CELLULAR NEUROBIOLOGY: A SERIES

EDITED BY SERGEY FEDOROFF

THE NODE OF RANVIER
Edited by JOY C. ZAGOREN AND SERGEY FEDOROFF

THE RETINA: A MODEL FOR CELL BIOLOGY STUDIES, PART I
Edited by RUBEN ADLER AND DEBORA FARBER

THE RETINA: A MODEL FOR CELL BIOLOGY STUDIES, PART II
Edited by RUBEN ADLER AND DEBORA FARBER

THE RETINA
A Model for Cell Biology Studies
Part II

EDITED BY

RUBEN ADLER

The Michael M. Wynn Center for the Study of Retinal Degenerations
The Wilmer Ophthalmological Institute
The Johns Hopkins University
School of Medicine
Baltimore, Maryland

DEBORA FARBER

Jules Stein Eye Institute
UCLA School of Medicine
Los Angeles, California

1986

ACADEMIC PRESS, INC.
Harcourt Brace Jovanovich, Publishers
Orlando San Diego New York Austin
London Montreal Sydney Tokyo Toronto

ACADEMIC PRESS, INC.
Orlando, Florida 32887

United Kingdom Edition published by
ACADEMIC PRESS INC. (LONDON) LTD.
24–28 Oval Road, London NW1 7DX

Library of Congress Cataloging in Publication Data
(Revised for pt. 2)
Main entry under title:

The Retina : a model for cell biology studies.

Includes bibliographies indexes.
1. Retina–Cytology. I. Adler, Ruben. II. Farber, Debora. [DNLM: 1. Retina–Cytology. 2. Retina–physiology. WW 270 R43804]
QP479.R468 1986 599'.01823 85-23005
ISBN 0–12–044276–0 (pt. 2 : alk. paper)

PRINTED IN THE UNITED STATES OF AMERICA

86 87 88 89 9 8 7 6 5 4 3 2 1

This book is dedicated to
Alfred J. (''Chris'') Coulombre,
a pioneer of the field of retinal cell biology
and an outstanding human being

CONTRIBUTORS

Numbers in parentheses indicate the pages on which the authors' contributions begin.

Virginia M. Clark (129), Jules Stein Eye Institute, UCLA School of Medicine, Los Angeles, California 90024

Bert M. Glaser (215), Center for Vitreoretinal Research, The Wilmer Ophthalmological Institute, The Johns Hopkins University, School of Medicine, Baltimore, Maryland 21205

Bernice Grafstein (275), Department of Physiology, Cornell University Medical College, New York, New York 10021

Karl Herrup (245), Department of Human Genetics, Yale University School of Medicine, New Haven, Connecticut 06510

A. Tyl Hewitt (169), The Michael M. Wynn Center for the Study of Retinal Degenerations, The Wilmer Ophthalmological Institute, The Johns Hopkins University, School of Medicine, Baltimore, Maryland 21205

P. Michael Iuvone (1), Department of Pharmacology, Emory University School of Medicine, Atlanta, Georgia 30322

Robert W. Massof (73), Laboratory of Physiological Optics, The Wilmer Ophthalmological Institute, The Johns Hopkins University, School of Medicine, Baltimore, Maryland 21205

Jerry Silver (245), Department of Developmental Genetics and Anatomy, Case Western Reserve University School of Medicine, Cleveland, Ohio 44106

PREFACE

Retinal Cell Biology: Past, Present, and Future

It is difficult at times to define the field of cell biology. One need only attend the annual meeting of the American Society for Cell Biology to experience the enormous breadth of this field. Nonetheless, many would define cell biology in a very general way as a combination of microscopic anatomy, biochemistry, physiology, and pathology.

The confluence of these disciplines into a unifying field did not really occur until the 1950s, but we can trace the ancestry of the discipline to Matthias Schleiden, Theodor Schwann, and Rudolf Virchow, the botanist, histologist, and pathologist, respectively, who are generally credited with the development of the cell theory in 1839. Somewhat later in that century the study of retinal cells was initiated. Ultimately, Max Schultze (1867) set forth the duplicity theory for photoreceptor function through his observation that cones are the receptors for bright light and color and that rods function in dim light. Subsequently Franz Boll (1877) observed that the photochemical event underlying light detection was the bleaching of "visual purple" now known as rhodopsin. He also suggested that a carotenoid might be a precursor for this photopigment. His contemporary, Willy Kühne (1878) was the first to solubilize photopigments in bile salts thereby demonstrating their hydrophobic nature. Santiago Ramon y Cajal (1894) soon thereafter modified methods shared by fellow Nobel laureate Camillo Golgi and displayed the neuronal network of the retina in such breathtaking detail that his work serves as the standard even to this day. Therefore, by the turn of the century, the study of retinal cells by representatives of the separate disciplines of microscopic anatomy, biochemistry, and physiology was well established.

Retinal research was graced by investigators of equivalent intuition and creativity during the first half of this century. The disciplines of electrophysiology and biochemistry, exemplified by Ragnar Granit, Haldane Hartline, and George Wald, produced insights into retinal electrical activity, cell interactions, and photochemistry worthy of the Nobel Prize so appropriately bestowed upon them. The contributions of these gifted scientists and their contemporaries drew

into the field a new generation of capable scientists armed with new and powerful techniques. Fritiof Sjostrand, Eichi Yamada, Adolph Cohen, Brian Boycott, John Dowling, and many others began the systematic examination of retinal cells by electron microscopy. The application of tissue autoradiographic technique to retinal studies by Richard Sidman, Richard Young, and Bernard Droz in the early to mid 1960s added a dynamic component to microscopy. It was possible for the first time to mark retinal birth dates and to study the metabolism of individual cell types.

Particularly suitable for autoradiographic studies were the photoreceptors with their highly compartmentalized structure and their prodigious biosynthesis and assembly of membranes. By virtue of this technique, an entirely new role was discovered for retinal pigment epithelial cells, the phagocytosis and photoreceptor outer segment membranes. Furthermore, a new research strategy began to emerge. Whereas scientists of the past had adhered rather strictly to their chosen disciplines each of which drew upon a rather limited constellation of methods, the new approach was to combine the methods of multiple disciplines in order to address problems of ever-increasing complexity. This is the essence of cell biology and, with the advent of this approach, retinal cell biology came into full bloom.

A subject that benefited greatly from this philosophy was the study of membrane biosynthesis in photoreceptors. Michael Hall and I combined radiobiochemical and autoradiographic methods in the study of rhodopsin biosynthesis and assembly into outer segment disk membranes, and David Papermaster and Barbara Schneider applied high-resolution immunocytochemical methods to plot membrane trafficking from sites of synthesis to sites of assembly within the cell.

Another area that was aided tremendously by this combined approach was that of retinal neurotransmitter studies. Relatively little progress was made on this subject until the introduction of autoradiographic and immunocytochemical techniques. Autoradiographic analysis of high-affinity-uptake sites for neurotransmitters as initiated by Berndt Ehinger and Dominic Lam combined with immunocytochemical localization of enzymes involved in neurotransmitter synthesis have been very illuminating as have studies introduced by Harvey Karten and Nicholas Brecha on the localization of the myriad of peptides that are now known to exist either as neurotransmitters or neuromodulators in the amacrine cells of the retina. Immunocytochemical techniques are now employed by a growing number of retinal cell biologists and, when applied at high resolution in particular, will add significantly to our understanding of the molecular division of labor among retinal organelles.

Research during the 1970s has featured another group of exciting topics. The subject of photoreceptor transduction has been hotly debated among proponents who favor the hypothesis that calcium serves as the internal messenger during

signal amplification, as first proposed by William Hagins and Shuko Yoshikami, and the adherents of cyclic guanosine monophosphate, as initially suggested from the work of Mark Bitensky and collaborators. Whatever the outcome, it appears likely that the photoreceptors will be the first sensory cells in which the mystery of transduction is solved. Furthermore, the contributions of Debora Farber and Richard Lolley and their collaborators have shown how cyclic nucleotide metabolism, when perturbed, can lead to photoreceptor degeneration of the type observed in some animal models for retinitis pigmentosa.

Rhythmic phenomena in the retina, pioneered by Matthew LaVail through his observations on cyclic outer segment disk shedding, have also been a subject of intense investigation resulting in the discovery by several laboratories that these activities are controlled intraocularly rather than by remote tissues such as the pineal gland. The development of an *in vitro* preparation by Joseph Besharse for the study of the molecular basis of rhythmic activity has already moved us significantly toward an understanding of these processes. Superimposed upon these metabolic studies has been exciting progress in our understanding of the role of extracellular adhesion and matrix molecules in retinal development and trophic interactions. The recent work of Ruben Adler and associates now provides us with the capability to study the differentiation of photoreceptors in culture.

Another informational leap is now occurring in retinal cell biology, spawned by the technology that has impacted so favorably on other tissues as well, namely, recombinant DNA research. Some of the most exciting observations are so new that they have not yet appeared in print. The first application of recombinant DNA methods to retinal research has involved the amino acid sequencing of proteins involved in phototransduction. The sequencing of bovine rhodopsin by conventional methods was initiated by Paul Hargrave and his colleagues shortly after this protein was purified by Joram Heller in 1968.

About 15 years of intensive work were required before the primary structure of this hydrophobic membrane protein was solved. Utilizing this important sequence information, the fact that the vertebrate photopigments are well conserved, and rapid nucleotide sequencing developed independently by the laboratories of Frederick Sanger and Walter Gilbert, Jeremy Nathans and David Hogness have sequenced human rhodopsin and all of the human cone opsins in the space of just a few years. Additionally, the primary sequences of all of the subunits of the G protein and light-activated cyclic nucleotide phosphodiesterase thought to be involved in phototransduction will probably be solved in several laboratories by the time that these books (Parts I and II) appear in print. With this information in hand we will have, for the first time, the opportunity to determine the molecular basis of several forms of inherited photoreceptor degeneration that are thought to involve one or more of these proteins.

We are currently experiencing a fast-moving and exciting period for the retinal

cell biologist and the opportunities appear almost limitless at this time. It is therefore most fitting that a two-part series entitled *The Retina: A Model for Cell Biology Studies* should be made available to those who already have an interest in this subject and to others who may be inspired to join us. We hope that you find the subject as inviting and rewarding as we have during the past two decades.

DEAN BOK

NEUROTRANSMITTERS AND NEUROMODULATORS IN THE RETINA: REGULATION, INTERACTIONS, AND CELLULAR EFFECTS

P. MICHAEL IUVONE

Department of Pharmacology
Emory University School of Medicine
Atlanta, Georgia

I. Introduction

Intercellular communication in the nervous system of vertebrates is believed to occur primarily by chemical messengers. These messengers are usually categorized as neurotransmitters or neuromodulators. A "neurotransmitter" is generally considered to be a substance that is synthesized and released from a specialized part of a neuron, usually a presynaptic axon terminal. This substance diffuses across a relatively narrow synaptic cleft where it binds to a specific receptor located on the postsynaptic membrane. Binding of the neurotransmitter to the receptor elicits an electrophysiological response that typically has a rapid onset and a short duration (milliseconds). The action of the neurotransmitter is terminated by enzymatic degradation of the substance or by its reuptake into the presynaptic terminal. Identification of a substance as a neurotransmitter at a particular synapse requires a demonstration of (1) its synthesis and release, (2) its postsynaptic action, and (3) a mechanism for the rapid termination of its postsynaptic action. In addition, the pharmacological demonstration of synaptic mimicry and antagonism by selective receptor agonists and antagonists, respectively, provides powerful approaches to the identification of the transmitter. The term "neuromodulator" is used to describe endogeneous substances that are released from neurons, or possibly from glia, that produce effects on neural cells that are distinct from the rapid, short duration effects of neurotransmitters. For example, a neuromodulator might (1) act postsynaptically to alter the electrophysiological response to a neurotransmitter while having no electrophysiological effect of its own, (2) act presynaptically to modulate the synthesis, release, or reuptake of a neurotransmitter, or (3) produce long-term changes in cellular metabolism. A neuromodulator could act locally on adjacent cells or diffuse through the extracellular space to act on distant neurons in the tissue. A neuromodulator might also affect its cell of origin, modulating that cell's response to other transmitters.

The retina of vertebrates has many characteristics that make it a useful model for studying regulatory mechanisms involved in intercellular communication by neurotransmitters and neuromodulators. The retina contains several anatomically and functionally distinct classes of neurons that are arranged in distinctive layers. The tissue is accessable to microdissection. It can be dissociated into suspensions of single viable cells for the purpose of disrupting synaptic interactions, and the various cell types can be isolated from the suspensions to study their biochemical and electrophysiological properties. The retina is a relatively thin neural tissue which in many species receives its nutrients by diffusion through the extracellular fluid. As a consequence, the retina is accessible for *in vitro* as well as *in vivo* experimentation and can be readily manipulated chemically and pharmacologically by the administration of substances into the vitreous fluid *in vivo* or into incubation media. Perhaps most important is the ability to reproducibly

stimulate or inhibit various neurons in the tissue by a physiological stimulus, light.

The purpose of this article is to review the current evidence for the role of putative neurotransmitters at particular synapses in the retina. In addition, pre- and postsynaptic regulatory mechanisms and the interactions of neurotransmitter–neuromodulatory systems will be discussed. The article will conclude with a discussion of some novel cellular effects of putative neurotransmitters and neuromodulators that might influence the directions of future research into interneuronal communication and neural integration.

II. Putative Neurotransmitters and Neuromodulators of Retinal Neurons

The vertebrate retina is composed of six major classes of neurons: photoreceptors, horizontal cells, bipolar cells, amacrine cells, interplexiform cells, and ganglion cells (Fig. 1). Each of these classes has subtypes defined by their morphology, by physiology, and in some cases by their neurotransmitter. For many of the neuronal subtypes, there exists substantial evidence that a particular substance acts as a neurotransmitter. However, for other neuronal subtypes, and

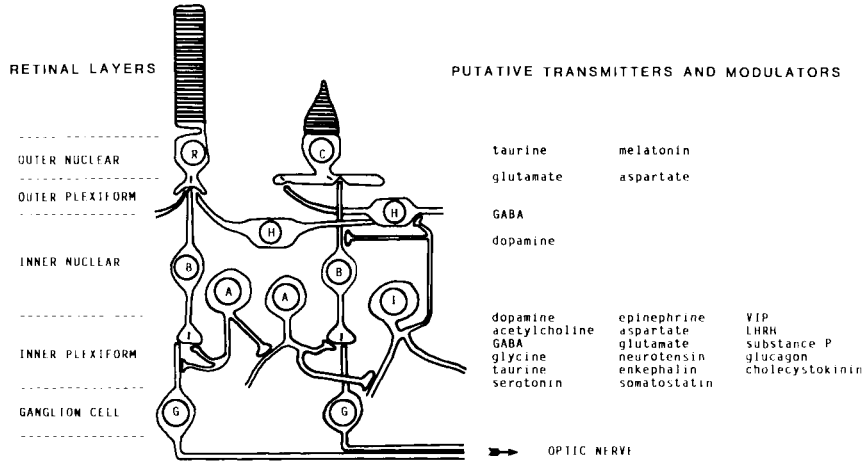


FIG. 1. Cellular organization and putative neurotransmitters and neuromodulators of the retina. The letters in the cell nuclei are abbreviations of the cell type as follows: A, amacrine cell; B, bipolar cell; C, cone photoreceptor cell; G, ganglion cell; H, horizontal cell; I, interplexiform cell; R, rod photoreceptor cell. The vertical position of the listed transmitters and modulators corresponds approximately to the retinal layer in which they are localized.

in some cases entire classes of neurons, no good transmitter candidate has been identified. Furthermore, there is considerable species variation in the cellular localization of putative retinal neurotransmitters that may be significant.

In addition to the six classes of neurons, the retina contains two other types of cells that play important roles in retinal function. Müller cells are the main type of glial cell in the retina. They are large cells that traverse most retinal layers, extending from the innermost border of the retina to the photoreceptor cells. The retinal pigment epithelial cells form a sheet of cells that surround the photoreceptor outer segments. They provide several support functions to retinal neurons, particularly to photoreceptor cells (see Clark, this volume, for a discussion of pigment epithelial cell function).

A. Photoreceptors

Photoreceptor cells in the vertebrate retina are subclassified into rods and cones on the basis of morphology and the visual pigments they contain. These cells make synapses with horizontal cells and bipolar cells in the outer plexiform layers (Fig. 1). In darkness, photoreceptors are partially depolarized (Hagins *et al.*, 1970) and the photoreceptor neurotransmitter(s) are apparently released continuously onto the bipolar and horizontal cells. Light hyperpolarizes the photoreceptors and, by suppressing transmitter secretion from their terminals (Dowling and Ripps, 1973; Ripps *et al.*, 1976; Schacher *et al.*, 1974, 1976), causes the hyperpolarization of horizontal cells and one subtype of biopolar cell, referred to as the hyperpolarizing bipolar or OFF bipolar cell. The other subtype of bipolar cell, the depolarizing bipolar or ON bipolar cell, depolarizes in response to light. Thus, a candidate for a photoreceptor neurotransmitter should be released from the photoreceptor in darkness and should depolarize horizontal and OFF bipolar cells while hyperpolarizing ON bipolar cells.

1. Aspartate and Glutamate

Several lines of evidence suggest that the acidic amino acids glutamate and aspartate may be neurotransmitters released from photoreceptors. For nearly three decades, it has been known that high concentrations of L-asparate eliminated the response of inner retinal neurons to light without blocking the hyperpolarizing response of the photoreceptor itself (Furukawa and Hanawa, 1955). L-Asparate and L-glutamate were subsequently found to mimic the natural transmitter on horizontal cells (Cervetto and MacNichol, 1972; Murakami *et al.*, 1972; Wu and Dowling, 1978) and bipolar cells (Murakami *et al.*, 1975). In these studies, which employed intact retinas, high concentrations (0.3–10 m*M*) of the amino acids were required to produce the effects. However, much lower

concentrations (20–50 μM) of L-glutamate are required to depolarize horizontal cells that are isolated from dissociated retinas (Lasater *et al.*, 1982; Ishida *et al.*, 1983). The difference in sensitivity to glutamate in the two preparations is probably due to the efficient removal of the amino acid from the extracellular space by a high-affinity transport mechanism. This hypothesis is supported by the observation that D-aspartate, an inhibitor of glutamate uptake, potentiates the effects of L-glutamate on horizontal cells of intact retina (Ishida and Fain, 1981).

The postsynaptic action of many neurotransmitters is terminated by removal of the transmitter from the synaptic cleft by high-affinity transport into the presynaptic terminal. Localization of high-affinity transport sites is often used to identify cells that utilize a particular putative transmitter. Thomas and Redburn (1978) reported the high-affinity uptake of L-glutamate and L-aspartate into a subcellar fraction from bovine retina that was enriched with synaptosomes from photoreceptors. Autoradiographic localization of high-affinity L-[^{3}H]glutamate and L-[^{3}H]aspartate uptake also supports the presence of such transport sites in photoreceptors. Marc and Lam (1981) demonstrated a clear accumulation of L-[^{3}H]glutamate and L-[^{3}H]aspartate into rods of goldfish retina. The uptake of L-glutamate into rods was approximately 30 times greater than that for L-aspartate indicating a high degree of selectivity. L-[^{3}H]Glutamate and L-[^{3}H]aspartate were also accumulated by cones, but the uptake mechanism for cones did not appear to discriminate between glutamate and aspartate. In most mammalian species examined, Bruun and Ehinger (1974) found uptake of [^{3}H]glutamate primarily into retinal glia; however, in retinas of cat, cynomolgus monkeys, and humans, there was also some accumulation of label in rods and cell bodies of the outer nuclear layer. Labeled D-aspartate was also accumulated by cones and rods of goldfish (Marc and Lam, 1981) and by cones in guinea pig and rabbit retinas (Ehinger, 1981). In human retina, [^{3}H]aspartate was accumulated by rods to a greater extent than by cones (Lam and Hollyfield, 1981). Identification of high-affinity transport sites in photoreceptors does not prove a transmitter role for these acid amino acids but does fulfill one of the criteria, a mechanism for terminating the action of synaptically released transmitter.

The localization of cells that utilize glutamate or aspartate as their neurotransmitter has been hampered by the lack of a specific histochemical marker for these substances. However, Altschuler *et al.* (1982) and Mosinger (1983) recently reported on the use of immunohistochemistry to localize aspartate aminotransferase (EC 2.6.1.1), the enzyme that catalyzes the reversible conversions of oxaloacetate to aspartate and of α-ketoglutarate to glutamate. A high concentration of aspartate aminotransferase-like immunoreactivity was localized to cones in guinea pig retina, while in the retina of cynomolgus monkeys aspartate aminotransferase-like immunoreactivity was found in rod photoreceptors. Future use of this immunohistochemical methodology may enhance attempts to localize retinal neurons that are rich in the glutamate- and aspartate-snythesizing enzyme.

As described above, the photoreceptor transmitter should be released in the dark, and its release should be suppressed by light. Neal *et al.* (1979) found that flashes of light (3 Hz) inhibited the release of aspartate from the rabbit retina, suggesting release from photoreceptor cells. However, the cellular source of the released aspartate was not identified. This is of some importance because aspartate-accumulating cells and cells with aspartate aminotransferase-like immunoreactivity have also been observed in the inner retina (see Section II,D). In order to identify putative neurotransmitters released from photoreceptor cells, Miller and Schwartz (1983) studied the K^+-evoked release of substances from toad (*Bufo marinus*) retinas that had been previously incubated with neurotoxins to destroy most inner retinal neurons while sparing the photoreceptors. The release of aspartate, glutamate, *N*-acetylhistidine, putrescine, and cadaverine was stimulated by K^+ in the presence of Ca^{2+}. The ability of these compounds to stimulate the release from isolated horizontal cells of γ-aminobutyric acid (GABA), a putative neurotransmitter of a subclass of horizontal cell, was examined to evaluate their possible postsynaptic action. Aspartate and glutamate, but not *N*-acetylhistidine, purtrescine, and cadaverine, stimulated the release of [^{3}H]GABA. These results support the involvement of one or both of these amino acids in synapses between photoreceptors and horizontal cells. The lack of effect of *N*-acetylhistidine, putrescine, and cadaverine on GABA release suggest that these compounds have no postsynaptic effect or that they are involved in synapses between photoreceptors and bipolar and/or horizontal cells that do not secrete GABA.

Additional evidence for aspartate and/or glutamate being a neurotransmitter in photoreceptors comes from pharmacological and electrophysiological studies. Wu and Dowling (1978) reported that L-glutamate and L-aspartate depolarized H_1 horizontal cells of carp retina. H_1 horizontal cells, also referred to as luminosity (L) cells, receive synaptic input from cones and respond to light with hyperpolarizing responses (Kaneko, 1970). Glutamate- and aspartate-induced depolarization was observed when cobalt was present to block synaptic transmission. Aspartate was considerably more potent than glutamate at depolarizing the H_1 horizontal cells, and the effect of aspartate was blocked by α-aminoadipate, a relatively selective antagonist of aspartate receptors. α-Aminoadipate also blocked the response to the endogenous cone neurotransmitter. In contrast, L-glutamic acid diethylester, a relatively selective antagonist at some glutamate receptors, had no effect on the H_1 cell membrane potential alone, nor did it block the effect of aspartate. From these observations, Wu and Dowling (1978) concluded that aspartate or an aspartate-like substance may be the transmitter at the synapse of cones onto H_1 horizontal cells.

In a series of experiments, Slaughter and Miller (1981, 1983a,b) have characterized the transmitter receptors mediating cone responses in the second-order

neurons of the mudpuppy (*Necturus maculosus*). Kainic acid, a glutamate analog, mimicked the photoreceptor transmitter at all three types of second-order neurons: horizontal, ON bipolar, and OFF bipolar cells. *N*-Methylaspartate was much less potent than kainate on the second-order neurons but did have effects in the inner retina (see Section II,D). The receptors on the horizontal and OFF bipolar cells are apparently distinct from those on the ON bipolar cells, although both types of receptors are sensitive to glutamate and kainic acid. *cis*-2,3-Piperidinedicarboxylic acid (PDA), an excitatory amino acid antagonist, blocks the cone input to sign-conserving second-order neurons (Slaughter and Miller, 1983a). PDA hyperpolarized horizontal and OFF bipolar cells, mimicking the effect of light, and blocked the depolarizing effect of kainic acid on the horizontal cell. In contrast, PDA had no effect on the depolarizing response of ON bipolar cells. Another glutamate analog, 2-amino-4-phosphonobutyric acid (APB), also distinguishes between the receptors on the ON bipolar cells and those on the OFF bipolar and horizontal cells (Slaughter and Miller, 1981). APB mimicked the effect of the photoreceptor transmitter on the ON bipolar cell, but had no effect on OFF bipolar cells, horizontal cells, or photoreceptors. In contrast to the effects of the glutamate analog APB, the corresponding analog of aspartate, 2-amino-3-phosphonopropionic acid, had no effect on the ON bipolar cells. In addition, α-aminoadipate, an aspartate antagonist, did not hyperpolarize horizontal cells or attenuate their response to light. These results have led the authors to suggest that glutamate rather than aspartate is the photoreceptor transmitter in mudpuppy and that the receptors for the photoreceptor transmitter on the sign-conserving and sign-inverting second-order neurons are different.

To summarize, aspartate and glutamate fulfill many of the requirements for neurotransmitter candidates at photoreceptor synapses onto second-order retinal neurons in some vertebrate species: (1) high concentrations of aspartate aminotransferase, an aspartate- and glutamate-synthesizing enzyme, appear to be localized in photoreceptor cells; (2) aspartate and glutamate have been shown to be released from toad photoreceptors by high concentrations of K^+, and the release of aspartate from the rabbit retina is decreased by light, a response that would be expected for a photoreceptor transmitter; (3) high-affinity uptake of aspartate and glutamate into rods and cones of several species has been demonstrated, suggesting a mechanism for terminating the action of the released transmitter; (4) postsynaptic receptors for these compounds have been identified on second-order retinal neurons, and the postsynaptic response of the three types of neurons to glutamate and aspartate are the same as those to the natural photoreceptor transmitter; and (5) relatively selective antagonists of glutamate or aspartate receptors have been reported to block responses to the natural transmitter. Thus, aspartate and/or glutamate are probably neurotransmitters in some types of photoreceptors.

2. Taurine

Taurine (2-aminoethanesulfonic acid) appears to play a role in photoreceptor function, possibly as a neuromodulator, but the exact function of this amino acid is not clear. Taurine is the most abundant amino acid in the vertebrate retina (Kubicék and Dolenék, 1958; Pasantes-Morales *et al.*, 1972; Cohen *et al.*, 1973; Starr, 1973). By microdissection of the frog retina, Kennedy and Voaden (1974) determined that 80% of the total retinal taurine content was localized to the photoreceptor layer. The photoreceptor layer also contains the highest concentration of taurine in retinas of cat, rat, rabbit, monkey, pigeon, and chicken (Orr *et al.*, 1976; Kennedy *et al.*, 1977; Voaden *et al.*, 1977). Iodoacetate-induced lesions of rat retinal photoreceptors significantly decreased the levels of taurine and the activities of the taurine-synthesizing enzymes cysteine oxidase and cysteine sulfinate decarboxylase (Salceda *et al.*, 1979), suggesting that photoreceptors in this species contain the synthetic machinery to make at least some of the taurine in these cells. However, treatment of rats with guanidinoethyl sulfonate (GES), an inhibitor of taurine transport, decreased the content of retinal taurine, presumably by blocking taurine transport across the blood–retinal barrier (Lake, 1981). This study and the observation that systemically administered taurine was accumulated by photoreceptors of several species (Lake *et al.*, 1977; Pourcho, 1977; Salceda *et al.*, 1979) indicate that a portion of the taurine in the photoreceptors is derived from systemically synthesized or nutritional sources. The nutritional source of taurine is particularly evident in the cat, which apparently does not synthesize taurine at appreciable rates (Schmidt *et al.*, 1976).

The importance of taurine in photoreceptor function is suggested by observations on the effects of taurine-free diets or treatment with GES. Feeding cats a taurine-free casein diet resulted in disruption of photoreceptor outer segment structure, photoreceptor cell death, and eventually complete loss of the electroretinogram (ERG) (Hayes *et al.*, 1975; Schmidt *et al.*, 1976). Similarly, treating rats with GES, the taurine transport inhibitor, decreased retinal taurine and caused photoreceptor degeneration and a progressive loss in the ERG (Lake, 1982). These findings suggest that taurine is involved in the maintenance of photoreceptor function and viability, but the mechanism of action of taurine in this regard is unknown.

3. Melatonin and Serotonin

Melatonin (5-methoxy-*N*-acetyltryptamine) may also function as neuromodulator that is secreted from photoreceptor cells. Melatonin-like immunoreactivity has been detected in the retinas of amphibians, birds, and mammals, including man (Bubenick *et al.*, 1976; Pang *et al.*, 1977, 1982; Hamm and

Menaker, 1980; Reiter *et al.*, 1981, 1983; Vivien-Roels *et al.*, 1981; Osol and Schwartz, 1984). In chicken retina, the identification of the melatonin-like immunoreactivity as authentic melatonin was confirmed by high-pressure liquid chromatography with electrochemical detection (HPLC-EC) (Reppert and Sagar, 1983). Identification of melatonin in human retina was confirmed by gas chromatography–mass spectrometry (Leino *et al.*, 1984).

Melatonin-like immunoreactivity in the retina persists after removal of the pineal gland (Hamm and Menaker, 1980; Yu *et al.*, 1981; Reiter *et al.*, 1983), the major source of circulating melatonin. The enzymes for the synthesis of melatonin from serotonin, serotonin *N*-acetyltransferase (EC 2.3.1.5) and hydroxyindole *O*-methyltransferase (EC 2.1.1.4), have been identified in retina (Baker *et al.*, 1965; Quay, 1965; Cardinali and Rosner, 1971a; Binkley *et al.*, 1979; Hamm and Menaker, 1980; Miller *et al.*, 1980; Iuvone and Besharse, 1983). Furthermore, the synthesis of radiolabeled melatonin from radiolabeled serotonin in retina has been demonstrated (Cardinali and Rosner, 1971b; Gern and Ralph, 1979). These studies strongly suggest that melatonin is synthesized in retina. Melatonin-like immunoreactivity has been localized primarily in the outer nuclear layer (Bubenik *et al.*, 1974, 1976; Vivien-Roels *et al.*, 1981). In addition, hydroxyindole *O*-methyltransferase-like immunoreactivity has recently been observed in the outer nuclear layer and photoreceptor inner segments (Wiechmann *et al.*, 1985). Based on the homology between retinal and pineal photoreceptors in certain lower vertebrates, Gern (1980) has suggested that photoreceptors are the site of retinal melatonin biosynthesis. The release of melatonin-like immunoreactivity from guinea pig retina has been reported (Yu *et al.*, 1982), and, consistent with the hypothesis that melatonin is released from photoreceptors, release was greater in darkness than in light. Pharmacological application of melatonin produces effects on rods, cones, and inner retinal neurons (see Sections IV,A, V,A, and V,B), supporting a neuromodulatory role for melatonin in the retina.

Recently, Redburn (1984) reported that photoreceptor terminals of hooded rats accumulate [^{3}H]serotonin. Accumulation of [^{3}H]serotonin was greater in light than in darkness. The rat retina synthesized [^{3}H]serotonin from [^{3}H]tryptophan and metabolized it to 5-[^{3}H]hydroxyindoleacetic acid. Synthesized [^{3}H]serotonin was released by depolarizing concentrations of K^+. The endogenous concentration of serotonin in retina increased when rats were exposed to constant light for 24 hr and decreased during a 24-hr period of darkness. The decreased accumulation of [^{3}H]serotonin and decreased levels of the endogenous amine in darkness suggest that the system is physiologically modulated. Thus, serotonin may be a candidate for a photoreceptor transmitter. Alternatively, serotonin may be present in the photoreceptors of the hooded rat as a precursor to melatonin. This question awaits further biochemical and electrophysiological analysis.

B. Horizontal Cells

Horizontal cells are involved in the lateral processing of information in the outer plexiform layer (Fig. 1). In certain fish, amphibians, and birds, GABA may be the transmitter of one of the subclasses of horizontal cells. Immunohistochemical studies on goldfish retina indicate that H_1 horizontal cells contain the GABA-synthesizing enzyme glutamic acid decarboxylase (GAD, EC 4.1.1.15), and the synthesis of GABA in isolated cone horizontal cells from dissociated goldfish retina has been demonstrated (Lam, 1975; Lam *et al.*, 1979). H_1 horizontal cells of goldfish accumulate [^{3}H]GABA by high-affinity transport, and evidence has been presented for the stimulation of GABA release from these cells in darkness (Marc *et al.*, 1978) and by glutamate (Yazulla and Kleinschmidt, 1983). Interestingly, the glutamate-induced release of GABA from horizontal cells is not Ca^{2+} dependent, but rather appears to occur by a Na^+-dependent carrier-mediated process (Yazulla and Kleinschmidt, 1983). This novel release mechanism may be related to the apparent absence of synaptic vesicles in the presynaptic processes of these horizontal cells (Stell, 1967; Witkovsky and Dowling, 1969).

Horizontal cells of frog, toad, catfish, pigeons, and chickens also accumulate [^{3}H]GABA (Marshall and Voaden, 1974; Voaden *et al.*, 1974; Lam *et al.*, 1978; Hollyfield *et al.*, 1979; Schwartz, 1982). Using a preparation of isolated horizontal cells prepared by lesioning other retinal neurons with veratridine and an antibody directed against photoreceptor outer segments, Schwartz (1982) demonstrated that horizontal cells from toad (*B. marinus*) retina release [^{3}H]GABA in response to stimulation with glutamate. This release, like that from goldfish horizontal cells (Yazulla and Kleinschmidt, 1983), is Ca^{2+} independent, and requires sodium or lithium. Glutamate also stimulates the release of [^{3}H]GABA from intact chicken retina in a Ca^{2+}-independent, Na^+-dependent manner (Tapia and Arias, 1982; Morán and Pasantes-Morales, 1983). While not directly demonstrated, it was suggested that the horizontal cells may be the site of the Ca^{2+}-independent GABA release in this species (Morán and Pasantes-Morales, 1983).

Electrophysiological studies support a role for GABA in horizontal cell-mediated feedback onto cones of fish retina. Application of GABA to isolated carp retina hyperpolarized cones and H_1 horizontal cells and reduced the response to light (Wu and Dowling, 1980). The GABA antagonist bicuculline depolarized H_1 horizontal cells and increased the magnitude of the light response. The effect of GABA on the horizontal cell was cobalt sensitive, suggesting that it was mediated by an action of GABA on photoreceptors. Inotophoretic application of GABA to the dendrites of bipolar cells in the carp retina produced a small depolarization of ON bipolar cells and a small hyperpolarization of OFF bipolar cells (Kondo and Toyoda, 1983). Similar to the effects on H_1 cells, these re-

sponses were concluded to be due to an action of GABA on photoreceptors because the responses were blocked by cobalt.

GABA does not appear to be the neurotransmitter of horizontal cells in mammalian retinas. Horizontal cells of the mammalian species examined to date neither accumulate [^{3}H]GABA nor possess GAD activity (e.g., Bruun and Ehinger, 1974; Brandon *et al.*, 1979; Pourcho, 1980). Thus, GABA may be the transmitter of some horizontal cells (H_1) in retinas of fish and possibly in those of other nonmammalian vertebrates. However, the identification of the transmitter in other types of horizontal cells in mammalian and nonmammalian retinas awaits further investigation.

Stone *et al.* (1983) reported that a subpopulation of horizontal cells in cat retina were stained with an antiserum against α-melanocyte-stimulating hormone (α -MSH). However, the antigen in the horizontal cells was not biochemically characterized and did not cross-react with other anti-α-MSH antisera. These results suggest that a compound similar, but not identical, in structure to α-MSH may be present in some horizontal cells. Recently, cholecystokinin-like immunoreactivity has been observed in cat horizontal cells, but this immunoreactivity has also not been biochemically characterized (Thier and Bolz, 1985).

C. Bipolar Cells

Bipolar cells transmit visual information from the outer plexiform layer to the inner plexiform layer (Fig. 1). They receive input from photoreceptors, horizontal cells, and interplexiform cells and have synaptic connections in the inner plexiform layer with dendrites of ganglion cells and processes of amacrine cells. The transmitter(s) of the bipolar cells is not known, but electrophysiological studies suggest that it is excitatory rather than inhibitory (Naka, 1977; Dacheux *et al.*, 1979). Ikeda and Sheardown (1982a) have suggested that in the cat retina aspartate is the transmitter released from both ON and OFF bipolar cells onto "sustained" ganglion cells (cells that respond to a stationary spot at the center of the visual field with a sustained increase in firing). Visually evoked excitation of all sustained ganglion cells recorded was mimicked by microiontophoretic application of aspartate, and the effects of applied aspartate and of photic stimulation were both antagonized by 2-amino-5-phosphonovalerate (APV), an antagonist of the excitatory amino acid receptor that is preferentially stimulated by *N*-methyl-D-aspartate. Application of L-glutamate did not have consistent stimulatory effects. The influence of L-aspartate and APV occurred in both ON-center and OFF-center sustained ganglion cells. However, neither L-aspartate nor APV influenced the activity of "transient" ganglion cells, leading to the suggestion that excitation of the transient ganglion cells involves another neurotransmitter. A subsequent study

(Ikeda and Sheardown, 1982b) suggested that some of those cells receive their excitatory input from acetylcholine-secreting amacrine cells (see Section II,D).

Slaughter and Miller (1983b,c) have suggested that an excitatory amino acid, possibly L-glutamate, is the neurotransmitter of both OFF and ON bipolar cells of the mudpuppy retina. *N*-Methylaspartate and kainic acid both depolarized amacrine and ganglion cells. PDA, the excitatory amino acid antagonist that blocked the photoreceptor input to horizontal cells and OFF bipolar cells but not that to the ON bipolar cell (Slaughter and Miller, 1981), was found to block light-evoked transmission from ON bipolar cells to amacrine and ganglion cells. This result suggested that ON bipolar cells secrete an excitatory amino acid transmitter. A low dose of PDA appeared to have a greater ability to block the OFF ganglion cell than the OFF bipolar, suggesting that PDA may also inhibit the effects of the transmitter released from the OFF bipolar cell. However, as the authors point out, this effect of PDA could be explained by other mechanisms. PDA does not distinguish between aspartate-preferring and glutamate-preferring receptors. Application of selective antagonists of the *N*-methylaspartate receptor, α-aminoadipate and α-aminosuberate, had no effect on light-evoked transmission to most ganglion cells but did block some sustained OFF ganglion cells (Slaughter and Miller, 1983b). These results suggest that most of the receptors involved in bipolar cell transmission are not of the aspartate-preferring type and, because they are stimulated by kainic acid, may be of the glutamate-preferring variety.

Both the studies of Ikeda and Sheardown (1982a) and those of Slaughter and Miller (1983b,c) suggest that aspartate and/or glutamate may be bipolar cell neurotransmitters. Furthermore, Mosinger (1983) recently reported the localization of aspartate aminotransferase-like immunoreactivity in bipolar cell terminals of the cynomolgus monkey retina. However, it should be emphasized that there is little histological evidence for aspartate- or glutamate-accumulating bipolar cells. Autoradiographic studies have indicated that these amino acids are accumulated by photoreceptors, glia, and some amacrine cells, but not by bipolar cells (Brunn and Ehinger, 1974; Ehinger, 1981; Marc and Lam, 1981). Thus, related compounds that can act on the same receptors as aspartate and glutamate may be the natural bipolar cell neurotransmitter(s).

D. Amacrine Cells

Amacrine cells are retinal neurons that have cell bodies in the proximal portion of the inner nuclear layer and processes that extend into the inner plexiform layer (Fig. 1). Amacrine cells have synaptic connections with bipolar cell terminals, other amacrine cells, interplexiform cells, and ganglion cells. In many cases, these connections represent reciprocal synapses. Morphological and physiologi-

cal studies indicate a wide variety of subtypes of amacrine cells, and the apparent heterogeneity of this class of retinal neurons is supported by the large number of putative neurotransmitters and neuromodulators that have been localized in amacrine cells.

1. Acetylcholine

There is considerable biochemical, morphological, and physiological evidence that acetylcholine (ACh) is the neurotransmitter of a subclass of amacrine cells. ACh and its synthetic enzyme choline acetyltransferase (EC 2.3.1.6) are present in significant amounts in the retinas of most vertebrate species, as is the ACh-degrading enzyme acetylcholinesterase (EC 3.1.1.7) (reviewed by Graham, 1974). Microdissection studies have demonstrated that choline acetyltransferase activity is highest in the inner plexiform layer (Graham, 1974; Ross and McDougal, 1976). Histochemical localization of acetylcholinesterase indicates that this enzyme is also concentrated in the inner plexiform and inner nuclear layers, principally within amacrine cells (Nicholas and Koelle, 1968). Retina possesses a high-affinity choline uptake mechanism (Neil and Gilroy, 1975; Baughman and Bader, 1977; Vivas and Drujan, 1980) which has been localized by autoradiography to photoreceptors, to cells in the inner nuclear and ganglion cell layers, and to processes in the inner plexiform layer (Baughman and Bader, 1977; Masland and Mills, 1979). The choline taken up by photoreceptor cells was converted principally to choline-containing phospholipids, while that taken up into neurons of the inner nuclear and ganglion cell layers was converted primarily to ACh. The heavily labeled cells in the inner nuclear layer appear to be amacrine cells (Masland and Mills, 1979), while those in the ganglion cell layer may be a subclass of amacrines referred to by Cajal (1893) as displaced amacrine cells (Baughman and Bader, 1977; Hayden *et al.*, 1980). Comparison of the choline-labeled amacrine and displaced amacrine cells with Golgi-stained cells suggests that ACh is the transmitter of an amacrine subtype referred to as "starburst" amacrine cells (Famiglietti, 1983). Similar cells in the inner plexiform and ganglion cell layers that stain with antisera directed against choline acetyltransferase have also been identified (Eckenstein *et al.*, 1983).

Both evoked release of ACh and the presence of functional cholinergic receptors in retina provide support for a neurotransmitter role for ACh in amacrine cells. Photic stimulation increased the biosynthesis and release of retinal ACh (Masland and Livingston, 1976; Massey and Neal, 1979; Vivas and Drujan, 1980), suggesting that the activity of cholinergic amacrine cells is modulated by light. Depolarizing concentrations of K^+ also evoked ACh release from retinal amacrine cells (Baughman and Bader, 1977), and both light- and K^+-evoked release were Ca^{2+} dependent, suggesting a specific synaptic event. Ligand-binding studies have provided evidence for the presence in retina of both mus-

carinic and nicotinic cholinergic receptors (Yazulla and Schmidt, 1977; Hruska *et al.*, 1978; Moreno-Yanes and Mahler, 1979). In addition, the presence of muscarinic and nicotinic cholinergic receptors is supported by electrophysiological experiments. Microiontophoretic application of ACh altered the activity of most ganglion cells of the carp retina (Negishi *et al.*, 1978). Of the cells that responded to ACh, the firing rate of 85% was increased and that of 15% was decreased. Excitation was blocked by hexamethonium, a nicotinic cholinergic antagonist, while inhibition was blocked by atropine, a muscarinic cholinergic antagonist. Light-evoked activity of ganglion cells was also inhibited by nicotinic antagonists (Glickman *et al.*, 1982). Rabbit ganglion cells with ON-center or directionally selective receptive fields were found to be activated by ACh (Masland and Ames, 1976). The spontaneous activity and response of ganglion cells to light was also enhanced by physostigmine, an acetylcholinesterase inhibitor, presumably by increasing the concentration of endogenously released ACh at its receptors (Masland and Ames, 1976; Ariel and Daw, 1982). Dihydro-β-erythroidine, a nicotinic receptor antagonist, inhibited the response of these cells to light, as well as those to exogenous ACh and to physostigmine. Similarly, the light-evoked excitation of a subpopulation of transient ganglion cells of cat retina was enhanced by ACh and suppressed by dihydro-β-erythroidine (Ikeda and Sheardown, 1982b).

Thus, ACh is probably an amacrine cell transmitter. It is synthesized in amacrine cells and is released as a consequence of physiological stimulation or K^+-induced depolarization. It acts on specific receptors to produce electrophysiological responses in other retinal neurons, and antagonists of these receptors have been shown to affect the response to light and to exogenous ACh in a similar fashion.

2. Dopamine

Most of the classical criteria for establishing dopamine (DA) as a retinal neurotransmitter have been met and recent evidence also supports a neuromodulatory role for this catecholamine in retinal function. Shortly after the development of the formaldehyde-induced histofluorescence method for visualizing catecholamine-containing cells (Falck, 1962), the presence of catecholamine-containing neurons in the retinas of rats and rabbits was discovered (Malmfors, 1963; Haggendal and Malmfors, 1964). Subsequently it was determined that DA was present in retinal neurons of many vertebrate species (DaPrada, 1977). Various methods have been used to localize the DA-containing retinal cells, including catecholamine histofluorescence and autoradiography following preincubation with [^{3}H]dopamine. In most species examined, including

rat, rabbit, chicken, dog, cat, mouse, frog, and several primates, DA is localized predominantly to a subclass of amacrine cells (Malmfors, 1963; Haggendal and Malmfors, 1965; Ehinger, 1966; Laties and Jacobowitz, 1966; Ehinger and Falck, 1969; Kramer *et al.*, 1971; Sarthy *et al.*, 1981). In other species, DA is localized primarily in interplexiform cells (see Section II,E). Retina contains the enzymes for the synthesis of DA from tyrosine, tyrosine hydroxylase (EC 1.14.16.2) and aromatic L-amino acid decarboxylase (EC 4.1.1.28) (Lam *et al.*, 1976; Schwarcz and Coyle, 1977; Iuvone *et al.*, 1978b; Parkinson *et al.*, 1981; Parkinson and Rando, 1983a; P. M. Iuvone, unpublished observations), and tyrosine hydroxylase-like immunoreactivity has been specifically localized to retinal cells with the same shape, size, and distribution as those observed with formaldehyde-induced histofluorescence (Nguyen-Legros *et al.*, 1981, 1984; Brecha *et al.*, 1984; Versaux-Botteri *et al.*, 1984).

Light stimulates the biosynthesis and turnover of retinal DA (DaPrada, 1977; Iuvone *et al.*, 1978a; Morgan and Kamp, 1980; Parkinson and Rando, 1983a,b). DA release from amacrine cells is enhanced by photic and electrical stimulation and by potassium-induced depolarization (Kramer, 1971; Thomas *et al.*, 1978; Sarthy and Lam, 1979; Bauer *et al.*, 1980; Pasantes-Morales *et al.*, 1980; Dubocovich and Weiner, 1981; Sarthy *et al.*, 1981). The light-evoked activation of DA-containing amacrine cells is apparently mediated transsynaptically by photoreceptors, bipolar cells, and GABA-containing amacrine cells (Morgan and Kamp, 1980, 1983a; Marshburn and Iuvone, 1981; Frucht *et al.*, 1982). A subpopulation of amacrine cells, presumably DA-secreting cells, possess a high-affinity uptake mechanism for the putative transmitter, providing a potential mechanism for terminating its postsynaptic action (Ehinger and Falck, 1971; Kramer *et al.*, 1971; Ehinger and Floren, 1978; Thomas *et al.*, 1978; Sarthy *et al.*, 1981). The enzymes for the degradation of DA are present in retina, and light increases the concentration of DA metabolites in this tissue (Mustakallio, 1967; Frucht *et al.*, 1982; Gibson *et al.*, 1982; Cohen *et al.*, 1983). Radioligand-binding studies with agonists and antagonists have provided evidence for the presence in the retina of DA receptors (Magistretti and Schorderet, 1979; Shaeffer, 1980; Makman *et al.*, 1980; Redburn and Keyes, 1980). These receptors appear to mediate the effects of dopamine on electrical activity of retinal neurons and on the regulation of adenylate cyclase activity (Brown and Makman, 1972; Schorderet, 1977; Spano *et al.*, 1977; Jagadeesh and Sanchez, 1981; Dyer *et al.*, 1981; Jensen and Daw, 1983).

Biochemical and electrophysiological studies indicate that DA influences transmission from the retina to the brain. Administration of the DA receptor agonist apomorphine, through an action on the retina, increased the uptake of 2-deoxyglucose into rat superior colliculus (McCulloch *et al.*, 1980), suggesting that stimulation of retinal DA receptors increased the metabolic activity in axon

terminals or postsynaptic cells in this brain area. In addition, haloperidol, a DA receptor antagonist, and α-methyl-*p*-tyrosine, an inhibitor of DA biosynthesis, increased the latency of early peaks in flash-evoked potentials recorded from visual cortex, lateral geniculate nucleus, and optic tract of the rat, but had no effect on evoked potentials caused by electrical stimulation of the optic tract (Dyer *et al.*, 1981). Application of DA to isolated rabbit retina increased the spontaneous activity of OFF-center ganglion cells and increased "breakthrough" during light inhibition (Ames and Pollen, 1969). In contrast, DA decreased the spontaneous activity of the majority of ON and ON–OFF cells. More recently, Jensen and Daw (1983) reported that apomorphine increased the spontaneous activity of OFF-center ganglion cells, while DA receptor antagonists, haloperidol and fluphenazine, decreased spontaneous activity and the "on" response of these cells to diffuse light. Recordings from ON–OFF directionally sensitive cells demonstrated a decreased response to the leading edge of a moving stimulus with haloperidol. Thus, retinal DA appears to be involved in the processing of visual information in the retina. The influence of DA on ganglion cell activity is probably indirect because DA-containing amacrine cells have been observed to synapse with other amacrine cells but not with ganglion cells (Dowling and Ehinger, 1978; Holgrem-Taylor, 1982). Interestingly, the DA-containing cells appear to receive input only from amacrine cells; no contacts with bipolar terminals have been observed. Thus, DA appears to be a transmitter involved in an interamacrine cell network.

DA may also function in retina as a neuromodulator, influencing the response of cells to other putative neurotransmitters. Using an *in vitro* preparation of acetylcholine-secreting amacrine cells grown on striated muscle cells, Yeh *et al.* (1983, 1984) examined the effects of DA and 8-bromo cyclic AMP on glutamate-evoked cholinergic transmission. At an early stage in the functional maturation of the retinal cell–muscle synapse, acetylcholine release, as determined by the electrophysiological response of the muscle cell, was spontaneous and could not be evoked by microiontophoretic application of L-glutamate. However, following microiontophoretic application of dopamine for 3–7 min, glutamate-evoked cholinergic transmission was reproducibly observed (Yeh *et al.*, 1984). The effect of DA on glutamate-evoked transmission was reversible and could be blocked by haloperidol. DA stimulated the accumulation of cyclic AMP in cultured retinal cells, and application of 8-bromo cyclic AMP mimicked the effect of DA on glutamate-evoked transmission (Yeh *et al.*, 1983, 1984), suggesting that dopamine produced its effect through a cyclic AMP-dependent mechanism. These studies demonstrate that DA can influence the response to another putative transmitter in a developing synaptic system *in vitro*, but it remains to be determined if it plays a similar role *in vivo* and if it can influence transmitter responsiveness at mature synapses.

3. Epinephrine and Norepinephrine

Recent studies have suggested that DA is not the only catecholamine present in bovine and rat retinas. In bovine retina, epinephrine and norepinephrine have been detected by HPLC-EC (Nesselhut and Osborne, 1982; Osborne and Nesselhut, 1983). The levels of norepinephrine and epinephrine in bovine retina were found to be similar to those of DA. The conversion of [^{14}C]DA into [^{14}C]norepinephrine in bovine retina was also demonstrated (Osborne, 1981a; Nesselhut and Osborne, 1982), as was the presence of phenylethanolamine *N*-methyltransferase (PNMT; EC 2.1.1.28) (Osborne and Nesselhut, 1983), the enzyme that converts norepinephrine to epinephrine. Binding sites for [^{3}H]norepinephrine and [^{3}H]clonidine, an adrenergic agonist, on bovine retinal membranes have been described, and pharmacological characterization of the binding sites suggests the presence of α_2-adrenergic receptors in retina (Bittinger *et al.*, 1980; Osborne, 1982a).

Norepinephrine and epinephrine have also been detected in rat retina (Wyse and Lorsheider, 1981; Hadjiconstantinou *et al.*, 1983). In contrast to bovine retina which contains similar levels of the three catecholamines (Nesselhut and Osborne, 1982a; Osborne and Nesselhut, 1983), the concentrations of norepinephrine and epinephrine in rat retina appeared to be approximately 20-fold lower than that of DA (Hadjiconstantinou *et al.*, 1983). Rat retina also contains PNMT activity. Photic stimulation caused a transient increase in the concentration of epinephrine (Hadjiconstantinou *et al.*, 1983), suggesting that the epinephrine-containing cells in rat retina are modulated by light. Pharmacological studies have provided evidence for the presence in rat retina of functional α_2-adrenergic receptors (Iuvone and Rauch, 1983; Hadjiconstantinou *et al.*, 1984a; see Section IV,A).

Most of the norepinephrine in rat retina is probably associated with sympathetic nerve terminals, while epinephrine is apparently contained in intrinsic retinal cells. Surgical removal of the superior cervical ganglion, which provides sympathetic input to the eye, decreased the concentration of norepinephrine in rat retina but had no effect on that of epinephrine or on the activity of PNMT (Hadjiconstantinou *et al.*, 1983). In addition, PNMT-like immunoreactivity has been localized to a subclass of amacrine cells in rat retina that are morphologically distinct from the DA-containing cells, providing strong evidence that the epinephrine-containing retinal cells are amacrine cells (Hadjiconstantinou *et al.*, 1984b). Norepinephrine-accumulating amacrine cells have been described in both rat and bovine retinas (Osborne, 1981a; Fukuda *et al.*, 1982), but it is not clear if these cells are epinephrine-containing cells or a separate subclass of amacrine cell.

These observations suggest that epinephrine may be a neurotransmitter or

neuromodulator in a subclass of amacrine cells in rat and possibly bovine retina. Its presence in amacrine cells of other vertebrate species awaits further investigation.

4. Serotonin

Retinas of most vertebrate species contain amacrine cells that accumulate serotonin and related indoleamines (e.g., Ehinger and Floren, 1976; Floren, 1979; Ehinger *et al.*, 1981; Negishi *et al.*, 1981; Redburn and Thomas, 1981; Mitchell and Redburn, 1985). The indoleamine-accumulating amacrine cells could usually be observed by either autoradiography or by formaldehyde-induced histofluorescence. However, the cells could be detected by the histofluorescence technique only after incubation with an indoleamine. No histofluorescence due to endogenous serotonin could be detected in these cells, even following pharmacological manipulations that increase serotonin fluorescence in the brain (Floren, 1979). Using a more sensitive immunohistochemical technique, amacrine cells with endogenous serotonin-like immunoreactivity have been observed in chicken, pigeon, frog, and goldfish, but not in any of several mammalian species (Osborne, 1982b; Osborne *et al.*, 1982; Tornqvist *et al.*, 1983). Similarly, quantitative analysis of the serotonin content in retina indicated high levels in chicken, frog, and pigeon, but low levels in rat, rabbit, and cow (Floren and Hanson, 1980; Osborne *et al.*, 1982; Tornqvist *et al.*, 1983; Mitchell and Redburn, 1984). Thus, the indoleamine-accumulating amacrine cells in retinas of nonmammalian species may contain endogenous serotonin. The cellular localization of endogenous serotonin in mammalian retina has not been determined, but dissection of bovine retina suggests that serotonin is localized to the inner nuclear–inner plexiform layers, possibly in indoleamine-accumulating amacrine cells (Osborne *et al.*, 1982b).

The synthesis of [^{3}H]serotonin from [^{3}H]tryptophan in bovine and chick retina has been demonstrated (Osborne, 1980; Parkinson and Rando, 1981), as has the K^+-stimulated, Ca^{2+}-dependent release of [^{3}H]serotonin previously accumulated by high-affinity uptake (Thomas and Redburn, 1979, 1980; Osborne, 1980, 1982b; Mitchell and Redburn, 1985). The high-affinity binding of [^{3}H]serotonin to membranes of bovine retina suggests the presence of serotonin receptors (Osborne, 1981b). Binding was enriched in a subcellular fraction containing synaptosomes from inner retina. Binding studies with rabbit retinal membranes also support the presence of serotonin receptors and suggest the presence of two subtypes of binding sites, 5-HT_1 and 5-HT_2 (Mitchell and Redburn, 1985).

There is very little electrophysiological data to support a neurotransmitter role for serotonin in retina. Application of serotonin to isolated rabbit retina increased spontaneous and light-evoked activity of ganglion cells (Ames and Pollen, 1969). In contrast, infusion of serotonin into the carotid artery inhibited the

activity of ganglion cells in cat retina (Straschill, 1968). This latter effect may have been indirect, as serotonin would not be expected to freely cross the blood-retinal barrier. Furthermore, microiontophoretic application of serotonin had no significant effect on cat retinal ganglion cells (Straschill and Perwein, 1969). Clearly, additional physiological studies are required to determine the role of serotonin in both mammalian and nonmammalian retinas.

5. GABA

The presence of high concentrations of GABA in the retinas of several vertebrate species was first reported by Kojima *et al.* (1958) and Kubicék and Dolenék (1958). As described in Section II,B, GABA is a transmitter candidate in a subpopulation of horizontal cells in retinas of some nonmammalian vertebrates. In addition, GABA is a neurotransmitter candidate of a subpopulation of amacrine cells in retinas of most vertebrates, including mammals. Microdissection studies have demonstrated that GABA and the biosynthetic enzyme GAD are concentrated in the inner plexiform layer (Kuriyama *et al.*, 1968; Graham, 1972; Berger *et al.*, 1977; Kennedy and Neal, 1977). Histochemical localization of GAD-like immunoreactivity has provided direct evidence for the presence of the GABA-synthesizing enzyme in both horizontal cells and amacrine cells in fish and frog retinas but only in amacrine cells in retinas of rabbits and rats (Lam *et al.*, 1979; Brandon *et al.*, 1979, 1980; Vaughn *et al.*, 1981; Zucker *et al.*, 1984). In rat retina, the GAD-positive amacrine cells receive synaptic input primarily from bipolar cells and synapse upon bipolar, amacrine, and ganglion cells (Vaughn *et al.*, 1981).

GABA is accumulated in amacrine cells by high-affinity uptake. In fish, frogs, and birds, [^{3}H]GABA was found to be accumulated by subpopulations of amacrine cells, as well as into horizontal cells (Voaden *et al.*, 1974; Marshall and Voaden, 1974; Marc *et al.*, 1978; Hollyfield *et al.*, 1979). In most mammalian species examined, [^{3}H]GABA was accumulated by Müller cells, obscuring the detection of neuronal uptake (Neil and Iversen, 1972; Bruun and Ehinger, 1974; Ehinger, 1977). However, subpopulations of amacrine cells that accumulate GABA have been detected in cat and guinea pig (Bruun and Ehinger, 1974; Pourcho, 1980; Freed *et al.*, 1983).

The release of previously accumulated GABA supports a transmitter role for the amino acid. Depolarizing concentrations of K^+ increase the Ca^{2+}-dependent release of [^{3}H]GABA from subcellular fractions enriched in synaptosomes from inner retina (Redburn, 1977). Glutamate and kainic acid also stimulate the Ca^{2+}-dependent release of GABA from isolated rat retina (Kamada *et al.*, 1981). The release of [^{3}H]GABA from preloaded rabbit retinas is increased by photic stimulation (Bauer and Ehinger, 1977), suggesting the presence of GABA-secreting amacrines that are activated by light. In addition, phar-

macological and electrophysiological experiments have presented evidence for GABA neurons in retinas of rabbit, rat, and mudpuppy that tonically release GABA in darkness (Miller *et al.*, 1981; Marshburn and Iuvone, 1981; Kamp and Morgan, 1981; Massey and Redburn, 1982; see Section IV,A and B). Thus, several subpopulations of amacrine cells may release GABA. This hypothesis is supported by the morphological identification of four different types of amacrine cells in cat retina that accumulate GABA (Pourcho, 1989; Freed *et al.*, 1983).

The neurons that accumulate a putative transmitter by high-affinity transport are usually considered to also be the neurons that synthesize and secrete that putative transmitter. This appears to be the case for the GABA-accumulating horizontal cells but, surprisingly, not for the GABA-accumulating amacrine cells of goldfish retina. Using a double-label technique to localize GAD-like immunoreactivity and [^{3}H]GABA accumulation, Zucker *et al.* (1984) noted very few amacrine cells in which the two histochemical markers were colocalized. In most cases, [^{3}H]GABA accumulation was found in amacrine cell processes that surround GAD-positive processes. Only when two GAD-positive processes were juxtaposed were [^{3}H]GABA uptake and GAD-like immunoreactivity colocalized. Thus, while identification of high-affinity uptake of a putative transmitter provides evidence for a mechanism to terminate the action of the compound, uptake sites may not always be good markers for localizing the neurons that synthesize and secrete a particular transmitter. Although the observations of Zucker *et al.* (1984) pertain directly only to GABA-accumulating amacrine cells of goldfish retina, they raise the possibility that the release of previously accumulated GABA from retinal cells of other species may not have occurred from the cells that secrete endogenous GABA.

High-affinity binding of [^{3}H]GABA and of [^{3}H]muscimol, a GABA receptor agonist, has been observed in mammalian retina, suggesting the presence of GABA receptors (Enna and Snyder, 1976; Redburn *et al.*, 1979; Redburn and Mitchell, 1981). Electrophysiological effects of GABA and GABA antagonists also support the presence of functional GABA receptors. GABA and its antagonists influence the ERG (Starr, 1975; DeVries and Friedman, 1978), and GABA inhibits the spontaneous and light-evoked activity of ganglion cells (Straschill and Perwein, 1969). Studies with GABA antagonists suggest that inhibition of ON-center ganglion cells is mediated by GABA (Ikeda and Sheardown, 1983), and that GABA bestows the properties of directional selectivity to directionally selective ganglion cells (Wyatt and Daw, 1976; Caldwell *et al.*, 1978).

Pharmacological effects of GABA receptor agonists and antagonists on other putative retinal transmitters also support a transmitter or modulatory role for GABA in the retina. GABA and the GABA receptor agonist muscimol were found to inhibit the light-evoked release of ACh from amacrine cells (Massey and Neal, 1979; Massey and Redburn, 1982). In contrast, the GABA antagonists

bicuculline and picrotoxin increased the light-evoked and spontaneous release of the putative transmitter, suggesting that GABA tonically inhibits ACh-containing amacrine cells. DA-containing amacrine cells also appear to be subject to tonic inhibition by GABA in darkness, but not in light (Morgan and Kamp, 1980; Marshburn and Iuvone, 1981; Kamp and Morgan, 1981; see Section IV,A).

In summary, the majority of evidence suggests that GABA is an amacrine cell transmitter. It is synthesized in and probably released from amacrine cells. It influences the activity of retinal neurons that receive synaptic input from GAD-positive amacrine cells, and specific GABA receptor antagonists influence the responses of retinal ganglion cells to physiological stimuli.

6. Glycine

This putative inhibitory neurotransmitter is present in the vertebrate retina at concentrations that are similar to those found elsewhere in the central nervous system (Pasantes-Morales *et al.*, 1972; Starr, 1973; Berger *et al.*, 1977; Kennedy *et al.*, 1977). The highest concentrations of glycine within the retina are found in the amacrine cell, inner plexiform, and ganglion cell layers (Berger *et al.*, 1977; Kennedy *et al.*, 1977; Chin and Lam, 1980). Retina accumulates [^{3}H]glycine by a high-affinity active uptake system (Bruun and Ehinger, 1972; Neal *et al.*, 1973), and autoradiographic analysis indicates that [^{3}H]glycine is concentrated primarily in amacrine cells, although in some species interplexiform cells are also labeled (Ehinger and Falck, 1971; Marshall and Voaden, 1974b; Voaden *et al.*, 1974; Bruun and Ehinger, 1974; Pourcho, 1980; Lam and Hollyfield, 1980; Marc and Lam, 1981b; Rayborn *et al.*, 1981). Potassium-stimulated calcium-dependent release from retina of accumulated [^{3}H]glycine and of endogenous glycine has been reported (Kennedy and Neal, 1978; Lopez-Colome *et al.*, 1978; Chin and Lam, 1980; Kong *et al.*, 1980; Coull *et al.*, 1981). In addition, photic stimulation has been observed to increase the release of [^{3}H]glycine from retinas of cats and rabbits (Ehinger and Lindberg, 1974; Ehinger and Lindberg-Bauer, 1976) and that of endogenous glycine from retinas of pigmented rats (Coull and Cutler, 1978). In goldfish retina, modulation of [^{3}H]glycine uptake into amacrine cells suggests that the glycine-accumulating amacrine cells may be red-hyperpolarizing/green-depolarizing neurons (Marc and Lam, 1981b).

Glycine influences the activity of retinal ganglion cells by a receptor-mediated mechanism. Application of glycine inhibits spontaneous and light-evoked activity of ganglion cells, while strychnine, a glycine antagonist, increases ganglion cell activity (Ames and Pollen, 1969). Glycine-secreting amacrine cells may provide inhibitory input to OFF-center ganglion cells (Wyatt and Daw, 1976; Miller *et al.*, 1981; Ikeda and Sheardown, 1983). The presence in retina of high-affinity-binding sites for [^{3}H]strychnine further supports the existence of synaptic glycine receptors (Borbe *et al.*, 1981). However, all of these results need to

be interpretted with caution because taurine also inhibits ganglion cells in a strychnine-sensitive manner (Cunningham and Miller, 1976).

Most evidence supports a neurotransmitter role for glycine in retinal amacrine cells. It is accumulated by specific amacrines, is released, and has physiological effects. Availability of specific antagonists of glycine receptors would greatly facilitate a more conclusive assessment of glycine's role in retinal function.

7. Other Amino Acids

Aspartate aminotransferase-like immunoreactivity has been localized to a subpopulation of amacrine cells in monkey and guinea pig retina (Altschuler *et al.*, 1982; Mosinger, 1983), suggesting that high concentrations of glutamate and/or aspartate may be present in these cells. In mudpuppy retina, application of *N*-methylaspartate depolarized amacrine cells and hyperpolarized sustained OFF ganglion cells (Slaughter and Miller, 1983b). The effects of *N*-methylaspartate were blocked by the antagonists α-aminoadipate and α-aminosuberate. In addition, α-aminosuberate inhibited the sustained light-evoked hyperpolarization of the sustained OFF ganglion cell. Because *N*-methylaspartate did not appear to mimic the bipolar cell transmitter, it was suggested that it mimicked an amacrine cell transmitter. Other than these observations, there is little to suggest that aspartate or glutamate are amacrine cell neurotransmitters at this time.

Taurine is accumulated by processes in the inner plexiform layer and by cell bodies in the amacrine cell layer (Lake *et al.*, 1977, 1978; Pourcho, 1981), and monosodium glutamate-induced lesions of inner retinal neurons decreased the activity of the taurine-synthesizing enzymes (Salceda *et al.*, 1979). Thus, taurine may be present in amacrine cells as well as in photoreceptors. The role of taurine in the inner retina is unknown. Taurine has been shown to inhibit the b wave of electroretinogram and the activity of retinal ganglion cells (Pasantes-Morales *et al.*, 1972, 1973; Bonaventure *et al.*, 1974; Starr, 1975; Cunningham and Miller, 1976). The electrophysiological effects of taurine were blocked by the antagonist strychnine, indicating that the effects were receptor mediated. However, it is not clear if taurine acted on specific taurine receptors or if its actions were mediated by glycine receptors.

8. Neuropeptides

In the past few years, immunohistochemical studies have provided evidence for the presence in subpopulations of amacrine cells of several neuropeptides, including enkephalin, vasoactive intestinal polypeptide (VIP), luteinizing hormone-releasing hormone (LHRH), substance P, glucagon, somatostatin, neurotensin, and cholecystokinin (e.g., Karten and Brecha, 1980; Brecha *et al.*, 1981, 1982; Eskay *et al.*, 1981; Fukuda *et al.*, 1981, 1982; Morgan *et al.*, 1981;

Osborne *et al.*, 1981, 1982b; Yamada *et al.*, 1981; Altschuler *et al.*, 1982b; Kuwayama *et al.*, 1982; Tornqvist *et al.*, 1982; Terubayashi *et al.*, 1983; Tornqvist and Ehinger, 1983). The substance P-like immunoreactivity of rat, monkey, and chick retinas was shown to comigrate with the synthetic substance P in HPLC (Eskay *et al.*, 1981). In contrast, the substance P-like immunoreactivity in frog and carp retina did not cochromatograph with the synthetic peptide. The somatostatin-like immunoreactivity purified from rat and human retinas had biological activity (inhibited growth hormone secretion from pituitary cells) (Rorstad *et al.*, 1979, 1980), and sequence analysis of the peptide from bovine retina indicated an apparent identity to authentic somatostatin-28 (Marshak *et al.*, 1983). The biosynthesis of a somatostatin-like substance from radiolabeled amino acids in frog retina has been demonstrated. The radiolabeled substance was shown to have somatostatin-like immunoreactivity and to cochromatograph with synthetic somatostatin-14 (Yamada and Basinger, 1982). The biosynthesis of cholecystokinin-like immunoreactivity has also been demonstrated to occur in frog retina (Yamada *et al.*, 1981).

Potassium-induced, Ca^{2+}-dependent release from frog retina of somatostatin-like and substance P-like immunoreactivities has been demonstrated (Eskay *et al.*, 1980). These studies suggest that retinal neuropeptides may have synaptic actions, but very little is known about the electrophysiological effects of the peptides in retina. Microiontophoretic application of neurotensin and substance P increased the firing rate of mudpuppy ganglion cells, while (D-Ala^2, Met^5)-enkephalinamide (DALA), an enkephalin analog, inhibited ganglion cell activity (Dick and Miller, 1981). In goldfish retina, DALA was observed to reduce the spontaneous and light-evoked activity of OFF-center ganglion cells, while it increased the activity of ON-center cells (Djamgoz *et al.*, 1981).

Several peptides appear to modulate adenylate cyclase activity in retina. Glucagon and VIP increase cyclic AMP in retina (Kuwayama *et al.*, 1982; Watling and Dowling, 1983). Some of the increase of cyclic AMP levels may have occurred in neuronal cells, because isolated horizontal cells responded to VIP with an increase of cyclic AMP and with membrane depolarization (Watling and Dowling, 1983; Lasater *et al.*, 1983). However, VIP and glucagon also increased cyclic AMP in isolated cultured Müller cells and retinal pigment epithelium (Koh and Chader, 1984; Koh *et al.*, 1984). Thus, some of these peptides may have modulatory effects on metabolism of several retinal cell types instead of or in addition to neurotransmitter roles.

E. Interplexiform Cells

The term "interplexiform" has been applied to a class of retinal neurons with cell bodies in the amacrine cell layer that have processes extending into the inner

plexiform layer and other processes that project centrifugally through the inner nuclear layer (Gallego, 1971; Fig. 1). These latter processes ramify in the outer plexiform layer where they are presynaptic to horizontal and bipolar cells. The interplexiform cell is unique among retinal neurons in its apparent ability to feedback information from the inner retina to the outer synaptic layer.

1. Dopamine

In some species, most notably in teleost fish and some New World monkeys, DA may be the neurotransmitter of some of the interplexiform cells. Examination of retinas from these species for formaldehyde-induced catecholamine histofluorescence indicated the presence of catecholamine-containing processes in both synaptic layers (Laties and Jacobowitz, 1966; Ehinger *et al.*, 1969; Laties, 1972). These interplexiform cells in goldfish and *Cebus* monkey retinas have been characterized by electron microscopy (Dowling and Ehinger, 1975). They were found both pre- and postsynaptic to amacrine cells in the inner plexiform layer, and presynaptic to bipolar and horizontal cells in the outer plexiform layer. They apparently do not receive synaptic input in the outer plexiform layer.

Binding studies with DA receptor antagonists have suggested the presence of dopamine receptors in teleost retina (Redburn *et al.*, 1980), and DA-sensitive adenylate cyclase activity in this tissue has been described (Watling *et al.*, 1979; Redburn *et al.*, 1980; Watling and Dowling, 1981). The DA receptors were also found on isolated horizontal cells (Van Buskirk and Dowling, 1981, 1982). Photic stimulation increased cyclic AMP accumulation in the teleost retina, and the light-evoked increase of cyclic AMP was blocked by the DA receptor antagonist haloperidol (Dowling and Watling, 1981).

DA modulates the receptive field properties of cells in the outer nuclear layer of teleost fish. Application of the putative transmitter augmented the central response to light of the bipolar cell, while depressing the surround response (Hedden and Dowling, 1978). DA produced a similar effect on cone-driven horizontal cells (Negishi and Drujan, 1979a). It also contracted the receptive field size of horizontal cells (Laufer *et al.*, 1981), while haloperidol and 6-hydroxydopamine, a neurotoxin that destroys the DA-containing interplexiform cells (Ehinger and Nordenfelt, 1977), increased the receptive field size (Teranishi *et al.*, 1983; Cohen *et al.*, 1983). Thus, it appears that DA is probably a neurotransmitter or neuromodulator of interplexiform cells of teleost fish.

Autoradiographic analysis of DA accumulation and tyrosine hydroxylase immunohistochemistry have provided evidence for dopamine-containing interplexiform cells in rat, mouse, and human retina (Nguyen-Legros *et al.*, 1981, 1984; Frederick *et al.*, 1982; Versaux-Botteri *et al.*, 1984). However, DA-containing amacrine cells appeared to predominate over the DA-containing interplexiform cells in retinas of these species, and there is no electrophysiological evidence to support a transmitter role for dopamine in their outer plexiform layers.

2. Glycine

In some species, other putative interplexiform cell transmitters have been identified. In retinas of goldfish and frog (*Xenopus laevis*), glycine was accumulated by cells with processes that ramify in both plexiform layers, and these cells have been considered to be a subtype of interplexiform cell (Chin and Lam, 1980; Marc and Lam, 1981b; Rayborn *et al.*, 1981). In the outer plexiform layer, the glycine-accumulating cells were found presynaptic to horizontal cells. In the goldfish, they were also postsynaptic to H_1 horizontal cells (Marc and Lam, 1981b), which distinguishes them from the DA-containing interplexiform cells of this species that appeared to receive synaptic input only in the inner plexiform layer (Dowling and Ehinger, 1975). Application of glycine hyperpolarized some goldfish horizontal cells and depressed their response to red light (Wu and Dowling, 1980), suggesting a possible functional role for glycine in the outer plexiform layer.

3. GABA

In cat retina, a subtype of interplexiform cell accumulated [^{3}H]GABA and [^{3}H]muscimol, a GABA analog, and it has been suggested that GABA may be the transmitter of this cell (Nakamura *et al.*, 1980; Pourcho, 1981b). The GABA-accumulating interplexiform cell synapsed on bipolar processes in the outer nuclear layer. In the inner plexiform layer, the cells appeared to be presynaptic to amacrine and bipolar cells and postsynaptic to amacrine cells. Localization of GAD to these cells would provide more direct evidence that these cells actually synthesize GABA.

F. Ganglion Cells

Retinal ganglion cells have their cell bodies located in the innermost cell layer of the retina (Fig. 1). Their dendrites synapse with bipolar cell terminals and amacrine cell processes in the inner plexiform layer, and their axons form the optic nerve, which projects to various visual areas of the brain. Ganglion cells are morphologically and physiologically heterogeneous and little is known about the transmitters secreted by them.

1. Glutamate and Aspartate

Several lines of evidence suggest that aspartate and/or glutamate may be neurotransmitters in some of these cells. Berger *et al.* (1977) found that the concentrations of aspartate and glutamate were higher in the ganglion cell layer of monkey retina than in any other retinal layer. However, the concentration of

these putative neurotransmitters was low in the optic nerve. Perhaps, these amino acids or their synthetic enzymes are rapidly transported from the cell bodies to the synaptic terminals. Alternatively, the high concentrations of aspartate might be localized in displaced amacrine cells or in Müller cells, which have a high proportion of their cytoplasm in the ganglion cell layer (Rasmussen, 1972).

The best evidence for aspartate or glutamate being a ganglion cell transmitter comes from work on the pigeon retinotectal projection. Approximately 5–10% of cells in the ganglion cell layer accumulated D-[^{3}H]aspartate following intravitreal injection (Ehinger, 1981), and the accumulated D-aspartate was transported to layers 1–7 of the contralateral optic tectum, confirming its accumulation by ganglion cells (Beaudet *et al.*, 1981). The transported D-aspartate could be released from the tectum by depolarizing concentrations of K^+, and the D-aspartate release was Ca^{2+} dependent. Ablation of the pigeon retina decreased the V_{max} of high-affinity L-glutamate uptake and the concentrations of endogenous glutamate and aspartate in the tectum (Henke *et al.*, 1976; Fonnum and Henke, 1979), suggesting the presence of high concentrations of these amino acids in ganglion cell terminals. In addition, optic nerve stimulation caused an increase in the release of endogenous aspartate and glutamate in optic tectum, and had no effect on the release of GABA (Canzek *et al.*, 1981). Furthermore, electrical stimulation of the optic nerve or microiontophoretic application of glutamate increased the firing of tectal neurons, and the effect of optic nerve stimulation was reduced by a glutamate receptor antagonist, glutamic acid diethylester (Wang *et al.*, 1978).

Similar ganglion cells may exist in retinas of other species. D-Aspartate-accumulating cells have also been observed in the ganglion cell layer of guinea pig and rabbit retinas (Ehinger, 1981). Aspartate aminotransferase-like immunoreactivity has been found in cells in the ganglion cell layer of guinea pig and monkey retinas (Altschuler *et al.*, 1982a; Mosinger, 1983). In addition, unilateral enucleation of chicks resulted in a large reduction in the ability of the tectum to accumulate L-glutamate (Bondy and Purdy, 1977).

2. Acetylcholine

Acetylcholine may be a ganglion cell transmitter in the toad *B. marinus*. The toad optic nerve and tectum were found to contain significant amounts of acetylcholine and choline acetyltransferase activity (Oswald *et al.*, 1979). The levels of these two markers for cholinergic neurons was decreased in the tectum following enucleation. Furthermore, antagonists of nicotinic cholinergic receptors, *d*-tubocurarine and α-bungarotoxin, decreased the presumed postsynaptic potential evoked by photic stimulation of OFF ganglion cells synapsing in the tectum (Freeman, 1977).

It is likely that many ganglion cells utilize transmitters other than acetylcho-

line, glutamate, or aspartate, and based on the proliferation in the number of putative transmitters identified in other retinal cell types, it is likely that other ganglion cell transmitter candidates will soon be identified. Indeed, a preliminary report indicates that a neuropeptide related to α-MSH may be localized in some ganglion cells of cat retina (Stone *et al.*, 1983).

G. Summary

Information regarding the nature of the neurotransmitters of the various retinal cell types is increasing rapidly, but at present our knowledge is fragmentary. Glutamate and/or aspartate appear to be good candidates for neurotransmitters in photoreceptors, but wide species variability may exist in use of these substances as photoreceptor transmitters. This is exemplified by observations on the accumulation of D-aspartate and the localization of aspartate aminotransferase-like immunoreactivity selectively in cones of some species and selectively in rods of others. In nonmammalian vertebrates, one subtype of horizontal cell appears to utilize GABA; the transmitter of other types of horizontal cells in both mammalian and nonmammalian retinas has not been identified. Excitatory amino acids such as aspartate and glutamate may also be transmitters in bipolar and ganglion cells while a large number of transmitter candidates have been identified in amacrine cells. An emerging picture is that at least some of the direct pathways from photoreceptor to visual centers in the brain may involve excitatory amino acids, while lateral processing of visual information within the retina is subserved by a wide variety of excitatory and inhibitory transmitter substances.

Visual function may also be influenced by neuromodulators, such as melatonin, and some substances may have both neurotransmitter-like and neuromodulator-like functions. This possibility will be discussed in more detail in Section V.

III. Pre- and Postsynaptic Regulatory Mechanisms

The retina provides unique opportunities for the study of synaptic regulatory mechanisms, primarily because of the ability to modulate the activity of neurotransmitter and neuromodulator systems with light. Despite this fact, the regulatory mechanisms of only a few systems have been examined in any detail. As examples of the potential of retina for regulatory studies, this section will discuss the pre- and postsynaptic regulatory mechanisms of the dopamine neuronal system of retina and will describe recent studies on the regulation of the biosynthesis of the putative retinal neuromodulator melatonin.

A. Regulation of Dopamine Biosynthesis

DA is synthesized from the amino acid tyrosine by the sequential actions of tyrosine hydroxylase (TH) and aromatic L-amino acid decarboxylase (Fig. 2). TH, which requires molecular oxygen and a reduced pteridine as cofactors, hydroxylates the 3 position of the phenol ring of L-tyrosine to form L-3,4-dihydroxyphenylalanine (DOPA) (Nagatsu *et al.*, 1964). Aromatic L-amino acid decarboxylase, a pyridoxyl-requiring enzyme, removes the carboxyl moiety from L-DOPA to form dopamine. The first reaction, the hydroxylation of tyrosine, appears to be the rate-limiting step in catecholamine biosynthesis (Levitt *et al.*, 1965).

In many vertebrate species, the DA-containing neurons of retina are transsynaptically activated by light. Photic stimulation increases the release and turnover of retinal DA and increases the levels of the DA metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) (Kramer, 1971; Iuvone *et al.*, 1978a; Morgan and Kamp, 1980; Bauer *et al.*, 1980; Frucht *et al.*, 1982; Cohen *et al.*, 1983; Parkinson and Rando, 1983a,b). However, light does not decrease the steady-state concentration of DA in the retina. Thus, an increase in DA biosynthesis occurs in the activated retinal DA-containing neuron. Measurements in rat retina demonstrated a fourfold increase of DA biosynthesis following exposure to white light at intensities of greater than 32 lux (Iuvone *et al.*, 1978; Proll *et al.*, 1982).

Concomitant with the increase of dopamine biosynthesis is an activation of TH that can be measured in homogenates following exposure of the retina to light (Iuvone *et al.*, 1978a,b). Figure 3 illustrates the activity of the enzyme measured *in vitro* in retinal homogenates prepared from rats sacrificed at various times during a 12-hr light : 12-hr dark cycle. Activity was relatively constant during the dark phase of the light–dark cycle, but increased rapidly following light onset and remained elevated throughout the light phase. Activation of the enzyme occurred rapidly and could be demonstrated to occur in isolated eyes within 60 sec of photic stimulation (Iuvone *et al.*, 1978a). Activation was not strictly dependent on photoperiod, as it was delayed by keeping the animals in the dark beyond the time of normal light onset and could be elicited by light exposure

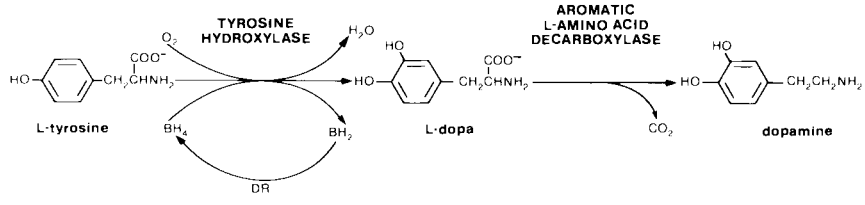


FIG. 2. Reactions in the biosynthesis of dopamine. BH_4, Tetrahydrobiopterin; BH_2, dihydrobiopterin; DR, dihydropteridine reductase.

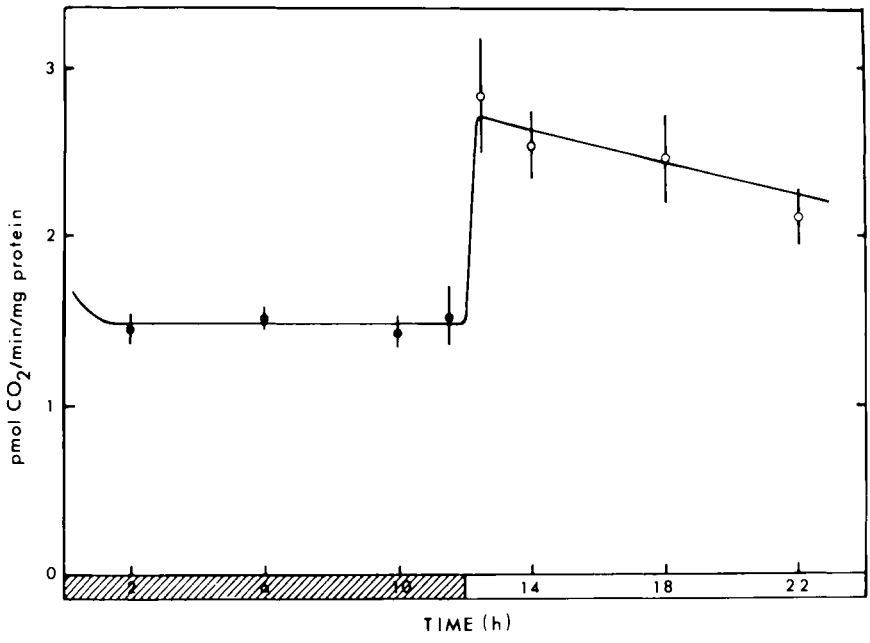


FIG. 3. Diurnal rhythm of retinal tyrosine hydroxylase activity. Lights were on from 12 to 24 hr. (Reproduced from Iuvone *et al.*, 1978a.)

during the dark phase (Iuvone *et al.*, 1978a; P. M. Iuvone, unpublished observations).

Light-evoked TH activation may involve synaptically induced depolarization of the DA-containing neurons. Activation was elicited by incubating suspensions of viable cells from dissociated retinas in medium containing depolarizing concentrations of potassium (Iuvone and Marshburn, 1982). The experiment was conducted on cell suspensions in which greater than 95% of the cells were present as single cells so that direct, rather than transsynaptic, effects of potassium might be examined. In addition, muscimol, which inhibits neurons that contain GABA receptors (Krogsgaard-Larsen *et al.*, 1975), decreased TH activity in the cell suspensions (Marshburn and Iuvone, 1981; see Section IV,A).

Kinetic characterization of the light-evoked activation of TH[1] indicated that light exposure decreased the apparent K_m of the enzyme for the synthetic pteridine cofactors 2-amino-4-hydroxy-6-methyl-5,6,7,8-tetrahydropterin ($6MPH_4$) and 2-

[1]Two types of TH activation in retina have been characterized. The type described here occurs in response to relatively short-term stimulation. The other type of activation is characterized by an increased V_{max} and occurs only following several days of constant light exposure (Iuvone *et al.*, 1978b, 1979).

amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropterin ($DMPH_4$) (Iuvone *et al.*, 1978b, 1982), as well as that for the presumed natural cofactor, *6(R)*-5,6,7,-8-tetrahydro-L-biopterin (BH_4) (P. M. Iuvone, unpublished observation). No significant effect on the apparent V_{max} of the reaction was observed with any of these cofactors. The apparent K_m for tyrosine was also not altered (Iuvone *et al.*, 1978b), but the apparent K_m for tyrosine was only determined with $DMPH_4$ as cofactor, and the possibility that it might be different when measured with the natural cofactor cannot be excluded. Nevertheless, the change of apparent K_m for the pteridine cofactor suggests that an increased affinity for the cofactor may be responsible for the light-evoked stimulation of DA biosynthesis.

For this mechanism to be operative, one would not expect the enzyme to be saturated with endogenous cofactor *in situ* in the dark-adapted state. To test these hypotheses, BH_4 was administered intravitreally and retinal DOPA accumulation following pharmacological inhibition of aromatic L-amino acid decarboxylase was measured as an index of *in vivo* tyrosine hydroxylation (Iuvone *et al.*, 1985). Administration of BH_4 during the last hour of the dark phase of the light–dark cycle increased retinal DOPA accumulation to a level that was comparable to that in vehicle-injected light-exposed eyes. In contrast, administration of BH_4 during the first hour of light had no significant effect on retinal DOPA accumulation. Thus, tyrosine hydroxylase appears not to be saturated with endogenous cofactor in dark-adapted retinas, while following light exposure the enzyme becomes more nearly saturated, probably as a consequence of the increased affinity for the cofactor. It is possible that a light-induced increase of endogenous BH_4 in the DA-containing cells might have contributed to the apparent saturation of the enzyme. However, measurements of BH_4 in whole retina indicated that light under these conditions had no effect on either the level of endogenous BH_4 or the concentration of BH_4 following intravitreal injection of the cofactor.

The disposition of the substrate tyrosine may also play a role in the regulation of retinal DA biosynthesis. Systemic administration of large doses of tyrosine to light-exposed rats increased the concentrations in retina of tyrosine and DOPAC and stimulated the accumulation of DOPA following decarboxylase inhibition (Gibson *et al.*, 1983; Fernstrom *et al.*, 1984, 1986). In contrast, tyrosine administration to dark-adapted rats had no effect on DOPAC levels or DOPA accumulation, although it did increase the concentration of tyrosine in retina. Thus, it appears that exogenous tyrosine can selectively stimulate tyrosine hydroxylation and DA metabolism in active DA-containing neurons, those which contain the activated form of TH. The mechanism responsible for this selective stimulation is not known. Kinetic analysis of activated TH demonstrated no change in the affinity for tyrosine that could explain these observations (Iuvone *et al.*, 1978b; P.M. Iuvone, unpublished observations). Exogenous tyrosine might act to replenish a strategic pool of tyrosine that is partially depleted by the activated TH in the light-exposed retina and that is sufficient in concentration to saturate the enzyme in the dark-adapted retina.

Several lines of evidence suggest that photic stimulation and presumably consequent depolarization of the DA-containing neurons elicit the activation of TH by a mechanism that involves protein phosphorylation. Dilution and gel filtration of retinal extracts do not diminish the activation of the enzyme elicited by prior exposure of the retina to light (P. M. Iuvone, unpublished), implicating strong hydrophobic interactions or covalent modification of the enzyme. TH purified from several tissues is a substrate for cyclic AMP (cAMP)-dependent protein kinase (APK) (Raese *et al.*, 1977; Joh *et al.*, 1978; Yamauchi and Fujisawa, 1979; Vulliet *et al.*, 1980; Markey *et al.*, 1980), and TH in extracts of dark-adapted retinas is activated by cAMP-dependent protein phosphorylation (Iuvone *et al.*, 1982; Iuvone and Marshburn, 1982). Figure 4A illustrates the effect of preincubating retinal extracts in the presence of cAMP, ATP, and magnesium. The activation produced by such preincubation required the presence of all three substrates for APK and was completely blocked by the heat-stable inhibitor of APK described by Walsh *et al.* (1971). TH activation could also be elicited by incubation of cell suspensions of dissociated retinas with 8-bromo cAMP (Fig.

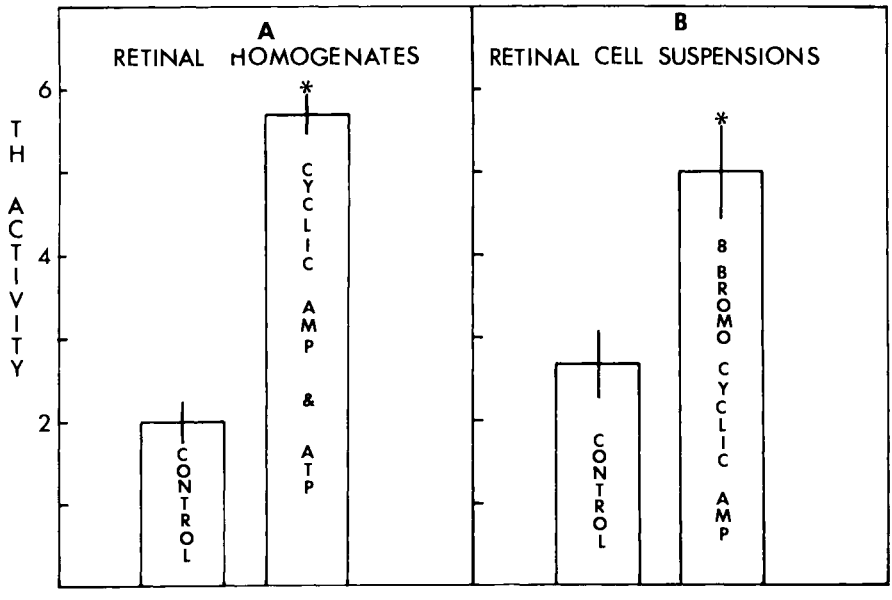


FIG. 4. Cyclic AMP-dependent activation of tyrosine hydroxylase. (A) Supernatant fractions of homogenized rat retina were preincubated for 7 min at 37°C under control (10 m*M* Mg^{2+}, 1 m*M* adenosine) or phosphorylating (10 m*M* Mg^{2+}, 1 m*M* ATP, 0.15 m*M* cAMP) conditions prior to determination of TH activity. (B) Cell suspensions were incubated for 30 min at 37°C under an atmosphere of 95% O_2/5% CO_2 with control Ringer's solution or Ringer's solution containing 1 m*M* 8-bromo cAMP. TH activity was subsequently assayed in cell extracts. Data are expressed as mean ± SEM in pmol/min/mg protein. $*p < 0.01$. (Reproduced from Iuvone and Marshburn, 1981).

4B), providing evidence that retinal DA-containing neurons contain sufficient APK in the appropriate subcellular compartment to activate the enzyme *in situ*.

The activation produced by photic stimulation *in vivo* was similar to that produced by cAMP-dependent protein phosphorylation *in vitro* (Iuvone *et al.*, 1982). Phosphorylation and photic stimulation decreased the apparent K_m for $6MPH_4$ to the same value and produced the same change in the relationship of assay pH to enzyme activity, that being to decrease the decline in activity with increasing pH above the optimum.

The activations produced by phosphorylation and photic stimulation were both reversible *in vitro*. Incubation of the extracts containing activated TH at 25°C resulted in a time-dependent inactivation of the enzyme that was complete in 50–60 min (Iuvone *et al.*, 1982). Following inactivation, the enzyme could be completely reactivated by phosphorylation. The inactivation of the enzyme following activation by photic stimulation or by phosphorylation was decreased by including in the incubation mixture sodium pyrophosphate, an inhibitor of phosphoprotein phosphatase. These observations, although simple correlations, support the hypothesis that retinal TH is regulated by a mechanism that involves phosphorylation and dephosphorylation. This hypothesis is also supported by the observation that the activation produced by cAMP-dependent phosphorylation is not additive to that elicited by light (Iuvone *et al.*, 1982). A demonstration of light-induced phosphorylation of the enzyme would provide more direct evidence for this hypothesis. The hypothesis and the observations supporting it do not exclude the involvement of protein kinases other than APK that produce a similar molecular modification. It is noteworthy in this regard that recent studies have demonstrated a calcium-dependent activation of TH in extracts of corpus striatum that presumably involves a calcium-dependent protein kinase (El Mestikawy *et al.*, 1983; Iuvone, 1984). Should a protein kinase prove to be involved in the activation of tyrosine hydroxylase by photic stimulation, it will be of interest to establish the molecular link between membrane depolarization and activation of the kinase.

The regulation of DA biosynthesis, release, and metabolism is also influenced by a dopamine receptor-mediated feedback mechanism. Administration of dopamine receptor antagonists to rats increased retinal DOPA accumulation, TH activity, and the concentrations of DOPA and HVA (Westerink and Korf, 1976a; DaPrada, 1977; Scatton *et al.*, 1977; Cohen *et al.*, 1981, 1983; Iuvone, 1983; Malamed *et al.*, 1983). In addition, DA receptor antagonists were found to increase electrical stimulation-induced release of [^{3}H]DA from isolated rabbit retina (Dubocovich and Weiner, 1981; Dubocovich, 1984). DA receptor agonists have been reported to inhibit all of these indices of dopaminergic function. The ability of some agonists and antagonists with a high degree of selectivity for the D_2 subtype of DA receptors to elicit these effects implicates D_2 receptors in this feedback mechanism, and it has been suggested that the effects are mediated by

"autoreceptors" that are located on the membranes of DA-containing neurons (Dubocovich, 1984). Selective stimulation of D_2 dopamine receptors in other tissues has been reported to decrease adenylate cyclase activity (Cote *et al.*, 1981; Onali *et al.*, 1981), and it is tempting to speculate that some of the effects of these drugs in retinal DA-containing neurons, particularly those on TH activity, may be due to changes in cAMP.

B. Regulation of Dopamine Receptors and Dopamine-Stimulated Adenylate Cyclase

According to the classification of Kebabian and Calne (1979), the D_1 subtype of DA receptors is linked to adenylate cyclase in a stimulatory manner. In the retina, DA is a potent stimulator of adenylate cyclase activity (Brown and Makman, 1972), and it has been suggested that D_1 receptors are the predominant subtype of DA receptor in the vertebrate retina (Watling *et al.*, 1979). In the carp retina, 6-hydroxydopamine, a neurotoxin that causes the degeneration of retinal DA-containing neurons, failed to decrease DA-stimulated cAMP accumulation, suggesting that the D_1 receptors are postsynaptic rather than autoreceptors (Watling *et al.*, 1982). Accumulation of cAMP in carp retina has been shown to be stimulated by DA, by depolarizing concentrations of potassium, and by flashing red light (Dowling and Watling, 1981). The effects of both potassium and photic stimulation were blocked by haloperidol, a DA receptor antagonist, suggesting that they were mediated by synaptically released dopamine. The effect of potassium was also blocked by cobalt, which inhibits neurotransmitter release.

Dopamine-stimulated adenylate cyclase activity in retinas of other vertebrate species is also influenced by photic stimulation. For example, the accumulation of cAMP in isolated rabbit retina was shown to be stimulated by incubation with the DA precursor L-DOPA, and the L-DOPA-stimulated accumulation was greater in retinas incubated in light than in darkness (Sovilla and Schorderet, 1982). The effect of L-DOPA on cAMP accumulation in intact retina was blocked by the decarboxylase inhibitor benzserazide, and L-DOPA had no direct effect on adenylate cyclase activity in tissue homogenates. These observations suggest that the accumulation of cAMP is mediated by DA which is synthesized from L-DOPA and released.

Dopamine receptor-stimulated andenylate cyclase activity is down-regulated by persistent occupation of the receptor by the putative transmitter. Incubation of cultured retinal cells from chick embryos with DA for 5–48 hr followed by washing with DA-free medium dramatically decreased the subsequent ability of DA to stimulate cAMP accumulation (DeMello *et al.*, 1982). The stimulation of cAMP accumulation by DA was also much lower in retinas of chicks that were exposed to constant light for several days than in retinas of light-deprived chicks,

and the effect of constant light was reversed by administration of fluphenazine, a DA receptor antagonist (DeMello *et al.*, 1982). Constant light exposure also decreased the number of binding sites for the dopamine receptor antagonist [^{3}H]spiroperidol. Thus, it appears that prolonged release of DA and subsequent occupation of postsynaptic receptors trigger regulatory processes in the postsynaptic cells that decrease the responsiveness to the putative transmitter. Conversely, light deprivation for 65 hr increased DA-stimulated cAMP formation in the rat retina (Spano *et al.*, 1977), suggesting that a lack of receptor occupancy leads to receptor supersensitivity.

A rapid down-regulation of DA-stimulated adenylate cyclase has recently been demonstrated in bovine retina (Gnegy *et al.*, 1984a). DA-stimulated adenylate cyclase activity was significantly lower in a washed particulate (membrane) fraction prepared from retinas that were preincubated in light for 20 min than in that prepared from retinas that were preincubated in darkness. Basal adenylate cyclase activity in this preparation was not influenced by prior exposure of the retina to light or dark. Preincubation in light or dark altered the apparent V_{max} of DA-stimulated activity without changing the K_a for DA. Addition of DA to the preincubation medium decreased DA-stimulated adenylate cyclase activity of samples preincubated in darkness to the same level as that in samples preincubated in light, and had no additive effect with light. Thus, transmitter-induced alterations in postsynaptic responsiveness can occur rapidly.

Calcium and calmodulin may be involved in the regulation of DA-stimulated adenylate cyclase activity. Microdissected inner plexiform layer of rabbit and ground squirrel retinas contains adenylate cyclase activity that is stimulated by dopamine and inhibited by the calcium chelator ethylene glycol tetraacetate (EGTA) (DeVries *et al.*, 1982). In the mouse retina, basal adenylate cyclase activity was inhibited by low concentrations of EGTA (50 μM), and DA-stimulated activity was inhibited by higher concentrations (>1 mM) of the chelator (Ferrendelli *et al.*, 1982).

Calcium significantly influences the DA receptor/adenylate cyclase complex in isolated horizontal cells of carp retina (Van Buskirk and Dowling, 1982). Raising the concentration of calcium in the culture medium increased DA-stimulated cAMP accumulation in the horizontal cells, presumably by increasing intracellular calcium levels. Calcium did not influence basal cAMP accumulation or the sensitivity to DA, but increased the magnitude of the maximal response to the putative transmitter. In addition, calcium decreased the sensitivity of the cells to DA receptor antagonists with respect to their ability to inhibit DA-stimulated cyclic AMP accumulation. L-Glutamate, which depolarizes and produces a prolonged Ca^{2+}-dependent action potential in isolated horizontal cells (Lasater and Dowling, 1981), also decreased the sensitivity to the antagonists, presumably by increasing intracellular Ca^{2+} (Van Buskirk and Dowling, 1982).

Calmodulin may play a role in modulating DA-stimulated adenylate cyclase

activity in retina. Calmodulin has been shown to stimulate, in a calcium-dependent manner, adenylate cyclase activity in an EGTA-washed particulate fraction of homogenized bovine retina (Gnegy *et al.*, 1984b). In addition, calmodulin doubled the maximal stimulation of cyclase activity by DA. Preincubation of retinas for 20 min in light or dark altered calmodulin-stimulated activity in a manner that was analogous to the effects of light and dark on DA-stimulated activity (Gnegy *et al.*, 1984a). The V_{max} values of calmodulin-stimulated activity and that of DA-stimulated activity were both lower in samples that had been preincubated in light than in those preincubated in darkness. Light and dark had no effect on the apparent K_a for calmodulin or on the sensitivity of the enzyme to DA. Furthermore, preincubation in darkness in the presence DA reduced the maximal stimulation of activity by calmodulin to the level of that in retinas preincubated in light, while addition of DA in light had no effect. These observations suggest that light, by stimulating dopamine release and receptor occupancy, decreased calmodulin-stimulated adenylate cyclase activity and indicate that calmodulin may be a modulator of DA-sensitive adenylate cyclase.

C. Regulation of Melatonin Biosynthesis

As described above, the putative neuromodulator melatonin is synthesized in the retina, and the site of synthesis has been proposed to be photoreceptor cells (see Section II,A). Melatonin is synthesized from serotonin by the sequential actions of serotonin *N*-acetyltransferase (NAT) and hydroxyindole *O*-methyltransferase (Fig. 5). In the pineal gland, NAT is thought to be the rate-limiting enzyme (Klein and Weller, 1971), and this enzyme may play a key role in the retina as well. In many mammalian and nonmammalian species, the concentration of melatonin and the activity of NAT fluctuate in the retina in a rhythmic fashion, with peak enzyme activity and melatonin content at night (Bubenick *et al.*, 1978; Binkley *et al.*, 1979, 1980; Hamm and Menaker, 1980; Miller *et al.*, 1980; Pang *et al.*, 1982; Iuvone and Besharse, 1983). In the chicken and the African clawed frog (*X. laevis*), and probably other species as well, the rhythm of retinal NAT activity is circadian (Hamm and Menaker, 1980; Binkley *et al.*, 1980; Iuvone and Besharse, 1983). The rhythm of NAT activity was shown to be entrained by light and dark, and following entainment, to persist for several days in constant darkness. In contrast, constant light for several days abolished the rhythm, and exposure to light during the dark phase of the light–dark cycle inhibited the rise in enzyme activity and melatonin levels in the retina.

Experiments have recently been conducted to determine if the biological clock that controls the rhythm is localized in the eye or if it is dependent upon endocrine or neural input. Frogs (*X. laevis*) were entrained to a 12-hr light–12-hr dark cycle. Eye cups were then prepared and cultured *in vitro* in cyclic light or

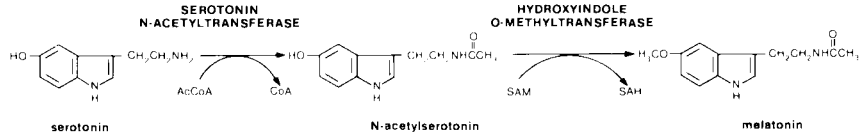


FIG. 5. Reactions in the biosynthesis of melatonin. AcCoA, Acetyl coenzyme A; CoA, coenzyme A; SAM, *S*-adenosylmethionine; SAH, *S*-adenosylhomocysteine.

constant darkness (Besharse and Iuvone, 1983). Figure 6 shows that a rhythm of NAT activity similar to that observed *in vivo* persisted *in vitro* in both cyclic light and in constant darkness. In another experiment, eye cups were cultured for 48 hr on a light–dark cycle that was the inverse of the original entrainment cycle. The rhythm of NAT activity was subsequently examined during a 30-hr period of constant darkness and was found to be completely shifted to the new entrainment cycle (Besharse and Iuvone, 1983). Thus, both the clock and the entrainment mechanism appeared to be localized to the posterior portion of the eye, possibly in the retina.

The eye cup preparation has also been used to study potential regulatory factors that influence the expression of NAT activity. Protein synthesis, possibly enzyme induction, may be involved in the stimulation of NAT activity. The protein synthesis inhibitors cycloheximide and puromycin blocked the nocturnal rise in NAT activity (Iuvone and Besharse, 1983). However, because of potential toxic effects of the protein synthesis inhibitors, these observations must be interpreted with caution.

Calcium plays an important role in the stimulation of NAT activity by darkness (Table I). The dark-induced increase of NAT activity was completely abolished when eye cups were incubated in calcium-free medium (Iuvone and Besharse, 1986a). The calcium channel blockers, nifedipine and D600, as well as magnesium and cobalt, which inhibit neurotransmitter release by interfering with depolarization-induced calcium influx, also inhibited the nocturnal rise in NAT activity. The site of the action of calcium is not known. Perhaps the NAT-containing cells have an endogenous rhythm of membrane potential with conse-

FIG. 6. Circadian rhythm of retinal serotonin *N*-acetyltransferase activity in eye cups maintained in culture. Eye cups of *X. laevis*, which had been entrained to a light cycle of 12 hr light–12 hr dark, were prepared 2 hr prior to the time of normal light offset (1200 hr). Eye cups were subsequently cultured in cyclic light or constant darkness for 1–3 days. Retinas were removed and assayed for NAT activity at 6-hr intervals. Open circles represent retinas prepared in light; solid circles represent those prepared in dim red light (Kodak Wratten No. 2 filter). The first time point (1200 hr) represents samples taken 2 hr after preparation of the cultures in A and B, and 50 hr after preparation in C. Statistically significant rhythms of NAT activity were observed under all three conditions ($p < 0.01$). (Reproduced from Besharse and Iuvone, 1983, by permission from *Nature*, Vol. 305, No. 5930, pp. 133–135. Copyright © 1983 Macmillan Journals Limited.)

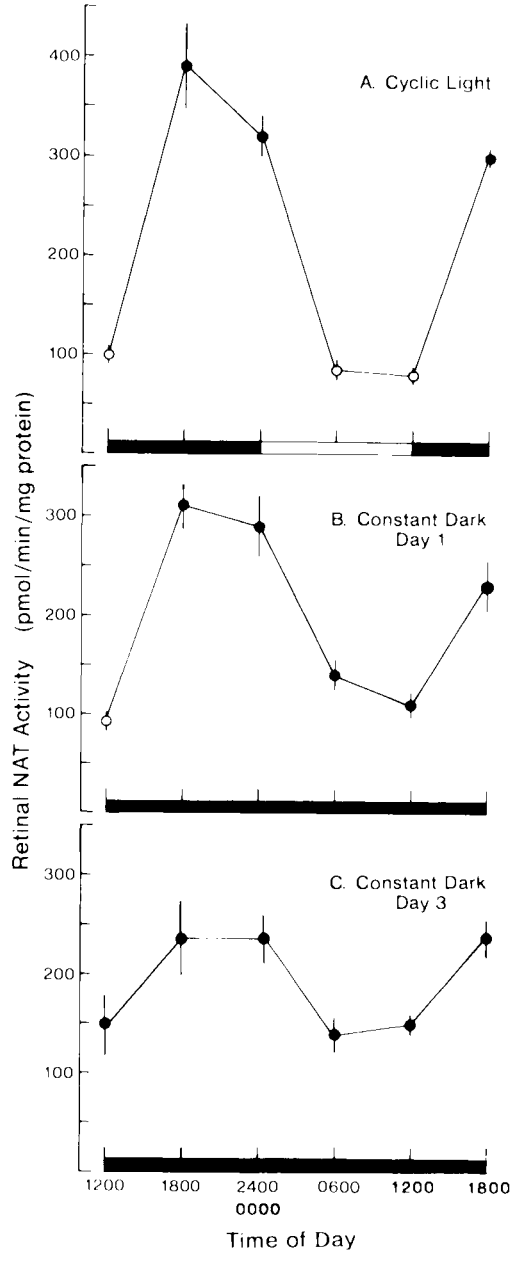

A. Cyclic Light
B. Constant Dark
Day 1
C. Constant Dark
Day 3
Retinal NAT Activity (pmol/min/mg protein)
400
300
200
100
300
200
100
300
200
100
1200
1800
2400
0000
0600
1200
1800
Time of Day

TABLE I

THE ROLE OF CALCIUM IN THE STIMULATION OF RETINAL *N*-ACETYLTRANSFERASE ACTIVITY[a]

Condition	*N*	Percentage stimulation
Control	15	150
Ca^{2+} omission	5	0
Nifedipine, 3 μM	5	49
D600, 0.1 m*M*	5	49
Cobalt, 0.5 m*M*	5	7
Magnesium, 15 m*M*	5	0

[a] Eye cups were incubated in the dark for 6 hr. Data are expressed as the stimulation of activity that occurred during the 6-hr period. Control medium and media with nifedipine, D600, cobalt, and magnesium contained 1.8 m*M* $CaCl_2$. All manipulations significantly inhibited stimulation of activity, $p < 0.01$.

quent calcium influx during depolarization. The rhythm might then be generated by a clock localized within the NAT-containing cell and could be independent of synaptic influences. Alternatively, calcium might be required to maintain neurotransmitter release, and the regulation of the enzyme might be subject to transsynaptic influences. Pharmacological evidence for neurotransmitter-mediated neural control of NAT activity and of photoreceptor metabolism will be discussed below (see Section IV,B, V,A, and V,B).

Retinal NAT activity is stimulated by increased levels of intracellular cyclic AMP. When eye cups were incubated under light during the subjective dark phase of the light–dark cycle, the rise in NAT activity was blocked (Iuvone and Besharse, 1983; Iuvone and Besharse, 1986b). Addition of dibutyryl cyclic AMP (db cAMP) or 8-bromo cAMP to the incubation medium under these conditions stimulated NAT activity, while addition of dibutyryl cyclic GMP, 5′-AMP, or adenosine was without effect. The stimulation of NAT activity by db cAMP was not additive to that produced by darkness (Fig. 7), suggesting the involvement of similar mechanisms. Increased levels of endogenous cyclic AMP elicited by forskolin, an activator of adenylate cyclase, or by 3-isobutylmethylxanthine, a phosphodiesterase inhibitor, also mimicked darkness by stimulating NAT activity. The effects of db cAMP and forskolin did not require calcium in the medium, suggesting that the site of action of cAMP was within the NAT-containing cell or in a cell that communicates with it in a Ca^{2+}-independent manner.

Based on these observations it is tempting to speculate that darkness or a

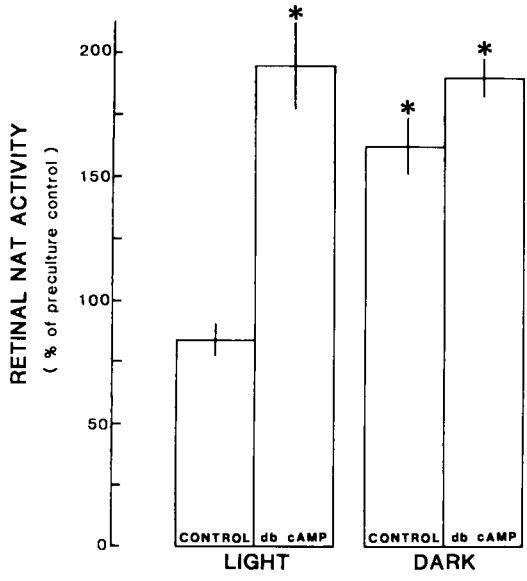


FIG. 7. Effects of db cAMP on serotonin *N*-acetyltranferase activity of retinas cultured in light or dark. Eye cups were prepared just prior to light offset (1200 hr) and were subsequently cultured for 4 hr in light or dark, with or without 2 m*M* db cAMP. Data are expressed as percentage of preculture control, which was the activity in retinas prepared at the time of light offset, but not subsequently cultured. $*p < 0.01$. (Reproduced from Iuvone and Besharse, 1983.)

stimulus provided by the clock elicits the Ca^{2+}-dependent release of a neurotransmitter or neuromodulator that stimulates adenylate cyclase activity in the NAT-containing cell. However, a candidate for such a mediator has not yet been identified. Alternatively, an endogenous rhythm of adenylate cyclase activity, possibly mediated by an endogenous rhythm of Ca^{2+} influx, might regulate NAT activity. The inhibitory effect of light might be a direct effect of light on the NAT-containing cell, which may be a photoreceptor, or could be mediated by a transmitter–modulator that is released by other cells in response to light. With respect to this latter possibility, dopamine has been identified as a possible mediator of the effect of light (see Section IV,B).

IV. Interactions of Neurotransmitters and Neuromodulators

With an increased understanding of the localization of putative transmitters and modulators in retina, it has been possible to examine pharmacologically the interactions of various synaptic mediators. Knowledge of these interactions

should facilitate the elucidation of the functional synaptic circuitry of the retina and the processing of visual stimuli. In addition, the utility of the retina as a pharmacological model with transmitter systems that can be experimentally modulated by a physiological stimulus cannot be overemphasized. To illustrate some approaches that can be used to study interactions in retina, this section will discuss studies that attempt to understand the nature of the input to the DA-containing and melatonin-containing cells of retina.

A. Dopamine

DA-containing neurons of retina are activated by photic stimulation, and a number of putative transmitters and modulators have been examined as potential mediators of the effects of light and dark on their neuronal activity. DA-containing neurons in the retina of teleost fish are primarily interplexiform cells (see Section II,E). They have been shown to alter the receptive field properties of bipolar and horizontal cells and to inhibit the carrier-mediated release of GABA from H_2 horizontal cells (Hedden and Dowling, 1978; Negishi and Drujan, 1979a; Yazulla and Kleinschmidt, 1982). Acetylcholine, carbachol, a cholinergic agonist, and substance P were found to produce a contraction of the receptive field of cone-driven horizontal cells that was similar to that elicited by DA (Negishi and Drujan, 1979b; Laufer *et al.*, 1981). The effect of these agents were blocked by phentolamine and haloperidol, suggesting that their influence on horizontal cells was mediated by DA released from interplexiform cells. This hypothesis is supported by histochemical studies that demonstrated a decreased catecholamine histofluorescence in retinas inbucated with carbachol or substance P (Negishi *et. al.*, 1980). There is also evidence for the involvement of serotonin in the regulation of DA-containing interplexiform cells of fish retina. Serotonin stimulated the release of [^{3}H]DA from a particulate fraction of fish retina (Kato *et al.*, 1982). The serotonin-induced release of [^{3}H]DA was Ca^{2+} dependent and could be simulated by serotonergic agonists, 5-methoxytryptamine, 5-methoxy-*N*,*N*-dimethyltryptamine, and tryptamine. In contrast, DA had no effect on the release of [^{3}H]serotonin. Thus, serotonin, substance P, and ACh may influence the activity of DA-containing interplexiform cells of fish retina.

In rat and rabbit retinas, where the DA-containing neurons are primarily amacrine cells (see Section II,D), ACh does not appear to influence DA neuronal activity. Carbachol had no effect on [^{3}H]dopamine release from rabbit retina (Bauer *et al.*, 1980). In rat retina, the cholinergic agonists carbachol, oxotremorine, and nicotine, as well as atropine, an antagonist, had no effect on TH activity (P. M. Iuvone, unpublished observations). Oxotremorine was also found to be without effect on the levels of the DA metabolite HVA (Westerink and Korf, 1976) and atropine had no effect on DA turnover (Kamp and Morgan,

1981). However, ACh may influence the postsynaptic response to dopamine. Carbachol and oxotremorine produced a large potentiation of DA-stimulated cyclic AMP accumulation in rat retina and the potentiation was blocked by atropine (Brown and Rietow, 1981). The effects of the muscarinic cholinergic agonists and DA were synergistic and did not require calcium in the incubation medium, suggesting that the cholinergic drugs were not producing their effects by stimulating the release of other neurotransmitters. The synergism was apparently due to an effect of the cholinergic agonists on cyclic AMP formation rather than breakdown, because it was not attenuated by high concentrations of phosphodiesterase inhibitors. These observations suggest that DA and ACh may interact to regulate adenylate cyclase activity in cells that are postsynaptic to both cholinergic and DA-containing amacrine cells.

Dopamine-containing amacrine cells are apparently activated by light (see Section II,D), but the nature of the stimulatory input to these cells is not known. α-MSH has been reported to increase the release of [^{3}H]dopamine from rabbit retina (Bauer *et al.*, 1980), but without specific α-MSH receptor antagonists, it is difficult to determine if this peptide plays a role in the light-induced activation. Quisqualate, a glutamate analog, stimulated *in vivo* tyrosine hydroxylation, an index of DA synthesis, in the dark-adapted rat retina (Kamp and Morgan, 1983). This stimulatory effect was not produced by the excitatory amino acid analogs kainate and *N*-methyl-D-asparate. Thus, glutamate or a related compound may play a role in the regulation of DA-containing amacrine cells. However, excitatory amino acid antagonists that specifically block the effects of quisqualate and of light on the dopamine neurons have not yet been identified.

Several types of receptors have been identified that mediate inhibition of the DA-containing amacrine cells. As described in Section III,A, DA synthesis and release are modulated by a DA receptor-mediated feedback mechanism that may involve inhibitory autoreceptors. In addition, α_2-adrenergic receptors, GABA receptors, and opiate receptors appear to be involved in the inhibitory input to the DA-containing amacrine cells.

