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*Phospholipid Composition and Genesis of
Myeloid Bodies of the Retinal Pigment
Epithelium*

by

Daniel Abran

Submitted in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

at

Dalhousie University
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October, 1991

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Table of Contents

	Page
Title Page	i
Signature Page	ii
Copyright Agreement Form	iii
Dedication	iv
Table of Contents	v
List of Figures	viii
List of Tables	x
Abstract	xi
Acknowledgments	xii
Introduction	1
I. Morphology of the Retinal Pigment Epithelium	3
a) Myeloid bodies of the retinal pigment epithelium	5
b) Phagocytosis of outer segments	11
II. Biochemistry of the Retinal Pigment Epithelium	21
a) Retinol in the retinal pigment epithelium	23
b) Lipid metabolism in the retinal pigment epithelium	26
III. Regeneration	38
Materials and Methods	44
a) Myeloid Body Isolation and Phospholipid Analysis	44
Isolation of retinal pigment epithelial cells	44
Purification of myeloid bodies	45

Isolation of retinal photoreceptor outer segments	47
Lipid extraction and phospholipid separation	47
Methyl ester formation and gas chromatography	48
b) Myeloid Body Morphogenesis in Regenerating Newt Retina	49
Surgical procedure	49
Routine fixation for electron microscopy	50
Morphometrics	51
c) Embryological Newt Retina	52
Results	53
a) Myeloid Body Lipid Composition	53
Myeloid body purification	53
Phospholipid composition of myeloid bodies and photoreceptor outer segments	56
Fatty acyl chain composition of myeloid body and photoreceptor outer segment phospholipids	56
b) Myeloid Body Morphogenesis in the Regenerating Newt Retinal Pigment Epithelium	58
General appearance of the newt retinal pigment epithelium	58
Observations on the regenerating newt retina	63
Observations on the embryogenesis in the newt retina	64
Myeloid body morphogenesis	64
Morphometric analysis of template and myeloid body	66
Discussion	84
a) Techniques Used to Complete this Thesis	84

b) Biochemistry of Chick Myeloid Bodies	86
c) Myeloid Bodies in the Regenerating Newt Retina	94
d) Concluding Remarks	106
References	107

List of Figures

		Page
Figure 1	Electron micrograph of a retinal pigment epithelial cell of a mature newt	8
Figure 2	Electron micrograph of a retinal pigment epithelial cell taken from a 28 days old white Leghorn chicken	8
Figure 3	Diagram showing the process of phagocytosis	16
Figure 4	Electron micrographs of chick retinal pigment epithelial cell subcellular fractions	55
Figure 5	Electron micrograph of the newt RPE after 6 days of regeneration	69
Figure 6	Electron micrograph of the newt RPE after 3 weeks of regeneration	69
Figure 7	Electron micrograph of the newt RPE after 4 weeks of regeneration	69
Figure 8	Electron micrograph of the newt RPE after 5 weeks of regeneration	69
Figure 9	Electron micrograph of the newt RPE after 6 weeks of regeneration	71
Figure 10	Electron micrograph of the newt RPE after 8 weeks of regeneration	71
Figure 11	Electron micrograph of the newt RPE after 14 weeks of regeneration	71
Figure 12	Montage of a series of electron micrographs showing a newt retina after six weeks of regeneration	73

Figure 13	Electron micrograph showing photoreceptor outer segments at six week of regeneration	75
Figure 14	Electron micrograph depicting the morphogenesis of myeloid bodies	77
Figure 15	Changes in the average myeloid body and template sizes in regenerating newt	79
Figure 16	Changes in myeloid body and template numbers in regenerating newt	81
Figure 17	Changes in the fraction of regenerated retinal pigment epithelial cells containing either myeloid bodies or myeloid body templates	83
Figure 18	Schematic representation of a functional hypothesis for retinal pigment epithelium myeloid bodies	96

List of Tables

		Page
Table A	Fatty acid nomenclature glossary	35
Table B	Pathway of linoleic acid series of desaturation and elongation	36
Table C	Pathway of linolenic acid series of desaturation and elongation	37
Table D	Phosphorus analysis of chick myeloid bodies and rod outer segments	59
Table E	Fatty acid composition of glycerophospholipids from chick myeloid bodies	60
Table F	Fatty acid composition of glycerophospholipids from chick rod outer segments	61
Table G	Comparison of fatty acid groups between myeloid bodies and photoreceptor rod outer segments	62

Abstract

Myeloid bodies (MBs) are organelles which are continuous with the smooth endoplasmic reticulum (SER) membranes within the retinal pigment epithelium (RPE) of a number of lower vertebrates. Also, MB numbers in the RPE of the newt have been temporally correlated with phagocytosis of outer segment (OS) disc membranes in previous studies. To test the hypothesis that MBs are directly involved in OS lipid metabolism, I compared the phospholipid composition of a MB-enriched subcellular fraction of chick retinal pigment epithelium (RPE) with that of chick rod outer segments (ROS). The major phospholipids found in MBs were phosphatidylcholine and phosphatidylethanolamine which represented 43% and 34% of the total MB lipids respectively. Sphingomyelin and phosphatidylinositol comprised the remaining detectable phospholipids in MBs. The fatty acyl chain composition of all detected phospholipids showed that the long-chain polyunsaturated fatty acids, 20:4 n-6, 22:5 n-3 and 22:6 n-3, accounted for more than 45% of the fatty acids in MB membranes. The ROS contained, in addition to the phospholipids found in MBs, phosphatidylserine and lyso-phosphatidyl-choline. Further, the distribution of the fatty acids in ROS phospholipids differed substantially from the ones in MBs, especially for 22:6 n-3 and 22:5 n-3. The results from this study demonstrated that chick MBs had a different phospholipid and fatty acid composition than chick ROS. However, they both represent a significantly enriched pool of essential polyunsaturated fatty acids.

I also examined the effects on MBs of eliminating the source of OS membrane lipids (neural retina removal) and of the subsequent return of OS (retinal regeneration) in the newt (*Notophthalmus viridescens*) in order to further characterize the origin and functional significance of these organelles. Light and electron microscopic analysis demonstrated that within six days of neural retina removal, MBs disappeared from the RPE. By the sixth week of regeneration, rudimentary photoreceptor OS were present MBs were still absent from the RPE. However, at this time, the SER in some areas of the RPE cells had become flattened, giving rise to small (0.5 μm long), two to four layer-thick lamellar units which we have described as MB templates. Small MBs were first observed one week later, at the seventh week of retinal regeneration.

This study has revealed that newt MBs are specialized areas of SER. It also showed that contact between functional photoreceptors and the RPE is essential to the presence of MBs in the RPE cell. Although the phospholipid and fatty acid analysis of chick MBs did not conclusively establish the expected link between MBs and ROS, it pioneered an isolation protocol for MBs and provided invaluable information on MB membrane composition as well as suggesting a possible role for MBs in the recycling of essential fatty acids from photoreceptor OS. These findings will, I hope, offer new directions to the search for MBs function in the RPE.

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INTRODUCTION

The retinal pigment epithelium (RPE) is a heavily pigmented single layer of hexagonally-shaped cuboidal cells situated between the choroid and the photoreceptor cells of the retina. The RPE is one of the most important cell layers in the visual system and is vital to the integrity of rod and cone outer segments and to the function of the photoreceptor cells. The RPE and the photoreceptors are intimately associated anatomically and developmentally, to the extent that photoreceptors will fail to form in the absence of the pigment epithelium (Hollyfield and Witkovsky 1974; LaVail and Hild 1971). The RPE also accomplishes many functions such as the absorption of light, the storage and conversion of vitamin A esters, the regulation of metabolites entering and leaving the retina, as well as phagocytosis of rod and cone outer segments. The structure of the RPE and its intimate relationship with the neural sensory retina is uniquely adapted to many of the diverse functions of this cell layer (Zinn and Benjamin-Henkind 1979).

In a number of fish, amphibia, reptiles and birds, the RPE contains lamellar membrane organelles called myeloid bodies (MBs). Most studies on MBs have been morphological in nature, basically describing the relationship of the organelle with other cytoplasmic elements of the RPE cell under varying conditions. There is some controversy with respect to the origin of the MBs, the consensus being that they arise from, and represent specialized areas of the smooth endoplasmic reticulum. However, no systematic study of the genesis of MBs has ever been attempted. Similarly, no attempt has

ever been made to describe the lipid composition of the membranes forming MBs.

This study employed a multidisciplinary approach to examine the structure, function and morphogenesis of MBs. Firstly, an analysis of the phospholipid and fatty acid composition of chick MBs using thin layer and gas chromatography was undertaken, subsequent to the development of a methodology to isolate MB membranes from other RPE cell organelles. By comparing the MB phospholipid composition to that of rod outer segment membrane phospholipids, I hoped to provide a direct relationship between the two membrane systems, and test the hypothesis that ROS and MBs have a similar phospholipid and fatty acid composition, and that this composition might explain the ultrastructural appearance of MBs. Further, it was anticipated that this phospholipid and fatty acid analysis of MBs would allow me to confirm the involvement of MBs in the recycling of ROS fatty acids. Secondly, using the regenerating newt retina as a model, I examined the development of MBs. I paid special attention to any ultrastructural changes occurring in the endoplasmic reticulum membrane organization, since this membrane system is believed to be the source of MBs in the RPE cell. By removing the neural retina of the newt, and allowing the subsequent retinal regeneration to occur, I attempted to demonstrate a relationship between the MBs found in the RPE and the presence of functional photoreceptor cells in the retina.

I. Morphology of the Retinal Pigment Epithelium

Historically, European anatomists referred to the pigment epithelium, Bruch's membrane and the choroidal capillary layer collectively as the tunica Ruyschiana after the early work of Frederick Ruysch in the late 16th century. The pigment epithelium and Bruch's membrane were thought to be part of the choroid. In 1833, Wharton Jones was the first to adequately describe the morphology of the retinal pigment epithelium (RPE), showing that it was a single layer of hexagonally shaped cells. Several years later, Bruch (1844) described a "structureless membrane" immediately beneath the RPE which eventually became known as Bruch's membrane. In 1861 Rudolf von Kölliker demonstrated that the pigment epithelium and Bruch's membrane originated embryologically from the outer layer of the secondary optic vesicle and therefore the pigment epithelium was, in reality, a part of the retina. He correctly referred to the pigmental cell layer as the RPE. In 1877, Kühnt inferred that each RPE cell was ultimately bound to its neighbor by a cementing substance that also bound the basal surface of the RPE to Bruch's membrane. He was also the first to note that the RPE cells had processes that invested the outer tips of the photoreceptor cells. Kühne (1879) used the term "fuscin", later changed to "melanin", to denote the pigment in the RPE. Usher (1906) was the first to point out that the RPE was darker in the macular region than elsewhere. Histological studies later revealed that the difference was largely due to the fact that macular RPE cells were taller and thus contained more pigment than elsewhere. Raehlmann (1907) described the pigment as consisting of crystalline needles 1.5 μm in length.

We have been particularly interested in the MBs of the newt RPE for many years, and more recently in chick RPE MBs. Although there is little variation in the general structure and function of the RPE cells, studies on the ultrastructure of these cells have revealed small but definite differences between the chick and the newt. Electron micrographs (Figure 1 and 2) as well as more detailed descriptions of their respective RPE cell ultrastructure are provided.

The ultrastructure of the chicken RPE has been reported by several authors (Nishida 1964; Matsusaka 1967; Kuwabara 1979). Although it appears to be a thick cell layer, the cells consist of an extremely small soma and elongated apical microvilli. The main cell body contains relatively sparse accumulations of endoplasmic reticulum apically, however the smooth endoplasmic reticulum (SER) is closely packed in the central area, together with numerous mitochondria and MBs, and occasional pigment granules. The endoplasmic reticulum observed in the RPE cells consists mostly of agranular elements. The microvilli contain SER, MBs and elongated melanin granules. Because of the flat shape of the RPE cell body, the tips of the outer segment are found in close proximity to the base of the cell. The basal cell membrane is markedly infolded to facilitate absorption of metabolites.

In the newt, the RPE is thick and has several unique features (Porter and Yamada 1960; Dickson and Hollenberg 1971; Keefe 1971; Kuwabara 1979). The basal cell membrane is sparsely infolded. The apical cytoplasmic compartment is conspicuously large and extends numerous large microvilli between the photoreceptor outer segments. The microvilli and the photoreceptor outer segments are closely associated with the tips of the microvilli reaching the level of the photoreceptor inner segments. The RPE

cell cytoplasm contains an abundance of SER, which is dense and uniformly distributed. However, the endoplasmic reticulum in the microvilli is sparse. Several small to medium sized perinuclear Golgi complexes are enmeshed in the SER, while numerous MBs, as well as elongated mitochondria are present throughout the RPE cell cytoplasm. Phagosomes, when present, as well as lysosomes and dark inclusion bodies are generally found in the apical region of the cell although they have been reported as deep as the basal infoldings (Keefe 1971). In addition, large membrane-bound oil droplets of various sizes, and scattered free ribosomes or polysomes may be seen in the cell cytoplasm. The lipid droplets are moderately electron-opaque and structureless. Small, round melanin granules are distributed throughout the RPE cell cytoplasm, being predominately located in the apical microvilli when the animals are light adapted, and basally located in the dark.

a) Myeloid Bodies of the Retinal Pigment Epithelium

More than a century ago, Angelucci (1878) and Kühne (1879) independantly reported a cytoplasmic structure in the frog retinal pigment epithelium which Kühne (1879) named the myeloid body. Arey (1928) described these structures under the light microscope as “colorless, colloidal bodies, spherical or cuboidal in shape and of wax-like luster...”. Porter (1956) confirmed the existence of MBs using the electron microscope and described their lamellar structure. Further studies by Porter and Yamada (1960) indicated that MBs represented a differentiated area of the smooth endoplasmic reticulum system of the RPE cell. Porter (1956) regarded these organelles as being equivalent to the "myeloid bodies" first described with

Figure 1.

Low magnification electron micrograph of the retinal pigment epithelium from a mature newt. The basal cell membrane (bm) is sparsely infolded. The apical cytoplasm is extensive, with numerous large microvilli (v) extending between the photoreceptor outer segments (OS), with which they are closely associated. The tips of the microvilli reach the level of the photoreceptor inner segments (IS). The RPE cell cytoplasm contains an abundance of smooth endoplasmic reticulum, melanin granules and mitochondria (m). Different myeloid body (MB) configurations ranging from elongated lenticular structures to circular organelles are observed. Bar = 2 μm .

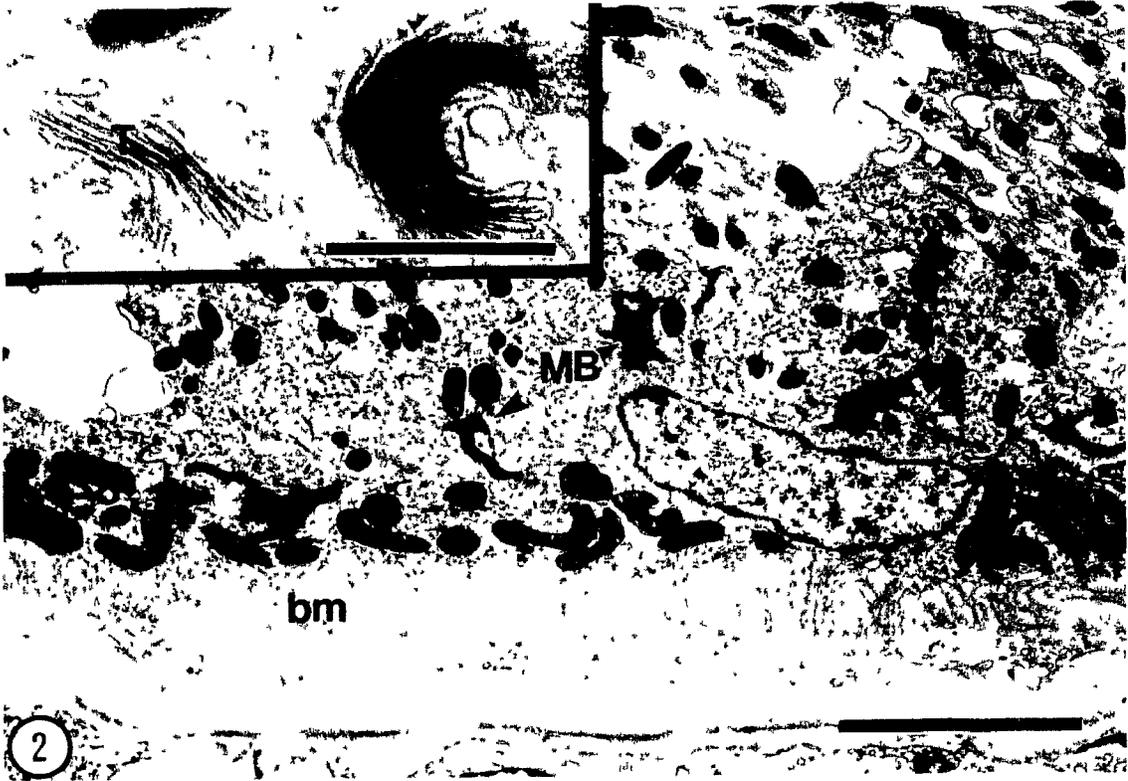
Insert. Higher magnification electron micrograph of a lenticular MB from the newt retinal pigment epithelium. Bar = 0.5 μm

Figure 2.

Low magnification electron micrograph of the retinal pigment epithelium from a 28 day old white Leghorn chicken. The basal cell membrane (BM) is highly infolded and thick. The apical cytoplasm is conspicuously extensive, with numerous small microvilli (v) extending between the photoreceptor outer segments. The RPE cell cytoplasm contains mainly smooth endoplasmic reticulum, numerous melanin granules and

mitochondria (m). Different configuration of myeloid bodies (MB) can also be observed but these are all variations on the lenticular shape. Bar = 2 μm .

Insert. Higher magnification of a curved MB and of flattened stacked of membranes resulting from ultrastructural changes of the SER, and which we have named template (T), from a 28 day old white Leghorn chicken. Bar = 0.5 μm



the light microscope by Kühne and Angelucci. Dowling and Gibbons (1962) suggested that the term myeloid body be reserved for membrane inclusions in non-mammalian species and that lamellar bodies be used for the comparable structures in mammals. It is evident that in these early studies, there was some confusion between MBs and the structures that ultimately became known as phagosomes. Myeloid bodies were not known to occur in any mammalian species at the time. In 1969, Young and Bok introduced the term RPE phagosome to clarify the fact that the “pigment epithelial cell inclusion bodies were fragments of photoreceptor outer segments”. Young and Bok (1969) also presented unequivocal autoradiographic evidence, in frogs, that the RPE was in fact phagocytizing rod outer segment tips. Using animals in which a group of rod outer segments discs had been labeled with radioactive amino acids, these authors observed that when the intensely labeled discs disappeared from the ends of the rod outer segments, highly radioactive inclusion bodies suddenly appeared in the cytoplasm of the pigment epithelium. Examination of the radioactive inclusions with the electron microscope revealed their close resemblance to rod outer segments. Further, they showed that phagosomes were surrounded by two separate cell membranes, the inner one was derived from the photoreceptor outer segment, while the outer membrane was of RPE cytoplasmic membrane origin. This observation was the first instance where phagosomes were differentiated from the MBs of RPE cells. Up to that time, confusion had existed and the term myeloid body had been used interchangeably for these different forms of lamellar inclusions in the pigment epithelium. Subsequently, MBs were found in a wide range of non-mammalian species (Nguyen-Legros 1975), and more recently, Tabor and Fisher (1983) observed a cytoplasmic organelle

in the RPE of the Eastern gray squirrel that closely resembles the MBs of lower vertebrates. In fact, Tabor and Fisher (1983) convincingly established that MBs are present in this mammalian species, although MBs were only observed in the dark.

