Regional distribution of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity and protein mass in the ocular lens

Honglan Shi and Richard J. Cenedella[1]

Department of Biochemistry, Kirksville College of Osteopathic Medicine, 800 West Jefferson, Kirksville, MO 63501

Abstract We have attempted to map the regional distribution of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) enzyme activity and protein mass along the radial axis of the ocular lens. Because lens plasma membrane is extremely rich in cholesterol and the lens must synthesize this cholesterol, the activity of HMGR could be a key factor controlling lens membrane formation. Lenses of young rats were divided into discrete fractions by gradual dissolution in a tergitol-containing buffer; each fraction was then equated to a specific arc of the radius based upon its protein content. Aliquots of each fraction were assayed for HMGR enzyme activity and protein mass. Relative protein mass was quantitatively estimated by Western blotting using a monoclonal antibody to HMGR with immunoreactivity detected by enhanced chemiluminescence. Lens HMGR possessed a molecular mass of about 97 kDa and localized in the cell's insoluble fraction. Peak levels of both HMGR enzyme activity and protein mass were found in the outer 5% of the lens radius; levels of both decreased precipitously from there to the outer 10% radius mark. This distribution paralleled synthesis of the membrane's cholesterol, phospholipid, and intrinsic protein. We conclude that the abrupt cessation of plasma membrane synthesis in the ocular lens could involve loss of HMGR activity over a narrow arc of the lens radius, and that this activity loss is due to disappearance of enzyme protein.—**Shi, H., and R. J. Cenedella.** Regional distribution of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity and protein mass in the ocular lens. *J. Lipid Res.* 1993. **34:** 2177–2182.

Supplementary key words plasma membrane • membrane synthesis • cholesterol

The ocular lens can provide a useful model for investigating questions about membrane assembly. The lens grows by continuous differentiation of epithelial cells, which cover the anterior surface as a monolayer, into greatly elongated fiber cells. One layer of fiber cells is laid down upon another throughout life and, with exception of the epithelial cells, the lens is composed entirely of fiber cells. Fiber cell elongation involves an estimated thousandfold increase in plasma membrane (1). Membrane synthesis continues until the ends of the fiber cell reach the anterior and posterior sutures of the lens; that is, until it extends from hemisphere to hemisphere (2). There net membrane synthesis stops. We are interested in the biochemical signals responsible for this cessation of membrane formation.

Regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) activity could be a key factor in controlling the initiation and cessation of membrane synthesis in the lens. Cholesterol biosynthesis is of special significance in lens biology because fiber cell plasma membrane is essentially the only subcellular organelle of this organ (2), the cholesterol to phospholipid molar ratio of this membrane is the highest of any eukaryotic cell (3), and the lens likely obtains all of its required cholesterol by de novo synthesis (4, 5). The ocular humors that surround this avascular organ are devoid of lipoproteins except for trace amounts of high density lipoproteins (6). In humans, lens cholesterol synthesis proceeds at a near constant rate from 8 to 95 years of age (7). Lens cholesterol biosynthesis has recently become an important topic relative to the therapeutic safety of lovastatin and other HMGR inhibitors (5, 8–12), since inhibition of lens cholesterol synthesis can produce cataracts and blindness in rats (13), dogs (10), and humans (14, 15).

Besides cholesterol, lens membrane is composed of phospholipid, and a 26-kDa protein (termed the main intrinsic protein or MP26) accounts for most of the intrinsic protein (16). We recently demonstrated that the synthesis and presumably assembly of the principal components of the fiber cell membrane, cholesterol, fatty acid (reflecting phospholipid) and MP26, is highly coordinated and confined to the outer 10% of the rat lens radius (17). The

Abbreviations: HMGR, 3-hydroxy-3-methylglutaryl coenzyme A reductase; MVL, mevalonic acid.

