Regulation of 3-Hydroxy-3-methylglutaryl Coenzyme A Reductase Activity in Avian Myeloblasts

MODE OF ACTION OF 25-HYDROXYCHOLESTEROL*

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25-Hydroxycholesterol inhibits cholesterol biosynthesis by inhibiting the activity of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase. Addition of 25-hydroxycholesterol to chicken myeloblasts caused a rapid inhibition of HMG-CoA reductase activity, producing approximately an 80% decrease in enzyme activity after 60 min. The mode of action of 25-hydroxycholesterol was determined by immunoprecipitating radiolabeled enzyme from 25-hydroxycholesterol-treated myeloblasts. The decline in enzyme activity due to addition of 25-hydroxycholesterol was not associated with increased levels of $[^{38}P]P_4$ incorporation into the immunoprecipitated reductase polypeptide ($M_r = 94,000$). Hence, 25-hydroxycholesterol did not appear to regulate reductase activity by enzyme phosphorylation, as observed for other modulators of HMG-CoA reductase. However, 25-hydroxycholesterol was shown to inhibit reductase activity by causing a 350% increase in the relative rate of reductase degradation and a 72% decrease in the relative rate of reductase synthesis. These alterations in the rates of degradation and synthesis occurred rapidly (within 10–30 min after addition of 25-hydroxycholesterol) and can account completely for the 25-hydroxycholesterol-induced inhibition of enzyme activity. The rapid decline in the rate of synthesis of HMG-CoA reductase in 25-hydroxycholesterol-treated cells was not associated with concomitant changes in the levels of reductase mRNA; therefore, suggesting that 25-hydroxycholesterol must inhibit the rate of reductase synthesis by translational regulation. We also present evidence that mRNA purified from chicken myeloblasts codes for two reductase polypeptides of $M_r = 94,000$ and 102,000.

3-Hydroxy-3-methylglutaryl coenzyme A reductase catalyzes the rate-controlling step in cholesterol biosynthesis (1). Addition of 25-hydroxycholesterol to cells causes suppression of cholesterol biosynthesis by inhibiting the activity of HMG-CoA reductase (2–4). Faust et al. (5) and Sinensky et al. (6) immunoprecipitated radiolabeled reductase ($M_r = 52,000–68,000$) from Chinese hamster ovary cells which were either adapted to grow in the presence of compactin (5) or were mutants auxotrophic for mevalonate (6) and concluded that the primary effect of 25-hydroxycholesterol was to suppress the synthesis of the enzyme. 25-Hydroxycholesterol also increased the rate of reductase degradation in the cells adapted to grow in compactin (5) but had no effect in mevalonate auxotrophs (7). A more recent report demonstrated that the molecular weight of the enzyme subunit from Chinese hamster ovary cells was approximately 90,000 (8). Thus, these data (5–7) derived by immunoprecipitation of a proteolytic fragment of the enzyme may not describe accurately the mode of action of 25-hydroxycholesterol. Consequently, the effect of 25-hydroxycholesterol on the intact enzyme has yet to be established.

The role of enzyme phosphorylation in the mode of action of 25-hydroxycholesterol is also unclear. Some modulators of HMG-CoA reductase are reported to inhibit enzyme activity by inducing phosphorylation of the enzyme (for review, see Ref. 9). However, studies which have examined the effect of 25-hydroxycholesterol on enzyme phosphorylation have used only indirect methods for assessing phosphorylation and further work is needed to clarify this point (10, 11). Therefore, in the present study, we have immunoprecipitated the native HMG-CoA reductase ($M_r = 94,000$) from chicken myeloblasts and determined whether treatment of cells with 25-hydroxycholesterol inhibits enzyme activity by changes in enzyme phosphorylation and/or by changes in the rates of synthesis and degradation of the reductase. We also report the effect of 25-hydroxycholesterol on the levels of reductase mRNA.

EXPERIMENTAL PROCEDURES

Materials—Chemicals were obtained from the indicated sources: [$^{35}$S]methionine and [$^{38}P$]PO$_4$ from Amersham Corp.; $^{14}$C molecular weight standards from New England Nuclear; eupepin, aprotinin, and phenylmethylsulfonfyl fluoride from Sigma; Pansorbin from Calbiochem-Behring; oligo(dT)-cellulose (type 3) from Collaborative Research; ultrapure urea from Schwarz/Mann; guanidine thiocyanate from Fluka; and 25-hydroxycholesterol from Steraloids. Mevinolin was a generous gift from A. Alberts (Merck Sharp and Dohme). Sources for all other materials have been previously described (12).

