The ocular crystalline lens is a transparent tissue that by means of altering its shape provides the ability for visible light to be transmitted unimpeded into the eye and focused onto the retina for proper visual sensation. The lens is an encapsulated structure consisting almost entirely of a large number of rigid, elongated cells known as lens fibers or fiber cells. These cells are produced via the terminal differentiation of a monolayer of epithelial cells located just beneath the anterior lens capsule (1). This process begins early in embryogenesis and continues throughout life, resulting in the deposition of one layer of fiber cells upon another. As new layers of fiber cells are produced, a region known as the lens nucleus; fiber cells peripheral to the nucleus, including the newly formed and metabolically active fiber cells, define the lens cortex.

During their progressive displacement toward the lens nucleus, fiber cells lose all subcellular organelles that would scatter light and thus impair vision (2). Consequently, plasma membrane becomes essentially the only organelle of the lens (1). The fiber cell plasma membrane is unique in that it contains only trace amounts of polyunsaturated fatty acid (3), very high concentrations of a 26-kDa water channel protein known as main intrinsic protein (MIP or MP26) (4), and, in the human lens, a phospholipid composition of over 50% sphingomyelin and sphingomyelin derivatives (5, 6). In addition, human lens fiber cell plasma membrane contains a relative concentration of cholesterol that is the highest found in nature (7). The cholesterol to phospholipid (C/P) mole ratio ranges from 1 to 2 in the cortex to 3 to 4 in the nucleus (8, 9). In contrast, plasma membranes of typical eukaryotic cells have C/P mole ratios between 0.5 and 1.0 (10).

Despite an advanced understanding of the lipid composition of the human ocular lens, there is little information about the structural organization of the lens plasma membrane. How does a membrane accommodate 3 to 4 cholesterol molecules per one phospholipid? The presence of cholesterol at such extremely high relative concentrations in lens membrane has led to the proposal that the fiber cell plasma membrane is “a mosaic of phospholipid bilayer and cholesterol patches” (8). A number of independent studies employing other biological membrane systems give credence to this possibility. Using model membrane systems, it has been determined that cholesterol tends to aggregate into clusters at C/P mole ratios in excess of 0.3 (11), and C/P mole ratios greater than 1.0 (i.e. 50 mole % sterol) can yield pure cholesterol phases (12). In well defined lipid monolayer systems, the addition of cholesterol produces lateral sterol domains, as characterized by microcopy approaches (13–15). The formation of distinct cholesterol domains has also been observed in various membrane bilayer systems. Using small angle x-ray diffraction, it has been shown that increasing the relative cholesterol content to 50% of total phospholipid in model membrane bilayers produces an immiscible cholesterol monohydrate phase with a unit cell periodicity of 34.0 Å, consistent with a cholesterol monohydrate bilayer. The dimensions of the sterol-rich domains remained constant over a broad range of temperatures (5–20 °C) and relative humidity levels (31–97%). In contrast, dimensions of the surrounding sterol-poor phase were significantly affected by experimental conditions. Similar structural features were observed in membranes reconstituted from fiber cell plasma membrane lipid extracts. The results of this study indicate that the lens fiber cell plasma membrane is a complex structure consisting of separate sterol-rich and -poor domains. Maintenance of these separate domains may be required for the normal function of lens fiber cell plasma membrane and may interfere with the cataractogenic aggregation of soluble lens proteins at the membrane surface.

Fiber cell layers that are compacted into the center of the lens during embryonic development and through adulthood comprise a region known as the lens nucleus; fiber cells peripheral to the nucleus, including the newly formed and metabolically active fiber cells, define the lens cortex.

