The differential binding of cholesterol and 25-hydroxycholesterol to cytosolic proteins in various types of cells was investigated. 25-Hydroxycholesterol taken up by six different established cell culture lines and by mouse spleen cells in primary culture was bound to cytosolic components which, during velocity gradient centrifugation, displayed sedimentation coefficients of approximately 5 S and 8 S. In contrast, cholesterol taken up by the cells was concentrated near the bottom (approximately 20 S) of the sucrose density gradient but was distributed throughout. Results with primary cultures of mouse fetal liver differed from those obtained with other cell cultures in that both sterols appeared principally in a 5 S band. Further characterization of the binding components from intact L cells indicated that binding of 25-hydroxycholesterol to the 8 S fraction was saturable and reversible, whereas binding to the 5 S band was not saturable. The 8 S 25-hydroxycholesterol complex involved a protein with a relatively long half-life. The complex was essentially stable at 0°C but dissociated slowly at 25°C. Sulphydral functions were not required for sterol binding, and formation of the complex was not dependent upon cAMP. Competition studies with intact cells and with isolated cytosol indicated that a number of other oxygenated sterols bind to 8 S sites occupied by 25-hydroxycholesterol. Those sterols which are potent suppressors of 3-hydroxy-3-methylglutaryl coenzyme A reductase (EC 1.1.1.34) activity competed for these binding sites, whereas those which do not suppress the reductase did not compete for them. These studies suggest that the binding of oxygenated sterols to the 8 S cytosolic component may be in some sense specific, while that to the 5 S component is nonspecific. The kinetics of formation and dissociation of the 8 S 25-hydroxycholesterol-protein complex and of the suppression of 3-hydroxy-3-methylthiol-CoA reductase in the presence of the diol was also consistent with a postulated role for the complex in the regulation of the enzyme.

Certain oxygenated sterols are potent inhibitors of cholesterol synthesis and of cell growth. They act specifically to lower the cellular level of 3-hydroxy-3-methylthiol-CoA reductase, the regulatory enzyme in the cholesterol biosynthesis pathway. How they do so is unknown, but it is known that certain structural configurations of the sterol molecule are essential (1, 2) and that certain of them are produced as precursors of cholesterol (1, 3, 4) or as products of its catabolism (1, 2, 5, 6). Inhibitory oxygenated sterols are, therefore, present in cells and may function therein to regulate the rate of cholesterol synthesis.

We have recently described intracellular L cell proteins which bind the inhibitory sterol, 25-hydroxycholesterol, but not cholesterol (7). In the present paper we describe in more detail the cellular distribution, specificities, and binding properties of the proteins. These characteristics of one such protein fraction, that which occupies the 8 S region of sucrose velocity sedimentation gradients, are consistent with the possibility that it plays a role in the regulatory actions of the oxygenated sterols.

MATERIALS AND METHODS

Sterols—[26,27-3H]25-Hydroxycholesterol, [1,2-3H]cholesterol, and [4-14C]cholesterol (New England Nuclear) were purified by thin layer chromatography before use (1, 2). The sources of unlabeled sterols and procedures used to purify them have been described (4-6, 8). Sodium tetrathionate dihydrate was from Fluka AG, Switzerland. L-[4,5-3H]Leucine and (R,S)-[3-14C]hydroxymethylglutaryl-CoA were from New England Nuclear.

Cell Cultures—L cell mouse fibroblasts (a subline of NCTC clone 929) were grown either as monolayers in plastic cell culture flasks or in spinner culture in serum-free Waymouth's 752/1 medium modified as described (9). Approximately 1 x 10^6 cells were pipetted into each of six 150-cm^2 plastic culture bottles (Corning) approximately 24 h before they were used in an experiment. The cells became attached and grew as monolayers under these conditions. Primary cultures of mouse fetal liver cells and an established line of fetal liver cells (FL83/B) were grown in serum-free medium as described (5). Chinese hamster lung (Dede) cells and Chinese hamster ovary (CHO) cells were grown in medium containing delipidated fetal bovine serum (4 mg/ml) as described previously (10). Mouse myeloma (MOPC2104E) cells were grown in Dulbecco's modified medium containing 5% horse serum (9).

Spleen cells were obtained from six adult C57BL/6J male mice. The spleens were pressed through a fine wire mesh, and the mesh was washed with Waymouth's 752/1 medium. Cells were sedimented by centrifugation at 600 x g at 20°C for 5 min and washed twice with medium. They were then suspended in 18 ml of medium and divided into two equal parts. One part was incubated with [3H]-cholesterol, the other with [3H]25-hydroxycholesterol under the conditions described below.

