5.3).

Radio-isotope	Specimen 2 (14 hrs)	Specimen 7 (21 hrs)	References	
Taurine			*Human (1);	Rat (1)
K_m	55	ND	49	51
V_{max}	0.08	ND	0.3-0.7	0.90
Glycine			Rabbit (2);	Frog (3)
K_m	40	45	28	17
V_{max}	0.15	0.14	3.3	0.13
GABA			Rat (4);	Frog (3)
K_m	52	50	40	25
V_{max}	0.44	0.40	3.7	0.15

(unit: K_m - uM, V_{max} - nmol/min/mg protein)

The human retinae were incubated for 20 minutes as described in the text, with substrate concentrations from 5 to 40 uM. The values of K_m and V_{max} were obtained from double reciprocal Lineweaver-Burk plots, in which each data point was derived from the mean of 3 estimations. ND = not detectable.

Sources:
(1) Schmidt and Berson, 1980: * data from human retinae 2 to 4.5 hours postmortem.
(2) Bruun and Ehinger, 1972.
(3) Voaden et al., 1974.
(4) Goodchild and Neal, 1970.
In order to facilitate comparison, all values of V_{max} in reference data have been converted into nmol/min/mg protein (cf. p.74).
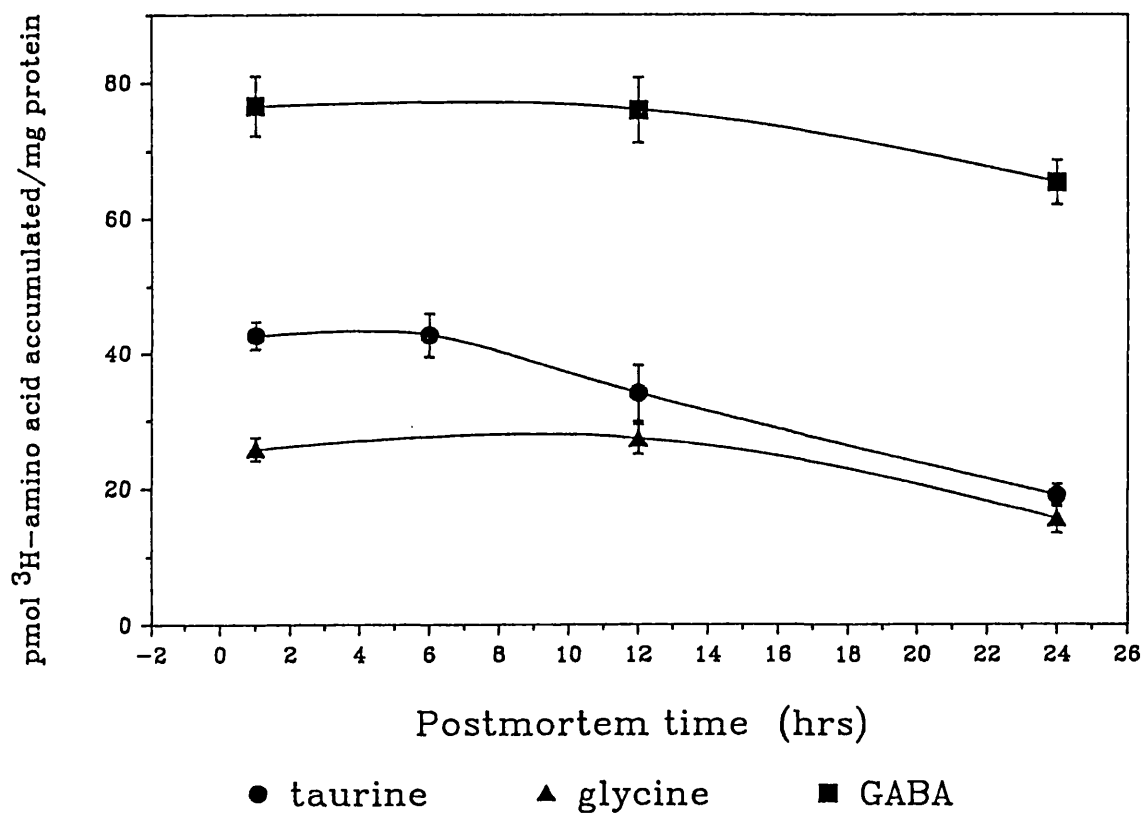
Fig.2.5.4 Survival of amino acid uptake in the baboon retina.

Freshly-enucleated baboon eyes were stored at 4°C for the times indicated. The retinae were then dissected and incubated at 37°C for 10 minutes with 1 uM ^{3}H-amino acid, as described in the text. Each point represents the mean $\pm$ SEM of at least 3 estimations.

2.5.4 DISCUSSION

Previous studies have shown that the cellular localization of high-affinity uptake of amino acids in the retina varies with the amino acid, the species, and, potentially, between _in vivo_ and _in vitro_ conditions (cf. 1.5, p.73). However, such studies in man are limited, no doubt because of the lack of availability of human tissue. The present study thus expands our understanding of amino acid uptake in the human retina, as well as establishing postmortem survival times for such mechanisms.

2.5.4.1 Sites of high affinity uptake of amino acids in the human retina

(1)   Taurine uptake

Although taurine has yet to be established as a retinal neurotransmitter, it exists in the retina in extremely high concentrations, has a powerful inhibitory effect on electrical responses, and is taken up via high-affinity mechanisms by specific cell types (for a review: Pasantes-Morales, 1985). It has been shown that taurine inhibits the activities of ON-bipolar, amacrine and ganglion cells but not those of photoreceptor and horizontal cells (cf. Cunningham and Miller, 1976, 1980a,b). In contrast to their lack of responsiveness to taurine, photoreceptors are the retinal cell type that predominantly accumulates it. In addition, although there are species variations, certain types of neurones in the INL also take up this amino acid (Pasantes-Morales, 1985). It should be noted, however, that the large pool of taurine located in the photoreceptors is not thought to relate to a neurotransmitter role (see p.73) and that these cells communicate through an excitatory synaptic transmitter (e.g., Ehinger and Dowling, 1987).

The current results show that, in the human retinae 5 and 17 hours postmortem, taurine was predominantly taken up by photoreceptors and some neurones located in the INL. The latter were possibly bipolar cells (Fig.2.5.1a,b).

These features of taurine uptake are similar to those observed by Lake et al. (1978) for taurine uptake by the baboon retina.

(2) Glycine uptake

The vertebrate retina contains endogenous stores of glycine in the millimolar range (for a review: Marc, 1985) and accumulates ^{3}H-glycine into particular subclasses of retinal neurones (for reviews: Ehinger and Dowling, 1987; Massey and Redburn, 1987). Although glycine exerts an inhibitory effects on a number of different types of retinal neurones, its most effective inhibition is seen in ganglion cells (Miller et al., 1981a,b). This possibly relates to the finding that glycine is predominantly accumulated by neurones presynaptic to ganglion cells, such as amacrine and bipolar cells (Ehinger and Dowling, 1987; Massey and Redburn, 1987). It is also interesting that glycine and taurine have been suggested to interact with the same synaptic receptor in the retina (for a review: Pasantes-Morales, 1985) but that their sites of accumulation are different.

The current results show that high-affinity uptake of glycine in human retinae 17 to 36 hours postmortem occurred predominantly into cells located in the INL and some synapses in the IPL and OPL (Fig.2.5.2). The anatomical distribution of labelled neurones in the INL indicates that they are predominantly bipolar cells but that they may include a limited number of amacrine and horizontal cells.

According to previously-published data, uptake of glycine by amacrine cells is the most consistent observation across species, including man (Frederick et al., 1984, 1 hour postmortem tissue; Marc and Liu, 1985, unknown postmortem time for preservation of donor confidentiality). The present autoradiographs do not show this clearly. In addition, glycine uptake into horizontal cells has not been reported previously. This appears to occur in the present study, although no cross check was performed with EM microscopy. The reasons for the above differences are unknown, but we have to consider the

following potential contributory factors: (1) retinal vacuolation resulting from the longer postmortem times in the present study and (2) retinal disruption, resulting in cellular displacement. Otherwise, there were no apparent differences in incubation time and temperature, and glycine concentration and specific activity between the present experiments and those of Frederick et al. (1984) and Marc and Liu (1985).

In the present study it was also found that photoreceptor cells accumulated glycine, although apparently less intensively than the neurones in the INL (Fig.2.5.2). Glycine differs from taurine and GABA in that it is an important constituent of proteins. Thus, it is not clear whether the label in photoreceptors represents free glycine, and/or amino acid incorporated into protein.

Glycine was also accumulated by some ganglion cells (Fig.2.5.2a). Previously, only a single report has appeared concerning uptake of glycine by ganglion cells. This was in the light-adapted frog retina (Voaden et al., 1974) and no label was seen in dark-adapted tissue. More studies are needed to see if the present observation is a postmortem artifact or reflects a functional attribute of a specific subgroup of human ganglion cells.

It was noteworthy that some cells surrounding retinal vessels also accumulated glycine (cf. Fig.2.5.2a). Voaden et al. (1974) also saw accumulation of glycine in the walls of vessels in the light-adapted frog retina but curiously, again, not in dark-adapted tissue. In the present results the cells accumulating glycine might be astrocytes (cf. Hogan and Feeney, 1963; Wise et al., 1971). Hogan and Feeney (1963) have described two types of astrocytes in the retina. One resembles fibrous astrocytes of the brain and is found most often around the walls of retinal arteries. The second type is likened to a protoplasmic astrocyte and is usually found in contact with retinal veins. Since, in the present results, the radiolabelling of the cells was seen both around the arteries and veins, it was possible that both types of astrocytes were accumulating glycine. It is unlikely that

the glycine uptake into these cells relates to
neurotransmission, because there are no vasomotor nerves
in the retina (Wise et al., 1971). It has been suggested
that the constituents of the basement membrane in retinal
vessels include collagen (Wise et al., 1971) and the
latter is extremely rich in glycine (Alberts et al.,
1983). Thus, it might be possible that the glycine
accumulation into these cells relates to collagen
synthesis. More studies are needed to understand the
significance of the above observations.

(3) GABA uptake

Previously-published data have shown that marked
variations of high-affinity GABA uptake exist across
species. In mammals, differences are also noted between
in vivo and in vitro studies (cf. Table 1.5.1, p.76). The
latter may be explained by the observations of Voaden and
colleagues (for a review: Voaden, 1988) that GABA taken up
by Muller cells is rapidly metabolised to glutamine, which
then moves freely through the tissue and is preferentially
converted back to neuroactive amino acids in the higher-
order neurones. A similar cycle exists also in the brain
(Kuffler et al., 1984). Given the difference in time
courses of in vivo (several hours) and in vitro (10 to 30
minutes) studies, it is perhaps not surprising that the
radioactive label is located in different cell types.

Previously-published findings have shown that, in
short-term incubation in vitro, GABA is predominantly
accumulated by Muller cells in mammalian retinae,
including guinea pig, rabbit, cat, goat, baboon (Marshall
and Voaden, 1975), rat, monkey and man (Bruun and Ehinger,
1974; Lam and Hollyfield, 1980). The current result shows
that GABA is predominantly taken up by Muller cells in the
human retina in vitro (Fig.2.5.3) and is thus consistent
with the previous results.

2.5.4.2 Survival of amino acid uptake in postmortem human retinae

High-affinity uptake of neuroactive amino acids in the CNS is mediated by Na^+-dependent, ATP-requiring processes, based on integral membrane carrier proteins. In the postmortem state, this energy-requiring process is inevitably impaired and, therefore, it is surprising that organised amino acid uptake remained in human retinae for several hours postmortem.

Schmidt and Berson (1980) have suggested that high-affinity taurine uptake in the human retina persists for at least 4.5 hours postmortem, as indicated by kinetic studies. The present observations show that the mechanism can survive for at least 17 hours postmortem, as demonstrated by both autoradiographic studies (Fig.2.5.1) and analysis of kinetics (Table 2.5.1). With longer postmortem time, however, taurine uptake by specific neurones progressively declined (Fig.2.5.1c; Table 2.5.1). This could reflect a specific vulnerability of the taurine carrier system.

However, it should be noted that, even when taurine uptake became abnormal, glycine and GABA were still specifically accumulated by neurones and Muller cells, respectively (cf. Figures. 2.5.1 to 2.5.3). The better preservation of glycine and GABA uptake was also observed in baboon tissue (Fig.2.5.4).

Like the observations with autoradiography, kinetic studies showed that the kinetic parameters for both glycine and GABA uptake compared well with the previously-published data obtained for various species (Table 2.5.1). In contrast, although the K_m for taurine uptake by the 14-hour-postmortem retina was comparable with that obtained by Schmidt and Berson (1980) for human retinae 2 to 4.5 hours postmortem, the V_{max} was much lower than those obtained by the latter authors (i.e., 0.08 against 0.3-0.7 nmol/min/mg protein). This probably means that the number of uptake sites is reduced postmortem, but that remaining ones retain normal characteristics (Garey and Heath, 1974).

From the comparison of survival of high-affinity

uptake of taurine, glycine and GABA, it would appear that the specific mechanism for taurine uptake was more readily damaged postmortem. Thus, the carrier system mediating taurine uptake may be quite different from those for glycine and GABA. It has been shown that a decrease in the proportion of unsaturated fatty acids in cultured retinoblastoma cells increases the K_m for taurine transport (Yorek et al., 1984). It is possible, therefore, that the taurine carrier is more dependent on membrane fluidity of the cells.

2.6 PROTEIN SYNTHESIS

2.6.1 INTRODUCTION

In general, body proteins undergo continual metabolic turnover, the rates varying enormously between tissues and molecules, depending on their functions. The retina is no exception and the extent, to which it can support such a fundamental process, provides a useful index of not only tissue survival but also the potential for continuation of function in vitro. Many studies have estimated protein synthesis in the retina by incorporation of ^{3}H-leucine in vivo or in vitro (e.g., Droz, 1963; Steiman and Ames, 1974; cf. p.77). In the present investigations protein, including opsin, synthesis has been monitored by this technique in postmortem human retinae.