[1]To whom correspondence should be addressed.

site where membrane synthesis stops approximates the location where the fiber cell should have become fully elongated. The purpose of the present work was to map the distribution of HMGR enzyme activity and protein mass in the lens with the intent of determining why cholesterol synthesis, and membrane formation, halt abruptly. The results indicate that neither HMGR enzyme activity nor protein mass exist much beyond the region of fiber cell elongation and imply that disappearance of HMGR could be a signal for terminating membrane formation.

MATERIALS AND METHODS

Materials

DL-[3-^{14}C]3-hydroxy-3-methylglutaryl coenzyme A ([^{14}C]HMG-CoA) (49.0 mCi/mmol) and *RS*-[5-^{3}H(N)] mevalonolactone ([^{3}H]MVL) (27.5 Ci/mmol) were purchased from New England Nuclear Corp. (Boston, MA). Murine A9 cells that produce monoclonal antibody to HMGR were obtained from the American Type Culture Collection (Rockville, MD) and cultured according to instructions. Monoclonal antibody was isolated from approximately 120 ml of media recovered from cultures at high cell density by the caprylic acid precipitation technique of Reik et al. (18). The protein concentration of the purified monoclonal antibody solution was 9.11 mg/ml. Peroxidase-conjugated goat anti-mouse IgG was a product of Jackson Immuno Research (West Grove, PA) and the enhanced chemiluminescence (ECL) Western blotting analysis system was obtained from Amersham (Arlington Heights, IL). All other chemicals were from Sigma Chemical Co. (St. Louis, MO).

Fractionation of lens cell homogenates

Rat lens soluble and insoluble fractions were isolated by methods similar to those used by Li et al. (19) for preparation of rat liver microsomes. Briefly, 70 fresh rat lenses from 9-day-old Sprague-Dawley rat pups (male and female, Hilltop Lab Animals, Scottdale, PA) were homogenized in ice-cold solution A, 0.25 M sucrose and 15 mM EDTA adjusted to pH 7.4 (20), using a Dounce homogenizer (0.1 ml buffer per lens). Aliquots of the whole homogenate were taken for protein determination (21) and measurements of HMGR enzyme activity and protein mass (methods described below). The remaining homogenate was centrifuged for 60 min at 100,000 *g* at 4°C. Supernatant (lens soluble fraction) and pellets (insoluble fraction) were recovered, and the pellets were washed with solution A and centrifuged as before. The recovered pellets were then resuspended in solution A (the volume of the suspension was about one-seventh of the original homogenate volume), frozen, and stored at −70°C.

Lens dissolution

We recently described a simple method for dividing the rat lens into numerous uniform fractions by gently stirring the decapsulated lens in buffer containing 0.2% sodium dodecylsulfate (SDS) (17). From the protein content of each fraction and published information on the radial distribution of protein in the young rat lens, each fraction could be equated to a specific percentage of the lens radius. Because HMGR enzyme activity cannot be measured in the presence of SDS, in the present study we choose to dissolve lenses in tergitol (Type 15-s-9, Sigma Co.), a nonionic detergent that can be substituted for Kryo-EOB in the HMGR enzyme assay (personal communication, Harry Rudney, University of Cincinnati). When tested, HMGR enzyme activity measured in the presence of tergitol was 73% of that measured in its absence. Fresh lenses collected from Sprague-Dawley rats (male and female, 21–37 days of age) were carefully decapsulated and uniformly dissolved in 3 ml buffer A (5 mM Tris, 1 mM EDTA, 5 mM 2-mercaptoethanol, 0.1 mM leupeptin, 0.25% tergitol, pH 8.0). The fractions were recovered by aspiration at specific times and immediately replaced with 3 ml of fresh buffer. After 30 min of dissolution (buffer replaced 4 to 9 times) only the lens nucleus remained. The nuclei were homogenized in buffer A using a Dounce homogenizer.

