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# A Requirement for Cholesterol and Its Structural Features for a Human Macrophage-like Cell Line

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The lipid requirements of a human macrophagelike cell line were studied. The cells grew only about one generation in a medium supplemented with delipidated serum; during the growth the cholesterol content of the cells was depleted. Growth was restored by including in the medium serum lipids subjected to alkaline hydrolysis or cholesterol. The extent of growth was dependent on cholesterol concentration. No growth was obtained with 5-cholestene, 5-cholesten-3-one, cholesteryl chloride, coprostanol, β-sitosterol, or stigmasterol. Very limited growth occurred with cholesterol methylether, epicholesterol, or β-cholestanol. Therefore, for optimal growth of these cells there is a stringent requirement for the structural features of cholesterol, which include a 3-βOH group, a $\Delta^5$-double bond, a *trans* ring A/B configuration, and freedom of the side chain from bulky groups. This stringency far exceeds what was previously reported for other cells. Of the six sterols that failed to support growth at all, five were incorporated into cells moderately to extensively. This suggests that assembly of a functional membrane is impaired when these sterols are used as substrates for growth.

**Key words: macrophagelike cell, cholesterol auxotroph, growth, sterol structure**

A number of mammalian cell lines, both murine [1-4] and human [5] have been used as models of macrophage function. These cells are attractive models because they are easily cultured in homogenous populations and in large quantities. There have been no studies of the lipid requirements for growth of these cells lines. A definition of these requirements will facilitate studies of the role of cholesterol, a major component of the plasma membrane of mammalian cells [6], and other lipids in membrane function in general and macrophage-related functions in particular. We report here that a human macrophagelike cell line requires cholesterol for growth, and we establish the structural features of sterols that are required for cell division. We have found that these requirements are more stringent than those reported for other cells [7–14], indicating that the requirement for the molecular features of the sterol is tailored to the lipid and protein of the membrane. Furthermore, for sterols

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that failed to support growth, we found no correlation between growth-promoting capacity and extent of incorporation into the cells. This suggests that for use as substrates for membrane synthesis, the rate-limiting step is not their uptake by the cell. Rather, they cannot fulfill the role of cholesterol in generating functional membrane.

## EXPERIMENTAL PROCEDURES

### Materials

Cholesterol, β-cholestanol, 7-dehydrocholesterol, fatty acids, crystalline bovine serum albumin (BSA), and fatty-acid-free BSA were products of Sigma Chemical Company. 5-Cholestene, 5-cholesten-3-one, cholesteryl chloride, cholesteryl methylether, and epicholesterol were purchased from Steraloids, Inc. Desmosterol was obtained from Supelco, and β-sitosterol and stigmasterol were purchased from Applied Science Laboratories. The purity of the sterols was examined by gas-liquid chromatography (GLC), and each was found to be more than 99% pure. Heat-inactivated fetal calf serum (FCS) and RPMi 1640 with glutamine (0.3 g/liter) were obtained from Flow Laboratories.

### Cell Strain and Growth Conditions

The human macrophagelike cell line U937 [5] was a gift of Dr Hillel S. Koren of Duke University Medical Center. The cells were grown at 37°C in suspension cultures in RPMi 1640 with glutamine (0.3 g/liter) supplemented with 10% heat-inactivated fetal calf serum, 1 mM Na pyruvate, 100 units per ml of pennicilin, and 78 units per ml of streptomycin in a humidified 5% $CO_2$ environment. Growth was monitored by diluting 0.5 ml of the culture with 9.5 ml of phosphate buffered saline, and counting the cells in a Coulter Counter.

### Delipidation of Serum

Lipids were removed from the heat-inactivated fetal calf serum according to the procedure of Rothblat et al [15]. Briefly, to an ice-cold mixture consisting of 250 ml each of acetone and ethanol was added, dropwise, 50 ml of serum and gently stirred at 0°C for 4 hr. The mixture was filtered through a Whatman No. 1 paper filter, and before the solvent was completely removed, 125 ml of precooled diethyl ether were added. Filteration was continued until the residue was dry. The residue was kept in a desicator overnight, then dissolved in 35 ml of $H_20$ and sterilized by filtering through a Millipore filter (0.45 μm). The level of cholesterol remaining in the delipidated serum was less than 30 ng/ml. This was demonstrated as follows: serum, before and after delipidation by the procedure indicated above, was extracted with chloroform and methanol according to the Bligh and Dyer method [16]. The chloroform extracts were dried under nitrogen and lipids were hydrolyzed in 0.1 M KOH for 1 hr at 75°C. After acidification to pH 1, lipids were extracted into hexane and used for quantification of cholestrol by GLC. Fatty acids were also quantitated by GLC after conversion to methyl esters [17]. The delipidation procedure described above resulted in removal of more than 90% of the total acyl chains of serum.