Myeloid bodies can be defined as lamellar specializations of RPE cells. These organelles may have a variety of morphological shapes, from lenticular to circular, but they are always composed of stacks of closely apposed flattened membranes. The branched tubular network of the surrounding smooth endoplasmic reticulum is often observed to be continuous with the membranes at the outer edge of MBs, although a few ribosomes (normally present on rough endoplasmic reticulum) may sometimes be associated with the outermost cisternae of MBs as well. Unlike the Golgi apparatus, myeloid bodies exhibit no structural polarization of their lamellar stacks such as that embodied by the cis/trans configuration of the Golgi. Furthermore, MBs are devoid of cytoplasmic vesicles such as those commonly associated with the Golgi cisternae.

The presence of MBs in vertebrate RPE cells, together with the now well established association between the endoplasmic reticulum membrane system and lipid synthesis (Goldblatt 1969), has led to the general belief that MBs are in some way involved in photoreceptor cell lipid renewal and recycling. Much of the experimental evidence gathered from an ever-increasing number of animal species support such an hypothesis. Myeloid bodies have been reported and studied in RPE cells of fish (Nicol et al. 1975; Kunz et al. 1983; Ennis and Kunz 1984; Kunz et al. 1985), amphibians (Porter and Yamada 1960; Matthes and Basinger 1980; Yorke and Dickson 1984; 1985a; 1985b; 1985c), reptiles (Nguyen-H-Anh 1972; Ngyen-Legros

1975; Maekawa and Mizuno 1976; Williams et al. 1990) and birds (Meyer et al. 1973; Dieterich 1975) were they were always continuous with the endoplasmic reticulum membrane system. MBs have also been observed in the pineal of fish (Herwig 1980), frogs (Kelly and Smith 1964), urodeles (Flight 1973; Flight and van Donselaar 1975) and even rats (Cos et al. 1987). Recent studies have demonstrated the presence of MBs in invertebrate retinas (Whittle 1976; White and Bennett 1989), where they have been linked with carotenoid deficiency. Immunocytochemical, autoradiographic and biochemical analyses performed on cephalopod retinas have also revealed an important role for MBs in membrane and photopigment renewal in these species (Robles et al. 1984; 1987; Aguilar et al. 1991).

b) Phagocytosis of Outer Segments

Phagocytosis is the process by which cells internalize solid particulate matter (Novikoff et al. 1964; DeDuve and Wattiaux 1966; Cohn 1972; Steinman and Cohn 1974). Pinocytosis is a qualitatively similar function involving a smaller amount of soluble material. Although the two are included in the more general term endocytosis, in both cases, the material is ingested in a membrane-bounded vacuole, so that it is isolated from the cytoplasm. Autophagy is a related mechanism for the segregation within a vacuole of organelles or a portion of the cell's own cytoplasm. All three are related functions, because the fate of the membrane-bound material in each instance is intracellular degradation by lysosomal hydrolytic enzymes.

The importance of the phagocytic properties of the retinal pigment epithelium to the present study cannot be understated. From the early

confusion which arose between phagosomes and MBs, to the observation, of a diurnal rhythm linking the shedding of outer segment discs to the number and size of MBs, evidence tends to indicate that the process of phagocytosis is a key factor in the presence of MBs in the RPE. An understanding of the phagocytic process in the pigment epithelium is therefore essential to this study.

The first indication that the pigment epithelium was capable of phagocytosis was provided by Karli in 1954. He observed in the rabbit that pigment epithelial cells could migrate away from Bruch's membrane and engulf necrotic debris resulting from the poisoning effects of sodium iodoacetate. In 1963, Bairati and Orzalesi reported their investigations of the human pigment epithelium where they offered an hypothesis concerning the significance of the lamellar inclusions found within the cells. Their observations led them to predict that photoreceptor outer segment discs might detach as small packets of membrane and that these were subsequently phagocytized by the retinal pigment epithelial cells. They further speculated that these outer segment fragments might be digested by the pigment epithelium, so that the highly ordered lamellar inclusions found near the apex of RPE cells could be considered the predecessors of the more disorganized inclusions that were often found deeper in the cells. Of equal importance was their prediction that a loss of discs from the apex of photoreceptor outer segments would require a compensatory addition of new discs at the base. A few years later, the elegant autoradiographic studies of Young (1965, 1967) on rat and frog retinas clearly demonstrated the concept of rod outer segment renewal. Using radioactive amino acids as markers, Young demonstrated that newly synthesized proteins in rod inner segments, migrated to the base of

the outer segment and travelled slowly in a discrete band along the entire length of the outer segment. When the labeled band reached the tip of a rod outer segment (approximately nine days in the rat and 28 days in the frog) it disappeared. From these results, Young concluded that it was not simply the protein component of the outer segments which was undergoing renewal, but that the outer segment discs themselves were continuously replaced. New disc material which was added at the base of the outer segment was balanced by removal of effete disc membrane from the tip. These studies lead to the work of Young and Bok (1969) which demonstrated, again using autoradiographic techniques, that phagocytosis of rod outer segment discs was effectively being accomplished by the retinal pigment epithelium.

At the earliest recognizable stage of the disc-shedding process, the edges of the discs at the tip of the outer segment become bent or curled, with the convex side commonly facing the remaining discs of the outer segment. The membrane that surrounds the rod outer segment begins to fold inward, separating the group of deformed apical discs from the remainder of the membrane stack. This pinching off of outer segment discs can be interpreted in two ways: it occurs by itself and is, in other words, an active outer segment process (Young 1971), or it results from pressure exerted by the apical RPE cytoplasmic processes (pseudopodia) which surround the outer segments (Spitznas and Hogan 1970; McLaughlin et al. 1983; Matsumoto et al. 1987). The exact process which leads to the shedding of outer segment discs may well involve both mechanisms. Next, the pigment epithelial pseudopodia separate the group of apically deformed discs from the rest of the stack, as they invaginate, meet and fuse. The newly created phagosome is then withdrawn into the main cytoplasmic compartment of the pigment epithelial

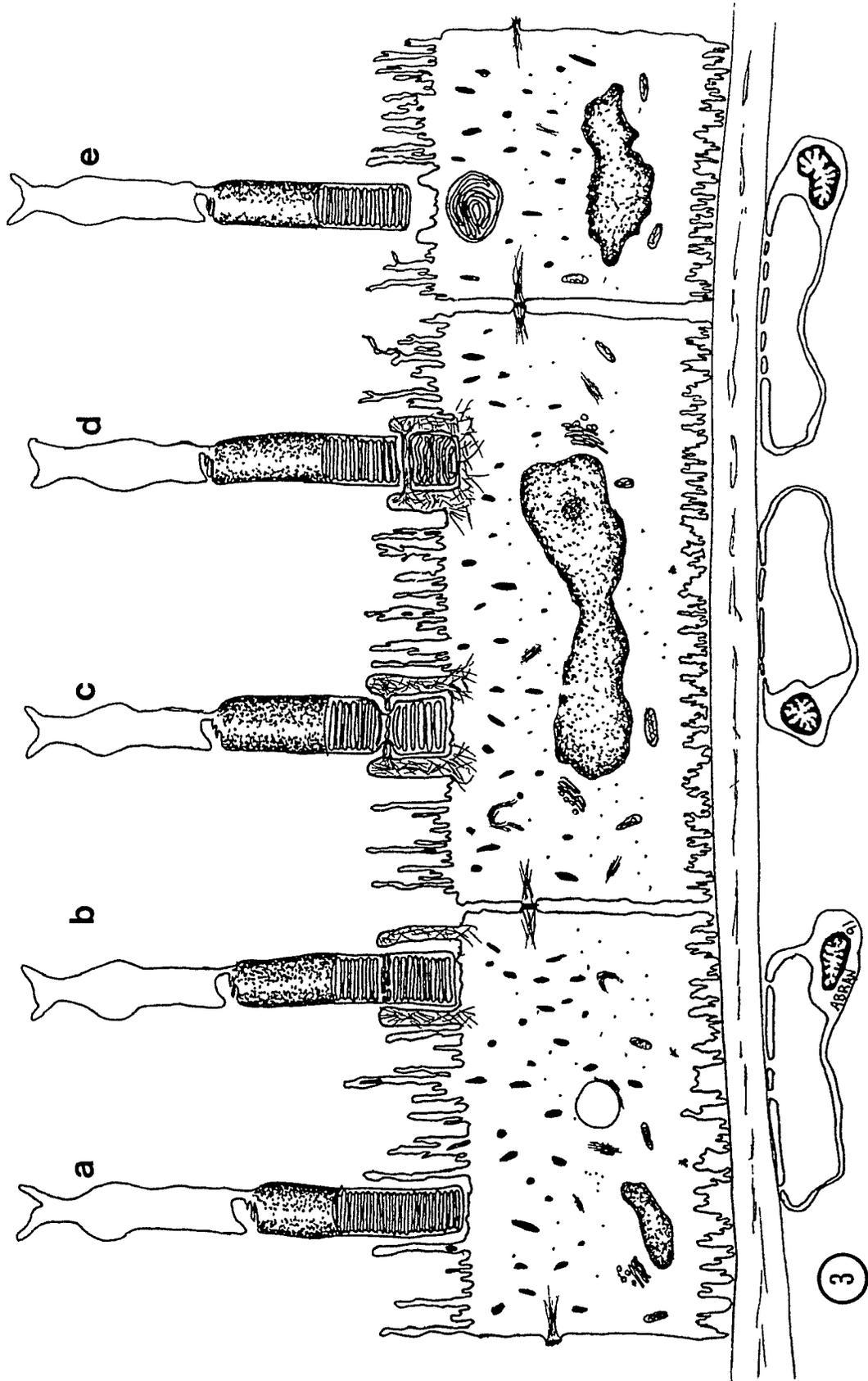
cell (Matsumoto et al. 1987). A schematic representation of the phagocytic process is provided in figure 3.

Digestion of the contents of the phagosome begins very soon after the outer segment membranes are ingested. As digestion continues, the contents of the phagosome bear less and less resemblance to disc membranes. Characteristic changes include, deformation and compaction of the discs; rolling up of the discs to yield a concentric, layered appearance; disorganization of the membranes, with homogenization of the contents; and, appearance of a granular matrix and dense cores. The smaller, more disorganized masses tend to be located in the basal cell compartment, close to Bruch's membrane, indicating a progressive movement of the phagosome toward the basal aspect of the cell as digestion proceeds.

The rapid rate of assembly of new outer segment disc membrane by rod visual cells, and the shedding of a concomitant amount of effete disc membrane, presents the pigment epithelium with a quantitatively significant disposal task. It had been estimated that it takes between one and four weeks to renew a rod outer segment (Young 1967). This represents a dai'y production of roughly 100 discs per rod, compensated by the shedding of an equivalent amount of discs. Since RPE cells can be in contact with

Figure 3.

Depiction of the process of phagocytosis of rod outer segments by the retinal pigment epithelium. (a) Stage one (resting); Only apical microvillous processes surround the rod outer segments in periods when no shedding occurs. (b) Stage two; In response to light, pseudopodia containing a high concentration of actin filaments arise from the apical surface of the RPE cell and surround the distal portion of the outer segment. Simultaneously, vesiculation of a few discs occurs in a small zone of the distal outer segment. (c) Stage three; The pseudopodia intrude into the outer segment. The vesiculated discs are also found to be opened to the extracellular matrix at the point where the pseudopodia are invading. (d) Stage four; Isolation and retraction of the severed outer segment tip is proceeding. The pseudopodia with their actin filaments are actively involved in the ingestion process. (e) Stage five. The pseudopodia and its actin filaments disappear and the outer segment tip, now a phagosome, is surrounded by plasma membrane. It will rapidly be digested by the RPE cell.



as many as 200 rods, this could represent an astounding 20 thousands discs consumed daily. One would have to conclude that the phagocytic capacity of a RPE cell is much greater than that of a normal cell. Marshall (1970) working on rabbits observed that each pigment epithelial cell contained an average 20 to 30 phagosomes. However, after ruby laser injury which resulted in mass destruction of outer segments, 250 to 400 phagosomes were found to be present in the adjacent RPE cells, in many cases occupying over 70 percent of the cell volume. Clearly, the pigment epithelium retains a very considerable capacity to phagocytize which is available for emergencies.

It has also been demonstrated that delivery of the phagocytic burden to the pigment epithelium, rather than being uniform throughout the day, proceeds according to a daily rhythm that is regulated either by light or by an intrinsic circadian clock (Besharse 1984). LaVail (1976) reported that rats maintained on a lighting cycle of 12 hours light and 12 hours darkness shed their rod discs shortly after the time when lights were turned on. However this shedding process also occurred on schedule over a period of three days even in the absence of a light stimulus, indicating that the phenomenon might follow a light entrained circadian rhythm. The rhythmicity of rod disc shedding is a feature which appears to be common to all vertebrate retinas studied thus far. However it is not a circadian event in all species. Basinger et al. (1976) demonstrated that the rod outer segment disc shedding in the leopard frog, *Rana pipiens*, is a diurnal event which does not continue in the absence of a light stimulus.

The shedding of rod and cone outer segments has also been shown to take place at different periods during a normal light-dark cycle. Young (1977, 1978) and O'Day and Young (1978) demonstrated that the pigment

epithelium of lizards, chickens and goldfish readily ingest the shed cone discs at the time of light off set and that the resulting phagosomes are degraded by morning. At light onset, rod outer segment tips are shed, and these discs are degraded during the day (Basinger et al. 1976; Young 1978). The concept of rod outer segment turnover has been further refined by the discovery that light also plays a basic role in disc assembly (Besharse et al. 1977a; Besharse et al. 1977b; Hollyfield et al.,1982). The rate of assembly of new discs at the base of the photoreceptor outer segments has been shown to increase in constant light (Besharse et al. 1977a; Besharse 1982; Hollyfield et al.,1982).

Futhermore, it has also been demonstrated in rats that the lipid composition of the discs is affected by the light history (Organisciak and Noell 1977; Penn and Anderson 1987).

Although a variety of conditions have been studied, the exact nature of the signal that allows phagosome recognition by the retinal pigment epithelial cell remains unanswered. Studies using frog (Hollyfield and Ward 1974a; 1974b), rat (Custer and Bok 1975 ; Reich-D'Almeida and Hockley 1975a), monkey (Funahashi et al. 1976), rabbit (Karli 1954), and human (Tso et al. 1973; Feeney and Mixon 1976) have shown that the pigment epithelium demonstrates a general phagocytic capacity by ingesting a variety of non-specific particles such as gold, carbon, polystyrene beads and dyes. On the other hand, frog pigment epithelial cells fail to phagocytize pasteurized and native micrococcus, although other phagocytic cells in the frog will ingest these organisms (Hollyfield and Ward 1974b; Hollyfield 1976). Apparently, the appropriate recognition sites for micrococcus are not present on the pigment epithelial cell outer membrane. When both micrococci and polystyrene spheres are provided simultaneously, the pigment epithelium will

engulf the spheres, while the micrococci are excluded (Hollyfield 1976). Even fragments of rod outer segments may not be recognized under certain circumstances, such as when outer segment fragments are isolated by sucrose flotation without precautions to preserve their integrity. When such fragmented outer segment membranes are presented to normal, intact pigment epithelial cells, they are frequently not phagocytized, although the same cells will ingest fragments of native photoreceptor outer segment that are damaged by mechanical manipulation (Custer and Bok 1975).

Outer segment membrane recognition appears to be important to successful phagocytosis by the RPE. O'Brien (1976) was the first to suggest that sugars on the surface of the outer segment plasma membranes, perhaps those bound to opsin, might serve as ligands that could be recognized by receptors on the apical surface of the RPE. Rhodopsin has been shown to be present in the rod outer segment plasma membranes, as well as in outer segment discs (Basinger et al. 1976; Kamps et al. 1982) and to contain both N-acetylglucosamine and mannose residues (Fukada et al. 1979). Recent studies using sugar-coated beads (Tarnowski and McLaughlin 1987) demonstrated specific interactions with the RPE in the absence or presence of a variety of ligand inhibitors. However, these studies and others (Lentrichia et al. 1987) have failed to provide convincing evidence supporting the role of rhodopsin as a ligand in photoreceptor outer segment and RPE cell interactions. Components of the interphotoreceptor matrix which surrounds photoreceptor inner and outer segments (Röhlich 1970), have also been implicated in the recognition and subsequent phagocytic process (Zimmerman and Eastham 1959; La Vail et al. 1981; Adler and Klucznick 1982; Tarnowski and McLaughlin 1987; Hollyfield et al. 1989). One major

component of the interphotoreceptor matrix, the proteoglycans, has been studied recently (Tawara and Hollyfield 1990) but appears to have a supporting role rather than be involved directly in the phagocytic process. The exact nature of RPE-outer segment recognition remains unclear at the present time.

II. Biochemistry of the Retinal Pigment Epithelium

Information which is now available regarding the biochemical transformations which take place in the retinal pigment epithelium indicates that the RPE plays an important, if not essential role in retinal function. In this introduction, discussion of RPE biochemistry because of its complex nature, has been restricted to include only the knowledge base which is essential to the discussion of the function of MBs.

The general lack of information on RPE biochemistry, specific to the maintenance of photoreceptor cells, is due in part to the unavailability of material in amounts suitable for accurate biochemical analyses. Although RPE cells can be isolated and purified from large eyes such as those of cattle and sheep, even with these large species, 20 to 30 eyes are usually needed for a complete metabolic experiment. This also explains why most of the information related to RPE biochemistry that has been obtained to date is specific to bovine RPE cells. Unfortunately, the eyes of animals that are known to have MBs are small by comparison. As a result, most of the biochemical information obtained on small animals such as the frog, the rat or the newt, has been extrapolated from the elegant histochemical and autoradiographic studies on bovine eyes. These techniques are however limited mainly to the study of proteins or enzymes and generally give qualitative results only.

Much of the success in the recent RPE cell biochemical studies relates to the refinement of techniques used to isolate these cells from the eyecup, to fractionate the cells, and to isolate specific cell organelles from the cell homogenate.

Whole cell suspensions or homogenates of the retinal pigment epithelium are the most commonly used tissue preparations in biochemical studies. The first published method for isolating pigment cells by brushing them from the optic cup, after removal of the retina, appeared in 1962 (Glocklin and Potts 1962). Although this technique has been used by the originating investigator and many others (Berman 1964; 1971; Glocklin and Potts 1965; Shichi 1969; 1973; Vento and Cacioppo 1973; Gonasun and Potts 1974; Zimmerman 1976), systematic examination of the resultant preparations under the microscope had not been carried out. As a result, there was no real assurance that the preparations were free of contamination originating from adjacent ocular tissues. These suspicions of contamination were confirmed in a study (Berman et al. 1974) that showed that only under carefully defined conditions of centrifugation, in solutions of known density or viscosity, can all of the impurities be effectively separated from isolated RPE cells. The principal contaminants present in unwashed RPE preparations are red blood cells, broken and intact outer segment, retinal neurons and broken RPE cells.

Whatever method is used for the purification of RPE cells, it is now also possible to fractionate homogenates of these cells and to isolate their subcellular organelles (Rothman et al. 1976; Feeney-Burns and Berman 1982). Although either density gradient centrifugation or differential pelleting can be used for fractionation, the latter method is somewhat simpler, and when the amount of tissue available is limited, it is usually the method of choice. However, in difficult circumstances, a combination of these methods may yield the best results at the cost of losing some cellular material.

a) Retinol in the Retinal Pigment Epithelium

As retinol and its derivatives play a central role in the visual cycle, measurement of the amount, and the state of this component in the RPE is of prime importance. The involvement of the retinal pigment epithelium in the process of rhodopsin regeneration, and in regulating the retinoid circulation to and from the photoreceptors, is fundamentally important in the visual cycle.