Assay of HMGR enzyme activity

HMGR enzyme activity was measured as we described before (22) by the method of Panini, Sexton, and Rudney (23) except that tergitol was substituted for Kryo-EOB in the assay system. Aliquots of lens whole homogenate, soluble and insoluble fractions, and the tergitol dissolution fractions were assayed for enzyme activity in a final volume of 75 μl. The mass of lens protein added ranged from 37 to 515 μg. Each assay tube contained 2.3 pmol of [^{14}C]HMG-CoA (109 dpm/pmol) and 50,000 dpm of [^{3}H]MVL. Incubation was carried out at 37°C in a controlled environment incubator (Napco 5100, Portland) for 1.5 or 3 h. The reaction was stopped by the addition of 20 μl of 6 N HCl and incubated for 30 min at 37°C. Along with the tubes containing a lens enzyme source, control tubes containing either no enzyme source or heat-denatured or acid-denatured enzyme were simultaneously incubated and assayed for product. The products of reaction were separated by thin-layer chromatography, recovered and assayed for ^{14}C and ^{3}H content by scintillation counting (22, 23). One unit of reductase activity was defined as 1 pmol of mevalonate formation per min at 37°C.

Estimation of HMGR protein mass

HMGR enzyme protein mass was estimated by Western blotting using the A9 monoclonal antibody to

HMGR and a recently developed nonisotopic enhanced chemiluminescence (ECL) detection system (Amersham Corp). Samples of lens protein fractions (15–65 μg protein) were first separated by routine SDS-polyacrylamide gel electrophoresis on 12% gels. The proteins were then electrotransferred to a ProBlott membrane (Applied Biosystems, Forster City, CA) for Western blotting. Coomassie blue staining of the gel after transfer showed virtually complete transfer of all proteins. Subsequent immunoreaction and detection were performed by using the ECL Western blotting system exactly as described by the manufacturer's instructions. ProBlott membranes bearing the transferred protein were briefly rinsed with TBS-T buffer (20 mM Tris-HCl, 137 mM NaCl, 0.1% Tween 20, pH 7.6). Nonspecific binding sites were then blocked by incubation for 1 h with 10% nonfat dried milk in TBS-T. Membranes were briefly rinsed twice with TBS-T buffer, washed once for 15 min, twice for 5 min with 50–100 ml of the same buffer, and incubated in either a 1:100 dilution of the monoclonal anti-HMGR antibody in TBS-T buffer or in TBS-T buffer without antibody (controls) for 1 h. Membranes were washed as above and then incubated for 1 h in TBS-T buffer containing peroxidase-conjugated goat anti-mouse IgG at a 1:80,000 dilution (50–100 ml). The membranes were then exhaustively washed with TBS-T buffer according to the manufacturer's instructions and incubated with 10 ml of detection reagent for 1 min. The wet membrane was placed on a glass plate (8 × 10 cm), covered with an air-tight layer of Saran Wrap® (Dow Chemical Co., Indianapolis, IN) and exposed to X-OMAT AT Diagnostic film (Kodak, Rochester, NY) for 30–60 min in a sandwich consisting of glass plate, membrane, film, glass plate. After development of the film, the intensities of the bands corresponding to HMGR were scanned with a GS 300 scanning densitometer (Hoefer Scientific Instruments, San Francisco, CA) and the area under the scan curves was estimated.

RESULTS

We determined the gross subcellular distribution and apparent molecular weight of lens HMGR. A protein of about 97 kDa selectively reacted with the monoclonal antibody and essentially all of the enzyme was recovered from the lens water-insoluble fraction (**Fig. 1**). The insoluble fraction would contain endoplasmic reticulum. Thus, the molecular weight and subcellular distribution of the enzyme appears identical to HMGR in other cells (24). Although the secondary antibody used in the detection system (peroxidase-conjugated goat anti-mouse IgG at 1:80,000 dilution) gave nonspecific reactions with several lens proteins, it did not react with protein equivalent to the 97 kDa form of either rat liver or lens HMGR (Fig. 1).

