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Cholesterol biosynthesis by the cornea. Comparison of rates of sterol synthesis with accumulation during early development

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Abstract The origin of the cholesterol needed by the cornea for growth and cell turnover was addressed by comparing absolute rates of sterol synthesis with rates of sterol accumulation during early development of the rabbit. Linearity of incorporation of ^3H_2O and [^{14}C]mevalonate into digitonin-precipitable sterols with time of incubation in vitro and a lack of accumulation of ^{14}C in intermediates of sterol biosynthesis indicated that tritiated water can validly be used to measure rates of sterol synthesis by the cornea. The rate of sterol synthesis per unit weight of rabbit cornea was constant between 14 and 60 days of age at an average 1.03 nmol of ^3H of ^3H_2O incorporated/mg dry cornea per 8 h. Essentially all of the synthesized cholesterol and most of the cholesterol mass was present in corneal epithelium. The cumulative sterol synthesized over the 46-day period studied exceeded the observed rate of cholesterol accumulation by sixfold. Cholesterol synthesized in excess of the growth requirement was likely used to support turnover of the epithelium which was estimated at 9 days. Removal of cholesterol from the cornea by excretion into tear fluid and clearance by high density lipoproteins are also considered. — **Cenedella, R. J., and C. R. Fleschner.** Cholesterol biosynthesis by the cornea. Comparison of rates of sterol synthesis with accumulation during early development. *J. Lipid Res.* 1989. **30:** 1079–1084.

Supplementary key words tritiated water • epithelial cell turnover HMG-CoA reductase

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The origin and transport of cholesterol in the cornea are subjects of interest because corneal opacification involving accumulation of cholesterol is associated with at least four separate disorders of the high density lipoprotein (HDL)/lecithin cholesterol acyltransferase (LCAT) system; LCAT deficiency (1), Tangier disease (2), fish eye disease (3), and familial apolipoprotein A-I and C-III deficiency variant II (4). These observations suggest that HDL has an important role in clearing cholesterol from the cornea. As a step toward understanding this relationship, we examined the origin of cholesterol in the cornea by measuring the capacity of this avascular organ to satisfy its cholesterol requirements by de novo synthesis. In addition to growth, the cornea requires cholesterol for membrane formation associated with the turnover of its epithelium and for wound healing (5, 6). The epithelium consists of five to seven layers of epithelial cells which cover the cornea's air-exposed surface. This region overlies the stroma, a thick layer of collagenous lamellae, and a monolayer of endothelial cells at the posterior surface.

Our results indicate that tritiated water can validly be used to estimate absolute rates of sterol synthesis by the rabbit cornea in vitro. Rates of synthesis during early development were compared to rates of cholesterol accumulation. The distribution of cholesterol synthesis, HMG-CoA reductase activity, and cholesterol mass in the cornea were also measured. The results show that the corneal epithelial cell population accounted for essentially all of the de novo synthesis and that the cornea synthesized much more cholesterol than needed to support growth. Most of the cholesterol synthesized in the cornea was likely needed for turnover of epithelial cells.

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MATERIALS AND METHODS

Incubation conditions

^3H_2O (100 mCi/mmol), DL-[2-^{14}C]mevalonic acid, DBED salt (48.6 mCi/mmol), and DL-[3-^{14}C]3-hydroxy-3-methyl-glutaryl coenzyme A (HMG-CoA) (55.5 mCi/mmol) were purchased from New England Nuclear Corp. (Boston, MA). DL-[2-^3H]Mevalonic acid lactone (1.3 Ci/mmol) was purchased from Amersham Corp. (Arlington Heights, IL). Most other reagents were from Sigma Chemical Co. (St. Louis, MO). Corneas with a trace of scleral ring (about 0.5 mm) were immediately dissected from the eyes of 7- to 60-day-old New Zealand variety

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Abbreviations: DPS, digitonin-precipitable sterol; TLC, thin-layer chromatography; HDL, high density lipoprotein.

albino rabbits of either sex (Wallenburn Rabbitry, Otterville, MO) after they were killed between about 6:30 AM and 9:00 AM by cervical dislocation. Pairs of corneas from individual rabbits were transferred to 4.0 ml of MK-199 media supplemented with 100 mg/l of L-glutamine, 10 mg/l gentamycin, 10 mg/l phenol red, 5 mM HEPES, 30 mM $NaHCO_3$, and 5 g/100 ml dextran 40 (7), and containing either 0.8 μCi/ml of [2-^{14}C]mevalonate (converted to the free acid from the DBED salt immediately before use) or 2 to 4 mCi/ml of ^3H_2O. Media containing ^3H_2O of the selected specific activity was obtained by preparing the sterile media at 2 × concentration and adding an equal volume of the ^3H_2O at double the final specific activity desired. Media was then bubbled with 95% O_2-5% CO_2 for 15-20 min and the pH was adjusted to 7.4 with a small volume of 1 N NaOH. Pairs of corneas (with the epithelial surface down) were incubated in sealed scintillation glass counting vials at either 37°C with gentle rotatory shaking or at 0°C in an ice bath.

