Regulation of Cytosolic Acetoacetyl Coenzyme A Thiolase, 3-Hydroxy-3-methylglutaryl Coenzyme A Synthase, 3-Hydroxy-3-methylglutaryl Coenzyme A Reductase, and Mevalonate Kinase by Low Density Lipoprotein and by 25-Hydroxycholesterol in Chinese Hamster Ovary Cells

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Removal of lipids from growth media of Chinese hamster ovary cells for 48 h resulted in significant increases in activities of the first four enzymes in the cholesterol biosynthetic pathway. In three experiments, the average maximal detectable increase in activity was 3.6-fold for cytosolic acetoacetyl-CoA thiolase, 16-fold for 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) synthase, 11-fold for HMG-CoA reductase, and 2.7-fold for mevalonate kinase. The activity increase of HMG-CoA reductase was shown to occur sooner than those of the other three enzymes, which is consistent with the concept that HMG-CoA reductase is the rate-limiting enzyme for sterol synthesis. Activity increases of all four enzymes were prevented largely by including low density lipoprotein (LDL) or 25-hydroxycholesterol in lipid-depleted serum medium. Moreover, increased activities of these four enzymes were all shown to be suppressed by adding either LDL or 25-hydroxycholesterol in the growth medium, implicating that HMG-CoA reductase is not the only enzyme under regulatory control by LDL. These results are consistent with the concept that 25-hydroxycholesterol mimics the intracellular action(s) of LDL in tissue culture cells. Two putative mutants (clones 25-RA and 25-RB) have been isolated for 25-hydroxycholesterol resistance from mutagenized Chinese hamster ovary cells. It was shown that 25-RA and 25-RB cells grown in 10% fetal calf serum medium had elevated levels of the first four sterolgenic enzymes as compared with those found in the wild type cells; moreover, activities of these enzymes were all shown to be more resistant to suppression by 25-hydroxycholesterol in 25-RA and 25-RB cells than those in wild type cells. These findings suggest the existence of a common cellular factor controlling the intracellular action(s) of 25-hydroxycholesterol on activities of the first four sterolgenic enzymes; the function of this common controlling factor is defective or abnormal in clones 25-RA and 25-RB. These results, coupled with the fact that 25-hydroxycholesterol mimics the intracellular action(s) of LDL, imply that this putative common controlling factor may also be involved in mediating the intracellular action(s) of LDL-bound cholesterol (or its metabolite) on various sterolgenic enzymes.

The abbreviations used are: LDL, low density lipoprotein; CHO, Chinese hamster ovary; De-S, delipidated fetal calf serum; Me2SO, dimethyl sulfoxide; HDL, high density lipoprotein; 25-OH cholesterol, 25-hydroxycholesterol; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; LP(-), lipoprotein-deficient serum; GLC, gas-liquid chromatography; TLC, thin layer chromatography.
first validate a specific procedure (12) for preparing the cyto-
sollic fraction which contains optimal cytosolic cholesterogenic
enzymes from intact CHO cell monolayers; we then demon-
strate that in CHO cells, the activities of first four enzymes
in the sterol biosynthetic pathway (for a review, see Ref. 13)
are all regulated by LDL and by 25-OH cholesterol. Moreover,
in two putative CHO cell mutants isolated for resistance to
cytotoxic action(s) of 25-OH cholesterol (designated as clone
25-RA and 25-RB), the activities of these four enzymes are all
found to be more resistant to suppression by 25-OH choles-
terol than those found in wild type cells. These findings
suggest the existence of a common cellular factor controlling
the intracellular action(s) of 25-OH cholesterol on activities of
the first four cholesterogenic enzymes; the function of this
cellular factor is defective or abnormal in clones 25-RA and
25-RB.

