Comparison of the metabolism of cholesterol, cholestanol, and β-sitosterol in L-cell mouse fibroblasts

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ABSTRACT The following data have been obtained from comparative studies on the metabolism of cholesterol, cholestanol, and β-sitosterol by L-cell mouse fibroblasts. (*1*) When the sterols are added to the growth medium under similar conditions, cellular incorporation of cholesterol > cholestanol > β-sitosterol; (*2*) only limited cellular esterification of these compounds occurs; (*3*) no metabolic products arising from the sterols could be detected; (*4*) influx of all sterols is dependent upon the concentration; and (*5*) exogenous cholesterol reduces mevalonate incorporation into cellular sterol to a lesser extent than acetate or glucose. The metabolism of these sterols is discussed in relation to their ability to influence de novo sterol biosynthesis.

SUPPLEMENTARY KEY WORDS desmosterol · sterol synthesis · feedback inhibition

L-CELL mouse fibroblasts grown in the absence of exogenous sterol can synthesize sterol at levels sufficient to allow continued growth (1–4). The major sterol synthesized by these cells is desmosterol (5). When exogenous cholesterol is added to the culture medium, this sterol is incorporated into the cells and elicits a sterol feedback response which blocks acetate incorporation into cellular sterols. Exogenous cholesterol then serves as a sterol source for cellular growth (1).

Some sterols, such as coprostanol and Δ^4-cholestenone, inhibit de novo sterol synthesis, as well as cell growth, whereas C_{28} and C_{29} phytosterols (β-sitosterol, stigmasterol, campesterol, and ergosterol) and cholestanol have no pronounced effect on either de novo synthesis of desmosterol or cell growth (6). The cellular metabolism of some sterols of this latter group has been studied in an effort to determine why such sterols do not inhibit sterol biosynthesis in L cells.

MATERIALS AND METHODS

Methods for the cultivation of L-cell mouse fibroblasts in a sterol-free medium containing delipidized proteins have been described previously (1, 5). All stock cultures were maintained in a medium containing 5 mg/ml of delipidized calf serum protein.

Cholesterol-4-^{14}C (50 mCi/mmole), cholesterol-7α-^{3}H (10 mCi/mmole), cholestanol-4-^{14}C (40 mCi/mmole), and β-sitosterol-22,23-^{3}H (30 Ci/mmole) were purchased from New England Nuclear Corp., Boston. All of the labeled sterols were purified by thin-layer chromatographic methods immediately prior to use. The labeled sitosterol used in these experiments contained 5–10% labeled stigmasterol. Unlabeled cholesterol and β-sitosterol were purified by crystallization three times from ethanol.

GLC analysis on XE-61 and SE-30 columns showed that β-sitosterol contained a second sterol (approximately 10%) corresponding to campesterol, while cholesterol

Systematic names of the sterols referred to in the text by their trivial names are as follows: cholesterol, cholest-5-en-3β-ol; 7-dehydrocholesterol, cholesta-5,7-dien-3β-ol; lathosterol, cholest-7-en-3β-ol; cholestanol, 5α-cholestan-3β-ol; coprostanol, 5β-cholestan-3β-ol; desmosterol, cholesta-5,24-dien-3β-ol; ergosterol, cholest-5,7,22-trien-24-methyl-3β-ol; stigmasterol, cholesta-5,22-dien-24-ethyl-3β-ol; sitosterol, cholest-5-en-24α-ethyl-3β-ol; campesterol, cholest-5-en-24β-ethyl-3β-ol; cholestanone, 5α-cholestan-3-one; Δ^4-cholestenone, cholest-4-en-3-one.

Abbreviations: GLC, gas–liquid chromatography; TLC, thin-layer chromatography.

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gave a single peak. Purified cholestanol exhibited only one peak upon GLC analysis. GLC analysis was conducted with a Glowall model 310 equipped with an argon ionization detector. The following columns were used: 80–100 mesh Supelcoport coated with 3% SE-30 (6-ft column, 235°C), and 100–120 mesh Supelcoport coated with 3% XE-61 (6-ft column, 235°C); the inlet pressure was 25 psi.

All lipid extractions were made with chloroform–methanol 2:1 (v/v) according to the method of Folch, Lees, and Sloane Stanley (7). Lipids were saponified in 10% ethanolic KOH at 60°C for 30 min, followed by extraction of the aqueous layer once with 3 vol of petroleum ether and twice with 3 vol of diethyl ether, and washing of the pooled extracts with water. Sterols were precipitated with digitonin by the method of Sperry and Webb (8). Digitonin precipitates were assayed for radioactivity by dissolving the dried precipitates in methanol (9) and counting in a Packard liquid scintillation spectrometer, using 0.6% 2,5-diphenyloxazole and 0.02% dimethyl-1,4-bis-2-(5-phenyloxazolyl)benzene in toluene as the scintillator.