### Growth of Cells in Media Containing Exogenous Lipids

Cells in the logarithmic phase of growth were harvested by centrifugation at 3,000g for 5 min at room temperature, washed once with RPMi 1640, and suspended

in the growth medium described above except that FCS had been replaced by an equivalent amount of delipidated FCS. Aliquots (10 ml) were added to tissue culture flasks (50 ml, 25 $cm^2$, Falcon) containing 0.27 ml of a sterile solution of 5% BSA in 0.14 M NaCl to which 30 $\mu$l of ethanol containing the desired amount of lipids had been added [18].

### Lipid Extraction and Sterol Analysis

Lipids were extracted from 0.5–5 $\times$ $10^7$ cells according to the Bligh and Dyer procedure [16] and, unless otherwise indicated, were subjected to alkaline hydrolysis, acidified, and extracted into hexane as described above. After evaporation of hexane, the residue was dissolved in ethanol, and aliquots were chromatographed in a Varian Series 1400 equipped with a glass column packed with 3% SP2300 on 100/120 supelcoport maintained at 220°C [19]. All of the sterols were well-resolved on this column. For quantification of cholesteryl esters, the alkaline hydrolysis step was eliminated and the lipid extracts were dissolved in chloroform and chromatographed on a 2-ft glass column packed with 1% poly S-179 on 100/120 mesh gas chrom Q (Applied Science Laboratories) maintained at 300°C. Cholesterol was quantified as follows: standard curves were obtained in which the instrument response was linear with respect to the mass of cholesterol (0–1 $\mu$g). Aliquots of lipids derived from the cells were chromatographed using a sample size whose cholesterol content fell within the linear range of the standard curve. Similar procedures were used for other sterols, taking into account the detector-sensitivity correction factor [20]. Samples containing as low as 0.01 $\mu$g sterol could be quantified. Protein was determined by the procedure of Lowry et al [21] after the cells were washed twice with phosphate-buffered saline, suspended in distilled water and sonicated for 1 min.

Experiments presented in Figures 1, 2, and 3 were each carried out twice; those presented in Figures 4, 5, and 6 were each carried out three times. The results were very similar in each case, and what are shown are representative experiments.

## RESULTS

### Growth of U937 Cell Line Requires Exogenous Lipids

The effect of delipidation of serum on growth of the cells is shown in Figure 1A. In the medium supplemented with delipidated FCS, the cells grew for only about one generation, while in the medium containing FCS, growth continued until a density of 2 $\times$ $10^6$ cell/ml was reached [after 160 hr]. Lipids extracted from FCS by the Bligh and Dyer procedure [16, 22] supported growth of the cells suspended in the medium containing delipidated FCS (data not shown). When the lipid extract from FCS was subjected to alkaline hydrolysis as described above, and then included in the medium containing delipidated FCS, full growth was obtained (Fig. 1B). These observations indicate that U937 is defective in biosynthesis of a lipid(s) that cannot be degraded by alkaline hydrolysis. It was suspected that this lipid may be cholesterol, and therefore, the effect of incubation of the cells in the medium containing delipidated FCS on cellular cholesterol content, including the free and esterified cholesterol, was determined (Fig. 2). The initial level of cholesterol in the cells was 12.5 $\mu$g/mg protein, which is essentially the same as that reported for murine peritonal machophages [23]. Incubation in delipidated serum led to depletion of cellular cholesterol such that after 24 hr it was decreased by 70%, and after 48 hr it was barely detectable. The cells, having been deprived of an exogenous source, were unable to replenish their normal cholesterol levels endogenously. It should be noted that the

