Proc. Natl. Acad. Sci. USA
Vol. 74, No. 6, pp. 2500–2503, June 1977
Cell Biology

Binding of 25-hydroxycholesterol and cholesterol to different cytoplasmic proteins

(regulation/sterol synthesis/cell cultures)

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Communicated by Elizabeth S. Russell, April 14, 1977

ABSTRACT **Studies were carried out to determine whether or not oxygenated derivatives of cholesterol (e.g., 25-hydroxycholesterol) that specifically suppress the activity of 3-hydroxy-3-methylglutaryl-CoA reductase [mevalonate:NADP$^+$ oxidoreductase (CoA-acylating), EC 1.1.1.34], bind to a soluble component of the cytoplasm different from that which binds the nonsuppressor, cholesterol. Density gradient fractionation of the cytosolic fraction isolated from L cell cultures that had been incubated with low concentrations of 25-hydroxy[*26,27*-^{3}H]cholesterol or [*1,2*-^{3}H]cholesterol provided evidence for the existence of at least two different sterol-binding proteins. Bound cholesterol sedimented in a sucrose density gradient as two or more broad bands with coefficients of approximately 9 S and 21 S. Two relatively narrow bands of bound 25-hydroxycholesterol had sedimentation coefficients of 5 S and 8 S. Preincubation of the cells with a relatively high concentration of unlabeled 25-hydroxycholesterol altered the banding pattern of the 25-hydroxy[^{3}H]cholesterol taken up during a subsequent incubation period by decreasing the size of the major (8S) band. Under these conditions, cholesterol did not affect the banding pattern of 25-hydroxy[^{3}H]cholesterol. The density gradient banding pattern of bound [^{3}H]cholesterol was only slightly affected by preincubating the cells with unlabeled cholesterol or 25-hydroxycholesterol. Both sterols appeared to be bound to proteins because the bound sterols were eliminated from cytosol that had been heated at 100°, and their sedimentation coefficients were altered by proteolysis.**

It has been established that certain derivatives of cholesterol that are produced by the introduction of a ketone or hydroxyl function in the steroid nucleus or in the side chain specifically depress the activity of the microsomal enzyme 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase [mevalonate: NADP$^+$ oxidoreductase (CoA-acylating), EC 1.1.1.34] in cultured cells when they are added to the medium at concentrations in the range of 0.05–5 μM (1–4). Whether or not pure exogenous cholesterol is itself capable of suppressing the enzyme activity in cell cultures is disputed. In other studies, pure cholesterol, at concentrations of 0.2 mM or higher, was taken into the cells but did not affect HMG-CoA reductase activity in cultures of human fibroblasts (5) or mouse fibroblasts or in primary or long-term cultures of fetal mouse liver cells (1–4) when an antioxidant was present in the medium to inhibit autoxidation reactions (4). On the other hand, the suppression of HMG-CoA reductase activity in cells grown in the presence of serum or serum lipoproteins was assumed by Brown and Goldstein (6, 7) and by Kirsten and Watson (8) to be due to the cholesterol component of the lipoproteins. Weak suppression of HMG-CoA reductase activity in fibroblast cultures by relatively high levels of nonlipoprotein cholesterol has also been reported from several laboratories (1, 9–11).

A probable explanation for these contrasting observations lies in our finding that inhibitory activity associated with commercial preparations of cholesterol, which had been recrystallized several months previously and which appeared to be pure by the usual chromatographic criteria, was in fact due to trace amounts of contaminating sterols (1). Furthermore, the most potent known suppressors of HMG-CoA reductase activity are readily produced by autoxidation of cholesterol, a reaction that may be expected to occur when serum lipoproteins or cholesterol preparations are exposed to air or are suspended in culture medium and incubated at 37° for prolonged periods of time in the absence of an antioxidant (1–3, 12, 13). Because the purity of the cholesterol was not established in most studies in which it appeared to be inhibitory and because the generation of cholesterol autoxidation products in the culture media was not excluded, it seems likely that the suppression of HMG-CoA reductase observed in those studies was due to compounds other than cholesterol.

