

TCOMHSL  
  
M81GW6

LEWIS LIBRARY  
University of North Texas Center  
377 Camp Bowie Blvd.  
Ft. Worth, Texas 76107-2699







Ke, Tai-Lee, Age Related Changes in Rabbit Cornea: Permeability and Membrane Properties. Doctor of Philosophy (Biochemistry), December, 1994, 139 pp., 26 tables, 13 illustrations, bibliography, 117 titles.

This investigation was designed to characterize age-related changes in corneal function and biochemical structure. The specific aims were to: 1) systematically assess changes in permeability to compounds of different molecular weights and lipophilicities, 2) examine differences in tissue binding by utilizing a theoretical transport model, and 3) evaluate the biochemical changes in lipid composition and distribution.

Experiments to compare young (six weeks) versus old (three to four years) rabbit corneal permeability were carried out utilizing an in vitro diffusion model. Changes in corneal transmembrane resistance, permeability to various compounds, and metabolic capability were examined by various analytical techniques. In addition, a theoretical penetration model which took into account stromal binding was studied. Corneal lipid composition and distribution were assessed by HPLC and GC.

Permeabilities of selected compounds with different physicochemical properties were evaluated in young and old intact and denuded (wounded) rabbit corneas. With age, the



membrane permeability significantly decreased in parallel with an increase in transmembrane resistance. Age-related changes in activities of esterase and phosphatase were also found. For some compounds, the aged corneas exhibited longer lag times in penetration studies. This suggested that the binding constant in the cornea from older animals was higher than in young animals. Maximum binding capacity from theoretical model calculations correlated well with experimental results in the young corneal stroma but correlation was less rigorous for old corneal stroma. Age-related changes in lipid composition and distribution in corneas were observed and provide indirect evidence for a decrease in membrane fluidity (decrease in the ratio of phosphatidylcholine/sphingomyelin) in the aged cornea.

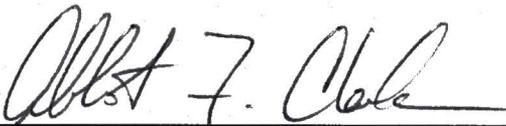
Results indicate that the aging process in the cornea is associated with changes in biochemical structural matrix including membrane lipid composition and physical properties such as fluidity (microviscosity). Functional correlations include changes in: 1) transmembrane resistance, 2) membrane permeability, 3) enzymatic activities (esterase and phosphatase), and 4) binding properties of the cornea. A possible mechanism for understanding and developing an intervention for aged-related changes in the cornea is postulated.



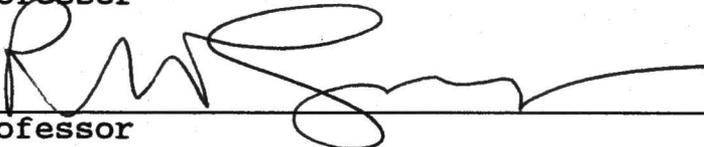
AGE RELATED CHANGES IN RABBIT CORNEA:  
PERMEABILITY AND MEMBRANE PROPERTIES

Tai-Lee Ke, B.S., M.S.

APPROVED:



Major Professor



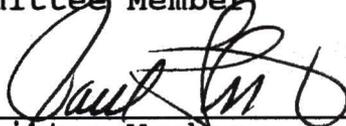
Minor Professor



Committee Member



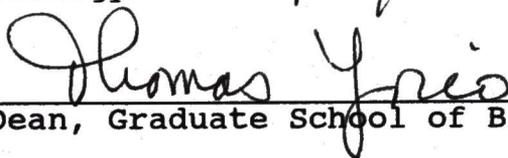
Committee Member



Committee Member



Chair, Department of Biochemistry and Molecular  
Biology



Dean, Graduate School of Biomedical Sciences



AGE RELATED CHANGES IN RABBIT CORNEA:  
PERMEABILITY AND MEMBRANE PROPERTIES

DISSERTATION

Presented to the Graduate Council of the  
Graduate School of Biomedical Sciences  
University of North Texas  
Health Science Center at Fort Worth  
in Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

By

Tai-Lee Ke, B.S., M.S.

Fort Worth, Texas

December, 1994



## ACKNOWLEDGEMENTS

I would like to express my gratitude and sincere appreciation to those who assisted me in this investigation:

To my advisors, Drs. A. F. Clark and R. W. Gracy, for their friendship, encouragement and guidance throughout my entire study.

To Dr. E. Cooper (Sterling Inc.) for the development of a theoretical corneal transport model.

To Dr. M. McCartney (Alcon Laboratories, Inc.) for his microscope work in the cornea.

To the other faculty of the Department of Biochemistry and Molecular Biology for sharing their individual expertise, especially Drs. P. Cook, B. Harris, and A. Lacko.

To my children, Glenda, Eugene, and Austin and especially to my husband, Victor, for their love, support, patience and encouragement.

Finally, this work was entirely funded by Alcon Laboratories, Inc. I deeply appreciate its commitment and support.



## TABLE OF CONTENTS

ACKNOWLEDGEMENTS . . . . .	iii
LIST OF TABLES . . . . .	vi
LIST OF ILLUSTRATIONS . . . . .	viii
LIST OF ABBREVIATIONS . . . . .	ix
I. INTRODUCTION . . . . .	1
A. Aging	
1. In General	
2. Membrane Lipids	
3. Membrane Fluidity	
4. Aging of Biological Membranes	
B. Cornea	
1. Anatomy and Physiology	
a. Epithelium	
b. Stroma	
c. Endothelium	
2. Drug Penetration, Deposition, and Metabolism	
3. Drug Binding	
4. Aging in the Cornea	
5. Aging in the Lens	
C. Purpose of This Study	
II. MATERIALS AND METHODS . . . . .	38
A. Materials	
1. Reagents and Chemicals	
2. Animals	
B. Methods	
1. Penetration Studies	
a. <u>In Vitro</u> Intact Cornea Penetration	
b. <u>In Vitro</u> Stromal/Endothelial Penetration of "Denuded Cornea"	
c. <u>In Vitro</u> Endothelial/Stromal Penetration	
d. Esterase and Phosphatase Activities	
2. Tissue Binding	
a. Penetration Experiments	
b. Membrane Partition Study	
c. Stromal Binding Experiments	



3. Lipid Analysis	
a. Lipid Extraction	
b. Lipid Fatty Acid Analysis	
4. HPLC Assays	
a. Penetration and Binding Studies	
b. Phospholipid and Cholesterol Analysis	
5. Fluorescence	
6. Transmembrane Resistance	
7. SEM and TEM	
8. Statistical Analysis	
III. RESULTS . . . . .	51
A. General Characterization and Transmembrane Resistance of Young and Aged Cornea	
B. Penetration Studies	
1. Age Related Changes in Permeability of Whole Cornea	
2. Age Related Changes in Permeability of "Denuded Cornea"	
3. Age Related Changes in the Rate of Enzymatic Hydrolysis	
C. Stromal Binding Constants	
1. Penetration Studies	
2. Partition Studies	
3. Stromal Binding Studies	
4. Theoretical Model for Stromal Penetration Study	
D. Lipid Analysis	
1. Fatty Acid Composition	
2. Phospholipid and Cholesterol Composition	
3. Membrane Fluidity	
IV. DISCUSSION . . . . .	107
A. Age-Related Changes in the Cornea	
1. Penetration Studies	
2. Enzymatic Activities	
3. Tissue Binding	
4. Lipid Composition and Membrane Fluidity	
B. Summary	
C. Future Studies	
APPENDIX . . . . .	127
REFERENCES . . . . .	129



## LIST OF TABLES

Table		Page
1.	Size and transmembrane resistance of young and aged corneas. . . . .	56
2.	Age related changes in whole corneal permeability to lipophilic compounds.. . . .	61
3.	Age related changes in whole corneal permeability to compounds of different molecular weight. . . . .	62
4.	Age related changes of denuded cornea to permeability of compounds with different lipophilicities. . . . .	64
5.	Age related changes in denuded corneal permeability of compounds with different molecular weight.. . . .	65
6.	Comparison of permeability changes between denuded and intact corneas on basis of compound's lipophilicity or molecular weight.. . . .	67
7.	Age related changes in endothelial/stromal permeability of basis of molecular weight.. . . .	69
8.	Effect of age on esterase and phosphatase activities in the intact cornea.. . . . .	71
9.	Effect of age on esterase and phosphatase activities in the stromal/endothelium (denuded cornea).. . . . .	72
10.	Suprofen lag time and permeability coefficient in the young cornea. . . . .	74
11.	Suprofen lag time and permeability coefficient in the old cornea. . . . .	75
12.	Suprofen lag time and permeability coefficient in the young stroma/endothelium. . . . .	76



13.	Suprofen lag time and permeability coefficient in the old stroma/endothelium.. . . . .	77
14.	Caffeine lag time and permeability coefficient in the young cornea.. . . . .	78
15.	Caffeine lag time and permeability coefficient in the old cornea.. . . . .	79
16.	The slope and intercept of 1/suprofen concentration versus lag time.. . . . .	85
17.	Fatty acid composition in young and old corneal epithelium. . . . .	91
18.	Fatty acid composition in young and old corneal stroma/endothelium . . . . .	92
19.	Fatty acid composition in young and old lens epithelium. . . . .	93
20.	Fatty acid composition in young and old lens cortex. . . . .	94
21.	Fatty acid composition in young and old lens nucleus.. . . . .	95
22.	Phospholipid and cholesterol composition in various ocular tissues.. . . . .	98
23.	Changes in phosphatidylcholine/sphingomyelin ratio of various ocular tissues.. . . . .	101
24.	Age related changes in phosphatidylcholine/ sphingomyelin ratio of various ocular tissues..	102
25.	Changes in cholesterol/phospholipid ratio of various ocular tissues.. . . . .	104
26.	Age related changes in cholesterol/phospholipid ratio of various ocular tissues. . . . .	105



## LIST OF ILLUSTRATIONS

Figure		Page
1.	Chemical structure of phosphatidylcholine and sphingomyelin. . . . .	6
2.	Chemical pathways for synthesis of phosphatidylcholine from A) CDP-choline and B) methylation of phosphatidylethanolamine.	12
3.	Cross-section view of the cornea. . . . .	15
4.	Artist's rendition of the structure of epithelium. . . . .	19
5.	The arrangement of collagens in the stroma .	22
6.	Structure of the rabbit corneal endothelium .	25
7.	A schematic drawing of a compound diffusing through the cornea. . . . .	40
8.	Scanning electron micrograph of the epithelium. . . . .	52
9.	Transmission electron micrograph of the cornea	54
10.	A typical set of data for transport process.	57
11.	The relationship between lag time and suprofen concentration in young animals. . . . .	81
12.	The relationship between lag time and suprofen concentration in old animals. . . . .	83
13.	Binding of suprofen with stroma in equilibrium studies. . . . .	86



## LIST OF ABBREVIATIONS

C12:0	Laurate
C14:0	Myristate
C16:0	Palmitate
C16:1	Palmitoleate
C18:0	Stearate
C18:1	Oleate
C20:4	Arachidonate
CHOL	Cholesterol
EPI	Epithelium
GBR	Glutathione bicarbonate Ringer solution
GC	Gas chromatography
HPLC	High performance liquid chromatography
M.W.	Molecular weight
NUCL	Nucleus
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PI	Phosphatidylinositol
PL	Phospholipids
PS	Phosphatidylserine
SEM	Scanning Electron Microscopy



SPH

Sphingomyelin

SOR

Superoxide radical

STR

Stroma

TEM

Transmission Electron Microscopy



## CHAPTER I

### INTRODUCTION

It is becoming very important to understand the effect of aging on ocular tissues. More people are living longer and ocular diseases are generally an affliction of the elderly (Young, 1992). Degenerative ocular diseases are generally described as being caused by or related to the aging processes. Age related cataract and macular degeneration are the major causes of blindness among the elderly. Therefore, as the population grows older, more ocular therapy will be needed.

The present study focuses on the effects of aging in the cornea since the majority of topically applied substances enter the eye by passing across the cornea (Benson, 1974). This chapter is divided into three sections: 1) Aging and its related membrane changes, 2) Cornea, corneal structure and membrane permeability, and 3) The purpose of this study.

#### Aging

Aging is a complex process that leads to many alterations involving major biological functions of physiological, biochemical and immunological importance. Aging appears to affect every tissue, organ, and



physiological function of an organism. There are two major theories of aging: the programmatic theory states that aging is an inherent genetic process, while the stochastic theory states that aging represents random environmental influences which result in wear and tear during the life span. Work on changes in membrane function, protein synthesis, DNA structure has lead to the conclusion that aging, at least in part, is a programmed process (Cutler, 1984). The physiological age-related changes are quite different; cells in different organs age at different rates, at different times and in different ways. Some investigators proposed that true age changes may be more easily demonstrated in the apparently younger cells than in older ones (Watkin, 1982 and Cutler, 1984).

It is believed that the aging process is associated with oxygen free radical reactions which lead to lipid and protein peroxidation (Hegner, 1980 and Wahnnon, 1989). Such reactions can promote crosslinking interactions in the lipid and protein moieties of the membrane, thereby contributing to changes in membrane properties. Several investigations (e.g., Shinitzky, 1984; Hamm, et al., 1985 and Keelan, et al., 1985) have suggested that age-dependent deterioration in cell functions can be related to molecular and functional changes in the properties of biological membranes.

Membrane integrity is a fundamental prerequisite for cellular homeostasis, and its structural intactness is a



prerequisite for optimal biological functioning of cells. The inseparable relationship between structure and function has been the subject of several studies (e.g., Hegner, 1980 and Yu, et al., 1992).

Recently, attention has been devoted to the age-dependent changes in membrane function and composition. Peroxidation of membrane components seems to be an ongoing physiological phenomenon *in vivo* and is cumulative with age (e.g. accumulation of lipofuscin in the central nervous system - Hochstein and Jain, 1981, Wolf, 1993). Lipid peroxidation increases with age due to the increased production of oxygen radicals (Schroeder, 1984). Lipid Peroxidation results in a change in lipid composition and a direct or an indirect decrease of lipid-dependent enzyme activities and transport capacities (Dobretsov, et al., 1977). Receptor losses are at least 50-70% over the lifespan, while enzyme activities decrease about 25% (Roth, 1979).

### Membrane Lipids

Membrane functions are influenced by the composition and physical state of lipids. The physicochemical properties of membrane lipids are largely governed by the nature of the fatty acid and phospholipid components. In biological membranes at physiological temperature, the bulk of lipids are in a liquid-crystalline phase in which the lipid hydrocarbon chains are relatively fluid (Zelenka, 1984). The



liquid-crystalline state is distinguished from a gel phase in which the hydrocarbon chains are rigid and closely packed. The liquid-crystalline fluid state of a biological membrane (membrane fluidity) is an essential condition for maintenance of different plasma membrane functions (Hegner, 1980), including membrane proteins such as receptors, enzymes, and ion channels, which are highly sensitive to the lipid environment.

Membrane lipids play a primary role in age-related changes in membrane function through alterations in lipid and phospholipid content and distribution, one of the major changes typical of aging in mammals. Lipid changes cause a rigidification of biological membranes, a dynamic parameter which influences the lateral mobility and organization of membrane components and domain structure. Accordingly, alterations in membrane lipid affect the biology of cells, tissues, organs, and the whole animal to a considerable extent (Marin, et al., 1990).

### Membrane Fluidity

Membrane fluidity is the dynamic physicochemical property of membranes and is important in regulating the sequence of events involved in such processes as signal transduction (Loh and Law, 1980; Shinitzky and Henkart, 1979). Plasma membranes are approximately as viscous as honey. The lipid bilayer is being held together by strong hydrophobic bonds that permit diffusional and rotational



movement within the membranes. The viscosity determines the availability of diffusible membrane components such as receptors and channels. The maintenance of a constant membrane lipid fluidity (microviscosity) within precisely determined limits has been identified as a necessity for proper functioning of almost all types of cells (Shinitzky, 1984).

The main factors that determine the fluidity of membrane lipids are: 1) the ratio of phosphatidylcholine to sphingomyelin (Svshinitzky and Barenholz, 1974; and Antonian, et al., 1987); 2) the ratio of cholesterol to phospholipid (Hegner, 1980); and 3) the interaction between the fatty acid side chains of membrane phospholipids (Watson, et al., 1975; and Janki, et al., 1975). An unidentified homeostatic mechanism regulates membrane fluidity, mainly by adjusting all of these factors.

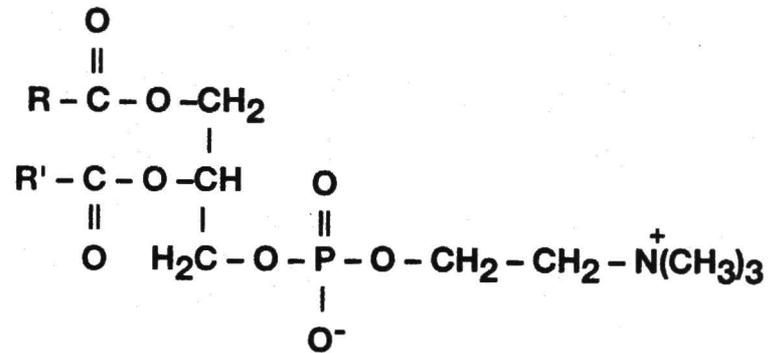
Phosphatidylcholine and sphingomyelin constitute more than 50% of the phospholipids in most mammalian membranes (Figure 1). Sphingomyelin is chemically more stable than glycerophospholipids (phosphatidylcholine). The high degree of saturation of the acyl side chains, as well as the trans double bond and the amide bond in the hydrophilic region of sphingomyelin, contribute to the rigidifying nature of membranes. In contrast, phosphatidylcholine forms highly fluid lipid regions (Zelenka, 1984). Aging is characterized by a substantial increase in the membrane sphingomyelin/



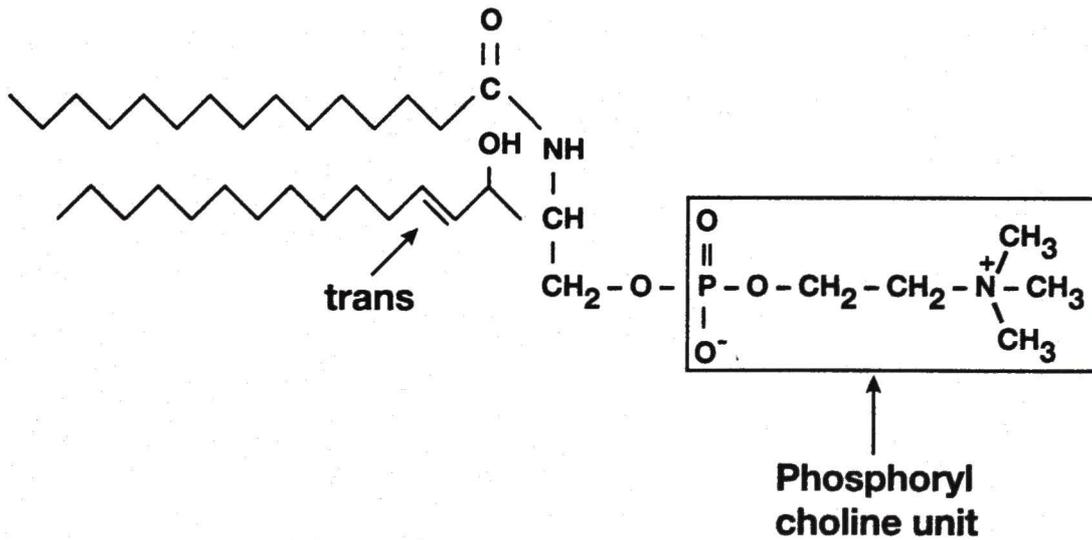
Figure 1. Chemical structures of (A) phosphatidylcholine and (B) sphingomyelin. Phosphatidylcholine is derived from glycerol. Sphingomyelin is derived from sphingosine and its residues pack tightly into the outer leaflet of the phospholipid bilayer, producing a membrane with low fluidity (Stryer, 1988).



## A. Phosphatidylcholine



## B. Sphingomyelin





phosphatidylcholine ratio and a decrease in membrane fluidity (Eisenberg et al., 1969, Antonian et al., 1987, Marin et al., 1990).

Due to the chemical structure, cholesterol prevents the transmission of motion in one fatty acid chain to its neighbor. The dampening of the cooperative motion of chains causes the changes in the membrane to remain relatively local. Thus, membranes with high cholesterol content do not undergo sharp phase transitions (from gel state to liquid state).

The interactions between the fatty acid side chains increase with increasing chain length and are hindered by the presence of double bonds which prevent close contacts between the acyl chains. The hydrocarbon chains of unsaturated fatty acids exhibit more freedom of motion due to their *cis* configuration of the double bond. Thus, high concentrations of long chain saturated fatty acids tend to form a more rigid lipid matrix.

Overall, membrane fluidity affects the accessibility of surface receptors to external ligands and modulates subsequent signal transduction by either the activation of adenylate cyclase or the microaggregation of ligand-receptor complexes. Lipid fluidity is important in changing membrane permeability, osmotic fragility, and the activity of certain membrane-bound enzymes and transport systems.



At the cellular level, reactive oxygen species have been found to be responsible for alterations in membrane fluidity by reacting more readily with unsaturated phospholipids. A rise in superoxide radical (SOR) levels was found with age (Hegner, 1984). SOR formation appears to be enzyme-mediated. Elevated levels of this oxygen radical could be involved in membrane breakdown in older animals. The rise in the SOR formation in the membrane was associated with an increase in lipid peroxidation, a decrease in membrane fluidity, and an elevation in cathepsin B activity in the brain and liver (O'Brien, 1987).

#### Aging of Biological Membranes

Several investigations have suggested that age-dependent deterioration in cell functions can be related to molecular and functional changes in the properties of biological membranes (Calderini, et al., 1983; Gastaldi, et al.; and Hegner, 1980). During tissue aging, cellular metabolic processes slow down. Aged cells compensate for their reduced ability to carry out normal processes by accumulating lipids, particularly cholesterol and sphingomyelin in their membranes. Consequently, the CHOL/PL and SPH/PC increase. These increased ratios result in decreased membrane fluidity (Antonian et al., 1987). Several alterations in lipid composition are known to occur with aging and are likely to reduce the membrane fluidity: a decrease in the degree of fatty acid unsaturation, an



increase in the content of cholesterol, and an increase in the content of SPH and thereby in the SPH and PC ratio.