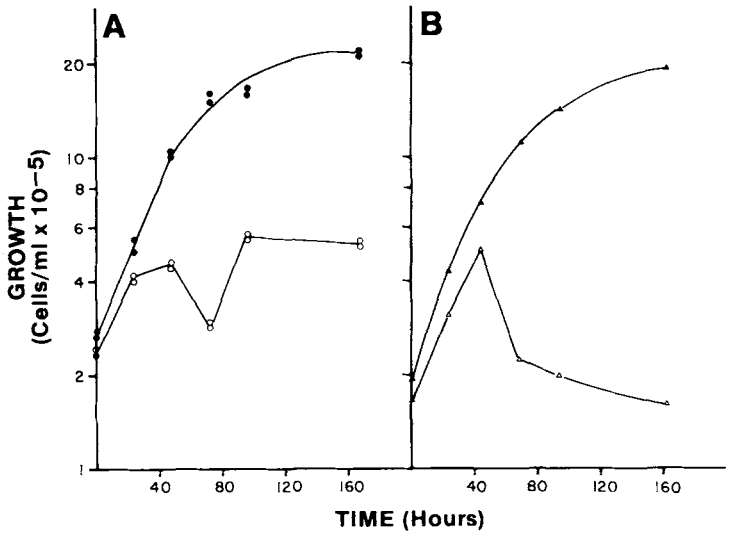


Fig. 1. Growth of U937 in medium containing delipidated FCS (A) or delipidated FCS supplemented with hydrolyzed serum lipids (B). Exponentially growing cells, were washed and resuspended in a medium containing 10% FCS(●), a medium containing an equivalent amount of delipidated FCS (○, △), or a medium containing delipidated FCS supplemented with lipids from an equivalent amount of FCS that had been hydrolyzed as described under Experimental Procedures (▲).

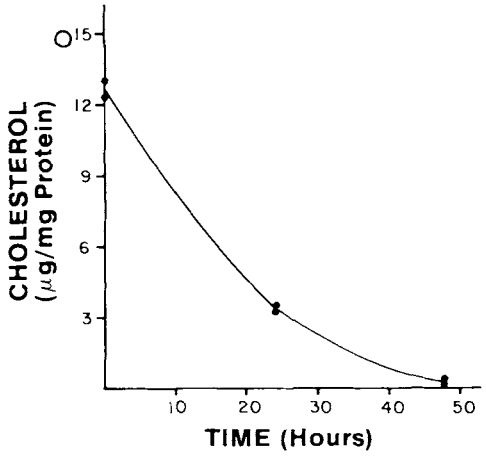


Fig. 2. Depletion of cellular cholesterol upon incubation in medium containing delipidated FCS. Exponentially growing cells were washed and incubated at 37°C in a humidified 5% $CO_2$ environment, in a medium containing delipidated FCS. At indicated times the cells were collected, washed once, and samples were taken for protein determination, or subjected to alkaline hydrolysis for GLC quantification of cholesterol as described under Experimental Procedures.

level of cholesterol esters in U937 was very low (less that 0.05 μg/mg protein) and become totally undetectable in cells incubated for 48 hr in the medium containing delipidated serum.

Two other lines of evidence also indicate that U937 is defective in cholesterol synthesis. First, as shown in Figure 3, growth was dependent on the concentration of