2.6.2 MATERIALS AND METHODS

2.6.2.1 Medium

Medium A: The fortified Eagle's medium (p.99).
Medium B: As medium A, but without leucine.

2.6.2.2 Incubation of the human retina

Postmortem human retinae, 5 to 48 hours postmortem, were dissected in medium B, using the techniques described previously (p.102). Approximately 20 mm^2 portions of the retina were then individually incubated in one ml of the same medium containing L-^{3}H-leucine (Amersham) at either 5 uCi/ml (final concentration 0.04 nM) or 100 uCi/ml (final concentration 0.8 nM). Incubations were in the dark at 37°C for the times designated, with gentle shaking, and the surface of the medium was gassed with a moistened mixture of 95% O_2/5% CO_2. For "pulse" and "chase" studies, the retina was first incubated with 100 uCi/ml ^{3}H-leucine for 1 hour ("pulse"), and then gently washed twice in medium A for a total of 2 minutes, followed by subsequent incubation in 1 ml of medium A for the times indicated in the text ("chase").

To see whether incubation in the fortified medium improved vegetative function in postmortem human tissue, a number of retinae were first incubated in medium A for 24 hours, followed by the incubation with ^{3}H-leucine as described above.

Radiolabelling of total protein and, specifically, of opsin by ^{3}H-leucine was monitored by scintillation spectrometry, fluorography of electrophoretic gel profiles, autoradiography of immunoblotted opsin and LM autoradiography.

2.6.2.3 Counting of incorporated radioactivity

At the end of each incubation, tissue was removed and washed twice in ice-cold medium A for a total of 2 minutes. Individual samples were disrupted in 0.25 ml of 10 mM ice-cold Tris-HCl, pH 7.4, by intermittent sonication for a total of 20 seconds (cf. p.106). Half of the resultant suspension was pipetted into a scintillation mini-vial containing 1 ml of 15% ice-cold trichloroacetic acid (TCA). The remainder was stored at -70°C for electro-phoresis and immunoassay.

The TCA-precipitated proteins were centrifuged at 8000 g for 5 minutes at 4°C and the supernatant discarded. The pellets were then washed twice with 1 ml aliquots of 12% ice-cold TCA and solubilized in 0.5 ml of 0.5 M NaCl overnight at 4°C. An aliquot of 0.05 ml of the resulting suspension was taken for protein estimation by the modified Lowry's method (p.103). The remainder was mixed with 4.5 ml of OptiPhase 'Safe' scintillant cocktail (LKB). Radioactivity was counted with a Packard Model 4000 liquid scintillation spectrometer.

2.6.2.4 SDS-PAGE and fluorography

In order to analyse the proteins synthesized in postmortem human retinae, one-dimensional sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and fluorography were employed.

Unless otherwise indicated, materials for electro-phoresis were obtained from Bio-Rad. Molecular weight

markers were obtained from Sigma (SDS-6H) and BDH (44264).

"ProtoGel", containing 30% acrylamide and 0.8% methylene bisacrylamide: 30.8% T (total) and 2.67% C (crosslinked), was obtained from National Diagnostics.

SDS-PAGE was performed using the techniques described by Laemmli (1970), with a separating gel of 12% and stacking gel of 4% acrylamide. Retinal proteins were dissolved in "Sample" buffer containing 2% SDS, 5% 2-beta-mercaptoethanol and 62.5 mM Tris-HCl (pH 6.8). A total of 20 ug of protein was loaded into each well. Gels were run at 200 volts for about 45 minutes. Some of the gels were fixed and stained with 0.1% Coomassie brilliant blue R250 in 40% methanol and 10% acetic acid; and the others, destined for immunoblotting, were treated as described below. The Coomassie blue-stained gels were dried onto absorbent papers (Whatman, 3 MM) using a gel dryer (Bio Rad, Model 483) and then fluorographed, using Autofluor (National Diagnostics) and X-ray film (Amersham, Hyperfilm-MP). The film was exposed at -70°C for 10 weeks. After development, the film was printed on Ilford "grade 3" photographic paper.

2.6.2.5 Immunoblotting and autoradiography

Western blotting was used to identify the opsin bands in the gels.

After electrophoresis, gels were fixed with a transfer buffer, consisting of 25 mM Tris-base, 192 mM glycine and 20% methanol (pH 8.3), and then electroblotted onto nitrocellulose paper, following the method of Towbin et al. (1979). The electroblotting was carried out at 0.1 mA at room temperature overnight. Opsin was identified using rabbit anti-bovine opsin antiserum (courtesy of Drs. A. Shallal and N. McKechnie; cf. Shallal et al., 1988) and goat anti-rabbit, peroxidase-conjugated IgG (ICN Biomedicals Ltd.). Autoradiography was performed on the immunoblotted nitrocellulose paper, using Hyperfilm-^{3}H (Amersham). Exposure was at -70°C for 10 weeks.

2.6.2.6 Measurement of radioactivity in electrophoresed proteins

The radioactivity incorporated into opsin and total protein in the dry gels and the nitrocellulose papers was released and then counted, using the method described by Dion and Pomenti (1985). Briefly, strips of dry gels or nitrocellulose papers were cut into segments (1 to 2 mm by 7 mm), and each segment was incubated at 50°C for 36 hours in 0.5 ml of 0.4% (w/v) pronase (Sigma) in 0.1 M Tris/HCl, pH 8.0, containing 10 mM $CaCl_2$ and 0.5 mM NaN_3. Additional pronase (50 ul of a 2% solution) was added at 12 and 24 hours for gels, or at 24 hours only for nitrocellulose papers. Radioactivity was counted as described above and calculated in relation to the amount of proteins loaded in each lane of the gels.

2.6.2.7 LM microscopy and autoradiography

LM microscopy and autoradiography were performed as described in 2.1.2.2 (p. 83) and 2.5.2.2 (p. 157).

2.6.3 RESULTS

2.6.3.1 The synthesis of opsin and total protein in postmortem human retinae

Fig.2.6.1 compares radioactivity incorporated into total protein in 5 human retinae. Protein synthesis in all samples increased with incubation time, and rates were well maintained after 24 hours retinal explant culture in the fortified medium.
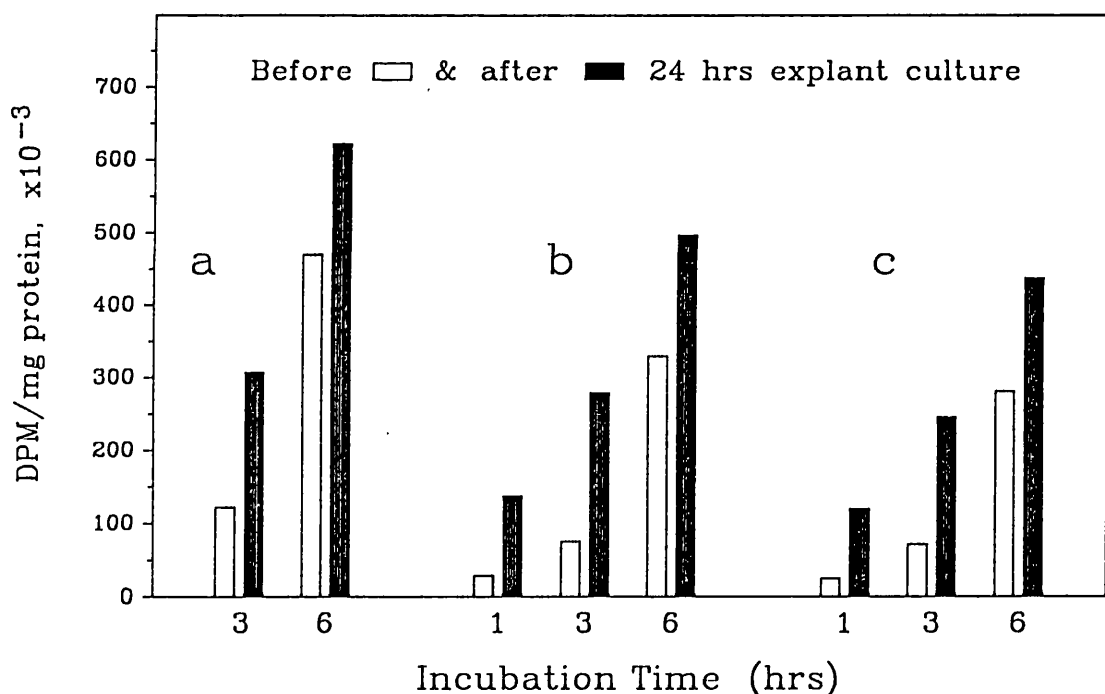
Fig.2.6.1 Protein synthesis in 5 postmortem human retinae (3 donors) before and after explant culture.
    The retinae, before and after culture, were incubated with 5 uCi/ml ^{3}H-leucine for the times indicated. The radioactivity incorporated into proteins was isolated and counted as described in the text.
    Sample a) was from an 85-year-old male, 24 hours postmortem and 5 hours between death and enucleation. Sample b) represents the mean of two estimations from the retinae of an 85-year-old male, 48 hours postmortem and 5 hours to enucleation, Sample c) is also from two retinae this time of a 66-year-old male, 48 hours postmortem and 7 hours to enucleation [cf. Specimens 11 (a), 23 (b) and 24 (c) in Table 2.1.1, p.89].

Fig.2.6.2 compares the total pattern, opsin immunoblot, fluorograph and autoradiograph of radio-labelled proteins in a 24-hour-postmortem human retina (Specimen 1, Fig.2.6.1) under various incubation conditions. Comparable results were obtained with a 36-hour-postmortem human retina (Specimen 17 in Table 2.1.1, p.89; data not shown).

It is evident from Fig.2.6.2 that:

1) Protein patterns were similar among all specimens (A, B), incubated for different periods.

2) Radiolabelling was increased in the specimens after 24 hours explant culture in the fortified medium (B', b'), as compared with uncultured tissue (A', a').

3) A doublet of opsin immunoreactivity was apparent at molecular weights of approximately 35 and 37 kDa (see a, b).

4) The opsin bands (Rh) were also radiolabelled (see, e.g., lanes IIb', IIIb').

5) Two unidentified protein bands, at approximately 75 and 23 kDa, were intensely labelled (see e.g. b').
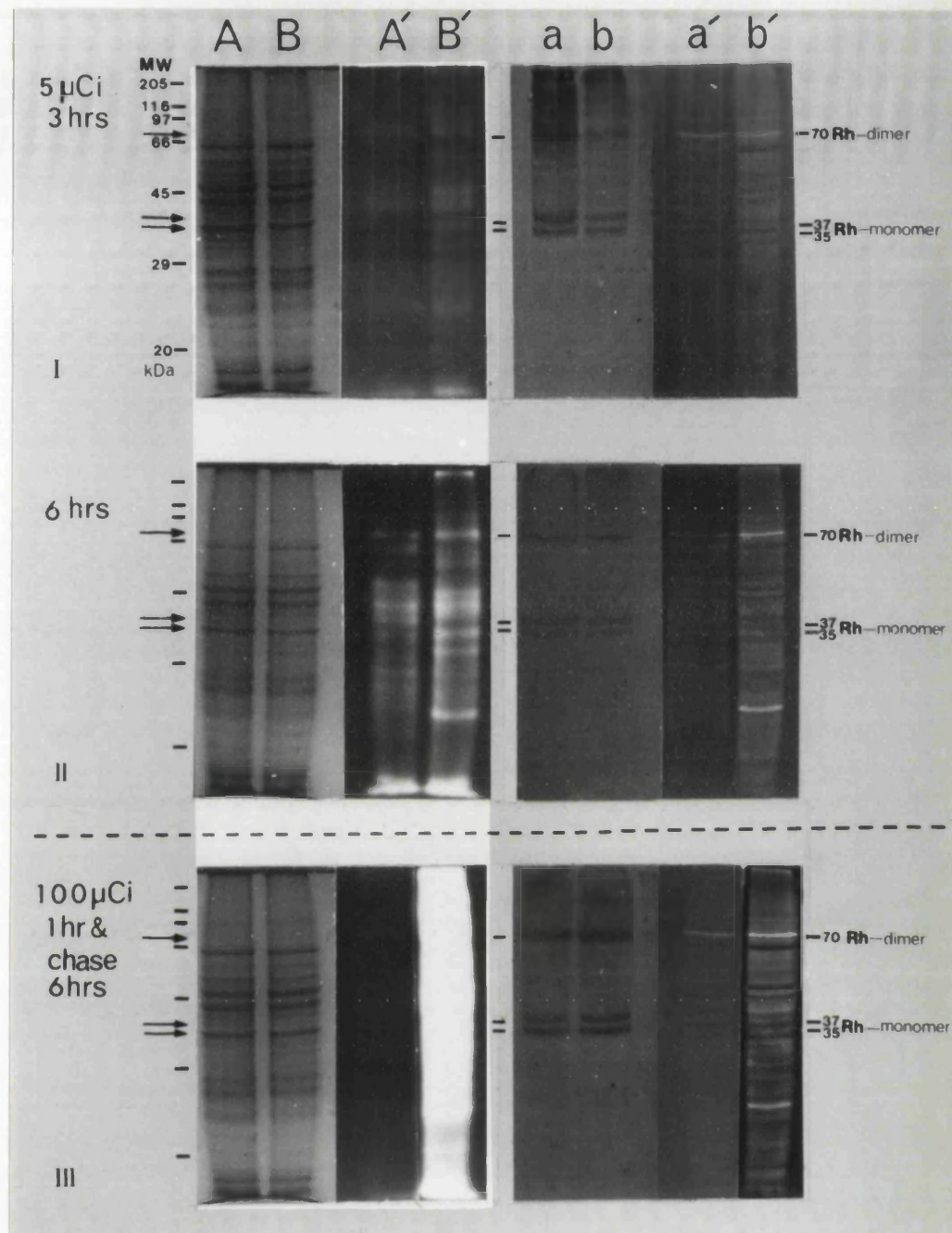
Fig.2.6.2 Protein profile plus fluorograph, and immunoblot plus autoradiograph of a 24-hour-postmortem human retina (Sample a, Fig.2.6.1).