If the results of our investigations with cultured cells are relevant to the regulation of cholesterol synthesis *in vivo*, then they imply that dietary cholesterol depresses hepatic HMG-CoA reductase activity because in the liver it is a precursor of an inhibitory sterol. They also imply that purified cholesterol did not suppress HMG-CoA reductase in the cell cultures because these cells did not metabolize it to any inhibitory sterol, which is consistent with what is presently known in this regard (3). The probability that oxygenated sterols—either precursors of cholesterol or products of its metabolism—function as regulators of sterol synthesis *in vivo* is enhanced by the observation that hydroxylated cholesterol derivatives suppress cholesterogenesis in rapidly proliferating intestinal crypt cells very effectively when they are administered orally to mice, whereas cholesterol does not (14). Intracellular synthesis of a regulatory sterol other than cholesterol seems to be a reasonable explanation for the cycle of sterol synthesis observed in lectin-stimulated lymphocytes cultered in medium containing lipoprotein cholesterol or cholesterol suspended with albumin (15).

The mechanism by which the cholesterol derivatives suppress HMG-CoA reductase activity has not been determined. Diurnal alterations in hepatic HMG-CoA reductase activity and induction of the enzyme activity by cholestyramine feeding or by injecting Triton WR 1339 involve alterations in the rate of enzyme synthesis (16–19). On the other hand, dietary cholesterol may result in inactivation of the enzyme as well as suppression of its synthesis (20). Because inhibitory sterols do not affect HMG-CoA reductase activity directly—i.e., when they are added to an enzyme reaction mixture (1–3)—it seems likely that they repress the synthesis of the enzyme or else they inactivate it through some indirect mechanism as has been suggested by Bell *et al.* (10).

It is reasonable to postulate that (*i*) either of the above mechanisms for suppressing HMG-CoA reductase activity involves the specific binding of the inhibitory sterol to some intracellular component other than the enzyme itself, and (*ii*) cholesterol, which is not inhibitory, either will not bind to the same component or will bind to it with lower affinity than does the inhibitory sterol. Previously reported evidence for the dif-

Abbreviation: HMG-CoA, 3-hydroxy-3-methylglutaryl CoA.

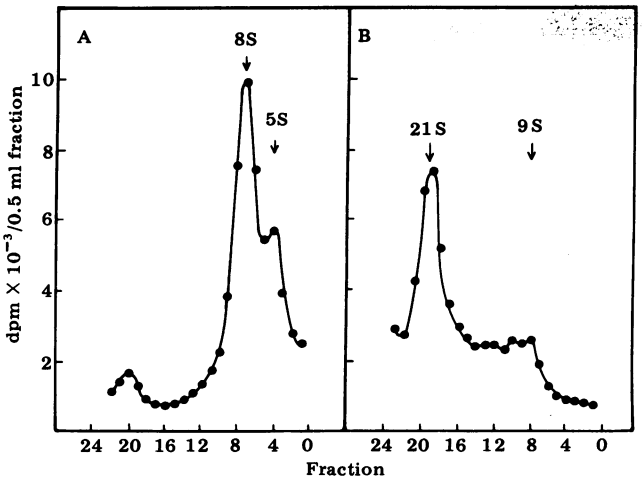

FIG. 1. Density gradient banding of 25-hydroxycholesterol–protein complexes and cholesterol–protein complexes after incubation of L cell cultures in 150-cm^2 flasks with low concentrations of the sterols for 2 hr at 37° in a shaking incubator (50 rpm). The cytosolic fraction of the cells was then isolated and fractionated by centrifugation at 4° in a 5–20% sucrose gradient underlaid with 1 ml of 30% sucrose. Aliquots (0.5 ml) were collected from the top of the gradient and fractions are correspondingly numbered from the top to the bottom. (*A*) With 25-hydroxy[*26,27*-^{3}H]cholesterol (40 ng, 1 μCi/ml) in 10 ml of medium. (*B*) With [*1,2*-^{3}H]cholesterol (40 ng, 1 μCi/ml) in 10 ml of medium.