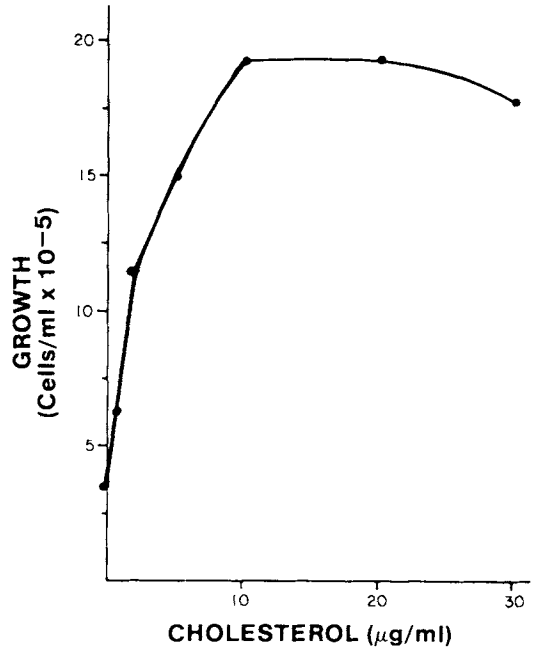


Fig. 3 Dependence of growth on external cholesterol concentration. Exponentially growing cells were harvested, washed, and resuspended in medium containing delipidated FCS. Ten-milliliter aliquots were transferred to flasks containing 0.27 ml of 5% BSA in 0.14 M NaCl and 30 μl of ethanol containing various amounts of cholesterol. Cells were incubated at 37°C, and cell division was monitored as described under Experimental Procedures. Growth refers to cell number after the stationary phase of growth was reached.

cholesterol in the medium. At 10 μg/ml of cholesterol a maximal cell yield of about $2 \times 10^6$ cells/ml was obtained, which is essentially the same extent of growth as that observed in the medium containing whole FCS or delipidated FCS plus hydrolyzed serum lipids (Fig. 1A,B). Second, since the alkaline hydrolysate used in the experiment of Fig. 1B also contained fatty acids, we also tested growth-promoting capacities of arachidonic acid (at 1 or 10 μg/ml), oleic acid (10 μg/ml), and palmitic acid (10 μg/ml). Neither the fatty acids individually nor a combination of oleic and palmitic acids supported growth. When cholesterol was also included, growth proceeded at the same rate and to the same extent as that observed in the medium containing only cholesterol except for arachidonic acid, which at 10 μg/ml was slightly inhibitory (data not shown). Substitution of fatty acid free BSA for crystalline BSA had no significant effects on growth (data not shown).

The structural features of cholesterol required for growth of U937 were established by comparing the growth of the cells on a number of cholesterol analogs at a concentration of 10 μg/ml, which had been found to be optimal for growth on cholesterol (Fig. 3).

### C-3 Substituent

The stringency of the requirement for 3 β-hydroxy group of cholesterol was tested by comparing the growth response of the cells to a number of analogs that

differ from cholesterol only in the C-3 substituent. These included 5-cholestene, 5-cholesten-3-one, 5-cholesten-3 α-ol (epicholesterol), cholesteryl chloride, and cholesteryl methylether (Fig. 4). When the C-3 substituent was H (5-cholestene), O (5-cholesten-3-one), or Cl (cholesteryl chloride), the sterol tested failed to support growth. When the C-3 substituent was methoxyl or αOH (ie, epicholesterol), growth was poor, and no more than 50% higher than that obtained in the medium lacking any sterols. Even then, this level was not maintained, since the initial limited increase in cell number was followed by a drop (Fig. 4) probably due to cell lysis. It should be noted that GLC anlalyis of the sterols of these cells revealed epicholesterol as the only sterol of cells grown on this analog, but composition of sterol in cells grown on cholesteryl methylether showed 17% cholesterol and 83% cholesteryl methylether.

### Unsaturation in Ring B and *cis/trans* Configuration in Ring A/B Juncture

The importance of the degree of unsaturation in ring B was evaluated by comparing the growth response of the cells with β-cholestanol (dihydrocholesterol), 7-dehydrocholesterol, and cholesterol (Fig. 5). Cholestanol was only about half as effective as cholesterol in supporting growth. GLC analysis of the sterol in these cells revealed that the cells were totally devoid of cholesterol and that cholestanol was the only sterol present in the cells. In contrast, the cells grew as efficiently on 7-dehydrocholesterol as cholesterol, but when sterol profile of the cells was analyzed, it showed 45% cholesterol and 55% 7-dehydrocholesterol. Therefore, because of the