Sodium acetate-2-^{14}C (2 mCi/mmole) was obtained from New England Nuclear Corp. and made to a final specific activity of 24.5 μCi/mmole. Glucose-U-^{14}C (3.1 mCi/mmole) and DL-mevalonic acid-2-^{14}C lactone (5.85 mCi/mmole) were purchased from Amersham/Searle Corp., Des Plaines, Ill. Glucose-^{14}C was added at a level of 5 μCi/20 ml to the tissue culture medium, which contained unlabeled glucose, to give a specific activity of 13.5 μCi/mmole. The glucose concentration was determined by use of glucose oxidase (Glucostat, Worthington Biochemical Corp., Freehold, N.J.). The mevalonic acid-2-^{14}C was prepared to give a final specific activity of 430 μCi/mmole. All solutions were filtered through a 0.45-μ Millipore filter before they were added to the growth medium. Egg lecithin (98% pure) was purchased from Supelco, Inc., Bellefonte, Pa. Protein was assayed by the method of Lowry et al. (10). All solvents were reagent grade, and all evaporation procedures were carried out under nitrogen.

Thin-layer chromatography was performed using silica gel H plates impregnated with 12.5% silver nitrate and a solvent system of chloroform–acetone 95:5 (v/v) (11) or on silica gel G plates developed with petroleum ether–diethyl ether–acetic acid 75:25:1 (v/v/v). Radioactive regions were detected by scanning the plates in a Packard radiochromatogram scanner. The quantity of radioisotope in each region was estimated by removal of the gel from a given region of the plate and elution of the labeled compounds with 20-ml volumes of benzene (2×) and of acetone (2×). Aliquots of the eluted lipid were added to a liquid scintillation mixture and the radioactivity was determined.

Sterol Uptake

The general procedures used for the preparation of tissue culture media containing radioactive sterols have been described previously (1, 6, 12). In all uptake experiments, the medium contained delipidized calf serum protein at a level of 5 mg/ml, to which was added 20 μg/ml of lecithin and labeled sterols of known specific activity, either individually or in combination at the levels indicated in the text. Phospholipid and sterols were added in ethanol, and the mixture was dialyzed against tissue culture medium, under aseptic conditions, to reduce the alcohol concentration to 0.5% (1).

Petri plates (60 mm) were seeded with an inoculum of 7.5×10^5 cells and incubated in an atmosphere of 5% CO_2 for 2 days in a medium containing delipidized protein. After the monolayers had been initiated, this medium was removed and replaced with fresh culture fluid containing labeled sterol. At intervals during the next 48 hr, duplicate plates were removed and the cells were assayed for sterol incorporation. The medium containing labeled sterol was removed and the cells were washed by addition of 5 ml of fresh medium to the monolayer. Cells were then detached from the plates by the addition of trypsin and were washed twice by centrifugation (5000 rpm for 5 min) in 10 ml of buffered salt solution (13). The cell pellet was suspended in 2.5 ml of distilled water and disrupted by sonication in a Branson Sonifier model S125 (setting 2 for 30 sec). Samples of this cell homogenate were removed for protein determinations and 1 ml of the homogenate was assayed for radioactivity by liquid scintillation techniques, using 10 ml of Bray's solution (14). All counts were corrected for quenching. The data are expressed as micrograms of the labeled sterol recovered from the cell per milligram of cellular protein.

Cellular Fractionation

Isotopically labeled cells were obtained for fractionation studies by growing the cells for 48 hr in medium containing combinations of either cholesterol-4-^{14}C and β-sitosterol-22,23-^{3}H, or cholesterol-7α-^{3}H and cholestanol-4-^{14}C, of known specific activity. Each sterol was added to the growth medium at a level of 20 μg/ml, together with 5 mg/ml delipidized calf serum protein and 20 μg/ml of lecithin. The levels of labeled sterols in the growth medium, whole cells, and subcellular fractions were assayed after extraction with chloroform–methanol 2:1 (v/v).