Isolation and analysis of corneal lipids

Following incubation, the media was aspirated and assayed for ^{3}H content; the corneas were washed five times with phosphate-buffered saline (pH 7.4), transferred to weighed tubes, freeze dried, reweighed, and saponified overnight at 90°C in 2.0 ml of 1 N KOH in 60% ethanol. In order to determine the regional distribution of the synthesized cholesterol, corneas were divided into either epithelial and stromal (plus endothelia) fractions by dissection (8) or into central and peripheral fractions using a sharpened borer (0.84-cm diameter) and the separated regions were saponified. The epithelial fraction contained considerable stroma since histological sections of intact corneas showed the epithelial cell layers to comprise 15 to 20% of the intact cornea's cross-sectional width; yet, this fraction comprised 35 to 40% of the cornea's total dry weight (see Tables 1, 2, and 4). Nonsaponifiable lipids were extracted into hexane and the digitonin-precipitable sterols (DPS) were prepared, recovered and assayed for ^{3}H content as previously described (9). The nonsaponifiable lipids were also separated by TLC on silica gel G into squalene, lanosterol, and C27 sterol fractions using a solvent of hexane-diethyl ether-glacial acetic acid 73:25:2. Carrier squalene, lanosterol, and cholesterol (400 μg of each) were added to the samples prior to saponification. The sterol content of intact and divided corneas from other age-matched rabbits was quantitated by gas-liquid chromatography using 5α-cholestane as internal standard as described before (10).

HMG-CoA reductase assay

Corneas were separated into epithelial and stromal (including the monolayer of endothelial cells) fractions by dissection. The fractions were weighed and pools of the separated fractions from eight corneas were homogenized on ice in 0.25 M sucrose plus 15 mM EDTA (pH 7.4) using a Tissumizer (Tekmar Corp., Cincinnati, OH). A microsomal fraction was isolated from each by centrifugation (11) and then assayed for reductase activity according to Panini, Sexton, and Rudney (12). One unit of reductase activity was defined as 1 pmol of mevalonate formed per min at 37°C.

Calculation of rates of cholesterol synthesis

Incorporation of tritium into DPS was corrected for bound ^3H_2O (incorporation at 0°C) and expressed as nmol (ng atoms) of ^{3}H of ^3H_2O incorporated per cornea or per mg of dry cornea. The specific activity of the media water was determined after incubation (to account for dilution with tissue water) by direct assay of the media and from the knowledge that the solids content of the complete media was measured at 6.35% (w/w). Absolute rates of cholesterol synthesis were estimated from the tritium incorporation assuming that either all of the NADPH required by the cornea for sterol synthesis equilibrated with ^{3}H of ^3H_2O (i.e., NADPH was generated totally by oxidative enzymatic processes and thus all 22 of the incorporated hydrogens become labeled) or none of the NADPH equilibrated with the ^3H_2O (i.e., NADPH was generated exclusively by the pentose phosphate pathway and thus only 7 of the 22 hydrogens incorporated into cholesterol become labeled) (9, 13, 14).

The nmol cholesterol synthesized/mg dry cornea per day = nmol ^{3}H incorporated/mg per 8 h × 3 ÷ 22 nmol ^{3}H incorporated per nmol of cholesterol synthesized or ÷ 7 nmol of ^{3}H incorporated per nmol of cholesterol synthesized.

RESULTS

Incorporation of both [^{14}C]mevalonate and ^3H_2O into DPS by the rabbit cornea was highly temperature-dependent and essentially linear over an 8-h incubation (**Fig. 1**). Incorporation was also proportional to the number of corneas incubated per vial, at least from one to four (data not shown). No significant diurnal variation in the rate of sterol synthesis was seen between corneas examined at the end of a 12-h dark cycle versus the end of the light cycle (data not shown). Our estimates of absolute rates of sterol synthesis per day are based upon an 8-h incubation and since there appeared to be a slight lag in incorporation of ^3H_2O into DPS during the first 1-2 h of this incubation (Fig. 1B), our estimates of total synthesis per 24 h could be low.