Cells—Chicken myeloblasts (BM-II cell line) were grown as described (13) in 75 cm$^2$ culture flasks. The media was supplemented with 10% tryptose phosphate broth, 5% bovine calf serum, and 5% chicken serum (Flow Laboratories). For methionine-free or phosphate-free media the specific component was omitted along with the tryptose phosphate broth, and the media was prepared with dialyzed calf and chicken sera. Cells were adapted to grow in the presence of mevinolin by first selecting for growth in normal media containing 15 $\mu$M mevinolin and then slowly adapting the cells to grow in the presence of higher concentrations of the drug.

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were removed at specified time intervals and centrifuged, and the ethanol in the control cells. Aliquots were removed at specified time intervals, phosphate buffer, pH 7.5, 0.1 mM leupeptin, aprotinin (26 pg/ml), with [\(^{35}S\)]methionine (specific activity 2.6 millicuries/mmol) and incubated with 25-hydroxycholesterol (final concentration 5 \(\mu\)g/ml). 25-Hydroxycholesterol was added in 95% ethanol and control cells received equivalent amounts of 95% ethanol. Aliquots were removed at specific time intervals and centrifuged, and the cell lysates were solubilized in buffer A (1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 0.01% NaN\(_3\), 0.1 M NaCl, 5 mM EDTA, 0.01% bromophenol blue, 50% glycerol) and the samples were applied to a SDS-polyacrylamide stacking gel and the 7.5% polyacrylamide separating gel. Electrophoresis buffers were those described by Laemmli (18). Electrophoresis was conducted for 16 h at 50 V (constant voltage), and the gels were processed for fluorography using sodium salicylate (19) and exposed to Kodak XAR-5 film at -76°C. Molecular weight standards were: myosin, M\(_r\) = 200,000; phosphorylase b, M\(_r\) = 92,500; and ovalbumin, M\(_r\) = 43,000. To determine the radioactivity present in a protein band, the appropriate segment was cut out of the gel and incubated overnight in 2.0 ml of 30% H\(_2\)O\(_2\) at 70°C, and the radioactivity was determined by liquid scintillation spectrometry after adding 10 ml of Aquasol.

**RNA Purification**—Cells (1 \(\times\) 10\(^7\) cells/ml) were incubated at 37°C for 120 min in media containing 25-hydroxycholesterol (5 \(\mu\)g/ml) or with an equivalent volume of ethanol for the control sample. Enzyme activity was assayed after 120 min and the 25-hydroxycholesterol-treated and control cells were treated and processed for purification of total RNA as described (20). Polyadenylated RNA was purified by oligo(dT)-cellulose chromatography (20).

In Vitro Translation of Poly(A\(^+\)) RNA—Rabbit reticulocyte lysates were prepared (21) and treated with 2.9 M, 0.1 M NaCl, 5 mM EDTA, 0.01% bromophenol blue, 50% glycerol were added, and the samples were incubated for 60 min and the reactions were stopped by addition of 550 \(\mu\)l of buffer B. Samples were immunoprecipitated as described above. Total incorporation of [\(^{35}S\)]methionine into protein was determined by trichloroacetic acid precipitation as previously described.

**RESULTS**

**Anti-reductase Antibody**—The specific activity of HMG-CoA reductase in microsomes prepared from chicken myeloblasts grown in sera was 0.144 ± 0.025 (S.D.) nmol/min/mg of microsomal protein (\(n = 9\)). Enzyme activity increased when cells were incubated for 48 h in media devoid of lipoteins (data not shown). Antibody prepared to the rat liver enzyme cross-reacted and inactivated approximately 80% of the reductase activity present in myeloblast microsomes (Fig. 1). Addition of normal rabbit serum IgG had no effect on enzyme activity (data not shown). Similar results were obtained when the anti-reductase antibody was added to microsomes prepared from fresh chicken liver (data not shown). The inability of the antibody to inactivate 100% of the reductase activity has also been observed in microsomal preparations from rat liver (16) and human liver (14).