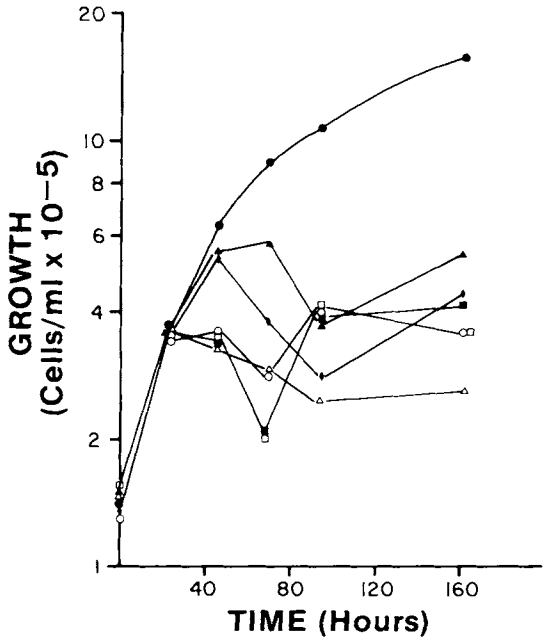


Fig. 4 Effect of C-3 substituent of the sterol on growth of U937. Cells were processed as described in the legend to Figure 3 except that 10-ml aliquots were transferred to flasks containing 0.27 ml of 5% BSA and 30 μl of ethanol containing 100 μg of a sterol. Cholesterol (●), epicholesterol (▲), cholesteryl methylether (◆), cholesteryl chloride (■), 5-cholestene (□), 5-cholesten-3-one (△), 0.27 ml of 5% BSA solution and 30 μl ethanol, no sterols (○).

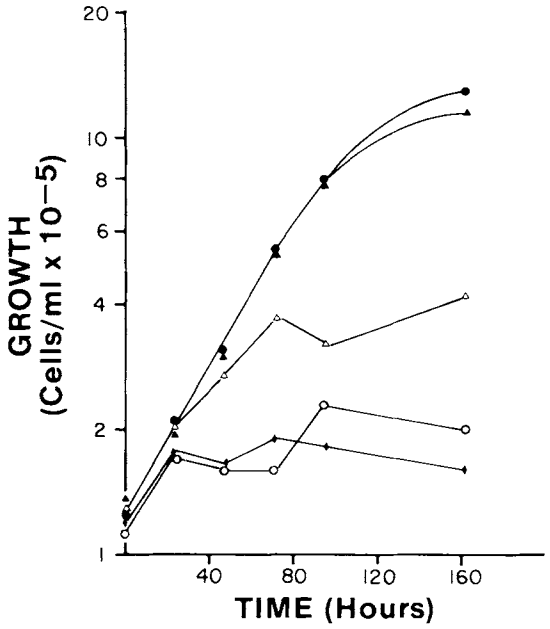


Fig. 5. Effect of degree of unsaturation in B ring and ring A/B configuration of sterols on growth of U937. Cells were processed as described in the legend of Figure 4. Cholesterol (●), 7-dehydrocholesterol (▲), cholestanol (△), coprostanol (◆), no sterols (○).

extensive conversion of 7-dehydrocholesterol to cholesterol, significance of C7-C8 bond order could not be evaluated. However, the results indicate that the defect in cholesterol synthesis in these cells must lie in steps prior to C7-C8 reduction.

The importance of planarity of the sterol nucleus in supporting growth was assessed by comparing the growth-promoting capacity of coprostanol (5 β-cholestan-3βol) to that of cholestanol (5 α-cholestan-3βol). The former has the ring A/B juncture in a *cis* configuration, while the latter has ring A/B in a *trans* configureation and, therefore, a more planar nucleus. As shown in Figure 5, coprostanol was totally ineffective in supporting growth.

## Structure of Side Chain

To establish the structural features of the side chain of cholesterol important for growth three analogs that differ from cholesterol only in the structure of the side chain—namely, desmosterol (24-dehydrocholesterol) and two plant sterols, β-sitosterol (5-cholesten-24b-ethyl-3β-ol) and stigmasterol (5, 22-cholestadien-24-ethyl-3β-ol)—were evaluated (Fig. 6). Desmosterol supported growth, though somewhat less effectively than cholesterol. However, when sterols of these cells were analyzed, cholesterol constituted 97% and desmosterol 3% of the sterol content. Thus, growth obtained on desmosterol must be attributed to its being converted to cholesterol precluding the possibility of assessing the significance of C24-C25 bond order. Nonetheless, the results demonstrate that, as noted above, reduction of the C=C of sterol precursors to cholesterol is not defective in U937. Introduction of an ethyl