The age-related changes in membrane lipid composition and fluidity appear as a constant finding and have been observed in a variety of tissues such as intestinal brush border (Wahnon, et al., 1989), lymphocytes (Huber, et al., 1991), liver cells (Benedetti, et al., 1988), red blood cells (Prisco, et al., 1991), macrophages (Alvarez, et al., 1993), erythrocytes (Tozzi-Ciancarelli, et al., 1989 and Martin, et al., 1990), and brain (Kessler, et al., 1985), in humans as well as in experimental animals. For example, the phospholipid content in red cell membranes of old donors is significantly lower than in young ones. Cholesterol and fatty acid compositions show no difference between young and old donor groups; however, the phospholipid/cholesterol ratio is decreased with age. This is associated with decreased membrane fluidity, a decrease of  $\text{Na}^+\text{-K}^+\text{-ATPase}$  activity, and a change in ion permeability (Hegner et al., 1979).

In rat brain, microsome membrane properties also undergo changes with increasing age (Calderini, et al., 1983, Lepagnol, et al., 1993). The rates of phosphatidylethanolamine (PE) and phosphatidylcholine (PC) synthesis, via CDP-choline metabolism decrease as a function of age. Conversely, the synthesis of PC through the



enzymatic methylation of PE appears to increase as a function of age (Figure 2).

In rat liver, the amount of phospholipids are significantly lower in old compared to young rats. The cholesterol content of plasma membranes increases during aging, thereby decreasing lateral diffusion and fluidity. The unsaturated fatty acid chains of rat plasma membranes decrease during aging. Old rat liver plasma membrane lipids show a decrease in membrane fluidity, resulting in a decrease in transport of cholic acid and thymidine as well as lipid carrier-mediated transport. In addition, the diffusion coefficient of cholic acid shows a significant decline with age (Hegner, 1980).

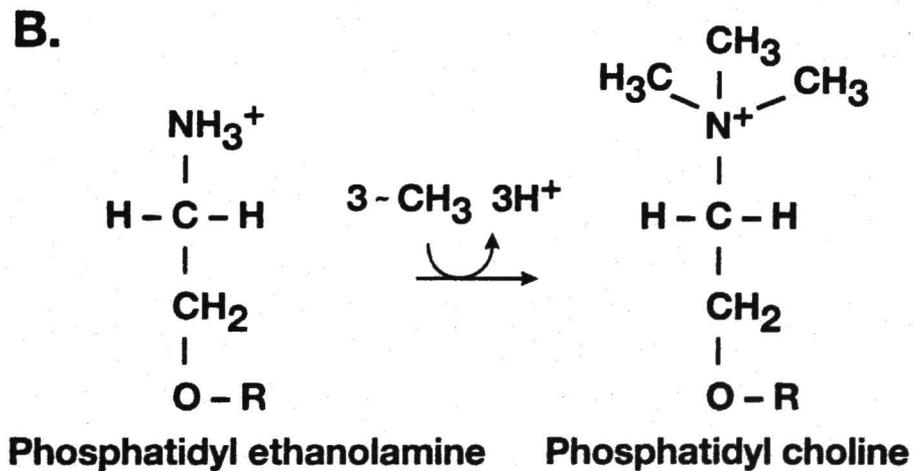
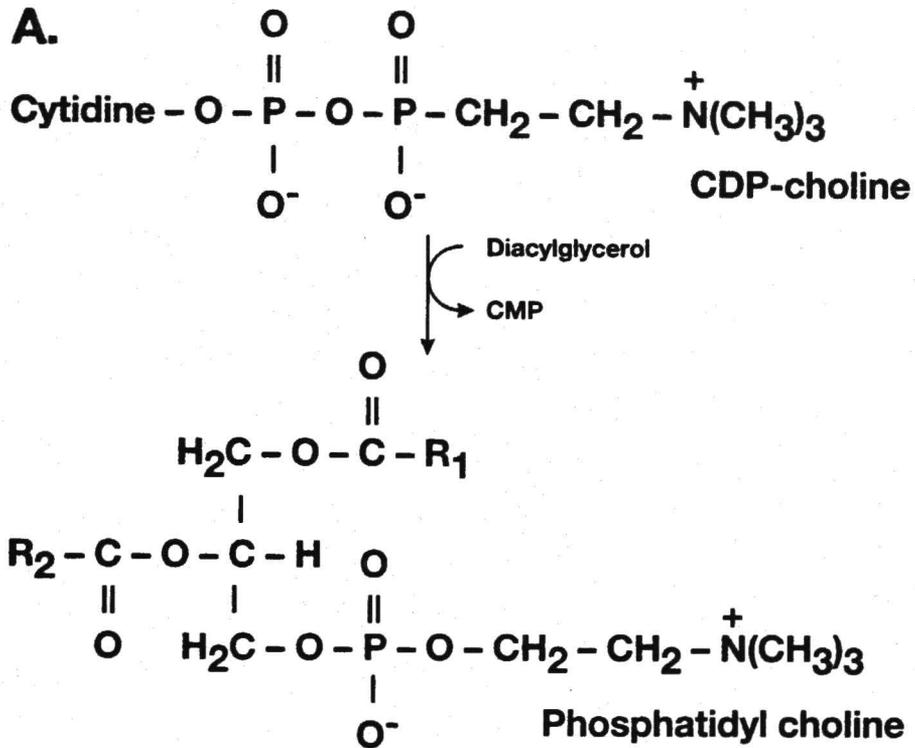
Immune function is another system that decreases with age. A decrease in membrane fluidity of lymphocytes has been closely associated with the decline in lymphocyte function during aging (Alvarez, et al., 1993).

The function of membrane bound proteins is known to be intimately associated with the dynamic state of the membrane lipids (Hegner, 1980). In recent years it has been suggested that aging might be accompanied by a decrease in membrane fluidity and in the function of membrane bound proteins (Wahnon, et al., 1989; Miyamoto, et al., 1990; and Yegutkin, et al., 1991). Overall, in aging tissue, the systems concerned with the maintenance of membrane fluidity become less efficient, and the membrane lipid layer becomes



Figure 2. Chemical pathways for synthesis of phosphatidylcholine from (A) CDP-choline and (B) methylation of phosphatidylethanolamine (Stryer, 1988).







progressively more viscous. Membrane fluidity is directly involved in diffusion. As a result of aging, passive membrane processes that relate to physiologic activity are retarded. Thus, a study of the effects of aging on corneal membrane properties can reveal if the cornea, like other systems in the body, is affected by the age-related changes.

### Corneal Anatomy and Physiology

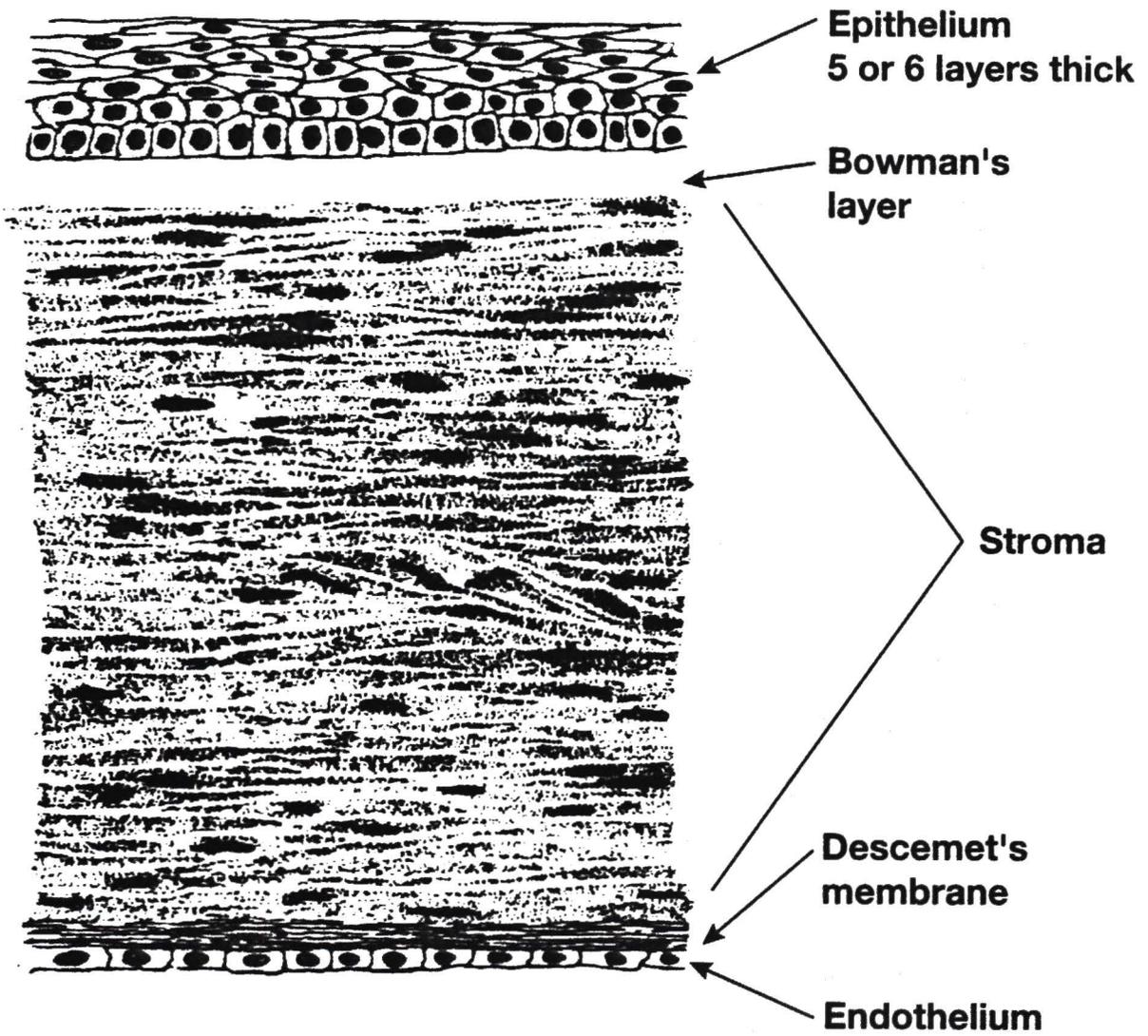
For most compounds derived from the tears and topically delivered pharmaceuticals to reach tissues in the anterior portion of the eye, they must first cross the cornea. The cornea consists of three major cellular layers: the epithelium, the stroma, and the endothelium (Figure 3). Unique anatomic features include the absence of blood vessels and pigment in the cornea, the regular arrangement of the epithelial and endothelial cells, and the scarcity of cell nuclei in the stroma. The cornea is transparent, richly innervated, and resistant to deformation and infection (Maurice, 1984).

The cornea serves as the major light refractor of the eye. Ninety-eight percent of incident light is transmitted by the cornea. The transparency of the cornea is due to: 1) its anatomic structure, including the smoothness of the epithelium, the lack of blood vessels, and the arrangement of the collagen, cells, and ground substance; 2) the tight junctions of the epithelial cells that are not permeable to aqueous solutions; and 3) the dynamic balance between ions



Figure 3. Cross-sectional view of the cornea depicting the three major anatomical layers of the cornea (Maurice, 1984). The epithelium, stroma, and endothelium also serve as major barriers to the penetration of molecules across the cornea. Bowman's layer is the collagen and ground substance lying immediately beneath the corneal epithelium in human and primate, but not in rabbit (Friend, 1994).







and water in the stroma that is maintained by an endothelial pump mechanism that controls corneal dehydration (Maurice, 1984). These structural features (chemical composition, state of metabolism, and hydration) are important in maintaining transparency. The nutrition of the central cornea depends on substances that enter through either the endothelium (single cell layer) from the aqueous humor or the epithelium (multilayers) through the tears. The cornea requires energy to maintain its degree of hydration, to provide its metabolic needs, and to provide for epithelial cell renewal. Energy in the form of adenosine triphosphate is provided by the metabolism of glucose (Friend, 1994). The epithelium is the site of most of the metabolic activity in the cornea as it contains 15 to 20 times more cells than the stroma or endothelium. Disease or injury to the epithelium or endothelium causes corneal edema. Damaged epithelium rapidly replicates itself and the swelling is therefore transient. In contrast, the endothelium does not replicate, and the existing cells increase in size to cover the defects (Wigham and Hodson, 1987).

The cornea acts as a primary biological barrier to the entry of foreign compounds into the eye (Maurice and Mishima, 1984). The epithelial and endothelial layers are lipophilic in nature, allowing lipid soluble compounds to pass more readily than hydrophilic compounds. Due to the presence of intercellular tight junctions, the epithelium



provides a more effective barrier than the endothelium. In contrast, the stroma is an hydrophilic extracellular matrix sparsely populated with keratocytes composed of 80% water, which is readily penetrated by hydrophilic compounds. Thus, in general, compounds must exhibit biphasic or differential solubility characteristics in order to cross the cornea (Havener, 1978). In addition, all three layers are penetration barriers for high molecular weight compounds (especially greater than 500 Daltons) (Pepose and Ubels, 1992). In general, when a compound comes in contact with the eye, only a small percentage of it penetrates the cornea. [The amount of compound able to penetrate the cornea is the first step in determining intraocular pharmacologic drug action (Doane et al., 1978).]

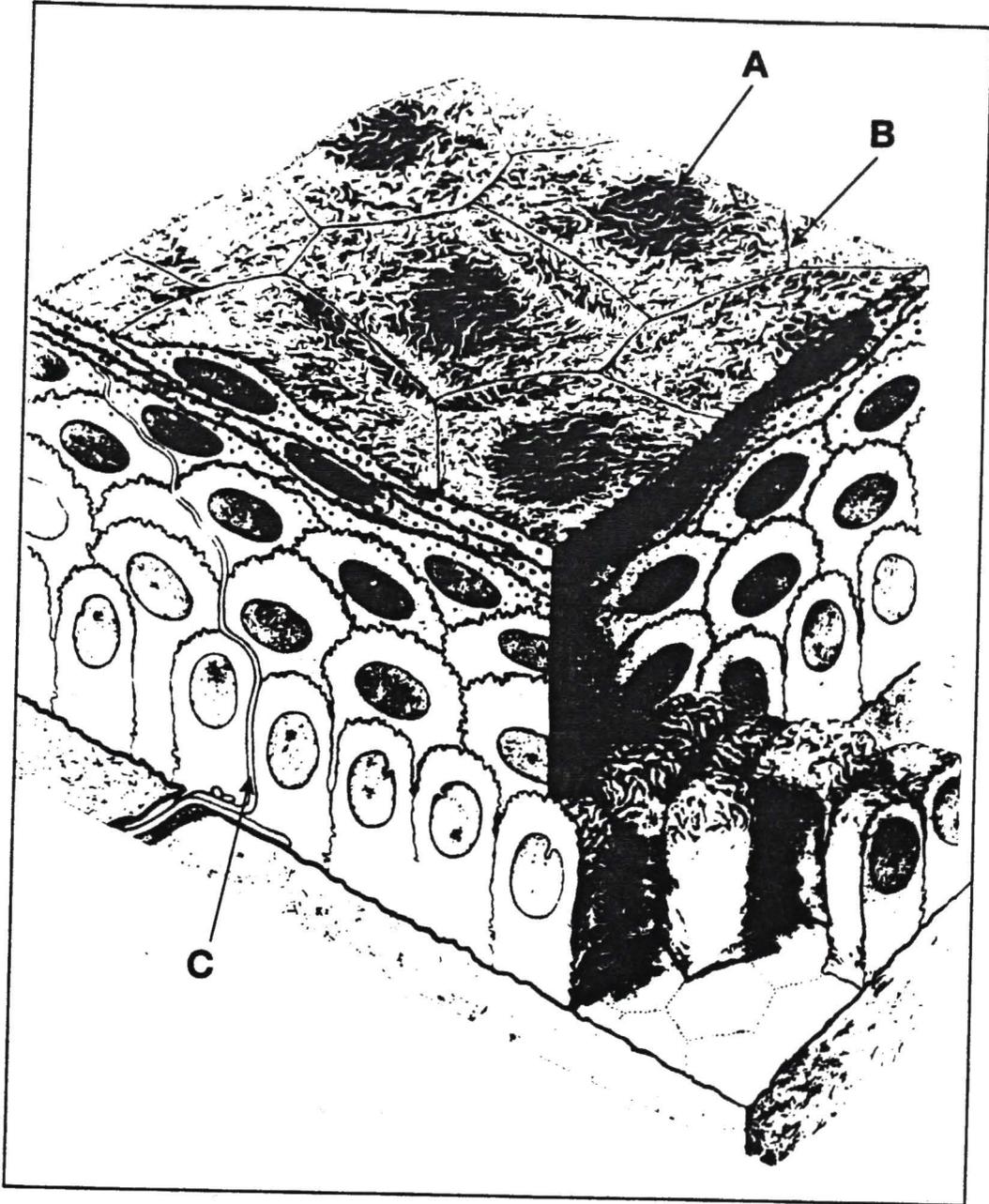
Due to the unique nature and physiological importance of these three layers of the cornea, each will be described in more detail.

The corneal epithelium (Figure 4) is 50 to 90  $\mu\text{m}$  thick and covers the stroma anteriorly. The surface of the epithelium is smooth and covered by a pre-corneal tear film about 7  $\mu\text{m}$  thick. The presence of microvilli on the outer surface of the external layer of cells may serve to hold the tear film against the cells. The epithelium consists of six layers of cells. The basal epithelial cells are columnar, and bound to a basement membrane containing many gap (leaky) junctions, while the more superficial layers become



Figure 4. Artist's rendition of the structure of epithelium. The surface of the epithelium is smooth and covered by microvilli (A). The two layers of flattened surface cells have tight junctions which seal off the intercellular spaces (B). The bottom layers contain many gap junctions (C) (Hogan et al., 1971).







progressively more flattened. The two outer layers of flattened surface cells have zonulae occludentes junctional complexes (tight junctions) which seal off the intercellular spaces. The corneal epithelium is in a constant dynamic equilibrium. Mitosis in the basal layer gives rise to cells which, over a period of several days, migrate anteriorly and are shed from the superficial layer and discarded into the tear film (Hanna et al., 1961).

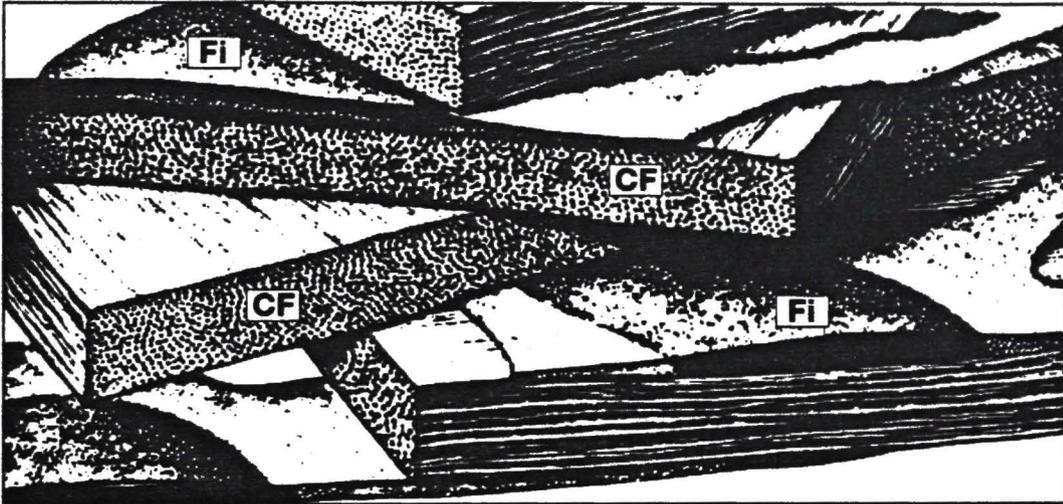
In keeping with its function as a barrier, the corneal epithelium has a low ionic conductance through its apical cell membranes and a high resistance to paracellular transport (Klyce and Crosson, 1985). Electrophysiologic experiments have shown that the loss of superficial epithelial cells is accompanied by a decrease in the transepithelial voltage due to the loss of the high resistance barrier formed by the apical tight junctions (Klyce, 1977). Loss of the corneal epithelium greatly enhances the penetration of hydrophilic compounds, which normally traverse the epithelium mostly via paracellular routes (Hull, et al. 1974; Mindel, et al., 1984).

The stroma (Figure 5) comprises 90% of the corneal thickness. The corneal stroma is the most highly organized and the most transparent of any connective tissue in the body. The molecular composition of the stroma includes primarily collagen, with associated proteoglycans, and soluble glycoproteins (Gipson, 1994). In corneal stroma,



Figure 5. The collagen fibrils (CF) in the stroma are uniformly distributed and interlaced with thin, flattened, and elongated stromal fibroblasts ( $F_i$ ) (Hogan et al., 1971).







type I collagen is the main collagenous product. A, B chains of collagen and type III collagen have also been recovered. Keratin sulfate is the principal glycosaminoglycan component (Yue and Baum, 1981; and Lee and Davison, 1981).

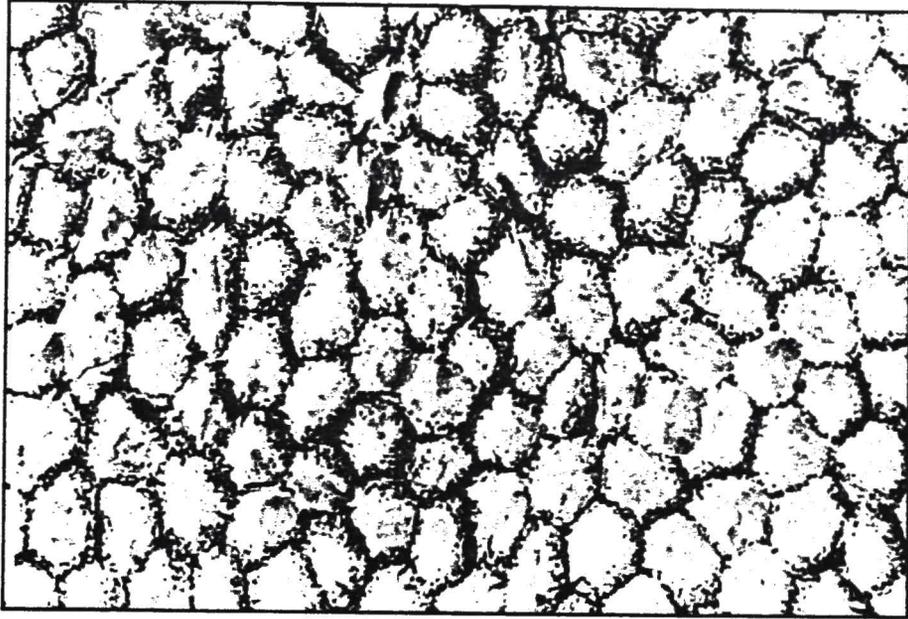
Stroma is composed of 78% water and 15% collagen, forming lamellae of collagen fibrils of uniform diameter and regular spacing that extend the entire width of the cornea (Cogan, 1951). The collagen fibrils in the stroma are uniformly distributed and separated by thin, flattened, and elongated stromal fibroblasts (keratocytes) (Zimmermann et al., 1986). The stroma provides the mechanical strength required to withstand intraocular pressure and external trauma. The stromal fibroblasts are embedded in ordered sheets of collagen fibrils parallel to the surface of the tissue which are attached to proteoglycans embedded in an acid polysaccharide matrix.