**Immunoprecipitation**—Immunoprecipitates from [\(^{35}S\)]methionine-labeled cells were analyzed on SDS, 8\% polyacrylamide gels and only one polypeptide was specifically precipitated by anti-reductase IgG (Fig. 2). This polypeptide had M\(_r\) = 94,000 (lanes 2 and 3). All other minor bands were also present in samples treated with normal serum IgG (data not shown). The relative amounts of the M\(_r\) = 94,000 protein...
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**Fig. 1.** Immunotitration of HMG-CoA reductase from chicken myeloblast. Antibody was made to the purified HMG-CoA reductase from rat liver (16) and the IgG fraction was isolated (17). The IgG concentration was 0.35 μg/μl. Microsomes from myeloblasts were prepared as stated in the text and preincubated for 30 min at 37°C with varying amounts of anti-reductase IgG. Reductase activity was determined by a radioassay (14) and the proteconcentration was determined by a Coomassie blue dye binding assay (16).

**Fig. 2.** Immunoprecipitation of HMG-CoA reductase from radiolabeled myeloblasts. Cells (1 x 10^6/ml) were incubated with [35S]methionine (1100 Ci/mmol, 26.4 μCi/1 x 10^6 cells) for 80 min at 37°C in the presence (lane 1) of 25-hydroxycholesterol (5 μg/ml) or an equivalent amount of ethanol (lanes 2 and 3). The cells shown in lane 3 were grown in the presence of 19 μM mevinolin. Mevinolin was not included during radiolabeling and the conditions for immunoprecipitation of HMG-CoA reductase from mevinolin-resistant cells precipitated only approximately 50% of the enzyme (data not shown). Normal cells (3.8 x 10^6/ml) were also incubated with [35P]PiPO_4 (100 μCi/ml) for 120 min at 37°C. Lane 4 shows the material immunoprecipitated with anti-reductase IgG and lane 5 shows the material immunoprecipitated with normal rabbit serum IgG. Conditions for radiolabeling, immunoprecipitation, and electrophoresis were those stated in the text. Immunoprecipitates were analyzed on SDS, 8% polyacrylamide gels and radiolabeled bands were visualized by fluorography (19). The relative positions of radiolabeled molecular weight standards (myosin, phosphorylase b, ovalbumin) are depicted on the left (see “Experimental Procedures”). The arrow on the right marks the relative position of the reductase polypeptide (M_r = 94,000). 46K, 92.5K, and 200K represent M_r = 46,000, 92,500, and 200,000, respectively.

**Fig. 3.** Immunoprecipitation of HMG-CoA reductase from radiolabeled myeloblasts in the presence or absence of pure rat liver HMG-CoA reductase. Myeloblasts were labeled with [35S]methionine as stated in the legend to Fig. 2. Immunoprecipitations were performed as stated in the text with the exception that only 1.9 μg of anti-reductase IgG were used and one sample received 31 μg of pure rat liver HMG-CoA reductase (lane 3) and the other sample received an equivalent amount of buffer (lane 2). Lane 1 is an immunoprecipitate from normal serum IgG (3.8 μg). Lane 4 is an immunoprecipitate from rat liver cells. Rat hepatocytes were prepared (16) and labeled with [35S]methionine in methionine-free Swinn’s media. Conditions for labeling and immunoprecipitation were similar to those stated above. The relative positions of the radiolabeled molecular weight standards are depicted on the left (see “Experimental Procedures”). The arrow on the right marks the relative position of the reductase polypeptide (M_r = 94,000).
activity was rapid and an approximate 80% inhibition was observed by 60-90 min after addition (Fig. 5). The rate of enzyme inactivation by the hydroxysterol was the same for cells incubated in the complete media or in media containing dialyzed serum and no tryptose phosphate broth (Fig. 5). Maximal inhibition after 120 min was approximately 83% of the initial reductase activity. Similar results were observed in rat hepatocytes treated with 25-hydroxycholesterol (data not shown).

