Regulation of Cholesterol Biosynthesis in Enucleated Cells*

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In cells that had been physically enucleated after treatment with cytochalasin B (cytoplasts) levels of cholesterol synthesis and 3-hydroxy-3-methylglutaryl coenzyme A reductase activity were nearly constant over a 6-h period of time. The ratio of the inactive to the active form of the reductase was unaltered by enucleation and did not change when the cytoplasts were incubated at 37°C. The addition to the medium of 25-hydroxycholesterol or serum, agents which specifically suppress the reductase activity in nucleated cells, or of cycloheximide, a general inhibitor of protein synthesis, did not affect cholesterol synthesis or reductase activity in the cytoplasts. In contrast compactin, a competitive inhibitor of 3-hydroxy-3-methylglutaryl-CoA reductase, rapidly suppressed reductase activity and cholesterol synthesis. 3-Hydroxy-3-methylglutaryl-CoA reductase activity was stable when protein synthesis was blocked by cycloheximide, indicating that degradation of the reductase did not occur in the cytoplasts. Evidence was obtained that other short-lived proteins also were not degraded in the cytoplasts. The stability of 3-hydroxy-3-methylglutaryl-CoA reductase activity levels in cytoplasts appeared, therefore, to be due to the absence of both synthesis and degradation of the reductase. The inability of 25-hydroxycholesterol to suppress reductase activity under these conditions appears to exclude mechanisms involving direct inactivation of the enzyme or indirect inactivation of it through alterations of membrane fluidity or through reversible phosphorylation.

These effects of enucleation upon the regulation of sterol synthesis differed from those observed in cells treated with actinomycin D to prevent RNA synthesis. Actinomycin D did not alter the level of 3-hydroxy-3-methylglutaryl-CoA reductase, consistent with the effects of enucleation. But the addition of 25-hydroxycholesterol to actinomycin D-treated cultures resulted in suppression of reductase activity.

The regulation of cholesterol synthesis has received a great deal of attention in recent years (reviewed in ref. 1), mainly as a consequence of the observation that dietary cholesterol elicits a decline of hepatic cholesterogenesis which is correlated with a reduction of the activity of the microsomal enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase [mevalonate:NADP⁺ oxidoreductase (CoA-acylating), EC 1.1.3.4]. These observations were extended to cultured cells and it was reported (2-4) that the inclusion of certain cholesterol preparations or of serum lipoproteins in the medium of such cultures suppressed sterol synthesis and HMG-CoA reductase activity. Other work (5-12) showed that certain oxysterols are potent suppressors of HMG-CoA reductase, whereas purified cholesterol was without effect in several lines of cultured cells. Variant cell lines selected for resistance to 25-hydroxycholesterol were also resistant to serum lipoproteins and to all oxysterol inhibitors tested, suggesting that all of the known inhibitory sterols act through a common mechanism (10-12).

The mechanism whereby these oxygenated sterols act is obscure but may be of importance since it has been suggested that some of them, particularly those which arise during the synthesis of cholesterol or during its catabolism, may function as regulators in vivo (9, 12). One mechanism of action that has been proposed for the oxysterols involves their insertion into cellular membranes, thus modulating the activity of HMG-CoA reductase (13-16). However, it should be noted in this regard that incubation of high levels of these inhibitory oxysterols with microsomal preparations of HMG-CoA reductase prior to, and during, the enzyme assay have no detectable inhibitory effects (5-7). Other mechanisms that have been proposed for the action of the oxysterols include increased enzyme degradation (17) and inhibition of enzyme synthesis (9).

Investigations with enucleated cells can help to clarify the mechanism by which the activity of HMG-CoA reductase is modulated. The enucleation of cells by treatment with cytochalasin B and centrifugation of the treated cells results in cytoplasts which attach to surfaces of culture flasks and exhibit many functions of normal cells (18, 19). Synthesis of HMG-CoA reductase mRNA is prevented by removal of the nucleus, and thus any action of oxysterols or other effectors at the level of transcription is not possible in cytoplasts. This system can, therefore, distinguish between mechanisms that involve the nucleus and those that do not. As described in the present report, sterol synthesis occurred at a nearly constant rate for at least 8 h in cytoplasts incubated at 37°C. Neither the rate of sterol synthesis nor the level of HMG-CoA reductase activity in the cytoplasts was affected by 25-hydroxycholesterol, serum lipoproteins, or cycloheximide. On the other hand compactin (ML-236B), a non-steroid, fungal metabolite which is a competitive inhibitor of HMG-CoA reductase (20, 21), effectively blocked sterol synthesis in the cytoplasts.