MATERIALS AND METHODS

Lipids were from Sigma, except 25-OH cholesterol was from Ster-
aloids. 25-OH [(24-3H)cholesterol was synthesized from desmosterol
by Dr. Albert F. T. Chen at Beckman Microbia Division of Beckman
Instruments using selective oxymercuration followed by NaBTd treat-
ment according to the published procedure (14). Both the unlabeled
and labeled 25-OH cholesterol and their acetate derivatives were
shown to be at least 98% pure by four different thin layer chromato-
graphic ( TLC) systems. Both the unlabeled and labeled 25-OH choles-
terol preparations contain no more than 6% detectable impurity
by gas-liquid chromatographic (GLC) analysis or by radio-GLC anal-
ysis as described (15). Other radioactive chemicals were from New
England Nuclear. Various coenzyme A derivatives were from Sigma
except acetoacetyl coenzyme A (99% pure) was from P-L Biochemi-
cals. All other chemicals were of analytical grade. Human low density
lipoprotein (LDL), d 1.019 to 1.063 g/ml, and high density lipoprotein
(HDL) contained 0.5 mg of cholesterol and 0.3 mg of fatty acid/mg of
protein.

Cells— Cultures of CHO cells (11, 15) were grown as monolayers
in 75-cm² Corning flasks in F-12 medium (linoleic acid-deleted) plus
either 10% fetal calf serum, or 6% human lipoprotein-deficient serum
(LP(-)), 4 mg of protein/ml. LP(-) and De-S sera were prepared according
to published procedures (16, 17). The composition of LDL and the relative
distribution of its lipid components agreed with published data (18). The
HDL contained 0.5 mg of cholesterol and 0.3 mg of fatty acid/mg of protein.

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Validity of sequential digitonin exposure treatment for releasing acetoacety-
coenzyme A thiolase activity and mevalonate kinase activity
from CHO cells

Table I

| Method       | Cytosolic acetoacetyl-
|              | coenzyme A thiolase |
|--------------|----------------------|
|              | Estimated mitochondrial enzyme release* |
|              | %          | µg | %          | µg |
| 1. Hypotonic shock | n.d.* | 1.51±0.12 | n.d. | 80 |
| 2. Sequential digitonin exposure treatment | | | | |
| 1 flask | 35±6 | 1.20±0.08 | 100 | 5 |
| 2 flasks | 43±3 | n.d. | n.d. | 240 |
| 3 flasks | 34±3 | 1.31±0.03 | n.d. | 328 |
| 4 flasks | 39±2 | 1.15±0.14 | n.d. | 394 |

* Determined according to published procedure (12) using lactate
dehydrogenase as cytoplasmic enzyme marker.

** Determined according to published procedure (12) using citrate
synthase as mitochondrial enzyme marker.

n.d., not determined.
titled back and forth continuously by hand during exposure. Before exposure, cell monolayers were covered with 10 ml of phosphate-buffered saline at 4°C. After the first exposure, the 1.0 ml of digitonin solution (containing cytosolic fraction from the first flask) was transferred to the second flask for the second exposure, and so on. The pooled cytosolic fraction was centrifuged at 10,000 x g for 10 min at 4°C to pellet the insoluble digitonin suspension; the supernatant was stored at −20°C before being used for assays. Control experiments indicated that, stored at −70°C, the acetoacetyl-CoA thiolase activity and mevalonate kinase activity were stable for at least 7 days. The freshly prepared supernatant fraction, if dialyzed as described (5), was also used for HMG-CoA synthase assay in some experiments reported in this paper. The specific activity of mevalonate kinase and HMG-CoA synthase prepared by hypotonic shock treatment described above or by digitonin exposure treatment agreed with each other (see Tables I and II under “Results”). Control experiments showed that the HMG-CoA synthase activity prepared by either method was not stable upon storage, and needed to be assayed immediately after dialysis. For acetoacetyl-CoA thiolase assay, the reaction was performed at pH 8.0; the reaction mixture was preincubated at 25°C for 2 min, after which the reaction was started by addition of cell extract. The reaction was continued for at least 2 min. Reaction rate was found to be linear for at least 4 min. A stoichiometry of 2 mol of acetyl-CoA formed/mol of acetoacetyl-CoA cleaved was confirmed by coupling acetyl-CoA formation to NAD+ reduction via the combined citrate synthase-malate dehydrogenase system as described (24). For the mevalonate kinase assay, the reaction rate was found to be linear for 12 min with no more than 10% substrate consumption. For all enzyme assays reported in this paper, two enzyme levels were used for each assay point to assure linearity between the two enzyme levels.