Effect of 25-Hydroxycholesterol on the Rate of Phosphorylation of HMG-CoA Reductase—Phosphorylation and subsequent inactivation of HMG-CoA reductase has been reported to be a mechanism for rapidly regulating reductase activity (9). To determine if this regulatory mechanism was involved in the 25-hydroxycholesterol-induced inhibition of enzyme activity, cells were preincubated for 60 min with [γ-32P]ATP and the cells were then incubated for 20, 40, or 60 min in the same media in the absence or presence of 25-hydroxycholesterol (5 μg/ml). The amount of [32P]PO4 incorporated into the reductase polypeptide was determined by immunoprecipitation. If the rapid decline in reductase activity were due to enzyme phosphorylation, we would predict a significant increase in the amount of [32P] associated with the (M, = 94,000) polypeptide. Short incubation periods (20, 40, and 60 min) with 25-hydroxycholesterol were used in this experiment to ensure that we would observe any rapid but possibly transient increase in reductase phosphorylation.

Addition of 25-hydroxycholesterol to the myeloblasts had no effect on the total rate of incorporation of [32P]PO4 into the cells (Fig. 6A). We conclude that 25-hydroxycholesterol did not affect the specific activity of the [γ-32P]ATP since the radiactive content of total cellular proteins was not affected by the hydroxysterol. The total cellular incorporation continuously increased in Fig. 6 because the cells were incubated with [32P]PO4 throughout the experiment. Similar results were observed for the incorporation of [32P]PO4 into the reductase polypeptide (M, = 94,000) immunoprecipitated from control cells (Fig. 6B, lanes 2-5). However, after addition of 25-hydroxycholesterol to the cells, there was no increase in the amount of [32P]PO4 incorporated into the enzyme (Fig. 6B, lanes 6-8). This is shown quantitatively in Fig. 6C, where the M, = 94,000 bands were cut out of the gel shown in Fig. 6B and the radioactivity was determined. The apparent decrease in the rate of phosphorylation of the reductase polypeptide in 25-hydroxycholesterol-treated cells was presumably due to the 25-hydroxycholesterol-induced changes in the rates of reductase degradation and synthesis (see below). Therefore, 25-hydroxycholesterol clearly did not enhance the level of reductase phosphorylation, and we conclude that 25-hydroxycholesterol does not inhibit enzyme activity by promoting enzyme phosphorylation.

Effect of 25-Hydroxycholesterol on the Rates of Reductase Degradation and Synthesis—Although the addition of 25-hydroxycholesterol did not affect the rate of total cellular protein degradation (Fig. 7A), it significantly increased the relative rate of degradation of HMG-CoA reductase, (Fig. 7B, compare lanes 3-5, and 6). The apparent half-life of the enzyme in control cells was approximately 196 min, while in the 25-hydroxycholesterol-treated cells the apparent half-life was only 55 min (Fig. 7C). Hence, 25-hydroxycholesterol caused an approximate 350% increase in the relative rate of reductase degradation.