MATERIALS AND METHODS

Sodium [1,14C]acetate (2.4 Ci/mol), R,S-3-hydroxy-3-methyl(3-14C)glutaryl-CoA (52 Ci/mol), R,S-[5-3H]mevalonic acid (3 Ci/...
Sterol Synthesis in Enucleated Cells

Cells were plated as described under "Materials and Methods." The cultures were then divided into three sets. The first set was enucleated by filling the flasks with warm medium containing 5 mg/ml cytochalasin B and centrifuging the flasks at 6500 rpm for 30 min as described under "Materials and Methods." The second set was incubated for an equivalent length of time in the presence of cytochalasin B but was not subjected to centrifugation. The third set was incubated for the same length of time in medium without the drug and was also not centrifuged. In one experiment, after the cultures were washed and supplied with fresh media and allowed to stabilize for 1 h, each set was pulsed for 1 h with 1 pCi/ml of [3H]leucine (171 Ci/mmol) and 7.5 pCi/ml of [14C]acetate to determine the rates of sterol and fatty acid synthesis.

**Table I**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Protein recovered (mg/flask)</th>
<th>Protein synthesis (dpm/100 μg protein)</th>
<th>Sterol synthesis (dpm/1.5 h/μg protein)</th>
<th>Fatty acid synthesis (dpm/1.5 h/μg protein)</th>
<th>HMG-CoA reductase activity (pmol/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No cytochalasin B, nucleated (not centrifuged)</td>
<td>2.63</td>
<td>92.6</td>
<td>26.12</td>
<td>62.83</td>
<td>262</td>
</tr>
<tr>
<td>Cytochalasin B, nucleated (not centrifuged)</td>
<td>2.65</td>
<td>101.0</td>
<td>25.36</td>
<td>60.56</td>
<td>270</td>
</tr>
<tr>
<td>Cytochalasin B, enucleated (centrifuged)</td>
<td>0.93</td>
<td>32.8</td>
<td>6.04</td>
<td>20.81</td>
<td>158</td>
</tr>
</tbody>
</table>
RESULTS

The Synthesis of Proteins, Sterols, and Fatty Acids in Enucleated Cells—The results shown in Table I indicate that treatment of cells with cytochalasin B followed by a recovery period of 1 h, without centrifugation to remove the nuclei, did not affect the total protein recovered from the flask, the level of HMG-CoA reductase activity, or the rates of incorporation of [1H]leucine into protein or of [14C]acetate into sterols or fatty acids. This treatment also did not alter the effectiveness of compactin serum, 25-hydroxycholesterol, or cycloheximide to suppress HMG-CoA reductase activity and sterol synthesis. Similar results (not shown) were obtained when cells were centrifuged in the absence of the drug. After enucleation of the treated cells by centrifugation followed by a 1-h recovery period, about 35% of the protein present in the whole culture was recovered in the cytoplast fraction. Rates of protein, sterol, and fatty acid synthesis/mg of cellular protein in the cytoplasts were approximately 30% of those in the nucleated controls. The loss of approximately 50% of the cellular protein and 50% of each of the several metabolic activities, was accounted for by the removal from the flask of whole cells and a relatively impure portion of the cytoplast layer as described under "Materials and Methods." The remainder of the total cellular protein was removed from the flask as the karyoplast fraction. The level of HMG-CoA reductase activity/mg of protein was approximately 50% of that in the control cultures. In other experiments (not shown) HMG-CoA reductase activity declined 15 ± 2% during the 1-h period allowed for stabilization of the cytoplast culture, following enucleation. A loss of about 35% of the HMG-CoA reductase activity initially present in the culture was not accounted for but may have been present in cytoplasmic fragments associated with the karyoplasts. After enucleation and stabilization of the cytoplast layer, the levels of these metabolic parameters declined very slowly, or remained essentially constant, over an 8-h incubation period. Inactive HMG-CoA reductase was present in the control cells and in the cytoplasts and could be activated by incubating broken cell preparations, under appropriate conditions (22-28). In the present study, a 20-min preincubation period usually preceded the assay of HMG-CoA reductase activity in order to convert any inactive enzyme into the active form (28). Therefore, values for reductase activity represent total activity unless stated otherwise. The results in Fig. 1 show that approximately 30% of the reductase