RESULTS

Validity of Sequential Digitonin Exposure Treatment in Preparing Cytosolic Cholesterogenic Enzymes in CHO Cells—We sought to develop an efficient and rapid procedure to prepare cytosplasmic fractions from monolayers of CHO cells with minimal contamination from mitochondrial leakage. This was necessary since Clinkenbeard et al. (7, 13) have

TABLE I

Validity of sequential digitonin exposure treatment for releasing HMG-CoA synthase activity from CHO cells

Wild type and 25-RA cells were plated at 0.2 x 10^6/75-cm^2 flask in 10 ml of F-12 + 10% fetal calf serum for 48 h. Medium was then renewed once and cells were grown for an additional 24 h. Afterwards, cytosolic fractions were prepared from cells by two different methods as described under “Materials and Methods.” Cells from two flasks were pooled and used for Method 1. The cell extracts were either assayed right away, or dialyzed for 24 h at 4°C as described (5) before being used for enzyme assay. HMG-CoA synthase activity was determined as described under “Materials and Methods.” Values shown represent the mean ± variation from the mean of two enzyme assays using a single source of cell extract.

| Method                  | Wild type cells            | 25-RA cells             |
|-------------------------|-----------------------------|-------------------------|
|                         | Before dialysis | After dialysis | Before dialysis | After dialysis |
| HMG-CoA synthase activity | nmol/min·mg^-1·protein | nmol/min·mg^-1·protein |
| 1. Hypotonic shock, Dounce homogenization, and ultracentrifugation | 0.48 ± 0.07 | n.d. | 5.5 ± 0.4 |
| 2. Sequential digitonin exposure treatment | n.d. | n.d. | 3.1 ± 0.4 | n.d. |
| 3. flasks | 0.47 ± 0.05 | 3.3 ± 0.4 | 6.5 ± 0.3 |
| 4. flasks | 0.53 ± 0.01 | 3.4 ± 0.4 | 6.0 ± 0.5 |

a N.L., enzyme activity was found to be nonlinear with time and nonlinear with protein levels.

b n.d., not determined.