Incubation of Sterols with Cell Cultures—Prior to incubation with a sterol the cultures were refed with 9 ml of fresh medium. Sterol suspended in a solution of 10% (v/v) ethanol and 4.5% (w/v) bovine serum albumin (Sigma, essentially fatty acid free) in culture medium was then added so that the final concentration of albumin was 0.45% and that of ethanol was 1% (5). Routinely the cultures were incubated at 37°C on a gyrating table (40 rpm) for an appropriate period of time after which, while the cells were still adhering as a monolayer, they were washed by flooding the dishes four times with 10-ml volumes of fresh medium free of any additions at 0°C.

In competition experiments with whole cells, after labeling with [3H]25-hydroxycholesterol as described above, the cultures were

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washed four times with 10-ml volumes of medium at 37°C. They were then reincubated with unlabeled sterol after which they were washed three times with 10-ml volumes of medium at 0°C. Cells were harvested by scraping them into 13 ml of medium and sedimented by centrifugation. All further operations were carried out at temperatures between 0°C and 4°C unless indicated otherwise. To obtain nuclear, microsomal, and soluble fractions, the cells were resuspended in hypotonic buffer, homogenized (50 passes) with a glass homogenizer with a Teflon plunger, and fractionated by differential centrifugation as described by Baxter et al. (11). Microscopic examination of the unfractonated homogenate in the presence of trypan blue indicated that approximately 96% of the cells were disrupted. When only the nuclear fraction of the cells was required, the cells were homogenized in a hypotonic buffer, pH 7.8 (Buffer A) containing 20 mM Tris, 1 mM MgCl₂, 2 mM CaCl₂, and 2 mM dithiothreitol. A solution of 30% sucrose (w/w) in Buffer A was then added to give a final concentration of 5% sucrose. Subcellular fractions were dissolved in Soluene (New England Nuclear) and assayed for 'H and 'C in toluene base scintillation fluid. Sucrose density gradient centrifugation (5% to 20% sucrose), collection of fractions, and assays of the fractions for ¹⁴C and 'H were carried out as described (7), except that the sample was centrifuged with the gradient for either 20 h at 40,000 rpm or 40 h at 35,000 rpm. Sterol concentrations in cells or in subcellular fractions were expressed in terms of protein or, when comparisons were between essentially identical cultures, as amounts derived from one culture.

Incubation of Sterols with Isolated Cytosol—Sterols dissolved in 10 μl of freshly distilled absolute ethanol were placed in the bottom of a 10-ml test tube siliconized by treatment with Siliclad (Dow Corning). The cytosolic fraction (1 ml), isolated from cultured cells as described above, containing from 1.2 to 1.8 mg of protein, was added to the tube, shaken gently, and incubated in an ice bath or, where indicated, in a water bath at 25°C. The mixture was then fractionated by sucrose density gradient centrifugation as described above.

Other Assays—HMG-CoA reductase activity in whole cell homogenates was determined as described previously (12). Protein was determined by a modification of the Lowry procedure (13) or by a dye-binding assay (14) using reagents purchased from Bio-Rad. The incorporation of [³⁵S]leucine into peroxisomal-acid precipitated proteins was determined by incubating cultures with 1.4,5-³H]leucine at concentrations of 2 μCi (0.03 mmol)/ml of medium for 20 min. The cultures were then scraped from the flasks, sedimented by centrifugation, and extracted three times with 5-ml volumes of 6% perchloric acid and once with 5 ml of 90% ethanol. The residue was then dissolved in a solution of 0.3 N NaOH containing 1.7% sodium dodecyl sulfate. The mixture was neutralized with HCl and aliquots were taken for protein and tritium assays.

RESULTS

Subcellular Localization and Cell Uptake Kinetics—The data shown in Fig. 1 represent the uptake by L cells of 25-hydroxycholesterol and cholesterol and their localization in some subcellular fractions as a function of time. The concentration of the sterols in the medium (50 μM) was that which results in the maximum rate of uptake of the two sterols (18). The relationship between the two curves illustrating total cell uptake with increasing time (Fig. 1A) is consistent with that reported previously; the uptake of 25-hydroxycholesterol was greater than that of cholesterol over the entire time period. The relative proportions of the two sterols at various time intervals in these crude soluble (cytosolic), nuclear, and microsomal fractions did not differ greatly from that in the whole cells, and the data, therefore, do not provide clear evidence for the preferential subcellular localization of either sterol. The concentrations of the two sterols were higher in the nuclear fraction than in the microsomal or cytosolic fractions on a microgram of sterol per mg of protein basis. The quantitative relationships between 25-hydroxycholesterol and cholesterol, found in whole cell homogenates and in different subcellular fractions shown in Fig. 1, are representative of several other experiments with L cells in which the concentration of the sterols in the medium was varied between 1.5 and 100 μM and the incubation period between 15 min and 2 h. Additional similar experiments with long-term fetal liver cell cultures gave results similar to those obtained with L cell cultures. The bulk of the recovered sterols was found in the particulate fractions, presumably because of their association with lipid-rich membrane elements. A relatively small proportion (1 to 2%) of the sterols taken up by the cells was recovered in the cytosolic fraction. In this fraction, however, was found a class of sterol-binding sites with potentially interesting properties, and it is these sites and their properties with which the remainder of this paper deals.