Plasma membranes were obtained from L cells after disruption of fluorescein–mercuric acetate-treated cells in a Dounce homogenizer fitted with a tight pestle (35 strokes) (15). Purification of the plasma membrane fraction by centrifugation in sucrose solutions was accomplished using the method of Warren, Glick, and Nass

(15). The material that sedimented through 45% sucrose upon the initial centrifugation at 550 *g* for 30 min was considered nuclear material and is designated "nuclear (FMA)" in Tables 3 and 4. A second nuclear fraction was obtained from untreated cells disrupted in a Dounce homogenizer (10×) in 10 mM hydroxymethyl(tris)-aminomethane (Tris) buffer, pH 7.4, containing 10 mM $MgCl_2$ and 2 mM $CaCl_2$. The material that sedimented through 50% sucrose after centrifugation at 550 *g* for 30 min (16) is designated "nuclear (Dounce)" in Tables 3 and 4.

A third nuclear fraction, together with microsomal, mitochondrial, and high-speed supernatant fractions, was obtained by nitrogen cavitation techniques (16). A cell homogenate in 10 mM Tris buffer (pH 7.4) containing 10 mM $MgCl_2$ and 2 mM $CaCl_2$ was obtained by N_2 cavitation (500 psi for 15 min) and was centrifuged at 800 *g* in a Sorvall SS-34 rotor for 5 min. The pellet obtained was layered over 50% sucrose and centrifuged at 21,500 *g* in a Sorvall HB-4 swinging bucket rotor. This pellet was designated "nuclear (N_2)." The 800 *g* supernatant fraction was recentrifuged at 16,000 *g* for 7 min in a Sorvall SS-34 rotor. The pellet was assumed to be the mitochondrial fraction. The supernatant fraction was then recentrifuged in a Spinco 40 rotor at 100,000 *g* for 45 min. The resulting microsomal pellet and high-speed supernatant were then analyzed for radioactivity.

RESULTS

Inhibition of Sterol Biosynthesis

Previous experiments have demonstrated that exogenous cholesterol effectively inhibits incorporation of acetate into cellular digitonin-precipitable sterol (1, 6). The present experiments were conducted to determine the effect of exogenous cholesterol on mevalonic acid, glucose, and acetate incorporation into sterols and thus to gain information on the nature of the metabolic block. The detailed procedures used in this study have been presented in previous publications (1, 6). Monolayers of L cells were exposed for 2 days to medium containing delipidized protein (5 mg/ml), lecithin (20 μg/ml), and cholesterol (30 μg/ml). Control cultures were grown in similar medium minus the sterol. The cells were incubated for the final 24 hr in the presence of labeled precursors of known specific activity at the levels indicated in Table 1. Preliminary experiments had demonstrated that the incorporation of the three precursors into cellular sterol was continuous throughout the 24-hr incubation period. A twofold increase in the concentration of precursor resulted in no significant increase in the incorporation of label from either acetate or mevalonate and only a 20% increase with glucose. At the end of this period, cells were harvested. The digitonin-precipitable sterols from the cells and media were prepared and assayed for radioactivity. The data indicate the amount of precursor incorporated by the cells into sterols during a 24-hr growth period, both in the absence and presence of exogenous cholesterol. Table 1 shows that exogenous sterol effectively inhibits the incorporation of acetate (64–84%) and glucose (70–77%) into cellular sterols. The inhibition of mevalonate incorporation was not as pronounced, with the values of the two experiments being 25 and 13%.

TABLE 1 INCORPORATION OF LABELED PRECURSORS INTO L-CELL STEROL WITH AND WITHOUT EXOGENOUS CHOLESTEROL

Precursor	Exogenous Cholesterol 30 μg/ml	Precursor in Synthesized Sterol	Inhibition
		μg/mg of protein	*%*
Expt. 1			
Acetate-2-^{14}C	−	6.16	0
	+	0.97	84.3
Glucose-U-^{14}C	−	5.33	0
	+	1.22	77.1
Mevalonate-2-^{14}C	−	0.24	0
	+	0.18	25.0
Expt. 2			
Acetate-2-^{14}C	−	5.29	0
	+	1.92	63.7
Glucose-U-^{14}C	−	2.87	0
	+	0.85	70.4
Mevalonate-2-^{14}C	−	0.24	0
	+	0.21	12.5

Values are averages of duplicate determinations. Glucose was present in all cultures at a level of 665 μg/ml. Acetate was present at 250 μg/ml and mevalonate at 33 μg/ml.

The pronounced inhibition of acetate and glucose incorporation into sterol could result not only from a specific block in the metabolic pathway, but also from less specific changes in glucose and acetate permeability, resulting in reduced cellular incorporation. To test this possibility, aliquots of whole cells (experiment 2, Table 1) were assayed for the total amount of labeled precursor in the cells. The results indicated that in the presence of exogenous cholesterol, incorporation of acetate into the cell was reduced by 16.2%, and that of glucose by 1.4%, whereas mevalonate incorporation showed no reduction.