Incorporation of [^{14}C]mevalonate into C27 sterols was linear over an 8-h incubation and much greater than in-

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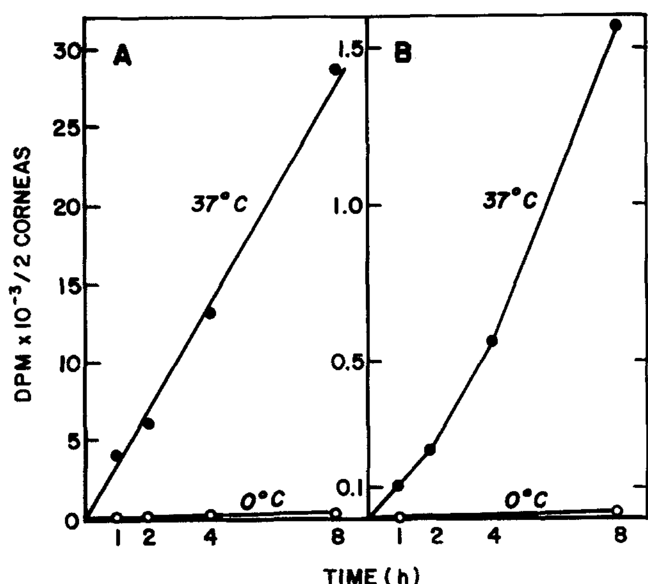

Fig. 1. Time course of incorporation of radiolabel into digitonin-precipitable sterols. Pairs of cornea from individual rabbits (28–30 days of age) were incubated at 0° or 37°C in 4 ml of MK-199 media containing either 0.8 μCi/ml of [2-^{14}C]mevalonate (A) or 2 mCi/ml of ^3H_2O (B). The corneas were then saponified and digitonin-precipitable sterols (DPS) were recovered and counted as described under Experimental Procedures. Each point represents the incorporation by an individual pair of intact corneas.

corporation into either squalene or lanosterol fractions (**Fig. 2**). This indicates that there is no significant block in the flow of carbon through the sterol biosynthetic pathway from mevalonate to cholesterol. This conclusion is important since Keller, Fliesler, and Nellis (15) were recently prevented from using tritiated water to estimate absolute rates of cholesterol synthesis by the retina due to a discovered block in the conversion of squalene to cholesterol in this tissue.

Incorporation of ^3H_2O into DPS by corneas from 14- to 60-day-old rabbits was directly proportional to cornea dry weight and averaged a constant 1.029 nmol of ^3H per mg dry weight per 8-h incubation (**Fig. 3**). The incorporation rate appeared higher in 7-day-old rabbits. Essentially all of the newly synthesized sterol (**Table 1**) and most of the total HMG-CoA reductase activity (**Table 2**) in the cornea was recovered from the epithelial fraction. These distributions likely underestimate the true differences between the epithelium and stroma since our epithelial fraction separated from the cornea by dissection contained considerable stroma. The smaller difference between epithelial and stromal fractions in the distribution of HMG-CoA reductase activity than in the distribution of synthesized sterols might be related to differences in the completeness of recovery of microsomal protein from the two fractions.

Absolute rates of sterol synthesis for the 14- to 60-day age interval were calculated from the average incorporation rate (Fig. 3) using the two extremes for the source of NADPH required for sterol synthesis. Twenty-two hydrogen atoms are incorporated per molecule of cholesterol synthesized, 7 enter from water and 15 from NADPH (13). Since the hydrogens of NADPH generated by the pentose phosphate pathway do not equilibrate with ^3H of ^3H_2O, only 7 nmol of ^3H would be incorporated per nmol of cholesterol synthesized if all the NADPH were generated by this pathway versus 22 if the NADPH were generated totally by oxidative reactions where complete equilibration occurs (14). Using the incorporation data and this information, we estimate that during 14 and 60 days of age the rabbit cornea synthesized between 0.44 and 0.14 nmol of cholesterol/mg dry cornea per day (**Table 3**).