Differential Binding of Cholesterol and 25-Hydroxycholesterol to Cytosolic Proteins in Various Lines of Cultured Cells—Although only a small fraction of each sterol taken up by L cells was found in the cytosol, previous studies (7) showed that the hydroxysterol and cholesterol were bound to different protein fractions. Fig. 2A shows that the sedimentation patterns for 25-hydroxycholesterol and cholesterol bound to the cytosolic proteins of an established fetal liver cell line were similar to those found with L cells (7). Two bands of 25-hydroxycholesterol with sedimentation coefficients of approximately 8S and 5S, as determined by comparison with 'H-labeled standards (7), were present in each cell type, whereas cholesterol was present throughout the gradient, with the highest concentrations near the bottom. Protein in the fractions assayed by measurements of absorbance at 2600 Å and 2800 Å (not shown) appeared as a single band with its peak corresponding approximately with that of the lighter (5S) band containing [³H]25-hydroxycholesterol. The presence of the 8S and 5S 25-hydroxycholesterol-binding proteins in a variety of cultured cell lines is illustrated in Fig. 2B. Amounts of cytosolic protein placed upon the gradient and the conditions of cell culture varied somewhat for the different cell types, so that precise quantitative comparisons between the patterns are not meaningful. However, Chinese hamster ovary (CHO) cells consistently showed the greatest proportion of the bound 25-hydroxycholesterol in the 5S band, whereas the 8S band was predominant in the cytoplasm.

![Fig. 1. Subcellular localization of sterols taken up by L cell cultures](image-url)
for the other nonhepatic cells. The banding patterns for cholesterol in cytosolic fractions from nonhepatic cells in Fig. 2B are not shown, but they were generally similar to that shown for the established liver cell in Fig. 2A and that shown previously for L cell cultures (7). In no case was the banding pattern for cholesterol in the cytoplasm of any of the cultures shown in Fig. 2B similar to that found for 25-hydroxycholesterol.

Only one of the types of cell cultures tested, namely primary cultures of mouse fetal liver cells, exhibited sedimentation patterns for bound sterols which appeared to be qualitatively different from those shown in Fig. 2. Cytosol from primary fetal liver cell cultures showed only one major band (approximately 5 S) with a slight shoulder corresponding roughly to an 8 S component, whether the cells were incubated either with [3H]25-hydroxycholesterol or [3H]cholesterol (Fig. 3A). Primary cultures of fetal liver cell differ from the long-term cell line and from other established cell cultures in many respects, including a lower sensitivity to the inhibitory effects of most oxygenated sterols upon cholesterol synthesis (2, 5, 6), a much greater capacity to take up cholesterol and oxygenated sterols from the medium (2-6), and a greater ability to metabolize the oxygenated sterols, at least in comparison to L cells (4). The differences in sensitivity to, and uptake of, sterols might be accounted for by the presence in the primary fetal liver cell cultures of one or more liver-specific proteins which bind both cholesterol and oxygenated sterols. The presence in liver of several different cytoplasmic proteins, each of which is capable of binding a variety of lipids including sterols, has been reported (16-19).

The presence of such proteins could also account for the distinctive binding pattern for 25-hydroxycholesterol in the cytoplasm of the primary liver cell culture shown in Fig. 3A, with little evidence for an 8 S and 5 S component, and for the apparent qualitative similarity between the binding of 25-hydroxycholesterol and cholesterol. In contrast to the primary fetal liver cell cultures, the banding patterns for the established fetal liver cell line (Fig. 2A) resembled those of other established cell lines, as does its capacity for sterol uptake and its sensitivity to the suppressive effects of oxysterols on HMG-CoA reductase (4, 6, 15).

Evidence that differences in banding of the two sterols between primary fetal liver cell cultures and established cell cultures were not due to adaptational changes of cells to the conditions of long-term culture was provided by the results obtained with mouse spleen cells (Fig. 3B). The banding pattern for 25-hydroxycholesterol was similar to that of the cultured cells shown in Fig. 2 with clearly distinguishable 8 S and 5 S components. The banding pattern for cholesterol also corresponded generally with that found in the established cell lines, sterol being present throughout the gradient but largely concentrated near the bottom.