![Fig. 1. The ratio of active to total HMG-CoA reductase activity in enucleated Chinese hamster ovary cells. Conditions for the enucleation of Chinese hamster ovary cells were identical with those described under "Materials and Methods" and in the legend to Table I. At the indicated times parallel cultures of nucleated (○—○) and enucleated (●—●) cells were washed three times with cold Dulbecco's phosphate-buffered saline, scraped into the same buffer, sedimented by centrifugation in the cold, and frozen at -90°C. HMG-CoA reductase activity was determined in sonicated lysates of the samples after a 20-min preincubation at 37°C (total reductase) and without preincubation (active reductase). The data are presented as the ratio of these values for each sample and represent the average values obtained in three experiments. At zero time the average total reductase activities (+ S.D.) in the nucleated and enucleated cultures were 224 ± 25 and 162 ± 21 pmol/min/mg of protein, respectively.](image1)

![Fig. 2. Effects of 25-hydroxycholesterol on sterol and fatty acid synthesis and HMG-CoA reductase activity in nucleated and enucleated Chinese hamster ovary cells. Enucleated and nucleated cell samples were prepared as described under "Materials and Methods" and in the legend to Table I. After a 1-h recovery period, the cultures were exposed to 1 pmol (7.5 μCi) of [14C]acetate/ml of culture medium for 1 h prior to the addition of KOH at the indicated times. At time zero the indicated cultures were supplemented with 1 μg/ml of 25-hydroxycholesterol. Sterol and fatty acid synthesis were measured as described under "Materials and Methods." HMG-CoA reductase activity was determined in the sonicated lysates of nucleated or enucleated cells which were not exposed to the radioisotope. ○, Δ, nucleated cells; ●, △, enucleated cells; ○, ○, controls; △, △, cultures exposed to 1 μg/ml of 25-hydroxycholesterol. Points represent the mean measurement of 2 to 6 experiments; bars are the standard deviation from the mean. Points without bars represent the results of a single experiment. The zero time values ± S.D. obtained with nucleated cells were: sterol synthesis, 57,141 ± 3,653 dpm/h/μg of protein (4 experiments); HMG-CoA reductase activity, 471 ± 26 pmol/min/mg of protein (3 experiments); and fatty acid synthesis, 250,940 ± 47,543 dpm/h/μg of protein (4 experiments). The corresponding values from enucleated cells were: sterol synthesis, 18,521 ± 5,684 dpm/h/μg of protein (6 experiments); HMG-CoA reductase activity, 260 ± 85 pmol/min/mg of protein (3 experiments); and fatty acid synthesis, 45,902 ± 5,982 dpm/h/μg of protein (6 experiments).](image2)
Sterol Synthesis in Enucleated Cells

Effects of Serum on Sterol and Fatty Acid Syntheses and HMG-CoA Reductase Activity in Nucleated and Enucleated Chinese Hamster Ovary Cells. Conditions were the same as those described in the legend to Fig. 1, except that at time zero the indicated cultures were supplemented with fetal bovine serum to a final concentration of 16% and the exposure time to [14C]acetate was 1.5 h. O, Δ, nucleated cells; ●, ▲, enucleated cells; ○, ○, controls; Δ, ▲, cultures exposed to serum. The zero time values ± S.D. obtained with nucleated cells were: sterol synthesis, 22,106 ± 2,822 dpm/1.5 h/µg of protein (3 experiments); HMG-CoA reductase activity, 258 ± 19 pmol/min/mg of protein (3 experiments); and fatty acid synthesis, 60,357 ± 3,890 dpm/1.5 h/µg of protein (3 experiments). The corresponding values from enucleated cells were: sterol synthesis, 5,264 ± 1,068 dpm/1.5 h/µg of protein (3 experiments); HMG-CoA reductase activity, 154 ± 14 pmol/min/mg of protein (3 experiments); and fatty acid synthesis, 15,561 ± 7,422 dpm/1.5 h/µg of protein (3 experiments).