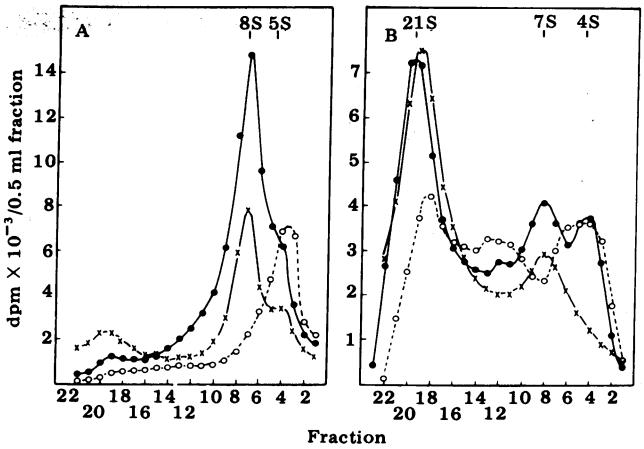

FIG. 2. Effect of incubation at 37° and of digestion with protease upon the density-gradient banding pattern of 25-hydroxycholesterol–protein complexes (*A*) and cholesterol–protein complexes (*B*). Three (150 cm^2) L-cell cultures were incubated with 25-hydroxycholesterol (40 ng, 1 μCi/ml) or [*1,2*-^{3}H]cholesterol (40 ng, 1 μCi/ml) for 2 hr at 37°, and 1-ml samples of the pooled cytosol were then placed in an ice bath (●—●), incubated at 37° (X—X), or incubated at 37° with bacterial protease (0.5 mg) for 30 min at 37° (O ··· O) before they were fractionated by density gradient centrifugation as described in Fig. 1. Fractions are numbered from the top of the gradient.

ferential uptakes of 25-hydroxycholesterol, 7-ketocholesterol, and cholesterol was taken as suggesting the existence of specific receptors for these sterols (3). Further evidence for the existence of distinct cytoplasmic proteins with specificity for 25-hydroxycholesterol or cholesterol is presented in this report.

MATERIALS AND METHODS

[*1,2*-^{3}H]Cholesterol and 25-hydroxy[*26,27*-^{3}H]cholesterol (New England Nuclear Corp.) were purified by thin-layer chromatography before use. Unlabeled cholesterol (Sigma, Ch-S grade) and 25-hydroxycholesterol (Steraloids) were purified as described previously (1, 2). [^{14}C]Formate was from New England Nuclear. Ovalbumin, human gamma globulin, and *Streptomyces griseus* protease (Type VI) were from Sigma.

Mouse fibroblasts (L cells) were grown as monolayers in 150-cm^2 Falcon flasks in chemically defined medium as described previously (1). Sterols were added to the cell cultures as solutions or suspensions in 5% (wt/vol) bovine serum albumin (Pentex, crystallized) containing 10% (vol/vol) ethanol as described before (1). The final concentrations in the medium were 0.45% for albumin and 1.0% for ethanol. After incubation with labeled sterols, the cultures were washed four times with 10-ml portions of medium and then scraped from the flask in 10 ml of medium. The cells were sedimented at room temperature, cooled to 0°, and disrupted by homogenization in 0.85 ml of ice-cold 20 mM Tris, pH 7.8/1 mM $MgCl_2$/2 mM $CaCl_2$/1 mM 2-mercaptoethanol. All further operations were carried out at 4°. Sucrose was added to the homogenate to a final concentration of 5% (wt/vol) and the homogenate was centrifuged at 100,000 × *g* for 1 hr. The growth of the cultures varied between experiments so that the yield of soluble (cytosolic) protein varied between 0.9 and 1.9 mg per flask, although only minor variations in yield between individual cultures within an experimental group were observed. The cytosol was fractionated by layering it over a 16.5-ml sucrose gradient (5–20%) buffered at pH 7.4 with 10 mM Tris/1.5 mM EDTA/0.3 M KCl, underlaid with 1 ml of 30% sucrose in the same buffer and centrifuging it for 18 hr at 35,000 rpm in an SW 41 Ti rotor. [^{14}C]Ovalbumin and human gamma [^{14}C]globulin prepared as described by Stancel and Gorsky (21) were added as sedimentation markers to some of the cytosol preparations before they were layered over the gradient. Fractions (0.5 ml) were collected from the top of the gradient with a Buchler Densiflow apparatus and aliquots were assayed for ^{3}H and ^{14}C by counting in a Triton-toluene scintillation mixture or in Tritosol (22). Protein was determined by absorbancy measurements at 280 and 260 nm.