Vitamin A is the term used for those retinoids that exhibit the biological activity of retinol. It is obtained in the diet as provitamin A carotenoids from vegetables and as retinyl esters from animal tissues. An excellent summary of the biosynthetic and metabolic pathways of carotenoids and retinol derivatives was given by Olsen (1964). A recent review of the absorption, transport and storage of vitamin A has been presented by Blomhoff, Green, Berg and Norum (1990). Vitamin A alcohol (all-trans retinol) is poorly soluble in water and so is transported in blood bound to a protein complex that is made up of retinol binding protein (RBP) and transthyretin (Goodman and Blaner, 1984). The mechanism by which retinol moves from the protein complex in blood to the cytosol of target cells, like the RPE, is not clear. It has been proposed that specific receptors for the RBP are found on the RPE basal plasma membrane and that they mediate the uptake of all-trans retinol (Heller, 1975; Bok and Heller, 1976; Chen and Heller, 1977; Ottonello and Maraini, 1981; Pfeffer et al., 1986), although recent observations indicate that retinol can spontaneously and freely move from the blood to the intracellular compartment of the RPE (Noy and Xu, 1990). Following the uptake of all-trans retinol by the RPE, the alcohol can either be esterified directly, or isomerized to 11-cis retinoid before

esterification, giving a mixture of stored retinoids. This isomerization is thought to be due to an esterase enzyme, which has been identified in frog and bovine RPE (Bernstein et al., 1987; Fulton and Rando, 1987) as well as in cultured human RPE (Flannery et al., 1990).

There is striking species variation in the amount of visual pigment chromophore present in the RPE. As well, the state of light and dark adaptation plays a role in the quantity observed. In both vertebrates and invertebrates, 11-cis retinal, which is the chromophore bound to the protein opsin within the outer segment disc membrane, is cleaved and isomerized when it is exposed to light and is then reduced and stored in the pigment epithelium (Dowling 1960; Noell et al. 1971; Bridges 1976). In the dark, visual pigment chromophore that is recycled from the photoreceptor and originating from the vascular system, flows back to the neural retina, where it is used for the regeneration of rhodopsin. The absolute necessity of the RPE in the regeneration of visual pigment was demonstrated initially by Ewald and Kühne in 1877, when they showed that neurosensory retina in contact with the RPE regenerated visual purple, whereas retina separated from the RPE did not. The dynamics of this process have been examined in detail in the albino rat by determining both the distribution and relative proportions of vitamin A compounds in the retina and in the RPE under various conditions of light adaptation (Zimmerman 1974, Zimmerman et al. 1974). During 60 minutes of light adaptation, the amount of vitamin A in the RPE of the albino rat increases nearly sixfold. In contrast to this, changes in retinol content in the frog RPE during light and dark adaptation are less dramatic. The reason for this appears to be the enormous capacity of the amphibian to store esterified vitamin A in the form of oil droplets in the cell cytosol (Bridges

1975). Even though 87 percent of the rhodopsin bleached in strong light finds its way to the RPE in frogs, less than half of the vitamin A stored in the RPE returns to the retina during dark adaptation (Bridges 1976). Thus the frog, unlike the rat, maintains a large reservoir of vitamin A in the RPE even under conditions of complete dark adaptation.

In all species examined to date, 95 percent or more of the retinol in the RPE is esterified (Krinsky 1958; Dowling 1960; Hubbard and Dowling 1962; Bridges 1976). Although the fatty acid components of retinol esters have not been identified with certainty in all species, there is some evidence suggesting that in cattle and rabbits, retinol is esterified mainly to palmitate (Krinsky 1958; Alvarez et al. 1981), a 16 carbon saturated fatty acid.

In summary, the RPE appears to be a major storage depot for retinol, where it serves as one source of the chromophore for rhodopsin regeneration as well as renewal of disc membrane. In addition, it is clear that retinoids are transported back and forth between the photoreceptor and the RPE. However, the identity of the mode of intercellular transfer has not been firmly established. One candidate is the interphotoreceptor retinoid binding protein (IRBP), a soluble protein that readily binds different chemical and isomeric forms of retinoid and is uniquely localized in the interphotoreceptor matrix compartment that separates photoreceptors and the apical surface of the RPE. This transport protein has been studied extensively over the last decade (Adler and Martin 1982; Lai et al. 1982; Liou et al. 1982; Chader et al. 1983; Pfeffer et al. 1983; Chader and Wiggert 1984; Fong et al. 1984; Redmond et al. 1985; Saari et al. 1985; Das and Gouras 1988; Flannery et al. 1988; Okajima et al. 1989; 1990; Das et al. 1990; Flannery et al. 1990) but its full function in

the visual cycle is still uncertain (Rando and Bangerter 1982; Fex and Johannesson 1987, 1988; Ho et al. 1989).

b) Lipid Metabolism in the Retinal Pigment Epithelium

The definition of a "lipid" is used rather loosely to define a group of compounds, having the common property of being insoluble in water but readily soluble in non-polar organic solvents such as chloroform, ethers and alcohols. Such a definition encompasses steroids, carotenoids, terpenes and bile acids, in addition to fatty acids and glycerolipids for example. This definition is unnecessarily broad and for the purpose of this thesis I will use the definition proposed by Christie (1987, 1989) which is that lipids are fatty acids and their derivatives, together with substances related biosynthetically or functionally to these compounds.

The three major kinds of membrane lipids are phospholipids, glycolipids and cholesterol. The phospholipids and glycolipids can themselves be further subdivided into glycerolipids and sphingolipids. Glycerolipids are derived from glycerol, a three-carbon alcohol, and sphingolipids from sphingosine, a more complex alcohol. A phosphoglyceride consists of a glycerol backbone, two fatty acid chains and a phosphorylated alcohol.

The fatty acids are compounds synthesized in nature via condensation of malonyl-coenzyme A units by a fatty acid synthetase complex, catalyzed by acetyl-coenzyme A carboxylase. The fatty acids of plants, animals and microorganisms generally contain even numbers of carbon atoms in straight chains, with a carboxyl group at one extremity and with double bonds of the

cis configuration in specific relation to this group. In animal tissues, the common fatty acids vary in chain length from 14 to 24 carbon atoms and may have one to six double bonds. The 16 and 18 carbon atom fatty acids are the most common ones. Fatty acids are usually expressed according to their common names or in an abbreviated form (see Table A for nomenclature). The fatty acid abbreviations consist of two numbers separated by a colon. The first number represents the number of carbon atoms forming the fatty acid chain, while the second number expresses the number of double bonds found on the chain. Abbreviations of unsaturated fatty acids are followed by a description of their family identity. There are three main families, the n-3, n-6 and n-9 presented in this thesis. The letter n express the terminal methyl group and the number defines the position of the double bond. The n-3 family of unsaturated fatty acids, for example, would have its first double bond starting at the third carbon atom from the methyl end of the fatty acid chain.

The total lipids of the RPE in general account for three percent, by weight, of the tissue, a value close to that of the retina but lower than the lipid content of liver for example. In the RPE of species that have been studied, neutral lipids are present in only trace amounts, while phospholipids represent the major lipid class in these cells. Although at first RPE phospholipids were thought to consist mainly of phosphatidylcholine (Berman et al. 1974), subsequent studies have shown that phosphatidylethanolamine is also present (Anderson et al. 1976; Berman and Feeney 1978). The molar ratio of phosphatidylcholine to phosphatidylethanolamine is approximately 1.5 to 1.0, and these two phospholipids account for 80 percent of the total RPE phospholipids, with sphingomyelin, phosphatidylinositol and

phosphatidylserine comprising another 16 percent (Anderson et al. 1976). While the relative concentrations of certain fatty acids (16:0, 16:1, 18:0 and 18:1) are nearly the same in RPE and rod outer segments, for several other fatty acids the differences are quite striking. For example, arachidonic acid (20:4) is found in much higher concentrations in the RPE than in the ROS. Linoleic acid (18:2) is present only in trace amounts in rod outer segment phospholipids (Anderson and Sperling 1971; Berman et al. 1974; Anderson et al. 1976), but accounts for 10 percent of the fatty acids in phosphatidylcholine of RPE. On the whole, the RPE has a higher level of saturated fatty acids (especially 16:0) than do rod outer segments (Anderson et al. 1976); moreover, the relative distribution of the fatty acids in individual phospholipids in the RPE is very different from that of rod outer segments.

The most striking difference in fatty acid composition between the RPE and rod outer segments is found in docosahexaenoic acid (22:6 n-3). This long-chain polyunsaturated fatty acid is unusually abundant, comprising 20 to 60 percent of the total fatty acids in phospholipids in the central nervous system, retina and brain (Fliesler and Anderson, 1983; Bazan and Reddy, 1985). It is also the most abundant fatty acid present in rod outer segments, accounting for 26 to 47 percent of the total fatty acids in various phospholipids of this tissue (Anderson et al. 1976; Bazan, 1989). In contrast, only small amounts of docosahexaenoate are detectable among the fatty acids of RPE phospholipids. LaVail (1976) reported that it was likely that lipid peroxidations catalyzed by free radicals in the presence of oxygen account for the extremely rapid disappearance of long-chain fatty acids, such as docosahexaenoic acid, from the membranes of phagocytized outer segments.

However, Bazan, as recently as 1985, has suggested a short-loop recycling mechanism from the RPE cells back to the photoreceptor for 22:6 n-3.

The pigment epithelium also regulates the exchange of fatty acids between the blood and the retina, and the supply of fatty acids (essential or not) to the retina follows a route that is very similar to the one previously described for vitamin A. Dietary fatty acids find their way into the blood supply where they are bound to serum albumin (Spector et al. 1969), with the remainder being attached to lipoproteins. Binding to albumin stabilizes fatty acids and allows them to be transported. However, fatty acids bound to serum albumin spend very little time in the blood. Normally, they are delivered to tissues in less than three minutes, but it is not yet clear how internalization into cells is accomplished. In the case of the RPE cells, fatty acids could be translocated across the basal membrane in a fashion similar to that of vitamin A. Once inside the cell, they are usually esterified with glycerol to form triglycerides (Donabedian and Karmen 1967), although free fatty acids have been detected in bovine pigment epithelium (Berman et al. 1974). Some of these free fatty acids may be in transit to the photoreceptors, presumably complexed with a carrier protein.

Within five minutes after intravenous injection of tritium-labeled fatty acids in frogs, a significant concentration of radioactivity can be detected within the RPE (Bibb and Young 1974). Autoradiography shows that incorporation into the pigment epithelium is greater than into any other type of retinal cell. This points to an important role for the pigment epithelium in retinal fatty acid metabolism. It also suggests that RPE cells are largely responsible for the unusual ability of retinal tissues to retain essential fatty acids when subjected to deficiency or disease.

Palmitic, stearic and arachidonic acid were the first fatty acids to be studied autoradiographically. Palmitic and stearic acid were shown to be most intensely concentrated in the large, vitamin-A containing oil droplets in frog RPE cells (Futterman and Andrews 1964). Presumably this was largely due to the esterification of vitamin A. On the other hand, arachidonic acid, which is not used for vitamin A esterification, was less concentrated in oil droplets. These fatty acids were heavily distributed throughout the cytoplasm, and nuclear uptake was found to be negligible. Phagosomes in the pigment epithelium showed immediate binding of the labeled fatty acids, as did rod outer segments. Since neither of these structures directly utilizes fatty acids, the uptake of radioactive fatty acids is probably due to exchange with the fatty acids already present within phospholipids of disc and phagosome membranes (Bibb and Young 1974). The variability observed in phagosome labeling was accounted for by the presence of membranes in different state of degradation.

Fatty acyl groups are important in that they form the hydrophobic interior of all biological membranes, and provide the impermeability to hydrophilic substances found at the boundaries of cells and cell organelles. Considerable metabolic energy is expended in ensuring that each membrane and each phospholipid class possesses a characteristic fatty acyl composition (White 1973). As described earlier, this is especially true of the photoreceptor outer segments. Increasing effort has been done in recent years to further understand the requirement for the specific fatty acyl group composition in membranes. Such attempts include investigations of cell membranes in which fatty acyl groups have been modified *in vivo* by dietary means, or *in vitro* by incubation with media of restricted lipid composition. The ultimate

purpose of these experiments is to correlate fatty acyl group composition, membrane physical properties and membrane functions.

My studies on MB membrane composition has attempted to establish a link between the phospholipid and fatty acyl groups composition and the membrane structure and function. Special interest has been paid to the unsaturated fatty acids, since these are found in large quantities in photoreceptor outer segment and MBs. Studies of microorganism (McElhaney 1989) and model membrane systems (Ehringer et al. 1990) have produced findings of major significance with respect to the role of unsaturated fatty acids in cell membranes (for a more complete review see, Stubbs and Smith 1984).

Because of the unique lamellar nature of MB membranes in the RPE, and in order to ascertain the potential functional significance of these organelles, a basic understanding of the influence of lipids on membrane properties and conformation is important.

The widespread acceptance of the fluid mosaic membrane model by Singer and Nicolson (1972) led, for a time, to the concept that membrane proteins have almost complete freedom of lateral motion within the lipid bilayer. Since then, awareness has grown with respect to the importance of the severe restrictions which cytoskeletal elements impose on protein mobility (Nicolson 1976). Recent refinements in our understanding of membrane structure have resulted in a vision of a complex membrane architecture with both lateral and transverse lipid-protein coupled regions, as well as lipid phase separation and domains (Israelachvili 1977). Nevertheless, the simpler concept of membrane 'fluidity' has persisted. Earlier, the term had been used to express an increased disorder of fatty acyl chains above the phase transition

(Chapman 1958) and in that sense it is accepted that biological membranes are required to be in a fluid state for normal functioning. Fluidity specifically refers to properties of the hydrophobic region of the membrane, and is essentially a semi-quantitative term. A quantitative analysis of membrane fluidity is more usefully served by a description of lipid motion as it relates either to the rate of motion of the fatty acyl chain, or to the orientation parameter of the acyl chain, where an increase in the rate of motion is related to the presence of double bonds. However, the effect on membrane fluidity is complicated by the lack of a formal relationship between this movement rate, the orientation of the acyl chain, and the observations made with different techniques.

The difficulty in deriving a satisfactory means of relating fatty acid unsaturation to membrane properties in all probability stems from the need for more detailed analyses of membrane lipids. It is well established that biological membranes contain considerable diversity in their lipid composition. Further, each lipid, when totally defined with regard to every functional group, represents a specific molecular species. Inherent in this definition is the identity of each fatty acid, aldehyde or alcohol, together with their position on the supporting molecule. Comparatively few reports include all this information, and this represents a serious disadvantage in any attempt to relate physical properties, function and composition of membranes. Although recent developments using high-performance liquid chromatography provide the means for quantitative analysis of phospholipid molecular species (Takamura and Kito 1991), the difficulties which are inherent in such comprehensive analyses forced me to adopt a simpler and more widely used approach. The determination of MB phospholipids and

their respective fatty acid content in the studies reported here was achieved using thin layer and gas chromatography. Knowing the phospholipid and fatty acid composition of MB membranes is important since MBs are a RPE subcellular specialization of unconfirmed function, and they are believed to be involved in photoreceptor outer segment lipid metabolism (Yorke and Dickson 1985a). Furthermore, fatty acids are one of the major contributing elements to lipid organization within membranes, together with the lipid headgroup composition, and their analysis in MB could help understand MB structure and function. Although the determination of molecular species is better suited to relate membrane structure with lipid and fatty acid composition, it is possible to determine the effect caused by fatty acids on membrane structure by simply measuring phase transition temperature changes on model systems. The experimental findings correlating the influence of fatty acyl substituents with the phase transition temperature have been summarized in a number of reviews (Chapman 1975; Melchior and Stein 1975; McElhanev 1982). Early observations by Chapman and co-workers (Chapman 1958; Chapman et al. 1966; Chapman 1982) of infra-red and other spectra indicated a marked decrease in the order of the fatty acyl chains as the phospholipids entered the liquid crystalline phase. The term 'order', as applied to fatty acyl chains, implies a restriction on mobility, and forms the basis for the interpretation of certain aspects of physical observations on membrane structure.

In this introductory section my objective has been to provide a level of background information, related to membrane structure, sufficient for the discussions which follow. A glossary of fatty acid nomenclature is included in Table A. A detailed description of fatty acid metabolic pathways and

associated enzymes, although relevant, are not essential to the understanding of this work. However, a summary of the pathways for docosapentaenoic and docosahexaenoic acid synthesis has been included (Table B and C), since these are discussed more extensively within the general context of RPE cell metabolism.

Table A

FATTY ACID NOMENCLATURE GLOSSARY

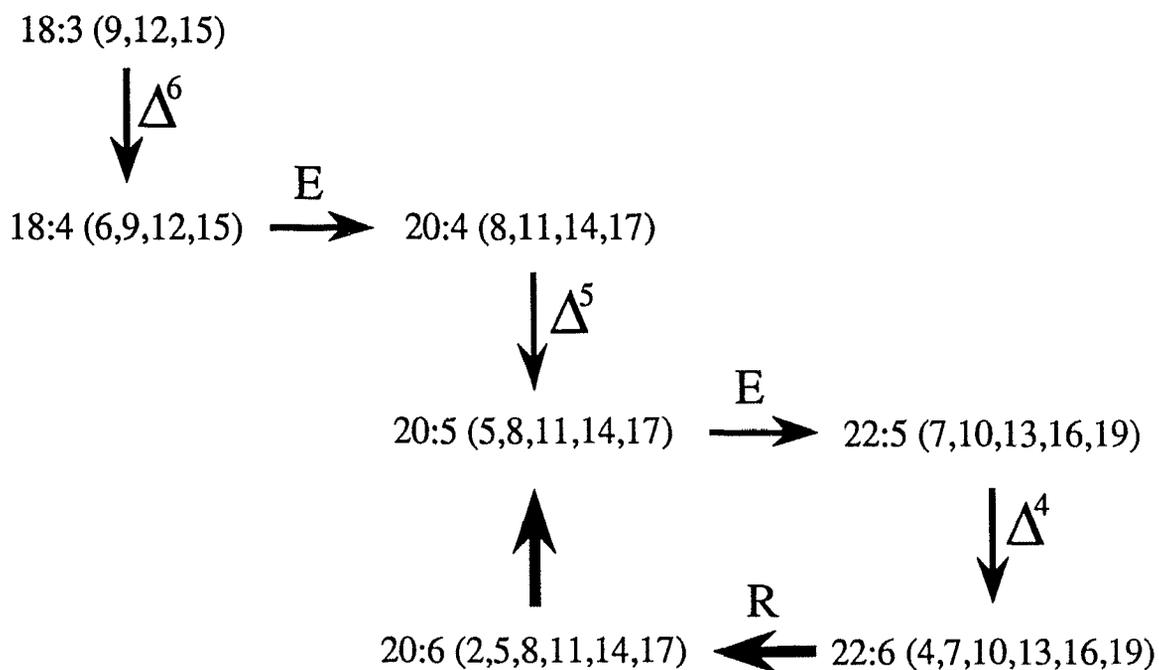
Common name	Systemic designation*	Abbreviations
Myristic		14:0
Palmitic	Hexadecanoic	16:0
Stearic	Octadecanoic	18:0
<u>Monoenoic</u>		
Palmitoleic	9-hexadecenoic	16:1 n-7
Oleic	9-octadecenoic	18:1 n-7
Vaccenic	11-octadecenoic	18:1 n-7
Gadoleic	9-eicosenoic	20:1 n-12
Petroselinic	6-octodecenoic	18:1 n-12
Cetoleic	11-docosenoic	22:1 n-11
Erucic	13-docosenoic	22:1 n-9
Nervonic	15-tetracosenoic	24:1 n-9
Elaidic	9- <i>trans</i> -octadecenoic	18:1 n-9 <i>trans</i>
<u>Dienoic</u>		
Linolenic	9,12-octadecatrienoic	18:2 n-6
<u>Trienoic</u>		
<i>a</i> -Linolenic	9,12,15-octadecatrienoic	18:3 n-3
<i>g</i> -Linolenic	6,9,12-octadecatrienoic	18:3 n-6
Dihomo- <i>g</i> -linolenic	8,11,14-eicosatrienoic	20:3 n-6
<i>a</i> -Eleostearic	9- <i>cis</i> ,11- <i>trans</i> ,13- <i>trans</i> -octadecatrienoic	18:3
Mead acid	5,8,11-eicosatrienoic	20:3 n-9
<u>Tetraenoic</u>		
Arachidonic	5,8,11,14-eicosatetraenoic	20:4 n-6
Parinaric	9,11,13,15-octadecatetraenoic	18:4
Adrenic	7,10,13,16-docosatetraenoic	22:4 n-6
<u>Pentaenoic</u>		
Timnodonic	5,8,11,14,17-eicosapentaenoic	20:5 n-3
Docosapentaenoic	4,7,10,13,16-docosapentaenoic	22:5 n-6
Clupanodonic	7,10,13,16,19-docosapentaenoic	22:5 n-3
<u>Hexaenoic</u>		
Cervonic (Docosahexaenoic)	4,7,10,13,16,19-docosahexaenoic	22:6 n-3

For more details, see Hilditch and Williams (1964), Gunstone (1967; 1986).