The molecular structure of human ocular lens fiber cell plasma membranes was examined directly using small angle x-ray diffraction approaches. A distinct biochemical feature of these membranes is their high relative levels of free cholesterol; the mole ratio of cholesterol to phospholipid (C/P) measured in these membranes ranges from 1 to 4. The organization of cholesterol in this membrane system is not well understood, however. In this study, the structure of plasma membrane samples isolated from nuclear (3.3 C/P) and cortical (2.4 C/P) regions of human lenses was examined using x-ray diffraction approaches. Meridional diffraction patterns obtained from the oriented membrane samples demonstrated the presence of an immiscible cholesterol domain with a unit cell periodicity of 34.0 Å, consistent with a cholesterol monohydrate bilayer. The dimensions of the sterol-rich domains remained constant over a broad range of temperatures (5–20 °C) and relative humidity levels (31–97%). In contrast, dimensions of the surrounding sterol-poor phase were significantly affected by experimental conditions. Similar structural features were observed in membranes reconstituted from fiber cell plasma membrane lipid extracts. The results of this study indicate that the lens fiber cell plasma membrane is a complex structure consisting of separate sterol-rich and -poor domains. Maintenance of these separate domains may be required for the normal function of lens fiber cell plasma membrane and may interfere with the cataractogenic aggregation of soluble lens proteins at the membrane surface.

The abbreviations used are: C/P, mole ratio of cholesterol to phospholipid; RH, relative humidity.

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plasma membranes under diseased, atherosclerotic conditions, a process characterized by an excessive accumulation of cholesterol in the vascular wall (18). In these diseased membranes, the C/P mole ratio approached 1.0, a level that is 3-fold higher than in normal membranes. Small angle x-ray diffraction analysis of these membranes provided evidence for the formation of membrane-restricted cholesterol domains in vivo under pathologic conditions.

In the present study, small angle x-ray diffraction approaches were used to characterize the structural organization of cholesterol within fiber cell plasma membranes isolated from human ocular lenses. Immiscible cholesterol monohydrate domains were present in lens cortical and nuclear membrane samples, and these domains remained stable over a broad range of temperatures and relative humidity levels. Additionally, membrane protein content did not affect the presence or molecular dimensions of the observed cholesterol domains. The structurally distinct cholesterol phase was found to be restricted within the surrounding sterol-poor membrane bilayer phase with dimensions that were highly affected by temperature, humidity, and protein content. These results provide direct evidence for the existence of immiscible cholesterol monohydrate domains in human ocular lens fiber cell plasma membranes under physiologic-like conditions. The structural stability of these domains suggests a well ordered membrane system that must be conserved to maintain lens transparency to visible light.

EXPERIMENTAL PROCEDURES

**Isolation of Human Lens Plasma Membrane and Lipid**—Six normal human lenses were obtained from the National Disease Research Interchange (Philadelphia). The ages of the lens sample donors ranged from 65 to 77 years. Lenses were decapitated with the concomitant removal of lens epithelial cells that adhere to the capsule. The decapsulated lenses were placed in a 20-cm² culture dish containing 8 ml of 5 mM Tris-HCl (pH 7.4), 5 mM EDTA, 10 µM β-mercaptoethanol (buffer A) and stirred on a rotatory mixer at 100 rpm for 2 h. Under these gentle stirring conditions, the lens cortex separated from the nucleus as clumps of fiber cells. Based on lens weight before and after fractionation, the removed cortex accounted for 48% of total lens volume.

Cortical and nuclear lens regions were separately homogenized in 8 ml of buffer A using a glass Dounce homogenizer. Plasma membranes were isolated using the methods described by Russell, et al. (19). Briefly, the homogenates were centrifuged at 10,000 × g for 20 min. The pellets were washed twice with buffer A, extracted twice with 7 M urea and stirred on a rotatory mixer at 100 rpm for 2 h. Under these gentle stirring conditions, the lens cortex separated from the nucleus as clumps of fiber cells. Based on lens weight before and after fractionation, the removed cortex accounted for 48% of total lens volume.