RESULTS

Density gradient fractionation of cytosol isolated from L cells is illustrated in Fig. 1. Consistently, in many similar experiments, bound 25-hydroxy[^{3}H]cholesterol was located in two narrow overlapping bands in the top half of the gradient. With [^{14}C]ovalbumin and gamma [^{14}C]globulin as markers, approximate sedimentation coefficients of 5 S and 8 S were calculated for the two bands of bound 25-hydroxycholesterol (Fig. 1*A*). In contrast to the results obtained with 25-hydroxycholesterol, cholesterol was widely distributed throughout the lower three-fourths of the gradient, usually as two or more broad, overlapping bands—the highest concentration of the sterol being near the bottom of the gradient (Fig. 1*B*). The approximate sedimentation coefficient calculated for the major band of bound cholesterol was 21 S and sedimentation coefficients for minor bands ranged between 4 S and 9 S (see Figs. 1–3). Measurements of protein in the fractions (not shown) showed a single band with its peak corresponding approximately with the position of the 5S band of bound 25-hydroxycholesterol near the top of the gradient.

A change in the sedimentation-banding pattern of 25-hydroxy[^{3}H]cholesterol that occurred when the cytosol was incubated for 30 min at 37° immediately after isolation from the cells is shown in Fig. 2*A*. Both the 5S and 8S bands of 25-hydroxy[^{3}H]cholesterol were diminished under these conditions. Exposure of the cytosol to a temperature of 50° for 5 min resulted in a banding pattern similar to that found after heating at 37°, except that the heights of the labeled bands were further diminished by about 20%. Incubation of L-cell cytosol con-

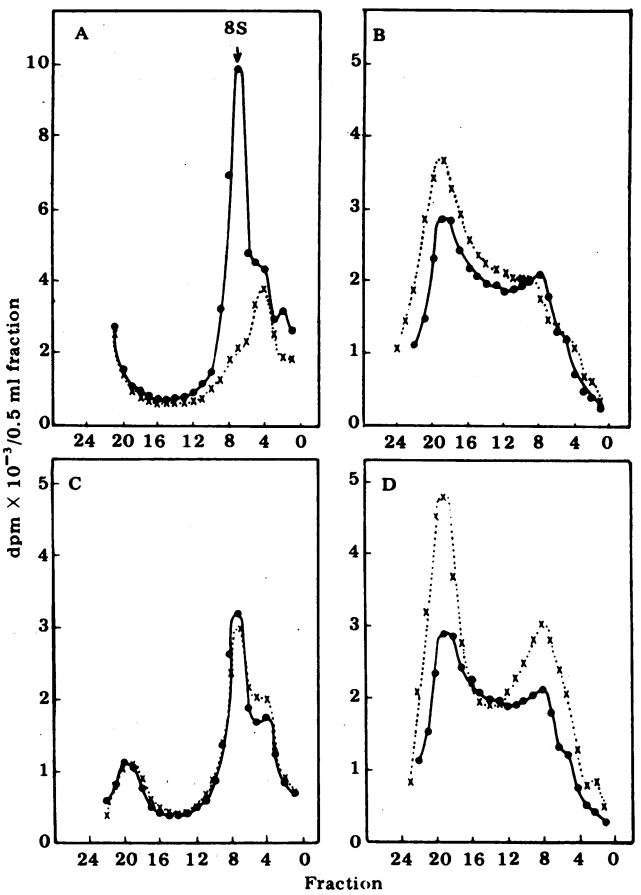

FIG. 3. Effects of preincubating L cells with unlabeled 25-hydroxycholesterol (*A*) at 5 μg/ml or 20 μg/ml (*D*) or cholesterol at 10 μg/ml (*B*) or 20 μg/ml (*C*) upon the density gradient banding patterns of the ^{3}H-labeled sterols. L cells were preincubated for 2 hr at 37° in a shaking incubator (50 rpm) with one of the unlabeled sterols (X- -X), or, as a control, with the ethanol/albumin solution used to suspend the sterols (●—●). The cultures were then washed four times with fresh medium and reincubated for 2 hr at 37° with 10 ml of medium containing 25-hydroxy[*26,27*-^{3}H]cholesterol or [*1,2*-^{3}H]cholesterol under conditions described in Fig. 1. The labeled sterol was 25-hydroxy[^{3}H]cholesterol in *A* and *C*; it was [^{3}H]cholesterol in *B* and *D*. Fractions are numbered from the top of the gradient.