The rate of increase of both cornea dry weight and cholesterol content was linear between 14 and 60 days of age (**Fig. 4**). The rabbit cornea accumulated 12.34 μg or 31.92 nmol of cholesterol per cornea over this 46-day growth period (Fig. 4). Most of the increase in cholesterol content likely occurred in the epithelial cell population since this fraction contained most of the cornea's cholesterol (**Table 4**). The cholesterol content of the rabbit cornea is similar to that recently reported by others (16). Because both the rate of increase of cornea dry weight and cholesterol content and the rate of incorporation of ^3H of ^3H_2O into DPS per unit of dry cornea were constant over the 46-day period, estimates of the total amount of cholesterol synthesized per cornea over this aging interval can be calculated by summing the synthetic contribution for each day between 14 and 60 days of age. Using the two

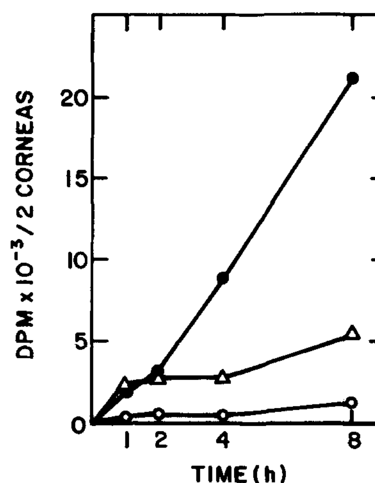

Fig. 2. Time course of incorporation of radiolabeled mevalonate into nonsaponifiable sterol fractions. Pairs of cornea from 28-day-old rabbits were incubated at 37°C in 4 ml of MK-199 media containing 0.8 μCi/ml of [2-^{14}C]mevalonate. Cornea were then saponified; nonsaponifiable lipids were recovered and fractionated into C27 sterols (●), lanosterol (△), and squalene (○) fractions by thin-layer chromatography. Each point represents the incorporation by an individual pair of corneas.

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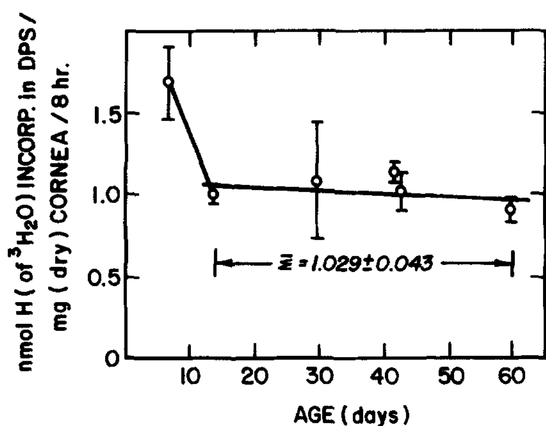

Fig. 3. Rates of incorporation of ^3H of ^3H_2O into sterols by the cornea with aging. Pairs of corneas from individual rabbits were incubated in MK-199 media for 8 h at 37°C containing ^3H_2O at specific activities of between 36.4 and 47.6 dpm/nmol of ^3H of ^3H_2O. Corneas were then saponified; DPS were recovered and counted. Incorporation of tritium into DPS at 37°C was corrected for incorporation by corneas incubated at 0°C. Each value is the mean ± SEM incorporation of four to five pairs of corneas.

extremes for the source of NADPH, we estimate that the rabbit cornea synthesized a total of between 62 and 194 nmol of cholesterol per cornea during this period (Table 3). These values are from 2 to 6 times greater than the amount of cholesterol which accumulated per cornea; i.e., one-half to five-sixths of the synthesized cholesterol was needed for a purpose other than corneal growth.

DISCUSSION

The results of the present study indicate that the rabbit cornea has the capacity to satisfy all of its sterol requirements by de novo synthesis and, in fact, appears to synthesize much more cholesterol than required to support growth between 14 and 60 days of age. The higher rate of synthesis estimated, the one assuming NADPH was generated exclusively by the pentose phosphate pathway, is probably more correct, since most of the cornea's total sterol synthesis occurred in the epithelial cell fraction and corneal epithelium has been reported to possess a very active pentose phosphate pathway as judged by high glucose-6-phosphate dehydrogenase activity and by two-thirds of epithelial glucose oxidation occurring by this pathway (17).

It is not surprising that the cornea can satisfy its cholesterol needs by de novo synthesis considering that the organ is avascular and largely inaccessible to circulating low density lipoproteins. The peripheral cornea which is in contact with the limbal blood supply is likely exposed to low density lipoprotein since aging and hypercholesterolemia can involve cholesterol deposition in this region and corneal arcus (18). Our finding that de novo sterol synthesis appeared slightly lower in the peripheral cornea compared to the central region (Table 1) supports the possibility that some exogenous cholesterol is normally delivered to the periphery. The results of the present study do not provide an explanation for the apparent synthesis of cholesterol by the cornea in great excess of its growth requirements. However, several explanations are possible.