25-Hydroxycholesterol did not affect the rate of total cellular protein synthesis (Fig. 8A). After addition of 25-hydroxycholesterol a rapid and significant decrease in the rate of enzyme synthesis was observed, and the effect was apparent 10 min after addition (Fig. 8B, compare lanes 2-7 and 8-13). When the radioactivity present in the M, = 94,000 polypeptide was determined (Fig. 8C), addition of 25-hydroxycholesterol decreased the relative rate of reductase synthesis by approximately 50%. The rates of synthesis were calculated from data on the 10- and 20-min time points in order to minimize any interference due to the effect of 25-hydroxycholesterol on enzyme degradation. The 50% inhibition of the rate of enzyme synthesis after 20 min may underestimate the actual value because the 25-hydroxycholesterol and [35S]methionine were added simultaneously and the 20-min incubation period would also include the time required for the 25-hydroxycholesterol to bind to the cells. To examine this point, cells were treated with 25-hydroxycholesterol for 120 min, then pulsed with [35S]methionine for either 10 or 20 min and the reductase was
FIG. 6. Effect of 25-hydroxycholesterol on the rate of phosphorylation of HMG-CoA reductase. Cells (3.8 x 10^7/ml) were preincubated 60 min at 37 °C in media containing [32P]P04 (100 µCi/ml). 25-Hydroxycholesterol was added (final concentration 5 µg/ml) to one-half of the cells and an equivalent amount of ethanol was added to the control cells. At specified time periods samples were removed and washed twice in phosphate-buffered saline, and cell lysates were prepared as stated in the text. A, total cellular incorporation of [32P]P04 was determined by trichloroacetic acid precipitation of 10 µl of cell lysate. Values plotted on the ordinate are the radioactivity present in 10-µl aliquots from control (●) and 25-hydroxycholesterol-treated (▲) cells. Time zero represents the time point when the 25-hydroxycholesterol or ethanol were added. B, the radiolabeled reductase was immunoprecipitated and analyzed by electrophoresis on SDS, 8-M urea gels as stated in the text. Radioactive bands were visualized by fluorography (19). The times given are those after addition of 25-hydroxycholesterol or ethanol. Lane 1 on the fluorograph corresponds to an immunoprecipitate using normal serum IgG, 60 min. The following lanes are immunoprecipitates using anti-reductase IgG: 2, time zero; 3, control, 20 min; 4, control, 40 min; 5, control, 60 min; 6, 25-hydroxycholesterol, 20 min; 7, 25-hydroxycholesterol, 40 min; and 8, 25-hydroxycholesterol, 60 min. The relative positions of radiolabeled molecular weight standards are depicted on the left (see “Experimental Procedures”). The arrow on the right marks the relative position of the reductase polypeptide (M_r = 94,000). C, the bands corresponding to a M_r = 94,000 were cut out of the gel (lanes 2–8) and incubated with 30% H_2O_2 overnight at 70 °C, and the radioactivity was determined as described. Values plotted on the ordinate represent the radioactivity present in control (●) and 25-hydroxycholesterol-treated (▲) cells.

FIG. 7. Effect of 25-hydroxycholesterol on the rate of degradation of HMG-CoA reductase. Cells (1 x 10^7/ml) were incubated with [35S]methionine for 90 min at 37 °C, washed, and incubated in media containing unlabeled methionine (5 µM) with or without 25-hydroxycholesterol (5 µg/ml). Samples were removed at specified time periods and cell lysates were prepared as stated in the text. A, total cellular incorporation of [35S]methionine was determined by trichloroacetic acid precipitation of 10 µl of cell lysate. Values plotted on the ordinate are the radioactivity present in 10-µl aliquots from control (●) and 25-hydroxycholesterol-treated (▲) cells. Time zero represents the time when the 25-hydroxycholesterol or ethanol was added. B, radiolabeled HMG-CoA reductase was immunoprecipitated and analyzed on SDS, 8-M urea gels. The radioactive bands were visualized by fluorography (19). Lane 1 on the fluorograph corresponds to an immunoprecipitate using normal serum IgG, time zero. The following lanes are immunoprecipitates using anti-reductase IgG: 2, time zero; 3, control, 20 min; 4, control, 40 min; 5, control, 60 min; 6, 25-hydroxycholesterol, 20 min; 7, 25-hydroxycholesterol, 40 min; and 8, 25-hydroxycholesterol, 90 min. Time zero represents the time when the 25-hydroxycholesterol or ethanol was added. The relative positions of radiolabeled molecular weight standards are depicted on the left (see “Experimental Procedures”). The arrow on the right marks the relative position of the reductase polypeptide (M_r = 94,000). C, the radioactivity present in the M_r = 94,000 bands was determined (lanes 2–8) as described in the legend to Fig. 6. Values plotted on the ordinate represent the radioactivity present in control (●) and 25-hydroxycholesterol-treated (▲) cells.

immunoprecipitated as previously described. The relative rate of reductase synthesis was decreased by approximately 72% in the 25-hydroxycholesterol-treated cells as compared to control cells treated in an identical manner without 25-hydroxycholesterol (data not shown).

Reductase mRNA—To determine if the 72% decrease in the relative rate of reductase synthesis was due to a decrease in the amount of reductase mRNA, the poly(A*) RNA was
The final concentration of RNA used in the translation was divided into two equal aliquots, and the reductase was immunoprecipitated from rat liver. The immunoprecipitates were analyzed by SDS, urea polyacrylamide gel electrophoresis and the radiolabeled molecular weight standards are depicted on the right marks the relative position of the reductase polypeptide.