**Properties of the 8 S and 5 S 25-Hydroxycholesterol-binding Proteins**—The 8 S and 5 S 25-Hydroxycholesterol-binding proteins from L cell cytosol were not strongly involved in the binding of the diol to the protein was shown by the following experiments. When diithiothreitol was omitted from the buffer, the recovery of 8 S and 5 S sterol in the cytosol fraction was diminished about 30%. However, the addition of p-hydroxymercuribenzoate (1 mM) to the buffer in the absence of diithiothreitol did not further depress the amounts of sterol recovered in these bands. Treatment of the isolated, labeled cytosol with sulphydryl-oxidizing

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**Fig. 2. Binding of sterols to cytoplasmic proteins in some established cell cultures.** A, fetal mouse liver cells (FL83B) were incubated for 20 min at 37°C with 0.1 μM (1 μCi/ml) of [26,27-3H]25-hydroxycholesterol (C—C) or with 0.1 μM (1 μCi/ml) of [1,2-3H]cholesterol (X—X). B, [26,27-3H]25-hydroxycholesterol was incubated under the same conditions with various cell cultures. The cytosol fraction was extracted from the cells and centrifuged in a 5 to 20% sucrose density gradient for 20 h at 40,000 X g as described in the text. Amounts of cytosolic protein placed upon the gradient ranged between 1.0 and 1.5 mg. Fraction 1 corresponds to the top of the gradient in this and all subsequent figures.

**Fig. 3. Binding of [1,2-3H]cholesterol and [26,27-3H]25-hydroxycholesterol to cytoplasmic proteins in primary cultures of mouse fetal liver cells (A) and primary cultures of mouse spleen cells (B).** Incubation conditions with [3H]25-hydroxycholesterol (C—C) and [3H]cholesterol (x—x) were as in Fig. 2. The amounts of cytosolic protein placed upon the sucrose gradients were 1.4 and 0.96 mg of protein in A and B, respectively.
terol in the incubation of L cell cultures for 22 h with 10 mM dibutyryl cyclic nucleotides did not appear to influence the binding of 25-hydroxycholesterol to the 8 S complex. However, the observed decline in the 5 S complex appeared to be relatively independent of the presence of albumin in the medium and also of time after the first 20 min of incubation. These results indicated that dissociation of the 8 S complex was related to efflux of sterol agents (5 mM o-phenanthroline plus 1 mM CuCl₂ or 2 mM tetrathionate) for 10 min at 25°C also did not affect the 8 S or 5 S complexes (data not shown).

Cyclic nucleotides did not appear to influence the binding of 25-hydroxycholesterol to the cytoplasmic proteins, since incubation of L cell cultures for 22 h with 10 mM dibutyrlyl cAMP, or 10 mM theophylline, alone or together, did not affect the subsequent uptake and binding of [³H]25-hydroxycholesterol to the cytosolic proteins.

The results of experiments with cycloheximide suggested that the half-lives of the 25-hydroxycholesterol-binding components are relatively long. In the presence of 30 μM cycloheximide, HMG-CoA reductase activity in L cells declined (T½ = approximately 5 h), whereas the amount of [³H]25-hydroxycholesterol (Table I) bound to the 8 S and 5 S cytosolic components appeared to increase somewhat. Under these conditions, incubation for 30 min with cycloheximide resulted in an 86% decrease in the incorporation of [³H]lucine into perchloric acid-precipitable material. In a single experiment, incubation of L cells for 5 h with actinomycin D (10 μg/ml) diminished HMG-CoA reductase activity to 18% of the control value but did not alter the amount of 25-hydroxycholesterol bound to the 8 S cytosolic components.

Kinetics of 25-Hydroxycholesterol Binding to Cytoplasmic Proteins—The results shown in Fig. 5 provide evidence that the binding of 25-hydroxycholesterol to the 8 S cytosolic protein is a saturable process. The proportion of added diol that was bound to the 8 S protein declined with increasing sterol concentration, whereas binding to the 5 S component was linear within the range of concentrations employed (Fig. 5). The linear relationship between the binding of 25-hydroxycholesterol to the 5 S component and sterol concentration, along with information regarding the kinetics of cellular 25-hydroxycholesterol uptake over a much wider range of concentrations (Fig. 1 and Ref. 14), is evidence that the hyperbolic curve shown for the 8 S band in Fig. 5 reflects the character of the binding reaction and is essentially independent of unrelated uptake processes.

A steady state equilibrium between free 25-hydroxycholesterol and that bound to the 8 S cytosolic protein appeared to be reached soon after the sterol, at a concentration of 0.1 μM, was added to the medium (Fig. 6A). Maximum binding to both the 8 S and 5 S components was attained after approximately 30 min of incubation at 37°C using a single concentration of sterol. Following the removal of exogenous [³H]25-hydroxycholesterol and incubation of the labeled cells in protein-free medium, or in medium containing 0.45% albumin, the amount of extractable 8 S complex declined slowly (Fig. 6B). The extent of dissociation of the 8 S complex after 20 min in protein-free medium and in medium containing 0.45% albumin was 19% and 30%, respectively. In contrast, 86% dissociation occurred after 20 min in medium containing 1% albumin. Apparent losses of labeled sterol from the 5 S band of as much as 50% occurred during the first 20 min of incubation in sterol-free medium. However, the observed decline in the 5 S complex appeared to be relatively independent of the presence of albumin in the medium and also of time after the first 20 min of incubation. These results indicated that dissociation of the 8 S complex was related to efflux of sterol.