The regional distribution of HMGR among concentric arcs of lens radius was investigated by gradually and uniformly dissolving lenses in buffer containing tergitol, a detergent that did not interfere with the assay for HMGR activity. Aliquots of each recovered fraction, which could be equated to specific segments of the lens radius based upon their total protein contents, were assayed for HMGR enzyme activity and relative enzyme protein mass. The outer 10% of the lens radius was found to account for 96–97% of the total HMGR enzyme activity in lens from 23- and 37-day-old rats (**Fig. 2**). Peak enzyme activity was detected in a zone corresponding to the outer 2–4% of the lens radius and enzyme activity abruptly decreased beyond this point toward the lens center.

Prior to attempting measurements of the regional distribution of HMGR enzyme protein mass in the lens, we established that Western blotting with the A9 monoclonal antibody can be used to provide a quantitative estimate of relative HMGR enzyme mass. Using an outer layer of

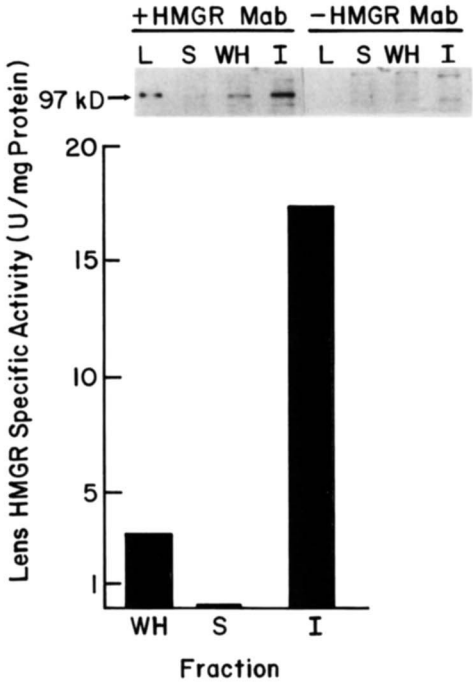

Fig. 1. Subcellular distribution of HMGR enzyme activity and protein mass in the lens. Lenses (70) for 9-day-old rats were homogenized in a sucrose–EDTA solution and centrifuged at 100,000 *g* for 1 h. Aliquots of the whole homogenate (WH), soluble supernatant (S), and insoluble pellet (I) fractions were assayed for HMGR activity and examined by Western blotting for relative HMGR protein content using the A9 monoclonal antibody against HMGR and detection by enhanced chemiluminescence. The HMGR enzyme assay systems (75 μl) contained 515 μg, 480 μg, and 48 μg of WH, S, and I protein, respectively, per reaction tube. One unit of enzyme activity equals one pmol of mevalonate formed per minute. In the Western blots, protein per gel lane was < 1 μg, 64 μg, 60 μg, and 15 μg for liver microsomes, lens WH, S, and I fractions, respectively. L, liver microsomes from 23-day-old rats treated for 4 days with lovastatin (0.1%, w/w, in the diet). HMGR Mab, A9 monoclonal antibody against HMGR.