Radioligand-binding studies have suggested the presence in retina of α_2-adrenergic receptors (Bittinger *et al.*, 1980; Osborne, 1982). Systemic administration of yohimbine, an α_2-adrenoceptor antagonist, increased the activity of TH (Table II), the turnover of DA, and the concentration of DOPAC in retinas of dark-adapted or light-exposed rats (Iuvone and Rauch, 1983; Hadjiconstantinou *et al.*, 1984a). Similar effects on TH activity and DOPAC levels were observed with another selective α_2-antagonist, piperoxan, but not with prazosin, an α_1-adrenoceptor antagonist, or propranolol, a β-adrenoceptor antagonist. The effect of the α_2-adrenoceptor antagonists were apparently mediated within the retina. A low dose (2 nmol) of yohimbine injected into the vitreous was extremely effective in activating retinal TH (Iuvone and Rauch, 1983). The maximally effective dose (10 mg/kg, ip) of systemically administered yohimbine and the EC_{50} (4.5

TABLE II

EFFECTS OF CLONIDINE AND YOHIMBINE ON RETINAL TYROSINE HYDROXYLASE ACTIVITY IN LIGHT AND DARK[a]

Condition	Tyrosine hydroxylase activity
Dark	
+ Saline	4.3 ± 0.3
+ Clonidine	4.7 ± 0.7
+ Yohimbine	11.0 ± 1.9
+ Clonidine and yohimbine	7.1 ± 0.8
Light	
+ Saline	9.8 ± 0.8
+ Clonidine	5.6 ± 0.5
+ Yohimbine	14.4 ± 0.5
+ Clonidine and yohimbine	11.5 ± 0.8

[a] Dark-adapted rats injected ip with clonidine HCl and yohimbine HCl 45 and 30 min, respectively, prior to sacrifice either in darkness or following 10–30 min of light exposure. Adapted from Iuvone and Rauch (1983).

mg/kg, ip) for activating retinal TH were similar to those observed for activating TH in noradrenergic neurons in brain (Iuvone and Rauch, 1983), where α_2-adrenergic autoreceptors are thought to be involved in the regulation of neuronal firing rate (Cedarbaum and Aghajanian, 1976, 1977). Administration of clonidine, an α_2-adrenoceptor agonist, inhibited the effect of yohimbine (Table II). Clonidine administered alone had no effect on retinal TH activity or DOPAC levels in darkness, but decreased these measures of dopaminergic activity in light (Iuvone and Rauch, 1983; Hadjiconstantinou *et al.*, 1984a). Two other relatively specific α_2-adrenoceptor agonists, naphazoline and oxymetazoline, were also effective in reducing retinal DOPAC in the light, while phenylephrine, which is relatively selective for α_1-adrenoceptors, had no effect (Hadjiconstantinou *et al.*, 1984a).

α_2-Adrenoceptors have also been reported to modulate the electrical stimulation-evoked release of [^{3}H]DA from rabbit retina (Dubocovich, 1984b). Clonidine, epinephrine, norepinephrine, dopamine, and apomorphine all inhibited the evoked release of [^{3}H]DA. The effect of clonidine was blocked by yohimbine. In contrast, the inhibitory effect of apomorphine, a DA receptor agonist, was blocked by DA receptor antagonists, but not by yohimbine (Dubocovich and Weiner, 1981; Dubocovich, 1984).

These observations provide evidence for an endogenous neurotransmitter or neuromodulator in the retina that acts through α_2-adrenoceptors to inhibit the activity of DA-containing amacrine cells. The ability of α_2-adrenoceptor agonists to decrease retinal TH activity and DOPAC levels in light but not in

darkness, combined with the effectiveness of the antagonist in both conditions (Iuvone and Rauch, 1983; Hadjiconstantinou *et al.*, 1984a), suggests that the relevant α_2-adrenoceptors may be maximally stimulated by the endogenous ligand in the dark and only partially occupied in the light. The recent discovery of epinephrine-containing amacrine cells in rat retina (Hadjiconstantinou *et al.*, 1983, 1984b; see Section II,D) has led to the suggestion that epinephrine, which is a potent activator of α_2-adrenoceptors (Starke *et al.*, 1975), may be the endogenous ligand of these receptors (Iuvone and Rauch, 1983; Hadjiconstantinou *et al.*, 1984a).

Enkephalin analogs and morphine have also been shown to inhibit the evoked release of [^{3}H]DA from isolated rabbit retina (Dubocovich and Weiner, 1983). Naloxone, an opiate receptor antagonist, had no effect alone on [^{3}H]DA release, but stereospecifically blocked the inhibitory effects of opiate peptides and morphine. Thus, an endogenous opiate-like compound might influence the activity of dopamine-containing amacrine cells in rabbit retina. However, the possible involvement of an opiate system in rat retina is less clear. A high dose of morphine had no effect on HVA levels and produced a small increase in the levels of DOPAC in rat retina (Westerink and Korf, 1976; Barbaccia *et al.*, 1982). In contrast, morphine elicited large increases of both DOPAC and HVA in the corpus striatum of rat brain (Westerink and Korf, 1976b; Barbaccia *et al.*, 1982), and the effective dose of morphine that elicited the increase in striatal DOPAC was substantially lower than that required to increase DOPAC in retina. The ability of naloxone to block the effect of morphine on retinal DOPAC levels was not reported. Thus, it is not clear that the effect of morphine on retinal DOPAC was mediated by specific opiate receptors.

Substantial evidence supports an inhibitory influence of GABA on the DA-containing amacrine cells in the dark-adapted rat retina. Systemic administration of muscimol, a GABA receptor agonist, inhibited the light-evoked increase of DA turnover in retina (Morgan and Kamp, 1980). This effect was apparently mediated by retinal GABA receptors because intravitreal administration of GABA or muscimol inhibited the activation of retinal TH by light (Marshburn and Iuvone, 1981; Fig. 8), and the inhibitory effects of both systemically administered or intravitreally administered muscimol were blocked by the GABA antagonist picrotoxin. Increasing the concentration of endogenous retinal GABA in the extracellular space by administering inhibitors of GABA uptake and of the GABA-metabolizing enzyme GABA-transaminase also inhibited *in vivo* tyrosine hydroxylation in light-exposed retinas (Proll and Morgan, 1983). Intravitreal administration of the GABA antagonists bicuculline and picrotoxin (2 nmol or greater) activated TH and increased *in vivo* tyrosine hydroxylation and dopamine turnover in dark-adapted retinas (Marshburn and Iuvone, 1981; Kamp and Morgan, 1981; Morgan and Kamp, 1983). Thus, GABA-containing amacrine cells appear to exert a tonic inhibitory influence on the dopamine-containing neurons in the dark.

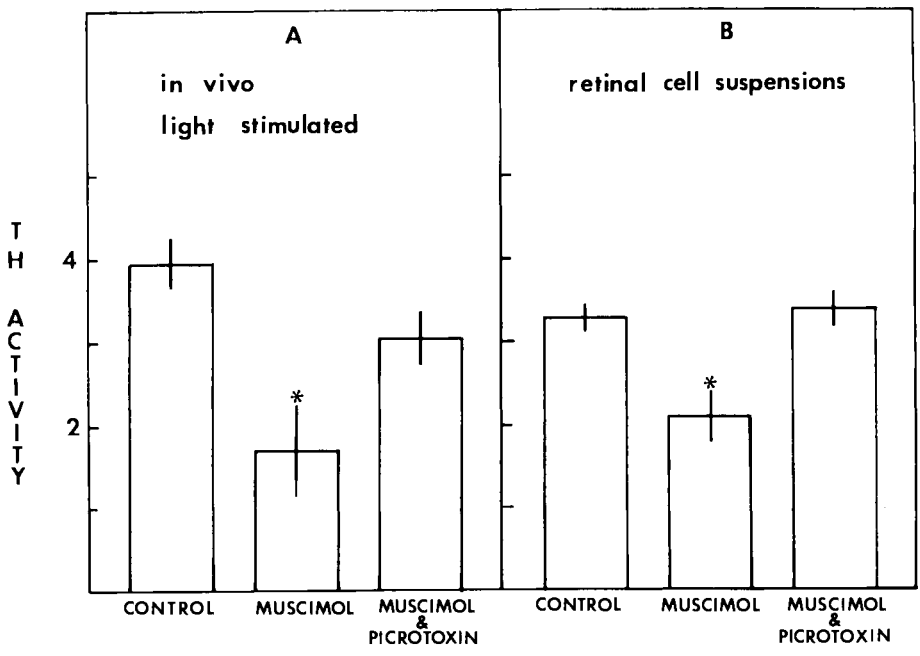


FIG. 8. Effect of muscimol and picrotoxin on retinal tyrosine hydroxylase activity. (A) Dark-adapted rats were injected subcutaneously with saline or picrotoxin (4 μmol/kg). Ten minutes after the subcutaneous injection, the subjects were injected intravitreally with saline or muscimol (0.4 nmol) in a volume of 5 μl. Retinas were dissected and frozen 25 min after intravitreal injection, following 12–18 min of light exposure. Tyrosine hydroxylase activity was measured at 37°C in the 32,000 *g* supernatant fraction of retinal homogenates (Marshburn and Iuvone, 1981). (B) Viable cell suspensions of dark-adapted retinas were prepared by a modification of the technique of Sarthy and Lam (1979). The cell suspensions were incubated for 30 min at 37°C under an atmosphere at 95% O_2/5% CO_2 in Ringer's solution with muscimol (1 μ*M*) and picrotoxin (20 μ*M*). Following incubation, the suspensions were centrifuged at 250 *g* to pellet the cells. The pellets were frozen, homogenized, and assayed for tyrosine hydroxylase activity. Activity is expressed as pmol/min/mg protein. $^*p < 0.01$. (Adapted from Iuvone and Marshburn, 1981, and unpublished data.)

In addition to this inhibitory role of GABA, there is some evidence that stimulation of some GABA receptors in retina may facilitate the light-evoked activation of DA-containing amacrine cells (Marshburn and Iuvone, 1981). Dose–response studies of the effects of intravitreally administered muscimol on light-evoked TH activation indicated that muscimol produced a dose-related biphasic effect. Low doses of the GABA agonist (35 and 60 pmol) enhanced the light-evoked activation of TH, while higher doses (>120 pmol) were inhibitory. A dose of 300 pmol completely inhibited the light-evoked activation of the enzyme. In contrast, neither low nor high doses of muscimol influenced TH

activity in dark-adapted retinas. The GABA antagonists bicuculline and picrotoxin at low doses (0.4–0.5 nmol) produced a small inhibition of the light-evoked activation of the enzyme, while at higher doses they activated the enzyme in the dark and attenuated the further activation of the enzyme by light.

In order to investigate the possibility that the inhibitory or the facilitatory actions of muscimol might be mediated by receptors located on the DA-containing neurons, dark-adapted retinas were dissociated into suspensions of viable cells (Marshburn and Iuvone, 1981). TH activity in these suspensions, which were composed largely of single cells, was higher than in dark-adapted retinas, suggesting that dissociation released the enzyme from a tonic inhibitory influence. Addition of muscimol over a large concentration range produced a concentration-dependent decrease in TH activity. Augmentation of activity was not observed. As was the case *in vivo*, the inhibitory effect of muscimol was blocked by picrotoxin (Fig. 8).

These observations suggest that the dopamine-containing amacrine cells possess inhibitory GABA receptors. The significance of the apparently indirect facilitatory effect of muscimol is not known. Immunohistochemical localization of GAD- and GABA-containing amacrine cells in rat retina indicate that they are postsynaptic to bipolar cells and provide synaptic inputs to bipolar cells, to unlabeled amacrine cells, and to other GAD-positive amacrine cells (Vaughn *et al.*, 1981). Perhaps the low doses of muscimol act preferentially at GABA autoreceptors involved in synapses between GABA-containing processes and act to decrease the release of GABA onto the DA-containing neurons. Such a mechanism would be plausable if GABA autoreceptors are more sensitive than are the GABA receptors located on the DA-containing neurons. Regardless of the mechanism of the facilitatory effect of muscimol, it is clear that the DA-containing neurons of rat retina are subject to tonic inhibition in the dark that appears to be mediated by synaptic input from GABA-containing amacrine cells.

Dopamine-containing amacrine cells may also be subject to inhibition by the putative neuromodulator melatonin. Melatonin inhibited the stimulation-evoked release of [^{3}H]DA from isolated rabbit retina in a concentration-dependent manner (Dubocovich, 1983). Melatonin was remarkably potent and inhibited Ca^{2+}-dependent evoked release by 50% at a concentration of 9 p*M* (Fig. 9). The melatonin precursor *N*-acetylserotonin was approximately three orders of magnitude less potent than melatonin, while the putative neurotransmitter serotonin was ineffective as an inhibitor of [^{3}H]DA release. Of the other related methoxyindoles tested, only 5-methoxytryptamine inhibited [^{3}H]dopamine release, and it was substantially less potent than melatonin; 5-methoxy-*N*,*N*-dimethyltryptamine and 5-methoxytryptophol were inactive at the concentrations tested (Dubocovich, 1984c). Thus, a melatonin-like receptor appears to influence [^{3}H]DA release. Melatonin may participate in the inhibition of DA-containing amacrine cells in the dark, when melatonin release from retinal cells is appar-

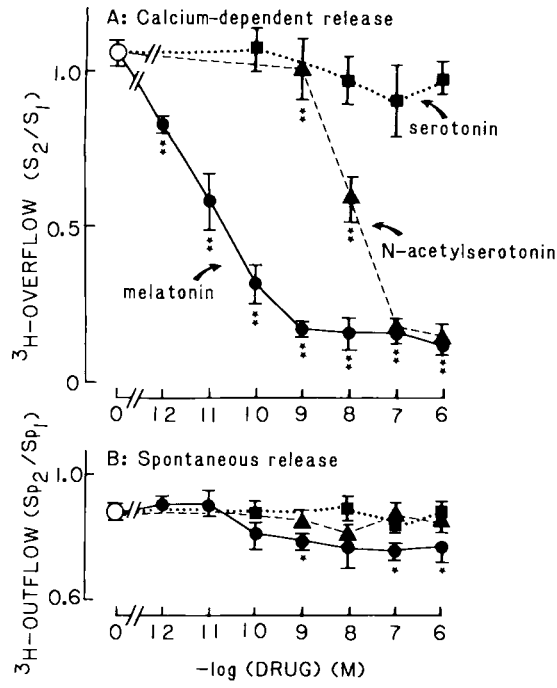


FIG. 9. Effect of serotonin, *N*-acetylserotonin, and melatonin on the spontaneous and calcium-dependent release of [^{3}H]dopamine from rabbit retina. Ordinates: (A) ^{3}H-labeled overflow is the percentage of total tissue radioactivity released by field stimulation above the spontaneous levels of release. Results are expressed as the ratio obtained between the second (S_2) and the first (S_1) stimulation periods within the same experiment. The calcium-dependent release of [^{3}H]dopamine was elicited by a 1-min period of field stimulation at 3 Hz (20 mA, 2 msec). In the controls, the percentage of the total tissue radioactivity released after the first 1-min period of stimulation (S_1) was 1.49 ± 0.21% (n = 8). The radioactivity retained by the control tissue after 120 min superfusion was 47.3 ± 4.8, nCi/chamber (n = 8). (B)^{3}H-labeled outflow is the percentage of total tissue radioactivity release in the 4-min sample preceding the second (Sp_2) and the first (Sp_1) period of field stimulation, expressed as the ratio SP_2 : SP_1. In the controls, the spontaneous outflow calculated as the percentage of total tissue radioactivity released during the 4-min preceding the first period of stimulation (Sp_1) was 1.65 ± 0.13% (n = 8) and the second stimulation (Sp_2) was 1.17 ± 0.14% (n = 8). The ratio SP_2 : SP_1 was 0.88 ± 0.02. Abscissas: Molar concentrations of the drugs (logarithmic scale). Drugs were added 20 min before S_2. Only one concentration of each drug was tested per experiment: ○, control; ●, melatonin; ▲, *N*-acetylserotonin; ■, serotonin. Shown are mean values ±SEM of three to eight experiments per group. $*p < 0.05$; $**p < 0.001$ when compared with the corresponding control. (Reprinted from Dubocovich, 1983, by permission from *Nature*, Vol. 306, No. 5945, pp. 782–784. Copyright © 1983 Macmillan Journals Limited.)

ently elevated (Yu *et al.*, 1982). It is not known if melatonin acts directly on the DA-containing cells or if its actions are mediated transsynaptically through the release of other neurotransmitters or modulators.

In summary, DA-containing amacrine cells have been shown to be activated by α-MSH and by quisqualate, a glutamate analog, and to be inhibited by DA, GABA, α_2-adrenergic agonists, opiate receptor agonists, and melatonin. The effect of GABA appears to be direct and appears to provide a tonic inhibitory input to the DA-containing cells in the dark. The synaptic regulation of the dopamine-containing amacrine cells of rabbits and rats show similarities, but interspecies differences may also exist.

B. Melatonin

Knowledge of the regulation of the retinal melatonin-synthesizing cells of retina is still relatively scarce; in fact the retinal cell type that synthesizes melatonin has not been conclusively identified. Based on the histochemical localization of melatonin-like immunoreactivity and on the homology between photoreceptors and pinealocytes of some lower vertebrates, a type of photoreceptor is a likely candidate for the melatonin-synthesizing cells of retina (see Section II,A). Although there is little evidence for synaptic input to photoreceptors, several lines of evidence suggest that the regulation of retinal serotonin *N*-acetyltransferase activity is influenced by humoral factors, possibly neurotransmitters or neuromodulators. For example, the stimulation of NAT activity in darkness is a calcium-dependent process that may involve voltage-gated Ca^{2+} channels, as does neurotransmitter release, and the effects of darkness are mimicked by increasing levels of cyclic AMP, which is a second messenger for several neurotransmitters and hormones (see Section III,C). Although other explanations for the calcium dependency and apparent involvement of cyclic AMP cannot be excluded, the involvement of neurotransmitters or neuromodulators in NAT regulation is an attractive hypothesis.

A candidate for a stimulatory modulator that increases NAT activity has not yet been identified, but the activity of the enzyme appears to be suppressed by a catecholamine receptor-mediated mechanism. Intraocular injection of a large dose (23 mg/kg) of isoproterenol, a β-adrenergic receptor agonist, decreased NAT activity in chick retina (Binkley *et al.*, 1980). In isolated eye cups of *X. laevis*, all three endogenous catecholamines, DA, epinephrine, and norepinephrine, were effective in inhibiting the dark-induced increase of retinal NAT activity when present in the incubation medium at a concentration 50 μ*M* (Besharse *et al.*, 1984; Table III). Tests with a low concentration (1 μ*M*) of several agonists that are selective for different types of catecholamine receptors indicated that

only the dopamine receptor agonists were effective in suppressing the stimulatory effect of darkness; selective agonists of α_1-, α_2-, and β-adrenergic receptors were without effect (Iuvone and Besharse, 1986c; Table III). In addition, the DA uptake inhibitor benztropine was an effective inhibitor of the dark-induced stimulation of enzyme activity, presumably by raising the extracellular concentration of endogenous DA.

Concentration–response studies indicated that exogenous DA inhibited the stimulation of NAT activity in eye cups by 50% at a concentration of approximately 0.3 μ*M*. In contrast, addition of 100 μ*M* DA to retinal homogenates had no effect on NAT activity, indicating that the catecholamine does not directly inhibit the enzyme. This observation, coupled with the pharmacological specificity of the response, suggests the involvement of a dopamine receptor in the inhibition of the dark-dependent increase of the enzyme in retina.

Pharmacological studies to characterize the subtype of dopamine receptor involved in the regulation of NAT suggest that the effect of dopamine is mediated by a D_2 dopamine receptor (Iuvone, 1986). LY171555, a selective D_2 receptor agonist, inhibits the dark-dependent increase of retinal NAT activity, with half-maximal inhibition at 75 n*M*. In contrast, SKF38393, a D_1 receptor agonist, had no effect on NAT activity at 1 μ*M*. Furthermore, the effect of dopamine on NAT activity was antagonized by spiroperidol, a D_2 receptor antagonist, but not by SCH23390, a D_1 receptor antagonist.

The effects of DA on the stimulation of NAT activity by dibutyryl cyclic AMP

TABLE III

INHIBITION OF THE DARK-INDUCED STIMULATION OF RETINAL SEROTONIN *N*-ACETYLTRANSFERASE BY CATECHOLAMINES AND RELATED DRUGS[a]

Drug	Mechanism	Percentage inhibition
Dopamine, 50 μ*M*	—	92[b]
Norepinephrine, 50 μ*M*	—	70[b]
Epinephrine, 50 μ*M*	—	70[b]
Apomorphine, 1 μ*M*	Dopamine receptor agonist	94[b]
Bromocriptine, 1 μ*M*	Dopamine receptor agonist	67[b]
Phenylephrine, 1 μ*M*	α_1-Adrenoceptor agonist	0
Clonidine, 1 μ*M*	α_2-Adrenoceptor agonist	0
Isoproterenol, 1 μ*M*	β-Adrenoceptor agonist	17
Benztropine, 0.1 m*M*	Dopamine uptake inhibitor	76[b]

[a] Eye cups were cultured for 6 hr beginning at the time of light offset. Data are expressed as percentage inhibition of the increase in activity that occurred in controls cultured in the dark. Each group had four to five eye cups.

[b] Significant inhibition, $^*p < 0.01$.

and by IBMX in eye cups incubated in light were also examined (Iuvone, 1986). DA was found to be effective in blocking the stimulatory effect of the phosphodiesterase inhibitor IBMX, which presumably is mediated by accumulation of endogenously synthesized cyclic AMP. However, DA had no effect on the increase in activity elicited by dibutyryl cyclic AMP. Thus, the effect of dopamine on NAT activity appears to be mediated by a decrease in cyclic AMP formation. Whether this effect of DA is produced by a direct interaction with DA receptors on the melatonin-synthesizing cell or through a transsynaptic mechanism is not known. There is no evidence for synaptic input to photoreceptors from DA-containing neurons, making a direct interaction seem unlikely. However, the possibility that dopamine diffuses through the extracellular space to its site of action should not be overlooked. It is noteworthy that DA is effective at blocking the effect of IBMX in medium from which Ca^{2+} has been omitted to block synaptic transmission. The functional role of DA in the regulation of serotonin *N*-acetyltransferase activity is unknown. As described above, light suppresses the nocturnal rise in enzyme activity and appears to activate dopamine neurons. Perhaps dopamine mediates the inhibitory effect of light.

V. Cellular and Morphological Effects of Neurotransmitters and Neuromodulators

The effects of neurotransmitters and neuromodulators are generally thought of in terms of changes in the electrical activity of single cells, usually through the opening or closing of selective ion channels in the neuronal plasma membrane. However, recent studies on first- and second-order retinal neurons indicate that transmitters and modulators may also elicit morphological changes in neurons that influence their function in the system. This section will deal with a few examples of the effects of putative neurotransmitters and neuromodulators on cellular structure and function, including studies on cone photoreceptor movement, outer segment membrane turnover of rod photoreceptors, and the regulation of gap junctions between horizontal cells.

A. Cone Retinomotor Movement

In retinas of several lower vertebrate species, cone photoreceptors elongate at night and contract during the day, presumably to position the photosensitive membrane for optimal function in dim or bright light (see Burnside, Part I for a detailed discussion of the mechanism and regulation of cone movement). These retinomotor movements persist in constant darkness, indicating regulation by a

circadian clock (Welsh and Osborne, 1937). In the sunfish retina, cone movement has been shown to be influenced by intraocular injection of catecholamines (Dearry and Burnside, 1984). Intraocular administration of DA or epinephrine caused cones in the dark-adapted retina to contract. DA also inhibits cone elongation induced by forskolin and IBMX in isolated cultured retinas (Dearry and Burnside, 1985).

The regulation of cone retinomotor movements in isolated *Xenopus* eye cups also is influenced by DA (Pierce *et al.*, 1984; Pierce and Besharse, 1985). In addition, there is evidence for the involvement of melatonin and GABA receptors. Frogs were exposed to constant light for 4 days to abolish the rhythm of melatonin biosynthesis. When eye cups prepared from constant-light-treated frogs were incubated for 3 hr in darkness their cones elongated and were more than twice the length of cones in eye cups that were incubated for 3 hr in light (Table IV). Addition of DA to the incubation medium prevented the elongation in darkness, and thus mimicked the effect of light (Table IV). Dopamine addition following preincubation in the dark, when cones were already partially elongated, caused the cones to contract.

Addition of melatonin to eye cups incubated in the light mimicked the effect of darkness, i.e., cone elongation (Table IV). Dopamine also inhibited the melatonin-induced elongation. Based on the observation that melatonin inhibited retinal DA release and decreased the concentration of DOPAC (Dubocovich, 1983; Pierce *et al.*, 1984), it has been suggested that melatonin may produce its

TABLE IV

EFFECTS OF DOPAMINE, MELATONIN, AND MUSCIMOL ON CONE RETINOMOTOR MOVEMENTS[a]

Conditions	Additions	Cone length (% dark control)
Dark	—	100
Light	—	41[b]
Dark	Dopamine, 50 μM	45[b]
Light	Melatonin, 0.5 μM	80[c]
Light	Melatonin + dopamine	44[d]
Light	Muscimol, 50 μM	95[c]
Light	Muscimol + dopamine	41[d]

[a] Eye cups were prepared from constant-light-treated *X. laevis* and were incubated for 3 hr under the conditions indicated.

[b] $p < 0.01$ vs dark control.

[c] $p < 0.01$ vs light control.

[d] $p < 0.01$ vs same condition without dopamine.

effect on cone length by preventing the action of endogenous DA (Pierce *et al.*, 1984).

In addition to melatonin, the GABA receptor agonist muscimol was observed to stimulate cone elongation in light-exposed eye cups, and, similarly the muscimol-induced elongation was inhibited by DA (Table IV). Muscimol appears to be an inhibitor of DA-containing neurons of retina (see Section IV,A). Thus, muscimol, and possibly endogenous GABA, may influence cone movement by modulating the release of DA. Alternatively, DA may directly block the postsynaptic or transsynaptic actions of muscimol on the cones or other cells that communicate with them.

B. Rod Outer Segment Disk Shedding

Membrane turnover of rod outer segments is a complex process that involves disk assembly at the base of the outer segment, displacement of the disks distally by newly formed disks, and finally shedding of the distal tips of the outer segments and their phagocytosis by the retinal pigment epithelium (see Besharse, Part I, for a review of these processes). The final process of rod disk shedding and phagocytosis occurs in the early morning shortly after light onset (LaVail, 1976). It has been suggested that changes in the threshold of the rod ERG observed in humans at this time may reflect this process (Birch *et al.*, 1984). In retinas of several vertebrates, including rat and *Xenopus*, rod disk shedding is a circadian process that occurs in constant darkness and is blocked by constant light (LaVail, 1976; Besharse *et al.*, 1977). In cyclic light, disk shedding appears to require processes that occur during darkness and is subsequently triggered by light onset (reviewed in Besharse, 1982). Disk shedding and the regulation of melatonin biosynthesis are similar in that they both are rhythmic and are influenced in the same manner by alterations in the light–dark cycle.

Addition of melatonin to isolated eye cups can stimulate disk shedding (Besharse and Dunis, 1983). Light-evoked disk shedding in eye cups prepared from *Xenopus* that had been maintained on a regimen of 12 hr light–12 hr dark was activated by melatonin (Besharse and Dunis, 1983; Besharse *et al.*, 1984; Fig. 10). Melatonin-activated shedding required preincubation with the methoxyindole in the dark prior to the stimulation of shedding by light. In addition, melatonin mimicked the effect of darkness in stimulating shedding in eye cups from constant-light-treated frogs (Besharse and Dunis, 1983). 5-Methoxytryptophol and 6-chloromelatonin were also effective in stimulating disk shedding, but the melatonin presursors serotonin and *N*-acetylserotonin were not. Initial studies in which media contained the solvent dimethylsulfoxide (DMSO) and no antioxidants suggested that 5-methoxytryptophol was more potent than melatonin (Besharse and Dunis, 1983). However, it was subsequently noted that

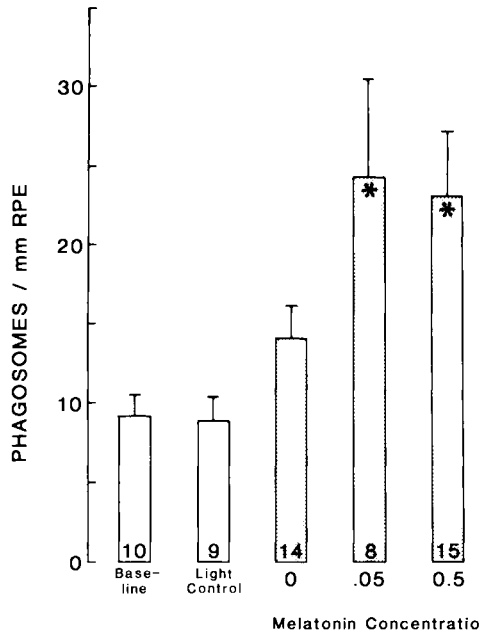


FIG. 10. Activation of disk shedding by melatonin. Eye cups were prepared in red light from *X. laevis* before light onset and were preincubated for 45 min before light treatment for 2.5 hr. Open bars represent samples cultured in normal medium (Besharse and Dunis, 1983), and stippled bars indicate normal medium supplimented with 10 μM ascorbic acid and various concentrations of melatonin (expressed as micromolar). Disk shedding was evaluated by counting large phagosomes within the retinal pigment epithelium (RPE) as described by Besharse *et al.* (1980). These phagosomes represent rod outer segments tips that were shed and internalized by the RPE. $*p < 0.05$. (Reproduced from Besharse *et al.*, 1984, with permission from *Federation Proceedings, Federation of American Societies for Experimental Biology.*)

melatonin was a significantly more potent activator of disk shedding in medium containing ascorbic acid, but lacking DMSO (Besharse *et al.*, 1984). These observations suggest that melatonin or a closely related compound may play a role in the regulation of disk shedding.

Light-evoked shedding may also be influenced by inner retinal transmitters or modulators. Disk shedding *in vitro* appears to require millimolar concentrations of calcium (Greenberger and Besharse, 1983), possibly to support the release of a mediator. In addition, Basinger and Gordon (1982) noted that stimulation of small areas of the retina with light elicited a shedding response that was uniform throughout the retina and considered the presence in retina of a "diffusable factor" as a possible initiator of shedding. To examine the possible involvement of the inner retina, the ability of aspartate to block light-evoked shedding was

investigated (Heath and Basinger, 1983). The use of aspartate was based on the rationale that because the amino acid abolishes the b wave of the ERG (Furakawa and Hanawa, 1955), it isolates photoreceptor activity from that of the inner retina. Aspartate did not abolish light-evoked shedding and it was suggested that synaptic activity in the inner retina is not necessary for shedding to occur (Heath and Basinger, 1983). However, aspartate and glutamate were later found to stimulate shedding (Greenberger and Besharse, 1985). The glutamate analog kainic acid was also a potent activator of disk shedding. These excitatory amino acids produced morphological signs of neurotoxicity in the inner retina, but no morphological effects on the photoreceptor–pigment epithelial complex were observed, other than the apparent increase in shedding. Although direct effects on the photoreceptor or pigment epithelium cannot be excluded, the observations raise the possibility that shedding may be stimulated by these amino acids as a consequence of their action on the inner retina.

C. Horizontal Cell Gap Junctions

Horizontal cells in retinas of lower vertebrates are electrotonically coupled (Naka and Rushton, 1967; Kaneko, 1971; Kaneko and Yamada, 1972; Simon, 1973). The electrical coupling is probably mediated by gap junctions that have been observed between horizontal cells of the same subclass (Witkovsky *et al.*, 1979). The functional connectivity of horizontal cells by gap junctions is also supported by the movement of intracellularly injected dye from injected horizontal cells to neighboring horizontal cells, a phenomenon termed "dye coupling" (Stewart, 1978), and by a correlation between the extent of dye coupling and the spatial properties of horizontal cells (Piccolino *et al.*, 1982; Teranishi *et al.*, 1983).

Recently, it has become apparent that electrical coupling resistance and dye coupling between horizontal cells may be modulated by neurotransmitters or neuromodulators. Bicuculline and picrotoxin, GABA antagonists, have been shown to contract the receptive field profile of large-field horizontal cells in turtle retina (Piccolino *et al.*, 1982). The effect of the GABA antagonists on receptive field properties was not accompanied by any significant change in the dark membrane potential of the horizontal cells, but was associated with an apparent increase in coupling resistance. GABA had an effect on receptive field that was opposite to that of the antagonists. GABA antagonists also caused a dramatic reduction in the diffusion of intracellularly injected Lucifer yellow between horizontal cells, but did not restrict diffusion of the dye within the injected cell. These observations are consistent with the hypothesis that endogenous GABA functions at least in part to regulate the receptive field properties of horizontal cells by increasing the coupling between cells via gap junctions.

As described above (Section II,E), DA-containing interplexiform cells modulate the receptive field properties of cone-driven horizontal cells in fish retina. Dopamine application contracted the receptive field, while treatment with 6-hydroxydopamine, which causes the DA-containing interplexiform cells to degenerate, increased receptive field size (Negishi and Drujan, 1979a; Teranishi *et al.*, 1983; Cohen *et al.*, 1983). The effects of DA on the receptive field properties were correlated with increases of membrane and coupling resistances (Laufer and Salas, 1981) and may be mediated by gap junctions. Lucifer yellow injected into a horizontal cell of control retinas spread into several cells (Teranishi *et al.*, 1983). However, following application of DA the dye was confined within a single cell. In contrast, the dye spread to a greater number of cells in 6-hydroxydopamine-treated retinas than in controls.

Haloperidol blocked both the DA-mediated receptive-field contraction and the elimination of dye coupling (Teranishi *et al.*, 1983), suggesting the involvement of dopamine receptors. DA receptors on horizontal cells are linked to adenylate cyclase (Van Buskirk and Dowling, 1981), and the effects of DA on horizontal cell coupling may be mediated by cyclic AMP. Application of dibutyryl cyclic AMP and IBMX had similar effects to DA on the spatial properties of horizontal cells and on dye coupling (Teranishi *et al.*, 1983). Thus, DA may alter the spatial properties of horizontal cells through a cyclic AMP-dependent mechanism that involves uncoupling gap junctions.

These examples illustrate that neurotransmitters and neuromodulators, in addition to their role in chemical synaptic transmission between cells, may also influence intercellular communication by altering electrotonic coupling of cells via gap junctions.

VI. Concluding Remarks

This review has summarized the current evidence that various putative neurotransmitters and neuromodulators function in the different cell types of the vertebrate retina. Many compounds that have been proposed to be transmitters or modulators in other parts of the central nervous system have been identified in amacrine cells, and excitatory amino acids may be transmitters in some photoreceptor, bipolar, and ganglion cells. However, putative transmitters have not been identified for many cell subtypes, including most subclasses of horizontal cells in submammalian vertebrates and all subclasses of horizontal cells in mammals. The number of putative transmitters and modulators identified in retina has increased dramatically in the last few years, and it is anticipated that more will be identified in the near future. The use of isolated cell types, such as isolated horizontal cells, should prove useful in the identification of new transmitter

candidates that might be overlooked using more complex systems. Studies have also been reviewed that examine the interactions of transmitter and modulator systems and the approaches that can be taken to examine direct and polysynaptic interactions and regulatory mechanisms involved in synaptic transmission.

Recent work conducted on the biology of intercellular communication in the retina suggests that chemical mediators may act in several ways to influence the flow of information through the system. Transmitters and modulators can apparently influence membrane turnover, cellular motility, gap junctions, and perhaps other cellular events that are important in the reception and transfer of information.

Acknowledgments

This review could not have been completed without the help of several people. I wish to especially thank Joe Besharse, Mary Pierce, and Margaret Tigges for their critical reading of drafts of this paper, Bonnie Butler for preparation of figures, Michael Bloom for his many trips to the library, Margarita Dubocovich for providing a copy of Fig. 9, and Marlise Casteel for processing this manuscript. I also thank the following people for making available preprints of manuscripts and discussing unpublished data: Joe Besharse, Beth Burnside, Margarita Dubocovich, Peggy Gnegy, Madelyn Fernstrom, Maria Hadjiconstantinou, Bill Morgan, Norton Neff, Mary Pierce, Don Puro, and Dianna Redburn. The recent work from my laboratory was supported by National Institutes of Health Grants NS17524 and EY04864.

References

Altschuler, R. A., Mosinger, J. L., Harmison, G. G., Parakkal, M. H., and Wenthold, R. J. (1982a). Aspartate aminotransferase-like immunoreactivity as a marker for aspartate/glutamate in guinea pig photoreceptors. *Nature (London)* **298,** 657–659.

Altschuler, R. A., Mosinger, J. L., Hoffman, J. L., and Parakkal, M. H. (1982b). Immunocytochemical localization of enkephalin-like immunoreactivity in the retina of the guinea pig. *Proc. Natl. Acad. Sci. U.S.A.***79,** 2398–2400.

Ames, A., III, and Pollen, D. A. (1969). Neurotransmission in central nervous tissue: A study of isolated rabbit retina. *J. Neurophysiol.* **32,** 424–442.

Ariel, M., and Daw, N. W. (1982). Effects of cholinergic drugs on receptive field properties of rabbit retina ganglion cells. *J. Physiol. (London)* **324,** 135–160.

Baker, P. C., Quay, W. B., and Axelrod, J. (1965). Development of hydroxyindole-*O*-methyltransferase activity in eye and brain of the amphibian, *Xenopus laevis*. *Life Sci.* **4,** 1981–1987.

Barbaccia, M. L., Lucchi, L., Kobayashi, H., Spano, P. F., Govani, S., and Trabucchi, M. (1982). Modulation of dopamine turnover in rat retina by opiates: Effects of different pharmacological treatments. *Pharmacol. Res. Commun.* **14,** 541–550.

Basinger, S. F., and Gordon, W. C. (1982). Local stimulation induces shedding throughout the frog retina. *Vision Res.* **22,** 1533–1538.

Bauer, B., and Ehinger, B. (1977). Light evoked release of radioactivity from rabbit retinas preloaded with (^{3}H)-GABA. *Experientia* **33,** 470–471.

Bauer, B., Ehinger, B., and Aberg, L. (1980). [^{3}H]-dopamine release from the rabbit retina. *Albrecht Von Graefes Arch. Klin. Exp. Ophthalmol.* **215,** 71–78.

Baughman, R. W., and Bader, C. R. (1977). Biochemical characterization and cellular localization of the cholinergic system in the chicken retina. *Brain Res.* **138,** 469–485.

Beaudet, A., Burkhalter, A., Reubi, J.-C., and Cuénod, M. (1981). Selective bidirectional transport of [^{3}H]D-aspartate in the pigeon retina-tectal pathway. *Neuroscience* **6,** 2021–2034.

Berger, S. J., McDaniel, M. L., Carter, J. G., and Lowry, O. H. (1977). Distribution of four potential transmitter amino acids in monkey retina. *J. Neurochem.* **28,** 158–163.

Besharse, J. C. (1982). The daily light–dark cycle and rhythmic metabolism in the photoreceptor–pigment epithelial complex. *In* "Progress in Retinal Research" (N. Osborne and G. Chader, eds.), Vol. 1, pp. 81–124. Pergamon, Oxford.

Besharse, J. C., and Dunis, D. A. (1983). Methoxyindoles and photoreceptor metabolism: Activation of rod shedding. *Science* **219,** 1341–1343.

Besharse, J. C., and Iuvone, P. M. (1983). Circadian clock in *Xenopus* eye controlling retinal serotonin *N*-acetyltransferase. *Nature (London)* **305,** 133–135.

Besharse, J. C., Hollyfield, J. G., and Rayborn, M. E. (1977). Turnover of rod photoreceptor outer segments. II. Membrane addition and loss in relationship to light. *J. Cell Biol.* **75,** 507–527.

Besharse, J. C., Dunis, D. A., and Iuvone, P. M. (1984). Regulation and possible role of serotonin *N*-acetyltransferase in retina. *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **43,** 2704–2708.

Binkley, S., Hryshchyshyn, M., and Reilly, K. (1979). *N*-Acetyltransferase activity responds to environmental lighting in the eye as well as in the pineal gland. *Nature (London)* **281,** 479–481.

Binkley, S., Reilly, K. B., and Hryshchyshyn, M. (1980). *N*-Acetyltransferase in the chick retina. I. Circadian rhythms controlled by environmental lighting are similar to those in the pineal gland. *J. Comp. Physiol. (B)* **189,** 103–108.

Birch, D. G., Berson, E. L., and Sandberg, M. A. (1984). Diurnal rhythm in the human rod ERG. *Invest. Ophthalmol. Visual Sci.* **25,** 236–238.

Bittinger, H., Heid, J., and Wigger, N. (1980). Are only α_2-adrenergic receptors present in retina. *Nature (London)* **287,** 645–647.

Bonaventure, N., Wioland, N., and Mandel, P. (1974). Antagonists of the putative inhibitory transmitter effects of taurine and GABA in the retina. *Brain Res.* **80,** 281–289.

Bondy, S. C., and Purdy, J. L. (1977). Putative neurotransmitters of the avian visual pathway. *Brain Res.* **119,** 417–426.

Borbe, H. O., Müller, W. E., and Wollert, U. (1981). Specific [^{3}H]strychnine binding associated with glycine receptors in bovine retina. *Brain Res.* **205,** 131–139.

Brandon, C., Lam, D. M. K., and Wu, J.-Y. (1979). The γ-aminobutyric acid system in rabbit retina: Localization by immunocytochemistry and autoradiography. *Proc. Natl. Acad. Sci. U.S.A.* **76,** 3557–3561.

Brandon, C., Lam, D. M. K., Su, Y. Y. T., and Wu, J.-Y. (1980). Immunocytochemical localization of GABA neurons in the rabbit and frog retina. *Brain Res. Bull. 5 Suppl.* **2,** 21–29.

Brecha, N. L., Oyster, C. W., and Takahashi, E. S. (1984). Identification and characterization of tyrosine hydroxylase immunoreactive amacrine cells. *Invest. Ophthalmol. Visual Sci.* **25,** 66–70.

Brecha, N., Sharma, S. C., and Karten, H. J. (1981). Localization of substance P-like immunoreactivity in the adult and developing goldfish retina. *Neurosci.* **6,** 2737–2746.

Brecha, N., Hendrickson, A., Florén, I., and Karten, H. J. (1982). Localization of substance P-like immunoreactivity within the monkey retina. *Invest. Ophthalmol. Visual Sci.* **23,** 147–153.

Brown, J. H., and Makman, M. H. (1972). Stimulation by dopamine of adenylate cyclase in retinal homogenates of adenosine-3′:5′-cyclic monophosphate formation in intact retina. *Proc. Natl. Acad. Sci. U.S.A.* **69,** 539–543.

Brown, J. H., and Rietow, M. (1981). Muscarinic–dopaminergic synergism on retinal cyclic AMP formation. *Brain Res.* **215,** 388–392.

Bruun, A., and Ehinger, B. (1972). Uptake of the putative neurotransmitter glycine into the rabbit retina. *Invest. Ophthalmol.* **11,** 191–198.

Bruun, A., and Ehinger, B. (1974). Uptake of certain possible neurotransmitters into retinal neurones of some mammals. *Exp. Eye Res.* **19,** 435–447.

Bubenik, G. A., Brown, G. M., Uhlir, I., and Grota, L. J. (1974). Immunohistochemical localization of *N*-acetylindoleamines in pineal, retina and cerebellum. *Brain Res.* **81,** 233–242.

Bubenik, G. A., Brown, G. M., and Grota, L. J. (1976). Differential localization of N-acetylated indolealkylamine in CNS and the Harderian gland using immunohistochemistry. *Brain Res.* **118,** 417–427.

Bubenik, G. A., Purtill, R. A., Brown, G. M., and Grota, L. J. (1978). Melatonin in the retina and the Harderian gland. Ontogeny, diurnal variations and melatonin treatment. *Exp. Eye Res.* **27,** 323–334.

Cajal, S. R. (1893). La rétinae des vertébrés. *Cellule* **9,** 17–257.

Caldwell, J. H., Daw, N. W., and Wyatt, H. J. (1978). Effects of picrotoxin and strychnine on rabbit retinal ganglion cells: Lateral interactions for cells with more complex receptive fields. *J. Physiol. (London)* **276,** 277–298.

Canzek, V., Wolfensberger, M., Amsler, U., and Cuénod, M. (1981). *In vivo* release of glutamate and aspartate following optic nerve stimulation. *Nature (London)* **293,** 572–574.

Cardinali, D. P., and Rosner, J. M. (1971a). Retinal localization of hydroxyindole-*O*-methyltransferase (HIOMT) in the rat. *Endocrinology* **86,** 301–303.

Cardinali, D. P., and Rosner, J. M. (1971b). Metabolism of serotonin by the rat retina *in vitro*. *J. Neurochem.* **18,** 1769–1770.

Cedarbaum, J. M., and Aghajanian, G. K. (1976). Noradrenergic neurons of the *locus coeruleus:* Inhibition by epinephrine and activation by the α-antagonist piperoxane. *Brain Res.* **112,** 413–419.

Cedarbaum, J. M., and Aghajanian, G. K. (1977). Catecholamine receptors on locus coeruleus neurons: Pharmacological characterization. *Eur. J. Pharmacol.* **44,** 375–385.

Cervetto, L., and MacNichol, E. F., Jr. (1972). Inactivation of horizontal cells in turtle retina by glutamate and asparate. *Science* **178,** 767–768.

Cervetto, L., and Piccolino, M. (1974). Synaptic transmission between photoreceptors and horizontal cells in the turtle retina. *Science* **183,** 417–419.

Chin, C.-A., and Lam, D. M. K. (1980). The uptake and release of [^{3}H]glycine in the goldfish retina. *J. Physiol. (London)* **308,** 185–195.

Cohen, A. I., McDaniel, M., and Orr, H. (1973). Absolute levels of some free amino acids in normal and biologically fractionated retinas. *Invest. Ophthalmol.***12,** 686–693.

Cohen, J., Iuvone, P. M., and Neff, N. H. (1981). Neuroleptic drugs activate tyrosine hydroxylase of retinal amacrine cells. *J. Pharmacol. Exp. Ther.* **218,** 390–394.

Cohen, J. C., Hadjiconstantinou, M., and Neff, N. H. (1983). Activation of dopamine-containing amacrine cells of retina: Light-induced increase of acidic dopamine metabolites. *Brain Res.* **260,** 125–127.

Cohen, J. L., and Dowling, J. E. (1983). The role of the retinal interplexiform cell: Effects of 6-hydroxydopamine on the spatial properties of carp horizontal cells. *Brain Res.* **264,** 307–310.

Cote, T. E., Grewe, C. W., and Kebabian, J. W. (1981). Stimulation of a D-2 dopamine receptor in the intermediate lobe of the rat pituitary gland decreases the responsiveness of the β-adrenoceptor: Biochemical mechanism. *Endocrinology* **108,** 420–426.

Coull, B. M., and Cutler, R. W. P. (1976). Light-evoked release of endogenous glycine into the perfused vitreous of the intact rat eye. *Invest. Ophthalmol. Visual Sci.* **17,** 682–684.

Coull, B. M., Owens, D. K., and Cutler, R. W. P. (1981). The release of endogenous amino acids

into the vitreous of the intact eye of the albino rat: Effect of light, potassium and ouabain. *Brain Res.* **210,** 301–309.

Cunningham, R., and Miller, R. F. (1976). Taurine: its selective action on neuronal pathways in the rabbit retina. *Brain Res.* **117,** 341–345.

Dacheux, R. F., and Miller, R. F. (1976). Photoreceptor–bipolar cell transmission in the perfused retina eyecup of the mudpuppy. *Science* **191,** 963–964.

Dacheux, R. F., Frumkes, T. E. and Miller, R. F. (1979). Pathways and polarities of synaptic interactions in the inner retina of the mudpuppy. I. Synaptic blocking studies. *Brain Res.* **161,** 1–12.

DaPrada, M. (1977). Dopamine content and synthesis in retina and *N. accumbens septi:* Pharmacological and light-induced modifications. *Adv. Biochem. Psychopharmacol.* **16,** 311–319.

Dearry, A., and Burnside, B. (1984). Influence of catecholamines on cone and RPE retinomotor movements in teleost fish. *Invest. Ophthalmol. Visual Sci. Suppl.* **25,** 65.

Dearry, A., and Burnside, B. (1985). Dopamine inhibits forskolin- and 3-isobutylmethylxanthine-induced dark-adaptive retinomotor movements in isolated teleost retinas. *J. Neurochem.* **44,** 1753–1763.

DeMello, M. C. F., Ventura, A. L. M., Decarvalho, R. P., Klein, W. L., and DeMello, F. G. (1982). Regulation of dopamine-dependent and adenosine-dependent adenylate cyclase systems of chicken embryo retina cells in culture. *Proc. Natl. Acad. Sci. U.S.A.* **79,** 5708–5712.

DeVries, G. W., and Friedman, A. H. (1978). GABA, picrotoxin and retinal sensitivity. *Brain Res.* **148,** 530–535.

DeVries, G. W., Campau, K. M., and Ferrendelli, F. A. (1982). Adenylate cyclases in the vertebrate retina: Distribution and characteristics in rabbit and ground squirrel. *J. Neurochem.* **38,** 759–765.

Dick, E., and Miller, R. F. (1981). Peptides influence retinal ganglion cells. *Neurosci. Lett.* **26,** 131–135.

Djamgoz, M. B. A., Stell, W. K., Chin, C.-A., and Lam, D. M. K. (1981). An opiate system in the goldfish retina. *Nature (London)* **292,** 620–623.

Dowling, J. E., and Ehinger, B. (1975). Synaptic organization of the amine-containing interplexiform cells of the goldfish and *Cebus* monkey retinas. *Science* **188,** 270–273.

Dowling, J. E., and Ehinger, B. (1978). Synaptic organization of the dopaminergic neurons in the rabbit retina. *J. Comp. Neurol.* **180,** 203–220.

Dowling, J. E., and Ripps, H. (1973). Effect of magnesium on horizontal cell activity in the skate retina. *Nature (London)* **242,** 101–103.

Dowling, J. E., and Watling, K. J. (1981). Dopaminergic mechanisms in the teleost retina. II. Factors affecting the accumulation of cyclic AMP in pieces of intact carp retina. *J. Neurochem.* **36,** 569–579.

Dubocovich, M. L. (1983). Melatonin is a potent modulator of dopamine release in the retina. *Nature (London)* **306,** 782–784.

Dubocovich, M. L. (1984). Modulation of ^{3}H-dopamine release from rabbit retina. *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **43,** 2714–2718.

Dubocovich, M. L. (1984b). Alpha-2 adrenoceptors modulate [^{3}H]dopamine release from rabbit retina. *J. Pharmacol. Exp. Ther.* **230,** 149–155.

Dubocovich, M. L. (1984c). Pharmacological characterization of the melatonin receptor site modulating dopamine release from rabbit retina. *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **43,** 457.

Dubocovich, M. L., and Weiner, N. (1981). Modulation of the stimulation-evoked release of ^{3}H-dopamine in the rabbit retina. *J. Pharmacol. Exp. Ther.* **219,** 701–707.

Dubocovich, M. L., and Weiner, N. (1983). Enkephalins modulate [H-3]dopamine release from rabbit retinas *in vitro*. *J. Pharmacol. Exp. Ther.* **224,** 634–639.

Dyer, R. S., Howell, W. E., and MacPhail, R. C. (1981). Dopamine depletion slows retinal transmission. *Exp. Neurol.* **71,** 326–340.

Eckenstein, F., Baughman, R. W., Sofroniew, M. V., and Thibault, J. (1983). A comparison of the distribution of choline acetyltransferase and tyrosine hydroxylase immunoreactivities in rat retina. *Abstr. Soc. Neurosci.* **9,** 80.

Ehinger, B. (1966). Adrenergic nerves to the eye and to related structures in men and in cynomolgus monkey (*Macaca irus*). *Invest. Ophthalmol.* **5,** 42–52.

Ehinger, B. (1977). Glial and neuronal uptake of GABA, glutamic acid, glutamine and glutathione in the rabbit retina. *Exp. Eye Res.* **25,** 221–234.

Ehinger, B. (1981). [^{3}H]-D-aspartate accumulation in the retina of pigeon, guinea-pig and rabbit. *Exp. Eye Res.* **33,** 381–391.

Ehinger, B., and Falck, B. (1969). Morphological and pharmacohistochemical characteristics of adrenergic retinal neurons of some mammals. *Albrect von Graefes Arch. Klin. Exp. Ophthalmol.* **178,** 295–305.

Ehinger, B., and Falck, B. (1971). Autoradiography of some suspected neurotransmitter substances: GABA, glycine, glutamic acid, histamine, dopamine and L-DOPA. *Brain Res.* **33,** 157–172.

Ehinger, B., and Florén, I. (1978). Quantitation of the uptake of indoleamines and dopamine in the rabbit retina. *Exp. Eye Res.* **26,** 1–11.

Ehinger, B., and Lindberg, B. (1974). Light-evoked release of glycine from the retina. *Nature (London)* **251,** 727–728.

Ehinger, B., and Lindberg-Bauer, B. (1976). Light-evoked release of glycine from cat and rabbit retina. *Brain Res.* **113,** 535–549.

Ehinger, B., and Nordenfelt, L. (1977). Destruction of retinal dopamine-containing neurons in rabbit and goldfish. *Exp. Eye Res.* **24,** 179–187.

Ehinger, B., Falck, B., and Laties, A. M. (1969). Adrenergic innervation of teleost retina. *Z. Zellforsch. Mikrosk. Anat.* **97,** 285–297.

Ehinger, B., Hansson, C., and Tornqvist, K. (1981). 5-Hydroxytryptamine in the retina of some mammals. *Exp. Eye Res.* **33,** 663–672.

El Mestikawy, S., Glowinski, J., and Hamon, M. (1983). Tyrosine hydroxylase activation in depolarized dopaminergic terminals—involvement of Ca^{2+}-dependent phosphorylation. *Nature (London)* **302,** 830–832.

Enna, S. J., and Snyder, S. H. (1976). Gamma-aminobutyric acid (GABA) receptor binding in mammalian retina. *Brain Res.* **115,** 174–179.

Eskay, R. L., Long, R. T., and Iuvone, P. M. (1980). Evidence that TRH, somatostatin, and substance P are present in neurosecretory elements of vertebrate retina. *Brain Res.* **196,** 554–559.

Eskay, R. L., Furness, J. F., and Long, R. T. (1981). Substance P activity in the bullfrog retina: Localization and identification in several vertebrate species. *Science* **212,** 1049–1051.

Falck, B. (1962). Observations on the possibilities of the cellular localization of monoamines by a fluorescence method. *Acta Physiol. Scand.* **56,** (Suppl.) (197), 1–25.

Famiglietti, E. V. (1983). "Starburst" amacrine cells and cholinergic neurons: Mirror-symmetric ON and OFF amacrine cells of rabbit retina. *Brain Res.* **261,** 138–144.

Fernstrom, M. H., Volk, E. A., and Fernstrom, J. D. (1984). *In vivo* tyrosine hydroxylatin in the diabetic rat retina: Effect of tyrosine administration. *Brain Res.* **298,** 167–170.

Fernstrom, M. H., Volk, E. A., Fernstrom, J. D., and Iuvone, P. M. (1986). Effect of tyrosine administration on tyrosine hydroxylation in light- and dark-adapted retinas from normal and diabetic rats. *J. Pharmacol. Exp. Ther.*, submitted.

Ferrendelli, J. A., Compau, K. M., and DeVries, G. W. (1982). Adenylate cyclases in vertebrate retina: Enzymatic characteristics in normal and distrophic mouse retina. *J. Neurochem.* **38,** 753–758.

Florén, I. (1979). Arguments against 5-hydroxytryptamine as neurotransmitter in the rabbit retina. *J. Neural Transmission* **46,** 1–15.

Florén, I., and Hansson, H. C. (1980). Investigations into whether 5-hydroxytryptamine is a neurotransmitter in the retina of rabbit and chicken. *Invest. Ophthalmol. Visual Sci.* **19,** 117–125.

Fonnum, F., and Henke, H. (1979). Effects of retinal ablation on the pool of some amino acids in different layers of the pigeon tectum. *Experientia* **35,** 919.

Frederick, J. M., Rayborn, M. E., Laties, A. M., Lam, D. M. K., and Hollyfield, J. G. (1982). Dopaminergic neurons in the human retina. *J. Comp. Neurol.* **210,** 65–79.

Freed, M. A., Nakamura, Y., and Sterling, P. (1983). Four types of amacrine in the cat retina that accumulate GABA. *J. Comp. Neurol.* **219,** 295–304.

Freedman, J. A. (1977). Possible regulatory function of acetylcholine receptor in maintenance of retinotectal synapses. *Nature (London)* **269,** 218–222.

Fukuda, M., Kuwayama, Y., Shiosaka, S., Inagaki, S., Ishimoto, I., Shimizu, Y., Takagi, H., Sakanaka, M., Takatsuki, K., Senba, E., and Tohyama, M. (1981). Localization of vasoactive intestinal polypeptide and neurotensin immunoreactivities in avian retinas. *Curr. Eye Res.* **1,** 115–118.

Fukuda, M., Ishimoto, I., Kuwayama, Y., Shimizu, Y., Shiosaka, S., Takagi, H., and Tohyama, M. (1982a). Monoamine accumulating neuron system in the rat retina with special reference to noradrenaline accumulating neurons. *Exp. Eye Res.* **34,** 487–491.

Fukuda, M., Ishimoto, I., Shiosaka, Y., Kuwayama, Y., Shimizu, Y., Inagaki, S., Sakanaka, M., Takagi, H., Senba, E., Takatsuki, K., Umegaki, F., and Tohyama, M. (1982b). Localization of LH-RH immunoreactivity in the avian retina. *Curr. Eye Res.* **2,** 71–75.

Furakawa, T., and Hanawa, I. (1955). Effects of some common cations on electroretinogram of the toad. *J. Jpn. Physiol.* **5,** 289–300.

Frucht, Y., Vidauri, J., and Melamed, E. (1982). Light activation of dopaminergic neurons in rat retina is mediated through photoreceptors. *Brain Res.* **249,** 153–156.

Gallego, A. (1971). Horizontal and amacrine cells in the mammals retina. *Vision Res.* **3,** (Suppl.), 33–50.

Gern, W. A. (1980). Evolution of melatonin function: A hypothesis. *In* "Melatonin: Current Status and Perspectives" (N. Birau and W. Schloot, eds.), pp. 85–87. Pergamon, Oxford.

Gern, W. A., and Ralph, C. L. (1979). Melatonin synthesis by the retina. *Science* **204,** 183–184.

Gibson, C. J., Watkins, C. J., and Wurtman, R. J. (1983). Tyrosine administration enhances dopamine synthesis and release in light-activated rat retina. *J. Neural Transmiss.* **56,** 153–160.

Glickman, R. D., Adolph, A. R., and Dowling, J. E. (1982). Interplexiform circuits in the carp retina: Effects of cholinergic agonists, GABA, and substance P on the ganglion cells. *Brain Res.* **234,** 81–99.

Gnegy, M. E., Muirhead, N., and Harrison, J. K. (1984a). Regulation of calmodulin- and dopamine-stimulated adenylate cyclase activities by light in bovine retina. *J. Neurochem.* **42,** 1632–1640.

Gnegy, M. E., Muirhead, N., Roberts-Lewis, J. M., and Treisman, G. (1984b). Calmodulin stimulates adenylate cyclase activity and increases dopamine activation in bovine retina. *J. Neurosci.* **12,** 2712–2717.

Graham, L. T., Jr. (1972). Intraretinal distribution of GABA content and GAD activity. *Brain Res.* **36,** 476–479.

Graham, L. T. (1974). Comparative aspects of neurotransmitters in the retina. *In* "The Eye, Comparative Physiology" (H. Davson and L.T. Graham, eds.), Vol. 6, pp. 283–342. Academic Press, New York.

Greenberger, L. M., and Besharse, J. C. (1983). Photoreceptor disc shedding in eye cups: Inhibition by deletion of extracellular divalent cations. *Invest. Ophthalmol. Visual Sci.* **24,** 1456–1464.

Greenberger, L. M., and Besharse, J. C. (1985). Stimulation of photoreceptor disc shedding and phagocytosis by glutamate, aspartate, and other amino acids. *J. Comp. Neurol.* **239,** 361–372.

Hadjiconstantinou, M., Cohen, J., and Neff, N. H. (1983). Epinephrine: A potential neurotransmitter in retina. *J. Neurochem.* **41,** 1440–1444.

Hadjiconstantinou, M., Cohen, J., Rubenstein, J.R., and Neff, N. H. (1984a). An endogenous ligand modulates dopamine-containing neurons in retina via alpha-2 adrenoceptors. *J. Pharmacol. Exp. Ther.* **229,** 381–385.

Hadjiconstantinou, M., Mariani, A. P., Panula, P., Joh, T. H., and Neff, N. H. (1984b). Immunohistochemical evidence for epinephrine-containing retinal amacrine cells. *Neurosci.* **13,** 547–551.

Haggendal, J., and Malmfors, T. (1965). Identification and cellular localization of the catecholamines in the retina and choroid plexus of the rabbit. *Acta Physiol. Scand.* **64,** 58–66.

Hagins, W. A., Penn, R. D., and Yoshikami, S. (1970). Dark current and photocurrent in retinal rods. *Biophys. J.* **10,** 380–412.

Hamm, H. E., and Menaker, M. (1980). Retinal rhythms in chicks—circadian variation in melatonin and serotonin *N*-acetyltransferase activity. *Proc. Natl. Acad. Sci. U.S.A.* **77,** 4998–5002.

Hayden, S. A., Mills, J. W., and Masland, R. M. (1980). Acetylcholine synthesis by displaced amacrine cells. *Science* **210,** 435–437.

Hayes, K. C., Carey, R. E., and Schmidt, S. Y. (1975). Retinal degeneration associated with taurine deficiency in the cat. *Science* **188,** 949–951.

Heath, A. R., and Basinger, S. F. (1983). Frog rod outer segment shedding *in vitro:* Histologic and electrophysiologic observations. *Invest. Ophthalmol. Visual Sci.* **24,** 277–284.

Hedden, W. L. Jr., and Dowling, J. E. (1978). The interplexiform cell systems. II. Effects of dopamine on goldfish retinal neurones. *Proc. R. Soc. London* **201,** 27–55.

Henke, H., Schenker, T. M., and Cuénod, M. (1976). The effects of retinal ablation on uptake of glutamate, glycine, GABA, proline and choline in pigeon tectum. *J. Neurochem.* **26,** 131–134.

Hollyfield, J. G., Rayborn, M. E., Sarthy, P. V., and Lam, D. M. K. (1979). The emergence, localization and maturation of neurotransmitter systems during development of retina in *Xenopus laevis*. I. γ-Aminobutyric acid. *J. Comp. Neurol.* **188,** 587–598.

Holmgren-Taylor, I. (1982). Ultrastructure and synapses of the [^{3}H]dopamine-accumulating neurons in the retina of the rabbit. *Exp. Eye Res.* **35,** 555–572.

Ikeda, H., and Sheardown, M. J. (1982a). Aspartate may be an excitatory transmitter mediating visual excitation of sustained but not transient cells in the cat retina: Iontophoretic studies *in vivo*. *Neurosci.* **7,** 25–36.

Ikeda, H., and Sheardown, M. J. (1982b). Acetylcholine may be an excitatory transmitter mediating visual excitation of "transient" cells with the periphery effect in the cat retina: Iontophoretic studies *in vivo*. *Neurosci.* **7,** 1299–1308.

Ikeda, H., and Sheardown, M. J. (1983). Transmitters mediating inhibition of ganglion cells in the cat retina: Iontophoretic studies *in vivo*. *Neurosci.* **8,** 837–853.

Ishida, A. T., and Fain, G. L. (1981). D-Aspartate potentiates the effects of L-glutamate on horizontal cells in goldfish retina. *Proc. Natl. Acad. Sci. U.S.A.* **78,** 5890–5894.

Ishida, A. T., Kaneko, A., and Tachibana, M. (1983). Solitary horizontal cells in culture. II. A new tool for examining effects of photoreceptor neurotransmitter candidates. *Vision Res.* **23,** 1217–1220.

Iuvone, P. M. (1983). Short-term regulation of tyrosine hydroxylase in tonically-active and in tonically-inactive dopamine neurons: Effects of haloperidol and protein phosphorylation. *Life Sci.* **33,** 1315–1324.

Iuvone, P. M. (1984). Calcium, ATP and magnesium activate soluble tyrosine hydroxylase from rat striatum. *J. Neurochem.* **43,** 1359–1368.

Iuvone, P. M. (1986). Evidence for a D2 dopamine receptor in frog retina that decreases cyclic AMP accumulation and serotonin *N*-acetyltransferase activity. *Life Sci.* **38,** 331–342.

Iuvone, P. M., and Besharse, J. C. (1983). Regulation of indoleamine *N*-acetyltransferase activity in the retina: Effects of light and dark, protein synthesis inhibitors and cyclic nucleotide analogs. *Brain Res.* **273,** 111–119.

Iuvone, P. M., and Besharse, J. C. (1986a). Involvement of calcium in the regulation of retinal serotonin *N*-acetyltransferase activity in retina. *J. Neurochem.*, **46,** 82–88.

Iuvone, P. M., and Besharse, J. C. (1986b). Cyclic AMP stimulates serotonin *N*-acetyltransferase activity in *Xenopus* retina *in vitro*. *J. Neurochem.* **46,** 33–39.

Iuvone, P. M., and Besharse, J. C. (1986c). Dopamine receptor-mediated inhibition of serotonin *N*-acetyltransferase activity in retina. *Brain Res.*, in press.

Iuvone, P. M., and Marshburn, P. B. (1982). Regulation of tyrosine hydroxylase activity in retinal cell suspensions: Effects of potassium and 8-bromo cyclic AMP. *Life Sci.* **30,** 85–91.

Iuvone, P. M., and Rauch, A. L. (1983). Alpha$_2$-adrenergic receptors influence tyrosine hydroxylase activity in retinal dopamine neurons. *Life Sci.* **33,** 2455–2466.

Iuvone, P. M., Galli, C. L., Garrison-Gund, C. K., and Neff, N. H. (1978a). Light stimulates tyrosine hydroxylase activity and dopamine synthesis in retinal amacrine neurons. *Science* **202,** 901–902.

Iuvone, P. M., Galli, C. L., and Neff, N. H. (1978b). Retinal tyrosine hydroxylase: Comparison of short-term and long-term stimulation by light. *Mol. Pharmacol* **14,** 1212–1219.

Iuvone, P. M., Joh, T. H., and Neff, N. H. (1979). Regulation of retinal tyrosine hydroxylase: Long-term exposure to light increased the apparent V_{max} without a concomitant increase of immunotitratable enzyme molecules. *Brain Res.* **178,** 191–195.

Iuvone, P. M., Rauch, A. L., Marshburn, P. B., Glass, D. B., and Neff, N. H. (1982). Activation of retinal tyrosine hydroxylase *in vitro* by cyclic AMP-dependent protein kinase: Characterization and comparison to activation *in vivo* by photic stimulation. *J. Neurochem.* **39,** 1632–1640.

Iuvone, P. M., Reinhard, J. F., Abou-Donia, M. M., Viveros, O. H., and Nichol, C. A. (1985). Stimulation of retinal dopamine biosynthesis *in vivo* by exogenous tetrahydrobiopterin: Relationship to tyrosine hydroxylase activation. *Brain Res.* **359,** 392–396.

Jagadeesh, J. M., and Sanchez, R. (1981). Effects of apomorphine on the rabbit electroretinogram. *Invest. Ophthalmol. Visual Sci.* **21,** 620–625.

Jensen, R. J., and Daw, N. W. (1983). Towards an understanding of the role of dopamine in the mammalian retina. *Vision Res.* **23,** 1293–1298.

Joh, T. H., Park, D. H., and Reis, D. J. (1978). Direct phosphorylation of brain tyrosine hydroxylase by cyclic AMP-dependent protein kinase: Mechanism of enzyme activation. *Proc. Natl. Acad. Sci. U.S.A.* **75,** 4744–4748.

Kamada, Y., Mizuno, A., and Matsuda, M. (1981). Effect of L-glutamic acid on [^{14}C]GABA release from isolated rat retina. *Brain Res.* **229,** 251–255.

Kamp, C. W., and Morgan, W. W. (1981). GABA antagonists enhance dopamine turnover in the rat retina *in vivo*. *Eur. J. Pharmacol.* **69,** 273–279.

Kamp, C. W., and Morgan, W. W. (1983). Effects of excitatory amino acids on dopamine synthesis in the rat retina. *Eur. J. Pharmacol.* **92,** 139–142.

Kaneko, A. (1970). Physiological and morphological identification of horizontal, bipolar, and amacrine cells in goldfish retina. *J. Physiol. (London)* **207,** 673–633.

Kaneko, A. (1971). Electrical connections between horizontal cells in the dogfish retina. *J. Physiol (London)* **213,** 95–105.

Kaneko, A., and Yamada, M. (1972). S-potentials in the dark-adapted retina of the carp. *J. Physiol. (London)* **227,** 261–274.

Karten, H. J., and Brecha, N. (1980). Localization of substance P immunoreactivity in amacrine cells of the retina. *Nature (London)* **283,** 87–88.

Kato, S., Teranishi, T., Kuo, C. H., and Negishi, K. (1982). 5-Hydroxytryptamine stimulated [^{3}H]dopamine release from the fish retina. *J. Neurochem.* **39,** 493–498.

Kennedy, A. J., and Neal, M. J. (1978). The effect of light and potassium depolarization on the release of endogenous amino acids from isolated rat retina. *Exp. Eye Res.* **26,** 71–75.

Kennedy, A. J., and Voaden, M. J. (1974). Free amino acids in the photoreceptor cells of the frog retina. *J. Neurochem.* **23,** 1093–1095.

Kennedy, A. J., Neal, M. J., and Lolley, R. N. (1977). The distribution of amino acids within the rat retina. *J. Neurochem.* **29,** 152–159.

Klein, D. C., and Weller, J. L. (1970). Indole metabolism in the pineal gland: A circadian rhythm in *N*-acetyltransferase. *Science* **169,** 1093–1095.

Koh, S.-W., and Chader, G. J. (1984). Agonist effects on the intracellular cyclic AMP concentration of retinal pigment epithelial cells in culture. *J. Neurochem.* **42,** 287–289.

Koh, S.-W. M., Kyritsis, A., and Chader, G. J. (1984). Interaction of neuropeptides and cultured glial (Müller) cells of the chick retina: Elevation of intracellular cyclic AMP by vasoactive intestinal peptide and glucagon. *J. Neurochem.* **43,** 199–203.

Kojima, K., Mizuno, K., and Mizazaki, M. (1958). Gamma-aminobutyric acid in ocular tissue. *Nature (London)* **181,** 1200–1201.

Kondo, H., and Toyoda, J. (1983). GABA and glycine effects on bipolar cells on the carp retina. *Vision Res.* **23,** 1259–1264.

Kong, Y.-C., Fung, S.-C., and Lam, D. M. K. (1980). Postnatal development of glyclinergic neurons in the rabbit retina. *J. Comp. Neurol.* **193,** 1127–1135.

Kramer, S. G. (1971). Dopamine: A retinal neurotransmitter. I. Retinal uptake, storage, and light stimulated release of H3-dopamine *in vivo*. *Invest. Ophthalmol.* **10,** 438–452.

Kramer, S. G., Potts, A. M., and Mangnall, Y. (1971). Dopamine: A retinal neurotransmitter. II. Autoradiographic localization of H3-dopamine in the retina. *Invest. Ophthalmol.* **10,** 617–624.

Krogsgaard-Larsen, P., Johnston, G. A. R., Curtis, D. R., Game, C. J. A., and McCulloch, R. M. (1975). Structure and biologic activity of a series of conformationally restricted analogues of GABA. *J. Neurochem.* **25,** 803–809.

Kubicék, R., and Dolének, A. (1958). Taurine et acids aminos dans la retine des animaux. *J. Chromatogr.* **1,** 266–268.

Kuriyama, K., Siskin, B., Haber, B., and Roberts, E. (1968). The γ-aminobutyric acid system in rabbit retina. *Brain Res.* **9,** 165–168.

Kuwayama, Y., Ishimoto, I., Fukuda, M., Shiosaka, Y. S. S., Inagaki, S., Senba, E., Sakanaka, M., Takagi, H., Takasuki, K., Hara, Y., Kawai, Y., and Tohyama, M. (1982). Overall distribution of glucagon-like immunoreactivity in the chicken retina: An immunohistochemical study with flat-mounts. *Invest. Ophthalmol. Visual Sci.* **22,** 681–686.

Lake, N. (1981). Depletion of retinal taurine by treatment with guanidinoethyl sulfonate. *Life Sci.* **29,** 445–448.

Lake, N. (1982). Is taurine an essential amino acid? *Retina* **2,** 261–262.

Lake, N., Marshall, J., and Voaden, M. J. (1977). The entry of taurine into the neural retina and pigment epithelium of the frog. *Brain Res.* **128,** 497–503.

Lake, N., Marshall, J., and Voaden, M. J. (1978). High affinity uptake sites for taurine in the retina. *Exp. Eye Res.* **27,** 713–718.

Lam, D. M. K. (1975). Biosynthesis of γ-aminobutyric acid by isolated axons of cone horizontal cells in the goldfish retina. *Nature (London)* **254,** 345–347.

Lam, D. M. K. (1976). Synaptic chemistry of identified cells in the vertebrate retina. *Cold Spring Harbor Symp. Quant. Biol.* **XL,** 571–576.

Lam, D. M. K., and Hollyfield, J. G. (1980). Localization of putative amino acid neurotransmitters in the human retina. *Exp. Eye Res.* **31,** 729–732.

Lam, D. M. K., Lasater, E. M., and Naka, K.-I. (1978). γ-Aminobutyric acid: A neurotransmitter candidate for cone horizontal cells of the catfish retina. *Proc. Natl. Acad. Sci. U.S.A.* **25,** 6310–6313.

Lam, D. M. K., Su, Y. Y. T., Swain, L., Marc, R. E., Brandon, C., and Wu, J.-Y. (1979). Immunocytochemical localization of L-glutamic acid decarboxylase in the goldfish retina. *Nature (London)* **278,** 565–567.

Lasater, E. M., and Dowling, J. E. (1982). Carp horizontal cells in culture respond selectively to L-glutamate and its agonists. *Proc. Natl. Acad. Sci. U.S.A.* **79,** 936–940.

Lasater, E. M., Watling, K. J., and Dowling, J. E. (1983). Vasoactive intestinal peptide alters membrane potential and cyclic nucleotide levels in retinal horizontal cells. *Science* **221,** 1070–1072.

Laties, A. M. (1972). Specific neurohistology comes of age: A look back and a look forward. *Invest. Ophthalmol.* **11,** 555–584.

Laties, A., and Jacobowitz, D. (1966). A comparative study of the autonomic innervention of the eye in monkey, cat and rabbit. *Anat. Rec.* **156,** 383–396.

Laufer, M., and Salas, R. (1981). Intercellular coupling and retinal horizontal cell receptive field. *Neurosci. Lett.* **24,** S339.

Laufer, M., Negishi, K., and Drujan, B. D. (1981). Pharmacological manipulation of spatial properties of S-potentials. *Vision Res.* **21,** 1657–1660.

LaVail, M. M. (1976). Rod outer segment disk shedding in rat retina: Relationship to cyclic lighting. *Science* **194,** 1071–1074.

Leino, M. (1984). 6-Methoxy-tetrahydro-β-carboline and melatonin in the human retina. *Exp. Eye Res.* **38,** 325–330.

Levitt, M., Spector, S., Sjoerdsma, A., and Udenfriend, S. (1965). Elucidation of the rate-limiting step in noradrenaline biosynthesis in the perfused guinea-pig heart. *J. Pharmacol. Exp. Ther.* **148,** 1–8.

López-Colomé, A. M., Tapia, R., Salceda, R., and Pasantes-Morales, H. (1978). Potassium-stimulated release of GABA, glycine and taurine from the chick retina. *Neurochem. Res.* **3,** 431–441.

McCulloch, J., Savaki, H. E., McCulloch, M. C., and Sokoloff, L. (1980). Retina-dependent activation by apomorphine of metabolic activity in the superficial layer of the superior colliculus. *Science* **207,** 313–315.

Magistretti, P., and Schorderet, M. (1979). Dopamine receptors in bovine retina: Characterization of the ^{3}H-spiroperidol binding and its use for screening dopamine receptor affinity of drugs. *Life Sci.* **25,** 1675–1686.

Makman, M. H., Dvorkin, B., Horowitz, S. G., and Thal, L. J. (1980). Properties of dopamine agonist and antagonist binding sites in mammalian retina. *Brain Res.* **194,** 403–418.

Malmfors, T. (1963). Evidence of adrenergic neurons with synaptic terminals in the retina of rats demonstrated with fluorescence and electron microscopy. *Acta Physiol. Scand.* **58,** 99–100.

Marc, R. E., and Lam, D. M. K. (1981a). Uptake of aspartic and glutamic acid by photoreceptors in goldfish retina. *Proc. Natl. Acad. Sci. U.S.A.* **78,** 7185–7189.

Marc, R. E., and Lam, D. M. K. (1981b). Glycinergic pathways in the goldfish retina. *J. Neurosci.* **1,** 152–165.

Marc, R. E., Stell, W. K., Bok, D., and Lam, D. M. K. (1978). GABA-ergic pathways in the goldfish retina. *J. Comp. Neurol.* **182,** 221–246.

Markey, K., Kondo, S., Shenkman, L., and Goldstein, M. (1980). Purification and characterization of tyrosine hydroxylase from a clonal pheochromocytoma cell line. *Mol. Pharmacol.* **17,** 79–85.

Marshak, D. W., Reeve, J. R., Shively, J. E., Hawke, D., Takami, M. S., and Yamada, T. (1983). Structure of somatostatin isolated from bovine retina. *J. Neurochem.* **41,** 601–606.

Marshall, J., and Voaden, M. (1974a). An autoradiographic study of the cells accumulating ^{3}H γ-aminobutyric acid in the isolated retinas of pigeons and chickens. *Invest. Ophthalmol.* **13,** 602–607.

Marshall, J., and Voaden, M. J. (1974b). A study of γ-[^{3}H]aminobutyric acid and [^{3}H]glycine accumulation by the isolated pigeon retina utilizing scintillation radioautography. *Biochem. Soc. Trans.* **2,** 268–270.

Marshburn, P. B., and Iuvone, P. M. (1981). The role of GABA in the regulation of the dopamine tyrosine hydroxylase-containing neurons of the rat retina. *Brain Res.* **214,** 335–347.

Masland, R. H., and Ames, III, A. (1976). Responses to acetylcholine of ganglion cells in an isolated mammalian retina. *J. Neurophysiol.* **39,** 1220–1235.

Masland, R. H., and Livingstone, C. J. (1976). Effect of stimulation with light on synthesis and release of acetylcholine by an isolated mammalian retina. *J. Neurophysiol.* **39,** 1210–1219.

Masland, R. H., and Mills, J. W. (1979). Autoradiographic identification of acetylcholine in the rabbit retina. *J. Cell Biol.* **83,** 159–178.

Massey, S. C., and Neal, N. J. (1979). The light-evoked release of acetylcholine from rabbit retina *in vivo* and its inhibition by γ-aminobutyric acid. *J. Neurochem.* **32,** 1327–1329.

Massey, S. C., and Redburn, D. A. (1982). A tonic γ-aminobutyric acid-mediated inhibition of cholinergic amacrine cells in rabbit retina. *J. Neurosci.* **2,** 1633–1643.

Melamed, E., Durst, R., Frucht, Y., and Globus, M. (1983). Differential effect of acute and chronic haloperidol administration on dopamine turnover in rat nigrostiatal and retinal dopaminergic neurons. *Eur. J. Pharmacol.* **89,** 279–282.

Miller, A. M., and Schwartz, E. A. (1983). Evidence for the identification of synaptic transmitters released by photoreceptors of the toad retina. *J. Physiol. (London)* **334,** 325–349.

Miller, L., Stier, M., and Lovenberg, W. (1980). Evidence for the presence of *N*-acetyltransferase in rat retina. *Comp Biochem. Physiol.* **66C,** 213–216.

Miller, R. F., Frumkes, T. E., Slaughter, M., and Dacheux, R. F. (1981). Physiological and pharmacological basis of GABA and glycine action on neurons of mudpuppy retina. II. Amacrine and ganglion cells. *J. Neurophysiol.* **45,** 764–782.

Mitchell, C. K., and Redburn, D. A. (1985). Analysis of pre- and postsynaptic factors of the serotonin system in rabbit retina. *J. Cell Biol.* **100,** 64–73.

Morán, J., and Pasantes-Morales, H. (1983). Effects of excitatory amino acids, and of their agonists and antagonists on the release of neurotransmitters from chick retina. *J. Neurosci. Res.* **10,** 261–271.

Moreno-Yanes, J. A., and Mahler, H. R. (1979). Subcellular distribution of [^{3}H]quinuclidinyl benzylate binding activity in vertebrate retina and its relationship to other cholinergic markers. *J. Neurochem.* **33,** 505–516.

Morgan, I. G., Oliver, J., and Chubb, I. W. (1981). Discrete distributions of putative cholinergic and somatostatinergic amacrine cell dendrites in chicken retina. *Neurosci. Lett.* **27,** 55–60.

Morgan, W. W., and Kamp, C. W. (1980). A GABAergic influence on the light-induced increase in dopamine turnover in the dark-adapted rat retina *in vivo*. *J. Neurochem.* **34,** 1082–1086.

Morgan, W. W., and Kamp, C. W. (1983a). Extended exposure to continuous low intensity light abolishes the photosensitivity of retinal dopamine neurons. *Life Sci.* **33,** 1419–1426.

Morgan, W. W., and Kamp, C. W. (1983b). Effect of strychnine and bicuculline on dopamine synthesis in retinas of dark-maintained rats. *Brain Res.* **278,** 362–365.

Mosinger, J. L. (1983). Aspartate aminotransferase-like immunoreactivity as a marker for aspartate/glutamate in the monkey retina. *Soc. Neurosci. Abstr.* **9,** 801.

Murakami, M., Ohtsu, K., and Ohtsuka, T. (1972). Effects of chemicals on receptors and horizontal cells in retina. *J. Physiol. (London)* **227,** 889–913.

Murakami, M., Ohtsuka, T., and Shimazaki, H. (1975). Effects of aspartate and glutamate on the bipolar cells in the carp retina. *Vision Res.* **15,** 456–458.

Mustakallio, A. (1967). Monoamine oxidase activity in the various structures of the mammalian eye. *Acta Ophthal. Suppl.* **93,** 1–62.

Nagatsu, T., Levitt, M., and Udenfriend, S. (1964). Tyrosine hydroxylase. The initial step in norepinephrine biosynthesis. *J. Biol. Chem.* **239,** 2910–2917.

Naka, K. (1977). Functional organization of catfish retina. *J. Neurophysiol.* **40,** 22–43.

Naka, K. I., and Rushton, W. A. (1967). The generation and spread of S-potentials in fish (Cyprinidae). *J. Physiol. (London)* **192,** 437–462.

Nakamura, Y., McGuire, B. A., and Sterling, P. (1980). Interplexiform cell in cat retina: Identification by uptake of γ-[^{3}H]aminobutyric acid and serial reconstruction. *Proc. Natl. Acad. Sci. U.S.A.* **77,** 658–661.

Neal, M. J., and Gilroy, J. (1975). High affinity choline transport in the retina. *Brain Res.* **93,** 548–551.

Neal, M. J., and Iversen, L. L. (1972). Autoradiographic localization of ^{3}H-GABA in rat retina. *Nature (London) New Biol.* **235,** 217–218.

Neal, M. J., Peacock, D. G., and White, R. D. (1973). Kinetic analysis of amino acid uptake by the rat retina *in vitro*. *Br. J. Pharmacol* **47,** 656–657.

Neal, M. J., Collins, G. G., and Massey, S. C. (1979). Inhibition of aspartate release from the retina of the anesthetized rabbit by stimulation with light flashes. *Neurosci. Lett.* **14,** 241–245.

Negishi, K., and Drujan, B. D. (1979a). Effects of catecholamines and related compounds on horizontal cells in the fish retina. *J. Neurosci. Res.* **4,** 311–334.

Negishi, K., and Drujan, B. D. (1979b). Similarities in effects of acetylcholine and dopamine on horizontal cells in fish retina. *J. Neurosci. Res.* **4,** 335–349.

Negishi, K., Kato, S., Teranishi, T., and Laufer, M. (1978). An electrophysiological study of the cholinergic system in the carp retina. *Brain Res.* **148,** 85–93.

Negishi, K., Laufer, M., and Drujan, B. D. (1980). Drug-induced changes in catecholaminergic cells of the fish retina. *J. Neurosci. Res.* **5,** 599–610.

Negishi, K., Kato, S., and Teranishi, T. (1981). Indoleamine-accumulating cells and dopaminergic cells are distributed similarly in carp retina. *Neurosci. Lett.* **25,** 1–5.

Nesselhut, T., and Osborne, N. N. (1982). Is noradrenaline a major catecholamine in the bovine retina? *Neurosci. Lett.* **28,** 41–45.

Nguyen-Legros, J., Berger, B., Vigny, A., and Alverez, C. (1981). Tyrosine hydroxylase-like immunoreactive interplexiform cells in the rat retina. *Neurosci. Lett.* **27,** 255–259.

Nguyen-Legros, J., Botteri, C., LeHoang, P., Vigny, A., and Gay, M. (1984). Morphology of primate's dopaminergic amacrine cells as revealed by TH-like immunoreactivity on retinal flat mounts. *Brain Res.* **295,** 145–153.

Nicholas, C. W,, and Koelle, G. B. (1968). Comparison of the localization of acetylcholinesterase and non-specific cholinesterase activities in mammalian and avian retinas. *J. Comp. Neurol.* **133,** 1–15.

Onali, P., Schwartz, J. P., and Costa, E. (1981). Dopaminergic modulation of adenylate cyclase stimulation by vasoactive intestinal peptide in anterior pituitary. *Proc. Natl. Acad. Sci. U.S.A.* **78,** 6531–6534.

Orr, H. T., Cohen, A. I., and Lowry, O. H. (1976). The distribution of taurine in the vertebrate retina. *J. Neurochem.* **26,** 609–611.

Osborne, N. N. (1980). *In vitro* experiments on the metabolism, uptake and release of 5-hydroxytryptamine in bovine retina. *Brain Res.* **184,** 283–298.

Osborne, N. N. (1981a). Noradrenaline, a transmitter candidate in retina. *J. Neurochem.* **36,** 17–27.

Osborne, N. N. (1981b). Binding of [^{3}H]serotonin to membranes of the bovine retina. *Exp. Eye Res.* **33,** 371–380.

Osborne, N. N. (1982a). Binding of (−) ^{3}H-noradrenaline to bovine membrane of the retina. Evidence for the existence of alpha$_2$ receptors. *Vision Res.* **22,** 1401–1407.

Osborne, N. N. (1982b). Uptake, localization and release of serotonin in the chick retina. *J. Physiol. (London)* **331,** 469–479.

Osborne, N. N., and Nesselhut, T. (1983). Adrenaline: Occurrence in the bovine retina. *Neurosci. Lett.* **39,** 33–36.

Osborne, N. N., Nicholas, D. A., Cuello, A. C., and Dockray, G. J. (1981). Localization of cholecystokinin immunoreactivity in amacrine cells of the retina. *Neurosci. Lett.* **26,** 31–35.

Osborne, N. N., Nesselhut, T., Nicholas, D. A., Patel, S., and Cuello, A. C. (1982a). Serotonin-containing neurons in vertebrate retinas. *J. Neurochem.* **39,** 1519–1528.

Osborne, N. N., Nicholas, D. A., Dockray, G. J., and Cuello, A. C. (1982b). Cholecystokinin and substance P immunoreactivity in retinas of rats, frogs, lizards and chicks. *Exp. Eye Res.* **34,** 639–649.

Osol, G., and Schwartz, B. (1984). Melatonin in the human retina. *Exp. Eye Res.* **38,** 213–215.

Oswald, R. E., Schmidt, D. E., and Freeman, J. A. (1979). Assessment of acetylcholine as an optic nerve neurotransmitter in *Bufo marinus*. *Neurosci.* **4,** 1129–1136.

Pang, S. F., Brown, G. M., Grota, L. J., Chambers, J. W., and Rodman, R. L. (1977). Determination of *N*-acetylserotonin and melatonin activities in pineal gland, retina, Harderian gland, brain and serum of rats and chickens. *Neuroendocrinol.* **23,** 1–13.

Pang, S. F., Yu, H. S., and Tang, P. L. (1982). Regulation of melatonin in the retinae of guinea pigs: Effect of environmental lighting. *J. Exp. Zool.* **222,** 11–16.

Parkinson, D., and Rando, R. (1981). Evidence for a neurotransmitter role for 5-hydroxytryptamine in chick retina. *J. Neurosci.* **1,** 1211–1217.

Parkinson, D., and Rando, R. R. (1983a). Effects of light on dopamine metabolism in the chick retina. *J. Neurochem.* **40,** 39–46.

Parkinson, D., and Rando, R. R. (1983b). Effect of light on dopamine turnover and metabolism in rabbit retina. *Invest. Ophthalmol. Visual Sci.* **24,** 384–388.

Parkinson, D., Baughman, R., Masland, R. H., and Rando, R. R. (1981). Dopamine metabolism following irreversible inactivation of aromatic amino acid decarboxylase in retina. *J. Neurosci.* **1,** 1205–1210.

Pasantes-Morales, H., Klethi, J., Ledig, M., and Mandel, P. (1972a). Free amino acids of chicken and rat retina. *Brain Res.* **41,** 494–497.

Pasantes-Morales, H., Klethi, J., Urban, P. F., and Mandel, P. (1972). The physiological role of taurine in retina: Uptake and effect on electroretinogram (ERG). *Physiol. Chem. Phys.* **4,** 339–348.

Pasantes-Morales, H., Bonaventure, N., Wioland, N., and Mandel, P. (1973). Effect of intravitreal injections of taurine and GABA on chicken electroretinogram. *Int. J. Neurosci.* **5,** 235–241.

Pasantes-Morales, H., Salceda, R., and López-Colomé, A. M. (1980). The effect of colchicine and cytochalesin B on the release of taurine from the chick retina. *J. Neurochem.* **34,** 172–177.

Piccolino, M., Neyton, J., Witkovsky, P., and Gershenfeld, H. M. (1982). γ-Aminobutyric acid antagonists decrease junctional communtion between L-horizontal cells of the retina. *Proc. Natl. Acad. Sci. U.S.A.* **79,** 3671–3675.

Pierce, M. E., and Besharse, J. C. (1985). Circadian regulation of retinomotor movements: I. Interaction of melatonin and dopamine in the control of cone length. *J. Gen. Physiol.* **86,** 671–689.

Pierce, M. E., Iuvone, P. M., and Besharse, J. C. (1984). Melatonin and photoreceptor metabolism: Regulation of cone retinomotor movement by melatonin and dopamine. *Soc. Neurosci. Abst.* **10,** 19.

Pourcho, R. G. (1977). Distribution of [^{3}H]taurine in mouse retina after intravitreal and intravascular injection. *Exp. Eye Res.* **25,** 119–127.

Pourcho, R. G. (1980). Uptake of [^{3}H]glycine and [^{3}H]GABA by amacrine cells in the cat retina. *Brain Res.* **198,** 333–346.

Pourcho, R. G. (1981). [^{3}H]Taurine-accumulating neurons in the cat retina. *Exp. Eye Res.* **32,** 11–20.

Pourcho, R. G. (1981b). Autoradiographic localization of [^{3}H]muscimol in the cat retina. *Brain Res.* **215,** 187–199.

Proll, M. A., and Morgan, W. W. (1983). Use of γ-aminobutyric acid (GABA)-transaminase inhibitors and a GABA uptake inhibitor to investigate the influence of GABA neurons on dopamine-containing amacrine cells of the rat retina. *J. Pharmacol. Exp. Ther.* **227,** 627–630.

Proll, M. A., Kamp, C. W., and Morgan, W. W. (1982). Use of liquid chromatography with electrochemistry to measure effects of varying intensities of white light on DOPA accumulation in rat retinas. *Life Sci.* **30,** 11–19.

Raese, J. D., Edelman, A. M., Lazar, M. A., and Barchas, J. D. (1977). Bovine striatal tyrosine hydroxylase: Multiple forms and evidence for phosphorylation by cyclic AMP-dependent protein kinase. *In* "Structure and Function of Monoamine Enzymes" (E. Usdin, N. Weiner, and M. B. H. Youdim, eds.) pp. 383–400. Dekker, New York.

Rasmussen, K.-E. (1972). A morphometric study of the Müller cell cytoplasm in the rat retina. *J. Ultrastruct. Res.* **39,** 413–429.

Rayborn, M. E., Sarthy, P. V., Lam, D. M. K., and Hollyfield, J. G. (1981). The emergence, localization and maturation of neurotransmitter systems during development of the retina in *Xenopus laevis*. II. Glycine. *J. Comp. Neurol.* **195,** 585–593.

Redburn, D. A. (1977). Uptake and release of [^{14}C]GABA from rabbit retina synaptosomes. *Exp. Eye Res.* **25,** 265–275.

Redburn, D. A. (1984). Serotonin systems in the inner and outer plexiform layers of the vertebrate retina. *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **43,** 2699–2703.

Redburn, D. A., and Keyes, C. B. (1980). Localization and characterization of dopamine receptors within two synaptosome fractions of rabbit and bovine retina. *Exp. Eye Res.* **30,** 699–708.

Redburn, D. A., and Mitchell, C. K. (1981). ^{3}H-Muscimol binding in synaptosomal fractions from bovine and developing rabbit retinas. *J. Neurosci. Res.* **6,** 487–495.

Redburn, D. A., and Thomas, T. N. (1981). Evidence for a serotonin neurotransmitter system in bovine retina. *Vision Res.* **21,** 1673–1676.

Redburn, D. A., Clement-Cormier, Y., and Lam, D. M. K. (1970). Dopamine receptors in the goldfish retina: ^{3}H-spiroperidol and ^{3}H-domperidone binding; and dopamine-stimulated adenylate cyclase activity. *Life Sci.* **27,** 23–32.

Redburn, D. A., Kyles, C. B., and Ferkany, J. (1979). Subcellular distribution of GABA receptors in bovine retina. *Exp. Eye Res.* **28,** 525–532.

Reiter, R. J., Richardson, B. A., and Hurlbut, E. C. (1981). Pineal, retinal and Harderian gland melatonin in a diurnal species, the Richardson's ground squirrel (*Spermophilus richardonii*). *Neurosci. Lett.* **22,** 285–288.

Reiter, R. J., Richardson, B. A., Matthews, S. A., Lane, S. J., and Ferguson, B. N. (1983). Rhythms in immunoreactive melatonin in the retina and Harderian gland of rats: Persistence after pinealectomy. *Life Sci.* **32,** 1229–1236.

Reppert, S. M., and Sagar, S. M. (1983). Characterization of the day–night variation of retinal melatonin content in the chick. *Invest. Ophthal. Visual Sci.* **24,** 294–300.

Ripps, H., Shakib, M., and MacDonald, E. D. (1976). Peroxidase uptake by photoreceptor terminals of the skate retina. *J. Cell Biol.* **70,** 86–96.

Rorstad, O. P., Brownstein, M. J., and Martin, J. B. (1979). Immunoreactive and biologically active somatostatin-like material in the rat retina. *Proc. Natl. Acad. Sci. U.S.A.* **76,** 3019–3023.

Rorstad, O. P., Senterman, M. K., Hoyte, K. M., and Martin, J. B. (1980). Immunoreactive and biologically active somatostatin-like material in the human retina. *Brain Res.* **199,** 488–492.

Ross, C. D., and McDougal, D. B., Jr. (1976). The distribution of choline acetyltransferase activity in vertebrate retina. *J. Neurochem.* **26,** 521–526.

Rowe, J.-S., and Ruddock, K. H. (1982). Depolarization of retinal horizontal cells by excitatory amino acid neurotransmitter agonists. *Neurosci. Lett.* **30,** 257–262.

Salceda, R., Cárabez, A., Pacheo, P., and Pasantes-Morales, H. (1979). Taurine levels, uptake and synthesizing enzyme activities in degenerated rat retinas. *Exp. Eye Res.* **28,** 137–146.

Sarthy, P. V., and Lam, D. M. K. (1979a). The uptake and release of [^{3}H]-dopamine in the goldfish retina. *J. Neurochem.* **32,** 1269–1277.

Sarthy, P. V., and Lam, D. M. K. (1979b). Isolated cells from a mammalian retina. *Brain Res.* **176,** 208–212.

Sarthy, P. V., Rayborn, M. E., Hollyfield, J. G., and Lam, D. M. K. (1981). The emergence, localization and maturation of neurotransmitter systems during the development of the retina in *Xenopus laevis*. III. Dopamine. *J. Comp. Neurol.* **195,** 595–602.

Scatton, B., Dedek, J., and Korf, J. (1977). Effect of single and repeated administration of haloperidol on striatal and retinal dopamine turnover in the rat. *Brain Res.* **135,** 374–377.

Schacher, S. M., Holtzman, E., and Hood, D. C. (1974). Uptake of horseradish peroxidase by frog photoreceptor synapses in the dark and the light. *Nature (London)* **249,** 261–263.

Schacher, S., Holtzman, E., and Hood, D. C. (1976). Synaptic activity of frog retinal photoreceptors. A peroxidase uptake study. *J. Cell Biol.* **70,** 178–192.

Schaeffer, J. M. (1980). Identification of dopamine receptors in the rat retina. *Exp. Eye Res.* **30,** 431–437.

Schmidt, S.-Y., Berson, E. L., and Hayes, K. C. (1976). Retinal degeneration in cats fed casein. I. Taurine deficiency. *Invest. Ophthal. Visual Sci.* **15,** 47–52.

Schorderet, M. (1977). Pharmacological characterization of the dopamine-mediated accumulation of cyclic AMP in intact retina of rabbit. *Life Sci.* **20,** 1741–1748.

Schwarcz, R., and Coyle, J. T. (1976). Adenylate cyclase activity in chick retina. *Gen. Pharmacol.* **7,** 349–354.

Schwartz, E. A. (1982). Calcium-independent release of GABA from isolated horizontal cells of the toad retina. *J. Physiol. (London)* **323,** 211–227.

Simon, E. J. (1973). Two types of luminosity horizontal cells in the retina of the turtle. *J. Physiol. (London)* **230,** 199–211.

Slaughter, M. M., and Miller, R. F. (1981). 2-Amino-4-phosphonobutyric acid: A new pharmacological tool for retina research. *Science* **211,** 182–185.

Slaughter, M. M., and Miller, R. F. (1983a). An excitatory amino acid antagonist blocks cone input to sign-conserving second-order retinal neurons. *Science* **219,** 1230–1232.

Slaughter, M. M., and Miller, R. F. (1983b). The role of excitatory amino acid transmitters in the mudpuppy retina: An analysis with kainic acid and *N*-methylaspartate. *J. Neurosci.* **3,** 1701–1711.

Slaughter, M. M., and Miller, R. J. (1983c). Bipolar cells in the mudpuppy retina use an excitatory amino acid neurotransmitter. *Nature (London)* **303,** 537–538.

Sovilla, J.-Y., and Schorderet, M. (1982). L-DOPA mediated accumulation of cyclic AMP in isolated rabbit retina *in vitro*. Effects of light and/or pharmacological factors. *Life Sci.* **31,** 2081–2092.

Spano, P. F., Govoni, S., Hofmann, M., Kumakura, K., and Trabucchi, M. (1977). Physiological and pharmacological influences on dopaminergic receptors in the retina. *Adv. Biochem. Psychopharmacol.* **16,** 307–310.

Starke, K., Endo, T., and Taube, H. D. (1975). Relative pre- and postsynaptic potencies of α-adrenoceptor agonists in the rabbit pulmonary artery. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **291,** 55–78.

Starr, M. S. (1973). Effect of dark-adaptation on the GABA system in retina. *Brain Res.* **59,** 331–338.

Starr, M. S. (1975). The effects of various amino acids, dopamine and some convulsants on the electroretinogram of the rabbit. *Exp. Eye Res.* **21,** 79–87.

Stell, W. K. (1967). The structure and relationships of horizontal cells and photoreceptor–bipolar synaptic complexes in goldfish retina. *Am. J. Anat.* **120,** 401–424.

Stewart, W. W. (1978). Functional connections between cells as revealed by dye-coupling with a highly fluorescent napthalimide tracer. *Cell,* **14,** 741–759.

Stone, R., Laties, A., and Sterling, P. (1983). Specific types of horizontal and ganglion cell in cat retina stained by antiserum raised against alpha-MSH. *Soc. Neurosci. Abstr.* **9,** 803.

Straschill, M. (1968). Action of drugs on single neurons in the cat's retina. *Vision Res.* **8,** 35–47.

Straschill, M., and Perwein, J. (1969). The inhibition of retinal ganglion cells by catecholamines and γ-aminobutyric acid. *Pflugers Arch.* **312,** 45–54.

Tapia, R., and Arias, C. (1982). Selective stimulation of neurotransmitter release from chick retina by kainic and glutamic acids. *J. Neurochem.* **39,** 1169–1178.

Teranishi, T., Negishi, K., and Kato, S. (1983). Dopamine modulates S-potential amplitude and dye-coupling between external horizontal cells in carp retina. *Nature (London)* **301,** 243–246.

Terubayashi, H., Tsuto, T., Fukui, K., Obata, H. L., Okamura, H., Fujisawa, H., Itoi, M., Yanaihara, C., Yanaihara, N., and Ibata, Y. (1983). VIP (vasoactive intestinal polypeptide)-like immunoreactive amacrine cells in the retina of the rat. *Exp. Eye Res.* **36,** 743–749.

Thier, P., and Bolz, J. (1985). Cholecystokinin in the cat retina. Action of exogenous CCK8 and localization of cholecystokinin-like immunoreactivity. *Invest. Ophthalmol. Visual Sci.* **26,** 266–272.

Thomas, T. N., and Redburn, D. A. (1978). Uptake of [^{14}C]aspartic acid and [^{14}C]glutamic acid by retinal synaptosomal fractions. *J. Neurochem.* **31,** 63–68.

Thomas, T. N., and Redburn, D. A. (1979). 5-Hydroxytryptamine—A neurotransmitter of bovine retina. *Exp. Eye Res.* **28,** 55–61.

Thomas, T. N., and Redburn, D. A. (1980). Serotonin-uptake and release by subcellular fractions of bovine retina. *Vision Res.* **20,** 1–8.

Thomas, T. N., Clement-Cormier, Y. C., and Redburn, D. A. (1978). Uptake and release of [^{3}H]dopamine and dopamine-sensitive adenylate cyclase activity in retinal synaptosomal fractions. *Brain Res.* **155,** 391–396.

Tornqvist, K., and Ehinger, B. (1983). Simultaneous immunohistochemistry and autoradiography of peptides and 5-hydroxytryptamine in bird retina. *Neurochem. Int.* **5,** 579–586.

Tornqvist, K., Uddman, R., Sundler, F., and Ehinger, B. (1982). Somatostatin and VIP neurons in the retina of different species. *Histochem.* **76,** 137–157.

Tornqvist, K., Hansson, C. L., and Ehinger, B. (1983). Immunohistochemical and quantitative analysis of 5-hydroxytryptamine in the retina of some vertebrates. *Neurochem. Int.* **5,** 299–308.

Van Buskirk, R., and Dowling, J. E. (1981). Isolated horizontal cells from carp retina demonstrate dopamine-dependent accumulation of cyclic AMP. *Proc. Natl. Acad. Sci. U.S.A.* **78,** 7825–7829.

Van Buskirk, R., and Dowling, J. E. (1982). Calcium alters the sensitivity of intact horizontal cells to dopamine antagonists. *Proc. Natl. Acad. Sci. U.S.A.* **79,** 3350–3354.

Vaughn, J. E., Famiglietti, Jr., E. V., Barber, R. P., Saito, K., Roberts, E., and Ribak, E. E. (1981). GABAergic amacrine cells in rat retina: Immunocytochemical identification and synaptic connectivity. *J. Comp. Neurol.* **197,** 113–127.

Versaux-Botteri, C., Nguyen-Legros, J., Vigny, A., and Raoux, N. (1984). Morphology, density and distribution of tyrosine hydroxylase-like immunoreactive cells in the retina of mice. *Brain Res.* **301,** 192–197.

Vivas, I. M., and Drujan, B. D. (1980). Certain aspects of acetylcholine metabolism in teleost retina. *Neurochem. Res.* **5,** 817–829.

Vivien-Roels, B., Pévet, P., Dubois, M. P., Arendt, J., and Brown, G. M. (1981). Immunohistochemical evidence for the presence of melatonin in the pineal gland, the retina and the Harderian gland. *Cell Tissue Res.* **217,** 105–115.

Voaden, M. J., Marshall, J., and Murani, N. (1974). The uptake of [^{3}H]γ-aminobutyric acid and [^{3}H]glycine by isolated retina of the frog. *Brain Res.* **67,** 115–132.

Voaden, M. J., Lake, N., Marshall, J., and Morjaria, B. (1977). Studies on the distribution of taurine and other neuroactive amino acids in the retina. *Exp. Eye Res.* **25,** 249–257.

Vulliet, P. R., Langan, T. A., and Weiner, N. (1980). Tyrosine hydroxylase—a substrate of cyclic AMP-dependent protein kinase. *Proc. Natl. Acad. Sci. U.S.A.* **77,** 92–96.

Walsh, D. A., Ashby, C. D., Gonzalez, C., Calkins, D., Fishcer, E. H., and Krebs, E. G. (1971). Purification and characterization of a protein inhibitor of adenosine 3′,5′ -monophosphate dependent protein kinase. *J. Biol. Chem.* **246,** 1977–1985.

Wang, S. J., Felix, D., and Frangi, U. (1978). The role of glutamate in pigeon optic tectum. *Brain Res.* **157,** 360–363.

Watling, K. J., and Dowling, J. E. (1981). Dopaminergic mechanisms in the teleost retina. I. Dopamine-sensitive adenylate cyclase in homogenates of carp retina; effects of agonists, antagonists and ergots. *J. Neurochem.* **36,** 559–568.

Watling, K. J., and Dowling, J. E. (1983). Effects of vasoactive intestinal peptide and other peptides on cyclic AMP accumulation in intact pieces and isolated horizontal cells of the teleost retina. *J. Neurochem.* **41,** 1205–1213.

Watling, K. J., Dowling, J. E., and Iversen, L. L. (1979). Dopamine receptors in the retina may all be linked to adenylate cyclase. *Nature (London)* **281,** 578–580.

Watling, K. J., Parkinson, D., and Dowling, J. E. (1982). Effects of intraocular injections of 6-hydroxydopamine on dopamine-dependent cyclic AMP accumulation in intact pieces of carp retina. *Brain Res.* **253,** 334–336.

Welsh, J. H., and Osborne, C. M. (1937). Diurnal changes in the retina of the catfish, *Ameiurus nebulosus*. *J. Comp Neurol.* **66,** 349–359.

Westerink, B. H. C., and Korf, J. (1976). Effects of drugs on the formation of homovanillic acid in the rat retina. *Eur. J. Pharmacol.* **40,** 175–178.

Westerink, B. H. C., and Korf, J. (1976b). Comparison of effects of drugs on dopamine metabolism in the substantia nigra and the corpus striatum of rat brain. *Eur. J. Pharmacol.* **40,** 131–136.

Wiechmann, A. F., Bok, D., and Horwitz, J. (1985). Localization of hydroxyindole *O*-methyltransferase in the mammalian pineal gland and retina. *Invest. Ophthalmol. Visual Sci.* **26,** 253–265.

Witkovsky, P., and Dowling, J. E. (1969). Synaptic relationships in the plexiform layers of carp retina. *Z. Zellforsch.* **100,** 60–82.

Witkovsky, P., Burkhardt, D. A., and Nagy, A. R. (1979). Synaptic connections linking cones and horizontal cells in the retina of the pikefish (*Stizostedion vitreum*). *J. Comp. Neurol.* **186,** 541–560.

Wu, S. M., and Dowling, J. E. (1978). L-Aspartate: Evidence for a role in cone photoreceptor transmission in the carp retina. *Proc. Natl. Acad. Sci. U.S.A.* **75,** 5205–5209.

Wu, S. M., and Dowling, J. E. (1980). Effects of GABA and glycine in the distal cells of cyprinid retina. *Brain Res.* **199,** 401–414.

Wyatt, H. J., and Daw, N. W. (1976). Specific effects of neurotransmitter antagonists on ganglion cells in rabbit retina. *Science* **191,** 204–205.

Wyse, J. P. H., and Lorscheider, F. L. (1981). Low retinal dopamine and serum prolactin levels indicate an inherited dopaminergic abnormality in BW rats. *Exp. Eye Res.* **32,** 541–551.

Yamada, T., and Basinger, S. (1982). Biosynthesis of somatostatin-like immunoreactivity by frog retinas *in vitro*. *J. Neurochem.* **39,** 1539–1546.

Yamada, T., Brecha, N., Rosenquist, G., and Basinger, S. (1981). Cholecystokinin-like immunoreactivity in frog retina: Localization, characterization and biosynthesis. *Peptides* **2,** (Suppl. 2), 93–97.

Yamauchi, T., and Fujisawa, H. (1979). *In vitro* phosphorylation of bovine adrenal tyrosine hydroxylase by adenosine 3′ : 5′ -monophosphate dependent protein kinase. *J. Biol. Chem.* **254,** 503–507.

Yazulla, S., and Kleinschmidt, J. (1982). Dopamine blocks carrier-mediated release of GABA from retinal horizontal cells. *Brain Res.* **233,** 211–215.

Yazulla, S., and Kleinschmidt, J. (1983). Carrier-mediated release of GABA from retinal horizontal cells. *Brain Res.* **263,** 63–75.

Yazulla, S., and Schmidt, J. (1977). Two types of receptors for α-bungarotoxin in the synaptic layers of the pigeon retina. *Brain Res.* **138,** 45–57.

Yeh, H. H., Battelle, B.-A., and Puro, D. G. (1983). Maturation of neurotransmission at cholinergic synapses formed in culture by rat retinal neurons: Regulation by cyclic AMP. *Dev. Brain Res.* **10,** 63–72.

Yeh, H. H., Batelle, B.-A., and Puro, D. B. (1984). Dopamine regulates synaptic transmission mediated by cholinergic neurons of the rat retina. *Neurosci.* **13,** 901–909.

Yu, H. S., Pang, S. F., and Tang, P. L. (1981). Increase in the level of retinal melatonin and persistance of its diurnal rhythm in rats after pinealectomy. *J. Endocrinol.* **91,** 477–481.

Yu, H. S., Chow, P. H., Tang, P. L., and Pang, S. F. (1982). Effect of light and darkness on the *in vitro* release of *N*-acetylserotonin and melatonin by the retina of guinea pigs. *Neuroendocrinol.* **34,** 265–268.

Zucker, C., Yazulla, S., and Wu, J.-Y. (1984). Non-correspondence of [^{3}H]GABA uptake and GAD localization in goldfish amacrine cells. *Brain Res.* **298,** 154–158.

VISION PSYCHOPHYSICS AND RETINAL CELL BIOLOGY

ROBERT W. MASSOF

Laboratory of Physiological Optics
The Wilmer Ophthalmological Institute
The Johns Hopkins University
School of Medicine
Baltimore, Maryland

I. Introduction

To many biologists, psychophysics might appear to be a cryptic discipline that is protected from the unwashed by an impenetrable shield of eponyms, laws, and

mathematical models. To other biologists, who fail to be impressed or intimidated by these deterrents, psychophysics might seem to be a "soft science," a discipline that concerns itself with "subjective" phenomena rather than the hard "objective" stuff of the biological sciences. Although it is true that psychophysicists must solicit the cooperation of their experimental subjects in order to study the properties of vision, psychophysical observations are no less empirical, objective, or reproducible than other biological observations. There are inherent limitations, however, and probably more so than other aspects of biology, psychophysics leans heavily on theory to understand observed phenomena.

The major limitation of vision psychophysics is inherent in the fundamental question being addressed, "How do we see?" Seeing, or vision, is a personal, subjective, and private phenomenon about which each of us has knowledge only through our own experience and introspection. Vision is strictly a mental event, a phenomenon of the mind that has no physical properties. Thus, vision per se is not a proper subject of the physical sciences; it is a subject that falls within the purviews of psychology and philosophy. Only solipsists would deny that external and internal physical events play a crucial role in determining our visual experiences. However, vision, the phenomenon of interest, is inaccessible to public measurement and the only way we can obtain information about vision is to ask the experimental subject to describe or act on the basis of what is seen. Thus, the so-called subjective aspect of psychophysics is a necessary part of the science. Vision cannot be studied any other way because vision, by its nature, is not measurable in the traditional sense.

It is now taken for granted that the retina and the brain are responsible for transducing light into vision. This assumption, which we call *psychoneural congruency,* provides the basis for drawing conclusions about retinal cell function from psychophysical studies. By employing appropriate physiological–psychophysical linking hypotheses (Brindley, 1970), we can study cell system behavior *in vivo* using noninvasive techniques. The purpose of this article is to introduce vision psychophysics to the cell biologist and show how psychophysics can be used to study the functioning of cells in the retina.

II. The Systems Approach of Psychophysics

Psychophysics approaches the study of vision as an input–output problem (see Massof and Bird, 1978; Bird and Massof, 1978). That is, the input, light, is manipulated in order to determine its effect on the output, vision; the information obtained is used to deduce the properties of the visual system. Thus, psychophysics treats the visual system like a "black box." The aim of psychophysics is to understand and describe the logic of that black box, rather than to understand

and describe its structure. Necessarily, the logic of the visual system is represented and modeled mathematically. To make sense out of psychophysics, it will be necessary to venture somewhat into the mathematics. However, to be merciful we will take a predominantly intuitive approach in this presentation.

A. Inputs, Outputs, and Black Boxes

Light is the input to the visual system, but light alone is not enough. To be an effective stimulus to vision, light must be absorbed by the visual pigments located in the outer segments of the photoreceptors. There are two major types of photoreceptors in the human retina, the rods and the cones. The rods contain the visual pigment rhodopsin, and the cones contain one of three different cone visual pigments (see Boynton, 1979). One cone pigment, which we will call the S pigment, absorbs light maximally in the short wavelengths ($\lambda_{max} \approx 440$ nm). The second cone pigment, which we will call the M pigment, absorbs light maximally in the middle wavelengths ($\lambda_{max} \approx 530$ nm). The third cone pigment, which we will call the L pigment, absorbs light maximally in the long wavelengths ($\lambda_{max} \approx 570$ nm).

Light is quantal in nature, and one quantum absorption is sufficient to photoisomerize a visual pigment molecule and activate the chain of neural events leading to vision (Hecht *et al.*, 1942). To specify the number of light quanta that will be absorbed by the visual pigment, we simply multiply the number of incident quanta at each wavelength by the absorption coefficient of the photopigment at the respective wavelength. The number of incident quanta can be specified by the light energy at each wavelength, P_λ. The absorption coefficients of the photopigments at each wavelength are specified as L_λ, M_λ, and S_λ for the long- , middle- , and short-wavelength-sensitive cone pigments, respectively, and R_λ for rhodopsin. Thus, for example, the total quanta absorbed by the L pigment, Q_L, integrated over the spectrum, would be specified as

$$Q_L = \int_0^\infty P_\lambda L_\lambda \, d\lambda \tag{1}$$

The distribution of light energy will vary spatially and temporally over the retinal image. That is, spectral light energy is a function of space (x,y) and time (t), i.e., $P_\lambda(t,x,y)$. The spectral absorption coefficients of the photopigments also may vary with retinal position (x,y) and, because of pigment bleaching and regeneration, the absorption coefficients also may vary with time (t). Thus, with both light energy and photopigment absorption coefficients depending on space and time, the total quantum absorptions (integrated over wavelength) will be dependent on space and time

$$Q_{\mathrm{L}}(t,x,y) = \int_0^\infty P_\lambda(t,x,y)\, L_\lambda(t,x,y)\, d\lambda \tag{2a}$$

$$Q_{\mathrm{M}}(t,x,y) = \int_0^\infty P_\lambda(t,x,y)\, M_\lambda(t,x,y)\, d\lambda \tag{2b}$$

$$Q_{\mathrm{S}}(t,x,y) = \int_0^\infty P_\lambda(t,x,y)\, S_\lambda(t,x,y)\, d\lambda \tag{2c}$$

$$Q_{\mathrm{R}}(t,x,y) = \int_0^\infty P_\lambda(t,x,y)\, R_\lambda(t,x,y)\, d\lambda \tag{2d}$$

for the L, M, and S cone pigments and rhodopsin, respectively.

The advantages of Eqs. (2a)–(2d) is that the input to the visual system is made explicit in terms of the spatiotemporal distribution of the light energy in the retinal image and the absorption spectra of the visual pigments. Although these equations provide a compact and complete notation for specifying the visual stimulus, we can be even more succinct by recognizing that a given distribution of light energy in the retinal image will give rise to a set of quantum absorptions. Thus, the input to the visual system can be summarized with the vector field notation[1] (Fig. 1)

$$\mathbf{Q}(t,x,y) = [Q_{\mathrm{L}}(t,x,y),\ Q_{\mathrm{M}}(t,x,y),\ Q_{\mathrm{S}}(t,x,y),\ Q_{\mathrm{R}}(t,x,y)] \tag{3}$$

where the vector function $\mathbf{Q}(t,x,y)$ simply refers to the quantum absorptions occurring in a set of visual pigments as a function of space and time.

Turning to the other end of the problem, the output of the visual system is visual sensation. The objects we claim to see actually are not objects at all, but are patches of colors and brightness. Indeed, all of our visual experience can be reduced to the abstract concept of color and brightness varying over space and time. Spatial variations in color and brightness are responsible for our perceptions of shape and form; temporal variations in color and brightness are responsi-

[1]The term "vector field" is jargon designed to obfuscate an otherwise very simple concept. The "field" part of the term is what you might expect—a cornfield is a good example. The term "vector" simply means that there are components that can be independently manipulated. Anytime a system has components that can be independently manipulated the system can be represented geometrically, as illustrated in Fig. 1A. Each axis on the diagram represents a different component of the system. A set of values of the components will determine a unique direction and magnitude of a resulting vector in that diagram. Thus, a vector field is simply a field in which each point can be represented by a vector. If we were to specify the height, color, and number of worms for each cornstalk, we would be talking about a "vector cornfield." In the more general sense, fields need not be specified by only spatial coordinates; any variables, including time, will do.