**Table I**

| Incubation    | Concentration of cytosolic protein (mg/ml) | 25-Hydroxycholesterol bound (10⁻⁷ x dpm/mg cytosolic protein) | HMG-CoA reductase activity (pmol/min mg cell protein) |
|---------------|------------------------------------------|-------------------------------------------------------------|---------------------------------------------------|
| h             | 8 S                                      | 5 S                                                          |                                                  |
| 0             | 1.3                                      | 9.5                                                         | 4.2                                               |
| 2.5           | 1.3                                      | 13.7                                                       7.9                                               | 167                                               |
| 5.0           | 1.1                                      | 13.2                                                       6.7                                               | 116                                               |
| 14.0          | 0.7                                      | 17.4                                                       11.6                                              | 33                                                |

**Fig. 4.** Quantitation of bound [26,27-³H]25-hydroxycholesterol in the 8 S and 5 S bands of L cell cytosol. Conditions were similar to those in Fig. 2 except that the cytosol fraction was centrifuged in the sucrose gradient for 40 h at 35,000 × g.

**Fig. 5.** Binding of 25-hydroxycholesterol to L cell cytoplasmic proteins as a function of the concentration of sterol in the medium. Conditions were similar to those in Fig. 4 except that the [26,27-³H]25-hydroxycholesterol added to the medium was diluted with increasing amounts of unlabeled sterol. The area under the 5 S and 8 S peaks was estimated as indicated in Fig. 4.
Sterol-binding proteins in L cell cultures (A) and dissociation of the sterol-protein complexes upon reincubation in sterol-free medium (B) as functions of time. A. [26,27-3H]25-hydroxycholesterol (0.1 μM, 1 μCi/ml) was incubated at 37°C with L cell cultures for varying periods of time before assaying radioactivity in the cytosolic 8 S and 5 S complexes. B. Cultures incubated with labeled 25-hydroxycholesterol as described for A were washed four times with 10-ml volumes of medium (protein-free) and then reincubated at 37°C for varying periods of time in medium alone (●), medium containing 0.45% albumin (○), or medium containing 1% albumin (×). ---, radioactivity in the 8 S band; ---, radioactivity in the 5 S band.

from the cell which was, in turn, strongly influenced by the presence of albumin in the medium.

Temporal Relationships Between Dissociation of 25-Hydroxycholesterol-8 S Protein Complex and the Recovery of HMG-CoA Reductase Activity—If binding of 25-hydroxycholesterol to the 8 S protein(s) is involved in the mechanism leading to suppression of HMG-CoA reductase, the kinetics of complex formation and dissociation should be consistent with the kinetics of the suppression and recovery of reductase under the same conditions. The data in Figs. 2 through 6 show that binding of the diol to the 8 S protein(s) occurs rapidly, so that formation of the 8 S complex could be an intermediate step in the suppression of HMG-CoA reductase activity, since the rate at which the activity declines in the presence of relatively high levels of 25-hydroxycholesterol is approximately 1.5 h (6). The results in Fig. 7 show that, under conditions wherein reductase activity in L cells has been suppressed by a concentration of 25-hydroxycholesterol adequate to saturate the 8 S protein(s) (12.5 μM), subsequent dissociation of the 8 S complex following the removal of exogenous sterol is correlated with the recovery of HMG-CoA reductase activity. In Fig. 7A, unlabeled 25-hydroxycholesterol was added to the medium at zero time at a level of 12.5 μM, the sterol was removed after 20 min, and the culture was then incubated in protein-free medium. After various periods of 5 min after the addition to the medium of unlabeled 25-hydroxycholesterol at a concentration of 50 μM. Tritiated 25-hydroxycholesterol in the 5 S band was, on the other hand, unaffected by the unlabeled sterol (data not shown).

The effects of incubation with some other unlabeled sterols upon 8 S and 5 S protein-[3H]25-hydroxycholesterol complexes in L cells are illustrated in Fig. 8. The concentration of sterol chosen for these comparisons was 25 μM, and the incubation period with the unlabeled sterol was 20 min. A procedure involving sequential exposure of the cells, first to a low concentration of [3H]25-hydroxycholesterol, then to a relatively high concentration of unlabeled sterol, was chosen to avoid the possibility of competition for transport or of extra-
cellular interactions between sterols added together to the medium. Unlabeled 20a-hydroxycholesterol (a potent suppressor of HMG-CoA reductase activity) as well as 25-hydroxycholesterol competed for 8 S sites occupied by [3H]25-hydroxycholesterol. In contrast, the 25,26,27-nor homologue and reincubated with previously measured abilities to suppress HMG-CoA reductase activity. The failure of 5a-lanosta-7,22-dien-3p-ol at the top of the list do not suppress the reductase (6); results similar to those of 20a-hydroxycholesterol (1 pCi/ml), then washed four times with 10-ml volumes of medium and reincubated at 37°C for 20 min with the indicated unlabeled sterols at a concentration of 25 μM.