Incorporation of Exogenous Sterols

One reason some sterols, when added to the culture medium in the experimental conditions previously described (6), do not depress cellular sterol synthesis may be that these sterols are not incorporated by the cells. For this reason, the incorporation of cholestanol-4-^{14}C, cholesterol-4-^{14}C, and 3β-sitosterol-22,23-^{3}H into L cells over a 48-hr period was measured. The results from these experiments are shown in Fig. 1. Cholesterol is incorporated into washed cells to the greatest extent, while

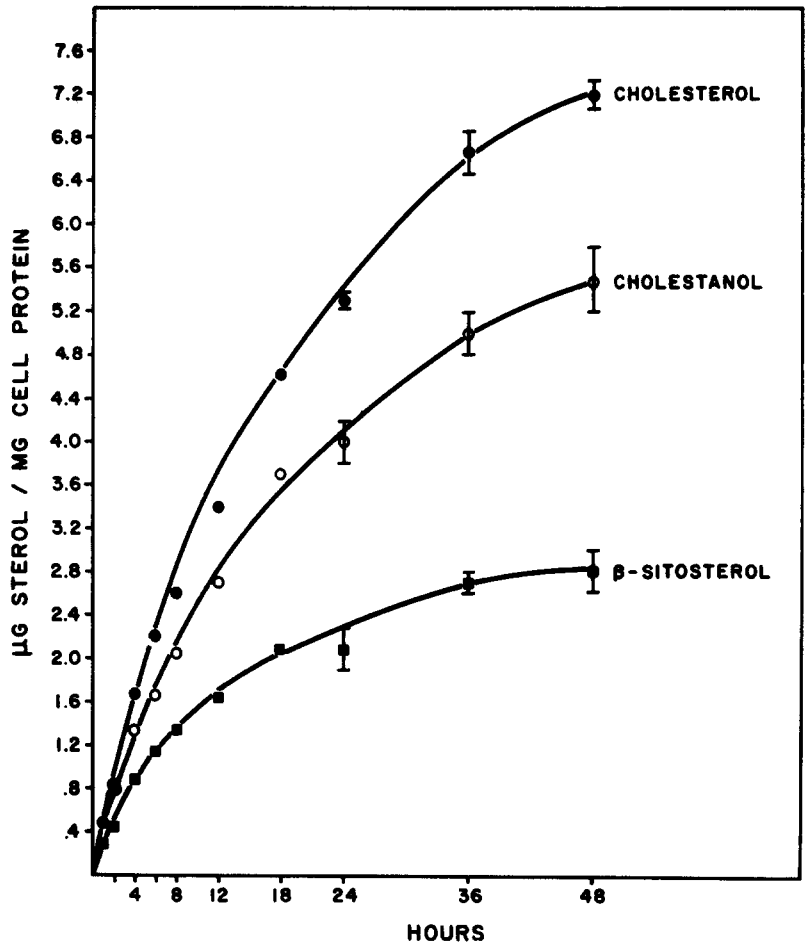

FIG. 1. Uptake of sterol by L cells. Incubation medium contained delipidized calf serum protein (5 mg/ml), lecithin (20 μg/ml), and sterol (20 μg/ml). Averages of four determinations. The symbol (I) indicates standard error.

cholestanol incorporation after 48 hr is about two-thirds that of cholesterol. β-Sitosterol is incorporated at the slowest rate, approximately one-third that of cholesterol after 48 hr.

Fig. 2 demonstrates the effect of increasing the concentration of exogenous sterol on cellular sterol accumulation. At all concentrations tested, the same pattern of results was obtained as previously described with cholesterol, cholestanol, and β-sitosterol. Increasing the sterol concentration in the medium resulted in an increase in cellular sterol incorporation for all three sterols tested.

When cells were grown in a medium containing cholesterol in combination with either cholestanol or β-sitosterol, there was a mutual reduction in sterol incorporation compared with the incorporation found when these sterols were present individually (Fig. 3). The most pronounced reductions were observed with the cholesterol/β-sitosterol combination and these reductions were evident after both 24- and 48-hr incubations.

Metabolism of Cellular Sterol

The inability of cholestanol and β-sitosterol to inhibit endogenous sterol synthesis could be related to the metabolism of these sterols, resulting in either oxidation or esterification.