Effects of Compounds on Sterol and Fatty Acid Syntheses and HMG-CoA Reductase Activity in Intact Cells—As shown in Fig. 2, the addition of 25-hydroxycholesterol to the medium of intact cells resulted in a decline in the rate of sterol synthesis (t1/2 = 1.8 h) and in the level of HMG-CoA reductase activity (t1/2 ≈ 1.7 h) but did not affect fatty acid synthesis. These results agree with previous studies of the specificity of the oxysterol's effect upon sterol synthesis (5-12). In contrast to these results, the addition of 25-hydroxycholesterol to cultures of cytoplasts did not significantly suppress sterol synthesis or HMG-CoA reductase activity.

The suppression of cholesterogenesis in cultured cells by serum or serum lipoproteins is well established (2-4). It is often assumed that the suppressive effect of lipoproteins is due to the cholesterol component, but this assumption is not firmly based since purified cholesterol itself is not inhibitory if autooxidation reactions are prevented (9, 29). Furthermore, we have shown that a mutant cell line selected for resistance to 25-hydroxycholesterol was also resistant to the suppressive effect of serum lipoproteins upon cholesterol synthesis, suggesting that the regulatory factor in the lipoproteins may have been an oxysterol (10, 11). The data in Fig. 3 show that the effect of serum upon nucleated control cells and upon enucleated cytoplasts was similar to that of 25-hydroxycholesterol; sterol synthesis and HMG-CoA reductase were largely suppressed in the control cells but were affected only slightly, or not at all in the cytoplasts.

In contrast to the results obtained with 25-hydroxycholesterol and serum, compactin effectively inhibited sterol synthesis in both the nucleated control and the cytoplast cultures (Fig. 4). This result was expected since compactin is a competitive inhibitor of the reductase even when added to isolated microsomes (20, 21). However, it demonstrates that the rate of cholesterol synthesis was a function of the level of HMG-CoA reductase activity in the cytoplasts, as in the nucleated controls, and that the biosynthetic pathway in enucleated cells was complete.

HMG-CoA Reductase Activity Is Stabilized in Enucleated Cells

Fig. 3. Effects of compactin (ML-236B) on sterol and fatty acid synthesis in nucleated and enucleated Chinese hamster ovary cells. Conditions were the same as those described in the legend to Fig. 1, except that at time zero the indicated cultures were supplemented with 5 µg/ml of compactin. O, Δ, nucleated cells; ●, ▲, enucleated cells; ○, ○, controls; Δ, ▲, cultures that were exposed to compactin. The points with bars represent the mean of four separate experiments ± S.D. The average zero time values ± S.D. obtained in three experiments with nucleated cells were: sterol synthesis, 192,580 ± 28,907 dpm/1.5 h/µg of protein; and fatty acid synthesis, 866,667 ± 125,033 dpm/1.5 h/µg of protein. The corresponding values obtained in three experiments with enucleated cells were: sterol synthesis, 22,770 ± 523 dpm/1.5 h/µg of protein; and fatty acid synthesis, 256,667 ± 5,774 dpm/1.5 h/µg protein.
Fig. 5. HMG-CoA reductase activity is sensitive to cycloheximide in nucleated but not in enucleated cells. Conditions for the enucleation of Chinese hamster ovary cells were the same as those described under "Materials and Methods" and in the legend to Fig. 1. Nucleated and enucleated samples were incubated in presence (△, △) or absence (○, ○) of 10 μg/ml of cycloheximide for the times indicated. A and C, 1 h prior to the times indicated, nucleated (○, △) or enucleated cells (●, △) were exposed to 1 μCi of [3H]leucine (171 Ci/mmol). At the indicated times the monolayers were washed three times with cold phosphate-buffered saline, twice with cold 10% trichloroacetic acid and once with absolute ethanol. The precipitates were dissolved in 0.1 N NaOH containing 0.5% sodium dodecyl sulfate and the radioactivity and protein content of the samples were determined. B and D, a parallel set of cultures were treated as described above and HMG-CoA reductase activity was determined on samples of nucleated (○, △) or enucleated (●, △) cells which had been incubated in the presence and absence of cycloheximide. Points represent the mean measurement from four experiments ± S.D. The average zero time values ± S.D. obtained with nucleated cells were: leucine incorporation, 76.5 ± 23.6 dpm/h/μg of protein; and HMG-CoA reductase activity, 282 ± 14 pmol/min/μg of protein. The corresponding values obtained with enucleated cells were: leucine incorporation, 29.5 ± 10.1 dpm/h/μg of protein; and HMG-CoA reductase activity, 153 ± 38 pmol/min/μg of protein.