taining bound [^{3}H]cholesterol at 37° or at 50° decreased the amount of bound sterol in the more slowly sedimenting bands but did not significantly affect the 21S band (Fig. 2*B*). When cytosol containing bound 25-hydroxy[^{3}H]cholesterol or [^{3}H]cholesterol was heated to 100° for 5 min and then centrifuged to remove denatured proteins before it was fractionated, no radioactivity was recovered in any of the fractions, indicating complete loss of the bound sterols. Fig. 2 also shows that digestion of the cytosol for 30 min with bacterial protease resulted in marked alterations in the banding patterns. The 8S band of bound 25-hydroxycholesterol was entirely eliminated, and all of the labeled diol appeared in a band with a sedimentation coefficient of approximately 5 S (Fig. 2*A*). After digestion with protease, the 21S band of bound cholesterol was diminished and new bands of lower density appeared (Fig. 2*B*).

The characteristic banding pattern of bound 25-hydroxycholesterol found when L cells were incubated with low concentrations of the labeled sterol was also markedly altered when the cells were preincubated with relatively high concentrations, 5 μg/ml (12.5 μM) or higher, of unlabeled 25-hydroxycholesterol (Fig. 3*A*). The major (8S) band was greatly diminished whereas the smaller 5S band remained unaltered. Diminution of the 8S band, similar to that indicated by the broken line in Fig. 3*A*, was also observed when the cells were incubated for a single 2-hr period with 25-hydroxy[^{3}H]cholesterol diluted at least 1:50 with unlabeled 25-hydroxycholesterol to give a final concentration of 2 μg/ml or higher or when the cells were incubated first for 2 hr with 25-hydroxy[^{3}H]cholesterol at a concentration of 40 ng/ml and then washed and reincubated for 2 hr with unlabeled 25-hydroxycholesterol at a concentration of 10 μg/ml. As shown in Fig. 3*C*, preincubation of the cells with unlabeled cholesterol at a concentration of 10 μg/ml did not alter the binding of 25-hydroxy[^{3}H]cholesterol to cytosolic components.

The sedimentation pattern obtained when a low concentration of [^{3}H]cholesterol was incubated with L cells was only slightly altered when the cells were preincubated with a high concentration (20 μg/ml) of unlabeled cholesterol or 25-hydroxycholesterol (Fig. 3 *B* and *D*). Preincubation with cholesterol resulted in a slight decrease in the diffuse band of [^{3}H]cholesterol with the slower sedimentation rate and an increased concentration of bound cholesterol at the bottom of the gradient. A similar change in the banding pattern was found when the [^{3}H]cholesterol was diluted with unlabeled cholesterol to give a final concentration of 20 μg/ml during a single incubation period. Preincubation of the cells with unlabeled 25-hydroxycholesterol did not alter the banding pattern of [^{3}H]cholesterol qualitatively but appeared to enhance either the uptake of cholesterol or its binding to the proteins.

DISCUSSION

The results indicate the existence of at least two different cytosolic components, one binding 25-hydroxycholesterol and the other binding cholesterol. 25-Hydroxycholesterol at low concentration in the culture medium became bound to L-cell cytosolic proteins, resulting in two bands of sterol–protein complex with sedimentation coefficients of approximately 5 S and 8 S. These two complexes differed in the way in which they were affected when the cells were incubated with higher concentrations of 25-hydroxycholesterol. When unlabeled 25-hydroxycholesterol was present in the culture medium in high concentration, either during a preincubation period or with the 25-hydroxy[^{3}H]cholesterol, very little of the labeled diol appeared in the 8S band while the amount in the 5S band was not altered. In contrast, preincubation of the cells with unlabeled cholesterol did not affect the amounts of 25-hydroxy[^{3}H]cholesterol in the 5S or 8S band.

Evidence that the 8S cytosolic component was able to bind other inhibitory sterols in addition to 25-hydroxycholesterol was obtained in further studies (data not shown). Preincubation with 7-ketocholesterol, 20α-hydroxycholesterol, 5α-cholest-3,6-dione, or 24-ketocholesterol at a concentration of 10 μg/ml caused an alteration in the banding pattern of 25-hydroxy[^{3}H]cholesterol qualitatively similar to that brought about by unlabeled 25-hydroxycholesterol as illustrated in Fig. 3*A*. All of these steroids have been shown to suppress sterol synthesis in culture cells by specifically depressing the activity of HMG-CoA reductase (1–3). Sterols that do not affect HMG-CoA reductase activity in cultured cells—cholesterol, cholestanol, and 20-propyl-5-pregnene-3β,20α-diol—did not alter the banding pattern of 25-hydroxy[^{3}H]cholesterol.