Because of the relatively small percentage of total cholestanol or β-sitosterol in the medium incorporated by the cells (<3%), quantitative recovery experiments utilizing lipid extraction, saponification, and digitonin precipitation were not attempted. Therefore, thin-layer chromatographic analyses were used to assay both the cells and medium for the appearance of labeled compounds other than the cholestanol-4-^{14}C or β-sitosterol-22,23-^{3}H present initially in the culture medium. Total lipid, nonsaponifiable lipid and digitonin-precipitable sterols from both cells and growth medium were monitored by TLC, using the silica gel H–silver nitrate system (11). Previous experiments had indicated that if 5% of the total counts applied to the plates had a mobility different from that of the original compound, it could be

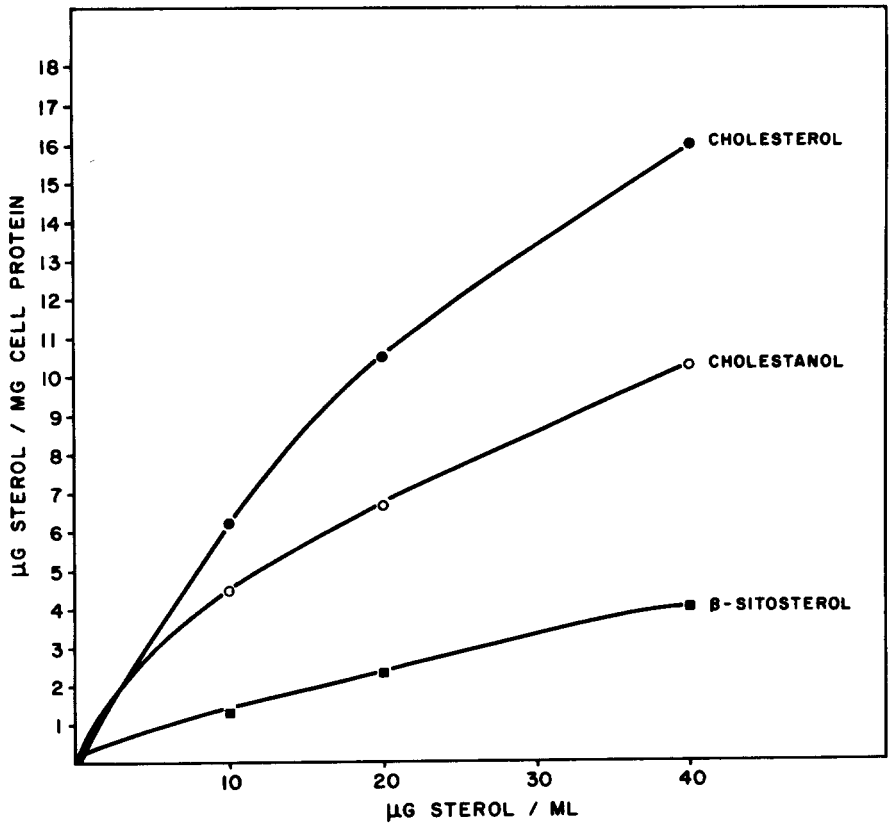

FIG. 2. Effect of increasing sterol concentration on cellular sterol incorporation. Medium contained delipidized serum protein (5 mg/ml), lecithin (20 µg/ml), and labeled sterol as indicated. Incubation time 48 hr. Averages of two determinations.