Fig. 8. Density gradient centrifugation patterns illustrating the abilities of some unlabeled sterols to compete for 8 S sites occupied by [26,27-3H]25-hydroxycholesterol in L cell cultures. Cultures were incubated for 20 min at 37°C with 0.1 μM [3H]25-hydroxycholesterol (1 µCi/ml), then washed four times with 10-ml volumes of medium and reincubated at 37°C for 20 min with the indicated unlabeled sterols at a concentration of 25 μM.

A survey of competition by various unlabeled sterols for 8 S sites in intact L cell cytosol was assayed as in Fig. 8. Some correlation between the abilities of the sterols to compete for the 8 S sites and their previously measured abilities to suppress HMG-CoA reductase activity is apparent. The two monofunctional sterols and the 25,26,27-nor diol at the top of the list do not suppress HMG-CoA reductase in cell cultures and showed little ability to compete for 25-hydroxycholesterol 8 S-binding sites. The competitive activities of the following 3β-hydroxy sterols functionalized in position 25, 15, 6, 20, 32, 22, or 7 were roughly consistent with their previously reported activities as inhibitors of HMG-CoA reductase activity. The failure of 5α-lanosta-7-ene-3β,22-diol to compete effectively in contrast to the greater activity of its Δ7 isomer may be noteworthy. The Δ7 sterol is converted into cholesterol in L cells more rapidly than the Δ7 isomer, but both sterols give rise to other products as well (4). Thus, competitive ability measured in the test with intact cells may be influenced by metabolism of the sterol being tested. A further uncertainty in tests with intact cells is due to a lack of information regarding the intracellular concentrations of the test sterol that are available for binding to the 8 S protein. It is possible that concentrations of weak competitors cannot be elevated high enough to give observa-

TABLE II

| Sterol                                | Concentration for 50% inhibition of HMG-CoA reductase (μM) | 8 S [% of [3H]25-hydroxycholesterol] | % control |
|----------------------------------------|------------------------------------------------------------|-------------------------------------|--------|
| Cholesterol                            | Not inhibitory                                             | 90                                  |        |
| 5a-Cholestan-3β-ol                     | Not inhibitory                                             | 80                                  |        |
| 25-Propyl-pregnene-3β,20α-diol          | Not inhibitory                                             | 84                                  |        |
| 25-Hydroxycholesterol                  | 0.05                                                       | 12                                  |        |
| 5α-Cholest-8(14)-en-3β-ol              | 0.3                                                        | 18                                  |        |
| 6-Ketocholesterol                      | 1.5                                                        | 21                                  |        |
| 20α-Hydroxycholesterol                 | 1.5                                                        | 25                                  |        |
| 5α-Lanost-7-ene-3β,32-diol              | 1.7                                                        | 49                                  |        |
| 22-Ketocholesterol                     | 3.2                                                        | 57                                  |        |
| 7β-Hydroxycholesterol                  | 2.5                                                        | 66                                  |        |
| 5α-Lanost-8-ene-3β,32-diol              | 2.5                                                        | 82                                  |        |

* Values reported in References 2, 4-6, and 8.

b Calculated from values for 8 S dpm/mg of cytosolic protein.

Fig. 9. Binding of 25-hydroxycholesterol to proteins in isolated cytosol. L cell cytosol (1 ml, containing 1.8 mg of protein) was incubated for 2 h at 0°C with [26,27-3H]25-hydroxycholesterol (1 μCi, 7.5 ng in 15 μl of ethanol) in a siliconized 10-ml test tube. Binding of the sterol to cytosolic proteins was assayed as in Fig. 4.
considerably diminished \(^{3}H\)-sterol throughout the gradient, including that in the 8 S complex.

Accurate quantitation of the 8 S band was not possible under the conditions shown in Fig. 9. Therefore, additional competition studies to examine the specificity of the 8 S-binding protein were carried out with cytosol isolated from L cells that had been previously incubated with \(^{[3}H\)-25-hydroxycholesterol. Preliminary experiments showed that the 8 S and 5 S complexes were nearly stable for as long as 24 h when the isolated cytosol was incubated at 0–4°C. Furthermore, the presence of unlabeled 25-hydroxycholesterol added to the cytosol as an ethanolic solution (15 μl/ml of cytosol) in concentrations as high as 750 nm (approximately a 600-fold excess over the labeled sterol present in the cytosol) had only a minor effect upon the amount of bound \(^{3}H\)-sterol in the 8 S band at these temperatures, decreasing it by only 10% at the highest sterol concentration tested. In contrast, at 25°C dissociation of the 8 S complex appeared to be biphasic. Approximately 10% of the bound \(^{3}H\)sterol was lost during the first hour after which a slower, linear rate of loss obtained for at least 9 additional h (Fig. 10). The 5 S complex also showed an initial rapid rate of dissociation during the first hour. However, further dissociation with continued incubation was not apparent. The inset in Fig. 10 shows that the addition of unlabeled 25-hydroxycholesterol to the labeled cytosol after it had been incubated for 1 h at 25°C increased the rate at which the \(^{3}H\)-sterol was lost from the 8 S band. Presumably, this effect of the unlabeled sterol was due to its ability to occupy vacant binding sites, thus inhibiting reassociation of released \(^{[3}H\)-25-hydroxycholesterol. The addition of the unlabeled sterol did not affect the \(^{[3}H\)-25-hydroxycholesterol-5 S complex (data not shown).