The air-exposed surface of the cornea is covered by five to seven layers of epithelial cells, with proliferation confined to the basal cell layer and cell density highest in this layer (6, 19, 20). Our results indicate that most of the cholesterol mass and essentially all of the de novo synthesis and HMG-CoA reductase activity are present in the epithelium. A high rate of turnover of these cells might account for the large sterol requirement. About one of 100 basal epithelial cells of the cornea from young rabbits became labeled following a 2.5-h incubation in vitro with [^{3}H]thymidine (19). Similar labeling rates were reported for the human and rat corneal epithelium (6, 20). Therefore, the basal layer of rabbit corneal epithelial cells could completely turn over in about 10 days (100 × 2.5 h/24 h). Following division, labeled basal epithelial cells were reported to move into the superficial layers and to be displaced out of the cornea in 2 to 4 days (20). Taken together, this information suggests that perhaps 2 weeks would be required for complete turnover of the corneal

TABLE 1. Regional distribution of newly synthesized sterol in the cornea

Corneal Fractions	Pairs of Corneas	Ratio (nmol ^3H incorporated in DPS/mg dry weight)
Central/peripheral	5	1.23 ± 0.10[a]
Epithelial/stromal	6	14.1 ± 4.2[b]

Pairs of corneas from individual rabbits (39–42 days of age) were incubated for 8 h at 37°C in 4 ml of MK-199 media containing ^3H_2O at specific activities of either 36.4 (central/peripheral) or 73.0 (epithelial/stromal) dpm/nmol of ^3H of ^3H_2O. Each pair of corneas was subsequently divided into central and peripheral fractions or into epithelial and stromal fractions and saponified, and the DPS were recovered and counted. Central cornea comprised 61 ± 2% of the total dry weight and the epithelial fraction comprised 40 ± 4% of the total dry weight.

[a]P(t) of difference from 1.00 < 0.09 by the paired *t*-test.

[b]P(t) of difference from 1.00 < 0.0005 by the paired *t*-test.

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TABLE 2. Regional distribution of HMG CoA reductase activity in the cornea

Fraction	Total Activity (units per fraction)	Specific Activity (units per mg microsomal protein)
Epithelial	3.69 ± 1.17	18.34 ± 7.8
Stromal	1.86 ± 0.60[a]	12.98 ± 4.7

Five pools of eight corneas each from 32-day-old rabbits were divided into epithelial and stromal (plus endothelium) fractions by dissection. The tissue was homogenized, and the microsomal fractions were isolated by differential centrifugation. Aliquots of the microsomal fractions were assayed for HMG-CoA reductase activity. One unit of enzyme activity is defined as formation of 1 pmol mevalonic acid per min at 37°C. The epithelial fraction accounted for 40.2 ± 2.3% of the total corneal wet weight, and contained 66.0 ± 3.6% of the total HMG-CoA reductase activity. Values are mean ± SEM (n = 5).

[a]P(t) of difference < 0.05 by the paired *t*-test.

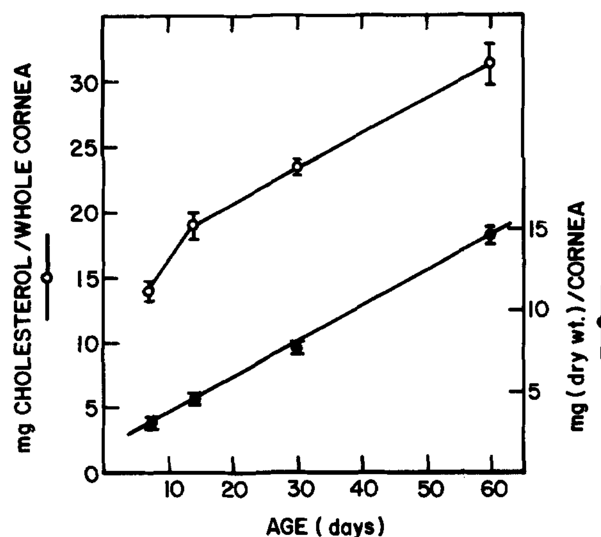

Fig. 4. Changes in cholesterol and dry weight content of the cornea with aging. Each cholesterol concentration is the mean ± SEM from pairs of corneas from four to six individual rabbits. Desmosterol accounted for about 5% of the total sterol in corneas of the 7-day-old rabbits. Each cornea weight is mean ± SEM from 10 to 12 rabbits.