(A and B) Proteins separated by SDS-PAGE:
- (A) Retinal explants were incubated with ^{3}H-leucine (5 uCi/ml or 100 uCi/ml) for the times indicated.
- (B) Explants were cultured in the fortified medium for 24 hours, followed by incubation as in (A).

(A' and B') Fluorographs of A and B, respectively (negative photographs).

(a and b) Immunoblots of identical gels to A and B, respectively.

(a' and b') Autoradiographs of a and b, respectively (negative photographs).

It is noted that the specimens subjected to explant culture for 24 hours (B' and b') were more heavily radio-labelled than uncultured ones (A' and a'), and that opsin bands (Rh, arrowed) were also labelled.

To compare quantitatively the newly-synthesized opsin in the samples described in Fig.2.6.2, the radioactivity of relevant proteins in the dried gels and nitrocellulose papers was recovered and counted. The results are shown in Fig.2.6.3. In line with total protein synthesis (Fig.2.6.1), opsin formation increased with time, both in the uncultured and cultured samples (Fig.2.6.3). Comparison between the pulse/chase incubation samples (i.e., Fig.2.6.2, section III) showed that the radioactivity in the cultured tissue was approximately 8 times higher than in the uncultured one (106,000 against 13,400 dpm/mg protein).
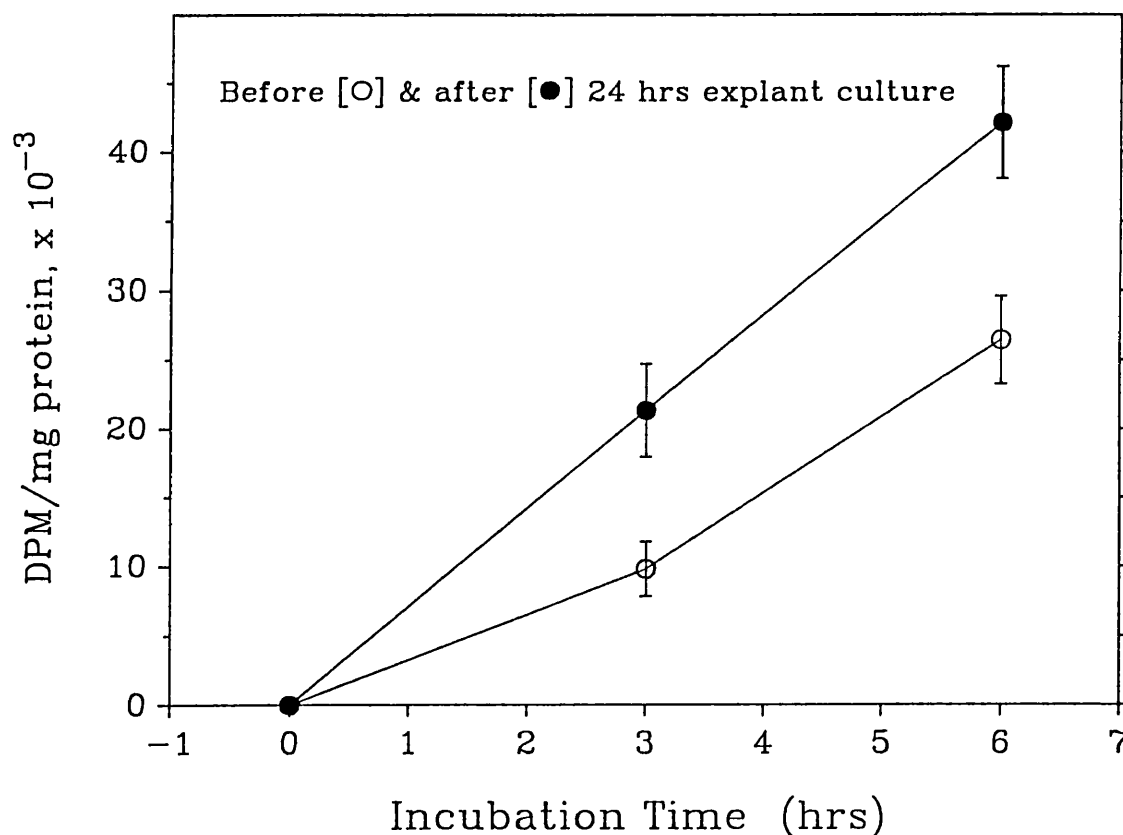
Fig.2.6.3 Opsin synthesis in a 24-hour-postmortem human retina before and after 24 hours explant culture (Specimen a in Fig.2.6.1, and Fig.2.6.2).

The retina, before and after culture, was incubated with 5 uCi/ml ^{3}H-leucine for 3 and 6 hours. Radioactivity was released and counted. Only the two monomer opsin bands of 35 and 37 kDa were included in the calculation of opsin, since the dimer was not easily distinguished from adjacent bands (cf. Fig.2.6.2). Each point represents the mean ± SEM of data obtained from 3 electrophoretic profiles.

2.6.3.2 Morphology of incubated retinae

To see if short-term culture in the fortified medium might allow improvement of morphology in postmortem tissue, human retinal explants (18 to 34 hours postmortem; Specimens 4, 7, 11 and 15 in Table 2.1.1, p.89) were examined with LM microscopy after incubation. Typical results are shown in Fig.2.6.4. It can be seen that initially there were vacuolation and cytoplasmic swelling in all retinal layers, except for photoreceptor outer and inner segments (Fig.2.6.4 a). After 1 hour of incubation (Fig.2.6.4 b), the general appearance of the retinae was similar, with the possible exception of a slight reduction in the degree of vacuolation in the inner retinal layers. However, a common finding at this stage was the migration of cone nuclei from the outer nuclear layer, passing through the outer limiting membrane. After 24 hours incubation (Fig.2.6.4 c), marked changes were seen in retinal anatomy, with an almost complete absence of vacuolation or perinuclear swelling. Much of the tissue reorganization is likely to relate to changes in retinal glia, with a degree of swelling of component Muller's fibres. This was most marked in the outer nuclear layer where the swelling of glial sheaths around the photoreceptor nuclei resulted in a change in internuclear spacing. Some cone cells were also swollen. After 24 hours of incubation, the number of cones with displaced nuclei was reduced and photoreceptor inner segment anatomy was still well preserved (Fig.2.6.4 c). However, swelling was evident in the outer segments.

Further studies showed that the reduction of retinal vacuolation usually started after about 3 hours' incubation. Fig.2.6.5 demonstrates structural improvement in a 24-hour-postmortem retina with increasing time of incubation. Similar results were obtained with two other specimens (Specimen 7, 21 hours postmortem; Specimen 15, 34 hours postmortem, Table 2.1.1, p.89). This is in line with the previous observations suggesting that the rate of protein synthesis was increased after 3 hours' incubation (Fig.2.6.1; Fig.2.6.3).
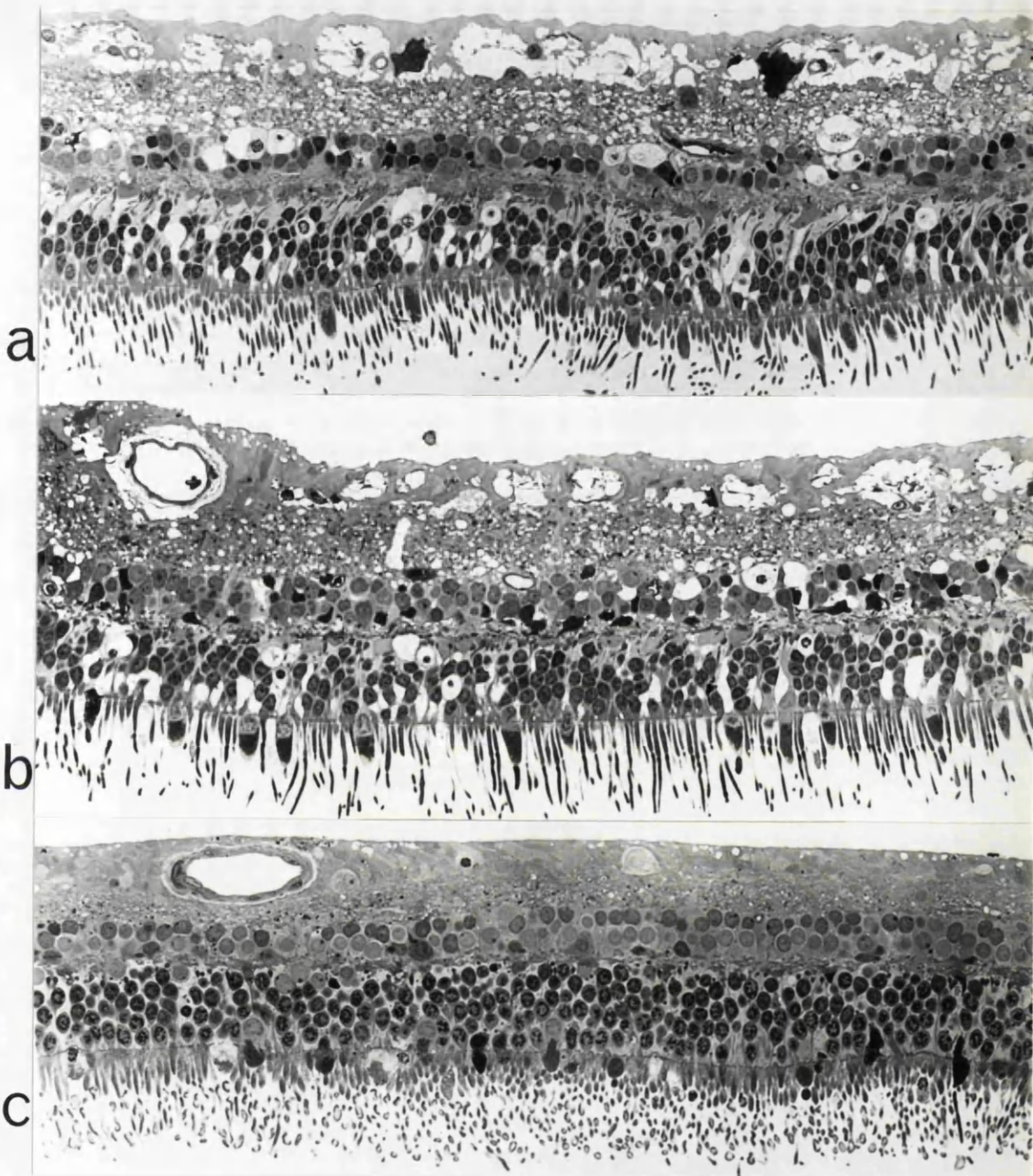
Fig.2.6.4 Light micrographs of an 18-hour-postmortem human retina (Specimen 4, Table 2.1.1, p.89).

a) 18 hrs postmortem; b) 18 hrs postmortem plus 1 hr incubation; c) 18 hrs postmortem plus 24 hrs incubation.

The typical distribution of postmortem vacuolation is illustrated in (a) and still present, although slightly reduced, in (b). However, it is very much reduced in (c), although there remains a slight swelling of retinal glial elements. This is particularly noticeable in the ONL, where swelling of Muller's fibre cytoplasm has resulted in an increase in the spatial separation of photoreceptor nuclei. There is also swelling of some cone cells and, in general, of photoreceptor OS. The bar marker: 100 um.
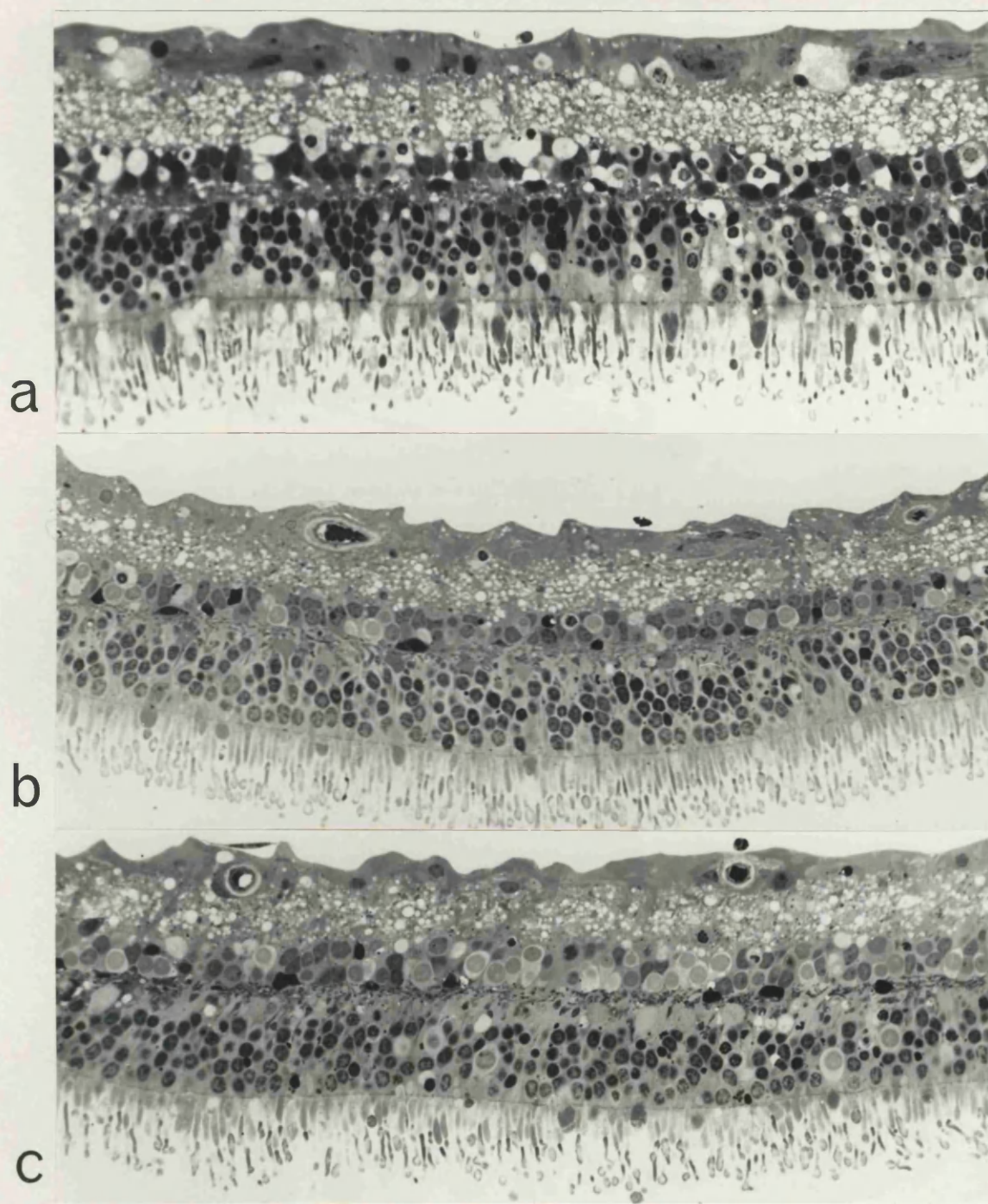
Fig.2.6.5 Light micrographs of a 24-hours-postmortem human
retina (Sample a, Fig.2.6.1).