The endothelium (Figure 6) is a single layer of mesothelium only 3  $\mu$ m thick and is thus not a major reservoir for solutes (Riley, 1982). It is lipid in nature and consists of both leaky and tight junctions. For small ions, the resistance offered by the endothelium is 100 to 200 times less than that of the epithelium (Kaye et al., 1963; Hirsch et al., 1977). A few short microvilli and a single cilium are frequently present on the surface of the endothelial cells exposed to the aqueous humor. The corneal endothelial cells ultrastructurally resemble cells engaged



Figure 6. Structure of the rabbit corneal endothelium in vitro as seen by specular microscope during perfusion with bicarbonate-Ringer's solution (Maurice, 1984). Note the hexagonal cell shape, regular appearance of the cell borders, and the smooth surface.







in active transport. For example, they possess numerous mitochondria and are thought to be involved in the movement of osmotically active substances. The endothelium is responsible for the dehydration of the corneal stroma. Injury to the endothelium results in edema of the stroma leading to corneal thickening and opacification.

#### Drug Penetration, Deposition, and Metabolism

It is accepted (Maurice and Mishima, 1984) that the vast majority of ophthalmic drugs permeate the cornea via a passive diffusion mechanism. Compounds penetrate either by going across cells or between cells. Lipophilic substances penetrate by going across cells (transcellular) and hydrophilic compounds by going between cells (paracellular). The corneal epithelium contributes to over 90% of the resistance to penetration for hydrophilic compounds, decreasing to about 50% for the moderately lipophilic and to less than 10% for the lipophilic (Schoenwald and Huang, 1983).

A measure of the corneal penetration efficiency of compounds is the permeability coefficient. This is generally on the order of  $0.1-4.0 \times 10^{-5}$  cm/sec for a variety of different compounds (Wang et al., 1991). Unless the substance is very lipophilic, at least a two-fold increase in the extent of penetration occurs when the corneal epithelium is absent. The cornea is assumed to be impermeable to the uptake and transport of macromolecules



due to the tight junction of epithelial barriers. However, Lee et al. (1986) reported on the ability of inulin (M.W. = 5000) to cross the cornea and suggested that the paracellular pathway plays an important role in the corneal absorption of macromolecules.

The eye, as a whole, is not a primary drug-metabolizing organ (Shichi and Nebert, 1980 and Cheng-Bennett, et al., 1990). Drugs that are modified by oxidation or reduction are less likely to be metabolized than those that are metabolized by hydrolysis in the eye. The corneal epithelium and the iris-ciliary body are the most metabolically active tissues in the anterior segment of the eye. Of the ocular drug metabolizing enzymes, the esterases (acetylcholinesterase, butyrylcholinesterase) are perhaps the most important (Lee and Li, 1989).

With the growing numbers of peptide and protein drugs entering development, there is an increasing interest in application of these drugs to the eye. The clinical uses of peptides are limited by their bioavailabilities to ocular tissues following topical application. There are currently insufficient data to estimate peptidase and protease enzymatic activities in the cornea (Harris, et al., 1992).

### Drug Binding

Passive diffusion, followed by cellular binding, has been postulated as a mechanism of drug uptake in many cells (Dalmark and Storm, 1981; Dalmark, 1981). Although *in vitro*



corneal penetration of ophthalmic drugs has been studied in many laboratories (Ahmed, et al., 1987; Huang, et al., 1983; Grass and Robinson, 1988; Igarashi, et al., 1984), little attention has focused on the lag time associated with corneal permeation. The lag time is the time required for the rate of transport of any compound to reach the steady-state during the transport process. The lag time is inversely related to the permeability coefficient. Consequently, more rapidly penetrating compounds will have shorter lag times and greater permeations. For most compounds, the lag times are often independent of initial concentrations. However, the *in vitro* investigation of corneal penetration shows that certain drugs such as the NSAIDS (non-steroid anti-inflammatory drugs), suprofen and flurbiprofen, exhibit rather long diffusional lag times in the cornea which are concentration-dependent, whereas their corneal permeabilities are independent of concentration. This concentration dependence of lag times appears to be a consequence of the binding of these substances to the cornea. Lag time is sensitive to tissue binding, whereas permeability is not generally affected by binding (Cooper, 1974). A long lag time generally leads to the delay of compound peak time in aqueous humor.

A mathematical model is used to explain the concentration dependence of the lag time in terms of tissue binding. Non-metabolizable model compounds such as suprofen



and caffeine demonstrate the principles of this model. Lag time studies were conducted for the stroma (modeled as a single membrane) as well as for the entire cornea to identify the origin of the concentration dependence. The endothelium was not considered in this model because the endothelium is thin and not a significant barrier [lag times are proportional to the square of the thickness (Burstein, 1979) and usually is ignored as a reservoir for drugs (Maurice and Mishima, 1984)]. From this model one can obtain information about drug binding in the cornea.

#### Aging in the Cornea

The aging processes in the cornea are not well characterized. Many structural and functional changes occur within the eye from birth to adulthood. However, the effect that such changes may have on overall permeability to the eye is not well understood. The cornea normally exists in a state of dynamic equilibrium. There is a continuous turnover of corneal epithelial cells with cell loss balanced by production. The turnover rate for both young and aged rabbit corneal epithelium is approximately the same (five to seven days) (Maurice, 1984). However, functional differences between young and old corneas have been reported. Miller and Patton (1981) documented that substantially different aqueous humor drug levels were observed in rabbits of different ages when the same dose of pilocarpine was instilled into the eye. Clayton and co-workers (1985) and



Green and Chapman (1986) reported a difference in the distribution of various surfactants (sodium lauryl sulphate and ethoxylated C18 alkyl sulphate) and benzalkonium chloride between young and old rabbit ocular tissues after topical administration. Young animals had a higher surfactant disposition in aqueous humor. This enhanced tissue uptake of compounds may reflect a greater permeability of the cellular membranes of younger animals and/or different properties of cells that make up these rapidly growing, differentiated epithelial tissues.

Other age-related changes in the cornea include diminished corneal endothelial pump capability, only partially compensated by an increase in barrier function and by a decrease in ionic permeability (Geroski et al., 1985; Wigham and Hodson, 1987). The permeability changes in the endothelium progress slowly with age. The loss of endothelial cells with age may have physiologically important consequences. As the number of endothelial cells decreases with age, the work load on the remaining cells increases and the control of corneal hydration becomes more difficult (Wigham and Hodson, 1987).

The major component of stroma besides water is collagen. Therefore, the aging effects on stroma may be closely related to the aging of collagen. This fibrous structural protein is synthesized in all tissues and deposited extracellularly. It is not renewed during cell



division and it exhibits very little turn-over. Age related alterations in collagen have long been recognized and characterized (Malik, et al., 1992).

In general, human collagens undergo progressive changes with age, including a decrease in solubility, elasticity and permeability, as well as an increase in thermal stability, resistance to enzymatic digestion and an accumulation of fluorescent pigments (Sell and Monnier, 1989). The precise chemical changes of these transformations are unknown. However, Malik, et al. (1992) used x-ray diffraction to suggest that the physical changes involve progressive cross-linking between the collagen molecules. They also demonstrated an age-related decrease in the interfibrillar spacing of collagen which may be related to changes in the proteoglycan composition of the interfibrillar matrix. An initial increase in glycation of collagen was observed between 6 months to 30 years of age and then leveled off showing no further significant change.

Collagen accumulates in tissues at the expense of cells and may cause a decline in the function of organs. Age-related changes in collagens may also directly or indirectly influence tissue binding capacity and permeability. The progressive wrinkling of the skin and the loss of bone and its increasing brittleness are examples of the consequences of aging collagen.



Although the cellular aging of cornea is not well characterized, aging in other cells is associated with a variety of changes (Schroeder et al., 1984). In general, both the function and composition of the cell surface membrane are altered. As cells age *in vivo*, several physicochemical properties undergo significant changes, including decreased enzyme activities, cellular dehydration, an increase in post-translational modification of proteins (for example, glycation) (Schroeder, 1984) and the accumulation of modified proteins (Gracy, et al., 1991). The current mechanisms proposed for altered regulation of membranes in aging include free radical damage to membrane lipids, proteins and other components. Thus, even though the molecular mechanisms of aging in the cornea remain unknown, a variety of evidence in other systems points to a primary involvement of membranes and changes in permeabilities.

#### Aging in the Lens

Unlike the cornea, the biochemical and structural changes that occur in the lens with age have been well characterized (Berman, 1991). Abnormalities in membrane structure and lipid biosynthesis are associated with human senile cataracts (Harding et al., 1980), diabetic cataracts in Rhesus monkey (Farnsworth, et al., 1980), and hereditary cataracts in Philly mice (Andrews, et al., 1984). In fact, lens lipid research began with the discovery of pathological processes that appear in lipid metabolism during cataract



formation (Murawski, et al., 1980). The above studies suggest that elevated intracellular levels of lipid could be detrimental to the clarity of the lens.

Throughout life, the lens continues to grow (Broekhuysse, 1969). The lens contains no vascular system and derives all its nutrients from the aqueous humor. New cells are formed in the lens epithelium and then differentiate at the equator, transforming into lens fibers. Gradually these fibers accumulate in the central part of the lens, forming the lens nucleus. Lens fiber cells are not shed, nor do they degenerate; rather, they are continually displaced inwardly toward the center as they mature. The majority of these cells are dead. There is no mechanism for eliminating damage that may occur in this protein-filled tissue. Hence, proteins in old nuclear lens fiber cells are as old as the organism itself.

About half of the lipids of the human lens are cholesterol (by weight) (Feldman, 1967). Sphingomyelin represents the major component of the phospholipids (Broekhuysse, 1973). The phospholipid composition of the lens depends on its age. This is not only true for the lens as a whole but also for individual cellular layers (Broekhuysse, 1971).

Previous investigation concerning the aging human lens showed that the ratio of sphingomyelin to phosphatidyl-choline increases with age (Broekhuysse, 1971). The



phospholipid composition of the lens also has been found to change with age. In addition, an increase in certain phospholipids and alterations in the membrane structure have been detected. Changes are mainly caused by a continuous net synthesis of sphingomyelin and by a decrease in concentration of other phospholipids with age (Broekhuysse, 1969). Changes are correlated with cell differentiation and the formation of the lens nucleus.

Sphingomyelin packs tightly into the outer leaflet of the phospholipid bilayer, producing a membrane with low fluidity (Van Blitterswijk, et al., 1981). Accumulation of sphingomyelin in the lens seems to begin as lens epithelial cells differentiate into lens fibers (Zelenka, 1978), and continues throughout life. In the lens nucleus, sphingomyelin is protected from enzymatic degradation, since sphingomyelinase is confined to the lysosomes of the epithelium and superficial cortical fiber cells (Zelenka, 1984). In relatively long-lived species such as man, the sphingomyelin content may reach 60% or more of the total lens phospholipid (Zelenka, 1984).

Measurements of the fluidity of human lens fiber cell membranes demonstrate that they are the least fluid of all eukaryotic membranes that have been studied (Gooden, et al., 1983). Endogenous lipid synthesis, in conjunction with uptake of exogenous cholesterol and certain fatty acids from the aqueous humor leads to the formation of a lens plasma



membrane that is especially rich in sphingomyelin, cholesterol, and long-chain saturated fatty acids which are responsible for its very low membrane fluidity. Molar ratios of cholesterol:phospholipid in most biological membranes are typically about 0.5 (Chapman, 1975), but in the adult human lens fiber membrane the ratio is between 2.0 and 2.7 (Gooden, et al., 1983).

The present study was designed to biochemically characterize the structural and functional alterations which occur in the cornea during aging. This study included determination of corneal membrane permeability and tissue binding of specific compounds as well as the effects of aging on the corneal lipid composition. The specific aims were to: 1) systematically determine changes in corneal permeability to compounds of different molecular weight and lipophilicity; 2) examine tissue binding and lag time by utilizing a theoretical transport model; and 3) examine the biochemical changes related to lipid composition and distribution.

In the first set of studies, an *in vitro* diffusion model analogous to the *in vivo* conditions was utilized to examine functional changes in permeabilities and metabolic capabilities between young and aged rabbit corneas. Experiments were carried out by means of steady-state passive transport conditions in corneal diffusion cells.



This permitted the comparison of the intrinsic membrane permeability properties of young and old corneas.

The second set of studies concentrated on a theoretical penetration model which took into account stromal binding as well as other penetration parameters. From this model one can obtain information about compound-tissue binding and penetration parameters within the cornea. In addition, age-related differences in binding constants could be determined and clarified.

The purpose of the third set of studies was to determine lipid composition and structural changes in young and old corneas. A similar approach was also applied to the lens in order to compare with previously published results and verify the experimental techniques used in this study.

The aim of the present investigation was to assess whether aging modifies corneal permeability and tissue binding of various compounds by inducing intrinsic alterations of the corneal membrane. Simply, by identifying the mechanism(s) of the aging process of the cornea, one may have the opportunity to control its consequences.



## CHAPTER II

### MATERIALS AND METHODS

#### Reagents and Chemicals

Betaxolol, atenolol, suprofen, caffeine, dexamethasone, dexamethasone phosphate, dexamethasone acetate and luteinizing hormone releasing hormone (LHRH) were purchased from Sigma Chemical Company. Fluorescein and fluorescein dextrans were obtained from Molecular Probe. Lipid standards - phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, sphingomyelin, cholesterol, and triglycerides were obtained from Avanti Polar Lipids, Inc. Fatty acids standards were purchased from Nu Chek Prep., Inc. All other chemicals were reagent grade.

#### Animals

All corneas and lenses were obtained from albino female rabbits which were either six weeks (young) or three to four years old (aged). These animals were maintained on a normal laboratory diet and water *ad libitum*. All animal care and use conformed to the ARVO Resolution on the Use of Animals in Research.



### Penetration Studies

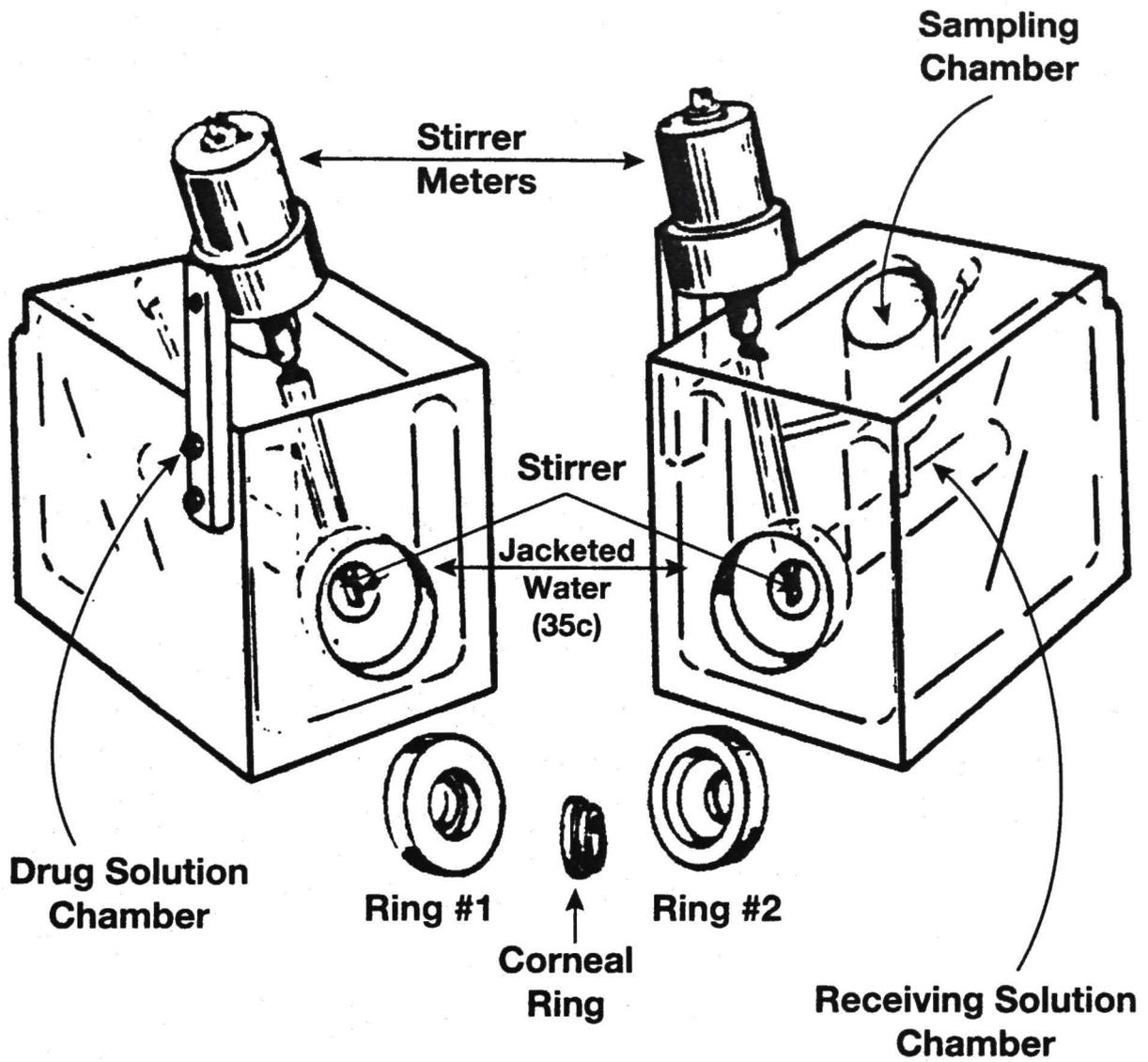
Model compounds were selected for investigation based on differences in either lipophilicity or molecular weight. Five compounds were tested based on their different lipophilic properties (distribution coefficient at pH=7.4). Betaxolol was the most lipophilic compound tested, while atenolol and fluorescein were the most hydrophilic compounds. Dexamethasone and suprofen were of intermediate lipophilicity. Four compounds were tested based on molecular size. All of these compounds are very hydrophilic. Fluorescein was chosen for its smaller size (M.W. = 374 Mass) compared to fluorescein dextrans (M.W. = 10,000 and 70,000 Mass) which served as larger size model polymers. LHRH is a small peptide (M.W. = 1284 Mass).

Corneal penetration experiments were conducted with lucite diffusion cells according to Schoenwald and Huang (1983). Rabbits were sacrificed with an overdose intravenous injection of pentobarbital. Corneas were mounted in the diffusion apparatus within 15 minutes after enucleation (Figure 7). The corneas were perfused with glutathione bicarbonate Ringers (GBR) solution which preserved tissue integrity for greater than six hours. Part 1 contained sodium chloride (13.039 g/liter), potassium chloride (0.719 g/liter), monobasic sodium phosphate monohydrate (0.216 g/liter), calcium chloride dihydrate (0.306 g/liter), and magnesium chloride hexahydrate (0.317 g/liter). Part 2



Figure 7. A schematic drawing of a compound diffusing from the well-stirred left chamber through the cornea to the well-stirred right chamber with a temperature controlled water jacket (Schoenwald and Huang, 1983).







contained sodium bicarbonate (4.908 g/liter), glucose (1.8g/liter) and oxidized glutathione (0.184 g/liter). GBR was prepared by mixing equal volumes of Parts 1 and 2 prior to use and gassed with an O<sub>2</sub>-CO<sub>2</sub> mixture (95:5) for at least 15 minutes. A volume of 7 ml GBR solution was added first to the corneal endothelial side of the chamber to serve as the receiver solution. An equal volume of GBR solution containing the various test compounds (1.0-10 mM) was then added to the epithelial side to serve as the donor solution. Both solutions were bubbled with O<sub>2</sub>-CO<sub>2</sub> mixture (95:5) to provide adequate mixing. Temperature was maintained at 35°C. Solution samples were withdrawn from each side of the cornea at selected times (from one minute to four or six hours) and analyzed by HPLC. All corneas were judged clear at the end of each four-to-six hour experiment, the water contents determined according to the procedure of Schoenwald and Huang (1983) and were within 74%-78% of total corneal wet weight.

Conditions for these studies were similar to those of the intact cornea, except penetration studies were conducted after scraping off the epithelial layers from the cornea with a corneal gill knife and exposing the stroma directly to the test solution. The water content of the denuded cornea after each experiment was measured and its value was approximately 78%-83% of the weight of stroma/endothelium.



Studies were conducted similarly to "denuded cornea" by scraping off the epithelial layers from the cornea, except that the endothelium was directly exposed to the test materials (fluorescein or fluorescein dextran). Samples were withdrawn from the stromal side of the cornea at selected times and were analyzed fluorophotometrically to determine the permeability coefficient across endothelial cells.

The permeabilities of dexamethasone and dexamethasone acetate or dexamethasone phosphate were compared in order to determine the rate of esterase and phosphatase activities in the cornea. Experiments were carried out with intact cornea or stromal/endothelial tissues (denuded cornea) for both the young and aged animals.

#### Tissue Binding

Penetration experiments and binding studies were conducted for the stroma to demonstrate the role of this ocular tissue in controlling the lag time of various drugs in both young and old animals.

The penetration experiments of whole or denuded cornea were conducted using identical procedures as described on pages 39 and 42 (*in vitro* intact cornea penetration and *in vitro* stroma/endothelial penetration), except that a GBR buffer solution containing different concentrations (from 20 to 1000 ug/ml) of suprofen or caffeine was used as the donor solution.



The study of suprofen partition between the GBR buffer solution and rabbit epithelium was conducted in both young and old corneas. The epithelium was scraped from the cornea, weighed, then equilibrated with various concentrations of suprofen (from 2 to 4,000 ug/ml) at 35°C for two to three hours. Samples of various suprofen solutions were withdrawn before and after the incubation. The amounts of suprofen partitioning between the epithelium and GBR solutions were determined by HPLC.