demonstrated that, in avian and rat liver homogenates, acetoacetyl-CoA thiolases exhibit dual mitochondrial and cytosolic localization; only the cytosolic thiolases are subject to feedback control by cholesterol. A mild procedure for the rapid release of cytosplasmic enzymes from monolayers of cultured animal cells has been developed by Mackall et al. (12). We have validated and extended this method in monolayers of CHO cells. Data presented in Table I indicate that, when a single flask is used, the digitonin exposure treatment causes rapid and efficient release of cytosolic enzymes with minimal release of enzymes from mitochondria. The specific activity of mevalonate kinase prepared by this method was shown to be similar to that prepared by the method of hypotonic shock followed by Dounce homogenization and ultracentrifugation. We have also found that this method can be used to extract sequentially cytosolic fractions from up to four separate 75-cm^2 flasks of monolayer cells into 1 ml of buffered digitonin solution with efficient recovery in total cytosolic protein contents, and with efficient and constant recoveries in mevalonate kinase and acetoacetyl-CoA thiolase activities (Table I). In Table II, we have validated this procedure further for releasing HMG-CoA synthase activities from both the wild type CHO cells and from a specific clone.
Fig. 2. Time course of derepression and suppression of HMG-CoA reductase (a), HMG-CoA synthase (b), cytosolic acetoacetyl-CoA thiolase (c), and mevalonate kinase (d) activities in wild type cells. Cells were trypsinized and replated in 20 ml of F-12 + 10% fetal calf serum (FCS) at 0.8 × 10^5, 0.3 × 10^5, 0.12 × 10^5, and 0.06 × 10^5 cells/75-cm² flask for Days 0 through 3, respectively. After 2 days, the cells were rinsed with 5 ml of saline and the medium was replaced with 15 ml of (1) F-12 + 10% fetal calf serum, (2) F-12 + 10% De-S with or without 0.3% Me₅S₀, (3) F-12 + 10% De-S + 25-OH cholesterol (0.3 µg/ml), or (4) F-12 + 10% De-S + 0.3% Me₅S₀ + 25-OH cholesterol (0.3 µg/ml). On Day 2, one group of cells grown in F-12 + 10% De-S was fed (5) LDL (50 µg of protein/ml); one group of cells grown in F-12 + 10% De-S + 0.3% Me₅S₀ was fed (6) 0.3% Me₅S₀ + 25-OH cholesterol (0.3 µg/ml). Media were renewed every day. Specific enzyme assays were as described under "Materials and Methods." For each time point, two flasks of cells were combined and used for both reductase and synthase assays, while one flask of cells was used for both thiolase and kinase assays. Each experimental point shows the mean of duplicate enzyme assays from a single source of cell extracts. For reductase, thiolase, and kinase assays, variations between duplicates were within 10% of the mean. Values for all four enzyme activities obtained from cells grown in F-12 + 10% De-S with Me₅S₀ differed by no more than 10% from those obtained from cells grown in the same medium without Me₅S₀. Each experimental point shown in c was confirmed by using a different thiolase assay by measuring the acetyl-CoA formation via the combined citrate synthase-malate dehydrogenase system as described (24).

Regulation of HMG-CoA Reductase, HMG-CoA Synthase, Cytosolic Acetoacetyl-CoA Thiolase, and Mevalonate Kinase Activities by LDL and by 25-OH Cholesterol—In three different experiments, switching growth medium from 10% fetal calf serum to 10% delipidated serum for 48 h invariably causes an increase in the activities of the first four cholesterogenic enzymes in wild type CHO cells. In these experiments, the average maximal detectable activity increase was found to be 11-fold for HMG-CoA reductase, 16-fold for HMG-CoA synthase, 3.6-fold for acetoacetyl-CoA thiolase, and 2.7-fold for mevalonate kinase. A typical experiment was shown in Fig. 2, a to d. The increases in activities of all four enzymes invariably were prevented largely by including either LDL or 25-OH cholesterol in the delipidated serum medium. Moreover, increased enzyme activities were all invariably suppressed by adding either LDL or 25-OH cholesterol in the growth medium. The prevention effects and suppression effects of 25-OH cholesterol on activities of all four enzymes persisted regardless of whether this agent was added to the growth medium from a stock ethanol solution or from a stock dimethyl sulfoxide (Me₅S₀) solution (data not shown). Data in Fig. 2 indicate that the activity increases of HMG-CoA reductase occur sooner than those of the other three enzymes.

Regulation of HMG-CoA Reductase, HMG-CoA Synthase, Cytosolic Acetoacetyl-CoA Thiolase, and Mevalonate Kinase Activities in CHO Cells—Removal of LDL from the growth medium causes rapid increase in activity of HMG-CoA reductase in CHO cells as shown in Fig. 1; the increased activity is rapidly suppressible in a dose-dependent manner by adding back LDL to the growth medium. A very high level of HDL caused only a slight suppression of HMG-CoA reductase activity. This suppression could occur by nonspecific endocytosis of HDL by these cells. This experiment confirms the reports by Brown and Goldstein that CHO cells express LDL receptors at the cell surface (1, 27).

