Absolute rates of sterol synthesis estimated from [^{3}H]water for bovine lens epithelial cells in culture

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Abstract All cells of the avascular ocular lens derive from a monolayer of epithelial cells located on only the anterior surface of this organ. The source of the cholesterol required for the growth and division of these cells was studied by using cultures of bovine lens epithelial cells. Cells were in active growth during the third to fourth day of subculture following seeding. Absolute rates of cholesterol synthesis were estimated for the cultured cells from incorporation of [^{3}H]water. Rates were estimated on the assumption that 0.81 atoms of ^{3}H of [^{3}H]water were incorporated into cholesterol per carbon atom of cholesterol, a situation where all of the NADPH would be generated by oxidative enzymatic processes. We tested this assumption by measuring the changes in sterol mass per dish of cells grown in lipoprotein-deficient media over day 3 to 4 of subculture and by simultaneously measuring the rates of incorporation of [^{3}H]water into sterols during this period. In this situation, the increases in sterol mass should be attributable solely to de novo sterol synthesis. We calculated that an average of 0.79 atoms of ^{3}H of [^{3}H]water were incorporated by these cells into cholesterol per carbon atom of cholesterol. Sterol synthesis was only modestly decreased (about 30%) when the cells were cultured in media prepared with whole calf serum. Growth rates of the cells were also little affected by the absence of lipoproteins. In spite of the capacity to furnish its sterol requirements by de novo synthesis, the lens epithelial cells readily degraded ^{125}I-labeled bovine LDL, and LDL greatly decreased sterol synthesis when added to the media at low levels. ^{125}I-Labeled HDL_3 was not significantly metabolized by the cultured lens cells, and HDL_3 stimulated sterol synthesis when added to the media, apparently by removing sterol from the cells. In conclusion, bovine lens epithelial cells in culture incorporated 0.79 atoms of ^{3}H of [^{3}H]water into synthesized cholesterol per carbon atom of cholesterol. — **Hitchener, W. R., and R. J. Cenedella.** Absolute rates of sterol synthesis estimated from [^{3}H]water for bovine lens epithelial cells in culture. *J. Lipid Res.* 1985. **26:** 1455–1463.

Supplementary key words ocular lens • tissue culture • cholesterol • digitonin-precipitable sterols • rates of sterol synthesis • lipoprotein metabolism • aqueous humor

All cells of the vertebrate ocular lens arise from a monolayer of epithelial cells that cover the anterior surface of the lens. These cells undergo a terminal differentiation in the equatorial zone of the lens to form the vastly elongated fiber cells, the only other cell type in this avascular organ (1). Formation of the fiber cell involves a tremendous increase in cell surface area and eventual disappearance of all subcellular organelles (2). Fiber cells are deposited one layer upon another throughout the life of the animal. Since the epithelial cell layer occupies such a central role in lens biology, factors that influence DNA replication and differentiation of this single monolayer of cells could exert control over the growth and development of the whole organ.

Our laboratory has been interested in the sterol metabolism of the lens and we now recognize that this avascular organ likely supplies most of the cholesterol that it requires by de novo synthesis (3). Some cholesterol might also be supplied from trace amounts of high density lipoproteins that we identified in aqueous humor (4). If cholesterol synthesis is blocked in the rat lens by the compound U18666A, growth of the lens is retarded and permanent nuclear cataracts can develop (5–7). Nonsterol isoprenes formed during cholesterogenesis appear to play a critical role in DNA replication and growth of perhaps all eukaryotic cells (8–10). This control mechanism might be especially important to the lens which seems committed to conducting cholesterogenesis. Essentially nothing is known of the regulation of sterol synthesis in the ocular lens. As a step toward understanding this regulation and its importance to controlling lens growth, we have investigated the ability of bovine lens epithelial cells in culture to supply their sterol needs by de novo synthesis. We also examined the potential of lipoproteins to influence cholesterogenesis and measured the ability of these unique cells to metabolize lipoproteins. To our knowledge, this study represents the first reported attempt to measure absolute rates of sterol synthesis by cultured cells using [^{3}H]water as substrate.

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; WM, whole media (DMEM plus 9% by volume of whole calf serum); LDM, lipoprotein-deficient media (DMEM plus 9% by volume of lipoprotein-deficient calf serum); LDL, low density lipoprotein; HDL_2, high density lipoprotein, class 2; HDL_3, high density lipoprotein, class 3; DPS, digitonin-precipitable sterols; dpm, disintegrations per minute; cpm, counts per minute; GLC, gas-liquid chromatography.