A number of other unlabeled sterols were tested for their abilities to compete with \(^{[3}H\)-25-hydroxycholesterol for 8 S sites in isolated cytosol under conditions similar to those in Fig. 10. This procedure for testing competition for binding sites with isolated cytosol is cumbersome and insensitive. The dissociation versus time plot is complex, and it is not known whether or not some part of the observed dissociation is due to denaturation of the binding protein(s) and, therefore, irreversible. For these reasons, the results of competition experiments with isolated cytosol as carried out, would not, by themselves, constitute strong evidence for the specific binding of oxysterols, and they are not presented in detail. The data do, however, confirm in general the conclusions drawn from competition experiments with intact cells. Unlabeled sterols were tested in amounts 200- to 2000-fold greater than the amount of \(^{[3}H\)-25-hydroxycholesterol present in the incubation mixture as an 8 S complex (approximately 70 fmol/mg of cytosolic protein). Differences between the sterols that were seen included the following. 5a-Cholest-8(14)-en-3β-ol-15-one and 5a-lanost-7-ene-3β,25-diol appeared to compete as effectively for \(^{[3}H\)-25-hydroxycholesterol-binding sites as did unlabeled 25-hydroxycholesterol. However, as in tests with intact cells, 5a-lanost-7-ene-3β,25-diol was a better competitor than the Δ\(^{5}\) isomer. The 7-functionalized sterols were relatively poor competitors, and two sterols which did not suppress HMG-CoA reductase (cholesterol and 20-propyl-pregnene-3β,20a-diol) were not effective competitors.

**DISCUSSION**

The present results extend in several directions our knowledge of cytosolic proteins that bind 25-hydroxycholesterol. The 8 S and 5 S 25-hydroxycholesterol-binding proteins were present in a variety of established cell culture lines and in a primary culture of mouse spleen cells. These proteins did not bind an appreciable amount of cholesterol, which was largely associated with complexes of greater density. Primary cultures of fetal mouse liver cells differed in that 25-hydroxycholesterol and cholesterol appeared to bind similarly to components of the cytosol which sedimented essentially as a single band of approximately 5 S with only a trace of 8 S material. It seems likely that the binding of both sterols in the same fraction of the cytosol of the primary fetal liver cultures reflects the presence of one or more liver proteins which are able to bind a wide range of lipids (19–22). Binding of the sterols to each protein(s) in the primary cultures of fetal liver may have masked any of the more specific 25-hydroxycholesterol-binding complex formed. If this explanation is correct, some of the proteins which bound 25-hydroxycholesterol nonspecifically were lost during the development of the established line of fetal mouse liver cells, unmasking the presence of the 8 S and 5 S binding proteins. It is also possible that the latter proteins only began to be expressed after the line became established.

Preliminary experiments indicate that the 8 S binding protein(s) is not identical with receptors for glucocorticoids or the sex steroids. First, as noted above, the 8 S oxygenated sterol-binding protein(s) is not particularly sensitive to reagents known to promote oxidation of thiol groups, whereas the steroid hormone receptors are exquisitely so (21, 22). Second, the sterol-binding protein(s) appears to be less thermolabile than are the steroid hormone receptors (21, 22). Third, under some conditions steroid hormone receptors are known to sediment as 8 S components on sucrose density gradients (21, 22). However, two types of experiments indicated that steroid hormones do not bind specifically to the oxysterol-binding 8 S component. Direct steroid hormone-binding studies and studies of the inhibition of \(^{[3}H\)-25-hydroxycholesterol binding gave no indication that cortisone, dexamethasone, testoster-
one, or 17β-estradiol bind to the 8 S oxysterol-binding protein(s) (data not shown). Furthermore, limited studies suggest that the 8 S binding protein(s) is not identical with the sterol-binding proteins previously described by others. Collaborative comparisons of the sedimentation rates of the sterol-binding proteins investigated by Dr. T. J. Scallen and his associates (18) and by Dr. J. L. Gaylor and his associates (19) indicated their nonidentity with the 8 S protein that binds oxygenated sterols.