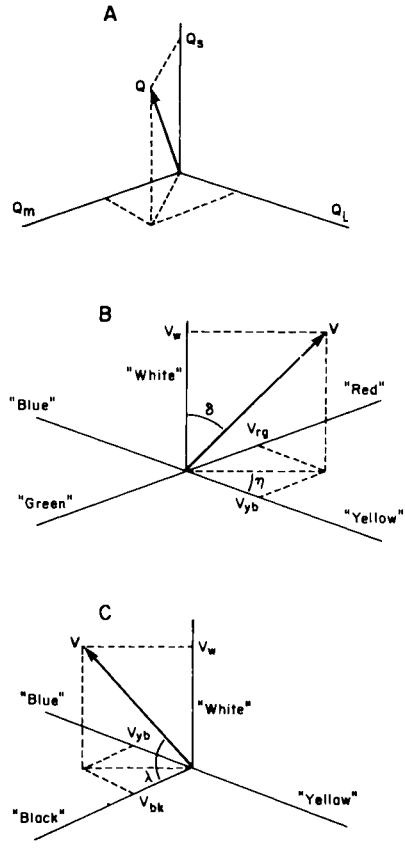


FIG. 1. (A) Three-dimensional vector space representing quantum absorptions among the three cone types. One dimension corresponds to the quantum absorptions in the L cone, Q_L, a second dimension corresponds to the quantum absorptions in the M cone, Q_M, and the third dimension corresponds to quantum absorptions in the S cone, Q_S. (The quantum absorptions in the rods would be represented by a fourth dimension.) Any particular light stimulus would produce some combination of quantum absorptions among the three cone types that can be summarized by the resulting vector, **Q.** The direction of the vector would be determined by the spectral composition of the light and the length of the vector would be determined by the intensity of the light. (B) Three-dimensional vector space representing the components of visual sensation (the fourth component is shown in C). One dimension, V_{rg}, corresponds to "red" for positive values and corresponds to "green" for negative values. A second dimension, V_{yb}, corresponds to "yellow" for positive values and corresponds to "blue" for negative values. A third dimension, V_w, corresponds to "white" and is only positive. "Black" is represented by a fourth positive dimension (C). Every visual sensation can be described in terms of some amount of "red" or "green," some amount of "blue" or "yellow," some amount of "white," and some amount of "black" (C). Thus, each visual sensation can be represented in a four-dimensional space by a vector **V,** whose direction corresponds to color and whose length corresponds to brightness. Color is decomposed into hue, saturation, and lightness which are represented by the angles η, δ, and λ (C), respectively.

ble for our perceptions of motion and change. Thus, color and brightness and their spatial and temporal dependence may be regarded as the elementary blocks out of which all of vision is built.

Brightness cannot be reduced to more elementary concepts, since it is simply a description of the strength of visual sensation. Color, on the other hand, is a complex component of visual experience that can be reduced to simpler categories, namely, hue, saturation, and lightness. Hue refers to the primary *chromaticness* of a color. For example, red and green differ in hue; red and pink do not. Saturation refers to the difference of a color from white. For example, baby blue is less saturated than royal blue, pink is less saturated than red; also, cutting across hue, pink is less saturated than royal blue. Lightness refers to the difference of a color from black. For example, yellow is lighter than brown and kelly green is lighter than olive (see Boynton, 1979; Hurvich, 1981).

Another way to specify color is to reduce it to elemental color names. All hues can be expressed as combinations of red, yellow, green, and blue. For example, lime green can be described in terms of yellowish-green, purple in terms of reddish-blue, aqua in terms of greenish-blue, and orange in terms of yellowish-red. Red and green are mutually exclusive sensations—we cannot experience reddish-green or greenish-red. Blue and yellow also are mutually exclusive sensations—we cannot experience bluish-yellow or yellowish-blue. These two pairs of mutually exclusive hues are called opponent colors (Hurvich, 1981).

Red, green, blue, and yellow are regarded to be elemental sensations; we cannot describe these sensations in simpler terms. Two other elemental sensations are white and black. Although white and black are often thought of as opposite, they are not mutually exclusive. For example, gray can be described as whitish-black (Heggelund, 1974). Because white and black are not hue names, they are called the achromatic colors.

The six elemental colors vary in space and time to make up the visual scene that we experience. However, the space and time that are used to describe visual sensations are *not* the same as physical space and time. Rather, they are sensory space and time, that is, coordinates that describe the ordering of sensory events. As far as we know, sensations per se do not necessarily occupy any physical space. To make the distinction between physical space and time and sensory space and time explicit, we employ the notation t, x, y for physical coordinates and t', x', y' for sensory coordinates.

At this point, we are skirting the edge of the mind–body problem, a topic that makes many of us uncomfortable when brought up in a serious scientific discourse. Most biologists enjoy the luxury of not having to deal with this issue, but for the psychophysicist, the problem must be squarely addressed. By necessity, and namesake, psychophysics must be dualistic in its approach. The input to the visual system is clearly physical, but the relevant output can be described only in psychological terms. To avoid nonproductive parlor room debates, the problem is converted to a mathematical one: visual sensation is treated as a vector field.

Each of the elementary color names makes up a component of visual sensation. Since red and green are mutually exclusive, they can be regarded as opposite poles of a single bipolar visual sensation component we will call V_{rg}. Similarly, the mutually exclusive yellow and blue elemental sensations can be regarded as opposite poles of a single bipolar visual sensation component we will call V_{yb}. The elemental colors of white and black each would be represented by a monopolar visual sensation component, V_w and V_{bk}, respectively. Using these visual sensation components as orthogonal axes, visual sensation at a given instant and point could then be represented geometrically as shown in Fig. 1B and C. That is, visual sensation is represented by the vector $\mathbf{V}$ with components V_w, V_{rg}, V_{yb}, and V_{bk}. Referring to Fig. 1, hue would correspond to the angle η, saturation to the angle δ, lightness to the angle λ, and brightness to the vector magnitude $|\mathbf{V}|$.

Each of the visual sensation components is a function of sensory space (x',y') and sensory time (t'). Thus, there would be a sensory vector $\mathbf{V}$ for each point of sensory space and each instant of sensory time. That is, the visual sensation vector is a function of sensory space and time

$$\mathbf{V}(t',x',y') = [V_w(t',x',y'),\ V_{bk}(t',x',y'),\ V_{rg}(t',x',y'),\ V_{yb}(t',x',y')] \quad (4)$$

Equation (4) is the notation for the vector field that represents the visual system output, visual sensation.

The input to the visual system, the set of quantum absorptions denoted by Eq. (3), is a vector field, and the output of the visual system, the set of visual sensation components denoted by Eq. (4), is a vector field. Thus, mathematically we may think about the visual system in terms of the operations required to transform the quantum absorption vector field into the visual sensation vector field. A function transforms one set of numbers into another set of numbers, whereas an operator transforms one set of functions into another set of functions. Since the visual system is transforming one set of functions, Eq. (3), into another set of functions, Eq. (4), we can substitute a mathematical black box for the visual system. That is, the visual system is thought of in terms of a set of operators. To remind us that these operators are stand-ins for the physiologic operations of the visual system, we employ the notation $\Phi\cdot$

Since visual sensation is represented by a vector field, there will be an operator that transforms the entire visual system input, the quantum absorption vector field, into each vector component of visual sensation. In mathematical notation,

$$V_w(t',x',y') = \Phi_w[\mathbf{Q}(t,x,y)] \quad (5a)$$

$$V_{bk}(t',x',y') = \Phi_{bk}[\mathbf{Q}(t,x,y)] \quad (5b)$$

$$V_{rg}(t',x',y') = \Phi_{rg}[\mathbf{Q}(t,x,y)] \quad (5c)$$

$$V_{yb}(t',x',y') = \Phi_{yb}[\mathbf{Q}(t,x,y)] \quad (5d)$$

where $\mathbf{Q}(t,x,y)$ is the quantum absorption vector field defined by Eq. (3) and $V_w(t',x',y')$, $V_{bk}(t',x',y')$, etc. are the visual sensation vector components that go into defining the visual sensation vector field; there is a separate operator on $\mathbf{Q}(t,x,y)$ for each visual sensation component. Thus, the visual system black box can be represented by a set of operators,

$$\mathbf{\Phi} = [\Phi_w, \Phi_{bk}, \Phi_{rg}, \Phi_{yb}] \tag{6}$$

For the psychophysicist, the entire problem of vision can be summarized by one simple equation derived from the above arguments,

$$\mathbf{V}(t',x',y') = \mathbf{\Phi}[\mathbf{Q}(t,x,y)] \tag{7}$$

which, in words, says that the space- and time-dependent components of visual sensation are equivalent to a set of operations on the space- and time-dependent components of light absorption in the visual pigments. Equation (7) not only obviates the mind–body problem, it also provides a compact notation for the most basic assumptions of visual science. However, Eq. (7) provides only a framework; it is the task of psychophysics to give substance to the theory by building and experimentally testing mathematical models of the physiological operators $\mathbf{\Phi}$.

B. Linking Neural Cellular Behavior to Psychophysical Phenomena

A guiding principle in linking psychophysical measures of vision to the properties of cells in the visual system is the concept that visual performance reflects the performance of visual system cellular components. Teller formalizes this concept by introducing psychophysical "linking propositions" (Teller, 1984). These propositions consist of categories of arguments that link psychophysically measured phenomena to physiologically measured cell behavior. The five major linking propositions elucidated by Teller are Identity, Similarity, Mutual Exclusivity, Simplicity, and Analogy.

The Identity proposition is perhaps the strongest argument for linking psychophysical phenomena to cell behavior. The Identity proposition states that stimuli that produce identical physiological responses produce identical sensations, or the converse, that identical sensations imply identical physiological responses at some level of neural processing. All experiments in which the observer is asked to report whether stimuli appear to be identical or appear to be different (i.e., threshold and discrimination experiments, which will be described later) are interpreted in terms of the Identity proposition.

The Similarity proposition asserts that similar physiological responses will

produce similar visual sensations, or the converse, that similar visual sensations imply similar physiological responses. The Mutual Exclusivity proposition makes the same assertions about the relationship between mutually exclusive sensations and mutually exclusive physiological responses. The Simplicity proposition asserts that simple neural states will result in simple sensations, and the converse implication. Similarity, Mutual Exclusivity, and Simplicity propositions are related to each other in that they all refer to the linking of visual sensation components to putative corresponding neural components. In a sense, these three propositions underlie the linking of physiological responses of cells to the physiological operators of Eq. (6).

The Analogy proposition is perhaps the most used proposition in vision psychophysics and, logically, it is perhaps the weakest proposition in vision psychophysics. The Analogy proposition asserts that if data from physiological studies "look like" data from psychophysical studies, then the physiological phenomena explain the corresponding psychophysical phenomena. That is, if the results of physiological studies of neural cellular behavior can be mapped onto the results of psychophysical studies of visual sensation, or the converse, then we tend to believe that the particular cellular behavior determines the corresponding properties of visual sensation. As discussed in detail by Teller, such a belief entails many other subsidiary beliefs, most of which are not made explicit by the theorist employing the Analogy proposition. Although the Analogy proposition is a powerful tool for developing models of the physiological operators in Eq. (6), care must be taken not to rely literally upon conclusions drawn from such reasoning without other supporting arguments for establishing the linking.

In principle, psychophysics is not formally concerned with the linking of visual sensation to physiological measures. Rather, psychophysics is concerned with deducing the operations required to explain the observed input–output relationships. However, physiological measures provide a rich source of hypotheses for modeling the physiological operators, and consequently linking propositions are of constant practical concern to psychophysicists. From the other point of view, physiologists who study the visual system with the aim of explaining the substrate of vision are working on a problem that requires them to employ linking propositions to explain how visual sensation depends upon the observed physiological phenomena. In this case, psychophysics provides the data base and the mathematical laws and models that constrain the physiologist's explanations. For example, to explain properties of color vision in terms of ganglion cell behavior, the physiologist must be concerned with the mutual exclusivity of the opponent colors, the simplicity of the elemental colors, and the space–time field nature of visual sensation, not to mention the various empirical laws. Most importantly, the physiologist must offer some suggestion on what the relevant signal from the cell under study is (e.g., spike frequency) and how that signal is mapped onto the sensory components.

C. *Mathematical Models of the Physiological Operators*

The most heavily relied-upon data in vision psychophysics are the results of visual threshold and visual discrimination measures.[2] These measurements, called class A measurements by Brindley (1970), require the observer to report that presented stimuli appear identical or that they appear different in the case of discrimination studies or to report the presence or absence of stimuli, in the case of threshold studies. All class A experiments require the observer to detect a perturbation. As with many other dynamic systems, the visual system tends to respond linearly to small perturbations, like the perturbations typically employed in threshold and discrimination studies (Massof, 1985).

If we consider the situation in which a steady and spatially uniform stimulus is imaged on the retina, the stimulus will produce a set of quantum absorptions in photoreceptors that we will call $\mathbf{Q}$. Assuming the receptor spectral sensitivities do not change over the space and time of interest, $\mathbf{Q}$ will be space–time constant; i.e., $\mathbf{Q}$ will be a set of numbers rather than a set of functions of space and time. If the physiological operations are invariant over the space and time of interest, then we expect the resulting set of visual sensations $\mathbf{V}$ also to be steady and spatially uniform. That is, under these circumstances, $\mathbf{V}$ also can be thought of as a set of numbers rather than as a set of functions of space and time. In this case, the physiological operators may be regarded as transforming one set of numbers into another set of numbers, which is the description of a function. Therefore, we may replace the operator notation of Eq. (7) with a simple functional notation

$$\mathbf{V} = \mathbf{f}(\mathbf{Q}) \tag{8}$$

where $\mathbf{f}(\ )$ refers to a set of functions that describes the behavior of the set of physiological operators under the constraints of steady and uniform stimulation leading to steady and uniform visual sensations. The forms of the functions will depend on the spatial parameters of the stimulus.

In the case of a discrimination experiment, the observer is asked to report when the sensations produced by one stimulus, say $\mathbf{Q}_1$, no longer appear identical to the sensations produced by a reference stimulus, say $\mathbf{Q}_0$. Using Eq. (8), the first stimulus will produce the set of visual sensations $\mathbf{V}_1$ and the reference stimulus will produce the set of visual sensations $\mathbf{V}_0$. The difference between these sensations, which forms the basis for the observer's judgment, is

$$\Delta\mathbf{V} = \mathbf{V}_1 - \mathbf{V}_0 = \mathbf{f}(\mathbf{Q}_1) - \mathbf{f}(\mathbf{Q}_0) \tag{9a}$$

[2]Threshold refers to the "threshold of visibility." For example, absolute threshold is defined as the lowest stimulus energy that produces a visual sensation. Discrimination refers to a "just noticeable difference" between stimuli. For example, color discrimination is the smallest difference between stimuli that would first make them appear perceptibly different in color.

or if we define $\Delta\mathbf{Q}$ as the difference between $\mathbf{Q}_1$ and $\mathbf{Q}_0$,

$$\Delta\mathbf{V} = \mathbf{f}(\mathbf{Q}_0+\Delta\mathbf{Q}) - \mathbf{f}(\mathbf{Q}_0) \tag{9b}$$

If the stimulus perturbation $\Delta\mathbf{Q}$ is sufficiently small then

$$\frac{\Delta\mathbf{V}}{\Delta\mathbf{Q}} \approx \lim_{\Delta Q \to 0} \frac{\mathbf{f}(\mathbf{Q}_0 + \Delta\mathbf{Q}) - \mathbf{f}(\mathbf{Q}_0)}{\Delta\mathbf{Q}} = \mathbf{f}'(\mathbf{Q}_0) \tag{10}$$

which says that the slopes of the lines describing $\mathbf{V}$ as a function of $\mathbf{Q}$, over the interval $\Delta\mathbf{Q}$ (i.e., $\Delta\mathbf{V}/\Delta\mathbf{Q}$), are approximately equal to the derivatives of $\mathbf{f}(\mathbf{Q})$ at $\mathbf{Q}_0$, i.e., $\mathbf{f}'(\mathbf{Q}_0)$. Equation (10) can be rewritten as

$$\Delta\mathbf{V} \approx \mathbf{f}'(\mathbf{Q}_0)\,\Delta\mathbf{Q} \tag{11}$$

which, since we are taking the derivative of a vector with respect to a vector, is a set of linear equations. The constant weighting coefficients in these equations, $\mathbf{f}'(\mathbf{Q}_0)$, are partial derivatives of each of the components of $\mathbf{f}(\mathbf{Q})$ with respect to each of the components of $\mathbf{Q}$. Since we are linearizing around a point determined by the reference stimulus, a change in the reference stimulus $\mathbf{Q}_0$ will produce a new set of weighting coefficients in the linear equations.

The Identity proposition would say that at detection or discrimination threshold $\Delta\mathbf{V}$ is not zero and that implies that the set of physiological responses are not the same for the two stimuli, i.e., $\mathbf{f}(\mathbf{Q}_1) \neq \mathbf{f}(\mathbf{Q}_0)$. From physiological measures, we might propose specific functions for $\mathbf{f}(\mathbf{Q})$. These proposed functions could then be evaluated psychophysically by collecting threshold or discrimination data that would test the specific predictions of Eq. (11). The linking between the cell behavior that inspired the choice of functions for $\mathbf{f}(\mathbf{Q})$ and the psychophysical results of threshold discrimination measures would then rely on the Analogy proposition. Several such examples linking retinal cell behavior to such psychophysical measures, using the above-described approach, will be discussed in detail in the next sections.

D. The Nature of Psychophysical Experiments

There are two major categories of psychophysical experiments. For one category, both the dependent and independent variables are physical parameters. For the other category, the independent variable is a physical parameter, and the dependent variable is a description by the observer of some aspect of visual sensation.

One example from the first category is the classic increment threshold experiment. This experiment consists of presenting the observer with a steady and uniform stimulus, say a circle subtending 5° visual angle,[3] for which the light intensity I is variable. This steady and uniform stimulus is called the "background." After the background has been on for some period of time to allow the visual system to achieve a steady state of adaptation, a second stimulus is presented, superimposed on the background. This second stimulus, say a 2° diameter circle centered on the background, is called the "test" stimulus. The test stimulus is physically added to the background; therefore, it produces an increment in the background intensity ΔI. The object of the experiment is to determine the minimum value of ΔI required for the test stimulus to be just visible on the background. In other words, an increment threshold is obtained. The independent variable in this experiment is the intensity of the background. Thus, ΔI for detection threshold is measured as a function of I. Figure 2 illustrates typical data from such an experiment, plotted on double logarithmic coordinates. The linear part of the function in Fig. 2 is called Weber's law, i.e., $\Delta I/I$ = constant.

The task of the experimental subject in the increment threshold experiment is simply to detect a perturbation in visual sensation $\Delta \mathbf{V}$ in response to a perturbation in stimulus intensity ΔI, as a function of I. Thus, the data in Fig. 2 would be understood in terms of Eq. (11). Further elaborations of the increment threshold experiment consist of manipulating the spectral composition of the background stimulus and the spectral composition of the test stimulus. Such manipulations of spectral parameters make it possible to study properties of the different components of the vector function in Eq. (8) (Guth *et al.*, 1980).

One model for Eq. (11) that accounts for the data in Fig. 2 is

$$f(I) = \frac{CI}{I + \sigma(I)} \tag{12}$$

where I is a scalar variable, since all quantum absorptions $\mathbf{Q}$ will scale with I, C is a constant, and $\sigma(I)$ is an adaption function (Geisler, 1981).

The function in Eq. (12) is only one of many functions that would produce a satisfactory description of the data. The choice of this particular function was inspired by physiological studies of retinal photoreceptor response characteristics (Boynton and Whitten, 1970). In order to link psychophysical measures of increment thresholds to photoreceptor response characteristics, we would have to assume that (1) Eq. (12) is an empirical description of the photoreceptor re-

[3]Visual angle refers to the size of an image on the retina. The visual angle is defined as the angle subtended by the image at the eye's posterior nodal point. The moon subtends a visual angle of 30′. A 35-cm-diameter circle will subtend a visual angle of 2° at 1 m. The same circle will subtend a visual angle of 60° at viewing distance of 20 cm.

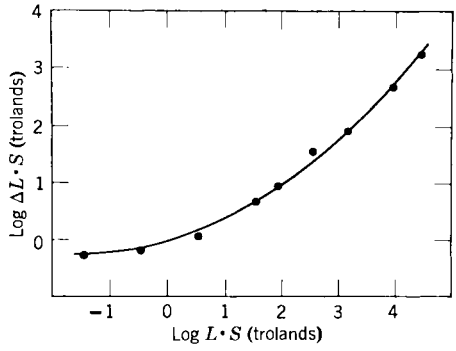


FIG. 2. Increment threshold function plotted as log ΔI, intensity increment for test stimulus threshold, versus log I, intensity of the continuous background stimulus. (The notation ΔL and L appear in this figure and refer to stimulus luminance, which is the same as intensity.) The portion of the function that is linear with unit slope is referred as Weber's law ($\Delta I/I$ = constant). (From Brown and Mueller, 1965.)

sponse, (2) the relevant component of visual sensation V upon which the observer's threshold response is based is a function of the photoreceptor response, i.e.,

$$V = g[f(I)] \tag{13}$$

and (3) the function $g[\ ]$, which characterizes the transformation of the receptor response by the rest of the visual system, is slowly varying with respect to the receptor response. Thus, from the linearization approximation, we would write for the increment threshold experiment

$$\Delta V \approx \frac{\partial g[f(I)]}{\partial f(I)} \frac{\partial f(I)}{\partial I} \Delta I \tag{14a}$$

which, from postulate 3, could be simplified to

$$\Delta V \approx k \frac{\partial f(I)}{\partial I} \Delta I \tag{14b}$$

Equation (14b) explicitly states that the visual perturbation ΔV as a function of background intensity I is determined by the response function of the photoreceptor, $f(I)$. The weakest assumption in the linking argument is the third postulate, which in effect is a restatement of the original wishful thought that inspired Eq. (12).

For the second category of psychophysical experiments, the independent vari-

able, as above, is a physical parameter of the stimulus, but the dependent variable is some subjective description by the observer of visual sensation. One classic example of an experiment in this category is magnitude estimation of brightness (see Marks, 1974). In the brightness magnitude estimation experiment, the observer is presented with only the background stimulus of variable intensity I and is asked to assign a number to the brightness produced by the stimulus. The only requirement of the observer is that the numbers be assigned in such a way that they maintain equal ratio relations to brightness. In other words, if one light appears twice as bright as another light, the number assigned to that light should be twice as large, etc. Therefore, no matter which numbers are used by different observers, the scales ought to all be identical for all observers when transformed to logarithms.

Typical data from a brightness magnitude estimation experiment are plotted on double logarithmic coordinates in Fig. 3. That is, the log brightness rating assigned by the observer is plotted as a function of log stimulus intensity. The straight-line fit to the data has a slope of 1/3 and implies the relation

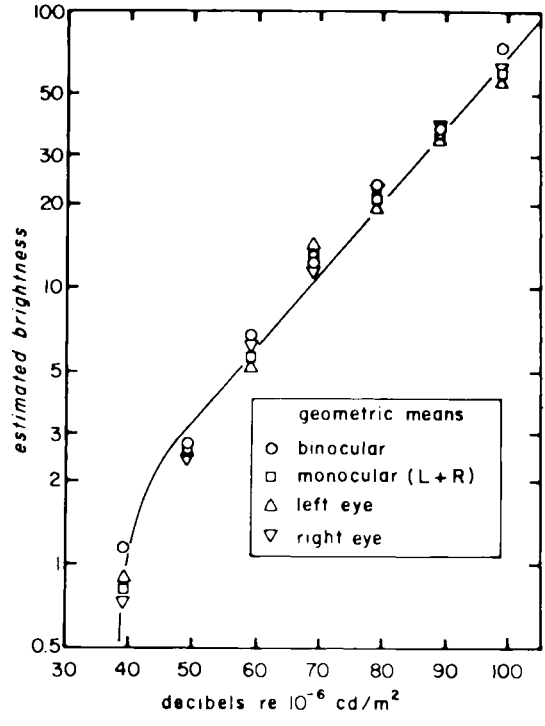


FIG. 3. Log estimated magnitude of brightness plotted as a function of log stimulus intensity. The straight-line fit to the data has a slope of 1/3. (From Stevens, 1967.)

$$\log V = \tfrac{1}{3} \log I + k \tag{15a}$$

which is the same as

$$V = CI^{1/3} \tag{15b}$$

Equation (15b) is the famous Stevens power law (Stevens, 1961) which many psychophysicists take to represent $f(I)$ in Eq. (8).

There is another aspect of psychophysical experiments that we have not yet discussed, an aspect that bears strongly on the above-mentioned issues. This aspect of psychophysics is not too different from a problem faced by other biologists. I have been told that there are gremlins in the cell biology laboratory who capriciously and mischievously alter the temperature of controlled temperature baths, add unknown ingredients to cell culture media, etc. Having to deal with these gremlins leaves one in constant doubt about whether experiments are revealing properties of nature or revealing properties of gremlins. Although control experiments often are designed to put in investigator at greater case, such controls rarely are successful at outwitting clever gremlins.

Psychophysicists tend not to have too many problems with gremlins. Gremlin activity generally is restricted to misaligning optical systems and forcing light sources out of calibration. However, psychophysicists do have to deal with a closely related problem—human subjects. The fact that human subjects have the capacity to behave like gremlins is a major source of the distrust of many types of psychophysical data. Psychophysical experiments in the second category, such as magnitude estimation of brightness, are the least trusted, because the human subject has the greatest control over the outcome of the experiment. Psychophysical experiments in the first category, such as increment threshold measures, are the most trusted because the experimenter can trick the subject by occasionally not presenting the stimulus, thereby checking the subject's honesty. There are, of course, intermediate types of experiments, such as matching the brightness of lights that are not the same color, that warrant intermediate levels of trust in the data.

Because psychophysicists have, in effect, invited gremlins into the laboratory, a great deal of attention has been given to characterizing the decision-making behavior of the subject. This concern over decision-making behavior constitutes the subdiscipline known as Signal Detection Theory.

Consider again the increment threshold experiment that I described earlier. The subject is viewing the steady background stimulus and is instructed to respond when he first sees the test stimulus. If the test stimulus is extremely bright, the subject will probably report seeing it on 100% of the stimulus presentation trials. If the test stimulus is extremely dim, the subject may never admit to

having seen it. At intermediate intensities, the subject may report seeing the test stimulus on 50% of the trials. Therefore, how do decide which intensity is the increment threshold? Obviously, it would be straightforward to choose the 50 or 75% point (like an LD_{50}), but this simple choice does not take into consideration the gremlin properties of the subject.

Imagine yourself as the subject in an increment threshold experiment. We have made an impression of your teeth in dental compound which is attached to an x,y,z positioner. This device is called a bite-bar and when you bite on it, your head does not move (assuming that you have your own teeth). Thus, you become a precisely aligned part of our precisely aligned optical system. Your hand is on a rocker switch and you have been instructed to press the switch to the right if you see the test stimulus flash on, and to press the switch to the left if you do not see it. The presentation of the test stimulus will be cued by a beep. After each of your responses you will be told whether or not the test stimulus actually was presented. The experiment begins and the first test stimulus flash is very obvious, you confidently press the switch to the right, and you are told what you already know—the test stimulus flash occurred. The beep sounds again, you thought you might have seen something, but maybe you had blinked at the time, or maybe you just imagined seeing something—whatever it was, it certainly didn't look like the first flash. You press the switch to the left. The voice says that there was a flash that time. You think to yourself, "I guess what I saw really was a flash. The next time I see something like that I will respond yes." Again the beep sounds, and again you think you might have seen something happen, but you just can't be sure. But, last time that same "something" occurred, it was the flash, so you press the switch to the right. You are told that you are wrong! There was no flash on that trial. You think to yourself, "Does the experimenter think I lied? Does he think I'm stupid?" You readjust your criterion for a yes response; you will wait next time until you are more certain. Eventually, you settle on a criterion with which you can live.

An example of your data is shown in Fig. 4A, in a frequency-of-seeing curve. You did very well despite all of your concerns. However, to be on the safe side we will try the experiment again, but now we are going to employ a different psychophysical technique. This technique is called the two-alternative forced-choice procedure. This time each stimulus presentation trial will have two beeps. You are told that the test stimulus flash will occur after either the first beep or after the second beep. Which beep the test stimulus flash follows will be determined randomly. You are instructed to press the switch to the right if you think that the test flash occurred after the first beep and press the switch to the left if you think that the test flash occurred after the second beep. You must respond by pressing the switch to the left or right for every trial, even if you are absolutely sure that you did not see a test flash in either interval. In this case, if does not

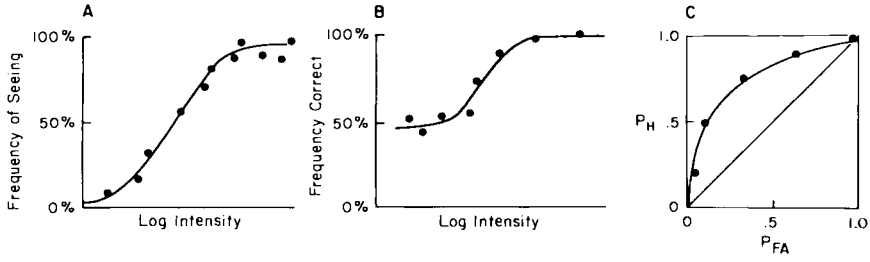


FIG. 4. (A) Frequency-of-seeing curve for an increment threshold experiment. The data points represent the percentage of test stimulus presentations reported seen by the subject as a function of log test stimulus intensity. As test stimulus intensity is increased, the frequency of detection increases. (B) Results of increment threshold measures using a forced-choice procedure. The data points represent the frequency of choosing the correct interval as a function of log test flash intensity. Chance performance is 50%. (C) Results of an increment threshold measure for one test stimulus intensity employing the rating scale procedure. The fraction of stimulus trials on which the subject responded with each rating, or greater, is plotted against the fraction of blank trials on which the subject responded with that rating or greater. The solid curve drawn through the data is an ROC curve.

matter what you think, in that you will end up choosing the interval with the most visual activity.

An example of your data is shown in Fig. 4B. The experimenter kept track of the number of correct responses that you made and the results in Fig. 4B are plotted as frequency correct as a function of log test stimulus intensity. At test stimulus intensity levels below vision threshold, you performed at chance. If you had acted mischievously, your performance might have fallen below chance.

Another technique we might have employed is called the rating scale method. In this case, you would have been given five switches with which to record your response. You would have been instructed to press switch 5 if you were certain that the test stimulus was presented; press switch 4 if you were not sure, but you think that the test stimulus probably was presented; press switch 3 if you could not decide whether or not the test stimulus was presented; press switch 2 if you were not sure, but you think the test stimulus probably was not presented; and press switch 1 if you were certain that the test stimulus was not presented. In this case, a large number of trials would be blanks, i.e., no test stimulus would be presented.

For each test stimulus intensity, a graph such as the one in Fig. 4C would be plotted. The frequency of responding with a certain rating or greater on stimulus trials is plotted on the ordinate, and the frequency of responding with a certain rating or greater on blank trials is plotted on the abscissa. Thus, the lowest point is the fraction of stimulus trials on which you responded with rating 5 versus the

fraction of blank trials on which you responded with the rating 5. The second point is the fraction of stimulus trials on which you responded with rating 4 or greater (i.e., 4 or 5), versus the fraction of blank trials on which you responded 4 or greater.The last point necessarily plots at 1.0 on both axes, because on all of the trials you would have responded with a rating of 1 or greater. The curve drawn through the data is called a receiver operating characteristic (ROC) curve.

All of these experiments can be understood and interrelated within the framework of signal detection theory (Nachmias, 1972). Following the same arguments that were used to derive Eqs. (8) and (9), let us say that the background light produces the value V_0 in visual sensation component V. However, in this case we recognize that there are likely to be fluctuations in neurophysiological response characteristics which will cause fluctuations in V_0. Thus we would consider V_0 to be a stochastic variable. The density function for V_0 might look like that shown in Fig. 5, with a mean of $\bar{V}_0$ and variance of σ_0^2. The test stimulus added to the background would produce a new value V_1, in visual sensation component V. As for V_0, V_1 also would be a stochastic variable. The density function for V_1 might look like that illustrated in Fig. 5, with a mean of $\bar{V}_1$ and variance of σ_1^2. The subject in the increment threshold experiment may now be thought of as having a statistical problem to solve: was the visual sensation on any given trial from the V_0 distribution or was it from the V_1 distribution?

In the first experiment, in which you were asked to press the switch to the right if you saw the test stimulus and press the switch to the left if you did not see the test stimulus, we would assume that you chose a criterion value for V, as shown in Fig. 5, and responded "not seen" for all values of V that were less than the criterion and responded "seen" for all values of V that were equal to or greater than the criterion. Referring to Fig. 5, the area under the V_1 distribution to the right of the criterion value corresponds to the true positive rate, i.e., the frequency of responding "seen" for that particular stimulus. The area under the V_0 distribution to the right of the criterion value corresponds to the false-positive rate, i.e., the frequency of responding "seen" on blank trials. It can be seen from this analysis that changes in the criterion will produce changes in both the true positive rate and the false-positive rate. That is, the rates of true positives and false-positives will covary with changes in the subject's criterion. This covariance can be summarized by an ROC curve.

For a given pair of visual sensation density functions V_0 and V_1, we can construct an ROC curve by continuously varying the criterion from zero to infinity. Conversely, we can deduce the parameters of the underlying density functions for V_0 and V_1 from experimentally determined ROC curves. Returning to the experiment that employed the rating scale method, each rating from 1 to 5 corresponds to a different criterion. Theoretically, we assume that the subject has partitioned the visual sensation continuum V into five zones. As illustrated in Fig. 6, when V is equal to or less than V_a, the subject would respond 1. When V

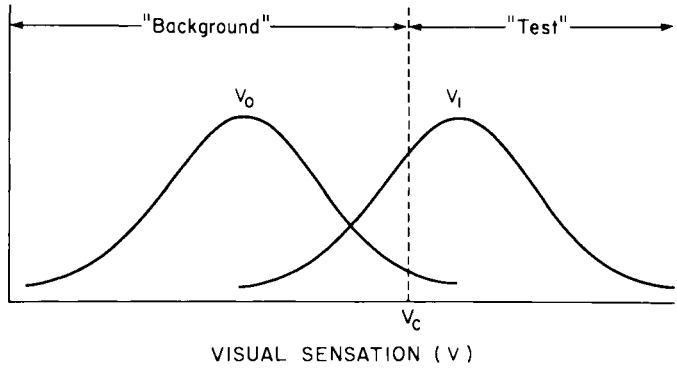


FIG. 5. Density functions for V_0, visual sensations produced by the background stimulus, and for V_1, visual sensations produced by the test stimulus added to the background. The means of the density functions are $\overline{V}_0$ and $\overline{V}_1$, respectively. The line V_c refers to the criterion visual sensation set by the subject. Values of V greater than or equal to V_c would be called "test stimulus" and values of V less than V_c would be called "background."

is greater than V_a, but less than or equal to V_b, the subject would respond 2. Similar descriptions would apply to ratings 3 and 4. When V is greater than V_d the subject would respond 5. The expected frequency of responding 5 on blank trials is the area under the V_0 density function from V_d to infinity, and the expected frequency of responding 5 on stimulus trials is the area under the V_1 density function from V_d to infinity. Similarly, the expected frequency of re-

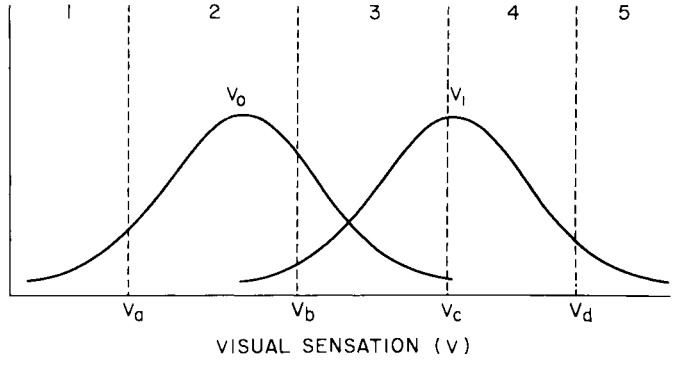


FIG. 6. In interpreting the rating scale experiment, it is assumed that the subject chooses four different criteria. The subject responds 1 when V is less than or equal to V_a, responds 2 when V is greater than V_a but less than or equal to V_b, responds 3 when V is greater than V_b but less than or equal to V_c, responds 4 when V is greater than V_c but less than or equal to V_d, and responds 5 when V is greater than V_d.

sponding 4 or 5 on blank trials is the area under the V_0 density function from V_c to infinity, and the expected frequency of responding 4 or 5 on stimulus trials is the area under the V_1 distribution from V_c to infinity, etc. Thus, the results of the rating scale experiment can be thought of as sampling different parts of the V_0 and V_1 distributions by using different criteria.

Because the true positive rate axis and the false-positive rate axis on the ROC curve may be thought of as cumulative frequency distributions of the V_1 and V_0 density functions, respectively, we can replot the ROC curve in terms of z scores. The z score corresponding to the true positive rate is defined as

$$z_1 = \frac{V - \overline{V}_1}{\sigma_1} \tag{16}$$

and the z score corresponding to the false-positive rate is defined as

$$z_0 = \frac{V - \overline{V}_0}{\sigma_0} \tag{17}$$

where σ_0 and σ_1 are the standard deviations of the distributions V_0 and V_1, respectively and assuming that both density functions are Gaussian. Since both Eqs. (16) and (17) are functions of V, we can rewrite those equations to yield an expression for the ROC curve

$$z_1 = \frac{\sigma_0}{\sigma_1}\left[z_0 - \frac{\overline{V}_1 - \overline{V}_0}{\sigma_0} \right] \tag{18}$$

Thus, the ROC curve is a straight line when plotted on normal deviate coordinates. The slope of the ROC curve is equal to the ratio of the standard deviations of the two distributions, σ_0/σ_1, and the horizontal intercept is proportional to the difference between the means of the two distributions, $(\bar{V}_1 - \bar{V}_0)/\sigma_0$.

This short introduction to signal detection theory is intended only to give the reader an appreciation for the approach employed to understand the decision-making characteristics of the experimental subject. However, we have not yet talked about the factors that control the subject's criterion, what signal detection theorists call costs and values. If we hold a gun to the subject's head and tell him that he will be shot if he says he sees the stimulus when none was presented, we can be sure the subject will adopt a very high criterion. Indeed, unless we balance this cost with some value, such as offering \$1000 for every correct response, the subject is likely to adopt a strategy of saying "not seen" on every trial. Conversely, if we took away the gun, but left the \$1000 reward, the subject is likely to adopt a strategy of saying "seen" on every trial. These extreme examples illustrate that costs can be used to lower the false-positive rate and values can be used to increase the true positive rate. The subject generally will

adopt a strategy that maximizes the payoff, i.e., minimizes the costs while maximizing the value.

In addition to consideration of the costs and values, the subject will employ knowledge of *a priori* probabilities in designing a strategy to maximize the payoff. For example, if the subject knows that the stimulus will be presented on 98% of the trials, a different strategy will be employed than the one that would be used if the subject knew that the stimulus would be presented on only 2% of the trials. Thus, the subject's knowledge of *a priori* probabilities of stimulus and blank trials (or guesses about these probabilities), as well as the relative costs assigned to false-positives and the relative values assigned to true-positives, will determine the subject's choice of a criterion.

The design of a psychophysical experiment must take into consideration the decision-making behavior of the subject. The yes–no paradigm of the first example will give a single point on the ROC curve, the rating scale method will allow one to construct the entire ROC curve, and the two-alternative forced-choice procedure is a criterion-free measure for which the resulting frequency of correct response corresponds to the area under the ROC curve (Green and Swets, 1966). Most subjects restrict their capricious activity to a personal choice of criterion, which can be determined by the experimenter. If the gremlin comes out in the subject and he chooses to act irrationally or destructively, this will be recognized by comparing the subject's performance against the expectations of signal detection theory. Appropriate manipulations of costs and values generally can bring even the most unruly subject into line. This control might be one reason why psychophysical research is sometimes so expensive.

III. Psychophysical Studies of Photoreceptor Physiology

One of the classic success stories of vision psychophysics has been the deduction of rod and cone visual pigment absorption spectra from psychophysical measures of spectral sensitivity and color mixture functions. Another important and more recent psychophysical contribution to the understanding of photoreceptor function is the evaluation of rod photoreceptor transduction kinetics, employing psychophysical measures of afterimages. In this section, I will elaborate on these two psychophysical contributions in order to illustrate the role psychophysics plays in the study of retinal cellular physiology.

A. Rod and Cone Pigment Absorption Spectra

We already have accepted the premise that light must be absorbed by the photopigments before it can be an effective stimulus to vision. This premise led

to the quantum absorption expressions of Eqs. (2a)–(2d). We also will accept without argument that there are three different cone photopigments (L, M, S) and the rod pigment, rhodopsin. This specific assumption is formally stated in Eq. (3). Finally, I will ask you to believe without proof that under dark-adapted conditions, for certain stimulus configurations, all vision is mediated by rods and under light-adapted conditions, for certain other stimulus conditions, the rods make no contribution to vision, only cone responses are relevant (see Boynton, 1979). These last premises are based primarily on a large body of psychophysical data.

In order to determine the absorption spectrum of rhodopsin, $R(\lambda)$, we will employ a variation on the increment threshold experiment described in the preceding section. In this case, the subject is allowed to adjust to the dark for approximately 1 hr, so only the rods will be used, the background intensity I is zero, and the test stimulus is monochromatic. In other words, an absolute threshold spectral sensitivity curve from such an experiment typically is plotted as log ΔI versus wavelength, as shown in Fig. 7.

To understand the spectral absolute threshold curve that is plotted in Fig. 7, we again turn to Eq. (11). In this case, ΔV refers to one sensory component (rod-mediated vision is achromatic) and ΔQ refers only to the rod component of the quantum absorptions. Thus, we would rewrite equation (11) as

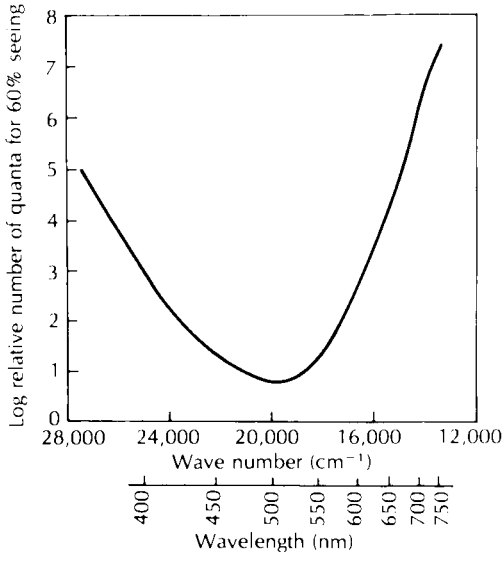


FIG. 7. Absolute threshold spectral sensitivity for rod-mediated vision. Log test stimulus intensity required for detection by the subject is plotted as a function of test stimulus wavelength. (From Wald, 1945, reproduced with permission of *Science*. Copyright 1945 by the AAAS.)

$$\Delta V = f'(Q_R)\,\Delta Q_R \tag{19a}$$

or, since $Q_R = 0$ for absolute threshold, and $\Delta Q_R = R(\lambda)\Delta I(\lambda)$ for monochromatic stimuli

$$\Delta V = kR(\lambda)\,\Delta I(\lambda) \tag{19b}$$

where k is $f'(Q_R)$ for the fully dark-adapted state of the visual system (singularities are not tolerated). Since $\Delta I(\lambda)$, as measured in the experiment, is not specified at the retina, we must take into consideration the spectral transmission of the eye, $\tau(\lambda)$, and modify Eq. (19b) to read

$$\Delta V = kR(\lambda)\,\tau(\lambda)\,\Delta I(\lambda) \tag{19c}$$

Regardless of which experimental method we employ, and regardless of the subject's criterion, from signal detection theory we presume that the measured threshold will correspond to a constant value of ΔV. Thus, the value of ΔV at threshold will be independent of wavelength, i.e., $\Delta V = C$. This strong assumption leads to the derivation of the rhodopsin absorption spectrum from Eq. (19c), using the absolute threshold spectral sensitivity data,

$$R(\lambda) = \frac{C}{k\tau(\lambda)\,\Delta I(\lambda)} \tag{20a}$$

or, in terms of log spectral sensitivity

$$\log R(\lambda) = -\log \Delta I(\lambda) - \log \tau(\lambda) + c \tag{20b}$$

Figure 8 illustrates the rhodopsin log absorption spectrum predicted from Eq. (20b), using the absolute threshold data plotted in Fig. 7 for $\Delta I(\lambda)$, and using known $\tau(\lambda)$ values. These predictions are plotted along with the log absorption spectrum of extracted rhodopsin, as ascertained by spectrophotometry (Cornsweet, 1970).

The important point of the comparison in Fig. 8 is not so much that the psychophysical predictions are in agreement with the measured absorption spectrum of rhodopsin, but the converse; the measured absorption spectrum of rhodopsin is in agreement with tbe psychophysical measures. If there were disagreement between these two measures, we would be forced to reexamine our estimates of $\tau(\lambda)$, or more importantly, question the relation of the absorption spectrum of extracted rhodopsin to the *in vivo* rhodopsin action spectrum. That is, there must be agreement with psychophysics if the measured properties of rhodopsin are to be used to explain the properties of vision.

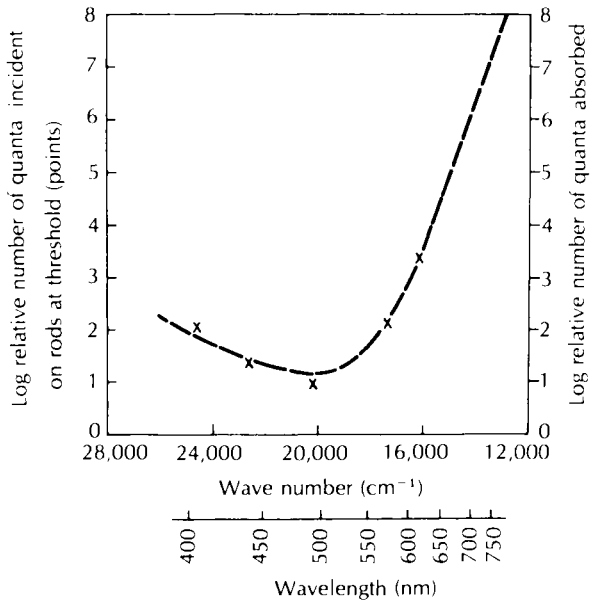


FIG. 8. Data points are log sensitivity (inverse of threshold intensity) plotted as a function of wavelength. The measured sensitivity values have been corrected for the spectral transmission of the eye. The solid curve is the log absorption spectrum of rhodopsin. (From Wald, 1945, reporduced with permission of *Science*. Copyright 1945 by the AAAS.)

It is not as straightforward as it was for the rod photopigment to derive the cone photopigment absorption spectra from psychophysical data. This is because there are three cone pigments, all of which contribute to vision under light-adapted conditions. However, there is an indirect way of deriving the cone photopigment absorption spectra by employing color mixture data from individuals with normal color vision and from different types of color-blind individuals (Vos and Walraven, 1971).

A fundamental characteristic of normal color vision is that any light, of arbitrary color and brightness, can be matched identically by a suitable mixture of red, green, and blue lights. This property of vision is called trichromacy. The red, green, and blue lights are called primaries and the only requirement that must be met by the lights chosen is that none of the three lights can be matched by a mixture of the other two. For example, red, yellow, and green lights would not constitute a set of primaries because the yellow light can be matched by a mixture of the red and green lights.

Two stimuli that match psychophysically, but differ physically are called metamers. When a spectral red is mixed with a spectral green to match a spectral

yellow, the match is metameric. An important set of empirical properties pertaining to color matching is summarized by Grassmann's laws (Boynton, 1979). These laws state that (1) metamers are additive, (2) metamers are invariant under scalar multiplication, and (3) three primaries are necessary and sufficient to make any metameric match.

The first law, additivity, refers to the fact that if the same stimulus is added to both sides of a metameric match, the resulting stimuli will remain matched. For example, if we have adjusted a mixture of red and green lights to identically match a yellow light, and then add some blue light to the red-green mixture, and add the same blue light to the yellow light, the resulting red + green + blue mixture will identically match the resulting yellow + blue mixture. The second law, scalar multiplication, refers to the fact that if both sides of a metameric match are changed in intensity by the same factor, the resulting stimuli will remain matched. Returning to our example, if we increase the intensity of the red + green mixture by a factor of 10 and also increase the intensity of the yellow light by a factor of 10 (for example, by removing one log unit neutral density filters from the optical paths) the resulting stimuli, although now much brighter, will still match identically. The third law refers to trichromacy. That is, as mentioned earlier, any stimulus can be matched identically with an appropriate mixture of three primaries. Two primaries are not sufficient, and a fourth primary would be superfluous.

Grassmann's laws allow us to treat color matching as a set of linear operations. In other words, if A_1, A_2, and A_3 represent three primary colors, and X is an arbitrary stimulus that we want to match, then we can write

$$a_1A_1 + a_2A_2 + a_3A_3 = X \tag{21}$$

where a_1, a_2, and a_3 refer to the intensities of the three primaries, respectively. Equation (21) is a restatement of trichromacy in which the pluses refer to physical mixtures of light, the coefficients refer to physical light intensities, and the equals sign refers to an identity match. For an arbitrary stimulus C, Grassmann's additivity law is written as

$$a_1A_1 + a_2A_2 + a_3A_3 + C = X+C \tag{22}$$

and for a change in intensity by an arbitrary factor k, Grassmann's law of scalar multiplication is written as

$$ka_1A_1 + ka_2A_2 + ka_3A_3 = kX \tag{23}$$

A new set of primaries, B_1, B_2, and B_3, can be written in terms of the old set of primaries as a system of linear equations

$$B_1 = a_{11}A_1 + a_{12}A_2 + a_{13}A_3 \tag{24a}$$

$$B_2 = a_{21}A_1 + a_{22}A_2 + a_{23}A_3 \tag{24b}$$

$$B_3 = a_{31}A_1 + a_{32}A_2 + a_{33}A_3 \tag{24c}$$

since each of the new primaries can be identically matched by a mixture of the old primaries. Thus, we say that any set of primaries is a linear transformation of any other set of primaries.

If we choose a set of primaries and perform matches to monochromatic stimuli spanning the spectrum, we will obtain a set of coefficients in Equation (21)

$$a_1(\lambda)A_1 + a_2(\lambda)A_2 + a_3(\lambda)A_3 = X(\lambda) \tag{25}$$

If we define $A_1(\lambda) = a_1(\lambda)A_1$, $A_2(\lambda) = a_2(\lambda)A_2$, and $A_3(\lambda) = a_3(\lambda)A_3$, then we can think of the spectral matching experiment as giving rise to functions that are analogous to spectral sensitivity functions. These functions, $A_1(\lambda)$, $A_2(\lambda)$, and $A_3(\lambda)$, are called spectral distribution functions. From Eqs. (24a)–(24c), we see that spectral distribution functions for one set of primaries are a linear transformation of spectral distribution functions for another set of primaries

$$B_1(\lambda) = a_{11}A_1(\lambda) + a_{12}A_2(\lambda) + a_{13}A_3(\lambda) \tag{26a}$$

$$B_2(\lambda) = a_{21}A_1(\lambda) + a_{22}A_2(\lambda) + a_{23}A_3(\lambda) \tag{26b}$$

$$B_3(\lambda) = a_{31}A_1(\lambda) + a_{32}A_2(\lambda) + a_{33}A_3(\lambda) \tag{26c}$$

If we could produce a set of primaries, F_1, F_2, and F_3, for which F_1 stimulated only the S cone, F_2 stimulated only the M cone, and F_3 stimulated only the L cone, then we would be able to compute the photopigment absorption from the spectral distribution functions. This set of primaries, which we will call fundamental primaries, is not physically possible to produce because the absorption spectra of the cone photopigments overlap each other. However, if they could be produced, the measured fundamental spectral distribution functions would be identical to the cone photopigment absorption spectra. For example, in a spectral color matching experiment each wavelength of intensity $I(\lambda)$ would be identically matched by a mixture of the F_1, F_2, F_3 primaries. For the identity match to occur, the quantum absorption vectors on the two sides of the match must be the same. Thus, Q_L for the monochromatic light is equal to Q_L for the F_3 primary (remember that F_1 and F_2 do not stimulate the L pigment). Similarly Q_M for the monochromatic light is equal to Q_M for the F_2 primary, and Q_S for the monochromatic light is equal to Q_S for the F_1 primary. If $Q_L^{(1)}$ refers to the quantum absorptions in the L pigment from the monochromatic light of intensity $I(\lambda)$ and $Q_L^{(2)}$ refers to the quantum absorptions in the L pigment from the F_3 primary, then we can write

$$Q_L^{(1)} = L(\lambda)\, I(\lambda) \tag{27a}$$

and

$$Q_L^{(2)} = a_3(\lambda)\, F_3 \tag{27b}$$

Since $Q_L^{(1)} = Q_L^{(2)}$ for a metameric match, from Eqs. (27a) and (27b)

$$L(\lambda)I(\lambda) = a_3(\lambda)F_3 \tag{28a}$$

or

$$L(\lambda) = \frac{a_3(\lambda)}{I(\lambda)}F_3 \tag{28b}$$

which is an expression of the L cone spectral absorption function in terms of the psychophysically and physically measured parameters, $I(\lambda)$ and $a_3(\lambda)$ (F_3 is a constant). Similar expressions for the M and S cone pigments are derived in the same way,

$$M(\lambda) = \frac{a_2(\lambda)}{I(\lambda)}F_2 \tag{29}$$

and

$$S(\lambda) = \frac{a_1(\lambda)}{I(\lambda)}F_1 \tag{30}$$

The only problem with the above argument is that we cannot perform the experiment that we so carefully designed because F_1, F_2, and F_3 are not physically realizable stimuli (they would require negative energy). However, F_1, F_2, and F_3 are mathematically real and, because they are primaries they can be derived from any set of physically realizable primaries. Thus, if $I(\lambda)$ is held constant (i.e., equal energy spectrum), then by definition [from Eqs. (28)–(30)] $L(\lambda)$, $M(\lambda)$, and $S(\lambda)$ are spectral distribution functions for the fundamental primaries. Since one set of primaries is a linear transformation of another set of primaries, we can conclude that

$$L(\lambda) = c_{11}A_1(\lambda) + c_{12}A_2(\lambda) + c_{13}A_3(\lambda) \tag{31a}$$

$$M(\lambda) = c_{21}A_1(\lambda) + c_{22}A_2(\lambda) + c_{23}A_3(\lambda) \tag{31b}$$

$$S(\lambda) = c_{31}A_1(\lambda) + c_{32}A_2(\lambda) + c_{33}A_3(\lambda) \tag{31c}$$

following the same reasoning that led to Eqs. (26a)–(26c). Thus, the problem is reduced to one of finding the values of the nine unknown coefficients c_{ij}, in order to solve Eqs. (31a)–(31c).

Over the past 100 years, many attempts have been made to solve the fundamental color mixture equations (Thomson and Wright, 1947; Vos and Walraven, 1971; Smith and Pokorny, 1975). Six of the nine required solution equations are obtained from data collected on color-blind individuals. There are three types of color blindness: protanopia, deuteranopia, and tritanopia (Boynton, 1979). Protanopes have the normal M and S cone pigments, but lack the L cone pigment. Deuteranopes have the normal L and S cone pigments, but lack the M cone pigment. Tritanopes have the normal L and M cone pigments, but lack the S cone pigment. Color matching data collected on these three types of color-blindness provide the six solution equations, two for each type of color blindness.

The remaining three solution equations for deriving the cone pigment absorption spectra are obtained from psychophysical measures of spectral sensitivity using a technique called heterochromatic flicker. In the heterochromatic flicker experiment, a monochromatic stimulus is alternated in time with a white reference stimulus at a frequency of about 15 Hz. At this frequency there is no color flicker, and the field appears to be a uniform color. However, there still is a brightness flicker. The subject's task is to adjust the intensity of the monochromatic stimulus so that the light appears steady. Because there is no color flicker, the resulting spectral sensitivity curve may be thought of as representing the weighted sum of the three cone photopigment absorption spectra. This interpretation of the heterochromatic flicker spectral sensitivity curve gives rise to the three solution equations for the derivation of the cone photopigment absorption spectra (Vos and Walraven, 1971).

Figure 9 illustrates the cone photopigment relative absorbance spectra derived from Eqs. (31a)–(31c) (Boynton and Wisowaty, 1980). As for the predicted rhodopsin absorption spectrum, these relative absorbance spectra are corrected for the spectral transmission of the eye, $\tau(\lambda)$. Also plotted in Fig. 9 are the relative absorbance spectra of human cone photopigments, as ascertained by microspectrophotometry (Dartnall *et al.*, 1983). The agreement between the two measures is encouraging. However, unlike the case for the derivation of the rhodopsin absorption spectrum, several vulnerable assumptions are required for the derivation of the nine transformation coefficients. Thus, the disagreements between the psychophysical predictions and microspectrophotometric measures can be blamed on either side. Yet, the psychophysics of color matching imposes a strong constraint on the interpretation of physical measures of cone photopigment absorption spectra; that is, the measured spectra must be a linear transformation of color mixture primaries if they are to be interpreted as relevant to the visual process.

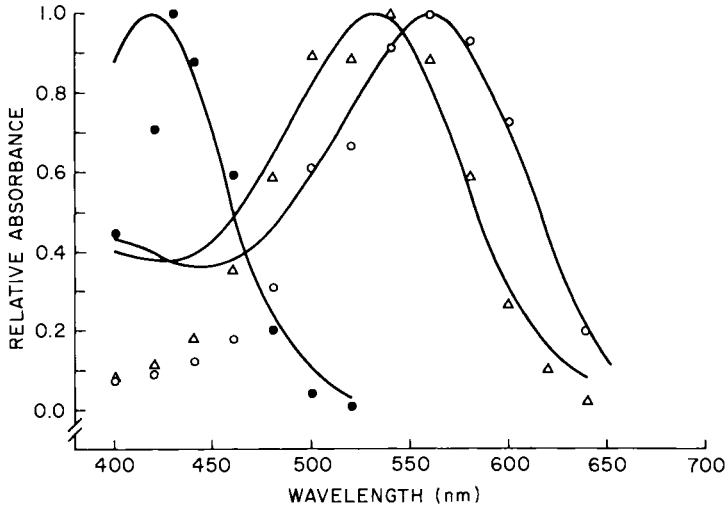


FIG. 9. Cone photopigment relative absorbance spectra derived from color mixture data for short- (●), middle- (△), and long- (○) wavelength-sensitive cones plotted along with cone photopigment relative absorbance spectra measured by microspectrophotometry (solid curves). The color mixture data were corrected for the spectral transmission of the eye.

B. Rod Internal Transduction Kinetics

Once light has been absorbed by the visual pigment, there must be a chain of events within the photoreceptor cell that is responsible for transducing the photon absorptions into neural signals. These internal transduction steps have been the subject of intensive physiologic research over the past several years and many of the known details are described in other articles in these two volumes (Farber and Shuster, Part I). We need not go too deeply into the specifics here; however, four fundamental assumptions are required in order to establish a linking between psychophysical studies and internal photoreceptor transduction. First, we assume that the photoreceptor membrane potential reflects the relevant photoreceptor neural signal. It is not necessary to assume that the membrane potential actually is the neural signal, but we assume that it determines, or parallels, the neural signal. Second, we assume that some blocking agent is released or created in response to a photon absorption. This agent travels to the photoreceptor membrane and blocks the ion channels. The term blocking agent may refer collectively to several different molecules and numerous biochemical steps. Third, we assume that the effects of the blocking agent on the membrane are reversible. That is, the blocking agent is degraded over time so that it no longer blocks the

ion channels, thereby producing recovery of the photoreceptor response. Fourth, we assume that the kinetics of photoreceptor response recovery is a rate-limiting process in the visual system. That is, none of the other components of the visual system are slower to recover than are the photoreceptors. This fourth assumption says that the dynamics of the relevant aspects of the visual process are governed by the dynamics of the photoreceptor.

Geisler and Adelson (Geisler, 1980; Adelson, 1982) independently employed the above-described linking postulates to develop a general model that explains psychophysical measures of rod afterimage kinetics in terms of the kinetics of internal photoreceptor transduction steps. Their arguments are interesting, elegant, and a model of simplicity. However, before getting into the esthetics, allow me to describe first the phenomenon and the psychophysical experiment that the model purports to explain.

Returning to the increment threshold experiment, we will make the following changes: (1) the background stimulus color is changed to red and the test stimulus color is changed to blue-green (these changes will maximize the contrast for rods and increase the likelihood that rods will subserve detection of the test stimulus), (2) the subject is allowed to fully dark-adapt before each stimulus trial, and (3) the background and test stimuli are flashed on and off together for an exposure duration of 100 msec. Depending on the intensity of the background and test stimuli, during the initial flash the subject will see only the background stimulus. For several seconds after the flash, the subject will see a persistent afterimage, such as is frequently experienced when someone takes your photograph using a flash camera. At first only the circular background is visible in the afterimage. However, after a few seconds, the square test stimulus becomes visible on the background. Then with additional time, the two images fade together. The greater the intensity of the test and background stimuli, the longer the delay before the test stimulus appears in the afterimage.

The object of the experiment is to measure the time required for the subject to see the test stimulus on the background (in the afterimage), as a function of the intensity of the background and test stimuli. However, the first step in the experiment is to determine the increment threshold. That is, we would first measure the minimum intensity of the test stimulus required to make the square just visible sometime in the afterimage. Once the increment threshold for detection of the square in the afterimage is determined, the ratio of test stimulus intensity (ΔI) to background intensity (I) is set to a fixed value, i.e., $\Delta I/I$ = constant. As the background intensity is varied during the course of the experiment, the test stimulus intensity is changed by the same factor. The rationale for holding the ratio of these two intensities constant will be made clear later in this section.

The main experiment consists of flashing the test and background stimuli on and off together for an exposure duration of 100 msec. The background stimulus

is set to some intensity I and the test stimulus is set to ΔI, which is a fixed proportion of I, as determined earlier by the increment threshold measurement (actually ΔI is set to about 0.1 log unit greater than the increment threshold so that the square will be clearly visible, rather than being at the threshold of visibility). Initially the subject will see only the background. However, the square eventually will become visible during the decay of the afterimage. The subject responds by pressing a switch, as quickly as possible, when the square is first seen. The experimenter records the time elapsed from the beginning of the flash until the subject presses the switch. This measure of the subject's reaction time is made over a wide range of stimulus intensities.

Geisler employed the above-described reaction time technique, Adelson employed a somewhat more elaborate procedure. In Adelson's experiment, a beep was presented at some time after the 100-msec flash. The subject was required to press a switch to the right if the beep occurred after the appearance of the square in the afterimage, and to press the switch to the left if the beep occurred before the appearance of the square in the afterimage. Depending on the subject's response, the delay of the beep was adjusted to a longer or shorter time. After repeated trials the beep eventually was made coincident with the appearance of the square in the afterimage. The delay time for the beep was then taken as the dependent measure in the experiment, that is, time to the appearance of the test stimulus in the afterimage.

Figure 10 illustrates the data from Adelson's experiment for three subjects. The results are plotted as time in seconds for detection of the square in the afterimage as a function of log background intensity. Remember that the test stimulus intensity was always a fixed proportion of the background intensity. The results of Geisler's experiments were similar to Adelson's results. Terry Benzschawel and I also performed this experiment (Benzschawel *et al.*, 1983) employing both the reaction time procedure and Adelson's coincident beep technique. As shown in Fig. 11, the two techniques yield identical results. This comparison of techniques is important because Adelson's approach, although considered to be more rigorous, is very time-consuming (e.g., an 8-hr experimental session for the coincident beep technique versus a 2-hr experimental session for the reaction time measures). Therefore, the coincident beep technique is not suitable for detailed parametric studies, especially with clinical patients, as will be described in the next section.

Various physiological experiments have led to the conclusion that the amount of blocking agent released, or manufactured is proportional to the number of rhodopsin molecules bleached (see Witkovsky, 1980). Consequently, the concentration of blocking agent is proportional to the stimulus intensity. The degradation of blocking agent, i.e., receptor response recovery, would scale with the blocking agent concentration; thus, receptor response recovery also will scale with stimulus intensity. After a flash of light, the buildup and degradation of

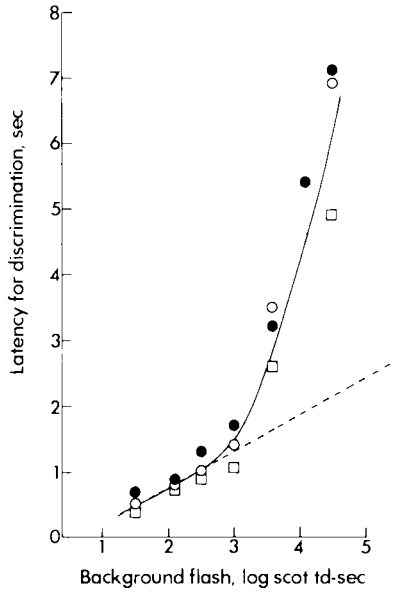


FIG. 10. Time (in seconds) before the square is seen in the afterimage as a function of log intensity of the background stimulus. Data are plotted for three different subjects. [Reprinted with permission from (*Vision Res.*, **22**, E. H. Adelson, The delayed rod afterimage), Copyright (1982), Pergamon Press.]

blocking agent will follow some time course. We can specify this time-dependent change in blocking agent concentration as

$$c(t) = Ig(t) \tag{32}$$

where I is the intensity of the light flash and $g(t)$ is the function describing the relative time course of blocking agent concentration following a flash (i.e., the function for a unit amount of blocking agent).

The change in receptor membrane potential will follow the concentration of the blocking agent, up to a limit. In other words, there are only a finite number of ion channels on the membrane that can be blocked, so the receptor response has a ceiling. Thus for examples illustrated in Fig. 12A, the membrane potential will follow the blocking agent concentration up to a limit and then stay at a constant level until the blocking agent falls below the critical level. As shown in Fig. 12b, we say that the receptor response follows a saturating nonlinearity. Empirically, this nonlinearity in the receptor response $R(t)$ has been expressed as

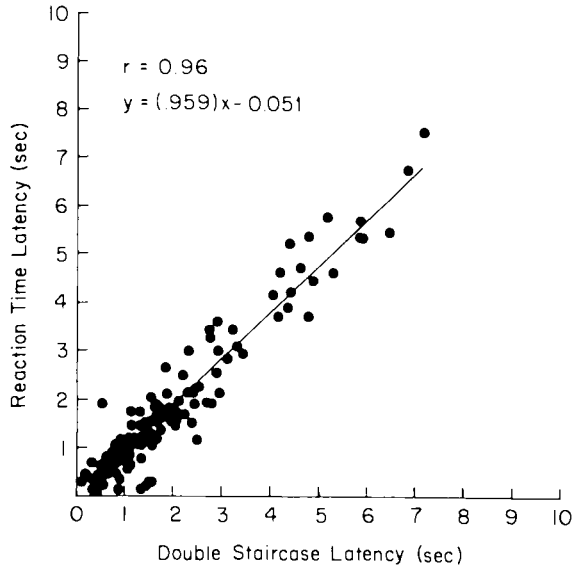


FIG. 11. Scatter plot of time to detection of test stimulus in the afterimage, using reaction time and the coincident beep (double staircase) techniques. The slope of the best fit line is 1.

$$R(t) = \frac{R_{max}c(t)}{Ic(t) + \sigma} \tag{33a}$$

where R_{max} is the ceiling or maximum receptor response and σ is the half-saturation constant [if $c(t) = \sigma$, then $R(t) = \frac{1}{2}R_{max}$]. Substituting Eq. (32) for $c(t)$ in Eq. (33a) we obtain

$$R(t,I) = \frac{R_{max}Ig(t)}{Ig(t) + \sigma} \tag{33b}$$

which is an explicit expression of the receptor response in terms of both time and stimulus intensity.

From our first linking postulate, we say that the relevant component of visual sensation is a function of the receptor response

$$V(t') = f[R(t,I)] \tag{34}$$

Similar to the line of reasoning leading to Eq. (11), we say that the red background intensity I_0 produces the visual sensation $V_0(t')$ and the blue-green square of intensity ΔI, superimposed on the red background, produces the visual sensa-

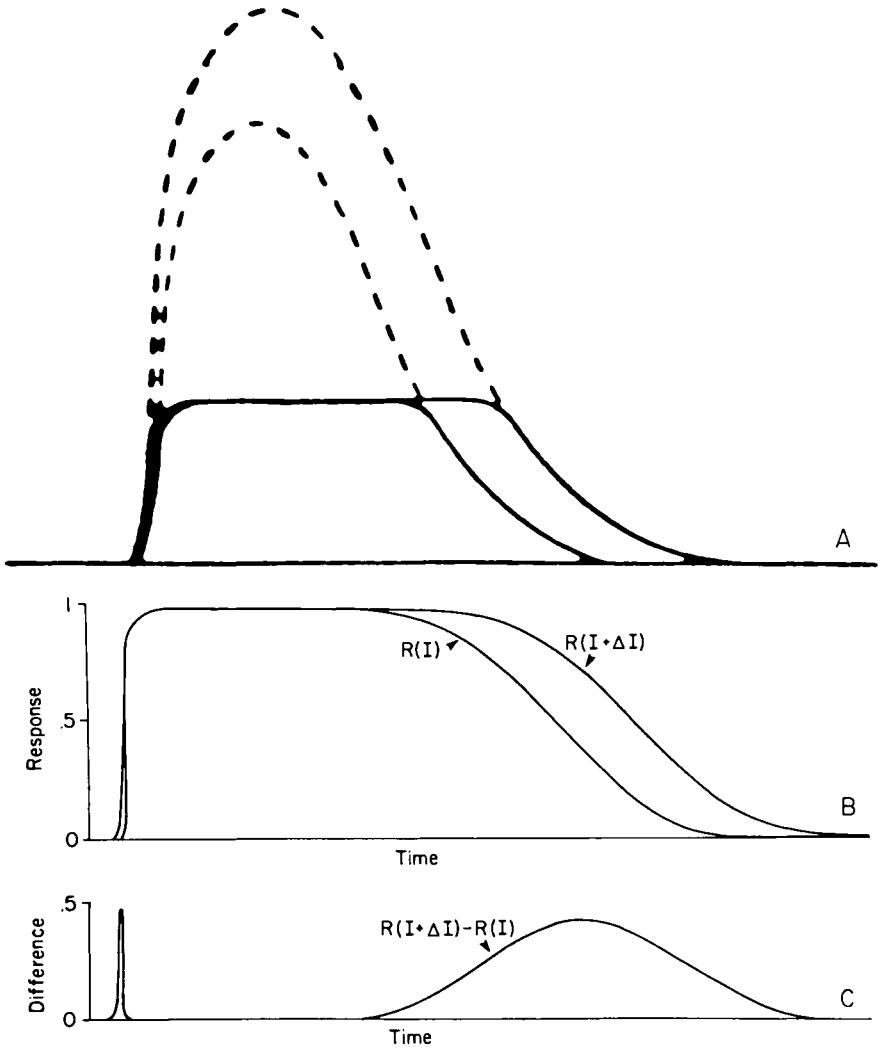


FIG. 12 (A) Schematic representations of rod membrane potential changes (solid curve) in response to flashes of stimuli of two different intensities. The dashed curves represent the changes in blocking agent concentration that exceed the response capacity of the rod membrane potential. The two curves describing the change in blocking agent concentration are identical in shape; they simply are scaled differently according to the fractional differences in intensities. (B) Receptor response as a function of log stimulus intensity. Because the response achieves an asymptote at high stimulus intensities, we say the receptor follows a saturating nonlinearity. (C) Differences between the pairs of receptor responses illustrated in (B), plotted as a function of time after the flash.

tion $V_1(t')$. Remembering that the rod system does not care about the color of the lights, except to the extent that it scales the intensity by a constant, we can write

$$\Delta V(t') = f[R(t,I_0 + \Delta I)] - f[R(t,I_0)] \tag{35a}$$

for the difference between the two visual sensations. From the assumption that we will keep $\Delta V(t')$ relatively small, we can employ the linear approximation

$$\Delta V(t') \approx \frac{\partial f[R(t,I)]}{\partial R} \frac{\partial R(t,I)}{\partial I} \Delta I \tag{35b}$$

which is identical to Eq. (14a). Taking the derivative of $R(t,I_0)$ as expressed in Eq. (33b), we can rewrite Eq. (35b) as

$$\Delta V(t') \approx \frac{\partial f[R(t,I_0)]}{\partial R} \frac{R_{\max}\sigma g(t)}{[I_0 g(t) + \sigma]^2} \Delta I \tag{35c}$$

Figure 12C illustrates a schematic of the time course of ΔV, which is simply the difference between the two receptor responses in Fig. 12B. We assume that during the first part of the experiment, when an increment threshold for detection of the square in the afterimage is measured, the subject is basing his decision on the maximum value of $\Delta V(t')$, i.e., the peak of the difference function in Fig. 12C. To locate the peak of $\Delta V(t')$, we differentiate Eq. (35c) with respect to time.

From the fourth linking postulate, we can say that $\partial f[R(t,I_0)]/\partial R = \text{constant}$; thus, Eq. (35c) becomes

$$\Delta V(t') \approx k \frac{R_{\max}\sigma g(t)}{[I_0 g(t) + \sigma]^2} \Delta I \tag{35d}$$

in which $g(t)$, the function describing the time-dependent change in the relative concentration of the blocking agent, is the only parameter that controls the time course of $\Delta V(t')$. Differentiating $\Delta V(t')$ with respect to time leads to

$$\frac{\partial \Delta V(t')}{\partial t} = kR_{\max}\sigma \frac{\partial g(t)}{\partial t} \left\{ \frac{\sigma^2 - I_0^2 g(t)^2}{[I_0 g(t) + \sigma]^4} \right\} \Delta I \tag{36}$$

At the maximum value of $\Delta V(t')$, $\partial \Delta V(t')/\partial t = 0$, which, from Eq. (36), leads to

$$g(t_0) = \sigma / I \tag{37}$$

where t_0 is time at which the maximum value of ΔV occurs.

If we substitute Eq. (37) for $g(t)$ in equation (35d), we obtain

$$\Delta V(t_0') \approx K(\Delta I/I) \tag{38}$$

where K is a collection of all the constants. Given the assumption that the subject will choose a criterion value of ΔV for threshold, Eq. (38) states that our linking postulates require Weber's law (i.e., $\Delta I/I$ = constant) to hold for increment thresholds measured on the basis of detecting the test stimulus in the afterimage. On double logarithmic coordinates, Weber's law requires a straight-line relationship between log I and log ΔI, with a slope of one. As illustrated in Fig. 13, this prediction from the linking postulates is verified.

Given the experimental verification of Weber's law, we now see that if we set ΔI to a fixed proportion of I, then the maximum value of $\Delta V(t')$ will not vary with I. However, the time at which the maximum value of ΔV occurs, t_0, is a function of I. Thus, by measuring the latency to detection of the square in the afterimage, while holding $\Delta I/I$ fixed, we obtain a direct measure of t_0 as a function of I. This interpretation of the experiment follows from Eq. (37), which can be reexpressed as

$$t_0 = g\,(\sigma/I)^{-1} \tag{39}$$

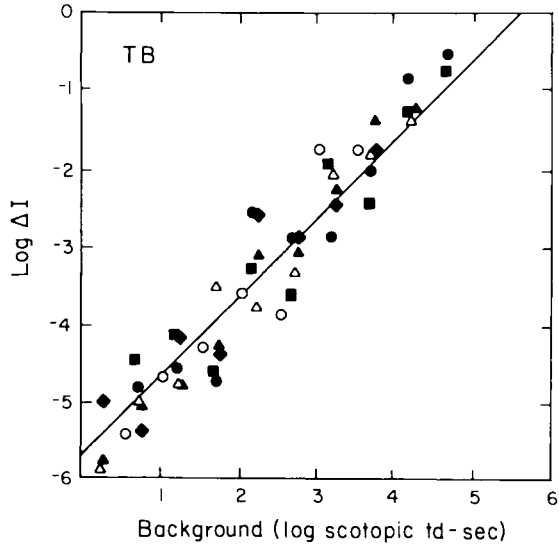


FIG. 13. Log test flash intensity (ΔI) for first detection of the square in the afterimage is plotted as a function of log background intensity. The solid line fit to the data is Weber's law ($\Delta I/I$ = constant).

where $g(\ )^{-1}$ is the inverse function of $g(t_0)$. For example, if $g(t)$ is an exponential decay,

$$g(t) = e^{-t/\tau} \tag{40a}$$

then from Eq. (37)

$$e^{-t_0/\tau} = \sigma/I \tag{40b}$$

and solving for t_0, by taking the natural logarithm of both sides of Eq. (40b) (note that the natural logarithm is the inverse function of the exponential), we obtain

$$t_0 = \tau \ln I + C \tag{40c}$$

Equation (40c) predicts a straight-line relationship between the time to detection of the square in the afterimage and log background intensity. The slope of this straight line is equal to the time constant τ (Geisler, 1980; Adelson, 1982).

The dashed line in Fig. 10 is the prediction of $g(t)$ expressed by Eq. (40a). To the extent that the results of the experiment are better characterized by two straight lines of different slopes, Adelson argued that $g(t)$ is better characterized by a double exponential

$$g(t) = A_1 e^{-t/\tau_1} + A_2 e^{-t/\tau_2} \tag{41}$$

where A_1 and A_2 are amplitude constants and τ_1 and τ_2 are the two time constants. The solid curve fit to the data in Fig. 10 was generated from Eq. (41) with time constant values of $\tau_1 = 180$ msec and $\tau_2 = 1.5$ sec. Thus, we would conclude from this interpretation of the data that the degradation of the blocking agent consists of a fast process (τ_1) and a slow process (τ_2).

Up to this point the linking arguments have followed mainly from the Identity proposition. That is, we assumed that while the rod response is saturated there is no difference between the responses of rods under the background stimulus and the responses of rods under the test stimulus superimposed on the background. Thus, we infer that there is no difference between the visual sensations, i.e., $\Delta V = 0$.

To further illustrate the linking arguments, we also can turn to the Analogy proposition. Penn and Hagins recorded electrical responses from rat rods to different light flash intensities (Penn and Hagins, 1972). By measuring the time required for the rod response to recover to a criterion value, as a function of stimulus intensity, Adelson was able to directly compare rat rod recovery to the psychophysical measures of human rod recovery. Figure 14 illustrates latency to

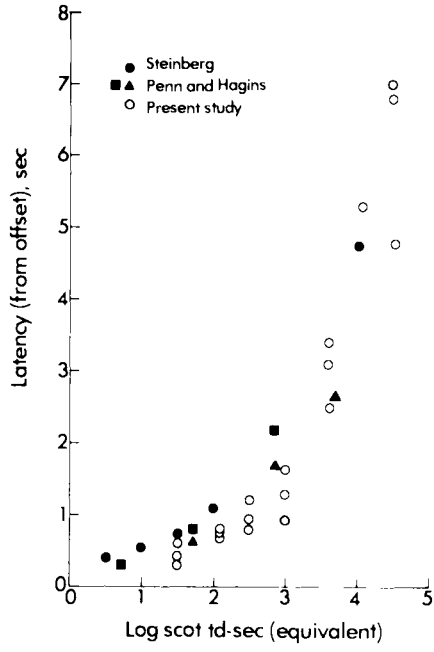


FIG. 14. Data from afterimage detection latencies shown in Fig. 10 (○) are compared to rat rod receptor responses (▲, ■) and cat horizontal cell responses (●). [Reprinted with permission from (*Vision Res.*, **22**, E. H. Adelson, The delayed rod afterimage), Copyright (1982), Pergamon Press.]

recovery of the rod response in seconds as a function log intensity for both rat rods (squares and triangles) and human rods (open circles). The intensity axis is expressed in units equivalent to quantum absorptions by rhodopsin, so the data can be compared on an absolute basis. Steinberg made similar electrophysiologic measures on cat horizontal cells (Steinberg, 1969). Those data also are plotted in Fig. 14 (solid circles). To the extent that the recovery of the rod response is a rate-limiting process, the dynamics of the horizontal cell response will reflect the dynamics of the rod response.

The comparisons in Fig. 14 show remarkable agreement between psychophysical and physiological measures. Although this agreeement does not formally prove the linking propositions, it does increase our confidence in accepting these propositions as true. Thus, we find ourselves on firm footing when interpreting results of psychophysical afterimage detection latency study in terms of internal photoreceptor transduction processes. In the next section, we will extend this experiment and analysis to the study of pathological processes in retinal disease.

IV. Psychophysical Studies of Abnormal Retinal Physiology in Retinitis Pigmentosa

Once we have established linkings between psychophysical and physiological properties of the visual process, we then can employ psychophysics to study new aspects of, or altered, retinal cellular function. This application of psychophysics has been of particular importance to the study of retinal pathophysiology. Retinal diseases, such as retinitis pigmentosa, do not lend themselves well to most laboratory studies of retinal cell biology because fresh tissue from such patients rarely is available. Even when donor tissue does come into the laboratory, the patient often is not well-described clinically and the disease often is in an advanced and less informative state. Furthermore, the systemic condition of the patient usually is poor prior to enucleation. That is, the patient typically has had a serious disease that caused death and has received pharmacological agents that frequently are retinotoxic. Except in a few extraordinary cases, the tissue is not acquired until several hours postmortem. This delay precludes most physiological studies of the retina.

Psychophysical measures that are linked to retinal cellular physiology can provide us with a means of studying the physiology, dynamics, and diversity of retinal disease in human patients. In order to illustrate what can be learned about retinal pathophysiology from psychophysics, I will review two studies that we conducted on patients with retinitis pigmentosa. These two studies employ the experiments and analyses presented in the preceding section on photoreceptor physiology.

A. Overview of Retinitis Pigmentosa

Before reimmersing ourselves in psychophysics, I would like to provide a brief overview of retinitis pigmentosa (RP) for the purpose of orientation. Many cell biologists are familiar with animal models of RP, such as the RCS rat or the *rd* mouse, and that familiarity can be misleading. We know far less about human RP than we know about most of the animal models for RP. In fact we know so little about RP that it is not clear which, if any, of the inherited retinal degenerations in laboratory animals is an appropriate surrogate for the human conditions. Indeed, with the recent surge of interest in the study of RP (recent in the sense that RP was first described in 1855 by Donders), much of what we thought we knew about RP has come under attack. For example, it was long widely accepted that all RP is inherited. There clearly are familial forms of RP with patterns that follow classic Mendelian genetics. In the United States, about 22% of all RP

patients have family histories of RP that are consistent with an autosomal dominant mode of inheritance, and about 6% of all RP patients have family histories consistent with X-linked recessive inheritance. Another 22% of all RP patients have only affected siblings or some scattered relatives with RP. This group of RP patients is labeled multiplex and often they are presumed to reflect an autosomal recessive mode of inheritance. However, 50% of all RP patients have no other family history of the disorder. These patients are called simplex, and although in the past these patients have been presumed to have autosomal recessive disease, formal tests of the genetic hypothesis through segregation analysis have led to a rejection of that presumption (Boughman *et al.*, 1980; Massof *et al.*, 1982).

The problem with RP is that as a clinical entity it is defined by a set of nonspecific characteristics. If a patient exhibits or is expected to exhibit night blindness, a progressive loss of peripheral vision while retaining relatively good visual acuity, abnormally narrow retinal arterioles, and the characteristic intraretinal, black, spicule-shaped pigment deposits that give RP its name, then the patient is a candidate for the RP diagnosis. The diagnosis is not established, however, until the clinician is confident that there is no known systemic metabolic, inflammatory, dietary, toxic, or traumatic cause of the signs and symptoms. That is, RP is defined by exclusion; it is idiopathic by definition.

B. The Relation of Rod Loss to Cone Loss in RP

Night blindness in RP indicates that rod-mediated vision is impaired. The progressive loss of peripheral and eventually central vision argues that cone function also becomes affected. The first question we asked about RP was, "How is rod function in RP lost relative to the loss of cone function?" We wanted to know (1) if rods and cones were affected together or if one receptor type was affected before the other, (2) if the retinal degeneration was diffuse throughout the retina or if it was regionalized and spread from specific retinal areas, and (3) if the relation of rod sensitivity loss to cone sensitivity loss was the same in all RP.

In order to psychophysically study rod loss relative to cone loss in RP, we exploited the difference between the absorption spectrum of the rod pigment, $R(\lambda)$, and the absorption spectra of the cone pigments, $L(\lambda)$, $M(\lambda)$, and $S(\lambda)$ (Massof and Finkelstein, 1979, 1981a,b). For the normal eye that is totally dark adapted, absolute visual thresholds will be determined by the rods. Consequently, absolute threshold spectral sensitivity measures, as discussed earlier, will be determined by the absorption spectrum of rhodopsin (see Fig. 8). On the other hand, if the rods were missing or nonfunctional, then absolute visual thresholds would be determined by the cones. In this case, absolute threshold spectral sensitivity measures would be determined by some combination of the

L, M, and S cone photopigment absorption spectra. Turning the argument around, if absolute visual threshold spectral sensitivity measures fit the rhodopsin absorption spectrum, we would conclude that visual detection was mediated by rods. If the absolute visual threshold spectral sensitivity measures fit some combination of the absorption spectra of the L, M, and S cones, then we would conclude that visual detection was mediated by cones.

Because of time and endurance constraints, it is not practical to measure complete absolute threshold spectral sensitivity functions at different retinal locations in RP patients. However, if the underlying rod-determined and cone-determined spectral sensitivity functions are known *a priori*, it is sufficient to measure absolute visual thresholds at only two wavelengths. As shown in Fig. 15A, rod-determined spectral sensitivity is about 3 log units greater for a 500-nm stimulus than it is for a 650-nm stimulus. As shown in Figure 15B, cone-determined spectral sensitivity is about 1 log unit greater for a 500-nm stimulus than it is for a 650-nm stimulus. Thus, if we measure absolute visual thresholds for a 500-nm stimulus and for 650-nm stimulus and find that there is a 3 log unit difference between the threshold intensities for the two stimuli, we would conclude that rods mediated visual detection. On the other hand, if we found a 1 log unit difference between the threshold intensities for the two stimuli, we would conclude that cones mediated visual detection. If we obtain a difference between the threshold intensities for the two stimuli that is greater than 1 log unit, but less than 3 log units, we would conclude that rods mediated visual detection of the 500-nm stimulus and cones mediated detection of the 650-nm stimulus. This conclusion assumes a relation between rod- and cone-mediated spectral sensitivities that is similar to the one shown by the overlapping curves in Fig. 15C.

Within limits, the difference between the log threshold intensities for the 500-nm and 650-nm stimuli can provide information about rod sensitivity relative to cone sensitivity. Figure 16 illustrates the predicted difference between the log threshold intensities for the two stimuli as a function of the difference between the maximum log sensitivity of rods and the maximum log sensitivity of cones.[4] The graph in Fig. 16 may be thought of as the result of sliding the rod and cone log spectral sensitivity curves in Fig. 15A and B up and down relative to each other, and determining the log threshold intensities for the 500-nm and 650-nm stimuli from the resulting composite curve. For example, the relation of maximum rod sensitivity (peak of the rod curve) to maximum cone sensitivity (peak of the cone curve) for the overlapping curves in Fig. 15C would plot at 0.5 log units on the abscissa and -0.1 log units on the ordinate of the graph in Fig. 16.

[4]We specify threshold intensities in luminance units. That is, the stimulus energy is weighted according to its effectiveness in stimulating cones. Thus, by definition the theshold luminances are the same for the two stimuli if detection is mediated by cones. For rods, the threshold luminance difference is 2 log units.

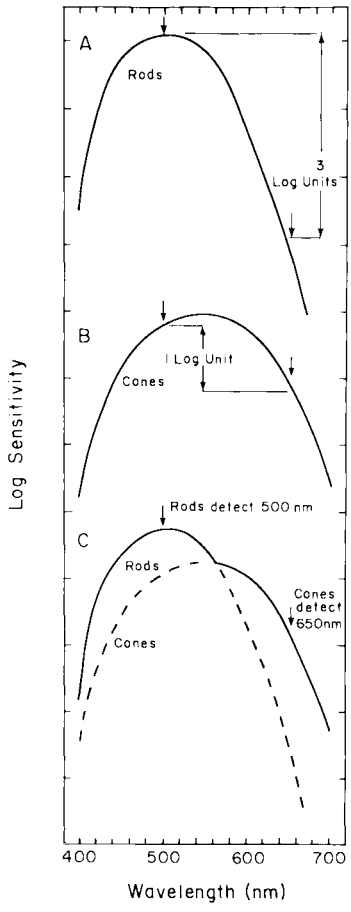


FIG. 15. (A) Schematic rod-determined spectral sensitivity curve. As shown by the arrows, rods are 3 log units more sensitive to a 500-nm stimulus than to a 650-nm stimulus. (B) Schematic cone-determined spectral sensitivity curve. This curve represents a weighted sum of the L, M, and S cone sensitivities. As shown by the arrows, the cone system is 1 log unit more sensitive to a 500-nm stimulus than it is to a 650-nm stimulus. (C) Schematic representation of a composite rod- and cone-determined spectral sensitivity. In this case the rods are more sensitive than the cones at 500 nm and the cones are more sensitive than the rods at 650 nm. (From Massof and Finkelstein, 1981b.)

Figure 17 illustrates how peak rod sensitivity and peak cone sensitivity change with retinal position. The abscissa of the graph in Fig. 17 is degrees of visual angle away from the center of fixation along a horizontal line extending from the extreme edge of the nasal visual field to the extreme edge of the temporal visual field. The point labeled 0° corresponds to fixation (i.e., the fovea), and the blank

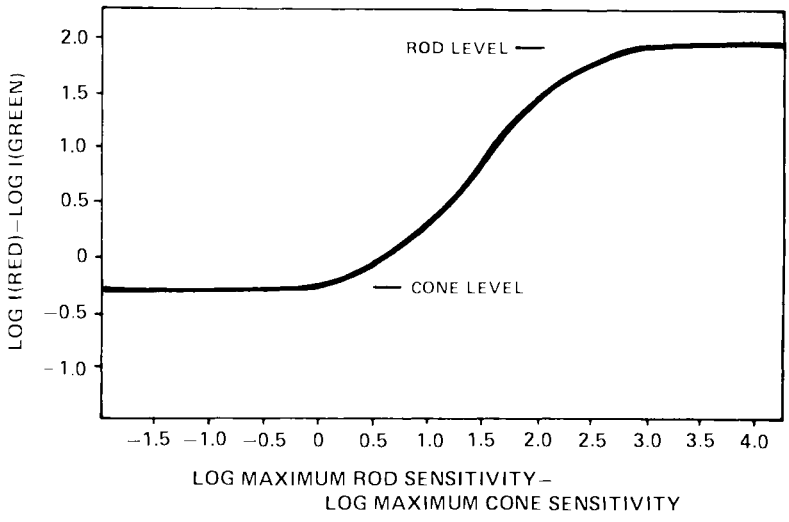


FIG. 16. Predicted difference between log threshold luminances for different relationships between the log sensitivities or rods and of cones. (From Massof and Finkelstein, 1981b.)

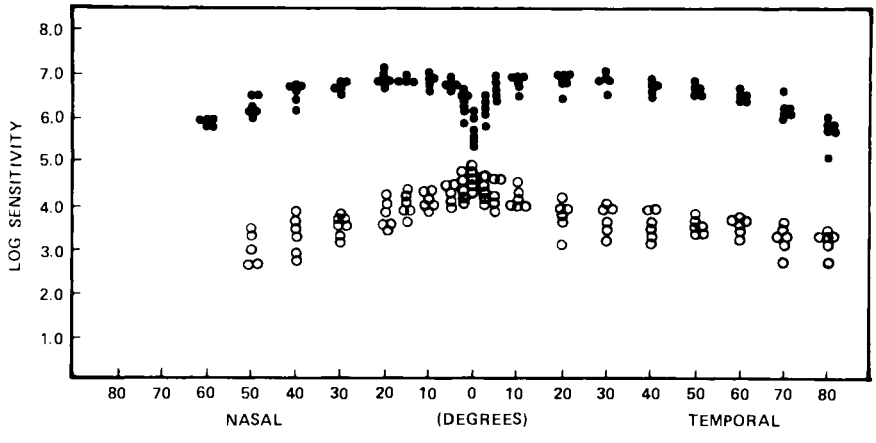


FIG. 17. Peak rod sensitivity (●) and peak cone sensitivity (○) for six normal subjects are plotted as a function of visual field eccentricity in degrees. (From Massof and Finkelstein, 1981b.)

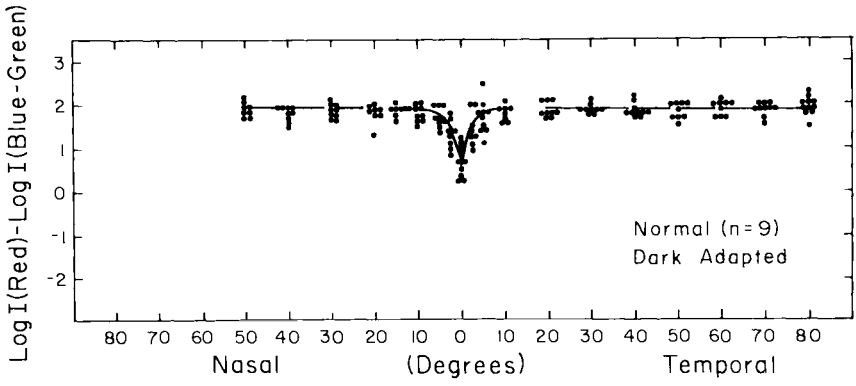


FIG. 18. Difference between log threshold luminances for the 650-nm stimulus and the 500-nm stimulus for nine normal subjects (points). The solid curve through the data is the prediction from the function in Fig. 16, using the average rod and cone sensitivity values from the data in Fig. 17. (From Massof and Finkelstein, 1981b.)

area from 10° temporal to 20° temporal is the blind spot and corresponds to the location of the optic nerve head. By taking the difference between points on the log sensitivity curve for rods and corresponding points on the log sensitivity curve for cones, we can generate a prediction from the curve in Fig. 16 of the difference between log threshold intensity for the 500-nm stimulus and log threshold intensity for the 650-nm stimulus as a function of retinal eccentricity. This prediction, along with the data from ophthalmologically normal subjects, is plotted in Fig. 18. These results verify the consequences of our reasoning and lend support to the assumptions that underlie our interpretation of data from RP patients.

Much to our surprise, we learned that there are two distinct categories of RP when considering only the relation of rod loss to cone loss. For one group, what we call type I RP, we measured cone-determined absolute threshold spectral sensitivity functions at all retinal positions. Figure 19 illustrates the sensitivity profile of one representative, a young type I RP patient. The cone sensitivities are near normal throughout the visual field and, as illustrated in the upper panel of Fig. 19A, we could not measure any rod contributions to visual detection. A full absolute threshold spectral sensitivity curve for this type I RP patient, measured at 50° in the temporal visual field is shown in Fig. 19B (solid circles). The curve drawn through the patient's data is the normal cone-determined spectral sensitivity function. The open circles in Fig. 19B are average normal spectral sensitivity values at the same visual field position. The curve drawn through the normal data is the normal rod-determined spectral sensitivity function.

For another group of patients, what we call type 2 RP, we measured rod-determined absolute threshold spectral sensitivity functions, despite very large (2–5 log unit) sensitivity losses. Figure 20 illustrates the rod and cone sensitivity profile of one representative, a young type 2 RP patient. In this case, both the rod and cone sensitivities are depressed in the affected parts of the visual field. Thus, as shown in the upper panel of Fig. 20A, the rods and cones are lost in the same proportion, thereby preserving the normal difference between the log absolute thresholds for the 500- and 650-nm stimuli. A full absolute threshold spectral sensitivity curve for this type 2 RP patient, measured at 40° in the temporal visual field, is shown in Fig. 20B (solid circles) along with the average normal values at the same visual field position (open circles). The normal rod-determined spectral sensitivity curve is drawn through both the patient's data and the average normal data.

We found for some RP patients that the absolute threshold profiles were different from the patterns shown in Figs. 19 and 20. However, by applying the same analysis to the results, we concluded that these patients fell on a continuum with type 1 RP. The two lower panels of Fig. 21A illustrates the rod and cone sensitivity profiles of one such young RP patient. In this case, as illustrated in the upper panel of Fig. 21A, all the retinal rods were depressed in sensitivity 2 log units more than the cones were depressed. Thus, at visual field positions outside of 10°, the rods mediated detection of the 500-nm stimulus and the cones mediated detection of the 650-nm stimulus. This result is further illustrated by the complete spectral sensitivity curve for this patient at 30° in the temporal visual field, which is plotted in Fig. 21B (solid circles). The two curves drawn through the data are the normal rod- and cone-determined spectral sensitivity functions. The average normal spectral sensitivity data at the same visual field position are plotted for comparison (open circles). The curve drawn through the normal data is the normal rod-determined spectral sensitivity function.

We have concluded that patients like the one whose data are illustrated in Fig. 21 belong to the type 1 subgroup of RP. Thus, we argue from our analysis that type 1 RP is characterized by a retinally diffuse and uniform loss of rod sensitivity, whereas type 2 RP is characterized by a combined and regionalized loss of rod and cone sensitivity. We now are using these measures and analyses to determine the nature of the progression of the retinal degeneration in these two types of RP.

C. Rod Internal Transduction Kinetics in RP

There has been considerable speculation that cyclic nucleotide metabolism may be abnormal in RP (Schmidt and Lolley, 1973). As described by Farber and

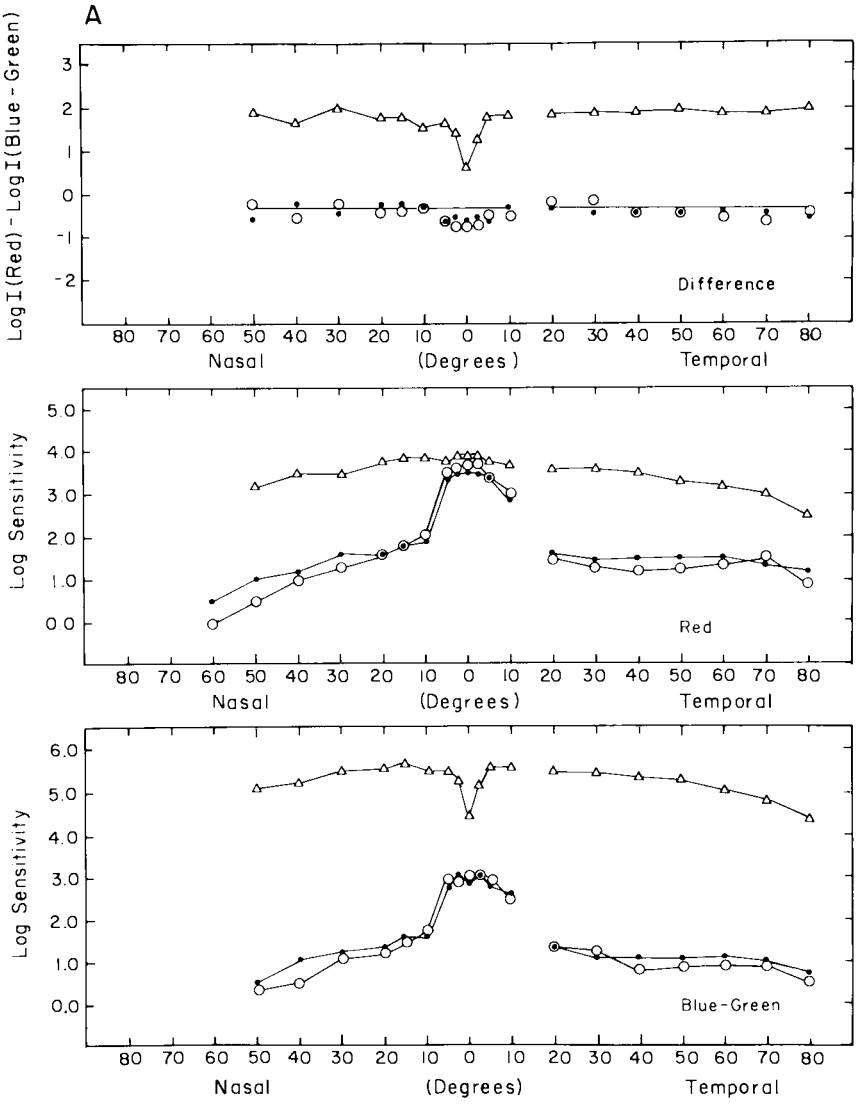
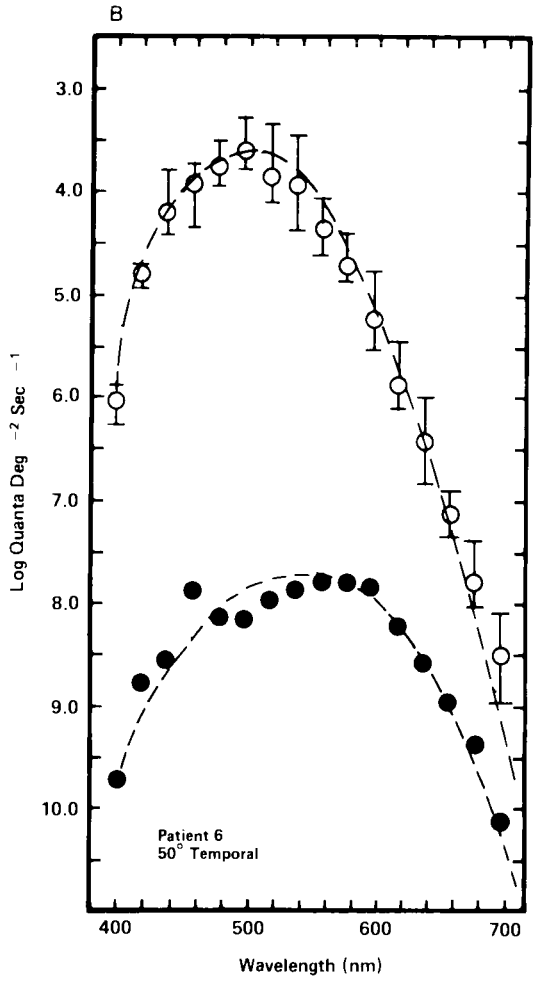


FIG. 19. (A) Log sensitivities for a young RP patient (○) as a function of visual field eccentricity for the 500-nm stimulus (lower panel) and the 650-nm stimulus (middle panel). Average normal log sensitivities (△) are plotted for comparison. The upper panel illustrates this patient's log threshold luminance differences for the two stimuli as a function of visual field eccentricity (points) in comparison to the average normal values (△). These difference values are at the cone level through-



out the visual field. (B) A complete spectral sensitivity function measured at absolute threshold at 50° in the temporal visual field for this same patient (●). The solid curve is the normal cone-determined spectral sensitivity function. The average normal spectral sensitivity for this same visual field position is plotted for comparison (○). The curve drawn through the normal data is the rod-determined spectral sensitivity function. (From Massof and Finkelstein, 1981b.)

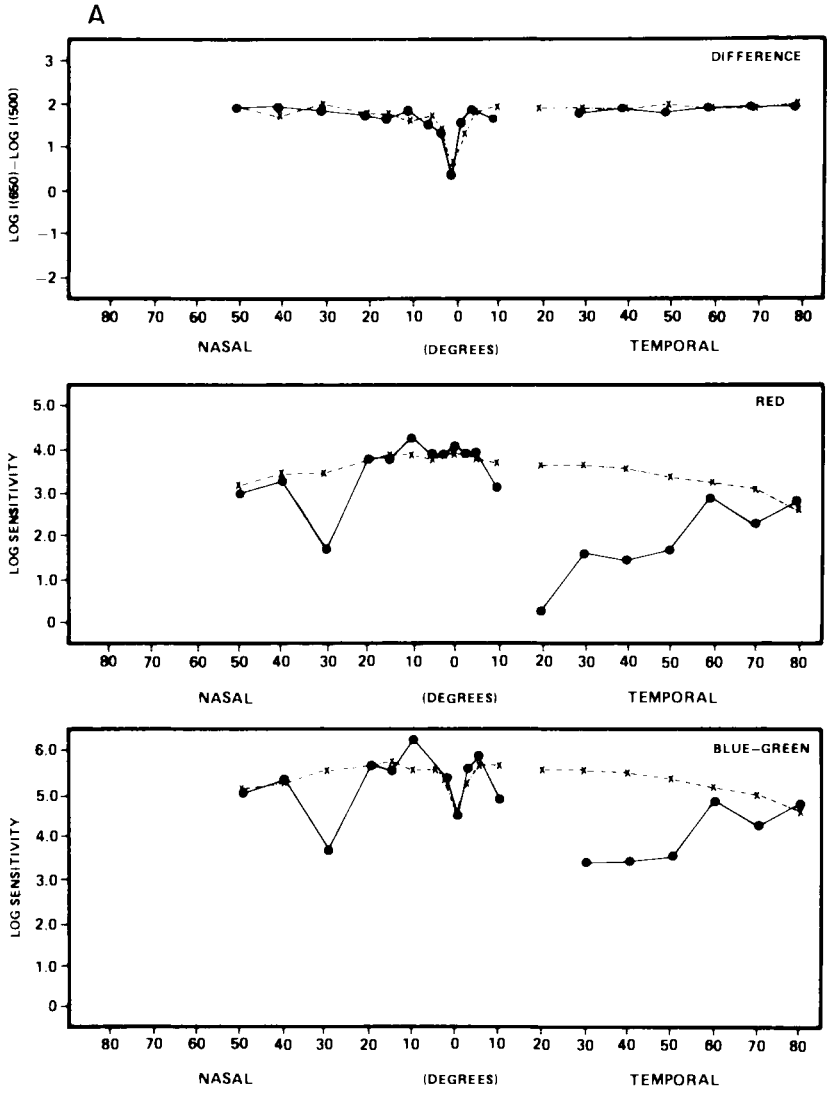
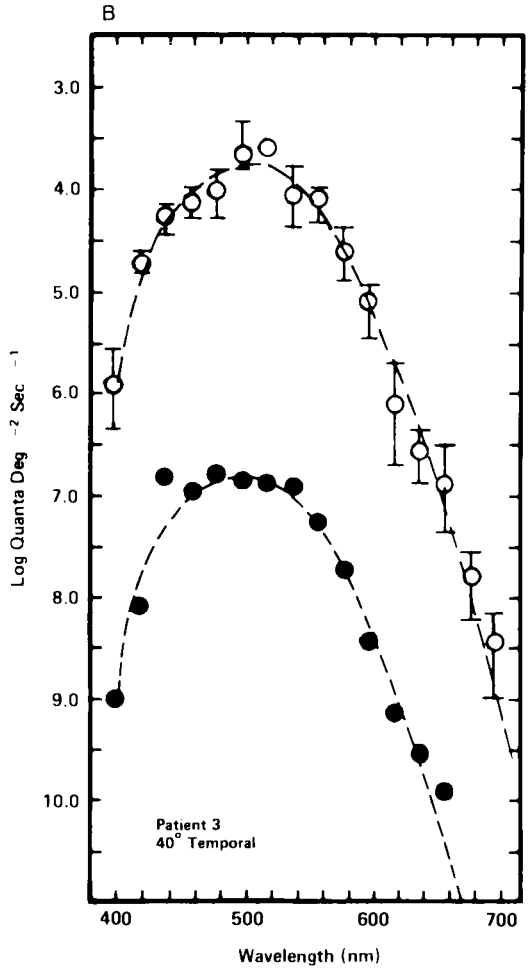


FIG. 20. Same as Fig. 19, but for a type 2 RP patient. Note that, unlike the patient data in Fig. 19, the log threshold luminance differences are nearly normal (points in upper panel of A). Thus, as reflected by this patient's full spectral sensitivity function measured at absolute threshold, rods still



mediate detection despite large losses in sensitivity (points in B). The solid curve fit to the patient's spectral sensitivity data is the rod-determined spectral sensitivity function. (From Massof and Finkelstein, 1981a.)

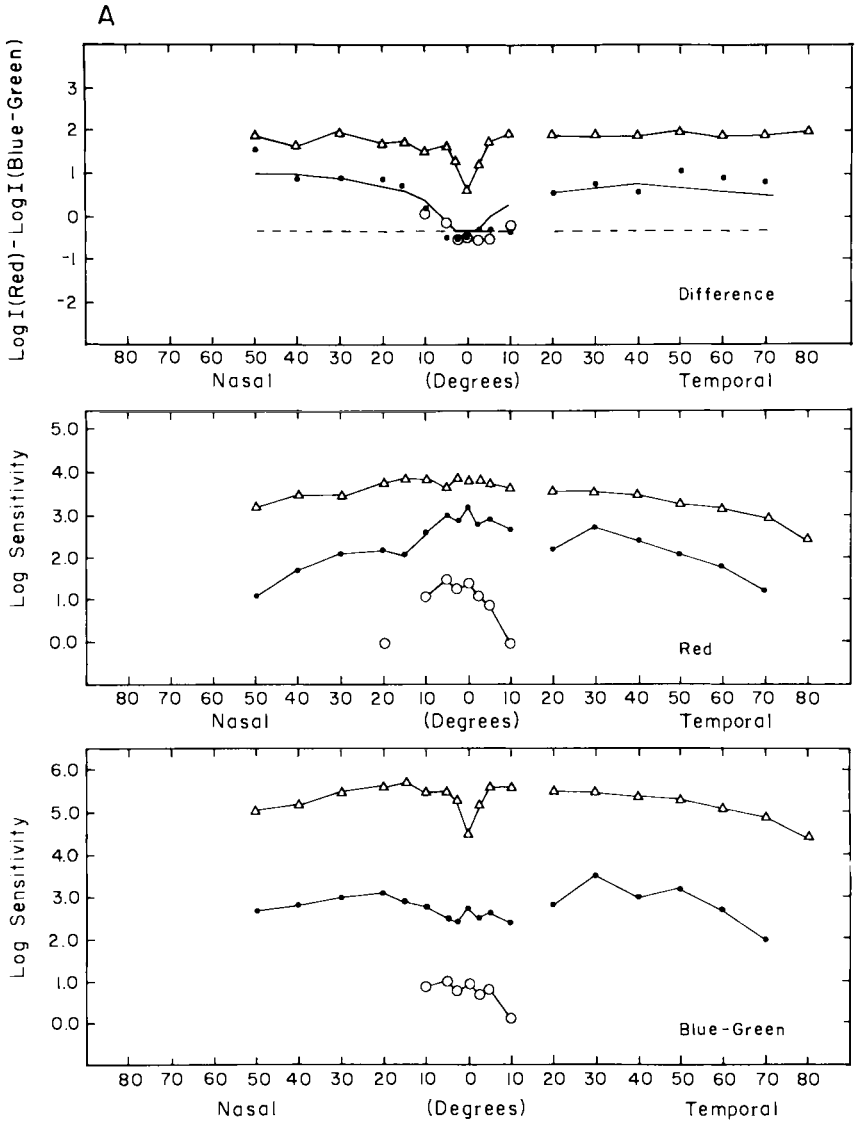
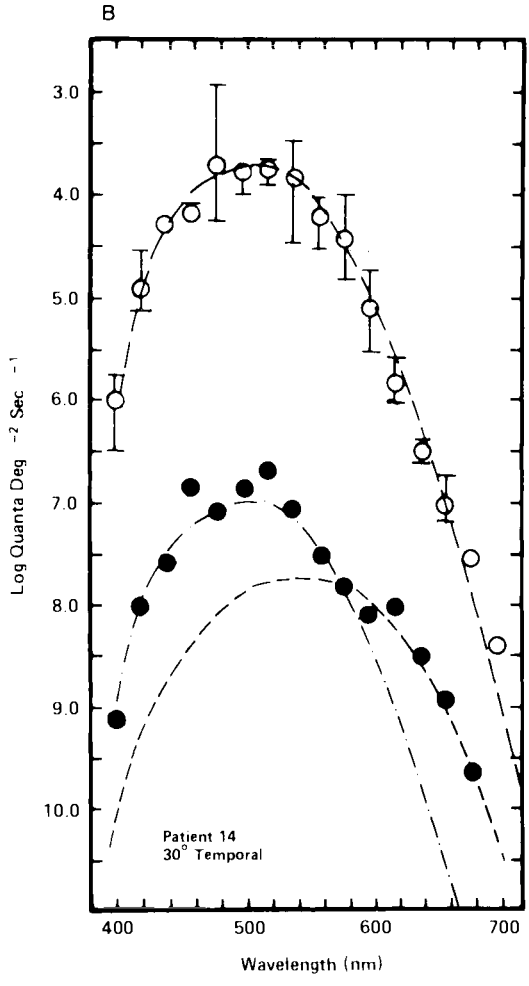


FIG. 21. Same as Fig. 19, but for a type 1 RP patient who exhibited intermediate rod sensitivity losses (●). As illustrated in the upper panel of (A), the log threshold luminance difference values ascended toward the rod level with increasing eccentricity (points). The solid curve drawn through the patient's data in this upper panel is the prediction of the function in Fig. 16 if all the retinal rods were depressed in sensitivity two log units more than the cones were depressed. (The open circles are



data for this patient's mother.) (B) An illustration of the complete spectral sensitivity function for this patient at absolute threshold at 30° temporal visual field (●). The two curves fit to the patient's data are the rod-determined and cone-determined spectral sensitivity functions. (From Massof and Finkelstein, 1981b.)

Shuster in Part I, there now is evidence that the activity of phosphodiesterase may be abnormal in the rods of mice with inherited retinal degenerations. If such an abnormality also occurs in human RP, then we might expect to see an abnormality in rod transduction kinetics.

We employed the afterimage experiment, described earlier, to obtain measures of rod afterimage latencies from type 2 RP patients (Benzschawel and Massof, 1984). Using the formal theoretical arguments specified by Eqs. (32)–(41), we felt that we could interpret RP afterimage detection latencies in terms of rod internal transduction kinetics. However, it was first necessary to establish that the linking postulates held for the RP patients.

From the arguments leading up to Eq. (38), the linking postulates require Weber's law (i.e., $\Delta I/I$ = constant) to hold for increment thresholds measured on the basis of detecting the test stimulus in the afterimage. Therefore, we obtained measures of increment thresholds for detection in the afterimage from each RP patient. As illustrated for a representative RP patient in Fig. 22, Weber's law (straight-line fit to the data) does hold.

We found that rod afterimage detection latencies for RP patients were significantly prolonged. As illustrated for an early RP patient in Fig. 23, the second branch of the function is considerably steeper than normal. Using Adelson's double exponential model in Eq. (41), we find that τ_1 = 180 msec and τ_2 = 3.5 sec for this RP patient, whereas τ_1 = 180 msec and τ_2 = 1.5 sec for the average normal rods. Thus, we would conclude that this RP patient's fast recovery

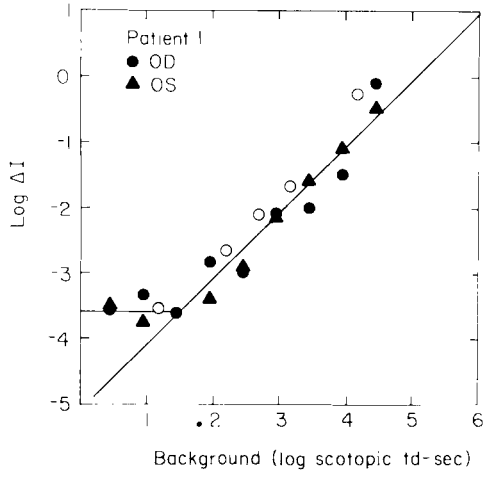


FIG. 22. Log test flash intensity (ΔI) for first detection of the square in the afterimage as a function of log background intensity for a representative RP patient. The straight-line fit to the data is Weber's law (i.e., $\Delta I/I$ = constant).

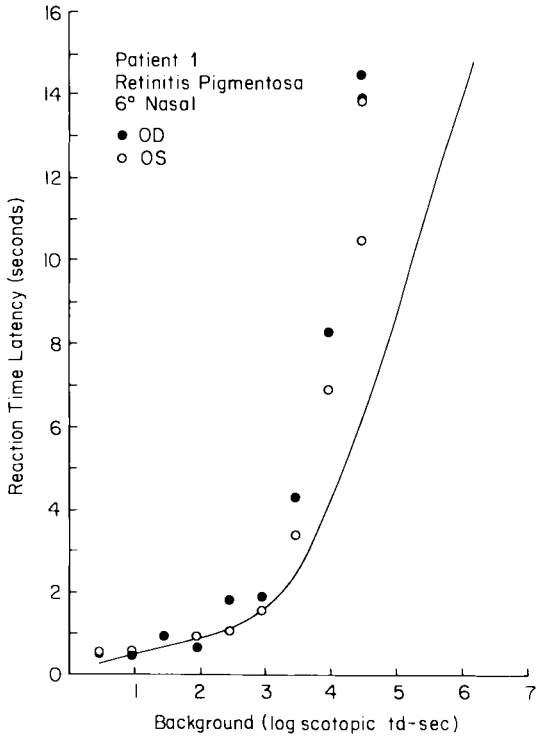


FIG. 23. Latency (seconds) to detection of the square in the afterimage as a function of log background intensity for a patient with early RP. The solid curve is the prediction of the double exponential model [Eq. (41)] with $\tau_1 = 180$ msec and $\tau_2 = 3.5$ sec.

process has normal kinetics, but the slow recovery process is markedly prolonged. Such a conclusion would be in agreement with a putative abnormality in phosphodiesterase activity.

V. Retinal Cell Biology from a Psychophysicist's Perspective

Within a very short period of time, our knowledge of the retina has grown from anatomical descriptions and studies of gross electrical potentials to detailed descriptions of specific biochemical processes within specific organelles of retinal cells. In the recent past, the retina was studied with the aim of describing the machinery of vision. Now, because of the remarkable advances in retinal cell biology, the retina has become an entity of study unto itself. That is, where once

vision was the prior concern of retinal anatomists and physiologists, now the retina is the prior concern. The objective of retinal cell biology is to understand the retina for its own sake.