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MATERIALS AND METHODS

Cell culture

Bovine lens epithelial cells were cultured by a modification of the method of Gospodarowicz et al. (11). The central zone of the anterior capsule of the bovine lens was placed capsule-surface down in a 5-cm plastic dish and cultured in 3 ml of Dulbecco's modified Eagle's media (DMEM), pH 7.4, supplemented with 9% (by final volume) of whole calf serum (Gibco Labs, Chagrin Falls, OH). Cells were cultured in a Napco 5100 incubator at 37°C in an atmosphere of 5% CO_2–95% air at saturating humidity. Within 8–12 days the cells had reached confluency and were recovered from dishes by mild trypsinization (0.005% trypsin, w/v, in isotonic saline containing 0.01% EDTA buffered to pH 7.4). Cells were pooled and replated in complete media at a split ratio of one to four. Subconfluent culture was obtained after 5–7 days. At this time, the cells were harvested by trypsinization and counted by hemacytometer. The second passage of cells was subcultured at a density of 3×10^5 cells per dish and fed every other day with 3 ml per dish of fresh media containing 9% whole calf serum. Subconfluent cultures possessing 1 to 2×10^6 cells/dish were obtained by day 4 and the cells were subcultured at this time. Following two to four subcultures, cells (3×10^5/dish) were replated in DMEM (3 ml) supplemented with either 9% whole calf serum or 9% lipoprotein-deficient calf serum and used for experiments on days 3 and 4 of this subculture. Thus, only during this terminal subculture were lens epithelial cells grown in lipoprotein-deficient media. New primary cultures were started after every three to five subcultures. The viability of cells released from dishes by trypsinization was tested by trypan-blue exclusion. No dye uptake was seen. One of the two major functions of bovine lens epithelial cells is retained when these cells are placed into culture. They continue to actively synthesize and secrete glycosaminoglycans (12), precursors of the lens capsule formed in vivo. The ability to differentiate into fiber cells is lost in culture.

Preparation of lipoprotein-deficient serum

Lipoprotein-deficient calf serum was prepared by adjusting the density of whole calf serum to 1.25 g/ml with solid KBr and centrifuging for 22–24 hr at 110,000 *g*. The upper approximate 20% of the total volume was removed and discarded. The infranatant fraction was resuspended and the centrifugation was repeated twice. After each spin the upper 20% of the total volume was removed. The recovered lipoprotein-deficient serum was exhaustively dialyzed against isotonic saline and aliquots of both whole serum and lipoprotein-deficient serum were extracted with 20 volumes of chloroform–methanol 2:1 (v/v). After adding 5α-cholestane as internal standard, the recovered lipids were saponified in alcoholic KOH. The concentration of cholesterol in the nonsaponifiable fraction, isolated by hexane extraction, was quantitated by gas–liquid chromatography (13). The concentration of cholesterol in the DMEM prepared with various batches of whole calf serum, 9% by vol (Gibco Labs), ranged from 53 to 166 μg/ml; the cholesterol content of the DMEM prepared with the lipoprotein-deficient calf serum (9% by vol) ranged from 1.3 μg/ml to less than 1 μg/ml. When whole calf serum was fractionated by ultracentrifugation at increasing densities, 12% of the total cholesterol was recovered from the fraction of density less than 1.063 g/ml, 53% from the fraction of density 1.063–1.10 g/ml, and 35% in the 1.10–1.21 g/ml density fraction.

Estimation of absolute rate of sterol synthesis by cultured lens epithelial cells

On the third day of subculture, cells grown in lipoprotein-deficient media (LDM) were refed LDM or LDM supplemented with various concentrations of bovine serum low density lipoproteins (LDL) or high density lipoproteins (HDL_2 or HDL_3). Bovine serum lipoproteins were prepared as previously described (4). HDL_2 was recovered at density 1.063–1.10 g/ml and HDL_3 at density 1.10–1.21 g/ml. As described below, the bovine HDL_2 fraction appeared to be a complex mixture of lipoproteins and therefore the effects of this fraction upon sterol synthesis are not reported. Aliquots of DMEM were lyophilized at −50°C and reconstituted with tritiated water (New England Nuclear Corp., Boston, MA) to give known specific activities of between 9,000 and 15,000 dpm/μg atom of ^3H of [^3H]water. The media were supplemented with whole calf serum or lipoprotein-deficient serum or with lipoprotein-deficient serum plus specific concentrations of the isolated lipoprotein fractions (added as μg of protein/ml). The pH of the media was adjusted to pH 7.4 with a small volume of sterile 1 N HCl.

Three ml of the radioactive media was added per dish of cells under an atmosphere of 95% O_2–5% CO_2. The additions were usually made at hour 14–15 of the 3rd to 4th day of culture. Each dish received medium identical to that added at day 3 except that now it contained [^3H]water. The dishes were vigorously flushed with 95% O_2–5% CO_2 for 15–20 sec and tightly sealed with strips of parafilm under this atmosphere; they were then incubated at either 37°C (in the CO_2 incubator) or O°C (on ice in the refrigerator) for 7 hr. Gassing and sealing of the dishes was performed with the dishes held inside an inverted 10-cm glass funnel (mounted on a ring-stand) which was being flushed through the neck with 15 liters per min of 95% O_2–5% CO_2. The dishes remained sealed during incubation as judged by the absence of ^3H radioactivity on the dish or incubator surfaces.

Following incubation, the media were aspirated and the

cell layer was washed six times with isotonic saline prepared with 0.067 M phosphate buffer (pH 7.4). The cell layer was dissolved in 2.0 ml of 0.5 N KOH at 37°C for 15 min. The dishes were washed with three 2-ml aliquots of 95% ethanol containing a total of 1 mg of cholesterol carrier. After adjusting the KOH concentration of the combined KOH–ethanol digest to 0.6 N, the lipids were saponified for 1 hr at 70°C. In a preliminary experiment, the cells were released from the dishes by trypsinization prior to digestion in the alcoholic KOH. Nonsaponifiable lipids were extracted into hexane and the hexane extracts were washed with water until the washes were free of radioactivity. The digitonin-precipitable sterols (DPS) were prepared from the recovered nonsaponifiable fraction as described before (3). Digitonin quantitatively precipitates sterols such as cholesterol, desmosterol, and 7-dehydrocholesterol which possess a beta-hydroxyl group attached to carbon three (14). Cholesterol alone or cholesterol plus desmosterol accounted for essentially all of the sterol in the cultured lens cells. Cholesterol and desmosterol were considered identical in calculating rates of sterol synthesis from [^{3}H]water incorporated into DPS. The ^{3}H content of the DPS was measured by scintillation counting (counting error, 2σ, was about 2% or less). Counting efficiencies were determined by internal standardization and averaged about 40%. The dpm incorporated into DPS per dish at 37°C was corrected for that incorporated at 0°C. The 0°C incorporations were always only a few counts above background. The release of newly synthesized ^{3}H-labeled DPS to the incubation media was measured in one series of experiments. Here the media were recovered, centrifuged at about 1000 *g* for 10 min, and exhaustively dialyzed against isotonic saline to remove [^{3}H]water. The media were adjusted to contain 0.6 N KOH and 60% ethanol, 1 mg of cholesterol carrier was added, and the mixture was saponified for 1 hr at 100°C. The ^{3}H content of the DPS recovered from the media was measured and the 0°C values were subtracted from the 37°C values.