In experiments not shown here, we have found that when 0.1 mM chloroquine was added to the culture medium concomitantly with LDL (at 10 to 200 µg/ml protein concentration) in a 6-h incubation, the suppression effect of LDL on HMG-CoA reductase activity was inhibited by at least 80% in every single case, suggesting that lysosomal hydrolysis of LDL is required for LDL-mediated regulation of cholesterol metabolism in CHO cells; these results are similar to what has been demonstrated in human fibroblasts (28) (J. Chin and T. Y. Chang, results to be published elsewhere).
this is consistent with the concept that HMG-CoA reductase is the "rate-limiting" enzyme for sterol biosynthesis (2-4); however, these data also stress the fact that HMG-CoA reductase is not the only enzyme under regulatory control by LDL. Furthermore, these results are consistent with the concept (10) that 25-OH cholesterol mimics the intracellular action(s) of LDL-bound cholesterol (or its metabolite). The maximal detectable increases in activities of cytosolic acetoacetyl-CoA thiolase and mevalonate kinase were not as large as those of HMG-CoA synthase and HMG-CoA reductase; the rates of activity increase of the former two enzymes were shown to be slower than the latter two enzymes. The less dramatic and slower increase of thiolase and kinase activities may explain why enhancement of their activities was not demonstrated in rat adrenal gland after cholesterol deprivation (5).

Characterization of 25-OH Cholesterol-resistant Clones 25-RA and 25-RB—Two different clones, designated as 25-RA and 25-RB, have been isolated for 25-OH cholesterol resistance from mutagenized CHO cells (see "Materials and Methods" for isolation procedure). Unlike wild type cells, these cells continued to grow (Fig. 3) and synthesize cholesterol (Fig. 4) in 25-OH cholesterol supplement medium (1 μg/ml). GLC-sterol analyses indicated similar steady state incorporations of 25-OH cholesterol by wild type, 25-RA, and 25-RB cells (Fig. 4). We have also employed 25-OH[14] cholesterol to examine the uptake of cell-bound 25-OH cholesterol in three cell types. The results shown in Table III along with

![Figure 3](image-url)  
**Fig. 3.** Growth curves of wild type, 25-RA, and 25-RB cells grown in F-12 + 10% De-S with or without 25-OH cholesterol (1 μg/ml). Cells grown in F-12 + 10% fetal calf serum were trypsinized and replated at 0.04 × 10⁶ cells/25-cm² flask in 10 ml of the same medium. After 3 days, the cells were rinsed with 5 ml of phosphate-buffered saline and the medium was replaced with 15 ml of F-12 + 10% De-S + 0.3% MeSO₄ (C, D) or F-12 + 10% De-S + 0.3% MeSO₄ + 25-OH cholesterol (1 μg/ml) (B, A, D). Media were renewed every day. Growth was followed by dissolving the cells in 1 ml of 2 M KOH and measuring protein. Each value represents the mean of duplicate cultures. Variation between the duplicates was within 5% of the mean.

![Figure 4](image-url)  
**Fig. 4.** Sterol contents in wild type, 25-RA, and 25-RB cells grown in F-12 + 10% De-S with 25-OH cholesterol (1 μg/ml). Cells were grown and harvested as described in Fig. 3. Sterols were analyzed by GLC as described (11). Solid bars: micrograms of cholesterol per 25-cm² flask. Open bars, micrograms of 25-OH cholesterol per mg of protein. Values represent means of duplicate cultures. Variation between duplicates was within 10% of the mean.

**TABLE III**

| Incorporation time | Wild type | 25-RA | 25-RB |
|-------------------|-----------|-------|-------|
| 1 h               | 0.38 ± 0.016 | 0.42 ± 0.004 | 0.37 ± 0.018 |
| 6 h               | 0.32 ± 0.004 | 0.39 ± 0.007 | 0.33 ± 0.014 |
| 8 h               | 0.33 ± 0.013 | 0.34 ± 0.004 | 0.34 ± 0.016 |