Cells—Since the half-life of HMG-CoA reductase is thought to be between 1 and 3 h (1), the loss in the cytoplasts of enzyme synthesis, with retention of a normal degradative system, would be expected to result in a rapid decline in reductase activity upon incubation. Conversely, loss of the degradative system with retention of a normal rate of enzyme synthesis would be expected to result in an increasing level of reductase activity. The data in Figs. 2, 3, and 4, indicate that the reductase activity neither increased nor decreased over an 8-h incubation period at rates commensurate with those predicted on the basis of estimates of its half-life. Therefore, it is unlikely that the exclusive loss of either reductase synthesis or degradation occurred in the cytoplasts. One possible explanation for the stability of the reductase activity in the cytoplasts was that reductase mRNA was stabilized and translated normally and that enzyme degradation also occurred normally. Another possibility was that the synthesis and degradation of the enzyme were equally blocked. In order to distinguish between these possibilities, we examined the effects of cycloheximide and puromycin upon the rate of [3H]leucine incorporation into protein and the level of HMG-CoA reductase activity in cytoplasts (Fig. 5). Cycloheximide reduced the rate of protein synthesis by 35 to 98% in both nucleated and enucleated cells, and it reduced the level of HMG-CoA reductase activity in nucleated cells (t½ = 2.6 h). However, this inhibitor had little effect on the activity of the reductase in enucleated cells. In other experiments, using puromycin as an inhibitor of translation (data not shown), results similar to those described above were obtained. Depression of reductase activity by cycloheximide and puromycin in enucleated cells indicates that the system that synthesizes the reductase is more sensitive than the degradative system to these inhibitors. The failure of either inhibitor to influence significantly the level of reductase activity in enucleated cells suggests that neither synthesis nor degradation of the reductase occurred in the enucleated cells.

Enucleation and the Degradation of Short-lived Proteins—The stability of the cytoplast reductase under conditions wherein any ability to synthesize it would have been blocked by cycloheximide (Fig. 5) indicates that the system which normally degrades HMG-CoA reductase was lost in the enucleated cells. This conclusion is consistent with the observation of Gopalakrishnan and Thompson (30) that the system(s) that degrades rapidly turning-over proteins is lost from cytoplasts. The data in Fig. 6 are also consistent with this conclusion. When the incorporation of [3H]leucine into total cellular proteins was allowed to proceed for a short time (1 h) before the rate of protein degradation was determined so that a relatively large fraction of the incorporated label was in rapidly turning-over proteins, there was a significant difference between the decay rates in nucleated cells and cytoplasts.
Approximately 6\% of the labeled protein was degraded/h in the nucleated cells, whereas in the cytoplasts the rate of degradation was much slower (2.6%/h). This reduction in the degradation of short-lived proteins in enucleated cells supports the idea that degradation of HMG-CoA reductase does not occur since the reductase is thought to have a short half-life between 1 and 3 h (1).

**DISCUSSION**

A variety of evidence supports the idea that some regulatory fluctuations in HMG-CoA reductase activity are brought about by changes in the rate of enzyme synthesis, although regulation at the level of enzyme degradation is probably not excluded in any case. This evidence includes the suppression of cyclic- and detergent-induced increases in reductase activity by inhibitors of protein synthesis (reviewed in Ref. 1). Measurements of immunoreactive hepatic HMG-CoA reductase in rats at different stages of the diurnal cycle, or after feeding with cholestyramine or cholesteryl, are also consistent with this mechanism because changes in the amount of enzyme protein under these conditions were correlated with changes in enzyme activity (31-33). In some of these cases, changes in the ratio of inactive to active enzyme may also have occurred (33).

