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Sterol to Phospholipid Molar Ratios of L Cells with Qualitative and Quantitative Variations of Cellular Sterol (38267)

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L cells can take up a variety of exogenous sterols from the growth medium with consequent changes in the amount and kinds of cellular sterol (1). When the level of cellular sterol changes, the cells respond with negative feedback regulation of *de novo* synthesis of sterol (2, 3). However, the feedback response does not operate to maintain a constant level of cellular sterol; in fact, cellular sterol can vary by more than 150% (3).

The cellular sterol of L cells growing in lipid-free medium is almost exclusively desmosterol, because Δ^{24}-reductase activity is missing in L cells (4). If additional desmosterol is available exogenously, then the cellular desmosterol increases. Alternatively, when cholesterol is supplied exogenously, then desmosterol synthesis is almost completely inhibited and the cellular sterol consists almost exclusively of cholesterol. Since sterols function together with phospholipids, it was of interest to see if coordinated changes of phospholipid occurred so as to maintain a constant sterol to phospholipid molar ratio. The L cell system was used because both the amount and kind of cellular sterol could be varied.

Materials and Methods. L cells were grown as monolayers on Eagle's minimal essential medium (Auto-Pow, Flow Laboratories) with twice the normal concentration of vitamins (BME vitamins, Flow Laboratories), and supplemented either with whole calf serum (7.5%) or with delipidized calf-serum protein (5 mg/ml) (3, 5). The latter provided a lipid-free growth medium which could be additionally supplemented with cholesterol or desmosterol at a predetermined concentration of sterol. The sterol was added to the medium in a 0.5% volume of warm ethanol that contained lecithin (4 mg/ml) in addition to sterol to yield final concentrations of either 0, 10, 20, 25, 30, or 40 μg/ml. After the L cells were equilibrated to a particular medium by several subcultivations over several weeks time, replicate cultures for an experiment were then grown in petri plates (60 × 15 mm area). For membrane studies larger quantities of cells were grown in roller bottles (680 cm^2 area). Details of the preparation of cellular material for analysis have been published previously (3). In brief, the monolayers were washed, released by trypsinization or by scraping with a rubber policeman, and the cells collected by centrifugation. The pellets were resuspended in distilled water and separate aliquots were analyzed for protein with Folins phenol reagent or the total lipid was extracted with chloroform and methanol (6). Nonlipid contaminants were removed by partition between upper and lower phases of chloroform–methanol–2*N* aqueous ammonia (8:4:3) as described by Rouser *et al.* (7). Prior to lipid extraction (6), coprostanol (5β-cholstan-3β-ol, Applied Science Laboratories) at 6 μg/mg cell protein and 10^5 cpm of (^{14}C)-phosphatidylcholine (New England Nuclear, 1.5 Ci/mmole) were added. The coprostanol was used as an

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internal standard for quantitative analysis of the cellular sterol (8) with a Hewlett Packard No. 402 gas chromatograph equipped with a hydrogen flame detector and a 6 ft column packed with 100–120 mesh Gas-Chrom Q coated with 3% OV-17 and operated at 245° with helium carrier gas flowing at 75 ml/min. The gas-liquid chromatogram was quantitated by proportionating the peak areas of cholesterol and desmosterol with the peak area of coprostanol. Peak areas were measured by an electronic integrator.

The (^{14}C)-phosphatidylcholine was used to correct for loss during extraction of phospholipid. More than 98% of the (^{14}C) was recovered in the chloroform phase. Separate aliquots of the chloroform phase were dried down under nitrogen gas and then analyzed for sterol and for total lipid phosphorus. The lipid residue used for quantitative phosphorus determination was digested with 0.25 ml H_2SO_4–60% $HClO_4$ (4:1, v/v), 180–190°, for 2 hr. After cooling the samples to room temperature, color proportional to the presence of inorganic phosphate was developed by adding 4.15 ml H_2O, 0.4 ml 5% ammonium molybdate, 0.2 ml 0.15% hydrazine sulfate, with thorough mixing between each addition (9). The mixture was heated at 100° for 20 min, and read against inorganic phosphate standards at either 640 nm for normal samples or 830 nm for more dilute samples (10). In some instances, phospholipid classes were resolved into three major fractions by one dimensional thin-layer chromatography of the total lipids extracted from about 2 mg of cellular protein using silica gel G (250 μm, Uniplate from Analtech Inc.). The thin-layer plates were developed 12 cm with chloroform–methanol–acetic acid–water (60:30:8:4 v/v/v/v) (11, 12), air dried, and the lipids visualized by iodine vapors. Lanes that contained standards to serve as markers for cholesterol, phosphatidylethanolamine, phosphatidylcholine, and sphingomyelin showed that complete resolution was achieved for these lipids. Iodine visualization of the sample and marker lanes showed the above lipid classes were the major components of the sample lipid extract. Other loci representing lysophosphatidylcholine, phosphatidylserine, and phosphatidylinositol were present in only trace amounts, as previously shown by Weinstein *et al.* (13). The material in the sample lanes that ran below cholesterol was divided completely into three fractions as determined by the three phospholipid markers, each fraction contained the locus of its corresponding marker phospholipid. The silica gel from each fraction was quantitatively transferred to an acid-washed conical centrifuge tube. Acid digestion of the lipid to release inorganic phosphate and the subsequent formation of phosphomolybdate color complex was carried out in the presence of the silica gel using the procedure described above (11). The silica gel was centrifuged to the bottom, and the optical density of the clarified solution was determined.