The retinal explants were incubated in the fortified
medium for a) 1, b) 3 and c) 6 hours, respectively.

Retinal vacuolation was extensive in a), but reduced
in b) and c). Whereas, swelling of OS was also evident in
b) and c). The bar marker: 100 um.

2.6.3.3 Sites of retinal protein synthesis

Pulse/chase incubations with ^{3}H-leucine were performed in human retinae 5 to 36 hours postmortem (Specimens 1, 2, 3, 11 and 17, Table 2.1.1, p.89). Typical patterns of radiolabelling are shown in the auto-radiographs of Fig.2.6.6. Protein synthesis in fresh retinae occurred in both neurones and glia (Fig.2.6.6.a), whilst with longer time postmortem, the label in Muller cells was predominant (Fig.2.6.6.b).

In all the samples subjected to 1-hour-pulse plus 6- to 20-hour-chase incubations, no discrete bands of labelled rod discs could be recognized. Instead, label was diffusely distributed over the OS (cf. Fig.2.6.6).
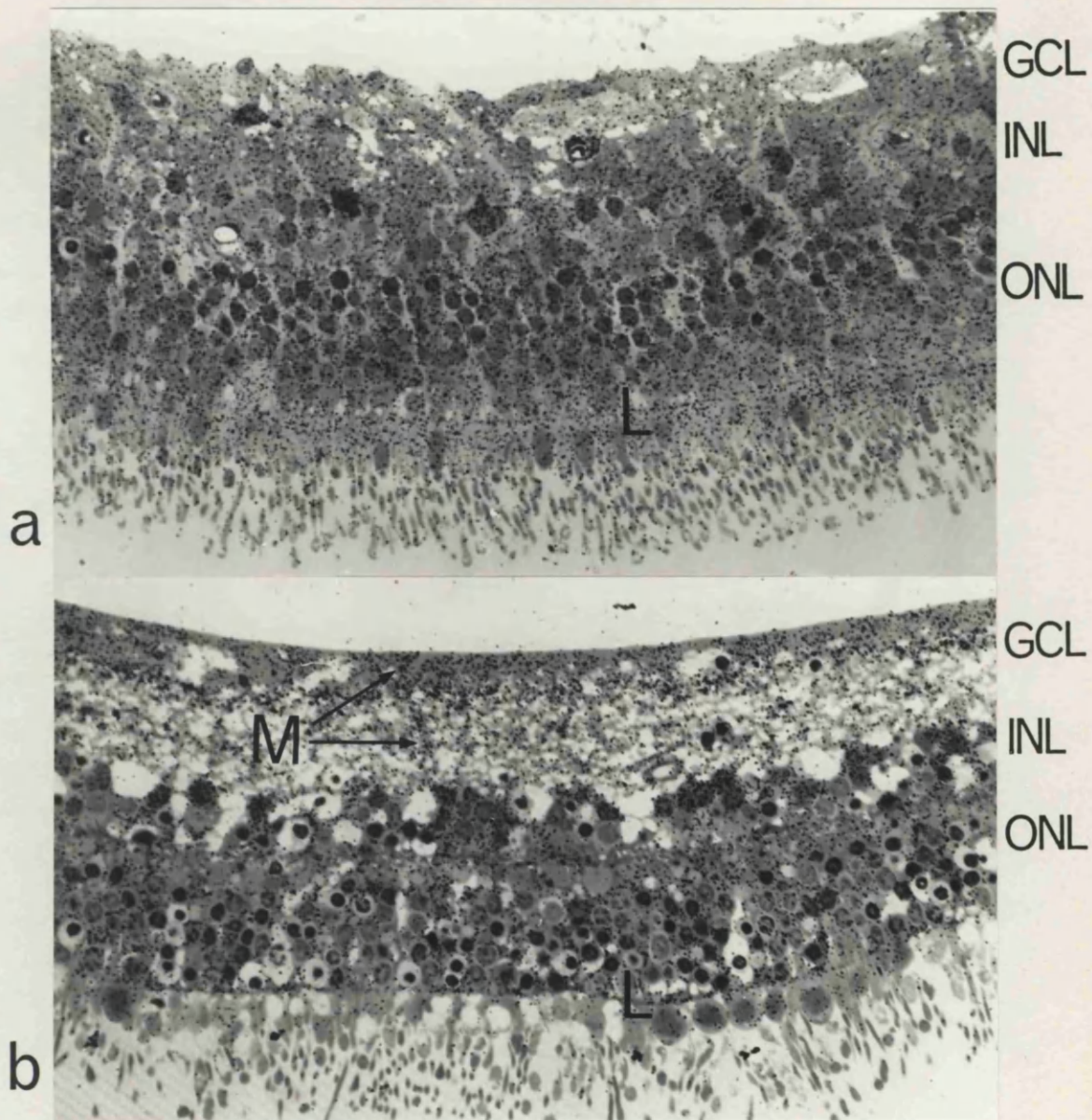
Fig.2.6.6 Autoradiographs of protein synthesis in two human retinae, a) 5 and b) 36 hours postmortem (Specimens 1 and 17 in Table 2.1.1, p.89).

The retinae were incubated with 100 uCi/ml ^{3}H-leucine for 1 hour, followed by chase incubations for 20 hours. Diffuse radio-labelling in both retinae indicates that proteins were synthesized in both neurones and glia. However, in sample (b) glia were predominantly labelled. L - outer limiting membrane, M - Muller cells, ONL - outer nuclear layer, INL - inner nuclear layer, GCL - ganglion cell layer. The bar marker: 100 um.

2.6.4 DISCUSSION

2.6.4.1 Survival of protein synthesis in postmortem human retinae

Structure survives well in the human retina postmortem (cf. 2.1.3.1, p.85), and tissue, explanted 30 hours after death, retains discernible photoreceptors and higher-order neurones for at least 4 months in culture (Kim and Takahashi, 1988). It is of considerable interest and importance, therefore, to ascertain the extent to which both function and vegetative metabolism persist and can be recovered in vitro.

Previously Schmidt and Berson (1980) have found that protein, including opsin, synthesis in the human retina persisted for at least 4 to 4.5 hours after death. The present results show that the processes can survive in the human retina for at least 48 hours postmortem (Fig.2.6.1). This is in line with the preceding observations of survival of structure, phototransduction and amino acid uptake in similarly-aged tissue. Furthermore, commencing after about three hours incubation, the rate of protein synthesis increased during culture in the fortified medium (Figures. 2.6.1 and 2.6.3).

It is likely that the profile of proteins produced is changing with time postmortem since, although initially it was occurring in both neurones and glia, at longer times postmortem Muller cells were predominantly labelled (Fig.2.6.6). This implies that glia survive better than neurones. Protein synthesis involves a series of processes including transcription of messenger RNA, and translation from mRNA to amino acids. It will be of considerable interest to ascertain the range of proteins being produced and to see if the activity is dependent on a pre-existing pool of messenger RNA or if new transcription has been initiated.

Rod disc renewal was abnormal or rod disc membrane stability was impaired as there was only a diffuse incorporation of tritiated leucine into OS proteins (cf. Fig.2.6.6), in spite of the fact that 6- to 20-hour-chase incubations should have been sufficient to allow labelled

discs to form some discrete bands (cf. p.27; Young, 1971; Bok, 1985). Observation of detached retinae as well as evidence from tissue culture has indicated that factor(s) derived from the RPE modulate rod disc formation (Machemer and Kroll, 1971; Anderson et al., 1983; Spoerri et al., 1988; Caffe et al., 1989). The effect of co-culture with the RPE or addition of, for example, likely factors derived from the IPM (Hewitt et al., 1990) must be tested.

2.6.4.2 Morphological changes in incubated retinae

In line with the survival of protein synthesis, concurrent improvement of retinal vacuolation during short-term culture was seen (Fig.2.6.4, Fig.2.6.5). This implies survival of membrane-based active transport systems in retinal cells. Furthermore, the reduction of vacuolation was found to start after about 3 hours of incubation (Fig.2.6.5), in parallel with the increase in the rate of protein synthesis (Fig.2.6.1, Fig.2.6.3). This suggests that a duration of several hours was required for the retina to build up energy and begin to restore its metabolic processes. In contrast to a general reduction in retinal vacuolation, swelling of photoreceptor OS was evident (Fig.2.6.4 and Fig.2.6.5), which might explain why, on the two occasions that the PIII has been measured after 24 hours' incubation, amplitudes were approximately halved (see the text, p.147). This, therefore, provides an additional challenge - to specifically protect and improve OS structure in culture.

2.7 CONCLUDING DISCUSSION AND SUMMARY

This study has utilized morphology, photochemistry, electrophysiology and biochemistry to investigate postmortem survival and the potential for recovery of structure and function in the human retina _in vitro_.

The details have been discussed previously in individual sections. Some general comments and a summary are presented below.

2.7.1 VIABILITY OF CNS NEURONES

Since the retina is part of the CNS and is often described as a model of certain aspects of the brain (e.g. Morgan, 1985), the knowledge obtained from retinal studies is likely to be applicable to other parts of the CNS.

It is well known that complete ischaemia for more than a few minutes will result in brain death, i.e., irreversible dysfunction of the neuronal contents of the intracranial cavity (Korein, 1986). Brain neurones have been thought to be the elements particularly vulnerable to anoxia (Siesjo, 1978). Most criteria for brain death include information from neurological examination and electro-physiological findings such as electro-encephalo-graphy (EEG) (Korein, 1986). The EEG normally represents the spontaneous electrical responses of the cortical neurones which receive afferent impulses from other parts of the brain. In the event of brain death, the EEG responses are absent. However, this might not necessarily mean the loss of excitability of individual neurones.

In the cat and monkey, the EEG, as well as brain energy metabolism and protein synthesis, were largely recovered when circulation was restored after 60 minutes of bloodless brain ischaemia (Hossmann and Kleihues, 1973; Hossmann and Ophoff, 1986; Bodsch et al., 1986). It has also been reported in monkeys that some neurological functions, such as sitting unassisted, can be recovered after up to 24 minutes of bloodless ischaemia (Miller and Myers, 1970). Another remarkable example is that the electro-corticogram (ECG) in the cat brain recovered after a complicated procedure of freezing for up to 203 days,

subsequent thawing and reperfusion (Suda et al., 1966). In addition, several studies on frozen brains many hours postmortem have shown partial recovery of metabolic activities, such as oxygen consumption, a variety of enzyme activities and high-affinity uptake of various neurotransmitters (for a review: Hardy and Dodd, 1983). The retina shows a similar recovery from anoxia. For example, it has been reported that, in dogs who suffered ischaemia for up to 60 minutes, the ERG disappeared within 4 minutes, but manifested again a few minutes after reperfusion, recovering its typical waveform after about 25 minutes (Sobotka and Gebert, 1970).

Thus, it seems likely that the neurones of CNS, including brain, are not exceptionally vulnerable to anoxia. The lack of neurological function and the loss of the spontaneous electrical responses, when brain death (as currently defined) occurs, may be attributed to temporary dysfunction of neuronal communication but not to death of the neurones themselves, especially in the early stages. This concept is supported by the present observations concerning continuation of several aspects of function in the human retina postmortem.

The inability of the brain to recover from anoxic damage is perhaps due to failure of restoration of the microcirculation (Suda et al., 1963; Kogure et al., 1985). In addition, the brain differs from the retina in that it is much thicker than the latter. This will prevent the possible diffusion of some deleterious agents, such as lactic acid and excitatory amino acid neurotransmitters, out of the tissue. This may also prevent in vitro survival by means of perfusion.

2.7.2 THE FEATURES OF POSTMORTEM HUMAN RETINA

The current results demonstrate that at least some aspects of function, albeit declining, can survive in the human retina for 2 to 3 days postmortem. Factors affecting survival are total time postmortem and, more importantly, the time between death and enucleation (p.95; p.154).

The central retina appears more vulnerable as regards morphological deterioration than the periphery, and the

typical changes are vacuolation and cytoplasmic swelling. Postmortem degenerative changes start in the nerve fibre and ganglion cell layers and finally involve photoreceptor IS and OS. In general, rods survive better than other neurones (p.87) but, overall, Muller cells survive the best, as indicated by their longer retainment of protein synthesis (p.184).