Rates of incorporation of ^{3}H of [^{3}H]water into total sterols were expressed as ng-atoms of ^{3}H incorporated into DPS per dish of cells per 7 hr. The rates of incorporation of ^{3}H into both DPS and total fatty acids were linear throughout the 7-hr incubation at 37°C (**Fig. 1**). This observation suggests that the availability of oxygen and other substrates to the cells was adequate to maintain constant metabolic activity throughout this period. Since NADPH generated by the pentose phosphate pathway does not become labeled with ^{3}H of [^{3}H]water (15), the observed linearity of ^{3}H incorporation into DPS over the 7-hr incubation also indicates that the balance of production of NADPH by the pentose phosphate pathway to production by oxidative enzymatic reactions was constant during this period. There was little difference in the amounts of ^{3}H incorporated into DPS between duplicate

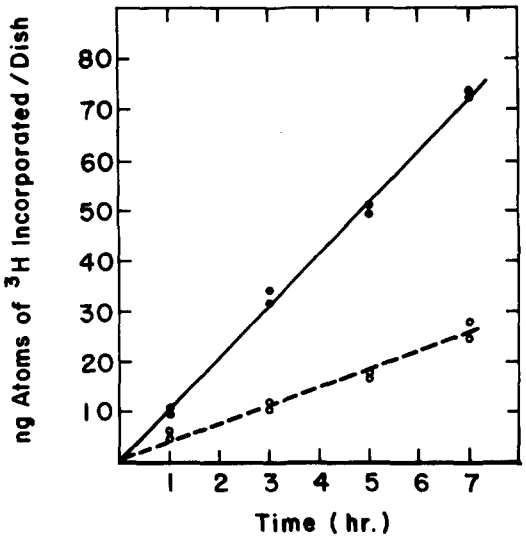

Fig. 1. Incorporation of [^{3}H]water into DPS (—●—) and total fatty acids (-- ○ --) versus time of incubation. Each point represents the incorporation by one dish of bovine lens epithelial cells (1.51 ± 0.06 (SEM) × 10^6 cells/dish, avg. eight dishes) cultured in DMEM containing 9% whole calf serum (by final volume). The specific activity of the media water was 10,910 dpm/μg atom of ^{3}H of [^{3}H]water.

dishes of cells (Fig. 1). At day 3 and day 4 of culture, cells released by trypsinization from dishes of cells identical to those used to measure sterol synthesis were counted by hemacytometer. Also, at these times, other identical dishes of cells not incubated with [^{3}H]water were digested in alcoholic KOH and saponified (as described above) and the recovered sterols were quantitated by gas–liquid chromatography (13). This chromatographic method readily separates cholesterol and desmosterol. The desmosterol peak in the cell sterol fractions was verified as being desmosterol by mass spectroscopy (data not shown). The changes in cell numbers and sterol content per dish were, therefore, estimated for the 24-hr interval during which the rates of sterol synthesis were simultaneously measured.

The absolute rates of sterol synthesis were estimated from the incorporation rates by making two assumptions. The first assumption was that the rates of incorporation of ^{3}H of [^{3}H]water measured over hours 14 to 21 of the 24-hr culture period were representative of the rates of sterol synthesis throughout the 24-hr interval. Since the number of cells per dish increased from about 30 to 60% over this 24-hr period (**Fig. 2**) and because the incorporation rates were not measured exactly in the middle of the 24-hr culture period, the calculated rates of sterol synthesis could be slightly high (we estimate 5 to 10% high). The second assumption is that 0.81 atoms of ^{3}H were incorporated into cholesterol per carbon atom of cholesterol. This ^{3}H/C incorporation ratio assumes that the cultured lens epithelial cells possess an active oxidative metabolism and thus all of the hydrogens of the NADPH generated