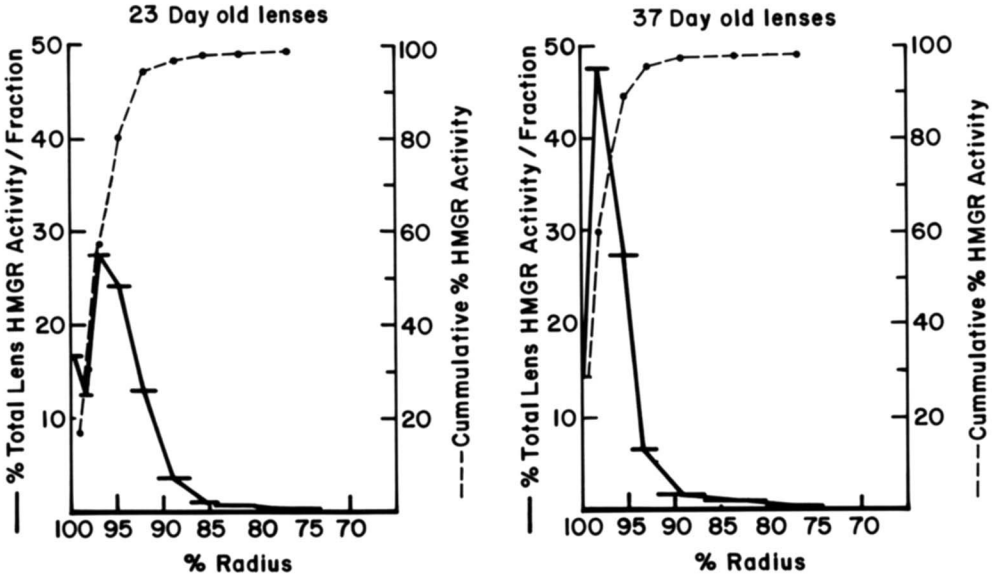

Fig. 2. Regional distribution of HMGR enzyme activity of the lens. Pools of decapsulated lenses (20 to 23) from either 23- or 37-day-old rats were uniformly "dissolved" in 3-ml aliquots of buffer containing 0.25% tergitol. At specific times (2, 4, 6, 8, 10, 15, 20, and 30 min) the buffer was rapidly replaced with 3 ml of fresh buffer. Aliquots of the recovered fractions were assayed for total protein content and HMGR activity. Between 37 μg and 256 μg of protein from each dissolution fraction was used per enzyme assay. HMGR activity was expressed as U activity (pmol mevalonate formed per min)/mg protein. Total activity per fraction equaled U × mg total protein per fraction. Based on the total protein content, each fraction was equated to a specific percentage of the lens radius. Bars equal the HMGR activity in a defined arc of the lens radius.

lens cortex as the source of enzyme protein, a good linear relationship was obtained between the intensity of HMGR immunostain and the concentration of total protein applied to the gel (**Fig. 3**). Aliquots of fractions obtained by dissolution of intact lenses were then examined for relative HMGR mass. The distribution of enzyme protein closely paralleled the distribution of enzyme activity (**Fig. 4**). About 90% of the total HMGR protein was identified in the outer 10% of the lens radius, with maximum concentration found in the outer 6%. The disappearance of 97 kDa enzyme from the deeper layers of the lens was not accompanied by the appearance of lower molecular weight proteins that reacted specifically with the monoclonal antibody.

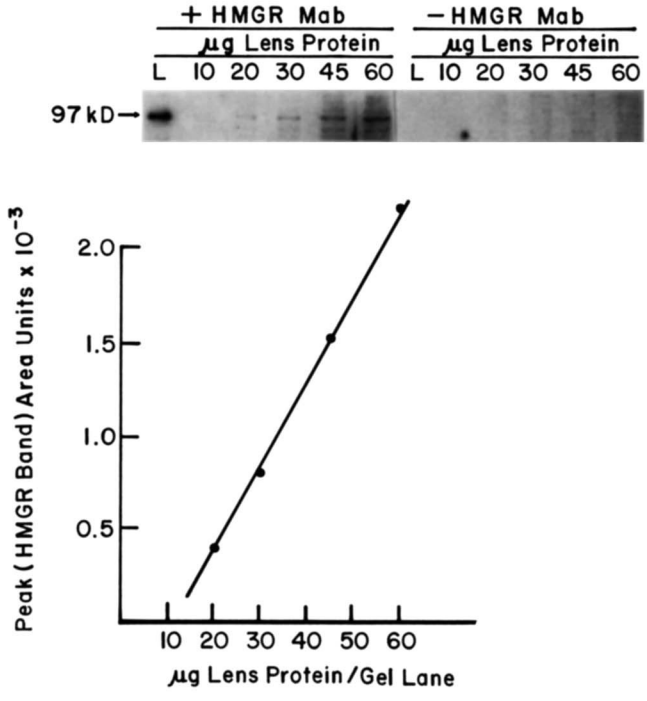

Fig. 3. Linearity of Western blot estimation of HMGR protein mass. Lens protein was from the outer cortex (first 15-min dissolution fraction) of 14-day-old rats. The absorbances of the HMGR bands (97 kDa) were estimated by scanning the ECL exposed film and calculating the relative areas of the generated curves. L, rat liver microsomes (< 1 μg protein/gel lane).