For binding studies, the stromal/endothelial layers were placed in GBR buffer solutions containing a wide range of concentrations of suprofen and were incubated at 35°C for three hours, which was adequate to obtain equilibrium. Two to four pieces of stromal/endothelial layers were employed in each experiment. The suprofen concentration before and after incubation with tissues was measured by HPLC assay. The amount of suprofen bound to the young and aged tissues was determined by difference calculations.

### Lipid Analysis

Cornea specimens (epithelium or stroma/endothelium) and lens (dissected into epithelium, cortex and nuclear portions) were pooled together from young (eight eyes) or old (four eyes) animals and were weighed and immersed in liquid nitrogen prior to being reduced to a fine powder using a liquid nitrogen chilled homogenizer. A simple Folch extraction of the tissue lipids was done (Folch et al.,



1957). To protect the samples from oxidative changes, they were maintained under a  $N_2$  atmosphere at  $-20^\circ C$  at all times. This procedure avoided the need for the addition of antioxidants to the samples (antioxidants may interfere with lipid detection at 205 nm).

Tissues were extracted by 20 weight-volume (g-ml) of chloroform/methanol, 2/1 v/v. Each extract was centrifuged at 3000 rpm for 10 minutes. The proteins were sedimented as a precipitate, and the supernatant containing the lipids was removed. The protein precipitates were re-extracted with chloroform:methanol (2:1 by volume) as above and added to the first lipid extract described above. The combined liquid extracts were washed with 0.2 volumes of 0.1 M KCl and allowed to separate thoroughly after being centrifuged. The chloroform phase was recovered and dried over anhydrous sodium sulphate and taken to dryness under reduced pressure at  $40^\circ C$ . These lipid extracts were analyzed for lipid composition using quantities of 0.01-10 mg per analysis.

Gas chromatography was used to analyze the individual fatty acids of the lipid extract. Fatty acid methyl esters were prepared from lipid extracts. Samples to be analyzed contained about 50 nM of lipid and were dried under nitrogen and suspended in 0.5 mL of petroleum ether. The  $BF_3$  - Methanol reagent (Supelco, Inc.), was added, sealed, and maintained in a heating block at  $95^\circ C$  for two hours. 0.2 ml of water was added to stop the reaction. The samples were



vortexed and centrifuged at 3000 rpm to yield two phases. The top phase, containing the fatty acid methyl ester, was removed with pipets and dried under nitrogen, and the samples were transferred to small vials for analysis.

Fatty acid methyl esters were analyzed on a Perkin-Elmer Sigma 2000 Gas-liquid Chromatography equipped with a flame ionization detector and interfaced with a Perkin-Elmer LC-100 Laboratory Computing Integrator. These analyses were performed using a DB-23 megabore column (30 m x 53 mm ID, 5  $\mu$  film) packed with 50%-cyanopropyl polysiloxane (J & W Scientific). Helium was used as the carrier gas at a flow rate of 15mL/min. Initial oven temperature was 50°C for the first 3 min, increased to 150°C at a rate of 25°C/min and then increased to 240°C at a rate of 5°C/min and held at 240°C for another 5 min. The temperature for injector and detector was at 250°C. Authentic fatty acid methyl esters were used as standards to identify retention times.

#### HPLC Analyses

The amounts of suprofen, caffeine, atenolol, betaxolol, dexamethasone, dexamethasone acetate, and dexamethasone phosphate in various samples were determined by HPLC, using a Waters Liquid Chromatography system which consisted of pumps (Waters 510), an injector (WISP 710B) and a detector (Waters Lambda-Max 481) with a 25 cm x 4.6 mm i.d. Spherosorb RP-18, 10  $\mu$ m column (Phenomenex Company). In all analyses, a flow rate of 2.0 ml/min was used, and the



absorbance sensitivity was 0.01 AFUS. The chromatographic conditions for each test compound were as follows: (1) Suprofen and caffeine: isocratic, methanol:0.05M phosphate buffer pH=3.0 (60:40) with detection at 287 nm; retention time for suprofen was 4.45 min and for caffeine was 2.35 min. (2) Betaxolol: isocratic, acetonitrile:0.05M phosphate buffer pH=3.0 (60:40) with detection at 220 nm; retention time was 4.56 min. (3) Atenolol: isocratic, acetonitrile :0.05M phosphate buffer pH=3.0 (10:90)with detection at 220 nm; retention time was 6.45 min. (4) Dexamethasone, dexamethasone acetate and phosphate: isocratic, methanol:water (55:45) with detection at 246 nm; retention times were 5.36 min, 8.15 min and 1.98 min, respectively. Chromatography of LHRH utilized a 25 cm x 4.6 mm RP-18 Hi-Pore Reversed Phase Column (BIO-RAD Company) with isocratic elution using 0.2% trifluoroacetic acid in water :acetonitrile (82.5:17.5) with detection at 220 nm; retention time was 7.42 min. The amount of test compound was determined by comparing its peak area with standard curves. Concentrations as low as 0.1  $\mu$ M could be easily detected and quantitated.

The phospholipids in various tissue samples were determined with a Perkin Elmer Liquid Chromatograph system which consisted of pumps (Series 410), an injector (Series 4), a LC-100 Laboratory Computing Integrator, and a detector (LC-235 Diode Array Detector) set at 205 nm. The



chromatographic conditions for each test compound were similar to those developed by Patton, et al. (1990) and were as follows: (1) PC and SPH used a 25 cm x 4.6 mm Brownlee 5  $\mu$ m Si-Column with isocratic elution (acetonitrile:methanol:water (50:45:5.5)) at a flow rate of 1.2 ml; retention times were 8 min (PC) and 10 min (SPH). (2) PI, PS and PE analysis used a 25 cm x 4.6 mm Si-Spherisorb 10  $\mu$ m column with isocratic elution (hexane:isopropanol: ethanol:25 mM  $\text{KH}_2\text{PO}_4$  pH=7.0:acetic acid (376:485:100:56.2:0.6) at a flow rate of 1.0 ml; retention times were 6.0 min (PE), 9.8 min (PI) and 14.4 min (PS). (3) Cholesterol analysis used a 25 cm x 4.6 mm Allteck 5  $\mu$ m Si-column with isocratic elution using hexane:isopropanol:acetic acid (100:2:0.02) at a flow rate 1.0 ml; retention time was 10.0 min.

### Fluorescence

The amounts of fluorescein and fluorescein dextrans transversing the cornea were determined by monitoring the increase in fluorescence intensity in the receiver chamber using a Perkin-Elmer Fluorescence Spectrophotomer (Model MPF-66) at an excitation wavelength of 493 nm, slit width = 4 nm, and an emission wavelength of 512 nm, slit width = 4.5 nm.

### Transmembrane Resistance

Membrane resistance of young and old corneas was determined using an Epithelial Voltohmmeter (Millcell-ERS,



Millipore Inc.). The setup was similar to that used in the perfusion studies with both sides of cornea bathed with 7 ml GBR solutions. The resistance was measured by inserting electrodes in either side of the solution 15 minutes after the cornea was mounted in the diffusion cells.

### SEM and TEM

Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) were performed by Dr. Mitch McCartney (Alcon Laboratories, Inc.). Briefly, corneas were fixed with a fixative containing 2% paraformaldehyde, 2.5% glutaraldehyde in a 0.1M sodium cacodylate buffer (pH=7.3) for two hours at room temperature. The corneas were divided into one half for SEM and the other half for TEM.

The cornea prepared for SEM was osmicated for one hour, dehydrated through an ascending series of alcohols and critical point dried. The tissue was then mounted with epithelium side uppermost on aluminum SEM stubs with silver paint, sputtered with 20 nm of gold and examined in a Zeiss OSM-940 scanning electron microscope.

The cornea used for TEM was osmicated for one hour, rinsed in buffer, stained with uranyl acetate, dehydrated through an ascending series of alcohols and embedded in a PolyBed 812/Araldite mixture. Samples were thin sectioned and examined in a Zeiss CEM-902 transmission electron microscope.



### Statistical Analysis

To assess the statistical significance of the data, means and standard deviations of the means were calculated, and student's t-tests of paired observations were done to obtain p values. P values less than 0.05 were considered statistically different.



## CHAPTER III

### RESULTS

#### General Characterization and Transmembrane Resistance of Young and Aged Corneas

Corneas from young and old animals were examined and characterized by transmission and scanning electron microscopy for general morphology and ultrastructure. No obvious alterations in corneal morphology with increasing age were observed (Figures 8 & 9). Young and old corneas showed the same thickness (approximately 0.05 cm). The membrane resistances of both are listed in Table 1. The area of the cornea from old rabbits was twice that of the young. The resistance for the young corneas was significantly lower than the resistance for the old corneas ( $p < 0.01$ ). These data suggest that small electrolytes should penetrate the young cornea more easily than the old cornea.

#### Penetration Studies

By monitoring the appearance of compounds in the receiving solution as a function of time, a transport process driven by a concentration gradient was observed. An illustration of this process is shown in Figure 10. A linear steady-state appearance of drug in the receiver solution was



Figure 8. Scanning electron micrograph of the anterior surface of the cornea illustrating the appearance of epithelium. The superficial epithelial cells are polygonal in shape, and there are populations of light (arrow) and dark (arrow heads) cells. There was no difference between the young and old corneas in regards to cell size, shape, or the distribution of light and dark cells (Bar = 10  $\mu$ m).

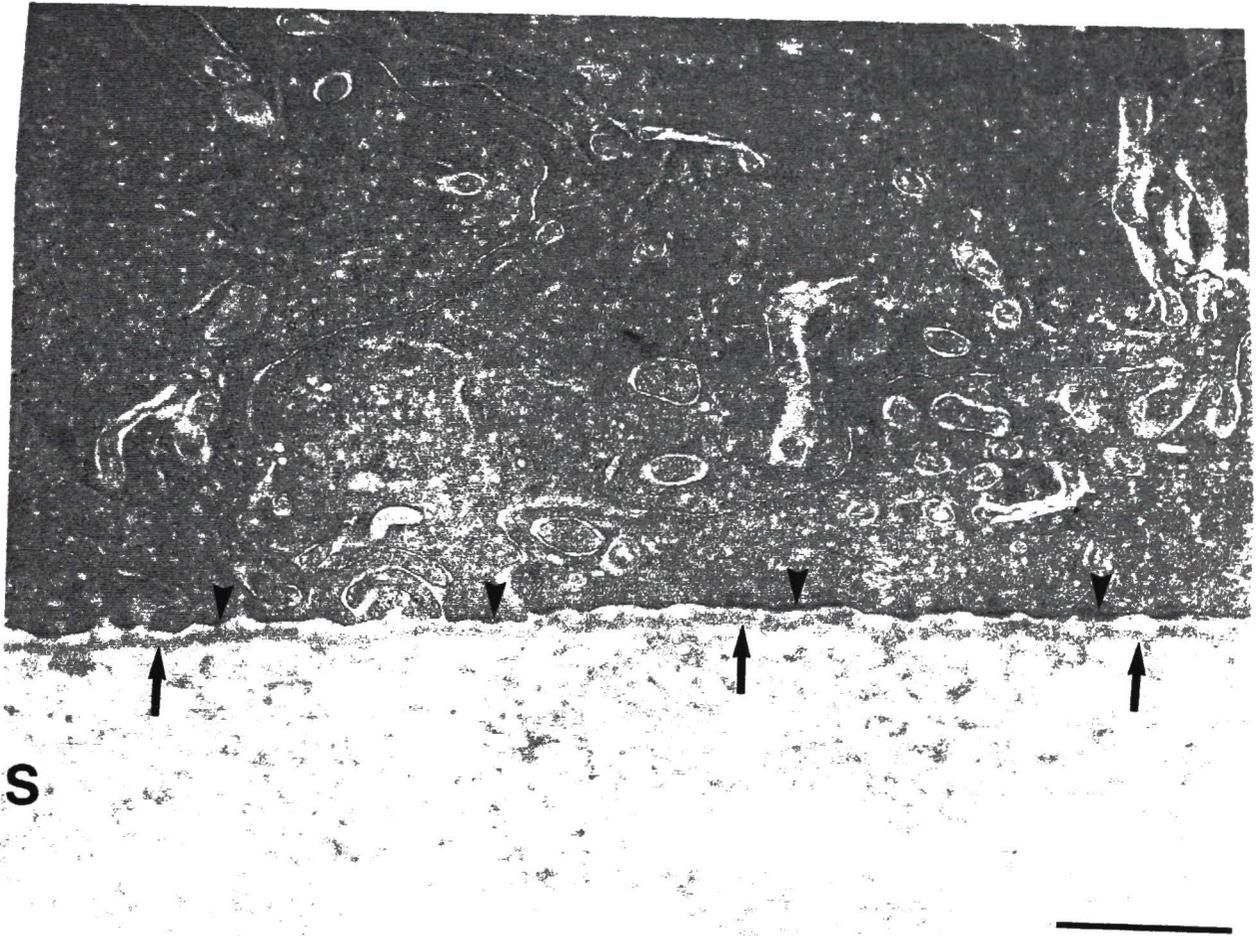






Figure 9. Transmission electron micrograph illustrating the appearance of the corneal epithelium and stroma in the young animal. The epithelium was typically 5-7 layers thick, and the cells showed the normal complement of cellular organelles. The basal cells were tightly adherent to the basement membrane (arrows) with hemi-desmosomes (arrow heads). The stroma (S) can be seen below the basal cells of the epithelium. There was no morphological difference between the young and old corneas. (Bar = 1  $\mu$ m).





S



Table 1

Size and Transmembrane Resistance of Young and  
Aged Corneas

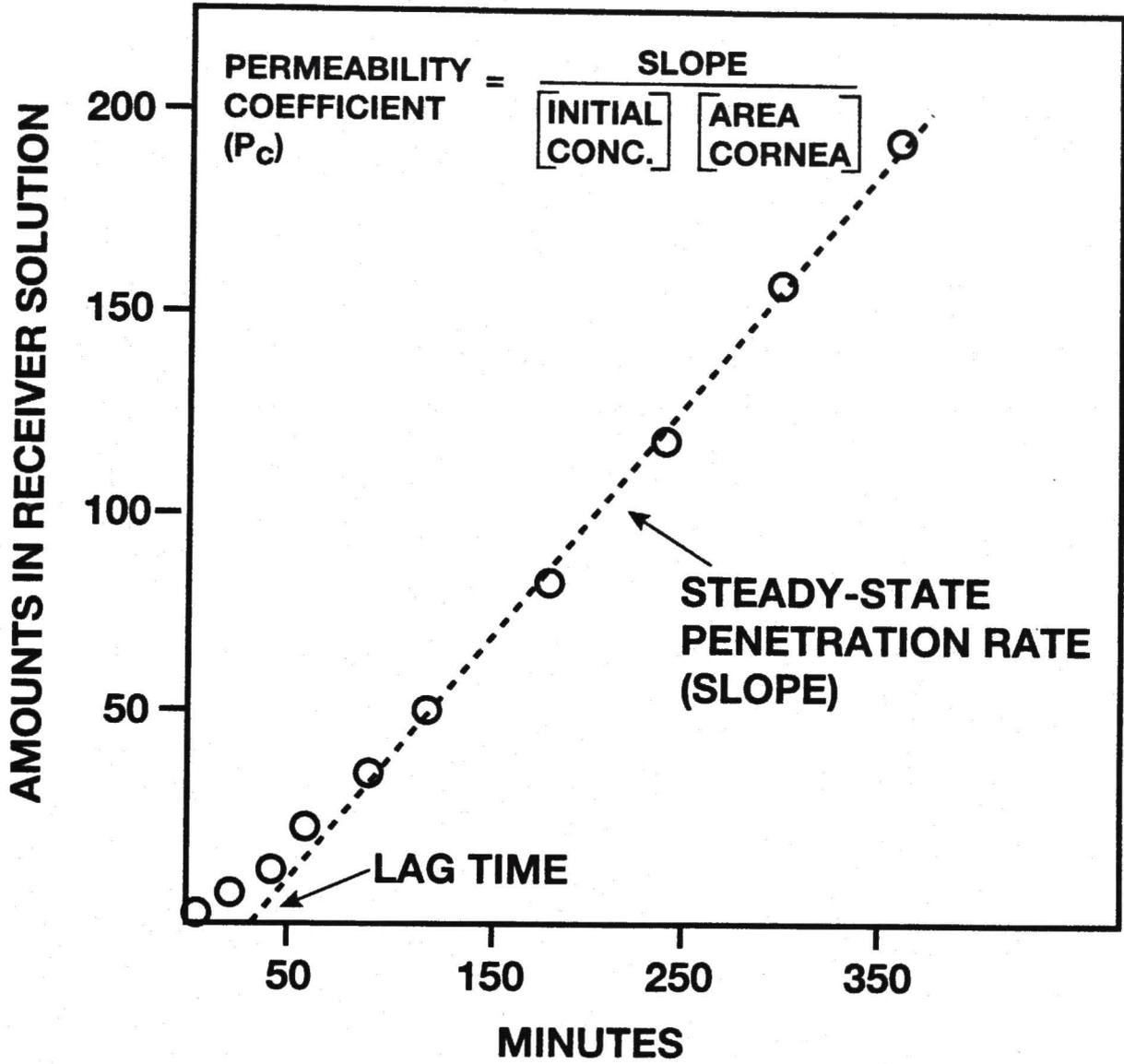
<u>Animal Age</u>	<u>Area</u> <u>(cm<sup>2</sup>)</u>	<u>Weight</u> <u>(gram)</u>	<u>Transmembrane</u> <u>Resistance</u> <u>(Kohmes-cm<sup>2</sup>)</u>	<u>Resistance</u> <u>Ratio</u>
Young	1.08	0.06±0.01*	5.2±0.5*	
Old	2.01	0.11±0.01*	8.7±0.4*	1.7±0.2

\*Each value represents the mean of five determinations ± SD. Statistical significance was determined using the student's t-test ( $p < 0.005$ ).



Figure 10. Illustration of the transport of a compound across the cornea. A representative set of data for transport process. The slope represents the steady-state penetration rate. The intercept in the X - axis represents the lag time for compound reaching steady-state diffusion.







observed over the time course of the experiments. The lag time ( $t_L$ ) was determined by extrapolating this linear slope to the x (time)- axis which reflected the time scale for the compound to reach steady-state diffusion. When dividing the slope values of the linear steady-state portions of the plots by the corneal surface areas and initial concentrations of the test compounds over which the permeation was occurring, steady-state membrane permeability values (P) for the test compounds were obtained. These data, presented as cm/sec (equation 1) depended only on chemical properties of tested compounds and the biological barriers of the cornea.

The amount of drug penetrating the membrane per unit time at steady-state is given by Fick's law of diffusion.

$$\frac{dQ}{dt} = \frac{\epsilon DA}{\tau h} (C_{ma} - C_{mb}) \quad (1)$$

$dQ/dt$  is the penetration rate of substance penetrating the membrane per unit time

A = surface area of the cornea

h = thickness of the cornea

D = diffusion coefficient

$\epsilon$  = porosity

$\tau$  = tortuosity

$C_{ma}$ ,  $C_{mb}$  = solute concentration



$C_{ma} = kC_a$ ,  $C_{mb} = kC_b$  and  $k$  is the membrane partition coefficient

$$\text{Thus, } \frac{dQ}{dt} = \frac{\epsilon DkA}{\tau h} (C_a - C_b) \text{ or } \frac{dQ}{dt} = PA(C_a - C_b),$$

$p$  is the permeability coefficient =  $\frac{\epsilon DK}{\tau h}$

Therefore,

$$P = \frac{dQ}{dtAC_a} \text{ when } (C_a \gg C_b). \text{ The unit for } P \text{ is cm/sec.}$$

The permeability of young and old corneas to test compounds of different lipophilicities (Table 2) and molecular weights (Table 3) were characterized and quantitated. The entry of lipophilic compounds into the eye is aided by the cell membrane of the corneal epithelium. The penetration of compounds was consistent with their relative octanol-buffer distribution coefficients (Table 2). These compounds are lipid soluble and may be able to penetrate the epithelium partly through the cell membrane. Betaxolol was the most permeable of the test compounds and was able to penetrate in appreciable quantities across cell membrane. This is compatible with its relatively high octanol-buffer partition coefficient. The permeabilities of hydrophilic atenolol and fluorescein were much lower, while suprofen and dexamethasone with intermediate lipophilicity showed intermediate corneal permeability. This relative ranking of permeabilities was found in both the young and old corneas. An age-dependent relationship of permeability of each compound was observed. In all cases the permeability



Table 2  
Age Related Changes in Whole Corneal Permeability  
to Lipophilic Compounds

COMPOUND	Log DC <sup>1</sup>	PERMEABILITY COEFFICIENT (cm/sec x 10 <sup>6</sup> )		Difference (fold)
		YOUNG*	OLD*	
Betaxolol	2.52	51±3**	36±1	1.4±0.1
Dexamethasone	1.90	9.0±1.0	3.6±0.3	2.5±0.4
Suprofen	-0.25	9.6±0.8	3.6±0.7	2.6±0.5
Atenolol	-1.52	2.3±0.4	0.7±0.1	3.2±0.8
Fluorescein	<-1.52	1.6±0.2	0.40±0.03	4.0±0.6

<sup>1</sup> DC = Distribution coefficient of compound in Octanol/Phosphate Buffer (pH=7.65)

\* Data are based on three separate determinations for all compounds in young rabbits and two in old rabbits. Values represent mean ± standard deviation.

\*\* Statistical significance was determined using the student's t test (*p* is at least < 0.02 for all tested compounds).



Table 3

Age Related Changes in Whole Corneal Permeability to  
Compounds of Differing Molecular Weight

COMPOUND	M.W. <sup>1</sup> (Mass)	PERMEABILITY COEFFICIENT (cm/sec x 10 <sup>6</sup> )		Difference (fold)
		YOUNG*	OLD*	
Fluorescein	375	1.6±0.2	0.40±0.03	4.0±0.6
LHRH	1284	0.12±0.02	0.015±0.003	8.3±1.4
Fluorescein Dextran	10000	0.6±0.2	0.069±0.003	8±2
Fluorescein Dextran	70000	0.04±0.01	0.005±0.001	8±2

<sup>1</sup> M.W. = Molecular Weight

\* Data were mean ± standard deviation (young n=3, old n=2) and analyzed as in Table 2 (p is at least < 0.03 for all compounds).



coefficients were significantly lower in cornea from old animals. For betaxolol, the most lipophilic compound, the difference between the young and old was smaller (1.4-fold) than for hydrophilic compounds such as fluorescein (4-fold). Table 3 shows the effect of age on the permeability of a series of compounds differing in molecular weight. These compounds were very hydrophilic and probably crossed the epithelial cell layers only through the paracellular space. An inverse relationship was observed between the molecular weight and permeability for both the young and aged corneas. The higher the molecular weight, the smaller the amount of compound that penetrated the cornea. One exception was LHRH, a small peptide (M.W.=1284 Mass), which showed a permeability considerably less than the fluorescein dextran of 10,000 molecular mass. Here, the epithelial barrier also served as the metabolic barrier for this peptide. Only a small percent of the intact LHRH could cross the cornea. There was a significant decrease in permeability of all of these hydrophilic compounds with age. The magnitude of this age-related decrease in permeability was greater with large molecules.