* All double bonds are in the *cis* configuration, unless stated otherwise.

Table C

PATHWAY OF LINOLENIC ACID SERIES DESATURATION AND
ELONGATION



Desaturation can occur at positions nine, six, five and four from the carboxyl end. Reactions are designated as desaturation (Δ), which are catalyzed by separate desaturase enzymes, termed the Δ^9 , Δ^6 , Δ^5 , and Δ^4 desaturases (James 1977). Reactions are also designated by elongation of two carbons, E, and less common pathway, the retroconversion, R (Gronn et al. 1991). Retroconversion is further enlightened by the use of thick arrows.

III. Regeneration

The phenomenon of regeneration has fascinated man since antiquity. The earliest historical allusion to regeneration may be that recorded by Aristotle in his *De generatione animalium* (see Platt, 1910), written two thousand years ago. Aristotle wrote,

"Hence, if the eyes of swallows still young be put out they recover their sight again, for the birds are still developing, not yet developed, when the injury is inflicted, so that the eyes grow and sprout afresh."

We know today, however, that although eyes of young swallows may recover rapidly from injurious insult, neither a swallow nor any other vertebrate is capable of regenerating a new eye, if by "put out" Aristotle meant complete removal of the eye.

Much has been learned since the time of Aristotle, including the discovery that not all animals are equally endowed with the capacity to regenerate lost parts. The lower vertebrates demonstrate an ability for regeneration that appears to exceed that found in higher vertebrates. This fact has led to the suggestion that regeneration may be a manifestation of phylogenetic primitiveness. Although there is some merit in this proposal, it is however difficult to generalize from available data, since there are some lower members that regenerate poorly, if at all.

The process of regeneration is in many respects analogous to embryonic development, especially in amphibia where the regenerating tissues rely on many of the molecular activities which are also found in developing systems. However, there is a major difference in the origin of regenerating adult tissues, compared to that of the multipotential mesenchymal and ectodermal cells of the embryo. For this reason, the analogy rather than the homology of these two distinctly different processes should be emphasized.

Defining regeneration is important if this phenomenon is to be clearly distinguished from other, closely related, biological processes such as tissue repair. Schmidt (1966) defined regeneration as the process by which organisms reconstitute whole lost parts of the body. In amphibians, regeneration is exemplified by the reconstruction of complex structures such as a limb or an eye, consisting of several tissues, and results in duplication, both structurally and functionally, of the lost original structure. In higher vertebrates, and especially in man, the regenerative capacity is highly restricted and is more appropriately referred to as tissue repair.

The phenomenon of regeneration in an amphibian species was formally described first by Spallanzani, a pioneer experimental morphologist, in 1768. Spallanzani, a perceptive investigator, amputated the limbs and tail of frog and toad tadpoles, and of larval and adult salamanders. From his several experiments, Spallanzani noted that young animals, particularly the tadpoles, regenerated amputated limbs much more rapidly than did older animals. He also showed that regeneration was affected by temperature, with the rate slowing considerably during the winter months, and that the amount of tissue that regenerated was related to the amount removed. However, the

resulting regenerated tissues never achieved the size of the portions removed. Repeated amputations resulted in repeated regenerations. Inanition affected the growth of the animals, but appeared not to have any effect on the capacity to regenerate.

In the late 17th century, it was found that regeneration of the eyes of salamanders was possible, but not if the entire eye was removed. Experimenting with locally gathered salamanders, Bonnet (1781), Blumenbach (1787), and later, Philipeaux (1880), and Colucci (1891) reported that animals with various amounts of excised eye tissue showed remarkable recovery in gross appearance through regeneration of the lost part. The first demonstration, with histological evidence, of regenerating retina occurred in 1889 when Griffini and Marchiò not only demonstrated neural retina and optic nerve regeneration in the adult newt, but stated that the pigment epithelium formed a layer from which the new neural retina differentiated. Wachs (1920) concluded that pigment epithelial cells were one source of origin for the regenerated retina in his experiments on European newt (*Triturus*) larvae, a conclusion that had been reached some 30 years before by Griffini and Marchiò (1889) experimenting on adult newt.

It has been demonstrated in many experiments on transplanted eyes of adult urodeles (Matthey, 1926; Stone and Zaur, 1940; Stone and Chace, 1941; Stone and Cole, 1943) that the temporary loss of blood supply to the eye induces degeneration of the whole neural sensory retina. An account of the degeneration of the neural portion of the retina and the subsequent regeneration of a new one, derived from the surviving pigment epithelial cells of the retina, was given by Stone (1950a, 1950b).

Stone (1950a) developed a series of descriptive stages to classify the degeneration and subsequent regeneration of the salamander retina. He reported that striking changes occur in the different layers of the retina as soon as the adult eye is deprived of its blood supply. Within a few days, cellular destruction becomes widespread, and is especially notable in the central portion of the retina. As degeneration spreads in the neural retina, marked changes take place in the cells of the pigment layer which survive to perform new functions. The pigment granules in these cells, first recede from the apical processes and accumulate in the cell basal compartment just as in the dark adapted state. These events characterize stage one.

Stage two consists of the preparation of the environment to receive a new retina. During the second and third weeks following surgery, neural retinal cells are destroyed. Absorption of cellular and pigment debris takes place within the vitreous chamber, and through a process of disintegration and absorption of the debris, the neural retina is rapidly eliminated. The pigment epithelial cells, on the other hand, survive and radically change their structural characteristics to temporarily take on a new function.

The proliferation of mitotic RPE cells characterizes stage three. During the third and fourth weeks, the pigmented cells transform into a low columnar epithelial cell layer, which retains varying amounts of pigment as they begin to undergo mitosis. One set of daughter cells migrates inward (vitrally) to establish a less-densely pigmented single layer of regenerating neural retina. The underlying cells proceed simultaneously to organize a new pigment epithelial cell layer. Stone (1950b) observed that there was no evidence of another source of daughter cells beside those originating from the original retinal pigment epithelium. Later studies carried out by Levine (1975)

suggested a contribution by the pigmented cells of the iris and ciliary body, as well as by the RPE, in his experimental paradigm. However, in both systems, the new neural retina gradually becomes thicker due to a rapid mitosis of the cells in this layer.

By six to eight weeks of regeneration, differentiation (stage four) of the various layers of the neural retina starts to take place. Stone (1950b) noticed that the onset of differentiation was not simultaneous in all areas of the neural retina, but rather seemed to occur sporadically in "patches". Between eight and 12 weeks, differentiation of the new neural retina is completed and the ganglion cell axons which comprise the optic nerve make their exit from the globe to create functional connections within the brain. After three months, the functional characteristics of the retinal pigment epithelial cells are again demonstrated, with the migration of pigment granules into the apical processes occurring in the light-adapted eye.

The use of the regenerating newt as a model to study MBs allowed me to answer some of the questions surrounding the relationship of MBs with the photoreceptor cells, and to refine the hypotheses which had been developed with respect to MB function in the RPE. The observation by Yorke and Dickson (1984) that the fluctuation in MB size and number was correlated temporally with the phagocytic process, suggested a possible relationship between MBs and the shedding of photoreceptor outer segment discs. To further test this hypothesis, I decided to eliminate the photoreceptor cells from the eye by removing the neural sensory retina of the newt, and thus eliminate the source of outer segment disc membrane for RPE phagocytosis. In addition, the use of an animal like the newt, with regenerating capacities, allowed me to determine if MBs would reappear when the neural retina

regenerated and became functional. And finally, since the regeneration process proceeds at a much slower pace than embryological development of the retina, by carefully monitoring the endoplasmic reticulum throughout the regeneration process, it should be possible to observe all of the ultrastructural changes which lead to the formation of myeloid bodies.

It was clear from Stone's work (1950b) that there is significant individual variation between animals in the rapidity with which the cells degenerate and then regenerate. Further studies by Keefe (1973a; 1973b; 1973c; 1973d) established a more detailed picture of the regeneration process in two species of urodeles, *Notophthalmus viridescens* and *Triturus cristatus carnifex*. He demonstrated that retinal pigment epithelial cell proliferation following differentiation occurred at different rates throughout the retina. Furthermore, using colchicine and radiolabeled thymidine, he showed that the areas with maximum rates of proliferation coincided with the pattern of ocular blood vessels. This patchy development of the retinal pigment epithelial cells during regeneration has been demonstrated recently using monoclonal antibodies selective for newt retinal pigment epithelium (Klein et al. 1990). Further, the studies by Levine (1975) demonstrated a clear antero-posterior pole gradient in rate of differentiation of the regenerating RPE, with the most anterior pigment epithelial cells differentiating first. The studies done by Stone in the 50's, and by Keefe and Levine in the 70's, established crucial information essential to the understanding of MB morphogenesis in the regenerating eye of the newt *Notophthalmus viridescens*.

Materials and Methods

a) Myeloid Body Isolation and Phospholipid Analysis

Isolation of retinal pigment epithelial cells

The isolation and purification of retinal pigment epithelial (RPE) cells is a two-step process. The first step involves removal of the neural sensory retina from the underlying RPE layer, followed by the separation of the RPE cells or sheets of cells from the basement (Bruch's) membrane. In the second step, the detached crude preparation of RPE cells is separated from impurities such as outer segments, red blood cells, and pigment granules. The standard detachment procedure is accomplished by careful brushing of the eyecup with a camel-hair brush after removal of the retina (Berman et al. 1974; Anderson et al. 1976; Rothman et al. 1976; Heller and Jones 1980). Other approaches, such as scraping the RPE free of the basement membrane, introduce unacceptable levels of choroid contamination that cannot be separated from the RPE by most methods of RPE purification. The second step, cell purification, can be carried out in a variety of ways, although the most successful of these employs multiple washes using differential centrifugation (Berman et al. 1974; Anderson et al. 1976; Rothman et al. 1976) to remove non-RPE impurities. A detailed description of the steps leading to purified chick RPE cell preparations follows.

In excess of two hundred chick eyes were used for each myeloid body (MB) purification experiment. Chick heads (light entrained on 12L:12D cycle, seven day old White Leghorn) were obtained from a nearby laboratory within

15 min of decapitation, performed at approximately 10 a.m. (sampling window at 5 to 6 hr after light onset) at a time when the daytime maximum amount of MB material had previously been reported in the RPE of the newt (Yorke and Dickson 1984). The eyes were enucleated, placed in a RPMI 1640 tissue culture medium (Sigma) containing 0.25 M sucrose, and kept on ice during tissue collection. Each eye was then opened at the ora serrata, and the vitreous and lens were removed. To facilitate removal of the neural sensory retina, the eyecups were left on ice in RPMI 1640 tissue culture medium at pH 6.0 with 0.25 M sucrose for 0.5 to 1 hr. All subsequent manipulations were performed on ice and the centrifugations were carried out at a temperature of 5 °C. The neural retina was removed using camel-hair brush and forceps, and the RPE cells were collected by gentle brushing. The collected RPE cells were pooled and washed 4 to 6 times by differential centrifugation (floatation at 200 x g, sedimentation at 3600 x g) to remove broken cells and any attached outer segment tips. At this stage, the RPMI 1640 medium was replaced by a tris buffer (Buffer A - 0.05 M Tris-base, 0.25 M potassium chloride and 0.005 M magnesium chloride with 0.25 M sucrose at pH 7.0). The purity of the RPE cell preparation was verified from wet mounts by light microscopy.

Purification of myeloid bodies

The purity of subcellular fractions is normally assessed by electron microscopy in combination with the characterization of certain specific enzymes or by chemical analyses of the isolated fractions. Implicit in the definition of a marker enzyme is its exclusive localization in a single intracellular organelle. However in the case of MBs, lack of detailed

knowledge with respect to the biochemistry and function of these organelles precluded the use of any form of chemical assays to assess the purity of these preparations. Assessment of MB purity thus relied exclusively on electron microscopic observations of pelleted material. The fractionation of chick retinal pigment epithelial cells was carried out as follows.

The RPE cells were resuspended in tris buffer A outlined above and were thoroughly homogenized with a tissue homogenizer (Sorval Omni Mixer). The homogenate was centrifuged for 30 min at 10,000 x g to remove nuclei, melanin granules, and large sheets of apical and basal plasma membranes. The pellet was resuspended in tris buffer A, vigorously handshaken, vortexed on a mixer (Thermolyne, type 37600 mixer) at the maximum setting and then centrifuged, as outlined above. The combined supernatants from the two centrifugations were then put on top of a discontinuous sucrose gradient buffered with 0.05 M Tris-base, and containing 0.25 M potassium chloride and 0.005 M magnesium chloride at pH 7.0. The gradient consisted of incremental density layers of 1.110, 1.126, 1.160 and 1.180 g/ml at 20 °C. The loaded gradient was centrifuged for 90 min at 100,000 x g. At the end of this period, a band had formed at the interface of each of the three density gradients, and a pellet was observed at the bottom of the tube. The three bands were removed and pelleted, and all four pellets were fixed and embedded for electron microscopy. The pellets were then processed as described later in the sub-section "Routine fixation for electron microscopy" of the section entitle "Myeloid Body Morphogenesis in Regenerating Newt Retina".

Isolation of retinal photoreceptor outer segments

Chick outer segments were isolated following the modifications made by Papermaster and Dreyer (1974) to a method originally described by Saito (1938) and McConnell (1965), and used for the isolation of bovine photoreceptor outer segments. The following modifications were made to the methods of Papermaster and Dreyer (1974) in order to successfully isolate chick rod outer segments. A Tris-hydrochloric acid buffer was used instead of Tris-acetate buffer throughout the experiment. Approximately 10 chick retinas instead of one bovine retina were added to one ml of homogenizing solution, and a freeze-thaw step was added to help shear the ROS at the junction of the inner and outer segments before the first homogenization. The resuspended crude ROS preparation was not re-homogenized but rather it was placed directly on top of the discontinuous sucrose gradient in order to isolate intact ROS. The purity of the ROS preparation was verified by phase contrast microscopy, and the final preparation consisting of intact ROS, and a few small stacks of membranes of cone or rod origin, used for the phospholipid and fatty acid analysis. The final preparation also contained a minor oil droplet contaminant from cone inner segment.

Lipid extraction and phospholipid separation

The outer segment and enriched MB samples were next subjected to lipid extraction with a solution of chloroform/methanol/water (2:2:1.8(vol:vol:vol)). This extraction process produces two phases, the lower phase contained the lipids and the upper phase the water. The lower phase was

pipetted to a screw-cap tube and the aqueous phase and interphase were re-extracted with chloroform. The combined total lipid extracts were then dried under nitrogen gas, resuspended in chloroform/methanol (20:1 (vol:vol) for faster evaporation) and spotted on a thin-layer chromatography plate (LK6D Whatman silica gel 60A). Phospholipids were developed using a solvent system consisting of chloroform/ethanol/water/triethylamine (30:35:8:34(vol:vol:vol:vol)) (slightly modified from Touchstone et al. 1979). Individual phospholipids known to be present in rod outer segments were obtained from Serdary Research Laboratory (London, Canada). These were used as references and were spotted on different channels of all thin-layer chromatographic plates used in this study. The individual phospholipids, after visualization with the aid of a modified molybdenum spray reagent, were scraped off the plate and quantitated by measurement of their phosphorus content following the procedure outlined by Bartlett (1959).

Methyl ester formation and gas chromatography

After scraping MB and outer segment phospholipids from the chromatographic plates, the fatty acid methyl esters were prepared by transmethylation using boron-trifluoride in methanol (Morrison and Smith 1964) for 30 min. The fatty acid methyl esters were extracted with petroleum ether (3 X 2 ml) and stored at -70°C under nitrogen until the analysis could be performed.

Prior to gas chromatography (GC) analysis, the fatty acid methyl esters were dried under nitrogen and resuspended in appropriate amounts of carbon disulfide (approximately 1.5 µl). The samples were injected into a 1.7 m X 2

mm (internal diameter) glass column packed with 15% Silar 10C on Gaschrom R and analyzed on a Hewlett-Packard model 5710A gas chromatograph equipped with flame ionization detectors. The column was subjected to a programmed temperature range from 195 °C to 230 °C over 30 min with a nitrogen carrier flow-rate of 20 ml/min. The computerized integration was performed by the MAXIMA Integration System (Dynamic Solutions, Division of Millipore) which was programmed to quantify fatty acyl chains in the samples using relative response factors for each fatty acid and comparison to standards used for calibration.

b) Myeloid Body Morphogenesis in Regenerating Newt Retina

Sexually-mature newts (*Notophthalmus viridescens*) were used throughout this portion of the study. Animals were maintained in glass aquaria at 15-18 °C and fed brine shrimp supplemented occasionally with *Xenopus* tadpoles. Illumination of 60 ft-c (624 Lux) was provided by two 20-Watt, "cool-white" fluorescent lights (General Electric Co.), cycled to provide a strict 12 h light/12 h dark lighting regime.

Surgical procedure

Animals were anaesthetized in a solution of 0.1% MS 222 (Tricaine methanesulphanate, Syndel Laboratories Ltd, Vancouver, Canada) buffered in amphibian Ringer's solution. An opening of approximately 180° was made at the corneo-limbal junction of both eyes with the aid of a dissecting microscope using a small scalpel. The cornea was then partially retracted and

the lens, vitreous and the neurosensory retina were carefully removed from both eyes. The cornea was repositioned and the animals were left to recover in a shallow dish on a bed of moist gauze. Sterile techniques were not used, as it is impossible to maintain a sterile field when operating on aquatic species. Antibiotics (tetracycline • hydrochloric acid, Aquarium Pharmaceuticals Inc, USA) were added to the aquaria water in order to prevent post-operative infection.

Routine fixation for electron microscopy

A minimum of three animals were sacrificed at each of the following post-operative time points: days 3 and 6; and 3, 4, 5, 6, 7, 8, 10, 12, 14, 16, 18 and 20 weeks. Three adult non-operated animals were also used as a standard for comparative purposes. All animals were sacrificed by decapitation at 10:00 hr in the morning (three hr after light onset), and both eyes were removed. Whole eyes were punctured at the ora serrata and fixed for 30 min at room temperature in an aqueous fixative solution composed of 2.0% glutaraldehyde and 1.0% paraformaldehyde in 0.15M sodium cacodylate buffer with 2.0 mM CaCl₂ and 1.0% sucrose, at pH 7.2. The anterior segment was then removed and the posterior portion of each globe was quartered and returned to the fixative for an additional two hours. After primary fixation, tissues were washed twice for 10 min in 0.15 M sodium cacodylate and post-fixed in 1% aqueous OsO₄ for 30 min. After another wash, the tissue was stained *en bloc* using saturated aqueous uranyl acetate for 30 min and dehydrated with acetone prior to infiltration and embedding in TAAB low-viscosity epoxy resin (TAAB Laboratories, Reading, England).