DISCUSSION

Although there has been much interest in the ocular safety of the vastatin class of therapeutic HMGR inhibitors (8–12), lens HMGR has been little investigated. The HMGR activity of cultured bovine lens epithelial cells has been assayed and shown to be well correlated with simultaneously measured absolute rates of sterol synthesis (22). However, Kalinowski, Tanaka, and Mosley (11) reported the only measurement of HMGR activity in whole lens. They found about 1.4 U activity (pmol mevalonate formed per min) per mg of total protein in lenses of

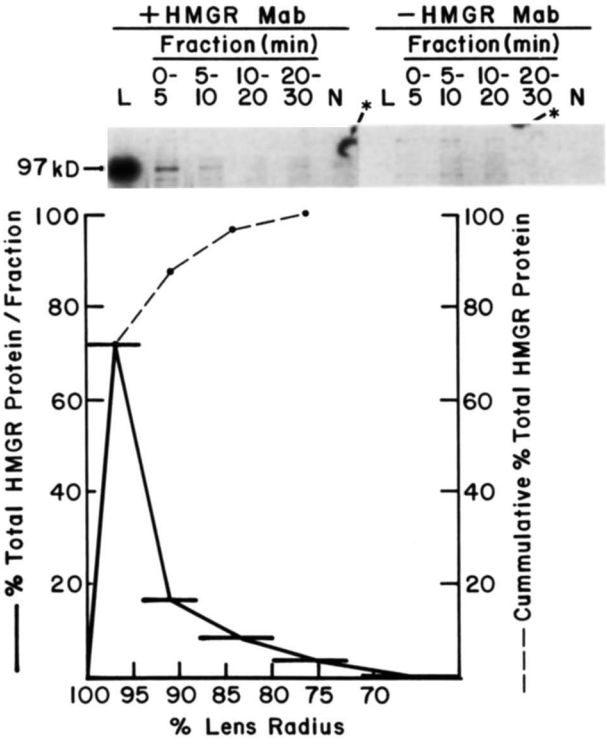

Fig. 4. Regional distribution of HMGR protein mass in the lens. Pooled lenses, 58, from 21-day-old rats were dissolved by stirring in 3-ml aliquots of buffer containing 0.25% tergitol. Buffer was replaced at 5, 10, and 20 minutes. The lens fraction remaining after 30 min, the nucleus (N), was homogenized in 10 ml of buffer. Fifty μg of protein from each fraction was applied per gel lane. Relative HMGR protein mass was estimated by Western blotting using the A9 monoclonal antibody and ECL detection. HMGR band intensities were estimated by scanning the exposed film. L, rat liver microsomal protein (< 1 μg protein/gel lane). *Static electricity artifact.

21-day-old rats. We found about 3.2 U/mg total lens protein in 9-day-old rats (Fig. 1). The present results also show that, like liver, the lens enzyme had a molecular mass of about 97 kDa and was water-insoluble, presumably reflecting its association with endoplasmic reticulum.