Permeabilities in stromal/endothelial tissues (denuded cornea) were determined and are shown in Tables 4 and 5 for compounds of differing lipophilicity and molecular weight. In each of these studies the epithelial layer had been removed (simulating corneal abrasion) and permeability



**Table 4**  
**Age-related Changes of Denuded Cornea to Permeability of**  
**Compounds with Different Lipophilicities**

COMPOUND	PERMEABILITY COEFFICIENT (cm/sec x 10 <sup>6</sup> )		Difference (fold)
	YOUNG*	OLD*	
Betaxolol	45±1	17.8±0.4	2.5±0.1
Dexamethasone	36±2	21.8±0.3	1.7±0.1
suprofen	37.7±0.5	18.3±0.5	2.07±0.06
Atenolol	22.4±0.6	10.3±0.4	2.18±0.13
Fluorescein	17±1	8.6±0.4	2.0±0.1

\* Data were mean ± standard deviation (young n=3, old n=2) and analyzed as in Table 2 (  $p < 0.005$  for all five compounds).



Table 5  
Age Related Changes in Denuded Corneal Permeability  
of Compounds with Different Molecular Weights

COMPOUND	PERMEABILITY COEFFICIENT (cm/sec x 10 <sup>6</sup> )		Difference (fold)
	YOUNG*	OLD*	
Fluorescein	17±1	8.6±0.4	2.0±0.1
LHRH	5.13±0.03	3.49±0.06	1.46±0.03
Fluorescein Dextran(10000)	3.22±0.05	1.9±0.2	1.7±0.1
Fluorescein Dextran(70000)	0.44±0.02	0.20±0.03	2.2±0.4

\* Data were mean ± standard deviation (young n=3, old n=2) and analyzed as in Table 2 (  $p < 0.005$  for all compounds).



was assessed across the stromal/endothelial layers.

Differences in permeability among the different lipophilic compounds still existed but the differences were much less than those observed from the intact cornea. This phenomenon was observed in both young and old tissues, indicating that epithelium is the major selective barrier for these tested compounds.

The difference between ratios in permeabilities of young and old damaged corneas was essentially the same for all of these test compounds (about 2-fold). Old tissues were less permeable than young ones and the difference was independent of lipophilicity.

A correlation between molecular weight and permeability in the stromal/endothelial cells (denuded cornea) was also found (Table 5). This relationship existed for both young and old animals. The compounds with greater molecular size had lower permeabilities. The ratio between the young and old was essentially the same as that for the different lipophilic compounds (about 2-fold).

Differences in permeabilities before and after removal of epithelium were compared and are shown in Table 6 (Tables 2 and 3 versus Tables 4 and 5). Lipophilic betaxolol readily penetrated both intact and damaged corneas. For the more hydrophilic compounds as well as the high molecular weight compounds upon removal of the epithelium an increase in permeability could be as great as several to one hundred



Table 6

Comparison of Permeability Changes Between Denuded and Intact Corneas on Basis of Compound's Lipophilicity or Molecular Weight

COMPOUND	PERMEABILITY CHANGES OF DENUDED/INTACT CORNEA (Ratio)	
	YOUNG*	OLD*
Betaxolol	0.88±0.06	0.50±0.02
Dexamethasone	4.0±0.5	6.0±0.6
Suprofen	3.9±0.3	5.0±0.9
Atenolol	10±2	15±3
Fluorescein	11±2	22±2
LHRH	43±7	232±5
Fluorescein Dextran (10000)	5±2	28±3
Fluorescein Dextran (70000)	11±3	40±6

\* Statistical significance between denuded and intact cornea ( $p < 0.05$  for all eight compounds)



fold. For fluorescein, which penetrates the cornea poorly because of its hydrophilicity, the degree of enhancement was as 10-fold in the young 21-fold in the old. For LHRH, which penetrates the cornea poorly mainly because of susceptibility to peptidases hydrolysis and hydrophilicity a 41-fold in the young and 233-fold in the old could be found. For dextran (M.W.=70,000), which penetrates the cornea poorly because of its size and hydrophilicity a 10-fold in the young and 40-fold in the old could be detected. Thus, for these compounds the resistance of the corneal epithelium contributed substantially to penetration across the cornea.

The endothelial/stromal permeabilities (reverse permeation direction) of two model compounds are shown in Table 7. No difference in membrane transport/permeability for endothelial/stromal or stromal/endothelial layers was observed (i.e., compare Table 5 with Table 7). The permeabilities of these two model compounds were essentially the same in either direction (stroma to endothelium or endothelium to stroma). Evidently, the endothelial layer contributes much less resistance for compound penetration than the epithelium. There was a difference in permeabilities between the young and the old tissues but the difference was independent of molecular weight as in the stromal/endothelial permeability.

It is well known that the activities of some enzymes change with age. It is worthwhile to determine the metabolic



Table 7

Age Related Changes in Endothelial/Stromal Permeability on  
Basis of Molecular Weight

COMPOUND	PERMEABILITY COEFFICIENT (cm/sec x 10 <sup>6</sup> )		Difference (fold)
	YOUNG*	OLD*	
Fluorescein	15±1	11.1±0.1	1.4±0.1
Fluorescein Dextran(10000)	2.9±0.1	1.87±0.06	1.6±0.1

\* Data were mean ± standard deviation (young n=3, old n=2)  
and analyzed as in Table 2 ( $p < 0.02$  for both compounds).



capability of normal and damaged (denuded) corneas with respect to esterases and phosphatases. Results are shown in Tables 8 and 9. During the process of penetrating the cornea, dexamethasone acetate and dexamethasone phosphate were completely converted into dexamethasone by esterases and phosphatases, respectively. The magnitude of changes in the amount of dexamethasone across the cornea due to this biotransformation was determined and expressed as a difference in permeability. This difference directly reflects the rate of hydrolysis (Table 8). The ratio of this difference in enzymatic hydrolysis between young and old corneas was calculated and represented a difference in the rate of hydrolysis between these two tissues. (i.e., a number greater than one indicates increased hydrolysis). Thus, results suggest that decreased phosphatase and increased esterase activities in the aged cornea. The age-related differences in phosphatase and esterase activities appear to play less of a role in the penetration of a compound than the intrinsic permeability of the compound.

Although the epithelium appeared to have the highest hydrolytic activity in the cornea, there were still sufficient esterases and phosphatases in the stroma/endothelium to completely hydrolyze dexamethasone acetate and phosphate into dexamethasone (Table 9). None of the intact dexamethasone acetate or dexamethasone phosphate was detected in the receiver solutions for denuded corneas.



Table 8

Effect of Age on Esterase and Phosphatase Activities in the Intact Cornea

COMPOUND	PERMEABILITY COEFFICIENT (cm/sec x 10 <sup>6</sup> )		Difference (fold)		Ratio Young/old
	Young*	Old*	Young	Old	
Dexamethasone	9.0±0.9	3.6±0.3	(1.0)	(1.0)	(1.0)
Dexamethasone Acetate	42±5	24±6	4.7±0.7	7±2	0.7±0.2
Dexamethasone Phosphate	2.1±0.4	0.58±0.08	0.23 ±0.04	0.16 ±0.03	1.5±0.4

\* Data were mean ± standard deviation (young n=3, old n=2) and analyzed as in Table 2 ( $p < 0.05$  for all three compounds).



Table 9

Effect of Age on Esterase and Phosphatase Activities in the stromal/Endothelium (Denuded Cornea)

COMPOUND	PERMEABILITY COEFFICIENT (cm/sec x 10 <sup>6</sup> )		Difference (fold)		Ratio Young/old
	Young*	Old*	Young	Old	
Dexamethasone	37±2	22±1	(1.0)	(1.0)	(1.0)
Dexamethasone Acetate	40±2	16±2	1.1 ±0.1	0.7 ±0.1	1.6±0.3
Dexamethasone Phosphate	10±2	5.3±0.3	0.27 ±0.05	0.24 ±0.02	1.1±0.2

\* Data were mean ± standard deviation (young n=3, old n=2) and analyzed as in Table 2 ( $p < 0.05$  for all three compounds).



Results from the denuded corneas were compared with the data obtained from intact corneas. The damaged cornea preparations had lower esterase activity while the rate of phosphate hydrolysis remained the same in the aged stromal/endothelial tissues. This was opposite to that observed in the intact cornea.

### Stromal Binding Constants

In these studies, steady-state (pseudo-sink) conditions were maintained (i.e. the concentration in the donor chamber was much higher than that in the receiver chamber). The quantity of drug transported across the excised cornea was determined as plotted in Figure 1. The steady state flux was determined by dividing the slope of the time course plot by the corneal surface area ( $1.089 \text{ cm}^2$  for the young animals and  $2.010 \text{ cm}^2$  for the old animals). The permeability coefficient was obtained by dividing the steady state flux by the drug concentration on the donor side. The lag time was determined by extrapolation of the slope of the line to the X-axis. The permeability coefficients and lag times for suprofen through both young and old corneas are listed in Tables 10 and 11. The permeability coefficient and lag times for suprofen in the stroma/endothelium are shown in Tables 12 and 13. The permeability coefficient and lag times for caffeine in the cornea and stroma/endothelium are presented in Tables 14 and 15. The average corneal and stromal permeability coefficients for suprofen are shown in Tables 2



Table 10

Suprofen Lag Time and Permeability Coefficient in the Young  
Cornea

Donor Concentration (ug/ml)	Lag Time (min)	Permeability Coefficient (cm/sec) x 10 <sup>6</sup>
1000	36 <sup>a</sup>	9.3 (± 0.8) <sup>b</sup>
126	57	11 (± 3)
62	91	10.4 (± 0.6)
40	113	13 (± 1)

<sup>a</sup>Data point is a mean value from five separate determinations, and standard deviation is within ± 8.4% of the mean value.

<sup>b</sup>Numbers in parentheses represent standard error of the mean for each study.



Table 11

Suprofen Lag Time and Permeability Coefficient in the Old  
Cornea

Donor Concentration (ug/ml)	Lag Time (min)	Permeability Coefficient (cm/sec) x 10 <sup>6</sup>
952	79 <sup>a</sup>	5.0 (± 0.9) <sup>b</sup>
121	113	3.9 (± 0.1)
82	124	3.0 (± 0.4)
32	156	3.2 (± 0.1)

<sup>a</sup>Data point is mean value from five separate determinations, and standard deviation is within ±18.0% of the mean value.

<sup>b</sup>Numbers in parentheses represent standard error of the mean for each study.



Table 12

Suprofen Lag Time and Permeability Coefficient in the Young  
Stroma/Endothelium

Donor Concentration (ug/ml)	Lag Time (min)	Permeability Coefficient (cm/sec) x 10 <sup>5</sup>
886	7	3.0 (± 0.1) <sup>a</sup>
108	9	3.8 (± 0.1)
74	15	4.3 (± 0.1)
43	23	5.2 (± 0.6)
23	39	4.9 (± 0.1)

\*Numbers in parentheses represent standard error of the mean  
for each study.



Table 13

Suprofen Lag Time and Permeability Coefficient in the Old  
Stroma/Endothelium

Donor Concentration (ug/ml)	Lag Time (min)	Permeability Coefficient (cm/sec) x 10 <sup>5</sup>
84	21	1.9 (± 0.1) <sup>a</sup>
65	25	2.1 (± 0.1)
24	46	2.2 (± 0.1)

<sup>a</sup> Numbers in parentheses represent standard error of the mean for each study.



Table 14

Caffeine Lag Time and Permeability Coefficient in the Young  
Cornea

Donor Concentration (ug/ml)	Lag Time (min)	Permeability Coefficient (cm/sec) x 10 <sup>5</sup>
1000	6	5.8 (± 0.1) <sup>a</sup>
157	8	6.8 (± 0.2)
46	7	6.8 (± 0.5)

<sup>a</sup> Numbers in parentheses represent standard error of the mean for each study.



Table 15

Caffeine Lag Time and Permeability Coefficient in the Old  
Cornea

Donor Concentration (ug/ml)	Lag Time (min)	Permeability Coefficient (cm/sec) x 10 <sup>5</sup>
1488	7	3.2 (± 0.1) <sup>a</sup>
59	9	3.1 (± 0.1)

<sup>a</sup> Numbers in parentheses represent standard error of the mean for each study.



and 4. The average corneal permeability coefficient of caffeine was  $6.5 \times 10^{-5}$  cm/sec for the young and  $3.1 \times 10^{-5}$  cm/sec for the old.

The lag time of suprofen for the whole cornea as well as for stroma/endothelium was proportional to the inverse of the donor drug concentration as shown in Figure 11 for the young tissues and Figure 12 for the old tissues. The lag time increased as the donor concentration decreased. The slopes and intercepts of these plots are listed in Table 16.

The lag time of caffeine for the whole cornea was independent of its concentration for both the young and old animals. The slope of lag time versus drug concentration was close to zero (as shown in Tables 14 and 15), indicating no drug binding in either the young or old corneas.

Results of partition studies between rabbit epithelium and GBR suprofen solution gave a partition coefficient of  $3.9 \pm 0.4$  for the young and  $4.6 \pm 0.7$  for the old over a concentration range of 2.6 ug/ml to 4000 ug/ml. There was no significant difference between the young and aged animals. Suprofen binding to the stroma exhibits a typical, saturable binding as illustrated in Figure 13 for both young and old tissues. If one assumes that this binding fits a simple isothermal curve then the binding should fit the following equation:

$$A_I = k \left( \frac{k_2 C}{1 + k_2 C} \right) \quad (2)$$



Figure 11. The relationship between lag time and suprofen concentration in young animals. Plot  $1/\text{donor concentration}$  of suprofen versus the lag time obtained from penetration studies to illustrate the binding effect. A) whole cornea and B) stromal/endothelial layers.



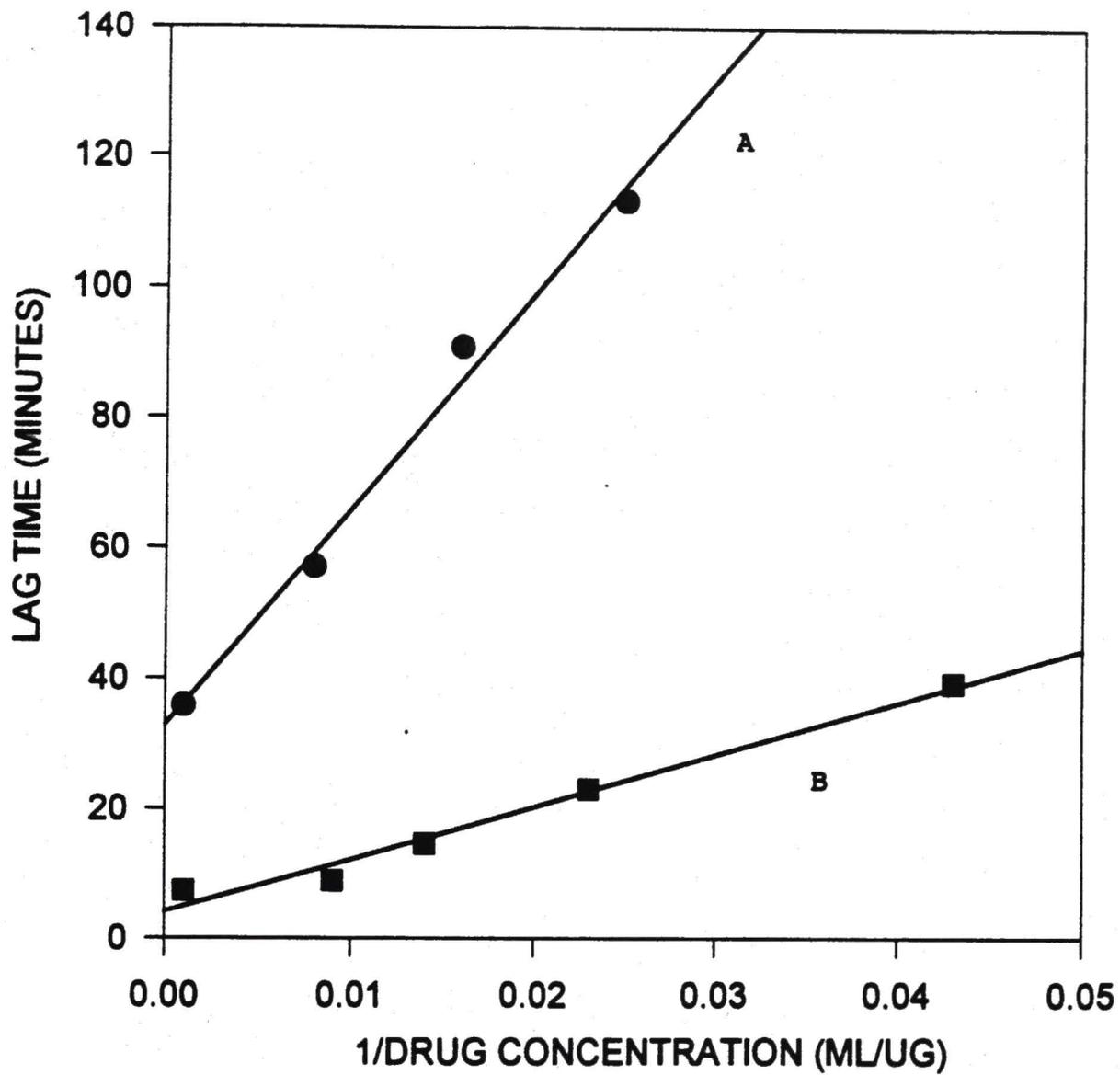




Figure 12. The relationship between lag time and suprofen concentration in old animals. Plot  $1/\text{donor concentration of suprofen}$  versus the lag time obtained from penetration studies to illustrate the binding effect. A) whole cornea and B) stromal/endothelial layers.



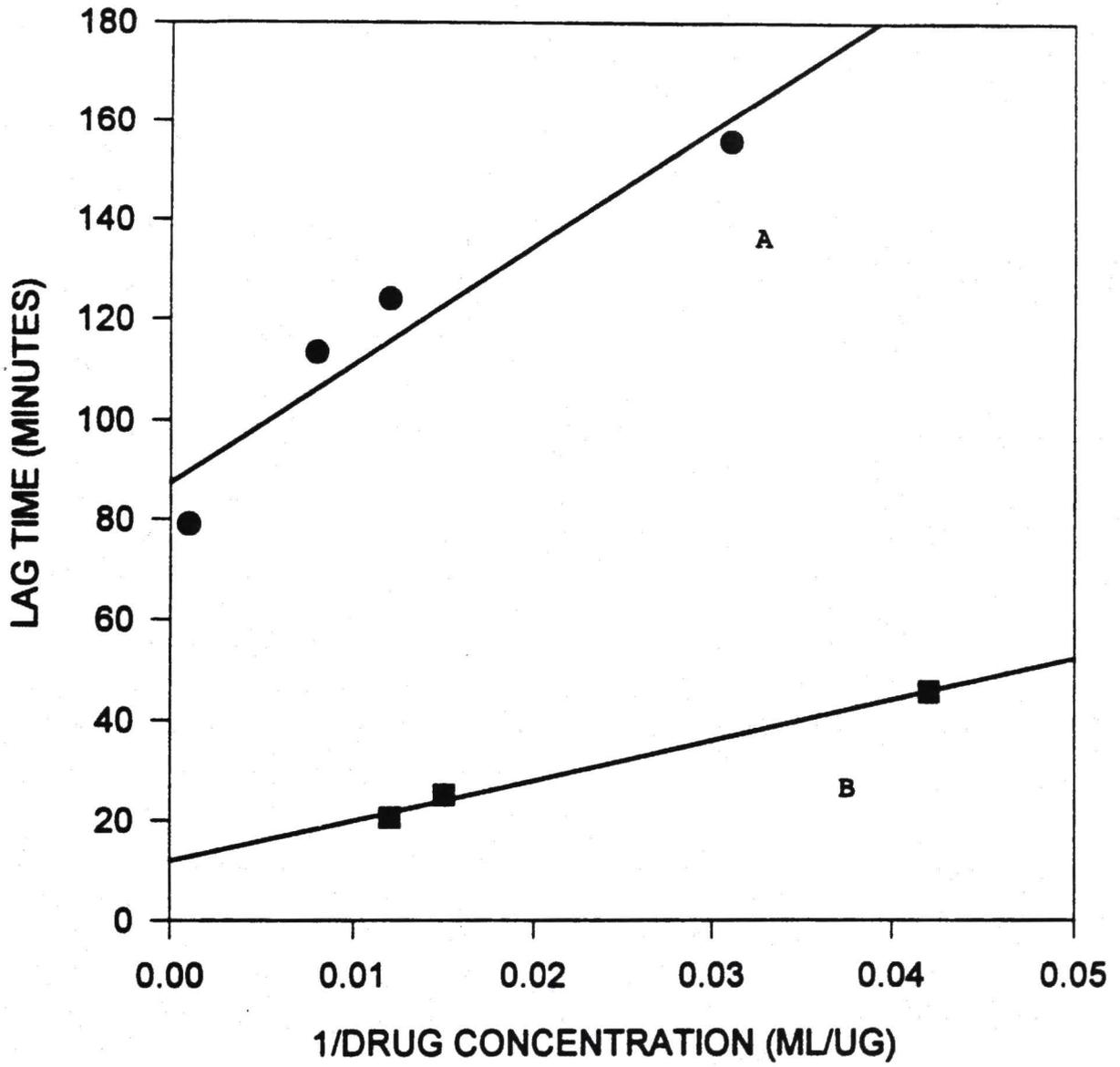




Table 16

The Slope and Intercept of 1/Suprofen Concentration versus  
Lag Time

## A. Young Animals

Tissue	Permeability Coefficient (cm/sec) $\times 10^6$	Slope(secxg/ml)	Intercept (minutes)	Regression
Stroma	37.7	$4.9 \times 10^2 (\pm 0.4)^a$	4 ( $\pm 1$ )	$R^2=0.99$
Cornea	9.6	$20 \times 10^2 (\pm 1)$	33 ( $\pm 3$ )	$R^2=0.98$