Thus, presently available data indicate that the 8S cytosolic component is specific for steroids that repress HMG-CoA reductase activity. Because, after digestion with protease, all of the bound 25-hydroxy[^{3}H]cholesterol appeared in a 5S band, it is possible that the 5S band of bound diol found in cytosol that had not been exposed to protease represents a degradation product of the 8S complex. Alternatively, the 5S band may represent nonspecific binding of 25-hydroxycholesterol to cy-

tosolic proteins, the greatest proportion of which exhibited a sedimentation coefficient of about 5 S. The protein nature of the cytosolic components in both the 5S and 8S bands is indicated by their lability to heat. Furthermore, the 8S band was eliminated by proteolysis.

The nature of the change in the banding pattern of the 25-hydroxycholesterol–protein complexes, brought about by preincubating the cells with high levels of unlabeled 25-hydroxycholesterol, was not established by the present studies. The unlabeled sterol may have saturated the 8S protein so that 25-hydroxy[^{3}H]cholesterol taken up subsequently may have been unable to bind to it. Alternatively, the unlabeled sterol–protein complexes may have aggregated or may have become bound to nuclear or other particulate components of the cell so that they were not recovered with the cytosol.

The banding pattern of bound cholesterol varied somewhat from experiment to experiment. The lability of the bands to heat and the effect upon them of proteolysis indicated that they included protein. However, the results obtained did not show that the cholesterol binding sites were saturable because the banding pattern for [^{3}H]cholesterol was only slightly altered by preincubating the cells with high concentrations of cholesterol. We have shown previously (3, 4) that the uptake of cholesterol by L cells is limited and it is possible that intracellular concentrations of the unlabeled sterol did not become high enough during the preincubation period to saturate the protein to which [^{3}H]cholesterol was subsequently bound. Presumably, the proteins present in some, or in all, of the bands of cholesterol–protein complex represent sterol-binding proteins that have already been studied by other investigators using procedures other than those described herein. The "sterol-carrier protein" described by Dempsey (23) has been characterized as a polypeptide, of molecular weight 16,000, which forms large aggregates when cholesterol or other related monohydroxysterols are bound to it and which functions as a carrier in the terminal stages of steroid biosynthesis. Another protein which has been labeled "sterol-carrier protein$_1$" by Scallen *et al.* (24) functions as a carrier protein in the squalene epoxidase reaction (25). It binds squalene but appears not to be an effective carrier for sterols (26).

We have characterized the 25-hydroxycholesterol- and cholesterol-binding proteins further in many studies in which the sterols were incubated with isolated L-cell cytosol at 0° [conditions similar to those commonly used to investigate the binding of steroid hormones to cytoplasmic receptors (27)] before they were fractionated by density gradient centrifugation. These studies provide further evidence for the presence of separate binding proteins for the two sterols and illustrate clearly that relatively low molecular weight proteins that bind cholesterol are present in the cytosol and that they aggregate when the concentration of cholesterol in the incubation mixture is increased. However, the possibility that nonspecific associations between sterols and proteins may develop is much greater under these conditions than when the sterols are taken up by live cells, and these results require a more cautious analysis than those presented herein.

The binding of 25-hydroxycholesterol and cholesterol to cytosolic components in long-term cultures of fetal mouse liver cells has also been investigated. Under conditions similar to those shown in Figs. 1 and 3, the results obtained with the liver cells were in close agreement with those shown for L cells, indicating that the binding proteins were present in more than one cell type.

Studies of cellular sterol-binding proteins and of their functions are still in an early stage and only tentative conclusions can be drawn from the results presented in this report. However, the evidence for a protein with specificity for sterols that suppress HMG-CoA reductase represents a significant step toward elucidation of the mechanism through which cholesterol synthesis is regulated. Also by demonstrating a cellular means for distinguishing between inhibitory and noninhibitory sterols, it provides support for the hypothesis that regulation of HMG-CoA reductase is accomplished by sterols other than cholesterol.