Modern retinal cell biology is the natural consequence of a reductionistic or top-down approach. If we trace the history of work on the fundamental question of visual science, "How do we see?," we find that it took until 800 AD to determine that the retina is the relevant structure for transducing the light image into vision (prior to that time, the lens was considered to be the relevant structure). With both light and attention focused on the retina, the next step was to obtain a more detailed microscopic description of this ocular tunica. With the discovery of interconnected retinal cells, of a pigment in the retina that bleaches upon exposure to light, and of light-controlled electrical potentials that are generated by the retina, we were provided with better explanations of how the retina could serve to transduce light into vision. The amazing accomplishments of this century have given us detailed descriptions of the ultrastructure and function of individual retinal cells, Now, as evidenced by the work described in this book, retinal cell biology has carried us into the next level of understanding. That is, we are explaining the retina in molecular terms. From an extrapolation of this reductionistic approach, we might expect that sometime in the future our level of understanding could be described as an elementary particle physics of the retina.

An alternative to the reductionistic approach is the systems approach. Whereas retinal cell biology is a consequence of the reductionistic approach applied to the fundamental question of "How do we see?," psychophysics is a consequence of the systems approach applied to the same question. The finding that the retina, not the lens, is the relevant structure to vision is of no formal concern to psychophysics, just as long as we agree on the equations for transducing light into vision.

Although vision psychophysics and retinal cell biology seem to have parted company several hundred years ago, a complete visual science would require the establishment of formal connections between the disciplines. That is, the objective of visual science would be to describe the physiological operators of psychophysics in terms of physiological subsystems. To meet this objective, it would be necessary to develop a systems theory for each level of understanding. For example, retinal network behavior would be described in terms of cellular subsystems and individual retinal cell behavior would be described in terms of biochemical subsystems. Ultimately, through such nested descriptions, the physiological operators of psychophysics could be described in terms of the biochemical subsystems. To a small extent, we already have done this in our modeling of rod afterimages using the blocking agent postulates.

Retinal cell biology is still largely descriptive. As more information is acquired, emergent properties of the biochemical processes will become evident. It is these emergent properties that constitute a system. Thus, it is likely that retinal

cell biology will soon become more concerned with the logic of the cell, in addition to being concerned with detailed descriptions of cell structure and physiology. Descriptions of the logic at a biochemical level will provide building blocks for describing the logic of the next level of organization and so on. Ultimately, we would achieve a level of organization where psychophysically measured properties of vision could be connected to the finest level of understanding.

References

Adelson, E. H. (1982). The delayed rod afterimage. *Vision Res.* **22,** 1313–1328.

Benzschawel, T., and Massof, R. (1984). Rod photoreceptor kinetics in retinitis pigmentosa. *Invest. Ophthalmol. Visual Sci. Suppl.* **25,** 197 (Abstr.).

Benzschawel, T., Massof, R. W., and Kays, D. L. (1983). Delayed rod afterimage in a rod monochromat. *J. Opt. Soc. Am.* **73,** 1915 (Abstr.).

Bird, J. F., and Massof, R. W. (1978). A general zone theory of color and brightness vision. II. The space–time field. *J. Opt. Soc. Am.* **68,** 1471–1481.

Boughman, J. A., Connelly, M., and Nance, W. E. (1980). Population genetic studies of retinitis pigmentosa. *Am. J. Hum. Genet.* **32,** 223–235.

Boynton, R. M. (1979). "Human Color Vision." Holt, New York.

Boynton, R. M., and Whitten, D. N. (1970). Visual adaptation in monkey cones: Recordings of late receptor potentials. *Science* **170,** 1423–1426.

Boynton, R. M., and Wisowaty, J. J. (1980). Equations for chromatic discrimination models. *J. Opt. Soc. Am.* **70,** 1471–1476.

Brindley, G. S. (1970). "Physiology of the Retina and Visual Pathway," 2nd Ed. Arnold, London.

Brown, J. L., and Mueller, C. G. (1965). Brightness discrimination and brightness contrast. *In* "Vision and Visual Perception," (C. H. Graham, ed.), pp. 208–250. Wiley, New York.

Cornsweet, T. N. (1970). "Visual Perception," pp. 100–105. Academic Press, New York.

Dartnall, H. J. A., Bowmaker, J. K., and Mollon, J. D. (1983). Human visual pigments: Microspectrophotometric results from the eyes of seven persons. *Proc. R. Soc. London Ser. B* **220,** 115–130.

Geisler, W. S. (1980). Increment threshold and detection latency in the rod and cone systems. *Vision Res.* **20,** 981–994.

Geisler, W. S. (1981). Effects of bleaching and backgrounds on the flash response of the cone system. *J. Physiol. (London)* **312,** 413–434.

Green, D. M., and Swets, J. A. (1966). "Signal Detection Theory and Psychophysics." Wiley, New York.

Guth, S. L., Massof, R. W., and Benzschawel, T. (1980). Vector model for normal and dichromatic color vision. *J. Opt. Soc. Am.* **70,** 197–212.

Hecht, S., Shlaer, S., and Pirenne, M. H. (1942). Energy, quanta, and vision. *J. Gen. Physiol.* **25,** 819–840.

Heggelund, P. (1974). Achromatic color vision. I. Perceptive variables of achromatic colors. *Vision Res.* **14,** 1071–1079.

Hurvich, L. M. (1981). "Color Vision." Sinauer, Sunderland, Massachusetts.

Marks, L. E. (1974). "Sensory Processes: The New Psychophysics." Academic Press, New York.

Massof, R. W. (1985). Color vision theory and linear models of color vision. *Color Res. Appl.* **10,** 133–146.

Massof, R. W., and Bird, J. F. (1978). A general zone theory of color and brightness vision. I. Basic formulation. *J. Opt. Soc. Am.* **68,** 1465–1471.

Massof, R. W., and Finkelstein, D. (1979). Rod sensitivity relative to cone sensitivity in retinitis pigmentosa. *Invest. Ophthalmol. Visual Sci.* **18,** 263–272.

Massof, R. W., and Finkelstein, D. (1981a). Subclassifications of retinitis pigmentosa from two-color scotopic static perimetry. *Doc. Ophthalmol. Proc. Ser.* **26,** 219–225.

Massof, R. W., and Finkelstein, D. (1981b). Two forms of autosomal dominant primary retinitis pigmentosa. *Doc. Ophthalmol.* **51,** 289–346.

Massof, R. W., Finkelstein, D., and Boughman, J. A. (1982). Genetic analysis of subgroups within simplex and multiplex retinitis pigmentosa. *In* "Genetic Eye Diseases: Retinitis Pigmentosa and other Inherited Eye Disorders" (E. Cotlier, I. H. Maumenee, and E. R. Berman, eds.), pp. 161–166. Liss, New York.

Nachmias, J. (1972). Signal detection theory and its applications to problems in vision. *In* "Visual Psychophysics" (D. Jameson and L. Hurvich, eds.), Handbook of Sensory Physiology VII/4, pp. 56–77. Springer-Verlag, Berlin and New York.

Penn, R. D., and Hagins, W. A. (1972). Kinetics of the photocurrent of retinal rods. *Biophys. J.* **12,** 1073–1094.

Schmidt, S. Y., and Lolley, R. N. (1973). Cyclicnucleotide phosphodiesterase. An early defect in inherited retinal degeneration of C3H mice. *J. Cell Biol.* **57,** 117–123.

Smith, V. C., and Pokorny, J. (1975). Spectral sensitivity of the foveal cone photopigments between 400 and 500 mm. *Vision Res.* **15,** 161–171.

Steinberg, R. H. (1969). The rod after-effect in s-potentials from the cat retina. *Vision Res.* **9,** 1345–1355.

Stevens, J. C. (1967). Brightness function: Binocular versus monocular stimulation. *Percept. Psychophys.* **2,** 451–454.

Stevens, S. S. (1961). To honor Fechner and repeal his law. *Science* **133,** 80–86.

Teller, D. Y. (1984). Linking propositions. *Vision Res.* **24,** 1233–1246.

Thomson, L. C., and Wright, W. D. (1947). The colour sensitivity of the retina within the central fovea of man. *J. Physiol. (London)* **105,** 316–331.

Vos, J. J., and Walraven, P. L. (1971). On the derivation of the foveal receptor primaries. *Vision Res.* **II,** 799–818.

Wald, G. (1945). Human vision and the spectrum. *Science* **101,** 653–658.

Witkovsky, P. (1980). Excitation and adaptation in the vertebrate retina. *Curr. Top. Eye Res.* **2,** 1–66.

Wyzecki, G., and Stiles, W. S. (1982). "Color Science," 2nd Ed. Wiley, New York.

THE CELL BIOLOGY OF THE RETINAL PIGMENT EPITHELIUM

VIRGINIA M. CLARK

Jules Stein Eye Institute
UCLA School of Medicine
Los Angeles, California

I. Summary of RPE Cell Structure and Functions

The retinal pigment epithelium (RPE) is situated between the choroidal blood supply and the photoreceptor cell layer. When viewed enface, the RPE cell appears to be hexagonally shaped and darkly pigmented (Fig. 1). However, most of the important structural features of the cell can be best appreciated by examining it in cross section (Fig. 2). Like other epithelial cells, the RPE cell displays a differentiation of its plasma membrane or cell asymmetry. This asymmetry is essential for many of the functions carried out by the cell. At its apical surface (that which faces the retina), the RPE cell elaborates both long and short microvilli. Short microvilli ensheath rod and cone outer segments, while the long ones

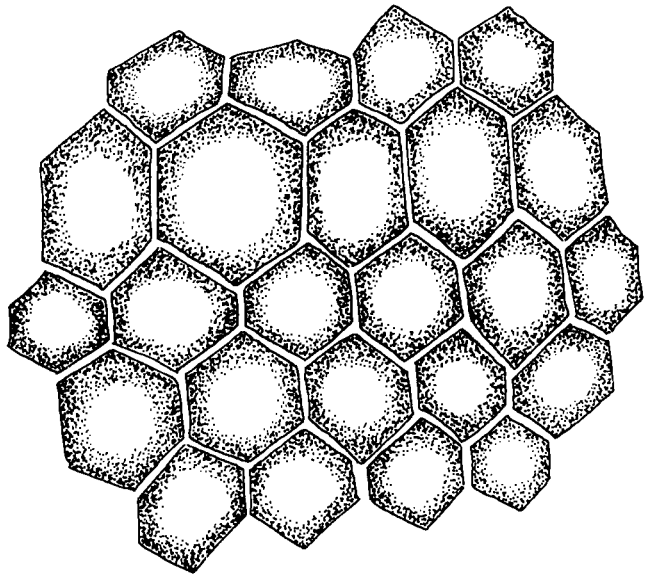


FIG. 1. RPE cells, enface view.

project like fingers along the lengths of outer segments. The plasma membranes of the photoreceptor and RPE cells do not have any points of contact, but rather are separated by a very thin layer of extracellular matrix material, called the interphotoreceptor matrix. Although the mechanism of retinal adhesion is still a mystery, it is currently believed that the interdigitation of photoreceptor and RPE cell plasma membranes, together with keeping the space between the membranes very small, are essential for good adhesion. There is evidence that the RPE maintains this thin layer by actively transporting ions and water out of the subretinal space. Close proximity of a large surface area of the photoreceptor and RPE cell plasma membranes facilitates exchange of nutrients and wastes between the two cell types.

At the lateral surface, each RPE cell is joined to adjacent cells by a continuous belt of tight intercellular junctions. This junctional complex (also called the terminal bar) is located near the apical region of the plasma membrane and prevents diffusion of large molecules through the space between adjacent RPE cells. The terminal bar is thought to be the main structure responsible for the blood–retinal barrier at the level of the choroidal blood supply.

The basal plasma membrane of the RPE cell contains many infoldings. As with the apical surface, these infoldings increase the surface area of the membrane, thus facilitating exchange of wastes and nutrients between the RPE cell and the choroidal blood supply. At its basal surface, the RPE cell secretes a

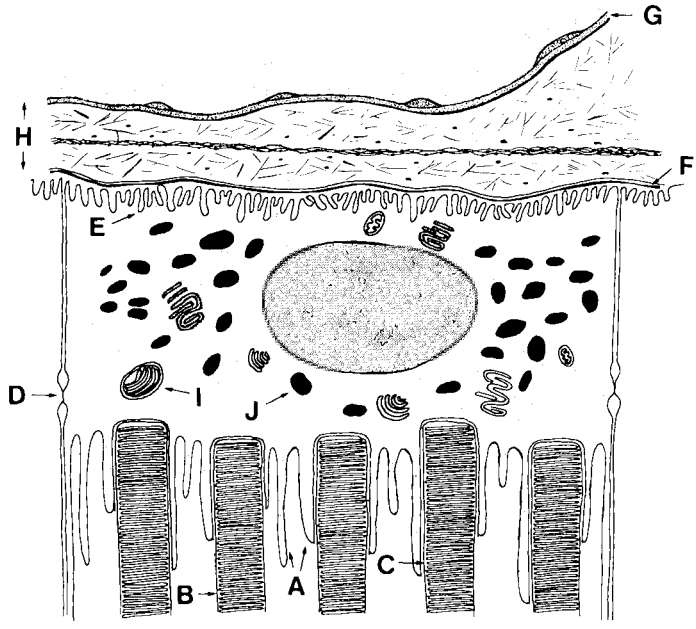


FIG. 2. RPE cell, cross-sectional view. A, Microvilli of apical plasma membrane; B, photoreceptor outer segment; C, interphotoreceptor matrix; D, tight junctions; E, infoldings of basal plasma membrane; F, basement membrane of the RPE cell; G, basement membrane of a choroidal capillary; H, Bruch's membrane; I, phagosome; J, melanin granule.

basement membrane to which it is firmly attached. The RPE basement membrane is separated from the basement membranes of the choroidal capillaries by a loose network of collagen fibers and other structural proteins. This composite structure of two basement membranes, separated by a layer of collagen, has been traditionally referred to as Bruch's membrane. Bruch's membrane is permeable to small molecules and some macromolecules. Recent studies suggest that it, too, may contribute to the blood–retinal barrier, at least in rats (Pino *et al.*, 1981, 1982).

There are some distinctive features of the RPE cytoplasm. Phagosomes, containing recently ingested photoreceptor membranes, are characterized by the lamellar appearance of their contents. As lysosomal digestion of a phagosome proceeds, residual contents may form a lipofuscin granule. RPE cells of many species contain oil droplets in which a reserve of vitamin A (retinol), esterified to fatty acids, is stored. Melanin (pigment) granules are thought to increase photoreceptor efficiency by absorbing excess, scattered light.

Very generally, the main functions of the RPE cell are (1) to participate in the

nurturing and renewal of the photoreceptor cell layer, (2) to promote retinal adhesion, and (3) to absorb scattered light. In this article, I have selected topics from categories 1 and 2 that serve as good illustrations of how the RPE cell might be used as a model for studying biological problems of current interest. Some of these problems include phagocytosis (encompassing cell–cell recognition, cytoskeletal structure and function, and lysosomal systems), aging, transcellular transport, the secretion and function of specialized extracellular matrix components, and the structure and function of blood–tissue barriers. Furthermore, I have emphasized areas of new developments since the publication of *The Retinal Pigment Epithelium* (1979), edited by Zinn and Marmor.

I. Cellular Processes That Have Been Studied in the RPE

A. Phagocytosis

An important RPE cell function is to remove wastes from the photoreceptor cells. The best characterized example of this process is the phagocytic uptake of shed photoreceptor outer segment (OS) membranes by the RPE cell (Young and Bok, 1969). By analogy with the model put forth for the macrophage (Rabinovitch, 1967), phagocytosis of OS membranes can be described as a multistep process (Fig. 3).

The first step is binding of the OS tip to the RPE cell plasma membrane. In the macrophage, particles are bound for phagocytosis by two basic mechanisms.

1. Particles coated (opsonized) with IgG, IgM-complement (C3b), or fibronectin are bound to the macrophage surface via specific plasma membrane receptors that recognize IgG (Fc region), C3b, and fibronectin, respectively (Mantovani *et al.*, 1972; Griffen, *et al.*, 1975a,b, 1976; Gudewicz *et al.*, 1980). Uptake of particles bound via specific cell surface receptors is called receptor-mediated phagocytosis.
2. Particles, such as latex and some nonopsonized biological materials, are bound to the cell surface by a little understood mechanism that is unlikely to be mediated by specific receptors (Griffen and Silverstein, 1974). This mode of binding may possibly be mediated by favorable hydrophobic or charge characteristics of the particle surface (van Oss, 1978). Uptake of particles bound in this manner is sometimes referred to as nonspecific phagocytosis.

In the macrophage, receptor-mediated and nonspecific phagocytosis are metabolically distinct processes (Michl *et al.*, 1976). Similarly, recent evidence suggests that the RPE phagocytizes latex spheres and other inert substances by a nonspecific mechanism that is distinct from the mechanism (receptor mediated?) by which OS are phagocytized (Philp and Bernstein, 1981).

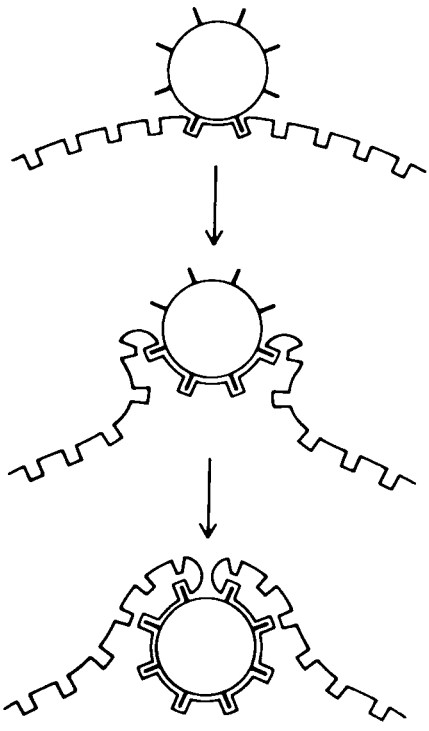


FIG. 3. Model for receptor-mediated phagocytosis (Silverstein and Loike, 1980).

Binding is followed by firm attachment and ingestion of the OS membranes. Once inside of the RPE cell, ingested OS membranes (now called phagosomes) combine with lysosomes and digestion of the phagosome contents occurs.

Since the phagocytosis of shed OS membranes is a multistep process, interruption of any of these steps can have dire consequences for the health of the retina. A dramatic example of this point is found in the RCS (dystrophic) rat (Bourne *et al.*, 1938; Dowling and Sidman, 1962). The RCS mutation affects the RPE cells (Mullen and LaVail, 1976) such that their ability to phagocytize OS membranes is impaired (Herron *et al.*, 1969; Bok and Hall, 1971; Goldman and O'Brien, 1978). As OS continue to elongate in the limited subretinal space, the tips become distorted, creating a layer of OS debris between the photoreceptor and RPE cell layers. It has been proposed that the accumulation of OS debris in the subretinal space causes an interruption of flow of nutrients between the RPE and photoreceptor cell and hence contributes to photoreceptor death (Herron *et al.*, 1969). The RCS mutant should prove to be a valuable tool for studying the molecular basis for the mechanism of phagocytosis.

In the past 10 years, there have been many studies of phagocytosis by the RPE. Often, it is difficult to compare the results of these studies because of the

variety of particles that have been chosen for the phagocytic assay and the condition of the RPE cells at the time of feeding. The various particles have included OS, latex spheres, carbon, and bacteria to name a few. These particles have been injected into the subretinal space (*in vivo* RPE) or incubated with cultured RPE explants or monolayers of pure RPE cells, cultured on plastic. Studies of phagocytosis by RPE cells *in vivo* and by RPE cell explants are discussed in the following section, while studies employing cultured RPE cell monolayers are primarily discussed in Section III,B,1.

1. Binding/Recognition

a. Particle Specificity. One question that early studies of binding/recognition addressed was whether the process of phagocytosis of OS by the RPE is a highly specific process or whether the RPE is a generalized phagocytic cell that ingests OS as well as other particles. Hollyfield and Ward (1974a) injected polystyrene spheres into the subretinal space of developing *Rana pipiens* and found that large numbers of the spheres were phagocytized by the RPE. In a subsequent study, Hollyfield and Ward (1974b) injected both polystyrene spheres and the bacteria *Sarcina subflava* into the subretinal space of *R. pipiens*. Under these circumstances, the polystyrene spheres were phagocytized, while the bacteria were not. This was the first evidence that the RPE displays selectivity with regard to the type of material that it will engulf. These results further showed that, like the macrophage (Griffen and Silverstein, 1974), the RPE cell is capable of engulfing one type of particle in the presence of an unpalatable particle without coingesting the second particle nonspecifically.

Custer and Bok (1975) injected carbon particles into the subretinal space of both normal and dystrophic rats and compared phagocytic uptake by the RPE of the two animals. They found that the RPE of dystrophic rats (which displays impaired ability to phagocytize OS) phagocytized carbon particles in the same quantities as did the RPE of normal rats. Similarly, Reich-D'Almeida (1976) found that the RPE of dystrophic rats was capable of phagocytizing carbon particles. These studies suggested for the first time that the RPE, like the macrophage, possesses two types of phagocytic capabilities: (1) a nonspecific process by which the RPE may phagocytize various particles (such as latex) very slowly (2–3/hr) and (2) a specific process by which the RPE cell phagocytizes 50–300 OS tips (Bok and Young, 1979) within 1–2 hr of availability (LaVail, 1976a,b; Basinger *et al.*, 1976).

This point was clearly made by Philp and Bernstein (1981), who presented a careful comparison and characterization of the two types of phagocytosis [nonspecific uptake of latex and specific uptake of rod outer segments (ROS)] in cultured RPE cell explants from normal rats. The first point that they made is that uptake of OS by RPE of adult rats occurs much more rapidly than uptake of latex

beads. In the RPE explants from adult rats, phagocytosis of OS peaked at 2 hr after onset of feeding and then declined. At the peak of this phagocytic activity, the RPE ingested 9 times more OS than latex beads. In contrast, phagocytosis of latex displayed a lag for the first 6 hr of feeding, followed by a slowly increasing rate of uptake for the next 24 hr of explant culture. [An even longer lag, 12–17 hr, in the rate of latex ingestion was observed by Essner *et al.* (1978) in explants of rabbit and calf RPE.]

Philp and Bernstein also compared the phagocytic capabilities of RPE from neonatal rats and adult rats. They found that RPE from adult rats phagocytized about 80 times more OS than did RPE from 10-day-old neonates. In contrast, RPE from 10- and 15-day-old rats phagocytized almost 2 times as many latex spheres as did RPE from adults. Therefore, since (1) the capabilities of the RPE to phagocytize latex and OS are expressed at different times in development and (2) phagocytosis of latex and OS occur at different rates in adult rat RPE, these results strongly support the conclusion that latex spheres and OS are phagocytized by two different mechanisms.

b. The Possible Role of Carbohydrates in Binding/Recognition. With regard to the processes of shedding and phagocytosis of OS membranes by RPE cells, there are at least two proposed mechanisms. One model suggests that shedding and phagocytosis of OS tips are one and the same process, i.e., that RPE cells pinch off OS tips in response to a chemical change in the environment of the photoreceptor–RPE complex (Spitznas and Hogan, 1970). The second model (Young, 1971) proposes that shedding and phagocytosis occur sequentially. The second model implies that the RPE is capable of phagocytizing OS membranes at all times and that only the shedding process is under cyclic modulation (LaVail, 1976a,b; Basinger *et al.*, 1976). (A more detailed exploration of these concepts may be found in the article by Besharse in Part I). Since RPE cells display selectivity with regard to phagocytosis (Section II,A,1,a), both models require that there be some sort of mechanism by which the RPE recognizes OS membrane material in order to bind and ingest it.

In 1976, O'Brien reported that a small fraction of rhodopsin molecules in bovine ROS can be labeled with [^{3}H]fucose and [^{3}H]galactose by endogenous glycosyl transferases (i.e., this is apparently a "post-inner segment processing" modification). O'Brien suggested that modification of rhodopsin in the highly localized region of the plasma membrane at the tip of the OS by addition of terminal sugars (fucose and galactose) might be the signal which initiates shedding and phagocytosis of the OS tip by the RPE. O'Brien's suggestion that carbohydrates might be the key to this recognition process, together with accumulated evidence that carbohydrates play a role in other cell–cell recognition phenomenon, led some investigators to explore this question more closely.

Initial studies focused on simply characterizing exposed sugar residues on the

external surfaces of both OS and RPE cell plasma membranes with the aid of plant lectins (Hall and Nir, 1976; Nir and Hall, 1979; Bridges and Fong, 1979; McLaughlin and Wood, 1980). Hall and Nir (1976) compared binding of ferritin-conjugated concanavalin A (Con A) to plasma membranes of both RPE and ROS in normal and dystrophic (RCS) rats. They found no differences in the density of Con A (specific for α-D-Man, α-D-Glc, and α-D-GlcNAc) binding in mutant and normal tissues. McLaughlin and Wood (1980) carried out a more extensive study, using peroxidase-conjugated wheat germ agglutinin (WGA), *Ricinis communis* agglutinin (RCA), Con A, and *Lens culinaris* agglutinin (LCA). The results of this study suggested that the surfaces of microvilli of normal RPE contain more LCA-binding sites than microvilli of dystrophic RPE. Due to the fact that the RPE of the dystrophic rats fails to phagocytize OS membranes, the authors suggested that LCA (specific for α-D-Man, α-D-Glc, and α-D-GlcNAc) binding sites on the RPE plasma membrane may be involved in the specific phagocytic uptake of ROS. The apparent discrepancy between the findings of Hall and Nir (1976) and McLaughlin and Wood (1980) may be due to differences in the microspecificities of Con A and LCA. LCA binds preferentially to mannose chains that contain fucose (Kornfeld, 1981).

Plant lectins were subsequently used to block exposed sugars on the surfaces of OS and RPE in order to study the effect on phagocytosis. Hall and Quon (1980) found that WGA, Con A, RCA, and soybean agglutinin (SBA) significantly reduced phagocytosis of ROS in cultured rat RPE cells only when both the RPE and ROS surfaces were coated with lectin prior to the phagocytic assay. Philp and Bernstein (1980) reported that pretreatment of isolated ROS with Con A inhibited subsequent ingestion by rat RPE explants by 50%. They further found that pretreatment of RPE with Con A had no effect on the phagocytosis of OS, but did inhibit uptake of latex beads.

It is reasonable to assume that if the RPE recognizes specific sugars on the surface of the OS, then it should be possible to inhibit this recognition by adding an excess of the sugar to the subretinal space or millieu in which the RPE and OS are making contact. Philp and Bernstein (1980) reported that treatment of RPE cell explants (rat) with 0.1 *M* mannose or 100 μg of yeast mannan/ml resulted in a 50% reduction in the ability of the cells to phagocytize ROS. This treatment had no effect on the phagocytosis of latex, suggesting a possible specificity for the effect. Heath and Basinger (1983) examined the effect of various simple sugars on disc shedding and phagocytosis in *in vitro* eyecup preparations from *R. pipiens*. They found that L-fucose, α-methyl-D-mannopyranoside, and D-mannose (at 50 m*M*) each inhibited disk shedding and phagocytosis, with L-fucose being the most potent inhibitor. This inhibition seemed to be a specific effect since a variety of other sugars (including D-fucose and L-mannose) were also tested and found to have no significant effect on shedding and phagocytosis. The authors emphasized that the inhibitory sugars affected both shedding and phago-

cytosis. If only phagocytosis were inhibited, then one would expect to find a number of shed but uningested disc packets (or an increase of what the authors called "incipient phagosomes") in the subretinal space. Such was not the case. Therefore, this study lends support to a model proposing that shedding and phagocytosis are inseparable processes.

It was mentioned earlier that normal RPE cells phagocytize latex spheres more slowly than OS. Seyfried-Williams and McLaughlin (1983) coated latex spheres with mannose and fucose in order to enhance the "OS-like" quality of surfaces of the spheres. This, they expected, might stimulate uptake of the latex by the rapid mechanism by which OS are phagocytized. Surprisingly, sugar-coated latex spheres were ingested at a reduced rate as compared to uncoated latex spheres. Mannose-coated beads were taken up at about half the rate of uncoated beads, while fucose-coated beads were not ingested at all. The authors interpreted these results in the following way. Latex was ingested by phagocytic cells by a mechanism that may involve a hydrophobic interaction between the spheres and cell membranes (van Oss, 1978). When the hydrophobic sites were covered with fucose, the beads were not ingested. Therefore, simple fucose is not "recognized" by a specific phagocytic process that may be responsible for ingestion of OS. Mannose-coated beads were ingested (at a slower rate) because either the hydrophobic sites were incompletely masked by the mannose coating or mannose is recognized by a specific phagocytic process. The former explanation is more likely, since the rate of latex ingestion was depressed, not stimulated by mannose coating.

If carbohydrates are involved at all in recognition of OS by RPE, it is most likely that a complex carbohydrate (possibly containing mannose and fucose) is the moiety that is recognized. By using high (but not toxic) concentrations of simple sugars, Heath and Basinger (1983) may have been forcing some interference with the fucose- or mannose-binding region of a carbohydrate recognizing receptor for OS. At the relatively low concentrations of latex-bound simple sugars used by Seyfried-Williams and McLaughlin, they would have found little or no "forced recognition." In future pursuits of this line of research, it may be fruitful to examine the ability of various oligosaccharides (containing mannose and fucose in common linkages) to competitively inhibit the phagocytosis of OS by RPE.

c. Possible Role of Serum Components in Binding/Recognition. Analogies between the macrophage and the RPE cell led to the speculation that some opsonizing factor might also be responsible for specific binding of OS to the RPE cell. Newsome and Bowles (1979) demonstrated the presence of Fc receptors on canine RPE cells. In 1981, Elner *et al.* published a more detailed report which confirmed Newsome's findings. RPE cells were isolated from monkey eyes and allowed to attach to a glass surface for 2 hr. Attached cells were presented with

human erythrocytes which had been (1) untreated, (2) pretreated with anti-erythrocyte IgG, or (3) pretreated with $F(ab)_2$ fragments (lacking an Fc region) of anti-erythrocyte IgG. The IgG-coated erythrocytes bound to the RPE cell surfaces in a rosette formation, while untreated or $F(ab)_2$-treated erythrocytes did not bind. IgG, but not $F(ab)_2$ fragments, were effective in preventing rosette formation. Some phagocytic uptake of IgG-coated erythrocytes was also observed. Similar experiments showed that the RPE cell also bound IgM- and C5-coated erythrocytes with apparent specificity. Thus, the RPE cell surface contains receptors which specifically recognize the Fc region of IgG and the C5 fragment of complement.

In a similar vein of thought, McCann *et al.* (1983) examined the effect of tuftsin on phagocytosis of latex beads by RPE cell explants. [Tuftsin is derived from the Fab portion of the immunoglobin molecule by the action of leukokinase, a protease bound to the surface of neutrophils. Tuftsin acts more like a hormone than an opsinin in its effect of stimulating phagocytosis by neutrophils (Najjar and Nishioka, 1970).] McCann *et al.* reported that tuftsin caused a fourfold stimulation of binding and ingestion of latex spheres by RPE cell explants. It will be of interest to know if tuftsin stimulates uptake of OS as well, or if tuftsin only stimulates the "nonspecific" process by which latex beads are ingested. As yet, there is no evidence that IgG, complement, or tuftsin are normal components of the interphotoreceptor matrix.

2. Ingestion

Once the OS is specifically bound to and recognized by the RPE cell surface, some sort of transmembrane-signaling mechanism must tell the RPE to begin the ingestion process. Nothing is known about this hypothetical signaling mechanism and little is known about the parameters controlling the ingestion process itself. The information that is available comes mainly from morphological observation of the ingestion process. Burnside (1976) first suggested a role for the RPE cytoskeleton in the ingestion phase of phagocytosis. She reported evidence (transmission electron microscopy) that RPE microvilli, which ensheath OS, contain microfilaments. Furthermore, RPE processes surrounding a newly ingested packet of shed OS discs near the apical RPE cell surface were shown to contain microfilaments. Recently, Besharse (1984) has further documented the role of the cytoskeleton in the formation of special processes (distinct from microvilli) which form at the apical surface of aspartate-treated RPE (*in vitro* eyecup preparation of *Xenopus laevis*) and appear to play a role in shedding and phagocytosis of the OS tip.

McLaughlin *et al.* (1983) examined by scanning electron microscopy (SEM) the morphology of microvilli in surface replicas of RPE cells (RPE explants) in the process of ingesting latex spheres. As the beads were engulfed, microvilli

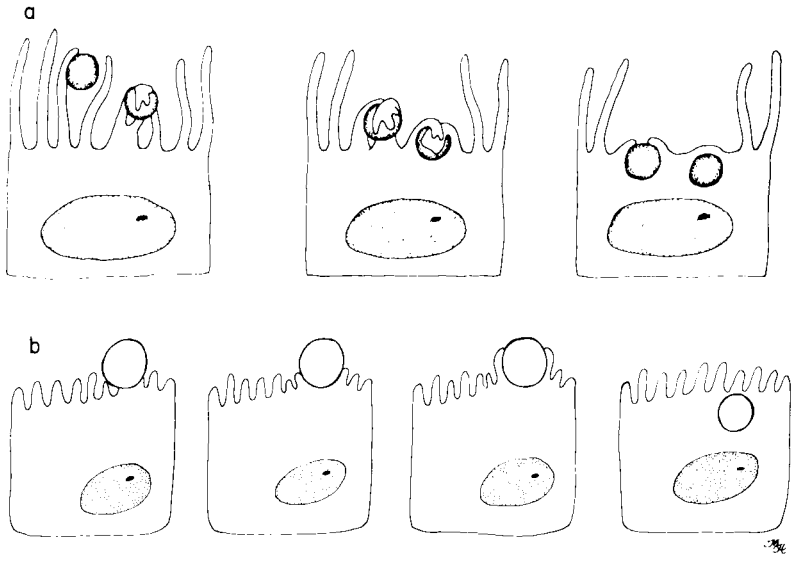


FIG. 4. Ingestion of particles by rat RPE cells. (a) Surface replicas of rat RPE ingesting latex beads in explant culture, showing "Venus' flytrap array" of microvilli (McLaughlin *et al.*, 1983). (b) Rat RPE cells ingesting isolated ROS in cell culture, showing saucer-shaped cell surface structure (Chaitin and Hall, 1983).

flattened and formed a "Venus' flytrap array" around the latex sphere. This is in contrast to the report of Chaitin and Hall (1983b), who observed by SEM a saucer-shaped structure forming on the surface of cultured rat RPE cells during the ingestion of isolated ROS (Fig. 4). The difference may be due to use of cultured cells vs RPE cell explants or to differences in preservation of the surface obtained by surface replica vs standard SEM sample preparation. However, it may also reflect a difference inherent in the mechanisms by which latex and OS are ingested (Philp and Bernstein, 1981).

Future research will no doubt delve into the biochemical control mechanisms that are responsible for the mobilization of the RPE cytoskeleton for the ingestion phase of phagocytosis. It is possible that these mechanisms are the same for both specific and nonspecific phagocytosis, but that they can be triggered either by receptor-mediated transmembrane signaling or by charge-related disturbances of the plasma membrane.

3. Degradation of Phagosome Contents

a. Specialized Hydrolase Activities? Studies of the digestion phase of phagocytosis in the retinal pigment epithelium began with attempts to identify

hydrolytic enzymes in subcellular organelles, both by electron microscopic histochemistry (Ishikawa and Yamada, 1970; Hollyfield and Ward, 1974c) and by assay of subcellular fractions (Berman, 1971; Swartz and Mitchell, 1973; Hayasaka *et al.*, 1975, 1977; Rothman *et al.*, 1976). Many of the hydrolytic enzymes that have been identified in lysosomes of other tissues have also been found in the RPE. (This work was reviewed in 1979 by Berman.)

More recently, work in this line has been directed toward identifying systems of hydrolases that might be specially adapted for degrading the rather unique protein and lipid constituents of photoreceptor outer segments. The possible existence of such a specialized degradative machinery in the RPE was proposed by Feeney (1973). Regan *et al.* (1980) showed that rhodopsin in disc membranes is degraded by a protease with cathepsin D-like activity, which copurifies with a "dense" (special?) lysosomal fraction in sucrose gradients. Zimmerman *et al.* (1983) isolated lysosomes from bovine RPE, liver, and retina. They compared the relative hydrolytic activities of these lysosomal fractions, using ROS membranes and hemoglobin as substrates. Whereas lysosomes from liver and retina degraded rhodopsin in ROS and hemoglobin at the same rate, lysosomes from the RPE degraded ROS-bound rhodopsin at twice the rate of hemoglobin. Therefore, lysosomes from the RPE seem to be specially adapted for the degradation of rhodopsin in ROS membranes. The RPE lysosomal fraction also displayed greater phospholipase A_1 plus A_2 activities when compared to lysosomes from the liver and retina. The authors tentatively proposed that it is the combination of high phospholipase A_1 plus A_2 activity together with cathepsin D (Regan *et al.*, 1980) that is responsible for the enhanced ability of RPE lysosomes to degrade membrane-bound proteins (e.g., rhodopsin).

Hara *et al.* (1983) have identified a rhodopsin-cleaving enzyme in homogenates of bovine RPE. This protease activity degraded purified rhodopsin more readily than bovine serum albumin (BSA). The specific activity of the rhodopsin-cleaving enzyme was greatest in the RPE when compared to other tissues (ROS, liver, and spleen). Whether the rhodopsin-cleaving activity is due to a specialized protease or the cathepsin D activity reported by Regan *et al.* (1980) is not clear. This same research group analyzed the carbohydrate structure of an acidic glycopeptide that was produced by the action of the rhodopsin-cleaving enzymes in RPE cell homogenates upon purified rhodopsin (Kean *et al.*, 1983). The oligosaccharide chains in the glycopeptide fragment were identical to those in intact purified rhodopsin. The authors speculated that the carbohydrate structure of rhodopsin is perhaps conserved during degradation. However, the activities of various sugar hydrolases is likely to be quite different in RPE cell homogenates than in the special environment of the phagolysosomal compartment. Perhaps these questions will be better answered by analyzing the contents of purified rhodopsin-containing phagolysosomes.

b. Role of Microtubules. Burnside (1976) has proposed that microtubules might be involved in movement of phagosomes through the RPE cytoplasm and fusion of phagosomes with lysosomes. Recently, Herman and Steinberg (1982a,b) reported the effects of colchicine on the movement and degradation of phagosomes in the tapetal RPE of opossum. They observed that soon after ingestion, phagosomes were moved from the apical to the basal portion of the RPE cell. This movement was blocked by treatment of the tissue with colchicine, suggesting that phagosome movement is mediated by microtubules. The authors also reported that phagosomes were degraded only within the basal half of the RPE cell. Acid phosphatase activity also appeared to be localized in the basal RPE. Phagosomes that were trapped near the apical surface of RPE cells by colchicine treatment did not appear to undergo degradation. In summary, the study showed that fusion of phagosomes with lysosomes is mediated by microtubules and occurs only in the basal portion of opossum RPE.

c. Lipofuscin Formation. Recent evidence suggests that the oxidative state of the retina (specifically, photoreceptor outer segments) can ultimately affect the ability of the pigment epithelium to degrade phagocytized OS membranes. The predominant fatty acid in ROS membranes is the polyunsaturated 22:6 docosahexaenoate (Anderson and Risk, 1974; Berman *et al.*, 1974). Polyunsaturated lipids are susceptible to cross-linking in the presence of free radicals that may, in the retina, be produced by light or high oxygen tension. Chio *et al.* (1969) demonstrated that autooxidative damage to polyunsaturated fatty acids in membranes contributes to formation of the autofluorescent age pigment, lipofuscin. It has been proposed that autooxidized, cross-linked macromolecules in photoreceptor membranes cannot be completely degraded by the RPE's phagolysosomal system. In the aging RPE, undigestible contents of phagolysosomes form residual bodies, which under some circumstances, have the fluorescent property of lipofuscin (for a review, see Feeney and Berman, 1976).

Vitamin E is present in photoreceptor OS (Dilley and McConnell, 1970) and provides protection against oxidative damage to OS membranes *in vitro* (Farnsworth and Dratz, 1976). There is evidence that vitamin E may also prevent oxidation of vitamin A (Robison *et al.*, 1980) in photoreceptor membranes. In 1974, Hayes reported that monkeys fed vitamin E-deficient diets developed disruption of disc membrane structure in photoreceptor OS and degeneration of photoreceptor cells in the macular area only. Additionally, the RPE of the experimental animals accumulated large quantities of residual bodies. In studies of the interaction of vitamins E and A in maintaining retinal health, Robison *et al.* (1979) observed a similar disruption of disc structure and accumulation of residual bodies in the RPE of rats fed a vitamin E-deficient diet. These authors have also presented evidence that the fluorescent property of lipofuscin granules is contrib-

uted by the vitamin A (retinol) content of ingested OS membranes (Robison *et al.*, 1980). Thus, it appears that vitamin E deficiency can accelerate formation of lipofuscin (a normal effect of aging) in the RPE of relatively young animals.

Katz *et al.* (1978) have taken these studies further by attempting to create a more extreme antioxidant deficiency in rats. Young rats were fed diets not only deficient in vitamin E (which blocks autooxidation of polyunsaturated lipids directly) but also lacking selenium, sulfur-containing amino acids, and chromium (dietary components that are necessary for proper functioning of the glutathione peroxidase system for converting oxidized lipids back to hydroxy fatty acids). After 32 weeks on the deficient diet, retinal vitamin E levels were significantly reduced, and the RPE accumulated autofluorescent lipofuscin pigment. Control rats of the same age lacked the fluorescent pigment.

Recently, Katz *et al.* (1982) carried out a morphological study of the eyes of rats fed the deficient diet to determine if they developed other signs of aging besides the accumulation of lipofuscin. Young albino rats fed the deficient diet developed a buildup of residual bodies with a concomitant accumulation of fluorescent material in the RPE. The experimental rats also developed some classic pathological changes found in aging eyes, such as irregularity of RPE cell height, the separation of some RPE cells from Bruch's membrane, and the separation of the RPE from the photoreceptor cell layer. The last change occurred especially in the macular region of the retina. Control animals of the same age did not display any of this pathology. These diet-induced alterations in the rat retina and RPE bear a striking similarity to changes that accompany macular disease in the aging human eye (Hogan, 1972; Sarks, 1976; Green and Key, 1977).

Katz *et al.* (1982) proposed that the accumulation of lipofuscin in aging RPE may be due to a gradual failure, with age, of the retina's mechanisms for dealing with oxidized lipids. Lipofuscin accumulation in the RPE is associated with some hereditary retinal degenerative diseases, also (Kolb and Gouras, 1974; Aguirre and Laties, 1976). Katz *et al.* suggested that in the future, it may be possible to slow or stop the progress of some retinal degenerative diseases by stimulating or augmenting the retina's natural antioxidative protection mechanisms.

Although lipofuscin accumulation is associated with the occurrence of senile macular degeneration, a cause and effect relationship between the two has not been established. It has been proposed that when the amount of lipofuscin-free cytoplasmic space falls below a critical point, the aging RPE can no longer carry out many of the functions (e.g., transcellular transport of nutrients and wastes and phagocytosis) that are essential to the health of the retina. However, since all aging human RPE accumulate lipofuscin (Feeney *et al.*, 1963) but not all aging human eyes develop senile macular degeneration, the etiology of the disease must be more complex. The painstaking characterization of age-related auto-

fluorescent pigments in human RPE that has been carried out by Feeney *et al.* (1978, 1984) may ultimately shed more light on the relationship, if any exists, between incomplete digestion of phagosomes and retinal degeneration.

Feeney *et al.* (1978) pointed out the distinction between lipofuscin granules (residual bodies or incompletely digested phagosomes) and melanolipofuscin (formed by the fusion of melanin wth lipofuscin), both of which have autofluorescent properties. A third nonfluorescent particle, resulting from the fusion of melanin with lysosomes (melanolysosomes), was also identified. This study also showed that lipofuscin present in the RPE of individuals over 50 years of age may contain more extensively cross-linked macromolecules than lipofuscin in younger RPE. More recently, Feeney-Burns *et al.* (1984) carried out a computer-assisted morphometric analysis of postmortem human eyes spanning 10 decades of life. The amount of lipofuscin, complex granules, melanin, and free cytoplasmic space was quantitated in macular, equatorial, and peripheral RPE. The results suggest that, while the numbers of lipofuscin and complex granules (melanolipofuscin and melanolysosomes) increase with increasing age, the number of normal melanin granules decreases. The authors proposed that the loss of melanin could make the retina more susceptible to free radical formation from light. Macular areas of the RPE contained the most complex granules, while lipofuscin accumulation was more evenly distributed in macular, equatorial, and peripheral RPE. The study also revealed that lipofuscin accumulation is well under way in the second decade of life. This information reinforces the importance of characterizing both kinds and quantities of RPE pigment granules when examining the relationship between RPE pigment content and disease.

B. Transport

1. Retinol Transport and Storage

This topic will be summarized briefly due to the recent proliferation of very thorough reviews (Goodman and Blaner, 1984; Goodman, 1984; Chytil and Ong, 1984; Bridges *et al.*, 1983; Chader *et al.*, 1983; Chader, 1982). The RPE cell acts as a mediator in the transport of dietary vitamin A (retinol) from the liver to the photoreceptor cells, where (after being converted to 11-*cis*-retinaldehyde) it becomes incorporated into the light-capturing pigment, rhodopsin. Retinoids are obtained from the diet, either in the form of β-carotene from yellow vegetables or directly from meats. Dietary retinol is stored in the liver, esterified to fatty acids, until it is released into the bloodstream in complex with a carrier protein (synthesized in the liver) called serum (or plasma) retinol-binding protein (RBP) (Muto *et al.*, 1972; Hirosawa and Yamada, 1973; Smith *et al.*, 1973). In complex with transthyretin (previously called prealbumin), serum RBP trans-

ports all-*trans*-retinol to various tissues in the body, including the RPE (Kanai *et al.*, 1968).

The retinol–RBP complex leaves the blood supply of the choriocapillaries via the diaphragmed fenestrae of the endothelial cells, travels by diffusion through Bruch's membrane, and apparently binds to a specific receptor at the lateral and basal plasma membranes of the RPE cells (Heller, 1975; Bok and Heller, 1976). There is evidence to suggest that RBP facilitates transport of retinol across the plasma membrane into the RPE cell cytoplasm without entering the RPE cell directly (Maraini and Gozzoli, 1975; Chen and Heller, 1977). Presumably, RBP returns to the bloodstream without transthyretin and is lost by filtration through the kidney.

What happens to the retinol once it enters the RPE cytoplasm has not yet been completely worked out. It is known that retinol, normally very toxic to cells due to lytic action on membranes, is stored in RPE cells in the form of relatively inert fatty acid esters (Bridges, 1975; Alvarez *et al.*, 1980; Bridges *et al.*, 1982). In invertebrate RPE cells and some vertebrates, these esters accumulate in oil droplets (Robison and Kuwabara, 1977). There is evidence that in mammalian RPE cells, fatty acid esters of retinol are stored in "chylomicron-like" protein–lipid complexes (Heller, 1976; Heller and Bok, 1976). Similar complexes, displaying retinol transfer and esterase activity, have been isolated from rat liver cytosol (Chen *et al.*, 1981). Additionally, there are the cytosolic retinoid-binding proteins: CRBP, which binds all-*trans*-retinol (Wiggert and Chader, 1975; Saari *et al.*, 1977), and CRALBP, which binds 11-*cis*-retinal (Saari *et al.*, 1982). It has been proposed that the cytosolic retinoid-binding proteins shuttle retinol and retinal from the basal side to the apical side of the RPE cell and back. To date, the process by which retinol is transferred across the apical plasma membrane to the subretinal space is unknown. Evidence of a RPE plasma membrane-associated retinoid-binding protein has been reported by Maraini *et al.* (1977). The interphotoreceptor retinoid-binding protein (IRBP) may play a role in shuttling retinol across the subretinal space to the photoreceptors (for regeneration of bleached rhodopsin and synthesis of new rhodopsin) and back to the RPE cell (after a strong bleach of rhodopsin) (Bridges, 1976; Chader *et al.*, 1983).

2. Na^+,K^+-ATPase, Water Transport, and Retinal Adhesion

Na^+,K^+-ATPase is confined to the apical plasma membrane of the RPE cell (that which faces the retina). Indirect evidence for the asymmetric distribution of Na^+,K^+-ATPase in the RPE cell plasma membrane came from the observation made by Steinberg and Miller (1973) that ouabain, when applied to the apical

RPE cell surface, altered membrane potentials (depolarized the apical plasma membrane). This effect was not seen when ouabain was applied to the basal (choroidal) cell surface. Additionally, net flux of Na^+ from the choroidal to retinal side of the RPE was inhibited by application of ouabain to the apical cell surface (Miller and Steinberg, 1977). Flux of K^+ from the retinal to choroidal surface was also inhibited (Miller *et al.*, 1978). Ouabain did not affect transcellular Na^+ and K^+ fluxes when applied to the basal surface of the RPE.

More direct evidence has confirmed the localization of Na^+,K^+-ATPase on the apical RPE cell plasma membrane. Ostwald and Steinberg (1980) microdissected the RPE cell layer in frog and assayed both apical and basal portions for Na^+,K^+-ATPase activity. Seventy-five to 100% of total cellular Na^+,K^+-ATPase activity was consistently found in the apical portion of the RPE cell layer (80% of the total cellular activity was associated with pelletable membrane fractions). Additionally, Bok (1982) has demonstrated by electron microscopic autoradiography that [^{3}H]ouabain binds predominantly to the apical plasma membrane of bullfrog RPE cells.

Current theory proposes that the RPE cell contributes to retinal adhesion, due to its ability to actively transport ions (and accompanying water) out of the subretinal space. Removal of water from the interphotoreceptor matrix is essential for keeping the space between the RPE cell and photoreceptor outer segment plasma membranes very thin, thus promoting retinal adhesion. This activity also influences the overall composition of the interphotoreceptor matrix in ways that could influence communication between the RPE cell and photoreceptor cell (Miller *et al.*, 1978).

The ability of the RPE to remove water from the subretinal space is dramatically illustrated by the phenomenon of subretinal fluid resorption. Net flow of water from the subretinal space to the choroid occurs relatively quickly and appears to be an energy-dependent process (Marmor *et al.*, 1980; Miller *et al.*, 1982; Negi and Marmor, 1983). The theory to explain this fluid flux proposes that ion pumps, located in the RPE cell plasma membrane, are responsible for active transport of ions (and accompanying water) from the subretinal space to the basal surface of the RPE. The asymmetric distribution of Na^+,K^+-ATPase in the RPE cell apical plasma membrane is opposite to that found in other epithelia (for example, Stirling, 1972; Stirling *et al.*, 1973). Therefore, Na^+,K^+-ATPase is unlikely to be responsible for the net movement of fluid from the retinal to the choroidal side of the RPE. In support of this contention, Miller *et al.* (1982) have found that the net fluid movement across the RPE from the retina to the choroid is not affected by ouabain. Transport of ions and fluid across the RPE is inhibited by elevated intracellular concentrations of cAMP, suggesting that cAMP may modulate the rate of ion and water transport (Miller *et al.*, 1982; Miller and Farber, 1984; Hughes *et al.*, 1984).

3. Taurine

Taurine is a sulfur-containing amino acid that is actively taken up and concentrated by the retina (Lake *et al.*, 1975; Kennedy and Voaden, 1976). Although the physiological role of taurine in the retina is currently unknown, it seems to be essential for retinal function. In the cat, the photoreceptor cells are dependent upon dietary taurine (Schmidt *et al.*, 1976, 1977; Berson *et al.*, 1976). Autoradiographic studies suggest a role for the RPE in delivery of dietary taurine to the photoreceptor cell layer (Young, 1969). Additionally, there has been a report that release of taurine into the subretinal space is triggered by light (Pasantes-Morales, 1973). Therefore, it is of interest to determine what role is played by the RPE in mediating the flux of taurine to and from the retina.

Miller and Steinberg (1976) demonstrated that the RPE in frogs is capable of mediating a net flux of taurine from the retina to the choroid. Taurine flux is inhibited by ouabain (when applied to the apical cell surface), suggesting that transport of taurine from the apical to basal surface of the RPE is dependent upon Na^+,K^+-ATPase activity. This conclusion was further supported by the observation that taurine flux is affected by extracellular Na^+ and K^+ concentrations (Miller and Steinberg, 1979). Ostwald and Steinberg (1981) have studied various components of taurine flux across the RPE (inward and outward fluxes at both apical and basal plasma membranes). The results of this work supported previous evidence that taurine is cotransported with Na^+ (down the Na^+ gradient that is created by the activity of Na^+,K^+-ATPase) through the apical plasma membrane into the RPE cell. Ostwald and Steinberg presented additional data suggesting that flux of taurine into the RPE through the basal plasma membrane occurs via a K^+-dependent mechanism.

Net fluxes of other ions (Ca^{2+}, HCO_3^-, Cl^-) across the RPE have been observed, but these systems are not well characterized (for a summary see Steinberg and Miller, 1979).

C. Extracellular Matrix Biosynthesis and Composition

The RPE cell secretes components for two highly specialized extracellular matrices: the interphotoreceptor matrix at the apical cell surface and a basement membrane at the basal cell surface.

1. The Interphotoreceptor Matrix

The interphotoreceptor matrix (IPM) normally fills the subretinal space, extending from the apical surface of the RPE to the outer limiting membrane of the

neural retina. As was mentioned in Section I, the plasma membranes of RPE cells and photoreceptor cell outer segments do not have any points of contact. Therefore, molecules (nutrients and wastes) that are exchanged by the RPE and photoreceptors must pass through the IPM. It is generally believed now that the IPM is not simply an inert space-filling substance, but that the molecules comprising the IPM play a role in facilitating exchange of materials between the RPE and photoreceptors. Additionally, the IPM may contribute slightly to the overall mechanism of retinal adhesion. Thus, there has been intermittent interest in analyzing the chemical composition of the IPM.

Early theories to explain retinal adhesion placed great emphasis upon the adhesive properties of the IPM. Therefore, initial studies of the composition of the IPM focused upon the "sticky" glycosaminoglycan components. Berman and Bach carried out detailed analyses of the mucopolysaccharides that could be gently rinsed from isolated retinas in saline solution (Berman, 1965; Berman and Bach, 1968; Bach and Berman, 1971a,b). This work culminated with the identification and characterization of various types and quantities of glycosaminoglycans in the IPM (mainly bovine). No particular biological function for any of these molecules was uncovered. New information about the role played by glycoproteins in cell recognition and adhesion has recently stimulated interest in purification and characterization of glycoprotein components of the IPM. These studies have been hampered by the technical difficulty of extracting IPM that is not contaminated by proteins from damaged photoreceptor or RPE cells.

Adler and Severin (1981) analyzed protein and glycoprotein components of bovine IPM by one-dimensional SDS gel electrophoresis. The authors attempted to rule out possible contamination by proteins from other tissues by comparing the protein pattern of the IPM with that of vitreous, serum, homogenized ROS, and homogenized RPE. The major protein components of their IPM preparation appeared to be unique (based upon R_f in SDS gels) when compared to those of surrounding tissues. Eleven polypeptides were detected, two of which (140,000 and 90,000 MW) were glycoproteins. In a subsequent report, Adler and Klucznik (1982) showed that the 140,000-MW glycoprotein could be purified by affinity chromotography on concanavalin A–Sepharose. Soon afterward, the 140,000-MW protein was demonstrated to be a subunit of a 7 S retinoid-binding protein (RBP), first discovered by Wiggert *et al.* (1976) in "retinal homogenates." The IPM localization of the 7 S RBP was reported by Adler and Martin (1982) in cattle and by Lai *et al.* (1982) in rabbits. The native molecule has an estimated MW of 290,000 (Pfeffer *et al.*, 1983) and has been named IRBP (Lai *et al.*, 1982). IRBP contains a greater quantity of endogenous retinol when purified from light-adapted retinas than when purified from dark-adapted retinas (Wiggert *et al.*, 1979; Adler and Martin, 1982; Lai *et al.*, 1982). This observation suggests that IRBP may bind retinol that is released from bleached photoreceptors and mediate the well-known

transport of retinol to the RPE (Dowling, 1960). It has been demonstrated that IRBP is synthesized by the retina in monkey (Wiggert *et al.*, 1984), *Xenopus* (Rayborn *et al.*, 1984), human (Hollyfield *et al.*, 1984), and rat (Gonzalez-Fernandez *et al.*, 1984) tissues.

As yet, no specific functions have been assigned to any other IPM proteins. Adler (1982) detected a 17,000-MW RBP in her preparations of bovine IPM. This IPM preparation was obtained by rinsing the apical surface of the RPE and retinas, following separation of the two tissue layers. Pfeffer *et al.* (1983) subsequently developed a method for rinsing IPM from the subretinal space without mechanically detaching the retina from the RPE. When this procedure (which is less likely to result in breakage of RPE or photoreceptor cells) was used, IRBP was the only retinoid-binding protein that could be found in the extracted IPM. These authors argued that the 17,000-MW RBP reported by Adler was CRBP (cytosolic RBP) which leaked out of damaged RPE into the IPM. However, species differences (cow vs monkey) may also have contributed to the discrepancy.

There has been some effort to determine which IPM components are synthesized by the RPE. Berman (1964) showed that freshly isolated bovine RPE cells, incubated in the presence of [^{14}C]glucose, secreted labeled mucopolysaccharides and glycoproteins. Adler (1981) compared the glycoproteins secreted by freshly isolated bovine RPE and retinas during brief incubation in the presence of [^{3}H]glucosamine or [^{3}H]leucine. She found that 6 of the 11 putative IPM proteins were synthesized by the RPE. A difficulty with these studies is the ever-present question of tissue integrity. Further information about the contribution of RPE to the IPM may come from study of the secretion of IPM components by isolated RPE cells cultured *in vitro* (see Section III,B,2).

The discovery of a protein component of the IPM with seemingly real biological activity is only the beginning of an exciting new chapter of research into mechanisms by which the RPE and photoreceptor cells communicate. Future research efforts will, no doubt, be directed toward determining if other IPM proteins display enzymatic, adhesive, or ligand-carrying activities. The RCS (*rdy*) rat may prove to be a useful tool for identifying a biological function for one or more IPM proteins and/or glycosaminoglycans. LaVail *et al.* (1981) has shown that the IPM of the RCS rat is deficient in colloidal iron-staining component(s) at postnatal day 12 (the time at which photoreceptor shedding begins, but before any morphologically observable degeneration of the retina has occurred). These results suggest that changes in the IPM of the RCS rat may be a contributory cause (rather than an ultimate effect) of retinal degeneration. Such changes may either directly or indirectly interfere with the ability of RPE to phagocytize OS membranes. It will be of interest to determine which components of the RCS rat IPM are altered, and if these are synthesized by the RPE or retina.

2. Basement Membrane Components

As was mentioned in Section I, Bruch's membrane consists of two basement membranes (one that is elaborated by the RPE and another that is synthesized by the choriocapillaris endothelium) that are separated from each other by a loose meshwork of structural proteins. Recent evidence suggests that this structure has molecular-sieving properties and that it may help to regulate the flow of materials from the choroidal circulation to the RPE (see Section II,D,2) (Lyda *et al.*, 1957; Pino and Essner, 1981; Pino *et al.*, 1982a). Furthermore, firm attachment of the RPE must surely be influenced by the composition of its basement membrane. Some pathological conditions involve changes in the RPE basement membrane (Farkas *et al.*, 1971). In order to identify the molecular basis of these pathological changes, it will be necessary to characterize the molecular structure of normal RPE basement membrane.

One approach to this problem has been the identification of anionic-binding sites in Bruch's membrane of the rat (Pino *et al.*, 1982b). By utilizing the cationic probes ruthenium red and native lysozyme, the authors demonstrated that anionic sites are prevalent on the basement membranes of both the RPE and choriocapillaris endothelium (EN). Heparinase preferentially removed anionic sites from the RPE basement membrane, suggesting that heparin is a major constituent. Anionic sites in the EN basement membrane were more sensitive to the activity of chondroitinase. These experiments demonstrated a distinct difference in the chemical compositions of the basement membranes of the RPE and EN.

Robey and Newsome (1983) analyzed the proteoglycan composition of Bruch's membrane from monkey eyes and found that heparin sulfate-containing proteoglycans were the most abundant. Smaller amounts of chondroitin and/or dermatin sulfate and hyaluronic acid were also found. Biosynthesis of these proteoglycans was examined by incubating eye organ cultures in media containing [^{3}H]glucosamine and $Na_2{}^{35}SO_4$. The labeled proteoglycans that could be extracted with 4 *M* guanidine–HCl ranged from 100,000 to 150,000 in MW. Removal of the protein component by treatment with papain yielded a 44,000-MW glycosaminoglycan structure. The newly synthesized proteoglycans were 65% chondroitin and/or dermatin sulfate and only 35% heparin sulfate. If the glycosaminoglycan composition of Bruch's membrane of the monkey is similar to that of the rat, then these results imply that the basement membrane of the EN turns over more quickly than that of the RPE. Preliminary studies by the same research group have revealed changes in Bruch's membrane proteoglycans, associated with aging and retinitis pigmentosa (Hewitt *et al.*, 1984). Analysis of the composition of the RPE basement membrane may be facilitated by growth of pure, highly differentiated RPE cells in tissue culture (see Section III,B,2).

D. Blood–Tissue Barriers—the Blood–Retinal Barrier

1. The Junctional Complex of the RPE

The junctional complex (or terminal bar structure) contributes to the ability of RPE to regulate the flow of molecules from the choroidal circulation to the retina in two ways. First, it prevents diffusion of most substances between adjacent RPE cells. Second, the junctional complex of epithelial cells, in general, may be responsible for maintaining the "macromolecular differentiation" of the apical and basal plasma membranes (Pisam and Ripoche, 1976), which is essential for epithelial cell transport activities. Breakdown of the RPE component of the blood–retinal barrier is associated with many retinal diseases (Cunha-Vaz, 1976, 1979). The RCS rat has proven to be a useful model for studying the pathology of the RPE cell junctional complex. This animal, which bears an inherited retinal degeneration, experiences a loss of tight junction integrity (evidenced by increased permeablity to extracellular tracers) (Bok in LaVail, 1979). Caldwell *et al.* (1982) carried out freeze-fracture analysis of the structure of tight junctions in RCS rats at various lengths of time after birth. They observed morphological changes in the junctional complex of RPE cells as early as 3 weeks postnatal (while photoreceptor degeneration is still in progress). At later stages of retinal degeneration, tight junctional strands break and form an unordered array; gap junctions are no longer concentrated in the junctional complex area, but migrate to other areas of the RPE cell plasma membrane. RPE cell tight junctions also become permeable to horseradish peroxidase and lanthanum tracers as early as 3 weeks (pink-eyed animals) postnatal, just as degenerating photoreceptors begin to appear (Caldwell and McLaughlin, 1983).

Owaribe and Masuda (1982) have isolated cirumferential microfilament bundles from chicken RPE cells. The authors believe that these bundles are associated with the zonula adherens of the cells. The isolated filament bundles were composed primarily of three proteins having molecular weights of 200,000, 55,000, and 42,000. In the future, similar techniques may be used to study the biochemical changes that underly the morphological changes observed in RPE cell junctional complex pathology.

2. Filtration by the Choriocapillaris Endothelium and Bruch's Membrane

Historically, the blood–retinal barrier at the level of the choroidal circulation has been attributed solely to the RPE junctional complex. However, studies of the relative permeabilities of Bruch's membrane and choriocapillaris endothelium in rats, to various proteins, has shown that there is a distinct size cutoff for free passage of macromolecules across these structures (Lyda, 1957). A more

recent study was conducted by injecting various-sized heme proteins into the bloodstreams of rats, and then examining the choriocapillaris, Bruch's membrane, and RPE cell layer for evidence of penetration of each probe (Pino and Essner, 1981; Pino *et al.*, 1982a). Horseradish peroxidase (MW of 40,000, radius of 30 Å) passed through Bruch's membrane, and penetrated the basal infoldings of the RPE cell plasma membrane. Hemoglobin (MW 68,000, 32 Å diameter) only passed partially through Bruch's membrane, while catalase (MW 240,000, diameter 52 Å) failed to pass through the choriocapillaris endothelial cells. Controls demonstrated that these observations were most likely due to the size rather than the net charge of the probes chosen.

These results showed that the rat, at least, is an exception to the rule about unlimited permeability of the choriocapillaris and Bruch's membrane. The authors make an interesting speculation concerning the possible advantage of size selectivity of the fenestrated endothelium of the choriocapillaris and Bruch's membrane. They propose that the size selectivity could function to regulate the flow of carrier proteins from the blood to the RPE. To illustrate their hypothesis, the authors chose the example of transport of dietary retinol from the blood to the RPE. The retinol–RBP–transthyretin complex has a radius of 37 Å, and thus would be retarded by Bruch's membrane in the rat. Therefore, only retinol–RBP that dissociates from transthyretin would be able to cross Bruch's membrane and reach the basal surface of the RPE. In this way, the selectivity of the diaphragmed fenestrae of the choriocapillaris endothelium and Bruch's membrane could serve to regulate the supply of retinol–RBP to the RPE. A similar mechanism for selective filtration of blood components by the kidney was put forth by Farquhar (1975). Therefore, Pino *et al.* have suggested that the RPE–Bruch's membrane–choriocapillaris endothelium be considered a complex structural unit that is responsible for selective filtration of blood components that ultimately reach the retina.

III. Use of Cultured RPE Cells to Study Cellular Processes

A. Update on Culturing RPE Cells

The ability to culture pure colonies of RPE cells has opened new possibilities for the study of the aforementioned cellular processes on a molecular level. Primary cultures of pure RPE cells from rat (Edwards, 1977a; Mayerson *et al.*, 1984), human (Albert *et al.*, 1972; Mannagh *et al.*, 1973; Flood *et al.*, 1980; DelMonte and Maumenee, 1980; Aronson, 1983; Boulton *et al.*, 1983; Pfeffer and Newsome, 1983), monkey (Pfeffer and Newsome, 1983), swine (Francois *et al.*, 1971; Dorey *et al.*, 1983), cat (Stramm *et al.*, 1983), cattle (Basu *et al.*,

1983), and chick (Newsome and Kenyon, 1973; Newsome *et al.*, 1974) appear to retain a morphologically differentiated state *in vitro*. Cultured RPE cells have been reported to elaborate microvilli, basal infoldings, intercellular junctions, and pigment granules. The morphological appearance (i.e., maintenance of differentiation) of cultured RPE cells has been improved by growth of the cells on collagen matrices (Hall and Quon, 1981) and by utilization of defined growth medium (Pfeffer and Newsome, 1983). More importantly, RPE cells continue to express some specialized biological activities *in vitro*. There have been reports that cultured RPE cells continue to melanize under certain conditions (Newsome *et al.*, 1974; Aronson, 1983; Dorey *et al.*, 1983). It has been observed that confluent monolayers of cultured RPE cells form "domes" (patches of monolayer which are raised up from the culture dish), suggesting that the cells continue to carry out unidirectional transport of ions and water in cell culture (Pfeffer and Newsome, 1983; Aronson, 1983). [In support of this possibility, Misfeldt *et al.* (1976) have demonstrated that cultured intestinal epithelial cells are capable of unidirectional movement of water to the basal cell surface.]

B. Processes That Are Being Studied in Cell Culture

1. Phagocytosis

Another activity of interest which RPE cells continue to express *in vitro* is specific phagocytosis of OS membranes. This process is being studied quite extensively in cultured rat and chick RPE cells.

a. Specificity. RPE cells display selectivity with regard to particle binding and phagocytosis *in vitro,* as they do *in vivo*. Effron *et al.* (1981) examined the ability of cultured RPE from normal and dystrophic (RCS) rats to phagocytize synthetic phospholipid vesicles and latex beads. They found that dystrophic RPE is capable of phagocytizing latex and phospholipid vesicles containing phosphatidylinositol and phosphatidylserine. However, dystrophic cells reject vesicles containing phosphatidylcholine and phosphatidylethanolamine, the most abundant phospholipids in bovine ROS (Anderson and Maude, 1970). In contrast, normal RPE phagocytize phospholipid vesicles of all composition. These data suggest that RPE cells continue to express *in vitro* the capacity for both "nonspecific" phagocytosis (e.g., of latex) and the very selective phagocytosis of OS-like membranes, defective in the dystrophic RPE (Edwards and Szamier, 1977) (see Section II,A,1,a).

Mayerson and Hall (1983) have performed quantitative analyses of the ability of cultured normal rat RPE to bind and ingest various non-OS particles as compared to OS. They found that cultured RPE cells bind (to the cell surface)

25–44 times more rat ROS than red blood cells, 30 times more OS than algae (*Botrycoccus brauni*), and 5 times more rod OS than yeast (*Saccharomyces cerevisiae*), bacteria (*Staphylococcus aureus*), or latex. Furthermore, very few algae or bacteria were ingested as compared to ROS (<1% of the total). Yeast were ingested to some extent (29% of the total), possibly due to the high concentration of mannose in the cell coat. Mannose has been implicated as a possible recognition component in the phagocytosis of ROS (Philp and Bernstein, 1980; Heath and Basinger, 1983) (see Section II,A,1,b).

b. The Dystrophic Rat RPE. In cell culture, dystrophic RPE cells continue to express (as *in vivo*) a greatly reduced capacity for phagocytosis of photoreceptor OS membranes (Edwards and Szamier, 1977). By employing a double immunofluorescent staining procedure, which allows differential visualization of externally bound vs already ingested ROS, Chaitin and Hall (1983a) found that cultured dystrophic RPE cells carry out the first stage of phagocytosis normally. The dystrophic cells bind purified rat ROS to the plasma membrane as avidly as do normal RPE cells. Therefore, the genetic lesion in the dystrophic RPE must affect the ingestion phase of phagocytosis.

In macrophages, the uptake of particles by phagocytosis requires a mobilization of cytoskeletal elements in order to form pseudopodia which engulf the particle. Burnside and Laties (1976) proposed that the actin filaments which they found in the apical processes of human and monkey RPE cells might play a role in the phagocytic ingestion of shed OS membranes. Chaitin and Hall (1983b) investigated the structure of microfilaments in cultured normal and dystrophic RPE cells by immunofluorescent staining of the cells with anti-actin antibodies. Within the limitations of the technique, the results showed that dystrophic RPE cells form normal microfilament arrays. Furthermore, dystrophic RPE cells form an actin "feltwork" cup structure beneath the plasma membrane at the site of ROS binding, as do the normal cells. [Similarly, Irons and Kalnin (1984) examined the microtubule arrays of normal and dystrophic rat RPE *in vitro* and found essentially no differences that would be discernible by immunofluorescent staining techniques.] The authors concluded that the dystrophic RPE cells are able at least to carry out the initial cytoskeletal reorganization required for phagocytosis of ROS. Therefore, the dystrophic RPE cell may be deficient in some component of a transmembrane-signaling mechanism which initiates ingestion of plasma membrane-bound OS. This defective signaling mechanism might involve an internal second messenger and enzymatic amplification. Alternatively, the dystrophic RPE cell may lack or carry a defective version of a plasma membrane receptor needed for initiation of a transmembrane signal.

Further research is required to identify plasma membrane components involved in specific binding and phagocytosis of OS by cultured RPE cells. Cohen and Nir (1983) have carried out a quantitative analysis of anionic sites on the

surfaces of normal and dystrophic rat RPE cells *in vitro*. Anionic sites were labeled with cationized ferritin and quantitated in electron micrographs. RPE cells from the RCS rat contain 20% more cationized ferritin-binding sites at the apical cell surface than do RPE from normal rats. Some, but not all, of the excess anionic sites may be contributed by sialic acid. The authors speculated that an increased surface charge density in the dystrophic RPE might diminish the ability of the RPE to phagocytize OS membranes.

Efforts to map, catalog, and identify biological functions for proteins of cultured RPE cells are now under way. Haley and Flood (1983) have analyzed total cellular proteins from cultured human RPE cells by two-dimensional electrophoresis and have determined that a major cellular protein is actin. Three major plasma membrane proteins in cultured rat RPE have been partially characterized by cell surface radioiodination and subcellular fractionation (Clark *et al.*, 1984). These three iodinatable cell surface proteins (MW of 152, 138, and 123K) become cross-linked to (or tightly associated with) the Triton X-100-insoluble cytoskeleton when cells are treated with anti-RPE cell antiserum or concanavalin A. The three cell surface proteins also become labeled when [^{3}H]fucose or [^{3}H]glucosamine are included in the growth medium, indicating that they are glycoproteins. Whether they are involved in binding and ingestion of OS remains to be determined. Additionally, the major glycoprotein components of a plasma membrane-enriched fraction from cultured rat RPE have been mapped by two-dimensional electrophoresis and autoradiography (Clark and Hall, 1986). Cell surface labeling by lactoperoxidase-catalyzed radioiodination has confirmed the plasma membrane location of a number of these glycoproteins (Fig. 5). Recently developed methods for the isolation of plasma membranes from freshly isolated RPE cells (Braunagel *et al.*, 1985) may also be applicable to purification and characterization of plasma membrane components in cultured RPE cells.

c. Cyclic Nucleotides. An early study of the effect of Bu_2cAMP on phagocytosis of latex spheres by bovine RPE–choroid explants suggested that cAMP stimulates phagocytic uptake of the spheres (Feeney and Mixon, 1976). Edwards and Bakshian (1980) examined the effect of cAMP and cGMP on phagocytosis of rat ROS by cultured rat RPE cells. They reported that phagocytosis is inhibited by cAMP and unaffected by cGMP. A criticism of both of these studies is that very high (nonphysiological and possibly toxic) concentrations of cyclic nucleotides were employed (1–25 m*M*).

A more recent investigation of the effect of cyclic nucleotides on phagocytic uptake of latex spheres by cultured chick RPE cells showed that cAMP inhibits phagocytosis and that cGMP can reverse the effect of cAMP (Ogino *et al.*, 1983). The effect of cAMP was observed in the presence of extracellular concentrations as low as 10^{-10} *M*, while cGMP reversed the cAMP effect at an

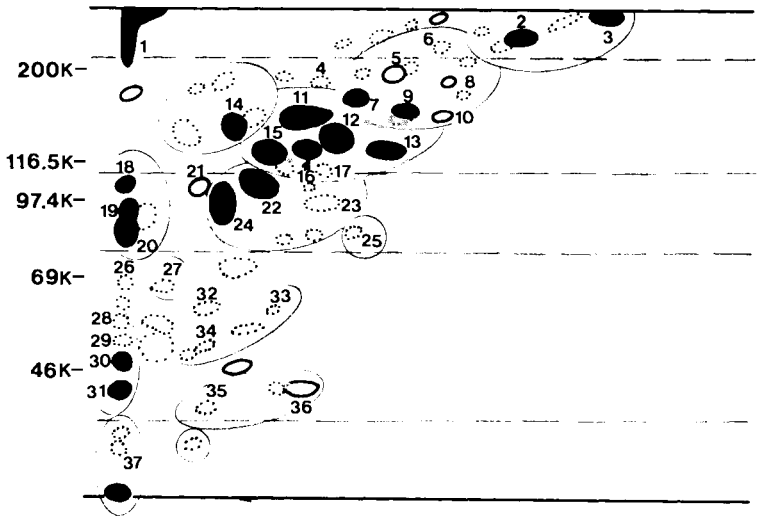


FIG. 5. Two-dimensional electrophoretic separation of [^{3}H]glucosamine labeled glycoproteins in a plasma membrane-enriched fraction from cultured rat RPE. Spot numbers 2–13, 15, 31, and 37 were also labeled by surface radioiodination of intact cells, indicating that they are located at the cell surface. Positions of MW markers are indicated to the left. The highly reproducible spots were assigned numbers. Solid lines and spots represent major spots, while dotted lines represent lighter spots in the autoradiogram.

extracellular concentration of 10^{-6} *M*. In this same paper, it was also reported that melatonin (at 10^{-16} *M*) inhibits phagocytosis by 50%. The effect of melatonin could be potentiated by IBMX (an inhibitor of phosphodiesterase; see article by Farber and Shuster, Part I), to produce a final inhibition of 75%. This IBMX effect suggested that melatonin inhibits phagocytosis by stimulating an increase in intracellular concentrations of cAMP in the RPE. Additionally, taurine effectively reversed the inhibition of phagocytosis by melatonin or cAMP. It will be important to determine if these reported effects of cyclic nucleotides upon phagocytosis of latex spheres apply also to phagocytosis of OS. It is possible that the mechanisms for phagocytosis of latex and OS differ only at the level of the cell surface and that common intracellular amplification pathways are utilized after transmembrane signaling has occurred.

d. Energy Requirements. Masterson and Chader (1981) investigated the relative contributions of three major glucose-degrading metabolic pathways to energy that is utilized during phagocytosis of bovine ROS by cultured chick RPE cells. Their results suggest that the tricarboxylic acid (TCA) cycle and cytochrome pathway provide the bulk of the energy consumed during phagocytosis

(by cultured chick RPE cells). The glycolytic pathway alone did not provide sufficient energy to support maximal phagocytic activity. Stimulation of the hexose monophosphate shunt decreased phagocytosis, possibly by diminishing the supply of glucose to the other pathways. Phagocytosis did not increase the rate of transport of 3-*O*-methyl-D-glucose into the cultured cells, indicating that the cells do not increase uptake of glucose during phagocytosis. The authors point out that further studies are required to determine if these observations apply to the RPE cell *in vivo*.

2. Synthesis and Function of the Extracellular Matrix

Pure cultures of RPE cells are now being used to investigate the synthesis and production of extracellular matrix (ECM) on a molecular level. Production of ECM at the basal surface of cultured chick embryo RPE has been described by Newsome and Kenyon (1973). RPE cells isolated from younger embryos produced basal ECM more quickly (within 4–14 days of *in vitro* culture) than those isolated from 21-day-old embryos, which synthesized no basal ECM until 3 weeks of *in vitro* culture. The authors observed the formation of collagen-like fibrils in the basal ECM of some of the cultured RPE cells. Identification of collagen was based upon morphological appearance of the fibers; no chemical characterization of the "basement membrane" was reported.

More recently, Li *et al.* (1984) have chemically and morphologically characterized the collagen produced at the basal surface of feline RPE cells *in vitro*. Like RPE cells isolated from older chick embryos (Newsome and Kenyon, 1973), RPE cells from adult cats produced a basal ECM very slowly *in vitro*. Although the cultured cells were confluent at 14 days *in vitro*, synthesis of an ECM which filled the space between the basal plasma membrane of the RPE and the tissue culture dish was not complete until 145 days *in vitro*. For chemical characterization, cultured RPE cells were labeled metabolically with [^{14}C]proline and the labeled collagen species (pepsin solubilized) were identified by carboxymethyl cellulose chromatography and SDS gel electrophoresis. The analysis showed that the cat RPE produces α_1(IV) and α_2(IV) collagen *in vitro*. In contrast, cultured choroidal–scleral fibroblasts (possible contaminants of RPE cell cultures) synthesized α_1(I), β_{12}(I), and α_2(I) collagen. These results showed that (1) cultured feline RPE cells continue to synthesize and secrete new collagen *in vitro* and (2) the collagen deposited at the basal surface of feline RPE *in vitro* is primarily type IV, a common component of basement membranes *in vivo*.

Closely related to the problem of extracellular matrix production and secretion is the synthesis of interphotoreceptor matrix (IPM) components. As the components of the IPM (e.g., carrier proteins) are identified and characterized, it will be of interest to know which are synthesized by the RPE, as opposed to the neural retina. Use of cultured RPE cells may simplify the investigation of this

question if the cells continue to secrete IPM proteins *in vitro*. In a recent preliminary report, Edwards and Brandt (1984) presented a partial characterization of a 31,000-MW glycoprotein, of unknown function, that is secreted into the growth medium by cultured human RPE cells.

Glycosaminoglycans are components of both the RPE basement membrane and the IPM. Edwards (1982) has studied the production of glycosaminoglycans (hyaluronic acid, chondroitin sulfate, and dermatin sulfate) by cultured human RPE cells. These preliminary studies have shown that human RPE cells continue to produce the same types and relative amounts of glycosaminoglycans through 18 passages. Therefore, expression of this trait is stable in cell culture. Furthermore, types and quantities of glycosaminoglycans synthesized by the RPE cells were quite distinct from those produced by cultured choroidal cells and fibroblasts.

3. Retinol Uptake and Storage

Flood *et al.* (1983) have studied the ability of cultured human RPE cells to take up extracellular all-*trans*-retinol and to synthesize and store retinyl esters. Comparison of vitamin A content in freshly isolated human cells with that of cultured RPE cells showed that cultured cells rapidly lost endogenous vitamin A. After 48 hr *in vitro*, vitamin A content decreased to 20% of that found in freshly isolated cells, and by 11 days fell to 1% of normal levels. Vitamin A content could be restored to normal levels in primary cultures by supplementing the medium with all-*trans*-retinol bound to bovine serum albumin. Subcultured cells, when grown in retinol-supplemented medium, were able to achieve only 5–10% of normal cellular levels of all-*trans*-retinyl esters.

Freshly isolated RPE cells contained primarily the palmitate ester of retinol, while cultured cells made relatively more oleate ester (proportion of oleate ester in subcultured cells > primary cultured cells > freshly isolated cells). These results showed that cultured human RPE cells undergo changes both in the ability to take up and/or store vitamin A and in the relative usage of various fatty acid substrates for the esterification reaction. The degree of change seemed to correlate with length of time in culture. Therefore, young primary cultures of human RPE cells most closely resembled the *in vivo* situation with respect to chemistry of vitamin A storage. Cultured cells did not produce any of the 11-*cis*-retinyl esters, which are found in freshly isolated cells.

It will be of interest to study receptor-mediated uptake of retinol, delivered by its natural carrier, serum retinol-binding protein (RBP) in cultured RPE cells. The type of retinol uptake observed by Flood *et al.* was likely due to nonspecific endocytosis of BSA-bound retinol at the apical cell surface. Whereas the retinol uptake observed by Flood *et al.* was very slow (18–24 hr), Maraini and Gozzoli (1975) reported that during a 2-min incubation, freshly isolated bovine RPE cells

took up 1000 times more [^{3}H]retinol when retinol was presented to the cells bound to RBP, than when [^{3}H]retinol was presented in the free state. Following the initial rapid uptake, bovine RPE cells continued to take up [^{3}H]retinol from RBP more slowly during a further 2-hr incubation. The initial rapid uptake of [^{3}H]retinol, stimulated by RBP, was likely to be mediated by the RBP receptors that are located on the basal plasma membrane of the RPE cell (Bok and Heller, 1976). Pfeffer *et al.* (1984) have recently demonstrated that cultured human RPE cells express serum RBP receptors at the basal cell surface. With the use of cultured RPE cells, it should be possible to study the mechanism of RBP receptor-mediated transport of vitamin A across the basal RPE cell plasma membrane. Receptor-mediated uptake of retinol through the apical RPE plasma membrane may also occur *in vivo*. The availability of cultured RPE cells should eventually make it possible to study both modes of transmembrane retinol transport on a molecular level.

IV. Summary and Future Research

The RPE cell plays a pivotal role in the nourishment and renewal of the photoreceptor cell layer. As such, it carries out many complex activities that make it a good model system for the study of cell biological problems of current interest. Some questions that future research may address are the following:

1. What is the biochemical basis for the cell surface recognition process that results in specific binding and phagocytic uptake of OS membranes by the RPE cell? What is the mechanism by which recognition at the cell surface signals a cytoskeletal rearrangement, resulting in ingestion of bound OS membranes? Do cyclic nucleotides play a role in this signaling process?
2. Is there a direct relationship between complex pigment granule accumulation in aging RPE and some forms of retinal degeneration?
3. What are the molecular mechanisms by which ions, nutrients, and water are transported across the RPE? These activities are integral to the process by which the RPE exchanges nutrients and wastes with the photoreceptor cell layer and promotes retinal adhesion.
4. Do glycoprotein components of the interphotoreceptor matrix (other than IRBP) possess enzymatic, adhesive, or ligand-carrying activities that are important for communication between the RPE and photoreceptor cells? If so, by which cell type are they synthesized, and how is synthesis regulated?
5. How does the composition of the RPE basement membrane affect firm attachment of the RPE and contribute to the regulation of flow of materials from the choriocapillaries to the RPE? Can changes in the RPE basement membrane composition contribute to some kinds of retinal pathology?

6. What is the biochemical composition of structural proteins that make up the tight intercellular junctions between adjacent RPE cells? How does this structure maintain the molecular differentiation of the RPE cell plasma membrane? Are changes in these proteins involved in junction breakdown in some forms of retinal pathology?

Investigation of these and other questions on a molecular level will be aided by recent improvements in techniques for culturing very pure, highly differentiated RPE cells *in vitro*.

References

Adler, A. J., and Klucznik, K. M. (1982). Proteins and glycoproteins of the bovine interphotoreceptor matrix: Composition and fractionation. *Exp. Eye Res.* **34,** 423–434.

Adler, A. J., and Martin, K. J. (1982). Retinal binding proteins in bovine interphotoreceptor matrix. *Biochem. Biophys. Res. Commun.* **108,** 1601–1608.

Adler, A. J., and Severin, K. M. (1981). Proteins of the bovine interphotoreceptor matrix: Tissues of origin. *Exp. Eye Res.* **32,** 755–769.

Aguirre, G. D., and Laties, A. (1976). Pigment epithelial dystrophy in the dog. *Exp. Eye Res.* **23,** 247–256.

Albert, D. M., Tso, M. O. M., and Rabson, A. S. (1972). *In vitro* growth of pure cultures of retinal pigment epithelium. *Arch. Ophthalmol.* **88,** 63–69.

Alvarez, R. A., Bridges, C. D. B., and Fong, S.-L. (1980). High pressure liquid chromotography of fatty acid esters of retinol isomers—analysis of retinyl esters stored in the eye. *Invest. Ophthalmol. Visual Sci.* **20,** 304–313.

Anderson, R. E., and Maude, M. B. (1970). Phospholipids of bovine rod outer segments. *Biochemistry* **9,** 3624–3628.

Anderson, R. E., and Risk, M. (1974). Lipids of ocular tissues. IX. The phospholipids of frog photoreceptor membranes. *Vision Res.* **14,** 129–131.

Aronson, J. F. (1983). Human retinal pigment cell culture. *In Vitro* **19,** 642–650.

Bach, G., and Berman, E. R. (1971a). Amino sugar-containing compounds of the retina. I. Isolation and identification. *Biochim. Biophys. Acta* **252,** 453–461.

Bach, G., and Berman, E. R. (1971b). Amino sugar-containing compounds of the retina. II. Structural studies. *Biochim. Biophys. Acta* **252,** 461–471.

Basinger, S., Hoffman, R., and Matthes, M. (1976). Photoreceptor shedding is initiated by light in the frog retina. *Science* **194,** 1074–1076.

Basu, P. K., Sarker, P., Menon, I., Carre, F., and Persad, S. (1983). Bovine retinal pigment epithelial cells cultured *in vitro:* Growth characteristics, morphology, chromosomes, phagocytosis ability, tyrosinase activity and effect of freezing. *Exp. Eye Res.* **36,** 671–683.

Berman, E. R. (1964). The biosynthesis of mucopolysaccharides and glycoproteins in pigment epithelial cells of bovine retina. *Biochim. Biophys. Acta* **83,** 371–373.

Berman, E. R. (1965). Isolation of neutral sugar-containing mucopolysaccharides from cattle retina. *Biochim. Biophys. Acta* **101,** 358–360.

Berman, E. R. (1971). Acid hydrolases of the retinal pigment epithelium. *Invest. Ophthalmol.* **10,** 64–68.

Berman, E. R. (1979). Biochemistry of the retinal pigment epithelium. *In* "The Retinal Pigment

Epithelium" (K. M. Zinn and M. F. Marmor, eds.), pp. 83–102. Harvard Univ. Press, Cambridge, Massachusetts.

Berman, E. R., and Bach, G. (1968). The acid mucopolysaccharides of cattle retina. *Biochem. J.* **108,** 75–88.

Berman, E. R., Schwell, H., and Feeney, L. (1974). The retinal pigment epithelium. Chemical composition and structure. *Invest. Ophthalmol.* **13,** 675–687.

Berson, E. L., Hayes, K. C., Rabin, A. R., Schmidt, S. Y., and Watson, G. (1976). Retinal degeneration in cats fed casein. II. Supplementation with methionine, cysteine, or taurine. *Invest. Ophthalmol.* **15,** 52–58.

Besharse, J. C., Forestner, D. M., and Greenberger, L. M. (1984). Mechanism of rod disc shedding: Induction of pigment epithelial ensheathing processes and phagocytosis in eyecups. *Invest. Ophthalmol. Visual Sci. Suppl.* **25,** 243.

Bok, D. (1982). Autoradiographic studies on the polarity of plasma membrane receptors in retinal pigment epithelial cells. *In* "The Structure of the Eye" (J. G. Hollyfield, ed.), pp. 247–256. Elsevier, Amsterdam.

Bok, D., and Hall, M. O. (1971). The role of the pigment epithelium in the etiology of inherited retinal dystrophy in the rat. *J. Cell Biol.* **49,** 664–682.

Bok, D., and Heller, J. (1976). Transport of retinol from the blood to the retina: An autoradiographic study of the pigment epithelial cell surface receptor for plasma retinol-binding protein. *Exp. Eye Res.* **22,** 395–402.

Bok, D., and Young, R. W. (1979). Phagocytic properties of the retinal pigment epithelium. *In* "The Retinal Pigment Epithelium" (K. M. Zinn and M. F. Marmor, eds.), pp. 148–174. Harvard Univ. Press, Cambridge, Massachusetts.

Boulton, M. E., Marshall, J., and Mellerio, J. (1983). Retinitis pigmentosa: A preliminary report on tissue culture studies of retinal pigment epithelial cells from eight affected human eyes. *Exp. Eye Res.* **37,** 307–313.

Bourne, M. C., Campbell, D. A., and Tansley, K. (1938). Hereditary degeneration of the rat retina. *Br. J. Ophthalmol.* **22,** 613–623.

Braunagle, S. C., Organisciak, D. T., and Wang, H. M. (1985). Isolation of plasma membranes from bovine retinal pigment epithelium. *Biochim. Biophys. Acta* **813,** 183–194.

Bridges, C. D. B. (1975). Storage, distribution and utilization of vitamin A in the eyes of adult amphibians and their tadpoles. *Vision Res.* **15,** 1311–1323.

Bridges, C. D. B. (1976). Vitamin A and the role of the pigment epithelium during bleaching and regeneration of rhodopsin in the frog eye. *Exp. Eye Res.* **22,** 435–455.

Bridges, C. D. B., and Fong, S.-L. (1979). Different receptors for distribution of peanut and ricin agglutinins between inner and outer segments of rod cells. *Nature (London)* **282,** 513–515.

Bridges, C. D. B., Alvarez, R. A., and Fong, S.-L. (1982). Vitamin A in human eyes—amount, distribution and composition. *Invest. Ophthalmol. Visual Sci.* **22,** 706–714.

Bridges, C. D. B., Fong, S.-L., Liou, G. I., Alvarez, R. A., and Landers, R. A. (1983). Transport, utilization and metabolism of visual cycle retinoids in the retina and pigment epithelium. *In* "Progress in Retinal Research" (N. Osborne and G. Chader, eds.), Vol. 2, pp. 137–162. Pergamon, Oxford.

Burnside, M. B. (1976). Possible roles of microtubules and actin filaments in retinal pigmented epithelium. *Exp. Eye Res.* **23,** 257–275.

Burnside, M. B., and Laties, A. M. (1976). Actin filaments in apical projections of the primate pigmented epithelial cell. *Invest. Ophthalmol.* **15,** 570–575.

Caldwell, R. B., and McLaughlin, B. J. (1983). Permeability of retinal pigment epithelial cell junctions in the dystrophic rat retina. *Exp. Eye Res.* **36,** 415–427.

Caldwell, R. B., McLaughlin, B. J., and Boykins, L. G. (1982). Intramembrane changes in retinal

pigment epithelial cell junctions of the dystrophic rat retina. *Invest. Ophthalmol. Visual Sci.* **23,** 305–318.

Chader, G. J. (1982). Retinoids in ocular tissues: Binding proteins, transport, and mechanism of action. *In* "Cell Biology of the Eye" (D. S. McDevitt, ed.), pp. 377–433. Academic Press, New York.

Chader, G. J., Wiggert, B., Lai, Y.-L., Lee, L., and Fletcher, R. T. (1983). Interphotoreceptor retinol-binding protein: A possible role in retinoid transport to the retina. *In* "Progress in Retinal Research" (N. Osborne and G. Chader, eds.), Vol. 2, pp. 163–189. Pergamon, Oxford.

Chaitin, M. H., and Hall, M. O. (1983a). Defective ingestion of rod outer segments by cultured dystrophic rat pigment epithelial cells. *Invest. Ophthalmol. Visual Sci.* **24,** 812–820.

Chaitin, M. H., and Hall, M. O. (1983b). The distribution of actin in cultured normal and dystrophic rat pigment epithelial cells during the phagocytosis of rod outer segments. *Invest. Ophthalmol. Visual Sci.* **24,** 821–831.

Chen, C.-C., and Heller, J. (1977). Uptake of retinol and retinoic acid from serum retinol-binding protein by retinal pigment epithelial cells. *J. Biol. Chem.* **252,** 5216–5221.

Chen, C.-C., Heller, J., Ding, L.-L., and Horwitz, J. (1981). Retinol transfer from rat liver cytosol retinyl ester lipoprotein complex to serum retinol-binding protein. *Arch. Biochem. Biophys.* **207,** 392–398.

Chio, K. S., Reiss, U., Fletcher, B., and Tappel, A. L. (1969). Peroxidation of subcellular organelles: Formation of lipofuscin-like fluorescent pigments. *Science* **166,** 1535–1536.

Chytil, F., and Ong, D. E. (1984). Cellular retinol binding proteins. *In* "The Retinoids" (M. B. Sporn, A. B. Roberts, and D. S. Goodman, eds.), Vol. 2, pp. 89–123. Academic Press, Orlando, Florida.

Clark, V. M., and Hall, M. O. (1986). 2D electrophoresis of rat PE plasma membrane proteins. *Invest. Ophthalmol. Visual Sci.*, in press.

Clark, V. M., Hall, M. O., Mayerson, P. L., and Schechter, C. (1984). Identification of some plasma membrane proteins of cultured rat pigment epithelial cells. *Exp. Eye Res.* **39,** 611–628.

Cohen, D., and Nir, I. (1983). Cytochemical evaluation of anionic sites on the surface of cultured pigment epithelium cells from normal and dystrophic RCS rats. *Exp. Eye Res.* **37,** 575–582.

Cunha-Vaz, J. G. (1976). The blood–retinal barriers. *Doc. Ophthalmol.* **41,** 287–327.

Cunha-Vaz, J. G. (1979). The blood–ocular barriers. *Surv. Ophthalmol.* **23,** 279–296.

Custer, N. V., and Bok, D. (1975). Pigment epithelium–photoreceptor interactions in the normal and dystrophic rat retina. *Exp. Eye Res.* **21,** 153–166.

DelMonte, M. A., and Maumenee, I. H. (1980). New technique for *in vitro* culture of human retinal pigment epithelium. *Birth Defects Orig. Artic. Ser.* **16,** 327–338.

Dilley, R. A., and McConnell. (1970). Alpha-tocopherol in the retinal outer segment of bovine eyes. *J. Membr. Biol.* **2,** 317–323.

Dorey, C. K., Bekhor, C., Sorgente, N., and Ryan, S. J. (1983). Porcine RPE cells maintain differentiated characteristics *in vitro*. *Invest. Ophthalmol. Visual Sci. Suppl.* **24,** 142.

Dowling, J. (1960). Chemistry of visual adaptation in the rat. *Nature (London)* **168,** 114–118.

Dowling, J. E., and Sidman, R. L. (1962). Inherited retinal dystrophy in the rat. *J. Cell Biol.* **14,** 73–109.

Edwards, R. B. (1977). Culture of rat retinal pigment epithelium. *In Vitro* **13,** 301–304.

Edwards, R. B. (1982). Glycosaminoglycan synthesis by cultured human retinal pigment epithelium from normal postmortem donors and a postmortem donor with retinitis pigmentosa. *Invest. Ophthalmol. Visual Sci.* **23,** 435–446.

Edwards, R. B., and Szamier, R. B. (1977). Defective phagocytosis of isolated rod outer segments by RCS rat retinal pigment epithelium in culture. *Science* **197,** 1001–1003.

Edwards, R. B., and Bakshian, S. (1980). Phagocytosis of outer segments by cultured rat pigment

epithelium. Reduction by cyclic AMP and phosphodiesterase inhibitors. *Invest. Ophthalmol. Visual Sci.* **19,** 1184–1188.

Edwards, R. B., and Brandt, J. T. (1984). A 31,000 dalton protein synthesized and released by cultured human retinal pigment epithelium. *Invest. Ophthalmol. Visual Sci. Suppl.* **25,** 274.

Effron, J. T., Szamier, R. B., and Edwards, R. B. (1981). Selective phagocytosis of liposomes by cultured RCS rat pigment epithelium. *Invest. Ophthalmol. Visual Sci.* **21,** 611–616.

Elner, V. M., Schaffner, T., Taylor, K., and Glasgov, S. (1981). Immunophagocytic properties of retinal pigment epithelium cells. *Science* **211,** 74–76.

Essner, E., Roszka, J. R., and Schreiber, J. H. (1978). Phagocytosis and surface morphology in cultured retinal pigment epithelial cells. *Invest. Ophthalmol. Visual Sci.* **17,** 1040–1048.

Farkas, T. G., Sylvester, V., and Archer, D. (1971). The ultrastructure of drusen. *Am. J. Ophthalmol.* **71,** 1196–1205.

Farnsworth, C. C., and Dratz, E. A. (1976). Oxidative damage of retinal rod outer segment membranes and the role of vitamin E. *Biochim. Biophys. Acta* **433,** 556–570.

Farquhar, M. G. (1975). The primary glomerular filtration barrier—basement membrane or epithelial slits? *Kidney Int.* **8,** 197–211.

Feeney, L. (1973). The phagolysosomal system of the pigment epithelium: A key to retinal disease. *Invest. Ophthalmol.* **12,** 635–638.

Feeney, L. (1978). Lipofuscin and melanin of human pigment epithelium: Fluorescence, enzyme cytochemical and ultrastructural studies. *Invest. Ophthalmol. Visual Sci.* **17,** 583–600.

Feeney, L., and Berman, E. R. (1976). Oxygen toxicity: Membrane damage by free radicals. *Invest. Ophthalmol.* **15,** 789–792.

Feeney, L., and Mixon, R. N. (1976). An *in vitro* model of phagocytosis in bovine and human retinal pigment epithelium. *Exp. Eye Res.* **22,** 533–548.

Feeney, L., Grieshaber, J. A., and Hogan, M. J. (1963). Studies on human ocular pigment. *In* "Eye Structure II. Symposium" (J. W. Power, ed.), pp. 535–548. Schottauer, Stuttgart.

Feeney-Burns, L., Hilderbrand, E. S., and Eldridge, S. (1984). Aging human RPE: Morphometric analysis of macular, equatorial and peripheral cells. *Invest. Ophthalmol. Visual Sci.* **25,** 195–200.

Flood, M. T., Gouras, P., and Kjeldbye, H. (1980). Growth characteristics and ultrastructure of human retinal pigment epithelium *in vitro*. *Invest. Ophthalmol. Visual Sci.* **19,** 1309–1320.

Flood, M. T., Bridges, C. D. B., Alvarez, R. A., Blaner, W. S., and Gouras, P. (1983). Vitamin A utilization in human retinal pigment epithelial cells *in vitro*. *Invest. Ophthalmol. Visual Sci.* **24,** 1227–1235.

Francois, J., Matton-van Leuven, M. Th., Verschraegen-Spae, M. R., and Netto, J. A. (1971). Tissue culture study of the retinal pigment epithelium of the adult pig. *Ophthalmic Res.* **2,** 86–95.

Goldman, A. I., and O'Brien, P. J. (1978). Phagocytosis in the retinal pigment epithelium of the RCS rat. *Science* **201,** 1023–1025.

Gonzalez-Fernandez, F., Landers, R. A., Liou, G. I., Glazebrook, P., Lam, D. M. K., and Bridges, C. D. B. (1984). Interstitial retinol binding protein (IRBP) in RCS rats: Immunocytochemical localization, amount and synthesis. *Invest. Ophthalmol. Visual Sci. Suppl.* **25,** 276.

Goodman, D. S. (1984). Plasma retinol-binding protein. *In* "The Retinoids" (M. B. Sporn, A. B. Roberts, and D. S. Goodman, eds.), Vol. 2, pp. 41–88. Academic Press, Orlando, Florida.

Goodman, D. S., and Blaner, W. S. (1984). Biosynthesis, absorption and hepatic metabolism of retinol. *In* "The Retinoids" (M. B. Sporn, A. B. Roberts, and D. S. Goodman, eds.), Vol. 2, pp. 1–39. Academic Press, Orlando, Florida.

Green, W. R., and Key, S. H., III. (1977). Senile macular degeneration: A histologic study. *Trans. Am. Ophthalmol. Soc.* **75,** 180–254.

Griffen, F. M., and Silverstein, S. C. (1974). Segmental response of the macrophage plasma membrane to a phagocytic stimulus. *J. Exp. Med.* **139,** 323–336.

Griffen, F. M., Bianco, C., and Silverstein, S. C. (1975a). Characterization of the macrophage receptor for complement and demonstration of its functional independence from the receptor for the Fc portion of immunoglobin G. *J. Exp. Med.* **141,** 1269–1277.

Griffen, F. M., Griffen, J. A., Leider, J. E., and Silverstein, S. C. (1975b). Studies on the mechanisms of phagocytosis. I. Requirements for circumferential attachment of particle bound ligands to specific receptors on the macrophage plasma membrane. *J. Exp. Med.* **142,** 1263–1282.

Griffen, F. M., Griffen, J. A., and Silverstein, S. C. (1976). Studies on the mechanism of phagocytosis. II. The interaction of macrophages with anti-immunoglobulin IgG coated bone marrow derived lymphocytes. *J. Exp. Med.* **144,** 788–809.

Gudewicz, P. W., Molnar, J., Zong Lai, M., Beezhold, D. W., Siefring, G. E., Jr., Credo, R. B., and Lorand, L. (1980). Fibronectin-mediated uptake of gelatin-coated latex particles by peritoneal macrophages. *J. Cell Biol.* **87,** 427–433.

Haley, J. E., Flood, M. T., Gouras, P., and Kjeldbye, H. M. (1983). Proteins from human pigment epithelial cells: Evidence that a major protein is actin. *Invest. Ophthalmol. Visual Sci.* **24,** 803–811.

Hall, M. O., and Nir, I. (1976). The binding of concanavalin A to the rod outer segments and pigment epithelium of normal and RCS rats. *Exp. Eye Res.* **22,** 469–476.

Hall, M. O., and Quon, D. S. (1980). Plant lectins inhibit phagocytosis of ROS by cultured PE cells. *Invest. Ophthalmol. Visual Sci. Suppl.* **21,** 162.

Hall, M. O., and Quon, D. S. (1981). Tissue culture of rat pigment epithelium on different supporting media. *Vision Res.* **21,** 127–132.

Hara, S., Plantner, J. J., and Kean, E. L. (1983). The enzymatic cleavage of rhodopsin by the retinal pigment epithelium. I. Enzyme preparation, properties and kinetics: Characterization of the glycopeptide product. *Exp. Eye Res.* **36,** 799–816.

Hayasaka, S., Hara, S., and Mizuno, K. (1975). Distribution and some properties of cathepsin D in the retinal pigment epithelium. *Exp. Eye Res.* **21,** 307–313.

Hayasaka, S., Hara, S., Takaku, Y., and Mizuno, K. (1977). Distribution of acid lipase in the bovine retinal pigment epithelium. *Exp. Eye Res.* **24,** 1–6.

Hayes, K. C. (1974). Retinal degeneration in monkeys induced by deficiencies of vitamin E or A. *Invest. Ophthalmol.* **13,** 499–510.

Heath, A. R., and Basinger, S. F. (1983). Simple sugars inhibit rod outer segment disc shedding by the frog retina. *Vision Res.* **23,** 1371–1377.

Heller, J. (1975). Interactions of plasma retinol-binding protein with its receptor. Specific binding of bovine and human retinol-binding protein to pigment epithelium cells from bovine eyes. *J. Biol. Chem.* **250,** 3613–3619.

Heller, J. (1976). Intracellular retinol-binding proteins from bovine pigment epithelial and photoreceptor cell fractions. *J. Biol. Chem.* **251,** 2952–2957.

Heller, J., and Bok, D. (1976). Transport of retinol from the blood to the retina: Involvement of high molecular weight lipoproteins as intracellular carriers. *Exp. Eye Res.* **22,** 403–410.

Herman, K. G., and Steinberg, R. H. (1982a). Phagosome movement and the diurnal pattern of phagocytosis in the tapetal retinal pigment epithelium of the opossum. *Invest. Ophthalmol. Visual Sci.* **23,** 277–290.

Herman, K. G., and Steinberg, R. H. (1982b). Phagosome degradation in the tapetal retinal pigment epithelium of the opossum. *Invest. Ophthalmol. Visual Sci.* **23,** 291–304.

Herron, W. L., Riegel, B. W., Meyers, D. E., and Rubin, M. L. (1969). Retinal dystrophy in the rat—a pigment epithelial disease. *Invest. Ophthalmol.* **8,** 595–604.

Hewitt, A. T., Krawczewicz, L., and Newsome, D. A. (1984). Changes in Bruch's membrane

proteoglycans associated with age and retinitis pigmentosa. *Invest. Ophthalmol. Visual Sci. Suppl.* **25,** 57.

Hirosawa, K., and Yamada, K. (1973). The localization of vitamin A in the mouse liver as revealed by electron microscope radioautography. *J. Electron Microsc.* **22,** 337–346.

Hogan, M. J. (1972). Role of the retinal pigment epithelium in macular disease. *Trans. Am. Acad. Ophthalmol. Otolaryngol.* **76,** 64–80.

Hollyfield, J. G., and Ward, A. (1974a). Phagocytic activity in the retinal pigment epithelium of the frog *Rana pipiens*. I. Uptake of polystyrene spheres. *J. Ultrastruct. Res.* **46,** 327–338.

Hollyfield, J. G., and Ward, A. (1974b). Phagocytic activity in the retinal pigment epithlium of the frog *Rana pipiens*. II. Exclusion of *Sarcina subflava*. *J. Ultrastruct. Res.* **46,** 339–350.

Hollyfield, J. G., and Ward, A. (1974c). Phagocytic activity of the pigmented retinal epithelium. III. Interaction between lysosomes and ingested polystyrene spheres. *Invest. Ophthalmol.* **13,** 1016–1023.

Hollyfield, J. G., Fliesler, S. J., Rayborn, M. E., Fong, S.-L., Landers, R. A., and Bridges, C. D. (1985). Synthesis and secretion of interstitial retinol-binding protein by the human retina. *Invest. Ophthalmol. Visual Sci.* **26,** 58–67.

Hughes, B. A., Miller, S. S., and Machen, T. E. (1984). Effects of cyclic AMP on fluid absorption and ion transport across frog retinal pigment epithelium. Measurements in the open-circuit state. *J. Gen. Physiol.* **83,** 875–899.

Irons, M. J., and Kalnins, V. I. (1984). Distribution of microtubules in cultured RPE cells from normal and dystrophic RCS rats. *Invest. Ophthalmol. Visual Sci.* **25,** 434–439.

Ishikawa, T., and Yamada, E. (1970). The degradation of photoreceptor outer segments within the pigment epithelial cell of the rat retina. *J. Electron Microsc.* **19,** 85–94.

Kanai, M., Raz, A., and Goodman, D. S. (1968). Retinol binding protein: The transport protein for vitamin A in human plasma. *J. Clin. Invest.* **47,** 2025–2044.

Katz, M. L., Stone, W. L., and Dratz, E. A. (1978). Fluorescent pigment accumulation in retinal pigment epithelium of antioxidant-deficient rats. *Invest. Ophthalmol. Visual Sci.* **17,** 1049–1058.

Katz, M. L., Parker, K. R., Handleman, G. J., Bramel, T. L., and Katz, E. A. (1982). Effects of antioxidant nutrient deficiency on the retina and retinal pigment epithelium of albino rats: A light and electron microscopic study. *Exp. Eye Res.* **34,** 339–369.

Kean, E. L., Hara, S., Mizoguchi, A., Matsumoto, A., and Kobata, A. (1983). The enzymatic cleavage of rhosopsin by the retinal pigment epithelium. II. The carbohydrate composition of the glycopeptide cleavage product. *Exp. Eye Res.* **36,** 817–825.

Kennedy, A. J., and Voaden, M. J. (1976). Stdies on the uptake and release of taurine by the frog retina. *J. Neurochem.* **27,** 131–137.

Kolb, H., and Gouras, P. (1974). Electron microscopic observations of human retinitis pigmentosa, dominantly inherited. *Invest. Ophthalmol.* **13,** 487–498.

Kornfeld, K., Reitman, M. L., and Kornfeld, R. (1981). The carbohydrate-binding specificity of pea and lentil lectins. *J. Biol. Chem.* **256,** 6633–6640.

Lai, Y. L., Wiggert, B., Liu, Y. P., and Chader, G. J. (1982). Interphotoreceptor retinol-binding proteins: Possible transport vehicles between compartments of the retina. *Nature (London)* **298,** 848–849.

Lake, N., Marshall, J., and Voaden, M. J. (1975). Studies on the uptake of taurine by the isolated neural retina and pigment epithelium of the frog. *Biochem. Soc. Trans.* **3,** 524–525.

LaVail, M. M. (1976a). Rod outer segment disc shedding in relation to cyclic lighting. *Exp. Eye Res.* **23,** 277–280.

LaVail, M. M. (1976b). Rod outer segment disc shedding in rat retina: Relationship to cyclic lighting. *Science* **194,** 1071–1074.

LaVail, M. M. (1979). The retinal pigment epithelium in mice and rats with inherited retinal

degeneration. *In* "The Retinal Pigment Epithelium" (K. M. Zinn and M. F. Marmor, eds.), p. 372. Harvard Univ. Press, Cambridge, Massachusetts.

LaVail, M. M., Pinto, L. H., and Yasumura, D. (1981). The interphotoreceptor matrix in rats with inherited retinal dystrophy. *Invest. Ophthalmol. Visual Sci.* **21,** 658–668.

Li, W., Stramm, L. E., Aguirre, G. D., and Rickey, J. H. (1984). Extracellular matrix production by cat retinal pigment epithelium *in vitro:* Characterization of type IV collagen synthesis. *Exp. Eye Res.* **38,** 291–304.

Lyda, W., Erikson, N., and Krishna, N. (1957). Studies of Bruch's membrane. Flow and permeability studies in a Bruch's membrane-choroid preparation. *Am. J. Ophthalmol.* **44,** 362–370.

McCann, P. M., Stevens, G., Jr., and Fisher, L. J. (1983). Tuftsin stimulates phagocytosis of latex spheres by retinal pigment epithelium explants. *Invest. Ophthalmol. Visual Sci. Suppl.* **24,** 288.

McLaughlin, B. J., and Wood, J. G. (1980). The localization of lectin binding sites on photoreceptor outer segments and pigment epithelium of dystrophic retinas. *Invest. Ophthalmol. Visual Sci.* **19,** 728–742.

McLaughlin, B. J., Boykins, L. B., and Seyfried, R. S. (1983). Surface-replica topography of retinal pigment epithelium during phagocytosis. *Exp. Eye Res.* **36,** 827–838.

Mannagh, J., Arya, D. V., and Irvine, A. R., Jr. (1973). Tissue culture of human retinal pigment epithelium. *Invest. Ophthalmol.* **12,** 52–64.

Mantovani, B., Rabinovitch, M., and Nussenzweig, V. (1972). Phagocytosis of immune complexes by macrophages. Different roles of the macrophage receptor sites for complement (c3) and for immunoglobulin (IgG). *J. Exp. Med.* **135,** 780–792.

Maraini, G., and Gozzoli, F. (1975). Binding of retinol to isolated retinal pigment epithelium in the presence and absence of retinol binding protein. *Invest. Ophthalmol.* **14,** 785–787.

Maraini, G., Ottonello, S., Gozzoli, F., and Merli, A. (1977). Identification of a membrane protein binding the retinol in retinal pigment epithelium. *Nature (London)* **265,** 68–69.

Marmor, M. F., Abdul-Rahim, A. S., and Cohen, D. S. (1980). The effect of metabolic inhibitors on retinal adhesion and subretinal fluid resorption. *Invest. Ophthalmol. Visual Sci.* **19,** 893–903.

Masterson, E., and Chader, G. J. (1981). Pigment epithelial cells in culture. Metabolic pathways required for phagocytosis. *Invest. Ophthalmol. Visual Sci.* **20,** 1–7.

Mayerson, P. L., and Hall, M. O. (1983). Rat pigment epithelial cells show specificity of phagocytosis *in vitro*. *Invest. Ophthalmol. Visual Sci. Suppl.* **24,** 289.

Mayerson, P. L., Clark, V. M., Abrams, T. A., and Hall, M. O. (1984). A new technique for the isolation and culture of rat retinal pigment epithelial cells. *Invest. Ophthalmol. Visual Sci. Suppl.* **25,** 286.

Michl, J., Ohlbaum, D. J., and Silverstein, S. C. (1976). 2-Deoxyglucose selectively inhibits Fc and complement receptor-mediated phagocytosis in mouse peritoneal macrophages. I. Description of inhibitory effect. *J. Exp. Med.* **144,** 1465–1483.

Miller, S., and Steinberg, E. H. (1976). Transport of taurine, L-methionine, and 3-*O*-methyl-D-glucose across frog retinal pigment epithelium. *Exp. Eye. Res.* **23,** 177–189.

Miller, S. S., and Steinberg, R. H. (1977). Active transport of ions across frog retinal pigment epithelium. *Exp. Eye Res.* **25,** 235–248.

Miller, S. S., and Steinberg, R. H. (1979). Potassium modulation of taurine transport across the frog retinal pigment epithelium. *J. Gen. Physiol.* **74,** 237–259.

Miller, S. S., Steinberg, R. H., and Oakley, B., II. (1978). The electrogenic sodium pump of the frog retinal pigment epithelium. *J. Membr. Biol.* **44,** 259–279.

Miller, S. S., Hughes, B. A., and Machen, T. E. (1982). Fluid transport across retinal pigment epithelium is inhibited by cyclic AMP. *Proc. Natl. Acad. Sci. U.S.A.* **79,** 2111–2115.

Miller, S., and Farber, D. (1984). Cyclic AMP modulation of ion transport across frog retinal pigment epithelium. *J. Gen. Physiol.* **83,** 853–874.

Misfeldt, D. S., Hamamoto, S. T., and Pitelka, D. R. (1976). Transepithelial transport in cell culture. *Proc. Natl. Acad. Sci. U.S.A.* **73,** 1212–1216.

Mullen, R. J., and LaVail, M. M. (1976). Inherited retinal dystrophy. A primary defect in pigment epithelium determined with experimental rat chimeras. *Science* **192,** 799–801.

Muto, Y., Smith, J., Milch, P., and Goodman, De. W. (1972). Regulation of retinol-binding protein metabolism by vitamin A status in the rat. *J. Biol. Chem.* **247,** 2542–2550.

Najjar, V. A., and Nishioka, K. (1970). "Tuftsin": A natural phagocytosis stimulating peptide. *Nature (London)* **228,** 672–673.

Negi, A., and Marmor, M. F. (1983). The resorption of subretinal fluid after diffuse damage to the retinal pigment epithelium. *Invest. Ophthalmol. Visual Sci.* **24,** 1475–1479.

Newsome, D. A., and Bowles, C. (1979). Retinal pigmented epithelial cells exhibit immune receptors. *Invest. Ophthalmol. Visual Sci. Suppl.* **20,** 51.

Newsome, D. A., and Kenyon, K. R. (1973). Collagen production *in vitro* by the retinal pigmented epithelium of the chick embryo. *Dev. Biol.* **32,** 387–400.

Newsome, D. A., Fletcher, R. T., Robison, W. G., Jr., Kenyon, K. R., and Chader, G. J. (1974). Effects of cyclic AMP and Sephadex fractions of chick embryo extract on cloned retinal pigmented epithelium in tissue culture. *J. Cell Biol.* **61,** 369–382.

Nir, I., and Hall, M. O. (1979). Ultrastructural localization of lectin binding sites on the surfaces of retinal photoreceptors and pigment epithelium. *Exp. Eye Res.* **29,** 181–194.

O'Brien, P. J. (1976). Rhodopsin as a glycoprotein: A possible role for the oligosaccharide in phagocytosis. *Exp. Eye Res.* **23,** 127–137.

Ogino, N., Matsumura, M., Shirakawa, H., and Tsukahara, I. (1983). Phagocytic activity of cultured retinal pigment epithlial cells from chick embryo: Inhibition by melatonin and cyclic AMP, and its reversal by taurine and cyclic GMP. *Ophthalmic Res.* **15,** 72–89.

Ostwald, T. J., and Steinberg, R. H. (1980). Localization of frog retinal pigment epithelium Na,K-ATPase. *Exp. Eye Res.* **31,** 351–360.

Ostwald, T. J., and Steinberg, R. H. (1981). Transmembrane components of taurine flux across frog retinal pigment epithelium. *Curr. Eye Res.* **1,** 437–443.

Owaribe, K., and Masuda, H. (1982). Isolation and characterization of circumferential microfilament bundles from retinal pigmented epithelial cells. *J. Cell Biol.* **95,** 310–315.

Pasantes-Morales, H., Urban, P. I., Klethi, J., and Mandel, P. (1973). Light stimulated release of [^{35}S]-taurine from chicken retina. *Brain Res.* **51,** 375–378.

Pfeffer, B., Wiggert, B., Lee, L., Zonnenberg, B., Newsome, D., and Chader, G. (1983). The presence of a soluble interphotoreceptor retinol-binding protein (IRBP) in the retinal interphotoreceptor space. *J. Cell. Physiol.* **117,** 333–341.

Pfeffer, B. A., and Newsome, D. A. (1983). Proliferation and differentiation of human and monkey RPE in culture. *Invest. Ophthalmol. Visual Sci. Suppl.* **24,** 143.

Pfeffer, B. A., Clark, V. M., and Bok, D. (1984). Cultured human retinal pigment epithelial cells express surface receptors for serum retinol binding protein. *J. Cell Biol.* **99,** 62a.