Comparable variability in cell survival, or vulnerability to the neurotoxic insults that potentially arise during anoxia or postmortem (p. 95), is found in the brain (Scholz, 1963; Kogure et al., 1985). For example, Purkinje cells are more vulnerable than granular cells, whereas Golgi II cells are among the most resistant. In turn, astrocytes are better preserved than all the neurones. The variation in cell thresholds is likely to relate to differences in cell chemistry (including surface receptors) and metabolism (Myers, 1979; Kogure et al., 1985).

Consistent with structural survival, the fundamental function of phototransduction persists in the human retina for at least 2 days postmortem (cf. p.146). Thus, not only is the ability of opsin to recombine with 11-cis retinal preserved (to the extent that it occurs faster than in freshly-isolated rat tissue; p.118), but also the resultant molecule is photosensitive, with, presumably, light initiating changes in the conformation of opsin that can trigger the transduction cascade. In line with survival of the enzymes involved in the latter, the increase in cGMP concentration, occurring during incubation in the dark, implies that the enzyme systems concerned with cGMP homeostasis are responding to their control mechanisms. The presence of photoresponses after incubation indicates the prior recovery of the photoreceptor dark current. The PIII complex reflects electrical activities of both photoreceptors and Muller cells, and the b-wave implies neurotransmission between photoreceptors and ON-bipolar cells. All these demonstrate that phototransduction and photoreceptor neurotransmission can survive in the human retina for a relatively long period postmortem, and they emphasize the

likely productiveness of more studies along these lines.

Direct investigation of electrical responses of higher-order neurones several hours postmortem has yet to be undertaken. Nevertheless, investigations concerning high-affinity uptake of neuroactive amino acids have shown that these specific, membrane-based transport functions remain in some inner retinal neurones and the Muller cells, for at least 36 hours (p.159). As well as demonstrating the ability of the retina to remove neurotransmitters from the extracellular space, this observation suggests the survival of ATP production in the active cells.

Survival of ATP generation and, potentially, mitochondrial function is also indicated by retention of protein synthesis and the reduction in tissue vacuolation observed in culture (2.6.3, p.176). The results are consistent with the observation of good structural survival of mitochondria (Fig.2.1.3c, p.87).

2.7.3 POSSIBLE IMPROVEMENTS TO METHODS OF RETINAL CULTURE

The present study has shown that incubation in the fortified medium used can improve structure and at least one aspect of metabolism in postmortem human retinae (cf. 2.6.4, p.185). However, it is likely that the current incubation conditions could be improved further, as photoreceptor OS and cone cells were swollen and the photoresponse was reduced after 24 hours' incubation. Thus, more studies are needed. Based on published observations and the results of the present studies, some possible improvements can be proposed.

(1) The retina could be incubated in a collagen-coated mini-cell, with photoreceptor OS placed downwards onto the collagen. Kim and Takahashi (1988) have shown that this system can maintain discernible neurones in human retinal explants cultured for up to 4 months.

(2) It may be desirable to co-culture the retina with RPE. For this purpose, the RPE/choroid complex could

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be from the human globe itself or obtained from, e.g., a fresh bovine eye. This system is likely to produce a more physiological environment for retinal survival. It is of interest that cultured rat RPE cells will phagocytose isolated rod OS (Edwards and Szamier, 1977). Phagocytosis of the old discs in a culture system might facilitate survival of photoreceptor OS. Furthermore, the RPE cells should provide the retina with some essential materials which modulate rod disc formation (Spoerri et al., 1988; Caffe et al., 1989) as well as giving mechanical protection for the OS and IS of photoreceptors.

(3) Provision of IPM may enhance the exchange of materials between the RPE and retina and ensure the presence of factors which facilitate survival of the photoreceptors (Hewitt et al., 1990; p.33).

(4) Vitamin A derivatives are essential for photoreceptor integrity and function (Pepperberg et al., 1989) and opsin synthesis is significantly increased following addition of 11-cis retinal ($\leq 10^{-7}$ M), or all-trans retinol or retinoic acid ($\leq 10^{-8}$ M) to cultured mouse and chick photoreceptor cells (Adler and Politi, 1989). Therefore, supplementation with retinoids may improve photoreceptor function and also provide for regeneration of visual pigment.

2.7.4 CONCLUSION

This study has provided the first evidence that major functions of human retina can persist for a relatively long period postmortem. Moreover, considered alongside existing knowledge, the present findings show it highly probable that some reversal of postmortem deterioration and longer-term maintenance of the isolated retina in explant culture will be possible. Thus, more experimentation and system development must be worthwhile, as this will ultimately provide a powerful preparation in which basic studies can be performed on both normal and diseased human retinae.

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Survival of structure and function in postmortem rat and human retinas: rhodopsin regeneration, cGMP and the ERG

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ABSTRACT

Procedures for regenerating visual pigment and restoring phototransduction have been established with freshly isolated, bleached rat retinas. Phosphatidyl choline liposomes containing a 500 µM mixture of retinal isomers, including the 9-cis and 11-cis forms, were employed and the results compared with dark-adapted retinas, incubated similarly but without retinal. The following were recovered in a 60 min incubation, rhodopsin (plus isorhodopsin) to 91% of the original rhodopsin concentration, 87% of cGMP and 89% of PIII amplitude at saturation. PIII amplitude vs. log intensity curves gave values of n between 0.6 and 1.0 and σ between 85 and 439 quanta/μm^2.

Human retinas, ranging from 18 to 58 hours postmortem and treated as above, also produced photoresponses. Of the 7 retinas studied so far, rhodopsin has been regenerated to 0.1 - 0.35 nmol/mg protein, cGMP to 23.5 - 49.2 pmol/mg protein, and PIII to 20 - 50 µV: in some cases a b-wave was also seen. Values of n varied between 0.6 and 1.0, and σ between 132 and 3700 quanta/μm^2. PIII responses were also seen after retinas, approximately 30 hours postmortem, were incubated for a further 24 hours in fortified medium. After incubation, retinal vacuolation was reduced.

INTRODUCTION

Although there are broad similarities in retinal structure and function amongst vertebrates, wide ranging species differences exist not only as regards regional variation in anatomy and relative cell numbers but also in circuitry, the neurotransmitters employed, extent of vascularization and general metabolic organization. Thus, although much information of relevance to man can and has been obtained from studies of other species, ultimately the human retina must be investigated.

Human retina is available mainly from the discarded portions of eyes donated for corneal grafting. Inevitably, eyes are received by the eye bank many hours postmortem and are then stored for a further period, usually at about 4°C, until corneas are removed. The aim of the present investigation was to see if, following regeneration of rhodopsin with exogenously applied, active isomers of retinal, phototransduction would occur in such tissue. The ERG, cGMP and morphology have been monitored and studies done with the rat retina to establish procedures for rhodopsin regeneration. We have found that phototransduction will occur in human retinas for at least 58 hours postmortem and that vacuolation and other morphological changes are lessened or reversed if tissue is cultured for 24 hours in fortified medium. A preliminary report has appeared (1).

MATERIALS AND METHODS

Female albino Wistar rats, weighing 200 to 300 grams, were killed by cervical dislocation after ether anesthesia. Some had been maintained in total darkness overnight before use and the retinas subsequently dissected out under dim red

light, others were light adapted and the retinas isolated under normal room lighting. Exposure of the latter to ambient light was continued for a further 5 minutes and rhodopsin was then undetectable by difference spectroscopy at 500 nm (see below). The care and use of rats in the present study have adhered to The Guiding Principles in the Care and Use of Animals (DHEW publication, NIH 80-23).

Normal human eyes, donated for corneal grafting, were obtained from the eye bank, Moorfields Eye Hospital. Typically, they were enucleated 5-8 hours after death, and were suspended by thread in a moist, sterile container. At the hospital they were stored at $4^{\circ}C$, but during transport to the hospital and for about 3 hours before laboratory investigation, they were at room temperature. Corneas were removed from between 5 and 18 hours before the studies began. Retinas were dissected in ambient light and cut into approximately 20.0 mm^2 portions for investigation. Samples were predominantly from the periphery and equator and never included the posterior pole.

Earle's medium (composition in mM - NaCl 116, KCl 5.4, $NaHCO_3$ 26.2, NaH_2PO_4 1.0, $MgSO_4$ 0.8, $CaCl_2$ 1.8 and glucose 5.5) was used for all studies on the rat retina and for electrophysiological investigation of human tissue. Eagle's MEM (Sigma M4526), supplemented with 100 μM taurine, 2.0 mM ascorbate, 1.7 μM insulin, 0.125 μM transferrin, 11 mM glucose, 1 mM pyruvate, 10% foetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin and 250 ng/ml amphotericin B and sterilized by ultrafiltration, was used for all other studies on the human retina. Both media were pregassed with a moistened mixture of 95% O_2/5% CO_2 and had a final pH of 7.2 at room

temperature. Incubations were at $37^{\circ}C$ with gentle shaking, and media were gassed throughout. One rat retina or approximately 20 mm^2 of human retina was incubated per ml of incubation medium.

Mixed isomers of retinal (principally 13-cis, 11-cis, 9-cis and all-trans), prepared by photo-isomerization of the all-trans form (2), were encapsulated in phosphatidyl choline liposomes before application to a retina (3,4). Only the 11-cis and 9-cis isomers of retinal form photoreactive complexes with opsin, yielding rhodopsin and isorhodopsin, respectively. The pigments have similar absorption characteristics and both will initiate transduction (5,6): in the following discussion they will be referred to solely as rhodopsin. After extraction and solubilization in 1% octyl glucoside in 0.1 M phosphate buffer, pH 7.4, they were quantified by difference spectroscopy at 500 nm in the presence of 0.1 M hydroxylamine, using an extinction coefficient of 40,000/M/cm (7). For comparative studies, some 11-cis retinal was purified from crude photoisomerate by preparative HPLC, using a Water's μ porasil column (7.8mm X 30cm) (2).

Cyclic GMP was radioimmunoassayed as described by Doshi et al (8) using a kit supplied by Amersham International plc., and protein was measured by the method of Lowry et al (9), as modified by Miller (10).

Photoresponses were recorded with cotton wick and Ag/AgCl electrodes, connected to a high input impedance preamplifier with a band pass of 0 - 200 Hz. A 1.5 mm diameter L.E.D. stimulus, wavelength 550 nm, was used. Duration was 0.2 sec. and maximum intensity 2.0 X 10^4 quanta/μm^2. Isolated retinas were placed ganglion cells downwards onto Whatman no. 1 filter paper. Both surfaces of the
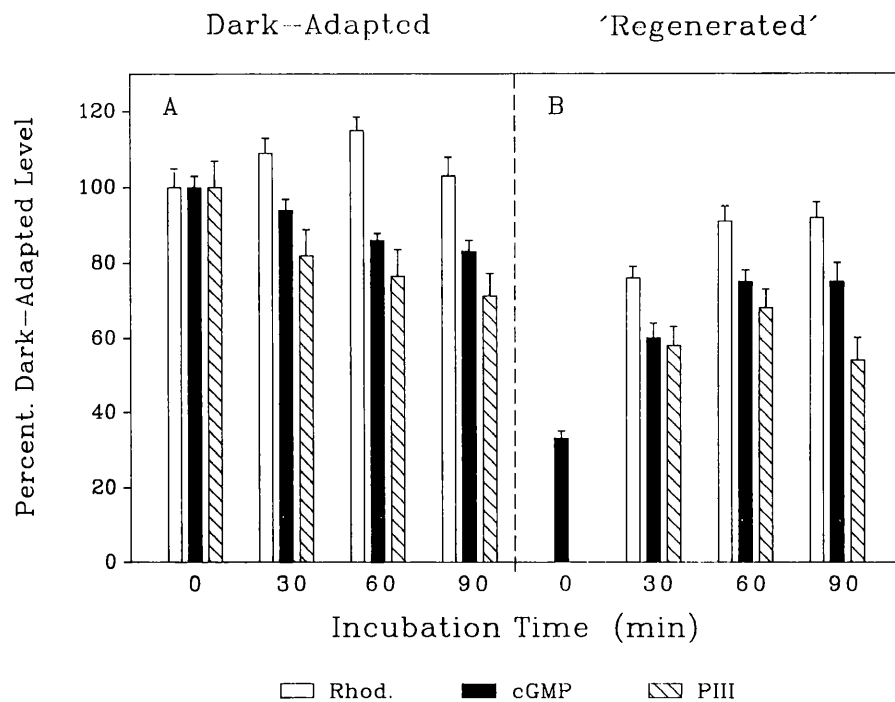
Figure 1 Rhodopsin, cGMP and the photoresponse (PIII) in rat retinas A) normally dark-adapted and B) bleached and then incubated with 500 μM retinal photoisomerate in phosphatidyl choline liposomes. The tissue in A and B was incubated, as described in the text, for the times indicated. Each point represents the mean ± S.E.M. of at least four estimations.

tissue/filter paper preparation were perfused at 7 ml/min with 400 ml of recirculating Earle's medium, containing 2% foetal calf serum. The medium was gassed continuously with a moistened mixture of 95% O_2/5% CO_2 and maintained at 35°C. Human ERGs were recorded intact in order to assess b-wave survival, whereas, for the rat, PIII was isolated by the addition of 10 mM (final concentration) sodium glutamate to the medium: tonicity was maintained by omission of an equivalent amount of sodium chloride. A 3 μV response was taken as threshold and response sensitivity defined as the inverse of the threshold value.

For EM and LM microscopy, portions of peripheral retina from 21 human eyes at varying age of donor and postmortem time were fixed in 2.5% glutaraldehyde, buffered to pH 7.4 with 0.1 M sodium cacodylate-HCl, containing 1.0 mg/ml $CaCl_2$. They were then washed in 7.5% (w/v) sucrose in 0.25 M cacodylate buffer and post-fixed in 2% osmium tetroxide in 0.2 M cacodylate/HCl, pH 7.4. The tissue was then dehydrated through increasing concentrations of ethanol in water and embedded in araldite.