## B) Old Animals

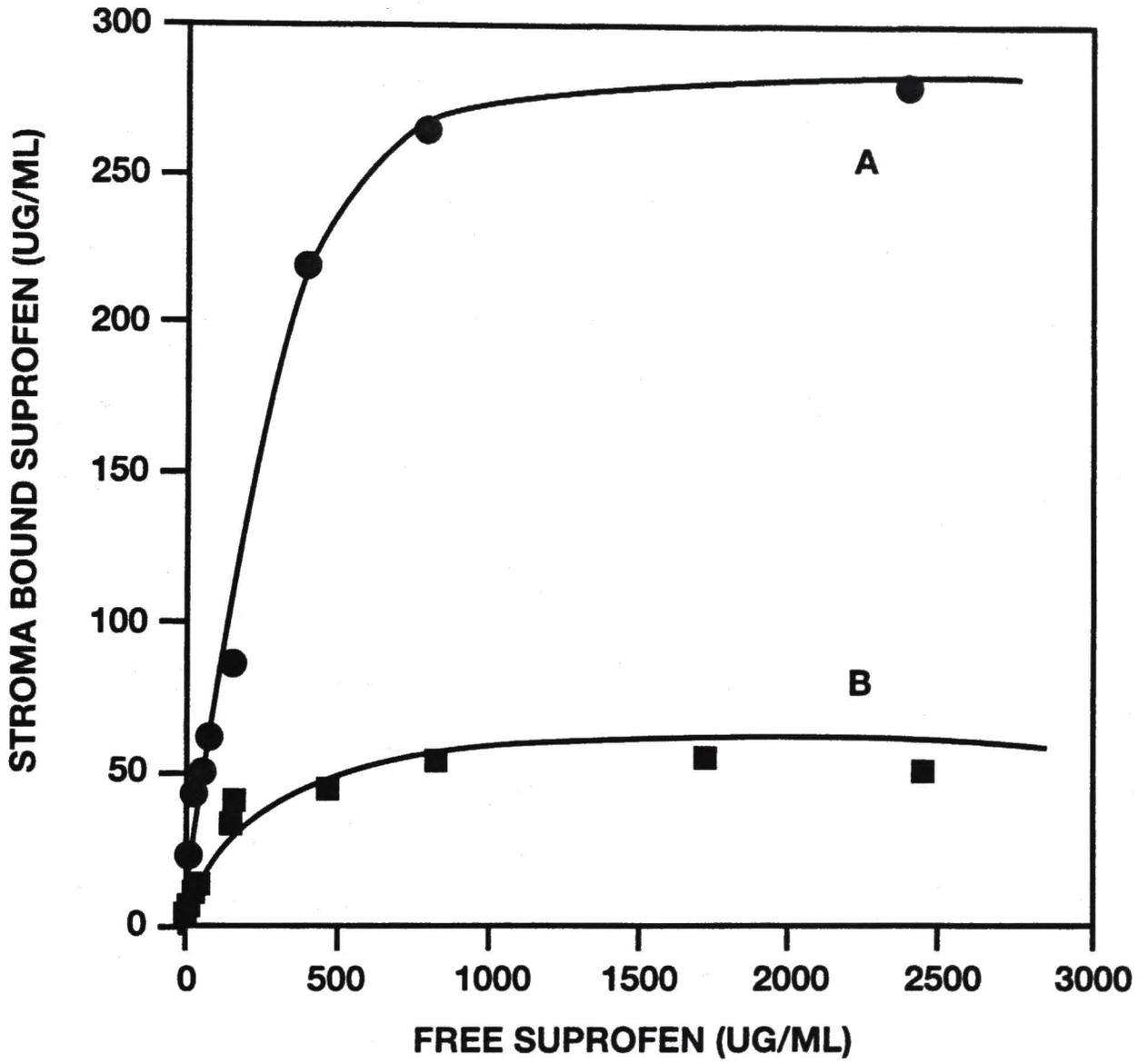
Tissue	Permeability Coefficient (cm/sec) $\times 10^6$	Slope(secxg/ml)	Intercept (minutes)	Regression
Stroma	18.3	$4.9 \times 10^2 (\pm 0.6)^a$	12 ( $\pm 1$ )	$R^2=0.99$
Cornea	3.6	$14 \times 10^2 (\pm 3)$	87 ( $\pm 9$ )	$R^2=0.92$

<sup>a</sup> Numbers in parentheses represent the standard error of the mean



Figure 13. Binding of suprofen with stroma in equilibrium studies. Free suprofen versus bound suprofen is plotted to demonstrate the saturable tissue binding curve for A) young and B) old tissues.







Where  $A_1$  is the amount bound (ug of suprofen/unit volume of nonpermeable stroma - calculated to be 20% of the weight of the stroma, which is 80% water),  $C$  is the concentration of free suprofen,  $k$  is the maximum amount bound, and  $k_2$  reflects the strength of the binding or the binding constant. When  $C$  is small, the initial slope of the curve represented by  $dA_1/dC$  equals to  $k \times k_2$ . When  $C$  is large,  $A_1 = k$ . The value of  $k$  was determined from these plots and was 280 ug/ml for the young and 56 ug/ml for the old corneas. The binding constant  $k_2$  was  $4.89 \times 10^{-3}$  ml/ug for the young and  $26.5 \times 10^{-3}$  ml/ug for the old. The binding constant in the old was about five fold higher than the young.

The lag time,  $t_L$ , for transport across a single membrane with binding is given by

$$t_L = t_{LO} + t_{LB} \quad (3)$$

Where  $t_{LO} = \frac{l_B^2}{6D_B}$  is the lag time with no binding and

$$t_{LB} = \frac{k V_b}{C_0 V_f} \left( \frac{P l_B^2}{2D_B} \right) \text{ is the lag time with binding for}$$

large donor concentration. Here

$l_B$  = thickness of the stroma

$D_B$  = diffusion coefficient of suprofen across stroma (unit  $\text{cm}^2/\text{sec}$ )

$P$  = distribution coefficient between stroma and the aqueous media = 1



$V_b$  = volume fraction of nonpermeable materials in the stroma  
= 0.2

$V_f$  = volume fraction of continuous phase (water) = 0.8

$k$  = maximum binding ( mass per unit volume)

Thus one predicts a dependence of the lag time on the inverse of the donor concentration with the intercept being  $t_{L_0}$ . The intercept lag time for the stroma gives  $l_B^2/6D_B$ , and the slope gives the quantity  $\frac{l_B^2}{2D_B} \cdot \frac{kV_b}{V_f}$ .

The permeability coefficient,  $k_{per}$  is given by

$$k_{per} = \frac{D_B}{l_B} = 3.8 \times 10^{-5} \text{ cm/sec}$$

Where the partition coefficient is assumed to be unity between the stroma and the phosphate buffer for both the young and old tissues. From these three equations, one can determine  $l_B = 0.054 \text{ cm}$ ,  $D_B = 2.04 \times 10^{-6} \text{ cm}^2/\text{sec}$ , and  $k = 273 \text{ ug/ml}$  for the young and  $l_B = 0.076 \text{ cm}$ ,  $D_B = 1.39 \times 10^{-6} \text{ cm}^2/\text{sec}$ , and  $k = 95.8 \text{ ug/ml}$  for the aged tissue.

The age-related changes in corneal permeability for a variety of compounds as well as the tissue binding phenomenon represent a basic alteration in corneal physiological function. A determination of the biochemical basis for these altered functions is required for a complete understanding of these changes and was the subject of the next studies.

### Lipid Analysis

All of the above observations clearly indicated the



necessity of more biochemical information in order to elucidate the mechanism of age-dependent changes in the cornea. Previous researchers have shown that during the aging process lipid peroxidation leads to a decrease in membrane permeability as well as changes in the physical state of membrane lipids, properties of transport and characteristics of enzymes in the liver, brain and lens plasma membranes.

The present studies concentrated on the determination of fatty acid composition, phospholipids, and cholesterol in the cornea and lens of young and old rabbits for the following reasons: 1) The interactions between the fatty acid side chains of membrane phospholipids affect membrane fluidity; 2) Usually, old cells contain more sphingomyelin and less phosphatidylcholine compared to the young. This affects the degree of lipid structural order; and 3) Cholesterol is thought to intercalate between the phospholipid residues, reducing the fluidity of the liquid crystal state by interacting with the fatty acid side chains. The molar ratio of cholesterol:phospholipid in biological membranes is "typically" about 0.5, the value varies depending on cell type.

The fatty acid compositions of ocular tissues in young (6 weeks) and old (3-4 years) rabbits were determined by GC assays. Results are listed in Tables 17 and 18 (cornea) as well as in Tables 19, 20, and 21 (lens). Among the different



Table 17

## Fatty Acid Composition in Young and old Corneal Epithelium

FAME*	PERCENTAGE OF TOTAL	
	Young	Old
C12:0	2.9	n.d.
C14:0	4.9	7.3
C16:0	30	28
C16:1	6.8	7.8
C18:0	8.4	6.9
C18:1	47	50.0
C20:4	n.d.	n.d.

Data are the mean values from two separate determinations, and standard deviation is within  $\pm 6.3\%$ .

\*FAME = Fatty acid methyl ester chain length and saturation.

Results are expressed as % of total.

n.d.= not detected



Table 18  
Fatty Acid Composition in Young and Old Corneal  
Stroma/endothelium

FAME*	PERCENTAGE OF TOTAL	
	Young	Old
C12:0	2.5	6.5
C14:0	5.5	9.2
C16:0	36	37
C18:0	20	13
C18:1	26	28
C20:4	10	6.3

Data are the mean values from two separate determinations, and standard deviation is within  $\pm 7.6\%$ .

\*FAME = Fatty acid methyl ester chain length and saturation. Results are expressed as % of total.



Table 19

## Fatty Acid Composition in Young and Old Lens Epithelium

FAME*	PERCENTAGE OF TOTAL	
	Young	Old
C14:0	6.5	5.2
C16:0	37	31
C16:1	9.6	5.1
C18:0	13	14
C18:1	29	35
C20:4	4.9	9.7

Data are the mean values from two separate determinations, and standard deviation is within  $\pm 4.3\%$ .

\*FAME = Fatty acid methyl ester chain length and saturation.

Results are expressed as % of total.



Table 20

## Fatty Acid Composition in Young and Old Lens Cortex

FAME*	PERCENTAGE OF TOTAL	
	Young	Old
C14:0	16	8.0
C16:0	42	36
C16:1	6.8	2.0
C18:0	4.2	8.0
C18:1	31	46
C20:4	n.d.	n.d.

Data are the mean values from two separate determinations, and standard deviation is within  $\pm 7.9\%$ .

\*FAME = Fatty acid methyl ester chain length and saturation.

Results are expressed as % of total.



Table 21

## Fatty Acid Composition in Young and Old Lens Nucleus

FAME*	PERCENTAGE OF TOTAL	
	Young	Old
C14:0	6.3	n.d.
C16:0	50	47
C18:0	7.7	14
C18:1	36	39
C20:4	n.d.	n.d.

Data are the mean values from two separate determinations, and standard deviation is within  $\pm 8.3\%$ .

\*FAME = Fatty acid methyl ester chain length and saturation.

Results are expressed as % of total.



tissue samples (corneal epithelium versus stroma /endothelium and lens epithelium, cortex, and nucleus), there were marked differences in fatty acid composition. In corneal epithelium (Table 17), oleic acid (18:1) accounted for about half of the fatty acids and palmitic acid (16:0) was the second most abundant. However, in stroma/endothelium (Table 18) palmitic acid was the most abundant and oleic acid was the second most abundant fatty acid. The stroma/endothelium also contained polyunsaturated arachidonic acid (20:4). No significant difference was observed between different age groups in the major fatty acid compositions for corneal epithelium and stroma/endothelium. Only very minor changes were detected in the overall composition.

For lens tissues, palmitic acid and oleic acid are the two major fatty acids (Tables 19, 20 and 21). No significant quantities of acids with retention times greater than oleic acid were observed in lens cortex and nucleus. There was an appreciable amount of arachidonic acid (20:4) in the lens epithelium. In aged lens, there was a decrease in the shorter chain length fatty acids (C14 and C16) and an increase in the longer chain length fatty acids (C18 and C20). Usually, interactions between the fatty acid side chains increase with increasing chain length and thus form a more rigid lipid matrix.



Phospholipid and cholesterol profiles of various ocular tissues were determined by HPLC analysis. Results are listed in Table 22. Clearly, there were large differences in the phospholipid and cholesterol profile among the various ocular tissues. Based on wet tissue weight, the corneal epithelium had the highest amount of phospholipids. Phosphatidylcholine was the major phospholipid of the corneal epithelium and stroma/endothelium. PE was the next most abundant. There was a small difference in sphingomyelin between young and old corneal epithelium ( $p < 0.05$ ). A bigger difference of sphingomyelin could be detected between young and old stroma/endothelium ( $p < 0.03$ ).

The composition of phospholipids was also determined in separate parts of the young and old lens. Lens epithelium had a higher phospholipid content and relatively more PC and PE than the inner lens. Lens tissue in which the turn over rate was slow showed a much greater difference in phospholipid composition comparing young and old tissues. In the young lens, the major phospholipids found in the cortical and nuclear fraction were PC and PE. In the old cortex and nucleus, the predominant PL was SPH. This change in the PL composition and the lower total PL content found in the old cortex and nucleus suggested that the lipids of the newly formed lens fibers were age-dependent. All of



Table 22

Phospholipid and Cholesterol Composition in Various Ocular  
Tissues

SAMPLE	PE	PI	PS	PC	SPH	CHOL
CORNEA EPI (Y)	2.58	1.78	0.50	10.9	2.35	10.7
CORNEA EPI (O)	2.80	1.71	0.39	10.7	2.81	10.4
CORNEA STR (Y)	0.74	0.32	0.07	2.8	0.20	1.40
CORNEA STR (O)	0.35	0.12	0.09	2.8	0.30	0.72
LENS EPI (Y)	3.1	1.08	1.01	8.7	0.34	3.6
LENS EPI (O)	1.03	0.44	0.29	3.6	0.20	1.17
LENS CORTEX(Y)	0.9	0.1	0.3	1.3	0.4	3.3
LENS CORTEX(O)	0.45	0.03	0.18	0.62	2.1	5.0
LENS NUCL (Y)	0.32	n.d.	0.10	0.27	0.21	2.7
LENS NUCL (O)	0.06	n.d.	0.02	0.01	0.79	5.6

The unit for each sample is  $\mu\text{M}$  of lipid per mg of wet tissue weight.

Data point is the mean value from two separate determinations and the standard deviation is within 2.9% for corneal epithelium, 4.0% for corneal stroma, 5.8% for lens epithelium, 12.7% for lens cortex, and 10.5% for lens nucleus, respectively.

n.d.= not detected



PE - phosphatidylethanolamine

PI - phosphatidylinositol

PS - phosphatidylserine

PC - phosphatidylcholine

SPH - sphingomyelin

CHOL - cholesterol

(Y) - young animals

(O) - old animal



these results on the lens agree well with previous studies (Anderson et al., 1969; Li and So, 1987).

Among the lipid classes, the ratio of PC/SPH is a marker for membrane fluidity. The higher the ratio the less rigid the membrane becomes. PC/SPH values of various tissues were determined and are listed in Table 23. Membrane fluidity is a function of the degree of structural ordering of lipid bilayers and is an important feature in regulating functional properties of the membrane. Results indicated that there were differences in PC/SPH ratio among different ocular tissues. The epithelium had a higher PC/SPH ratio indicative of a less rigid (more fluid) structure. The lens nucleus had the lowest ratio implying that it was the least fluid tissue in the eye. Differences in PC/SPH between young and aged animals were calculated and are listed in Table 24. The results clearly indicated that a relationship between the PC/SPH ratio and age of the animal. The younger tissues exhibited higher values which indicated a less rigid structure. The difference in the oldest tissue (lens nucleus) between different age groups exceeded 100 fold.

A slight difference in the cholesterol content of the cornea and lens epithelium was observed between the two different age groups. A significant increase in cholesterol was observed in the old lens cortex and nucleus. Cholesterol is thought to intercalate between the phospholipid residues, reducing the fluidity of the liquid crystal state by



Table 23

## Changes in PC/SPH Ratio of Various Ocular Tissues

SAMPLE	PC	SPH	PC/SPH (fluidity index)
CORNEA EPI (Y)	10.9	2.35	4.6±0.1
CORNEA EPI (O)	10.7	2.81	3.8±0.1
CORNEA STR (Y)	2.8	0.20	14±1
CORNEA STR (O)	2.8	0.30	9.3±0.5
LENS EPI (Y)	8.7	0.34	26±2
LENS EPI (O)	3.6	0.20	18±1
LENS CORTEX (Y)	1.3	0.4	3.3±0.4
LENS CORTEX (O)	0.62	2.1	0.29±0.04
LENS NUCL (Y)	0.27	0.21	1.3±0.1
LENS NUCL (O)	0.01	0.79	0.01±0.00

(Y) - Young Rabbits

(O) - Old Rabbits

EPI - Epithelium

STR - Stroma

NUCL - Nucleus



Table 24  
Age-Related Changes in PC/SPH Ratio  
of Various Ocular Tissues

Young/Old Ratio	PC/SPH
Cornea Epithelium	1.2±0.1
Cornea Stroma	1.5±0.1
Lens Epithelium	1.4±0.1
Lens Cortex	11±2
Lens Nucleus	130±10



interacting with the fatty acid side chains. The molar ratio of cholesterol/phospholipids (CHOL/PL) is shown in Table 25. In rapid turn over biological membranes it is typically about 0.5. Only the results from corneal epithelium were close to this number. The CHOL/PL ratio, another measurement of membrane rigidity, is higher in the old than in the young for lens cortex and nucleus but not other tissues, due to lower PL levels and higher CHOL levels in the older lens. In the nucleus of the old lens, the CHOL/PL value exceeded 6.0. The ratio between different age groups is shown in Table 26. The young lens cortex and nucleus had a lower CHOL/PL ratio than the old lens, indicating a more rigid structure in the old tissues. It is possible that in extremely slow turnover tissues such as the lens this ratio could distinguish between different age groups. The difference in the CHOL/PL ratio was not as great as the PC/SPH ratio suggesting that the PC/SPH ratio is a more sensitive indicator of changes in membrane fluidity than the CHOL/PL ratio. Overall, changes in these lipid classes have been demonstrated to be the main factors that affect the biophysical structural order of aged corneal and lens cell membranes. Age-related decreases in the ratio of phosphatidylcholine to sphingomyelin were observed in aged cornea and lens. These results provide evidence for a decrease in membrane fluidity in both aged cornea and lens tissues. The results also suggest that the small decrease in membrane fluidity of aged cornea



Table 25

## Changes in CHOL/PL Ratio of Various Ocular Tissues

SAMPLE	PL	CHOL	CHOL/PL
CORNEA EPI (Y)	18.1	10.7	0.59±0.02
CORNEA EPI (O)	18.4	10.4	0.56±0.02
CORNEA STR (Y)	4.13	1.40	0.34±0.01
CORNEA STR (O)	3.66	0.72	0.20±0.01
LENS EPI (Y)	14.2	3.6	0.25±0.02
LENS EPI (O)	5.56	1.17	0.21±0.01
LENS CORTEX (Y)	3.00	3.3	1.1±0.1
LENS CORTEX (O)	3.38	5.0	1.5±0.2
LENS NUCL (Y)	0.90	2.7	3.0±0.3
LENS NUCL (O)	0.88	5.6	6.4±0.7

PL = Total Phospholipids

CHOL = Cholesterol

(Y) = Young Rabbits

(O) = Old Rabbits



Table 26  
Age-Related Changes in CHOL/PL Ratio  
of Various Ocular Tissues

Young/Old Ratio	CHOL/PL
Cornea Epi	1.00±0.05
Cornea Stroma	1.7±0.1
Lens Epi	1.2±0.1
Lens Cortex	0.7±0.1
Lens Nucleus	0.47±0.07



may be a part of the mechanisms that affect membrane permeability and tissue binding because these two biochemical features are usually affected by membrane rigidity.



## CHAPTER IV

### DISCUSSION

#### Age-Related Changes in the Cornea

The delivery of compounds to target tissues in the eye is difficult because of the multiple protective barriers imposed by the eye against the entry of compounds. In the evaluation of a rational therapeutic approach in ophthalmology, the age of the patient may be another important consideration. The rabbit has served as an animal model for the study of age-related factors which affect ocular drug disposition. The overall objective of the present study was to conduct a systematic study of membrane permeabilities and biochemical changes in young and aged corneas in order to gain fundamental information on the effects of aging of the cornea and to develop the strategy for optimal delivery of ophthalmic drugs in elderly patients. The results clearly indicated that the aging process in the cornea is associated with changes in several biochemical and functional (physiological) parameters. These changes are discussed below in more detail.

Penetration experiments were carried out by means of passive transport of specific compounds under steady-state



conditions using *in vitro* perfused cornea diffusion cells. Permeabilities of a variety of compounds with different physicochemical properties were determined in young and old intact and denuded rabbit corneas. These studies clearly demonstrated age-related differences in the corneal permeability of a variety of compounds. A significant decrease in permeability with age was observed. The degree of difference depended on the lipophilicity and molecular weight of the compound and the integrity of the corneal epithelial cell layer. The difference was more pronounced for large hydrophilic than for small lipophilic compounds in the intact corneas.

Compounds differing in molecular weight exhibited greater differences in permeability compared to compounds differing in lipophilicity in both young and old intact corneas (8-fold versus 2-fold). These age-related permeation changes in the corneal epithelium suggest that the epithelial membrane becomes more lipophilic or more rigid with age due to changes in membrane partition coefficients or in membrane fluidity. Compounds, therefore, encounter more resistance in penetrating the aged intact cornea.

Compounds like fluorescein dextrans (10,000 and 70,000) and LHRH are very hydrophilic and probably cross the epithelial cell layers only through the paracellular space. Therefore, their entry is governed by the state of the intercellular junctional complexes. Usually, epithelial



permeability is a combined function of transfer through the paracellular spaces between the epithelial layers (paracellular) and penetration across the epithelial cell membranes (transcellular). Probably, a compound which penetrates the cell membrane with more difficulty (high molecular weight hydrophilic compounds) may be more sensitive to changes in the barrier function. Therefore, age-related permeability changes in these compounds (eight-fold) are much more pronounced than small lipophilic compounds (about two-fold).