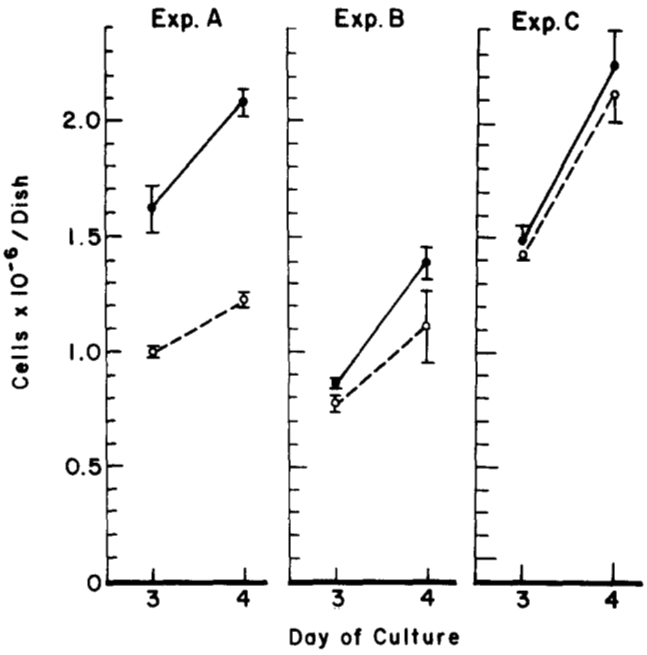

Fig. 2. Cell densities of lens epithelial cells cultured for 3–4 days in DMEM containing 9% whole calf serum, WM, (—●—) or 9% lipoprotein-deficient calf serum, LDM, (- - ○ - -). Cells were not exposed to lipoprotein-deficient media prior to this subculture. Each value is the mean ± SEM cell count of three or four dishes. The LDM contained 1.3 μg of cholesterol per ml in experiments A and B and <1 μg per ml in experiment C. The WM contained 103, 168, and 53 μg of cholesterol per ml in experiments A, B, and C, respectively. When whole calf serum was fractionated by ultracentrifugation at increasing densities, 12% of the total cholesterol was recovered from the fraction of density <1.063 g/ml, 53% was recovered in the d 1.063–1.10 g/ml fraction, and 35% in the d 1.10–1.21 g/ml density fraction. Rates of sterol synthesis by identical dishes of cells were measured usually over hours 14–21 of the 24-hr interval (Table 2). Cells were seeded on day zero and fed on days 1 and 3.

by these cells would equilibrate with ^3H of the [^3H]water substrate (15). Dietschy and Spady (16) report in their recent review that values of 0.84 and 0.81 were obtained for sterol synthesis by isolated hepatocytes and ovarian cells, respectively, from [^3H]water. The validity of our assumption was tested by comparing the rates of accumulation of sterol mass with the rates of incorporation of ^3H of [^3H]water into sterol by lens epithelial cells grown in the absence of lipoproteins. In this situation all of the sterol accumulated per dish of cells should come from de novo synthesis; the concentration of LDL-cholesterol in the lipoprotein-deficient media was negligible (about 0.1 μg cholesterol/ml). Using this approach, an experimentally estimated ratio of 0.79 was obtained (see Results).

Metabolism of lipoproteins by cultured lens epithelial cells

Bovine lipoprotein fractions corresponding to human LDL, HDL_2, and HDL_3 were radioiodinated with ^{125}I (New England Nuclear Corp., Boston, MA) by the method of Fidge and Poulis (17). Between 96% and 98% of the ^{125}I incorporated into all three fractions was protein-bound; 0.8 to 1.6% was free, and the remainder was bound to lipids. The specific activity of the preparations ranged from 94 to 137 cpm per ng of protein. Aliquots of each radioiodinated lipoprotein fraction were reduced with mercaptoethanol (5%, v/v) in 2% sodium dodecyl sulfate (w/v) and subjected to electrophoresis in 10% polyacrylamide gel rods with 4% stacking gels (4). The gels were either stained with Coomassie blue or sliced for determining distribution of ^{125}I among the apoprotein bands. Electrophoresis of the ^{125}I-labeled HDL_2 fraction, even after repurification, revealed an apoprotein pattern that suggested the presence of significant LDL contamination. Thus, the results of measurements of ^{125}I-labeled HDL_2 degradation are not reported. The apoprotein profiles of bovine LDL and HDL_3 were similar to those of their human counterparts (data not shown) (18).

Dishes of bovine epithelial cells subcultured since day zero in LDM were incubated on day 4 for 7 hr at 37°C in LDM supplemented with various concentrations of ^{125}I-labeled LDL or HDL_3. ^{125}I-Labeled lipoprotein-containing medium was also added to blank dishes and incubated at 37°C to measure cell-independent degradation. Degradation of labeled lipoproteins was measured by the appearance of non-iodide, non-trichloroacetic acid-precipitable ^{125}I in the media (19). The measurements were expressed as ng of lipoprotein-protein degraded per dish per 7 hr. Cell-independent degradation was subtracted from that measured for cells incubated at 37°C. No cell-dependent degradation of LDL and HDL was detected when lens epithelial cells were incubated at 4°C. Degradation of ^{125}I-labeled bovine LDL and HDL_3 was taken as an overall estimate of the ability of the cultured lens cells to metabolize these lipoproteins. Results of measurements of binding of the labeled lipoproteins by cells attached to the dishes are not reported in view of the difficulty in distinguishing between lipoproteins bound to the cells versus those nonspecifically bound to the plastic dishes.

Distribution of cholesterol among lipoprotein fractions of bovine aqueous humor

Duplicate 100-ml pools of bovine aqueous humor, collected as described previously (4), were concentrated to about 50 ml by Amicon filtration through a YM30 membrane. The density of the concentrated aqueous was initially adjusted to 1.063 g/ml with solid KBr–NaCl. Lipoprotein fractions were collected from the aqueous humor following serial ultracentrifugations (SW28 rotor at 28,000 rpm for 22–24 hr) at increasing densities; 1.063 g/ml, then 1.10 g/ml, then 1.21 g/ml, and finally 1.27 g/ml. The supernatant fraction (approximate upper 15% of the total volume) was collected after each centrifugation, dialyzed against isotonic saline, and lyophilized. The dried fractions were directly suspended in 2 ml of 95%

ethanol plus 0.25 ml of 6 N KOH containing 10 μg of 5α-cholestane as internal standard and saponified overnight at 70°C. The nonsaponifiable lipids were extracted into hexane and the cholesterol content was measured by gas-liquid chromatography (13).