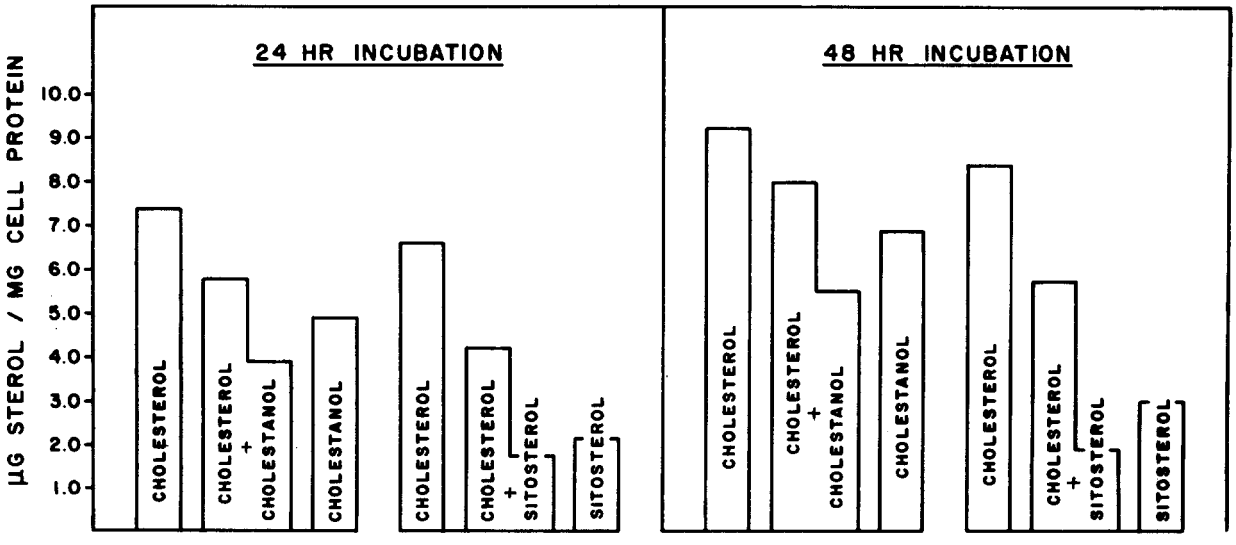

FIG. 3. Uptake of sterol mixtures by L cells. Incubation medium contained delipidized calf serum protein (5 mg/ml), lecithin (20 µg/ml), and sterols (20 µg/ml). Averages of two determinations.

detected. Only a single radioactive region was found, which corresponded to the original sterol when both cholestanol and β-sitosterol were employed. Previous studies had indicated that cholesterol is not metabolized by L cells (17).

TLC techniques were used to determine the extent of cellular esterification of exogenous sterols by L cells. In these experiments, the total lipid from cells grown in medium containing labeled cholesterol, cholestanol, or β-sitosterol was applied to silica gel G plates and developed

in a solvent system consisting of petroleum ether–diethyl ether–acetic acid 75:25:1. After development of the plates the silica gel in the regions corresponding to free and esterified sterol were removed, and the amount of labeled sterol was determined after elution of the sterol with benzene and acetone. In a separate set of experiments the amount of synthesized sterol recovered as ester was determined in cells grown for 48 hr in the presence of sodium acetate-2-^{14}C. In these latter experiments the free and esterified sterols were separated by TLC, saponified, and precipitated with digitonin prior to liquid scintillation spectrometry.

The degree of esterification, as indicated in Table 2, was low in all cases. These results indicate that active oxidation or extensive esterification of the sterols is not taking place in this system, and the lack of a response therefore cannot be attributed to metabolic alteration of the sterol molecule.

Sterol Distribution in Subcellular Fractions

Since it was established that β-sitosterol and cholestanol were incorporated by the cells at rates slower than cholesterol, it was of interest to determine if any of the sterols under study were preferentially incorporated into various subcellular fractions. The preferential exclusion of β-sitosterol and cholestanol from a subcellular fraction could explain the lack of sterol feedback response to these sterols. In these studies, cells were grown for 48 hr in medium containing either cholesterol-4-^{14}C and β-sitosterol-22,23-^{3}H, or cholesterol-7α-^{3}H and cholestanol-4-^{14}C, of known specific activity. In each experimental medium the two sterols were present at levels of 20 μg/ml. The ratios of cholesterol-^{14}C to β-sitosterol-^{3}H and cholesterol-^{3}H to cholestanol-^{14}C in the original incubation medium, whole cells, and various subcellular fractions were determined, and the data are presented in Tables 3 and 4.

As can be seen in Table 3, whole cells had a cholesterol/cholestanol ratio of 1.34, again indicating the preferential incorporation of cholesterol from the growth medium. All subcellular fractions, with the exception of the 100,000 *g* supernatant fraction, had a similar ratio, demonstrating that the preferential incorporation of cholesterol compared with cholestanol was similar throughout all the subcellular fractions. A slightly higher value of 1.49 was obtained with the 100,000 *g* supernatant fraction.

The results of similar studies using the cholesterol–β-sitosterol combination are presented in Table 4. The whole cells had a ratio of 1.75, whereas plasma membrane, mitochondrial, and microsomal fractions were further enriched in cholesterol, yielding ratios greater than 2.00. The nuclear fractions prepared by three separate methods had ratios less than that of the whole cells and plasma membranes, indicating less preferential incorporation of cholesterol.

TABLE 2 ESTERIFICATION OF STEROLS BY L CELLS

Sterol	Esterification*	Range	No. of Determinations
	%		
Cholesterol†	2.01	0.88–3.40	5
Cholestanol†	1.82	1.60–2.34	4
β-Sitosterol†	6.46	3.67–8.86	5
Desmosterol‡	1.06	0.22–2.06	5

* Average % of total labeled sterol recovered from cells as steryl ester.

† Cells grown for 48 hr in medium containing 20 μg of the labeled sterol per ml.

‡ Cells grown in medium containing 250 μg of sodium acetate-2-^{14}C per ml.