No clear evidence regarding the mechanism by which oxysterols suppress HMG-CoA reductase activity has been presented. Reports that cholesterol and oxysterols act by different mechanisms have appeared (17, 34). However, in none of these was there adequate precaution to ensure that the action attributed to cholesterol was, in fact, due to this sterol and not to a product of its autoxidation. Results indicating that 25-hydroxycholesterol suppressed HMG-CoA reductase more rapidly than did cycloheximide have been put forward as evidence that the oxysterols do not suppress the synthesis of the reductase (34, 35). However cycloheximide might suppress both the synthesis and the degradation of the reductase, thus giving a false measure of the half-life of the enzyme. This seems especially a matter of concern since the present results with enucleated cells suggest that the system which normally degrades HMG-CoA reductase may have a short half-life of the order of 1 h.

Other investigators have stressed the possibility that sterols might regulate the activity of HMG-CoA reductase by insertion into the lipid environment of the reductase, thereby altering its structure and activity (13-16). The demonstrated failure of oxysterols to alter the activity of the reductase when they were added to microsomal enzyme preparations (5, 6) is not conclusive evidence against this possibility because considerable changes in microsomal membranes may result from homogenization and fractionation of the cells. The present results seem to provide clear evidence that the oxysterols do not act by altering the structure of the reductase either directly, by binding to it, or indirectly, by altering its milieu. Furthermore, a system for reversible inactivation via phosphorylation and dephosphorylation appeared not to be involved. The (ATP,Mg\(^{2+}\))-dependent system for inactivating the enzyme was present in the cytoplasts and the ratio of active to inactive enzyme was not altered. Elimination of these possible modes of action adds strength to the hypothesis that the oxysterol:s act by altering the rate of synthesis and/or degradation of HMG-CoA reductase. The failure of 25-hydroxycholesterol to alter HMG-CoA reductase activity in the cytoplasts is consistent with this hypothesis since neither synthesis nor degradation of the reductase was apparent in the enucleated cells. The present results do not provide any indication as to whether inhibition of enzyme synthesis or enhancement of its degradation by 25-hydroxycholesterol is the more probable mechanism of action in nucleated cells.

However, in our view, evidence presented previously for the existence of a cytosolic binding protein with specificity for inhibitory oxysterols (36, 37) offers an analogy with proposed models for the mechanisms by which steroid hormones regulate protein synthesis. This evidence, along with that indicating that modulation of reductase synthesis is involved in the regulation of sterol synthesis under some conditions (e.g. the diurnal cycle, cholesterol feeding), favors the idea that oxysterols regulate reductase synthesis.

The deletion in the cytoplasts of the system for degrading HMG-CoA reductase is consistent with the general observation first made by Gopalakrishnan and Thompson (30), that degradation of short-lived proteins is blocked following enucleation of cells. Whether the system that degrades HMG-CoA reductase is specific, and is therefore a possible site for the regulation of cholesterol synthesis, or whether it is a mere general system for degrading several (or all) short-lived proteins, is not yet known.

It should be noted that the effects of enucleation upon HMG-CoA reductase are different from those observed when RNA synthesis is inhibited by actinomycin D. Kirsten and Watson (38) found that actinomycin D had little effect upon the level of reductase activity in cultured hepatoma (HTC) cells. However, the addition of serum lipoproteins to actinomycin D-treated cultures suppressed reductase activity by approximately 50\%. Similarly, we have observed (data not shown) that under conditions wherein Chinese hamster ovary cells were incubated at 37°C for 6 h with actinomycin D over a concentration range from 2 to 10 \(\mu\)g/ml of medium, the level of HMG-CoA reductase did not change significantly. However, when 25-hydroxycholesterol was added after the first 2 h of incubation with the various concentrations of actinomycin D, reductase activity was diminished by 60 to 70% over the following 4-h incubation period.

The basis for the difference between the effects of enucleation and chemical suppression of RNA synthesis are not known. Known, however, variable and controversial effects of actinomycin D upon the level of another enzyme with a short half-life, tyrosine aminotransferase, in cell cultures are well documented, as reviewed by Thompson (39). A possible explanation that is consistent with all of our observations is that actinomycin D partially, and equally, inhibits both the synthesis and degradation of HMG-CoA reductase so that no net change in enzyme activity is observed. In the presence of actinomycin D, 25-hydroxycholesterol acts, as it does in its absence, to suppress synthesis of the reductase without affecting its degradation, thereby causing the enzyme level to decline.

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**REFERENCES**

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