All electron micrographs to be analyzed morphometrically were prepared from thin sections having an interference color of silver to gray. The sections were cut with a diamond knife (Diatome, Switzerland) on a Reichert ultramicrotome (model Om U3) and subsequently fully expanded with chloroform vapors to reduce compression artifacts. The sections were mounted on Pioloform-coated 2 x 1 mm slotted copper grids, stained with uranyl acetate and lead citrate (Reynolds 1963), and viewed with a Zeiss 10A electron microscope. Micrographs were recorded on 70 mm film (Scientia Film, Agfa-Gevaert, Belgium) and analyzed by projecting the negative image onto the digitizing tablet of a Zeiss Videoplan 2 image analysis system (Version 2.2, January 1990).

Morphometrics

I used morphometric methods that followed the basic stereological principles described by Weibel et al. (1966) for the determination of quantitative parameters on cellular and tissue structure obtained from thin sections. A cascade sampling design (Cruz-Orive and Weibel, 1981) was used for estimating cell features. Following embedding, randomly selected blocks from two, three or four animals were oriented so that the plane of section passed through the long axis of the photoreceptor layer and through the RPE. Low magnification electron micrographs, encompassing the full thickness of the RPE, were taken of all of the cells (10-18) in the section from one animal, and of five randomly-selected cells from subsequent animals at each time point. A minimum of 15 cells from at least two different animals were analyzed at each time point studied. Each RPE cell was then carefully

surveyed at higher magnification (17, 279x) and electron micrographs of MBs and of their precursors were taken for further morphometric analysis.

The MBs and their precursors were counted and their surface area measured. The percentage of the area covered by the precursors and MBs in relation to the pigment epithelial cell area was calculated. Apical cell processes were excluded from the pigment cell area calculations.

c) Embryological Newt Retina

To compare the regeneration process with embryonic development, newt embryonic specimens processed in 1969 were obtained from Dr. D. H. Dickson. The fixation protocol for the newt embryos was as follows: all solutions had been prepared in 0.2M Sørensen's (1909) Buffer at pH 7.4. Whole embryos were fixed for five hr at room temperature in a 3.0% glutaraldehyde solution. After fixation, the specimens were washed in a 5% sucrose-buffer solution for 12 hr. The samples were then post-fixed in an aqueous 2% OsO₄ solution for six hr and dehydrated in a graded alcohol series prior to infiltration and embedding in Epon 812 resin at a final concentration of two part resin, one part alcohol. For electron microscopic analysis, the embedded material was sectioned, stained and photographed as described previously. The embryos which were examined ranged from seven days old, when the retinal layers start differentiating, to 11 days old (one day post-hatch), when the photoreceptor outer segments are well developed in the posterior retina.

Results

a) Myeloid Body Lipid Composition

Myeloid body purification

The electron microscopic analysis of the four pellets obtained through the last stage of the MB isolation procedure described in the Materials and Methods section is shown in figure 4. Identification of the membrane fractions found at the different densities was accomplished by comparison with electron micrographs from published RPE fractionation techniques (Feeney-Burns and Berman 1982). Figure 4A is taken from the fraction which formed at the interface of the 1.110 and 1.126 g/ml density gradients. This fraction contains membranes with an appearance similar to the mitochondrial and lysosomal membrane fractions (Feeney-Burns and Berman 1982). Figure 4B demonstrates the presence of lamellar membrane configurations with an ultrastructure comparable to the of MBs from intact RPE cells, together with other, lamellar membranes which are believed to be of MB origin. There was also smooth endoplasmic reticulum membrane continuous with MBs. This fraction was obtained at the interface of the 1.126 and 1.160 g/ml density gradients. Figure 4C was taken from the 1.160/1.180 g/ml density interface and contains a small number of melanin granules, as well as small microsomes. Figure 4D was taken from the discontinuous gradient pellet and is seen to contain mainly melanin granules and some large microbodies.

Figure 4.

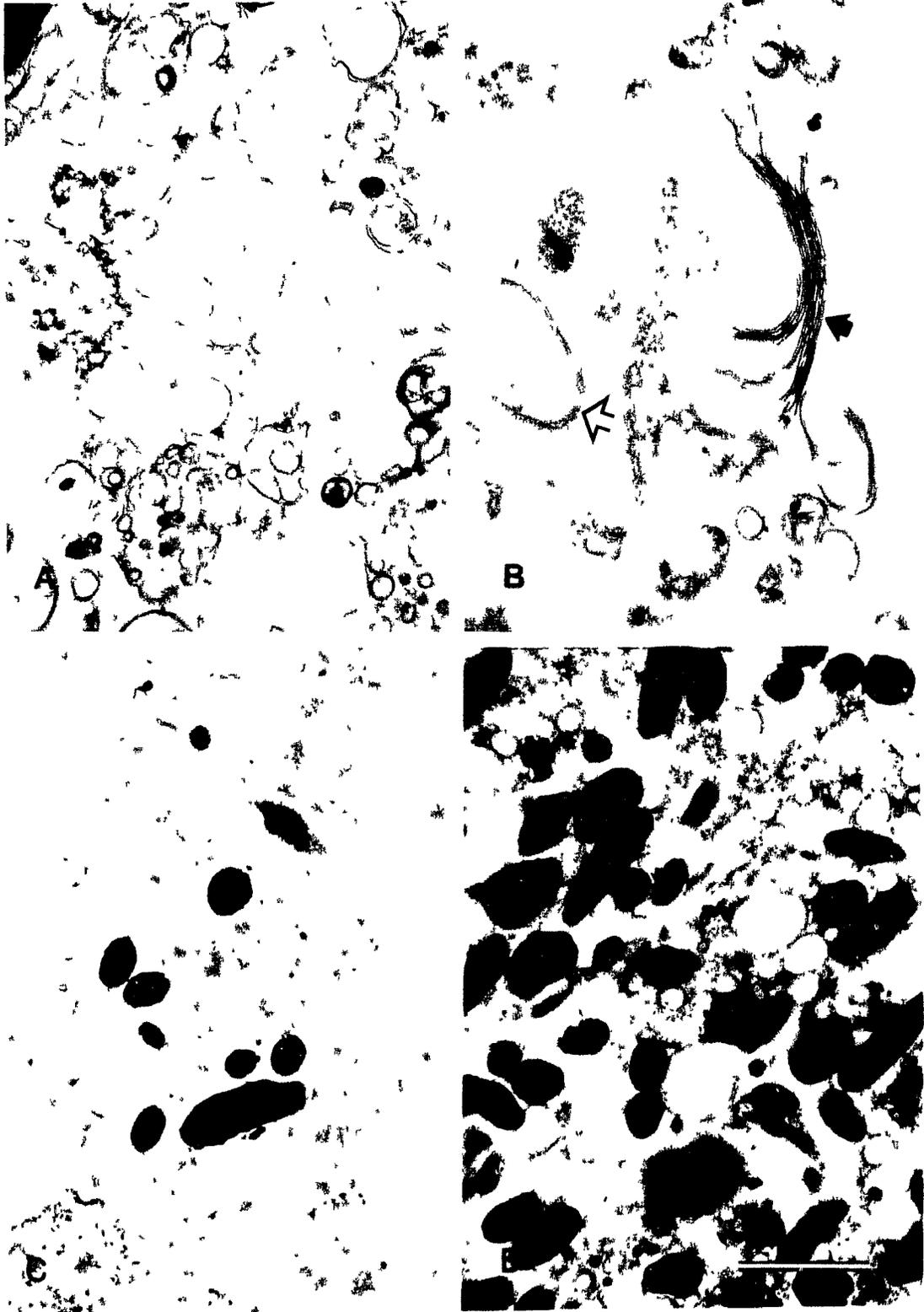
Electron micrographs taken of subcellular fractions of chick retinal pigment epithelium isolated by differential and sucrose gradient centrifugation as described in Materials and Methods. Bar. 1 μ m.

(A) Fraction forming the band at the interface of the 1.110 and 1.126 g/ml density gradients, which contains predominantly mitochondrial and lysosomal membranes.

(B) Fraction forming the band at the 1.126 and 1.160 g/ml density gradient interface containing MBs (arrow) and myeloid body membranes (open arrow)

(C) Fraction forming the band at the 1.160 and 1.180 g/ml density gradient interface containing a few melanin granules, as well as membranes which are probably of smooth endoplasmic reticulum origin.

(D) Pellet from the discontinuous sucrose gradient containing predominantly melanin granules, microbodies and some membranes.



4

Phospholipid composition of myeloid bodies and photoreceptor outer segments

When purified chick photoreceptor outer segments were analyzed for phospholipid content, these samples were shown to be composed of 43.9% phosphatidylcholine (PtdCho) and 35.8% phosphatidylethanolamine (PtdEtn) (Table D). Four other phospholipids, sphingomyelin (SPM), phosphatidylinositol (PtdIns), phosphatidylserine (PtdSer) and lyso-phosphatidylcholine (lyso-PtdCho) were also present in outer segments, but in much smaller amounts. These six phospholipids were taken to represent 100% of the phospholipid content of chick outer segment membranes.

When the MB-enriched subcellular fraction from chick RPE was subjected to phosphorus analysis, PtdCho and PtdEtn were found in similar percentages to outer segments (42.5 and 34.0% respectively), and together represented just under 80% of the total phospholipid content of these membranes (Table D). Sphingomyelin and PtdIns accounted for the remainder (less than 25%) of the MB phospholipids. The other two phospholipids found in chick outer segments, PtdSer and lyso-PtdCho, were undetected in the MB preparation.

Fatty acyl chain composition of myeloid body and photoreceptor outer segment phospholipids

Following MBs phospholipid analysis, the fatty acyl chain composition for each of the isolated MB phospholipid classes was determined by gas chromatography. These results are summarized in Table E. As a comparison,

the fatty acyl chain composition of chick outer segment phospholipids as determined in our laboratory is shown in Table F. In Table G, I compare the four phospholipids which are common to both rod outer segment membranes and MBs. I divided the fatty acid distribution into three groups: saturated, unsaturated and polyunsaturated with 20 carbons or more. I observed that the difference between MB and rod outer segment saturated and unsaturated fatty acids is less than 6 mol% for PtdIns and PtdEtn. However, the quantity of unsaturated fatty acids, with 20 carbons or more for those two same species, is higher in MBs (13.7 and 13.9 mol% respectively) than for outer segments.

When saturated and unsaturated fatty acids are examined in sphingomyelin (Table G), the saturated fatty acid are 13.2 mol% lower and the unsaturated fatty acid 13.2 mol% higher in MB when compared to rod outer segment membranes. For those polyunsaturated fatty acids with more than 20 carbons, the difference is 36.1 mol% more in MBs over rod outer segment membranes.

The most dramatic differences however are observed with PtdCho (Table G). While saturated and unsaturated represent 64.8 and 35.2 mol% of rod outer segment fatty acids respectively, a reversal occurs in MBs, with saturated fatty acids accounting for 30.2 mol% and unsaturated fatty acids for 69.8 mol%. The amount of 20 carbon or more polyunsaturated fatty acids represent a mere 8.5 mol% in rod outer segments while it represent 51.8 mol% in MBs.

Based on my calculations, approximately 22% of unsaturated fatty acid in chick rod outer segments are of 20 carbons or more compare to approximately 51% in MBs.

I noted that docosahexaenoic acid (22:6 n-3) is the major essential polyunsaturated fatty acid in rod outer segment while docosapentaenoic acid (22:5 n-3) dominates in MBs (Table E and F). I also noted an almost total absence of essential fatty acid precursors (18:2 n-6 and 18:3 n-3) in both rod outer segments and MBs.

b) Myeloid Body Morphogenesis in the Regenerating Newt Retinal Pigment Epithelium

General appearance of the newt retinal pigment epithelium

The retinal pigment epithelium (RPE) of the newt is comprised of a monolayer of large cuboidal cells containing numerous melanin granules, mitochondria and a dense accumulation of smooth endoplasmic reticulum (SER). Different configurations of MBs and lipid droplets are also present (Fig. 1).

Myeloid bodies appear as pleomorphic stacks of small membrane sacs, flattened at their center and slightly dilated at the edge where they are continuous with the surrounding SER (Fig. 1 insert). Myeloid bodies take on a variety of appearances, from straight lenticular arrays (Fig. 1 insert), to circular configurations with concentric layers of flattened membrane lamellae (see Nguyen H. Anh 1971; Yorke and Dickson 1984, for a complete description of MB morphology). While larger MBs are generally found around and at the level of the RPE cell nucleus, smaller MBs can be found anywhere in the cell.

TABLE D

**PHOSPHORUS ANALYSIS OF THE MAJOR
PHOSPHOLIPIDS IN MYELOID BODIES AND ROD OUTER
SEGMENTS FROM 7 DAY OLD CHICKS**

The phospholipids were extracted, separated and then determined, as described under Materials and Methods. The data are the mean values \pm S.E.M. for myeloid bodies (n=4) and the average value of two preparations for rod outer segments (n=2). The R_f for each phospholipids is given in parentheses. ND = Non Detectable

Phospholipid	mol%	
	myeloid bodies	rod outer segments
PtdCho (0.28)	42.5 \pm 2.8	43.9
PtdEtn (0.51)	34.0 \pm 1.2	35.8
PtdIns (0.45)	10.2 \pm 2.3	6.4
PtdSer (0.41)	ND	3.4
SPM (0.24)	13.8 \pm 1.8	7.3
lyso-PtdCho (0.19)	ND	3.0

TABLE E

**FATTY ACID COMPOSITION OF GLYCEROPHOSPHOLIPIDS
FROM CHICKS MYELOID BODIES**

Myeloid body phospholipids were isolated by thin layer chromatography and their fatty acids analyzed by gas chromatography. Figures represent mean values \pm S.E.M. (mol %) from six different MB preparations. T = trace ND = Non Detectable

Fatty acid	Phospholipid			
	PtdCho	PtdEtn	PtdIns	SPM
16:0	15.2 \pm 3.4	14.4 \pm 1.4	6.0 \pm 1.3	20.8 \pm 6.3
16:1 n-9	9.1 \pm 2.2	2.2 \pm 1.2	7.3 \pm 1.7	4.2 \pm 2.0
17:0	1.4 \pm 0.7	2.1 \pm 0.9	3.3 \pm 1.6	0.9 \pm 0.5
18:0	13.6 \pm 1.2	27.0 \pm 1.4	28.2 \pm 1.6	10.5 \pm 0.7
18:1 n-9	7.2 \pm 2.9	1.6 \pm 0.4	1.7 \pm 1.2	7.8 \pm 2.3
18:2 n-6	1.7 \pm 0.8	1.4 \pm 0.3	T	1.5 \pm 0.7
20:3 n-6	1.1 \pm 0.4	0.3 \pm 0.2	0.7 \pm 0.3	0.7 \pm 0.3
20:4 n-6	8.5 \pm 1.7	26.1 \pm 1.6	19.4 \pm 2.1	10.4 \pm 2.8
20:5 n-3	5.3 \pm 3.0	ND	T	T
22:4 n-6	1.1 \pm 0.5	2.5 \pm 0.3	0.5 \pm 0.3	1.5 \pm 0.8
22:5 n-3	30.9 \pm 3.4	7.6 \pm 1.4	26.1 \pm 4.3	32.6 \pm 12.9
22:6 n-3	5.0 \pm 0.7	12.5 \pm 0.9	6.2 \pm 0.6	6.2 \pm 0.9
24:0	T	2.5 \pm 0.3	T	0.8 \pm 0.4

TABLE F

**FATTY ACID COMPOSITION OF GLYCEROPHOSPHOLIPIDS
FROM CHICKS ROD OUTER SEGMENTS**

Rod outer segment phospholipids were isolated by thin layer chromatography and their fatty acids analyzed by gas chromatography. Figures represent mean values \pm S.E.M. (mol %) for three ROS preparations. T = trace ND = Non-Detectable

Fatty acid	Phospholipid					
	PtdCho	PtdEtn	PtdIns	SPM	PtdSer	lyso-PtdCho
16:0	49.2 \pm 0.7	7.1 \pm 0.9	3.0 \pm 0.2	17.1 \pm 0.9	5.2 \pm 1.3	13.8 \pm 1.3
16:1 n-9	3.7 \pm 0.3	0.9 \pm 0.2	0.5 \pm 0.1	2.0 \pm 0.3	1.1 \pm 0.5	2.3 \pm 0.6
17:0	ND	T	ND	ND	T	1.2 \pm 1.2
18:0	15.7 \pm 0.3	41.3 \pm 2.0	37.8 \pm 0.8	29.1 \pm 0.7	38.5 \pm 2.1	23.3 \pm 2.7
18:1 n-9	23.0 \pm 0.8	14.5 \pm 2.1	16.6 \pm 1.7	35.8 \pm 2.1	18.5 \pm 3.8	39.4 \pm 4.9
18:2 n-6	T	T	T	0.6 \pm 0.1	T	0.5 \pm 0.3
18:3 n-3	ND	T	T	T	T	T
20:3 n-6	ND	0.4 \pm 0.1	T	T	T	0.9 \pm 0.3
20:4 n-6	2.5 \pm 0.4	8.6 \pm 0.8	5.8 \pm 0.4	6.3 \pm 0.7	6.4 \pm 1.7	6.9 \pm 1.4
22:4 n-6	0.6 \pm 0.1	2.9 \pm 0.2	4.5 \pm 0.3	3.5 \pm 0.4	3.3 \pm 0.8	6.3 \pm 1.6
22:5 n-3	T	1.4 \pm 0.1	3.0 \pm 0.2	5.7 \pm 1.1	4.0 \pm 1.7	6.3 \pm 0.9
22:6 n-3	5.4 \pm 1.2	21.8 \pm 1.0	26.2 \pm 0.8	T	17.2 \pm 0.5	3.5 \pm 0.4
24:0	ND	1.1 \pm 0.1	2.5 \pm 0.3	ND	2.7 \pm 2.4	2.0 \pm 0.8

TABLE G

**COMPARISON OF FATTY ACID GROUPS BETWEEN
MYELOID BODY AND PHOTORECEPTOR ROD OUTER
SEGMENT**

Fatty acids from phospholipids common to both myeloid body and photoreceptor outer segment were grouped as follows : saturated (S); unsaturated (U); and polyunsaturated with chain lengths of 20 carbons (20C+) or more. Figures for myeloid bodies, rod outer segment and difference are expressed in mol %. The minus sign represent a reduction of fatty acid percentcontent in myeloid bodies.

Phospholipid		mol %	mol %	mol %
		Myeloid body	Outer segment	Difference*
PtdEtn	S	45.9	49.4	-3.5
	U	54.1	50.6	3.5
	20C+	49.0	35.1	13.9
PtdCho	S	30.2	64.8	-34.6
	U	69.8	35.2	34.6
	20C+	51.8	8.5	43.3
PtdIns	S	37.8	43.4	-5.6
	U	62.2	56.6	5.6
	20C+	53.2	39.5	13.7
SPM	S	33.0	46.2	-13.2
	U	67.0	53.8	13.2
	20C+	51.6	15.5	36.1

* Difference = Myeloid body mol% - Outer segment mol%

Observations on the regenerating newt retina

The first response caused by the removal of the neural retina was the migration of a few pigmented epithelial cells into vitreal space (Fig. 5). Here, these cells, as well as macrophages, were observed scavenging blood cells and pieces of neural retina. Also, I observed that 6 days after surgery, there were no MBs present in the RPE cells surveyed. Once the debris were cleared, the regeneration of the neural retina started. Over the first two to three weeks, there was a migration of depigmenting and mitotically active RPE cells over the top of non-dividing pigmented epithelial cells (Fig. 6 and 7). The stationary pigment epithelial cells appeared to retain some phagocytic ability with melanin granules filling most of the cytoplasmic compartment by the third week. Regeneration of the retina from a simple epithelial cell layer was achieved through a process of RPE cell division resulting in the formation of a thick disorganized and undifferentiated neuroblastic layer as seen after five weeks of regeneration (Fig. 8). Gradually, the neural retina became stratified, with the first differentiating photoreceptor inner segments being observed at six weeks post-surgery in the peripheral (anterior) retina (Fig. 9). At that time, the RPE apical processes were few in number, very thin and only occasionally contained small melanin granules. The RPE cell began to extend a large number of apical microvilli between photoreceptor cells after 8 weeks of regeneration (Fig. 10). At this stage, developing photoreceptor outer segments were generally present throughout the retina. By the 14th week of regeneration, all animals presented well differentiated photoreceptor outer segments (Fig. 11).