**Fig. 6**. Time zero represents the time when the 25-hydroxycholesterol or ethanol was added. The relative positions of the radiolabeled molecular weight standards are depicted on the left (see “Experimental Procedures”). The arrow on the right marks the relative position of the reductase polypeptide (M, 94,000). C, the radioactivity in the M, 94,000 bands (lanes 2–13) was determined as described in the legend to Fig. 6. Values on the ordinate represent the radioactivity present in control (●) and 25-hydroxycholesterol-treated (▲) cells.

**Fig. 7**. In vitro translation of poly(A) RNA from chicken myeloblasts and analyses of the immunoprecipitated translation products. Poly(A) RNA was purified from myeloblasts and translated in vitro using a rabbit reticulocyte lysate translation system as described. The final concentration of RNA used in the in vitro translation was 41 μg/ml. Conditions for in vitro translation and immunoprecipitation were those stated in the text. The sample was divided into two equal aliquots, and the reductase was immunoprecipitated in the presence or absence of 8.0 μg of pure HMG-CoA reductase from rat liver. The immunoprecipitates were analyzed by SDS, 8 M urea polyacrylamide gel electrophoresis and the radiolabeled polypeptides were visualized by fluorography. The relative positions of the radiolabeled molecular weight standards are depicted on the left (see “Experimental Procedures”). Translation products immunoprecipitated in the absence (lane 1) or presence (lane 2) of pure rat liver reductase are shown above. Samples treated with equivalent amounts of normal serum IgG contained bands identical to those present in lane 2 (data not shown).

**Fig. 8**. Effect of 25-hydroxycholesterol on the rate of synthesis of HMG-CoA reductase. Cells (1 × 10⁶/ml) were incubated in media containing [³⁵S]methionine and 25-hydroxycholesterol (final concentration 5 μg/ml) or in media containing [³⁵S]methionine and an equivalent amount of ethanol. The [³⁵S]methionine and 25-hydroxycholesterol (or ethanol in the control cells) were added at the same time. Aliquots were removed at specified time periods, and cell lysates were prepared as stated in the text. A, total cellular incorporation of [³⁵S]methionine was determined by trichloroacetic acid precipitation of 10 μl of cell lysate. Values plotted on the ordinate represent the radioactivity present in 10-μl aliquots from control (●) and 25-hydroxycholesterol-treated (▲) cells. Time zero represents the time when the 25-hydroxycholesterol or ethanol was added. B, radiolabeled HMG-CoA reductase was immunoprecipitated and analyzed on SDS, 8 M urea gels as described. Lane 1 on the fluorograph corresponds to an immunoprecipitate using normal serum IgG, 100 min. The following lanes are immunoprecipitates using anti-reductase IgG: 2, control, 10 min; 3, control, 20 min; 4, control, 40 min; 5, control, 60 min; 6, control, 80 min; 7, control, 100 min; 8, 25-hydroxycholesterol, 10 min; 9, 25-hydroxycholesterol, 20 min; 10, 25-hydroxycholesterol, 40 min; 11, 25-hydroxycholesterol, 60 min; 12, 25-hydroxycholesterol, 80 min; 13, 25-hydroxycholesterol, 100 min. Time zero represents the time when the 25-hydroxycholesterol or ethanol was added. The relative positions of the radiolabeled molecular weight standards are depicted on the left (see “Experimental Procedures”). The arrow on the right marks the relative position of the reductase polypeptide (M, 94,000). C, the radioactivity in the M, 94,000 bands (lanes 2–13) was determined as described in the legend to Fig. 6. Values on the ordinate represent the radioactivity present in control (●) and 25-hydroxycholesterol-treated (▲) cells.

**Fig. 9**. In vitro translation of poly(A) RNA from chicken myeloblasts and analyses of the immunoprecipitated translation products. Poly(A) RNA was purified from myeloblasts and translated in vitro using a rabbit reticulocyte lysate translation system as described. The final concentration of RNA used in the in vitro translation was 41 μg/ml. Conditions for in vitro translation and immunoprecipitation were those stated in the text. The sample was divided into two equal aliquots, and the reductase was immunoprecipitated in the presence or absence of 8.0 μg of pure HMG-CoA reductase from rat liver. The immunoprecipitates were analyzed by SDS, 8 M urea polyacrylamide gel electrophoresis and the radiolabeled polypeptides were visualized by fluorography. The relative positions of the radiolabeled molecular weight standards are depicted on the left (see “Experimental Procedures”). Translation products immunoprecipitated in the absence (lane 1) or presence (lane 2) of pure rat liver reductase are shown above. Samples treated with equivalent amounts of normal serum IgG contained bands identical to those present in lane 2 (data not shown). The ratio of the radioactivity present in the 102,000-dalton polypeptide/94,000-dalton polypeptide was 1.00 ± 0.16 (S.D.), n = 6. The total radioactivity present in the two polypeptides (M, 102,000 and 94,000) represented approximately 0.1% of the total trichloroacetic acid-precipitable radioactivity of the sample prior to immunoprecipitation.