RESULTS

Effect of serum lipoproteins on growth

Cells plated at 3×10^5 per dish on day zero reached about 1 to 1.5×10^6 cells per dish by day 3 and continued in rapid growth between days 3 and 4 of culture (Fig. 2). Plating and subculturing cells in media containing lipoprotein-deficient serum resulted in a slight (Fig. 2, Exp. B and C) to a significant reduction (Exp. A) of cell densities relative to cells cultured in whole media (WM); i.e., media containing 9% whole calf serum. Growth rates, percent increase in cell number per dish between days 3 and 4, were very similar for WM- and LDM-grown cells. Cells grown in WM accumulated more total sterol per dish than the LDM-grown cells and this difference was generally proportional to the higher densities of the WM-grown cells (**Table 1**). There were no statistically significant differences in the total sterol content per 10^6 cells between the WM- and LDM-grown cells at either day 3 or 4 of culture. However, desmosterol accounted for between 20 to 30% of the total sterol in the LDM-grown cells at both the third and fourth day of culture. Cholesterol was the only significant sterol present in the WM-grown cells, except in one experiment (Table 1, Exp. C, day 4) where desmosterol comprised about 12% of the total. The increase in total sterol mass by this group of WM-grown cells was also extremely large.

Rates of sterol synthesis

Incorporation of ^3H of [^3H]water into digitonin-precipitable sterols (DPS) was measured over a 7-hr interval between the third to fourth day of culture. Assuming a $^3H/C$ incorporation ratio (tritium-hydrogen atoms incorporated per carbon atom of the cholesterol molecule) of 0.81 and assuming that the rate of sterol synthesis measured over the 7-hr interval was descriptive of the rate over the day 3 to 4 culture period, absolute rates of sterol synthesis per 24-hr period of culture were estimated. There values were compared to the measured increases in sterol mass between day 3 to 4 of identical dishes (Table 1). Using this approach, we estimated that an average of 98% of the total sterol accumulated by the LDM-grown cells could be accounted for by de novo synthesis (**Table 2**). The validity of using the 0.81 $^3H/C$ ratio can be tested. Since biosynthesis is assumed to be the only source of sterol for the LDM-grown cells, an experimentally determined $^3H/C$ ratio can be calculated using the formula described in footnote *c* of Table 2 and substituting in the measured rates of incorporation of ^3H into DPS per dish of LDM-grown cells and the measured rates of accumulation of sterol mass per dish of these cells (difference between day 3 and 4). A ratio of 0.79 was thus obtained from the averages of experiments A, B, and C (0.73, 0.88, and 0.77, respectively). We assume that this $^3H/C$ ratio also applies to the cells incubated in whole media. In contrast to the LDM-grown cells, 73%, 82%, and 33% of the sterol mass accumulated by cells cultured in the presence of lipoproteins (WM) could be accounted for by de novo synthesis. When the synthesis was expressed per 10^6 cells, the WM-grown cells incorporated less ^3H into DPS in all three experiments (51.1, 42.5, and 51.5 ng atoms per 10^6 cells; Exp. A, B, and C, respectively) than the LDM-grown cells (72.8, 66.0, and 73.3 ng atoms per 10^6 cells; Exp. A, B, and C, respectively). The lower rates of sterol synthesis by the WM-grown cells presumably reflect the availability of lipoprotein-cholesterol in the whole calf serum. The ability of lipoproteins to affect the rate of sterol synthesis was directly tested.

TABLE 1. Sterol content of lens epithelial cells cultured in the absence and presence of lipoprotein-deficient serum

Culture Conditions[a]	n	μg Sterol/Dish[b] Day 3	n	Day 4	μg Accumulated per Dish per 24 hr
Exp. A					
WM	4	25.94 ± 0.29 (0)	4	34.82 ± 1.01 (0)	8.88
LDM	3	12.18 ± 1.37 (33)	4	18.20 ± 1.44 (29)	6.02
Exp. B					
WM	3	11.55 ± 0.19 (0)	3	15.89 ± 0.75 (2)	4.34
LDM	3	6.03 ± 0.16 (21)	3	10.09 ± 0.23 (22)	4.06
Exp. C					
WM	3	17.43 ± 1.29 (0)	3	38.86 ± 3.18 (12)	21.43
LDM	3	18.25 ± 1.01 (28)	3	28.14 ± 1.27 (32)	9.89

[a] Cells were cultured in DMEM containing 9% (by final volume) whole calf serum (WM) or 9% (by final volume) lipoprotein-deficient calf serum (LDM).
[b] Each value is the means ± SEM of three to four dishes. The values in parentheses show the percent of total sterol that eluted as desmosterol in GLC.