Observations on the embryogenesis in the newt retina

I have also observed in this study some aspects of photoreceptor development in regenerating retina which need to be presented here. I found differences between the rate of differentiation of photoreceptors in the central area of the eye (near the optic nerve head) and the rate in the peripheral retina. The peripheral photoreceptor outer segments appeared to differentiate first, well before the outer segments closer to the optic nerve head. The regional variation in regeneration across the retina is illustrated in figure 12 and is the reverse of the process which occurs in the embryological development of the retina in the newt, where retinal differentiation is initiated in the posterior pole of the eye and proceeds in a radial fashion anteriorly. Figure 13 illustrates fully differentiated photoreceptor cells in the central retina of a one-day post-hatch newt tadpole, while the peripheral retina remains undifferentiated.

Myeloid body morphogenesis

The development of MBs in the newt retina appears to parallel photoreceptor differentiation during retinal regeneration, with RPE cells in the peripheral retina in close apposition to well developed photoreceptor outer segments demonstrating both reorganized areas of smooth endoplasmic reticulum (SER) and small MBs. However, in the RPE cells of the central retina, where outer segments were just beginning to develop, only ultrastructural changes in the form of lamellar areas of the SER were observed. In areas near the optic nerve head, where photoreceptor outer segments were totally absent, neither MBs nor modifications of the SER were detected.

The ultrastructural changes in the SER were first observed in RPE cells six weeks after surgery, at a time when photoreceptor outer segments were beginning to develop. They first appeared as small (0.5 μm long) areas where the normally tubular SER membranes had become flattened, and consisted of a series of two to four flattened saccules in close apposition (Fig. 14A). Subsequently, these SER membrane areas doubled in size by increasing their length (Fig. 14B) and through the addition of more cisternae at the periphery. These larger structures had from five to ten unfused cisternae, with ribosomes often associated with peripheral cisternal membranes (Fig. 14C). The maturation of these flattened saccules into MBs appears to involve the fusion of the inner leaflet of the cisternal membranes, as demonstrated in figures 14D and 14E. This membrane fusion was first found to occur in cells where phagocytosis of photoreceptor outer segment membrane was underway, as demonstrated by the presence of phagosomes within these cells.

By the seventh week, in addition to the reorganized membrane structures associated with the SER, small MBs were also observed in approximately 60% of the cells surveyed. However, the occurrence of MBs was more prevalent in the peripheral retina where the regeneration process was first initiated and the photoreceptor outer segment development occurred first. By the 12th week, well differentiated photoreceptor outer segments, as well as MBs and stacked membrane saccules, were observed throughout the whole of the retina of all sampled animals. It was not uncommon at this later stage of regeneration, or in normal, unoperated adults, to find large MBs as well as these stacked membrane saccules within the same RPE cell. This is clearly demonstrated in figure 14F, where a number of mature MBs are seen in close proximity to two well structured stacks of flattened saccules having little or no cisternal

membrane fusion. Later in the regeneration process, when phagocytosis was well under way throughout the retina (after the 14th week of regeneration), the various stages of the MB maturation process, as described in figure 14, were still observed.

Morphometric analysis of template and myeloid body

The first occurrence of lamellar conformational changes in the SER was observed at week six of retinal regeneration, and the average size of the resulting stack of unfused flattened saccules (referred to as templates in the graphs) throughout the 14 weeks (week six to 20 of regeneration) of the study remained relatively constant at $0.67 \mu\text{m}^2$ (Fig. 15). Mature MBs on the other hand were first observed at week seven of regeneration, one week after the first sign of SER membrane reorganization. Although there appeared to be an increase in MB size in the early weeks of the regeneration process, the inherent variation in the size of individual MBs throughout the sampling period resulted in large standard deviations for the data obtained at most sample points (Fig. 15). However, after the eighth week, the average MB size was two to three times that of the stacks of unfused flattened saccules at each sample point, with values close to that of the non-operated controls (1.8 times).

A graphic summary of the unfused flattened saccules and MB numbers during retinal regeneration is presented in figure 16. At six weeks of regeneration when the first stacked saccules appeared, there was on average less than one of these per $100 \mu\text{m}^2$ of RPE cell cytoplasm. No MBs were observed in the 25 RPE cells surveyed from three different animals at this early time point. Over the following four week period (week seven to 12 of

regeneration), the number of MBs and of stacks of unfused flattened saccules increased and then remained constant throughout the remaining sample period at values similar to those encountered in the adult non-operated control animals. In controls, the ratio of MB to stacked saccules was approximately 2:1.

Because the retinal regeneration process does not occur in a uniform fashion throughout the retina, MB and stacked saccules data have been presented in figure 17 as percentage of RPE cells containing either MBs or the stacked saccules at each sample point studied. The rapid rate of increase of both MBs and stacked saccules appears to be similar, although the MB increase occurs later. The number of RPE cells with stacked saccules peaks at approximately 16 weeks with 75% of the RPE cells studied containing these reorganized SER membrane. At week 20 of regeneration, the percentage of RPE cells containing these stacked saccules diminishes slightly, while 90% of the cells at this stage contained MBs. In non-operated animals, 100% of the cells sampled contained MBs while the reorganized SER membrane areas were only observed in 60% of these cells.

Figure 5.

Electron micrograph of the newt RPE at 6 days of regeneration, after surgical removal of the neural retina, showing the RPE cell layer and adjacent vitreal space (V). Portions of two RPE macrophages (arrow heads) can be observed in the upper left corner of the micrograph. Bar = 10 μ m.

Figure 6.

Electron micrograph of the newt RPE and retina at 3 weeks of regeneration. The regenerating neural retina is incomplete and shows some dividing cells (arrow head) adjacent to the pigment epithelium. Bar = 10 μ m.

Figure 7.

Electron micrograph of a newt retina at 4 weeks of regeneration. Two RPE cells (arrows) appear to be migrating from the RPE through the developing neural retina. Bar = 10 μ m.

Figure 8.

Electron micrograph of a newt retina and RPE at 5 weeks of regeneration. Although the neural retina layer is thicker, stratification or differentiation of these cells has not yet started. Bar = 10 μ m.

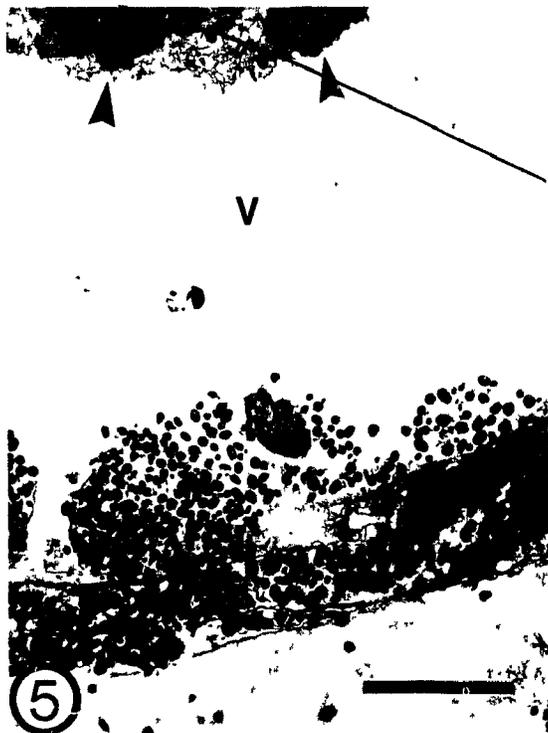


Figure 9.

Electron micrograph of a newt retina and RPE cell layer after 6 weeks of regeneration. Three differentiating photoreceptor inner segments are indicated by arrow heads. Bar = 10 μm .

Figure 10.

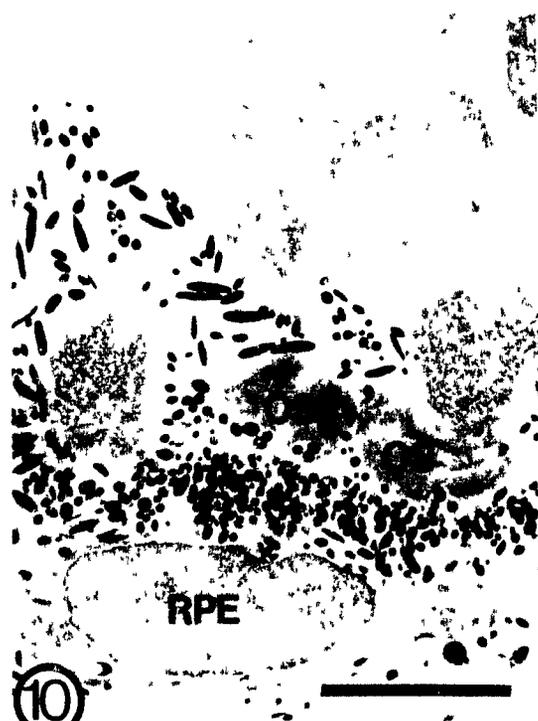
Electron micrograph of a newt photoreceptor and RPE cell layer after 8 weeks of regeneration. Developing photoreceptor outer segments (OS) are present. The RPE cells at this stage of regeneration are beginning to extend apical microvilli between the photoreceptor cells. Bar = 5 μm .

Figure 11.

Electron micrograph of the newt retina after 14 weeks of regeneration. At this stage of regeneration photoreceptor outer segments (OS) have elongated and appear to be fully developed. The RPE cells often appear to override one another at this stage. Bar = 5 μm .



9



10



11



Figure 12.

Montage of a series of electron micrographs showing a newt retina after six weeks of regeneration. This cross section through the neural retina and retinal pigment epithelium illustrate the poorly developed photoreceptor inner segments (arrows) and the absence of photoreceptor outer segments towards the back of the eyecup. However, the peripheral photoreceptor cells are differentiated and a few are showing outer segments (box). C = Central retina; M = Middle; P = Periphery; CN = ciliary nerve. Bar = 50 μm .

Insert. Higher magnification of photoreceptor outer segments found in the six week regenerating newt retina. The photoreceptor inner segments (IS) are relatively well developed and small outer segments (OS) can easily be observed. Bar = 10 μm .



12

Figure 13.

Montage of a series of electron micrographs showing a 1 day post-hatch newt tadpole. This cross section through the neural retina and retinal pigment epithelium illustrate the poorly developed photoreceptor inner segments (arrow heads) and the absence of photoreceptor outer segments in the peripheral area. At the posterior pole of the newt eye, photoreceptor outer segments are well developed and retinal pigment epithelial cell apical processes (AP) can be observed. Lens = L. Bar = 50 μm .

Insert. Higher magnification of the photoreceptors found in the central retina in the 1 day post-hatch newt embryo. Corresponding photoreceptor cells have been identified with a dot (•). Bar = 10 μm .



Figure 14.

Electron micrographs depicting myeloid body (MB) morphogenesis in the retinal pigment epithelium following surgical removal of the retina and its subsequent regeneration in the newt. Bar = 0.5 μm

A. Electron micrograph of an area of structurally reorganized smooth endoplasmic reticulum (SER) membranes (T) found in the cytoplasm of a six-week regenerating newt retina. These reorganized SER membranes formed a structure consisting of four apposed flattened cisternae. This structure is approximately 0.5 μm long.

B. Electron micrograph of two reorganized SER membranes structures (T) with increased length at eight week of regeneration.

C. Electron micrograph of SER flattened membrane structure (T) demonstrating additional saccules and an association of ribosomes with peripheral cisternae (arrows).

D and E. Electron micrographs of the SER stack of flattened saccules (T) demonstrating some fusion of the inner cisternal leaflets (open arrows).

F. Electron micrograph from a newt RPE cell after 12 weeks of retinal regeneration. The retinal pigment epithelial cell shows a number of mature myeloid bodies (MB) in close proximity to two SER membranes structure (T) which have little or no fusion of their cisternea. A few ribosomes are present on peripheral cisternea of mature MBs(arrows).

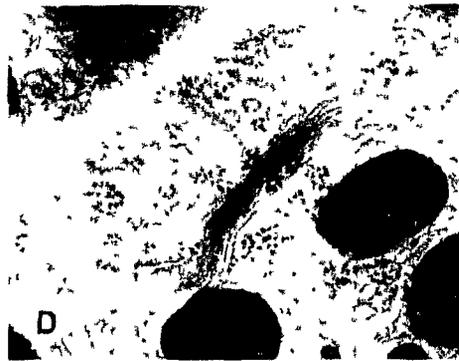
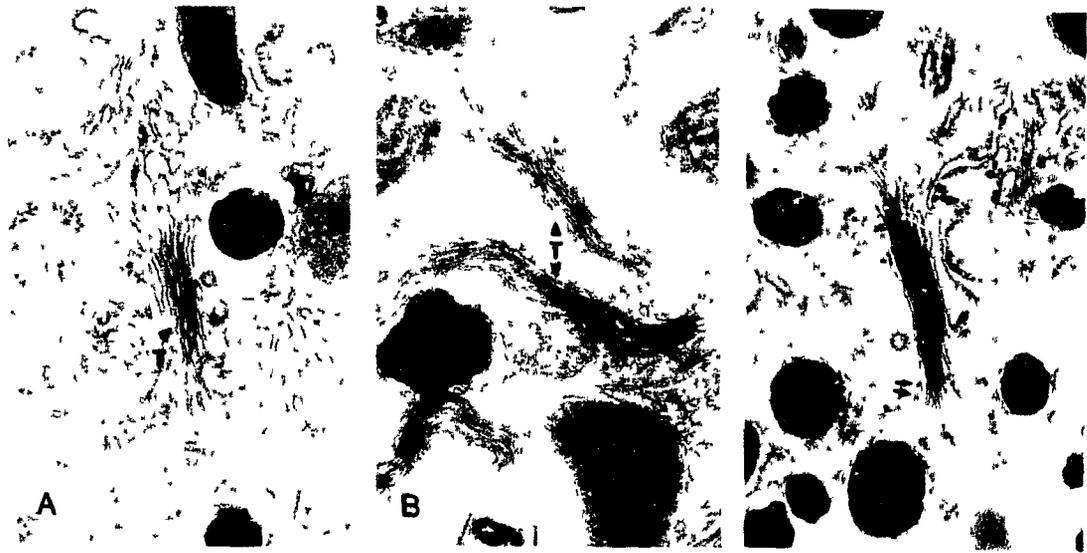


Figure 15.

Changes in average myeloid body (MB) and stack of unfused flattened saccules (template) area within regenerating newt retinal pigment epithelium observed throughout the regeneration process and in the adult non-operated animals. The average template size over the 14 weeks (week six to 22 of regeneration) of the study was $0.67 \mu\text{m}^2$ and this was relatively constant. The inherent variation in the size of individual MB at each sampling point resulted in large standard deviations. However, when present, MBs were on average two to three times larger than the templates at all sample points during regeneration.

Myeloid body and template size during retinal regeneration in the newt

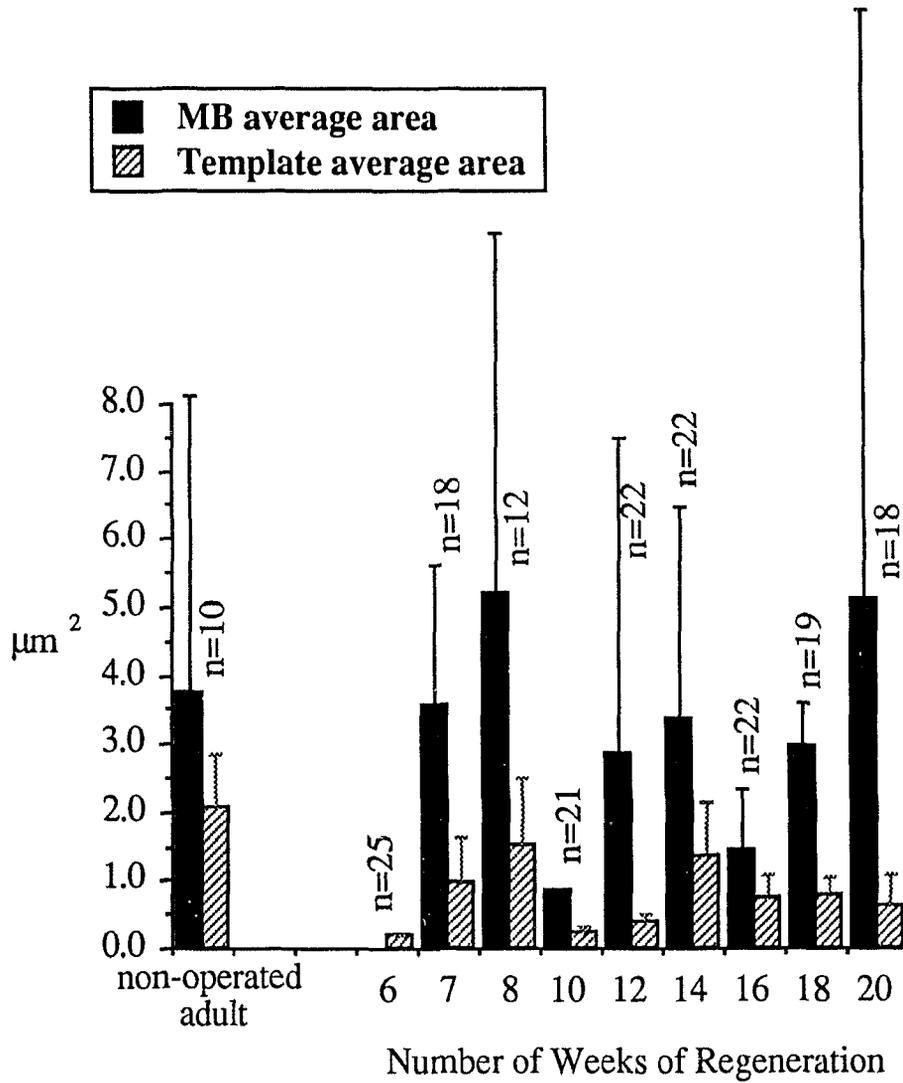


Figure 16.

Changes in the number of myeloid bodies and unfused flattened saccules structure (template) per $100 \mu\text{m}^2$ of retinal pigment epithelial cell cytoplasm during retinal regeneration and in the adult, non-operated newts. At six weeks of regeneration, there was on average less than one template per $100 \mu\text{m}^2$ of RPE cell cytoplasm. No MBs were observed in the 25 RPE cells surveyed from three different animals at this early time point. Numbers of MBs and template peaked at 12 weeks and remained constant throughout the remaining eight weeks of this study.