Philp, N. J., and Bernstein, M. H. (1980). Receptor specificities in RPE phagocytosis. *J. Cell Biol.* **87,** 91a.

Philp, N. J., and Bernstein, M. H. (1981). Phagocytosis by retinal pigment epithelium explants in culture. *Exp. Eye Res.* **33,** 47–53.

Pino, R. M., and Essner, E. (1981). Permeability of rat choriocapillaris to hemeproteins. *J. Histochem. Cytochem.* **29,** 281–290.

Pino, R. M., Essner, E., and Pino, L. C. (1982a). Permeability of the neonatal rat choriocapillaris to hemeproteins and ferritin. *Am. J. Anat.* **164,** 333–341.

Pino, R. M., Essner, E., and Pino, L. C. (1982b). Location and chemical composition of anionic sites in Bruch's membrane of the rat. *J. Histochem. Cytochem.* **30,** 245–252.

Pisam, M., and Ripoche, P. (1976). Redistribution of surface macromolecules in dissociated epithelial cells. *J. Cell Biol.* **71,** 907–920.

Rabinovitch, M. (1967). The dissociation of the attachment and ingestion phases of phagocytosis by macrophages. *Exp. Cell Res.* **46,** 19–28.

Rayborn, M. E., Bridges, C. D., Landers, R. A., and Hollyfield, J. G. (1984). Synthesis secretion, localization and immunoreactivity of interstitial retinol-binding protein in *Xenopus laevis*. *Invest. Ophthalmol. Visual Sci. Suppl.* **25,** 275.

Regan, C. M., DeGrip, W. J., Daemen, F. J. M., and Bonting, S. L. (1980). Degradation of rhodopsin by a lysosomal fraction of retinal pigment epithelium: Biochemical aspects of the visual process XLI. *Exp. Eye Res.* **30,** 183–191.

Reich-D'Almeida, F. B., and Hockley, D. J. (1976). Primary retinal degeneration: Evidence of normal phagocytosis in the retinal pigment epithelium. *Invest. Ophthalmol. Visual Sci.* **15,** 757–760.

Robey, P. G., and Newsome, D. A. (1983). Biosynthesis of proteoglycans present in primate Bruch's membrane. *Invest. Ophthalmol. Visual Sci.* **24,** 898–905.

Robison, W. G., Jr., and Kuwabara, T. (1977). Vitamin A storage and peroxisomes in retinal pigment epithelium and liver. *Invest. Ophthalmol. Visual Sci.* **16,** 1110–1117.

Robison, W. G., Kuwabara, T., and Bieri, J. G. (1979). Vitamin E deficiency and the retina: Photoreceptor and pigment epithelial changes. *Invest. Ophthalmol. Visual Sci.* **18,** 683–690.

Robison, W. G., Kuwabara, T. K., and Bieri, J. G. (1980). Deficiencies of vitamins A and E in the rat: Retinal damage and lipofuscin accumulation *Invest. Ophthalmol. Visual Sci.* **19,** 1030–1037.

Rothman, H., Feeney, L., and Berman, E. R. (1976). The retinal pigment epithelium. Analytical subcellular fractionation with special reference to acid lipase. *Exp. Eye Res.* **24,** 1–6.

Saari, J. C., Bunt, A. H., Futterman, S., and Berman, E. R. (1977). Localization of retinol-binding protein in bovine retina and retinal pigment epithelium, with a consideration of the pigment epithelium isolation technique. *Invest. Ophthalmol. Visual Sci.* **16,** 797–806.

Saari, J. C., Bredberg, L., and Garwin, G. G. (1982). Identification of the endogenous retinoids associated with three cellular retinoid-binding proteins from bovine retina and retinal pigment epithelium *J. Biol. Chem.* **257,** 13,329–13,333.

Sarks, S. H. (1976). Ageing and degeneration in the macular region: A clinicopathological study. *Br. J. Ophthalmol.* **60,** 324–341.

Schmidt, S. Y., Berson, E. L., and Hayes, K. C. (1976). Retinal degeneration in cats fed casein. I. Taurine deficiency. *Invest. Ophthalmol.* **15,** 47–52.

Schmidt, S. Y., Berson, E. L., Watson, G., and Huang, C. (1977). Retinal degeneration in cats fed casein. III. Taurine deficiency and ERG amplitudes. *Invest. Ophthalmol. Visual Sci.* **16,** 673–678.

Seyfried-Williams, R., and McLaughlin, B. J. (1983). The use of sugar-coated beads to study phagocytosis in normal and dystrophic retina. *Vision Res.* **23,** 485–494.

Silverstein, S. C., and Loike, J. D. (1980). Phagocytosis. *In* "Mononuclear Phagocytes" (R. van Furth, ed.); Part II, p. 899. Nijhoff, The Hague.

Smith, J. E., Muto, Y., Milch, P. O., and Goodman, D. S. (1973). The effects of chylomicron vitamin A on the metabolism of retinol-binding protein in the rat. *J. Biol. Chem.* **248,** 1544–1549.

Spitznas, M., and Hogan, M. J. (1970). Outer segments of photoreceptors and retinal pigment epithelium. *Arch. Ophthalmol.* **84,** 810–819.

Steinberg, R. H., and Miller, S. S. (1973). Aspects of electrolyte transport in frog pigment epithelium. *Exp. Eye Res.* **16,** 365–372.

Steinberg, R. H., and Miller, S. S. (1979). Transport and membrane properties of the retinal pigment

epithelium. *In* "The Retinal Pigment Epithelium" (K. M. Zinn and M. F. Marmor, eds.), pp. 205–225. Harvard Univ. Press, Cambridge, Massachusetts.

Stirling, C. E. (1972). Radioautographic localization of sodium pump sites in rabbit intestine. *J. Cell Biol.* **53,** 704–714.

Stirling, C. E., Karnaky, K. J., Kinter, L. B., and Kinter, W. B. (1973). Autoradiographic localization of ^{3}H-ouabain binding by Na-K ATPase in perfused gills of *Fundulus heteroclitus*. *Bull. Mt. Desert. Isl. Lab.* **13,** 117–120.

Stramm, L. E., Haskins, M. E., McGovern, M. M., and Aguirre, G. D. (1983). Tissue culture of cat retinal pigment epithelium. *Exp. Eye Res.* **36,** 94–101.

Swartz, J. G., and Mitchell, J. E. (1973). Phospholipase activity of retina and pigment epithelium. *Biochemistry* **12,** 5273–5278.

van Oss, C. J. (1978). Phagocytosis as a surface phenomenon. *Annu. Rev. Microbiol.* **32,** 19–39.

Wiggert, B. O., and Chader, G. J. (1975). A receptor for retinol in the developing retina and pigment epithelium. *Exp. Eye Res.* **21,** 143–151.

Wiggert, B. O., Bergsma, D. R., and Chader, G. J. (1976). Retinol receptors of the retina and pigment epithelium: Further characterization and species variation. *Exp. Eye Res.* **22,** 411–418.

Wiggert, B., Derr, J., Fitzpatrick, M., and Chader, G. (1979). Vitamin A receptors of the retina: Differential binding in light and dark. *Biochim. Biophys. Acta* **582,** 115–121.

Wiggert, B., Lee, L., O'Brien, P., and Chader, G. J. (1984). Synthesis of interphotoreceptor retinoid-binding protein (IRPB) by monkey retina in organ culture. *Invest. Ophthalmol. Visual Sci. Suppl.* **25,** 274.

Young, R. W. (1969). The organization of vertebrate photoreceptor cells. *In* "The Retina: Structure, Function and Clinical Characteristics" (B. Straatsma, R. Allen, M. Hall, *et al.*, eds.), pp. 177–209. Univ. of California Press, Berkeley.

Young, R. W. (1971). Shedding of discs from rod outer segments in the Rhesus monkey. *J. Ultrastruct. Res.* **34,** 190–203.

Young, R. W., and Bok, D. (1969). Participation of the retinal pigment epithelium in the rod outer segment renewal process. *J. Cell Biol.* **42,** 392–403.

Zimmerman, W. F., Godchaux, W., III, and Belkin, M. (1983). The relative proportions of lysosomal enzyme activities in bovine retinal pigment epithelium. *Exp. Eye Res.* **36,** 151–158.

EXTRACELLULAR MATRIX MOLECULES: THEIR IMPORTANCE IN THE STRUCTURE AND FUNCTION OF THE RETINA

A. TYL HEWITT

The Michael M. Wynn Center for the
Study of Retinal Degenerations
The Wilmer Ophthalmological Institute
The Johns Hopkins University
School of Medicine
Baltimore, Maryland

I. Introduction

Extracellular matrices are usually composed of three classes of molecules: collagens, proteoglycans, and glycoproteins. Once thought to be inert, it has now become apparent that these complex mixtures of molecules have important roles in development and maintenance of tissue integrity and function. Each group of molecules has specific functions within the matrix: collagens act as structural elements; proteoglycans provide spacing between collagens and thus influence matrix permeability; some glycoproteins are involved in cell attachment, while others transport molecules through the matrix. Molecules belonging to each of these groups may show certain structural similarities while still exhibiting chemical and structural differences. Consequently, extracellular matrices have a variety of physiological functions in mature tissue, based on their composition. For example, they may function as structural and support elements (bone and cartilage), filters (glomerular basement membrane, Bruch's membrane), or light-transmitting, transparent structures (extracellular matrix of the cornea). Extracellular matrices are also important for growth and phenotypic maintenance of the cells producing the matrix, and developmentally regulated changes in matrix composition may be essential for the formation of functionally normal adult tissues.

The last portion of this article will describe what is known about retinal extracellular matrices and the effects of matrix components on retinal neurons. While these topics are the main goal of this discussion, we will first provide some general background about extracellular matrix molecules and their influence on growth and differentiation. Most examples will unavoidably be from sources other than retina, but it is hoped that they will provide necessary insight to investigators involved in retinal research.

II. Extracellular Matrices

A. General Composition

1. Collagens

Collagenous proteins have a widespread distribution in the body. These proteins are unique in that every third amino acid is glycine, with hydroxyproline and proline also being abundant. This unusual composition allows collagen to form long, rigid, triple helical molecules (Bornstein and Sage, 1980; Kühn, 1982). Because of this structure, collagens are resistant to most proteases, al-

though they are degraded by specific proteases, the collagenases (Gross and Nagai, 1965; Peterkofsky and Diegelmann, 1971; Gross *et al.*, 1974; Miller *et al.*, 1976; Horwitz *et al.*, 1977; Liotta *et al.*, 1979, 1981; Mainardi *et al.*, 1980). Collagens are, for the most part, insoluble under physiological conditions. They are secreted as longer, soluble molecules (procollagen) and then enzymatically processed for deposition as fibers in the extracellular matrix (Lapière *et al.*, 1971; Prockop *et al.*, 1979; Kühn, 1982). There are five well-characterized, genetically distinct collagen types, each composed of three polypeptides, called α-chains, arranged in a helical configuration (Table I). The most abundant is type I collagen, found in skin, tendon, bone, and most other tissues. It consists of two identical chains and a chemically different third chain, and is designated as $[\alpha 1\ (I)]_2\ \alpha 2$. It forms large, highly ordered, extensively cross-linked fibers arrayed in parallel bundles (Gross, 1974; Gay and Miller, 1978). Type II collagen consists of three identical α-chains, $[\alpha 1\ (II)]_3$, which differ from those found in type I (Miller and Matukas, 1969). It occurs as thin, short, randomly arrayed fibers and is found primarily in cartilage (Miller and Matukas, 1969; Hay *et al.*, 1978; Kühn and von der Mark, 1978), although a type II-like collagen has been identified in the vitreous (Swann *et al.*, 1972; Smith *et al.*, 1976; Schmut *et al.*, 1979). Type III collagen, $[\alpha 1\ (III)]_3$, is found as thin fibers, usually associ-

TABLE I

COLLAGEN TYPES

Designation	Representative associated cell types	Tissue distribution
Type I	Fibroblasts, osteoblasts, stromacytes	Skin, bone, tendon, cornea, widespread in interstitial connective tissues
Type II	Chondrocytes, notochord cells, neural retinal cells	Cartilage, vitreous, notochord, chick cornea, nucleus pulposus
Type III	Fibroblasts, muscle cells	Skin (more prominent in fetal skin), blood vessels, sclera, parenchymal organs, "reticulin fibers" (?)
Type IV	Epithelial and endothelial cells, Schwann cells	Basement membranes
Type V	Smooth muscle cells, skeletal muscle cells, osteoblasts	Widespread (may be pericellular), placental membranes, smooth muscle, skeletal muscle, blood vessels, some basement membranes, corneal stroma, bone

ated with cells in blood vessels, in the parenchyma of internal organs, and in skin and sclera (Chung and Miller, 1974; Epstein, 1974; Schmut, 1978). It probably corresponds to the "reticular fibers" of classical literature. Type IV collagen has been found only in basement membranes (Timpl *et al.*, 1978; Kefalides *et al.*, 1979) and is composed of two distinct chains (Kresina and Miller, 1979; Robey and Martin, 1981). However, unlike type I collagen, the α1 (IV) and α2 (IV) chains may occur in separate triple helical molecules (Robey and Martin, 1981). These molecules are often considered procollagen-like because they are longer and more soluble than the interstitial collagens and are incorporated into the basement membrane without extensive processing (Timpl *et al.*, 1978, 1981; Tryggvason *et al.*, 1980). Type IV collagen is more sensitive to proteases because its helical portion is interrupted by globular domains. This structure allows for a more open continuous network rather than a fibrous structure (Timpl *et al.*, 1981; Kühn, 1982). Type V collagen is also composed of two types of chains and is found in skin, placenta, bone, cartilage, and smooth muscle cells (Burgeson *et al.*, 1976; Chung *et al.*, 1976; Rhodes and Miller, 1978; Sage and Bornstein, 1979), often associated with the cell surface (Gay *et al.*, 1981). [For a more detailed discussion of these collagens and the increasing number of minor collagens, see reviews by Linsenmayer (1981), Kühn (1982), Timpl (1982), and Mayne (1984).]

2. Proteoglycans

These macromolecules are ubiquitous in extracellular matrices and have also been demonstrated in association with cell membranes (Kjellén *et al.*, 1980). They are composed of a core protein to which sulfated polysaccharides, called glycosaminoglycans, are covalently bound (Table II). Sulfation makes these molecules highly negatively charged (Hascall and Sajdera, 1970; Antonopoulous *et al.*, 1974). Glycosaminoglycans are polymers of repeating disaccharide subunits, the composition of which defines the glycosaminoglycan as chondroitin sulfate, dermatan sulfate, keratan sulfate, or heparan sulfate (Hascall and Hascall, 1981). Proteoglycans are often named on the basis of their glycosaminoglycan portion. Thus, "chondroitin sulfate proteoglycans" can be found in cartilage, cornea (Hassell *et al.*, 1979), brain (Kiang *et al.*, 1981), retina (Aquino *et al.*, 1984a), and Bruch's membrane (Robey and Newsome, 1983). However, most of these proteoglycans are not identical since their core proteins represent different gene products. Normally, only one type of glycosaminoglycan is found attached to a core protein. The exception (once thought to be the rule) is the cartilage proteoglycan which contains both chondroitin sulfate and keratan sulfate attached to the same core protein (Hascall and Hascall, 1981).

The size, type, degree of sulfation, number of side chains, and chemical nature of the core protein give a great deal of diversity to these macromolecules,

TABLE II

EXAMPLES OF PROTEOGLYCANS

Type/tissue	Molecular weight[a]		
	Overall	Core protein	GAG[b]
Chondroitin sulfate			
Cartilage[c]	$2–3 \times 10^6$	350,000	20,000
Cornea	150,000	80,000	55,000
Keratan sulfate			
Cornea	80,000	40,000	6,000
Heparan sulfate			
EHS-sarcoma	$0.5–1 \times 10^6$	350,000	70,000
Glomerular basement membrane	130,000	26,000	26,000
Liver plasma membrane	80,000	24,000	14,000

[a] Values represent approximate average molecular weights.

[b] Glycosaminoglycan.

[c] Cartilage proteoglycan is predominantly chondroitin sulfate, although keratan sulfate is often present attached to the same core protein. In other tissues, only one type of glycosaminoglycan is linked to a core protein.

which are often tissue specific (e.g., see Stow *et al.*, 1985, for a striking example of diversity and differential distribution of heparan sulfate proteoglycans). These properties permit specific interactions of proteoglycans with other matrix components (Hascall, 1977; Kleinman *et al.*, 1983; Woodley *et al.*, 1983), which may be important in defining whether they function as structural, hydration, or filtration elements. [For a more extensive review of structure and function, see Hascall and Hascall (1981).]

3. GLYCOPROTEINS

This is probably the largest, most varied, and least understood and characterized category of extracellular matrix components. While many potentially important glycoproteins have been found in different matrices, their functions frequently remain obscure. Attachment proteins, which anchor cells to their matrix, represent one family of glycoproteins with a defined function (Hewitt and Martin, 1984). Fibronectin is a large (M_r 440,000), elongated glycoprotein composed of two disulfide-linked chains (Mosesson *et al.*, 1975; Yamada *et al.*, 1977). Various domains along the fibronectin molecule interact with other molecules, such as collagen, heparin, and cell surface molecules (Balian *et al.*, 1979; Hahn and Yamada, 1979a,b; Kleinman *et al.*, 1979; Ruoslahti *et al.*, 1979; Ruoslahti and Engvall, 1980; Yamada *et al.*, 1980, 1981; Spiegel *et al.*, 1985).

Besides being found in serum (Yamada and Olden, 1978), fibronectin is found in many connective tissues (Linder *et al.*, 1975; Stenman and Vaheri, 1978; Wartiovaara *et al.*, 1979), where it functions as an attachment protein for cells, such as fibroblasts (Klebe, 1974; Pearlstein, 1976), myoblasts (Chicquet *et al.*, 1979), or neural crest cells (Greenberg *et al.*, 1981). Fibronectin is a chemoattractant for a number of cell types, including fibroblasts, neural crest, and smooth muscle cells (Gauss-Müller *et al.*, 1980; Seppä *et al.*, 1981; Greenberg *et al.*, 1981; Grotendorst *et al.*, 1981, 1982). This function may have important implications during development and wound healing.

Laminin (M_r 1×10^6) is produced by epithelial, endothelial, and glial cells and is found in basement membranes (Timpl *et al.*, 1979; Foidart *et al.*, 1980a,b; Laurie *et al.*, 1982; Liesi *et al.*, 1983; Cornbrooks *et al.*, 1983; McGarvey *et al.*, 1984). It consists of two different-sized chains (M_r 400,000 and 200,000), arranged in a cross configuration (Timpl *et al.*, 1979; Engel *et al.*, 1981). Different binding sites localized in specific, distinct domains allow laminin to interact with other matrix components and with cells (Terranova *et al.*, 1980, 1983; Sakashita *et al.*, 1980; Del Rosso *et al.*, 1981; Woodley *et al.*, 1983; Kleinman *et al.*, 1983). Laminin mediates endothelial and epithelial cell attachment to type IV (basement membrane) collagen (Terranova *et al.*, 1980).

Chondronectin (M_r 175,000), the chondrocyte attachment factor, is found pericellularly in cartilage, as well as in serum and in vitreous (Hewitt *et al.*, 1980, 1982a). Although all three attachment proteins interact with proteoglycans, only chondronectin appears to have a specific proteoglycan requirement for its attachment activity (Hewitt *et al.*, 1982b). It is likely that there are other "adhesion" molecules with varying cell and substrate specificities. [For a more thorough discussion of attachment proteins, see Kleinman *et al.* (1981), Ruoslahti *et al.* (1981), Hynes (1981), Hynes and Yamada (1982), Timpl (1982), and Hewitt and Martin (1984).]

A number of other glycoproteins, some of which may be tissue specific, have been identified, but the functions of only a few have been well characterized. For example, cartilage link protein stabilizes huge proteoglycan aggregates formed by proteoglycan monomers and hyaluronic acid (Hascall, 1977). Osteonectin, a bone-specific protein that interacts with both collagen and hydroxyapatite, may be involved in initiating mineralization of skeletal tissue (Termine *et al.*, 1981). Directly relevant to the retina is the retinol-binding protein of the interphotoreceptor matrix (see Section III,B). These are just a few examples of the large number of glycoproteins in different extracellular matrices. Many of these vary during development, or may be quantitatively or qualitatively altered in genetic mutants or in pathological conditions. However, to date, most matrix glycoproteins are no more than bands on the biochemists' gels and, consequently, they represent an exciting area of investigation.

B. Correlation of Supramolecular Structure and Function

Stoichiometrically specific interactions of these different macromolecules are important because they give matrices their characteristic organization, structure, and function. For example, in the cornea, the properties of the small keratan sulfate and chondroitin sulfate proteoglycans enable them to fit within the highly ordered pattern of type I collagen. This allows for the hydration of this tissue and possibly also for its transparency (Trelstad and Coulombre, 1971; Hassell *et al.*, 1979; Hascall and Hascall, 1981). In basement membranes, the specific interaction of a large heparan sulfate proteoglycan intercalated between laminin and the large open network of type IV collagen creates a negatively charged shield against the passage of similarly charged macromolecules (Kanwar and Farquhar, 1979; Hassell *et al.*, 1980; Kanwar *et al.*, 1980; Timpl *et al.*, 1981). Pathological conditions can result from disruptions of these interactions caused by impaired synthesis, injury, or some other insult. For example, increased degradation of the basement membrane heparan sulfate proteoglycan in diabetes causes compensatory synthesis not only of the proteoglycan, but also of laminin and of type IV collagen, resulting in an abnormally thick basement membrane (Rohrbach *et al.*, 1982).

C. Cellular Responses to Extracellular Matrices

1. Attachment and Growth

Most normal cells require anchorage to a substratum in order to proliferate. Attachment and proliferation are influenced by extracellular matrices which in some cases are produced by the cells themselves. For example, spreading and proliferation of connective tissue cells are inhibited if they are cultured on tissue culture plastic in the presence of *cis*-hydroxyproline, a proline analog that prevents collagen secretion. This is not a toxic effect, since growth is not affected by *cis*-hydroxyproline if the cells are plated on a preformed collagenous substrate (Liotta *et al.*, 1978). Other ECM molecules are probably also capable of supporting proliferation. Some cells grow much faster when cultured on complex extracellular matrices elaborated by other cells than on either tissue culture plastic or collagen-coated plastic (Gospodarowicz *et al.*, 1978, 1980). In certain cases, attachment factors, such as laminin, will enhance the growth of cells dependent on that factor for attachment (McGarvey *et al.*, 1984). Regulation of growth has also been attributed to certain heparan sulfate proteoglycans which are synthesized by postconfluent smooth muscle cells and have an antiproliferative effect (Fritze *et al.*, 1985).

2. Development

It is becoming increasingly apparent that matrix components, either individually or in appropriate combinations within a supramolecular complex, can influence developmental processes. In some cases, these effects can be regionally specific. Thus, cartilage formation by cranial neural crest cells is stimulated by the extracellular matrix derived from retinal pigmented epithelium (Newsome, 1976). Similarly, differentiation of catecholamine-containing cells is stimulated by somite-produced ECM, which neural crest cells encounter during their migration (Cohen, 1972; Norr, 1973), as well as by fibronectin alone (Sieber-Blum *et al.*, 1981), which has been demonstrated along crest migration routes (Newgreen and Thiery, 1980). Collagen can act as a promoter and stabilizer of stroma production by corneal epithelium (Dodson and Hay, 1974; Meier and Hay, 1974) and of cartilage formation by embryonic chick somites (Kosher and Church, 1975; Lash and Vasan, 1978). In other cases (i.e., salivary gland and lung morphogenesis), both collagen and basal lamina proteoglycans are involved in epithelial–mesenchymal intereactions (Grobstein and Cohen, 1965; Bernfield and Banerjee, 1978; Spooner and Faubion, 1980).

Heterotypic cell interactions are often important in establishing an ECM and in regulating development. For example, laminin stimulates Schwann cell attachment and proliferation (McGarvey *et al.*, 1984) and will also elicit neurite outgrowth by sensory ganglia and retinal neurons in culture (Baron-Van Evercooren *et al.*, 1982; Adler and Hewitt, 1983; Adler *et al.*, 1985; Manthorpe *et al.*, 1983; Rogers *et al.*, 1983; Smalheiser *et al.*, 1984). Since the neuronal cells do not produce laminin, it is possible that the laminin produced by adjacent glial-like cells is inducing this response in neurons.

In many organs and tissues, there appears to be a distinct developmental timetable for the regional appearance and disappearance of some matrix molecules. For example, fibronectin is present at early stages of cerebellar development, but is lost at later stages (Hatten *et al.*, 1982). There are also developmentally controlled alterations in the distribution of other ECM molecules, such as the chondroitin sulfate proteoglycan in the brain (Aquino *et al.*, 1984b). It is possible that the presence or removal of one ECM molecule acts as a cue for the next stage of a differentiative sequence, as appears to be the case for myogenesis (Chen, 1977; Furcht *et al.*, 1978; Podleski *et al.*, 1979; Foidart *et al.*, 1980b; Tomesek *et al.*, 1982).

3. Maintenance of Phenotypic Expression

Cell behavior can be modified by disruption of normal interactions between the cells and their extracellular matrix or by alterations in the molecular composition of the latter. This is particularly evident when cells, removed from their *in*

vivo microenvironment and plated on tissue culture plastic, undergo alterations of their phenotypic expression (i.e., loss of marker enzymes, switching of collagen type or other ECM molecules synthesized, and/or altered morphology).

In recent years, some success has been achieved in restoring or maintaining normal phenotypic expression by modifying the substrate on which cells are grown. Phenotypic expression of chondrocytes is maintained more efficiently when grown on an exogenous ECM than on tissue culture plastic (Kata and Gospodarowicz, 1985). Also, in several neuronal systems, substrate-bound molecules, such as PNPF, laminin, and fibronectin, are capable of stimulating neurite production (see Section IV,B) (Akers *et al.*, 1981; Adler, 1982; Manthorpe *et al.*, 1981, 1983; Rogers *et al.*, 1983). "Biomatrix," a substratum prepared from extracts of the parent tissue, is also useful for the long-term culture of some types of differentiated cells (Rojkind *et al.*, 1980). This would suggest that there are tissue-specific matrix components which are important for the long-term retention of phenotype in culture and, presumably, the maintenance of phenotypic expression and localization of cells *in situ* as well.

III. Retinal Extracellular Matrices

In this section, we will review extracellular matrices associated with the retina. Studies in this area are relatively scant, but growing interest in extracellular matrices and their effects on differentiation and maintenance of phenotypic expression suggest that this aspect of retinal research will provide much needed information in the near future.

A. Bruch's Membrane

While Bruch's membrane is not universally accepted to be part of the retina, this structure is strategically located between the outer retina–RPE complex and their primary source of nutrition, the choriocapillaris. It functions as a support element and attachment site for pigmented epithelium cells and also appears to provide a selective filtration barrier for nutrients reaching the retina via the choriocapillaris.

1. Structure

Bruch's membrane can be ultrastructurally divided into five parts (Fig. 1): (1) the retinal pigmented epithelium basement membrane, (2) the inner collagenous zone, (3) the central elastin-bearing layer, (4) the outer collagenous zone, and (5)

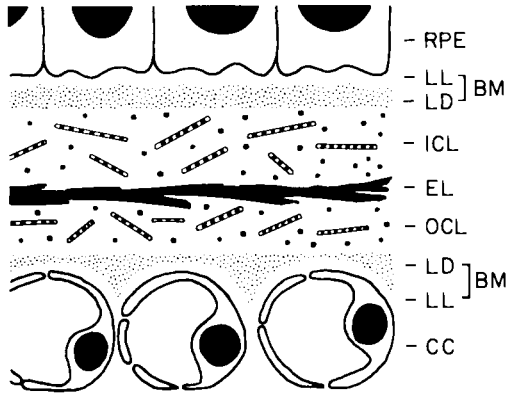


FIG. 1. Schematic representation of Bruch's membrane. This complex structure between the retinal pigmented epithelium (RPE) and the choriocapillaris (CC) is composed of two basement membranes (BM), each with its component lamina lucida (LL) and lamina densa (LD), an inner and an outer collagenous layer (ICL and OCL, respectively), and a thin elastin-bearing layer (EL). [Reprinted from Robey and Newsome (1983). *Invest. Ophthalmol. Visual Sci.* **24**, 898–905, with permission.]

the choriocapillaris basement membrane. The same basic structure has been found in all animals studied, with some alterations in animals bearing a tapetum lucidum (Nakaizumi, 1964a,b).

a. Light Microscopy and Immunocytochemistry. Bruch's membrane is periodic acid–Schiff stain (PAS) positive, the reaction becoming more intense with increasing age (Hogan and Alvarado, 1967; Hogan *et al.*, 1971; Feeney, 1973b); this increase appears to be correlated with increased ground substance seen with EM (Hogan *et al.*, 1971). In man, areas of basophilia also become evident with age. Immunofluorescent studies in human Bruch's membrane (Fig. 2) have demonstrated the ubiquitous basement membrane components, type IV collagen, laminin, and the basement membrane heparan sulfate proteoglycan. Similar studies detect collagen types I and III between the basement membrane portions of Bruch's membrane (P. G. Robey and D. A. Newsome, unpublished observations). Antibodies against the brain-derived chondroitin sulfate proteoglycan (Kiang *et al.*, 1981) also react with murine Bruch's membrane (Aquino *et al.*, 1984a).

b. Electron Microscopy. The outer layers of Bruch's membrane have the ultrastructural appearance of typical basement membranes (Nakaizumi, 1964a,b; Nakaizumi *et al.*, 1964; Hogan *et al.*, 1971; Feeney, 1973b). They stain with phosphotungstic acid while the collagenous layers do not (Feeney, 1973b). Both

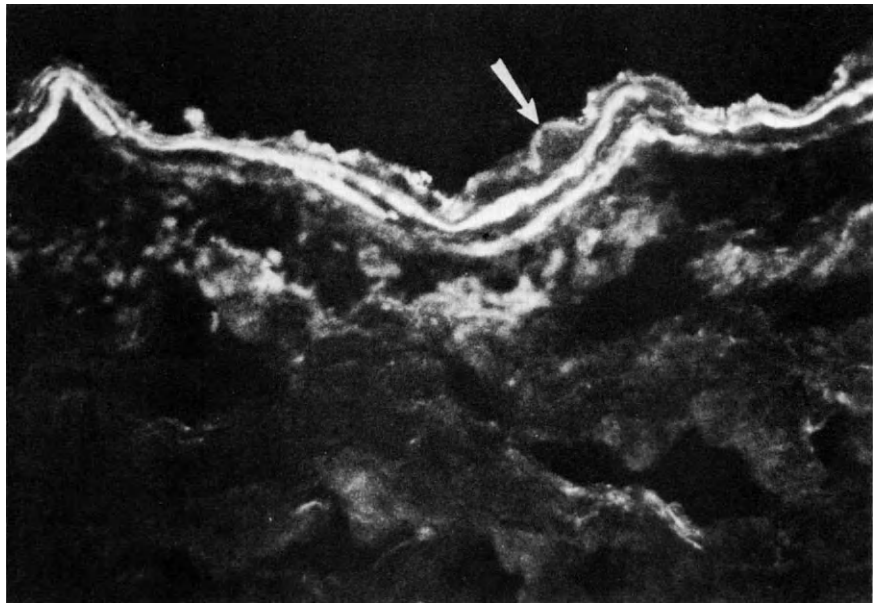


FIG. 2. Laminin immunoreactivity in human Bruch's membrane. Frozen section of Bruch's membrane from the eye of a 76-year-old donor was reacted with antibodies against laminin. Note the dual lines of fluorescence, representing the two basement membranes of Bruch's membrane. Note that the surface of drusen (arrow) also react with the antibodies. Fluorescence in the choroid is associated with the basement membranes in vessel walls. ×200. (Courtesy of Dr. David A. Newsome.)

collagenous layers are composed of loosely arranged, interwoven collagen fibrils generally, but not exclusively, oriented parallel to the outer retina. Collagen fibrils appear to be continuous through the elastic layer. Within the collagenous zones, collagen fibrils are embedded in granular ground substances (Nakaizumi, 1964a; Hogan *et al.*, 1971). Cationic markers, such as polyethyleneimine and ruthenium red, bind at regular intervals along the length of the collagen fibrils (Pino *et al.*, 1982; Essner and Pino, 1982), suggesting the presence of polyanionic molecules, such as proteoglycans.

The elastic layer of Bruch's membrane is a continuous, regular meshwork of long, thin, straight fibers. Numerous vesicles, electron-dense structures, and collagen fibrils lie in the spaces between and are closely associated with the intersecting elastin fibers (Hogan *et al.*, 1971).

Bruch's membrane undergoes as much as a threefold thickening with age (Mishima *et al.*, 1978), apparently resulting from an accumulation within the collagenous layers of short-segment, long-spacing collagen and other unknown

substances (Hogan and Alvarado, 1967; Hogan *et al.*, 1971). In man, crystal formation and calcification in the macular portion of the elastic layer are often associated with aging (Nakaizumi *et al.*, 1964; Hogan and Alvarado 1967; Sarks, 1980).

2. Biochemistry

There have not been many direct biochemical studies of Bruch's membrane components, although, as we have seen, some information has been inferred by histochemical and immunochemical procedures. In confirmation of the immunofluorescence studies, extracts of human and monkey Bruch's membranes contain collagen types I, III, and IV as well as small amounts of type V collagen (P. G. Robey and D. A. Newsome, unpublished observation).

Both heparan sulfate and chondroitin sulfate proteoglycans have been extracted from human and nonhuman primate Bruch's membrane. As expected for a basement membrane, the heparan sulfate-containing proteoglycans are predominant (greater than 60%) in unlabeled human and monkey Bruch's membrane, with the remainder being chondroitin/dermatan sulfate (Robey and Newsome, 1983). However, when Bruch's membrane proteoglycans are radiolabeled *in vitro*, chondroitin sulfate represents 75–80% of the labeled proteoglycans with the remainder being heparan sulfate (Robey and Newsome, 1983; Hewitt and Newsome, 1983, 1985a). In contrast, heparan sulfate predominates in glomerular basement membranes and Reichert's membrane labeled under similar conditions (Kanwar *et al.*, 1981; Hogan *et al.*, 1982). This difference, which probably reflects variations between the two systems in rates of synthesis and/or turnover, emphasizes the uniqueness of Bruch's membrane as a basement membrane. The synthesis of Bruch's membrane proteoglycans is altered in patients with retinitis pigmentosa. Radiolabeled Bruch's membranes show a higher molecular weight proteoglycan as well as a two- to threefold increase in the proportion of heparan sulfate (Hewitt *et al.*, 1985a). It is possible that altered proteoglycan synthesis could adversely affect the flow of nutrients from the choriocapillaris to the RPE and outer retina. Consequently, proteoglycans and Bruch's membrane should not be overlooked when studying RP and other pathological conditions of the retina.

3. Function

The location of Bruch's membrane between the RPE and the choriocapillaris suggests that this structure might act as a filtration barrier. Canine Bruch's membrane–choroid preparations (Lyda *et al.*, 1957) and murine choriocapillaris (Pino and Essner, 1981) have been shown to have a net negative charge and act as a molecular barrier. EM histochemical studies with a number of cationic probes have established that these anionic sites, due to the presence of at least

two proteoglycans, can be visualized as a regular staining pattern along both of the basement membrane components, as well as in a semiregular network within the collagenous layers (Pino *et al.*, 1982; Essner and Pino, 1982). Differential enzyme treatments suggested that heparan sulfate is associated primarily with the RPE basement membrane and chondroitin sulfate with the basement membrane of the choriocapillaris and the collagenous layers (Pino *et al.*, 1982).

Bruch's membrane also functions as a support element and attachment site for retinal pigmented epithelial cells. The presence of type IV collagen and laminin juxtaposed to the RPE is structurally significant, since these cells use laminin to attach to type IV collagen (Terranova *et al.*, 1980). Collagen from the inner collagenous layer merges with filaments coming from the RPE basement membrane. Fine filaments on the inner surface of the RPE basement membrane in turn merge with the plasma membrane of the RPE cells (Hogan *et al.*, 1971). All of these interactions could explain the strong bond between the RPE and Bruch's membrane. Perturbation of this firm interaction could affect phenotypic expression by RPE cells and lead to RPE cell migration and loss of differentiated function as seen in massive periretinal proliferation (Machemer *et al.*, 1978; Newsome *et al.*, 1981), abnormal periretinal collagen deposition (Laqua, 1981), and retinitis pigmentosa (Szamier and Berson, 1977). Such changes in the RPE could have far-reaching effects on the retina, since it is thought that, when the RPE or its normal function is lost, so are overlying photoreceptors (Sarks, 1976; Green, 1980).

B. Interphotoreceptor Matrix

1. Structure

a. Light Microscopy. The interphotoreceptor matrix (IPM) lies in the space between the pigmented epithelium and the outer limiting membrane, an unusually large extracellular space for neural tissues. In 1909, Kolmer (as discussed in Röhlich, 1970) described an intercellular substance in the layer of rods and cones. Metachromatic staining of a substance between the photoreceptors (Wislocki and Sidman, 1954) as well as positive staining with alcian blue (Sidman, 1958; Zimmerman and Eastham, 1959) and colloidal iron (Fine and Zimmerman, 1963; Hall *et al.*, 1965; Ocumpaugh and Young, 1966) suggested the presence of glycosaminoglycans in the interphotoreceptor matrix.

b. Electron Microscopy. The interphotoreceptor matrix can have either an amorphous or filamentous appearance in the electron microscope depending on the fixation and staining procedures used (Röhlich, 1970). Lanthanum binding of the extracellular space around rods indicates the presence of amorphous material

(Cohen, 1968). In contrast, uniform staining of the cell surface and strands of material in the interphotoreceptor space can be seen in the murine eye with colloidal iron and with ruthenium red, a cationic dye that binds to polysaccharide-rich macromolecules. Alcian blue, on the other hand, shows intermittent dense regions on cell surfaces and matrix materials. With periodic acid–silver methanamine stain, there is a reaction with strands within the matrix and on the surfaces of cells bordering the space in the adult, but not in the early murine embryo (Feeney, 1973b). The binding of these reagents to the interphotoreceptor matrix and to cell surfaces (Cohen, 1968; Röhlich, 1970; Feeney, 1973b) can be compared to the sialic acid-containing coating of many cells (Benedetti and Emmelot, 1967; Rambourg and Leblond, 1967). Alcian blue and colloidal iron staining also indicates the presence of acidic groups in the matrix, probably representing sulfated molecules, such as glycosaminoglycans (Zimmerman, 1958; Fine and Zimmermen, 1963; Röhlich, 1970; Feeney, 1973b). The presence of glycosaminoglycans and sialic acid-containing molecules has been confirmed by biochemical analyses (see below).

2. Biochemical Analysis

While there is only a small amount of interphotoreceptor matrix per eye [from less than 10 μl in murine eyes to 100 μl in bovine eyes (Hogan *et al.*, 1971; Röhlich, 1970; Adler and Severin, 1981)], it is easily extracted using phosphate-buffered saline. This can be done by rinsing either dissected retinas and RPE surfaces (Berman and Bach, 1968; Bach and Berman, 1970, 1971a,b; Adler and Severin, 1981; Adler and Klucznik, 1982a; Berman, 1982) or by cannulation and lavage of the interphotoreceptor space (Pfeffer *et al.*, 1983), the latter being particularly useful when large amounts of material are not required. Using these procedures, a great deal of information has been obtained regarding the carbohydrate and glycoprotein composition of the IPM.

a. Complex Carbohydrates. Approximately 75% of the polysaccharides in bovine IPM contain uronic acid and are hyaluronidase sensitive [15% hyaluronic acid, 45% partially sulfated chondroitin, and 15% fully sulfated chondroitin (Berman and Bach, 1968; Bach and Berman, 1971a)]. Human IPM has a similar composition, but lacks fully sulfated chondroitin (Edwards, 1982). IPM sulfated glycosaminoglycans also contain the neutral sugars galactose and xylose (Bach and Berman, 1971b), which are normally found in the carbohydrate–core protein linkage region of proteoglycans. With their low amino acid content and an average molecular weight of 9200 (Bach and Berman, 1971b), a size suggesting free glycosaminoglycans, it would appear that what are thought of as intact proteoglycans are not being extracted by the procedures used.

The remaining 25% of IPM complex carbohydrates are unsulfated and are

resistant to hyaluronidase. They lack uronic acid and are composed of galactosamine, glucosamine, sialic acid, fucose, and neutral sugars, with sialic acid and fucose being localized in terminal positions. They have an average molecular weight of approximately 5000. Alkaline-labile *O*-glycosidic bonds are the predominant linkages between the carbohydrate and peptide portions of these molecules (Berman and Bach, 1968; Bach and Berman, 1970, 1971a,b). This group of molecules was originally classified as sialoglycans because of their low protein and high sialic acid content. However, because of their size and structure, this group would appear to represent the oligosaccharide portions and small peptide fragments of the glycoproteins found in the interphotoreceptor matrix.

b. Glycoproteins: General Findings. When extracted from dissected eyes with saline and analyzed by SDS–polyacrymide gel electrophoresis under reducing conditions, the interphotoreceptor matrix is composed of 11 major proteins and a number of minor proteins ranging in molecular weight from about 20,000 to 140,000. In unreduced gels, only one protein (M_r 64,000) is affected, indicating that it probably is not albumin and also that the other proteins are not disulfide-linked subunits of larger molecules. Only the PAS-positive protein with M_r 140,000 binds to a Con A–Sepharose affinity column (Adler and Severin, 1981; Adler and Klucznik, 1982a). In this glycoprotein the oligosaccharides are linked through asparagine (Adler and Klucznik, 1982a) rather than serine and threonine as was found for most IPM carbohydrates (Bach and Berman, 1970). Fewer proteins are extracted with *in situ* lavage of the interphotoreceptor space (Pfeffer *et al.*, 1983). The reason for this is not clear, although it is possible that either the *in situ* procedure is too gentle to extract more tightly bound molecules or that the standard procedure extracts contaminants from broken cells and/or the vitreal surface of the retina. Regardless of the procedure used, however, the extracted proteins are distinct from those found in homogenates of either rinsed pigmented epithelium or retina as well as from those found in pathological subretinal fluid (Adler and Severin, 1981). Bovine IPM is also distinct from serum proteins (Adler and Severin, 1981), although human IPM appears to contain some serum protein contaminants (Adler and Evans, 1985a).

The interphotoreceptor matrix contains approximately 10% of the acid proteases in the retina–IPM–RPE functional unit (Adler and Martin, 1983). While their exact function in the IPM is not clear, there is some histochemical evidence that photoreceptor outer segment disk membranes may be partially hydrolyzed prior to phagocytosis by the RPE (Bernstein, 1980).

Neither rhodopsin nor fibronectin are components of the IPM (Adler and Klucznik, 1982a). Interestingly, collagen also appears to be absent. Although collagen would not be soluble under the conditions used to extract this matrix, EM studies also do not show any typical collagen fibrils in the interphotoreceptor matrix. Pigmented epithelial cells in culture have been shown to synthesize

collagen (Newsome and Kenyon, 1973; Li *et al.*, 1984) and to release it into the medium (Li *et al.*, 1984). RPE cells will also deposit collagen under certain pathological conditions when they become detached from Bruch's membrane (Machemer *et al.*, 1978; Laqua, 1981). It is possible that, under normal conditions *in vivo*, any collagen synthesized is directed basally toward Bruch's membrane and not toward the interphotoreceptor matrix.

c. Interphotoreceptor (Interstitial) Retinol-Binding Protein (IRBP). The IRBP is the most abundant and best characterized IPM glycoprotein. Its presence was hypothesized based on changes in retinol levels in the retina and RPE during the light–dark cycle (Dowling, 1960; Berman *et al.*, 1979), the lability of free retinol, and the potentially toxic effects of free vitamin A (Dewar *et al.*, 1975; Meeks *et al.*, 1981).

IRBP corresponds to the Con A-binding glycoprotein mentioned in the previous section (Adler and Klucznik, 1982a; Adler and Evans, 1983). It accounts for approximately 5% of the total IPM protein extracted from bovine eyes (Adler and Evans, 1985a). Molecular weight determinations have varied depending on the technique used, with estimates of approximately 140,000 from SDS–gel electrophoresis (Adler and Klucznik, 1982a; Adler and Evans, 1983; Hollyfield *et al.*, 1984; Rayborn *et al.*, 1984; Redmond *et al.*, 1985; Saari *et al.*, 1985) and between 250,000 and 290,000 from gel filtration (Adler and Martin, 1982; Liou *et al.*, 1982; Lai *et al.*, 1982). Sedimentation equilibrium centrifugation analyses resolved this discrepancy with molecular weight estimates between 103,000 and 134,000 (depending on species) and by indicating that IRBP is an elongated molecule, a property which can influence behavior on a gel filtration column (Adler and Evans, 1984, 1985b; Redmond *et al.*, 1985; Saari *et al.*, 1985). This may also, in part, explain why IRBP cannot penetrate the external limiting membrane which is permeable to some proteins (Bunt-Milam and Saari, 1984).

Binding studies using [^{3}H]retinol and IPM derived from washing dissected retinas and analyzed either by sucrose density gradients or by gel filtration give two peaks of retinol-binding activity, at 2 S and 7 S (Adler and Martin, 1982; Lai *et al.*, 1982; Liou *et al.*, 1982). The 2 S activity corresponds to the cellular retinol-binding protein (M_r 17,000) that is common to many tissues including retina and RPE (Wiggert and Chader, 1975; Saari and Futtermen, 1976; Goodman, 1980) and is not present when IPM is obtained by *in situ* cannulation and lavage (Pfeffer *et al.*, 1983). The retinol-binding activity due to the larger molecule is specific for the IPM and is associated with the photoreceptor outer segment, since it is not present either before outer segment development (Wiggert *et al.*, 1978) or when outer segments are absent as in retinitis pigmentosa (Bergsma *et al.*, 1977). In addition, the concentration increases when outer segments are partially purified (Lai *et al.*, 1982). These findings and the immunological localization of IRBP selectively to the interphotoreceptor matrix (Bunt-

Milam and Saari, 1983, 1984) suggest that this glycoprotein may represent a peripheral protein which is loosely associated with the outer surface of the rod outer segments.

While the amount of IRBP in the IPM is not altered by light or dark adaptation (Adler and Evans, 1985a), retinol binding to IRBP is sensitive to light adaptation with more ligand being bound in the light and being depleted of bound retinol in the dark (Adler and Martin, 1982; Lai *et al.*, 1982; Liou *et al.*, 1982; Adler and Evans, 1983, 1985a). Retinol binds less tightly to IRBP (dissociation constant, 10^{-6} *M*) than to the cellular retinol binding protein (dissociation constant of 1.6×10^{-8} *M*) (Adler and Evans, 1983, 1984), indicating that retinol can be transferred from the matrix retinol-binding protein to the cellular retinol-binding protein during the visual cycle. In addition to binding retinols, IRBP also binds retinals with more of the latter being bound in dark-adapted eyes (Adler and Spencer, 1985). Consequently, IRBP is capable of binding all of the retinoid isomers involved in the visual cycle.

3. Origin of the IPM

a. General Findings. The interphotoreceptor space forms between the walls of the optic cup following invagination of the optic vesicle and, consequently, corresponds to the ventricular space in the brain. Early in development, the extracellular matrix corresponds primarily to a cell coat, although weblike strands accumulate as the slit undergoes rapid expansion and the photoreceptor outer segments project into the space (Feeney, 1973a). In the adult, there are three possible sources for the matrix, since the space is bordered by the pigmented epithelium, the photoreceptors, and the slender processes of Müller cells. Over the years, studies using a variety of cytochemical techniques have attempted to elucidate the source of the IPM based on either the close proximity of stained material to a cell or the presence of similarly reacting material both in the interphotoreceptor space and intracellularly. Consequently, both the retina (e.g., Johnson, 1934; Fine and Zimmerman, 1963) and the RPE (e.g., Kolmer, 1909; Zimmerman and Eastham, 1959; Rohlich, 1970) have been cited as contributors to the IPM. Indeed, all three cell types lining the space may contribute to the IPM, since their cell coats lie in the interphotoreceptor space (Feeney, 1973b).

Autoradiography of murine neural retina incubated with $^{35}SO_4$ showed an initial incorporation into photoreceptors followed at later stages by movement of label toward extracellular spaces. This study indicated that photoreceptor inner segments can synthesize glycosaminoglycans and secrete them into the interphotoreceptor space (Ocumpaugh and Young, 1966). This study, however, did not include the pigmented epithelium for comparison. Similar studies labeling with [^{3}H]fucose indicated a rapid labeling of all retinal layers and the pigmented epithelium. With time, there was a decrease of label within the pig-

mented epithelium and the inner segments, suggesting that labeled material moved to the cell coats within the interphotoreceptor matrix (Feeney, 1973c).

The concept that cells on both sides of the interphotoreceptor space contribute to the matrix is supported by several biochemical studies. The glycosaminoglycans synthesized by cultured RPE cells are similar to those found in the interphotoreceptor matrix and include hyaluronate and undersulfated chondroitin sulfate (Edwards, 1982). Chick neural retinas labeled in organ culture also produce chondroitin sulfate (Morris *et al.*, 1977), although less than 2% of the total glycosaminoglycans are undersulfated chondroitin (Morris and Ting, 1981). Thus, it is possible that the glycosaminoglycans found in the IPM are synthesized both by the pigmented epithelial cells as well as in the neural retina. All of the IPM proteins were accounted for as being produced by either the pigmented epithelium or the neural retina, by comparing the IPM proteins to those in either retina- or RPE-conditioned medium. While initial comparisons were based on size and do not necessarily assure identity, they suggested that the neural retina and RPE contribute different glycoproteins to the IPM (Adler and Severin, 1981).

b. Synthesis and Retrieval of IRBP. Immunoprecipitation of metabolically labeled IRBP from separate cultures of retina and pigmented epithelial cells have demonstrated that this molecule is synthesized by the retina (Bridges *et al.*, 1983; Fong *et al.*, 1984a,b). Pulse–chase autoradiography with [^{3}H]fucose demonstrated uptake by many cells within the retina. There was little or no loss of label from Müller cells with time. However, the intense labeling over the Golgi apparatus of rod inner segments was displaced with time into the interphotoreceptor matrix and could be immunoprecipitated from the medium, indicating that IRBP is synthesized by the inner segments of the photoreceptors (Hollyfield *et al.*, 1984, 1985a).

Apropos of its role in the transport of retinoids across the IPM, both photoreceptors and RPE cells internalize colloidal gold-coated IRBP. In the photoreceptors, IRBP colocalizes with acid phosphatase activity in multivesicular bodies, suggesting that it is not recycled by these cells. In RPE cells, however, IRBP is found in small, acid phosphatase-negative vesicles near the apical surface. It is tempting to speculate that IRBP is delivering and/or acquiring its ligand in these small vesicles before returning to the photoreceptors for its final utilization (Hollyfield *et al.*, 1985b).

4. Functions

Because of its location between the photoreceptors and the pigmented epithelium, the interphotoreceptor matrix could be important for the normal development and maintenance of the visual process. Nutrients or other important

factors from the choroidal vessels must cross the space. It has been proposed that rod outer segments may need a coating of interphotoreceptor matrix in order to be phagocytized by the pigmented epithelium cells (Feeney and Mixon, 1976; Mixon and Feeney, 1977). Also, it has been demonstrated that the pigmented epithelium is required for the development of the retina (Hollyfield and Witkovsky, 1974). What molecules are required and whether or not these molecules exert a "trophic" influence in the adult eye is not clear, although there is a loss of retinal function in areas where the retina has become detached from the pigmented epithelium (Kroll and Machemer, 1968).

A number of studies have focused on the role of the interphotoreceptor matrix in the attachment of the retina to the pigmented epithelium. The energy required to separate bovine retina from the RPE is greater in pigmented areas compared to regions with a tapetum lucidum (Zauberman and Berman, 1969) and is lower in tissue treated with hyaluronidase compared to buffer-treated controls (Berman, 1969). This suggested that glycosaminoglycans, or more correctly proteoglycans, may influence the strength of adhesion between the retina and pigmented epithelium, perhaps by interactions with other macromolecules in the interphotoreceptor matrix.

Other studies disagree with these traction experiments regarding the role matrix components on adhesion. Adler and Klucznik (1982b) used rotary aggregation of pigmented epithelium and rod outer segments to test the effect of interphotoreceptor matrix components on reaggregation of RPE cells and photoreceptors. Lack of quantitative differences suggested that the glycoproteins and glycosaminoglycans of the interphotoreceptor matrix are not involved in rod outer segment–pigmented epithelium interactions, although it should be emphasized that these studies investigated the initiation of adhesion and not its maintenance. It was hypothesized that adhesion proteins exist in the adult as integral membrane components similar to the developmentally regulated retina-specific mediators of cell–cell interactions (Hausman and Moscona, 1975, 1976; Thiery *et al.*, 1977).

While this is entirely possible, the role of interphotoreceptor matrix molecules should not be ruled out for, despite the lack of quantitative affects, there were a number of qualitative effects on the cell interactions. For example, when incubated at 37°C for 24 hr, IPM will form long strands of a gelatin-like precipitate (Adler and Klucznik, 1982a). If the precipitate is allowed to form before adding cells, there appears to be an increased rate of aggregate formation, primarily with the strands as focal points. If the precipitate is removed, the supernatant had no affect on aggregation. Interestingly, the presence of intact rod outer segments consistently reduced the size of the pigmented epithelium cell aggregates even though the outer segments did not bind to the pigmented epithelial cells under these conditions (Adler and Klucznik, 1982b). These results are certainly suggestive that the interphotoreceptor matrix can influence cell interactions. It is

possible that other experimental designs may provide more clear-cut evidence regarding the role of IPM molecules in adhesion interactions.

The functions of most IPM components are not clear. A powerful tool for investigating these molecules is to radiolabel them either metabolically or by iodination. The latter procedure has been used to demonstrate the specific binding of a 20,000-Da protein specifically to RPE cells (Varner and Hollyfield, 1985). The use of molecules metabolically labeled either in whole eye organ culture (Robey and Newsome, 1983; Hewitt and Newsome, 1985a) or in cell culture (Edwards, 1982, 1984; Hewitt and Newsome, 1985b) could also prove useful in answering such questions as whether the interaction of interphotoreceptor matrix proteins with cell membranes is sensitive to light and dark (as has been demonstrated for some photoreceptor disk membrane proteins; Kühn, 1978, 1980), and whether the synthesis of a component by one cell type is dependent on the interaction with a component produced by another cell type.

C. Internal Limiting Membrane

There appear to be three horizontal, membrane-like structures within the retina: the internal, the middle, and outer limiting membranes. Of these, the middle and outer limiting membranes are indicative of synapses and cell junctions, respectively. Only the internal (or inner) limiting membrane (ILM) is a true extracellular matrix, with the ultrastructural appearance of a basement membrane in close association with collagenous fibrils of the cortical vitreous on one side and the branch processes of the Müller cells on the other (Sigelman and Ozanics, 1982).

1. Structure

a. Light Microscopy and Immunocytochemistry. The ILM is PAS positive and stains deep blue with the Mallory triple stain, suggesting the presence of glycoproteins and collagen, respectively (Hogan *et al.*, 1971). While it is difficult to do biochemical analyses on these structures, immunocytochemical studies have been helpful in elucidating its composition. Laminin has been localized by immunofluorescence in bovine (Kohno *et al.*, 1983) and chick (Jerdan *et al.*, 1984; Adler *et al.*, 1985) internal limiting membrane (Fig. 3). Antibodies to fibronectin also react with the ILM as well as with fine filaments projecting from the ILM into the vitreal cortex (Kohno *et al.*, 1983). The fine reactive fibrils within the vitreoretinal juncture may provide links between hyaluronate and collagen in the vitreous and ILM collagens. The ILM is also positive for the rat brain chondroitin sulfate proteoglyean (Aquino *et al.*, 1984) and, at least in the embryonic chick retina, for type II collagen as well (von der Mark *et al.*, 1977).

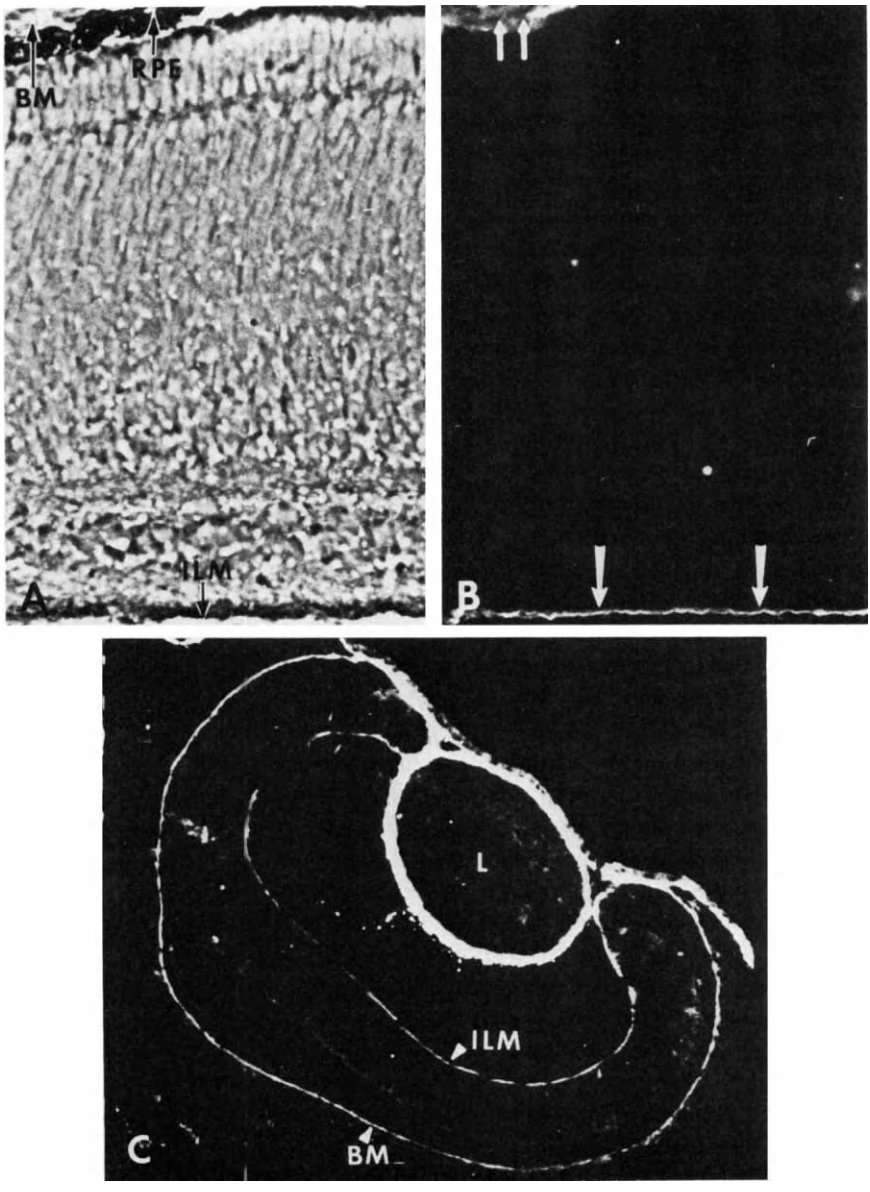


FIG. 3. Laminin immunoreactivity in embryonic chick internal limiting membrane. (A) Phase-contrast microscopy of an 8-day retina showing the retinal neuroepithelium, the internal limiting membrane (ILM), the retinal pigmented epithelium (RPE), and associated Bruch's membrane (BM). (B) Same section reacted with antibodies against laminin. Note immunoreactivity in the ILM (long arrows) and Bruch's membrane (short arrows). (C) Section through the eye of a 3-day embryo, demonstrating positive immunoreactivity in the ILM and Bruch's membrane (BM). There is also strong reactivity around the developing lens (L). (A, B) ×443; (C) ×110. (Reprinted from Adler *et al.*, 1985, with permission.)

In a study analyzing the molecular development of ocular basement membranes, a series of four monoclonal antibodies were reacted with ocular tissues from chick embryos at different developmental stages (Fitch and Linsenmayer, 1983). These antibodies showed different patterns of reactivity with the ILM: two antigens were continually reactive throughout development, one was not present at all, and the fourth made only transitory appearances. Moreover, for comparison, Bruch's membrane showed a strikingly different pattern of reactivity. Although the exact chemical nature of the antigens recognized by these antibodies is not known, it is apparent that different retinal-related basement membranes differ in composition and that they undergo developmentally regulated changes that are recognizable immunocytochemically.

b. Electron Microscopy. The general EM appearance of the ILM resembles that of other basement membranes. It is composed of a lamina rara interna which lies between the Müller cells and the lamina densa. A poorly defined layer, the lamina rara externa, lies on the vitreal side of the lamina densa. While the vitreal surface of the ILM is smooth, the surface facing the retina follows the terminal extensions of Müller cells to form the uneven, but continuous, border of attachment between the basement membrane and the cell surface (Hogan *et al.*, 1971; Tolentino *et al.*, 1976).

There is a great deal of topographic variation between species. The rabbit ILM has a uniform thickness over the entire retinal surface (Matsumoto *et al.*, 1984). This is in contrast to the regional variations in thickness seen in human (Hogan *et al.*, 1971; Tolentino *et al.*, 1976) and cynomolgus monkey (Matsumoto *et al.*, 1984). In primate eyes, the internal limiting membrane is thin in the area of the ora serrata and thickens as it extends toward the equatorial and posterior zones, but is markedly thin in the fovea (Foos, 1972; Matsumoto, 1984).

The internal limiting membrane is closely associated with sparse, thin collagen fibers and a relatively thick layer of complex carbohydrates, both along the vitreoretinal juncture in the cortical vitreous (Magalhães and Coimbra, 1972; Rhodes, 1982; Matsumoto *et al.*, 1984). The complex carbohydrates appear to be of two types. One class is represented by a fine filamentous network (*Streptomyces* hyaluronidase resistant), which is often associated with collagen fibrils and probably represents a network of noncollagenous glycoproteins. The other is hyaluronic acid of intermediate electron density (*Streptomyces* hyaluronidase sensitive) (Rhodes, 1982). Since large amounts of hyaluronic acid are a characteristic of some embryonic basement membranes that is lost in the mature structure (Trelstad *et al.*, 1974; Cohn *et al.*, 1977), the presence of hyaluronic acid on the surface of the ILM suggests that this structure differs from other mature basement membranes (Riepe and Norenburg, 1977; Rhodes, 1982).

2. Function

a. Attachment Site for Vitreous. The retina and vitreous are adherent to varying degrees along their entire interface, with the vitreous base and optic disc being the areas of strongest attachment. Attachment in the posterior fundus is the most tenuous so that vitreous detachment can easily occur in this area due to trauma or aging (Rhodes, 1982). Inflammatory retinal conditions affecting Müller cell morphology or conditions resulting in cortical vitreous contraction can cause wrinkling of the ILM due to these interactions. The most logical mechanism for this association is the insertion of the vitreal collagen into the ILM. Indeed, several studies on the human eye have shown collagen-like fibers from the vitreous penetrating the ILM to about one-half the thickness of the basement membrane (Hogan *et al.*, 1971; Foos, 1972; Tolentino *et al.*, 1976). However, there may be regional and species differences with regard to collagen orientation. Cynomolgus vitreal collagen was not inserted into the lamina densa of the ILM, and the majority of the collagen was excluded from the ILM altogether and ran parallel to the retinal surface. In the rabbit eye, collagen fibrils in the posterior fundus also run parallel to the retinal surface and only appear to be inserted into the lamina densa within the pars plana. In regions with a parallel orientation of cortical vitreal collagen, there appear to be fine strands of Alcian blue-reactive material joining the collagen fibrils to the ILM (Matsumoto *et al.*, 1984).

In human and nonhuman primate, there is an age-related thickening of the ILM. The thin ILM of young individuals gives rise to a thicker membrane characterized by complex foldings along the surface of the Müller cells (Foos, 1972; Matsumoto *et al.*, 1984). This is found primarily in the posterior fundus (the region of the weakest interaction) so that the thickening may represent an attempt at strengthening the interaction.

b. "Barrier" Functions. It appears appropriate to define the functional unit separating the vitreous from the retina as the structure comprising the ILM, a layer of hyaluronic acid, and a network of glycoproteins and collagen fibers (Rhodes, 1982). This region could be involved in control of solute flow (Fowlks, 1963; Rodriquez-Peralta, 1968) and of regulating factors involved in healing processes (Foos and Gloor, 1975; Constable *et al.*, 1981).

It has been suggested that this vitreoretinal juncture may represent a negatively charged barrier to sequester the vitreous from general circulation or to act as a filter in the vitreous–retina–blood route of solutes (Rodriquez-Peralta, 1968; Rhodes, 1982). Its effectiveness as a barrier is questionable, however, since 100 Å electron-dense thorium dioxide particles injected into the vitreous can penetrate the ILM and diffuse into the narrow intercellular clefts between Müller cells

down to synaptic processes (Smeltzer *et al.*, 1965). This would suggest that there is essentially no chemical barrier to prevent the free exchange between retinal cells and the vitreous of metabolites or noxious agents. On the other hand, the structure could prevent cell movement through the high concentrations of hyaluronic acid (Morris, 1979). Localized trauma removing part of this barrier could promote migration of cells involved in inflammatory reactions and healing or in the formation of epiretinal membranes (Rhodes, 1982).

D. Neural Retina

1. Structural Observations

a. General Comments. The central nervous system (CNS) has long been considered to be essentially devoid of an ECM because of the relatively small amount of intercellular space. By electron microscopy, however, these spaces, which appear as only narrow clefts between closely packed cells, are filled with material of detectable density (Wald and deRobertis, 1961). The retina is no exception, being composed of closely packed neurons and nonneuronal cells, such as Müller cells (Smeltzer *et al.*, 1965; Magalhães and Coimbra, 1972). Müller cells extend from the inner surface of the retina, where they are involved in the formation of the internal limiting membrane, to the retinal border of the interphotoreceptor space. They also display many structural similarities to brain astrocytes, such as straight bundles of microfilaments, numerous glycogen particles, dense bodies, and a marked plasticity of the cell membrane to fit the irregularities of neighboring cells. In addition to providing mechanical support and physiological insulation, these cells also have important metabolic functions, such as carbohydrate storage, glycogen and protein synthesis, and various enzymatic activities (Magalhães and Coimbra, 1972; Moscona and Linser, 1983). It is certainly possible that Müller cells have the ability to provide an environment permissive to the expression of phenotype by retinal neurons. Considering the small intercellular spaces in the retina, molecules found in these spaces are theoretically of great interest for functions other than structure.

b. Microscopy. Research done to date has been dominated by studies of proteoglycans found in the retina. Electron microscopic and histochemical observations on the vertebrate retina have indicated the presence of glycosaminoglycans not only in the interphotoreceptor matrix but also in the synaptic layers and on both surfaces of the retina (Röhlich, 1970). Autoradiographic and histological analyses of murine retinas suggest that glycosaminoglycans are present in the plexiform and optic nerve fiber layers, but are most heavily concentrated in the photoreceptor zone where production becomes significant when the outer seg-

ments start to develop. Enzyme susceptibility suggests that most of the labeling is due to chondroitin sulfate (Hall *et al.*, 1965; Ocumpaugh and Young, 1966).

While one does not normally think of proteoglycans as being intracellular, a CNS chondroitin sulfate proteoglycan (Kiang *et al.*, 1981) has been localized intracellularly in both the brain and the retina (Aquino *et al.*, 1984a), although it is also found extracellularly during development (Aquino *et al.*, 1984b). Cytoplasmic chondroitin sulfate has also been demonstrated histochemically in neurons of the human central nervous system (Alvarotto and Castejón, 1984). These authors have suggested that, in addition to an extracellular function, proteoglycans may serve some physiological function within cells of the central nervous system, perhaps as neurotransmitter storage sites, in synaptic transmission, or in maintaining cell turgor.

It is unlikely that proteoglycans are the only matrix molecules within the retina. However, because of the small amounts of material present intercellularly, it is likely that more sensitive EM cytochemical techniques will be required for their detection.

2. Biochemical Analyses

a. Proteoglycans. Unlike the proteoglycans found in most tissues, which require guanidine for extraction, a significant proportion of CNS proteoglycans can be extracted in saline. Consequently, in addition to a heparan sulfate proteoglycan and a chondroitin sulfate proteoglycan extracted from brains using standard procedures (White, 1977, 1978; White and Hudson, 1977), a different chondroitin sulfate proteoglycan can be isolated from brains using only phosphate-buffered saline (Kiang *et al.*, 1981).

Similar studies on the retina have demonstrated a partitioning of proteoglycans into three subfractions (Morris and Ting, 1981). When embryonic chick retinas (day 14) were labeled in organ culture with $^{35}SO_4$ and [^{3}H]glucosamine, 25% of the labeled material was released into the medium, 39% was extracted by homogenization in phosphate-buffered saline, 32% was released by subsequent extraction with guanidine, and 4% was unextracted. Analysis of the glycosaminoglycan portions in each of these fractions indicated that the ratio of chondroitin sulfate to heparan sulfate was four to seven times higher in the saline-soluble fractions than in the guanidine extracts, indicating an enrichment for heparan sulfate in the latter (Morris and Ting, 1981). The retinal proteoglycans are predominantly of low-buoyant density and do not form aggregates with hyaluronic acid (Morris *et al.*, 1984). Some of the retinal proteoglycans did form large complexes of low-buoyant densities with most of the label in unsulfated molecules, suggesting that some of these proteoglycans associate with glycoproteins, but because neither associative nor dissociative conditions had

any effect on the buoyant density of the proteoglycans, there is probably a low ratio of glycosaminoglycan to protein in these molecules (Morris *et al.*, 1984).

These studies have shown that the retinal proteoglycans released into the medium and those extracted with balanced salt solutions are rich in chondroitin sulfate. It is possible that those proteoglycans in the medium represent molecules of the extracellular matrix, while those extracted by homogenization in saline represent loosely associated extracellular proteoglycans as well as cytoplasmic proteoglycans (see above and Aguino *et al.*, 1984a). The guanidine extracts of retinal residues are enriched in heparan sulfate. These heparan sulfate proteoglycans are probably associated with the cell membranes as has been shown in other cell types (Kjellén *et al.*, 1980). Cell-associated proteoglycans may be important for interactions between cells within the retina.

The biochemical and microscopic investigations on adult retinal are in general agreement that chondroitin sulfate is the dominant glycosaminoglycan in mature retina. However, proteoglycan synthesis does undergo changes during retinal development. Heparan sulfate is the predominant glycosaminoglycan in the young, highly mitotic chick embryo retina (days 5–7). As tissue specialization progresses, the proportion of heparan sulfate decreases. At the same time, the proportion of chondroitin-4-sulfate relative to chondroitin-6-sulfate also increases (Morris *et al.*, 1977). This change is inhibited by 5-bromo-2′-deoxyuridine, a thymidine analog that interferes with cell differentiation (Morris and Dorfman, 1976). It is also possible that there are developmental changes in the compartmentalization of these proteoglycans (Aquino, 1984b).

b. Collagens. It has been stated that there is no collagen *within* the retina based on the absence of hydroxyproline (Schmut, 1978). This may be due to low sensitivity of the procedure because collagen has been detected in retinal vessels by immunofluorescence (Jerdan and Glaser, 1985) and in the ILM by histological and immunofluorescence techniques (Hogan *et al.*, 1971; von der Mark *et al.*, 1977). However, the immunofluorescence studies appear to confirm the absence of collagen in the retina per se. This is surprising considering the demonstrated ability of the retina to synthesize collagenous proteins. In studies with embryonic chick eyes, the retina was found to contribute the majority of the type II-like collagen found in the vitreous (Newsome *et al.*, 1976; Smith *et al.*, 1976) as well as vitreal glycosaminoglycans (Smith and Newsome, 1978). It is also now generally assumed that the Müller cells contribute vitreal collagen in the adult as well (Swann, 1980). This seems reasonable considering the ability of glia in the peripheral nervous system (PNS) to synthesize several collagen types (Bunge *et al.*, 1983). The ability of cells within the retina to direct the secretion of synthetic products in a specific direction is not an uncommon trait and can be found in such polarized cells as RPE. Indeed, we have already discussed the

synthesis of IRBP by photoreceptors and its directed secretion into the IPM (Hollyfield *et al.*, 1984, 1985).

Consequently, it appears that retinal cells can synthesize collagen, but do not deposit it within the retina. The absence of collagen from the neural retina is probably quite fortuitous, since this no doubt contributes to the transparency of the normal retina. This becomes apparent in certain pathological conditions, such as lattice degeneration and retinal detachments, which are characterized by "retinal sclerosis" in the area of the lesions (Witschel, 1981; Laqua, 1981). In many cases, the deposition of collagen within the retina appears to be contributed by glia and has the EM appearance of vitreal collagen.

IV. Effects of Extracellular Matrix Molecules on Retinal Neurons

A. General Comments

As we have seen in the preceding sections, there is an increasing body of evidence regarding the presence of extracellular matrix molecules in the retina. However, the functions of most of these molecules and, more specifically, their effects on retinal neurons are not known with great certainty. There have been more studies along this line in the PNS because of the abundance of extracellular matrix material found there compared to the CNS. Particularly well worked out are the Schwann cell–neuron interactions required for the establishment of the basal lamina characteristic of the PNS. Schwann cells *in vivo* exhibit basal lamina only when in contact with axons (Billings-Gagliardi *et al.*, 1974), and basal lamina production by Schwann cells *in vitro* initially requires the presence of nerve cells (Bunge *et al.*, 1982). Schwann cells in culture, even in the absence of neurons, will produce laminin (Cornbrooks *et al.*, 1983). However, type IV collagen is not secreted unless the Schwann cells are cultured in the presence of neurons or an established extracellular matrix (Carey and Bunge, 1981; Carey *et al.*, 1983), although this molecule is present intracellularly in the absence of neurons (McGarvey *et al.*, 1984). The haptotactic effect of laminin on certain types of cells in the PNS (McCarthy *et al.*, 1983) as well as the effects of fibronectin and laminin on PNS neurons have important implications both during development and in regeneration. It is possible that the framework, which is laid down by Schwann cells, can interact with axons during regeneration as they grow through the spaces previously filled by damaged axons (Bunge and Bunge, 1983).

By analogy, ECM molecules could have similar effects in the CNS. The

general absence of regeneration in adult CNS might be explained by the diminished capacity of cells in the central nervous system to synthesize certain extracellular matrix molecules. Astrocytes, for example, lose their ability to synthesize and secrete laminin with age (Liesi *et al.*, 1983). Although regeneration is not a characteristic of CNS neurons, similar mechanisms for neurite extension, migration, and guidance may take place during development when there are abundant spaces throughout the CNS. For example, fibronectin is present during cerebellar development along the route of granual cell migration, but disappears after formation of the external granular layer. Attachment of these cells to fibronectin is also stage specific (Hatten *et al.*, 1982).

An example of extracellular matrix effects specifically regarding the retina is the involvement of the retinal pigmented epithelium in photoreceptor outgrowth during development (Hollyfield and Witkovsky, 1974). Since these two layers of the developing eye are not in contact prior to photoreceptor outgrowth (Feeney, 1973a), it is likely that the RPE cells are elaborating a component for the interphotoreceptor matrix that is required for the outgrowth of the photoreceptors. In some animal mutants with RPE/IPM defects, such as the RCS rat, there can be associated retinal degeneration (LaVail, 1981). It is also possible that some component in the interphotoreceptor matrix is important for maintenance of the photoreceptors since retinal detachment will lead to a loss of photoreceptor function (Kroll and Machemer, 1968).

It has been proposed that extracellular matrix molecules may be involved in axonal guidance during development. A highly structured extracellular matrix is present ahead of growing axons in embryonic optic tectum, and it may have an effect on the guidance of optic fibers, although a more specific mechanism than mechanical guidance may be at work (Krayanek, 1980). Unidirectional factors in the chick retina may guide fibers toward the optic nerve (Goldberg, 1977), and components of the inner limiting membrane may be important for the guidance of axons in the retina (Halfter *et al.*, 1983; Faster *et al.*, 1984). The presence of laminin in the chick embryonic ILM (Fig. 3) (Jerdan *et al.*, 1984; Adler *et al.*, 1985) and the ability of this molecule to promote neurite extension from retinal neurons (see next section) support this hypothesis and suggest a dynamic role for laminin in retinal development as opposed to simply a structural role.

B. Neurite Extension

One possible effect of matrix molecules is the guidance of neuronal processes. Perhaps more fundamental than the ability to guide neuronal processes is, in fact, the ability of matrix components to induce neurite extension. Many factors have been found which will promote neurite extension in PNS and CNS neurons since

the finding that modification of a substratum *in vitro* can influence the ability of cells to adhere and to extend neurites (Letourneau, 1975). Fibronectin, for example, has been found to be effective in promoting neurite extension by PNS neurons (Baron-Van Evercoorens, 1982; Rogers *et al.*, 1983), but is without effect on CNS neurons (Manthorpe *et al.*, 1983; Rogers *et al.*, 1983; Adler and Hewitt, 1983; Adler *et al.*, 1985). Its ability to elicit neurite outgrowth from retinal aggregates (Akers *et al.*, 1981) probably is due to the influence of heterotypic cell–cell interactions within the aggregate. One might not expect fibronectin to have an influence on CNS neurons since, with the exception of its transient presence in developing cerebellum (Hatten *et al.*, 1982), fibronectin does not appear to be associated with either neurons or glia in the CNS in general (Linder *et al.*, 1975; Schachner *et al.*, 1978) or more specifically in the developing neural retina (Kurkinen *et al.*, 1979).

Laminin, on the other hand, is efficient in stimulating neurite outgrowth in both PNS and CNS neurons to include retinal neurons (Fig. 4) (Adler and Hewitt, 1983; Adler *et al.*, 1985; Manthorpe *et al.*, 1983; Rogers *et al.*, 1983; Smalheiser *et al.*, 1984; Liesi *et al.*, 1984). This response on retinal neurons is dose dependent and inhibited by antibodies against laminin. When these *in vitro* experiments are done under serum-free conditions, laminin will only induce extension of neurites from retinal neurons when bound to a polyornithine substratum. Laminin bound to tissue culture plastic is not effective even though similar amounts of laminin bind under both conditions (Table III) (Adler and Hewitt, 1983; Adler *et al.*, 1985). It is possible that the presence of the highly charged polyornithine induces a conformational change in the laminin molecule which is required for it to interact with the surface of the neurons.

The laminin effect is similar to that obtained using Schwannoma-conditioned medium which contains, in addition to laminin (Palm and Furcht, 1983), a polyornithine-binding, neurite-promoting factor (PNPF) (Manthorpe *et al.*, 1981). It is effective on both PNS and CNS neurons, although its distribution within the CNS as well as whether it is a matrix or cell surface component has not been determined. The conditioned medium effect does not appear to be due to laminin, since it is not inhibited by anti-laminin antibodies (Manthorpe *et al.*, 1983; Adler and Hewitt, 1983; Adler *et al.*, 1985). However, these antibodies do precipitate a molecule the size of the smaller laminin subunit (Davis *et al.*, 1984). PNPF is found in medium conditioned by several types of cells including heart, glia, and Schwannoma cells (Collins, 1978; Adler and Varon, 1980; Manthorpe *et al.*, 1981; Adler *et al.*, 1981). This molecule is a large, highly negatively charged, protein-containing molecule (Manthorpe *et al.*, 1981). The charged properties of PNPF suggest that it contains a proteoglycan portion. Recent evidence suggests that there is at least one molecule with neurite-promoting activity that is a heparan sulfate proteoglycan (Lander *et al.*, 1982). Howev-

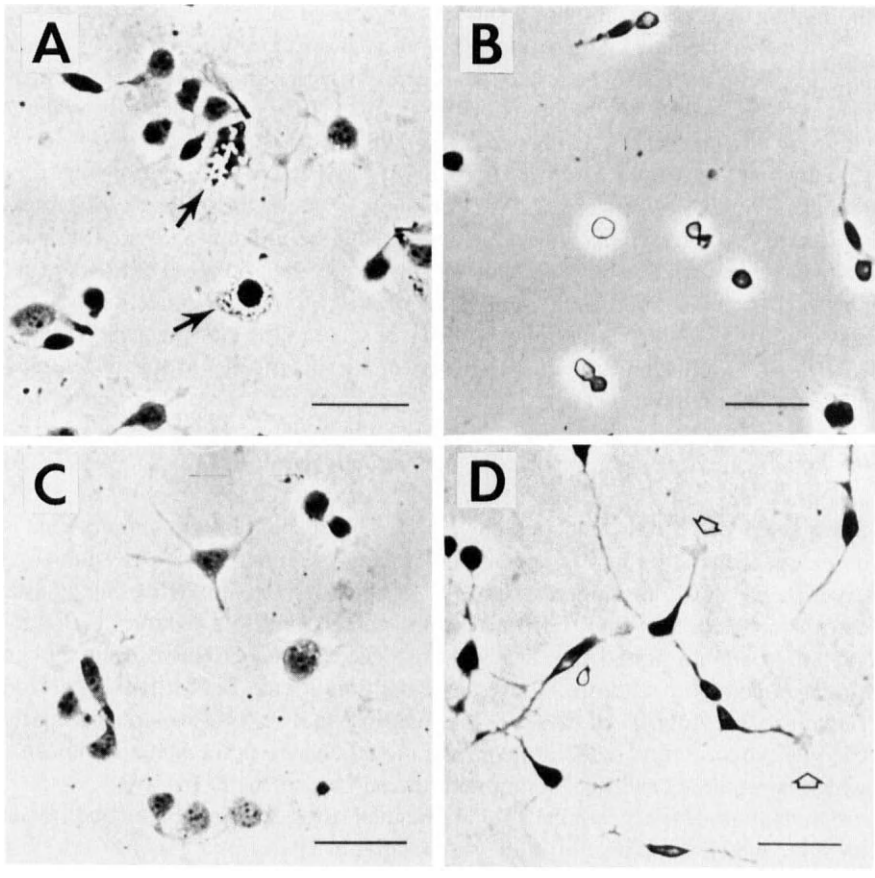


FIG. 4. Effect of modified substrata on the projection of neurites from 8-day chick embryo neural retina cells. Dissociated cells were cultured for 6 hr in N-1 supplemented, serum-free medium on (A) untreated polyornithine, (B) fetal calf serum-treated polyornithine, (C) fibronectin-treated polyornithine, and (D) laminin-coated polyornithine. Only laminin-treated polyornithine supported extensive neurite development and the presence of growth cones and filopodial processes (open arrows). Neurite extention was minimal or absent in the other conditions. Note the degree of cell spreading on fibronectin (C) and untreated polyornithine (A) and the extensive vacuolization of some cells in the latter (solid arrows). Bar, 25 μm. (Reprinted from Adler *et al.*, 1985, with permission.)

er, it is possible that such an active molecule is actually an aggregate of a proteoglycan and some other molecule (Matthew and Patterson, 1983). This type of interaction between a protein and a heparan sulfate proteoglycan has also been demonstrated as a mediator of chick neural retina cell–substratum adhesion (Cole *et al.*, 1985).

TABLE III

EFFECT OF SUBSTRATUM ON LAMININ-INDUCED RETINAL NEURON NEURITE EXTENSION[a]

	Tissue culture plastic		Polyornithine	
	1 μg Ln[b]	5 μg Ln[b]	1 μg Ln[b]	5 μg Ln[b]
Laminin bound per well	0.16 μg	1.00 μg	0.20 μg	0.85 μg
Number of cells attached ($\times 10^{-5}$)	6.0	1.0	6.1	5.8
Attached cells with neurites (percentage of maximum)	0	0	70	100

[a] Tissue culture plastic and polyornithine-coated wells (16 mm) were incubated with various concentrations of ^{125}I-labeled laminin for 3 hr at 37°C. Wells were washed extensively to remove unbound laminin before removing bound laminin with 1 *N* NaOH. Samples were assayed in a liquid scintillation counter and compared to a dilution series of labeled laminin. In other experiments, both with and without labeled laminin, the number of cells attached and percentage of cells with neurites were also determined. Experiments were performed at least twice in triplicate, and the numbers shown represent the means of values which did not differ by more than 10% (adapted from Adler *et al.*, 1985).

[b] Amount of laminin (Ln) added per well in 0.5 ml of medium.

C. *Comments on Future Research on Retinal ECM*

As this area of research becomes more popular and retinal extracellular matrix molecules are identified and characterized, one might expect more experimental approaches to define the roles of ECM molecules in development and maintenance of phenotypes within the retina. While retinal organ culture systems might be expected to be useful in determining the types of molecules that are being synthesized by cells within the retina, the use of "purified" cell cultures will be more important in determining the effects of ECM molecules on specific types of retinal cells. This is becoming a more practical approach with the advent of defined medium culture systems for specific cell types. Within just the past few years, it has become possible to separate the neuronal from nonneuronal cells in embryonic retinas (Adler *et al.*, 1982). Conditions have also been worked out to further subdivide the neuronal cells by establishing conditions which favor the expression of photoreceptors (Adler *et al.*, 1984). The prospect of testing the effects of known ECM molecules, subfractions of interphotoreceptor matrix, medium conditioned by flat cells, etc. on purified cultures of retinal neurons is quite exciting. Will components of the interphotoreceptor matrix influence the outgrowth of photoreceptor outer segments? Will these molecules influence such putative behavior as neurotransmitter uptake and/or storage? Will depletion of intracellular proteoglycans affect the ability of neurons to maintain neuritic projections or to store neurotransmitters? (See article by Ruben Adler in Part I).

The use of animal mutants with degenerative retinal conditions may also be useful in gaining an understanding of normal processes. A number of mutants have been described, one of which, the RCS rat with inherited retinal dystrophy, is manifested by the reduced ability of RPE cells to phagocytize outer segments and an associated degeneration of photoreceptors. This particular mutant has an obvious extracellular matrix defect, since altered staining characteristics of the interphotoreceptor matrix become evident before the degeneration of the photoreceptors takes place (LaVail *et al.*, 1981). This is followed by a decrease in IRBP (Gonzalex-Fernandez *et al.*, 1984).

Many of the other mutants, however, such as *rd* (Blanks *et al.*, 1974; LaVail, 1981) and *pcd* (LaVail *et al.*, 1982; Blanks *et al.*, 1982) mice, may not have ECM differences which are as readily apparent as those in the RCS rat. In the *rd* mouse, for example, Alcian blue staining of the interphotoreceptor matrix remains unaltered even after photoreceptor degeneration has begun (Zimmerman and Eastham, 1959). Even in cases such as these, however, there may be a defect in a histochemically "invisible" matrix factor that is important for either differentiation or maintenance of photoreceptors. The use of various cell culture techniques, such as either those described above, the coculturing of cells from normal and mutant animals, or the culturing of cells in the presence of medium conditioned by normal or mutant cells, may be useful in determining if extracellular matrix molecules are altered in these degenerative conditions and, consequently, if they are important for normal differentiation. An important caveat in such studies is that the matrix molecules produced may be normal but the cells may not be properly equipped to respond to their microenvironment because of abnormal surface properties. Consequently, studies of cell surface properties also offers an exciting area of research.

V. Concluding Comments

In this article we have described the various classes of molecules found in extracellular matrices and have discussed their roles not only in providing strength, stability, and structure to tissues but also in their abilities to influence morphology, growth, and phenotypic expression of cells. Also described is what is known regarding extracellular matrices found in association with the retina. In some cases, such as within the retina itself, very little is known about the molecules found intercellularly and even less is known about their functions. With today's rapidly advancing technology, the isolation and characterization of molecules from the small amounts of material available, although challenging, provide an exciting area of research for the biochemist. However, as has been found in many areas of endeavor, no one specialty will provide the final answers.

As was so elegantly demonstrated by the work on IRBP, it will take a concerted effort by those in all subspecialities now included under the term "cell biology" to elucidate the role of ECM molecules in the retina.

Acknowledgments

The author is appreciative of the helpful discussions and patient editorial advice of Dr. Ruben Adler as well as for the efficient secretarial assistance of Mrs. Doris Golembieski.

References

Adler, A. J., and Evans, C. D. (1983). Rapid isolation of bovine interphotoreceptor retinol-binding protein. *Biochim. Biophys. Acta* **761,** 217–222.

Adler, A. J., and Evans, C. D. (1984). Molecular properties of bovine interphotoreceptor retinol-binding protein. *Invest. Ophthalmol. Visual Sci. Suppl.* **25,** 274.

Adler, A. J., and Evans, C. D. (1985a). Some functional characteristics of purified bovine interphotoreceptor retinol-binding protein. *Invest. Ophthalmol. Visual Sci.* **26,** 273–282.

Adler, A. J., Evans, C. D., and Stafford, W. F., III. (1985b). Molecular properties of bovine interphotoreceptor retinol-binding protein. *J. Biol. Chem.* **260,** 4850–4855.

Adler, A. J., and Klucznik, K. M. (1982a). Proteins and glycoproteins of the bovine interphotoreceptor matrix: Composition and fractionation. *Exp. Eye Res.* **34,** 423–434.

Adler, A. J., and Klucznik, K. M. (1982b). Interaction of bovine pigment epithelium cells, photoreceptor outer segments, and interphotoreceptor matrix: A model for retinal adhesion. *Curr. Eye Res.* **1,** 579–589.

Adler, A. J., and Martin, K. J. (1982). Retinol-binding proteins in bovine interphotoreceptor matrix. *Biochem. Biophys. Res. Commun.* **108,** 1601–1608.

Adler, A. J., and Martin, K. J. (1983). Lysosomal enzymes in the interphotoreceptor matrix: Acid proteases. *Curr. Eye Res.* **2,** 359–366.

Adler, A. J., and Severin, K. M. (1981). Proteins of the bovine interphotoreceptor matrix: Tissues of origin. *Exp. Eye Res.* **32,** 755–769.

Adler, A. J., and Spencer, S. A. (1985). Retinals and retinols carried endogenously by bovine interphotoreceptor retinol-binding protein. *Invest. Ophthalmol. Visual Sci. Suppl.* **26,** 16.

Adler, R. (1982). Regulation of neurite growth in purified retina neuronal cultures: Effects of PNPF, a substratum-bound, neurite-promoting factor. *J. Neurosci. Res.* **8,** 165–177.

Adler, R., and Hewitt, A. T. (1983). Effects of extracellular matrix molecules on neural retinal cell attachment, survival and differentiation. *Soc. Neurosci. Abstr.* **9,** 209.

Adler, R., and Varon, S. (1980). Cholinergic neuronotrophic factors: V. Segregation of survival- and neurite-promoting activities in heart conditioned medium. *Brain Res.* **188,** 437–448.

Adler, R., Manthorpe, M., Skaper, S. D., and Varon, S. (1981). Polyornithine-attached neurite-promoting factors (PNPF's): Culture sources and responsive neurons. *Brain Res.* **206,** 129–144.

Adler, R., Magistretti, P. J., Hyndman, A. G., and Shoemaker, W. J. (1982). Purification and cytochemical identification of neuronal and non-neuronal cells in chick embryo retina cultures. *Dev. Neurosci.* **5,** 27–39.

Adler, R., Lindsey, J. D., and Elsner, C. L. (1984). Expression of cone-like properties by chick embryo neural retina cells in glial-free monolayer cultures. *J. Cell Biol.* **99,** 1173–1178.

Adler, R., Jerdan, J., and Hewitt, A. T. (1985). Responses of cultured neural retinal cells to substratum-bound laminin and other extracellular matrix molecules. *Dev. Biol.* **112,** 100–114.

Akers, R. M., Mosher, D. F., and Lilien, J. E. (1981). Promotion of retinal neurite outgrowth by substratum-bound fibronectin. *Dev. Biol.* **86,** 179–188.

Alvarado, M. V., and Castejón, H. V. (1984). Histochemical demonstration of cytoplasmic glycosaminoglycans in the macroneurons of the human central nervous system. *J. Neurosci. Res.* **11,** 13–26.

Antonopoulous, C. A., Axelsson, A., Heinegård, D., and Gardell, S. (1974). Extraction and purification of proteoglycans from various types of connective tissue. *Biochim. Biophys. Acta* **338,** 108–119.

Aquino, D. A., Margolis, R. U., and Margolis, R. K. (1984a). Immunocytochemical localization of a chondroitin sulfate proteoglycan in nervous tissue. I. Adult brain, retina, and peripheral nerve. *J. Cell Biol.* **99,** 1117–1129.

Aquino, D. A., Margolis, R. U., and Margolis, R. K. (1984b). Immunocytochemical localization of a chondroitin sulfate proteoglycan in nervous tissue. II. Studies in developing brain. *J. Cell Biol.* **99,** 1130–1139.

Bach, G., and Berman, E. R. (1970). Characterization of a sialoglycan isolated from cattle retina. *Ophthalmol. Res.* **1,** 257–272.

Bach, G., and Berman, E. R. (1971a). Amino sugar-containing compounds of the retina. I. Isolation and identification. *Biochim. Biophys. Acta* **252,** 453–461.

Bach, G., and Berman, E. R. (1971b). Amino sugar-containing compounds of the retina. II. Structural studies. *Biochim. Biophys. Acta* **252,** 461–471.

Balian, G., Click, E. M., Crouch, E., Davidson, J., and Bornstein, P. (1979). Isolation of a collagen binding fragment from fibronectin and cold-insoluble globulin. *J. Biol. Chem.* **254,** 1429–1432.

Baron-Van Evercooren, A., Kleinman, H. K., Ohno, S., Marangos, P., Schwartz, J. P., and Dubois-Dalcq, M. E. (1982). Nerve growth factor, laminin, and fibronectin promote neurite growth in human fetal sensory ganglia cultures. *J. Neurosci. Res.* **8,** 179–193.

Benedetti, E. L., and Emmelot, P. (1967). Studies on plasma membranes. IV. The ultrastructural localization and content of sialic acid in plasma membranes isolated from rat liver and hepatoma. *J. Cell Sci.* **2,** 499–512.

Bergsma, D., Wiggert, B., Funahashi, M., Kuwabara, T., and Chader, G. (1977). Vitamin A receptors in normal and dystrophic human retina. *Nature (London)* **265,** 66–67.

Berman, E. R. (1969). Mucopolysaccharides (glycosaminoglycans) of the retina: Identification, distribution and possible biological role. *Mod. Probl. Ophthalmol.* **8,** 5–31.

Berman, E. R. (1982). Isolation and characterization of the interphotoreceptor matrix. *In* "Methods in Enzymology" (L. Packer, ed.), Vol. 81, pp. 77–85. Academic Press, New York.

Berman, E. R., and Bach, G. (1968). The acid mucopolysaccharides of cattle retina. *Biochem. J.* **108,** 75–88.

Berman, E. R., Segal, N., and Feeney, L. (1979). Subcellular distribution of free and esterified forms of vitamin A in the pigment epithelium of the retina and in liver. *Biochim. Biophys. Acta* **572,** 167–177.

Bernfield, M. R., and Banerjee, S. D. (1978). The basal lamina in epithelial-mesenchymal morphogenetic interactions. *In* "Biology and Chemistry of Basement Membranes" (N. A. Kefalides, ed.), pp. 137–148. Academic Press, New York.

Bernfield, M. R., Banerjee, S. D., and Cohn, R. H. (1972). Dependence of salivary epithelial morphology and branching morphogenesis upon acid mucopolysaccharide-protein (proteoglycan) at the epithelial surface. *J. Cell Biol.* **52,** 674–689.

Bernstein, M. H. (1980). Phagocytosis in retinal pigmented epithelium: Early changes with light. *Invest. Ophthalmol. Visual Sci. (Suppl.)* **19,** 162.

Billings-Gagliardi, S., Webster, H. deF., and O'Connell, M. F. (1974). *In vivo* and electron microscopic observations on Schwann cells in developing tadpole nerve fibers. *Am. J. Anat.* **141,** 375–391.

Blanks, J. D., Adinolfi, A. M., and Lolley, R. N. (1974). Photoreceptor degeneration and synaptogenesis in retinal-degenerative (*rd*) mice. *J. Comp. Neurol.* **156,** 95–106.

Blanks, J. C., Mullen, R. J., and LaVail, M. M. (1982). Retinal degeneration in the *pcd* cerebellar mutant mouse. II. Electron microscopic analysis. *J. Comp. Neurol.* **212,** 231–246.

Bornstein, P., and Sage, H. (1980). Structurally distinct collagen types. *Annu. Rev. Biochem.* **49,** 957–1003.

Branford White, C. J. (1977). The isolation of proteoheparan sulphate from sheep brain. *Biochem. Soc. Trans* **5,** 1083–1085.

Branford White, C. J. (1978). Proteoglycans in ovine brain. *Experientia* **34,** 1036–1037.

Branford White, C. J., and Hudson, M. (1977). Characterization of a chondroitin sulphate proteoglycan from ovine brain. *J. Neurochem.* **28,** 581–588.

Bridges, C. D. B., Fong, S.-L., Liou, G. I., Landers, R. A., Gonzalez-Fernandez, F., Glazebrook, P., and Lam, D. M. K. (1983). A retinol-binding glycoprotein synthesized and secreted by mammalian neural retina. *Soc. Neurosci. Abstr.* **9,** 345.

Bunge, M. B., Williams, A. K., and Wood, P. M. (1982). Neuron–Schwann cell interaction and basal lamina formation. *Dev. Biol.* **92,** 449–460.

Bunge, M. B., Bunge, R. P., Carey, D. J., Cornbrooks, C. J., Eldridge, C. F., Williams, A. K., and Wood, P. M. (1983). Axonal and nonaxonal influences on Schwann cell development. *In* "Developing and Regenerating Vertebrate Nervous Systems" (P. N. Coates, R. R. Markwald, and A. D. Kenny, eds.), pp. 71–105. Liss, New York.

Bunge, R. D., and Bunge, M. B. (1983). Interrelationships between Schwann cell function and extracellular matrix production. *Trends Neurosci.* **6,** 499–505.

Bunt-Milam, A. H., and Saari, J. C. (1983). Immunocytochemical localization of two retinoid-binding proteins in vertebrate retina. *J. Cell Biol.* **97,** 703–712.

Bunt-Milam, A. H., and Saari, J. C. (1984). Determination of pore size of the external limiting membrane in rabbit retina. *Abstr. Int. Congr. Eye Res., 6th, Alicante* **3,** 10.

Burgeson, R. E., El Aldi, F. A., Kaitela, I. I., and Hollister, D. W. (1976). Fetal membrane collagens: Identification of two new collagen alpha chains. *Proc. Natl. Acad. Sci. U.S.A.* **73,** 2579–2583.

Carey, D. J., and Bunge, R. P. (1981). Factors influencing the release of proteins by cultured Schwann cells. *J. Cell Biol.* **91,** 666–672.

Carey, D. J., Eldridge, C. F., Cornbrooks, C. J., Timpl, R., and Bunge, R. P. (1983). Biosynthesis of type IV collagen by cultured rat Schwann cells. *J. Cell Biol.* **97,** 473–479.

Chen, L. B. (1977). Alteration in cell surface LETS protein during myogenesis. *Cell* **10,** 393–400.

Chicquet, M., Puri, E. C., and Turner, D. C. (1979). Fibronectin mediates attachment of chicken myoblasts to a gelatin-coated substratum. *J. Biol. Chem.* **254,** 5475–5482.

Chung, E., and Miller, E. J. (1974). Collagen polymorphism: Characterization of molecules with the chain composition $[\alpha 1(III)]_3$ in human tissues. *Science* **183,** 1200–1203.

Chung, E., Rhodes, R. K., and Miller, E. J. (1976). Isolation of three collagenous components of probable basement membrane origin from several tissues. *Biochem. Biophys. Res. Commun.* **71,** 1167–1174.

Cohen, A. I. (1968). New evidence supporting the linkage to extracellular space of outer segment saccules of frog cones but not rods. *J. Cell Biol.* **37,** 424–444.

Cohen, A. M. (1972). Factors directing the expression of sympathetic nerve traits in cells of neural crest origin. *J. Exp. Zool.* **179,** 167–182.

Cohn, R. H., Banerjee, S. D., and Bernfield, M. R. (1977). Basal laminae of embryonic salivary epithelia. Nature of glycosaminoglycan and organization of extracellular materials. *J. Cell Biol.* **73,** 464–478.

Cole, G. J., Schubert, D., and Glaser, L. (1985). Cell–substratum adhesion in chick neural retina depends upon protein–heparan sulfate interactions. *J. Cell Biol.* **100,** 1192–1199.

Collins, F. (1978). Induction of neurite outgrowth by a conditioned medium factor bound to culture substratum. *Proc. Natl. Acad. Sci. U.S.A.* **75,** 5210–5213.

Constable, I. J., Horne, R., Slatter, D. H., Chester, G. H., and Cooper, R. L. (1981). Regenerations of retinal limiting membranes after choroiretinal biopsy in dogs. *Invest. Ophthalmol. Visual Sci.* **20,** 246–251.

Cornbrooks, C. J., Carey, D. J., MacDonald, J. A., Timpl, R., and Bunge, R. P. (1983). *In vivo* and *in vitro* observations on laminin production by Schwann cells. *Proc. Natl. Acad. Sci. U.S.A.* **80,** 3850–3854.

Davis, G. E., Manthorpe, M., and Varon, S. (1984). Purification of rat Schwannoma neurite-promoting factor. *Soc. Neurosci. Abstr.* **10,** 38.

DelRosso, M., Cappelletti, R., Viti, M., Vannucchi, S., and Chiarugi, V. (1981). Binding of the basement membrane glycoprotein, laminin, to glycosaminoglycans. *Biochem. J.* **199,** 699–704.

Dewar, A., Barron, G., and Reading, H. (1975). The effect of retinol and acetylsalicylic acid on the release of lysosomal enzymes from rat retina *in vitro*. *Exp. Eye Res.* **20,** 63–72.

Dodson, J. W., and Hay, E. D. (1974). Secretion of collagen by corneal epithelium. Effect of the underlying substratum on secretion and polymerization of epithelial products. *J. Exp. Zool.* **189,** 51–72.

Dowling, J. (1960). Chemistry of visual adaptation in the rat. *Nature (London)* **188,** 114–118.

Easter, S. S., Bratton, B., and Scherer, S. S. (1984). Growth-related order of the retinal fiber layer in goldfish. *J. Neurosci.* **4,** 2177–2190.

Edwards, R. B. (1982). Glycosaminoglycan synthesis by cultured human retinal pigmented epithelium from normal postmortem donors and a postmortem donor with retinitis pigmentosa. *Invest. Ophthalmol. Visual Sci.* **23,** 435–446.

Edwards, R. B. (1983). The use of tissue culture techniques to study normal and diseased retinal pigment epithelium. *In* "Progress in Retinal Research" (N. N. Osborn and G. J. Chader, eds.), Vol. 2, pp. 51–66. Pergamon, Oxford.

Edwards, R. B. (1984). A 31,000 dalton protein synthesized and released by cultured human retinal pigment epithelium. *Invest. Ophthalmol. Visual Sci. Suppl.* **25,** 274.

Engel, J., Odermatt, E., Engel, A., Madri, J. A., Furthmayr, H., Rohde, H., and Timpl, R. (1981). Shapes, domain organization and flexibility of laminin and fibronectin: Two multifunctional extracellular matrix proteins. *J. Mol. Biol.* **150,** 97–120.

Epstein, E. H., Jr. (1974). $[\alpha 1(III)]_3$ human skin collagen. Release by pepsin digestion and preponderance in fetal life. *J. Biol. Chem.* **249,** 3225–3231.

Essner, E., and Pino, R. M. (1982). Distribution of anionic sites in Bruch's membrane of the rabbit eye. *Eur. J. Cell Biol.* **27,** 251–255.

Feeney, L. (1973a). The interphotoreceptor space: I. Postnatal ontogeny in mice and rats. *Dev. Biol.* **32,** 101–114.

Feeney, L. (1973b). The interphotoreceptor space: II. Histochemistry of the matrix. *Dev. Biol.* **32,** 115–128.

Feeney, L. (1973c). Synthesis of interphotoreceptor matrix: I. Autoradiography of ^{3}H-fucose incorporation. *Invest. Ophthalmol.* **12,** 739–751.

Feeney, L., and Mixon, R. N. (1976). An *in vitro* model of phagocytosis in bovine and human retinal pigment epithelium. *Exp. Eye Res.* **22,** 533–548.

Fine, B. S., and Zimmerman, L. E. (1963). Observations on the rod and cone layer of the human retina. *Invest. Ophthalmol.* **2,** 446–459.

Fitch, J. M., and Lindsenmeyer, T. F. (1983). Monoclonal antibody analysis of ocular basement membranes during development. *Dev. Biol.* **95,** 137–153.

Foidart, J.-M., Berman, J. J., Paglia, L., Rennard, S. I., Abe, S., Perantoni, A., and Martin, G. R. (1980a). Synthesis of fibronectin, laminin, and several collagens by a liver-derived epithelial cell line. *Lab. Invest.* **42,** 525–532.

Foidart, J.-M., Bere, E. W., Yaar, M., Rennard, S. I., Gullino, M., Martin, G. R., and Katz, S. I. (1980b). Distribution and immunoelectron microscopic localization of laminin, a non-collagenous basement membrane glycoprotein. *Lab. Invest.* **42,** 336–342.

Fong, S. L., Liou, G. I., Landers, R. A., Alvarez, R. A., Gonzalez-Fernandez, F., Glazebrook, P. A., Lam, D. M. K., and Bridges, C. D. B. (1984a). The characterization, localization and biosynthesis of an interstitial retinol-binding glycoprotein in the human eye. *J. Neurochem.* **42,** 1667–1676.

Fong, S.-L., Liou, G. I., Alvarez, R. A., and Bridges, C. D. B. (1984b). Purification and characterization of a retinol-binding glycoprotein synthesized and secreted by bovine neural retina. *J. Biol. Chem.* **259,** 6534–6542.

Foos, R. (1972). Vitreoretinal junction. Topographical variations. *Invest. Ophthalmol.* **11,** 801–808.

Foos, R. Y., and Gloor, B. P. (1976). Vitreoretinal juncture: Healing of experimental wounds. *Albrecht von Graefes Arch. Klin. Exp. Ophthalmol.* **196,** 213–230.

Fowlks, W. L. (1963). Meridional flow from the corona ciliaris through the pararetinal zone of the rabbit vitreous. *Invest. Ophthalmol.* **2,** 63–71.

Fritze, L. M. S., Reilly, C. F., and Rosenberg, R. D. (1985). An antiproliferative heparan sulfate species produced by post confluent smooth muscle cells. *J. Cell Biol.* **100,** 1041–1049.

Furcht, L. T., Mosher, D. F., and Wendelschafer-Craab, G. (1978). Immunocytochemical localization of fibronectin (LETS protein) on the surface of L_6 myoblasts: Light and electron microscopic studies. *Cell* **13,** 263–271.

Gauss-Müller, V., Kleinman, H. K., Martin, G. R., and Schiffmann, E. (1980). Role of attachment factors and attractants in fibroblast chemotaxis. *J. Lab. Clin. Med.* **96,** 1071–1080.

Gay, S., and Miller, F. J. (1978), "Collagen in the Physiology and Pathology of Connective Tissue." Fisher, Stuttgart.

Gay, S., Rhodes, R. K., Gay, R. E., and Miller, E. J. (1981). Collagen molecules comprised of α1(V)-chains (B-chains): An apparent localization in the exoskeleton. *Collagen Res.* **1,** 53–58.

Goldberg, S. (1977). Unidirectional, bidirectional, and random growth of embryonic optic axons. *Exp. Eye Res.* **25,** 399–404.

Gonzalez-Fernandez, F., Landers, R. A., Glazebrook, P. A., Fong, S.-L., Liou, G. I., Lam, D. M. K., and Bridges, C. D. B. (1984). An extracellular retinol-binding glycoprotein in the eyes of mutant rats with retinal dystrophy: Development, localization, and biosynthesis. *J. Cell Biol.* **99,** 2092–2098.

Goodman, D. S. (1980). Vitamin A metabolism. *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **39,** 2716–2721.

Gospodarowicz, D. G., Greenburg, G., and Birdwell, C. R. (1978). Determination of cellular shape by the extracellular matrix and its correlation with the control of cellular growth. *Cancer Res.* **38,** 4155–4171.

Gospodarowicz, D., Delgrado, D., and Vlodavsky, I. (1980). Permissive effect of the extracellular matrix on cell proliferation *in vitro*. *Proc. Natl. Acad. Sci. U.S.A.* **77,** 4094–4098.

Green, W. R. (1980). Clinicopathologic studies of senile macular degeneration. *In* "Ocular Pathology Update" (D. H. Nicholson, ed.), pp. 115–144. Masson, New York.

Greenberg, J. H., Seppä, S., Seppä, H., and Hewitt, A. T. (1981). Role of collagen and fibronectin in neural crest cell adhesion and migration. *Dev. Biol.* **87,** 259–266.

Grobstein, C., and Cohen, J. (1965). Collagenase: Effect on the morphogenesis of embryonic salivary epithelium *in vitro*. *Science* **150,** 626–628.

Gross, J. (1974). Collagen biology: Structure, degradation and disease. *Harvey Lect.* **68,** 351–432.

Gross, J., and Nagai, Y. (1965). Specific degradation of the collagen molecule by tadpole collagenolytic enzymes. *Proc. Natl. Acad. Sci. U.S.A.* **54,** 1197–1201.

Gross, J., Harper, E., Harris, E. D., Jr., McCroskery, P., Highberger, J. H., Corbett, C., and Kang, A. H. (1974). Animal collagenases, specificity of action and structure of the substrate cleavage site. *Biochem. Biophys. Res. Commun.* **61,** 605–612.

Grotendorst, G. R., Seppä, H. E. J., Kleinman, H. K., and Martin, G. R. (1981). Attachment of smooth muscle cells to collagen and their migration toward platelet-derived growth factor. *Proc. Natl. Acad. Sci. U.S.A.* **78,** 3669–3672.

Grotendorst, G. R., Kleinman, H. K., Rohrbach, D. H., Hewitt, A. T., Varner, H. H., Horigan, E. A., Hassell, J. R., Terranova, V. P., and Martin, G. R. (1982). Role of attachment factors in mediating the attachment, distribution, and differentiation of cells. *In* "Growth of Cells in Hormonally Defined Media" (G. H. Sato, A. B. Pardee, and D. A. Sirbasky, eds.), pp. 403–413. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Hahn, L.-H. E., and Yamada, K. M. (1979a). Identification and isolation of a collagen-binding fragment of the adhesive glycoprotein fibronectin. *Proc. Natl. Acad. Sci. U.S.A.* **76,** 1160–1163.

Hahn, L.-H. E., and Yamada, K. M. (1979b). Isolation and biological characterization of active fragments of the cell adhesive glycoprotein fibronectin. *Cell* **18,** 1043–1051.

Halfter, W., Newgreen, D. F., Sauter, J., and Schwartz, U. (1983). Oriented axon outgrowth from avian embryonic retina in culture. *Dev. Biol.* **95,** 56–64.

Hall, M. O., Ocumpaugh, D. E., and Young, R. W. (1965). The utilization of ^{35}S-sulfate in the synthesis of mucopolysaccharides by the retina. *Invest. Opthalmol.* **4,** 322–329.

Hascall, V. C. (1977). Interaction of cartilage proteoglycans with hyaluronic acid. *J. Supramol. Struct.* **7,** 101–120.

Hascall, V. C., and Hascall, G. K. (1981). Proteoglycans. *In* "Cell Biology of Extracellular Matrix" (E. D. Hay, ed.), pp. 39–63. Plenum, New York.

Hascall, V. C., and Sajdera, S. W. (1970). Physical properties and polydispersity of proteoglycan from bovine nasal cartilage. *J. Biol. Chem.* **245,** 4920–4930.

Hassell, J. R., Newsome, D. A., and Hascall, V. C. (1979). Characterization and biosynthesis of proteoglycans of corneal stroma from rhesus monkey. *J. Biol. Chem.* **254,** 12346–12354.

Hassell, J. R., Robey, P. G., Barrach, H.-J., Wilczek, S. I., and Martin, G. R. (1980). Isolation of a heparan sulfate-containing proteoglycan from basement membrane. *Proc. Natl. Acad. Sci. U.S.A.* **77,** 4494–4498.

Hatten, M. E., Furie, M. B., and Rifkin, D. B. (1982). Binding of developing mouse cerebellar cells to fibronectin: A possible mechanism for the formation of the external granular layer. *J. Neurosci.* **2,** 1195–1206.

Hausman, R. E., and Moscona, H. A. (1975). Purification and characterization of the retina-specific cell-aggregating factor. *Proc. Natl. Acad. Sci. U.S.A.* **72,** 916–920.

Hausman, R. E., and Moscona, A. A. (1976). Isolation of retina-specific cell-aggregating factor from membranes of embryonic neural retina tissue. *Proc. Natl. Acad. Sci. U.S.A.* **73,** 3594–3598.

Hay, E. D., Hasty, D. L., and Kiehnau, K. L. (1978). Fine structure of collagens and their relation to glycosaminoglycans. *In* "Collagen-Platelet Interactions" (H. Gastpar, K. Kühn, and R. Marx, eds.), pp. 129–151. Schattauer, Stuttgart.

Hewitt, A. T., and Martin, G. R. (1984). Attachment proteins and their role in extracellular matrices. *In* "The Biology of Glycoproteins" (R. Ivatt, ed.), pp. 65–93. Plenum, New York.

Hewitt, A. T., and Newsome, D. A. (1983). Characterization of proteoglycans isolated from human Bruch's membrane. *Proc. 7th Int. Symp. Glycoconjugates, 7th, Lund, Sweden* pp. 90–91.

Hewitt, A. T., and Newsome, D. A. (1985a). Altered synthesis of Bruch's membrane proteoglycans associated with dominant retinitis pigmentosa. *Curr. Eye Res.* **4,** 169–174.

Hewitt, A. T., and Newsome, D. A. (1985b). Altered proteoglycan synthesis in cultures of human retinitis pigmentosa (RP) retinal pigmented epithelial cells. *Invest. Ophthalmol. Visual Sci. Suppl.* **26,** 132.

Hewitt, A. T., Kleinman, H. K., Pennypacker, J. P., and Martin, G. R. (1980). Identification of an adhesion factor for chondrocytes. *Proc. Natl. Acad. Sci. U.S.A.* **77,** 385–388.

Hewitt, A. T., Varner, H. H., Silver, M. H., Dessau, W., Wilkes, C. M., and Martin, G. R. (1982a). The isolation and partial characterization of chondronectin, an attachment factor for chondrocytes. *J. Biol. Chem.* **257,** 2330–2334.

Hewitt, A. T., Varner, H. H., Silver, M. H., and Martin, G. R. (1982b). The role of chondronectin and cartilage proteoglycan in the attachment of chondrocytes to collagen. *In* "Limb Development and Regeneration, Part B" (R. O. Kelley, P. F. Goetinck, and J. A. MacCabe, eds.), pp. 25–33. Liss, New York.

Hogan, B. L., Taylor, A., and Cooper, A. R. (1982). Murine parietal endoderm cells synthesize heparan sulphate and 170K and 145K sulphated glycoproteins as components of Reichert's membrane. *Dev. Biol.* **90,** 210–214.

Hogan, M., and Alvarado, J. (1967). Studies on the human macula. IV. Aging changes in Bruch's membrane. *Arch. Ophthalmol.* **77,** 410–420.

Hogan, M. J., Alvarado, J. A., and Weddell, J. E. (1971). "Histology of the Human Eye." Saunders, Philadelphia.

Hollyfield, J. G., and Witkovsky, P. (1974). Pigmented retinal epithelium involvement in photoreceptor development and function. *J. Exp. Zool.* **189,** 357–378.

Hollyfield, J. G., Fliesler, S. J., Rayborn, M. E., Fong, S.-L., Landers, R. A., and Bridges, C. D. (1984). Interstitial retinol-binding protein is synthesized and secreted by rod photoreceptors in the human retina. *Invest. Ophthalmol. Visual Sci. Suppl.* **25,** 275.

Hollyfield, J. G., Fliesler, S. J., Rayborn, M. E., Fong, S.-L., Landers, R. A., and Bridges, C. D. (1985a). Synthesis and secretion of interstitial retinol-binding protein by the human retina. *Invest. Ophthalmol. Visual Sci.* **26,** 58–67.

Hollyfield, J. G., Varner, H. H., Rayborn, M. E., Liou, G. I., and Bridges, D. C. (1985b). Endocytosis and degradation of interstitial retinol-binding protein: Differential capabilities of cells that border the interphotoreceptor matrix. *J. Cell Biol.* **100,** 1676–1681.

Horwitz, A. L., Hance, A. J., and Crystal, R. G. (1977). Granulocyte collagenase: Selective degradation of type I relative to type III collagen. *Proc. Natl. Acad. Sci. U.S.A.* **74,** 897–901.

Hynes, R. O. (1981). Fibronectin and its relation to cellular structure and behavior. *In* "Cell Biology of the Extracellular Matrix" (E. D. Hay, ed.), pp. 295–334. Plenum, New York.

Hynes, R. O., and Yamada, K. M. (1982). Fibronectins: Multifunctional modular glycoproteins. *J. Cell Biol.* **95,** 369–377.

Jerdan, J. A., and Glaser, B. M. (1986). Retinal microvessel extracellular matrix: An immunofluorescent study. *Invest. Ophthalmol. Visual Sci.*, in press.

Jerdan, J., Lindsey, J. D., Adler, R., and Hewitt, A. T. (1984). Laminin immunoreactivity and extracellular spaces in chick embryo neural retina. *Soc. Neurosci. Abstr.* **10,** 40.

Johnson, M. L. (1935). Visual cells of the amphibian retina in the absence of the epithelial pigment layer. *Anat. Rec.* **63,** 53–75.

Kanwar, Y. S., and Farquhar, M. G. (1979). Presence of heparan sulfate in the glomerular basement membrane. *Proc. Natl. Acad. Sci. U.S.A.* **76,** 1303–1307.

Kanwar, Y. S., Linker, A., and Farquhar, M. G. (1980). Increased permeability of the glomerular

basement membrane to ferritin after removal of glycosaminoglycans (heparan sulfate) by enzyme digestion. *J. Cell Biol.* **86,** 688–693.

Kanwar, Y. S., Hascall, J. C., and Farquhar, M. G. (1981). Partial characterization of newly synthesized proteoglycans isolated from the glomerular basement membrane. *J. Cell Biol.* **90,** 527–532.

Kato, Y., and Gospodarowicz, D. (1985). Effect of exogenous extracellular matrices on proteoglycan synthesis by cultured rabbit costal chondrocytes. *J. Cell Biol.* **100,** 486–495.