All results are expressed as the mean ± S.E.M. (n), and Student's t-test applied to determine the significance of differences.

RESULTS

Figure 1A compares rhodopsin, cGMP and PIII in dark-adapted rat retinas, freshly-isolated or incubated for up to 90 mins at 37°C in the dark. Rhodopsin levels were unchanged on incubation but
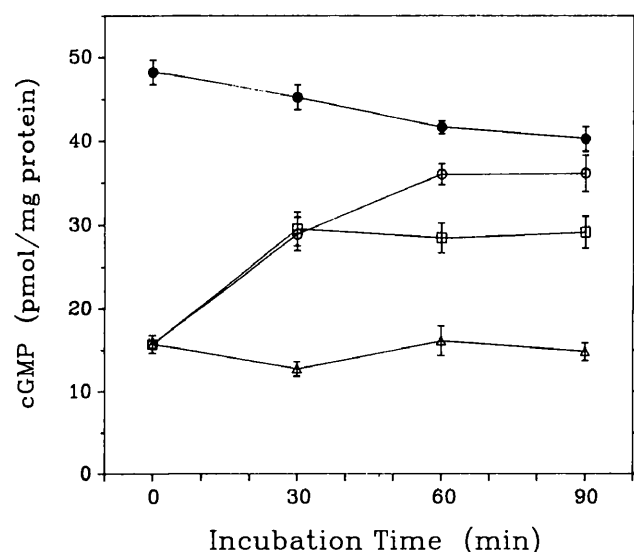
Figure 2 The concentration of cGMP in dark-adapted, bleached and 'regenerated' rat retinas.
●————● normally dark-adapted and incubated in the dark, △ ·········△ bleached and incubated in ambient light, □————□ bleached and incubated in the dark, ○———— ○ bleached and incubated in the dark with 500 µM retinal photoisomerate in phosphatidyl choline liposomes to regenerate rhodopsin. Each point represents the mean ± S.E.M. of at least four estimations.

Figure 3 Rhodopsin regeneration in bleached rat retinas incubated in the dark for 60 mins with various concentrations of 11-cis retinal or a photoisomerate of all-trans retinal.
●————● 11-cis retinal,
■————■ retinal photoisomerate,
□······□ estimated 9-cis and 11-cis retinal content of the preceding retinal photoisomerate.

there was a significant decrease in cGMP (14%, $P < 0.025$, at 60 mins.) and a decline in PIII, the latter falling from an initial value of 579 ± 41 (8) to 443 ± 40 (4) µV after 60 minutes incubation. In contrast, when bleached retinas were incubated in the dark, there was an 88% increase in the endogenous concentration of cGMP from 15.7 to 29.5 pmol/mg protein within the first 30 mins (Figure 2), and the higher level was then maintained for at least another 60 mins. Thus, although in vivo the levels of cGMP vary from 15.7 pmol/mg protein in the light adapted retina to 48.2 pmol/mg protein in the dark (a 3-fold difference), in vitro, under the conditions used here, the values at 60 mins for light and dark-adapted retinas, incubated in the dark, differ by only 1.5 fold (Figure 2). It is
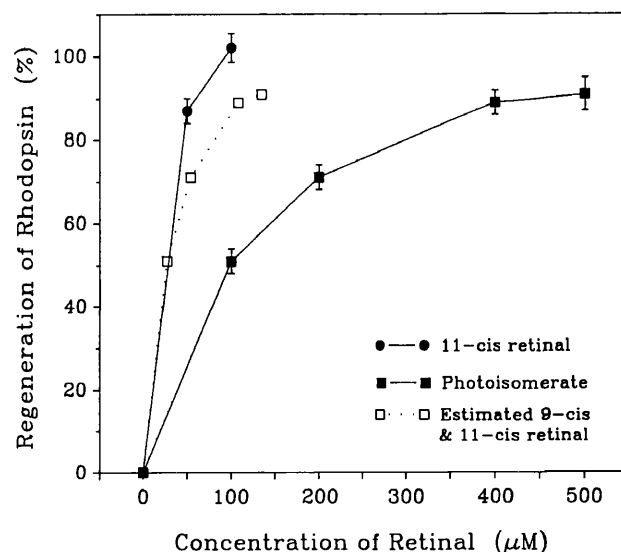
noteworthy that cGMP did not increase in bleached retinas incubated in the light (Figure 2).

Visual pigment regeneration in initially-bleached rat retinas, incubated for one hour with various concentrations of 11-cis retinal or crude photoisomerate, is shown in Figure 3. Although full regeneration was achieved with 100 µM 11-cis retinal, it was not attained with the isomerate at the concentrations studied here. Nevertheless, considering the quantity of 9-cis plus 11-cis retinal present in a photoisomerate (estimated by HPLC as $27 + 2$%, n=6), it can be seen from Table 1 and Figure 1B that, overall, the crude photoisomerate serves well in regenerating pigment. Moreover, our current results suggest equal efficiency in inducing an increase in the concentration of cGMP and the photoresponse. Thus, retinas incubated for 60 mins with 500 µM

Table 1 Visual pigment, cGMP and the photoresponse in rat retinas incubated with 11-cis retinal or a retinal photoisomerate.

Additions	Rhodopsin nmol/mg protein	cGMP pmol/mg protein	PIII μV
None			
Bleached retina:	zero	28.4 ± 1.5 (6)	
Dark-adapted retina:	1.33 ± 0.04 (4)	41.6 ± 0.8 (5)	443 ± 38 (4)
11-Cis retinal			
50 μM:	1.01 ± 0.03 (6)	35.6 ± 2.2 (4)	
100 μM:	1.18 ± 0.04 (5)	36.0 ± 1.9 (4)	407 ± 26 (4)
Retinal photoisomerate			
100 μM:	0.59 ± 0.03 (4)	24.7 ± 1.2 (3)	
200 μM:	0.82 ± 0.03 (4)	37.1 ± 1.9 (3)	
400 μM:	1.03 ± 0.03 (4)	39.8 ± 1.5 (3)	
500 μM:	1.05 ± 0.04 (4)	36.0 ± 1.3 (5)	394 ± 39 (4)
Initial (no incubation) dark-adapted levels:	1.16 ± 0.06 (5)	48.2 ± 1.5 (10)	579 ± 41 (8)

Retinas were incubated in the dark for 60 minutes as described in the text. PIII responses were elicited with the maximum stimulus of 2×10^4 quanta/μm^2. Results are expressed as the mean ± SEM (n).

photoisomerate have 87% and 89% of the levels of cGMP and PIII respectively present in dark-adapted retinas, incubated similarly but without retinal, and tissue incubated with 100 μM 11-cis retinal has 87% (cGMP) and 92% (PIII) of the dark-adapted levels (Figure 1B, Table 1).

The thresholds for PIII responses were as follows: 2.3 ± 0.5 (11) quanta/μm^2 for the normal dark-adapted retina, 2.7 ± 0.5 (6) quanta/μm^2 for the dark-adapted retina, incubated in the dark for 60 mins, and 3.2 ± 0.6 (4) quanta/μm^2 for the retina, regenerated with 500 μM mixed isomers for 60 mins. PIII amplitude vs. log intensity curves, fitted by the equation $V/V_{max} = I^n/(I^n + \sigma^n)$, where σ is the stimulus intensity for a half maximal response, gave values of n for the above retinas of between 0.7 - 0.8, 0.6 - 1.0 and 0.6 - 1.0 respectively, and σ between 53 - 216, 73 - 313 and 85 - 439 quanta/μm^2, respectively.

The human retina

Histopathological studies of post-mortem human retina showed a reasonable state of anatomical preservation of photoreceptor cells (Figure 4). In almost all specimens there was some perinuclear vacuolation, particularly in cone cells, coupled with a degree of swelling of the inner connecting fibres of cones. In contrast damage to rod photoreceptors was rare. The standard of preservation of inner and outer segments of both rods and cones was excellent (Figure 4 b,c,d). Of particular interest was the high degree of preservation of mitochondrial membranes in the photoreceptor inner segments, and of disc membranes in rod outer segments.

In line with these observations, tissue, 18 - 58 hours postmortem and incubated for 60 mins with 100 μM 11-cis retinal or 500 μM photoisomerate, produced photoresponses (Table 2, Figure 5), with PIII varying from 20 - 50 μV: on two occasions a small b-wave of 5 - 10 μV was also seen (Table 2, Figure 5). Values of n varied between 0.6 and 1.0, and σ between 103 and 3130 quanta/μm^2. Table 2
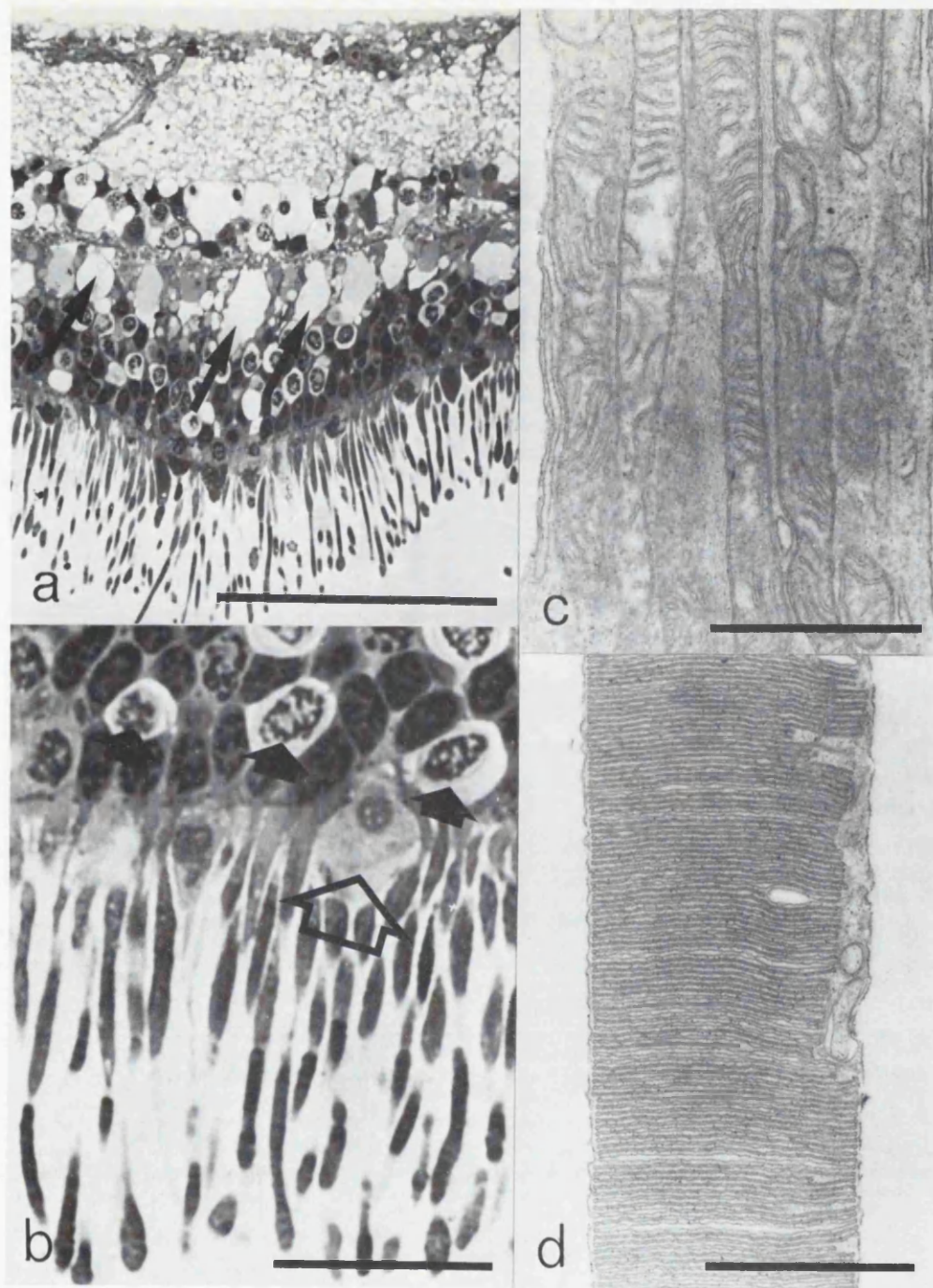
Figure 4 Light (a,b) and electron (c,d) micrographs of a 40 year old human retina, 34 hours postmortem (Specimen 5, Table 2).

a) Vacuolation can be identified in all retinal layers with the exception of the inner and outer segments of the photoreceptor cells. Within the photoreceptor cell layer, some perinuclear vacuolation was noted together with a marked swelling of the inner connecting fibres of some cones (arrowed).

b) High power light micrograph of inner and outer segments of photoreceptor cells showing a good state of anatomical preservation. In some cones, perinuclear vacuolation had occurred (solid arrows) whilst in others nuclei had migrated through the outer limiting membrane (open arrow).

c) Electron micrograph of an inner segment of a rod cell showing a high degree of preservation of mitochondrial membranes.

d) Electron micrograph showing the typical state of preservation of the disc membranes of rod outer segments.

The bar markers are: a) 100 µm; b) 25 µm; c) and d) 1 µm.

Table 2 Recovery of visual pigment and function in postmortem human retinas incubated with 9-cis and 11-cis retinal.