TABLE 2. Correlation of rates of sterol synthesis with rates of sterol accumulation by bovine lens epithelial cells in culture

Culture Conditions[a]	ng-Atom of ^3H of [^3H]Water Incorporation[b]	μg of Sterol Synthesized[c]	$\frac{\mu g \text{ Synthesized/Dish}}{\mu g \text{ Accumulated/Dish}}$[d]
	DPS per dish per 7 hr	*per dish per 24 hr*	
Exp. A[e]			
WM	106.2 ± 3.8	6.44	$0.73 \left(\frac{6.44}{8.88}\right)$
LDM	89.2 ± 4.3	5.41	$0.90 \left(\frac{5.41}{6.02}\right)$
Exp. B[e]			
WM	57.9, 59.9 } 58.9	3.57	$0.82 \left(\frac{3.57}{4.34}\right)$
LDM	73.9, 71.9 } 72.9	4.42	$1.09 \left(\frac{4.42}{4.06}\right)$
Exp. C[f]			
WM	116.7, 116.9 } 116.8	7.08	$0.33 \left(\frac{7.08}{21.43}\right)$
LDM	155.1 ± 1.3	9.40	$0.95 \left(\frac{9.40}{9.89}\right)$

[a] Culture conditions were identical to those described in Table 1.

[b] Values given as dpm ^3H incorporated into DPS ÷ specific activity of media water; 9440 dpm/μg atom of ^3H of [^3H]water (Exp. A), 9730 dpm/μg atom ^3H (Exp. B), 10,200 dpm/μg atom ^3H (Exp. C). Values are the mean ± SEM of three dishes or the average of two dishes (individual values shown).

[c] Assuming all of the NADPH generated by the cells equilibrated with ^3H of [^3H]water, 0.81 atom of ^3H would be incorporated per C atom of cholesterol. Rates of synthesis per 24 hr would be the ng-atom of ^3H incorporated per 7 hr × 3.429 ÷ (0.81 × 27) × 0.38664 μg per nmol of cholesterol.

[d] Values from Table 1.

[e] The rates of incorporation of ^3H into DPS were measured between hour 14–15 to 21–22 of the 3rd to 4th day of culture (24-hr period).

[f] The rates of incorporation of ^3H into DPS were measured between hour 22 to 29 of the 3rd to 4th day of culture (24-hr period). Cells were released from the plates by mild trypsinization prior to digestion in alcoholic KOH in this experiment.

Effect of lipoproteins on sterol synthesis: metabolism of lipoproteins

Lens epithelial cells were cultured in LDM supplemented with various concentrations of bovine lipoprotein fractions. The fractions corresponded to human LDL (d 1.02–1.06 g/ml) and HDL_3 (d 1.10–1.21 g/ml). Sterol synthesis was markedly decreased in the presence of LDL (**Fig. 3**). Twenty-five μg (apoprotein)/ml of LDL reduced incorporation of [^3H]water into DPS by about 50% (Fig. 3). In contrast, sterol synthesis was increased in the presence of 25 μg/ml of HDL_3 and decreased at only high HDL_3 levels (>100 μg/ml). The ability of these lipoprotein fractions to inhibit sterol synthesis by the lens epithelial cells correlated closely with the ability of the cells to degrade these lipoproteins. Cells cultures in LDM readily degraded ^{125}I-labeled LDL (**Fig. 4**). No appreciable degradation of HDL_3 was observed except at the highest concentration studied and even here it was only one-tenth of that seen with LDL. Adding a 20-fold excess of bovine LDL to the ^{125}I-labeled LDL (10 μg/ml) decreased degradation by 89%.

Release of newly synthesized sterol

When the lens epithelial cells were incubated in media prepared with whole calf serum, about 20% of the total ^3H-labeled DPS formed by the cells was recovered from the media (**Table 3**). Incubating cells with 50 μg/ml of HDL_3 (expressed as protein) resulted in a sharp enhancement of sterol synthesis and also in a significant recovery of labeled sterol from the media. In contrast to these situations, little ^3H-labeled DPS was found in the media following incubation of the cells in media prepared with lipoprotein-deficient serum.

Distribution of lipoprotein classes in aqueous humor

Bovine aqueous humor was found to contain about 0.7 μg of cholesterol per ml (**Table 4**); we previously reported a cholesterol content of 1 μg/ml (4). About 95% of the total cholesterol in aqueous humor was recovered at densities corresponding to high density lipoproteins. This is also similar to our earlier finding. We now recognize that HDL_3 and very high density lipoprotein fractions