No precursor-product relationship could be demonstrated between the M, 102,000 polypeptide and the M, 94,000 polypeptide. In vitro translations of the RNA were conducted for 60 min as described above, except the reactions were not stopped by adding buffer B. Instead, unlabeled methionine was added (final concentration 5 mM), and the reaction continued for an additional 120 min before addition of buffer B. Immunoprecipitation and analysis of the translation products revealed that both polypeptides (M, 102,000 and 94,000) were still present in the same relative ratio (data not shown). We conclude that the M, 94,000 polypeptide does not result from proteolysis of the M, 102,000 polypeptide.
The subunit molecular weight of the reductase purified from rat liver was identical to the molecular weight of the enzyme from chicken myeloblasts \( (M_r = 94,000) \). Previous investigators have reported the molecular weight of the rat liver enzyme to be approximately 52,000 \( (16, 26) \). Proteolysis during enzyme solubilization and purification may also explain the apparent discrepancies in molecular weight. We have recently reported that the molecular weight of the enzyme subunit from rat liver is 94,000 \( (27, 28) \).

Phosphorylation of HMG-CoA reductase has been proposed as a putative mechanism for rapidly regulating reductase activity \( (9) \). However the effect of 25-hydroxycholesterol on reductase from chicken myeloblasts was not correlated with enzyme phosphorylation. Our results actually show a slight decrease in reductase phosphorylation (Fig. 6C) after 25-hydroxycholesterol treatment, but this probably results from the rapid 25-hydroxycholesterol-induced alteration in the relative rates of reductase degradation and synthesis. Work by Erickson et al. \( (10) \) also noted that phosphorylation was not associated with 25-hydroxycholestrol-induced changes in rat liver HMG-CoA reductase. These investigators found that the decreased levels of reductase activity following 25-hydroxycholesterol administration to rats were not reversed by treatment of the microsomes with phosphatase. Similarly Cavenee et al. \( (11) \) reported the failure of 25-hydroxycholesterol to suppress reductase activity in enucleated Chinese hamster ovary cells even though the \( (ATP-Mg^{2+}) \)-dependent system for activating the reductase was present. They also concluded that 25-hydroxycholesterol did not decrease enzyme activity by inducing phosphorylation of the reductase. Both of these reports \( (10, 11) \) utilized relatively indirect methods for determining phosphorylation, however, our results from immunoprecipitation of \( [32P]PO_4 \)-labeled HMG-CoA reductase provide conclusive evidence for the absence of enhanced enzyme phosphorylation in 25-hydroxycholesterol-treated cells.

Bell et al. \( (29) \) and Erickson et al. \( (30) \) had proposed, from measurements of changing enzyme activity, that 25-hydroxycholesterol enhanced the rate of reductase degradation. Our direct studies confirm and extend their observations. The 350% increase in the relative rate of reductase degradation following 25-hydroxycholesterol treatment (Fig. 7) was similar to results reported by Faust et al. \( (5) \) for Chinese hamster ovary cells. In the latter study the conditions used for immunoprecipitation did not prevent proteolysis of the reductase and the rate of degradation was determined primarily from a \( M_r = 62,000 \) proteolytic fragment of the enzyme.