TABLE 3 RATIO OF CHOLESTEROL TO CHOLESTANOL INCORPORATED INTO SUBCELLULAR FRACTIONS

Material	μg Cholesterol-^{3}H/μg Cholestanol-^{14}C			
	Experiment			
	1	2	3	Average
Incubation medium	1.00	1.00	1.01	1.00
Whole cells	1.30	1.38	1.33	1.34
Plasma membrane (FMA)	1.29	1.28	1.33	1.30
Mitochondria		1.29	1.32	1.31
Microsomes		1.35	1.29	1.32
Nuclear (FMA)	1.31	1.32	1.34	1.33
Nuclear (N_2)		1.39	1.39	1.39
Nuclear (Dounce)	1.38		1.39	1.39
Supernate		1.45	1.53	1.49

L cells incubated 48 hr in medium containing delipidized calf serum protein (5 mg/ml), lecithin (20 μg/ml), cholesterol-^{3}H (20 μg/ml), and cholestanol-^{14}C (20 μg/ml).

TABLE 4 RATIO OF CHOLESTEROL TO SITOSTEROL INCORPORATED INTO SUBCELLULAR FRACTIONS

Material	μg Cholesterol-^{14}C/μg Sitosterol-^{3}H			
	Experiment			
	1	2	3	Average
Incubation medium	1.00	1.05	1.05	1.03
Whole cells	1.71	1.78	1.77	1.75
Plasma membrane (FMA)	2.00	2.11	2.31	2.14
Mitochondria	2.11	2.00	2.05	2.05
Microsomes	2.12	2.07	2.11	2.10
Nuclear (FMA)	1.36	1.43	1.27	1.35
Nuclear (N_2)	1.50	1.55	1.47	1.51
Nuclear (Dounce)			1.20	1.20
Supernate	1.57	1.65	1.76	1.66

L cells incubated 48 hr in medium containing delipidized calf serum protein (5 mg/ml), lecithin (20 μg/ml), cholesterol-^{14}C (20 μg/ml), and sitosterol-^{3}H (20 μg/ml).

DISCUSSION

In the absence of exogenous sterol, L cells are capable of synthesizing all of the sterol necessary for growth. The synthesized sterol has been identified as desmosterol (5), and its synthesis has been shown to be effectively inhibited by the addition of cholesterol to the culture medium (1, 6). Table 1 demonstrates that the incorporation of both glucose and acetate into desmosterol is blocked by the presence of cholesterol, whereas mevalonate incorporation is not reduced to a similar extent. This observation indicates that a major block in the desmosterol biosynthetic pathway occurs after acetate but prior to mevalonate, with additional inhibition occurring after mevalonate. This result is consistent with data obtained for liver which indicate a block after β-hydroxy-β-methylglutarate (HMG) (18) and prior to mevalonate. These results are, however, somewhat different from those of Bailey (4), who observed that glucose incorporation into cellular sterol in L cells was reduced to a lesser extent than was acetate. The difference between the present study and the earlier studies on L-cell sterol synthesis may be related to the difference in the two culture media employed, the presence of serum proteins, differences in the methods of adding exogenous cholesterol, or differences in cell growth in L cells adapted to grow in a chemically defined medium.

A number of sterols, when substituted for cholesterol in the culture medium, do not block desmosterol biosynthesis. It was of interest, therefore, to study the uptake and metabolism of these sterols and compare them with cholesterol to clarify the differences in their modes of action. Sterols which do not elicit the feedback response are cholestanol and the C_{28} and C_{29} phytosterols, ergosterol, β-sitosterol, campesterol, and stigmasterol. In the present investigation, β-sitosterol was studied as a representative of the sterols containing extra carbons in the sterol side chain.

The most obvious reason for the failure of an exogenous sterol to elicit biosynthetic feedback response would be the lack of incorporation of sterol into the cell. The data presented in Fig. 1 and 2 indicate that there is a distinct difference in the rate of incorporation of the sterols (cholesterol > cholestanol > β-sitosterol). This difference is consistent at all concentrations of the sterols in the culture medium. The markedly slower rate of β-sitosterol influx compared with cholesterol is similar to the results of numerous studies on absorption of cholesterol and β-sitosterol in the intestine (19–22). The data presented in Fig. 3 indicate a mutual reduction of incorporation of cholesterol and β-sitosterol when these sterols are together in the culture medium. Because of the limitations of the present system in solubilizing large amounts of sterol in the culture medium, cholesterol incorporation by the cells in the presence of a large excess of β-sitosterol could not be determined. The data suggest, however, that if cholesterol were presented to the cells in the presence of an excess of β-sitosterol, the incorporation of cholesterol could be greatly reduced.