The binding of 25-hydroxycholesterol to form 8 S and 5 S complexes involves the participation of proteins (7). The results of studies with cycloheximide suggest that the half-lives of the 8 S and 5 S binding proteins are longer than 14 h. The formation of the complexes within the cells did not appear to involve cAMP. Several lines of evidence indicate that binding of oxygenated sterols to the 8 S protein(s) differs qualitatively from their binding to the 5 S band. First, the kinetics of 25-hydroxycholesterol binding to the 8 S protein in intact cells indicate that it is saturable and reversible. In contrast, binding to the 5 S proteins was not saturable at the same concentrations of sterol. Second, evidence that the 8 S sites to which 25-hydroxycholesterol was bound were specific for oxygenated sterols which can suppress HMG-CoA reductase activity through two kinds of competition assays. Strong suppressors of HMG-CoA reductase, which are generally good competitors for the 8 S, 25-hydroxycholesterol-binding sites in intact L cell cultures. However, 5α-lanost-8-ene-3β,3β-diol, which was 50 times less potent than 25-hydroxycholesterol as a suppressor of HMG-CoA reductase activity, showed little or no ability to compete for sites occupied by the labeled diol under these conditions. The failure of the Δ5-lanosterol derivative to compete effectively in assays with intact cells might be due to its metabolism by the cell to inactive sterols, including cholesterol (4). Results obtained in assays with isolated cytosol were generally similar to those obtained with intact cells, and again the Δ5-derivative failed to compete well, although it is unlikely that significant metabolism of the sterols occurred in the cytosolic fraction under the conditions of the assay. No evidence for metabolism of either of the lanosterol derivatives was obtained in an experiment wherein the 2-3H2-labeled compounds (15,500 dpm for the Δ5-sterol, 31,600 dpm for the Δ7 isomer) were incubated at a concentration of 2.3 μM in medium containing 2.2 mg of protein for 7 h at 25°C, then extracted and analyzed by thin layer chromatography as described previously (4). Clearly, the sensitivities of both competition assays may be limited by the solubilities (unknown in most instances) of the sterols in aqueous solutions and by their propensities to bind nonspecifically to various substances including glass and plastic. It may, therefore, have been impossible in these experiments to achieve sufficiently high concentrations of, or appropriate incubation conditions for, some sterols, which have low affinities for binding sites, to demonstrate competition with 25-hydroxycholesterol.

With the aforementioned exception, oxygenated sterols which suppress HMG-CoA reductase activity in cultured cells were able to compete for 8 S 25-hydroxycholesterol-binding sites in at least one of the two assay systems. Sterols which do not suppress the reductase, including the 25,26,27-nor homologue of the potent inhibitor, 20α-hydroxycholesterol, did not compete for 8 S sites. Thus, the 8 S protein fraction appears to bind inhibitory sterols relatively specifically, a property consistent with a role in the regulation of HMG-CoA reductase activity. The kinetics of the formation and dissociation of the 25-hydroxycholesterol-8 S complex were also consistent with its involvement in the regulation of the reductase. Formation of the complex in intact cells was rapid enough to precede any appreciable decline in HMG-CoA reductase activity, and dissociation of the complex preceded any rise in reductase activity from a depressed level.

The actual role, if any, of the 8 S sterol-protein complex in the regulation of HMG-CoA reductase is presently only a matter for conjecture, since it has not been established whether oxygenated sterols suppress the synthesis of HMG-CoA reductase, accelerate its degradation, or inactivate it by some indirect mechanism (reviewed in Ref. 1). 25-Hydroxycholesterol does not inactivate the enzyme directly (5, 6) nor does it reversibly inactivate the enzyme indirectly via a system that appears to involve reversible phosphorylation, which is currently under study in several laboratories (12). Furthermore, L cell microsomal HMG-CoA reductase activity was not affected by preincubation with cytosol containing 25-hydroxycholesterol-protein complexes. Activity values were not significantly different when the microsomal fraction (880 μg) of protein was preincubated for 20 min at 37°C with cytosol (225 μg of protein) from control L cells or from L cells that had been allowed to take up 25-hydroxycholesterol, present in the medium at concentrations of 40 ng/ml or 10 μg/ml (data not shown). On the other hand, a good deal of evidence indicates that most of the known regulatory fluctuations in HMG-CoA reductase activity that occur in vivo involve changes in the rate of enzyme synthesis (see Refs. 1 and 23 for reviews). In our experience temporal changes in reductase activity following the addition of oxygenated sterols to the medium are consistent with alterations in the synthesis of an enzyme with a half-life of 1 to 2 h (6); 1/2 values as low as 1 h for the rat liver enzyme have been reported (24). If further studies demonstrate that the oxygenated sterols affect the rate of reductase synthesis then we expect that models for the action of steroid hormone receptors may be relevant to that of the 8 S oxygenated sterol-binding protein. 

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