Administration of 25-hydroxycholesterol also caused a 50-72% inhibition of the relative rate of reductase synthesis (Fig. 8C). The rate of synthesis was determined using short labeling times \( (10-20 \text{ min}) \) in order to minimize the effect of the 25-hydroxycholesterol-induced 3.5-fold increase in the rate of degradation. 25-Hydroxycholesterol altered the rate of synthesis as early as 10 min after addition. After 60 min the amount of reductase in the cells appears to be in a steady state (Fig. 8C) which may reflect the approximately 17% of the reductase activity which could not be suppressed by addition of 25-hydroxycholesterol (Figs. 4 and 5). Faust et al.
reported that 25-hydroxycholesterol inhibited the rate of reductase synthesis by approximately 98% in Chinese hamster ovary cells which were adapted to grow in the presence of compactin. The reason for the higher level of inhibition of reductase synthesis noted by these investigators is not known, but it may be due to proteolysis during immunoprecipitation or to different cell types.

The combined effects of the approximately 350% increase in the relative rate of reductase degradation and the 50–72% inhibition in the relative rate of reductase synthesis would be predicted to cause approximately a 86–92% inhibition in enzyme activity. This prediction approximates the observed 83% inhibition of the total reductase activity following 25-hydroxycholesterol treatment (Figs. 4 and 5). Therefore, the relative changes in the rates of reductase degradation and synthesis can account completely for the decrease in HMG-CoA reductase activity due to 25-hydroxycholesterol treatment.

The 25-hydroxycholesterol-induced inhibition of the rate of reductase synthesis was not correlated with concomitant changes in the levels of reductase mRNA. The amount of reductase mRNA was not decreased in cells treated with 25-hydroxycholesterol for 120 min and containing only 20% of the initial reductase activity (Fig. 10). To ensure that the in vitro translation system was able to detect 2-fold changes in reductase mRNA, several concentrations of mRNA were tested (Fig. 10). The amount of the total immunoprecipitable reductase (M, = 102,000 and 94,000) was proportional to the amount of mRNA added to the translation system, and there were no significant differences in the amount of reductase mRNA present in the control or 25-hydroxycholesterol-treated cells. These data clearly suggest that 25-hydroxycholesterol inhibited the rate of reductase synthesis by translational regulation. Translational regulation of HMG-CoA reductase was also reported by Koizumi et al. (31) for increases in enzyme activity in rat hepatocytes due to addition of compactin. These investigators noted that the increases in enzyme activity could be prevented by addition of puromycin, an inhibitor of protein synthesis, but not by addition of either actinomycin D or a-amanitin. Post-transcriptional control of lipoprotein-induced HMG-CoA reductase was also reported in hepatoma cells (92) and human lymphocytes (93). All of these studies (31–33) utilized inhibitors of RNA synthesis and can only indirectly monitor the levels of reductase mRNA. Therefore, our results clearly demonstrate for the first time that HMG-CoA reductase activity can be controlled by translational regulation. The mechanisms involved in this regulation are still unknown.

In vitro translation of the poly(A+) RNA produced two reductase polypeptides (M, = 102,000 and 94,000). The presence of two polypeptides might be due to proteolysis during translation of the RNA. This appears unlikely since no precursor-product relationship could be demonstrated and the relative amounts of the M, = 102,000 and M, = 94,000 polypeptides synthesized were always approximately equivalent in all in vitro translations which utilized a wide range of RNA concentrations. The higher molecular weight polypeptide could represent a contaminating polypeptide with similar antigenic sites. This would seem unlikely since we used polyclonal antibody in our experiments, but further work is needed to characterize both polypeptides. The possibility that the two polypeptides (M, = 102,000 and 94,000) represent two forms or subunits of HMG-CoA reductase appears intriguing. Chin et al. (8) recently cloned the HMG-CoA reductase from Chinese hamster ovary cells and reported that their cloned DNA hybridized to two different sizes of RNA present in the cells. These investigators postulated that this may reflect either two different forms of the enzyme or perhaps two different sized mRNAs which code for the same polypeptide.

Chin et al. (8) reported that the molecular weight of the reductase synthesized by in vitro translation of RNA from Chinese hamster ovary cells was predominantly 90,000, which was identical to the molecular weight of the reductase immunoprecipitated from radiolabeled cells. This differs from our results with chicken myeloblast RNA where one of the immunoprecipitated translation products had a higher molecular weight (M, = 102,000) than the reductase immunoprecipitated from cells (M, = 94,000). These data may suggest that in chicken myeloblasts, one form of HMG-CoA reductase may be synthesized as a larger sized proenzyme which is post-translationally processed to the M, = 94,000 polypeptide.

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