Unless drug is very lipophilic (for example betaxolol), generally a two- to 100-fold increase in corneal penetration occurs when the corneal epithelial barrier is absent. Without the epithelial barrier, the stromal/ endothelial layers become the sole barrier for large hydrophilic compounds. This barrier provides more resistance in the old than in the young corneas as indicated by a two fold decrease in permeability for all compounds tested. For betaxolol, the epithelial layer actually appeared to promote its penetration, since the amount of betaxolol entering the stroma was slightly decreased after removal of the epithelial barrier for both the young and aged corneas. This higher permeability may have been related to betaxolol's greater ability in promoting the fluidity of phospholipid membranes as suggested by Shi and Tien (1986).



The data on permeability changes in denuded cornea suggest age-related changes in the biochemical and structural matrix of stromal/endothelial cells in addition to and distinctly different from the aging changes of the corneal epithelium. Furthermore, the molecular weight (size)-dependent permeability differences (>ten-fold) are greater than the lipophilic-dependent differences (<ten-fold). This indicates that, in addition to the lipophilic epithelial barriers, the stromal/endothelial layers are also important permeability barriers for large hydrophilic compounds.

The penetration of small electrolytes is dependent on transmembrane resistance. Old corneas exhibit a higher resistance (1.7-fold), indicating that small electrolytes do not penetrate the old cornea as easily as the young cornea. Such differences may reflect changes in the properties of cells that make up the epithelial and stromal/endothelial tissues. The changes may be due to alterations in cellular lipid content and/or in cell- to cell- junctional structures in the cornea.

Fick's laws of diffusion state that the flux across a membrane is a function of the concentration gradient and the inherent ability of a compound to diffuse across the membrane (diffusivity) (Dalmark and Storm, 1981). As seen in equation 1 (page 57), the value of the permeability coefficient is directly proportional to the ratio of  $\epsilon DK/\tau h$ .



No apparent difference in  $h$  (membrane thickness) was observed from the morphology studies. In addition, no significant difference in  $K$  (membrane partition coefficient) of one model compound, suprofen, was found between these two age groups. Therefore, differences in permeability most likely reflect differences in  $\epsilon$  (membrane porosity),  $D$  (diffusion coefficient), and  $\tau$  (tortuosity) between the young and old corneas. The permeability differences due to age can probably be attributed to structural variations which affect  $\epsilon$ ,  $D$ , and  $\tau$  in the two age groups. Thus, the amount of any material diffusing per unit time per unit area across a biological membrane depends upon the physicochemical properties of the molecule and its interaction with the membrane (Lee, 1975). Any changes in membrane components associated with aging may result in a difference in permeability of the compound. In summary, changes in corneal permeability do occur with age and have a potential to alter ocular drug bioavailability.

In general, the corneas of older eyes had a decreased permeability to these test compounds which suggested alterations in both the drug flux and the membrane system. A similar finding was observed in the small intestinal microvillous vesicles of rat. The passive permeability coefficients of thiamin transport decreased with increasing age (Gastaldi, et al., 1992) which suggested that aging was



associated with intrinsic alterations of the enterocytic plasma membrane.

It is well known that peptidases in the cornea are extremely active and the metabolic and permeation barriers to the ocular penetration of peptides are substantial (Stratford and Lee, 1985; Kashi and Lee, 1986). During and after crossing the cornea, LHRH (the model peptide for this study) was rapidly hydrolyzed by peptidases in the cornea and less of it was detected in the receiver solution. Therefore, its apparent permeability appeared less than the larger fluorescein dextran (M.W.=10,000 Mass). In the denuded cornea, the smaller LHRH was more permeable than the larger fluorescein dextran (M.W.=10,000 Mass) which suggests that the corneal epithelium contains the highest peptidase activities. Without peptidases in the epithelial layer to break down LHRH, the intact compound can cross the stroma/endothelium barrier better and follows the inverse relationship between permeability and molecular weight. These results demonstrate that the epithelium is the major penetration and metabolic barrier for hydrophilic proteins and peptides for both the young and old animals.

There also appear to be differences in the esterase and phosphatase hydrolytic activities in corneal tissues using dexamethasone acetate and dexamethasone phosphate as model compounds. With the epithelium removed, results from these *in vitro* techniques were very similar to results of other



investigators using *in vitro* (Hull et al., 1974 and Cox et al., 1972) and *in vivo* (Kupferman and Leibowitz, 1975) models of corneal penetration. The permeability coefficients of dexamethasone and dexamethasone sodium phosphate increased in the denuded corneas about four-fold in the young and six- to nine- fold in the old. By comparison, permeability of dexamethasone acetate was unaffected in both the young and the old denuded corneas. The age-related changes in the rate of enzymatic hydrolysis are different in the intact and denuded corneas (0.71 versus 1.53 for esterases; 1.44 versus 1.13 for phosphatase). The rate of hydrolysis is an important criterion determining the distribution and half-life of many prodrugs (activation by hydrolysis) which come into contact with the cornea.

Within each class of enzymes, the activities of only certain enzymes of old tissues are lower than those of the young; others are higher. All the enzymes within a class do not show a specific type of change with age (Erlanger and Gershon, 1970 and Barja and Freire, 1992). Furthermore, certain enzymes that decrease in one tissue with age may increase in another (Lee et al., 1983). Thus, the activities of enzymes located in different cell compartments do not show any predictable pattern of change. For example, 70% of esterase activity resides in the corneal epithelium. The esterase activity in the corneal epithelium is predominantly microsomal. In contrast, almost all of the esterase activity



in the stroma is cytosolic or extracellular. The levels of a variety of enzymes have been studied as a function of age, but the data do not give any clue to the mechanism of aging. It is clear, however, that alterations in the activities of enzymes with age could affect the physiological/ pharmacological functions of specific tissues. For example, in prodrug delivery systems, changes in enzymatic activities will affect the bioavailability of these compounds in the body (Lee and Robinson, 1986).

It has been suggested that the lipophilicity of the membrane barriers largely determines the selectivity of penetration of certain compounds through the cornea (Lee and Robinson, 1986). The present studies show that the corneal epithelium is the most important structure in determining both the quality and quantity of penetration and metabolism, and that these parameters are markedly altered with age.

In the past, little attention has focused on the lag time associated with *in vitro* corneal penetration. The investigation of corneal penetration of suprofen shows that, for both young and old corneas suprofen has a long diffusional lag time which is concentration dependent. The overall corneal permeability remains the same for both young and old corneas (independent of concentration). The results from these penetration studies show that suprofen binds to the stroma and thus has an extended lag time which is concentration-dependent. The theoretical model presented in



this thesis accounts for the overall dependence of lag time on concentration. One can easily obtain the necessary information about compound binding with the cornea as well as other penetration parameters from this theoretical model in addition to some limited experimental results. The maximum binding capacity ( $k$ ) obtained from transport studies is remarkably similar to the one obtained from equilibrium binding studies (under the assumption that the binding is a simple isothermal phenomenon).

The value of  $k$  (273 ug/ml) for suprofen from the application of the theoretical model to the transport data was found to be in good agreement with the value obtained directly from the binding experiment determined in the young tissue (280 ug/ml). However, the value of  $k$  (95.8 ug/ml) from the theoretical calculations was considerably higher than the experimental one (60 ug/ml) in the aged tissue. Most likely, the tissue binding in the old stroma is more complicated than simple isothermal binding. It is worthwhile to study this binding effect in more detail. Overall, the binding constant for suprofen in the old stroma ( $26.5 \times 10^{-3}$  ml/ug) is much higher than that in the young ( $4.89 \times 10^{-3}$  ml/ug).

The normal thickness of the corneal stroma/endothelium is about 0.04 cm (Maurice and Mishima, 1984). A theoretical calculation of corneal thickness from the model gives a thickness of 0.05 cm in the young and 0.08 cm in the old,



indicating that although both young and old corneas had similar thickness from ultrastructure analysis, the old tissue apparently had more complex and tortuous pathways and compounds took a longer time to pass through the old cornea. The value for the diffusion coefficient for both young ( $2.04 \times 10^{-6} \text{ cm}^2/\text{sec}$ ) and old ( $1.39 \times 10^{-6} \text{ cm}^2/\text{sec}$ ) is quite reasonable for a gel matrix (stroma).

In addition, if one assumes that binding takes place predominantly in the corneal stroma (not in the epithelium), then one can analyze the lag time data for the entire cornea according to a model for transport across a two-layer membrane with binding in the second membrane (stroma). (See Appendix A for the mathematical model). In addition to the stromal binding data, one must consider the corneal permeability and partition coefficient as well as the lag time. The intercept and slope between the lag times and concentration for suprofen in the cornea were shown in Table 16. The corneal permeability  $k_{\text{per}}$  for suprofen can be written as :

$$K_{\text{per}} = \frac{P}{\frac{l_B}{D_B} + \frac{Pl_A}{D_A}} \quad (4)$$

Where  $l_A$  and  $l_B$  are the thickness of the epithelium and stroma respectively, and  $D_A$  and  $D_B$  are the diffusion coefficients of the epithelium and stroma respectively. The



partition coefficient between GBR buffer and the lipid component of the epithelium is given by P (3.7 for the young and 4.2 for the old).

The lag time (see Appendix A) is given by

$$t_L = t_{LO} + t_{LB}$$

where  $t_{LO}$  is now given by

$$t_{LO} = \left[ \frac{l_A}{l_B} + \frac{Pl_B}{D_B} \right]^{-1} \left[ \frac{l_A^2}{D_A} \left( \frac{l_A}{6D_A} + \frac{Pl_B}{2D_B} \right) + \frac{l_B^2}{D_B} \left( \frac{Pl_B}{6D_B} + \frac{l_A}{2D_A} \right) \right] \quad (5)$$

and  $t_{LB}$  in its high-binding limit is given by

$$t_{LB} = \frac{kV_B}{C_oV_f} \left[ \frac{l_A l_B}{D_A} + \frac{Pl_B^2}{2D_B} \right] \quad (6)$$

Using the values of  $l_B$  (0.04 cm for nonswollen stroma) and  $D_B$  from the stroma transport studies one calculates  $l_A = 0.018$  cm,  $D_A = 6.23 \times 10^{-8}$  cm<sup>2</sup>/sec and  $k = 161.8$  ug/ml for the young tissue and  $l_A = 0.025$  cm,  $D_A = 2.47 \times 10^{-8}$  cm<sup>2</sup>/sec and  $k = 57.2$  ug/ml for the old. The value of  $D_A$  is smaller than that of  $D_B$ , as expected, and the value for  $k$  is reasonable but somewhat smaller than the binding result for the young but close to the binding results for old animals. The value for  $l_A$  for both young and old is larger than the actual thickness of the epithelium (0.005 to 0.009 cm) and may reflect a tortuous pathway in the corneal epithelial membranes. The fact that the lag times and permeability coefficients give a reasonable diffusion coefficient and



most penetration parameters leads one to believe that this simple model is sufficient to describe the transport phenomenon.

Few pharmacokinetical reports have dealt with how cornea binding affects the overall disposition of drugs in the eye. Conceivably, the rate at which a drug is released from the cornea can markedly influence the pharmacological response and may vary from patient to patient. For compounds that bind, like suprofen, and thus have long lag time, it is expected that the peak time in aqueous humor would be much longer than the usual twenty minutes as suggested by Sieg and Robinson (1976). According to Leibowitz et al. (1986) and Tang-Liu et al. (1984), topical dosing with suprofen or flurbiprofen gives an aqueous humor concentration peak time of 60 minutes and 2-4 hours respectively. These results indicate that the time course of *in vivo* aqueous humor drug concentration should be determined in order to optimize the drug delivery process. One would expect many anionic drugs too have rather long lag times for corneal penetration and reach peak aqueous humor concentrations at a time longer than the usual 20 minutes. The lag time can be measured conveniently *in vitro* and be used to estimate the time for peak aqueous humor concentration.

Since the old tissue has a higher binding constant, it may take longer for the drug to be released from the old corneas. The difference between young and aged stromal



binding constants may indicate structural changes in collagen and the proteoglycan matrix. For example, during aging the solubility of collagen decreases which may reflect the higher binding constant in the old tissue.

Since most compounds penetrate the cornea by simple diffusion, one of the specific biochemical mechanisms responsible for different permeabilities between young and old corneas may be attributable to changes in membrane lipid composition and structure. Phospholipid analysis of the rabbit cornea reveals that only the SPH content has a significant positive correlation with age; all other phospholipid species remain basically unchanged. These results provide evidence that a decrease in membrane fluidity of the cornea (as measured by the ratio of PC/SPH) occurs with age and is an important mechanism that accounts for the modified corneal membrane function with age. Here, membrane fluidity was not directly measured, but estimated indirectly based on the corneal membrane phospholipid composition.

When similar approaches are applied to the lens, more pronounced age-related changes in phospholipid compositions and membrane fluidity can be found. In the aged lens, the most marked change in phospholipid content was the age-dependent increase of SPH and the decrease of PC. During aging there was a considerable decrease in C14:0 acid and C16:0 acid. In contrast, an increase in the levels of C18:1



acid was observed in the old lens. In addition, 20:4 content almost doubled in the old lens epithelium making the peroxidation index increase with age. All changes found in this study are very close to previously published results (Zelenka, 1984). It is well known that lens is the oldest tissue in the body, and membrane permeability is much lower in aged lens.

Changes in the ratio of phosphatidylcholine/sphingomyelin have been demonstrated to be the main factors that affect the biophysical structural order of cell membranes. In the rapid turnover tissues (corneal epithelium and lens epithelium), the extent of modification of the membrane fatty acid composition is limited. It is not surprising that the physical properties, in terms of bulk or average membrane fluidity parameters, can also change only to a small degree. In contrast, for aged tissues (lens cortex and nucleus), age-related changes in fatty acids are more pronounced because they tend to accumulate over time. Therefore, differences in membrane fluidity between young and old tissues are much greater. These findings of decreased fluidity with age in the cornea and lens are in accordance with other studies carried out by different techniques on the membrane fluidity of rat hepatocyte and human and mouse lymphocytes (Benedetti, et al. 1988 and Huber, et al. 1991).



Phosphatidylcholine and sphingomyelin are located primarily in the outer extracellular monolayer of the membrane while anionic phospholipids such as phosphatidylethanolamine, phosphatidylserine, and phosphatidylinositol are distributed in the inner monolayer. During the aging process, membrane lipid polyunsaturated fatty acids may undergo peroxidation, resulting in the accumulation of cross-linked products such as malondialdehyde. Repair, replacement of cross-linked lipids, or inhibition of lipid peroxidation may prevent this membrane aging process. Lipid peroxidation can increase the rigidity (decrease fluidity) of the monolayer of phospholipid model membranes. In summary, the alterations in lipid content may be an important mechanism that modulates corneal membrane function with age.

Age-dependent changes of membrane constituents, such as phospholipids, proteins, cholesterol, etc., may result in the modification of important membrane properties such as fluidity, activity of membrane bound enzymes, receptor mobility, excitability, and transduction of biological messages. The aging process may result in altered protein and lipid synthesis, degradation, or modification, leading to changes in membrane channels and/or charges, and thus altering the permeability of various compounds. Age-related changes of physicochemical properties of lipid phase might affect protein molecular conformation and function through



either changes in bulk lipid fluidity or through specific lipid-protein interactions. It is possible that the change in permeability to drugs is a reflection of age-related altered cell membrane properties. Results suggest that the membrane fluidity alterations of aged cornea can be a significant part of the mechanism of altered corneal permeability.

From these data, it appears that the dynamic features of membrane monitored by drug transport are significantly affected by aging. Results also provide indirect evidence of a decrease in membrane fluidity in the aged cornea. This modification could be considered one of the mechanisms which contributes to altered corneal permeability properties. Particularly remarkable is the decrease in the apparent fluidity of the corneal epithelium and stroma/endothelium. Such a perturbation could be induced by peroxidation of lipid in the membrane of the aged tissues. Free radical reactions, considered a key factor of the complex phenomena occurring during aging, could augment the differences in the membrane fluidity altering, as a consequence, the mobility of the membrane proteins.

Overall, the present study indicates that in cornea, aging is associated with changes in membrane constituents (lipids) and membrane physical properties which lead to decreased membrane fluidity (i.e. increased viscosity). The functional correlations associated with these physical



alterations are increased transmembrane resistance, decreased membrane permeability, altered enzymatic activities (esterases and phosphatase), and altered corneal drug binding.

Changes in the corneal permeabilities have both basic and applied importance. Obviously, considerations of drug delivery through the cornea for treatment of ocular diseases must take into account these changes in both the selectivity and sensitivity of penetration with age. For example, the transfer of molecules through cornea decreases with age due to the decreased permeability, so that delivery of nutrients or therapeutic agents to the interior of the aged eye becomes progressively less efficient. Consideration of such changes could lead to altered drugs, altered dosages or altered modes of drug delivery for the elderly. Thus, an attempt to develop rational age-related dosage modifications for ophthalmic drugs must include a consideration of functional difference as well as age effects. Secondly, the basic biochemical factors associated with the aging cornea have yet to be clearly sorted out and are, currently, a subject of considerable interest. For example, the nature of the age-related changes in the corneal membranes may also reflect age-related changes in membranes of other tissues. The plasma membrane is, in fact, the structure through which a cell recognizes specific biological signals, communicates with its environment, controls the intake of nutrients,



releases wastes, and allows the flux of ions which determine its electric state. Clearly a fundamental understanding of the age-related changes in cell membrane permeability is most basic because it governs cells' basic physiology and homeostasis.

As our understanding of the biochemical/physiological events in the cornea during aging deepens, how to rejuvenate the aging cornea becomes an intriguing challenge. Since the rate of the aging process is variable and depends on the effects of several different inherent and outside factors that accelerate or retard this deterioration. Some of these factors may be accessible to intervention. For example, it is well known that malnutrition exerted a protective effect on cell membranes of old animals by slowing down the increase in membrane microviscosity (Pieri et al., 1991; Levin et al., 1992 and Yu et al., 1992).

Antoniano and his associates (1987) suggested that the age-dependent decrease in membrane fluidity precedes irreversible changes in the chemical composition of the membrane proteins. They proposed that a therapeutic lipid mixture (AL 721) could restore proper membrane fluidity before membrane aging had reached an advanced stage. They claimed that physiologically diminished cell function due to increased rigidity of its membrane was reversible both *in vitro* and *in vivo* by AL 721. Fluidization of aged membranes with AL 721 has been shown to: 1) restore brain serotonin



receptor function both *in vivo* and *in vitro* (Antonian, et al., 1987); 2) restore deficient immune responsiveness of lymphocytes to mitogen stimulation in aged subjects (Rabinowich, et al., 1987 and Provinciali, et al., 1990); and 3) markedly reduce withdrawal symptoms from drug tolerance to morphine (Antonian, et al. 1987).

These results correlate the hypothesis that a rectification of rigidified cell membranes may represent a valuable approach to restore proper physiological function in old age (Provinciali, et al., 1990). It is important to determine if the age-dependent decrease in corneal permeability can, in fact, be reversed by AL 721. The prevention or modulation of the aging process in cornea remains an exciting research goal and merits exploration.

In addition to studying the rejuvenation of aged cornea by chronic treatment with AL 721 to reverse those age-related changes in function, several other experiments are proposed to address some unanswered questions.

- 1) Examine protein components in young and old corneal epithelium and stroma by gel electrophoresis to explore the possibility of post-translational protein modification. An understanding of the molecular structure of corneal proteins including collagen is necessary to appreciate the changes in its properties as a function of age.



- 2) Study corneal membrane fluidity by two direct fluorescence methods. The first of these, fluorescence polarization, is based on the microviscosity dependence of the rotational diffusion rate of a fluorophore, diphenylhexatriene (DPH), in membranes. The second method, pyrene excimer fluorescence, measures the microviscosity-dependence of the lateral diffusion rate of the probe pyrene in the membrane plane.
- 3) Determine whether a similar pattern of tissue binding is observed for other anionic compounds (cromolyn and prostaglandins etc.). At physiological pH, suprofen exists primarily as an anionic compound, and it is hypothesized that anionic drugs in general may exhibit a similar tissue binding phenomenon.



## APPENDIX

The lag time  $t_L$ , for transport across a membrane with two layers, A and B, where binding occurs in membrane B is given by

$$t_L = t_{LO} + t_{LB}$$

where the component without binding,  $t_{LO}$ , is given by equation and  $l_B^2/6D_B$  whereas the binding component is

$$t_{LB} = \frac{Kl_B V_b}{C_0 V_f} \left[ \frac{l_A}{D_A} + \frac{Pl_B}{2D_B} - \frac{l_A}{\alpha D_B} \ln |1+\alpha| + \frac{Pl_B}{\alpha D_B} - \frac{(1+\alpha)Pl_B}{D_B \alpha^2} \ln |1+\alpha| \right] \quad (A1)$$

Where

$$\alpha = \frac{k_2 C_0}{P + \frac{l_A D_B}{l_B D_A}} \quad (A2)$$

The limit as  $C_0$  or  $\alpha$  becomes large is the result given by equation 6.

The method for obtaining the lag time is that of Frisch (1957). The application of this method for laminated membrane can be found in Crank and Park (1968) and the application for binding can be found in reference (Cooper,



1974). The result given by equation A2 was obtained by a combination of these two methods.



## REFERENCES

- Ahmed, I., Gokhale, R. D., Shah, M. V. and Patton, T. F.: Physicochemical determinants of drug diffusion across the conjunctiva, sclera, and cornea. *J. Pharm. Sci.* 76: 583-586, 1987.
- Alvarez, E., Ruiz-Grtierrez, V., Maria, C. S. and Machado, A.: Age-related modification of lipid composition and lipid structural order parameter of rat peritoneal macrophage membranes. *Mech. Aging Dev.* 71: 1-12 (1993).
- Anderson, R. E., Maude, M. B. and Feldman, G. L.: Lipids of Ocular Tissues I. The phospholipids of mature rabbit and bovine lens. *Biochim. Biophys. Acta.* 187: 345-353, 1969.
- Andrews, J. S., Leonard-Martin, T. and Kador, P. F.: Membrane lipid biosynthesis in the Philly mouse lens. I. The major phospholipid classes. *Curr. Eye Res.* 3: 279-285, 1984.
- Antonian, L., Shinitzky, M., Samuel, D. and Lipka, A. S.: AL 721, a novel membrane fluidizer. *Neuroscience & Biobehavioral Rev.* 11: 399-413, 1987.
- Barja, P. and Freire, M.: Distribution of serine esterase activity in the lymphoid system of C57BL/6 mice, effect of aging on the enzymatic activity. *Int. J. Biochem.* 25: 551-555, 1993.
- Benedetti, A., Ferretti, G., Curatola, G., Jezequel, A. M. and Orlandi, F.: Age and sex related changes of plasma membrane fluidity in isolated rat hepatocytes. *Biochem Biophys. Res. Comm.* 156: 840-845, 1988.
- Benson, H.: Permeability of the cornea to topically applied drugs. *Arch. Ophthalmol.*, 91: 313-327, 1974.
- Berman, E. R.: Lens in "Biochemistry of the Eye". Plenum Press, New York. 201-274, 1991.
- Broekhoyse, R. M.: Phospholipids in tissues of the eye III. Composition and metabolism of phospholipids in human lens in relation to age and cataract formation. *Biochim. Biophys. Acta.* 187: 354-365, 1969.