Myeloid body and template number during
retinal regeneration in the newt

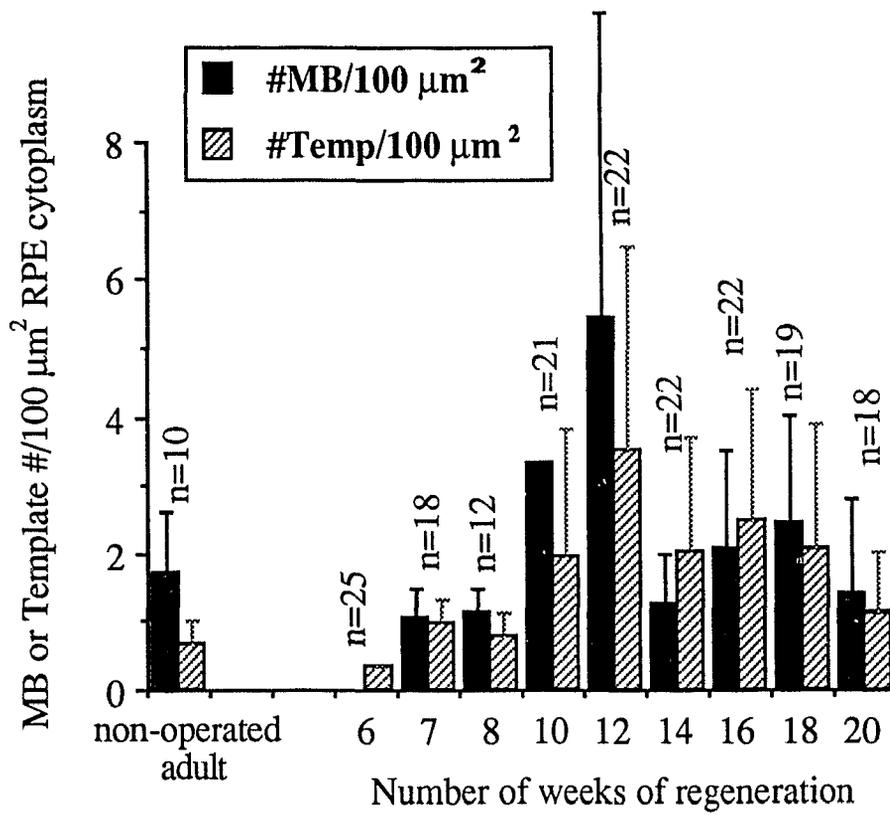
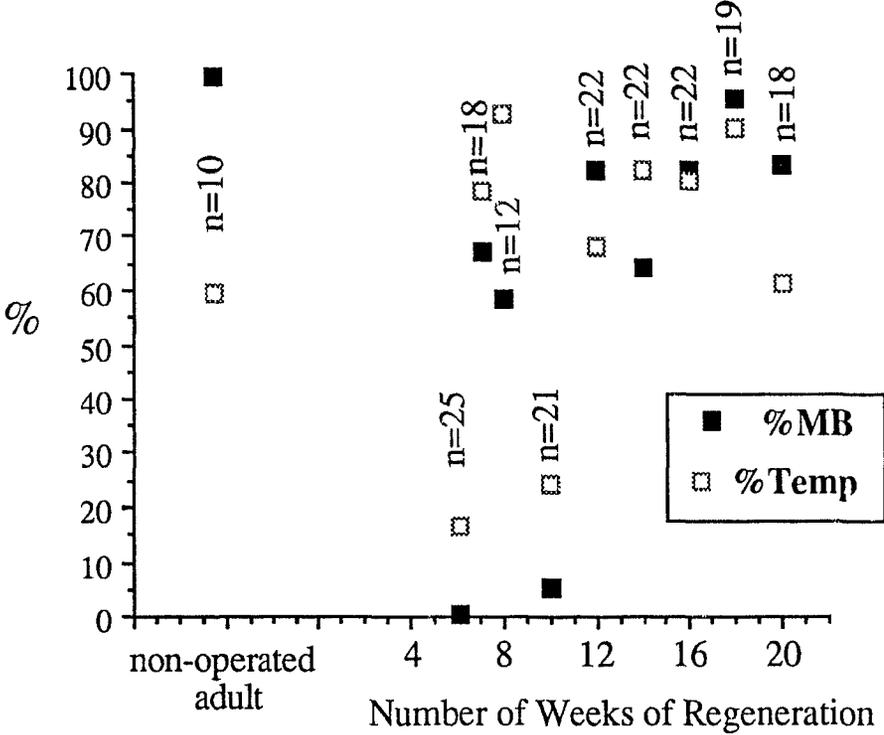


Figure 17.

Data for MB and unfused flattened saccules structure (template) appearance in the retinal pigment epithelium of the newt during retinal regeneration. The data has been plotted as a percentage of cells containing these organelles at each sample point during regeneration , as well as for the adult, non-operated controls. The expression of template by the SER preceeded that of MBs and was maximal at 75% of RPE cells at 16 weeks of regeneration, after which only 60% of the RPE cells expressed these structures at 20 weeks of regeneration and in the adult, non-operated animal. The expression of MBs, on the other hand, began later and reached a maximum of 95% at 18 weeks of regeneration. Templates = dashed box. Myeloid bodies = full box.

Percentage of RPE cells with either MB templates or MBs during retinal regeneration in the newt



Discussion

a) Techniques Used to Complete this Thesis

Recent advances in retinal cell biology have significantly enhanced our understanding of both the structural (Pfeffer and Fisher 1981; McLaughlin et al. 1983; Matsumoto et al. 1987) and functional (Greenberger and Besharse 1985; Lentricchia et al. 1987; Tarnowski and McLaughlin 1987; Akeo et al. 1988) relationships between retinal photoreceptors and the pigment epithelium. However, understanding the nature of myeloid bodies (MBs) continue to represent a challenge, both functionally and structurally. In spite of recent electron microscopic and morphometric analyses that have linked outer segment phagocytosis with MB occurrence (Yorke and Dickson 1984), and histochemical evidence that suggests an involvement of outer segment membrane lipids in MB formation (Yorke and Dickson 1984; 1985a), a detailed biochemical analysis of MB has never been reported or undertaken. The major hurdle to be overcome prior to such an analysis was the development of a cell separation technique that would provide an enriched MB membrane preparation. Since no specific method was available for the isolation of a MB-rich subcellular fraction from RPE cells, a procedure employing differential centrifugation was initially developed in this laboratory (Boylan and Dickson 1987). However, this process yielded fractions with only a moderate concentration of MBs. Accordingly, I undertook to improve the quality of isolated MB material using both differential and sucrose-gradient centrifugation.

My first objective was to obtain a retinal pigment epithelial cell preparation from seven day old White Leghorn chick eyes without contaminants of neural retina origin. As tissue culture conditions have been shown to have dramatic effects on pigment epithelial cell morphology (Israel et al. 1980; Opas and Dziak 1988), the RPE isolation protocol developed here uses culture media techniques which reduced the normal close association between the RPE and the photoreceptor outer segments and allowed the neural retina to be removed without leaving outer segment tip contamination on the RPE apical surface. By removing contaminants of neural retina (photoreceptor) origin, the new protocol which has been designed especially for chick retina, ensures the recovery of a highly purified preparation of RPE cells. To check for the possibility of outer segment contamination, RPE cells were examined for purity by wet-mount phase contrast microscopy and were shown to be free of outer segment contaminants before being homogenized. Any contaminants encountered in MB preparations are most probably of smooth endoplasmic reticulum origin and may be the result of the continuity of these two membrane systems. The current procedure appears to be reliable and yields consistent results. However, one major difficulty was the small amount of MB material that was ultimately isolated. This understandably strained the analytical portion of the research.

Without the development of a MB isolation protocol, it would have been impossible to obtain the lipid analysis results found in this thesis. A large number of experiments were required simply to establish a reliable method for the isolation of MBs, and since MBs can not be observed at the light microscope level, all the pellets from these experimental trials had to be examined under the electron microscope. From these observations of gradient interface pellets (Fig. 4), I established that I had obtained an enriched preparation of MBs. However,

because pelleted particles of differing sedimentation coefficients or densities result in a distribution in the pellets that is far from homogeneous, my observations were of a qualitative nature. While working on the redaction of this thesis, I came across a method described by Baudhuin et al. in 1967, which I would recommend to quantitatively estimate the purity of MBs preparations in future attempts to improve the MB isolation protocol. This method allows for the transfer of pelleted fractions to a flat surface, using a filtration technique, and provides specimens that are truly representative of the whole preparation and satisfies requirements for random sampling. The technique is applicable to minute quantities of material and could be truly invaluable for analysis of myeloid bodies.

b) Biochemistry of Chick Myeloid Bodies

The role played by RPE cells in maintaining the integrity and function of the photoreceptors, especially in the avascular retina of species with MBs, is very important. Retinal pigment epithelial cells are directly implicated in the biochemistry of vitamin A and its derivatives, as well as the essential fatty acids. Elucidating the involvement of MBs in the different biochemical processes taking place in RPE cells is essential to the overall understanding of photoreceptor-RPE interactions. This study has partially resolved the role played by MBs in the biochemistry of essential fatty acids of the RPE in chicks.

The sensitivity of the phosphorus analysis allowed us to measure four major phospholipids in MB preparations; phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, and sphingomyelin (Table D).

The amount of sphingomyelin and phosphatidylinositol in chick MBs is high when compared to literature values for chick outer segments, chick whole retina (Johnston and Hudson 1974), bovine rod outer segments (ROS) (Poincelot and Abrahamson 1970a; 1970b; Daemen 1973), frog ROS (Eichberg and Hess 1967; Daemen 1973), and rat ROS (Daemen 1973), as well as whole retina of dog, pig, human, sheep and rabbit (Anderson 1970). Furthermore, phosphatidylserine and lyso-phosphatidylcholine, which were present in small quantities in ROS preparations in this study, remained undetected in chick MBs.

I also analyzed the phospholipid composition of purified chick ROS as a method of standardizing my procedure and in order to be able to compare MB and ROS phospholipids and fatty acids analysis with identical protocol. When compared to cited values for chicks (Johnston and Hudson 1974), my results for rod outer segments are in agreement with the exception of modest differences in PtdIns and SPM. I am confident that my protocol allowed me to obtain results which adequately reflect the phospholipid and fatty acid compositions of both MBs and ROS.

To date, a number of observations made on MBs suggest a relationship between MB occurrence and outer segment phagocytosis (Yorke and Dickson 1984; 1985a; 1985b). The MB membrane phospholipid and fatty acid determinations carried out here provide further evidence of such a relationship, in that MBs and outer segment membranes appear to share an enriched pool of essential polyunsaturated fatty acids (Table E and F). However, the long-chain polyunsaturated fatty acids which I measured in chick MBs are present in proportions typical of the rod outer segments of a number of species (Eichberg and Hess 1967; Poincelot and Abrahamson 1970a; 1970b; Anderson and

Sterling 1971; Daemen 1973), but, not of the chick. Although the fatty acid composition of MB phospholipids is clearly different from both whole chicken retina (Johnston and Hudson 1974) and chick rod outer segments, as determined in this study, there is no documented analysis of cone outer segment phospholipids from any animal species. The most striking of the differences in essential polyunsaturated fatty acid between MBs and rod outer segments is the greater concentration of docosapentanoic acid (22:5 n-3) in MBs. Both my results and those of Johnston and Hudson (1974) show a relatively small amount of this fatty acid, as well as other long-chain polyunsaturated fatty acids in chick rod outer segments. In general, the quantities of long-chain polyunsaturated fatty acids present in bovine, frog, rabbit and goldfish rod outer segments, as well as in squid photoreceptors, are much greater (Poincelot and Abrahamson 1970b; Daemen 1973; Anderson et al. 1978; Fliesler et al. 1983) and sometimes reaches or surpasses 50% of the total fatty acid content of these membranes (Anderson et al. 1978), as is the case in MBs (51%). Docosahexaenoic acid (22:6 n-3) on the other hand is much more prevalent in chick rod outer segments than in MBs. However, Gordon and Bazan (1990) and Rodriguez de Turco et al. (1990) have shown a differential uptake of ^3H -docosahexaenoic acid by rods and cones of frog, monkey and human retina, suggesting differences in metabolism and/or phospholipid fatty acid composition in rods and cones of these species. Given that chick photoreceptors are composed of 60-70% cones and 30-40% rods (Meyer and May 1973), and assuming that MBs arise from phagocytized outer segments of both rods and cones, the observed low concentration of 22:6 n-3 and high concentration of 22:5 n-3 in MBs suggest two possibilities. The first could be a reversal of 22:6 n-3 to 22:5 n-3 acid in MBs supported by RPE

peroxisomes, organelles which are known to participate in both degradative and synthetic pathways of lipid metabolism (Lazarow 1987; Beard et al. 1988). Although such a metabolic pathway, described as retroconversion, is known (Grønn et al. 1991), the quantitative significance of the pathway is as yet unclear. Furthermore, the combined amount of 22:5 n-3 and 22:6 n-3 is greater in MBs than in rod outer segment in all but one phospholipid. If reversal were possible, one would have expected the combined amount of 22:5 n-3 and 22:6 n-3 in MBs to be less or equal to the combined amount in rod outer segments; this is not the case. Given the observed difference in concentration of 22:5 n-3 and 22:6 n-3 between MBs and rod outer segments, together with the unclear significance of the retroconversion pathway, the second possibility could be that the reduced 22:6 n-3 concentration in MBs (when compared to rod outer segments) could be accounted for on the basis of an inherent high concentration of 22:5 n-3 in chick cone photoreceptors. If a link is to be made between photoreceptor outer segment shedding and MB lipids, my results then suggests that the source of MB phospholipids and fatty acids in the chick is essentially of cone outer segment origin, although the presence of 22:6 n-3 in MBs also indicates a contribution from rod outer segments. The presence of cone outer segment phospholipids and fatty acids in MBs is not surprising however, since the occurrence of myeloid bodies in the lizard *Scleropus occidentalis* (Western fence swift), an animal with a pure cone retina, has already been demonstrated (Young 1977). Knowing the phospholipid and fatty acyl chain composition of chick cone outer segments might clarify this issue. Unfortunately, although cone visual pigments from chicken have been purified (Fager and Fager 1982), I am unaware of any

means of selectively isolating cone outer segments in chicken or any other animals retinas.

The significance of phospholipid and fatty acid composition on the ultrastructure of MBs is also worthy of consideration. It is well established that the lipid composition of membranes is responsible to a large extent for their conformation and organization. It is now accepted that phospholipids can have specific effects, both on the general structure of biological membranes, and on internal membrane organization as well (Buchheim et al. 1979; Cullis and De Kruijff 1979; Cullis et al. 1980; Borovjagin et al. 1982; Devaux 1982; Spector and Yorek 1985; Lindblom and Rilfors 1989; McElhaney 1989). For example, phospholipids with a polar head group containing either a net negative or positive charge would be expected not to pack closely and have more motion due to repulsion between the head groups. It was found that approximately 80% of the phospholipids in MBs are phosphatidylcholine and phosphatidylethanolamine, both with a net neutral charge, and thus more likely to form more compact structures, as demonstrated in lipid monolayer studies (Phillips and Chapman 1968).

Phosphatidylinositol has been shown to be an effective agent in stabilizing bilayer structures organized in a lamellar configuration at low concentrations (Nayar et al. 1982). The presence of a large fraction of phosphatidylinositol in MBs could help explain the change from a tubular smooth endoplasmic reticulum arrangement to the lamellar membrane organization that I observe in the formation of MBs.

Long-chain polyunsaturated fatty acids, such as 22:5, have also been suggested to facilitate the compaction of biological membranes (Haines 1979), as well as lowering transition temperature and enhancing fluidity within membranes.

The compactness of MBs, as well as their apparent crystalline membrane organization has been demonstrated by Yorke and Dickson (1984) and might also be explained by the phospholipid (PtdCho and PtdEtn) and fatty acid composition of these organelles. Myeloid bodies could be a prime example of the compacting effect caused by the incorporation of large quantities of phospholipids with long-chain polyunsaturated fatty acids, into a system that is normally organized in a tubular configuration.

It is also well established that the percentage of interactive lipids in biological membranes can also be related to membrane function. For example, most plasma membranes have a relatively low PtdEtn content, while intracellular membranes that are metabolically more active, or with special functions such as mitochondrial membranes, have a high PtdEtn content (Reviewed by Boggs 1980). The high content of PtdEtn in MBs may then indicate a high metabolic involvement of these organelles.

The great variety of different membrane lipids, with modifications in their hydrocarbon chains, polar groups, and backbone structure, suggests that each modification may play a unique role in membrane function. Acidic groups on lipids are clearly important since they allow interaction with basic groups on proteins and with ions. Another important property of certain lipids, which is not yet clearly understood in the membrane context, is their ability to interact with other lipids through hydrogen bonds. These interactions would occur mainly at the level of the fatty acid chains. In considering the various fatty acids that occur in a living cell, the presence of long-chain polyunsaturated fatty acids is questionable if one considers that the principal effect of unsaturation is to modify the fluidity of membranes. Indeed, it has been demonstrated that palmitoate (16:1) or oleate (18:1) in lipids, appear to be

capable of providing the whole range of fluidity required by membranes (Lands and Davis 1984). However, it has been previously demonstrated that lipids with a shorter chain length may impair or modify normal protein functions while lipids with fatty acid of longer chain length restores proper functioning (Abran et al. 1989). This may indicate two important aspects of fatty acid functions in lipids, in addition to their ability to alter membrane fluidity. Firstly, enzymatic reduction of the chain length of the lipid fatty acids in the microdomain surrounding a protein may render the protein inactive. Secondly, in myeloid bodies, the longer fatty acid chain length content of the phospholipids in these organelles may imply the presence of active proteins whose proper functioning depends on the presence of microdomains containing phospholipids with these long chain polyunsaturated fatty acids.

The difficulty of deriving an overall relationship between fatty acid saturation and membrane function relates to the fact that comparatively few reports include all the information necessary to establish such functional relationships. It is well known that biological membranes contain considerable diversity of phospholipids, and each of these, when totally defined with regard to every functional group, represents a molecular species. Molecular species analysis is inherently difficult and was not attempted in this study. Yet, if greater progress is to be made towards understanding the relationship between lipid composition, membrane function and the physical properties of MBs, a full analysis at the level of the molecular species will be needed.

A possible function for MBs could be an active involvement in the supply of both retinoids and polyunsaturated fatty acids to the photoreceptor by way of retinol-binding proteins. It is well established that the initial event of the visual cycle results in the reduction of 11-*cis* retinal to all-*trans* retinal in the

photoreceptor following absorption of light (Hubbard and Wald 1952). All-trans retinaldehyde is carried off to the pigment epithelium where it, together with retinoids originating from the blood supply, is esterified and reisolomerized to produce 11-*cis*-retinoids (Deigner et al. 1989). However, neither the identity of the 11-*cis*-retinoids returned from the RPE to the photoreceptor, nor the subcellular elements responsible for the all-trans to 11-*cis*-retinoid conversion, has been firmly established. Beinstein et al. (1987) demonstrated that membrane preparations from frog RPE cells which contained large and numerous MBs, could transform all-trans retinol into a mixture of 11-*cis*-retinoids. It is possible that MB could be involved in a mechanism that would account for the reisolomerization of all-*trans*-retinoids to 11-*cis*-retinoids. Previous work from this laboratory (Yorke and Dickson, 1985a) also suggested an involvement of MBs in lipid esterification. Myeloid bodies may then act as a site where all-trans retinoid is esterified, isomerized, stored or bound to proteins, and then sent to the apical RPE plasma membranes via cellular retinoid binding proteins (CRBP) to be transferred to the photoreceptor cells via interphotoreceptor retinol-binding proteins (IRBP) (Chader 1989) (see figure 18). Indirect evidence to that effect was provided by Flight and von Donselaar (1976) when they demonstrated the incorporation of ³H-vitamin A into MBs of the pineal of the urodele, *Diemictylus viridescens viridescens*.

Myeloid bodies may also be implicated in the recycling of polyunsaturated fatty acids to photoreceptor cells (Fig. 18). Bazan et al. (1985) have demonstrated that both fatty acids and retinol are transported by interphotoreceptor retinol-binding proteins in the monkey retina. Long-chain polyunsaturated fatty acids, like docosahexaenoic and docosapentaenoic acids, which are found in MBs, could be dealt with in the RPE in the same way as

retinoids. Myeloid bodies may then have the dual role of regulating retinoids and polyunsaturated fatty acid circulation between the RPE and photoreceptor cells of some vertebrate species.