Kefalides, N. A., Alper, R., and Clark, C. C. (1979). Biochemistry and metabolism of basement membranes. *Int. Rev. Cytol.* **61,** 167–228.

Kiang, W-L., Margolis, R. U., and Margolis, R. K. (1981). Fractionation and properties of a chondroitin sulfate proteoglycan and the soluble glycoproteins of brain. *J. Biol. Chem.* **256,** 10509–10537.

Kjellén, L., Oldberg, Å., and Höök, M. (1980). Cell-surface heparan sulfate: Mechanisms of proteoglycan-cell association. *J. Biol. Chem.* **255,** 10407–10413.

Klebe, R. J. (1974). Isolation of a collagen-dependent cell attachment factor. *Nature (London)* **250,** 248–251.

Kleinman, H. K., Martin, G. R., and Fishman, P. H. (1979). Ganglioside inhibition of fibronectin-mediated cell adhesion to collagen. *Proc. Natl. Acad. Sci. U.S.A.* **76,** 3367–3371.

Kleinman, H. K., Klebe, R. J., and Martin, G. R. (1981). Role of collagenous matrices in the adhesion and growth of cells. *J. Cell Biol.* **88,** 473–485.

Kleinman, H. K., McGravey, M. L., Hassell, J. R., and Martin, G. R. (1983). Formation of a supramolecular complex is involved in the reconstitution of basement membrane components. *Biochemistry* **22,** 4969–4974.

Kohno, T., Sorgente, N., Patterson, R., and Ryan, S. J. (1983). Fibronectin and laminin distribution in bovine eye. *Jpn. J. Ophthalmol.* **27,** 496–505.

Kosher, R. A., and Church, R. L. (1975). Stimulation of *in vitro* chondrogensis by procollagen and collagen. *Nature (London)* **258,** 327–330.

Krayanek, S. (1980). Structure and orientation of extracellular matrix in developing chick optic tectum. *Anat. Rec.* **197,** 95–109.

Kresina, T., and Miller, E. J. (1979). Isolation and characterization of basement membrane collagen from human placental tissues—evidence for the presence of two genetically distinct chains. *Biochemistry* **18,** 3089–3097.

Kroll, A. J., and Machemer, R. (1968). Experimental retinal detachment in the owl monkey. III. Electron microscopy of retina and pigment epithelium. *Am. J. Ophthalmol.* **66,** 410–427.

Kühn, H. (1978). Light-regulated binding of rhodopsin kinase and other proteins to cattle photoreceptor membranes. *Biochemistry* **17,** 4389–4395.

Kühn, H. (1980). Light- and GTP-regulated interaction of GTPase and other proteins with bovine photoreceptor membranes. *Nature (London)* **283,** 587–589.

Kühn, K. (1982). Chemical properties of collagen. *In* "Immunochemistry of the Extracellular Matrix" (H. Furthmayr, ed.), Vol. 1, pp. 1–29. CRC Press, Boca Raton, Florida.

Kühn, K., and von der Mark, K. (1978). The influence of proteoglycans on the macromolecular structure of collagen. *In* "Collagen–Platelet Interaction" (H. Gastpar, K. Kühn, and R. Marx, eds.), pp. 123–127. Schattauer, Stuttgart.

Kurkinen, M., Alitalo, K., Vaheri, A., Stenman, S., and Saxen, L. (1979). Fibronectin in the development of embryonic chick eye. *Dev. Biol.* **69,** 589–600.

Lai, Y. L., Wiggert, B., Liu, Y. P., and Chader, G. J. (1982). Interphotoreceptor retinol-binding proteins: Possible transport vehicles between compartments of the retina. *Nature (London)* **298,** 848–849.

Lander, A. D., Fujii, D. K., Gospodarowicz, D., and Reichardt, L. F. (1982). Characterization of a

factor that promotes neurite outgrowth: Evidence linking activity to a heparan sulfate proteoglycan. *J. Cell Biol.* **94,** 574–585.

Lapière, C. M., Lenaers, A., and Kohn, L. D. (1971). Procollagen peptidase: An enzyme excising the coordination peptides of procollagen. *Proc. Natl. Acad. Sci. U.S.A.* **68,** 3054–3058.

Laqua, H. (1981). Collagen formation by periretinal cellular membranes. *Dev. Ophthalmol.* **2,** 396–406.

Lash, J. W., and Vasan, N. S. (1978). Somite chondrogenesis *in vitro*. Stimulation by exogenous extracellular matrix components. *Dev. Biol.* **66,** 151–171.

Laurie, G. W., Leblond, C. P., and Martin, G. R. (1982). Localization of type IV collagen, laminin, heparan sulfate proteoglycan and fibronectin to the basal lamina of basement membranes. *J. Cell Biol.* **95,** 340–344.

LaVail, M. M. (1981). Analysis of neurological mutants with inherited retinal degenerations. *Invest. Ophthalmol. Visual Sci.* **21,** 630–657.

LaVail, M. M., Pinto, L. H., and Yasurmura, D. (1981). The interphotoreceptor matrix in rats with inherited retinal dystrophy. *Invest. Ophthalmol. Visual Sci.* **21,** 658–668.

LaVail, M. M., Blanks, J. C., and Mullen, R. J. (1982). Retinal degeneration in the *pcd* cerebellar mutant mouse. I. Light microscopic and autoradiographic analysis. *J. Comp. Neurol.* **212,** 217–230.

Letourneau, P. C. (1975). Possible roles for cell to substratum adhesion in neuronal morphogenesis. *Dev. Biol.* **44,** 77–91.

Li, W., Stramm, L. E., Aguirre, G. D., and Rockey, J. H. (1984). Extracellular matrix production by cat retinal pigment epithelium *in vitro:* Characterization of type IV collagen biosynthesis. *Exp. Eye Res.* **38,** 291–304.

Liesi, P., Dahl, D., and Vaheri, A. (1983). Laminin is produced by early rat astrocytes in primary culture. *J. Cell Biol.* **96,** 920–924.

Liesi, P., Dahl, D., and Vaheri, A. (1984). Neurons cultured from developing rat brain attach and spread preferentially to laminin. *J. Neurosci. Res.* **11,** 241–251.

Linder, E. A., Vaheri, A., Ruoslahti, E., and Wartiovaara, J. (1975). Distribution of fibroblast surface antigen in the developng chick embryo. *J. Exp. Med.* **142,** 41–49.

Linsenmayer, T. F. (1981). Collagen. *In* "Cell Biology of the Extracellular Matrix" (E. D. Hay, ed.), pp. 5–37. Plenum, New York.

Liotta, G. A., Vembu, D., Kleinman, H. K., Martin, G. R., and Boone, C. (1978). Collagen required for proliferation of cultured connective tissue cells but not their transformed counterparts. *Nature (London)* **272,** 622–624.

Liotta, L. A., Abe, S., Robey, P. G., and Martin, G. R. (1979). Preferential digestion of basement membrane collagen by an enzyme derived from a metastatic murine tumor. *Proc. Natl. Acad. Sci. U.S.A.* **76,** 2268–2272.

Liotta, L. A., Lanzer, W. L., and Garbisa, S. (1981). Identification of a type V collagenolytic enzyme. *Biochem. Biophys. Res. Commun.* **98,** 184–190.

Liou, G. I., Bridges, C. D. B., Fong, S.-L., Alvarez, R. A., and Gonzalez-Fernandez, F. (1982). Vitamin A transport between retina and pigment epithelium. An interstitial protein carrying endogenous retinol (interstitial retinol-binding protein). *Vision Res.* **22,** 1457–1467.

Lyda, N., Eriksen, N., and Krishna, N. (1957). Studies of Bruch's membrane. Flow and permeability studies in a Bruch's membrane-choroid preparation. *Am. J. Ophthalmol.* **44,** 362–369.

McCarthy, J. B., Palm, S. L., and Furcht, L. T. (1983). Migration by haptotaxis of a Schwann cell tumor line to the basement membrane glycoprotein laminin. *J. Cell Biol.* **97,** 772–777.

McGarvey, M. L., Baron-Van Evercooren, A., Kleinman, H. K., and Dubois-Dalcq, M. (1984). Synthesis and effects of basement membrane components in cultured rat Schwann cells. *Dev. Biol.* **105,** 18–28.

Machemer, R., Van Horn, D., and Aaberg, T. M. (1978). Pigment epithelial proliferation in human retinal detachment with massive periretinal proliferation. *Am. J. Ophthalmol.* **85,** 181–191.

Magalhães, M. M., and Coimbra, A. (1972). The rabbit retina Müller cell: A fine structural and cytochemical study. *J. Ultrastruct. Res.* **39,** 310–326.

Mainardi, C. L., Seyer, J. N., and Kang, A. H. (1980). Type specific collagenolysis of type V collagen degrading enzyme from macrophages. *Biochem. Biophys. Res. Commun.* **97,** 1108–1115.

Manthorpe, M., Varon, S., and Adler, R. (1981). Neurite-promoting factor in conditioned medium from RN22 Schwannoma cultures: Bioassay, fractionation, and properties. *J. Neurochem.* **37,** 759–767.

Manthorpe, M., Engvall, E., Ruoslahti, E., Longo, F. M., Davis, G. E., and Varon, S. (1983). Laminin promotes neuritic regeneration from cultured peripheral and central neurons. *J. Cell Biol.* **97,** 1882–1890.

Matsumoto, B., Blanks, J. C., and Ryan, S. J. (1984). Topographic variations in the rabbit and primate internal limiting membrane. *Invest. Ophthalmol. Visual Sci.* **25,** 71–82.

Matthew, W. D., and Patterson, P. H. (1983). Isolation of a monoclonal antibody that inhibits the *in vitro* neurite-promoting activity of a heparan sulfate proteoglycan. *Soc. Neurosci. Abstr.* **9,** 206.

Mayne, R. (1984). The different types of collagen and collagenous peptides. *In* "The Role of Extracellular Matrix in Development" (R. L. Trelstad, ed.), pp. 33–42. Liss, New York.

Meeks, R., Zaharevitz, D., and Chen, R. (1981). Membrane effects of retinoids: Possible correlation with toxicity. *Arch. Biochem. Biophys.* **207,** 141–147.

Meier, S., and Hay, E. D. (1974). Control of corneal differentiation by extracellular materials. Collagen as a promoter and stabilizer of epithelial stroma production. *Dev. Biol.* **38,** 249–270.

Miller, E. J., and Matukas, V. J. (1969). Chick cartilage collagen: A new type of $\alpha 1$ chain not present in bone or skin of the species. *Proc. Natl. Acad. Sci. U.S.A.* **64,** 1264–1268.

Miller, E. J., Harris, E. D., Chung, E., Finch, J. E., Jr., McCroskery, P. A., and Butler, W. T. (1976). Cleavage of type II and III collagens with mammalian collagenase: Site of cleavage and primary structure at NH_2 terminal position of the smaller fragments released from both collagens. *Biochemistry* **15,** 787–792.

Mishima, H., Hasebe, H., and Kondo, K. (1978). Age changes in the fine structure of the human retinal pigment epithelium. *Jpn. J. Ophthalmol.* **22,** 476–485.

Mixon, R. N., and Feeney, L. (1977). Phagocytosis of liposomes and isolated rod outer segments by bovine retinal pigment epithelium. *Invest. Ophthalmol. Visual Sci. Suppl.* **16,** 111.

Morris, J. E. (1979). Steric exclusion of cells. A mechanism of glycosaminoglycan-induced cell aggregation. *Exp. Cell Res.* **120,** 141–153.

Morris, J. E., and Dorfman, A. (1976). Inhibition by 5′-bromo-2′-deoxyuridine of differentiation-dependent changes in glycosaminoglycans of the retina. *Biochem. Biophys. Res. Commun.* **69,** 1065–1072.

Morris, J. E., and Ting, Y.-P. (1981). Comparison of proteoglycans extracted by saline and guanidinium chloride from cultured chick retinas. *J. Neurochem.* **37,** 1594–1602.

Morris, J. E., Hopwood, J. J., and Dorfman, A. (1977). Biosynthesis of glycosaminoglycans in the developing retina. *Dev. Biol.* **58,** 313–327.

Morris, J. E., Ting, Y.-P., and Birkholz-Lambrecht, A. (1984). Low buoyant density proteoglycans from saline and dissociative extracts of embryonic chicken retinas. *J. Neurochem.* **42,** 798–809.

Moscona, A. A., and Linser, P. (1983). Developmental and experimental changes in retinal glia cells: Cell interactions and control of phenotype expression and stability. *Curr. Top. Dev. Biol.* **18,** 155–188.

Mosesson, M. W., Chen, A. B., and Huseby, R. M. (1975). The cold-insoluble globulin of human plasma: Studies of its essential structural features. *Biochim. Biophys. Acta* **386,** 509–524.

Nakaizumi, Y. (1964a). The ultrastructure of Bruch's membrane. I. Human, monkey, rabbit, guinea pig and rat eyes. *Arch. Ophthalmol.* **72,** 380–387.

Nakaizumi, Y. (1964b). The ultrastructure of Bruch's membrane. II. Eyes with a tapetum. *Arch. Ophthalmol.* **72,** 388–394.

Nakaizumi, Y., Hogan, M. J., and Feeney, L. (1964). The ultrastructure of Bruch's membrane. III. The macular area of the human eye. *Arch. Ophthalmol.* **72,** 395–400.

Newgreen, D., and Thiery, J. P. (1980). Fibronectin in early avian embryo: Synthesis and distribution along the migration pathways of neural crest cells. *Cell Tissue Res.* **211,** 269–291.

Newsome, D. A. (1976). *In vitro* stimulation of cartilage in embryonic chick neural crest cells by products of retinal pigmented epithelium. *Dev. Biol.* **49,** 496–507.

Newsome, D. A. and Kenyon, K. R. (1973). Collagen production *in vitro* by the retinal pigmented epithelium of the chick embryo. *Dev. Biol.* **32,** 387–400.

Newsome, D. A., Rodriques, M. M., and Machemer, R. M. (1981). Human massive periretinal proliferation. *In vitro* characteristics of cellular components. *Arch. Ophthalmol.* **99,** 837–880.

Norr, S. C. (1973). *In vitro* analysis of sympathetic neuron differentiation from chick neural crest cells. *Dev. Biol.* **34,** 16–38.

Ocumpaugh, D. E., and Young, R. W. (1966). Distribution and synthesis of sulfated mucopolysaccharides in the retina of the rat. *Invest. Ophthalmol.* **5,** 196–203.

Palm, S. L., and Furcht, L. T. (1983). Production of laminin by a rat Schwannoma cell line: Cell–protein interactions *in vitro* and protein localization in peripheral nerve *in vivo*. *J. Cell Biol.* **96,** 1218–1226.

Pearlstein, E. (1976). Plasma membrane glycoprotein which mediates adhesion of fibroblasts to collagen. *Nature (London)* **262,** 497–500.

Peterkofsky, B., and Diegelmann, R. F. (1971). Use of a mixture of proteinase-free collagenases for the specific assay of radioactive collagen in the presence of other proteins. *Biochemistry* **10,** 988–994.

Pfeffer, B., Wiggert, B., Lee, L., Zonnenberg, B., Newsome, D., and Chader, G. (1983). The presence of a soluble interphotoreceptor retinol-binding protein (IRBP) in the retinal interphotoreceptor space. *J. Cell. Physiol.* **117,** 333–341.

Pino, R. M., and Essner, E. (1981). Permeability of rat choriocapillaris to hemeproteins. Restriction of tracers by a fenestrated endothelium. *J. Histochem. Cytochem.* **29,** 281–290.

Pino, R. M., Essner, E., and Pino, L. C. (1982). Location and chemical composition of anionic sites in Bruch's membrane of the rat. *J. Histochem. Cytochem.* **30,** 245–252.

Podleski, T. R., Greenberg, I., Schlessinger, J., and Yamada, K. M. (1979). Fibronectin delays the fusion of L_6 myoblasts. *Exp. Cell Res.* **122,** 317–326.

Prockop, D. J., Kivirikko, K. I., Tuderman, L., and Guzman, N. A. (1979). The biosynthesis of collagen and its disorders. *N. Engl. J. Med.* **301,** 13–23, 77–85.

Rambourg, A., and Leblond, C. P. (1967). Electron microscope observations on the carbohydrate-rich cell coat present at the surface of cells in the rat. *J. Cell Biol.* **32,** 27–53.

Rayborn, M. E., Bridges, C. D., Landers, R. A., and Hollyfield, J. G. (1984). Synthesis, secretion, localization and immunoreactivity of interstitial retinol-binding protein in *Xenopus laevis*. *Invest. Ophthalmol. Visual Sci. Suppl.* **25,** 275.

Redmond, T. M., Wiggert, B., Robey, F. A., Nguyen, N. Y., Lewis, M., Lee, L., and Chader, G. J. (1985). Isolation and characterization of monkey interphotoreceptor retinoid-binding protein, a unique extracellular matrix component of the retina. *Biochemistry* **24,** 787–793.

Rhodes, R. H. (1982). An ultrastructural study of the complex carbohydrates of the mouse posterior vitreoretinal juncture. *Invest. Ophthalmol. Visual Sci.* **22,** 460–477.

Rhodes, R. K., and Miller, E. J. (1978). Physiochemical characterization and molecular organization of the collagen A and B chains. *Biochemistry* **17,** 3442–3448.

Riepe, R. E., and Norenburg, M. D. (1977). Müller cell localization of glutamine synthetase in rat retina. *Nature (London)* **268,** 654–655.

Robey, P. G., and Martin, G. R. (1981). Type IV collagen contains two distinct chains in separate molecules. *Collagen Res.* **1,** 27–38.

Robey, P. G., and Newsome, D. A. (1983). Biosynthesis of proteoglycans present in primate Bruch's membrane. *Invest. Ophthalmol. Visual Sci.* **24,** 898–905.

Rodriquez-Peralta, L. A. (1968). Hematic and fluid barriers of the retina and vitreous body. *J. Comp. Neurol.* **132,** 109–123.

Rogers, S. L., Letourneau, P. C., Palm, S. L., McCarthy, J., and Furcht, L. T. (1983). Neurite extension by peripheral and central nervous system neurons in response to substratum-bound fibronectin and laminin. *Dev. Biol.* **98,** 212–220.

Röhlich, P. (1970). The interphotoreceptor matrix: Electron microscopic and histochemical observations on the vertebrate retina. *Exp. Eye Res.* **10,** 80–96.

Rohrbach, D. H., Hassell, J. R., Kleinman, H. K., and Martin, G. R. (1982). Alterations in the basement membrane (heparan sulfate) proteoglycan in diabetic mice. *Diabetes* **31,** 185–188.

Rojkind, M., Gatmaitan, Z., Mackensen, S., Giambrone, M-A., Ponce, P., and Reid, L. (1980). Connective tissue biomatrix: Its isolation and utilization for long-term cultures of normal rat hepatocytes. *J. Cell Biol.* **87,** 255–263.

Ruoslahti, E., and Engvall, E. (1980). Complexing of fibronectin glycosaminoglycans and collagen. *Biochim. Biophys. Acta* **631,** 350–358.

Ruoslahti, E., Hayman, E. G., Kuusela, P., Shively, J. E., and Engvall, E. (1979). Isolation of a tryptic fragment containing the collagen-binding site of plasma fibronectin. *J. Biol. Chem.* **254,** 6054–6059.

Ruoslahti, E., Engvall, E., and Hayman, E. G. (1981). Fibronectin: Current concepts of its structure and functions. *Collagen Res.* **1,** 95–128.

Saari, J. C., and Futterman, S. (1976). Retinol-binding protein in bovine retina: Isolation and partial characterization. *Exp. Eye Res.* **22,** 425–433.

Saari, J. C., Teller, D. C., Crabb, J. W., and Bredberg, L. (1985). Properties of an interphotoreceptor retinoid-binding protein from bovine retina. *J. Biol. Chem.* **260,** 195–201.

Sage, H., and Bornstein, P. (1979). Characterization of a novel collagen chain in human placenta and its relation to AB collagen. *Biochemistry* **18,** 3815–3822.

Sakashita, S., Engvall, E., and Ruoslahti, E. (1980). Basement membrane glycoprotein, laminin, binds to heparin. *FEBS Lett.* **116,** 243–246.

Sarks, S. H. (1976). Aging and degeneration in the macular region: A clinico-pathological study. *Br. J. Ophthalmol.* **60,** 324–341.

Sarks, S. H. (1980). Drusen and their relationship to senile macular degeneration. *Aust. J. Ophthalmol.* **8,** 117–130.

Schachner, M., Schoonmaker, G., and Hynes, R. O. (1978). Cellular and subcellular localization of LETS protein in the nervous system. *Brain Res.* **158,** 149–158.

Schmut, O. (1978). The organization of tissues of the eye by different collagen types. *Albrecht von Graefes Arch. Klin. Exp. Ophthalmol.* **207,** 189–199.

Schmut, O., Reich, M. E., and Hoffman, H. (1979). Isolation of different hydroxyproline-containing proteins from bovine vitreous body collagen. *Albrecht von Graefes Arch. Klin. Exp. Ophthalmol.* **211,** 329–332.

Seppä, H. E. J., Yamada, K. M., Seppä, S. T., Silver, M. H., Kleinman, H. K., and Schiffman, E. (1981). The cell binding fragment of fibronectin is chemotactic for fibroblasts. *Cell Biol. Int. Rep.* **5,** 813–820.

Sidman, R. L. (1958). Histochemical studies on photoreceptor cells. *Trans. N.Y. Acad. Sci.* **74,** 182–195.

Sieber-Blum, M., Sieber, F., and Yamada, K. M. (1981). Cellular fibronectin promotes adrenergic differentiation of quail neural crest cells *in vitro*. *Exp. Cell Res.* **133,** 285–295.

Sigelman, J., and Ozanics, V. (1982). Retina. *In* "Ocular Anatomy, Embryology and Teratology" (F. A. Jakobiec, ed.), pp. 441–506. Harper, New York.

Smalheiser, N. R., Crain, S. M., and Reid, L. M. (1984). Laminin as a substrate for retinal axons *in vitro*. *Dev. Brain Res.* **12,** 136–140.

Smelser, G. K., Ishikawa, T., and Pei, Y. F. (1965). Electron microscopic studies of intraretinal spaces: Diffusion of particulate materials. *In* "Structure of the Eye. II. Symposium" (J. W. Rohen, ed.), pp. 109–120. Schattauer, Stuttgart.

Smith, G. N., Jr., and Newsome, D. A. (1978). The nature and origin of the glycosaminoglycans of the embryonic chick vitreous body. *Dev. Biol.* **62,** 65–77.

Smith, G. N., Linsenmeyer, T. F., and Newsome, D. A. (1976). Synthesis of type II collagen *in vitro* by embryonic chick neural retina tissue. *Proc. Natl. Acad. Sci. U.S.A.* **73,** 4420–4423.

Spiegel, S., Yamada, K. M., Hom, B. E., Moss, J., and Fishman, P. H. (1985). Fluorescent gangliosides as probes for the retention and organization of fibronectin by ganglioside-deficient mouse cells. *J. Cell Biol.* **100,** 721–726.

Spooner, B. S., and Faubion, J. M. (1980). Collagen involvement in branching morphogenesis of embryonic lung and salivary gland. *Dev. Biol.* **77,** 84–102.

Stenman, S., and Vaheri, A. (1978). Distribution of a major connective tissue protein, fibronectin, in normal human tissue. *J. Exp. Med.* **147,** 1054–1064.

Stow, J. L., Kjellén, L., Unger, E., Höök, M., and Farquhar, M. G. (1985). Heparan sulfate proteoglycans are concentrated on the sinusoidal plasmalemmal domain and in intracellular organelles of hepatocytes. *J. Cell Biol.* **100,** 975–980.

Swann, D. A. (1980). Chemistry and biology of the vitreous body. *Int. Rev. Exp. Pathol.* **22,** 1–64.

Swann, D. A., Constable, I. J., and Harper, E. (1972). Vitreous structure. III: Composition of bovine vitreous collagen. *Invest. Ophthalmol.* **11,** 735–738.

Szamier, R. B., and Berson, E. C. (1977). Retinal ultrastructure in advanced retinitis pigmentosa. *Invest. Ophthalmol. Visual Sci.* **16,** 947–958.

Termine, J. D., Kleinman, H. K., Whitson, S. W., Conn, K. M., McGarvey, M. L., and Martin, G. R. (1981). Osteonectin, a bone-specific protein linking mineral to collagen. *Cell* **26,** 99–105.

Terranova, V. P., Rohrbach, D. H., and Martin, G. R. (1980). Role of laminin in the attachment of PAM 212 (epithelial) cells to basement membrane collagen. *Cell* **22,** 719–726.

Terranova, V. P., Rao, C. N., Kalebic, T., Margulies, I. M., and Liotta, L. A. (1983). Laminin receptor on human breast carcinoma cells. *Proc. Natl. Acad. Sci. U.S.A.* **80,** 444–448.

Thiery, J-P., Brackenbury, R., Rutishauser, U., and Edelman, G. M. (1977). Adhesion among neural cells of the chick embryo. II. Purification and characterization of a cell adhesion molecule from neural retina. *J. Biol. Chem.* **252,** 6841–6845.

Timpl, R. (1982). Components of basement membranes. *In* "Immunochemistry of the Extracellular Matrix: Vol. II Applications" (H. Furthmayr, ed.), pp. 119–150. CRC Press, Boca Raton, Florida.

Timpl, R., Martin, G. R., Bruchner, P., Wick, G., and Wiedemann, H. (1978). Nature of the collagenous protein in a tumor basement membrane. *Eur. J. Biochem.* **84,** 43–52.

Timpl, R., Rohde, H., Robey, P. G., Rennard, S. I., Foidart, J.-M., and Martin, G. R. (1979). Laminin—a glycoprotein from basement membranes. *J. Biol. Chem.* **254,** 9933–9937.

Timpl, R., Wiedermann, H., Van Delden, V., Furthmayr, H., and Kühn, K. (1981). A network model for the organization of type IV collagen molecules in basement membranes. *Eur. J. Biochem.* **120,** 203–211.

Tolentino, F. I., Schepens, C. L., and Freeman, H. M. (1976). "Vitreoretinal disorders. Diagnosis and Management," pp. 13–14. Saunders, Philadelphia, Pennsylvania.

Tomasek, J. J., Mazurkiewicz, J. E., and Newman, S. A. (1982). Non-uniform distribution of fibronectin during avian limb development. *Dev. Biol.* **90,** 118–126.

Trelstad, R. L., and Coulombre, A. J. (1971). Morphogenesis of the collagenous stroma in the chick cornea. *J. Cell Biol.* **50,** 840–858.

Trelstad, R. L., Hayashi, K., and Toole, B. P. (1974). Epithelial collagens and glycosaminoglycans in the embryonic cornea. Macromolecular order and morphogenesis in the basement membrane. *J. Cell Biol.* **62,** 815–830.

Tryggvason, K., Robey, P. G., and Martin, G. R. (1980). Biosynthesis of type IV procollagens. *Biochemistry* **19,** 1284–1289.

Varner, H. H., and Hollyfield, J. G. (1985). Selective binding of an interphotoreceptor matrix protein to the retinal pigment epithelium. *Invest. Ophthalmol. Visual Sci. Suppl.* **26,** 198.

von der Mark, K., von der Mark, H., Timpl, R., and Trelstad, R. L. (1977). Immunofluorescent localization of collagen types I, II, and III in the embryonic chick eye. *Dev. Biol.* **59,** 75–85.

Wald, F., and de Robertis, E. (1961). The action of glutamate and the problem of the "extracellular space" in the retina. *Z. Zellforsch.* **55,** 649–661.

Wartiovaara, J., Leivo, I., and Vaheri, A. (1979). Expression of the cell surface-associated glycoprotein, fibronectin, in the early mouse embryo. *Dev. Biol.* **69,** 247–257.

Wiggert, B. O., and Chader, G. J. (1975). A receptor for retinol in the developing retina and pigment epithelium. *Exp. Eye Res.* **21,** 143–151.

Wiggert, B., Mizukawa, A., Kuwabara, T., and Chader, G. J. (1978). Vitamin A receptors: Multiple species in retina and brain and possible compartmentalization in retinal photoreceptors. *J. Neurochem.* **30,** 653–659.

Wislocki, G. B., and Sidman, R. L. (1954). The chemical morphology of the retina. *J. Comp. Neurol.* **101,** 53–99.

Witschel, H. (1981). Intraretinal collagen formation in lattice degeneration. *Dev. Ophthalmol.* **2,** 369–384.

Woodley, D. T., Rao, C. N., Hassell, J. R., Liotta, L. A., Martin, G. R., and Kleinman, H. K. (1983). Interactions of basement membrane components. *Biochim. Biophys. Acta* **761,** 278–283.

Yamada, K. M. (1981). Fibronectin and other structural proteins. *In* "Cell Biology of the Extracellular Matrix" (E. D. Hay, ed.), pp. 95–114. Plenum, New York.

Yamada, K. M., and Olden, K. (1978). Fibronectins—adhesive glycoproteins of cell surface and blood. *Nature (London)* **275,** 179–184.

Yamada, K. M., Schlesinger, D. H., Kennedy, D. W., and Pastan, I. (1977). Characterization of a major fibroblast cell surface glycoprotein. *Biochemistry* **16,** 5552–5559.

Yamada, K. M., Kennedy, D. W., Kimata, K., and Pratt, R. M. (1980). Characterization of fibronectin interactions with glycosaminoglycans and identification of active proteolytic fragments. *J. Biol. Chem.* **255,** 6055–6063.

Yamada, K. M., Kennedy, D. W., Grotendorst, G. R., and Momoi, T. (1981). Glycolipids: Receptors for fibronectin? *J. Cell. Physiol.* **109,** 343–351.

Zauberman, H., and Berman, E. R. (1969). Measurement of adhesive forces between sensory retina and pigment epithelium. *Exp. Eye Res.* **8,** 276–283.

Zimmerman, L. E. (1958). Applications of histochemical methods for the demonstration of acid mucopolysaccharides to opthalmic pathology. *Trans. Am. Acad. Ophthalmol.* **62,** 697–701.

Zimmerman, L. E., and Eastham, A. B. (1959). Acid mucopolysaccharide in the retinal pigment epithelium and visual cell layer of the developing mouse eye. *Am. J. Ophthalmol.* **47,** 488–498.

CELL BIOLOGY AND BIOCHEMISTRY OF ENDOTHELIAL CELLS AND THE PHENOMENON OF INTRAOCULAR NEOVASCULARIZATION

BERT M. GLASER

Center for Vitreoretinal Research
The Wilmer Ophthalmological Institute
The Johns Hopkins University
School of Medicine
Baltimore, Maryland

I. Introduction

The process of new blood vessel formation and growth (commonly termed neovascularization) has been found to play an important role in an ever increasing number of biological phenomena. Neovascularization may be found at vari-

ous sites within the eye, depending upon the nature of the underlying disorder (Henkind, 1978). Retinal and optic disk neovascularization occur in diabetic retinopathy, venous occlusive disorders, sickle cell retinopathy, retrolental fibroplasia, and chronic inflammatory disorders. Subretinal pigment epithelial neovascularization occurs in disorders, such as senile macular degeneration and the presumed ocular histoplasmosis syndrome. Iris neovascularization (rubeosis irides) is most commonly found associated with proliferative diabetic retinopathy and central retinal vein occlusion.

A great deal has been learned about the factors that influence the formation and growth of new blood vessels. However, many questions still remain. In the following pages, we will review the current concepts and controversies regarding the biology of endothelial cells, key elements in the phenomenon of intraocular neovascularization.

II. New Vessels from Preexisting Vessels

One of the earliest questions to be addressed by scientists studying neovascularization was to determine the origin of the new blood vessels found in various situations. In the earliest stages of embryologic development, new blood vessels must, no doubt, arise *de novo*. The origin of new blood vessels which form during later stages of development and under various circumstances in the mature animal has been the subject of considerable study. At the end of the eighteenth century, it was thought that new blood vessels arise *de novo* within wounds without relation to preexisting vessels. John Hunter (1812), in a treatise published in 1812, reported observing many spots of red blood within wounds which he could not inject with mercury. He concluded that these were areas of *de novo* synthesis of vessels and red blood cells, independent of the existing circulation. The theory of *de novo* synthesis of new blood vessels within wounds was challenged in 1826 by Gendrin. He performed a series of experiments in which he surgically created skin flaps. These flaps were promptly reapplied and allowed to heal. After various periods of time, the edges of the flaps were recut in such a way that they remained attached to the surrounding skin only by the newly formed tissue filling the wound. Serial histologic studies demonstrated branches from adjacent preexisting vessels extending through the wound and into the skin flaps.

In 1852, Joseph Meyer studied the process of capillary formation and found that the growth of capillaries proceeds from preexisting vessels. He also felt that the red dots and lines thought by previous investigators to be site of *de novo* new blood vessel formation were extravasates. In addition, Meyer observed that arteries and veins always develop from simple capillary tubes. Auerbach in 1865

observed that capillaries are cellular tubes. Golubew (1869), Arnold (1871), and Rouget (1873) are among the many investigators who subsequently performed microscopic studies of the vasculature of the transparent fin expansion of the tadpole tail. These investigators all observed that new blood vessels begin as tiny cellular sprouts extending from existing capillaries. These sprouts gradually extend until they meet and anastomose with other sprouts or capillaries. As these sprouts extend, a lumen gradually forms. This process occurs entirely without the interposition of other cells, such as fibroblasts or macrophages. Numerous subsequent studies supported these findings (Thomas, 1893; Ziegler, 1905; Evans, 1909). Therefore, by the beginning of the twentieth century, it was well established that, except for the earliest stages of embryologic development, new blood vessels form from preexisting vessels.

There are several key steps that have been found to occur during the development of new capillary sprouts. One of the earliest steps is the localized fragmentation of the basement membrane surrounding the parent capillary, presumably at the sight where the new sprout is to develop. Secondly, vascular endothelial cells from the wall of the parent vessel migrate through openings in the surrounding basement membrane to form a capillary bud. This capillary bud then gradually extends into the surrounding tissue. The advancing tip of this sprout consists of migrating endothelial cells which do not seem to be dividing. The more proximal portions of the advancing sprout, which contain dividing cells, provide building blocks to link the advancing tip to the parent vessel. A discussion of these important aspects of capillary sprout formation follows.

III. Steps in Capillary Formation

A. Basement Membrane Fragmentation

The presence of a condensed zone of connective tissue completely surrounding mature capillaries was first described more than a century ago. The term "basement membrane" was applied to this condensation of connective tissue (Krakower and Greenspon, 1959). The basement membrane is composed of a tightly matted array of fine (30–40 Å) fibrils embedded in a finely granular matrix (Palade and Burns). Several investigators have isolated protein components from basement membranes which meet the chemical and physical criteria for collagens. However, basement membrane collagen (type IV) (Kefalides, 1973) appears to have some characteristics which differentiate it from the so-called interstitial collagens (types I, II, and III). More recently a glycoprotein, laminin, has been isolated from murine basement membrane (Timpl *et al.*, 1979; see also the article by Hewitt, this volume).

In 1963, Schoefl reported an extensive series of electron microscopic studies of neovascularization in muscle wounds and silver nitrate-induced corneal burns. One of the most important aspects of this study was the discovery of numerous gaps or discontinuities in the basement membrane surrounding the capillaries, seen very early in the course of new blood vessel development. Cytoplasmic extrusions arising from the outer aspect of the vascular wall and projecting through the gaps in the basement membrane were common. In 1976, Ausprunk and Folkman studied tumor-induced capillary proliferation in the rabbit cornea. In these studies, V2 carcinoma was implanted into the clear cornea of the rabbit, 1 mm from the vascular plexus. Within 1 day of tumor implantation, before capillary sprouts developed, the normally continuous basement membrane surrounding existing vessels became fragmented. Within 2 days, endothelial cells extended large pseudopods through gaps in the basement membrane. On the basis of these observations, it has been concluded that fragmentation of capillary basement membranes constitutes one of the earliest steps in the formation of new capillary sprouts. It has been hypothesized that initial gaps in the basement membrane provide exit sites through which the endothelial cells will later migrate to form capillary buds and sprouts.

Recent studies (Kalebic *et al.*, 1983) have demonstrated that fetal bovine aortic endothelial cells *in vitro,* when stimulated by angiogenic preparations, are capable of producing an enzyme which can fragment type IV collagen. Under similar conditions, fibroblasts and smooth muscle cells are unable to produce such an enzyme. The endothelial cell type IV collagenase may play a role in basement membrane fragmentation early in the course of new blood vessel formation.

The early occurrence of basement membrane fragmentation during capillary sprout formation makes this phenomenon an important subject for future research.

B. Capillary Endothelial Cell Migration

The following experimental evidence has led to the conclusion that after gaps develop in the basement membrane surrounding a parent capillary, vascular endothelial cells migrate from the vessel wall to form new capillary buds. The migratory activity of the vascular endothelial cells continues to be important as the capillary sprout elongates.

In 1955, Van den Brenk made detailed observations of new blood vessel formation in rabbit ear chambers. After prolonged observation, he was unable to demonstrate any mitotic activity in the vascular endothelium of newly formed capillary sprouts. He observed, additionally, that the vascular endothelium was capable of migrating with great facility. From these observations, he concluded

that endothelial cell *migration* plays a primary role in neovascularization. Schoefl (1963) observed numerous endothelial cell cytoplasmic processes at the distal end of capillary sprouts. These processes were interpreted as pseudopodia, which were thought to indicate active cellular migration at the advancing capillary tip. In 1970, Yamagami performed an electron microscopic study of corneal neovascularization in response to alkali burns. Careful observation of serial sections revealed that the advancing tips of new capillary sprouts are composed of a closely associated group of endothelial cells. Tight junctions were observed between cells composing this distal tip of the capillary sprout. However, endothelial cells located 40–60 μm proximal to the tip were only loosely approximated to one another and allowed the extravasation of blood cells and Throtrast particles. Mitotic figures were frequently found in the endothelial cells of this more proximal portion of the capillary sprout. In contrast, mitotic figures were never observed in the cells of the distal tip of the capillary sprout. These observations were felt to suggest that new capillary sprouts are composed of two regions, a distal region composed of migrating cells and a more proximal region composed of dividing cells. Ausprunk and Folkman (1977), in their electron microscopic studies of neovascularization found that vascular endothelial cell migration precedes tritiated thymidine incorporation and cell division during the formation of new capillaries.

The importance of vascular endothelial cell migration in the formation of new capillary sprouts has prompted several investigators to develop methods for studying vascular endothelial cell migration *in vitro*. Albrecht-Buehler (1977, 1975) has devised a system to analyze the movement of cultured fibroblasts *in vitro*. In this system, fibroblasts are first plated onto glass coverslips that have previously been coated with tiny particles of gold. As the cells move on the surface of the coverslips, they remove the gold, leaving bare areas or tracks that serve as a record of their movement. The phenomenon thus measured is termed phagokinesis. McAuslan (1979) has recently modified this technique to study vascular endothelial cell phagokinesis. He found that various fractions from Walker carcinoma extracts are capable of stimulating adult bovine aortic endothelial cell phagokinesis. Zetter (1980) used the above technique to study the phagokinetic activity of capillary endothelial cells.

Since the ability of cells to form detectable tracks depends on both phagocytosis and migration, it is difficult to determine which of these two components is being affected by a given substance under study. Investigators have therefore developed assays which measure endothelial cell migration more directly. Glaser *et al.* (1980) have developed an assay which measures the ability of vascular endothelial cells to migrate through a thin porous membrane. The apparatus used for this assay consists of two wells (an upper well and a lower well) separated by a porous membrane. The lower well of this apparatus is filled with tissue culture medium containing a known concentration of the substance to be tested. The

upper well is filled with tissue culture medium in which is suspended a predetermined number of vascular endothelial cells. The apparatus is then incubated for 7 hr at 37°C during which time the cells attach to the upper surface of the membrane, migrate through the pores, and then attach to the lower surface of the membranes. At the end of the experiment, the cells on the upper surface of the membrane are removed with a cotton swab. The endothelial cells which have migrated through the membrane attach to the lower surface where they are fixed and stained. Migration is quantified by counting the number of cell nuclei on the lower surface of each membrane.

Utilizing this technique, Glaser *et al.* (1980) have shown that adult mammalian retina contains a stimulator (or stimulators) of vascular endothelial cell migration. During the development of new blood vessels, the capillary sprouts appear to be oriented so that they advance toward the inciting stimulus (Ausprunk and Folkman, 1977). Very few capillary sprouts are ever seen to advance in a direction away from the inciting stimulus. It has been suggested that the migration of endothelial cells at the tip of the capillary sprout toward the stimulus may be due to a preferential migration of cells from lower to higher concentrations of a mediator. Such concentration gradient-dependent cellular migration has been termed chemotaxis. Glaser *et al.* (1980) have utilized the migration apparatus described above to study vascular endothelial cell chemotaxis *in vivo*. Various amounts of a substance to be tested for chemotactic activity are added to both the upper and the lower wells of the migration chambers to produce a series of concentration gradients across the membranes. Migration is then studied as a function of the concentration gradient across the membrane. In this manner, adult mammalian retina was found to contain a substance (or substances) which is chemotactic for fetal bovine aortic endothelial cells *in vivo*.

Vascular endothelial cell migration appears to play an important role in neovascularization. Further study of this phenomenon in both *in vivo* and *in vitro* systems will, hopefully, shed additional light on the factors and conditions which modulate new blood vessel formation.

C. Proliferation of Capillary Endothelial Cells

Wise *et al.* (1971) studied the replication rate of cells composing the retinal capillary bed of the adult rat and found this to be approximately 0.09%. Engerman *et al.* (1967) performed a similar study in mice and found a replication rate of 0.01%. With the onset of new capillary sprout formation, Ausprunk and Folkman (1977) have demonstrated an increase of the capillary endothelial cell replication rate to a peak of 8%. It is interesting to note that Yamagami (1970) has demonstrated that these dividing endothelial cells are confined to the proximal regions of the advancing capillary sprouts. The tip of the capillary sprouts

appears to be composed of nondividing endothelial cells with tight intercellular junctions and an ameboid shape. In contrast, the proximal region of the capillary sprout, where cell division occurs, is also the area where the endothelial cells have lost their tight junctions and have become separated by intercellular gaps (Yamagami, 1970). Ausprunk and Folkman (1977) have suggested that the cell division found in this region might be secondary to a loss of contact inhibition rather than the effect of specific growth stimulators. They base this hypothesis on studies (Ross and Glomset, 1976) of the mitotic behavior of endothelial cells in large vessels, which show that a group of endothelial cells adjacent to an area of desquamation will continue to divide until the defect becomes covered by a continuous monolayer of endothelial cells. The cessation of replication when cells reach a confluent monolayer, with each cell completely bordered by cell membranes of adjacent cells, is termed contact inhibition. Endothelial cells grown in tissue culture have also been found to cease replicating when a confluent monolayer of cells develops (Haudenschild *et al.*, 1976).

The study of the factors and conditions which affect vascular endothelial cell replication with subsequent comparison of the effects of these factors on neovascularization will help shed light on the role of vascular endothelial cell replication in new blood vessel formation.

IV. Maturation of the Vascular Bed

A. Capillaries Mature to Form Arteries and Veins

We have thus far described the process of capillary sprout formation and growth. The tip of the capillary sprout advances until it encounters another capillary sprout. When this occurs, the two capillary sprouts merge to form a capillary loop (Clark, 1918, 1939). Capillary loop formation leads to the development of a extensive capillary plexus. The subsequent maturation of this newly formed capillary bed to include arteries and arterioles connected by a capillary plexus to venules and veins has been studied by several investigators. In 1893, Thomas reported his studies of the extraembryonic yolk sac vessels of chick embryos. From a series of injected specimens, he concluded that the capillaries within the newly formed plexus, which are located so that the greatest amount of blood flows through them, enlarge to become arteries. Later studies by Mall (1906) and Evans (1909) utilizing injected mammalian embryos confirmed the observation that arteries and veins are derived from previously formed capillaries.

Up to this point, most of the observations of the development of arteries and veins were made utilizing fixed tissue specimens. Clark, in 1918, was one of the

first to study the complete growth of a portion of the vascular system by observing the same vessel from day to day over a period of several weeks. Clark (1918) studied the developing vessels of the transparent tail fin of the tadpole and made the following observations regarding maturation of the vascular plexus. A capillary, once formed, may have one of several fates; it may enlarge to form an arteriole or venule, which then may become an artery or a vein, or it may remain a capillary or it may retract (Clark, 1918). The size of the vessel which the capillary ultimately develops into was felt to be dependent upon the volume of blood flow through it. Ashton presented data suggesting that the periarteriolar capillary free zone found in mature retina develops by a process of capillary regression which probably occurs by the same mechanism described by Clark (1918).

Therefore, the mature vascular pattern develops by successive stages beginning with capillary sprout formation from existing vessels. These capillary sprouts link to form a dense immature capillary plexus. The capillaries, which are positioned within the plexus so that they carry greater blood flow, enlarge to form arterioles and venules and later arteries and veins. Those capillaries, which are positioned so that they carry less blood flow, eventually regress. The factors and conditions that dictate which vascular channels in the immature capillary plexus carry greater flow and which carry a lesser flow are not yet fully understood.

B. Capillary Endothelial Cells vs Large Vessel Endothelial Cells: Differences and Similarities

A major unanswered question related to the study of neovascularization is whether capillary endothelial cells are intrinsically different from endothelial cells lining large blood vessels, such as arteries and veins. The fact that the endothelial cells lining the large blood vessels are derived from capillary endothelial cells, as discussed previously, would suggest that the endothelial cells in both these locations have many similarities. Indeed, both capillary and large blood vessel endothelial cells form tight intercellular junctions (Engerman *et al.*, 1967), contain Weibel-Palade bodies (Weibel and Palade, 1964; Folkman *et al.*, 1979), contain factor VIII antigens (Folkman *et al.*, 1979; Bloom *et al.*, 1973; Hoyer *et al.*, 1973; Tuddenham *et al.*, 1974; Jaffee *et al.*, 1973), produce angiotensin-converting enzyme (Folkman *et al.*, 1979; Hill *et al.*, 1979), form a thromboresistance vascular lining (Spaet and Stemerman, 1972), and have basement membrane adjacent to the basal cell surface (Weiss and Greep, 1977). In addition to the observation that capillaries can develop to form arteries and veins, there exists data to suggest that endothelial cells lining large vessels can form capillaries. Michaelson (1948) has presented data which suggests that, during the development of the retinal vasculature, venules can be the source of new capil-

lary sprouts. In addition, the source of capillaries growing into a thrombus occluding a vein, although never definitively identified, are most likely derived from the endothelium lining the wall of the vein (Flane, 1968).

The differences between endothelial cells from capillaries and those from large blood vessels have been determined predominately from studies of their *in vitro* behavior. Although techniques for the long-term culture of endothelial cells from large blood vessels have become well established in recent years, attempts to establish long-term growth of capillary endothelial cells in culture have met with less success (Folkman *et al.*, 1979). Indeed, those techniques which have proved successful for culturing capillary endothelial cells require considerably more stringent conditions than are necessary for the growth of large blood vessel endothelial cells (Folkman *et al.*, 1979; Buzney and Massicotte, 1979; Frank and Kinsey, 1979). This may either be due to an intrinsic difference between the endothelial cells from these two sources or it may be secondary to anatomically determined difficulties in harvesting large numbers of capillary endothelial cells uncontaminated by other cell types. Large blood vessels, lined by a sheet composed of thousands of cells separated from other cell types by a continous basement membrane, provide an ideal situation for harvesting large numbers of cells for tissue culture. Techniques for harvesting equally large numbers of capillary endothelial cells uncontaminated by other cell types have yet to be developed. Therefore, the stringent growth requirements needed to establish capillary endothelial cells in culture may be secondary to the small number of cells available. Several investigators have shown that when large blood vessel endothelial cells are grown at low densities, they too require very stringent conditions for growth (Gospodarowicz *et al.*, 1976; McAuslan *et al.*, 1980).

Folkman *et al.* (1979) and Zetter (1980) have recently reported that the growth and migration of established lines of capillary endothelial cells are affected differently by various factors than are established lines of large vessel endothelial cells. Again, it is not clear whether these differences arise from intrinsic differences in the endothelial cells derived from the two sources or if they result from a selection process created by the more stringent conditions necessary to establish the capillary cell lines. For instance, Zetter (1980) reported that capillary endothelial cells *in vitro* are stimulated to migrate by tumor-conditioned media. This may be due to intrinsic differences between capillary and large blood vessel endothelial cells or it may be due to the fact that the techniques used to harvest and isolate capillary endothelial cells may select for endothelial cells which migrate in response to tumor-conditioned media. Zetter (1980) harvested capillary endothelial cells by placing isolated capillary segments in culture dishes with tumor-conditioned medium. Those capillary endothelial cells which were able to migrate from the capillary onto the surface of the culture dish were then grown to confluence. These conditions may select for cells which migrate in response to tumor-conditioned media.

Folkman and Haudenschild (1980) have recently reported the exciting obser-

vation that under certain conditions, cloned capillary endothelial cells in culture form tubes which by light and electron microscopy resemble capillaries *in vivo*. However, large blood vessel endothelial cells in culture also form capillary tubes (Maciag *et al.*, 1982; Sato *et al.*, 1984, 1985). Further studies of these phenomena may prove invaluable in advancing our understanding of capillary formation. Recently Buzney and Massicotte (1979) and Frank have independently developed techniques to isolate and grow retinal capillary endothelial cells from fetal and newborn animals. The extension of these techniques to the long-term culture of adult retinal and choroidal capillaries from human eye bank eyes with various ocular disorders will be of great help in the study of these disorders.

Whether capillary endothelial cells are the same or different from endothelial cells of large blood vessels has not as yet been determined.

V. Factors Influencing Capillary Sprout Formation

The control of new blood vessel formation is likely to be dependent upon a shift in the balance between stimulating and inhibitory factors. A discussion of the factors thought to participate in this balance follows.

A. Stimulators of Neovascularization

1. Mechanical Factors

The identification of factors which modulate the development and growth of new capillary sprouts has long been a central issue in the study of neovascularization. In the nineteenth century, several reports appeared which suggested that new blood vessel growth is largely controlled by mechanical factors. Gendrin, in 1826, suggested that neovascularization during wound healing resulted from the reopening of the cut ends of arterioles, so that blood is forced into the clot and, thereby, hollows out a channel. This "tunneling" process would allow the vessels to extend throughout the substance of the wound. In 1893, Thomas proposed that the growth of new capillary sprouts depends upon the pressure within them. His hypothesis was challenged by studies of developing embryos which documented new blood vessel development before the onset of a heartbeat. Sabin, in 1917, found that part of the aorta, the two vitelline veins next to the heart, and parts of the cardinal veins were formed in chick embryos prior to the onset of circulation. In addition, several reports appeared at the turn of the century describing studies performed on embryos whose heartbeat had been eliminated experimentally by either surgical removal or chemical inhibition

(Loeb, 1893; Knower, 1951; Patterson, 1909; Stockard, 1915). All of these studies found that embryologic neovascularization can proceed for several days following the cessation of circulation.

In 1918, Clark presented his studies on the developing vasculature of the transparent tail fin of the tadpole. In one series of experiments, he removed the heart of tadpoles before it began to beat. He subsequently studied the development of the tail fin vasculature for at least 12 days after the removal of the heart. He observed that vascularization proceeded by the usual process of capillary sprouting and that the resultant capillary plexus was not significantly different from what was observed in normal tadpoles with an actively beating heart. The one difference he did find was that the capillaries, once formed, showed no tendency to further differentiate into arterioles or venules. He concluded from these observations that capillary sprout formation is not dependent upon blood pressure or flow but that the later development into larger vessels is probably a function of these mechanical factors.

Lewis (1931) studied explants of skin and subcutaneous tissues from 7- and 8-day-old chick embryos grown in tissue culture. In 35% of these cultures, outgrowths appeared which, on microscopic examination, appeared to be capillary sprouts. In some instances, he was able to trace a direct connection of these outgrowths to blood vessels in parts of the explants. Motion pictures reportedly showed peripheral migration of the endothelial cells in these capillary sprouts in a manner similar to what had been observed by other investigators *in vivo*. Additional evidence suggesting that intraluminal blood pressure and flow are probably not key factors modulating the formation of new capillary sprouts comes from the recent studies demonstrating that vascular endothelial cells grown in tissue culture are capable of forming tubes which, on light and electron microscopic examination, appear similar to capillaries (Folkman and Haudenschild, 1980; Sato *et al.*, 1984, 1985). These observations argue against the notion that blood pressure is the driving force in new blood vessel development.

Wolbarsht and Landers (1980) have recently hypothesized that vascular tone has a role in modulating intraocular neovascularization. Landers and Wolbarsht (1980) measured tissue oxygen levels at the vitreous surface of the retina in normal and photocoagulated eyes of rhesus monkeys and cats. In both cats and monkeys, the oxygen levels of the normal and photocoagulated retinas were approximately the same. However, when the central retinal artery was blocked by pressure on the disk, with the choroidal circulation remaining intact, the oxygen level in both parts of the retina dropped, but was much lower in the normal nonphotocoagulated portion of the retina than in the laser photocoagulated area. From this, they suggested that panretinal photocoagulation in the diabetic may result in an increase in retinal oxygen tension. This increased oxygen tension is hypothesized to constrict the retinal vasculature, including those vessels which feed neovascular fronds. The investigators suggest that

vasoconstriction may be responsible for the regression of neovascularization. Significantly more data must be accumulated before the validity of this hypothesis can be determined.

2. Chemical Factors

Over the years, numerous investigators have speculated on the role of chemical mediators in the formation and growth of new blood vessels. Leber in 1888 suggested that new capillaries may be formed in response to the action of a specific "chemotactic" substance or substances. In 1893, Loeb suggested that chemical mediators might explain the growth of the new blood vessels that he observed in fish embryos whose heartbeat was eliminated. Michaelson in 1948 proposed that a chemical factor might be responsible for controlling the growth and development of the retinal vasculature. According to Michaelson (1948), the proposed "factor" would have the following characteristics:

1. The factor is present in the extravascular tissue of the retina.
2. It is present in a gradient of concentration such that it differs in arteriole and venous neighborhoods. The factor possibly is, therefore, of a biochemical nature.
3. Its action is on the retinal veins predominately.
4. The factor initiating capillary growth from veins probably determines the distance to which the capillary growth will extend, the initiation and cessation of growth depending on variation and concentration of the factor.

a. Role of O_2 and Retinal Perfusion. In 1952, Patz *et al.* (1952) reported the results of a controlled study designed to determine the effects of high oxygen therapy on the eyes of newborn infants. They discovered that grade III and grade IV retrolental fibroplasia occurred in 25% of those infants receiving constant high oxygen. In contrast, none of the 37 infants receiving lower oxygen concentrations progressed beyond grade II changes. Ashton and co-workers, in 1954, reported the experimental production of retrolental fibroplasia in kittens. Patz *et al.*, in 1953, produced retrolental fibroplasia in several species, indicating the general susceptibility of the immature retina to oxygen. Ashton's group discovered that oxygen in high concentrations exerted an obliterative effect on the developing retinal vessels of newborn kitten (Ashton *et al.*, 1954). These obliterated vessels reopened only partially when the animal was transferred to air. If the animal was kept in air over the ensuing weeks, a disordered and profuse proliferation of new vessels extended into the retina and the vitreous from the junction between the obliterated and patent retinal vasculature. This pathological process is comparable to what has been observed in the early stages of retrolental fibroplasia. In order to explain these findings, Ashton *et al.* (1954) postulated that, upon transfer to air after oxygen exposure, the retina served by

the obliterated vessels becomes anoxic. In response to this anoxia, the retina releases a vasoformative factor. They suggested that this factor might be related to the substance proposed by Michaelson (1948) which was thought to be responsible for the normal growth of new blood vessels in the developing mammalian retina. In 1956, Wise reviewed the available information regarding retinal neovascularization and presented his theory on its pathogenesis. He concluded that "retinal neovascularization is stimulated by the presence of what we may call retinal tissue factor X, intimately associated with tissue anoxia."

In the 1970s, observations utilizing the techniques of fundus fluorescein angiography expanded our understanding of intraocular neovascularization. Goldberg (1971) and Raichand *et al.* (1977), in a series of fluorescein angiographic studies of eyes with various stages of sickle retinopathy, showed that peripheral vasoocclusion always precedes the development of peripheral neovascularization. Furthermore, the neovascular fronds were almost always found to be adjacent to the areas of capillary nonperfusion. Shilling and Kohner (1976) found that optic disk and retinal neovascularization are almost always associated with pronounced retinal capillary nonperfusion in eyes with vein occlusions. They suggested that the areas of nonperfused retina liberate a vasogenic substance as proposed earlier in the case of retrolental fibroplasia. Kohner *et al.* (1976) studied fluorescein angiograms of patients with diabetes mellitus and observed that retinal capillary nonperfusion seemed to be associated with the development of new vessels. More recently, Shimizu *et al.* (1981) found a good correlation between the presence of retinal capillary nonperfusion and the occurrence of proliferative diabetic retinopathy. As mentioned previously, many investigators have proposed the existence of a retina-derived vasoproliferative substance released by ischemic retina to explain the common association of intraocular neovascularization with retinal capillary nonperfusion. Henkind (1978) gave a comprehensive and critical review of ocular neovascularization in his Krill Memorial Lecture published in 1978. Gartner and Henkind (1978) have provided an in-depth review of iris neovascularization, and Jampol and Goldbaum (1980) have recently reviewed the peripheral proliferative retinopathies.

Several recent studies have documented that scatter photocoagulation of retinal tissue can result in the regression of intraocular neovascularization in eyes with proliferative diabetic retinopathy (Taylor and Folkman, 1982; Jampol and Goldbaum, 1980) and in eyes with iris neovascularization associated with central retinal vein occlusions. The relationship of these observations to the pathogenesis of intraocular neovascularization has not yet been determined. Retinal photocoagulation has been found to have its major destructive effect on the outer retina, the retinal pigment epithelium, and the choriocapillaris (Diabetic Retinopathy Study Research Group, 1978). In contrast to the site of action of photocoagulation, retinal capillary nonperfusion associated with intraocular neovascularization primarily affects the inner retinal layers.

Several hypotheses have been proposed to reconcile these observations. One

explanation is based upon the finding that the photoreceptor–retinal pigment epithelial complex accounts for two-thirds of the total oxygen consumption of the retina (Little *et al.*, 1976). The destruction of the photoreceptors by photocoagulation may allow more oxygen to reach the inner retinal layers and thereby counteract the effects of capillary nonperfusion. The improved ability of inner retinal tissue to receive oxygen and nutrients from the choroid might then shut off the stimulus to produce a vasogenic substance. Another explanation for the effect of retinal photocoagulation is based upon the observation that the normal retinal pigment epithelial barrier to the diffusion of various substances is altered after photocoagulation (Zweng *et al.*, 1966). It has been hypothesized that this altered barrier allows the escape of vasogenic substances thought to be responsible for intraocular neovascularization. Obviously, considerably more data must be accumulated to evaluate these hypotheses further.

b. Tumor Angiogenic Factors. Studies of the vascularization of growing tumors have also suggested that chemical mediators may play a role in modulating new blood vessel development. In 1945, Algire *et al.* reported their observations on the vascularization of tumor transplants in the transparent skin chamber of mice. They demonstrated that tumor cells had the capacity to continuously elicit the growth of new capillaries. In 1971, Folkman *et al.* reported a series of experiments which suggested that a soluble factor capable of stimulating neovascularization *in vivo* is extractable from human and animal tumors. This factor was named tumor angiogenesis factor (TAF) (Folkman *et al.*, 1971). Subsequent studies (Folkman, 1974; Brem *et al.*, 1972; Klagsbrun *et al.*, 1974; Gimbrone and Gullino, 1976; Weiss *et al.*, 1979) demonstrated that the substance (substances) extractable from several different tumors is capable of stimulating neovascularization on the chick embryo chorioallantoic membrane and in the rabbit cornea. These methods [i.e., the chick chorioallantoic membrane (CAM) assay and the corneal pocket assay] have since been used repeatedly by many investigators to study the vasoproliferative activity of numerous substances. More detailed descriptions are available in the literature (Folkman, 1974; McAuslan and Hoffman, 1979; Gimbrone *et al.*, 1974).

The development of these relatively simple, reproducible assays of vasoproliferative activity represented a great advancement in the study of neovascularization. Utilizing these assays it became possible to study the effects of numerous substances on the process of neovascularization. Recently, Weiss *et al.* (1979) utilized the chick chorioallantoic membrane assay to isolate a low molecular weight angiogenic factor from rat Walker tumors. The active molecule has a molecular weight of approximately 200 and is stated not to be a prostaglandin, a protein, a peptide, or a nuclei acid. McAuslan and Hoffman (1979) have recently isolated a similar substance from the same tumor using a somewhat different assay. Fenselau *et al.* (1981) and Fournier *et al.* (1981) have recently

identified a tumor-derived small molecular weight substance capable of stimulating neovascularization when implanted in the cornea. In addition, this substance stimulates vascular endothelial cell proliferation *in vitro* (Ryu and Albert, 1979; Fournier *et al.*, 1981). Recently, investigators have found that a tumor-derived growth factor that stimulates capillary endothelial cell proliferation has a strong affinity for heparin (Shing *et al.*, 1980). This heparin affinity has made it possible to purify the growth factor to a single-band preparation. The purified growth factor is a cationic polypeptide with a molecular weight of about 18,000.

c. The Search for Intraocular Factors Stimulating Endothelial Cell Proliferation. Finkelstein *et al.* (1977) became interested in the recently identified tumor-derived vasoproliferative substance (or substances) and began to investigate its action on retinal vessels. Nodules of rabbit V2 carcinoma were implanted into the vitreous cavity of the rabbit eye. Retinal vessels invaded only those tumor nodules which actually touched a vascularized area of the retina. Tumor nodules separated from the retinal vessels even by only a small distance did not cause retinal neovascularization. This is an interesting contrast to studies performed earlier in which tumor nodules were implanted in the rabbit cornea at a distance of 1–2 mm from the limbal vascular plexus and were able to induce new blood vessels to grow through the avascular cornea and infiltrate the tumor nodule (Folkman, 1974). A possible explanation for the inability of tumor nodules to induce neovascularization at a distance when placed in the vitreous cavity was that the vitreous itself might contain an inhibitor of neovascularization. Studies to investigate this possibility will be discussed in the following section.

To begin the study of retina-derived vasoproliferative substances and their relationship to proliferative ocular disorders, Glaser *et al.* (1980) studied the vasoproliferative activity of extracts of mammalian retina. They obtained fresh bovine eyes, removed the retinas, and placed the intact retinas in balanced salt solution for 2–3 hr. The suspended retinas were then removed and the resultant balanced salt solution retinal extract was tested for vasoproliferative activity on the chick chorioallantoic membrane. These retinal extracts proved to be potent stimulators of neovascularization. Similar extracts of adult bovine skeletal muscle, heart muscle, and liver failed to produce significant neovascularization on the chick chorioallantoic membrane. Retinal extract was also found to stimulate the replication of fetal bovine aortic endothelial cells in culture. This response was found to be dose dependent. Studies are currently underway to further purify and characterize the active substance or substances. The relationship of this retina-derived vasoproliferative substance (or substances) to human ocular disease must be determined by further investigations in progress.

Federman *et al.* (1980) have recently implanted pieces of retina obtained from one eye of a rabbit into the cornea of the fellow eye and observed the resultant vascular response. They found that the vascularized portion of the rabbit retina

stimulated corneal neovascularization, but the avascular portion of the rabbit retina did not cause corneal neovascularization. Since the anatomy of the rabbit retina differs significantly from that of human retina, it is difficult to interpret the relationship of these findings to human disease. Further study of this phenomenon, however, may provide important information about the mechanism of neovascularization.

Tano *et al.* (1981) have recently shown that the intravitreal injection of autologous fibroblasts in the rabbit results in intravitreal neovascularization. The relationship of this finding to human ocular disease is currently unknown.

Although a great deal has been learned utilizing the *in vivo* assay of neovascularization described previously, there exist certain inherent limitations in the use of these assays. These *in vivo* assays do not provide a way to determine if a given substance is acting directly on the vascular endothelial cells to induce sprouting or if the substance under consideration is acting via adjacent cells, such as inflammatory cells or fibroblasts, that then produce secondary substances which elicit the neovascular response. In other words, utilizing *in vivo* assays one cannot differentiate between a substance which acts directly on the vascular endothelial cells or indirectly via neighboring cells. In order to overcome this limitation, several investigators have developed *in vitro* assays which are designed to measure the direct effect of substances on the endothelial cell functions known to be essential to new blood vessel formation. The two most commonly tested functions have been endothelial cell migration and proliferation. Assays designed to study these functions have been detailed in Sections III,B and C.

As a result of studies which demonstrate a close association of mast cells with capillary sprouts invading solid tumors, Azizkhan *et al.* (1980) have studied the effect of several isolated mast cell products. Only heparin reproducibly stimulated phagokinesis of capillary endothelial cells. The addition of protamine consistently inhibited this heparin-induced phagokinesis. Glaser *et al.* (1980) have recently demonstrated that extracts of adult mammalian retina contain a substance or substances with chemotactic activity for fetal bovine aortic endothelial cells *in vitro*. In subsequent studies, Glaser *et al.* (1980) have shown that intraocular fluid samples obtained from patients with proliferative diabetic retinopathy stimulated fetal bovine aortic endothelial cell migration *in vitro* and neovascularization on the CAM, whereas intraocular fluid samples obtained from control eyes without evidence of intraocular neovascularization lacked similar activity.

In addition to tumor and retina, several other sources of vasoproliferative activity have been investigated over the past several years. Corneal neovascularization has often been associated with the corneal inflammatory disorders, such as interstitial keratitis, alkali burns, and graft rejection (Henkind, 1978). In addition, disk neovascularization has been found in patients with chronic uveitis and no evidence of retinal nonperfusion (Glaser *et al.*, 1981). Fromer and Klintworth

(1975, 1976) performed a series of experiments which demonstrated that polymorphonuclear leukocytes could elaborate chemical mediators capable of inducing new blood vessel formation when implanted into the cornea. Polverini *et al.* (1977) have demonstrated that activated macrophages, when injected into the guinea pig cornea, can stimulate neovascularization. Ben-Ezra (1978) has recently demonstrated that intracorneal pellets containing prostaglandins stimulate neovascularization. Whether the prostaglandins act directly on the limbal vessels to induce capillary sprout formation or whether they act via intermediary cells, such as white blood cells of fibroblasts, has yet to be determined. Shabo and Maxwell (1976) have shown that intravitreal injections of insulin cause intraocular inflammation and associated neovascularization in monkeys which were previously sensitized by repeated intradermal injections of insulin. The relationship of this observation to human ocular disease is so far unknown. Further investigations are necessary to elucidate the exact factors responsible for the neovascularization associated with inflammation.

Numerous other sources of vasoproliferative stimulators have been identified in recent years. Among them are corpus luteum (Shabo and Maxwell, 1976), salivary gland (Gospodarowicz and Thakral, 1978), and epidermis (Hoffman *et al.*, 1976). Purification and characterization of vasoproliferative factors from the numerous sources discussed in this section will most likely advance our understanding of the process of new blood vessel formation.

B. Inhibition of Neovascularization

The ability to control new blood vessel formation will, no doubt, have applications in many branches of medicine. Folkman (1974, 1971) has shown that the ability to limit the growth of new blood vessels supplying tumors may result in the ability to control tumor growth. There are numerous ocular disorders in which neovascularization has devastating effects. The list of such disorders includes corneal neovascularization associated with conditions, such as alkali burns and graft rejection, iris neovascularization and its associated rubeotic glaucoma found predominately in central vein occlusion and proliferative diabetic retinopathy, and intravitreal neovascularization associated with proliferative diabetic retinopathy, venous occlusive disorders, retrolental fibroplasia, and sickle cell retinopathy. Additionally, neovascularization has an important role in the disciform macular disorders, such as senile macular degeneration associated with drusen and the presumed ocular histoplasmosis syndrome. The ability to control neovascularization in these ocular disorders would, most likely, play a significant role in combating blindness. For these reasons, many investigators have been studying the factors and conditions which show promise of controlling and inhibiting neovascularization.

1. Cartilage-Derived Inhibitors

In 1973, Eisenstein *et al.* reported a series of experiments which were designed to determine if normally avascular tissues, such as hyaline cartilage or cornea, are more resistant to capillary invasion than are tissues which are normally vascularized. When small pieces of tissues, such as skeletal muscle, cardiac muscle, or kidney, were implanted on the chick chorioallantoic membrane (CAM), they rapidly became vascularized. However, when small pieces of hyaline catilage were implanted on the CAM, no blood vessel invasion occurred. Interestingly, corneal stroma was readily invaded by new blood vessels, but Descemet's membrane appeared to be resistent to capillary invasion. In later experiments, they found that, after extraction with guanidine, cartilage becomes susceptible to blood vessel invasion. The guanidine extract of cartilage was found to inhibit growth of vascular endothelial cells in culture (Eisenstein *et al.*, 1973; Sorgente *et al.*, 1975).

Subsequently, Brem and Folkman (1975) implanted pieces of cartilage in rabbit corneas so that they were positioned between the limbal vasculature and an implanted piece of tumor. The cartilage inhibited the tumor-induced neovascularization. If the cartilage was boiled prior to implantation in the cornea, neovascularization proceeded toward the tumor pellet in a normal fashion. Several laboratories are now working to purify and characterize the putative cartilage inhibitor of vasoproliferation. Langer *et al.* (1980) have recently infused partially purified cartilage inhibitor into the bloodstream of rabbits with corneal tumor implants. The infusion of the partially purified inhibitor causes a significant inhibition of the tumor-induced corneal neovascularization. This is the first demonstration that the infusion of a substance into the bloodstream can inhibit neovascularization occurring at a remote site. Eisenstein *et al.* (1979) have recently identified a low molecular weight fraction extracted from bovine aorta which is capable of inhibiting corneal neovascularization in rabbits when administered either subconjunctively or topically as long as 48 hr after injury. Langer *et al.* (1976) isolated a fraction from cartilage which inhibits the proliferation of blood vessels in the rabbit cornea and also inhibits the activity of the enzyme trypsin. Sorgente and Dorey (1980) extracted a substance from cartilage which inhibits the growth of vascular endothelial cells in culture. This substance did not inhibit trypsin. They did isolate a trypsin inhibitor from cartilage, but it had no effect on endothelial cell proliferation.

2. Vitreous-Derived Inhibitors

As discussed previously, the observation that tumor nodules implanted within the vitreous cavity of the rabbit could only induce neovascularization if they were touching the vascularized portion of the retina suggested that the surrounding

vitreous may contain inhibitors of neovascularization. Following up on this observation, Brem and co-workers (1977) carried out the following experiments. Vitreous was removed from adult normal rabbit eyes, centrifuged, dialyzed, and lyophilized. The lyophilized powder was then impregnated into Elvax pellets. Elvax is a polymer which allows the gradual and sustained release of macromolecules which are dissolved within it (Brem *et al.*, 1977). Elvax pellets containing vitreous extracts were placed in a corneal pocket between the limbus and an implanted piece of tumor. As was observed in the case of cartilage implants, the pellets containing vitreous extract inhibit tumor-induced corneal neovascularization. Control pellets without vitreous extract do not interfere with tumor-induced corneal neovascularization. More recently, these investigators have repeated these experiments using retinal extract as the vasoproliferative stimulator and obtained similar results (Langer and Folkman, 1976). Characterization and purification of this vitreous-derived angiogenic inhibitor are currently underway.

3. Chemically Defined Substances

As discussed previously, Azizkhan *et al.* (1980) have performed *in vitro* studies which suggest that heparin plays a role in enhancing the rate of migration of capillary endothelial cells. It is well known that protamines, a group of arginine-rich peptides, avidly bind to heparin, and they have been used clinically to counter the anti-coagulative effect of heparin. Taylor and Folkman (1982) recently performed studies to determine if protamine could interfere with neovascularization *in vivo*. Taylor and Folkman (1982) have shown that slow-release pellets containing protamine placed between a tumor implant and the corneal limbus almost completely inhibits the tumor-induced blood vessel growth. In another study, these investigators inoculated mice intramuscularly with melanoma cells. These mice were then injected subcutaneously each day with protamine or saline at a remote site. All tumors grew at the same rate in both protamine- and saline-treated mice until the tumors were large enough to require vascularization. The protamine-treated tumors then gradually stopped growing and all of them eventually regressed. In saline-treated controls, tumors grew continuously. These same melanoma cells in tissue culture were not affected by large doses of protamine. These data suggest that protamine administered subcutaneously acts to inhibit the tumor-induced neovascularization necessary for the continued growth of the melanoma.

Unfortunately, these data do not rule out the possibility that protamine may be acting predominantely via another mechanism in this experiment. To determine whether protamine could inhibit neovascularization that was not induced by tumor, Taylor and Folkman implanted silica particles in the cornea of rabbits. The implantation of silica particles results in a marked inflammatory and neo-

vascular response. In eyes which received a pellet containing protamine implanted between the silica and the corneal limbus, a marked inhibition of vessel growth occurred. These data suggest that protamine may be capable of inhibiting new blood vessel formation regardless of its cause. Further research will be necessary to determine the mechanism of action of protamine and its true effectiveness as an inhibitor of neovascularization.

4. Lens-Derived Inhibitors

Williams *et al.* (1984) have recently demonstrated that inhibition of vascular endothelial cell growth can be extracted from the ocular lens.

5. Retinal Pigment Epithelial Cell-Derived Inhibitors

As discussed above, most inhibitors of neovascularization so far identified have been extracted from tissues that are avascular, i.e., cartilage, vitreous, and lens. Unfortunately, the study of these inhibitors is severely limited by the fact that only small quantities of active material can be extracted from these sources (Williams *et al.*, 1984).

It has been suggested that diabetic intraocular neovascularization is less likely to occur in eyes with chorioretinal scars (Lee and Langer, 1983). This has led to the widespread use of argon laser and xenon photocoagulation to therapeutically induce chorioretinal scar formation. The production of these scars often results in the rapid regression of intraocular neovascularization in eyes with proliferative diabetic retinopathy (Lee and Langer, 1983; Beetham *et al.*, 1969). Regression occurs even when photocoagulation and resultant chorioretinal scaring are located in areas remote from the new blood vessels. Retinal pigment epithelial cells (RPE) are one component of these scars. It has recently been discovered that human RPE cells in culture release a substance (or substances) that causes the regression of new blood vessels on the chick embryonic yolk sac and inhibits vascular endothelial cell proliferation *in vitro* (Maciag *et al.*, 1982).

Chorioretinal scars occurring in response to photocoagulation are mainly composed of astrocytes, retinal pigment epithelial cells (RPE), and possibly fibroblasts (Doft and Blankenship, 1984; Wallow *et al.*, 1973). The ability of these cell types to release substances that cause new blood vessels to regress was therefore tested on the growing embryonic vessels of the chicken egg, using a modification of the technique described by Lutty *et al.* (1980). Capillaries appear in the yolk sac of the chicken egg at 48 hr and grow rapidly over the next 6–8 days. Disks of filter paper soaked in medium conditioned by RPE cells (RPE-CM) when placed on the surface of the vascularized yolk sac caused regression of adjacent capillaries, resulting in a localized avascular zone. Histologic examination of yolk sac vessels adjacent to RPE-CM soaked filters showed vessels

occluded by platelets and packed RBC. This is similar to the appearance of regressing vessels in the cornea (Wallow and Davis, 1979). In contrast, media conditioned by either astrocytes (astro-CM) or fibroblasts (fibro-CM) did not affect the adjacent vasculature. Before testing, the conditioned media were concentrated fivefold using ultrafiltration membranes with a 10,000 molecular weight cutoff. To determine if RPE-CM is toxic to vascular endothelial cells, fetal bovine aortic endothelial cells (FBAE) were grown in concentrated RPE-CM for 24 hr. RPE-CM, at concentrations used on the chick vasculature, showed no toxicity for FBAE as determined by cell counts and trypan blue exclusion. The ability of RPE-CM to cause regression of vessels is lost after boiling for 10 min and trypsin treatment.

Extracts of cartilage, vitreous, lens, and aorta have been found not only to inhibit neovascularization, but also to inhibit the growth of vascular endothelial cells *in vitro*. Therefore the effect of RPE-CM on vascular endothelial cell proliferation was studied. It has previously been demonstrated that an extract of adult bovine retina stimulates both neovascularization on the chick chorioallantoic membrane and proliferation of fetal bovine aortic endothelial cells (FBAE) in culture (Patz, 1980). RPE-CM inhibits the proliferative response of FBAE to retinal extract. In contrast, astro-CM and fibro-CM not only fail to inhibit FBAE proliferation in response to retinal extract, but act to enhance the proliferative response. Furthermore, astro-CM itself stimulates FBAE proliferation. The ability of RPE-CM to inhibit FBAE proliferation in response to retinal extract is enhanced by pretreating the FBAE with RPE-CM for 24 hr prior to adding the retinal extract.

The removal of RPE-CM restored the rate of FBAE proliferation to that of control cultures growing without RPE-CM. Therefore, the inhibitory effect of RPE-CM is reversible. In another series of experiments, the effect of RPE-CM on FBAE prelabled with [^{14}C]thymidine was studied. The loss of [^{14}C]thymidine from prelabeled cells provides an estimate of cell death (Ausprunk *et al.*, 1978). The addition of RPE-CM to FBAE did not result in the loss of label. In order to study the effect of RPE-CM on a cell type other than FBAE, bovine corneal fibroblasts were studied. In contrast to its effect on FBAE, RPE-CM causes a marked stimulation of fibroblast proliferation. The previous series of experiments suggests that RPE-CM does not act as a cell toxin, but acts as a relatively specific inhibitor of vascular endothelial cell proliferation.

Cell–cell interactions play an important role in a large number of biologic processes including those occurring during development, wound healing, and tumor growth and spread. The establishment and control of an adequate blood supply has a role in all of these processes. Cell–cell interactions are also likely to be involved in controlling new blood vessel formation and regression during these processes. The inhibition of vascular endothelial cell proliferation and the regression of new blood vessels caused by a substance released by RPE lie

between the extremely vascular choroid and the avascular outer retina. RPE in a laser-induced chorioretinal scar may release the same substance into the vitreous cavity to cause regression of intraocular new blood vessels.

The fact that we are dealing with a population of cells that can be maintained in culture and continuously release the active substance(s) provided a significant advantage over most previous studies of factors capable of inhibiting or causing regression of new blood vessels. First of all, the amount of material for study can be significantly increased using currently available techniques for mass cell culture. Second, the ability to culture the cell producing the inhibitor provides the opportunity to isolate the DNA coding for the active substance, introduce it into bacteria or other vehicles, and achieve large-scale synthesis of the active molecule. Therefore, our current finding allows investigators to overcome a major obstacle (i.e., lack of significant quantities of active substance) that has impeded research into factors causing the regression of new blood vessels (Williams *et al.*, 1984).

VI. Conclusion

The major currently available pieces to the puzzle of neovascularization outlined in this review are as follows. New blood vessels arise from preexisting blood vessels except in the earliest stages of embryologic development. Furthermore, there is good evidence to suggest that one of the earliest steps in the formation of new blood vessels from preexisting vessels is the localized breakdown of the vascular basement membrane. Basement membrane fragmentation appears to be followed by the migration of endothelial cells from the wall of the parent vessel into the surrounding interstitial space. These migrating endothelial cells form capillary sprouts. The tips of these newly formed capillary sprouts appear to be composed of migrating vascular endothelial cells, whereas the more proximal regions contain dividing endothelial cells. Therefore, vascular basement membrane fragmentation, vascular endothelial cell migration, and proliferation all seem to play an important role in new blood vessel formation. The relative importance of these individual processes must await further study. There is some evidence that basement membrane fragmentation and endothelial cell migration occur at an earlier stage than endothelial cell proliferation.

Another important piece to the puzzle is the finding that capillaries mature to form arteries and veins. The question naturally arises as to whether capillary endothelial cells are intrinsically the same as large blood vessel endothelial cells. It is known from *in vivo* studies, that capillary endothelial cells and large blood vessel endothelial cells occasionally perform different functions. Whether this is a result of intrinsic differences between these two endothelial cell types, or

whether their different function is dictated by their different environments has yet to be determined.

Many investigators have studied the effect of chemical and physical factors on neovascularization. Neovascularization has been found to be associated with inflammation, tumors, and ischemia. The interrelationships among these three conditions are yet to be determined. Substances from tumors and inflammatory cells have been shown to stimulate neovascularization under specific experimental conditions. Furthermore, substances from these sources have been shown to affect vascular endothelial cell migration and proliferation. Substances from other tissues, among them retina, have also been shown to have similar activities. It is also of interest that fluid samples from eyes of patients with intraocular neovascularization have been found to stimulate neovascularization and vascular endothelial cell migration and proliferation. The relationship among the substances from these various sources remains to be determined. Hopefully, the study of these various phenomena will improve our understanding and perhaps our ability to control neovascularization.

Substances which appear to inhibit neovascularization have been extracted from various sources, among which are cartilage, vitreous, lens, aorta, and RPE. The relationship of these substances to *in vivo* neovascularization also needs further clarification. These inhibitors will hopefully lead us to new ways to control the blinding complications of neovascularization which occur in several disorders.

We currently have several important pieces of the puzzle of neovascularization. New pieces are constantly being added. We believe that the future holds the promise of being able to pharmacologically control new blood vessel formation, so that clinicians may be able to enhance neovascularization when beneficial, and inhibit neovascularization when harmful to the patient.

References

Algire, G. H., Chalkley, H. W., Legallais, F. Y., and Park, H. D. (1945). Vascular reactions of normal and malignant tissues *in vivo*. I. Vascular reactions of mice to wounds and to normal and neoplastic transplants. *J. Natl. Cancer Inst.* **6,** 73–85.

Albrecht-Buehler, G. (1977). The phagokinetic tracks of 3T3 cells. *Cell* **11,** 395–404.

Albrecht-Buehler, G. (1975). Daughter 3T3 cells. Are they mirror images of each other? *J. Cell Biol.* **72,** 595–603.

Arnold, J. (1871). Experimentelle Untersuchungen über die Blutkapillaren. *Virchow's Arch.* **53,** 70–92.

Ashton, N. (1954). The mode of development of the retinal vessels in man. *In* "The William MacKenzie Centenary Symposium on the Ocular Circulation in Health and Disease" (J. S. Cant, ed.), pp. 7–17. Mosby, St. Louis, Missouri.

Ashton, N., Ward, B., and Serpell, G. (1954). Effect of oxygen on developing retinal vessels with particular reference to the problems of retrolental fibroplasia. *Br. J. Ophthalmol.* **38,** 397–432.

Auerbach, L. (1865). Über den Bau der Lymph- und Blutcapillaren. *Zbl. Med. Wiss.* **12,** 177–179.

Ausprunk, D. H., and Folkman, J. (1977). Migration and proliferation of endothelial cells in preformed and newly formed blood vessels during tumor angiogenesis. *Microvasc. Res.* **14,** 53–65.

Ausprunk, D. H., Falterman, K., and Folkman, J. (1978). The sequence of events in the regression of corneal capillaries. *Lab. Invest.* **38,** 284–294.

Azizkhan, R. G., Azizkhan, J. C., Zetter, B. R., and Folkman, J. (1980). Mast cell heparin stimulate migration of capillary endothelial cells *in vitro*. *J. Exp. Med.* **152,** 931–944.

Beetham, W. P., Aiello, L. M., Balodimos, M. C., and Koncz, L. (1969). Ruby-laser photocoagulation of early diabetic neovascularization retinopathy: Preliminary report of a long-term controlled study. *Trans. Am. Ophthalmol. Soc.* **67,** 39–67.

Ben-Ezra, D. (1978). Neovascularization ability of prostaglandins, growth factors and synthetic chemoattractants. *Am. J. Ophthalmol.* **86,** 455–461.

Bloom, A. L., Giddings, J. C., and Wilks, C. J. (1973). Factor VIII on the vascular intima: Possible importance in hemostasis and thrombosis. *Nature (London)* **241,** 217–219.

Brem, S., Cotran, R., and Folkman, J. (1972). Tumor angiogenesis: A quantitative method for histologic grading. *J. Natl. Cancer Inst.* **48,** 347–356.

Brem, H., and Folkman, J. (1975). Inhibition of tumor angiogenesis mediated by cartilage. *J. Exp. Med.* **141,** 427–439.

Brem, S., Preis, I., Langer, R., Brem, H., Folkman, J., and Patz, A. (1977). Inhibition of neovascularization by an extract derived from vitreous. *Am. J. Ophthalmol.* **84,** 323–328.

Buzney, S. M., and Massicotte, S. J. (1979). Retinal vessels: Proliferation of endothelium *in vitro*. *Invest. Ophthalmol. Visual Sci.* **18,** 1191–1195.

Clark, E. R. (1918). Studies on the growth of blood vessels in the tail of the frog larva by observation and experiment on the living animal. *Am. J. Anat.* **23,** 37–88.

Clark, E. R. (1939). Microscopic observations on the growth of blood capillaries in the living mammal. *Am. J. Anat.* **64,** 251–301.

Cogan, D. G. (1948). Vascularization of the cornea. Its experimental induction by small lesions and a new theory of its pathogenesis. *Trans. Am. Ophthalmol. Soc.* **46,** 457–471.

Cogan, D. G. (1949). Studies on the clinical physiology of the cornea. *Am. J. Ophthalmol.* **32,** 625–634.

D'Amore, P. A., Glaser, B. M., Brunson, S. K., and Fenselav, A. H. (1981). Angiogenic activity from bovine retina: Partial purification and characterization. *Proc. Natl. Acad. Sci. U.S.A.* **78,** 3068–3072.

Diabetic Retinopathy Study Research Group. (1976). Preliminary report on effects of photocoagulation therapy. *Am. J. Ophthalmol.* **81,** 383–396.

Diabetic Retinopathy Study Research Group. (1978). Photocoagulation treatment of proliferative diabetic retinopathy. Second report from the Diabetic Retinopathy Study. *Ophthalmology* **85,** 82.

Doft, B. H., and Blankenship, G. (1984). Retinopathy risk factor regression after laser panretinal photocoagulation for proliferative diabetic retinopathy. *Ophthalmology* **91,** 1453–1457.

Evans, H. M. (1909). On the earliest blood vessels in the anterior limb buds of birds and their relation to the primary subclavian artery. *Am. J. Anat.* **9,** 281–317.

Engerman, R. L., Pfaffenbach, D., and Davis, M. D. (1967). Cell turnover of capillaries. *Lab. Invest.* **17,** 738–743.

Eisenstein, R., Sorgente, N., Soble, L. W., Miller, A., and Kuettner, K. E. (1973). The resistance of certain tissues to invasion. Penetrability of explanted tissues by vascularized mesenchyme. *Am. J. Pathol.* **73,** 765–774.

Eisenstein, R., Kuettner, K. E., Neapolitan, C., Soble, W., and Sorgente, N. (1975). The resistance of certain tissues to invasion. III. Cartilage extracts inhibit the growth of fibroblasts and endothelial cells in culture. *Am. J. Pathol.* **81,** 337–348.

Eisenstein, R., Goren, S. B., Shumacher, B., and Choromokos, E. (1979). Inhibition of corneal vascularization with aortic extracts in rabbits. *Am. J. Ophthalmol.* **88,** 1005–1012.

Eckel, R. H., and Fujimoto, W. Y. (1981). Quantification of cell death in human fibroblasts by measuring the loss of ^{14}C-thymidine from prolabelled cell monolayers. *Biochemistry* **114,** 118–124.

Federman, J. L., Brown, G. C., Felberg, N. T., and Felton, S. M. (1980). Experimental ocular angiogenesis. *Am. J. Ophthalmol.* **89,** 231–237.

Fenselau, A., and Mello, R. (1976). Growth stimulation of cultured endothelial cells by tumor cell homogenates. *Cancer Res.* **36,** 3269–3273.

Fenselau, A., Watt, S., and Mello, R. (1981). Tumor angiogenic factor: Purification from the Walker 256 rat tumor. *J. Biol. Chem.* **256,** 9605–9611.

Finkelstein, D., Brem, S., Patz, A., Folkman, J., Miller, S., and (Chung) Ho-Chen, C. (1977). Experimental retinal neovascularization induced by intravitreal tumors. *Am. J. Ophthalmol.* **83,** 660–664.

Flanc, C. (1968). An experimental study of the recanalization of arterial and venous thrombi. *Br. J. Surg.* **55,** 519–524.

Folkman, J., Merler, E., Abernathy, C., and Williams, G. (1971a). Isolation of a tumor factor responsible for angiogenesis. *J. Exp. Med.* **133,** 275–288.

Folkman, J. (1971b). Tumor angiogenesis: Therapeutic implications. *N. Engl. J. Med.* **285,** 1182–1186.

Folkman, J. (1974a). Tumor angiogenesis. *Adv. Cancer Res.* **19,** 331–358.

Folkman, J. (1974b). Tumor angiogenesis factor. *Cancer Res.* **34,** 2109–2113.

Folkman, J., Haudenschild, C. C., and Zetter, B. R. (1979). Long-term culture of capillary endothelial cells. *Proc. Natl. Acad. Sci. U.S.A.* **76,** 5217–5221.

Folkman, J., and Haudenschild, C. (1980). Angiogenesis *in vitro*. *Nature (London)* **288,** 551–556.

Foulds, W. S. (1980). The role of photocoagulation in the treatment of retinal disease. *Trans. Ophthalmol. Soc. N.Z.* **32,** 82–90.

Fournier, G. A., Lutty, G. A., Watt, S., Fenselau, A., and Patz, A. (1981). A corneal micropocket assay for angiogenesis in the rat eye. *Invest. Ophthalmol. Visual Sci.* **21,** 351–357.

Frank, R. N., Kinsey, V. E., Frank, K. W., Mikus, K. P., and Randolph, A. (1979). *In vitro* proliferation of endothelial cells from kitten retinal capillaries. *Invest. Ophthalmol. Visual Sci.* **18,** 1195–1200.

Fromer, C. H., and Klintworth, G. K. (1975a). An evaluation of the role of leukocytes in the pathogenesis of experimentally induced corneal vascularization. I. Comparison of experimental models of corneal vascularization. *Am. J. Pathol.* **79,** 537–554.

Fromer, C. H., and Klintworth, G. K. (1975b). An evaluation of the role of leukocytes in the pathogenesis of experimentally induced corneal vascularization. II. Studies on the effect of leukocytic elimination on corneal vascularization. *Am. J. Pathol.* **81,** 531–544.

Fromer, C. H., and Klintworth, G. K. (1976). An evaluation of the role of leukocyte in the pathogenesis of experimentally induced corneal vascularization. III. Studies related to the vasoproliferative capability of polymorphonuclear leukocytes and lymphocytes. *Am. J. Pathol.* **82,** 157–170.

Gartner, S., and Henkind, P. (1978). Neovascularization of the iris. *Surv. Ophthalmol.* **22,** 291–312.

Gendrin, A. N. (1826). Histoire anatomique des inflammations. Paris.

Gimbrone, M. A., Cotran, R. S., Leapman, S. B., and Folkman, J. (1974). Tumor growth and neovascularization: An experimental model using the rabbit cornea. *J. Natl. Cancer Inst.* **52,** 413–419.

Gimbrone, M. A., and Gullino, P. M. (1976). Neovascularization induced by intraocular xenografts of normal, preneoplastic, and neoplastic mouse mammary tissues *J. Natl. Cancer Inst.* **56,** 305–318.

Glaser, B. M., D'Amore, A. P., Seppa, H., Seppa, S., and Schiffman, E. (1980a). Adult tissues contain chemoattractants for vascular endothelial cells. *Nature (London)* **288,** 483–484.

Glaser, B. M., D'Amore, P. A., Michels, R. G., Patz, A., and Fenselau, A. (1980b). Demonstration of vasoproliferative activity from mammalian retina. *J. Cell Biol.* **84,** 298–304.

Glaser, B. M., D'Amore, P. A., Michels, R. G., Brunson, S. K., Fenslau, A., Rice, T., and Patz, A. (1980c). The demonstration of angiogenic activity from ocular tissues: Preliminary report. *Ophthalmology* **87,** 440–446.

Glaser, B. M., D'Amore, P. A., Lutty, G. A., Fenslau, A. H., Michels, R. G., and Patz, A. (1980d). Chemical mediators of intraocular neovascularization. *Trans. Ophthalmol. Soc. U.K.* **100,** 369–373.

Glaser, B. M., D'Amore, P. A., and Michels, R. G. (1981). The effect of human intraocular fluid on vascular endothelial cell migration. *Ophthalmology* **88,** 986–991.

Glaser, B. M. (1985). *Arch. Ophthalmol.*, submitted.

Goldberg, M. R. (1971). Classification and pathogenesis of proliferative sickle retinopathy. *Am. J. Ophthalmol.* **71,** 649–665.

Golubew, A. (1869). Beiträge zur Kenntnis des Baues und der Entwickelungsgeschichte der Capillargefässe des Frosches. *Arch. Mikrosk. Anat.* **5,** 49–89.

Gospodarowicz, D., Moran, J. S., Braun, D., and Birdwell, C. (1976). Clonal growth of bovine vascular endothelial cells in tissue culture: Fibroblast growth factor as a survival agent. *Proc. Natl. Acad. Sci. U.S.A.* **73,** 4120–4124.

Gospodarowicz, D., and Thakral, K. (1978). Production of a corpus luteum angiogenic factor responsible for proliferation of capillaries and neovascularization of the corpus luteum. *Proc. Natl. Acad. Sci. U.S.A.* **75,** 847–851.

Haudenschild, C. C., Zahniser, D., Folkman, J., and Klagsbrun, M. (1976). Human vascular endothelial cells in culture. Lack of response to serum growth factors. *Exp. Cell Res.* **98,** 175–183.

Henkind, P. (1978). Ocular neovascularization. The Krill Memorial Lecture. *Am. J. Ophthalmol.* **85,** 287–301.

Hial, V., Gimbrone, M. A., and Peyton, M. P. (1979). Angiotensin metabolism by cultured human vascular endothelial and smooth muscle cells. *Microvasc. Res.* **17,** 314–329.

Hoffman, H., McAuslan, B., Robertson, D., and Burnett, E. (1976). An endothelial growth-stimulating factor from salivary glands. *Exp. Cell Res.* **102,** 269–275.

Hoyer, L. W., de los Santos, R. P., and Hayer, J. R. (1973). Antihemophilic factor antigen. Localization in endothelial cells by immunofluorescent microscopy. *J. Clin. Invest.* **52,** 2737–2744.

Hunter, J. (1812). A treatise on the blood, inflammation and gunshot wounds. Published in London.

Jaffe, E. A., Hoyer, L. W., and Nachman, R. L. (1973). Synthesis of antihemophilic factor antigen by cultured human endothelial cells. *J. Clin. Invest.* **52,** 2757–2764.

Jampol, L. M., and Goldbaum, M. H. (1980). Peripheral proliferative retinopathies. *Surv. Ophthalmol.* **25,** 1–14.

Kalebic, T., Garbisa, S., Glaser, B. M., and Liotta, L. A. (1983). Basement membrane collagen: Degradation by migrating endothelial cells. *Science* **221,** 281–283.

Kefalides, N. A. (1973). Structures and biosynthesis of basement membranes. *Int. Rev. Connet. Tissue Res.* **6,** 63–65.

Klagsbrun, M., Knighton, D., and Folkman, J. (1972). Tumor angiogenesis activity in cells grown in tissue grading. *J. Natl. Cancer Inst.* **48,** 347–356.

Klagsbrun, M., Knighton, D., and Folkman, J. (1974). Tumor angiogenesis activity in cells grown in tissue culture. *Cancer Res.* **36,** 110–114.

Knower, H. (1951). Effects of early removal of the heart and arrest of the circulation on the development of the frog embryos. *Anat. Rec.* **1,** 161–165.

Kohner, E. M., Shilling, J. S., and Hamilton, A. M. (1976). The role of avascular retina in new vessel formation. *Metab. Ophthalmol.* **1,** 15–23.

Krakower, C. A., and Greenspon, S. A. (1959). Localization of the nephrotoxic antigen within the isolated renal glomerulus. *Arch. Pathol.* **52,** 629.

Langer, R., Brem, H., Falterman, K., Klein, M., and Folkman, J. (1976a). Isolation of a cartilage factor that inhibits tumor neovascularization. *Science* **193,** 70–72.

Langer, R., and Folkman, J. (1976b). Polymers for the sustained release of proteins and other macromolecule. *Nature (London)* **263,** 797–800.

Langer, R., Conn, H., Vacanti, J., Haudenschild, C., and Folkman, J. (1980). Control of tumor growth in animals by infusion of an angiogenesis inhibitor. *Proc. Natl. Acad. Sci. U.S.A.* **77,** 4331–4335.

Langham, M. (1953). Observations on the growth of blood vessels into the cornea. Application of a new experimental technique. *Br. J. Ophthalmol.* **37,** 210–222.

Lee, A., and Langer, R. (1983). Shark cartilage contains inhibitors of tumor angiogenesis. *Science* **221,** 1185–1187.

Lewis, W. H. (1931). The outgrowth of endothelium and capillaries in tissue culture. *Bull. Johns Hopkins Hosp.* **48,** 242–253.

Little, H. L., Rosenthal, A. R., Dellaporta, A., and Jakobson, D. R. (1976). The effect of panretinal photocoagulation on rubeosis iridis. *Am. J. Ophthalmol.* **81,** 804–809.

Loeb, J. (1983). Ueber die Entwickelung von Fisch Embryonen ohne Kreislauf. *Pfluger's Arch.* **54,** 525.

Lutty, G. A., Thompson, D. C., Gallup, J. G., *et al.* (1980). Inhibition of neovascularization by bovine vitreous in the CAM assay. *Invest. Ophthalmol. Visual Sci.* **19** (Suppl.), 138.

Maciag, T., Kadish, J., Wilkins, L., Stemerman, M. B., and Weinstein, R. (1982). Organizational behavior of human umbilical vein endothelial cells. *J. Cell Biol.* **94,** 511–520.

Mall, F. P. (1906). A study of the structural unit of the liver. *Am. J. Anat.* **5,** 227–313.

Marchand, F. (1901). Der Process der Wundheilung, mit Einschluss der Transplantation. Stuttgart.

Maximow, A. (1902). "Experimentelle Untersuchungen über die entzündliche Neubildung vom Bindegewebe." Gustav Fischer, Jena.

McAislan, B. R. (1979a). A new theory of neovascularization based on identification of an angiogenic factor and its effect on cultured endothelial cells. *EMBO Workshop Specific Growth Factors,* Rome, Italy.

McAuslan, B. R., and Hoffman, H. (1979b). Endothelium stimulating factor from walker carcinoma cells. *Exp. Cell Res.* **119,** 181–190.

McAuslan, B. R., Reilly, W., and Hannah, G. N. (1980). Stimulation of endothelial cell proliferation by precursors of thymidylate. *J. Cell. Physiol.* **100,** 87–94.

McNaugh, E. I., Foulds, W. S., and Johnson, N. F. (1977). The effect of photocoagulation on the barrier functions of the pigment epithelium. Proc. 4th Wm. Mackenzie Symposium. *Trans. Ophthalmol. Soc. U.K.* **97,** 634–640.

Meyer, J. (1852). Über die Neubildung von Butgefaben in plastischen exsudaten seroser Membrane und in Hautwunden. *Ann. Charité (Berlin)* **4,** 41.

Michaelson, I. C. (1948). The mod of development of the vascular system of the retina: With some observations on its significance for certain retinal diseases. *Trans. Ophthalmol. Soc. U.K.* **68,** 137–180.

Palade, G. E., and Burns, R. R. *In* "Small Blood Vessel Involvement in Diabetes Mellitus" (M. D. Spierstein, A. R. Colwell, and K. Meyer, eds.), p. 39. Amer. Inst. Biol. Sci., Washington, D.C.

Patterson, J. T. (1909). An experimental study on the development of the area vasculosa of the chick blastoderm. *Biol. Bull.* **16,** 83–90.

Patz, A., Hoech, L. E., and de la Cruz, E. (1952). Studies on the effect of high oxygen administration in retrolental fibroplasia. I. Nursery observations. *Am. J. Ophthalmol.* **35,** 1248–1253.

Patz, A., Eastham, A., Higginbotham, D. H., and Kleh, T. (1953). Oxygen studies in retrolental fibroplasia. II. The production of the microscopic changes of retrolental fibroplasia in experimental animals. *Am. J. Ophthalmol.* **36,** 1511–1522.

Patz, A. (1980). Studies on retinal neovascularization. Friedenwald Lecture. *Invest. Ophthalmol. Visual Sci.* **19,** 1133–1138.

Polverini, P. J., Cotran, R. S., Gimbrone, M. A., and Unanue, E. R. (1977). Activated macrophages induce vascular proliferation. *Nature (London)* **269,** 804–806.

Raichand, M., Goldberg, M. R., Nagpal, K. C., Goldbaum, M. H., and Asdouyian, G. K. (1977). Evolution of neovascularization in sickle cell retinopathy. *Arch. Ophthalmol.* **95,** 1543–1552.

Ross, R., and Glomset, J. A. (1976). The pathogenesis of atherosclerosis. *N. Engl. J. Med.* **295,** 369–377.

Rouget, C. (1873). Memoirs sur les Capillaires Sanguins et Lymphatiques. *Arch. Physiol.* **5,** 603–663.

Ryu, S., and Albert, D. (1979). Evaluation of tumor angiogenesis factor with the rabbit cornea model. *Invest. Ophthalmol. Visual Sci.* **18,** 831–841.

Sabin, F. R. (1917). Origin and development of the primitive vessels of the chick and of the pig. *Contrib. Embryol.* No. 18, Publication 226 of the Carnegie Institution of Washington.

Sandison, J. C. (1926). Observations on the growth of blood vessels as seen in the transparent chamber introduced into the rabbit's ear. *Am. J. Anat.* **41,** 475–503.

Sato, M., Adler, R., and Glaser, B. M. (1984). Reaggregated endothelial cells from capillary-like structures *in vitro*. *ARVO Annu. Spring Meet.*

Sato, M., Adler, R., and Glaser, B. M. (1985). Histogenesis in aggregates of vascular endothelial cells co-cultivated with smooth muscle cells and fibroblasts. *ARVO Annu. Spring Meet.*

Schoefl, G. I. (1963). Studies on inflammation. III. Growing capillaires: Their structure and permeability. *Virchows Arch.* **337,** 97–141.

Shabo, A. L., and Maxwell, D. S. (1976). Insulin-induced immunogenic retinopathy resembling the retinitis proliferans of diabetes. *Trans. Am. Acad. Ophthalmol. Otolaryngol.* **81,** 497–508.

Shilling, J. S., and Kohner, E. M. (1976). New vessel formation in retinal branch vein occlusion. *Br. J. Ophthalmol.* **60,** 810–815.

Shimizu, K., Kobayashi, Y., and Muraoka, K. (1981). Midperipheral fundus involvement in diabetic retinopathy. *Ophthalmology* **88,** 601–612.

Shing, Y., Folkman, J., Sullivan, R., Butterfield, C., Murray, J., and Klagsbrun, M. (1984). Heparin affinity: Purification of a tumor-derived capillary endothelial cell growth factor. *Science* **223,** 1296–1299.

Shorb, S. R., Irvine, A. R., Kimura, S. J., and Morris, V. W. (1976). Optic disc neovascularization associated with chronic uveitis. *Am. J. Ophthalmol.* **82,** 175–178.

Simionescu, M., Simionescu, N., and Palade, G. E. (1976). The blood vessel wall, hemorheology, and hemodynamics. *Thromb. Res. Suppl. II* **8,** 247–256.

Sorgente, N., Kuettner, K. E., Soble, L. W., and Eisenstein, R. (1975). The resistance of certain tissues to invasion. II. Evidence for extractable factors in cartilage which inhibits invasion by vascularized mesenchyme. *Lab. Invest.* **32,** 217–222.

Sorgente, N., and Dorey, C. K. (1980). Inhibition of endothelial cell growth by a factor isolated from cartilage. *Exp. Cell Res.* **128,** 63–71.

Spaet, T. H., and Stemerman, M. B. (1972). Platelet adhesion. *Ann. N.Y. Acad. Sci.* **201,** 13–21.

Stockard, C. R. (1915). The origin of blood and vascular endothelium. *Am. J. Anat.* **18,** 227–327.

Tano, Y., Chandler, D. B., and Machemer, R. (1981a). Retinal neovascularization after intravitreal fibroblast injection. *Am. J. Ophthalmol.* **92,** 103–109.

Tano, Y., Chandler, D. B., and Machemer, R. (1981b). Vascular casts of experimental retinal neovascularization. *Am. J. Ophthalmol.* **92,** 110–120.

Taylor, S., and Folkman, J. (1982). Protamine is an inhibitor of angiogenesis. *Nature (London)* **297,** 307–312.

Thoma, R. (1893). Untersuchungen über die Histogenese und Histomechanik des Gefasssystems. Stuttgart.

Thoma, R. (1911). Über die Histomechanik des Gefasssystems und die Pathogenese der Angiosklerose. *Virchow's Arch.* **204,** 1–74.

Timpl, R., Rohde, H., Robey, P. G., Rennard, S. I., Soidart, J. M., and Martin, G. R. (1979). Laminin—a glycoprotein from basement membranes. *J. Biol. Chem.* **254,** 9933–9937.

Tuddenham, E. G. D., Shearn, S. A. M., Peake, I. R., Giddings, J. C., and Bloom, A. L. (1974). Tissue localization and synthesis of factor-VIII-related antigen in the human foetus. *Br. J. Haematol.* **26,** 669–677.

van den Brenk, H. A. S. (1955). Studies in restorative growth processes in mammalian wound healing. *Br. J. Surg.* **43,** 525–550.

Versari, R. (1904). La Morfogenesi dei Vasi Sanguigni della Retina Umana. *Ric. Labor Anat. Norm. (Rome)* **10,** 25–62.

Wallow, I. H. L., Tso, M. O. M., and Fine, B. S. (1973a). Retinal repair after experimental xenon arc photocoagulation. I. A comparison between rhesus monkey and rabbit. *Am. J. Ophthalmol.* **75,** 32–52.

Wallow, I. H. L., and Tso, M. O. M. (1973b). Repair after xenon arc photocoagulation. 3. An electron microscopic study of the evolution of retinal lesions in rhesus monkeys. *Am. J. Ophthalmol.* **75,** 957–972.

Wallow, I. H. L., and Davis, M. D. (1979). Clinicopathologic correlation of xenon arc and argon laser photocoagulation procedure in human diabetic eyes. *Arch. Ophthalmol.* **97,** 2308–2315.

Weibel, E. R., and Palade, G. E. (1964). New cytoplasmic components in arterial endothelia. *J. Cell Biol.* **23,** 101–112.

Weiss, L., and Greep, R. O. (1977). "Histology," p. 381. McGraw-Hill, New York.

Weiss, J. B., Brown, R. A., Kumar, S., and Phillips, P. (1979). An angiogenic factor isolated from tumors: A potent low-molecular-weight compound. *Br. J. Cancer* **40,** 493–496.

Weiter, J. J., and Zuckerman, R. (1980). The influence of the photoreceptor–RPE complex on the inner retina. An explanation for the beneficial effects of photocoagulation. *Ophthalmology* **87,** 1133–1139.

Williams, G. A., Eisenstein, R., Schumacher, B., and (L. U.-Chuan Hsiao) Grant, D. (1984). Inhibitor of vascular endothelial cell growth in the lens. *Am. J. Ophthalmol.* **97,** 366–371.

Wise, G. N. (1956). Retinal neovascularization. *Trans. Am. Ophthalmol. Soc.* **54,** 729–826.

Wise, G. N., Dollery, C. T., and Henkind, P. (1971). "The Retinal Circulation," p. 47. Harper, New York.

Wolbarsht, M. L., and Landers, M. B. (1980). The rationale of photocoagulation therapy for proliferative diabetic retinopathy: A review and a model. *Ophthalmol. Surg.* **11,** 235–245.

Wolf, J. E., and Harrison, R. G. (1973). Demonstration and characterization of an epidermal angiogenic factor. *J. Invest. Dermatol.* **61,** 130–141.

Yamagami, I. (1970). Electromicroscopic study on the cornea. I. The mechanism of experimental new vessel formation. *Jpn. J. Ophthalmol.* **14,** 41–72.

Zetter, B. R. (1980). Migration of capillary endothelial cells is stimulated by tumor-derived factors. *Nature (London)* **285,** 41–43.

Ziegler, E. (1876). Untersuchungen über pathologische Bindegewebse und Gefassneubildung. Staudinger, Würzburg, Federal Republic of Germany.

Zweng, H. C., Flocks, M., and Peabody, R. (1966). Histology of human ocular laser coagulation. *Arch. Ophthalmol.* **76,** 11–15.

GENETIC MOSAICS AS TOOLS FOR THE STUDY OF THE RETINA

KARL HERRUP

Department of Human Genetics
Yale University School of Medicine
New Haven, Connecticut

JERRY SILVER

Department of Developmental Genetics and Anatomy
Case Western Reserve University School of Medicine
Cleveland, Ohio

The development of any nervous structure and its functional integration with the rest of the adult nervous system appear to the observer as an often bewildering cascade of interrelated and interdependent events. The clear delineation of cause from effect is rarely easy and always tempered with uncertainty. The other articles in these volumes deal with the various surgical, biochemical, and physiological approaches to the task of untangling these interconnections as they apply to the form and function of the retina. The goal of this article is to illustrate the ways in which genetic mutations and genetic mosaics can be used to complement these other studies.

The philosophy of this approach is founded in the tenet that, ultimately, the genome of an organism must contain all of the molecular instructions needed to build an adult eye and to maintain its function. The hope is to be able to view the eye from the perspective of the DNA of the cell rather than solely from the vantage point of its three-dimensional cellular structure or final physiological performance. Since the genome is large but finite [$\sim 3 \times 10^9$ base pairs in human beings, which could code for roughly $2–20 \times 10^5$ genes (see discussion in Lewin, 1980)] and since the organism must also build and maintain two kidneys, a liver, two gonads, millions of hair follicles, etc., one might reasonably expect that a manageable number of genetic instructions (i.e., genes) would be sufficient to shape an eye from the clay of the developing organism.

The existence of a normal gene is usually discovered by the occurrence of a mutation or variant. These genetic lesions alter or eliminate the coding function of a given stretch of DNA, and therein lies the essence of the genetic approach. By observing the consequences (i.e., the phenotype) of violating a single "ground rule," insight is gained into the genetic logic of the form and function of the eye. The challenge then becomes to accumulate as comprehensive a set as possible of these violations and to assemble them into subsets based on function. It cannot be stressed enough that this is not a quick and easy, foolproof way to unlock the mysteries of the eye. Even with the application of the awesome power of molecular genetics (e.g., Sorge *et al.*, 1984), the answers to some of our most pressing questions could well have epigenetic explanations. That is to say, many of the events we study may result from the interactions of gene products created at earlier times for other specific reasons. The genetic approach is best viewed not in isolation, but as one of several ways to approach the study of the retina.

I. A Modest Catalog of Known Mutations Affecting the Eye

From the preceding discussion, it should be clear that neurological mutants affecting eye development are valuable neurobiological resources. What is probably less well appreciated is that a substantial collection of mutations already exists. The reasons for the length of the list in Table I probably include the fact

TABLE I

MUTATIONS AFFECTING THE DEVELOPMENT OF THE EYE

Broad-spectrum developmental disorders[a]

blebbed (*bl*), chromosome 5
Undescribed since the 1970 personal communication in *Mouse News Letter* **42,** 26[b]

blind (*Bld*), chromosome 15
Watson (1968).[b] Incomplete penetrance. Homozygotes die before midgestation

blind sterile (*bs*), chromosome 2
Undescribed since the 1977 personal communication in *Mouse News Letter* **56,** 40; **57,** 21[b]

cataract and small eye (*Cts*), unknown linkage
Harata *et al.* (1978).[b] Semidominant

coloboma (*Cm*), chromosome 2
Undescribed since the 1966 personal communication in *Mouse News Letter* **35,** 27; **37,** 34[b]
May be an allele of *Dey*

Dickie's small eye (*Dey*), chromosome 2
Theiler *et al.* (1978).[b] Homozygotes die before midgestation

eye blebs (*eb*), chromosome 10
Beasly and Crutchfield (1969).[b] Incompletely penetrant with a wide range of defects

eye lens obsolescence (*Elo*), chromosome 1
Oda *et al.* (1980).[b] Homozygotes have not been described

eye opacity (*Eo*), unknown linkage
Undescribed since the 1953 personal communication in *Mouse News Letter* **8** (Suppl.), 14[b]

eyeless (*ey-1; ey-2*), unknown linkage
Chase and Chase (1944); Silver and Robb (1979).[b,c] Incompletely penetrant. Uncertain whether these are formally two single genes

gaping lids (*gp*), unknown linkage
Kelton and Smith (1964).[b] Lens hypertrophy followed by eye opacity

eye ear reduction (*Ie*), X chromosome
Noted only in *Mouse News Letter* **50,** 51, as personal communication. *Ie/Ie* and *Ie/Y* are anophthalmic[b]

lid gap (*lg*), unknown linkage
Ricardo and Miller (1967).[b] Incomplete penetrance. Several alleles known

micropinnia–microphthalmia (*Mp*), unknown linkage
Undescribed since 1965 personal communication in *Mouse News Letter* **32,** 68.[b] Heterozygotes have microphthalmia. Homozygotes are anophthalmic

microphthalmia (*mi*), chromosome 6
Interallelic heterozygotes have variable changes in eye defects
microphthalmia (*mi*)
Gruneberg (1948).[b] Semidominant; heterozygotes have iris pigment defects. Eye defects correlated with hyperplasia of pigment epithelium (Packer, 1967)
red-eyed white (Mi^{rw})
Undescribed since the 1974 personal communication in *Mouse News Letter,* **51,** 23[b]
white (Mi^{wh})
Gruneberg (1953); Deol (1967)[b]

ocular retardation (or^J and *or*), unknown linkage
Theiler *et al.* (1976); Truslove (1962).[b,c] See further discussion in text (Section V,B)

open eye lids with cleft palate (*oel*), unknown linkage
Undescribed since the 1961 personal communication in *Mouse News Letter,* **25,** 12[b]

(*continued*)

TABLE I—(*Continued*)

sightless (*Sig*), chromosome 6
Deol (1976).[b] Homozygotes presumed to die at birth
small eye (*Sey*), unknown linkage
Roberts (1967).[b] Homozygotes die before birth. Heterozygote expression is variable

Defects of cornea or lens[d]

recessive cataract (*cac*), unknown linkage
Konyukhov and Wachtel (1963)[b]
dysgenetic lens (*dyl*), unknown linkage
Sanyal and Hawkins (1979)
etopic (*ec*), unknown linkage
Beasley (1963)[b]
lens opacity (*Lop*), chromosome 10
Lyon *et al.* (1981)
lens rupture (*lr*), unknown linkage
Fraser and Herer (1948)[b]
Nakano cataract (*nct*), unknown linkage
Hamai *et al.* (1974)[b]
nuclear cataract (*nuc*), unknown linkage
Reported only in a 1981 personal communication in *Mouse News Letter* **64,** 59
vacuolated lens (*vl*), chromosome 1
Undescribed since the 1967 personal communication in *Mouse News Letter* **36,** 39[b]

Defects of neural retina

cribriform degeneration (*cri*), chromosome 4
Green *et al.* (1972).[b] Broad spectrum of CNS defects. Retina shows intracellular vacuoles in inner nuclear layer
Purkinje cell degeneration (*pcd*), chromosome 13
Mullen and LaVail (1975).[b] Slow photoreceptor degeneration; the mutant was originally isolated because of its cerebellar ataxia
nervous (*nr*), chromosome 8
Mullen and LaVail (1975).[b] Slow photoreceptor degeneration; the mutant was originally isolated because of its cerebellar ataxia
retinal degeneration (*rd*), chromosome 5
Tansley (1954).[b,c] Common in many inbred lines of mice. Described in text (Section III, B)
retinal degeneration slow (*rds*), chromosome 17
Demant *et al.* (1979); Van Nie *et al.* (1978)[a]

[a] These mutations lead to defects ranging from total anophthalmia to microphthalmia. Many of these mutations have pleiotrophic effects elsewhere in the organism. A substantial group of mutations leading to anencephaly and other major structural defects have not been listed.
[b] Described in Green (1981).
[c] Described in Sidman *et al.* (1965).
[d] Most defects in this group are adequately described by their name.

that disturbances in the structure of the eye are easily seen by casual observation of the animal, and the fact that the loss of visual function is not life threatening in the laboratory and often not even noticeable. Most people are surprised to learn that perfectly "normal" inbred lines of mice, such as many strains of C3H, are missing nearly all of their photoreceptors. The listing in Table I is not meant to provide a comprehensive description of each mutant. Comments are added only if the name of the gene is insufficient description. Any mutant for which there is uncertainty whether it is extinct has not been included. The references provided are usually to the earliest reference to the mutation. For more details, the reader is referred to *Mouse News Letter,* Vol. 70 (February, 1984). Other general references which may be of value in obtaining additional information are Sidman *et al.* (1965), Green (1966), and Green (1981).

Those mutations for which the position on the map of the mouse genome is known have been illustrated in Fig. 1. This figure illustrates that, as with mutations of all other organ systems, the eye-related genes appear to scatter randomly

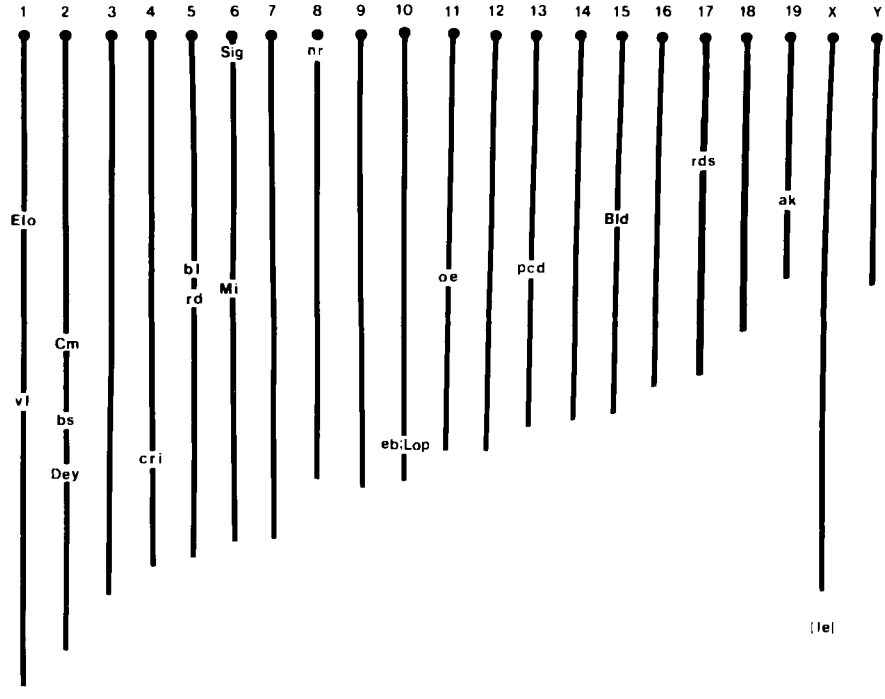


FIG. 1. Map position of the genes affecting eye structure for which linkage information is available. The solid lines represent the chromosomes and the length of each bar is proportional to the observed cytological lengths. The details of the means by which these genes have been positioned is contained in Roderick and Davisson (1981).

across the 1600 cM of mouse genome. The position for eye–ear reduction (*le*) is not known; as it is X linked, however, it is noted at the bottom of the chromosome. The linkage data illustrated here are taken from Roderick and Davidson (1981).