Sample No.	1	2	3	4	5	6	7
Age of donor (years)	18	28	44	66	40	82	72
Hours to enucleation	6	5	8	5	5	5	5
Total hrs postmortem	18	24	29	30	34	43	58
Rhodopsin (nmol/mg protein)	0.34	0.35	NE	0.15	0.27	0.10	NE
cGMP (pmol/mg protein)							
before:	18.5	15.5	NE	16.2	17.0	16.5	NE
after:	49.2	36.4	NE	23.5	31.5	34.0	NE
ERG							
PIII:							
threshold (quanta/μm^2)	53	53	35	85	8.8	504	1340
σ (quanta/μm^2)	313	132	295	1000	132	2530	3700
maximum response (μV)	35	45	50	25	34	20	20
b-wave (μV):	-	-	-	-	10	5	-

Retinas were incubated in the dark for 60 minutes, as described in the text. Samples are tabulated in order of the time elapsing between death and the start of laboratory investigation. Sample 6 was incubated with 100 μM 11-cis retinal and all others with a 500 μM retinal photoisomerate, containing 27% of the 9-cis and 11-cis isomers. Maximum stimulus was 2 x 10^4 quanta/μm^2. NE = not estimated.

shows that, as might be expected, fresher retinas in general exhibited higher rhodopsin and cGMP levels, greater sensitivity and larger amplitudes.

To see if incubation in fortified medium might allow improvement of morphology and function in postmortem tissue, peripheral retina from 4 eyes 18 - 30 hours postmortem was explant cultured for 24 hours. Typical results are shown in Figure 6. All retinas examined more than 10 hours postmortem have demonstrated vacuolation or cytoplasmic swelling in all retinal layers. Changes appeared most marked in the ganglion, inner plexiform and inner nuclear layers (Figure 6a). After 1 hour of incubation (Figure 6b), the general appearance of the retinas was similar, with the possible exception of a slight reduction in the degree of vacuolation in the inner retinal layers. However, a common finding at this stage was the migration of cone nuclei from the outer nuclear layer and passing through the outer limiting membrane. After 24 hours incubation (Figure 6c), marked changes were seen in retinal anatomy, with an almost complete absence of vacuolation or perinuclear swelling. Much of the tissue reorganization is likely to relate to changes in retinal glia, with a degree of swelling of component Mueller's fibres. This was most marked in the outer nuclear layer where the swelling of glial sheaths around the photoreceptor nuclei resulted in a change in internuclear spacing. Some cone cells were also swollen. After 24 hours incubation, the number of cones with displaced nuclei was reduced and photoreceptor inner segment anatomy was still well preserved (Figure 6c). However, swelling was evident in outer segments - perhaps explaining why,
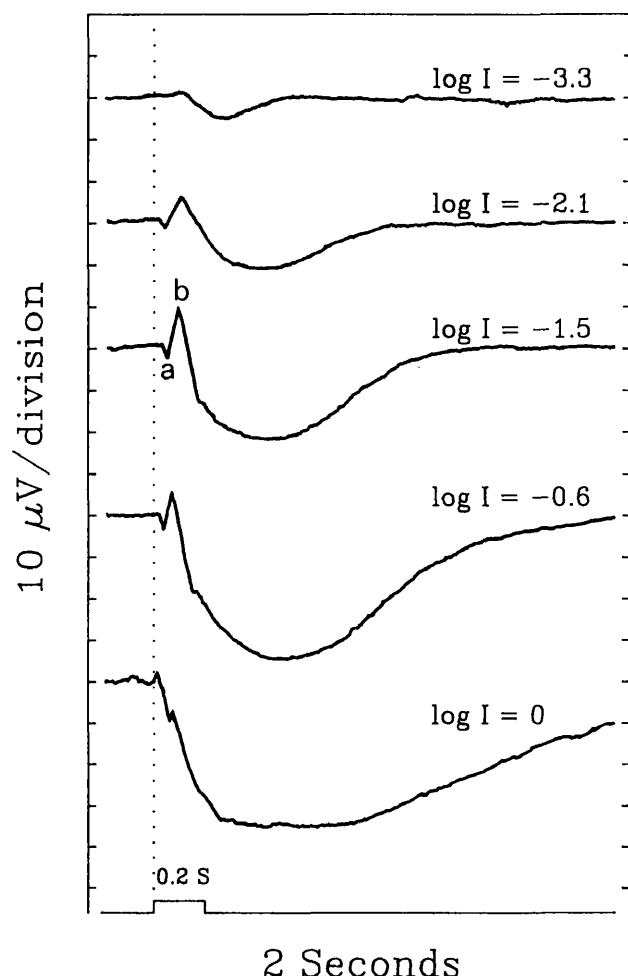
Figure 5 The photoresponse present in a 34 hour postmortem human retina (Specimen 5, Table 2) after regeneration of visual pigment. The sweep duration was 2 seconds and stimulus duration 0.2 seconds. Intensity: $\log I = -3.3$ is equivalent to 8.8 quanta/μm^2 and $\log I = 0$ to 2×10^4 quanta/μm^2. a = a-wave; b = b-wave.

on the two occasions that PIII has been measured, amplitudes were approximately 50% of those seen before incubation.

DISCUSSION

Several studies in which rhodopsin has been regenerated in isolated, bleached tissue have employed 9- or 11-cis retinal, formed by photoisomerization of the all-trans form and then purified by thin layer chromatography or HPLC (e.g. 2,6,7). However, an aim of the present investigation was to develop simple procedures that could be employed readily to investigate available human tissue. We, therefore, began our studies by comparing the efficiencies of a crude photoisomerate and of 11-cis retinal in pigment regeneration. Our results suggest that the former is adequate for investigating general photoreceptor function. However, increasing the amount of photoisomerate employed in regeneration does not give a proportional increase in efficiency (Figure 3), perhaps because inactive isomers are competitively inhibiting pigment regeneration.

Consistent with the higher level of cGMP normally seen in dark- as compared with light-adapted retinas, intracellular cGMP was also increased in 'regenerated' tissue. However, comparison with normal values is complicated by the changes that occurred in 'non-regenerated' levels on incubation. For, whereas the endogenous content in dark-adapted retinas, incubated in the dark, decreased by 6.6 pmol cGMP/mg protein, the content in bleached retinas, similarly incubated, increased by 13.8 pmol/mg protein, thus lessening the light/dark difference.

Although it is well recognized that medium composition can modulate the endogenous level of cGMP in photoreceptor cells (e.g. 11,12), this is not likely to explain the current observation on bleached retinas as the concentration of cGMP in similar tissue, incubated in the light, was stable (Figure 2). Partial recovery of sensitivity, through a process believed to be independent of pigment regeneration, is a common observation when light-exposed, rhodopsin-containing retinas are maintained in the dark (e.g. 13,14). It is possible that the present observation connects with this phenomenon.
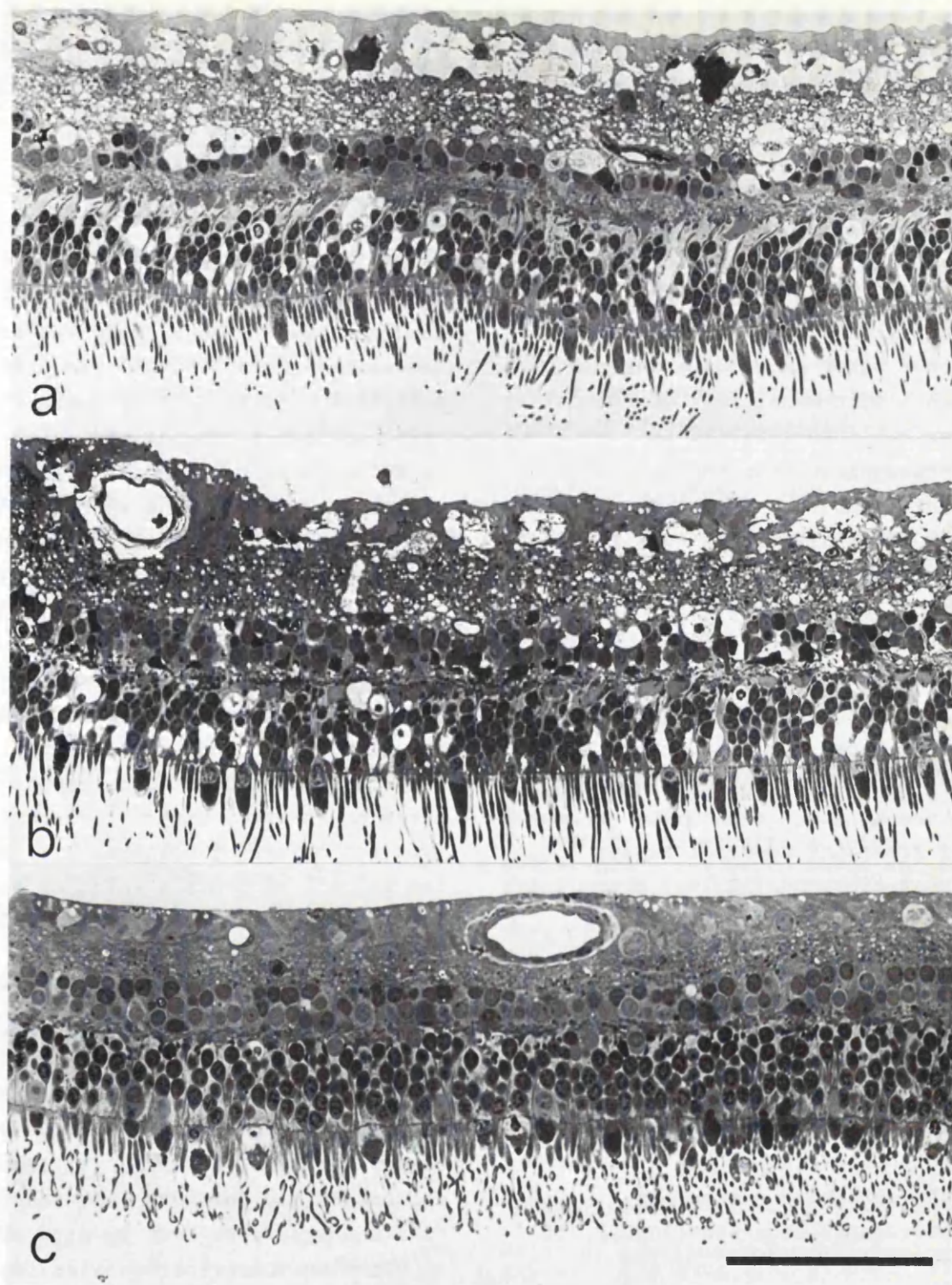
Whatever the factors leading to the

Figure 6 Light micrographs of an 18 year old human retina (Specimen 1, Table 2).

a) 18 hrs postmortem; b) 18 hrs postmortem plus 1 hr incubation; c) 18 hrs postmortem plus 24 hrs incubation.

The typical distribution of postmortem vacuolation is illustrated in (a) and still present, although slightly reduced, in (b). However, it is very much reduced in (c), although there remains a slight swelling of retinal glial elements. This is particularly noticeable in the outer nuclear layer, where swelling of Mueller's fibre cytoplasm has resulted in an increase in the spatial separation of photoreceptor nuclei. There is also swelling of some cone cells and, in general, of photoreceptor outer segments. The bar marker is 100 μm.

final cGMP level reached in rat retinas, 'regenerated' with a retinal photoiso-merate, Figure 1 shows that, in general, there is a close correspondence between the endogenous concentration of cGMP and the height of the photoresponse. The correspondence may be fortuitous since both are only indirect measures of their 'key' components. The central role of cGMP in determining the conductance of photoreceptor outer segments and hence the dark (or light-sensitive) current is now well established (15). It is generally recognised that the bulk of retinal cGMP is present in photoreceptors (16,17) but it is also known that less than 10% is free and contributing to the dark current (15). We know little of the factors determining the equilibrium between free and bound cGMP. The binding constants for cGMP at specific sites on PDE and other outer segment proteins (15) are likely to be important but there is also evidence to suggest that the concen-tration of free cGMP does not always parallel that of bound (18,19). Thus, the possibility that the determinants may change, particularly in tissue several hours postmortem, must be bourne in mind. Similarly, although it is common practice to block photoreceptor synaptic trans-mission with extracellular glutamate or aspartate and then to use the extra-cellular potential gradient as a measure of the light-sensitive current in photoreceptor outer segments (20,21), it may not always serve this purpose. Secondary factors that potentially contribute to the response are K^+ flux through Mueller cells in response to changes in extracellular K^+ (slow PIII; 22,23) and voltage-sensitive changes in inner segment Na^+ conductance (24). Current evidence suggests that, in general, these are proportional to the light sensitive current and do not significantly distort the relationship between potential and conductance changes but, again, the relationship may not always hold, particularly in postmortem tissue.

The human retina

The range of rhodopsin concentrations, found in the regenerated human retinas studied here (Table 2) compares well with the mean value of 280 pmol/mg protein, determined for 12 - 24 hr postmortem tissue by opsin immunoassay (25). As yet, we do not have a value for fresh human retina but applying the same techniques as used here, baboon tissue yields 430 ± 60 (7) pmol rhodopsin/mg protein (26).

Concentrations of cGMP in bleached and 'regenerated' human retinas (Table 2) should be compared with the values of 14 and 36 pmol/mg protein, found previously by us for light and dark-adapted tissue, respectively, processed between one and three hours after surgical removal of the eyes for malignant melanoma (1), and with the 10 pmol/mg protein found by Newsome et al (27) in peripheral tissue from light-exposed eyes up to 20 hours post-mortem. In the present investigations, light-adapted/bleached levels appeared constant for at least 40 hrs postmortem. In contrast, Newsome and colleagues found that levels declined after only 1 day in tissue 'obtained at autopsy'. The difference may relate to the length of time the eyes were left in situ before enucleation. Our findings also suggest that for at least 24 hours, dark-adapted levels can be regained. The apparently normal levels of cGMP, found in the 30 - 43 hour postmortem retinas (samples 4 - 6 in Table 2) are consistent with the longer term survival of outer segment morphology (cf. Figure 4) but contrast with the reduction in assayable

rhodopsin. Immunoassay is needed in order to determine if the latter represents a loss of opsin or of its capacity to combine with the cis isomers of retinal, as occurs when fresh retinas are exposed to high light levels (28).