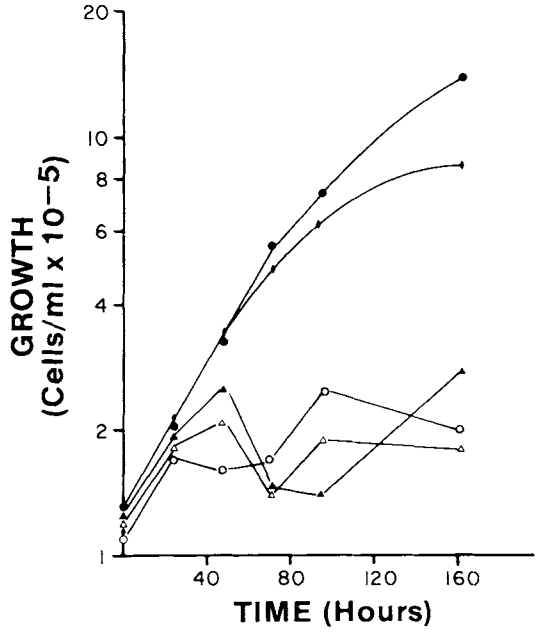


Fig. 6 Effect of structure of the side chain of sterols on growth of U937. Cells were processed as described in the legend of Figure 4. Cholesterol (●), desmosterol (◆), stigmasterol (▲), β -sitosterol (△), no sterols (○).

group at C-24 (stigmasterol) or the ethyl group at C-24 plus a double-bond at C22 eliminates the growth-promoting capacity of the sterol (Fig. 6).

### Effect of Sterol Structure on Its Uptake by the Cells

A sterol does not support growth either (1) because it is poorly incorporated into the cells or (2) because it is easily incorporated into the cells but cannot function in the membrane. The data of Table I suggest that the first possibility may be ruled out, since of the sterols that failed to support growth at all, only one—ie, cholesteryl chloride—was poorly incorporated into the cells. For the rest, compared to cholesterol, the extent of incorporation ranged from approximately 60% for 5-cholesten-3-one to 250% for corprostanol. This is even higher than the extent of uptake of cholestanol that permitted some growth. Thus, at least for these sterols, defective uptake by the cells is ruled out as the rate-limiting step for membrane synthesis.

As shown in Table I, there is virtually a total replacement of cholesterol by various analogues; that is, depletion of cholesterol occurs more rapidly than can be accounted for by growth and dilution. Therefore, depletion does not totally depend on the extent of growth, which is very limited in many instances.

## DISCUSSION

The present investigation was undertaken to define a system that would allow alteration of sterol content and structure of a mammalian cell so that membrane properties could be studied. The cell line U937 we employed is a human histiocytic lymphoma established by Sundstrom and Nilsson [24] and adapted to rapid growth in

**TABLE I. Effect of Sterol Structure on Its Uptake by U937***

| Sterol in medium | Uptake (μg/mg protein) |
|---|---|
| Cholesterol | 10.7 ± 0.7 |
| Cholesteryl chloride | 0.9 ± 0.2 |
| Cholesteryl methylether | 1.3 ± 0.7 |
| 5-cholestene | 7.6 ± 0.2 |
| 5-cholesten-3-one | 6.1 ± 0.2 |
| Cholestanol | 5.5 ± 0.2 |
| Coprostanol | 26.9 ± 1.9 |
| Epicholesterol | 5.9 ± 0.5 |
| β-Sitosterol | 18.4 ± 5.1 |
| Stigmasterol | 24.6 ± 3.3 |

*Cells were processed as described in legend to Figure 3 except that flasks (in duplicates), each containing 10 μg/ml of the desired sterol, were incubated for 48 hr at 37°C. Cells were harvested, washed and used for protein and sterol determination as described under Experimental Procedures except that the alkaline hydrolysis step was eliminated.

vitro by Lackman et al [25]. It exhibits macrophage characteristics [5, 26–28] and has been activated for antibody-dependent cellular cytotoxicity [26], phagocytosis [5], chemotaxis [27], and pinocytosis [28]. It has all the characteristics that have made the use of macrophagelike cell lines attractive as models of macrophage function [1–5, 26–28].