**Preparation of Oriented Intact Lens Plasma Membrane Samples for X-ray Diffraction**—Intact lens plasma membrane samples were oriented for x-ray diffraction analysis as described previously (24). Briefly, 200 µg (phospholipid) of plasma membrane samples (in buffer B) were loaded into Lucite sedimentation cells. Each sedimentation cell contained an aluminum foil substrate upon which the membrane pellets were collected. The membrane samples were centrifuged in a Sorvall AH-65 ultracentrifuge at 35,000 rpm for 50 min at 5 °C. Samples were washed three times with buffer (0.5 mM HEPES, 150 mM NaCl, pH 7.3). After the final washing cycle, the supernatants were removed, and the aluminum foil substrates, containing the membrane pellets, were removed from the sedimentation cells and mounted on curved glass supports. The samples were then placed in hermetically sealed brass canisters in which temperature and relative humidity were controlled during x-ray diffraction experiments.

**Preparation of Oriented Lens Membrane Lipid Samples for X-ray Diffraction**—Aliquots of lens plasma membrane total lipid, initially solubilized in chloroform (as described above), were added repeatedly to 13 × 100 mm glass test tubes to yield 230 µg of phospholipid. The samples were then dried down under a steady stream of nitrogen gas to the sides and bottom of the test tubes while vortex mixing. Residual solvent was removed under vacuum. A volume of diffusion buffer was added to each test tube to yield a final phospholipid concentration of 0.38 mg/ml. Multilamellar vesicles were formed by vortex mixing the buffer and lens membrane lipids for 3 min at ambient temperature. Volumes yielding the equivalent of 200 µg of phospholipid for each sample were loaded into sedimentation cells. Oriented membrane multilayers were prepared by centrifugation, as described above for the intact membrane samples.

**Small Angle X-ray Diffraction Analysis**—The oriented lens plasma membrane samples were aligned at grazing incidence with respect to a collimated, monochromatic x-ray beam (Cu Kα = 1.54 Å) produced by a Rigaku RotaFlex RU-200, a high brilliance rotating anode microfocus generator (Rigaku USA, Danvers, MA). The fixed geometry beamline utilized a single Frank's mirror providing nickel-filtered radiation (Kα, Kβ, unresolved) at the detection plane. Diffraction data were collected on a one-dimensional, position-sensitive electronic detector (Inel, Newburyport, MA), the calibration of which was verified using cholesterol monohydrate crystals. The sample-to-detector distance used in these experiments was 150 mm. Representative three-dimensional diffraction patterns for each sample were also collected on a two-dimensional PhosphorImager system (Molecular Dynamics, Sunnyvale, CA) at a sample-to-detector distance of 70 mm.

The unit cell periodicity, or d-space, of the membrane lipid bilayer is the measured distance from the center of one lipid bilayer to the next, including surface hydration. The d-spaces for the membrane multilayer samples were calculated using Bragg’s Law,

\[ h \lambda = 2d \sin \theta \]  

in which h is the diffraction order number, \( \lambda \) is the wavelength of the x-ray radiation (1.54 Å), d is the membrane lipid bilayer unit cell periodicity, and θ is the Bragg angle equal to one-half the angle between the incident beam and scattered beam.

Saturated salt solutions were used to define the relative humidity (RH) levels employed in these x-ray diffraction analyses. The following salt solutions (with associated RH in parentheses) were used in these experiments: MgCl₂6H₂O (33%), Mg(NO₃)₂6H₂O (52%), K₂C₂H₄O₆·H₂O (74%), (NH₄)₂SO₄ (79%), Na₂Cr₂O₇·2H₂O (87%), Na₂C₂H₄O₂·2H₂O (92%), NH₄HPO₄ (93%), K₂SO₄ (97%).