- Broekhooyse, R. M.: Lipids in tissues of the eye. IV. Influence of age and species difference on the phospholipid composition of the lens. *Biochim. Biophys. Acta.* 218: 546-548, 1970.
- Broekhooyse, R. M.: Membrane lipids and proteins in aging lens and cataract. In *The Human Lens in Relation to Cataract* Ciba Foundation Symp. (Elliot, K. and Fitzsimmons, D. K. eds.), Elsevier, New York. 135-149, 1973.
- Burstein, N. L.: Corneal Endothelial permeability to electron opaque tracers demonstrated by modified propane jet cryofixation. *J. Cell. Biol. Suppl.* 83, 300a: 1979.
- Calderini, G., Bonetti, A. C., Battistella, A., Crews, F. T. and Toffano, G.: Biochemical changes of rat brain membranes with aging. *Neurochem. Res.* 8: 483-492, 1983.
- Chapman, D.: Phase transitions and fluidity characteristics of lipids and cell membranes. *Quart. Rev. Biophys.* 8: 185-235, 1975.
- Cheng-Bennett, A. Poyer, J., Weinkam, R. J. and Woodward, D. F.: Lack of prostaglandin  $F_{2\alpha}$  metabolism by human ocular tissues. *Invest. Ophthalmol. Vis. Sci.* 31: 1389, 1990.
- Clayton, R. M., Green, K., Wilson, M., Zehir, A., Jack, J. and Searle, L.: The penetration of detergents into adult and infant eyes: possible hazards of additives to ophthalmic preparations. *Fd. Chem. Toxic.* 23: 239-246, 1985.
- Cogan, D. G.: Applied anatomy and physiology of the cornea. *Trans. Am. Acad. Ophthalmol.* 55: 329, 1951.
- Cooper, E.: Effect of adsorption of membrane diffusion. *J. Coll and Inter. Sci.* 48: 516-518, 1974.
- Cox, W. V., Kupferman, A. and Leibowitz, H. M.: Topically applied steroids in corneal disease I. The role of inflammation in stromal absorption of dexamethasone. *Arch. Ophthal.* 88: 308-313, 1972.
- Crank, J. and Park, G. S.: *Diffusion in Polymers.* Acad. Press, New York. 1968.



- Cutler, R. G.: Evolutionary biology of aging and longevity in mammalian species in "Aging and Cell Function". (Johnson, J. E. ed), Plenum Press, New York, 1-147, 1984.
- Dalmark, M. and Storm, H. H.: A Fickian diffusion transport process with features of transport catalysis. *J. Gen. Physiol.* 78: 349-364, 1981.
- Dalmark, M.: Characteristics of doxorubicin transport in human red blood cells. *Scand. J. Clin. Lab. Invest.* 41: 633-639, 1981.
- Doane, M. G., Jensen, A. D. and Dohlman, C. H.: Penetration routes of topically applied eye medications. *Am J. Ophthalmol.* 85: 383-386, 1978.
- Dobretsov, G. E., Borschevskaya, T. A., Petrov, V. A. and Vladimirov, Y. A.: The increase of phospholipid bilayer rigidity after lipid peroxidation. *FEBS Letters.* 84: 125-128, 1977.
- Eisenberg, S., Stein, Y. and Stein, O.: Phospholipases in arterial tissue. IV. The role of phosphatide acyl hydrolase, lysophosphatide acyl hydrolase, and sphingomyelin choline phosphohydrolase in the regulation of phospholipid composition in the normal human aorta with age. *J. Clin. Invest.* 48: 2320-2329, 1969.
- Erlanger, M. and Gershon, D.: Studies on aging in Nematodes. II. Studies of the activities of several enzymes as a function of age. *Expt. Gerontology*, 5: 13-19, 1970.
- Farnsworth, P. N., Burke, P. A., Wagner, B. J., Fu, S. C. J. and Reagan, T.: Diabetic cataracts in the Rhesus monkey lens. *Metab. Pediatr. Ophthalmol.*, 4: 31-42, 1980.
- Feldman G. L.: Human ocular lipids: their analysis and distribution. *Survey Ophthalmol.* 19: 207-243, 1967.
- Folch, J., Lees, M. and Sloane-Stanley, G. H.: A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* 226: 497-509, 1957.
- Friend, J.: Biochemistry of the Cornea, in: *The Cornea, Scientific Foundations and Clinical Practice*, 3rd ed. (G. Smolin and R. A. Thoft, eds.), Little, Brown, Boston, 47-68, 1994.



- Frisch, H. L.: The time lag in diffusion. *J. Phys. Chem.* 61: 93-95, 1957.
- Gastaldi, G., Laforenza, U., Ferrari, G., Casasco, A. and Rindi, G.: Age-related thiamin transport by small intestinal microvillous vesicles of rat. *Biochimica et Biophysica Acta.* 1105: 271-277, 1992.
- Geroski, D. H., Matsuda, M. and Edelhauser, H. F.: Pump function of the human corneal endothelium; effect of age and corneal guttata. *Ophthalmol.* 92: 759, 1985.
- Gipson, I. K.: Anatomy of the conjunctiva, cornea, and limbus, in: *The Cornea, Scientific Foundations and Clinical Practice*, 3rd ed. (G. Smoli and R. A. Thoft, eds). Little, Brown, Boston, 3-25, 1994.
- Gooden, M. M., Takemoto, L. J. and Rintoul, D. A.: Evidence for reduced lipid order in plasma membranes from cataractous human lenses. *Curr. Eye Res.* 2: 367-375, 1983.
- Gracy, R. W., Yuksel, K. U., Jacobson, T. M., Chapman, M. L., Hevelone, J. C., Wise, G. E. and Dimitrijevic, S. D.: Cellular models and tissue equivalent systems for evaluating the structures and significance of age-modified proteins. *Gerontology* 37: 113-27, 1991.
- Green, K. and Chapman, J.: Benzalkonium chloride kinetics in young and adult albino and pigmented rabbit eyes. *J. Toxicol.-Cut. & Ocular Toxicol.* 5: 133-142, 1986.
- Hamm, M. W., Winick, M. and Schachter, D.: Macrophage phagocytosis and membrane fluidity in mice: the effect of age and dietary protein. *Mech. Aging Dev.* 32: 11-20, 1985.
- Hanna, C., Bicknell, D. S. and O'Brien, J.: Cell turnover in the adult human eye. *Arch. Ophthalmol.* 65: 695, 1961.
- Harding, C. V., Chylack, L. T., Susan, S. R., Decker, J. G. and Lo, W. K.: Morphological changes in the cataract: the ultrastructure of human lens opacities localized by Cooperative Cataract Research Group Procedures. In "Red Blood Cell and Lens Metabolism", (ed. Srivastara, S. K.). Elsevier/North Holland, Inc., N. Y. Pp. 27-39, 1980.
- Harris, D., Liaw, J.-H. and Robinson, J. R.: (D) Routes of Delivery: Case Studies (7) Ocular delivery of peptide and protein drugs. *Adv. Drug Delivery Rev.* 8: 331-339, 1992.



- Havener, W. H.: Ocular Pharmacology. Mosby, St. Louis. 19-38, 1978.
- Hegner, D., Platt, D., Heckers, H., Schloeder, U. and Breuninger, V.: Age-dependent physicochemical and biochemical studies of human red cell membranes. Mech. Ageing Dev. 10: 117-130, 1979.
- Hegner, D.: Age-dependence of molecular and functional changes in biological membrane properties. Mech. Ageing Dev. 14: 101-118, 1980.
- Hirsch, M., Renard, G., Faure, J. P. and Poullquen, Y.: Study of the Ultrastructure of the rabbit corneal endothelium by the freeze-fracture technique: apical and lateral junctions. Exp. Eye. Res. 25: 277-288, 1977.
- Hochstein, P. and Jain, S. K.: Association of lipid peroxidation and polymerization of membrane proteins with erythrocyte aging. Fed. Proc. 40: 183-188, 1981.
- Hogan, J. A.; Alvarado, J. A. and Weddell, J. E. Histology of the Human Eye. W. B. Saunders. 1971.
- Huang, H. S. Schoenwald, R. D. and Lach, J. L.: Corneal penetration behavior of beta-blocking agents II: assessment of barrier contributions. J. Pharm. Sci. 72: 1272-1279, 1983.
- Huber, L. A., Xu, Q.-B., Jurgens, G., Bock, G., Buhler, E., Grey, K. F., Schonitzer, D., Traill, K. N. and Wick, G.: Correlation of lymphocyte lipid composition membrane microviscosity and mitogen response in the aged. Eur. J. Immunol. 21: 2761-2765, 1991.
- Hull, D. S., Hine, J. E., Edehauser, H. F. and Hyndiuk, R. A.: Permeability of the isolated rabbit cornea to corticosteroids. Inv. Ophthalmol. 13: 457-459, 1974.
- Igarashi, H., Sato, Y., Hamada, S. and Kawasaki, T.: Studies on rabbit corneal permeability of local anaesthetics. Jap. J. Pharmacology. 34: 429-434, 1984.
- Janki, R. M., Aithal, H. N., Tustanoff, E. R. and Bull, A.: The biogenesis of mitochondrial membranes in the yeast saccharomyces cerevisiae. Biochim. Biophys. Acta. 375: 446-461, 1975.



- Kashi, S. D. and Lee, V. H. L.: Hydrolysis of enkephalins in homogenates of anterior segment tissues of the albino rabbit eye. *Inv. Ophthalmol. Vis. Sci.* 27: 1300-1303, 1986.
- Kaye, G. I., Sibley, R. C., and Hoefle, F. B.: Recent studies on the nature and function of the corneal endothelial barrier. *Exp. Eye Res.* 15: 585-613, 1973.
- Keelan, M., Walker, K. and Thomson, A. B. R.: Intestinal morphology, marker enzymes and lipid content of brush border membranes from rabbit jejunum and ileum: effect of aging. *Mech. Aging Dev.* 31: 49-68, 1985.
- Kessler, A. R., Kessler, B. and Yehuda, S.: Changes in cholesterol level and membrane lipid microviscosity in rat brain induced by age and a plant oil mixture. *Biochem. Pharmacol.* 34: 1120-1124, 1985.
- Klyce, S. D.: Electrical profiles in the corneal epithelium. *J. Physiol.* 226: 407, 1977.
- Klyce, S. D. and Crosson, C. E.: Transport processes across the rabbit corneal epithelium: a review. *Curr. Eye Res.* 4: 323-331, 1985.
- Kupferman, A. and Leibowitz, H. M.: Anti-inflammatory effectiveness of topically administered corticosteroids in the cornea without epithelium. *Invest. Ophthalmol.* 14: 252, 1975.
- Lee, A. G.: Functional properties of biological membranes: a physical-chemical approach. *Prog. Biophys. Mol. Biol.* 29: 3-56, 1975.
- Lee, R. E. and Davison, P. F.: Collagen composition and turnover in ocular tissues of rabbit. *Exp. Eye Res.* 32: 737-745, 1981.
- Lee, V. H. L., Carson, W. and Takemoto, K. A.: Macromolecular drug absorption in the albino rabbit eye. *Int. J. Pharm.* 29: 43-51, 1986.
- Lee, V. H. L. and Li, H. K.: Prodrugs for improved ocular drug delivery. *Adv. Drug. Deliv. Rev.* 3: 1-38, 1989.
- Lee, V. H. L., Stratford, R. E. and Morimoto, K. W.: Age-related changes in esterase activity in rabbit eyes. *Int. J. of Pharm.* 13: 183-195, 1983.



- Lee, V. H. L. and Robinson, J. R.: Topical ocular drug delivery: Recent developments and future challenges. *J. Ocular Pharm.* 2: 67-108, 1986.
- Leibwitz, H. M., Ryan, W. J., Kupferman, A and DeSantis, L.: Bioavailability and corneal anti-inflammatory effect of topical suprofen. *Invest. Ophthalmol. Vis. Sci.* 27: 628-631, 1986.
- Lepagnol, J. M. and Heidet, V.: Comparative effects of aging process on phosphatidylcholine biosynthesis pathway: a key role for CTP-phosphocholine cytidyltransferase?. *Annals of the New York Academy of Science* 695: 86-90, 1993.
- Levin, G., Cogan, U. and Mokady, S.: Food restriction and membrane fluidity. *Mech. Aging and Dev.* 62: 137-141, 1992.
- Li, L-K. and So. L.: Age dependent lipid and protein changes in individual bovine lenses. *Curr. Eye Res.* 6: 599-605, 1987.
- Loh, H. H. and Law, P. Y.: The role of membrane lipids in receptor mechanism. *Ann. Rev. Pharmacol. Toxicol.* 20: 201-234, 1980.
- Malik, N. S., Moss, S. J., Furth, A. J., Wall, R. S., and Meek, K. M.: Aging of the human corneal stroma: structural and biochemical changes. *Biochimica et Biophysica Acta* 1138: 222-8, 1992.
- Marin, M. S., Fernandez, A., Sanchez-Yague, J., Cabezas, J. A., and Llanillo, M.: Changes in the phospholipid and fatty acid composition in normal erythrocytes from sheep of different ages. *Aminophospholipid organization in the membrane bilayer. Biochimie* 72: 745-750, 1990.
- Maurice, D. M.: The cornea and sclera, in: *The Eye*, 3rd ed. (Davson, H. ed.), Academic Press, New York. 1-158 1984.
- Maurice, D. M., and Mishima, S.: Ocular pharmacokinetics. In "Pharmacology of the Eye. Handbook of Experimental Pharmacology". (Sears, M. L. ed), Springer-Verlag, Berlin. 19-116, 1984.
- Mindel, J. S., Smith, H., Jacobs, M., Kharlamb, A. B. and Friedman, A. H.: Drug reservoirs in topical therapy. *Invest. Ophthalmol. Vis. Sci.* 25: 346-350, 1984.



- Miller, S. C. and Patton, T. F.: Age-related difference in ophthalmic drug disposition I. Effect of size on the intraocular tissue distribution of pilocarpine in albino rabbits. *Biopharmaceutics & Drug Disposition* 2: 215-233, 1981.
- Miyamoto, A., Araiso, T., Koyama, T., and Ohshika, H.: Membrane viscosity correlates with alpha 1-adrenergic signal transduction of the aged rat cerebral cortex. *J. Neurochem.* 55: 70-75, 1990.
- Murawski, U., Baumgarten, H. and Koch, H.-R.: Lipids of the rat lens during the early postnatal phase. In "Aging of the lens". (ed. Regnault, F., Hockwin, O. and Courtois, Y.). Elsevier/North Holland, Inc. N. Y. Pp. 109-119, 1980.
- O'Brien, P. J.: Oxidation of lipids in Biological Membranes and Intracellular Consequences in Autoxidation of Unsaturated lipids (Chan, H.W.-S. ed), Academic Press, London. 233-280, 1987.
- Patton, G. M., Fasulo, J. M. and Robins, S.: Analysis of lipids by high performance liquid chromatography: Part I. *J. Nutr. Biochem.* 1: 493-500, 1990.
- Pepose, J. S. and Ubels, J. L.: The Cornea. In "Adler's Physiology of the Eye". (Hart, W. M. ed), Mosby, St. Louis. 63-64, 1992.
- Pieri, C., Falasca, M., Moroni, F., Recchioni, R. and Marcheselli, F.: Diet restriction, body temperature and physicochemical properties of cell membranes. *Arch. Gerontol. Geriatr.* 12: 179-186, 1991.
- Prisco, D., Rogasi, P. G., Paniccia, R., Abbate, R. and Gensini, G. F.: Age-related changes in red blood cell lipids. *Angiology* 42: 316-322, 1991.
- Provinciali, M., Fabris, N. and Pieri, C.: Improvement of natural killer cell activity by in vitro active lipids (AL 721) administration in old mice. *Mech. aging and Dev.* 52: 245-254, 1990.
- Rabinowich, H., Lyte, M., Steiner, Z. and Klajman, A.: Augmentation of mitogen responsiveness in the aged by a special lipid diet AL 721. *Mech. Aging and Dev.* 40: 131-138, 1987.
- Riley, M. V.: Transport of ions and metabolites across the corneal endothelium. In "Cell Biology of the Eye". (McDevitt, D.S. ed), Academic, New York. 53-95, 1982.



- Roth, G. S.: Hormone receptor changes during adulthood and senescence: Significance for aging research. *Fed. Proc.* 38: 1919-1914, 1979.
- Schinitzky, M.: Membrane fluidity and cellular functions. in "Physiology of membrane fluidity". (Schinitzky, M. ed), CRC Press, Boca Raton. 1: 1-51, 1984.
- Schinitzky, M. and Henkart, P.: Fluidity of cell membranes-current concepts and trends. *Int Rev. Cytol.* 60: 121-147, 1979.
- Schoenwald, R. and Huang, H.: Corneal penetration behavior of  $\beta$ -blocking agents I: Physicochemical factors. *J. Pharm. Sci.* 72: 1266-1272, 1983.
- Schroeder, F., Goetz, I. and Roberts, E.: Sex and age alter plasma membranes of cultured fibroblasts. *Eur. J. Biochem.* 142: 183-191, 1984.
- Schroeder, F.: Role of membrane lipid asymmetry in aging. *Neurobiology of Aging.* 5: 323-333, 1984.
- Sell, D. R. and Monnier, V. M.: Isolation, purification and partial characterization of novel fluorophores from aging human insoluble collagen-rich tissue. *Connective Tissue Res.* 19: 77-92, 1989.
- Shi, B. and Tien, H. T.: Action of calcium channel and beta-adrenergic blocking agents in bilayer lipid membranes. *Biochim. Biophys. Acta.* 859: 125-134 (1986).
- Shichi, H. and Nebert, D. W.: Drug metabolism in ocular tissues, in "Extrahepatic Metabolism of Drugs and Other Foreign Compounds " (Gram, T. E. ed.) Spectrum, New York. p. 333, 1980.
- Sieg, J. W. and Robinson, J. R.: Mechanistic studies on transcorneal permeation of pilocarpine. *J. Pharm. Sci.* 65: 1816-1822, 1976.
- Stratford, R. E. and Lee, V. H. L.: Ocular aminopeptidase activity and distribution in the albino rabbit. *Curr. Eye Res.* 4: 995-999, 1985.
- Stryer, L. *Biochemistry.* 3rd edition. W. H. Freeman, NY. 1988.
- Svhinitzky, M and Barenholz, Y.: Dynamics of the hydrocarbon layer in liposomes of lecithin and sphingomyelin containing deacetylphosphate. *J. Bio. Chem.* 249: 2652-2657, 1974.



- Tang-Liu, D. D-S. Liu, S. S. and Weinkancy, R. J.: Ocular and systemic bioavailability of ophthalmic flurbiprofen. *J. Pharmacokinetics and Biopharm.* 12: 611-626, 1984.
- Tozzi-Ciancarelli, M. G., Dalfonso, A., Tozzi, E., Troiani-Semi, E. and de Matteis, G.: Fluorescence studies of the aged erythrocyte membrane. *Cell. Mol. Biol.* 35: 113-118, 1989.
- Van Blitterswijk, W. J., Van Hoeven, R. P. and Van Der Meer, B W.: Lipid structural order parameters (reciprocal of fluidity) in biomembranes derived from steady state fluorescence depolarization measurements. *Biochim. Biophys. Acta* 644: 323-332, 1981.
- Wahnon, R., Mokady, S. and Cogan, U.: Age and membrane fluidity. *Mech. Aging. Dev.* 50: 249-255, 1989.
- Wang, W., Sasaki, H., Chien, D. S. and Lee, V. H. L.: Lipophilicity influence on conjunctival drug penetration in the pigment rabbit: A comparison with corneal penetration. *Curr. Eye Res.* 10: 571-579, 1991.
- Watkin, D. M.: The physiology of aging. *Am. J. Clin. Nutr.* 36: 750-758, 1982.
- Watson, K., Houghton, R. L., Bertoli, E. and Griffiths, D.: Membrane-lipid unsaturation and mitochondrial function in *Saccharomyces cerevisiae*. *E. Biochem. J.* 146: 409-416, 1981.
- Wigham, C. G. and Hodson, S. A.: Physiological changes in the cornea of the ageing eye. *Eye* 1: 190-196, 1987.
- Wolf, G.: Lipofuscin, the age pigment. *Nutrition Reviews* 51: 205-206, 1993.
- Yegutkin, G. G., Sambursky, S. S., Zhitkovitch, A. V., and Gatsko, G. G.: Evaluation of age-related changes of physicochemical properties and functional activity of rat adipose plasma membranes and their possible relationship. *Mech. Aging Dev.* 59: 1-16, 1991.
- Young, R. G.: Sunlight and age-related eye disease. *J. Natl. Med. Assn.* 84: 353-358, 1992.
- Yu, B. P., Suescun, E. A. and Yang, S. Y.: Effect of age-related lipid peroxidation on membrane fluidity and phospholipase A<sub>2</sub>: Modulation by dietary restriction. *Mech. Aging Dev.* 65: 17-33, 1992.



Yue, B. J. T. and Baum, J. Studies of cornea in vivo and in vitro. *Vis. Res.* 21: 41-43, 1981.

Zelenka, P.: Phospholipid composition and metabolism in the embryonic chick lens. *Exp. Eye Res.* 26: 267-74, 1978.

Zelenka, P.: Lens lipids. *Curr. Eye Res.* 3: 1337-1359, 1984.

Zimmermann, D. R., Trueb, B., Winterhalter, K. H., Witmer, R., and Fischer, R. W.: Type VI collagen is a major component of the human cornea. *FEBS Lett.* 197: 55-58, 1986.











HECKMAN  
BINDERY INC.



**AUG 95**

Bound-To-Pleas® N. MANCHESTER,  
INDIANA 46962