Surface membrane enriched fractions were prepared from L cell monolayers by the two-phase system developed and evaluated by Brunette and Till for the isolation of surface membranes of L cells (14). Examination by phase microscopy of the cellular fragments, after Dounce homogenization in hypotonic zinc chloride, showed that more than 60% of the outer portions of the cells were cleanly separated from the still intact nuclei. The final pellet of surface membrane rich material was sonicated and analyzed for protein, sterol and phospholipid as described above for whole cell pellets. In comparison to whole cells, this material showed an enrichment in both sterol and phospholipid per mg protein and an increased sterol/phospholipid molar ratio.

Desmosterol (5,24-cholestadien-3β-ol, Applied Science Laboratories) was purified by chromatography on a column of silica gel G (15). The fraction isolated for use when analyzed by GLC showed a main peak area exceeding 90% of the total sum of peak areas.

Results. L cells were grown to equilibrium in media that differed only in the concentration of exogenous cholesterol which ranged from 0 to 40 μg/ml. The chloroform–methanol extracts were analyzed for sterol and phospholipid, and the data were normalized with respect to cellular protein.

TABLE I. Whole Cell Analysis: Phospholipid and Sterol were Determined for Cells Grown for Several Passages on Various Concentrations of Exogenous Cholesterol. For Each Concentration of Cholesterol, Determinations were Made on Duplicate Petri Dishes Harvested at Three Different Dates to Give Six Replicate Determinations. For Each Petri Dish, Sterol, Phospholipid and Cell Protein was Determined. Use of *F*-test on the Values of Lipid Phosphorus Showed No Statistical Significance of the Differences.[16]

Cholesterol concentration of medium***	Cell content μg/mg cell protein (mean ± SE)		Ratio*
	Sterol (% cholesterol; % desmosterol)	Lipid phosphorus	sterol/lipid P (mole/mole)
0	13.75 ± 0.13 (3; 97)	5.25 ± 0.18	0.21 ± 0.01**
10	15.90 ± 0.63 (58; 42)	4.95 ± 0.33	0.26 ± 0.02**
20	16.50 ± 0.70 (78; 22)	4.45 ± 0.29	0.30 ± 0.02**
30	17.95 ± 0.78 (84; 16)	4.48 ± 0.32	0.32 ± 0.03**
40	19.81 ± 1.29 (91; 9)	4.85 ± 0.45	0.33 ± 0.04**

* To convert to molar ratio the following approximations were assumed: mol wt cholesterol is ½ mol wt phospholipid and mol wt phospholipid is 25 times mol wt phosphorus.

** The estimate of error of the ratio (r) is related to the standard errors of sterol (S) and lipid phosphorus (P) by the following:

$$\Delta \text{ error (r)} \approx \left[\left(\frac{\partial r}{\partial S}\right)^2 \times (\text{SE of S})^2 + \left(\frac{\partial r}{\partial P}\right)^2 \times (\text{SE of P})^2\right]^{1/2}$$

*** MEM + Ethanol (0.5%) + lecithin (20 μg/ml) + cholesterol as shown above (μg/ml).

The data (Table I) show that as the concentration of exogenous cholesterol increases from 0 to 40 μg/ml the amount of cellular sterol increases from 13 to 19 μg/mg cell protein and the composition of the cellular sterol changes from 97% desmosterol to 91% cholesterol. This qualitative and quantitative transition of cellular sterol is accompanied by values of cellular phospholipid shown no statistically significant differences. As a result, the sterol to phospholipid molar ratio shows an increase ranging from 0.21 ± 0.01 to 0.33 ± 0.04 as the cellular sterol increases.

When L cells were grown in a variety of media that differed in components other than exogenous sterol a similar pattern was observed (Table II). While cellular sterol was directly correlated with the concentration and kind of exogenous sterol, the cellular phospholipid was more stable so that again the sterol to phospholipid molar ratio was dependent on the cellular sterol which in turn reflected the amount of sterol available exogenously.