epithelium in the young rabbit. If all of the cholesterol synthesized in excess of that required for growth (a maximum of about 5 times too much) was needed to form epithelial cell membranes for cell turnover, we estimate that between 14 and 60 days of age the rabbit corneal epithelium would have completely turned over a maximum of 5 times, giving a turnover rate of once every 9 days. The capacity for this rapid cell turnover is an important factor in healing of injuries to the corneal epithelium (19).

Secretion of cholesterol by the cornea epithelium might also contribute to the high rate of biosynthesis. Blumcke et al. (21) observed that the outermost layer of corneal epithelial cells, ones that develop a squamoid appearance, possess a plasma membrane that is covered with a lipid layer (10 nm thick) on its exposed surface. The authors speculate that this lipid layer could protect the epithelial cells from the osmotic effect of the hypotonic tear fluid. The extent to which this lipid layer contributes to the total sterol requirement of the cornea is unknown. However, a gentle swab of the surface of the rabbit cornea with chloroform–methanol 2:1 removed the equivalent of only about 3% of the total cholesterol in the epithelium (data not shown).

Cholesterol formed in excess of that required for growth, epithelial cell turnover, and lipid secretion might be cleared from the cornea by HDL-mediated reverse

TABLE 3. Rates of sterol synthesis compared with rates of sterol accumulation by the rabbit cornea between 14 and 60 days of age

Item	F = 0.26[a]	F = 0.81[b]
Cholesterol synthesized nmol/mg cornea/day[c]	0.44	0.14
Total cholesterol synthesized nmol/cornea/46-day interval[d]	194	62
Total nmol cholesterol synthesized/cornea[e]	6.1	1.9
Total nmol cholesterol accumulated/cornea		

[a]F = 0.26; nmol ^{3}H incorporated/nmol of carbon (of cholesterol) assuming all the NADPH comes from the pentose phosphate pathway (7 nmol ^{3}H incorporated/nmol of cholesterol synthesized).

[b]F = 0.81; nmol ^{3}H incorporated/nmol of carbon (of cholesterol) assuming all NADPH comes from oxidative enzymatic reactions (22 nmol ^{3}H incorporated/nmol of cholesterol synthesized).

[c]nmol Cholesterol synthesized/mg cornea/24 h (14- to 60-day interval) = nmol ^{3}H incorporated per 8 h (1.029) × 3 ÷ (0.26 × 27) or (0.81 × 27); see Materials and Methods for more detail.

[d]Since the rate of increase of cornea dry weight was constant over day 14 to 60 (0.218 mg per cornea per day, see Fig. 4) and the rate of sterol synthesized per mg of dry cornea was constant over the interval (Fig. 3), the cumulative total cholesterol synthesis can be estimated by summing the contribution of each day over the 46-day interval.

[e]The cholesterol increase per cornea between day 60 and 14 equaled 31.4 μg per cornea ÷ 0.38664 μg/nmol cholesterol on day 60 minus 19.06 μg per cornea ÷ 0.38664 on day 14 or 32 nmol of cholesterol (see Fig. 4).

TABLE 4. Regional distribution of cholesterol in the cornea

Fraction	Pairs of Corneas	μg Cholesterol/mg (Dry) Weight
Central[a]	6	1.99 ± 0.06
Peripheral		2.01 ± 0.08
Epithelial[b]	6	3.04 ± 0.13
Stromal		0.95 ± 0.05

Pairs of cornea from individual rabbits (40 days of age) were separated into central and peripheral fractions or into the epithelial and stromal (plus endothelium) fractions. The separated regions were saponified and cholesterol was quantitated by gas-liquid chromatography.

[a]Central cornea comprised 63 ± 4% of cornea dry weight and contained 63 ± 4% of the total cholesterol.

[b]The epithelial fraction comprised 37 ± 1% of the cornea dry weight and contained 65 ± 2% of the total cholesterol.

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cholesterol transport (22). Winder et al. (1) speculated that opacification of the human cornea associated with a variety of disorders of the HDL/LCAT system probably arises by decreased clearance of lipid from the cornea. The exchange of cholesterol between the cornea and lipoproteins remains to be investigated.

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