RESULTS

**Analysis of Human Lens Fiber Cell Plasma Membrane Structure**—Small angle x-ray diffraction approaches were used to characterize directly the structural organization of plasma membranes isolated from human ocular lens fiber cells in the presence and absence of membrane protein. Representative x-ray diffraction profiles generated from oriented fiber cell plasma membranes at 20 °C, 92% RH are shown in Fig. 1. All samples yielded meridional diffraction patterns consistent with two structurally distinct membrane domains or phases: a sterol-poor liquid crystalline membrane bilayer phase, corresponding to diffraction orders 1 and 2, and an immiscible cholesterol monohydrate domain, defined by diffraction orders 1' and 2' . Calculation of d-space values corresponding to these diffraction orders revealed distinct structural features of these separate lipid domains. The width of the sterol-poor membrane bilayer region varied with each sample, from intact plasma membranes isolated from the cortex and nucleus (Fig. 1, A and C, respectively) yielding d-space values of 80.6 and 88.8 Å, respectively. In the absence of membrane protein (reconstituted membrane samples), the cortical and nuclear membrane lipid bilayer d-space was 79.1 Å (Fig. 1, B and D, respectively). In contrast, the d-space for the cholesterol domain remained un-
Effects of Temperature on Membrane Structure—Intact and reconstituted cortical and nuclear plasma membranes were examined over a temperature range of 5 to 40 °C (Fig. 3). Diffraction peaks corresponding to an immiscible cholesterol domain were observed at every temperature level. The calculated width of the cholesterol domains was unaffected by temperature changes, with a reproducible d-space of 34.0 Å. In contrast, the surrounding lipid membrane bilayer phase was significantly affected by sample temperature. Consistent with a disordering effect with increasing temperature, elevating the temperature from 5 °C to 40 °C caused an overall decrease in membrane unit cell periodicity. Intact cortical and nuclear plasma membrane bilayer d-spaces decreased by 5.2 Å (7%) and 3.3 Å (5%), respectively.

Effects of Relative Humidity on Membrane Structure—As observed for temperature, changes in relative humidity did not affect the presence of cholesterol monohydrate phases in each of the samples tested (Fig. 4). Over a range of 33 to 97% RH, the cholesterol domains remained highly organized and stable with a consistent d-space value of 34.0 Å. However, the surrounding membrane bilayer phase for each sample was significantly altered as a function of relative humidity. With increasing relative humidity, d-space values increased by 28.2 Å (54%) and 30.9 Å (60%) for intact and reconstituted lens cortical plasma membrane bilayer phases, respectively. Over the same relative humidity range, nuclear sample d-space values increased by 40.1 Å (79%) and 24.0 Å (44%) for intact and reconstituted samples, respectively.

**DISCUSSION**

Cholesterol is asymmetrically distributed within typical eukaryotic cells, and it is estimated that more than 90% of cellular cholesterol associates with the plasma membrane (25). This estimate is probably low for lens fiber cell plasma membranes, however, because mature fiber cells lack internal organelles, particularly in the lens nucleus (8). Plasma membrane accounts for only ~1% of total lens volume (26, 27), and surface area calculations have indicated that phospholipid accounts for only ~1/3 of the plasma lens membrane (8). Because essentially all lens cholesterol is confined to this small portion of the total lens volume, the C/P mole ratios for native lens membranes are extremely high. In these studies, the C/P mole ratios ranged from 2.42 to 3.27 (Table I), consistent with previous reports (8, 9). These ratios are significantly greater than the C/P values of 0.5–1.0 reported for the typical cell membrane (10).

It is well established that C/P mole ratios in excess of 1 (i.e. 50 mole % sterol) can promote the formation of separate cholesterol domains in the cell membrane (12). Numerous theoretical and model monolayer and bilayer studies have demon-

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**FIG. 1.** Representative x-ray diffraction patterns obtained from oriented human ocular lens fiber cell plasma membrane samples. Data were collected on a one-dimensional, position-sensitive electronic detector at 20 °C and 92% RH. Typical diffraction profiles were generated from intact plasma membrane samples (A) and reconstituted lipid membrane samples (B) isolated from the lens cortex and from intact plasma membrane samples (C) and reconstituted lipid membrane samples (D) isolated from the lens nucleus. In each panel, diffraction peaks labeled as 1' and 2' correspond to immiscible cholesterol domains (periodicity of 34.0 Å); peaks labeled as 1 and 2 correspond to the surrounding membrane lipid bilayer phase.