Cell fractions enriched in surface membranes were obtained for cells grown in the same kinds of media as in Table II (no surface membrane fractions were examined from cells grown with exogenous desmosterol). Sterol and phospholipid analysis of such membrane enriched material (Table III) showed variation of the sterol to phospholipid molar ratio from 0.27 ± 0.02 to 0.47 ± 0.04.

An attempt was made to detect a change in the distribution of phospholipid classes resulting from changes in growth media. Table IV shows the relative distribution of three major fractions of phospholipid that were resolved by thin-layer chromatography. The two classes of cells selected for analysis were extreme in the sense that in one case (from line 4, Table II) the cells had a low

TABLE II. Whole Cell Analysis: Phospholipid and Sterol were Determined in L Cells Grown for Several Passages in the Media Shown. Replicate Determinations were Made from Cells in Separate Petri Dishes or from Aliquots of Cells Harvested from Roller Bottles Used for the Membrane Isolation Experiment Shown in Table III.

Medium MEM with supplement as stated	Replicates	Cell content μg/mg cell protein (mean ± SE)		Ratio* sterol/lipid P (mole/mole)
		Sterol (% chol; % desm.)	Lipid phosphorus	
1. Serum medium 7.5% calf serum	10***	13.75 ± 0.72 (92; 8)	4.48 ± 0.21	0.24 ± 0.06**
2. Cholesterol medium delipidized serum protein 5 mg/ml lecithin—20 μg/ml cholesterol—25 μg/ml ethanol—0.5% (v/v)	6	13.30 ± 0.30 (92; 8)	4.42 ± 0.21	0.24 ± 0.03**
3. Desmosterol medium delipidized serum protein 5 mg/ml lecithin—20 μg/ml desmosterol—25 μg/ml ethanol—0.5% (v/v)	7	18.61 ± 0.52 (9; 91)	5.67 ± 0.11	0.22 ± 0.04**
4. Lipid-free medium delipidized serum protein 5 mg/ml	5	7.86 ± 0.30 (2; 98)	3.93 ± 0.12	0.16 ± 0.03**

* See note * of Table I.

** See note ** of Table I.

*** Coordinate values of phospholipid, sterol and protein from only four replicates; additional six replicates were analyzed only for phospholipid and protein.

TABLE III. Analysis of Surface Membrane Enriched Fractions: Phospholipid and Sterol were Determined in Surface Membrane Enriched Fractions (14). The Cell Monolayers from 6 Roller Bottles Grown to Confluency on a Given Medium were Washed with 0.85% Saline, Released by Scraping with a Rubber Policeman and Pooled. After Additional Washing by Centrifugation, the Resuspended Cells were Divided Into Two Samples Which were Processed Independently for the Isolation of Surface Membrane Fractions and Their Subsequent Analysis.

Medium MEM with additions shown		Replicates	Content of surface membrane enriched fractions μg/mg protein (mean ± SE) Sterol (% chol.; % desm.)	Lipid P	Ratio* Sterol/lipid P (mole/mole)
7.5% calf serum		4	37.02 ± 5.9 (98; 2)	5.25 ± 0.43	0.47 ± 0.04**
Delipidized serum protein at 5 mg/ml and added lipid (μg/ml) as shown					
Lecithin	Cholesterol				
20	25	5	39.78 ± 2.8 (98; 2)	8.19 ± 0.89	0.40 ± 0.04**
0	0	3	25.18 ± 1.72 (2; 98)	7.97 ± 0.51	0.27 ± 0.02**

* See note * of Table I.
** See note ** of Table I.

level of cellular sterol consisting almost exclusively of desmosterol and in the other (from line 2, Table II) the cells contained a twofold higher level of cellular sterol consisting almost exclusively of cholesterol. No gross differences in phospholipid composition were apparent.

Discussion. This study has attempted to determine if change in cellular phospholipid occur after L cells were grown in medium known to induce changes in the levels and kinds of cellular sterol. No such changes in the quantitative levels of cellular phospholipid were apparent. Nor were differences apparent in the distribution of three major fractions of phospholipid classes that were reproducibly resolved by reference to markers of sphingomyelin, phosphatidylcholine,

TABLE IV. Percentage Composition of Major Phospholipid Classes in L-cells Grown with and without Exogenous Sterol.