In addition to the three groups listed in Table I, there is a fourth group that deserves mention without listing. A large number of genetic loci are known that affect the pattern of pigmentation in the mouse (Silvers, 1979). As shown by many authors, nonpigmented animals tend to have more or less abnormal visual projections from the retina. Specifically, the number of fibers projecting ipsilaterally (i.e., uncrossed) is greatly reduced. The precise mechanism by which the pigment disorder relates to axonal projections is unknown, but it is the degree of pigmentation in the eye rather than the coat that varies most consistantly with the number of crossed or uncrossed fibers (LaVail *et al.*, 1978). The focus of this book is the cell biology of the retina. Thus while the pigmentation mutants, in a sense, affect retinal ganglion cell development, a thorough discussion of this fascinating problem of axon guidance will be left to others.

II. Genetic Mosaics: A Primer

When studying a genetic mutation, questions arise whether any one observed effect is produced directly by the intracellular action of the mutation or indirectly through interference with the function of a distant cell. These questions can often be approached through the study of genetic mosaics. Experiments such as these have been described by Mullen (1975) as searching for the "site of gene action." With a few known exceptions, all of the genes of a mouse are physically present in all cells. Clearly, the site of mutant gene action is not defined simply by the presence of the mutated DNA sequence. Less obvious, however, is that the presence of gene expression (RNA transcription of the gene) does not, in and of itself, define the site of gene action either. This is an important distinction. While the mutant gene may be expressed in many cells, only those cells in which this leads to a malfunction important in the creation of the mutant phenotype are considered a site of gene action. For example, if a mutation leads to a severalfold reduction in the activity of an enzyme, this reduction will be expressed in all cells that normally contain the enzyme. If, because of the differences in normal physiology, this reduction produces a significant pathology in only one cell type, then this is the only cell type that we would consider to be a primary site of gene action. This is, therefore, a functional rather than a molecular distinction.

Because the cells of the CNS interact extensively with each other and with other cell types, a second important distinction concerning the site of gene action is made. The mutant phenotype may include one or more obviously affected cell

types. Each of these aspects of the phenotype must be individually assessed to determine whether the effect on a particular cell is the direct result of the genotype of that cell. If so, that cell type is a primary site of gene action. If the phenotype of a cell (mutant or normal) can be uncoupled from the genotype of that cell, then the phenotype must be caused by factors influencing events outside of the affected cell itself and thus that cell would not be a primary site of gene action.

Two different types of genetic mosaics have been used to study the role of the genome in the biology of the eye: X-inactivation mosaics and chimeric mice. Early in the development of most eutherian mammals, genetic females undergo a process known as X inactivation (Lyon, 1961; Gartler and Riggs, 1983). In each cell of the embryo proper, one of the two X chromosomes, chosen at random, becomes condensed (heterochromatic), late replicating, and transcriptionally inactive. Since this event happens early, is apparently random in any one embryonic cell, and is stable over many mitoses, the adult female will be a functional mosaic for any allele that differs on her two X chromosomes. If a small piece of an autosome is translocated to the X, the inactivation process spreads into the autosome and inactivates the autosomal genes. One such event, Cattenach's translocation, carries the wild-type gene for the albino locus. If a female mouse carries only copies of the mutant albino gene in the rest of her genome, then she will display albino-pigmented mosaicism. This condition is readily visible in the coat. It is also useful in the study of the retina, as it is visible in the pigment epithelium, the sclera, and the iris (see Deol and Whitten, 1970). This type of mosaic is unfortunately limited in its application to only those genes located on the X chromosome.

The second type of genetic mosaic, which has been used far more extensively in the study of the retina, is the experimental aggregation chimera. These animals are created by the aggregation of two embryos that differ at one or many loci in their genetic makeup. The manipulation is performed when the embryos are preimplantation and consist of only eight cells. Maintained at 37°C, the two embryos adhere and after overnight culture they develop into blastocysts which are of normal proportions but double in size. These are returned to a receptive uterus where they implant in the uterine wall, adjust in size, and finish development normally. The resulting chimeric animals are fine-grained mosaics of cells for any genetic trait that differed between the two original embryos.

It should be evident that in studying the site of mutant gene action with a genetic mosaic it is important to have some way to determine the genotype of a cell regardless of its phenotype. To do this effectively an independent genetic cell marker is needed. One ideal cell marker is the albino mutation. In albino $\leftrightarrow$ pigmented mosaic animals, the presence or absence of melanin serves as an unambiguous, cell-autonomous indication of the genotype of any normally pigmented cell. As we shall see below, this marking system is quite useful if one

needs to know the genotype of the retinal pigment epithelium. Unfortunately, it cannot be used to mark any of the neuronal or glial cell populations of the retina. Of the other marking systems available, most have only limited utility in the retina. Differences in β-glucuronidase activity between Gus^b/Gus^b (high activity) and Gus^h/Gus^h (low activity) alleles as well as antigenic differences in glucose phosphate isomerase allozymes coded for by the $Gpi\text{-}1^a$ and $Gpi\text{-}1^b$ alleles have been the two markers used predominantly in the CNS. Both are most successfully applied to larger cells (both enzymes are cytoplasmic), whereas in cells such as photoreceptors with only scant amounts of cytoplasm in their perikarya, detection of the enzymes is more elusive, especially for the glucuronidase marker. To date, neither marker has been used in prenatal experiments.

In spite of these difficulties, significant advances in our understanding of retinal genetics have been made through mosaic analysis. The remainder of the article describes studies on the mechanism of action of four genes, three in mouse and one in rat. Also discussed is our current knowledge of the role of cell lineage in retinal development. Finally, preliminary results of studies of a fifth mutant (from the mouse) are described.

III. The Study of the Site of Gene Action

A. The Retinal Dystrophy (rdy) Rat

After the discussion of mouse mutants in the previous section, it may be a bit surprising to begin this section with a discussion of a rat mutation. This choice is made in order to introduce the techniques of mosaic analysis with a gene defect whose interpretation is fairly straightforward. The RCS rat strain carries a mutation for inherited retinal dystrophy (*rdy*) as well as the mutation pink-eye (*p*). The latter results in the absence of pigment in the cells of the retinal pigment epithelium, but normal pigmentation in coat melanocytes. Rats which are homozygous *rdy*/*rdy* show an initially normal development of the retina. Beginning on about postnatal day 20, however, and continuing until the end of the second postnatal month, the population of photoreceptor cells completely degenerates. To understand how the mutant gene brings about this dramatic phenotype, one must first engage in a bit of forensic genetics. Is the observed death a suicide or a murder? In this case, circumstantial evidence implicates a malfunction in the pigment epithelium as the cause of the demise of the photoreceptor. Preceding their degeneration, the rod outer segments become distorted due to the accumulation of whorls of membrane debris. As emphasized in other articles in this volume (see especially Clark), the pigment epithelium in normal animals phagocytizes the distal rod outer segments as they are shed (Young and Bok, 1969),

while in *rdy/rdy* animals this process appears absent (Herron *et al.*, 1969; LaVail and Sidman, 1972; Bok and Hall, 1969).

It is tempting to speculate from these observations that the gene acts by disabling the pigment epithelial cell in such a way that it can no longer phagocytize the rod outer segments. This, in turn, produces the observed accumulation of debris and finally the death of the photoreceptor cells. Such a sequence of causes must be assigned cautiously, however, since it is equally possible that the rod outer segments are altered by the mutation in such a way that the otherwise normal cells of the pigment epithelium no longer recognize them as candidates for phagocytosis. Alternately, a circulating toxin might poison the interaction between the two cell types. In this case, the primary site of gene action would be the cells that were the source of the toxin.

This mystery has been solved most convincingly by an astute pair of genetic detectives, Mullen and LaVail (1976). By aggregating wild-type and *rdy/rdy* rat embryos and allowing the composite embryos to develop to term, they created two *p/p rdy/rdy* ↔ +/+ +/+ chimeras. (Recall that the RCS rat is also homozygous pink-eye.) Each of the four eyes examined showed evidence of mosaicism in both the pigment epithelium and neural retina. The neural retina consisted of regions in which retinal cytoarchitecture was indistinguishable from the wild type and other regions in which the photoreceptors were either missing or appeared highly abnormal. Likewise, the pigment epithelium was a mixture of nonpigmented (*rdy/rdy p/p*) and pigmented (+/+ +/+) cells.

The key to the analysis was the spatial relationship between the two phenotypes in the neural and pigment retina. In the words of Mullen and LaVail, "Most strikingly . . . patches of abnormal and degenerated photoreceptors were present only opposite *rdy/rdy* pigment epithelial cells and not opposite normal pigment epithelial cells. . . . Furthermore, of the more than 200 patches of mutant pigment epithelium examined that were greater than 2 cells in length, we found degenerated and abnormal photoreceptors opposite all." The conclusions are clear. The phenotype of the photoreceptor layer (normal or degenerate) is tightly coupled to the genotype of the immediately adjacent pigment epithelium. This situation is illustrated in Fig. 2. Given the way in which the eye develops, the odds of this apposition occurring by chance are infinitesimal. Therefore, hypotheses that either the photoreceptor cell or a circulating toxin causes the disease must be discarded. The pigment epithelial cell emerges clearly as a primary site of *rdy* gene action. This knowledge is an important step in our understanding of the mechanism of gene action. It does not, however, reveal why the accumulation of shed outer segments leads to the photoreceptor cell death.

It is important to point out early in this discussion the careful way in which sentences concerning site of gene action must be phrased. Notice that we described the pigment epithelial cell as *a*, not *the*, primary site of gene action. This

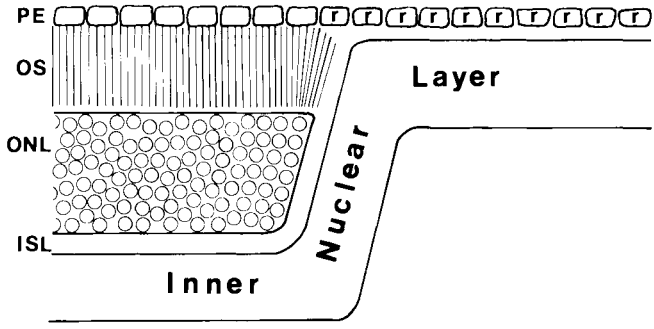


FIG. 2. A schematic representation of the retina in a rat *rdy*/*rdy* ↔ +/+ chimera. The genotype of the cells in the pigment epithelium (PE) is denoted by the presence (*rdy*/*rdy*) or absence (+/+) of an "r." The genotype of these cells is assessed by the presence (+/+) or absence (*rdy*/*rdy p*/*p*) of melanin pigment. Note the perfect correspondence between the phenotype of degeneration of the photoreceptor and the genotype of the overlying PE. See text for further details. OS, Outer segments; ONL, outer nuclear layer; ISL, inner synaptic layer.

is done to remind both the reader and ourselves that (1) knowledge of whether a gene acts directly or indirectly must be gained independently for each affected cell type and (2) knowledge that one or a few cell types are affected by a mutation does not prove that no other cells are involved elsewhere in the organism (i.e., the gene is pleiotrophic). For the *rdy* gene we can say that, of all the known affected cell types (two) in the *rdy*/*rdy* rat, the pigment epithelial cell is the only one that is a primary site of gene action. These distinctions may seem petty in the context of the *rdy* mutation; however, as we discuss the mouse mutants that have been studied via mosaic analysis, these issues become increasingly important.

B. The Retinal Degeneration (rd) Mouse

Superficially, the *rd* mouse mutation closely resembles the rat *rdy* disease. Relatively late in the development of the eye (postnatal day 10) the photoreceptor cells begin to degenerate such that by the end of the third postnatal week the outer nuclear layer is reduced to an incomplete row of photoreceptor cells which, during the next few weeks, will also disappear. The cytological details of the degeneration, however, are totally different from those in the RCS rat. No whorls of debris are found and the pigment epithelial cells seem capable of phagocytosis (Sanyal and Bal, 1973). Further, abnormalities are detected in the photoreceptor cells of *rd*/*rd* mice before the period of degeneration (Sanyal and Bal, 1973). Tissue culture experiments demonstrate that the defect is intrinsic to the eye (Sidman, 1961), but, given the interrelatedness of the cells, it is difficult

to be sure from these data which cell type(s) in the eye is a primary site of gene action.

The *rd* mutation has been examined in chimeric animals by a number of laboratories (Mintz and Sanyal, 1970; Mintz, 1974; Wegmann *et al.*, 1971; LaVail and Mullen, 1976; West, 1976). The work has helped to narrow the possible site of gene action to the neural retina, but the answers to many questions concerning further details of the action of the *rd* gene remain elusive. All of the above-cited authors agree that in *rd*/*rd* ↔ +/+ chimeras the outer nuclear layer is patchy; that is, regions of normal or nearly normal retina are found in the same eye together with regions in which no outer nuclear layer exists. This pattern of mosaicism makes it unlikely that the mutant phenotype is caused by a widely circulating toxin (or absent nutrient). If this were the cause, the expected pattern would be a uniformly normal, mutant, or intermediate eye. Beyond this conclusion, the only other direct piece of information concerns the involvement of the pigment epithelium in the etiology of the disease.

The detailed study of LaVail and Mullen (1976) carefully examined the relationship between the genotype of the cells of the pigment epithelium (using the absence of pigment in albino cells as a cell marker) and the phenotype of the underlying photoreceptor cells. Mintz and Sanyal (1970), Mintz (1974), and Sanyal and Zeilmaker (1976) had previously noted in single-section analyses that pigment epithelial cells of both *rd*/*rd* and +/+ genotypes could be found over both normal and degenerate retina. LaVail and Mullen added important information based on a reconstruction of one entire chimeric retina from a set of serial 10 μm wax sections. The animal whose eye they examined was a C57BL/10 ↔ SJL chimera. The SJL strain is both albino (*c*/*c*) and *rd*/*rd;* C57BL/10 is wild type at both loci. The reconstructed chimeric retina was found to have patches that could be hundreds of micrometers in size in which wild-type pigment epithelial cells overlay a totally degenerated outer nuclear layer. Similarly, there were large areas consisting of exclusively mutant pigment epithelial cells overlying either normal or intermediate neural retina (one to four rows of photoreceptor nuclei). Further, both mutant and wild-type pigment epithelial cells were found to contain phagosomes and hence both genotypes seem capable of clearing shed rod outer segments.

The total absence of correlation between the genotype of the pigment epithelium and the phenotype of the underlying photoreceptors is illustrated in Fig. 3. Note that the only difference between this arrangement and that shown in Fig. 2 is the placement of the mutant (''r'') pigment epithelial cells with respect to the degenerated and normal outer nuclear layer. This observation has several implications. First, it reemphasizes the importance of the perfect correlation between pigment epithelium genotype and retinal phenotype observed in the *rdy*/*rdy* ↔ +/+ rat chimeras. For the *rd* mutation, these results eliminate the possibility that the pigment epithelium is a primary site of gene action. The

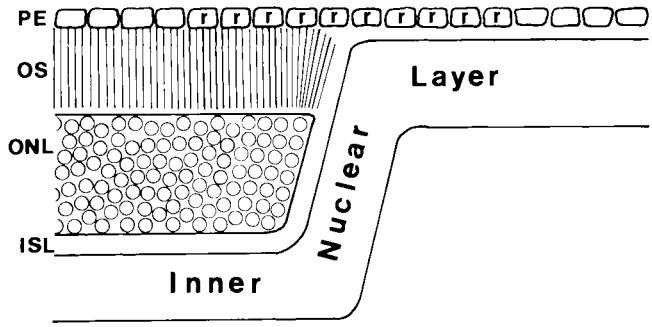


FIG. 3. A schematic representation of the retina of a mouse *rd/rd* ↔ +/+ chimera. The symbols and abbreviations have the same meaning as in Fig. 2. Note the *absence* of correlation between the genotype of the pigment epithelium (PE) and the phenotype of the photoreceptor layer. A normal ONL can be found under either wild-type mutant ("r") PE, and degenerated ONL is found under both genotypes as well. See text for further discussion.

presence of normal neural retina underlying mutant pigment epithelium combined with the presence of degenerate neural retina adjacent to wild-type pigment epithelium shows that the genotype of the pigment epithelium is neither necessary nor sufficient to assure the presence of mutant phenotype in the neural retina. Combined with the tissue culture experiments (Sidman, 1961), these data narrow the possibilities for site(s) of gene action to the neural retina. Whether the photoreceptor cell is the primary target or whether its degeneration results from a failed interaction with a second cell type in the neural retina cannot be deduced from the data.

As is frequently the case in the examination of mosaic tissue, these observations raise additional questions that were not anticipated originally. A good case in point is the observation of "intermediate" retina, areas in which the outer nuclear layer is thin but not absent. Mullen (1978) discusses two ways in which these intermediate patches could arise. In the first, discrete patches of *rd/rd* cells exist in the beginning but, following their death, the resulting gap in the outer nuclear layer is filled by the lateral spreading of cells from the surrounding normal tissue. A second mechanism that would explain the occurrence of intermediate retina is that there is an early intermingling of +/+ and *rd/rd* cells during development (i.e., before the photoreceptor degeneration). In areas where cells of both genotypes coexist, the degeneration of one genotype (*rd/rd*) would produce a region of intermediate phenotype. A set of similar possibilities can be generated if one assumes that the photoreceptor is not a primary site of gene action. While the most direct way to address this issue would be with an independent cell marker appropriate for photoreceptor cells, none exists. The evidence that has been accumulated to date strongly suggests that both developmental cell mixing and postnatal cell spreading occur in the chimeric eye. Cell mixing is

discussed in greater detail below. Suffice it to say here that its existence compels one to consider that cells can interact during retinal development in a fashion that would be difficult to predict solely from the study of nonmosaic tissue.

The existence of cell spreading is argued for most compellingly by West (1976). In his analysis of six chimeric eyes, West examined the entire neural retina and noticed that its depth was "fairly uniform throughout." This situation existed even in the presence of patchiness in the outer nuclear layer. In regions where the photoreceptor layer thinned, "the underlying inner nuclear area was corresponding thicker." In regions where the photoreceptor layer was normal, the inner nuclear layer "often appeared thinner than usual." If these observations are correct, they suggest that despite the highly ordered radial organization of the developing retina, cell movement occurs in the tangential direction, at least in the inner nuclear layer. West shows neither pictures nor measurements to document these provocative conclusions, and the numerous photomicrographs of LaVail and Mullen (1976) largely crop the inner nuclear layer. Some informative figures (e.g., Fig. 10 in LaVail and Mullen, 1976) support West's assertions while others (e.g., Fig. 3) appear contradictory. The resolution is important, but the issue is raised here primarily to illustrate the point that the juxtaposition of mutant and normal cells during the development of a single organism often reveals cellular potentials that are neither intuitively obvious nor easily addressed in other experimental systems.

C. The Purkinje Cell Degeneration (pcd) Mouse

This mutant was originally isolated because of its locomotor difficulties. The mutation causes a remarkable collection of postnatal neuronal degenerations including cerebellar Purkinje cells (as its name suggests), olfactory bulb mitral cells, certain thalamic neurons, and photoreceptor cells (Mullen *et al.*, 1976; Mullen and LaVail, 1975; O'Gorman and Sidman, 1980). In addition, male *pcd/pcd* animals are sterile due to severe abnormalities of their sperm (Mullen *et al.*, 1976). The retinal degeneration in *pcd/pcd* is slow, taking approximately a year to complete. The progress of the retinal disease has been described in detail by LaVail *et al.* (1982) and Blanks *et al.* (1982). At the ultrastructural level, abnormalities are present in both the photoreceptor cell (beginning with the inner segments) and the Müller cells (Blanks *et al.*, 1982). Once again the assignment of site(s) of gene action through analysis of chimeras is hampered by the absence of a good independent cell marker. As with the *rd* mutation, the use of albino as a pigment epithelial cell marker revealed no correlation between the genotype of the pigment epithelium and the phenotype (degenerate or normal) of the underlying photoreceptors. (Mullen, 1984). Thus the degeneration is not caused by a defect intrinsic to the *pcd/pcd* pigment epithelial cells.

D. The Nervous (nr) Mouse

The nervous mutation, like *pcd*, was originally detected because of its ataxia. Affected animals lose most, but not all, of their Purkinje cells (Sidman and Green, 1970), and the loss continues at a slow rate throughout the life of the animal (Sotelo and Triller, 1979). The photoreceptor cells also undergo a slow and progressive loss over the course of the first year of life. One hallmark of the disease in both Purkinje cells (Landis, 1973a,b) and photoreceptors (Mullen and LaVail, 1975) is the presence of abnormal, swollen mitochondria. Whorls of outer segment membrane are present, but the mutant pigment epithelial cells appear to be capable of phagocytosis (White *et al.*, 1982). Those chimeras that have been examined to date (Mullen, 1984) have illustrated how difficult determining a site of gene action can be. The chimeric outer nuclear layer does not appear patchy but instead it seems to be either uniformly normal or abnormal. This observation, as well as others, has led Mullen (1984) to suggest that the defect may be caused by a circulating toxin or nutrient deficiency. The only certainty about the disease process is that further study is needed.

IV. The Study of Cell Lineage

A. Cell Lineage: A Primer

When one attempts to view the retina from the perspective of the DNA of a cell, it becomes apparent that an important means of conveying information both during and after development is through lineage. Early in development when the entire nervous system is made up of only a few thousand of cells, profound but invisible changes take place that prescribe much of the fate of the progeny of these early cells. The eye, as other nervous structures, can trace its origin back to small regions of the neural plate. Two bilaterally symmetrical regions are fated to produce the eyes if left undisturbed (Jacobson, 1959), and in addition the mitotic progeny of these cells will develop into optic structures even if they are moved to a different location (e.g., Boterenbrood, 1970). Thus, it appears as if many of the cell biological events that unfold during the development and function of the eye are consequences of a regulatory decision or decisions made in a small number of progenitor cells and passed stably to the mitotic progeny of these cells.

The molecular bases of these regulatory events are not even dimly perceived. In recent years, it has been demonstrated that the DNA of many vertebrates can be covalently modified (e.g., by methylation) or frankly rearranged (e.g., in the genes of the immune system). These modifications are found to be developmentally regulated, they are passed stably to all the mitotic progeny of any cell in

which the modification occurs, and they are often associated with significant alterations in the activity of the gene involved. While DNA modifications are an attractive hypothesis, consistent with the known facts, noncovalent modifications or cytoplasmic factors could easily be involved. Nonetheless, from a functional standpoint and regardless of the mechanism, the lineage history of a cell must be one factor in the determination of its final form and function. The question facing students of the cell biology of the retina is what are those properties of retinal cells that can be explained by an understanding of their lineage.

This question does not have a known answer. What follows is a brief overview of what information has been assembled on the patterns of cell lineage in the eye, and some comments on what role for cell lineage is suggested by these patterns. In the cell types examined to date, there is a strong tendency for a basic pattern of radiating sectors to be observed. In and of itself this is not surprising, given the manner in which the retina develops. As one looks closer, however, it is equally clear that the sector organization of the retina is blurred considerably by the occurrence of areas of substantial intermingling of the two genotypes. Both patterns are evident in the iris of the pigmented ↔ albino chimera shown in Fig. 4. Since the pigment cells of the iris are also neuroectodermal in origin, their deployment serves to illustrate the situation found in pigment retina. Clear sections are seen in which the predominant genotype is albino, yet, even in the center of these sections, cells of the pigmented genotype can be found. This coexistence of order and chaos has been documented in sensory and pigment retina as well. The interpretation of the pattern has been the subject of some debate.

B. The Pigment Retina

It should be no surprise, given the ease with which albino/pigmented differences in genotype can be seen, that the pigment retina is perhaps the best studied of all neural tissues. (Mystkowska and Tarkowski, 1968; Mintz and Sanyal, 1970; Deol and Whitten, 1972; Sanyal and Zeilmaker 1976, 1977; Mullen and LaVail, 1976; West, 1976, 1978). Of these studies, the most detailed is that of Sanyal and Zeilmaker (1977). Figure 5 is a representation of 2 of the 20 chimeric eyes that were serially reconstructed by these authors to show the relationship of the pigmented and albino pigment epithelial cells. The observation of broad sectors of tissue consisting of significantly different ratios of the two genotypes is repeated here. In each of these eyes, the pigmented cells appear only in a restricted area of the pigment epithelial layer. Yet even in the regions where pigmented cells are most dense, albino cells are commonly found.

Different authors emphasize different features of this pattern. Mintz and Sanyal (1970), Mintz (1974), and Sanyal and Zeilmaker (1977) emphasized the

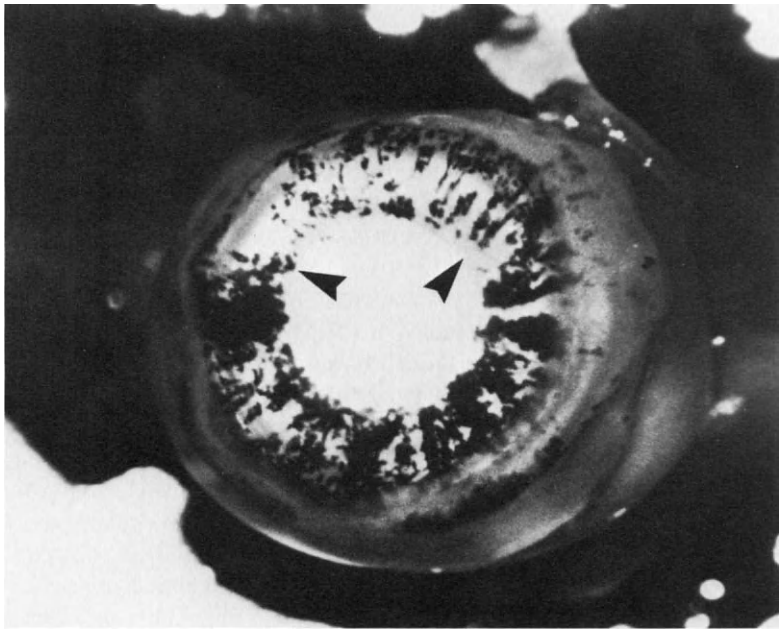


FIG. 4. Pattern of pigmentation in the iris of an AKR/J (c/c) ↔ C57BL/6 ($+/+$) chimera. Although the iris melanocytes are derived from neural crest, they emulate the pattern of mosaicism reported in both pigmented and neural retina. A broad sector organization is apparent with a predominantly albino (c/c) region shown between the arrowheads. This organization is significantly disrupted by cells of the opposite genotype, i.e., albino cells in pigmented patches and pigment cells in albino patches.

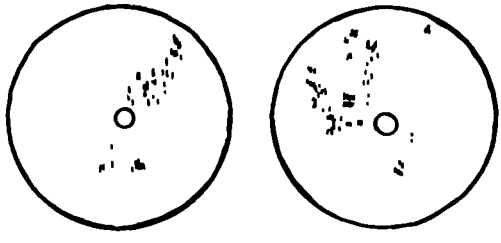


FIG. 5. A representation of the cells of the pigment epithelium from the two eyes of a mouse albino ↔ pigmented chimera, specifically the left and right eyes of chimera number 2 of Sanyal and Zeilmaker (1977). These eyes illustrate the features of broad sector organization combined with significant mixing of albino cells into mostly pigmented areas.

broad sectors. In this view, each sector represents a clone of cells; no one clone is a perfect pie-shaped wedge but the composite image is that the pigment epithelium consists of about a dozen of these wedges and therefore descends from about a dozen progenitor cells. The lack of congruence between the broad sectors in the pigment retina and similar sectors in the neural retina has led to the suggestion (Mintz, 1974) that "the pigment and visual layers of the retina are probably determined in separately compartmented groups of clonal lineages."

West (1976, 1978), rather than emphasizing the broad sectors, emphasized the mixing. Based on a one-dimensional statistical analysis applied to wax sections of chimeric retina, he suggested that, at the time in embryogenesis when the pigment epithelium first accumulates melanin, the distribution of the cells of the two genotypes is close to random. A similar analysis in the adult suggested that the average coherent clone (contiguous cells of like genotype beyond what is expected from a random mixture) is approximately six to eight cells. The suggestion is that extensive mixing is followed by a period of limited clonal growth. To these data, West (1978) added Sanyal and Zeilmaker's (1977) observation of chimeras with contributions of one genotype as small as 0.4% of the cells of the pigment epithelium. He argued that, taken together, these results suggest a much larger number of clones (~250) of smaller size as being most representative of the clonal organization of the eye. While McLaren (1976) and Mullen (1978) have thoughtfully discussed these two views, no acceptable resolution of these fundamentally opposite pictures has emerged.

C. The Neural Retina

The pattern of cell lineage in the neural retina is no clearer than that of the pigment epithelium. The same two research groups argue from the same basic difference in perspective. Based on a comprehensive study in which 20 chimeric retina were reconstructed from serial sections, Mintz and Sanyal (1970) and Mintz (1974) concluded that "an underlying developmental archetype or 'theme' with numerous 'variations' or development perturbations [appears]. The theme seemed to consist of 10 sectors of visual cells radiating from a center in each retina; each sector was interpreted as a separate cell lineage or clone in which specificity of differentiation was stable throughout a long proliferative history." The mixing is dismissed as "developmental 'noise.'"

West (1976) again stressed the mixing. As described above, there are considerable areas in *rd*/*rd* ↔ +/+ chimeras in which the outer nuclear layer appears intermediate, with 1–4 rows of photoreceptor nuclei instead of 8–10 as in the wild type. West concluded from the resulting cytoarchitecture that cell movement occurs *after* the degeneration of the *rd*/*rd* cells. "If cell movement does occur in this way, retinal degeneration [*rd*/*rd*] is clearly not a suitable marker for the analysis of clonal development in the chimeric neural retina, using adult eyes."

D. Discussion

One almost has the impression of the *rd/rd* ↔ +/+ and *c/c* ↔ +/+ mice grinning like Cheshire cats while various groups of scientists argue over their essence. Although we cannot add anything to help resolve the basic dilemma, we add a few thoughts that should also be considered. The first concerns the analysis of neural retina using the *rd* mutation. As discussed above, the evidence to date only eliminates the pigment epithelium as a primary site of gene action and narrows the possible site(s) to the neural retina. There is not any direct evidence that the photoreceptor cell itself is *the* site of gene action. Most authors assume that this is the case, but we feel that this uncertainty, more than any other, makes *rd/rd* an unsuitable choice for analysis of retinal cell lineage. If for example the Müller cell turns out to be the primary site of gene action in the retina, all of the preceding discussion is rendered moot.

A second comment concerns whether cell lineage is used by the CNS as a source of information in the creation neuronal specificity. The cells of the ganglion cell layer project with remarkable specificity to highly restricted locations in the diencephalon, based on their position in the retina. As described in preceding sections, it is apparent (whether or not sectors exist) that there is a profound mixing of cells of the various lineages in the retina (pigment and neural). This is reminiscent of other nervous structures analyzed in chimeras, in which the cells of the two genotypes appear highly dispersed (e.g., the Purkinje cells; Mullen, 1978; Oster-Granite and Gearhart, 1981). Since the lineage relationships among cells appear to restrict only broadly their spatial position, and since spatial location is a key element in receiving the proper neuronal connections, it follows that it is fairly unlikely that specific connections of a cell are determined by its lineage. This is not to say that the broad pattern of connectivity (retina projects to thalamus) is unrelated to lineage. Rather it seems unlikely that the highly specific retinotopic map is based primarily on the mitotic ancestry of the cells involved. As we stated in the introduction, many problems of retina biology will be solved most satisfactorily through epigenetic explanations. What little we know about cell lineage relationships in the retina suggests that retinal target specificity may well be one of those.

V. The Study of Embryonic Retinal Development

Studies of adult mosaics, as we have seen, can lead to important conclusions concerning sites of gene action and the contributions of cell lineage to a given nervous structure. Genetic mosaics can also be used, however, to learn about the genetic events that are needed to form the eye during embryogenesis. While

some information can be gained from analysis of adult tissue, observations made on embryonic chimeric retina can be a powerful additional source of insights into the cell biology of ocular development. This final section deals with some preliminary observations we have made of embryonic wild-type ↔ ocular retardation (or^J) chimeras. These studies have only just begun and yet they are already providing important clues to the logic of several important embryonic cell–cell interactions.

A. Features of Normal Development

In a general sense, the very early developing retina, like many other primitive brain regions, contributes two major classes to the neural apparatus, neurons and glia. In the retina, neuroepithelial cells that become neuroblasts differentiate as photoreceptors as well as the various other light stimulus-transducing elements (Sidman, 1961). Neuroepithelial cells that are destined to become retinal glioblasts differentiate most often as Müller cells (a form of radial astrocyte) (Lemmon and Rieser, 1983); however, depending on the species, retinal glioblasts can differentiate into a variety of other types (Ramon y Cajal, 1893). Importantly, neuroepithelial cells in the disk region of the retina and optic stalk mature solely into glia (Raff *et al.*, 1983; Juurlink and Federoff, 1980). Within recent years it has become apparent that in very young embryos the presumptive retinal glia in the region of the optic disk (in embryos this region is known as the optic fissure) have a specific interaction with the optic nerve axons as they exist the eye. Remarkably, one particular part of the primitive glial cell, its vitreal end-foot, becomes specialized during development to carry out this important function (Silver and Sidman, 1980; Silver, 1984).

Three observations suggest that retinal ganglion cell growth cones adhere preferentially to primitive glial end feet at all levels along their pathway, even in the presence of other axons. First, within any given developing optic fascicle still in the process of accumulating axons, growth cones are usually positioned immediately adjacent to the outer limiting glial membrane (Silver and Sapiro, 1981; Krayanek and Goldberg, 1981). Growth cones are never found suspended in the extracellular space or extracellular matrix; i.e., they always touch membranes of other cells. Second, in tissue fixed in severely hypertonic solutions, axons and end feet remain more tightly apposed to one another than do other surrounding tissues (Krayanek and Goldberg, 1981; Holley and Lasek, 1986). Third, after chemical digestion of the vitreal end-foot margin, growing axons become totally disoriented (Halfter and Deiss, 1984). It has recently been suggested that the tight association that occurs between optic axons and end feet may be mediated by a well-characterized neural cell adhesion molecule (N-CAM) (Silver and Rutishauser, 1985). The N-CAM molecule is localized on growth cones as well

as on glial end feet located along the presumptive route of axonal migration. Furthermore, injections of anti-NCAM Fab into the early developing eye of chicks causes the optic fibers to dislocate from the glial limitans as they exit from the globe (Silver and Rutishauser, 1985).

These observations illustrate the close developmental interaction between the retinal ganglion cells (through their axonal growth cones) and the embryonic glia (through their end feet). The significance of this association is increased by recent three-dimensional analyses of primitive glial end feet at the disk region of the retina. These studies have revealed a terrain with a strikingly well-ordered geometry, even at preaxonal stages (Silver and Sidman, 1980). Here, at the first critical junction where optic fibers must turn in one correct direction to successfully enter their nerve, the N-CAM-coated end feet are arranged in multiple parallel rows, forming a radiating network of channels oriented in the proper direction. Importantly, although end feet throughout the optic pathway contain N-CAM, the optic disk (i.e., optic fissure) is the only region along the entire route that has such a well-organized cellular alignment. N-CAM is not distributed in an obvious gradient fashion, in the direction of the tectum.

Another unusual phenomenon suggests that the glial cells may actually prepare in advance for their interaction with the ganglion cell axons. Associated only with end feet in the vicinity of the preaxonal fissure is the elaboration of very large amounts of extracellular space. After routine fixation of embryonic retinas in mixed aldehydes, the size of the extracellular spaces between end feet is always greatest in this region. In the most satisfactorily fixed tissue, much of the extracellular space produced as an artifact elsewhere in the tissue and at the margin can be eliminated. However, even in the best fixed specimens, the widest bored spaces always remain along the glial end-foot margin where axons will eventually grow. After the first wave of optic fibers is generated and (while clinging only to end feet) passes through the extracellular openings to exit the eye, these spaces become obliterated, leaving each fascicle ensheathed on either flank by radially arranged columns of glial partitions. A similar columnar type of axon–glia topology has also been reported in the developing folia of the mouse cerebellum (DeBlas, 1984). Is the extracellular space between young end feet real, or might some substance fill these openings during normal development? Although most routine fixatives tend to dissolve extracellular matrices, when embryonic tissue is processed though solutions that are designed to perserve extracellular material, a fibrous or flocculent substance can be maintained. In chick, such material has been shown along the route of the developing optic projection (Krayanek, 1980; Krayanek and Goldberg, 1981).

Many important questions remain concerning the role of glia–neuron interactions in providing growth and guidance cues to axons. What determines the amount and localized distribution of the extracellular material between glial end

feet? What role, if any, does the matrix, in addition to N-CAM, play in growth cone–glial adhesion? Does the matrix itself provide any guidance information beyond its potential ability to structure water (Bondareff and Pysh, 1968) and thereby maintain a portal large enough through which growth cones may migrate freely? Finally, and perhaps most important, are axons still capable of growing when the extracellular space or its matrix is experimentally or genetically eliminated?

B. The Ocular Retardation Mutant

Ocular retardation (*or* and *or^J*) is a recessive gene mutation in mice which causes micro- or anophthalmia associated with aplasia of the optic nerve and a progressive cataractous degeneration of the lens. The first isolated allele at this locus (*or*) was described by Truslove (1962), who observed that *or/or* neonates lacked optic nerve fibers from birth. However, it was not until recently that studies of *or^J/or^J* embryos have begun to shed light on the developmental mechanisms that might be involved initially in creating the optic nerve aplasia during embryonic stages. Theiler *et al.* (1976) and Silver and Robb (1979) observed that the first morphological deviation from normal in *or^J/or^J* embryos was a conspicuous reduction in the regular number of necrotic cells within the optic anlage. In the absence of normal cell resorption, there is a continuing increase in the number of cells within the eye rudiment but, interestingly, without a concurrent increase in the outer diameter of the globe. This results in a 30% increase in cell density per unit area and a progressive decrease in the amount of extracellular space to levels well below normal. The reduction in extracellular space is especially noticeable at preaxonal stages and is most striking in the region of the optic fissure.

In the *or^J/or^J* eye, although ganglion cells are generated on schedule and axons are produced intraocularly, the fibers repeatedly fail to exit from the globe. Instead, they usually take an irregular course through the retina and often appear in great whorls within the retina pigment epithelium interface, resulting in expansive areas of retinal detachment. The total absence of nerve outgrowth from the retina is thus correlated with the absence of cell death and with the much diminished volume of extracellular space (or, more likely, the extracellular material that usually fills these openings during normal development). The genetic removal of extracellular space in the mutant and the devastating consequences of its removal on axon outgrowth show how vitally important the normal extracellular material may be. Indeed, the *or^J/or^J* mutation is far more detrimental to the optic system than is the intraocular injection of anti-NCAM Fab. In eyes injected with anti-NCAM the early optic nerve fibers, although devoid of normal

neighborly topography, still maintain the ability to exit from the retina (Silver and Rutishauser, 1985). It remains to be determined if N-CAM (as well as the extracellular space between glial end feet) is also absent in the or^J/or^J mutant.

C. *Ocular Retardation Chimeras and the Study of Axon–Glia Interactions*

Initially, our interest in generating chimeras of or^J/or^J and wild-type embryos was to answer some very broad but intriguing questions about retinal development and the nature of the or^J gene. Would such chimeric offspring have a nerve or eye when even the smallest number of or^J/or^J cells were present? If no eye or nerve ever formed, this might suggest that or^J/or^J cells release a very potent deleterious diffusible substance into the retinal anlage. If small or normal eyes formed, would they contain nerves and if nerves were generated would they be constituted by normal or or^J/or^J ganglion cells? We were most interested to learn what the preaxonal terrain might look like, especially in regard to the presence of extracellular space and cell death. In chimeric embryos, would extracellular openings be generated in centrifugally radiating groups that reflect a clonal history of retinal cell generation (Fig. 4) or would the channels assume an intermediate size but with a uniform distribution? Would the pattern of cell death also reflect the distribution of or^J/or^J and $+/+$ cells and, in turn, the channel pattern, and, finally, the configuration of the nerve fascicles themselves? Clearly, whatever patterns emerged we believed that such chimeras might shed some light on the guidance mechanisms that axons normally use as they exit from the eye.

1. Cell Death

Serial 1-μm plastic section analyses of $or^J/or^J \leftrightarrow +/+$ chimeric embryos at various pre- and postaxonal stages have revealed that at preaxonal stages (E11.0–11.5), cell deaths within chimeric retinas tend to be located within discrete necrotic zones around the optic fissure (Fig. 6A and B). The size and distribution of the pycnotic centers vary from small, fulminant patches, to necrotic hemiretina, and finally to normal symmetric patterns of cell death surrounding the entire fissure region. Such patchy collections of necrotic debris have not been reported in either $+/+$ or or^J/or^J embryos.

The observation of patches of cell death rather than uniformly intermediate amounts of necrosis suggests that the cell death is a localized event and is not caused by a widely circulating substance. To understand the site(s) of or^J gene action, it is critical to know whether the dying cells are exclusively one genotype or whether the phenotype of death does not respect genotype in these animals.

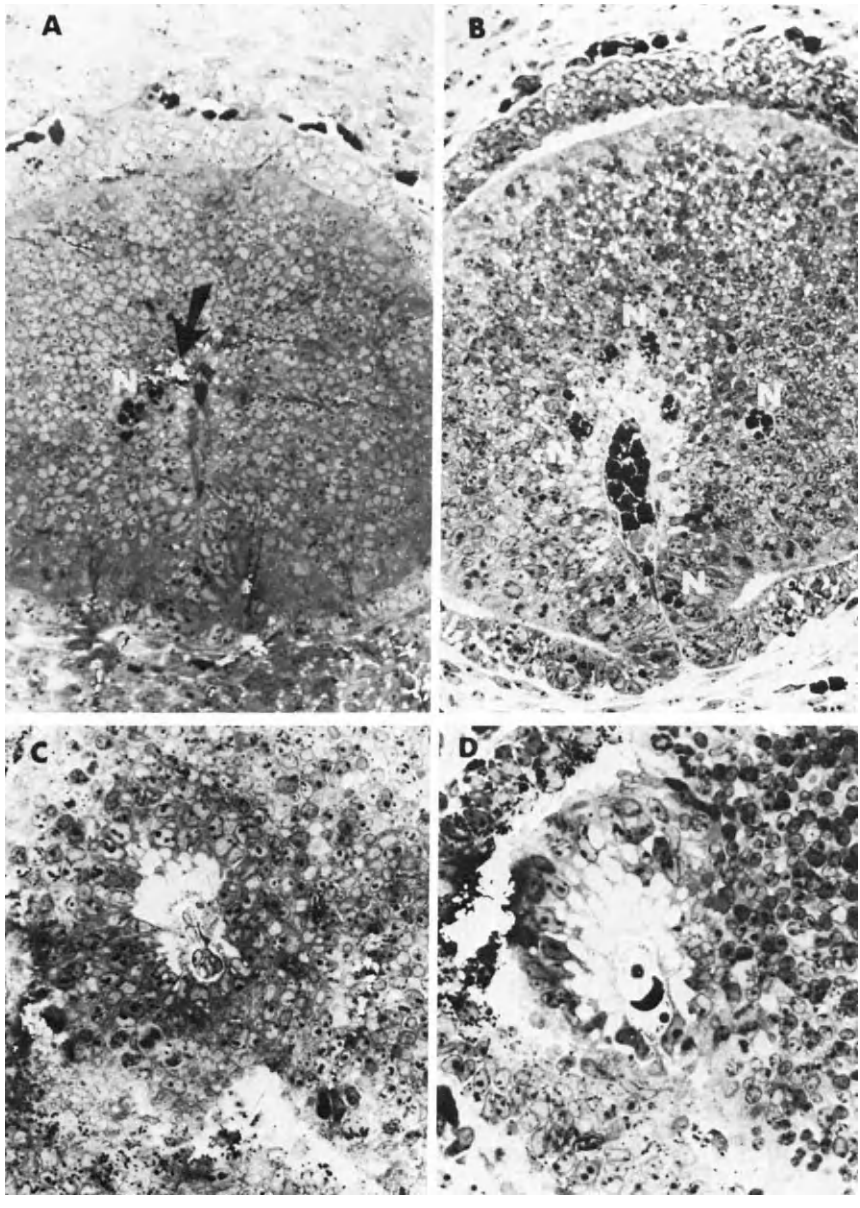


FIG. 6. A and B are coronal sections through the posterior retina of chimeric embryos (A) and control embryo (B) at E11.0, a preaxonal stage. In A, note the single cluster of necrotic cells (N) adjacent to a large extracellular opening (arrow). In B, note the increase in size of extracellular spaces and scattered clusters of necrotic cells. C and D are coronal sections through the posterior retina of a "chimeric" embryo (C) and control embryo (D) on day 13 of gestation. Note the smaller number of fibers in the "chimeric" eye. Except for the reduction in nerve fibers, all other morphological features of the two eyes were comparable.

This question unfortunately requires an embryonic retinal cell marker and none exists. Even in the absence of this marker, however, the appearance of the chimeric retinas provides several additional insights into retinal development.

2. Extracellular Space

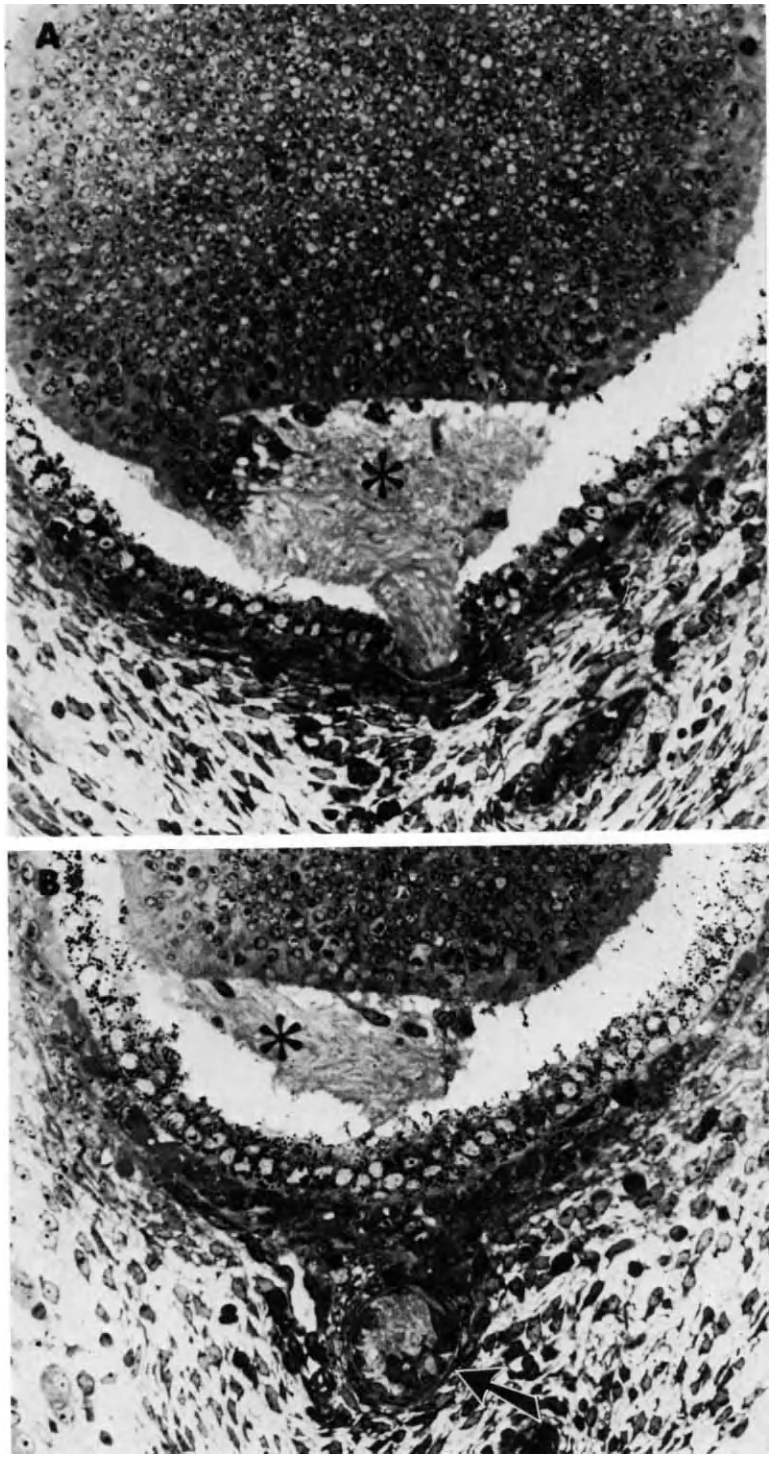
The distribution and size of the retinal extracellular spaces in chimeric embryos also varied from animal to animal. The presence of large extracellular openings was sometimes, but not always, strictly correlated with areas that were undergoing necrosis (Fig. 6A). Characteristically, however, the overall amount of extracellular space in chimeras (i.e., animals with patchy zones of cell resorption) was reduced. In most chimeric animals, the extracellular space tended to be evenly distributed around the fissure but intermediate in volume between or^J/or^J and normal embryos fixed under identical conditions (Fig. 6A and B).

These observations suggest that there may be an indirect relationship between cell death and the elaboration of retinal extracellular space or extracellular material. Also, if one hypothesizes that the absence of cell death is an adequate marker for or^J/or^J cells, then it would follow that or^J/or^J cells in chimeric retinas (where the normal cell population would undergo appropriate cell death) do have the ability to elaborate extracellular space or extracellular substances. Exactly how the production of necrotic cells or their removal is geared to the processes which lead to the formation of extracellular space is unknown. Further analysis $or^J/or^J \leftrightarrow +/+$ chimeras may shed light on this question.

3. Nerve Fiber Patterns

Some chimeric eyes did contain optic nerves, when examined at two postaxonal stages (E13 and E15). Such eyes and their retinas were classified as being chimeric because the nerves that they generated were quite unusual. Presumed chimeric optic nerves were obviously small when compared to controls of the same developmental stage (Fig. 6C and D). In some embryos at E15, a large neuroma was present in the same eye with a small, developing nerve (Fig. 7A and B). The small nerves usually emerged through the pigment epithelium in a region immediately adjacent to the point where the neuroma was forming (Fig. 7A). Exhaustive serial-section analysis of the retinas of such animals revealed that nerve fibers originated from all regions of the retina, and not from radially oriented slices. However, as fibers approached the disk region (i.e., optic fissure) many began to meander and, instead of exiting the eye, became incorporated into the neuroma. Other fibers (possibly those generated earliest) passed

FIG. 7. Coronal section through a chimeric eye on day 15 of gestation. Note the unusual presence of a nerve (arrow in B) and a neuroma (asterisk in A and B) in the same eye.


A
B

through the neuroma to enter the nerve. Although the optic fibers in the nerve appeared to be completely scrambled, they were able to grow along the stalk and as far as the tract by E15. In some "chimeric" animals, no nerves and only neuromas were present whereas, in others, normal-looking nerves occurred. Preliminary analysis of adult chimeras suggests that at least some or^J/or^J ganglion cells are capable of forming an appropriate central connection.

These results, taken together with the previous observations that channel size is variably reduced in chimeric mice, suggest that there may be a threshold amount of extracellular space or material needed to provide a portal large enough to accommodate the entire contingent of optic axons normally produced in the first few days of axonogenesis. It is conceivable, of course, that other factors besides a decline in the available amount of extracellular space causes optic axons to whorl. Since it has been established that optic growth cones adhere preferentially to neuroepithelial end feet (Silver and Rutishauser, 1985) as they exit from the eye, it might be suggested that a critical mass of this specialized growth-promoting membrane is constantly required during axonal exodus. Thus, it is possible that surface features of the end-feet membranes, in addition to the extracellular molecules they produce, are abnormal in or^J animals or underproduced in chimeric animals. In other words, several features of the end feet may be essential to build a normal nerve.

4. A Prospectus

Once a suitable or^J or normal retina ganglion cell marker is developed, one can determine definitively whether all or^J/or^J ganglion cells in chimeras have, indeed, grown to the brain. The preliminary data show that it is not only the wild-type ganglion cells in chimeric retinas that exit the globe, and such marker studies will be necessary to better resolve this issue. If it is determined that or^J/or^J ganglion cells do maintain their normal potential to constitute an optic nerve, then the reason they normally do not must reside in the nonneuronal environment.

What the exact nature of the or^J guidance abnormality is remains unknown, but we believe that the $or^J/or^J \leftrightarrow +/+$ chimera can provide a useful tool with which to study axon–glia interactions during development. For example, how do the central connections of the small, scrambled nerves sort out? Does the pattern of ipsi- vs contralateral projection change? Why does the failure of optic axon outgrowth lead to the total degeneration of the orb?

VI. General Conclusions

We have presented a view of the large (and growing) number of mutations that disrupt the development and function of the eye. Alone these congenital defects

are a fascinating glimpse at the way in which the genome views the eye. Coupled with the ability to produce mutant ↔ wild-type chimeras they offer a powerful analytical tool to aid in the dissection of the mechanisms that are responsible for creating and maintaining ocular function. Genetic mosaics offer both the potential for uncovering a site or sites of gene action as well as the ability to create unique juxtapositions of cells of two genotypes during normal development and thus allow the more thorough assessment of those epigenetic factors that are involved in the cell biology of the retina.

References

Bok, D., and Hall, M. O. (1971). The role of the pigment epithelium in the etiology of inherited retinal dystrophy in the rat. *J. Cell Biol.* **49,** 664–682.

Beasley, A. B. (1963). Inheritance and development of a lens abnormality in the mouse. *J. Morphol.* **112,** 1–11.

Beasley, A. B., and Crutchfield, F. L. (1969). Development of mutant with abnormalities of the eye and extremities. *Anat. Rec.* **163,** 293 (Abstr.).

Blanks, J. C., Mullen, R. J., and LaVail, M. M. (1982). Retinal degeneration in the *pcd* cerebellar mutant mouse. II. Electron microscopic analysis. *J. Comp. Neurol.* **212,** 231–246.

Bondareff, W., and Pysh, J. (1986). Distribution of the extracellular space during postnatal maturation of rat cerebral cortex. *Anat. Rec.* **160,** 773–780.

Boterenbrood, E. C. (1970). Differentiation in small grafts of the median region of the presumptive prosencephalon. *J. Embryol. Exp. Morphol.* **23,** 751–759.

Chase, H. B. (1944). Studies on an anophthalmic strain of mice. IV. A second major gene for anophthalmia. *Genetics* **29,** 264–269.

Chase, H. B., and Chase, H. B. (1941). Studies on an anophthalmic strain of mice. I. Embryology of the eye region. *J. Morphol.* **68,** 279–301.

DeBlas, A. L. (1984). Monoclonal antibodies to specific astoglial and neuronal antigens reveal the cytoarchitecture of the Bergmann glia fibers in the cerebellum. *J. Neurosci.* **4,** 265–273.

Demant, P., Ivanyi, D., and Vannie, R. (1979). Map position of the rds gene on the 17th chromosome of the mouse. *Tissue Antigens* **13,** 53–55.

Deol, M. S. (1976). *Colloq. Int. Cont. Nat. Rech. Sci.* **226,** 163.

Deol, M. S. (1967). The neural crest and the acoustic ganglion. *J. Embryol. Exp. Morphol.* **17,** 533–542.

Deol, M. S., and Whitten, W. K. (1972). Time of X-chromosome inactivation in retinal melanocytes in the mouse. *Nature (London) New. Biol.* **238,** 159–160.

Derer, P., and Nakanishi, S. (1983). Extracellular matrix distribution during neocortial wall ontogenesis in normal and reeler mice. *J. Hirnforsch.* **24,** 209–224.

Fraser, F. C., and Herer, M. L. (1948). Lens rupture, a new recessive gene in the house mouse. *J. Hered.* **39,** 149.

Gartler, S. M., and Riggs, A. D. (1983). Mammalian X-chromosome inactivation. *Annu. Rev. Genet.* **17,** 155–190.

Green, E. L., ed. (1976). "Biology of the Laboratory Mouse." Dover, New York.

Green, M. C. (1966). Mutant genes and linkages. In "Biology of the Laboratory Mouse" (E. L. Green, ed.), pp. 87–150. Dover, New York.

Green, M. C. (1981). "Genetic Variants and Strains of the Laboratory Mouse." Fischer, Stuttgart.

Green, M. C., Sidman, R. L., and Pivetta, O. H. (1972). Cribriform degeneration (*cri*): A new recessive neurological mutation in the mouse. *Science* **176,** 800–803.

Gleucksohn-Waelsch, S. (1961). Cited in M. C. Green (1966).

Gower, J. S. (1953). Cited in M. C. Green (1966).

Gruneberg, H. (1953). The relations of microphthalmia and white in the mouse. *J. Genet.* **51,** 359–362.

Gruneberg, H. (1948). Some observations on the microphthalmia gene in the mouse. *J. Genet.* **49,** 1–13.

Halfter, W., and Deiss, S. (1984). Axon growth in embryonic chick and quail retinal whole mounts *in vitro*. *Dev. Biol.* **102,** 344–355.

Hamai, Y., Fukui, H. N., and Kuwabana, T. (1974). Morphology of hereditary mouse cataract. *Exp. Eye Res.* **18,** 537–546.

Hankin, M. H., and Silver, J. (1985). Mechanisms of axonal guidance: The problem of intersecting fiber systems. *In* "Developmental Biology: A Comprehensive Synthesis" (L. Browder, ed.), Vol II, pp. 565–603. Plenum, New York, in press.

Harata, M. R., Shoji, R., and Semba, R. (1978). Genetic background and expressivity of congenital cataract in mice. *Jpn. J. Genet.* **53,** 147–152.

Herron, W. L., Reigel, B. W., Meyers, O. E., and Ruhin, M. L. (1969). Retinal dystrophy in the rat—a pigment epithelial disease. *Invest. Ophthamol.* **8,** 595–604.

Holley and Lasek. (1986). In preparation.

Hummel, K. P., and Chapman, D. B. (1963). Cited in M. C. Green (1966).

Jacobson, C. O. (1959). The localization of the presumptive cerebral regions in the neural plate of the axolotl larva. *J. Embryol. Exp. Morphol.* **7,** 1–21.

Juurlink, B. H. J., and Federoff, S. (1980). Differentation capabilities of mouse optic stalk in isolation of its immediate *in vivo* environment. *Dev. Biol.* **78,** 215–221.

Keeler, C. E. (1927). Rodless retina, an ophthalmic mutation in the house mouse, *Mus musculus*. *J. Exp. Zool.* **46,** 355–407.

Keeler, C. E. (1928). Blind mice. *J. Exp. Zool.* **51,** 495–508.

Kelton, D. E., and Smith, V. (1964). Gaping, a new open-eyelid mutation in the house mouse. *Genetics* **50,** 261–262 (Abstr.).

Konyukhov, B. V., and Wachtel, A. W. (1963). Electrophoretic studies of proteins in normal lenses and cataracts of inbred and mutant mice. *Exp. Eye Res.* **2,** 325–330.

Krayanek, S. (1980). Structure and orientation of extracellular matrix in developing chick optic tectum. *Anat. Rec.* **197,** 95–109.

Krayanek, S., and Goldberg, S. (1981). Oriented extracellular channels and axonal guidance in the embryonic chick retina. *Dev. Biol.* **84,** 41–50.

Landis, S. C. (1973a). Ultrastructural changes in the mitochondrial of cerebellar Purkinje cells of nervous mutant mice. *J. Cell Biol.* **57,** 782–797.

Landis, S. C. (1973b). Changes in neuronal mitochondrial shape in brains of nervous mutant mice. *J. Hered.* **64,** 193–196.

LaVail, J. H., Nixon, R. A., and Sidman, R. L. (1978). Genetic control of retinal ganglion cell projections. *J. Comp. Neurol.* **182,** 399–421.

LaVail, M. M., Blanks, J. C., and Mullen, R. J. (1982). Retinal degeneration in the *pcd* cerebellar mutant mouse. I. Light microscopic and autoradiographic analysis. *J. Comp. Neurol.* **212,** 217–230.

LaVail, M. M., Sidman, R. L., and O'Neil, D. (1972). Photoreceptor–pigment epithelial cell relationships in rats with inherited retinal degeneration. Radioautographic and electron microscope evidence for a dual source of extra lamellar material. *J. Cell Biol.* **53,** 185–209.

LaVail, M. M., and Mullen, R. J. (1976). Role of the pigment epithelium in inherited retinal degeneration analyzed with experimental mouse chimeras. *Exp. Eye Res.* **23,** 227–245.

Lemmon, V., and Rieser, G. (1983). The developmental distribution of vimentin in the chick retina. *Dev. Brain Res.* **11,** 191–197.

Lewin, B. (1980). "Gene Expression, Vol. 2, Eukaryotic chromosomes." Wiley, New York.

Lyon, M. F. (1961). Gene action in the X chromosome of the mouse (*Mus musculus*). *Nature (London)* **190,** 372–373.

Lyon, M. F., Jarvis, S. E., Sayers, I., and Holmes, R. S. (1981). Lens opacity. A new gene for congenital cataract on chromosome 10 of the mouse. *Genet. Res.* **38,** 337.

Mackensen, J. A. (1960). "Open eyelids" in newborn mice. *J. Hered.* **51,** 188–190.

McLaren, A. (1976). "Mammalian Chimeras." Cambridge Univ. Press, London and New York.

Mintz, B. (1974). Gene control of mammalian differentiation. *Annu. Rev. Genet.* **8,** 411–470.

Mintz, B., and Sanyal, S. (1970). Clonal origin of the mouse visual retina mapped from genetically mosaic eyes. *Genetics* **64,** 43–44.

Mullen, R. J. (1975). Neurological mutants: Use of chimeras to determine site of gene action. *Genetics* **80** (Suppl.), 56.

Mullen, R. J. (1978). Mosaicism in the central nervous system of mouse chimeras. *In* "The Clonal Basis of Development" 36th Symp. Soc. Devel. Biol. (S. Subtelny and I. M. Sussex, eds.), pp. 83–101. Academic Press, New York.

Mullen, R. J., and LaVail, M. M. (1975). Two new types of retinal degeneration in cerebellar mutant mice. *Nature (London)* **258,** 528–530.

Mullen, R. J., and LaVail, M. M. (1976). Inherited retinal dystrophy: Primary defect in pigment epithelium determined with experimental rat chimeras. *Science* **192,** 799–801.

Mullen, R. J. (1984). Sites of gene action in rodents with hereditary retinal degenerations. *In* "Heredity and Visual Development" (S. R. Hilfer and J. Sheffield, eds.). Springer-Verlag, Berlin and New York, in press.

Mystkowska, E. T., and Tarkowski, A. K. (1968). *J. Embryol. Exp. Morphol.* **20,** 33–52.

Nardi, J. B. (1983). Neuronal pathfinding in developing wings of the moth, *Manduca sexta*. *Dev. Biol.* **95,** 163–174.

Oda, S. I., Watanabe, T., and Kondo, K. (1980). New mutation, eye lens obsolescence, Elo, on chromosome 1 in the mouse. *Jpn. J. Genet.* **55,** 71–75.

O'Gorman, S., and Sidman, R. L. (1980). Cell loss in diencephalic nuclei of Purkinje cell degeneration (*pcd*) mutant mice. *Soc. Neurosci. Abstr.* **6,** 81.

Oster-Granite, M. L., and Gearhart, J. (1981). Cell lineage analysis of cerebellar Purkinje cells in mouse chimeras. *Dev. Biol.* **85,** 199–208.

Packer, S. O. (1967). The eye and skeletal defects of two mutant alleles at the microphthalmia locus of *Mus musculus*. *J. Exp. Zool.* **165,** 21–45.

Paget, O. E. (1953). *Cataracta hereditaria subcapsularis:* Ein neues, dominates Allel bei der Hausmaus. *Z. Indukt. Abstamm. Vererb.* **85,** 238–234.

Raff, M. C., Miller, R. H., and Noble, M. (1983). A glial progenitor cell that develops *in vitro* into an astrocyte or an oligodendrocyte depending on culture medium. *Nature (London)* **303,** 390–396.

Ramon y Cajal, S. (1893). The vertebrate retina. *In* "The Vertebrate Retina" (R. Rodiek, ed.). Freeman, San Francisco, California.

Ricardo, N. S., and Miller, J. R. (1967). Further observations on the *lg^{ml}* (lid-gap-Miller) and other open-eye mutants in the house mouse. *Can. J. Genet. Cytol.* **9,** 596–605.

Roberts, R. C. (1967). *Small-eyes,* a new dominant mutation in the mouse. *Genet. Res.* **9,** 121–122.

Roderick, T. H., and Davisson, M. T. (1981). Linkage map. *In* "Genetic Variants and Strains of the Laboratory Mouse" (M. C. Green, ed.) pp. 280–281. Fischer, Stuttgart.

Sanyal, S., and Bal. A. K. (1973). Comparative light and electron microscopic study of retinal histogenesis in normal and *rd* mutant mice. *Z. Anat. Entwicklungsgesch.* **142,** 219–38.

Sanyal, S., and Hawkins, P. K. (1979). Dysgenetic lens (*dyl*)—a new gene in the mouse. *Invest. Ophthamol. Visual Sci.* **18,** 642–645.

Sanyal, S., and Zeilmaker, G. H. (1976). Comparative analysis of cell distribution in the pigment epithelium and the visual layer of chimeric mice. *J. Embryol. Exp. Morphol.* **36,** 425–438.

Sanyal, S., and Zeilmaker, G. H. (1977). Cell lineage in retinal development of mice studied in experimental chimeras. *Nature (London)* **265,** 731–733.

Sidman, R. L. (1961). Histogenesis of mouse retina studied with thymidine-H^3. *In* "The Structure of the Eye" (G. Smelzer, ed.), pp. 487–506. Academic Press, New York.

Sidman, R. L. (1961). Tissue culture studies of inherited retinal dystrophy. *Dis. Nerv. Syst.* **22,** 14–20.

Sidman, R. L., and Green, M. C. (1970). "Nervous" a new mutant mouse with cerebellar disease. *In* "Les Mutants Pathologiques chez l'Animal. Leur Interet dans Recherche Biomedical" (M. Sabourdy, ed.), pp. 69–79. CNRS, Paris.

Silver, J. (1984). Studies on the factors that govern directionality of axonal growth in the embryonic optic nerve and at the chiasm of mice. *J. Comp. Neurol.* **223,** 238–251.

Silver, J., and Robb, R. M. (1979). Studies on the development of the eye cup and optic nerve in normal mice and in mutants with congenital optic nerve aplasia. *Dev. Biol.* **68,** 175–190.

Silver, J. and Rutishauser, U. (1984). Guidance of optic axons by a preformed adhesive pathway on neuroepithelial endfeet. *Dev. Biol.* **106,** 485–499.

Silver, J., and Sapiro, J. (1981). Axonal guidance during development of the optic nerve: The role of pigmented epithelia and other extrinsic factors. *J. Comp. Neurol.* **202,** 521–538.

Silver, J., and Sidman, R. L. (1980). A mechanism for the guidance and topographic patterning of retinal ganglion cell axons. *J. Comp. Neurol.* **189,** 101–111.

Silvers, W. K. (1979). The Coat Colors of Mice. Springer Verlag, Berlin and New York.

Singer, M., Nordlander, R. H., and Egar, M. (1979). Axonal guidance during embryogenesis and regeneration in the spinal cord of the newt. The blueprint hypothesis of neuronal pathway patterning. *J. Comp. Neurol.* **185,** 1–22.

Sorge, L. K., Levy, B. T., and Maness, P. F. (1984). *pp60 c-src* is developmentally regulated in the neural retina. *Cell* **36,** 249–257.

Sotelo, C., and Triller, A. (1979). Fate of presynaptic afferents to Purkinje cells in the adult nervous mutant mouse: A model to study presnaptic stabilization. *Brain Res.* **175,** 11–36.

Tansley, K. (1954). An inherited retinal degeneration in the mouse. *J. Hered.* **45,** 123–127.

Theiler, K., Varnum, D. S., and Stevens, L. C. (1978). Development of Dickies small eye, a mutation in house mouse. *Anat. Embryol.* **155,** 81–86.

Theiler, K., Varnum, D. S., Nadeau, J. H., Stevens, L. C., and Caginaut, B. (1976). A new allele of ocular retardation: Early development and morphogenetic cell death. *Anat. Embryol.* **150,** 85–97.

Truslove, G. M. (1962). A gene causing ocular retardation in the mouse. *J. Embryol. Exp. Morphol.* **10,** 652–660.

Van Nie, R., Ivanyi, D., and Demant, P. (1978). New H-2 linked mutation, *rds,* causing retinal degeneration in mouse. *Tissue Antigens* **12,** 106–108.

Varnum, D. S., and Stevens, L. C. (1968). Aphakia, a new mutation in the mouse. *J. Hered.* **59,** 147.

Watney, M. J., and Miller, J. R. (1964). Prevention of a genetically determined congenital eye anomaly in the mouse by the administration of cortisone during pregnancy. *Nature (London)* **202,** 1029–1031.

Watson, M. L. (1968). Blind—a dominant mutation in mice. *J. Hered.* **59,** 60.

Wegmann, T. G., LaVail, M. M., and Sidman, R. L. (1971). Patchy retinal degeneration in tetraparental mice. *Nature (London)* **230,** 333–334.

White, M. P., Gorrin, G. M., LaVail, M. M., and Mullen, R. J. (1982). Photoreceptor degeneration in the nervous mutant mouse. *Invest. Ophthalmol. Visual Sci. (Suppl.)* **22,** 259.

West, J. D. (1976). Distortion of patches of retinal degeneration in chimeric mice. *J. Embryol. Exp. Morphol.* **36,** 145–149.

West, J. D. (1978). Analysis of clonal growth using chimeras and mosaics. *In* "Development in Mammals" (M. H. Johnson, ed.), Vol. 3, pp. 413–460. Elsevier, Amsterdam.

Young, R. W., and Bok, D. (1969). *J. Cell Biol.* **42,** 392.

THE RETINA AS A REGENERATING ORGAN

BERNICE GRAFSTEIN

Department of Physiology
Cornell University Medical College
New York, New York

I. Introduction

Although the retina is a central nervous system (CNS) derivative, and CNS neurons, it is well known, do not regenerate readily, the retina represents an opportune system for the study of regeneration. Even in mammals, the retinal

ganglion cells can regenerate their axons (Ramon y Cajal, 1928; Goldberg and Frank, 1980; So and Aguayo, 1985). A much more impressive capacity for regeneration, however, is seen in the retinal ganglion cells of some lower species, including fish and amphibians, in which regenerating optic axons can reestablish their appropriate pattern of synaptic connections with the neurons of the optic tectum, culminating in the recovery of vision (Sperry, 1943, 1948; Attardi and Sperry, 1963; Jacobson and Gaze, 1965). In these species, even regeneration of the whole retina may occur in some circumstances (Burgen and Grafstein, 1962; Grafstein and Burgen, 1963; Grafstein, 1964; Reyer, 1977; Schmidt *et al.*, 1978; Maier and Wolburg, 1979). The presence of this high degree of regenerative capacity may be related to the fact that the retina in these animals continually enlarges with increasing age and body size. This requires not only the generation of new retinal neurons in adult life (Johns and Easter, 1977; Meyer, 1978), but also the development of new synaptic connections in the retina (Fisher and Easter, 1979) and continual reorganization of the retinotectal connections (Meyer, 1978). Consideration of the adult retina as a regenerating organ therefore leads us to confront some fundamental problems of ontogenetic development as well as nervous system repair.

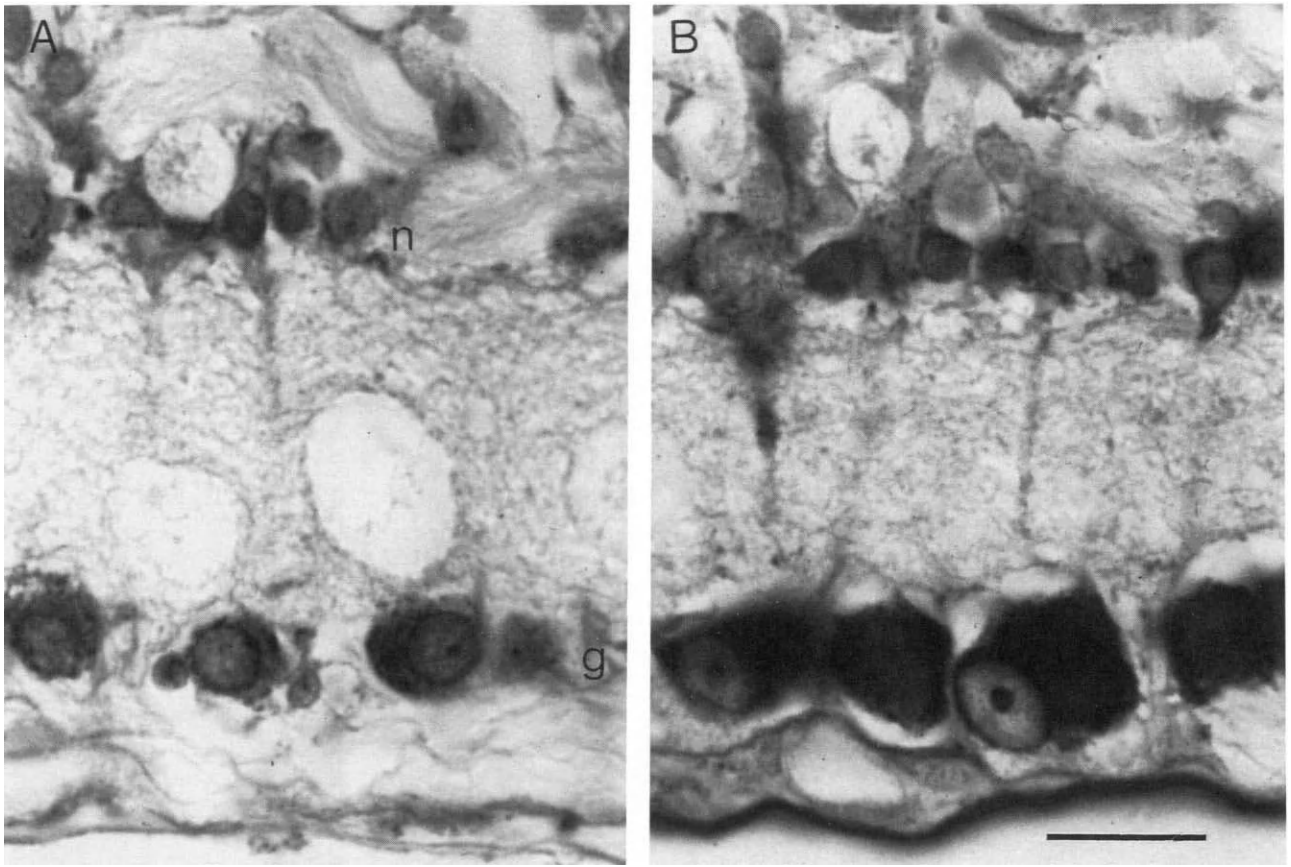
The present article is concerned primarily with one such issue, which will be considered mainly in the context of neuronal responses to injury, but which is equally important as a basic element of neuronal differentiation during ontogenetic development—the relationship between axonal outgrowth and the metabolic activities of the nerve cell body.

When an axon is injured, the part separated from the cell body degenerates. Reconstitution of the axon proceeds from the point of injury, with the requisite materials being supplied for the most part by axonal transport from the cell body (Grafstein and Forman, 1980), because the axon itself has only a very limited capacity, if any, for protein synthesis. Regeneration may therefore entail significant alterations in the exchange of materials and metabolic signals between the cell body and the axon. Thus the response to injury in a neuron capable of axonal regeneration involves not the axon alone, but also the cell body. Another important element in regeneration is the environment in which the emerging axon finds itself. The axons of the retinal ganglion cells offer a dramatic demonstration of the importance of the environmental factor: in most mammalian species, these axons do not ordinarily regenerate after injury (Grafstein and Ingoglia, 1982), but successful outgrowth may be seen when their environment is altered by a graft of peripheral nerve (Politis and Spencer, 1982; So and Aguayo, 1985).

For studies of interactions among members of this "regenerative triad"—axon, cell body, and axonal environment (Grafstein, 1983)—the ganglion cells of the retina have the advantage that they represent an anatomically well-defined system which is readily accessible for morphological, biochemical, and functional studies. For example, the cell bodies of the retinal ganglion cells can be

reproducibly subjected to pharmacological treatment by intraocular injection and can be readily located for histological examination. The axons of these cells, which constitute the optic nerve, run for most of their length in a separate physical compartment from the cell bodies and hence can be sampled or subjected to various experimental manipulations independently of the cell bodies. The properties of the glial cells surrounding the optic axons can likewise be independently studied in an excised length of optic nerve, and if necessary, the optic axons can be eliminated from this sample if they are caused to degenerate by removal of the retina. Finally, the terminal field of the optic axons, being confined to relatively circumscribed regions of the brain, can be separately sampled or experimentally perturbed; moreover, the functional status of the terminals can be assayed by electrophysiological or behavioral tests. These are some of the features that make the visual pathway especially suitable for studies on the correlation of events occurring in different parts of the regenerating system.

The present review will focus mainly on the characteristics of regeneration in the optic axons of the goldfish. Numerous studies of regeneration in the visual system of other species have contributed greatly to our understanding of the process of regeneration. For example, some outstanding studies have been carried out in amphibians, particularly with respect to the problem of reestablishment of the retinotectal connections (e.g., Gaze, 1970). Recent success in promoting regeneration of axons from mammalian retina (So and Aguayo, 1985) promises that our understanding of this system will soon also increase significantly. Nevertheless, it is studies in the goldfish that have provided the most coherent picture of the morphological and biochemical changes in both the axon and the cell body of a regenerating neuron. One reason is that the goldfish retinal ganglion cells (Fig. 1) show an unusually dramatic reaction to axotomy (Murray and Grafstein, 1969; Grafstein and Murray, 1969), which makes it easier to relate the features of this reaction to the progress of regeneration. In addition, the normal parameters of axonal transport have been extensively explored in this system (McEwen *et al.*, 1971; Elam and Agranoff, 1981; McQuarrie, 1984), providing an essential background for corresponding studies during regeneration. Most of these investigations of axonal transport have been based on the method (introduced by Taylor and Weiss, 1965) of injecting a radioactively labeled precursor into the eye and then measuring the rate of appearance of labeled transported materials in the optic nerve and optic tectum (Grafstein, 1975). This technique has not only been applied in elucidating the dynamics of transport during regeneration, but has also been used in measuring axonal outgrowth (McQuarrie and Grafstein, 1981; Sparrow *et al.*, 1984), supplementing outgrowth measurements based on morphological criteria (Grafstein, 1971; McQuarrie and Grafstein, 1981; McQuarrie, 1985). The study of factors that influence axonal outgrowth has also been advanced by observations on fragments of


A
n
g
B

the retina maintained in tissue culture (Agranoff *et al.*, 1976; Landreth and Agranoff, 1979), a technique that owes much to the fact that the survival and outgrowth capability of goldfish retinal ganglion cells are greatly enhanced by a prior ("conditioning") lesion (McQuarrie and Grafstein, 1973, 1981) made a few days before the retina is explanted. Thus there is now a wide array of data available about the characteristics of regeneration and the metabolic changes accompanying regeneration in the goldfish retinal ganglion cell. This leads us to attempt to define the conditions that are essential for the success of this neuron as a regenerating system, particularly as a basis for comparison with the corresponding cell in mammals.

II. Characteristics of Regenerating Goldfish Retinal Ganglion Cells

A basic strategy in the study of the regenerating neuron is to correlate the sequence of events in the growing axon itself with the concurrent changes in the cell body, which are presumably essential for the production of materials necessary for regeneration, and with changes in axonal transport, which is essential for conveying these materials to the site at which they will be utilized.

A. Axonal Regeneration

The goldfish retinal ganglion cells are remarkable not only because they show vigorous axonal outgrowth under conditions in which the corresponding mammalian neurons do not, but also because this outgrowth results in the restoration of a correct pattern of point-to-point connections between the retina and the optic tectum (Attardi and Sperry, 1963). The mechanisms involved in the reestablishment of the retinotopic map, however, are largely outside the scope of the present article, since the correlation between the topography-determining mechanisms (e.g., Stuermer and Easter, 1984; Edwards *et al.*, 1985) and metabolic events in the cell body is still relatively unexplored. The present treatment of the characteristics of axonal regeneration is therefore largely confined to those features that are related to the physical reconstruction of the axon and its terminals, with relatively little attention being given to the problems of direction of out-

FIG. 1. Inner layers of the retina from (A) normal goldfish optic nerve and (B) 28-day regenerating nerve. The retinal ganglion cell layer (g) consists of cells that are greatly enlarged and hyperchromic in the regenerating condition, whereas cells of the inner nuclear layer (n) do not show any marked differences. Bar, 10 μm.

growth (Sperry, 1963) or the ultimate modifications of structure and function that may be required for recovery of vision.

1. Initiation of Outgrowth

Injury of the goldfish optic nerve results in rapid retrograde degeneration of the axons ("traumatic" degeneration; Ramon y Cajal, 1928) for a short distance from the site of the lesion. This distance probably does not exceed 250 μm (McQuarrie and Grafstein, 1981), except for the small number of axons in the largest size group, the tips of which can usually be found about 300 μm further back toward the eye (Grafstein, 1971). Within 24 hr after the lesion, the axons develop terminal bulbs, containing a tangle of neurofilaments surrounded by an accumulation of various cellular organelles (Grafstein, 1971; Lanners and Grafstein, 1980a). Most of the terminal bulbs have disappeared by 2–4 days, but they may persist on the large axons in an altered form for several months (Grafstein, 1971).

By 48 hr, some signs of regeneration may be detected by electron microscopy, including the presence of some small bundles of unmyelinated axons, which are up to 1–2 μm in diameter and closely apposed to one another (Lanners and Grafstein, 1980a). These processes are unusual not only because they are relatively large in size, but also because they appear to be filled with intermediate filaments and have few microtubules, in contrast to the usual picture of developing or regenerating optic axons, which are a fraction of a micrometer in diameter and contain a prominent array of microtubules, but very little filamentous material (Peters and Vaughan, 1967; Murray, 1976). In fact, bundles of regenerating axons of this latter type are detectable by 3 days, although they are not common before 6 days (Lanners and Grafstein, 1980a). This sequence suggests that the plump filament-filled processes represent an initial response to injury, which is superseded by the thin microtubule-filled configuration as the axons elongate. With light microscopy bundles of regenerating axons can be detected in silver-stained sections by 3–4 days (Grafstein 1971), but it is not clear whether the stain can reveal the initial filament-filled processes. Growth cones of the type commonly observed at the tips of growing axons (Ramon y Cajal, 1928; Bunge, 1973) are not usually seen within the goldfish optic nerve. However, under conditions of greatly accelerated outgrowth, as may occur during regeneration following a series of two lesions (see Section III,A,1) it has been possible to observe prominent terminal structures resembling growth cones in their morphology (particularly their content of smooth endoplasmic reticulum) and in their capacity for pinocytosis (Lanners and Grafstein, 1980b).

2. Axonal Elongation

The advancing axons are initially grouped in small bundles, with the most rapidly extending axons often arranged in isolated pairs, although individual

axons may sometimes be seen penetrating the crush zone in the earliest stages (McQuarrie, 1985). Each bundle of axons is closely surrounded by glial cells and degenerating debris (Murray, 1982). In the course of regeneration, the number of axons in the optic nerve increases considerably above normal (Murray, 1982). At a point 0.5 mm from the site of the nerve crush (on the cranial side of the lesion), nearly three times the normal number of axons is observed at 3–5 weeks after the lesion, and a maximum increase to nearly fourfold normal is seen at 6–12 weeks. This suggests that the regenerating axons undergo multiple branching, especially at the level of the lesion.

Initially, the regenerating axons constitute a very small proportion of the nerve mass. The total protein content of the optic nerve, which decreases by 45% within 1 week of optic nerve crush as the axons degenerate (Perry *et al.*, 1985), only begins to recover after 2 weeks. Some individual axon proteins may continue to decrease until 5 weeks.

Following an initial delay of 4–5 days, the regenerating axons normally advance at a rate of about 0.2–0.4 mm per day (measured at 20°C) (Grafstein and Murray, 1969; McQuarrie and Grafstein, 1981; Edwards *et al.*, 1981; McQuarrie, 1985). The extension of the axons involves the insertion of newly synthesized glycoproteins into the membrane just behind the growth cone (Feldman *et al.*, 1981), as well as the addition of subunits to the cytoskeleton (McQuarrie, 1984). The axons begin to enter the contralateral lobe of the optic tectum, their principal site of synaptic termination, at about 12–18 days after the optic nerve has been crushed a few millimeters behind the eye (Ingoglia *et al.*, 1973; Edwards *et al.*, 1981; Heacock and Agranoff, 1982), or about 10–14 days after the optic tract has been cut (Murray, 1976; Edwards *et al.*, 1981; McQuarrie and Grafstein, 1981). In the subsequent 2 weeks, the axons extend over the whole of the optic tectum (Meyer, 1980; Schmidt *et al.*, 1982).

The regenerative process is very temperature sensitive and when the temperature is raised from 20 to 30°C, the regeneration rate approximately doubles, with some innervation of the tectum seen as early as 7–8 days after optic nerve crush, and coverage of the whole tectum occurring by 12–15 days (Springer and Agranoff, 1977; Springer, 1980; Sbaschnig-Agler *et al.*, 1984). (All values given in the remainder of this article will apply to regeneration at 20°C, unless otherwise indicated.)

3. Growth within the Optic Tectum

As the axons traverse the optic tectum, they progress only about half as fast as in the optic nerve, and their growth cones are readily detectable (Gambetti *et al.*, 1978). Initially, the axons in the tectum, as in the optic nerve and tract, are arrayed in bundles, closely circumscribed by glial cells (Murray, 1976). The formation of these bundles appears to be dominated by axon-to-axon interactions, rather than by confinement of the axons to preformed glial channels

(Edwards and Murray, 1985). Within the tectum profuse axonal branching occurs, resulting in a large increase in the total volume of retinal axons (Murray and Edwards, 1982; Murray, 1976). At its maximum, seen at 3–5 weeks after nerve crush, the total axon volume appears to be about nine times normal (calculation based on data of Murray and Edwards, 1982). The disparity between axon numbers in the normal and regenerating conditions is even greater, about 20-fold, because of the relatively smaller mean size of the regenerated axons. Most of these axons are unmyelinated at this stage, whereas the normal population of axons is almost entirely myelinated. Most of the axons are still unmyelinated at 5 months. The mean diameter of the regenerating axons is about 0.1 μm when they first enter the tectum and increases to about 0.5 μm at 42 days and 0.7 μm at 90 days (Murray, 1976).

4. Synapse Formation and Recovery of Vision

a. Tectal Innervation. Axons begin to form some synapses as soon as they enter the tectum. Terminals may be seen in the rostral pole of the tectum as early as 17 days after optic nerve section (Stuermer and Easter, 1984). Some terminals apparently become functional very rapidly, since postsynaptic potentials evoked by stimulation of the optic nerve may be detectable in the rostral pole by 20 days and throughout the tectum by about 28 days after optic nerve crush (Schmidt *et al.*, 1982). The development of synaptic effectiveness may also be gauged from the recovery of food localization, a behavioral pattern that depends on the optic tectum. At 20°C, this behavior usually has a mean recovery time of about 35–40 days following optic nerve crush (Edwards *et al.*, 1981; Springer and Agranoff, 1977), which presumably indicates that by this time there are not only effective synapses, but some degree of retinotopic order in their arrangement. At about the same time, an orderly retinotopic projection can be discerned by electrophysiological mapping (Schmidt and Edwards, 1983; for additional references see Edwards *et al.*, 1985), in spite of the fact that there appear to be many incorrectly projecting axons (Meyer, 1980; Stuermer and Easter, 1984). Further improvement of the topographical precision of the connections apparently continues for some time thereafter (Meyer, 1980; Edwards *et al.*, 1985). It has been proposed that this progressive refinement is the result of coordinated electrophysiological activity in functionally related groups of retinal ganglion cells (see Section III,B,1). This phase of elimination of inappropriate axonal connections may be associated with a reduction in the number of excess unmyelinated axon profiles (Murray *et al.*, 1982; Murray and Edwards, 1982). Axonal regression, rather than autolytic degeneration, is thought to be responsible for the elimination of the excess branches (Edwards and Murray, 1985).

While the reestablishment of the pattern of retinotopic connections depends on mechanisms impinging on the presynaptic axons, it appears that the postsynaptic

elements determine the final synaptic density. Even if the volume of target tissue available to each retinal axon is decreased by removal of part of the tectum, the final density of synapses in the tectal remnant has been found to be normal (Murray *et al.*, 1982). In the absence of tectal lesions, recovery of normal numbers of retinotectal synapses requires 3 months or more from the time of optic nerve crush (Murray and Edwards, 1982). A similar extended time course has been observed for the recovery of α-bungarotoxin-binding sites in the optic tectum (Schechter *et al.*, 1979). These receptors are thought by some workers to represent nicotinic cholinergic receptors involved in transmission at retinotectal synapses (Freeman *et al.*, 1980), but the view that acetylcholine is the principal transmitter at these synapses is not unanimously accepted (Tumosa and Stell, 1986).

b. Innervation of Nontectal Centers. Some visual reflexes that do not involve the participation of the optic tectum recover earlier than food localization, e.g., a mean of about 12–14 days for the startle reaction to a bright light or shadow and about 20 days for the dorsal light reflex (Springer and Agranoff, 1977; Edwards *et al.*, 1981). The early recovery of these reflexes may indicate that they are mediated by pretectal centers which are innervated earlier than the tectum or that these reflexes do not require a very highly ordered innervation pattern. Some diencephalic centers become innervated later than the tectum (Murray, 1977) in spite of their shorter distance from the site of the lesion, indicating that the time course of regeneration is not identical for all axons. It has been suggested (Edwards *et al.*, 1981) that various populations of retinal ganglion cells may each have a characteristic recovery time before they can become functional following a lesion (see Section III,A,1).

B. Features of the Cell Body Reaction to Axotomy

Chromatolysis, i.e., dispersal of the rough endoplasmic reticulum (Nissl, 1892), which has been considered to be a hallmark of the reaction to axotomy in many neurons (Lieberman, 1971), does not occur in its typical form in goldfish retinal ganglion cells. Instead, the sequence of changes initiated by axotomy in these neurons, whether viewed from a morphological or a biochemical perspective, may be seen as a series of events leading to increased protein synthesis.

1. Morphological Changes

a. Nucleolar Mass. The earliest morphological change thus far recognized in these cells is an increase in the amount of nucleolar material. This may be readily detected, for example, by determining the incidence of nucleoli seen in

the light microscope. Normally, only about 20–40% of the cells can be seen to contain nucleoli, but by 3 days after a lesion of the optic nerve or optic tract, this proportion has risen to over 50% and continues to rise until about 7 days, when nucleoli may be seen in virtually all the cells (Murray and Grafstein, 1969; McQuarrie and Grafstein, 1982b). Even after this, however, the nucleolar mass continues to increase as the individual nucleoli enlarge to about twice their normal size and some cells acquire two, and occasionally three, nucleoli (Murray and Grafstein, 1969).

b. Cell Body Size. Like many axotomized neurons (Lieberman, 1971), the goldfish retinal ganglion cells show enlargement of their cell bodies. In the classical picture, as originally described for mammalian hypoglossal neurons (Brattgard *et al.*, 1957), the axotomized cell enlarges very rapidly, apparently as a result of increased water content. The goldfish retinal ganglion cells show a slower enlargement, which does not become obvious until about 6 days after axonal injury and which represents an increase in cell mass, with concomitant changes in organelle content (Murray and Grafstein, 1969; Murray and Forman, 1971; Whitnall and Grafstein, 1983). There is some evidence that an even earlier increase in size (possibly representing cell swelling) may occur (Marani and Ruigrok, 1984).

c. Cytoplasmic Features. Far from showing chromatolysis, the goldfish retinal ganglion cell manifests increasingly intense Nissl staining over a period of several weeks following axotomy (Murray and Grafstein, 1969). Ultrastructural analysis has revealed that this is due to an increase in polyribosomes. During the first week, the increase is primarily in free polyribosomes, and there may even be a slight reduction in the amount of rough endoplasmic reticulum (RER), although the RER does not show any major disturbance (Whitnall and Grafstein, 1983). Subsequently both the RER and the free polyribosomes increase in amount (Murray and Forman, 1971), but the relative volume of the RER remains constant; i.e., it keeps pace with the increase in cell size, whereas the increase in free polyribosomes is proportionately greater (Whitnall and Grafstein, 1982, 1983). The volume of the Golgi system also increases in tandem with the total volume of the cell. Non-Golgi smooth membranes, on the other hand, show a massive and rapid proliferation, doubling their relative volume within 6 days after axotomy. Somewhat later, large increases in dense-cored vesicles, microtubules and neurofilament bundles are also seen (Murray and Forman, 1971; Whitnall and Grafstein, 1982, 1983). The dense-cored vesicles, which become a prominent feature in the regenerating axons by 10 days after axotomy, reach a maximum at 28 days and have virtually disappeared by 42 days. They may play a role in axon elongation (Murray, 1976).

2. Biochemical Changes

The evidence from the morphological studies showing that injury to the optic axons elicits a massive increase in RNA synthesis (evident from the nucleolar enlargement) followed by an increase in protein synthesis (manifested in an increase in cell size and organelle proliferation) has been confirmed by biochemical determinations. Many of the biochemical changes are large enough to be detectable by measurements made on the whole retina, even though only the retinal ganglion cells have been injured. However, since the ganglion cell changes would be superimposed on the background of total synthesis in all retinal elements and since the retinal ganglion cells constitute a relatively small proportion of the retinal mass, whole retina measurements are not always adequate for evaluating the status of ganglion cell metabolism. Measurements of incorporation of radioactive precursors into retinal ganglion cells by means of autoradiography is usually a better approach, although it is difficult to analyze the behavior of specific molecules this way. Another approach is to examine the axonally transported proteins that appear in the axons of these neurons in the optic nerve (see Section II,C,2).

a. RNA Synthesis and Metabolism of RNA Precursors. An increase in the incorporation of [^{3}H]uridine into RNA in the retinal ganglion cells is detectable as early as 3 days after axotomy by means of autoradiography (Murray, 1973). Incorporation of [^{3}H]uridine into RNA in the whole retina has also been seen to be elevated beginning at 2–3 days (Burrell *et al.*, 1978; Dokas *et al.*, 1981). Peak incorporation occurs at 4–7 days (Murray, 1973; Dokas *et al.*, 1981), when the total retinal content of RNA also reaches a peak (Burrell *et al.*, 1978). This indicates that the increased precursor incorporation is due to a net increase in RNA synthesis rather than an increase in turnover. This increase persists for more than 28 days following optic nerve crush (Murray, 1973; Dokas *et al.*, 1981).

The increased RNA synthesis is precisely correlated with the onset of nucleolar enlargement (see Section II,B,1,a), which presumably reflects an increase in the supply of ribosomal RNA. In confirmation of this, isolation of ribosomal RNA reveals a corresponding time course of labeling, with the increase in the specific activity of labeled RNA in free ribosomes greatly exceeding that in membrane-bound ribosomes (Burrell *et al.*, 1978). Synthesis of cytoplasmic poly(A)-containing RNA, including the messenger RNA (mRNA) fraction coding for tubulin, shows a large increase (Burrell *et al.*, 1979). The increased synthesis of tubulin mRNA has also been demonstrated by the use of labeled cDNA probes (Neumann *et al.*, 1983). There is evidence that in addition to a change in mRNA production, there is an alteration in posttranscriptional processing of mRNA (Burrell *et al.*, 1978).

The increase in RNA synthesis is closely paralleled by an increase in accumulation of acid-soluble RNA precursors (Dokas *et al.*, 1981), which is characteristic of cells undergoing a growth response (reviewed by Plagemann and Richey, 1974; Levine *et al.*, 1974). Peak uptake of nucleoside, which occurs at 4–7 days, has been calculated to reflect a more than 10-fold elevation in nucleotide kinase activity in the ganglion cells, and even by 21 days, this effect still persists at a level of about 30% of its peak value (Dokas *et al.*, 1981). These changes are evidently not due to an increased transport of nucleosides across the cell membrane, since the uptake of a corresponding nonmetabolizable precursor was not altered (Dokas *et al.*, 1981). Rather, the enhanced nucleoside incorporation appears to be due to increased nucleoside phosphorylation (Kohsaka *et al.*, 1981a). This can in turn be attributed to increased synthesis of nucleotide kinases, since the enhancement of nucleoside uptake was blocked by intraocular injection of actinomycin D (Kohsaka *et al.*, 1981a).

b. Ornithine Decarboxylase Activity. Ornithine decarboxylase is the rate-limiting enzyme in the formation of polyamines, which appear to play a role in growth processes that require increased synthesis of macromolecules (Russell, 1980). The activity of this enzyme was found to be significantly enhanced at 3–7 days after a crush of one optic nerve in goldfish, with a maximum level of more than twice normal on day 5 (Kohsaka *et al.*, 1981b). Although these changes correlate well with the time course of changes in RNA synthesis in the axotomized cell (see previous section), inhibition of the enzyme did not have any effect on the morphological or biochemical events in the retinal ganglion cells or on regeneration of their axons (Kohsaka *et al.*, 1982). Moreover, an increase in enzyme activity (though of smaller magnitude) was also seen in the contralateral (unlesioned) retina in fish in which one nerve had been crushed or one eye had been enucleated and in other parts of the nervous system, as well as in the kidney. These changes appeared to be attributable to systemic effects arising from the nerve lesion rather than to surgical stress. It has been suggested that they may be a consequence of degenerative processes in the lesioned nerve (Kohsaka *et al.*, 1982), possibly leading to production of antibodies to nerve constituents, such as gangliosides (Schwartz *et al.*, 1982b).

c. Protein Synthesis. Most of the studies of protein synthesis in the retina depend on measurements of incorporation of radioactively labeled amino acids. An increase in incorporation may not necessarily indicate an increase in net synthesis. It may indicate an increase in the turnover of protein, or a change in the specific activity of the amino acid precursor pool. A study of how protein labeling in goldfish retinal ganglion cells varies with the concentration and specific activity of the applied precursor has revealed that during regeneration a larger proportion of the amino acid incorporated into protein comes from extra-

cellular sources (Whitnall and Grafstein, 1981). A change in the amino acid precursor pool can also be inferred from the increase in the labeling of the axonally transported amino acid fraction, which develops within 24 hr after an optic nerve crush (Grafstein and Alpert, 1976). Subsequently, however, this fraction does not show any marked changes whereas the labeling in the protein fraction continues to increase manyfold. These observations, together with the fact that the goldfish retinal ganglion cells increase in size and organelle content, indicate that the increase in incorporation of labeled amino acid during regeneration represents an increase in protein synthesis.

i. Total protein. Autoradiographic studies on the incorporation of radioactive amino acid into protein in individual ganglion cells have shown that an increase in incorporation begins 4 days after an optic tract lesion (Murray and Grafstein, 1969), i.e., a short time after the onset of the increases in RNA synthesis and accumulation of RNA precursors (see Section II,B,2,a). In the early stages, some of the increased incorporation represents increased synthesis of nucleoprotein in the nucleus and nucleolus (Whitnall and Grafstein, 1983). Peak protein synthesis, representing a three- to fivefold increase above normal, has been reported at 14–20 days (Murray and Grafstein, 1969; McQuarrie and Grafstein, 1982a) and normal levels are not restored for over 60 days (Murray and Grafstein, 1969).

From electron microscopic autoradiography, it is evident that the change in protein synthesis is qualitative as well as quantitative (Whitnall and Grafstein, 1983). For example, although the protein synthesis in both RER and free polysomes was seen to be increased, the proportion of protein synthesized in the RER relative to that in the free polysomes increased from 6 to 30 days. An increased proportion of newly synthesized protein was found to be routed through the Golgi apparatus, and very large amounts of label collected in the smooth membranes, particularly during the first week of regeneration. These changes indicate that, in addition to increased synthesis of cytoskeletal elements, there is a large increase in the synthesis and processing of membranous material that is presumably destined to be conveyed to the axons by fast axonal transport (see Section II,C,2,a). Also, there is a greatly increased turnover of the plasma membrane of the cell body, especially at 2 weeks after axotomy (Whitnall and Grafstein, 1982).

ii. Tubulin and associated proteins. A large proportion of the increased protein synthesis is attributable to the synthesis of tubulin, which occurs mainly on free polysomes (Gilbert *et al.*, 1981), and which begins to increase as early as 5 days after the lesion (Heacock and Agranoff, 1976; Giulian *et al.*, 1980). Measured in the whole retina, a maximum increase of about threefold was observed (Heacock and Agranoff, 1976), although isolated retinal ganglion cells showed a somewhat larger change (Giulian *et al.*, 1980). This increase includes tubulin in both the soluble fraction and the particulate (apparently membrane-

associated) fraction (Heacock and Agranoff, 1982). There are some indications that, at least in the soluble fraction, the β subunit of tubulin is increased more than the α subunit. This has been correlated with the possibility that β-tubulin may have a specific role in neurite extension (Neumann *et al.*, 1983). The increased synthesis of tubulin results from an increase in synthesis of mRNA for tubulin (see Section II,B,2,a).

The increased synthesis of tubulin has been shown to be accompanied by an increase in synthesis of two microtubule-associated proteins of the tau group with a molecular weight of about 70,000 (Neumann *et al.*, 1983).

iii. Other proteins. Increased synthesis of other retinal proteins has been demonstrated (Heacock and Agranoff, 1982), particularly a cluster of soluble proteins of 68,000–70,000 molecular weight and p*I* 4.8–4.9, features that resemble those of one of the subunits of neurofilaments in mammals.

C. Axonal Transport during Regeneration

Regeneration is likely to entail alterations in axonal transport: in addition to materials to be laid down to form the new axon, some transported material is required to compensate for metabolic turnover, which may be increased even in the undamaged portions of the regenerating neurons (Whitnall and Grafstein, 1982, 1983). Axonal transport may also convey various molecules that may play a role in regulation of regeneration (Willard and Skene, 1982) or in communication between the growing axons and contiguous cells (Ingoglia *et al.*, 1982).

Unlike some other neurons in which regeneration proceeds with little change in axonal transport (reviewed by Forman, 1983), goldfish retinal ganglion cells show marked changes in axonal transport during regeneration, including increases in the velocity and amount of both fast and slow transport. This elevation in transport may possibly be related to the fact that the mass of the new axons is much greater than normal, since multiple new branches are produced by each regenerating neuron (see Section II,A,2).

Changes in axonal transport *velocity* that occur during regeneration may reflect alterations in local axonal mechanisms involved in transport. Changes in the *amount* of transported material, on the other hand, may reflect either a change in the capacity of the transport system within the axon or a change in the supply of various materials that are to be conveyed from the cell body to the axon.

A critical point in evaluating the significance of regeneration-associated changes in axonal transport is whether such changes are being measured in the retinal stump—the nerve segment between the cell body and the lesion, which contains the original axon trunks that have remained connected to their cell bodies—or in the cranial stump—the nerve segment between the lesion and the synaptic target, which contains the newly formed portions of the regenerating

axons. Changes in the amount of axonal transport in the retinal stump represent a modification of the supply of materials made available by the cell body to the growing branches, e.g., a change in the synthesis of such materials or in their release into the axon. Measurements of axonal transport in the cranial stump represent an evaluation of the amount of material incorporated into the growing axons. In the goldfish, with optic axons only a few millimeters in length, optic nerve samples taken in experiments involving optic tract lesions will contain the retinal (original) axon segments, whereas in experiments involving optic nerve lesions, the nerve samples will contain the cranial (newly formed) segments, except when special precautions are taken to ensure that measurements are confined to the length of nerve between the lesion and the eye. In either kind of experiments, however, measurements made on the optic tectum will monitor changes in the growing portions of the axons.

1. RNA and Associated Materials

a. RNA. A striking characteristic of regeneration in goldfish optic neurons is the axonal transport of large amounts of 4 S RNA (Ingoglia *et al.*, 1975; Ingoglia, 1982). Although small amounts may be transported in normal axons (Ingoglia *et al.*, 1973), there is an increase of about 20-fold during regeneration, as measured in the optic tectum (Ingoglia, 1982). The material involved is probably transfer RNA (Zanakis *et al.*, 1984), which may be involved in post-translational modification of axonally transported proteins, a function that it appears to serve in the axoplasm of the giant axon of the squid (Ingoglia *et al.*, 1983).

b. RNA Precursors: Axonal Transport. The amount of radioactively labeled RNA precursors (including various nucleosides and nucleotides) that is delivered to the optic tectum by axonal transport is increased during regeneration (Ingoglia *et al.*, 1975, 1982). An average level of about three to four times normal is seen at 24–25 days after optic nerve crush, although this value varies somewhat for different nucleotides (Ingoglia, 1982; Ingoglia *et al.*, 1982). To some extent, this increase undoubtedly reflects an increase in total nucleoside accumulation in the retinal ganglion cells (see Section II,B,2,a); whether there is also a greater diversion of nucleosides to the regenerating axons remains to be determined.

c. RNA Precursors: Transcellular Transfer. Some of the axonally transported RNA precursors are released along the course of the optic axons and at the axon terminals and become incorporated into RNA in the periaxonal cells, including both neurons and glia in the tectum (Ingoglia *et al.*, 1975, 1982; Edwards and Grafstein, 1985). Since the transported material is less readily degraded than comparable material reaching the neurons via the circulation, the

transcellularly transferred material may serve a special function in providing the tectal cells with RNA precursors, particularly during some phases of regeneration (Ingoglia, 1982). It has been suggested, therefore, that this transport might participate in trophic regulation by the axons of adjacent glial cells and postsynaptic neurons (Ingoglia *et al.*, 1982).

d. Polyamines. Polyamines, which play a role in the synthesis of RNA and proteins, also show altered axonal transport during regeneration. In regenerating nerves, there is an increased transport of spermidine (and an increased conversion of spermine to spermidine) and also some transport of putrescine, which appears not to be transported in normal nerve (Ingoglia *et al.*, 1977). Some of the transported polyamines are transferred to surrounding cells. Spermidine is transferred to both neurons and glia, whereas putrescine appears to be transferred only to periaxonal glia and not to radial glia or neurons in the tectum. It has been proposed that polyamines, like nucleosides, may play a role in trophic regulation by axons of periaxonal glia and neurons (Ingoglia *et al.*, 1982).

2. Axonal Transport of Proteins

Axonally transported proteins necessary for reconstitution of the new axon would include membrane components, which are normally conveyed by fast axonal transport and cytoskeletal and cytoplasmic elements, normally associated with slow transport (Grafstein and Forman, 1980). Both types of transport are altered during regeneration of goldfish optic axons.

a. Fast Transport. An increase in the total amount of labeled protein in fast axonal transport may be detected in the intact segment of the optic axons on the retinal side of an optic tract lesion as early as 1–2 days after the lesion (Grafstein and Alpert, 1976; Perry *et al.*, 1986a). This change in transport may precede the increase in protein synthesis in the cell bodies (see Section II,B,2,c) and hence may represent an increased diversion of material into the axon.

The labeling of total transported protein reaches a maximum at 2 weeks, with an increase of 8–25 times normal reported with labeling intervals from 2 to 24 hr (McQuarrie and Grafstein, 1981; Heacock and Agranoff, 1982; Perry *et al.*, 1986a). The velocity of fast transport in the intact segments of the axons is also increased by a factor of about two (Grafstein and Murray, 1969; McQuarrie and Grafstein, 1982a), so that the increase in the rate of delivery of transported materials to the growing axons may be even greater than might be inferred from instantaneous determinations of axonal content of the labeled materials.

Quantitative analyses of the labeling of transported proteins separated by two-dimensional gel electrophoresis have shown that, in the goldfish optic axons, there do not appear to be any new proteins induced during regeneration and that

all proteins containing sufficient radioactivity to be measured in normal nerves show significantly increased labeling within 1 week after axotomy (Perry *et al.*, 1986a). The changes observed in various proteins, however, are different in time course and magnitude.

The largest change thus far reported has been an increase of close to 100-fold in a protein (or group of closely related proteins) with a molecular weight of about 45,000 and a p*I* of about 4.5 (Benowitz *et al.*, 1981; Benowitz and Lewis, 1983; Heacock and Agranoff, 1982; Perry *et al.*, 1985). A significantly increased labeling of this protein (designated *B* in Perry *et al.*, 1985, and no. *4* in Perry *et al.*, 1986a) is already evident in the original segment of the goldfish optic nerve by 2 days after an optic tract lesion. The labeling of this protein reaches its peak at about 2 weeks after the lesion, falls abruptly in the next week, and then declines more slowly, returning to about twice normal by 8 weeks. The increased labeling of this protein, indicating an elevated synthesis, is accompanied by an increase in its abundance in the regenerating segment of nerve (Perry *et al.*, 1985), indicating that it is probably an important constituent of newly formed axons. A similar protein, with 43,000 molecular weight and acidic p*I*, which shows greatly increased labeling during regeneration, has been detected in toad optic nerve (Skene and Willard, 1981a,b). Because it is present in very small amount in normal axons and its synthesis increases so dramatically during regeneration, this protein has been considered to be one of a small group of "growth-associated proteins" (GAPs), the induction of which may be critical for regeneration (Willard and Skene, 1982). In the goldfish optic axons, several other proteins show the same time course of labeling as the 45,000-molecular weight protein, although they do not show as large an increase relative to their normal levels (Perry *et al.*, 1986a). The early induction of changes in this group of proteins, the large magnitude of the changes, and the prominence of these proteins in both the original and regenerating axon segments during axonal outgrowth (Perry *et al.*, 1986a) suggest that they may be characteristic of the smooth endoplasmic reticulum (SER). This would be consistent with the early massive proliferation of smooth membranes in the regenerating neurons (Whitnall and Grafstein, 1983) and with the prominence of SER in growth cones of goldfish optic axons *in vivo* (Gambetti *et al.*, 1978; Lanners and Grafstein, 1980b) and in axonally transported organelles of neurites emerging from goldfish retinal explants (Koenig *et al.*, 1985).

Four other groups of axonally transported proteins showing coordinated changes during regeneration have been recognized, and it is possible that they may each be characteristic of a different type of organelle (Perry *et al.*, 1986a), if it is assumed that most of the proteins in the membrane of each type of organelle are unique. For example, specific groups of proteins may represent constituents associated with synaptic terminals or with plasma membrane. One of the groups of proteins contains the cytoskeletal proteins, α- and β-tubulin and actin. Al-

though these elements are usually assigned to slow transport (see Section II,C,2,b), their initial rate of appearance in the regenerating nerve would imply a much faster maximum transport velocity.

The question of whether there are any axonally transported proteins that may play a specific role in initiating or regulating regeneration (Levine *et al.*, 1981) is still unanswered. The fact that no new proteins appeared during regeneration (as has also been observed in regeneration of frog optic nerve; Szaro *et al.*, 1985) may reflect the fact that new retinal ganglion cells are constantly being added to the retina (Johns and Easter, 1977; Meyer, 1978), and hence, the optic nerve always contains a small proportion of regenerating axons. Nevertheless, the evidence for the presence of specific regulatory proteins is still not strong. In view of the multiple patterns of changes seen in various groups of proteins, it may be necessary to think in terms of the regulation of a series of different events, rather than a single regeneration-initiating mechanism.

b. Slow Transport. As in other systems (Hoffman and Lasek, 1975), slow axonal transport in goldfish optic axons consists of two subcomponents, SCa, which normally advances 0.02 mm/day, and SCb, which normally advances 0.2–0.4 mm/day (McQuarrie, 1984). SCa conveys neurofilament proteins, whereas SCb conveys actin and, unlike mammalian axons, also most of the axonal tubulin (McQuarrie, 1984).

Assessing the changes in the amount of transported material that occur during regeneration is more difficult for slow than for fast transport. A basic problem is that it takes much longer for the transported material to invade the nerve segment that is to be analyzed, so that the measurements cannot be very precisely timed in relation to particular phases of the regeneration cycle. Another problem is that a significant increase in the velocity of SCb is a characteristic of regeneration in goldfish optic axons (Grafstein and Murray, 1969; Grafstein, 1971), although it is not clear that this happens in other systems or species (Forman, 1983).

The velocity of SCb begins to increase at about 6–8 days after axotomy (Grafstein, 1971) and reaches a maximum of about 1 mm/day at 14–17 days (Grafstein and Murray, 1969). [It is not yet known whether all the proteins in this transport component are uniformly altered in velocity. Also, it is possible that there may be no change in the rate at which individual proteins traverse the axon, but a relative increase in the labeling of somewhat more rapidly moving constituents of the transported population (cf. Hoffman and Lasek, 1975).] The change in velocity, whether real or apparent, makes it difficult to determine whether the amount of material transported is independently affected. However, there is some increase in the amount of transported material beginning 1–3 days before the velocity changes (B. Grafstein, unpublished observations), and it seems unlikely that the more than fivefold increase in slow-transport protein labeling

that has been observed in the original segments of the axons at 4–5 weeks after axotomy (Perry *et al.*, 1986c) would be due to the velocity change alone.

Among individual transported proteins, tubulin has received the most attention, since it constitutes a large proportion of both the protein content of the nerve and the transported material (Heacock and Agranoff, 1982; Giulian *et al.*, 1980; Skene and Willard, 1981a). An increase of nearly 100-fold in labeling of tubulin has been observed in the intact segment of nerves sampled at 5 weeks after optic tract lesions with a 1-week labeling interval (Perry *et al.*, 1986c). In consequence of these changes, tubulin comes to represent about 70% of the total slow-transported label in regenerating axons, as compared with 25% in normal axons. Some recovery is seen by 7–8 weeks, but normal levels are not yet restored even at 11–12 weeks.

The labeling of the tubulin that becomes incorporated into the growing segment of the axons is also greatly increased. In 10- and 30-day labeling patterns from the tectum of animals receiving intraocular injections of [^{3}H]proline at 2 weeks after nerve crush, the amount of label in tubulin was 40–50 times normal (Heacock and Agranoff, 1982), but had returned to normal in animals injected at 7 weeks.

Labeling of other slowly transported proteins seen with a 1-week labeling interval (i.e., probably mostly SCb) is also increased, although not as much as for tubulin (Perry *et al.*, 1986c). These proteins include actin and also the ON proteins, which may be related to intermediate filaments (Quitschke and Schechter, 1983, 1984), as well as some other less well-characterized axonal proteins (Perry *et al.*, 1985; Heacock and Agranoff, 1982). It is difficult, however, to differentiate constituents labeled by slow axonal transport from those labeled by local incorporation of molecules released from the axons (Strocchi *et al.*, 1984; Perry *et al.*, 1985). Some proteins that are known to originate at least partially from local synthesis, e.g., the intermediate filament-like proteins ON3 and ON4 (Quitschke and Schechter, 1983; Perry *et al.*, 1985) and W2 and W4, which may be associated with myelin (Perry *et al.*, 1985), show increased labeling with a time course that is not obviously different from that of proteins known to be axonally transported.

The slowest transport component, SCa, has not yet been investigated in detail. Its velocity does not appear to increase during regeneration; the amount of labeling does not increase and may even decrease (McQuarrie and Lasek, 1981; McQuarrie, 1984). A decrease in labeling of the neurofilament fraction of SCa might be associated with a decrease in caliber of the axons between the cell body and the lesion (Hoffman *et al.*, 1984).

c. Retrograde Transport. Retrograde axonal transport conveys both exogenous constituents, which are mainly taken up by endocytosis at the axon

terminal, and endogenous constituents that are initially conveyed to the terminal by anterograde transport.

Retrograde transport in the goldfish optic axons has received relatively little attention, possibly because the short length of the axons makes it difficult to design appropriate quantitative experiments. In the normal nerve, about 40% of the glycoproteins reaching the optic tectum by fast anterograde transport return toward the cell body by retrograde transport with a lag time in the terminals of not more than 1–2 hr (Whitnall *et al.*, 1982). However, the material that enters the retrograde stream may differ in composition from that arriving by the anterograde transport (Bisby, 1984), and different proteins may be conveyed at different retrograde velocities (Williams and Agranoff, 1983).

During regeneration, an increase of about fivefold was observed in the amount of retrogradely transported material (Whitnall *et al.*, 1982). It is not yet clear whether this change is entirely accounted for by the increase in materials arriving via the enhanced anterograde transport.

3. Lipids

Lipids are conveyed by fast axonal transport in association with protein, possibly in the form of an assembled membrane structure (Grafstein *et al.*, 1975; Currie *et al.*, 1976). Unlike the protein, however, the transported lipid apparently originates from a large presynthesized pool and is therefore relatively slowly released from the cell body.

The transport of lipids is increased during regeneration. For example, the amount of [^{3}H]mannosamine-containing gangliosides transported to the optic tectum in regenerating axons was found to be increased to more than twice normal (Sbaschnig-Agler *et al.*, 1984). For glycerophospholipids, the increase was even larger (Sbaschnig-Agler *et al.*, 1984), particularly in the case of the serine- and inositol-glycerophospholipids, which normally contain a relatively small proportion of the total transported phospholipid label. During regeneration, they underwent a 20-fold increase in labeling in the tectum (Sbaschnig-Agler *et al.*, 1984, 1985). Corresponding determinations in the optic nerves (cranial stump, containing the newly formed portions of the regenerating axons) showed even larger changes than in the tectum. These changes, moreover, were consistently larger than the changes in glycoproteins under the same conditions (Sbaschnig-Agler *et al.*, 1984), which may be taken to support the hypothesis that the proportion of lipids to proteins in the membrane of growing axons is higher than in mature axons (Small *et al.*, 1984).