**FIG. 2.** Two-dimensional diffraction patterns obtained from oriented human ocular lens fiber cell plasma membrane samples. Data were collected at 40 °C, 74% RH. Meridional diffraction patterns were generated from intact plasma membrane samples (A) and reconstituted lipid membrane samples (B) isolated from the lens cortex. Diffraction bands are labeled as for Fig. 1.
strated that the systematic addition of cholesterol to biological membranes can eventually induce lateral phase separation, forming membrane-restricted immiscible sterol domains (11–16, 18, 28). Additionally, recent small angle x-ray diffraction studies in our laboratory have provided direct evidence for the formation of separate, membrane-restricted cholesterol domains in vascular smooth muscle cell plasma membranes isolated from animals modeling atherosclerosis, a process characterized by an excessive accumulation of cholesterol in the vascular wall (18). In these diseased membranes, the C/P mole ratio approached 1.0, a level 3-fold higher than in normal membranes (18). These studies confirm the physicochemical propensity of cholesterol to form separate membrane phases at high relative concentrations. Because the C/P mole ratios of ocular lens fiber cell plasma membranes are extremely high, it has been proposed that these membrane bilayers are a mosaic of cholesterol-rich and cholesterol-poor regions (8); however, direct evidence for the existence of separate cholesterol domains in lens fiber cell plasma membrane has not been previously provided.

Immediately evident from the results of this study is the presence of two structurally distinct membrane domains within the lens fiber cell plasma membrane: a sterol-poor liquid crystalline membrane bilayer phase and an immiscible sterol-rich monohydrate bilayer phase. A striking feature of the cholesterol monohydrate phase is its stability over a broad range of temperatures and relative humidity levels. In contrast, the sterol-poor liquid crystalline membrane bilayer phase was significantly influenced by temperature and humidity. The biochemical basis for these changes in membrane width may be attributed to the complex head group and acyl chain composition of the lens fiber cell plasma membrane. This question is being systematically evaluated in a separate study.

The high levels of cholesterol present in these membrane samples may contribute to the stability of the lateral chole-

### Table I

| Composition of lens membrane samples | Protein | Cholesterol | Phospholipid | C/P |
|-------------------------------------|---------|-------------|--------------|-----|
|                                     | µg/µl   | µg/µl       | µg/µl        |     |
| Intact lens membrane                |         |             |              |     |
| Cortex                              | 3.07    | 1.23        | 1.00         | 2.42|
| Nucleus                             | 4.80    | 1.66        | 1.00         | 3.26|
| Reconstituted lens membrane         |         |             |              |     |
| Cortex                              | 0.263   | 0.214       | 2.43         |     |
| Nucleus                             | 0.301   | 0.181       | 3.27         |     |

![Fig. 3. Effects of temperature on lens fiber cell plasma membrane structure.](image1)

![Fig. 4. Effects of relative humidity on lens fiber cell plasma membrane structure.](image2)
terol phases. Cholesterol is present at levels 2.5–3.5-fold higher than required to produce sterol domains in previous studies (18). In addition, the phospholipid constitution of the surrounding membrane bilayer may promote cholesterol domain stability. Sphingomyelin and its derivative, 4,5-dihydrophosphorylcholine, are the most abundant phospholipids in human ocular lens, accounting for more than 50% of total phospholipid (5). Cholesterol is known to have favorable molecular interactions with sphingomyelin (29–31), exhibiting greater affinity for sphingomyelin-enriched plasma membranes than for other cellular membranes (32, 33). Although the mechanisms responsible for the preferential interaction of cholesterol with sphingomyelin are not fully understood, it is believed that an increased probability of van der Waal’s forces may contribute to the strength of their interaction (14, 30, 34). It should be pointed out that sphingomyelin is not required for the formation of cholesterol domains since domains have been observed in systems composed exclusively of other lipids (28), including dimyristoylphosphatidylcholine (35), dipalmitoylphosphatidylcholine (13), N-palmitoylglactosylphosphingosine (16), and dimyristoylphosphatidylserine (28). However, recent experiments conducted in our laboratory suggest that cholesterol domains form more readily in cholesterol/sphingomyelin binary mixtures and exhibit stability characteristics similar to that of cholesterol domains formed in the lens fiber cell plasma membrane (unpublished data). Slottes (14) has published data suggesting that the high-affinity interactions of cholesterol with sphingomyelin may reduce the free energy needed to form the critical nuclei for the growth of cholesterol domains in cholesterol/sphingomyelin monolayers. In addition, the lateral surface pressure required to abolish lateral phase boundaries of cholesterol-rich domains appears to be significantly lower for cholesterol/dipalmitoylphosphatidylcholine monolayers than for mixtures of cholesterol/sphingomyelin (14). These data suggest that the sphingomyelin-rich lens fiber cell membrane provides the ideal lipid milieu for forming very stable cholesterol domains. It is also interesting to note that nuclear lens membranes contain greater amounts of sphingomyelin (36) and saturated fatty acids (9) than do cortical membranes. This observation would suggest that cholesterol domains occur more readily in the lens nucleus, possibly explaining the fact that the diffraction peaks corresponding to the cholesterol domains in the intact nuclear samples were more well defined than in the intact cortical samples (compare Fig. 1, A and C).