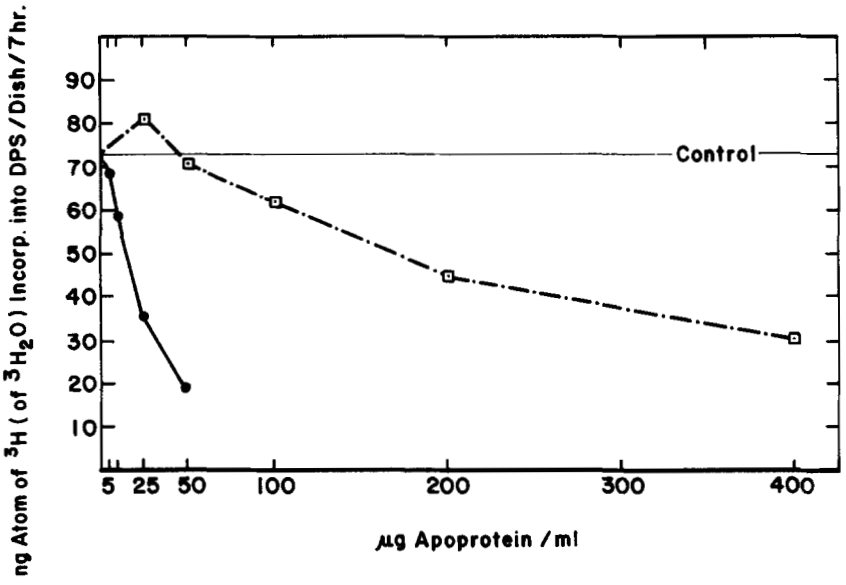

Fig. 3. Effects of bovine serum lipoprotein fractions upon incorporation of ^3H of [^3H]water into DPS by lens epithelial cells in culture. On the third day of culture in LDM (DMEM containing 9% lipoprotein deficient serum), bovine lipoproteins corresponding to LDL (—●—) or HDL_3 (- · - ⊡ - · -) were added at various concentrations. Fourteen-15 hr later the media were replaced with identical media prepared with [^3H]water (1.5 mCi/ml) and containing the same lipoproteins at the same concentrations used during the preincubation period. Each point represents the incorporation of ^3H into DPS by one dish of cells (avg. 1.11 ± 0.17 (SEM) × 10^6 cells/dish) during a 7-hr incubation at 37°C. These values were corrected for incorporation measured at 0°C. The dishes of control cells incorporated an average of 72.9 (73.9 and 71.9) ng atoms of ^3H of [^3H]water into DPS per dish.

account for about 50% and 30%, respectively, of the total lipoprotein cholesterol in bovine aqueous humor (Table 4).

DISCUSSION

This study describes a method for measuring absolute rates of sterol synthesis by cultured cells using [^3H]water. In using [^3H]water to estimate absolute rates of sterol synthesis by the cultured lens epithelial cell, we assumed that all the NADPH generated by the cultured cells equilibrated with the ^3H of the [^3H]water; a situation requiring that NADPH be formed by oxidative enzymatic reactions (15). In this situation 0.81 atoms of ^3H would be incorporated into cholesterol per carbon atom of cholesterol (15). We tested this assumption by measuring the changes in sterol mass per dish of cells grown in lipoprotein-deficient media over a 24-hr culture period and by simultaneously measuring the rates of incorporation of [^3H]water into digitonin-precipitable sterols for this period. The net accumulation of sterol per dish of cells cultured in the absence of lipoproteins should directly reflect the rate of sterol synthesis. We calculated that an average of 0.79 atom of ^3H of [^3H]water was incorporated per carbon atom of cholesterol. This value is similar to those obtained by others for tissues incubated in vitro with [^3H]water or heavy water. A ^3H/C ratio of 0.88 is obtained from the average of several studies of [^3H]water incorporation into cholesterol by liver slices (16). A ratio of 0.84 was calculated by Dietschy and Spady (16) for incorporation by isolated rat hepatocytes based upon data reported by Pullinger and Gibbons (20). Using D_2O to measure rates of cholesterol synthesis by ovarian cells in culture, Esterman, Cohen, and Javitt (21) recently estimated that deuterium was incorporated into all 22 theoretically labeled positions in cholesterol; i.e., 0.81 deuterium atoms were incorporated per carbon atom of cholesterol.

When lipoproteins were available to the cells from whole calf serum, the rates of incorporation of [^3H]water into total DPS by the cell layer decreased and, in two of three experiments, the estimated rates of synthesis were adequate to account for between 70 to 80% of the observed increase in sterol mass. The quantitative importance of biosynthesis as a source of sterol to cells grown in whole media (WM) will be underestimated unless one considers that about 20% of the labeled sterol formed by the cells cultured in WM was recovered from the media following a 7-hr incubation (Table 3); there was negligible release of ^3H-labeled sterol by cells to lipoprotein-deficient media (LDM). Even accounting for the release of newly synthesized sterol, the LDM-grown cells still incorporated about 30% more ^3H into total sterol than the WM-grown cells (Table 3). The higher rates of sterol synthesis by LDM-grown cells is also reflected by the presence of significant amounts of desmosterol in these cells (Table 2). The conversion of desmosterol to

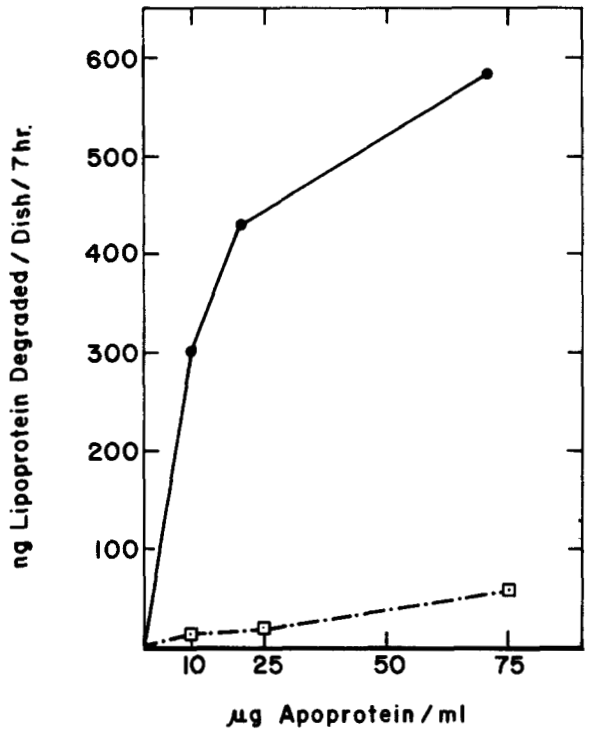

Fig. 4. Degradation of ^{125}I-labeled bovine lipoprotein fractions by lens epithelial cells in culture. Following 4 days of culture of bovine epithelial cells in LDM, the media was changed to LDM supplemented with various concentrations of ^{125}I-labeled bovine lipoprotein fractions of densities 1.02–1.063 g/ml (^{125}I-LDL, —●—) or 1.10–1.21 g/ml (^{125}I-HDL$_3$, -·-□-·-). Dishes containing an average of 1.03 ± 0.05 (SEM) × 10^6 cells and blank dishes were incubated for 7 hr at 37°C. Degradation was measured by the appearance of non-iodide, non-trichloroacetic acid-precipitable ^{125}I in the media. Cell-independent degradation was subtracted from cell-dependent degradation at 37°C. Each point is the average degradation by two dishes of cells. The specific activities of the lipoprotein fractions were 94 cpm/ng protein (LDL) and 137 cpm/ng protein (HDL$_3$). Between 96–98% of the ^{125}I in each lipoprotein fraction was protein-bound.