Growth medium	Phospholipid fraction (% of total) * Sphingomyelin**	Lecithin	Phosphatidylethanolamine***
Delipidized serum protein (5 mg/ml)	19 (14–23)	43 (41–44)	39 (37–42)
Delipidized serum protein (5 mg/ml) + Lecithin (20 μg/ml) + Cholesterol (25 μg/ml)	22 (19–28)	40 (34–44)	38 (37–39)

* Mean (range) of two experiments each carried out in duplicate.
** Sphingomyelin fraction includes trace amounts of lysophosphatidylcholine.
*** Phosphatidylethanolamine fraction includes trace amounts of phosphatidylserine.

and phosphatidylethanolamine. The data indicate that for L cells the phospholipid to protein ratio is a stable parameter. Other cell types may have different characteristic phospholipid to protein ratios (17). It has been postulated that a cell with a fairly constant level of phospholipid would have a level of cellular sterol that is then more passively influenced by the flux of sterol between medium and cells (18). Thus, when the medium contains increasing amounts of sterol, *de novo* synthesis of sterol would be successively reduced. Once sterol synthesis is near its lowest limit and the concentration of exogenous sterol is further increased, the equilibrium level of cellular sterol would then be dependent on something analogous to intracellular and extracellular partition coefficients. It is probable that the level of cellular phospholipid would influence the intracellular partition coefficient for sterol—and so would act to limit the ultimate amount of sterol in the cell. Some evidence for a limit to the level of sterol in the L cell has been published (3). So although cellular phospholipid may be fairly constant, a parameter such as the sterol to phospholipid molar ratio should not be considered as inherently constant but would be subject to changes induced by the medium.

Summary. In this study paired values of cellular sterol and phospholipid were determined after L cells were grown in medium known to induce changes in the level and kinds of cellular sterol. The sterol to phospholipid molar ratios computed from the paired values showed differences because, while the level of cellular sterol changed in response to exogenous sterol, no statistically significant differences in the levels of cellular phospholipid were apparent.

When L cells were grown in the presence of concentrations of exogenous cholesterol ranging from 0 to 40 μg/ml, the sterol to phospholipid molar ratio ranged from 0.21 ± 0.01 to 0.33 ± 0.04, and, although the cellular sterol ranged from a low level almost exclusively of desmosterol (97%) to a 60% higher level almost exclusively of cholesterol (91%), no statistically significant differences were observed in the amount of cellular phospholipid. In another instance of growth in lipid-free medium a sterol to phospholipid molar ratio as low as 0.16 ± 0.03 was observed. In cell fractions enriched for surface membrane material, sterol to phospholipid molar ratios of 0.27 ± 0.02 and 0.47 ± 0.04 were observed for cells grown respectively in lipid-free and 7.5% serum supplemented medium. Additional analysis by thin-layer chromatography of the total lipid extract showed no apparent differences in the relative amounts of phospholipid classes.

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1. Rothblat, G. H., and Burns, C. H., J. Lipid Res. **12**, 653 (1971).

2. Rothblat, G., J. Cell Physiol. **74**, 163 (1969).

3. Sokoloff, L., and Rothblat, G. H., Biochim. Biophys. Acta **280**, 172 (1972).

4. Rothblat, G. H., Burns, C. H., Conner, R. L., and Landrey, J. R., Science **169**, 880 (1970).

5. Rothblat, G. H., Buchko, M. K., and Kritchevsky, D., Biochim. Biophys. Acta **164**, 327 (1968).

6. Bligh, E. G., and Dyer, W. J., Canadian Journal of Biochim. Physiol. **37**, 911 (1959).

7. Rouser, G., Kritchevsky, D., Yamamoto, A., and C. F. Baxter, *in* "Advances in Lipid Research" (R. Paoletti and D. Kritchevsky, eds.), Vol. 10, pp. 261–360. Academic Press, New York (1972).

8. Bates, S. R., and Rothblat, G. H., J. Virol. **9**, 883 (1972).

9. Vogel, S. I., "A Text-Book of Quantitative Inorganic Analysis," 3rd ed., p. 810. John Wiley and Sons, Inc., New York (1961).

10. Bartlett, G. R., J. Biol. Chem. **234**, 466 (1959).

11. Brockmann, U., and Gercken, G., Clinica Chemica Acta **23**, 489 (1968).

12. Skipski, V. P., Peterson, R. F., Barclay, M., J. Lipid Res. 3, 467 (1962).

13. Weinstein, D. B., Marsh, J. B., Glick, M. C., Warren, L., J. Biol. Chem. 244, 4103 (1969).

14. Brunette, D. M., and Till, J. E., J. Membrane Biol. 5, 215 (1971).

15. Vroman, H. E., and Cohen, C. F., J. Lipid Res. 8, 150 (1967).

16. Snedecor, G. W., Cochran, W. G., "Statistical Methods," 6th ed., pp. 184, 258. The Iowa State University Press, Ames, Iowa (1967).

17. Ashworth, L. A. E., and Green, C., Science 151, 210 (1966).

18. Rothblat, G. H., *in* "Growth, Nutrition and Metabolism of Cells in Culture" (G. H. Rothblat and V. Cristofalo, eds.), Vol. 1, p. 297. Academic Press, New York (1972).

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