Protein content did not affect the presence of immiscible cholesterol domains within the lens fiber cell plasma membrane. However, the intensity of the cholesterol diffraction peaks was greater in the absence of membrane protein (reconstituted samples), which may be due to partial protein interference with diffraction of the cholesterol domains. It is also clear that the formation of cholesterol domains does not require lens membrane protein because cholesterol domains were present in reconstituted samples. Thus, lateral cholesterol domains occur within the phospholipid bilayer regions of the lens fiber cell plasma membrane.

These data support a model that is consistent with the existence of separate cholesterol monohydrate bilayers within the plane of the cell membrane (Fig. 5). Individual cholesterol molecules appear to align in a tail-to-tail fashion, as described previously in model bilayers and atherosclerotic vascular smooth muscle cell membranes (18, 37). X-ray crystallography approaches have determined that the long-axis dimension of an individual cholesterol monohydrate molecule is 17 Å (17); thus, a tail-to-tail orientation in the cholesterol bilayer yields a periodicity of 34.0 Å. The formation of cholesterol domains appears to be supported by direct interaction of cholesterol with the acyl chains of surrounding membrane phospholipids, independent of molecular interaction with membrane protein. This conclusion is supported by the observation of distinct cholesterol phases in bilayers reconstituted solely from lens fiber cell plasma membrane lipid.

The functional significance of cholesterol domains within the ocular lens fiber cell plasma membrane is not completely understood. However, the essential function of the lens fiber cell plasma membrane is to maintain lens transparency to visible light throughout life, and the unusually high membrane concentrations of cholesterol appear to be critical for supporting this function. Using infrared spectroscopy approaches, it has been determined that the progressive increase in membrane cholesterol concentration moving from the lens cortex toward the lens nucleus is necessary to buffer the structural order of these two regions to similar fluidity levels (38). This membrane ordering effect of cholesterol may be essential to maintaining lens transparency and is achieved only by significantly higher concentrations of cholesterol in nuclear versus cortical membranes. In addition to containing the highest relative levels of membrane cholesterol, the lens also contains high concentrations of soluble protein, known as lens crystallins. Association of crystallin, primarily α-crystallin, with the lens membrane has been shown to accompany the development of human and experimental animal cataracts (39–41). This association may be promoted by reductions in membrane cholesterol as the inhibition of cholesterol biosynthesis in the lens has been shown to induce the development of cataracts in rats (42), dogs (43), and humans (44, 45). Maintenance of high membrane concentrations of cholesterol may attenuate the interaction of α-crystallin with the lens fiber cell membrane (46), but the mechanism is not understood. Based on the findings from this study, it is proposed that the formation of separate sterol-rich and -poor domains may interfere with the ability of extrinsic proteins to aggregate at the membrane surface. This hypothesis is currently being investigated in our laboratory and may lead to new insights into the effects of fiber cell plasma membrane lipid organization on cataract formation.

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**Fig. 5. Schematic model of the proposed lipid organization of the human ocular lens fiber cell plasma membrane.** The lens fiber cell plasma membrane is characterized by separate cholesterol domains with a width of 34.0 Å surrounded by a sterol-poor liquid crystalline lipid membrane bilayer.

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