We are grateful to Dr. E. Brad Thompson, National Cancer Institute for advice and encouragement. This investigation was supported by Grant CA 02758 from the National Cancer Institute and by Grant GM 22900 from the General Medical Science Institute, Department of Health, Education, and Welfare.

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1. Kandutsch, A. A. & Chen, H. W. (1973), *J. Biol. Chem.* **248,** 8408–8417.
2. Kandutsch, A. A. & Chen, H. W. (1974) *J. Biol. Chem.* **249,** 6057–6061.
3. Kandutsch, A. A. & Chen, H. W. (1975) *J. Cell. Physiol.* **85,** 415–424.
4. Kandutsch, A.A. & Chen, H. W. (1977) *J. Biol. Chem.* **252,** 409–415.
5. Breslow, J. L., Lathrop, D. A., Spaulding, D. R. & Kandutsch, A. A. (1975) *Biochim. Biophys. Acta* **398,** 10–17.
6. Brown, M. S., Dana, S. E. & Goldstein, J. L. (1973) *Proc. Natl. Acad. Sci. USA* **70,** 2162–2166.
7. Brown, M. S. & Goldstein, J. L. (1976) *Science* **191,** 150–154.
8. Kirsten, E. S. & Watson, J. A. (1974) *J. Biol. Chem.* **249,** 6104–6109.
9. Brown, M. S., Brannan, P. G., Bohmfalk, H. A., Brunschede, G. Y., Dana, S. E., Helgeson, J. & Goldstein, J. L. (1975) *J. Cell. Physiol.* **85,** 425–436.
10. Bell, J. J., Sargeant, T. E. & Watson, J. A. (1976) *J. Biol. Chem.* **251,** 1745–1758.
11. Rothblat, G. H. & Buchko, M. K. (1971) *J. Lipid. Res.* **12,** 647–652.
12. Teng, J. I., Kulig, M. J., Smith, L. L., Kan, G. & van Lier, J. E. (1973) *J. Org. Chem.* **38,** 119–123.
13. van Lier, J. E., Da Costa, A. L. & Smith, L. L. (1975) *Chem. Phys. Lipids* **14,** 327–335.
14. Kandutsch, A. A., Heiniger, H.-J. & Chen, H. W. (1977) *Biochim Biophys. Acta,* **486,** 260–272.
15. Chen, H. W., Heiniger, H.-J. & Kandutsch, A. A. (1975) *Proc. Natl. Acad. Sci. USA* **72,** 1950–1954.
16. Higgins, M., Kawachi, T. & Rudney, H. (1971) *Biochem. Biophys. Res. Commun.* **45,** 138–144.
17. Higgins, M. J. P., Brady, D., & Rudney, H. (1974) *Arch. Biochem. Biophys.* **163,** 271–282.
18. Shapiro, D. J. & Rodwell, V. W. (1969) *Biochem. Biophys. Res. Commun.* **37,** 867–872.
19. Kandutsch, A. A. & Saucier, S. E. (1969) *J. Biol. Chem.* **244,** 2299–2305.
20. Higgins, M. & Rudney, H. (1973) *Nature New Biol.* **246,** 60–61.
21. Stancel, G. M. & Gorsky, J. (1975) in *Methods in Enzymology,* eds. O'Malley, B. O. & Hardman, J. G. (Academic Press, New York), Vol. 36, pp. 166–176.
22. Fricke, U. (1975) *Anal. Biochem.* **63,** 555–558.
23. Dempsey, M. E. (1974) *Annu. Rev. Biochem.* **43,** 967–990.
24. Scallen, T. J., Srikantaiah, M. V., Seetharam, B., Hansbury, E. & Gavey, K. L. (1974) *Fed. Proc.* **33,** 1733–1746.
25. Saat, Y. A. & Bloch, K. E. (1976) *J. Biol. Chem.* **251,** 5155–5160.
26. Srikantaiah, M. V., Hansbury, E., Laughran, E. D. & Scallen, T. J. (1976) *J. Biol. Chem.* **251,** 5496–5504.
27. Baxter, J. D. & Tomkins, G. M. (1971) *Proc. Natl. Acad. Sci. USA* **68,** 932–937.