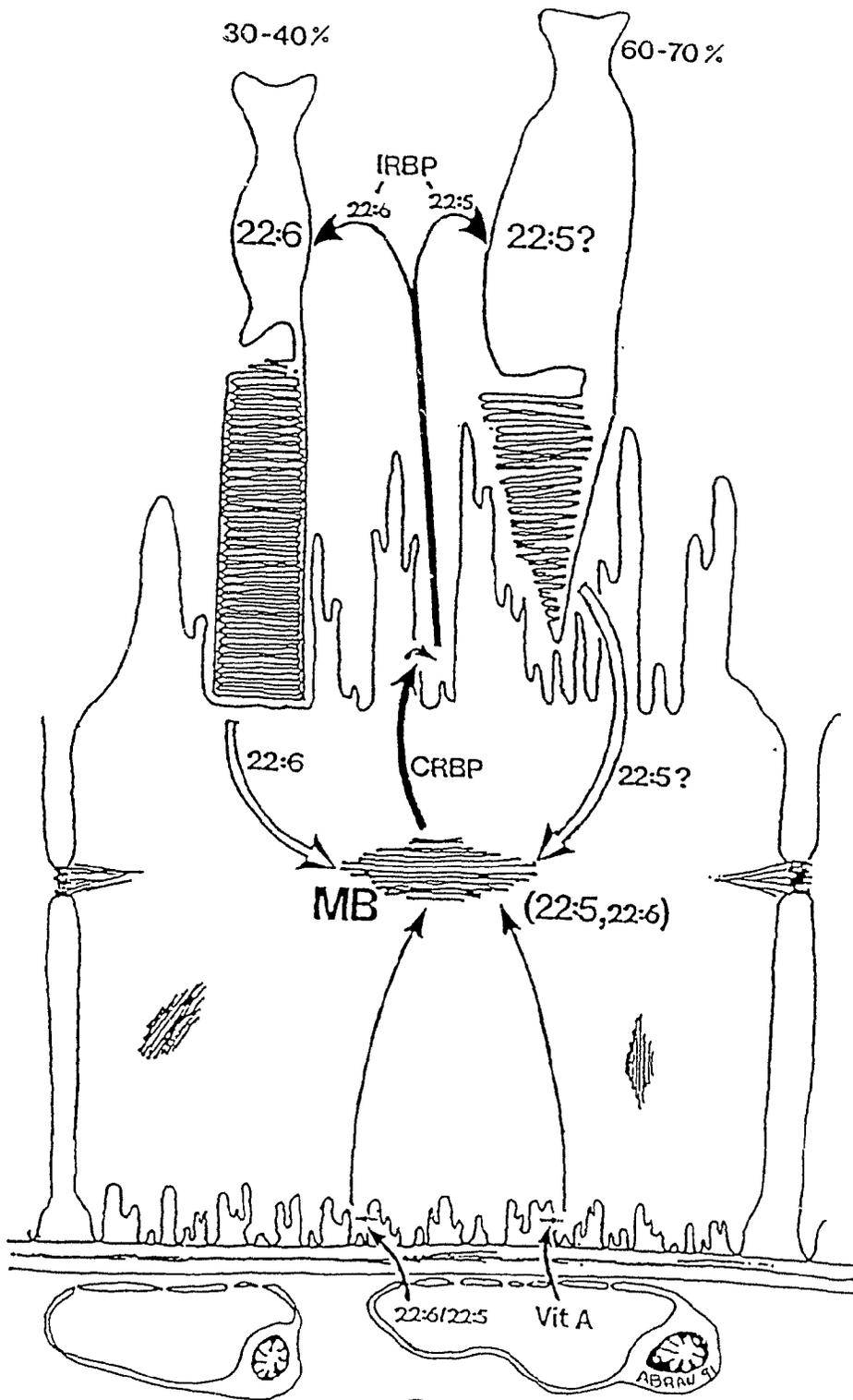
Although MBs have been shown to undergo cyclic changes in size, occurrence and relationship to other cytoplasmic organelles in the RPE over a 24-h light/dark cycle (Matthes and Basinger 1980; Tabor and Fisher 1983; Yorke and Dickson 1984; Yorke and Dickson 1985c), no direct link between MBs and other cyclic events, such as outer segment phagocytosis, has previously been made. The present investigation has attempted to provide this direct link by identifying the lipid composition of MBs and comparing it with that of other retinal components. These results have shown that the amounts of long-chain fatty acid material found in chick myeloid body membranes makes them different from chick rod outer segment membranes in this predominantly cone retina, and different from the general pool of retinal membranes as well. However, MBs are similar in their phospholipid and fatty acid composition to the rod outer segment of many other species. I believe that chick MBs concentrate long-chain polyunsaturated fatty acids in their membranes and that a significant proportion of these fatty acids in the chick may be of cone outer segment origin, in this predominantly cone retina.

c) Myeloid Bodies in the Regenerating Newt Retina

Although studies on the differentiation of photoreceptors in the developing retina have been carried out by Nilsson (1964) and Keefe et al. (1966), only Keefe (1973a; 1973b; 1973c; 1973d) has reported on the ultrastructural features of photoreceptor during urodelian retinal

Figure 18.

Schematic representation of a functional hypothesis for retinal pigment epithelium myeloid bodies (MBs). The drawing depicts from top to bottom; chick photoreceptor cells (30-40% rods and 60-70% cones), a pigment epithelial cell containing numerous MBs, Bruch's membrane and the choriocapillaris. The empty arrows demonstrate the main pathway by which polyunsaturated fatty acids could be incorporated into, and subsequently recycled by MBs. The solid arrows describe the normal supply pathway for vitamin A (vit A) as reviewed by Chader (1989), and polyunsaturated fatty acids as shown by Bazan (1985), to the retina. A MB is present as an intermediary in the pathway since it is hypothesized that MBs could have the dual role of regulating retinoids and polyunsaturated fatty acid circulation between RPE and photoreceptor cells. CRBP, cellular retinoid binding protein; IRBP, interphotoreceptor retinoid binding protein; 22:6, docosahexaenoic acid; 22:5, docosapentaenoic acid.



regeneration. In his studies, Keefe (1973a; 1973b; 1973c; 1973d) mentioned differences between the rate of differentiation of photoreceptors in different areas of the eye. He also paid particular attention to obtain results from one particular area of the retina (dorsal side of the posterior pole) and to get eyes with regenerated areas at approximately the same stage. Stone (1950c) reported that degeneration of the retina began first near the optic nerve head and proceeded outward toward the ciliary margin. However, he did not mention if there was a regional difference in the regeneration rate of the neural retina. Although I used a different technique to cause regeneration than Stone (1950c), who transplanted the eyes, Keefe (1973a; 1973b; 1973c; 1973d), who enucleated or retinectomized eyes without mentioning result differences between the two groups, I found that retinal differentiation was initiated first in the peripheral retina near the ora serrata and over time, proceeded radially to include the more central retinal areas. This same observation had also been made by Levine (1977) in the newt *Triturus cristatus*. He had surgically removed the retina of the newt *Triturus cristatus* with a technique similar to mine, but in addition, he rotated the anterior part of the eye (entire circumference, some distance behind the *limbus corneae*) and observed that under these circumstances, both the *pars ciliaris retinae* and the RPE participated in the regeneration of the new neural retina. However, he also stated that following a limbal incision, the regenerative response from the anterior part of the eye is inhibited (Levine 1975) while, after a post-limbal incision, there is a major regenerative response and a large part of the regenerated retina appears to be derived from the anterior complex (*pars ciliaris retinae*) (Levine 1977). My observations were made on newts, the retinas of which had been removed through a peripheral corneal-limbal incision (with

minimal disruption of the iris and ilial circulation). According to Levine (1975), such an incision would prevent the *pars ciliaris retinae* contribution to the regenerated retina, which meant that the regenerated neural retina in my study would only have RPE cells as precursors. The observation that the peripherally located photoreceptor OS are the first to differentiate and that this differentiation proceed radially towards the back of the eyes has never been reported elsewhere, and is supported by the different observations of the RPE differentiation made by Levine (1975, 1977) in his studies of the regenerating retina in the newt following surgical removal of the neural retina under various conditions. As a result, the last photoreceptor outer segments to regenerate were those closer to the optic nerve head. This positional difference in the regeneration of a new retina from the periphery to the optic nerve head is diametrically opposite to the normal pattern of retinal development in the embryo. This laboratory has studied the retinal development of the sea lamprey *Petromyzon marinus* L. (Dickson and Collard 1979), and the newt *Notophthalmus viridescens*, and found that retinal differentiation is initiated first at the posterior pole of the eye near the optic disc, and proceeds radially towards the periphery of the retina. This central to peripheral pattern of embryological development of the retina is typical of the vertebrate retinas in general (Grün 1982), including the human retina (Hollenberg and Spira 1973). My opinion is that the relatively undisturbed iris and ciliary arterial circle a critical vascular contribution that could be responsible for the faster differentiation process occurring in the RPE cells of the peripheral zone of the retina.

In this portion of the study, I have reported on the morphogenesis of myeloid bodies in the retina¹ pigment epithelium following surgical removal of

the retina and its subsequent regeneration in the newt. I have demonstrated that MBs disappear from RPE cells when photoreceptor outer segments are no longer present and in contact with the RPE. I have provided evidence to suggest that during regeneration, MBs find their origin in ultrastructural modifications of restricted areas of the normally tubular smooth endoplasmic reticulum (SER) membranes. These ultrastructural changes resulted first in the flattening of SER tubules, with the formation of unfused flattened saccules, which I have called templates. I believed the term template to be appropriate for a structure which eventually leads to the formation of MBs, since the term template means: "pattern or mold serving as a guide". In this study, I also demonstrated that photoreceptor outer segments must be present, and active outer segment phagocytosis must be occurring in order for MBs to reappear in the regenerating newt RPE. Further, the process of MB formation in the RPE during retinal regeneration is preceded by the formation of templates, which I have now documented, are continuously produced throughout the life of the animal, since they now have also been observed in adult non-operated controls. This latter observation suggests a dynamic process, with a turnover of MBs within the RPE cells. This finding is consistent with the morphometric analysis of Yorke and Dickson (1984) which outlined a diurnal fluctuation in MB numbers and lends support to the turnover hypothesis, although templates were not noted in that earlier study. In this MB renewal process, MB templates appear to be the initiator elements, followed by a gradual maturation and enlargement which gives rise to mature MBs, and subsequently, either a reversal to MB templates or the direct disappearance of MBs through elimination by the RPE cell.

The identification of MB templates has been somewhat problematical however, in that template numbers are low in the newt, with these precursor organelles being present in less than 60% of all RPE cells that have been observed. The scarcity of templates in regenerating retinal tissue, coupled with the nature of the regeneration process in general, contribute to the relatively high variation encountered at most data points. In spite of the scrupulous attention paid to the incorporation of morphometric sampling methods ensuring randomness in sampling, I was in fact not dealing with a homogeneous population of cells or organelles. Stratification of the retina was easily dealt with by simply sampling within the RPE cell layer. However, additional complexities were encountered in that the regeneration process does not proceed uniformly throughout the retina, but rather is initiated first within the peripheral retina at the region of the ora serrata and proceeds radially to include the posterior retina. As a result, for each regeneration time point studied, there was a variety of different stages of regeneration within the retina which contributes to the variation in morphometric data. Restricting the sampling to retinal areas of known regeneration status, as suggested by Keefe (1973a), would have seriously compromised the quantitative aspects of this study in that I would have introduced a significant sampling bias which would have predated against my goal to determine the overall timing of the genesis of MB formation in the regenerating retina. As a result, the predictable inter-animal variation was encountered, as well as a significant variation within each eye. Although a substantial augmentation in sample size may have reduced sample point variation, the enormous effort required did not seem to be warranted.

An additional concern associated with MB template morphogenesis relates to the size of these organelles and their similarity to the Golgi apparatus, allowing MB templates to be easily missed in routine surveys of this tissue. However, unlike the Golgi apparatus, MB templates exhibit no structural polarization of their lamellar stacks, such as the cis/trans configuration of the Golgi apparatus, and MB templates are devoid of cytoplasmic vesicles such as those commonly associated with Golgi cisternae. The fact that the occurrence of MB templates precedes the development of MBs, and that templates are present at all stages of the regeneration process following the differentiation of photoreceptor outer segments, as well as in the adult, supports the suggestion that templates are continuously formed and lead to the formation of mature MBs through a dynamic process of membrane addition and fusion.

Templates were first found to appear in the RPE at a time when photoreceptor outer segment disc formation had just begun in the regenerating retina, and in fact, MB template formation was found to precede photoreceptor outer segment disc shedding. This early occurrence of MB precursors seems contradictory to the earlier hypothesis that MBs arise as a result of the phagocytizing of specific outer segment lipids (Yorke and Dickson 1985a). However, since the appearance of MB templates clearly coincides with the development of photoreceptor outer segments, there could also be a link between the lipids required for the formation of photoreceptor outer segments and the lipids associated with MB templates. Although MB templates have not been analysed for their lipid content, nor is it likely that they could be isolated and analysed, on the basis of the lipid analysis of chick MBs which was undertaken in this laboratory, I would predict MB templates to be high in essential, long-chain fatty acids, like docosahexaenoic acid, which would of

necessity pass through the RPE of this avascular retina, by way of the choroidal vasculature, in order to reach and supply the growing photoreceptor outer segments.

Observations made in this study lead me to suggest that MBs undergo a process of maturation which includes the accumulation of additional, and elongation of existing lamellar cisternae, together with the formation of electron-dense band between adjacent membranes, which resulted from the fusion of the inner membrane leaflets of the template cisternae (Fig. 5 to 11). This apparent fusion of MB cisternal membranes is not a new observation, as it has been previously described in mature MBs of adult RPE cells (Nguyen H. Anh 1971; 1972a; Yorke and Dickson 1985a). The explanation of this phenomenon has however never been pursued in terms of either the structure or function of these unique RPE organelles. Evidence from this current study seems to indicate that the maturation process leading to the formation of these intracisternal dark bands is directly related to the phagocytosis of outer segment membranes, and although I observed the fusion of MB inner cisternal membrane leaflets early during the regeneration process, it did not occur until after outer segment phagosomes were detected in the RPE cells. Later, when the phagocytic process was well under way throughout the retina, the different morphological stages of the MB maturation process could still be observed. However, these same stages were not easily observed in the adult animal, as if the process was either too rapid or rarely occurring at the time when the adult control tissues were sampled. However, I believe that these electron-dense bands, which characterize template membrane fusion and MB maturation, represent a zone of strong interaction between membrane elements, resulting in

the formation of a membrane-membrane bridge, the explanation of which may relate to my analysis of MB membrane lipids.

In this study, I demonstrated that in relation to other membrane systems (Schwarzmann and Sandhoff 1990), chick MB membranes contained an unusually large quantity of sphingolipids in the form of sphingomyelin. This paragraph and the following one speculate on the potential significance of the presence of this phospholipid in MB membranes. It is well established (Kanfer et al. 1966) that sphingomyelin can be enzymatically degraded by sphingomyelinase, to give rise to a ceramide which consists of a sphingosine backbone and the associated fatty acid tail of the sphingomyelin molecule. This process has been well documented in a variety of mammalian tissues including rat liver (Kanfer et al. 1966), intestinal tract (Nilsson 1968) and brain (Gatt 1966), and is reversible (Shayman and Radin 1991). Interestingly, ceramides can give rise not only to sphingomyelin, which I have shown to be an important membrane constituent of MBs, but also to glycosphingolipids (Shayman and Radin 1991). As membrane components, glycosphingolipids are considered to have the potential to contribute to membrane physical properties by increasing rigidity and stability (Pascher 1976; Curatolo 1987a; Grant et al. 1990). In addition, they are known to have an established role as recognition sites that may mediate membrane interactions with the surroundings environment (Curatolo 1987b; Moran et al. 1991). I suggest that a gradual enzymatic transformation of sphingomyelin to ceramide, and the biosynthesis of glycosphingolipids which are thought to be catalysed within the endoplasmic reticulum (Shayman and Radin 1991), could account for the morphological transformation of templates into MBs. Since glycosphingolipids can have a variety of sugar head groups (Stein and Markus 1977), and there is evidence which suggests that the

glycosphingolipid head groups and oligosaccharide chains of glycolipids can interact cooperatively among themselves (Bunow and Bunow 1979; Skarjune and Oldfield 1979; Curatolo 1987a) through hydrogen bonding (Pascher 1976), such a network of hydrogen bonds at the level of glycosphingolipid head groups could serve as a basket for the heavy metal stain deposition which is observed in the MB maturation process.

Glycosphingolipids also possess another important physical attribute which may help to explain MB structural characteristics. The ceramide bridge in glycosphingolipids contains a unique amide bond that is known to enforce a rigid planar structure (Curatolo 1987a). In addition, lateral hydrogen bonding, as discussed earlier, is known to take place between lipid molecules and this is facilitated by the orientation of the hydrogen bond acceptors of the amide and hydroxyl groups (Pascher 1976; Karlsson 1977; Pascher and Sundell 1977). It is evident that the ability of these lipids to interact at both the ceramide bridge and head group levels makes glycosphingolipids prime candidates for the lateral segregation of lipids, and enhances their ability to form functionally important associations with the protein and glycoprotein membrane constituents. Schwarzmann and Sandhoff (1990) confirmed that glycosphingolipids cannot undergo transbilayer diffusion (flip-flop), and this could explain the fact that template membrane fusion occurs only on the intracisternal side of template saccules. It is conceivable that glycosphingolipids could create a lattice network within the inner leaflet of MB cisternae which could also explain the reported crystalline appearance of tangential sections of myeloid bodies under the electron microscope (Yorke and Dickson 1984). Such a model for MB membrane structure implies an asymmetry in lipid composition between leaflets of MB cisternal membranes,

with glycosphingolipids situated in the inner leaflet, away from the intracellular cytosol. In plasma membrane, sphingolipids are situated in the outer membrane leaflet (Karlson 1977), also away from the intracellular cytosol. Evidence for an asymmetric distribution of lipids across membranes is not new. Bretscher first demonstrated this phenomenon in the erythrocyte plasma membrane in 1972, and since then, the subject of lipid asymmetry has been extensively reviewed (Bergelson and Barsukov 1977; Rothman and Lenard 1977; Op den Kamp 1979; Etemadi 1980; van Deenen 1981; Benga and Holmes 1984; Schroeder 1984, 1985; Stubbs and Smith 1984; Sweet and Schroeder 1988).

In summary, this section of the thesis has presented a model for myeloid body formation, including a possible explanation of the ultrastructural changes involved in the maturation process of these organelles in the newt. It has also provided a morphometric analysis of the genesis of myeloid bodies and their template precursors throughout the regeneration process, as well as in the adult control RPE cells. It is also clear that the presence of photoreceptor outer segments and the process of RPE cell phagocytosis are essential to the formation of MBs. However, MB templates, the suggested precursors of MBs, seem to appear at an earlier stage, after the formation of rudimentary outer segments, but before phagocytosis has been initiated. I have found templates at all stages of the regeneration process, as well as in the adult unoperated control animals, a finding that could suggest an active role for myeloid bodies in the turnover and processing of lipids destined for outer segment renewal.

d) Concluding Remarks

It has become increasingly clear to me, that the Yorke and Dickson (1984) hypothesis which was developed regarding myeloid body function, was too simple. The discovery of essential fatty acids in the phospholipids which make up MBs also implies a very important role for this organelle in the metabolism and recycling of these fatty acids. The involvement of MBs in the wider context of the overall essential fatty acid metabolism in the retina should be further investigated. Also, the possible role of MBs in the recycling of vitamin A derivatives to the photoreceptor cells should not be forgotten. Understanding the functions of MBs in the lower vertebrate retinas may lead to important discoveries in the RPE of higher vertebrates, which do not have these organelles. There are still many questions to be answered in relation to the role played by MBs in the RPE. Hopefully, the work presented in this thesis has prepared new grounds for further studies on an enigmatic and very interesting organelle, the myeloid body.

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