We found that U937 cells grew for about one generation when suspended in the medium containing delipidated FCS, during which time there was a depletion of cellular cholesterol. When cholesterol was supplied, cell division progressed normally; cell yield was proportional to added cholesterol, reaching a maximum at 10 μg/ml of cholesterol. The dependence on serum lipids for growth can be explained in terms of a loss of capacity of U937 cells to synthesize cholesterol. The lesion(s) in the pathway of cholesterol biosynthesis in these cells is not known. It has been reported that murine macrophages cultivated in vitro were not active in conversion of acetate to cholesterol [23]. A possible interpretation of their results is that, rather than demonstrating a specific enzyme defect(s), their nondividing cells do not require cholesterol synthesis. U937 cells, on the other hand, divide actively and most likely are defective in an enzymatic or regulatory step(s).

The auxotrophic requirement of U937 for cholesterol permitted us to establish the structural features of the sterol for growth of a mammalian cell. Prior studies of structural specificity of sterols for growth have been extensively carried out for insects, protozoa, mycoplasma, and yeast [7–14, 29] but have been rare in mammalian cells except for studies of Chen et al [30], who compared the efficacy of desmosterol, cholesterol, 5 α-cholestanol, $\Delta^7$-cholestenol, cholest-4-en-3-one, and β-sitosterol in reversing inhibitory effects of 20 α-hydroxycholesterol on growth of L-cells. As noted by them [30], the inhibitory sterol might compete with desmosterol, the principle sterol of L-cells, for sites in the membrane, with resulting effects on membrane function complicating interpretation of the results. Consequently, little is known of the structural specificity of cholesterol for growth of mammalian cells and its importance in specific membrane functions beyond general roles in permeability and lipid fluidity.

U937 cells did not grow on 5-cholestene, 5-cholestene-3-one, cholesteryl chloride, coprostanol. stigmasterol, or β-sitosterol. Very limited growth was noted when

the cells were supplied with cholestanol, epicholesterol, or cholesteryl β-methylether. The extensive growth response to 7-dehydrocholesterol or desmosterol cannot be attributed necessarily to the exogenous sterols per se, since they were extensively converted to cholesterol. These observations indicate that for optimal growth of these cells the sterol must contain a β-OH group at C-3 a $\Delta^5$-double bond, a *trans* A/B ring configuration, and be free of bulky groups, such as an ethyl group, on the side chain. Except for the *trans* A/B ring configuration, the rest of the features of the sterol required for growth of U937 differ from those of other cells as follows: (1) M capriculum and S cerevisiae do not require an unblocked β-OH group at C-3 [12, 14]. (2) Mycoplasma 07 grows fully on cholestanol [9]; yeast grows as well on cholestanol as on cholesterol [13]; for pupitation of D vulpinus, cholestanol has full cholesterol-sparing effect [8] all indicating a lack of requirement for Δ-double-bond. (3) Our observation that U937 does not tolerate a bulky group on the side chain is at variance with previous findings wtih other cells that showed that, for D vulpinus, β-sitosterol had excellent cholesterol-sparing activity [7], T-strain mycoplasma grew well on β-sitosterol and to a lesser extent on stigmasterol [9], and β-sitosterol and stigmasterol were found to be as good as cholesterol for growth of yeast [11]. Taken together, it can be concluded that general rules governing the biological fitness of sterols cannot be drawn, at least as far as cell division is concerned. Rather, it appears that the need for particular structural features of sterols is tailored to the lipid and protein profile of the membrane or its particular function.

For a sterol to fit a biological membrane, it must first partition into the lipid-bilayer, and the resulting assembly should then be functional. Some of the sterols that failed to support growth could easily partition into the lipid-bilayer, but the resultant assembly might be nonfunctional. Our results support this view, since we found that, compared to cholesterol, 5-cholesten-3-one, 5-cholestene, coprostanol, β-sitosterol, and stigmasterol were moderately to extensively incorporated into the cells, and, therefore, should have been available for membrane synthesis. Since there is no evidence to indicate that once a sterol is taken up by the cell it is sequestered away from the lipid bilayer to any significant extent, we may assume that the above sterols partition into the lipid-bilayer but the resultant membrane is nonfunctional. In any case, U937 offers an opportunity to test the validity of this assumption and also to study the relationship between membrane sterol content and structure and macrophage-associated functions.

## ACKNOWLEDGMENTS

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