cholesterol can be a rate-limiting step in cholesterol formation by cultured cells that actively synthesize cholesterol (22). Although the lens epithelial cells responded to the absence of lipoproteins by increasing the rate of sterol synthesis, the increase was modest compared to other cell types (23, 24).

Finding slightly lower cell densities when lens epithelial cells werre cultured in LDM versus WM could reflect that sterol synthesis, although enhanced in the absence of lipoproteins, was not totally adequate to supply all of the sterol potentially required by these rapidly growing cells. Exogenous lipoproteins could be necessary to fill the gap between the requirement and that furnished by de novo synthesis. This possibility is supported by finding slightly higher cell densities when cells were cultured in whole media, by finding that sterol synthesis of the lens epithelial cell was inhibited in the presence of low concentrations of LDL, and by finding that the cells readily metabolize ^{125}I-labeled LDL. Cells grown in media prepared with whole serum were exposed to between about 50 to 150 µg of cholesterol per ml; LDL accounted for about 10% of the total lipoproteins in this media. Addition of 10 µg/ml of bovine LDL (as apoprotein) to the lipoprotein-deficient media produced about a 20% decrease in the rates of sterol synthesis (Fig. 3). Thus, the lower rates of sterol synthesis observed for the WM-grown cells could be totally explained by the LDL present in the whole calf serum used to prepare the whole media. Sterol synthesis by the cultured lens cells was also sensitive to the presence of bovine HDL$_3$. HDL$_3$ stimulated sterol synthesis by the lens cells (Fig. 3) perhaps secondary to enhancing removal of sterol from the cells (Table 3), an action of HDL$_3$ seen with cultured fibroblasts (25). Little HDL$_3$ was metabolized by the lens epithelial cells.

The results of this study demonstrate that lens epithelial cells in rapid growth can supply most of their sterol requirement by de novo synthesis and that the NADPH available for sterol synthesis is generated by oxidative enzymatic processes. The ability of the lens epithelial cell to thrive in a lipoprotein-deficient media might be expected for a cell population that must actively grow, divide, and differentiate in an environment naturally deficient in lipoproteins. Thus, we were surprised by the clear ability of the lens cells to metabolize LDL and to down-regulate its sterol synthesis in response to the availability of LDL. In view of the apparent commitment of the lens epithelia to supplying its required cholesterol by cholesterogenesis, the lens epithelial cell in culture could provide an interesting model for examining the relationship between cholesterogenesis and DNA replication and

TABLE 3. Release of newly synthesized cholesterol (digitonin-precipitable sterols) to the media in the presence and absence of lipoproteins

Culture Conditions[a]	ng-Atom of ^{3}H of [^{3}H]Water Incorporation[b]		
	Cells	Media	Total
	DPS per dish per 7 hr incubation		
WM	31.9	8.7 (21)	40.6
	37.0	8.7 (19)	45.7
LDM	55.2	2.3 (4)	57.5
	52.4	1.9 (3)	54.3
LDM + HDL$_3$[c]	70.0	6.9 (9)	76.9
	72.2	8.7 (11)	80.9

[a] Cells cultured in DMEM containing 9% whole calf serum, WM, (0.92 ± 0.06 × 10^6 cells/dish) or in DMEM containing 9% lipoprotein-deficient serum, LDM, (0.94 ± 0.05 × 10^6 cells/dish) were incubated for 7 hr in these respective media possessing [^{3}H]water at a specific activity of 15,210 dpm/µg atom of ^{3}H of [^{3}H]water. All sets of values are for individual dishes. Values in parentheses are % of total ^{3}H-labeled DPS synthesized.

[b] Cells and media were separately assayed for ^{3}H-labeled DPS content. The media were first exhaustively dialyzed to remove [^{3}H]water, saponified, and the nonsaponifiable lipids were extracted.

[c] The lipoprotein-deficient media were supplemented with 50 µg/ml of HDL$_3$ (as protein).

TABLE 4. Distribution of cholesterol in bovine aqueous humor by density fraction

Pool	Density Fractions, g/ml				
	<1.063	1.063–1.10	1.10–1.21	1.21–1.27	Total
	μg cholesterol/100 ml aqueous humor				
I	3.91	14.93	41.09	19.38	79.31
II	3.72	9.63	33.00	22.43	68.78
Average	3.82	12.28	37.05	20.91	74.05
	(5.2)[a]	(16.6)	(50.0)	(28.2)	

[a] Percent of total cholesterol.

cell division. Since development of the lens is dependent upon the activity of the monolayer of epithelial cells, factors that influence the growth and division of these epithelial cells could exert control over development of the entire lens. In addition to cholesterogenesis, other areas of lipid metabolism could participate in regulating lens growth. For example, Zelenka (26) demonstrated that the rate of turnover of phosphatidylinositol correlated with the rate of differentiation of epithelial cells of embryonic chick lens in vivo.

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