The data demonstrating the low level of esterification of the added sterols and of synthesized desmosterol (Table 2) confirm earlier studies on cholesterol esterification in L cells (23) and indicate no significant differences between the sterols tested in this study. The lack of esterification of sterols, together with the inability to demonstrate the presence of any metabolic products in cells or media, shows that the exogenous sterol incorporated by these cells is not extensively modified and should be available to provoke a feedback response.

Tables 3 and 4 present data on the intracellular compartmentalization of β-sitosterol and cholestanol compared with the distribution of cholesterol. These results indicate that the lack of a feedback response cannot be attributed to a highly selective exclusion of β-sitosterol or cholestanol from specific subcellular systems. With but two exceptions, the sterol ratios in the various cellular fractions showed a high degree of similarity. Experiments with liver and other tissue have shown that the enzymes responsible for sterol synthesis, including β-hydroxy-β-methylglutaryl CoA reductase, the enzyme thought to be the prime site of feedback control, are associated with the microsomal fraction (24). Although no detailed studies have been reported on the intracellular site of sterol synthesis in tissue culture cells, preliminary studies in this laboratory have indicated that much of the synthetic activity is associated with the microsomal fraction. The data in the present study indicate that β-sitosterol and cholestanol are incorporated into the microsomal fraction to the same degree as in other membrane-containing fractions, such as mitochondria and plasma membrane. Studies with liver have also shown the requirement for factors contained in the high-speed supernatant fraction necessary to obtain satisfactory synthesis of sterol in cell-free homogenates (25). Table 3 shows that the cholesterol/cholestanol ratio of this supernatant fraction is somewhat higher than that obtained in other subcellular material, indicating a more restricted incorporation of the saturated sterol into material recovered in the 100,000 *g* supernate. Ritter and Dempsey (26) have isolated a protein from the 100,000 *g* supernate of liver which may play a role in the activation of sterol intermediates. This material does not bind cholestanol to the same extent as sterol intermediates (27). It is possible that the higher ratio of cholesterol to cholestanol in the L-cell high-speed supernate is a reflection of a similar phenomenon. In addition, studies in adrenal homogenates have demonstrated a lack of conversion of cholestanol to steroid, which indicates an obligatory role of the Δ^5-bond of cholesterol (28) for such conversions. The less selective incorporation

of β-sitosterol into the nuclear fraction of L cells, compared with other subcellular fractions, cannot be explained at this time. The values obtained from material prepared by three different procedures show consistently greater incorporation than is seen in other membrane-containing fractions and may reflect distinct differences in the composition of the nuclear membrane.

The information obtained from the present investigation has not provided a precise reason for the inability of cholestanol and β-sitosterol to provoke feedback on sterol biosynthesis in L cells. In the case of β-sitosterol, the relatively low rate of incorporation could result in intracellular concentrations of β-sitosterol below those needed to provoke a feedback response. With cholestanol, however, this does not seem to be the case. Although uptake of cholestanol is less than that obtained with cholesterol, the difference does not seem sufficient to explain the lack of feedback observed (6). It is possible, then, that cholestanol fails to interact at the enzymatic level, and therefore could serve as a valuable compound in elucidating the mechanism of the sterol feedback phenomenon.

In these experiments the maximum sterol concentration in the medium was 40 μg/ml. This level of cholesterol was sufficient to reduce de novo synthesis by 80%. As indicated in Fig. 2, increasing the concentration of sterol in the medium resulted in increased cellular sterol incorporation. Recent studies with diploid skin fibroblasts demonstrated a reduction in sterol biosynthesis when these cells were grown in the presence of β-sitosterol and cholestanol at concentrations of 200–1000 μg/ml (29). This would suggest that many sterols, if present in sufficiently high concentrations, can inhibit cellular sterol synthesis.

It has been proposed that the initial steps in the incorporation of sterol by tissue culture cells are based on the partitioning of cholesterol between lipoprotein phases formed in a collision complex between serum lipoproteins and cell surface lipoproteins (17, 30). The extent to which the differences in uptake of the sterols used in this investigation are a reflection of their respective solubilities in the reconstituted lipoproteins of the medium or are due to a selectivity inherent in the cellular membrane lipoproteins cannot be assessed at this time. The reduced incorporation of cholestanol, as compared with cholesterol, is consistent with the observation that cholestanol is not incorporated into red blood cell membrane as readily as cholesterol (31), although cholestanol and cholesterol are solubilized by lecithin to a similar extent (31, 32).

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