What biochemical signals lead to the cessation of plasma membrane formation in the lens? We have chosen to focus on factors that control cholesterologenesis. The cholesterol content of the lens fiber cell plasma membrane is greater than that of any other eukaryotic cell membrane, reaching a molar cholesterol to phospholipid ratio of as high as 4 (3). And, the lens must likely synthesize all of this cholesterol (4, 5). The importance of cholesterol to lens membrane development and structure is reflected in the observation that inhibition of lens cholesterol synthesis can slow lens growth (13), and lead to cataracts in several species, including humans (10, 13–15). The signals that control lens cholesterol formation could thus contribute importantly to control of membranegenesis. As mentioned earlier, lens membrane formation abruptly stops once the elongating fiber cell reaches and extends from anterior to posterior poles. This terminal elongation is estimated to be reached in the rat lens at about 100 cell layers below the capsule; that is, at a point corresponding to about the outer 10% of the lens radius (17). This site also marks the approximate boundary limits of cholesterol synthesis (**Fig. 5**).

The present results show that a good correlation exists between the spatial distribution in lens of HMGR enzyme activity and protein mass, and these distributions parallel that of cholesterol biosynthesis as well as synthesis of MP26 and phospholipid (17), the other major components of lens membrane. Thus, the cessation of cholesterol synthesis could be explained by an abrupt disappearance of HMGR enzyme protein rather than inactivation of the enzyme. This interpretation assumes that the observed decrease of immunologically reactive HMGR represents a true loss of enzyme protein rather than only loss of epitopes recognized by the monoclonal antiserum. The disappearance of HMGR at about the 90% point of the lens radius could be due to a combination of cessation of enzyme synthesis at this location and proteolysis of the enzyme. Although the turnover rate of lens HMGR is unknown, in other cells it can be as brief as 1 h (25). Thus, a halt in enzyme synthesis coupled with even steady state proteolysis of HMGR could lead to rapid loss of enzyme protein. A halt to HMGR synthesis could be due to decreased transcription of the HMGR gene, as fiber cells lose their nucleus upon cell elongation. In addi-

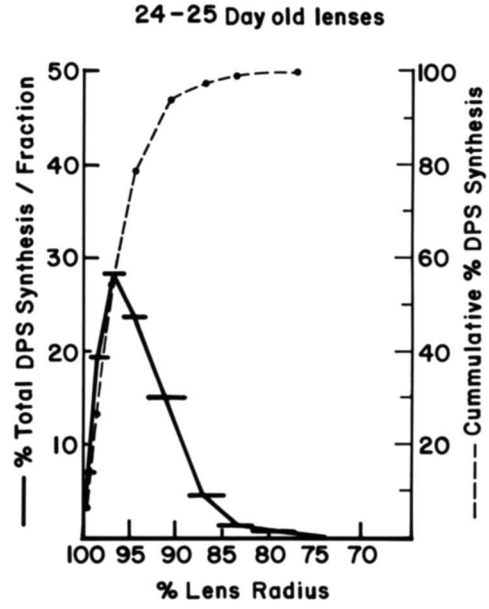

Fig. 5. Regional distribution of cholesterol synthesis in the lens. Lenses from 24- or 25-day-old rats were incubated with ^3H_2O and fractionated by stirring in buffer containing 0.2% SDS, and the [^{3}H]cholesterol was recovered by precipitation with digitonin and recovered as digitonin-precipitable sterols (DPS). Data are adapted from Fig. 2 of reference 17 with permission of the publisher.

tion to the cell nucleus, apparently all subcellular organelles of the fiber cell are eventually lost. Rafferty (2) estimated that, in the mouse, lens nuclei are present to a depth of about 50 fiber cells below the surface while rough and smooth endoplasmic reticulum are present to a depth of only a dozen layers. The loss of subcellular organelles, including the endoplasmic reticulum, implies activation of nucleases and proteases. At present, the relative importance of diminished HMGR synthesis versus proteolysis of the enzyme in accounting for the loss of HMGR over a narrow arc of the lens radius is unknown. Measurement of the regional lens distribution of the mRNA for HMGR might help to resolve this question. Regardless of the mechanism, the abrupt cessation of membrane formation in the ocular lens could involve a rapid halt in cholesterol synthesis due to the disappearance of HMGR enzyme protein.

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