Axonal transport of cholesterol shows about a fourfold increase in amount during regeneration, with no change in rate of accumulation (Heacock *et al.*, 1984).

III. Conditions That Modify Axonal Outgrowth

Among the various mechanisms that might regulate axonal outgrowth, some are indigenous to the nerve cell (including both cell body and axon), and others arise in the neuronal environment. Neuronal mechanisms that may regulate outgrowth include the following:

Synthesis of appropriate molecules. In addition to constituents necessary for reconstitution of the axon, regeneration may require the production of specific regulatory components, such as the postulated "growth-associated proteins" (Willard and Skene, 1982).

Axonal transport. The delivery of the requisite regulatory and structural materials to the site of growth is presumably mediated by axonal transport. The close correspondence between the rate of axonal outgrowth and the velocity of one component of slow axonal transport (SCb) in a number of regenerating systems has led to the suggestion that slow axonal transport of the cytoskeleton may be the primary rate-limiting process in axonal elongation (Lasek *et al.*, 1981; Wujek and Lasek, 1983). Fast transport must also play a role, however, at least to the extent of providing new membrane components, which are inserted not only at the axon tip, but all along the regenerating segment of axon (Griffin *et al.*, 1981; Tessler *et al.*, 1981), and even in the cell body (Whitnall and Grafstein, 1982).

Disposition of the available materials. Axonal elongation involves a continual reorganization of the tip of the axon, which contains its own mechanism of locomotion (Shaw and Bray, 1977). As long as the growth cone remains in continuity with the axon trunk, its advance depends on the insertion of new membrane elements into the axon immediately behind the tip (Feldman *et al.*, 1981), as well as on the assembly of microtubules (Daniels, 1972; Yamada *et al.*, 1970), a process that can apparently also be regulated by conditions at the site of growth (Letourneau, 1982; McQuarrie, 1983).

Environmental mechanisms of outgrowth regulation may include the following:

Surface contact relations. Contact guidance (Weiss, 1934) by other cells in the environment, including adjacent axons, is important in determining the rate of axonal outgrowth, as is evident, for example, in the acceleration of outgrowth that occurs on longitudinally oriented substrates. Another influence is the adhesivity of the substrate, which may affect the frequency of axonal branching, as well as the rate of elongation (Letourneau, 1975), and which may be determined by cell-surface glycoproteins (Fraser *et al.*, 1984). Finally, the presence of specific growth-directing surface components may be assumed (Sperry, 1963).

Diffusible agents. The nonneuronal cells in the vicinity of a growing axon are

likely to be the source of various factors that have either a *trophic* effect, promoting regeneration by stimulating neuronal metabolism, or a *tropic* effect, modifying the direction of outgrowth. An example of an agent that is apparently capable of both these kinds of effects is nerve growth factor (Gundersen and Barrett, 1979; Thoenen and Barde, 1980).

Electrical fields. A weak electrical field applied to neurites in tissue culture can accelerate their growth (Patel and Poo, 1982), possibly by affecting accumulation of axonally transported proteins at the axon tip (Mayes and Freeman, 1984). The presence of inward Ca^{2+} currents of considerable magnitude at the tips of regenerating axons (Freeman, 1985) may play a significant role in nerve growth.

The degree to which these various factors might contribute to goldfish optic nerve regeneration is not completely clear, but identification of some of the factors indigenous to the goldfish retinal ganglion cell that can modify outgrowth has come from studies on the effects of a "conditioning lesion" (which accelerates axonal outgrowth), the effects of abolishing electrophysiological activity (which interferes with outgrowth), and application of various biologically active materials. In addition, there is a growing body of evidence for significant influences exerted by nonneuronal elements in the optic nerve and by tectal cells.

A. Conditioning Lesion Effects

Regeneration in response to a lesion of the goldfish optic axons can be accelerated by a previous ("conditioning") lesion. It is evident, therefore, that the rate of outgrowth normally observed during regeneration is not the maximum that these neurons are capable of. This experimental paradigm offers us the opportunity to investigate some of the factors that may normally limit outgrowth.

1. Changes in Axonal Outgrowth

As a result of the conditioning lesion effect, the initial delay before the onset of axonal sprouting is reduced, and the rate of axonal elongation is accelerated (McQuarrie and Grafstein, 1981). The magnitude of the effect is related to the interval between the standard "testing" lesion (from which the rate of regeneration is measured) and the prior conditioning lesion. The minimum effective interval is between 2 and 7 days. The optimum interval, which has been determined to be 14 days (Edwards *et al.*, 1981), was found to reduce the initial delay by about half (i.e., from 4.3 to 2.5 days) and to double the rate of axonal elongation, from 0.34 to 0.74 mm/day (McQuarrie and Grafstein, 1981). Under conditions of accelerated growth, the initial axon sprouts are very thin and, unlike normally regenerating axons, have prominent growth cones that show

morphological features associated with vigorous pinocytosis (Lanners and Grafstein, 1980b). The time required for the axons to reach the optic tectum is shortened (McQuarrie and Grafstein, 1981), but the time between the arrival of the axons in the tectum and the onset of cell recovery is not reduced (J. Reich and D. Burmeister, unpublished observations), which suggests that the establishment of functional synapses in the tectum may not be accelerated by the conditioning lesion. Also, the time required for recovery of tectally mediated visual reflexes, as exemplified by the food localization response, is not reduced (Edwards *et al.*, 1981). One interpretation of this finding is that the reestablishment of synaptic function may require a constant period of recovery after each lesion. [The recovery of some visual reflexes that do not involve the tectum is accelerated in accordance with the faster axon outgrowth (Forman *et al.*, 1981; Edwards *et al.*, 1981). Possibly, the duration of the reaction to axotomy is shorter in populations of retinal ganglion cells involved in reflexes that recover more rapidly.]

2. Metabolic Alterations Associated with the Conditioning Lesion Effect

The enhanced axon outgrowth produced by the conditioning lesion is accompanied by increases in protein synthesis and in both fast and slow axonal transport of proteins. Although the cell body does not enlarge significantly beyond the maximum reached during normal regeneration, the rate of incorporation of labeled amino acids into the cell body is increased. Between 1 and 8 days after a testing lesion preceded by a conditioning lesion 14 days earlier, incorporation per ganglion cell increases to nearly twice that normally seen during regeneration (McQuarrie and Grafstein, 1982b). The fast axonal transport of labeled proteins increases within 1 day after the testing lesion by about 70% over the level at the time of the lesion (this increase in transport, which is seen before the synthesis has increased, presumably represents an increased diversion of material into the axon, as also occurs immediately following the initial axotomy; see Sections II,B,2,c and IV,A,2). By 8 days after the testing (second) lesion, transport has decreased again to the level seen at 8 days after a single lesion, and for at least 2 weeks thereafter, it follows the sequence of changes seen at the same intervals following a single lesion. This has led to the view that fast transport is unlikely to be a critical factor in the increased rate of elongation, which appears to be maintained even when fast transport has returned to the level seen with a single lesion. Slow transport, which has been less intensively investigated than fast transport, also shows an initial rapid two- to threefold increase in labeling following the second lesion, but remains elevated for at least a week thereafter (McQuarrie and Grafstein, 1982b). The velocity of slow transport is also increased beyond the elevated values seen during normal regeneration to reach values of up to 2 mm/day (McQuarrie, 1984). It has been suggested that the

increase in slow transport velocity, as well as the increase in transport amount, contributes to the enhanced rate of axonal elongation seen with a conditioning lesion (McQuarrie, 1984).

3. Conclusions from Experiments with Conditioning Lesions

The results of the conditioning lesion experiments represent a strong argument in favor of the view that the reaction of the cell body to axotomy is a direct determinant of the capacity for axonal outgrowth (Grafstein and McQuarrie, 1978). Many of the experiments on the goldfish optic axons and in other systems have been designed to eliminate the influence of local events at the site of the conditioning lesion, e.g., by placing this lesion some distance from the site of the testing lesion where the rate of outgrowth will eventually be determined. Thus, although there are some indications that axonal outgrowth may be accelerated if it occurs along a predegenerated pathway (Bisby, 1984), this does not fully account for the conditioning lesion effect. The basis for this effect, therefore, seems to be that the conditioning lesion alters the metabolism of the cell body, which is then able to respond more vigorously to the testing lesion.

The conclusion that the alteration in slow axonal transport ultimately determines the outgrowth rate is in line with evidence in other systems (reviewed by McQuarrie, 1984; Wujek and Lasek, 1983), showing that the rate of axonal elongation is proportional to the velocity of SCb, the component of slow transport that is responsible for conveying actin and much of the tubulin in regenerating axons (Hoffman and Lasek, 1975, 1980; McQuarrie, 1983). Nevertheless, the velocity of this component in the regenerating goldfish optic axons, as in some other systems, appears to be significantly faster than the elongation rate under equivalent circumstances. This suggests that these two processes may be regulated by the same underlying mechanism, even if they are not causally related.

B. Inhibition of Electrophysiological Activity by Application of Tetrodotoxin

In ontogenetic development, the importance of physiological activity has been unequivocally demonstrated. Although the basic patterns of neuronal organization may be established by a developmental timetable that is independent of functional activity, many interneuronal connections may break down or fail to develop completely in the absence of such activity (Jacobson, 1978, pp. 256–270, 422–433). Whether physiological activity also plays a significant role in regeneration is a relatively unexplored question. The regenerating optic nerve

lends itself particularly well to the investigation of this issue, since physiological activity can be relatively easily modified in the visual system. For example, physiological activity can be abolished for a controlled period of time by injecting tetrodotoxin (TTX) into the eye. In the goldfish, a single intraocular injection abolishes activity in the optic nerve for 2–3 days, and repeated injections can be used to keep activity blocked for several weeks (Edwards and Grafstein, 1983).

In normal nerves, TTX treatment has no discernible effect on either fast or slow axonal transport of proteins labeled with radioactive amino acids (Edwards and Grafstein, 1984; Grafstein *et al.*, 1981; Grafstein and Alpert, 1982; Grafstein and Edwards, 1982). However, the amount of transported glucosamine-labeled glycoproteins and, even more, glycolipids, is reduced, as is their rate of turnover in the optic axon terminals (Edwards and Grafstein, 1984). There is also a decrease in the axonal transport of nucleosides and nucleotides and in the transcellular transfer of these materials into tectal cells (Grafstein and Edwards, 1982; Edwards and Grafstein, 1986).

1. Effects of TTX on Regeneration

Intraocular TTX treatment interferes with goldfish optic nerve regeneration and probably in more than one way. One important long-term consequence of TTX treatment during regeneration is an abnormal organization of the terminal arbors of the optic axons (Schmidt and Edwards, 1983). This is evident from an increase in the size of multiunit visual receptive fields recorded in the tectum, which lasts for at least several months after the TTX treatment is discontinued. This effect was not observed if the TTX was injected during the early stages of regeneration, i.e., the first 2 weeks after a nerve crush, but was elicited if the TTX injections were confined to the 20- to 34-day period (Schmidt and Edwards, 1983; Schmidt and Eisele, 1983). Enlarged multiunit receptive fields have also been seen in fish that were maintained in either constant darkness or flashing light throughout regeneration (Schmidt and Eisele, 1983). A current hypothesis is that, if activity is abnormal during regeneration, the terminal arbors of the regenerating axons fail to undergo a final degree of refinement, which is normally produced by patterned physiological input (Schmidt and Edwards, 1982; Meyer, 1983). It has been suggested that this refinement may be a function of competition for synaptic space among the regenerating axons, in which asynchronously active retinal ganglion cells are at a disadvantage (Schmidt, 1982). The mechanism is apparently the same as that involved in formation of alternating bands of innervation from different eyes projecting to a single optic tectum, which appears to depend on some feedback from the activity of postsynaptic neurons in the tectum (Constantine-Paton and Law, 1982; Schmidt, 1982).

Although TTX treatment early in regeneration does not alter receptive field properties, it does affect other aspects of regeneration. One consequence is a

reduction in either the size or number of regenerating axons in the optic nerve, although the maximum rate of elongation is unaffected (Edwards and Grafstein, 1983). This deficit is presumably the basis for the increased latency of evoked electrophysiological activity in the optic tectum of the TTX-treated animals (Schmidt *et al.*, 1982) and also for the delayed recovery of behavioral responses, which is observed with either late or early TTX treatment (Edwards and Grafstein, 1983; Sparrow *et al.*, 1986).

2. Metabolic Effects

TTX treatment during regeneration had significant effects on axonal transport. For example, it reduced the amount of [^{3}H]proline-containing protein conveyed by fast axonal transport (Sparrow *et al.*, 1986), which was surprising in view of the fact that transport of this material in normal nerve is completely unaffected by TTX (Edwards and Grafstein, 1984). In the original segment of the regenerating nerves, the decrease in transport due to TTX treatment begun at the time of optic nerve crush became evident between 5 and 8 days after the lesion, and with maintained treatment, a maximum transport deficit of about 30–50% was seen, lasting at least 28 days (Sparrow *et al.*, 1986). There was no difference in the magnitude of the decrease produced by TTX treatment confined to either 0–13 days after the lesion or 20–33 days after the lesion, and in each case, the changes were no longer evident by 15–20 days after cessation of treatment. Incorporation of [^{3}H]glucosamine into axonally transported glycoproteins and glycolipids, which is reduced in normal nerves as a consequence of TTX treatment (Edwards and Grafstein, 1984), was also reduced in the regenerating nerves to about the same degree (Sparrow *et al.*, 1986). These deficits in protein and glycoprotein transport may explain the defects of axonal outgrowth and recovery of vision, which are produced by either early or delayed TTX treatment. Moreover, some recent experiments in which individual axonally transported proteins were examined by two-dimensional gel electrophoresis indicate that the delayed TTX treatment produces a relatively greater reduction in the synthesis of certain glycoproteins (E. Antonian, unpublished results). This suggests that altered transport of glycoproteins may be involved in the production of the long-term electrophysiological abnormalities that are caused by TTX treatment confined to the late period of regeneration.

3. Conclusions from Experiments with Inhibition of Physiological Activity

The TTX treatment appears to interfere with two separate events in regeneration, one occurring soon after the nerve lesion and the other during innervation of the tectum. At least the first of these events appears to involve a reduction in the

number or diameter of the regenerating axon branches in the optic nerve. This might also be associated with defective myelination, since the number of axons reaching the critical size for myelin formation (Berthold *et al.*, 1983) would be reduced.

The defect produced by TTX treatment during innervation of the tectum may likewise be attributable to a reduction in axonal branching, particularly within the optic tectum (Murray and Edwards, 1982). This might interfere with the refinement of the retinotectal connections by reducing the opportunity for competitive interactions among the regenerating axons, which are thought to be the basis for elimination of incorrect connections (see Section III,B,1). Reduction of the population of regenerating axons by other means has been shown to reduce the precision of the retinotectal map (Udin and Gaze, 1983).

A basic problem is how the effects of TTX on axonal outgrowth are related to its known properties in blocking physiological activity. It is to be expected that intraocular injection of TTX will block Na^+ channels mediating action potentials in the intraocular portion of the retinal ganglion cell axons, so that activity is eliminated from the whole axon, including the terminals. In addition, it is likely that the cell body and dendrites of the axotomized cells would develop electrogenic patches of membrane (Kuno and Llinas, 1970) that would presumably be blocked by the TTX. Thus the outgrowth-limiting effect of TTX might be due to an alteration in either the cell body or axon. In either case, the elimination of action potentials would result in a change in Na^+ fluxes, which might have a significant effect on axonal outgrowth by altering Na^+-linked processes, such as phosphorylation of proteins or membrane transport of organic solutes (Siegel *et al.*, 1981). Another possibility is that the elimination of action potentials may interfere with Ca^{2+} entry into the growing axon tip, since action potentials at the growth cone involve inward Ca^{2+} current (e.g., Spitzer, 1979). It has been postulated that such Ca^{2+} currents might have a direct effect on outgrowth (Llinas and Sugimori, 1979), although it has recently been found that Ca^{2+} influx is not essential for neurite elongation (Bixby and Spitzer, 1984).

A third possibility is that, since action potentials in the cell body of regenerating (as well as developing) neurons may involve Ca^{2+} (for references see Edwards and Grafstein, 1983), TTX treatment might affect cell body Ca^{2+} content. This could influence the passage of materials through the Golgi system and hence their availability for fast axonal transport (Hammerschlag *et al.*, 1982). This mechanism would explain the observation that TTX interferes with glycolipid and glycoprotein transport (Edwards and Grafstein, 1984). The fact that the Ca^{2+}-mediated action potentials are more prominent in the cell bodies of growing neurons than mature ones would presumably account for the greater efficacy of TTX on axonal transport in regenerating goldfish retinal ganglion cells, compared to normal ones. Although the most detailed observations of transport changes in regenerating neurons concern glycoproteins, it is possible that

changes in glycolipid transport may be at least as significant. The availability of gangliosides, in particular, might be an important determinant of the degree of branching of regenerating axons (see Section III,C,3,a).

C. *Application of Other Biologically Active Materials*

Various drugs have been applied to the regenerating goldfish visual system in an attempt to analyze the critical mechanisms involved in regeneration, as well as to assay various materials that might have a growth-promoting effect (Grafstein *et al.*, 1983). One of the advantages of the visual system in such studies is the possibility of testing the effects of various agents separately on the cell body (by intraocular injection) or at the site of the lesion (by local application). Because the goldfish optic nerve is only a few millimeters long, however, appropriate caution must be exercised to ascertain that the action of material applied by intraocular injection is confined to the cell body. This is especially critical in the case of an optic nerve lesion, which is quite close to the eye.

1. Inhibitors of Metabolism

a. Cycloheximide. Retinal protein synthesis can be blocked by intraocular injection of cycloheximide. A dose of cycloheximide sufficient to abolish the production of materials for fast axonal transport also arrests the advance of previously synthesized materials conveyed by slow axonal transport (Grafstein and Alpert, 1982). When this treatment was instituted at the time of optic nerve crush or within 2 days before or after the lesion, elongation of the axons proceeded at an apparently normal rate for 8 days after the lesion and then ceased (Sparrow *et al.*, 1985; C. M. McGuinness and B. Grafstein, unpublished results). Labeling of axonally transported protein in the regenerating axons was greatly reduced as a result of the cycloheximide treatment, although it was still about twice that seen in normal axons. Protein synthesis in the glial cells of the optic nerve, on the other hand, was not significantly affected. These results show that the increase in synthesis of axonally transported proteins that occurs during regeneration is not the limiting factor for axonal elongation during the early stage of regeneration, but that it becomes essential for outgrowth after about a week.

b. Monensin. Monensin, which interferes with glycosylation mechanisms, blocks fast axonal transport of proteins and lipids by preventing the newly synthesized material from gaining access to the fast transport system at the level of the Golgi apparatus (Hammerschlag *et al.*, 1982). Upon intraocular injection in the goldfish, monensin produces a long-lasting but reversible block of fast transport. In addition, either as a direct consequence of the monensin action or as

a result of inhibiting a fast-transported constituent required for maintenance of slow transport (Grafstein and Alpert, 1982), the drug causes a reduction in the amount of slowly transported protein, although with no change in transport velocity (J. Sparrow, unpublished results). With axonal transport thus reduced, axonal outgrowth following an optic nerve crush proceeded at a normal rate for about 8 days, but then was essentially completely arrested (Sparrow *et al.*, 1985; J. R. Sparrow and B. Grafstein, unpublished results). Even when outgrowth was accelerated by a conditioning lesion, presumably requiring a greater supply of transported material, axonal elongation was little impaired by the monensin treatment for at least 5 days after the lesion. These results, like those obtained with a block of protein synthesis by cycloheximide (see above), indicate that axonal outgrowth can be supported initially by the reserves of material in the axotomized neuron, but that a supply of newly synthesized materials eventually becomes necessary.

c. Tunicamycin. Tunicamycin inhibits glycosylation of proteins and lipids by blocking the assembly of the oligosaccharide core prior to its attachment to macromolecules (Heacock, 1982). It uniformly decreases the amount of carbohydrate incorporated into virtually all retinal glycoproteins, without grossly altering their pattern of synthesis (Heacock, 1982). Upon intraocular injection it blocks fast axonal transport to some extent, but more slowly and not as effectively as monensin (J. R. Sparrow, unpublished results). When tested on explants of goldfish retina *in vitro,* it was found to inhibit neurite outgrowth reversibly, and those neurites that were formed were deficient in glycoprotein (Heacock, 1982).

d. Diazacholesterol. Intraocular injection of 20,25-diazacholesterol, which eliminated both cholesterol synthesis in the retina and axonal transport of cholesterol, left the rate of axonal outgrowth *in vivo* unaffected (Heacock *et al.*, 1984). Axonal transport of proteins and glycoproteins was also unimpaired. However, inhibition of cholesterol synthesis in retinal explants by adding diazacholesterol to the culture medium inhibited neurite outgrowth. These findings suggest that cholesterol is essential for axonal outgrowth, but that cholesterol derived from sources other than axonal transport is available for regeneration *in vivo.*

2. Nerve Growth Factor

Nerve growth factor (NGF) applied by intraocular injection has been found to enhance axonal outgrowth following a crush of goldfish optic nerve (Yip and Grafstein, 1982; Grafstein *et al.*, 1983). NGF pretreatment *in vivo* is also effective in stimulating subsequent neurite outgrowth from goldfish retinal explants *in vitro* (Turner *et al.*, 1980). Moreover, the outgrowth *in vitro* is inhibited by anti-NGF (Turner *et al.*, 1981).

In vivo, the intraocularly injected NGF is effective only if it is given at the time of nerve crush, and direct application of NGF to the lesion site is also effective (Yip and Grafstein, 1982; Yip and Johnson, 1983). The NGF appears to be taken up into the goldfish optic axons at the site of the lesion (with intraocular injection, it probably reaches the lesion via the cerebrospinal fluid circulation in the optic nerve), and the NGF is then retrogradely transported to the retinal ganglion cell bodies, where it presumably exerts its effect. The uptake of NGF into injured goldfish optic axons is not dependent on the presence of NGF receptors in the membrane, and there is no specific NGF transport system (Yip and Johnson, 1983).

Although it has been reported that the NGF treatment may elicit some changes in the cell bodies of the goldfish retinal ganglion cells (Turner *et al.*, 1980), it has been found that the enhanced regeneration is not necessarily accompanied by hypertrophy of these cells beyond that normally observed during regeneration (Yip and Grafstein, 1982). This raises the possibility that the amount of material normally available for reconstruction of the axon during regeneration may be adequate to support the faster than normal rate of axonal elongation that is elicited by NGF treatment.

3. Gangliosides

Gangliosides constitute a considerable proportion of the lipids of the neuronal plasma membrane and are believed to play a significant role in axonal outgrowth, since it has been shown in a number of different systems that gangliosides can support neuronal survival and differentiation and also promote regeneration (reviewed by Ledeen, 1984). For example, gangliosides applied to the site of a lesion in a mammalian nerve may increase the number of axon branches produced, even though the maximum rate of axonal elongation is not enhanced (Sparrow and Grafstein, 1982).

a. Application of Gangliosides. Application of exogenous gangliosides has been found to enhance axonal outgrowth from goldfish retinal ganglion cells *in vivo.* Following a crush of the optic nerve, the rate of regeneration, as monitored by the rate of recovery of vision, was accelerated by treatment with small doses of gangliosides given either by intraocular injection or by direct application to the lesion site (Grafstein *et al.*, 1983). However, larger doses of ganglioside by either method of application were either deleterious to regeneration or had no effect. It is interesting that gangliosides have also been found to have a concentration-dependent bimodal effect in activation of Na^+,K^+-ATPase (Leon *et al.*, 1981). The reason for this bimodal action of gangliosides is not yet understood. One proposed mechanism of action is that the gangliosides may promote influx into the neuron of Ca^{2+} (Gorio *et al.*, 1983), which in small amounts may

promote regeneration (see Section III,C,4), but in large amounts may disturb the axoplasm (Schlaepfer and Zimmerman, 1984). Another possibility is that an increase in concentration of applied ganglioside may change its membrane-binding properties (Leon *et al.*, 1981).

It is not yet clear whether, as in the case of NGF, gangliosides are effective only at the site of the lesion, regardless of whether they are applied by either the intraocular or local route. It has been suggested (Gorio *et al.*, 1983, 1984) that gangliosides might have separate effects on metabolic processes in the cell body and on sprouting events in the axon.

b. Anti-Gangliosides. As might be predicted from the regeneration-promoting effects of exogenous gangliosides, antibodies to gangliosides interfere with outgrowth of goldfish optic axons *in vivo* following optic nerve crush (Sparrow *et al.*, 1984). Outgrowth from retinal explants *in vitro* was also inhibited by the anti-gangliosides, both when they were added to the culture medium (Spirman *et al.*, 1982) and when they were applied to the retina by intraocular injection before the explants were made (Spirman *et al.*, 1984). Antibodies to the GM1 ganglioside were the most effective (Spirman *et al.*, 1984; Sparrow *et al.*, 1984).

Here again, it is not clear whether the action is on the axon or the cell body. *In vivo,* a series of antibody treatments beginning close to the time of the lesion were not more effective than those beginning a few days later, suggesting that axonal elongation was impaired but the initial sprouting was probably not interfered with (Sparrow *et al.*, 1984). Intraocular injection of anti-ganglioside reduced retinal protein synthesis following optic nerve crush (Spirman *et al.*, 1984), although it increased axonal transport of glycoproteins and glycolipids in normal goldfish optic nerve (Sparrow *et al.*, 1984). These results suggest that the antibody can have an effect on synthetic mechanisms in the cell body (perhaps indirectly, via a feedback from ganglioside receptors on the plasma membrane). However, transport of glycosylated materials in the regenerating neurons was not significantly altered by the anti-ganglioside treatment (Sparrow *et al.*, 1984). It is clear, therefore, that the anti-ganglioside effect does not involve a gross inhibition of axonal transport of glycosylated materials, like that produced, for example, by monensin (see Section III,C,1,b).

4. Calcium Modulators

A possible role for calcium in goldfish optic nerve regeneration is evident from the observation that the ionophore A-23187, which permits free movement of calcium and other divalent ions across the cell membrane, decreased the time required following optic nerve crush for recovery of a startle reaction to a bright light (Grafstein *et al.*, 1983; Meiri and Grafstein, 1984). The ionophore was effective upon either intraocular injection or local application to the lesion site.

Local application of DMSO had a similar effect, and the effect of either agent was abolished by the simultaneous application of a calcium-chelating drug, suggesting that an increase in intracellular calcium was probably involved in both cases.

The mechanism by which the regeneration-promoting effects of the calcium modulating agents were produced is not known. Undoubtedly, regulation of intracellular calcium ions at the tips of developing and regenerating axons is important in neuronal growth and differentiation (for references see Meiri and Grafstein, 1984). Also, Ca^{2+} fluxes in the cell body are likely to affect the availability of axonally transported materials processed in the Golgi system (Hammerschlag *et al.*, 1982). However, the basis for the faster recovery produced by calcium-modulating agents applied to goldfish retinal ganglion cells is not clear, since there was no obvious increase in the rate of axonal outgrowth or delivery of axonally transported materials (Grafstein *et al.*, 1983; Meiri and Grafstein, 1984; H. Meiri, unpublished observations). Local application of these agents to the nerve might have affected primarily the nonneuronal elements in the nerve. This may be the reason for the increased packing density of the regenerating axons that was observed (Meiri and Grafstein, 1984), which might conceivably be sufficient eventually to cause a more efficient restoration of the synaptic pattern.

5. Cyclic Nucleotides

Cyclic AMP has been found to promote neurite formation in at least some types of nerve cells (for references, see McQuarrie and Grafstein, 1983). Also, forskolin, an activator of adenylate cyclase, has been found to promote regeneration of sensory axons in frog sciatic nerve (Kilmer and Carlsen, 1984), possibly via an action on the cell body. In the goldfish, intraocular injection of forskolin sufficient to produce a concentration within the eye of 10^{-8} *M* enhanced axonal outgrowth in the optic nerve (J. R. Sparrow, unpublished results), although it did not produce any alteration in fast axonal transport. However, 10- and 100-fold higher concentrations reduced both outgrowth and fast transport. This would be consistent with the finding that repeated intraocular injection of dibutyryl cyclic AMP on days 6–8 after an optic nerve crush inhibited axonal outgrowth (McQuarrie and Grafstein, 1983).

6. Conclusions from Experiments with Local Application of Biologically Active Materials

A number of agents, including NGF, gangliosides, a calcium ionophore, and an activator of adenylate cyclase, have been found to have some effect in pro-

moting regeneration. Most of these were effective both when they were applied to the retina and when they were applied to the site of the lesion, but, because of the limitations of the system, it cannot be assumed that they were capable of acting at both sites. Another complication was that some of the drugs ceased to be effective, or even reversed their effect, as the dose was increased. In some cases, the mechanism of action of the drug may be bimodal with respect to its effects on regeneration; another possibility is that the mechanism may be different at different sites on the neuron.

Nevertheless, these experiments, particularly those involving drugs that block metabolism, have led to the establishment of some general principles relating to the role of the cell body in regeneration:

1. The neuron normally contains sufficient material to sustain axonal outgrowth for about a week after the synthesis of new proteins has been arrested.
2. To maintain axonal outgrowth beyond the initial 1-week period, a level of protein synthesis considerably in excess of normal is required.
3. The metabolic changes that normally occur during regeneration are sufficient to sustain a faster rate of axonal outgrowth than is normally seen.

D. Factors in Extraretinal Tissues That May Influence Regeneration

There are now many indications that the normally vigorous regeneration of the goldfish optic axons may depend to some extent on factors other than local conditions within the axon or the metabolic status of the retinal ganglion cells. For example, it is possible that regeneration may be influenced by soluble factors released by the glial cells (or other elements in the optic pathway) into the extracellular environment.

1. Changes in Metabolic Activity of Nonneuronal Cells

The changing activity of nonneuronal cells during regeneration may affect axonal outgrowth. Cellular proliferation along the course of the optic axons (Giulian, 1978) and protein synthesis by these cells (Maxwell and Elam, 1980) increase in two phases, with maximum rates of change at 1–5 days and 10–14 days. Although it has been shown that glial cell metabolism may be stimulated by contact with regenerating axons (Bunge, 1983), both phases of increased synthesis are seen in the goldfish optic tract even in the absence of axonal regeneration (Maxwell and Elam, 1980). In the optic tectum, two corresponding phases of glial cell proliferation are seen in the layers containing the trunks of the optic axons (stratum opticum) and their principal site of termination (stratum

fibrosum et griseum superficiale, SFGS) (Stevenson and Yoon, 1978). Proliferation of the deeper-lying radial glia, on the other hand, occurs later and is dependent on the arrival of the regenerating axons (Stevenson and Yoon, 1978).

In addition to the changes in nonneuronal cell activity that occur independently of axonal regeneration, there are likely to be changes in the glial cells related to myelination, which begins very early (some axons in the optic nerve already show thin myelin sheaths at 1 week after nerve crush), and continues for up to 3–9 months (Murray, 1982). Most myelination occurs after the axons reach layer SFGS and may be associated with synaptogenesis, since the myelin appears to advance from the axon terminals toward the eye (Murray, 1976; Wolburg, 1978).

Axonal regeneration has been seen to be modified when the proliferation of the nonneuronal cells was abolished by X-irradiation. Increased outgrowth ability of the retina (tested *in vitro*) was seen with irradiation at 7 days after optic nerve crush, whereas irradiation at 10 days impaired the capacity for outgrowth *in vitro* and *in vivo* (Neuman *et al.*, 1983). The 10-day irradiation did not interfere qualitatively with the changes in protein synthesis (particularly increased tubulin synthesis) that characteristically occur in the retina during regeneration, but did reduce protein synthesis as well as cellular proliferation along the route of the optic axons (Neuman *et al.*, 1983). These experiments have been interpreted as indicating that axonal regeneration is affected by two phases of nonneuronal cell proliferation: the first, presumably at the site of injury, interferes with axonal elongation; the second, distal to the lesion zone, promotes regeneration, possibly by providing an appropriate physical substrate or trophic factors.

2. Extracellular Factors Released by Nonneuronal Cells during Regeneration

A number of specific proteins (probably glycoproteins) have been found to be synthesized by the cells of the goldfish optic nerve and released into the extracellular space beginning as early as 3–5.5 hr after nerve crush (Deaton and Freeman, 1983). These findings may be related to the demonstration that after nerve injury mammalian glial cells begin to release an acidic 37-kDa soluble protein which is also seen during development (Skene and Shooter, 1983; Politis *et al.*, 1983; Snipes and Freeman, 1984). At least one of the proteins originating from the cells of the goldfish nerve (with a molecular weight of 30,000) binds to a specific receptor on axonal membranes, suggesting that it might interact with injured axons and influence their growth. A few other polypeptides that show increased synthesis during regeneration, and several that show decreased synthesis, have been detected by *in vitro* translation of mRNA from the optic nerve (Rachailovich *et al.*, 1984).

The production of laminin, a basal lamina constituent that appears around

large axon bundles in the goldfish optic nerve, is increased during regeneration (Agranoff *et al.*, 1984). The potential importance of laminin in regeneration is emphasized by the finding that addition of laminin to the substrate on which goldfish retina explants are grown affects the direction and profusion of neurite outgrowth (Agranoff *et al.*, 1984).

An intriguing recent finding is that when regenerating goldfish optic nerves are excised and placed in a culture medium for a few hours, and this medium is then applied to a crushed rabbit optic nerve, changes are induced in the rabbit retinal ganglion cells that resemble those occurring during regeneration in the goldfish. Thus the rabbit retinas attached to nerves that have been crushed and treated with exudate from regenerating goldfish nerves show an increase in protein synthesis and an increased capacity for production of neurites when fragments of these retinas are placed in tissue culture, while the treated nerve itself shows an abundance of axon sprouts detectable by electron microscopy (Schwartz *et al.*, 1985). These results imply that the regenerating goldfish nerves may release a powerful growth-promoting factor (probably emanating from glial cells) that is capable of stimulating regeneration in a mammalian CNS pathway. This factor may be a polypeptide of less than 10,000 molecular weight (Schwartz *et al.*, 1985).

There is evidence that, in addition to growth-promoting factors, the glial cells in regenerating goldfish optic nerve may produce other factors that inhibit regeneration (Rachailovich and Schwartz, 1984), so that conditions must be carefully controlled in order to demonstrate maximum growth-promoting effects by optic nerve extracts.

3. Growth-Promoting Factors from the Optic Tectum

Evidence of diffusible growth-promoting factors in the tectum comes from the observation that, when fragments of goldfish tectum are cultured together with retinal explants, they promote the growth of neurites from the retinal tissue (Mizrachi and Schwartz, 1982). Also, outgrowth from retinal explants can be stimulated by extracts of tectum (Johnson and Turner, 1982; Schwartz *et al.*, 1982a). The possibility that nerve growth factor (NGF) or a closely related molecule might be involved is suggested by the following evidence: (1) goldfish brain extract gives a positive reaction in standard assays for NGF (Weis, 1968; Benowitz and Greene, 1979; Schwartz *et al.*, 1982), (2) NGF stimulates neurite outgrowth from goldfish retinal ganglion cells *in vivo* and *in vitro* (see Section III,C,2), and (3) *in vitro* outgrowth from retinal explants is impaired by antiserum to NGF (Turner *et al.*, 1980). The effects of the goldfish brain extract are not identical with those of NGF, however, since the brain extract can replace serum in supporting neurite outgrowth from explants of the retina, a property that is not shared by NGF (Schwartz *et al.*, 1982). (Extracts of retina also lack this

property.) Thus the outgrowth-promoting activity in goldfish brain extract may be due to a factor resembling NGF or in addition to NGF. One brain-derived factor that has been characterized to some extent is a 13-kDa glycoprotein which promotes neurite outgrowth even after it has been absorbed onto a tissue culture substrate; another factor, which may act without binding to the substrate, has also been recognized (Mizrachi *et al.*, 1986).

Extracts of optic tectum may also induce aggregation of acetylcholine receptors on cultured muscle cells (Schwartz *et al.*, 1981). This effect is maximal in tecta taken at 5–14 days after optic nerve crush, i.e., just prior to reinnervation of the tectum. Conceivably this may indicate the presence of a trophic factor that plays a role in optic nerve regeneration.

Recently it has been shown that a soluble peptide constituent produced by the optic tectum can stimulate protein synthesis in both the astrocytes and oligodendrocytes of the optic nerve (Giulian, 1984). The production of this material, which has a molecular weight of 14,000, is increased following optic nerve crush, whereas ablation of the tectum reduces the biosynthetic activity of the glial cells, presumably as a consequence of eliminating the critical peptide. It is not clear whether this factor acts directly on the glial cells, or indirectly by stimulating axonal growth, which then activates the glial cells.

IV. Regulation of the Cell Body Reaction

A. Initiation of the Reaction

In view of the evidence presented above showing that the cell body reaction to axotomy plays an essential role in regeneration, it becomes important to consider how this reaction is initiated. At present, we do not know the nature of the initiating event or how the cell body is informed that this event has occurred. A widely held current view is that the signal is conveyed to the cell body by retrograde axonal transport (Forman, 1983; Singer *et al.*, 1984). One hypothesis is that the reaction is triggered when the cell body is invaded by retrogradely transported exogenous materials that have entered the axon at the site of injury (Kristensson, 1984); another is that endogenous materials reaching the site of the lesion by anterograde transport are returned to the cell body altered in timing, amount, or composition, because the length of axon that they must traverse has been shortened (Bisby, 1984) or the axon terminals have been removed (Aletta and Goldberg, 1984); a third is that the interruption of the axon prevents the access to the cell body of trophic materials normally emanating from the target cells (Purves and Njå, 1976). Another mechanism that has been suggested is that the initiation of axon sprouting is the cell body-triggering event (Watson, 1969; Grafstein and McQuarrie, 1978).

A problem inherent in this issue is how to determine whether a critical change has been evoked in the cell body. In the past, the cell body alterations have frequently been equated with chromatolysis (e.g., Cragg, 1970). It is now clear, however, that chromatolysis is only one possible manifestation of the array of changes that may be elicited by axotomy (Grafstein, 1983) and that the presence of chromatolysis is not necessarily correlated with the initiation of changes essential for axonal regeneration. These regeneration-inducing changes have been categorized as "restorative" events, in distinction to (1) the "disruptive" events that represent the nonspecific consequences of cellular injury and (2) the "regulative" events that constitute a homeostatic response to the perturbation of cellular function produced by amputation of the axon (Grafstein, 1983).

The changes elicited by axotomy of the goldfish retinal ganglion cell appear to be strongly biased toward the restorative component. This is what makes this neuron particularly suitable for investigating the problem of the initiation of those aspects of the cell body reaction that are relevant for regeneration. In this cell, for example, the criteria to determine whether the restorative component of the cell body reaction has indeed been initiated would include some prominent changes, such as the increase in nucleolar size, the increase in synthesis and axonal transport of protein, and the capacity to support accelerated axonal outgrowth in response to a lesion made after the reaction to axotomy has already been initiated (conditioning lesion effect).

1. Relationship between the Cell Body Reaction and Axonal Sprouting

The onset of axonal sprouting may be identified with the appearance adjacent to the lesion of groups of closely apposed filament-filled processes, which begin to be seen at 2 days after the lesion (see Section II,A,1). Widespread axonal sprouting, however, only becomes evident by 3–6 days (Lanners and Grafstein, 1980). Thus, axonal sprouting appears to coincide very closely with the onset of changes in the cell bodies, which show clear evidence of altered RNA synthesis by 3 days after the lesion (see Sections II,B,1,a and II,B,2,a). Because of this close timing, it has been difficult to decide whether the onset of sprouting might be important in initiating the changes in the cell body. It is clear, however, that there is no significant degree of axonal elongation preceding the cell body changes.

2. Onset of Changes in Axonal Transport

Within 24 hr after axotomy, a significant increase in fast axonal transport of radioactively labeled proteins is detectable in the original segment of the optic axons (Grafstein and Alpert, 1976). Some of this effect may be due to alterations in the labeled amino acid pool (Whitnall and Grafstein, 1981) and in the altered

dynamics of transport resulting from the shortening of the axon (Bisby, 1984), but these do not completely account for the change. It appears, therefore, that there may be an increase in fast axonal transport preceding any change in protein synthesis. A similar increase in protein transport that cannot be ascribed to an increase in synthesis has been seen at 1 day after a testing lesion preceded 2 weeks earlier by a conditioning lesion (see Section III,A,1). These changes therefore appear to represent an increase in the diversion of cell body products into the axon. The changes in routing of axonal transport may be related to those that have been seen in the axon of an *Aplysia* neuron following amputation of one of its branches (Aletta and Goldberg, 1984).

3. Conditions That May Evoke or Modify the Cell Body Reaction

a. Application of Microtubule-Disrupting Agents. A cell body reaction resembling that produced by axotomy is elicited when vincristine, a drug that is known to depolymerize microtubules and cause aggregation of tubulin subunits (for references see Ben-Ze'ev *et al.*, 1979), is locally applied to the optic nerve or optic tract (White and Grafstein, 1974; C. M. McGuinness, unpublished results). The intensity and time course of this reaction are similar to that produced by axotomy: the nucleolar incidence may begin to increase within 4 days after the application, and by 5 days, cell size and incorporation of labeled amino acid may also be increased. Similar cell body changes are also seen after application of colchicine, a drug that disrupts the microtubules by a somewhat different mechanism, which results in an accumulation of unpolymerized subunits of tubulin (for references, see Ben-Ze'ev *et al.*, 1979). With either colchicine or vincristine, the initiation of the cell body reaction is not necessarily accompanied by axonal degeneration on the cranial side of the axonal transport block, even though the axons have presumably been deprived of transported material.

When vincristine is applied to the optic tract, the amount of labeling in fast axonal transport and the velocity of slow axonal transport have been found to be increased in the intact portion of nerve between the eye and the site of application of the drug (C. M. McGuinness, W. C. White, and B. Grafstein, unpublished results), as they are following axotomy. If vincristine is applied to the optic tract 2 weeks before an optic nerve crush, axonal outgrowth following the crush is greatly enhanced above normal; i.e., the vincristine application resembles axotomy in its ability to act as a conditioning event for regeneration.

When vincristine or colchicine is injected into the eye (White and Grafstein, 1974), the reaction it produces includes an increase in nucleoli, but little increase in cell size and no increase in protein synthesis. This may be due to the fact that microtubule-disrupting agents may inhibit protein synthesis in the retina (White and Grafstein, 1974).

b. Depolarization. Veratridine, which causes membrane depolarization by promoting Na^+ influx (Minchin, 1980), can block axonal transport (Grafstein and Forman, 1980). It did not, however, elicit a nucleolar reaction in goldfish retinal ganglion cells either upon local application to the optic nerve or upon intraocular injection, even though it produced a substantial block in fast anterograde transport (C. M. McGuinness, unpublished results). [The status of retrograde transport was not ascertained in these experiments, but it is known that batrachotoxin, which appears to act by the same mechanism as veratridine (Grafstein and Forman, 1980), can block retrograde transport as well as both fast and slow anterograde transport (Boegman and Riopelle, 1980).] Intraocular injection of veratridine potentiated slightly the nucleolar reaction produced by an optic nerve crush (C. M. McGuinness, unpublished results).

c. Interference with Axonal Sprouting. Taxol, a drug that "stabilizes" microtubules (Schiff and Horwick, 1980), inhibits sprouting of the goldfish optic axons if it is applied to the axons a few days prior to an optic nerve crush (C. M. McGuinness, unpublished results). Nevertheless, cell size and axonal transport are increased just as they are during normal regeneration. Also, delaying the time course of sprouting by excising the tip of the axon shortly after the initial lesion had no effect on the enlargement of the nucleoli (McGuinness and Grafstein, 1985). These experiments suggest that the sprouting of the axons is not a critical event in the initiation of the cell body reaction.

d. Conclusions from Experiments on Initiation or Modification of the Cell Body Reaction. Contrary to previous suggestions (Grafstein, 1975), the cell body reaction evoked by means other than axotomy (e.g., interruption of microtubules) is not a partial one, but appears to have the same characteristics whether or not axonal outgrowth occurs. This indicates that, once the reaction is initiated, its development requires little if any feedback from the growing axon, at least until the later stages (see Section IV,B,2). Apparently, the characteristics of the reaction are determined by the metabolic status of the nerve cell at the time that the initiation signal is received and by a (presumably) genetically determined pattern that governs the unfolding of the reaction. Also, the intensity of the reaction might be affected by concomitantly evoked cell body changes; e.g., depolarization of the cell body may potentiate the reaction.

As to the nature of the initiating signal, the experiments described here indicate that the initiation of a cell body reaction resembling the reaction produced by axotomy does not require (1) severance of the axon, (2) degeneration of the axon beyond the level of the lesion, (3) depolarization of the axon or cell body, or (4) axon sprout formation. Moreover, it has been shown that the initiation of the reaction does not necessarily occur when axonal transport, at least in the anterograde direction, is blocked (e.g., by veratridine). These observations appear to

eliminate many of the most obvious mechanisms that have been proposed for the initiation of the cell body reaction (Cragg, 1970; Grafstein, 1975; Grafstein and McQuarrie, 1978). One possibility that has not been previously considered, however, is that the critical event in the initiation of the reaction may be an alteration in the status of the microtubules at the lesion site, whether produced by axotomy or by the application of vincristine or colchicine. Conceivably, this might lead to a change in cell body metabolism via the mechanism that governs synthesis of microtubule protein, which is currently believed to be regulated by the concentration of tubulin monomer (Cleveland *et al.*, 1981; Olmstead *et al.*, 1984). How this mechanism functions in nerve cells, in which the concentration of monomer in the axon and in the cell body might both be important in regulating synthesis, is not yet clear—possibly retrograde transport of a sample of the axonal monomer pool might be involved. Another suggestive observation is that agents that interfere with microtubule function can cause fragmentation of the Golgi system (Wehland *et al.*, 1983; Sandoval *et al.*, 1983), which is reminiscent of the changes in this system that are frequently seen with axotomy (Lieberman, 1971). Further experiments are still required, however, to test the microtubule disruption hypothesis.

B. Termination of the Cell Body Reaction

1. Normal Time Course of Cell Body Reaction

The cell body reaction reaches its apex at 2–3 weeks after axotomy, as indicated by a number of criteria, e.g., overall level of protein synthesis, amount and velocity of fast and slow axonal transport, and cell size (Murray and Grafstein, 1969; Grafstein and Murray, 1976; McQuarrie and Grafstein, 1982a; Burmeister and Grafstein, 1985; Perry *et al.*, 1986a). The decline of the reaction begins at about the time that the axons begin to penetrate into the optic tectum, although recovery of the cell body is not complete until 12–26 weeks after the lesion (Burmeister and Grafstein, 1985; D. W. Burmeister, unpublished results), which corresponds to the time required for restoration of the normal number of retinotectal synapses (Murray and Edwards, 1982). This suggests that the process of tectal innervation may regulate the cell body reaction, at least in its declining phase.

In spite of the close correspondence between innervation of the tectum and regression of the cell body reaction, some features of the reaction begin to decline even before the regenerating axons reach the tectum. The peak of RNA synthesis, for example, has been observed at 4–7 days after the lesion (see Section II,B,2,a), and the labeling of some fast axonally transported proteins apparently shows a sharp decline after 1 week (Perry *et al.*, 1986a). These

observations suggest that the termination of the cell body reaction depends on a number of different mechanisms, some of which may be acting early in the course of the reaction.

2. Effects of Removal of the Target (Homotopic) Lobe of the Optic Tectum

a. Time Course of Regeneration. When one lobe of the optic tectum has been removed (Sharma, 1973; Lo and Levine, 1980; Burmeister and Grafstein, 1985), the regenerating axons that would ordinarily project to this lobe of the tectum (i.e., those originating in the contralateral eye) produce a very dense innervation of the pretectal regions on both sides and also form a large neuroma in the midbrain under the site of ablation (Burmeister and Grafstein, 1985). Some of the axons continue growing into the brainstem to reach several millimeters beyond their original length. A substantial proportion of the axons grow across the midline to innervate the remaining (heterotopic) tectal lobe, first appearing there about 3–4 weeks after the operation (Lo and Levine, 1980; G. W. Perry and D. W. Burmeister, unpublished results), and completely covering this lobe of the tectum by 6–8 weeks (Lo and Levine, 1980; Sharma, 1973; Levine and Jacobson, 1975). At least some of the retinotectal connections become functional within 4–6 weeks (Easter and Schmidt, 1977), but mature synapses (as judged by morphological criteria, including clustering of synaptic vesicles at the presynaptic membrane) are not frequently seen until later times (Airhart and Norden, 1985). Eventually the synaptic terminals formed by the regenerating axons become confined to narrow bands that alternate with similar bands of innervation formed by the axons indigenous to the heterotopic lobe (Levine and Jacobson, 1975; Spinger and Cohen, 1981); in our own studies this "banding" phenomenon did not appear until nearly 26 weeks following a crush of the optic nerve in animals kept at 20°C (Burmeister and Grafstein, 1985), i.e., long after most of the axons had established their initial synapses. The establishment of the banding pattern may resemble the process of refinement of the retinotectal projection by elimination of incorrect synaptic connections that normally occurs during reinnervation of the target (homotopic) lobe of the tectum (see Sections II,A,4 and III,B,1).

b. The Cell Body Reaction in the Absence of the Target Lobe of the Tectum. In the early stages of regeneration, changes in cell body size appear to be affected very little if at all, by the removal of the target lobe of the tectum (Burmeister and Grafstein, 1985). The enlargement of the cell body occurs with the same time course as during normal regeneration. The retinal ganglion cells remain enlarged, however, long beyond the time at which they would be expected to return to normal. They show little recovery in size by 10–12 weeks

(Burmeister and Grafstein, 1985), and they retain their enhanced capacity for axonal outgrowth when the retina is placed in tissue culture (Mizrachi *et al.*, 1984). Thus the cell body reaction, at least in its later stages, is normally modifiable by conditions encountered by the axon.

When only the homotopic lobe of the tectum has been removed, recovery of cell size to normal may be seen by 26–36 weeks at 20°C (D. W. Burmeister, unpublished results). This appears to correspond to the time required for formation of the banding pattern of retinotectal synapses in the remaining (heterotopic) lobe of the tectum (see Section IV,B,2,a). Recent experiments indicate that conditions that delay the formation of the banding pattern (e.g., injection of TTX into both eyes) also retard the time course of recovery, whereas removal of the indigenous innervation of the heterotopic lobe of the tectum (by removal of the heterotopic eye) accelerates the recovery of cell body size (D. W. Burmeister, unpublished results). In spite of these indications that recovery of the cell body depends on establishment of connections in tectal tissue, some degree of recovery is seen even when both lobes of the tectum have been removed (D. W. Burmeister, unpublished data), suggesting that another factor may be involved. It is not yet clear whether this represents recovery due to formation of abnormal synapses in other parts of the brain or long-term atrophy due to the failure to form synapses.

c. Changes in Fast Axonal Transport. Labeling of individual fast axonally transported proteins, as seen by two-dimensional gel electrophoresis, reveals virtually no effect of removal of the target lobe of the tectum for up to 3 weeks after axotomy (Perry *et al.*, 1986b). In the original axon segment, proteins showing a sharp peak at 1–2 weeks after the lesion, for example, also do so in the absence of the contralateral lobe of the tectum. After 3 weeks, however, the labeling declines more slowly than usual, or even shows a renewed increase, with a peak at 5–8 weeks. Almost all the proteins examined show significantly higher labeling than during normal regeneration until at least 12 weeks after the lesion. Highly elevated levels of labeling are also seen in the new segment of axon, especially after 5 weeks. Nearly all of the proteins that have been found to increase during regeneration can therefore be considered to be regulated by contact with the tectum. This includes even those proteins that show an early peak in labeling, presumably indicating early regulation by another mechanism as well. An example of this is seen with the 45,000-molecular weight protein described in Section II,C,2,a, which shows an early decrease in labeling that is independent of reconnection with the tectum (Benowitz *et al.*, 1983), but a subsequent slow decline that occurs only in the presence of the target lobe of the tectum (Perry *et al.*, 1986b).

Fast-transported proteins that show enhanced labeling only upon contact with the optic tectum, and hence might be specifically associated with synap-

togenesis, appear to be rare, but decreased labeling of some fast-transported proteins (140,000–155,000 MW) in the absence of the tectum has been reported (Benowitz *et al.*, 1983; Benowitz, 1984).

d. Changes in Slow Axonal Transport. Most of the individual proteins in slow axonal transport that show increased labeling during regeneration (see Section II,C,2,b) show an even higher level of labeling in the absence of the homotopic tectal lobe. The differences appear at 4–5 weeks after the lesion and last until at least 10–12 weeks (Perry *et al.*, 1986c). This includes tubulin, actin, and the ON proteins. It does not, however, include the W proteins (35–45 kDa, p*I* 6.5–7.0), the labeling of which is depressed below the levels normally seen during regeneration. These observations are consistent with the idea that the W proteins may be associated with myelin (see Section II,C,2,b), and that myelination, which is accelerated when the axons arrive in the tectum (Murray, 1976; Wolburg, 1978), may not proceed normally in the absence of the tectum. It has also been reported that labeling of a slowly transported 300-kDa protein, which normally appears during innervation of the tectum, fails to occur when the tectum has been removed (Giulian, 1984b).

e. Conclusions from Experiments on Target Removal. Cell body recovery normally begins with the entry of the regenerating axons into the optic tectum, and this recovery is delayed when the target lobe of the optic tectum has been removed. This indicates that the cell body reaction is normally not a self-limiting event, but depends on some extrinsic signal. However, the nature of the recovery signal is still not clear. The following conclusions can be drawn.

1. The reaction is not necessarily terminated when the axons regain their normal length.
2. Contact with the (heterotopic) tectal tissue is not sufficient for recovery. Hence a diffusible trophic factor produced by the normally innervated tectum cannot be responsible for recovery.
3. Synaptogenesis per se is not adequate for complete recovery, since the regenerating axons establish synapses (in the heterotopic tectal lobe) only a few weeks later than they normally would (in the homotopic lobe).

The apparently weak affect of the heterotopic tectal lobe in providing a signal for recovery may, however, be due to one or more of the following causes.

1. Many of the neurons with axons entering the heterotopic tectal lobe may also have branches that fail to find an appropriate target, so that the recovery signal from the tectum may be diluted or counteracted.
2. The recovery signal may be different on the two sides of the brain, the signal from the remaining tectal lobe being less effective for axons that belong on the other side.

3. The sensitivity of the regenerating axons to the recovery signal may have declined by the time they reach the heterotopic tectal lobe, because of the longer time required to get there.
4. Recovery-promoting trophic factors may be produced by the tectum only when it has been denervated.

The various possibilities that might account for delayed recovery of the retinal ganglion cells in the absence of the homotopic tectal lobe have not yet been completely explored. Nevertheless, the experiments showing that recovery of cell body size is correlated with the appearance of the banding pattern in the retinotectal projection is a persuasive argument in favor of the view that the competitive interactions thought to be involved in the establishment of banding pattern (see Section IV,B,2,a) are also responsible for the recovery of cell body characteristics. This is an important finding, because it provides for the first time a common ground for the analysis of problems of retinotectal specificity and retinal ganglion cell metabolism.

V. General Conclusions

A. Comparison of the Goldfish Retinal Ganglion Cell with Other Regenerating Neurons

Are the regeneration-associated changes displayed by the goldfish retinal ganglion cell unique to this neuron or do they occur in other regenerating neurons as well? The answer to this question requires a consideration of the salient characteristics of the regenerating goldfish retinal ganglion cell:

1. The goldfish retinal ganglion cell responds to axotomy with *very little disruption of the rough endoplasmic reticulum and little if any other evidence of deleterious consequences of the injury*. Thus this neuron may be expected to reveal the changes associated specifically with regeneration more clearly than neurons that show more prominent nonspecific injury effects (Grafstein, 1983).
2. The reaction to axotomy in the goldfish retinal ganglion cell involves *a rapid increase in the supply of virtually all major axonally transported proteins* (as well as lipids, nucleosides, polyamines, and probably other still-uninvestigated constituents). In other regenerating neurons, such a pervasive increase is not seen (reviewed by Forman, 1983). Frequently there is a decrease, at least in the synthesis of membrane-associated materials (Reis *et al.*, 1978) or neurofilaments (Hoffman *et al.*, 1984). In some neurons there is no net change in protein synthesis and axonal transport, possibly because the transport of some proteins

may be increased, while the transport of others is decreased; in some neurons a net decrease occurs. Sometimes increased synthesis appears only in association with synapse formation and maturation of the axon (Engh *et al.*, 1971). Thus, in many cases the normal level of transport may be adequate to support most of the materials needed for regeneration—conceivably the materials normally utilized for maintenance of the segment of the axon that has been amputated may be sufficient to supply the regenerating sprouts, or the deposition of materials in the undamaged part of the axon may be reduced. It is possible, however, that even when total protein synthesis is reduced, there may be an increase in materials specifically associated with regeneration (Skene and Willard, 1980a,b).

3. The regenerating goldfish retinal ganglion cell shows *increased transport of tRNA*. Although qualitatively similar changes have been observed in other regenerating axons (e.g., rat sciatic nerve; Lindquist and Ingoglia, 1979), the effect is much more prominent in the goldfish optic axons. It is not yet clear, however, whether these changes, which may be important in posttranslational modification of proteins, are critical for regeneration.

4. *The axonal transport of cytoskeletal elements, including tubulin and actin, is accelerated* during regeneration in goldfish retinal ganglion cells. This may represent either an increase in transport velocity of these elements or an increase in the amount of a relatively rapidly transported fraction of these constituents. In regenerating mammalian sciatic nerve, there is an increase in the magnitude of a relatively rapidly transported fraction of tubulin (Hoffman and Lasek, 1980), resulting in the association of a significant amount of tubulin with component SCb of slow transport, whereas most of the tubulin is conveyed in SCa under normal conditions (Hoffman and Lasek, 1975). In the goldfish retinal ganglion cell, much of the tubulin transport occurs in component SCb, even under normal conditions. It has been proposed that cotransport of tubulin with other SCb constituents, particularly actin, may be an important determinant of regeneration (McQuarrie, 1983).

5. In the goldfish retinal ganglion cell, as in other neurons, the mechanism that initiates the cell body reaction is still unknown. It has been suggested that *the initiating event may involve a disruption of the microtubules*, possibly leading to alterations in the Golgi system (see Section IV,A,3). Conceivably, one factor in the failure of mammalian CNS neurons to regenerate (Barron, 1983) may be the absence of this initiating event. Possibly, conditions at the site of the lesion in the CNS may be inappropriate for producing the requisite change in the microtubules. Or perhaps the microtubule change alone may not be sufficient to produce the cell body reaction, but may require, in addition, the presence of a trophic factor that may be produced by the nonneuronal cells of the injured goldfish optic nerve (see point 11, below), but not by mammalian glial cells. The idea that two independent conditions, namely, axon damage and the presence of

appropriate trophic factors, are required for successful regeneration of the axon has also been invoked to explain the phenomenology of growth of mammalian CNS axons into grafts of peripheral nerve (Aguayo, 1985).

6. The early stages of cell body reaction in the goldfish retinal ganglion cells (probably including the period in which the most rapid axon elongation normally occurs) are unaffected by whether or not axonal outgrowth takes place. Thus *the characteristics of the cell body reaction, at least during the early stages, are uninfluenced by feedback from the axon. They may be influenced, however, by other changes that might be concomitantly produced by the lesion, such as depolarization of the cell body.* If these properties are true also for other regenerating neurons, they might explain the differences in the reaction produced, for example, by lesions placed close to or far from the cell body (Watson, 1968).

7. Outgrowth of the goldfish optic axons can commence even when neuronal protein synthesis has been inhibited. This indicates that *all the proteins that are necessary for the transition from a normal neuron to one that is regenerating are probably already being synthesized under normal conditions.* Thus, it appears unlikely that new genes need to be turned on. Whether this is also true in other neurons remains to be determined.

8. Increased synthesis and axonal transport of proteins are essential if outgrowth of the goldfish optic axons is to continue beyond a few days. That *the cell body reaction is important for the maintenance of regeneration* has also been demonstrated in frog sciatic axons (Carlsen, 1984).

9. An enhanced rate of outgrowth of the goldfish optic axons resulting from a prior conditioning lesion is correlated with potentiation of the cell body reaction. These findings indicate that, although axonal sprouting may be initiated in the absence of any contribution from the cell body (Bray *et al.*, 1978), *the degree of metabolic support that the cell body provides eventually becomes an important determinant of the rate of regeneration.*

10. *Regeneration of the goldfish optic axons can be promoted by extracellular factors produced by the glial cells and other nonneuronal elements.* The isolation and identification of such growth-promoting factors (and also some growth-inhibiting factors) are likely to be accomplished very soon, and it will be interesting to see how the factors in goldfish optic nerve compare with corresponding factors released from the supporting cells in other regenerating systems (Varon and Adler, 1981). What still remains to be determined is how important such factors may be for the normal regeneration *in vivo,* relative to the intrinsic regenerative capacity of the nerve cell.

11. *Regeneration can be influenced by physiological activity.* Reduction of the level of physiological activity in goldfish retinal ganglion cells reduces the number (and/or size) of the regenerating axons, an effect that has not yet been described in other regenerating systems. It has also been found that the final stages of restoration of the retinotectal connections depend on the pattern of

electrophysiological activity in the retina. This mechanism has been shown to be important in normal development of the visual pathway in other species, including mammals (for references see Edwards and Grafstein, 1983).

12. Recovery of the axotomized goldfish retinal ganglion cells is not complete until the regenerating axons reestablish functionally appropriate connections in the optic tectum. The nature of the mechanisms involved is not yet clear, but this is an important finding in that it reveals *a possible role of physiological events at the axon terminals in modifying biochemical events in the cell body.* This possibility needs further careful exploration in normal as well as regenerating systems.

B. Comparison of the Effects of Axotomy on the Retinal Ganglion Cell in the Goldfish and in the Mammal

Injury of the optic nerve in adult mammals usually results in only very limited regeneration (Ramon y Cajal, 1928; Richardson *et al.*, 1982; Grafstein and Ingoglia, 1982). In the rat, for example, some axonal sprouting has been detected following an intracranial lesion of the optic nerve, including the formation of structures that resemble growth cones at 2 weeks after the lesion (Richardson *et al.*, 1982). The bundles of unmyelinated sprouts extended only to about 0.5 mm from the lesion, however, and even the axons that showed the regenerative response appeared to degenerate eventually.

Why does injury of the optic nerve in the mammal produce such poor regenerative results compared to the goldfish?

1. *Preservation of the blood supply of the nerve and survival of glial cells.* A lesion of the optic nerve in the mammal usually produces intense ischemic necrosis in the center of the retinal nerve stump, in some cases extending almost to the eye (Grafstein and Ingoglia, 1982; Richardson *et al.*, 1982). Even the relatively small proportion of optic axons that survive at the periphery of the nerve show evidence of poor blood supply in the failure of axonal transport at their tips (Grafstein and Ingoglia, 1982). In the goldfish, on the other hand, the glial cells and most of the axons remain viable almost up to the level of the lesion. The superior survival of the goldfish glial cells may be due to better preservation of the blood supply in the region of the lesion, and possibly also to better resistance of the glial cells to local ischemia. The importance of the vasculature in regeneration is also brought out by the fact that when a nerve is led to grow through an artifical conduit *in vivo,* the elongating axons follow a strand of vascular elements and supporting cells which forms before the axons arrive (Madison *et al.*, 1983). Conceivably, the successful regeneration of mammalian optic axons into peripheral nerve grafts (So and Aguayo, 1985) may be due to a

susceptibility of these grants to invasion by vascular elements. It is possible also that the Schwann cells are more resistant to anoxia than glial cells are.

2. *Production of growth-modulating factors by glial cells.* The glial cells of the goldfish optic nerve release factors that can promote regeneration of optic axons not only in goldfish but also in mammals. Thus when extracts of regenerating fish optic nerve are applied to the crushed optic nerve in adult rabbits, metabolic changes resembling those associated with regeneration are evoked in the rabbit retina (Schwartz *et al.*, 1985; see Section III,D,2), and axonal sprouting may be detected (Lavie *et al.*, 1985). Extracts of neonatal rabbit optic nerve can elicit a similar response, whereas extracts of the adult nerve cannot (Hadani *et al.*, 1984). It appears, therefore, that the ability of the optic glia to produce growth-promoting factors is present in mammals as well as lower vertebrates, but is lost in mammals during maturation, although the optic neurons retain their sensitivity to such factors. It is possible that similar factors liberated by Schwann cells are at least partly responsible for the ability of peripheral nerve grafts in the orbit to support mammalian optic nerve regeneration (So and Aguayo, 1985). These factors, however, are not able to sustain the long-term functional viability of the regenerating mammalian cells that remain disconnected from their synaptic target (Keirstead *et al.*, 1985).

Regeneration may be adversely affected by inhibitory factors produced by the glial cells. Even in the goldfish optic nerve such factors have been detected (Rachailovich and Schwartz, 1984), and there is some evidence that mammalian optic nerve glia release inhibitory factors *in vitro,* since the processes of cultured neurons avoid optic nerve explants (Schwab and Thoenen, 1985).

3. *Survival of neurons.* Almost all the goldfish retinal ganglion cells survive axotomy, whereas many of the mammalian retinal ganglion cells die. Some of the mammalian cells die almost immediately—about 20% disappear by 3 days after an intracranial optic nerve lesion in the mouse (Grafstein and Ingoglia, 1982). Others take longer to die—about 50% have disappeared by 2–3 months in the mouse (Grafstein and Ingoglia, 1982), and an even larger proportion may disappear in other species, although more slowly (for references see Misantone *et al.*, 1984). Those that survive usually shrink in size, at least initially. These changes, especially those that take a long time to occur, have been attributed to the lack of a trophic factor normally derived from the target tissues (e.g., Misantone *et al.*, 1984). Apparently, the goldfish neurons are less dependent on the putative trophic factor. Even when both lobes of the goldfish optic tectum have been removed and the retinal ganglion cells fail to make their normal connections, very few of them are lost over a period of months following the lesion (D. W. Burmeister, unpublished results). The loss of axotomized neurons from the retina probably does not account for the poor regenerative ability of mammalian optic nerve, since the cell loss is so long delayed. Nevertheless, the mechanisms leading to this loss may be related to those that interfere with regeneration.

4. *The cell body reaction to axotomy.* It is clear that the cell body changes elicited in goldfish retinal ganglion cells by axotomy contribute to the tremendous capacity for regeneration that these cells possess. Whether a similar reaction can be evoked in mammalian retinal ganglion cells still remains to be determined. Preliminary indications are that the mammalian neurons may enlarge during regeneration promoted by peripheral nerve grafts (So and Aguayo, 1985). It has not yet been established, however, whether even under optimal conditions the intense regenerative potential of the goldfish retinal ganglion cells will be matched by the mammalian neurons. It is likely that this problem will be resolved before long, with further development of techniques for promoting regeneration in the mammalian central nervous system.

Acknowledgments

I am grateful to the following colleagues for their comments and suggestions: Drs. D. Louise Edwards, David S. Forman, Nicholas A. Ingoglia, Marion Murray, Gary W. Perry, Janet R. Sparrow, and Mark H. Whitnall. I am indebted to various members of my laboratory for making their unpublished results available and to Ms. Julie Peyton for her patience, persistence, and perspicacity in the typing of the manuscript.

This article was written with the support of Research Grant NS-09015 from NINCDS. Previously unpublished research from the author's laboratory that is described herein was supported by this grant and also by Research Grant NS-14967 and Training Grant NS-07138 from NINCDS, as well as by grants from the Paralyzed Veterans of America Research and Technology Foundation, and from the Amyotrophic Lateral Sclerosis Society of America.

References

Agranoff, B. W., and Ford-Holevinski, T. S. (1984). Biochemical aspects of the regenerating goldfish visual system. *In* "Axonal Transport in Neuronal Growth and Regeneration" (J. S. Elam and P. Cancalon, eds.), pp. 69–86. Plenum, New York.

Agranoff, B. W., Field, P., and Gaze, R. M. (1976). Neurite outgrowth from explanted *Xenopus* retina: An effect of prior optic nerve section. *Brain Res.* **113,** 225–234.

Agranoff, B. W., Hopkins, J. M., Davis, R. E., Ford-Holevinski, T. S., and McCoy, J. P. (1984). Laminin: Role in goldfish optic nerve regeneration. *Soc. Neurosci. Abstr.* **10,** 282.

Aguayo, A. (1985). Axonal regeneration from injured neurons in the adult mammalian central nervous system. *In* "Synaptic Plasticity and Remodeling" (C. W. Cotman, ed.), pp. 457–483. Guilford, New York.

Airhart, M. J., and Norden, J. J. (1985). Retinotectal synapses formed by ipsilaterally projecting fibers in the doubly innervated goldfish tectum. *Brain Res.* **325,** 307–312.

Aletta, J. M., and Goldberg, D. J. (1984). Routing of transmitter and other changes in fast axonal transport after transection of one branch of the bifurcate axon of an identified neuron. *J. Neurosci.* **4,** 1800–1808.

Attardi, D. G., and Sperry, R. W. (1963). Preferential selection of central pathways by regenerating optic fibers. *Exp. Neurol.* **7,** 46–64.

Barron, K. D. (1983). Axon reaction and central nervous system regeneration. *In* "Nerve, Organ and Tissue Regeneration: Research Perspectives" (F. J. Seil, ed.), pp. 3–36. Academic Press, New York.

Benowitz, L. I. (1984). Target-dependent and target-independent changes in rapid axonal transport during regeneration of the goldfish retinotectal pathway. *In* "Axonal Transport in Neuronal Growth and Regeneration" (J. S. Elam and P. Cancalon, eds.), pp. 145–169. Plenum, New York.

Benowitz, L. I., and Greene, L. A. (1979). Nerve growth factor in the goldfish brain: Biological assay studies using pheochromocytoma cells. *Brain Res.* **162,** 164–168.

Benowitz, L. I., and Lewis, E. R. (1983). Increased transport of 44–49,000 Dalton acidic proteins during regeneration of the goldfish optic nerve: A 2-dimensional gel analysis. *J. Neurosci.* **3,** 2153–2163.

Benowitz, L. I., Shashoua, V. E., and Yoon, M. (1981). Specific changes in rapidly transported proteins during regeneration of the goldfish optic nerve. *J. Neurosci.* **1,** 300–307.

Benowitz, L. I., Yoon, M. G., and Lewis, E. R. (1983). Transported proteins in the regenerating optic nerve: Regulation by interactions with the optic tectum. *Science* **222,** 185–188.

Ben-Ze'ev, A., Farmer, S. R., and Penman, S. (1979). Mechanisms of regulating tubulin synthesis in cultured mammalian cells. *Cell* **17,** 319–325.

Berthold, C.-H., Nilsson, I., and Rydmark, G. (1983). Axon diameter and myelin sheath thickness in nerve fibres of the ventral spinal root of the seventh lumbar nerve of the adult and developing cat. *J. Anat.* **136,** 483–508.

Bisby, M. A. (1984). Retrograde axonal transport and nerve regeneration. *In* "Axonal Transport in Neuronal Growth and Regeneration" (J. S. Elam and P. Cancalon, eds.), pp. 45–67. Plenum, New York.

Bixby, J. L., and Spitzer, N. C. (1984). Early differentiation of vertebrate spinal neurons in the absence of voltage-dependent Ca^{2+} and Na^{+} influx. *Dev. Biol.* **106,** 89–96.

Boegman, R. J., and Riopelle, R. J. (1980). Batrachotoxin blocks slow and retrograde axonal transport *in vivo*. *Neurosci. Lett.* **18,** 143–147.

Boss, V. C., and Schmidt, J. T. (1984). Activity and the formation of ocular dominance patches in dually innervated tectum of goldfish. *J. Neurosci.* **4,** 2891–2905.

Brattgård, S.-O., Edström, J.-E., and Hydén, H. (1957). The chemical changes in regenerating neurons. *J. Neurochem.* **1,** 316–325.

Bray, D., Thomas, C., and Shaw, G. (1978). Growth cone formation in cultures of sensory neurons. *Proc. Natl. Acad. Sci. U.S.A.* **75,** 5226–5229.

Bunge, M. (1973). Fine structure of nerve fibers and growth cones of isolated sympathetic neurons in culture. *J. Cell Biol.* **56,** 713–735.

Bunge, R. P. (1983). Aspects of Schwann cell and fibroblast function relating to central nervous system regeneration. *In* "Spinal Cord Reconstruction" (C. C. Kao, R. P. Bunge, and P. J. Reier, eds.), pp. 261–270. Raven, New York.

Burgen, A. S. V., and Grafstein, B. (1962). Retinotectal connections after retinal regeneration. *Nature (London)* **196,** 898–899.

Burmeister, D. W., and Grafstein, B. (1985). Removal of optic tectum prolongs the cell body reaction to axotomy in goldfish retinal ganglion cells. *Brain Res.* **327,** 45–51.

Burrell, H. R., Dokas, L. A., and Agranoff, B. W. (1978). RNA metabolism in the goldfish retina during optic nerve regeneration. *J. Neurochem.* **31,** 289–298.

Burrell, H. R., Heacock, A. M., Water, R. D., and Agranoff, B. W. (1979). Increased tubulin messenger RNA in the goldfish retina during optic nerve regeneration. *Brain Res.* **168,** 628–632.

Carlsen, R. C. (1983). Regeneration of sciatic nerve in frogs maintained at 15°C: Failure to sustain regeneration after initiation. *Exp. Neurol.* **82,** 159–171.

Cleveland, D. W., Lopata, M. A., Sherline, P., and Kirschner, M. W. (1981). Unpolymerized tubulin modulates the level of tubulin mRNAs. *Cell* **25,** 537–546.

Constantine-Paton, M., and Law, M. I. (1982). The development of maps and stripes in the brain. *Sci. Am.* **247,** (12), 62–70.

Cragg, B. S. (1970). What is the signal for chromatolysis? *Brain Res.* **23,** 1–21.

Currie, J. R., Grafstein, B., Whitnall, M. H., and Alpert, R. (1978). Axonal transport of lipid in goldfish optic axons. *Neurochem. Res.* **3,** 479–492.

Daniels, M. P. (1972). Colchicine inhibition of nerve fiber formation *in vitro*. *J. Cell Biol.* **53,** 164–176.

Deaton, M. A., and Freeman, J. A. (1983). Glial cells secrete specific proteins during regeneration of the goldfish optic nerve which may play a role in axon growth. *Soc. Neurosci. Abstr.* **9,** 693.

Dokas, L. A., Kohsaka, S., Burrell, H. R., and Agranoff, B. W. (1981). Uridine metabolism in the goldfish retina during optic nerve regeneration: Whole retina studies. *J. Neurochem.* **36,** 1160–1165.

Easter, S. S., and Schmidt, J. T. (1977). Reversed visuomotor behavior mediated by induced ipsilateral retinal projections in goldfish. *J. Neurophysiol.* **40,** 1245–1254.

Edwards, D. L., and Grafstein, B. (1983). Intraocular tetrodotoxin in goldfish hinders optic nerve regeneration. *Brain Res.* **269,** 1–14.

Edwards, D. L., and Grafstein, B. (1984). Intraocular injection of tetrodotoxin in goldfish decreases fast axonal transport of [^{3}H]-glucosamine-labeled materials in optic axons. *Brain Res.* **299,** 190–194.

Edwards, D. L., and Grafstein, B. (1986). Intraocular tetrodotoxin reduces axonal transport and transcellular transfer of adenosine and other nucleosides in the visual system of the goldfish. *Brain Res.*, in press.

Edwards, D. L., Alpert, R. M., and Grafstein, B. (1981). Recovery of vision in regeneration of goldfish optic axons: Enhancement of axonal outgrowth by a conditioning lesion. *Exp. Neurol.* **72,** 672–686.

Edwards, M. A., and Murray, M. (1985). Selective retinal reinnervation of a surgically created tectal island in goldfish. II. Electron microscopic analysis. *J. Comp. Neurol.* **232,** 386–400.

Edwards, M. A., Sharma, S. C., and Murray, M. (1985). Selective retinal reinnervation of a surgically created tectal island in goldfish. I. Light microscopic analysis. *J. Comp. Neurol.* **232,** 372–385.

Elam, J. S., and Agranoff, B. W. (1971). Rapid transport of protein in the optic system of the goldfish. *J. Neurochem.* **18,** 375–387.

Engh, C. A., Schofield, B. H., Doty, S. B., and Robinson, R. A. (1971). Perikaryal synthetic function following reversible and irreversible peripheral axon injuries as shown by radioautography. *J. Comp. Neurol.* **142,** 465–480.

Feldman, E. L., Axelrod, D., Schwartz, M., Heacock, A. M., and Agranoff, B. W. (1981). Studies on the localization of newly added membrane in growing neurites. *J. Neurobiol.* **12,** 591–598.

Fisher, L. J., and Easter, S. S., Jr. (1979). Retinal synaptic arrays: Continuing development in the adult goldfish. *J. Comp. Neurol.* **185,** 373–379.

Forman, D. S. (1983). Axonal transport and nerve regeneration: A review. *In* "Spinal Cord Reconstruction" (C. C. Kao, R. P. Bunge, and P. J. Reier, eds.), pp. 75–86. Raven, New York.

Forman, D. S., McQuarrie, I. G., Grafstein, B., and Edwards, D. L. (1981). Effect of a conditioning lesion on axonal regeneration and recovery of function. *In* "Mechanisms of Recovery Following Lesions in Sensorimotor Systems" (W. Precht and H. Flohr, eds.), pp. 103–113. Springer-Verlag, Berlin and New York.

Fraser, S. E., Murray, B. A., Chuong, C.-M., and Edelman, G. M. (1984). Alteration of the

retinotectal map in *Xenopus* by antibodies to neural cell adhesion molecules. *Proc. Natl. Acad. Sci. U.S.A.* **81,** 4222–4226.

Freeman, J. A. (1985). Regenerating optic nerves produce steady currents at their tips. *Soc. Neurosci. Abstr.* **11,** 586.

Freeman, J. A., Schmidt, J. A., and Oswald, R. E. (1980). Effect of α-bungarotoxin on retinotectal synaptic transmission in the goldfish and the toad. *Neuroscience* **5,** 929–942.

Gambetti, P., Ingoglia, N. A., Autilio-Gambetti, L., and Weis, P. (1978). Distribution of [^{3}H]-RNA in goldfish optic tectum following intraocular or intracranial injection of [^{3}H]-uridine. Evidence of axonal migration of RNA in regenerating optic fibers. *Brain Res.* **154,** 285–300.

Gaze, R. M. (1970). "The Formation of Nerve Connections." Academic Press, New York.

Gilbert, J. M., Strocchi, P., Brown, B. A., and Marotta, C. A. (1981). Tubulin synthesis in rat forebrain: Studies with free and membrane-bound polysomes. *J. Neurochem.* **36,** 839–846.

Giulian, D. (1978). "A Study of Biochemical Events during Regeneration within the Visual System of *Carassius auratus*." Ph.D. thesis, Rockefeller University, New York.

Giulian, D. (1984a). Target regulation of protein biosynthesis in retinal ganglion cells during regeneration of the goldfish visual system. *Brain Res.* **296,** 198–201.

Giulian, D. (1984b). Peptides from the regenerating central nervous system of goldfish stimulate glia. *Proc. Natl. Acad. Sci. U.S.A.* **81,** 3567–3571.

Giulian, D., Des Ruisseaux, H., and Cowburn, D. (1980). Biosynthesis and intra-axonal transport of proteins during neuronal regeneration. *J. Biol. Chem.* **255,** 6494–6501.

Goldberg, S., and Frank, B. (1980). Will central nervous system axons in the adult mammal regenerate after bypassing a lesion? A study in the mouse and chick visual systems. *Exp. Neurol.* **70,** 675–689.

Gorio, A., Marini, P., and Zanoni, R. (1983). Muscle Reinnervation. III. Motoneuron sprouting capacity, enhancement by exogenous gangliosides. *Neuroscience* **8,** 417–429.

Gorio, A., Janigro, D., and Zanoni, R. (1984). Neuritogenesis and regeneration in the nervous system: An overview of the problem and on the promoting action of gangliosides. *In* "Ganglioside Structure, Function, and Biomedical Potential" (R. W. Ledeen, R. K. Yu, M. M. Rapport, and K. Suzuki, eds.), pp. 465–473. Plenum, New York.

Grafstein, B. (1964). Functional organization in regeneration of amphibian visual pathways. *Bol. Inst. Estud. Med. Biol. (Univ. Nac. Auton. Mex.)* **22,** 217–230.

Grafstein, B. (1971). Role of slow axonal transport in nerve regeneration. *Acta Neuropathol. Suppl.* **V,** 144–152.

Grafstein, B. (1975a). The eyes have it: Axonal transport and regeneration in the optic nerve. *In* "The Nervous System, Vol. 1, The Basic Neurosciences" (D. B. Tower, ed.), pp. 147–151. Raven, New York.

Grafstein, B. (1975b). The nerve cell body's response to axotomy. *Exp. Neurol.* **48,** 32–51.

Grafstein, B. (1977). Axonal transport: The intracellular traffic of the neuron. *In* "Cellular Biology of Neurons, Part 1" (Eric R. Kandel, ed.), Handbook of Physiology, Section 1: The Nervous System, Vol 1, pp. 691–717. Amer. Physiol. Soc., Bethesda, Maryland.

Grafstein, B. (1983). Chromatolysis reconsidered: A new view of the reaction of the nerve cell body to axon injury. In "Nerve, Organ, and Tissue Regeneration: Research Perspectives" (F. O. Seil, ed.), pp. 37–50. Academic Press, New York.

Grafstein, B., and Alpert, R. (1976). Early changes in the metabolism of goldfish retinal ganglion cells following axotomy. *Soc. Neurosci. Abstr.* **2,** 46.

Grafstein, B., and Alpert, R. M. (1982). Properties of slow axonal transport: Studies in goldfish optic axons. *In* "Axoplasmic Transport" (D. G. Weiss, ed.), pp. 226–331. Springer-Verlag, Berlin and New York.

Grafstein, B., and Burgen, A. S. V. (1964). Pattern of optic nerve connections following retinal regeneration. *Prog. Brain Res.* **6,** 126–138.

Grafstein, B., and Edwards, D. L. (1982). Effect of physiological activity in goldfish optic axons on axonal transport of protein and nucleosides. *In* "Axoplasmic Transport in Physiology and Pathology" (D. G. Weiss and A. Gorio, eds.), pp. 21–26. Springer-Verlag, Berlin and New York.

Grafstein, B., Edwards, D. L., and Alpert, R. M. (1981). Axonal transport and neuronal activity. *In* "International Cell Biology 1980–81" (H. G. Schweiger, ed.), pp. 728–736. Springer Verlag, Berlin and New York.

Grafstein, B., and Forman, D. S. (1980). Intracellular transport in neurons. *Physiol. Rev.* **60,** 1167–1283.

Grafstein, B., and Ingoglia, N. A. (1982). Intracranial transection of the optic nerve in adult mice: Preliminary observations. *Exp. Neurol.* **76,** 318–330.

Grafstein, B., and McQuarrie, I. G. (1978). The role of the nerve cell body in axonal regeneration. *In* "Neuronal Plasticity" (C. W. Cotman, ed.), pp. 155–195. Raven, New York.

Grafstein, B., Miller, J. A., Ledeen, R. W., Haley, J., and Specht, S. C. (1975). Axonal transport of phospholipid in goldfish optic system. *Exp. Neurol.* **46,** 261–281.

Grafstein, B., and Murray, M. (1969). Transport of protein in goldfish optic nerve during regeneration. *Exp. Neurol.* **25,** 494–508.

Grafstein, B., Murray, M., and Ingoglia, N. A. (1972). Protein synthesis and axonal transport in retinal ganglion cells of mice lacking visual receptors. *Brain Res.* **44,** 37–48.

Grafstein, B., Yip, H. K., and Meiri, H. (1983). Techniques for improving axonal regeneration: Assay in goldfish optic nerve. *In* "Nervous System Regeneration" (B. Haber, A. M. Giuffrida-Stella, J. R. Perez-Polo, and G. Hashim, eds.), pp. 105–118. Liss, New York.

Griffin, J. W., Price, D. L., Drachman, D. B., and Morris, J. (1981). Incorporation of axonally transported glycoproteins into axolemma during nerve regeneration. *J. Cell Biol.* **88,** 205–214.

Gundersen, R. W., and Barrett, J. N. (1979). Neuronal chemotaxis: Chick dorsal root axons turn toward high concentrations of nerve growth factor. *Science* **206,** 1079–1080.

Hadani, M., Harel, A., Solomon, A., Belkin, M., Lavie, V., and Schwartz, M. (1984). Substances originating from the optic nerve of neonatal rabbit induce regeneration-associated response in the injured optic nerve of adult rabbit. *Proc. Natl. Acad. Sci. U.S.A.* **81,** 7965–7969.

Hammerschlag, R., Stone, G. C., Bolen, F. A., Lindsey, J. D., and Ellisman, M. H. (1982). Evidence that all newly synthesized proteins destined for fast axonal transport pass through the Golgi apparatus. *J. Cell Biol.* **93,** 568–575.

Heacock, A. M. (1982). Glycoprotein requirement for neurite outgrowth in goldfish retina explants: Effects of tunicamycin. *Brain Res.* **241,** 307–315.

Heacock, A. M., and Agranoff, B. W. (1976). Enhanced labeling of a retinal protein during regeneration of optic nerve in goldfish. *Proc. Natl. Acad. Sci. U.S.A.* **73,** 828–832.

Heacock, A., and Agranoff, B. W. (1982). Protein synthesis and transport in the regenerating goldfish visual system. *Neurochem. Res.* **7,** 771–788.

Heacock, A. M., Klinger, P. D., Seguin, E. B., and Agranoff, B. W. (1984). Cholesterol synthesis and nerve regeneration. *J. Neurochem.* **42,** 987–993.

Hoffman, P. N., and Lasek, R. J. (1975). The slow component of axonal transport. Identification of major structural polypeptides of the axon and their generality among mammalian neurons. *J. Cell Biol.* **66,** 351–366.

Hoffman, P. N., Griffin, J. W., and Price, D. L. (1984). Control of axon caliber by neurofilament transport. *J. Cell Biol.* **99,** 705–714.

Ingoglia, N. A. (1982). 4S RNA in regenerating optic axons of goldfish. *J. Neurosci.* **2,** 331–338.

Ingoglia, N. A., Grafstein, B., McEwen, B. S., and McQuarrie, I. G. (1973). Axonal transport of radioactivity in the goldfish optic system following intraocular injection of labeled RNA precursors. *J. Neurochem.* **20,** 1605–1615.

Ingoglia, N. A., Weis, P., and Mycek, J. (1975). Axonal transport of RNA during regeneration of optic nerves of goldfish. *Brain Res.* **112,** 371–381.

Ingoglia, N. A., Sturman, J. A., and Eisner, R. A. (1977). Axonal transport of putrescine, spermidine and spermine in normal and regenerating goldfish optic nerves. *Brain Res.* **130,** 371–381.

Ingoglia, N. A., Sharma, S. C., Pilchman, J., Baranowski, K., and Sturman, J. A. (1982). Axonal transport and transcellular transfer of nucleosides and polyamines in intact and regenerating optic nerves of goldfish: Speculation on the axonal regulation of periaxonal cell metabolism. *J. Neurosci.* **2,** 1412–1423.

Ingoglia, N. A., Giuditta, S., Zanakis, M. F., Babigian, A., Tasaki, I., Chakraborty, G., and Sturman, J. A. (1983). Incorporation of ^{3}H-amino acids into proteins in a partially purified fraction of axoplasm: Evidence for transfer RNA-mediated, post-translational protein modification in squid giant axons. *J. Neurosci.* **3,** 2463–2473.

Jacobson, M. (1978). "Developmental Neurobiology," 2nd Ed. Plenum, New York.

Jacobson, M., and Gaze, R. M. (1965). Selection of appropriate tectal connections by regenerating optic nerve fibers in adult goldfish. *Exp. Neurol.* **13,** 418–430.

Johns, P. R., and Easter, S. S. (1977). Growth of the adult goldfish eye. II. Increase in retinal cell number. *J. Comp. Neurol.* **176,** 331–342.

Johnson, J. E., and Turner, J. E. (1982). Growth from regenerating goldfish retinal cultures in the absence of serum or humoral supplements: Tissue extract effect. *J. Neurosci. Res.* **8,** 315–329.

Keirstead, S. A., Vidal-Sanz, M., Rasminsky, M., Aguayo, A. J., Levesque, M., and So, K.-F. (1985). Responses to visual stimuli of rat retinal ganglion cells regenerating axons into peripheral nerve grafts. *Soc. Neurosci. Abstr.* **11,** 254.

Kilmer, S. L., and R. C. Carlsen (1984). Forskolin activation of adenylate cyclase *in vivo* stimulates nerve regeneration. *Nature (London)* **307,** 455–457.

Koenig, E., Kinsman, S., Repasky, E., and Sultz, L. (1985). Rapid mobility of motile varicosities and inclusions containing α-spectrin, actin, and calmodulin in regenerating axons *in vitro*. *J. Neurosci.* **5,** 715–729.

Kohsaka, S., Dokas, L. A., and Agranoff, B. W. (1981a). Uridine metabolism in the goldfish retina during optic nerve regeneration: Cell-free preparations. *J. Neurochem.* **36,** 1166–1174.

Kohsaka, S., Schwartz, M., and Agranoff, B. W. (1981b). Increased activity of ornithine decarboxylase in goldfish following optic nerve crush. *Dev. Brain Res.* **1,** 391–401.

Kohsaka, S., Heacock, A. M., Klinger, P. D., Porta, R., and Agranoff, B. W. (1982). Dissociation of enhanced ornithine decarboxylase activity and optic nerve regeneration in goldfish. *Dev. Brain Res.* **4,** 149–156.

Kristensson, K. (1984). Retrograde signaling after nerve injury. *In* "Axonal Transport in Neuronal Growth and Regeneration" (J. S. Elam and P. Cancalon, eds.), pp. 31–43. Plenum, New York.

Kuno, M., and Llinás, R. (1970). Enhancement of synaptic transmission by dendritic potentials in chromatolysed motoneurones of the cat. *J. Physiol. (London)* **210,** 807–821.

Landreth, G. E., and Agranoff, B. W. (1979). Explant culture of adult goldfish retina: A model for the study of CNS regeneration. *Brain Res.* **161,** 39–53.

Lanners, H. N., and Grafstein, B. (1980a). Early stages of axonal regeneration in goldfish optic tract: An electron microscopic study. *J. Neurocytol.* **9,** 733–751.

Lanners, H. N., and Grafstein, B. (1980b). Effect of a conditioning lesion on regeneration of goldfish optic axons: Ultrastructural evidence of enhanced outgrowth and pinocytosis. *Brain Res.* **196,** 547–553.

Lasek, R. J., McQuarrie, I. G., and Wujek, J. R. (1981). The central nervous system regeneration problem: Neuron and environment. *In* "Posttraumatic Nerve Regeneration: Experimental Basis and Clinical Applications" (A. Gorio, H. Millesi, and S. Mingrino eds.), pp. 59–70. Raven, New York.

Lavie, V., Sharma, S., Harel, A., Lobel, D., Solomon, A., Doron, A., Belkin, M., and Schwartz,

M. (1985). Substances originating from growing nerves induce morphological responses characteristic of regeneration in injured optic nerve of adult rabbit. *Soc. Neurosci. Abstr.* **11,** 1252.

Ledeen, R. W. (1984). Biology of gangliosides: Neuritogenic and neuronotrophic properties. *J. Neurosci. Res.* **12,** 147–159.

Leon, A., Facci, L., Toffano, G., Sonnino, S., and Tettamanti, G. (1981). Activation of (Na^+,K^+)-ATPase by nanomolar concentrations of GM_1 ganglioside. *J. Neurochem.* **37,** 350–357.

Letourneau, P. C. (1975). Cell-to-substratum adhesion and guidance of axonal elongation. *Dev. Biol.* **44,** 92–101.

Letourneau, P. C. (1982). Analysis of microtubule number and length in cytoskeletons of cultured chick sensory neurons. *J. Neurosci.* **2,** 806–811.

Levine, J., Skene, P., and Willard, M. (1981). GAPs and fodrin. Novel axonally transported proteins. *Trends Neurosci.* **4,** 273–277.

Levine, R., and Jacobson, M. (1975). Discontinuous mapping of retina onto tectum innervated by both eyes. *Brain Res.* **98,** 172–176.

Levine, R. L., Hogenraad, N. J., and Kretchmer, N. (1974). A review: Biological and clinical aspects of pyrimidine metabolism. *Pediatr. Res.* **8,** 724–734.

Lieberman, A. R. (1971). The axon reaction: A review of the principal features of perikaryal responses to axon injury. *Int. Rev. Neurobiol.* **14,** 49–124.

Lindquist, T. D., and Ingoglia, N. A. (1979). Evidence that 4S RNA is axonally transported in normal and regenerating rat sciatic nerves. *Brain Res.* **166,** 95–112.

Llinás, R., and Sugimori, M. (1979). Calcium conductances in Purkinje cell dendrites: Their role in development and integration. *Prog. Brain Res.* **51,** 323–334.

Lo, R. Y. S., and Levine, R. (1980). Time course and pattern of optic fiber regeneration following tectal lobe removal in the goldfish. *J. Comp. Neurol.* **191,** 295–314.

McEwen, B., Forman, D., and Grafstein, B. (1971). Components of fast and slow transport in the goldfish optic nerve. *J. Neurobiol.* **2,** 361–377.

McQuarrie, I. G. (1983). Role of the axonal cytoskeleton in the regenerating nervous system. *In* "Nerve, Organ and Tissue Regeneration: Research Perspectives" (F. J. Seil, ed.), pp. 51–88. Academic Press, New York.

McQuarrie, I. G. (1984). Effect of a conditioning lesion on axonal transport during regeneration. The role of slow transport. *In* "Axonal Transport in Neuronal Growth" (J. S. Elam and P. Cancalon, eds.), pp. 185–209. Plenum, New York.

McQuarrie, I. G. (1985). Stages of axonal regeneration following optic nerve crush in goldfish: Contrasting effects of conditioning nerve lesions and intraocular acetoxycycloheximide injections. *Brain Res.* **333,** 247–253.

McQuarrie, I. G., and Grafstein, B. (1973). Enhancement of axon outgrowth by a previous nerve injury. *Arch. Neurol.* **29,** 53–55.

McQuarrie, I. G., and Grafstein, B. (1981). Effect of a conditioning lesion on optic nerve regeneration in goldfish. *Brain Res.* **216,** 253–264.

McQuarrie, I. G., and Grafstein, B. (1982a). Protein synthesis and fast axonal transport in regenerating goldfish retinal ganglion cells. *Brain Res.* **235,** 213–223.

McQuarrie, I. G., and Grafstein, B. (1982b). Protein synthesis and axonal transport in goldfish retinal ganglion cells during regeneration accelerated by a conditioning lesion. *Brain Res.* **251,** 25–37.

McQuarrie, I. G., and Grafstein, B. (1983). Effect of acetoxycycloheximide and dibutyryladenosine cyclic 3′:5′-monophosphate on axonal regeneration in the goldfish optic nerve. *Brain Res.* **279,** 377–381.

McQuarrie, I. G., and Lasek, R. J. (1981). Axonal transport of labeled neurofilament proteins in goldfish optic axons. *J. Cell Biol.* **91,** 234a.

McQuarrie, I. G., Grafstein, B., and Gershon, M. D. (1977). Axonal regeneration in the rat sciatic nerve: Effect of a conditioning lesion and of db cAMP. *Brain Res.* **132,** 443–453.

Madison, R., Sidman, R. L., Chiu, T. H., and Nyllas, E. (1983). Bioresorbable nerve guides bridge transected optic nerve. *Soc. Neurosci. Abstr.* **9,** 770.

Maier, W., and Wolburg, H. (1979). Regeneration of the goldfish retina after exposure to different doses of ouabain. *Cell Tissue Res.* **202,** 99–118.

Marani, E., and Ruigrok, T. J. H. (1984). Enzyme histochemical changes in retinal ganglion cells and the optic nerve after goldfish optic nerve lesion. A regenerative and hypertrophic phenomena study. *Acta Morphol. Neerl. Scand.* **22,** 97–114.

Maxwell, J. K., and Elam, J. S. (1980). Amino acid incorporation into proteins of degenerating and regenerating goldfish optic tracts. *Exp. Neurol.* **67,** 118–130.

Mayes, B. N., and Freeman, J. A. (1984). Electric fields affect active transport of proteins in neurons. *Soc. Neurosci. Abstr.* **10,** 39.

Meiri, H., and Grafstein, B. (1984). Local application of calcium-modulating agents to a crushed goldfish optic nerve modifies visual recovery. *Exp. Neurol.* **83,** 403–413.

Meyer, R. L. (1978). Evidence from thymidine labeling for continuing growth of retina and tectum in juvenile goldfish. *Exp. Neurol.* **59,** 99–111.

Meyer, R. L. (1980). Mapping the normal and regenerating retinotectal projection of goldfish with autoradiographic methods. *J. Comp. Neurol.* **189,** 273–289.

Meyer, R. L. (1983). Tetrodotoxin inhibits the formation of refined retinotopography in goldfish. *Dev. Brain Res.* **6,** 293–298.

Minchin, M. C. W. (1980). Veratrum alkaloids as transmitter releasing agents. *J. Neurosci. Methods* **2,** 111–121.

Misantone, L. J., Gershenbaum, M., and Murray, M. (1984). Viability of retinal ganglion cells after optic nerve crush in adult rats. *J. Neurocytol.* **13,** 449–465.

Mizrachi, Y., and Schwartz, M. (1982). Goldfish tectal explants have a growth-promoting effect on neurites emerging from co-cultured regenerating retina explants. *Dev. Brain Res.* **3,** 502–505.

Mizrachi, Y., Neuman, D., Sharma, S., and Schwartz, M. (1984). Target-dependent and independent stages in regeneration. *Brain Res.* **322,** 115–118.

Mizrachi, Y., Rubinstein, M., Kimhi, Y., and Schwartz, M. (1986). Goldfish brain derived neurotrophic factor: Purification and characterization. *J. Neurosci. Res.*, in press.

Murray, M. (1973). ^{3}H-uridine incorporation by regenerating retinal ganglion cells of goldfish. *Exp. Neurol.* **39,** 489–497.

Murray, M. (1976). Regeneration of retinal axons into the goldfish optic tectum. *J. Comp. Neurol.* **168,** 175–196.

Murray, M. (1977). Delayed regeneration of the retino-diencephalic projections in the goldfish, *Carassius auratus*. *Brain Res.* **125,** 149–153.

Murray, M. (1982). A quantitative study of regenerative sprouting by optic axons in goldfish. *J. Comp. Neurol.* **209,** 352–362.

Murray, M., and Edwards, M. A. (1982). A quantitative study of the reinnervation of the goldfish optic tectum following optic nerve crush. *J. Comp. Neurol.* **109,** 363–373.

Murray, M., and Forman, D. S. (1971). Fine structural changes in goldfish retinal ganglion cells during axonal regeneration. *Brain Res.* **32,** 287–298.

Murray, M., and Grafstein, B. (1969). Changes in the morphology and amino acid incorporation of regenerating goldfish optic neurons. *Exp. Neurol.* **23,** 544–560.

Murray, M., Sharma, S. C., and Edwards, M. A. (1980). Target regulation of synaptic number in the compressed retinotectal projection of goldfish. *J. Comp. Neurol.* **209,** 374–385.

Neuman, D., Yerushalmi, A., and Schwartz, M. (1983). Inhibition of non-neuronal cell proliferation in the goldfish visual pathway affects the regenerative capacity of the retina. *Brain Res.* **272,** 237–245.

Neumann, D., Scherson, T., Ginzburg, J., Littauer, U. Z., and Schwartz, M. (1983). Regulation of mRNA levels of microtubule proteins during nerve regeneration. *FEBS Lett.* **162,** 270–276.

Nissl, F. (1892). Ueber die Veränderungen der Ganglienzellen am Facialiskern des Kaninchens nach Ausreissung der Nerven. *Allg. Z. Psychiatr.* **48,** 197–198.

Olmstead, J. B., Cox, J. V., Asnes, C. F., Parysek, L. M., and Lyon, H. D. (1984). Cellular regulation of microtubule organization. *J. Cell Biol.* **99,** 285–325.

Patel, N., and Poo, M.-M. (1982). Orientation of neurite growth by extracellular electric fields. *J. Neurosci.* **2,** 483–496.

Perry, G. W., Burmeister, D. W., and Grafstein, B. (1985). Changes in protein content of goldfish optic nerve during degeneration and regeneration following nerve crush. *J. Neurochem.* **44,** 1142–1151.

Perry, G. W., Burmeister, D. W., and Grafstein, B. (1986a). Labelling of proteins in fast axonal transport during regeneration of goldfish optic nerve. In preparation.

Perry, G. W., Burmeister, D. W., and Grafstein, B. (1986b). Effect of target removal on labelling of proteins in fast axonal transport during regeneration of goldfish optic nerve. In preparation.

Perry, G. W., Antonian, E., and Grafstein, B. (1986c). Labelling of proteins in slow axonal transport during regeneration of goldfish optic nerve. In preparation.

Peters, A., and Vaughan, J. E. (1967). Microtubules and filaments in the axons and astrocytes of early postnatal rat optic nerves. *J. Cell Biol.* **32,** 113–119.

Pilar, G., and Landmesser, L. (1972). Axotomy mimicked by localized colchicine application. *Science* **177,** 1116–1118.

Plagemann, P. G. W., and Richey, D. P. (1974). Transport of nucleosides, nucleic acid bases, choline and glucose by animal cells in culture. *Biochim. Biophys. Acta* **344,** 263–305.

Politis, M. J., Pellegrino, R. G., Oaklander, A. L., and Ritchie, J. M. (1983). Reactive glial protein synthesis and early disappearance of saxitoxin binding in degenerating rat optic nerve. *Brain Res.* **273,** 392–395.

Politis, M. J., and Spencer, P. S. (1982). Regeneration of rat optic axons into adjacent peripheral nerve grafts. *Soc. Neurosci. Abstr.* **8,** 761.

Purves, D., and Njå, A. (1976). Effect of nerve growth factor in synaptic depression after axotomy. *Nature (London)* **260,** 535–536.

Quitschke, W., and Schechter, N. (1983). *In vitro* protein synthesis in the goldfish retinotectal pathway during regeneration: Evidence for specific axonal proteins of retinal origin in the optic nerve. *J. Neurochem.* **41,** 1137–1142.

Quitschke, W., and Schechter, N. (1984). 58,000 Dalton intermediate filament proteins of neuronal and nonneuronal origin in the goldfish visual pathway. *J. Neurochem.* **42,** 569–576.

Rachailovich, I., and Schwartz, M. (1984). Molecular events associated with increased regenerative capacity of the goldfish retinal ganglion cells following X-irradiation: Decreased level of axonal growth inhibitors. *Brain Res.* **306,** 149–155.

Rachailovich, I., Stein-Izsak, C., and Schwartz, M. (1984). Regulation at the mRNA level of regeneration-associated changes in the synthetic products of non-neuronal cells surrounding goldfish optic nerve. *Soc. Neurosci. Abstr.* **10,** 1031.

Ramon y Cajal, S. (1928). "Degeneration and Regeneration of the Nervous System" (R. M. May, trans.), Vol. I. Oxford Univ. Press, London and New York.

Reis, D. J., Ross, R. A., Gilad, G., and Joh, T. H. (1978). Reaction of central catecholaminergic neurons to injury: Model systems for studying the neurobiology of central regeneration and sprouting. *In* "Neuronal Plasticity" (C. W. Cotman, ed.), pp. 197–226. Raven, New York.

Reyer, R. W. (1977). The amphibian eye: Development and regeneration. *In* "The Visual System in Vertebrates" (F. Crescitelli, ed.), pp. 331–390. Springer Verlag, Berlin and New York.

Richardson, P. M., Issa, V. M. K., and Shemie, S. (1982). Regeneration and retrograde degeneration of axons in the rat optic nerve. *J. Neurocytol.* **11,** 949–966.

Russell, D. H. (1980). Ornithine decarboxylase as a biological and pharmacological tool. *Pharmacology* **20,** 117–129.

Sandoval, I. V., Bonifacino, J. S., Klausner, R. D., Henkart, M., and Wehland, J. (1984). Role of microtubules in the organization and localization of the Golgi apparatus. *J. Cell Biol.* **99,** 113s–118s.

Sbaschnig-Agler, M., Ledeen, R. W., Grafstein, B., and Alpert, R. M. (1984). Ganglioside changes in the regenerating goldfish optic system: Comparison with glycoproteins and phospholipids. *J. Neurosci. Res.* **12,** 221–232.

Sbaschnig-Agler, M., Ledeen, R. W., Alpert, R. M., and Grafstein, B. (1985). Changes in axonal transport of phospholipids in the regenerating goldfish optic system. *Neurochem. Res.* **10,** 1499–1509.

Schechter, N., Francis, A., Deutsch, D. G., and Gazzaniga, M. S. (1979). Recovery of tectal nicotinic–cholinergic receptor sites during optic nerve regeneration in goldfish. *Brain Res.* **166,** 57–64.

Schiff, P. B., and Horwick, S. G. (1980). Taxol stabilizes microtubules in mouse fibroblast cells. *Proc. Natl. Acad. Sci. U.S.A.* **77,** 1561–1565.

Schlaepfer, W. W., and Zimmerman, U.-J. P. (1984). Calcium-activated protease and the regulation of the axonal cytoskeleton. *In* "Axonal Transport in Neuronal Growth and Regeneration" (J. S. Elam and P. Cancalon, eds.), pp. 261–273. Plenum, New York.

Schmidt, J. T. (1982). The formation of retinotectal projections. *Trends Neurosci.* **5,** 111–115.

Schmidt, J. T., and Edwards, D. L. (1983). Activity sharpens the map during the regeneration of the retinotectal projection in goldfish. *Brain Res.* **269,** 29–39.

Schmidt, J. T., and Eisele, L. E. (1983). Goldfish stroboscopic illumination: Regeneration in a flash fails to sharpen the retinotectal map. *Soc. Neurosci. Abstr.* **9,** 858.

Schmidt, J. T., Cicerone, C. M., and Easter, S. S. (1978). Expansion of the half retinal projection to the tectum in goldfish: An electrophysiological and anatomical study. *J. Comp. Neurol.* **177,** 257–277.

Schmidt, J. T., Edwards, D. L., and Stuermer, C. L. (1983). The re-establishment of synaptic transmission by regenerating optic axons in goldfish: Time course and effects of blocking activity by intraocular injection of tetrodotoxin. *Brain Res.* **269,** 15–27.

Schwab, M. E., and Thoenen, H. (1985). Dissociated neurons regenerate into sciatic but not optic nerve explants in culture irrespective of neurotrophic factors. *J. Neurosci.* **5,** 2415–2423.

Schwartz, M., Vogel, Z., and Kalcheim, C. (1981). Goldfish brain extract induces aggregation of acetylcholine receptors on cultured myotubes: Increased activity following optic nerve axotomy. *Neurosci. Lett.* **27,** 165–170.

Schwartz, M., Mizrachi, Y., and Eshhar, N. (1982a). Factor(s) from goldfish brain induce neuritic outgrowth from explanted regenerating retinas. *Dev. Brain Res.* **3,** 29–35.

Schwartz, M., Sela, B.-A., and Eshhar, N. (1982b). Antibodies to gangliosides and myelin autoantigens are produced in mice following sciatic nerve injury. *J. Neurochem.* **38,** 1192–1195.

Schwartz, M., Belkin, M., Harel, A., Solomon, A., Lavie, V., Hadani, M., Rachailovich, I., and Stein-Izsak, C. (1985). Regenerating fish optic nerves and a regeneration-like response in injured optic nerves of adult rabbits. *Science* **228,** 600–603.

Sharma, S. C. (1973). Anomalous retinal projection after removal of contralateral optic tectum in adult goldfish. *Exp. Neurol.* **41,** 661–669.

Shaw, B., and Bray, D. (1977). Movement and extension of isolated growth cones. *Exp. Cell Res.* **104,** 55–62.

Siegel, G. J., Stahl, W. L., and Swanson, P. D. (1981). Ion transport. *In* "Basic Neurochemistry" (B. W. Agranoff and R. Katzman, eds.), 3rd Ed., pp. 107–143. Little, Brown, Boston, Massachusetts.

Skene, J. H. P., and Shooter, E. (1983). Denervated sheath cells secrete a new protein after nerve injury. *Proc. Natl. Acad. Sci. U.S.A.* **80,** 4119–4173.

Skene, J. P., and Willard, M. (1981a). Changes in axonally transported proteins during axon regeneration in toad retinal ganglion cells. *J. Cell Biol.* **89,** 86–95.

Skene, J. P., and Willard, M. (1981b). Characteristics of growth-associated polypeptides in regenerating toad retinal ganglion cell axons. *J. Neurosci.* **4,** 419–426.

Singer, P. A., Mehler, S., and Fernandez, H. L. (1982). Blockade of retrograde axonal transport delays the onset of metabolic and morphologic changes induced by axotomy. *J. Neurosci.* **2,** 1299–1306.

Small, R. K., Blank, M., Ghez, R., and Pfenninger, K. H. (1984). Components of the plasma membrane of growing axons. II. Diffusion of membrane protein complexes. *J. Cell Biol.* **98,** 1434–1443.

Snipes, G. J., and Freeman, J. A. (1984). Characterization of the 37 kD protein associated with nerve development and injury. *Soc. Neurosci. Abstr.* **10,** 1029.

So, K.-F., and Aguayo, A. J. (1985). Lengthy regrowth of cut axons from ganglion cells after peripheral nerve transplantation into the retina of adult rats. *Brain Res.* **328,** 349–354.

Sparrow, J. R., and Grafstein, B. (1982). Sciatic nerve regeneration in ganglioside-treated rats. *Exp. Neurol.* **77,** 230–235.

Sparrow, J. R., McGuinness, C., Schwartz, M., and Grafstein, B. (1984). Antibodies to gangliosides inhibit goldfish optic nerve regeneration *in vivo*. *J. Neurosci. Res.* **12,** 233–243.

Sparrow, J. R., McGuinness, C. M., and Grafstein, B. (1985). Goldfish optic nerve regeneration with impaired axonal transport. *Anat. Rec.* **211,** 182A.

Sparrow, J. F., Edwards, D. L., McGuinness, C. M., Sloane, D. A., and Grafstein, B. (1986). Effect of intraocular TTX on fast axonal transport in regenerating goldfish optic nerve. In preparation.

Sperry, R. W. (1943). Visuomotor coordination in the newt (*Triturus viridescens*) after regeneration of the optic nerves. *J. Comp. Neurol.* **79,** 33–55.

Sperry, R. W. (1948). Patterning of central synapses in regeneration of the optic nerve in teleosts. *Physiol. Zool.* **21,** 351–361.

Sperry, R. W. (1963). Chemoaffinity in the orderly growth of nerve fiber patterns and connections. *Proc. Natl. Acad. Sci. U.S.A.* **50,** 703–710.

Spirman, N., Sela, B.-A., and Schwartz (1982). Antiganglioside antibodies inhibit neuritic outgrowth from regenerating goldfish retinal explants. *J. Neurochem.* **39,** 874–877.

Spirman, N., Sela, B.-A., Gitler, C., Calef, E., and Schwartz, M. (1984). Regenerative capacity of the goldfish visual system is affected by antibodies specific to gangliosides injected intraocularly. *J. Neuroimmunol.* **6,** 197–207.

Spitzer, N. C. (1979). Ion channels in development. *Annu. Rev. Neurosci.* **2,** 363–397.

Springer, A. D. (1980). Aberrant regeneration in goldfish after crushing one optic nerve. *Brain Res.* **199,** 214–218.

Springer, A. D., and Agranoff, B. W. (1977). Effect of temperature on rate of goldfish optic nerve regeneration: A radioautographic and behavioral study. *Brain Res.* **128,** 405–415.

Springer, A. D., and Cohen, S. M. (1981). Optic fiber segregation in goldfish with two eyes innervating one tectal lobe. *Brain Res.* **225,** 23–36.

Stevenson, J. A., and Yoon, M. G. (1978). Regeneration of optic nerve fibers enhances cell proliferation in the goldfish optic tectum. *Brain Res.* **153,** 345–351.

Strocchi, P., Gilbert, J. M., Benowitz, L. I., Dahl, D., and Lewis, E. R. (1984). Cellular origin and biosynthesis of rat optic nerve proteins: A two-dimensional gel analysis. *J. Neurochem.* **43,** 349–357.

Stuermer, C. A. O. (1984). Rules for retinotectal terminal arborizations in the goldfish optic tectum: A whole-mount study. *J. Comp. Neurol.* **229,** 214–232.

Stuermer, C. A. O., and Easter, S. S., Jr. (1984). A comparison of the normal and regenerated retinotectal pathways of goldfish. *J. Comp. Neurol.* **223,** 57–76.

Szaro, B. G., Loh, Y. P., and Hunt, R. K. (1985). Specific changes in axonally transported proteins during regeneration of the frog (*Xenopus laevis*) optic nerve. *J. Neurosci.* **5,** 192–208.

Taylor, A. C., and Weiss, P. (1965). Demonstration of axonal flow by the movement of tritium-labeled protein in mature optic nerve fibers. *Proc. Natl. Acad. Sci. U.S.A.* **54,** 1521–1527.

Tessler, A., Autilio-Gambetti, L., and Gambetti, P. (1981). Axonal growth during regeneration: A quantitative autoradiographic study. *J. Cell Biol.* **87,** 197–203.

Thoenen, H., and Barde, Y.-A. (1980). Physiology of nerve growth factor. *Physiol. Rev.* **60,** 1284–1335.

Tumosa, N., and Stell, W. K. (1986). Are the ganglion cells in the goldfish retina cholinergic? *J. Neurosci.*, in press.

Turner, J. E., Delaney, R. K., and Johnson, J. E. (1980). Retinal ganglion cell response to nerve growth factor and its antisera in the intact and regenerating visual system of the goldfish. *Brain Res.* **197,** 319–330.

Turner, J. E., Delaney, R. K., and Johnson, J. E. (1981). Retinal ganglion cell response to axotomy and nerve growth factor antiserum treatment in the regenerating visual system of the goldfish (*Carassius auratus*): An *in vivo* and *in vitro* analysis. *Brain Res.* **204,** 284–294.

Udin, S. B., and Gaze, R. M. (1983). Expansion and retinotopic order in the goldfish retinotectal map after large retinal lesions. *Exp. Brain Res.* **50,** 347–352.

Varon, S., and Adler, R. (1981). Trophic and specifying factors directed to neuronal cells. *Adv. Cell. Neurobiol.* **2,** 115–163.

Watson, W. E. (1968). Observations on the nucleolar and total cell body nucleic acid of injured nerve cells. *J. Physiol. (London)* **196,** 655–676.

Watson, W. E. (1969). The response of motor neurones to intramuscular injection of botulinum toxin. *J. Physiol. (London)* **202,** 611–630.

Wehland, J., and Willingham, M. C. (1983). A rat monoclonal antibody reacting specifically with the tyrosylated form of α-tubulin. II. Effects on cell movement, organization of microtubules, and intermediate filaments, and arrangement of Golgi elements. *J. Cell Biol.* **97,** 1476–1490.

Weis, J. J. (1968). The occurrence of nerve growth factor in teleost fishes. *Experientia* **24,** 736–737.

Weiss, P. (1934). *In vitro* experiments on the factors determining the course of the outgrowing nerve fiber. *J. Exp. Zool.* **68,** 393–448.

White, W. R., and Grafstein, B. (1974). Changes in goldfish retinal ganglion cells following intraocular injection of vincristine. *Soc. Neurosci., 4th Annu. Meet.*, 477.

Whitnall, M. H., and Grafstein, B. (1981). The relationship between extracellular amino acids and protein synthesis is altered during axonal regeneration. *Brain Res.* **220,** 362–366.

Whitnall, M., and Grafstein, B. (1982). Perikaryal routing of newly synthesized proteins in regenerating neurons: Quantitative electron microscopic autoradiography. *Brain Res.* **239,** 41–56.

Whitnall, M. H., and Grafstein, B. (1983). Changes in perikaryal organelles during axonal regeneration in goldfish retinal ganglion cells: An analysis of protein synthesis and routing. *Brain Res.* **272,** 49–56.

Whitnall, M., Currie, J. R., and Grafstein, B. (1982). Bidirectional axonal transport of glycoproteins in goldfish optic nerve. *Exp. Neurol.* **75,** 191–207.

Willard, M., and Skene, J. H. P. (1982). Molecular events in axonal regeneration. *In* "Repair and Regeneration of the Nervous System" (J. G. Nicholls, ed.), pp. 71–89. Dahlem Konferenzen, Springer-Verlag, Berlin and New York.

Williams, L. R., and Agranoff, B. W. (1983). Retrograde transport of goldfish optic nerve proteins labeled by *N*-succinimidyl [^{3}H]propionate. *Brain Res.* **259,** 207–216.

Wolburg, H. (1978). Growth and myelination of goldfish optic nerve fibers after retina regeneration and nerve crush. *Z. Naturforsch.* **33c,** 988–996.

Wujek, J. R., and Lasek, R. J. (1983). Correlation of axonal regeneration and slow component B in two branches of a single axon. *J. Neurosci.* **3,** 243–251.

Yamada, K. M., Spooner, B. S., and Wessells, N. K. (1970). Axon growth: Roles of microfilaments and microtubules. *Proc. Natl. Acad. Sci. U.S.A.* **66,** 1206–1212.

Yip, H. K., and Grafstein, B. (1982). Effect of nerve growth factor on regeneration of goldfish optic axons. *Brain Res.* **238,** 329–339.

Yip, H. K., and Johnson, E. M., Jr. (1983). Retrograde transport of nerve growth factor in lesioned goldfish retinal ganglion cells. *J. Neurosci.* **3,** 2172–2182.

Zanakis, M. F., Eskin, B., and Ingoglia, N. A. (1984). Evidence that multiple species of aminoacylated transfer RNA are present in regenerating optic axons of goldfish. *Neurochem. Res.* **9,** 249–262.

"Bountiful Harvest."

INDEX

D

E

F

G

H

I

S

T

V

W

X

Y