In the present study, a duration of 60 mins exposure to retinal has been used to regenerate visual pigment in the human retina, a choice based on results obtained with rat and mouse tissue (Figure 1B; 1). However, previous studies have suggested that rat opsin regenerates more slowly than other species (29). It is possible, therefore, that a considerably shorter time would suffice for man and this must be considered.

Although phototransduction has been observed in human retinas up to 58 hours postmortem, the longest time investigated (Table 2), comparison with the PIII responses recovered in the rat retina under similar incubation conditions (20 - 50 µV for man as compared with about 400 µV for rat), and the 1 - 3 log unit threshold increase in the human retina, suggests that survival was poor. Moreover, responses were not improved by 24 hours exposure to the fortified tissue culture medium employed here. The loss of sensitivity is likely to relate to a reduction in viable photoreceptors as well as to functional impairment. Cell loss is irreversible but it might be possible to improve function, and more studies are merited, particularly as Kim and Takahashi (30) have found that human photoreceptor cells and higher order neurones remain discernible in retinas explant cultured for up to 4 months.

It is noteworthy that b-waves were apparent in the ERGs obtained from two of the postmortem human retinas tested (Table 2, Figure 5), since this implies synaptic transmission between photo-

receptors and bipolar cells (23). However, since the b-wave is only an indirect measure of function and loss does not necessarily mean loss of neurotransmission (31), survival of ganglion cells and their responses must also be investigated.

Although we have not succeeded in the current investigation in improving the photoresponse by short-term explant culture of the retina, we did observe a reduction in vacuolation, implying some survival of tissue active transport mechanisms. In our subsequent investigations, we have also observed a doubling in protein synthesis, including opsin. This and other aspects of metabolic recovery will be the subject of our next communication.

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#377-95

PHOTOTRANSDUCTION AND OPSIN SYNTHESIS IN THE HUMAN RETINA POSTMORTEM AND IN EXPLANT CULTURE

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To promote studies on the human retina _in vitro_, we have investigated postmortem survival and recovery of phototransduction and protein synthesis in retinal explants.

Twelve normal human retinas (18 - 58 hours postmortem) were incubated in the dark for 60 minutes at 37°C in Eagle's MEM supplemented with taurine, insulin, transferrin, pyruvate, ascorbate, foetal calf serum, and 100 uM 11-cis retinal or 500 uM photoisomerate of all-trans retinal. Rhodopsin was regenerated from zero to 0.1 - 0.35 nmol/mg protein, and cyclic GMP increased from 15.5 - 18.8 pmol/mg protein to 23.5 - 49.2 pmol/mg protein. Photoresponses were obtained in 8 retinas up to 58 hours postmortem: PIII maximum amplitudes ranged from 20 - 50 uV, and thresholds from 8.8 - 1340 quanta/um^2. In two cases, b-waves were also seen.

Protein synthesis has been investigated in 6 bleached retinas 24 - 48 hours postmortem. Radio-labelling of total protein and specifically of opsin by ^{3}H-leucine was monitored by scintillation spectrometry, SDS-PAGE plus fluorography, and autoradiography of immunoblotted opsin. Incubation in the fortified medium outlined above (but without retinal) for 24 hours resulted in an increase in opsin and total protein synthesis of up to 400 and 500%, respectively. After incubation, retinal vacuolation was reduced.

These observations establish the feasibility of studying function of both normal and abnormal human retinas _in vitro_.

column temperature 80°C, isothermal for 3 min, increased by 4°C/min until 200°C, isothermal for 15 min followed by convection cooling; detector temperature 320°C.

In g.c.-m.s., the g.c. system was essentially the same; eluant from the column was fed directly into the chemical ionization chamber of a Varian MAT 112S mass spectrometer. The reagent gas was methane at 2×10^{-4} Torr with an ionizing voltage of 160 eV, an ionizing current of 0.7 mA and an ion source temperature of 200°C. The molecular ions and characteristic daughter ions of Thr, Ile, Asp and Trp, determined from standards, were monitored with respect to time.

Results and discussion

In *D. viviparus*, high proportions of Thr, Leu, Asx and Trp were found to consist of the D-enantiomers (Table 1). Similar or higher levels of D-Thr, D-Leu and D-Asx were observed in extracts of infected lung. No appreciable amounts of D-enantiomer were found in *N. brasiliensis* or normal lung except for the results of Orn, Lys and Arg which were due to column memory and decomposition of Arg to Orn.

G.c.-m.s. of lungworm and infected lung extracts confirmed that the percentages calculated from the original chromatograms were indeed true because the D- and L-enantiomer peaks were homogeneous.

The D-AA may have arisen from opportunistic infections of parasitized lung. A survey of the literature gave several bacterial peptides containing D-Asp (e.g. mycobacillin, bacitracin), D-Leu (e.g. gramicidin A, polymyxins), D-Trp (e.g. tyrocidins) and D-Thr (phallotoxins) but none which utilized more than one of the four D-AA [5]. Also, antibiotics were given to the parasitized cattle to suppress bacterial infections.

The structures and metabolites of these (oxytetracycline, chloramphenicol, tylosin, penicillin and streptomycin) do not easily yield any of the above D-AA or their metabolites.

The metabolism of nutrients may have been changed as a consequence of infection, so that D-AA was produced (normal lung was not found to have the same D-AA). This is mere speculation and has to be further investigated.

Other reasons for the D-AA levels are excretion/secretion of metabolite(s). Excretion is improbable as the total levels of Thr, Leu, Asx and Trp were not as high as some others in the worm or media. Therefore secretion of metabolite(s) seems the most likely cause for the high levels of D-AA observed.

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Functional recovery in human and rodent retinas *in vitro*

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Although broad similarities are seen in vertebrate retinal structure and function, there is considerable species variation in functional anatomy and the neurotransmitters employed. Thus, although much informatin of relevance to man has been obtained from studies of other species, ultimately the human retina must be investigated.

Structurally intact, although vacuolated, photoreceptors are still present in donated human eyes for at least 3 days *post mortem*; therefore, we have started a study of the potential for recovery of cell function in isolated human and rat retinas.

Most mammalian visual pigments consist of the chromophore, 11-*cis*-retinal, linked to a member of the opsin family of proteins. Phototransduction begins when the 11-*cis* isomer is photoisomerized to the all-*trans* form. For function to occur in a bleached, isolated retina, it is necessary for the visual pigments, e.g. rhodopsin in rods, to be regenerated.

Mixed isomers of retinal, prepared by photoisomerization of the all-*trans* form as described by Bridges & Alvarez (1982), were encapsulated in phosphatidylcholine liposomes before application to the retina (Perlman *et al.*, 1982). The contribution of the 13-*cis*, 11-*cis*, 9-*cis* and all-*trans* isomers, as measured by h.p.l.c. (Bridges & Alvarez, 1982), was approximately 15%, 17%, 7% and 60%, respectively. Only the 11-*cis* and 9-*cis* isomers of retinal form photoreactive complexes with opsin, yielding rhodopsin and isorhodopsin,

respectively: these were quantified spectrophotometrically in the presence of hydroxylamine (Hubbard *et al.*, 1971). Initial studies with freshly isolated rat and mouse retinas have established the feasibility of regenerating rhodopsin and restoring phototransduction *in vitro*.

High intracellular levels of cyclic GMP maintain open ionic channels in the plasma membrane of dark-adapted rod outer limbs (Pugh & Cobbs, 1986). Activation of a cyclic GMP phosphodiesterase in the transduction cascade, induced by light, lowers cyclic GMP and the channels close. The cell then hyperpolarizes and transmitter release ceases. Figure 1 shows that when rhodopsin is regenerated in isolated, bleached mouse retinas (taken initially from light-adapted animals and then exposed at room temperature to room light *in vitro* for 5 min), the concentration of cyclic GMP also increases, thus simulating dark-adaptation. Both rhodopsin and cyclic GMP reach levels only slightly below those typical of the dark-adapted retina. Similar results have been obtained with rat tissue and electroretinography has shown a 72% recovery of the PIII (photoreceptor) response with variable recovery of the b-wave.

We have also quantified cyclic GMP in human retinas from eyes removed surgically because of the presence of malignant melanoma; processing began between 1 and 3 h post-enucleation. Some eyes were maintained in the dark and the retinas dissected and processed under dim red light; these are referred to as dark-adapted. Three light-exposed and five dark-adapted retinas were investigated and the mean values for the individual tissues pooled to give overall means ($\pm$ s.e.m.) of 4.54 ± 0.69 pmol of cyclic GMP/5 mm disc light-exposed retina and 12.0 ± 0.81 pmol of cyclic
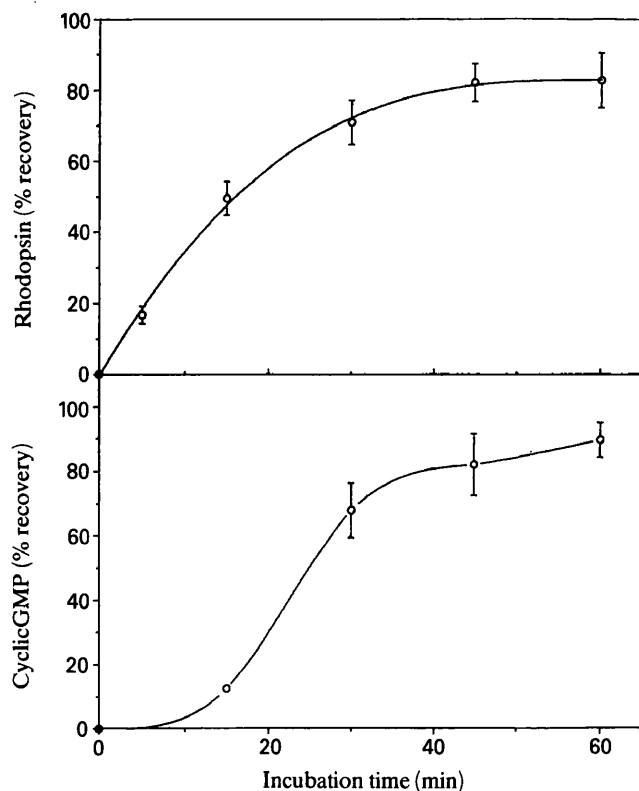
Fig. 1. *Rhodopsin regeneration and cyclic GMP recovery in the mouse retina in vitro*

Light-exposed retinas were incubated in Earle's medium containing 0.05 mg of photoisomerized retinal/ml in phosphatidylcholine liposomes. Rhodopsin was assayed spectrophotometrically and cyclic GMP by radioimmunoassay (Doshi *et al.*, 1985). Each point represents the mean of five separate estimations ± s.e.m.

GMP/5 mm disc dark-adapted retina. The 5 mm discs of retina from five eyes had an overall mean protein content of $332 \pm 37 \mu g$.

When a 30 h post-mortem human retina was incubated for 1 h at 37°C with 0.025 mg of photoisomerized retinal/ml in a liposome suspension, rhodopsin was regenerated to 0.27 ± 0.04 ($n = 3$) nmol/mg of protein and cyclic GMP rose from 2.28 ($n = 2$) to 6.03 ± 1.39 ($n = 3$) pmol/5 mm disc of retina. Moreover, electroretinography, performed on a 34 h post-mortem retina, treated similarly, gave a photoresponse (PIII) of 35 μV. Thus our observations establish the feasibility of studying function in human photoreceptor cells several hours after death. We shall now see if activity can be improved by explant culture in fortified media.

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A pathway linking NAD degradation and hypoxanthine formation associated with the tegument of the rat tapeworm *Hymenolepis diminuta*

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Using the rat tapeworm *Hymenolepis diminuta* as a model it has been clearly shown that the tegumental membranes determine a number of discrete processes which are essential for the survival and development of gut cestodes. These include: the transport of nutrients into and the removal of degradation products from the organism; the location of a series of enzymes linked to the catabolism of nucleic acids; the regulation of Ca^{2+}/ATP-ase and the release of free Ca^{2+} from calcareous corpuscles (Podesta & Mettrick, 1975; Gamble & Pappas, 1981; Hipkiss *et al.*, 1987).

In a recent study we have reported the utilization of exogenous NAD by the tegumental membrane which does not appear to involve a typical ADP-ribose (ADPR) transferase system. The incorporation of tritiated material from the NAD precursor into an acid-insoluble fraction (Alsharif *et al.*, 1988) was not inhibited by 3-acetamidobenzamide which is a potent competitive inhibitor of all nuclear ADPR trans-

Abbreviation used: ADPR, ADP-ribose.

ferases. The system was, however, sensitive to the calmodulin antagonist trifluoperazine (Stelazine) (Alsharif *et al.*, 1988). Extending these investigations, we report that NAD degradation occurs through a sequence of intermediates before incorporation as a purine-containing metabolite which is insoluble in trichloroacetic acid.

Adult *H. diminuta* (21 days) were used throughout. Tegument membranes were prepared following disruption with Triton X-100 and differential centrifugation (Siddiqui & Podesta, 1985; Hipkiss *et al.*, 1987). The activity was measured using a variety of radiolabelled substrates including NAD, adenosine and hypoxanthine (Nishizuka *et al.*, 1967). Radioactivity was measured in the acid-insoluble fraction following incubation and centrifugation.

The results in Fig. 1 show that the system is capable of incorporating radioactivity from NAD, adenosine and hypoxanthine into an acid-insoluble material. After a 5 h incubation period the rate of tritium uptake was significantly faster when hypoxanthine was the substrate. Over a 24 h period (data not shown), radioactivity levels showed little difference relative to the substrates used, indicating the absence of glycohydrolase activity which is commonly associated with ADPR-transferase. When 3 mM-mercuric acetate was included in the assays no incorporation of tritiated material was detected. This result suggests that the system is enzymic;