those shown in Fig. 4 indicate that the resistant phenotypes were not the result of a lack of permeability to 25-OH cholesterol. Data in Table III also indicate that the uptake of 25-OH cholesterol in three cell types reaches steady state within 3 h. This laboratory has shown previously that sterol synthesis in wild type CHO cells was minimal when cells were grown in 10% fetal calf serum (11). However, the [14] acetate pulse data
shown in Table IV indicate that 25-RA and 25-RB cells grown in 10% fetal calf serum possess elevated rates of sterol synthesis compared with that of wild type cells. The elevated rates of sterol synthesis in clones 25-RA and 25-RB were found not to correlate linearly with the elevated activities of HMG-CoA reductase found in these cells as compared with those of wild type cells; instead, these elevated rates can be explained much better by the finding that activities of the first four enzymes in cholesterol biosynthetic pathway in 25-RA and 25-RB type cells were all elevated in comparison with those found in wild type cells (Table IV). Rates of [14C]labeled fatty acid synthesis from [14C]acetate in these cells were shown to be only slightly elevated in comparison to that in the wild type cells (Table IV, Column 3). Previously, Chen et al. (29) and Sinensky et al. (30) reported the isolation of similar Chinese hamster mutant cells resistant to 25-OH cholesterol, and showed that the HMG-CoA reductase activities in these mutant cells were resistant to suppression by 25-OH cholesterol and by serum lipids (29, 30). The data presented in Table IV extend these findings. These data also indicate that, under certain conditions, large changes in activities of several enzymes catalyzing early steps in cholesterol biosynthetic pathway can significantly alter the rate of sterol synthesis in intact CHO cells without substantial changes in activities of HMG-CoA reductase.

### Table IV

**Elevated rates of sterol synthesis, and elevated cholesterogenic enzyme activities in clone 25-RA and 25-RB cells as compared with those in wild type cells grown in F-12 + 10% fetal calf serum.**

| Cell clone          | Relative rate of [14C]-sterol synthesis from [14C]acetate | Relative rate of [14C]-fatty acid synthesis from [14C]-acetate | Relative cytosolic mevalonate kinase activity | Relative HMG-CoA synthase activity | Relative HMG-CoA reductase activity | Relative HMG-CoA synthase activity |
|---------------------|------------------------------------------------------------|---------------------------------------------------------------|---------------------------------------------|-----------------------------------|-----------------------------------|-----------------------------------|
| Wild type           | 1.00                                                       | 1.00                                                          | 1.00                                        | 1.00                              | 1.00                              | 1.00                              |
| 25-RA               | (1.40 ± 0.07)                                             | (35.9 ± 0.2)                                                 | (47.8 ± 3.43)                               | (0.456 ± 0.023)                   | (0.056 ± 0.003)                   | (1.27 ± 0.064)                    |
| 25-RB               | (9.7 ± 0.4)                                               | (56.3 ± 0.4)                                                 | (121 ± 11.8)                               | (5.15 ± 0.45)                     | (0.140 ± 0.008)                   | (2.67 ± 0.045)                    |

* Values shown represent the mean from duplicate cultures in a single experiment. Values in parentheses represent the mean of the actual rates found in duplicate cultures ± variation from the mean. **Cells were seeded at 0.3 $\times 10^4$/75-cm² flask for this experiment. [14C]Acetate pulse (32 μCi/flask, 0.59 mM sodium acetate) done as described (11, 21). After the pulse, cells were dissolved in 2 ml of 1.5 M KOH.

* Values shown represent the mean of results from five different experiments. Values in parentheses represent the mean of the actual enzyme activities ± S.E. found in these five experiments.

* Values shown represent the mean of results from 11 different experiments. Values in parentheses represent the mean of the actual enzyme activities ± S.E. found in these 11 experiments.

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FIG. 5. Time course of derepression of HMG-CoA reductase (a), HMG-CoA synthase (b), cytosolic acetyl-CoA thiolase (c), and mevalonate kinase (d) of three cell types. Cells were grown and harvested for enzyme assays as described in Fig. 2. 1, 2, 3, enzyme activities of cells grown in 10% fetal calf serum. Circle, triangle, and square enzyme activities of cells grown in 10% De-S. Each point shows the results of a single cell extract in vitro. Variations in the duplicates were within 10% of the mean for reductase, thiolase, and kinase. For the synthase assays, variations were within 15% of the mean.
in 25-RA and 25-RB cells. The rates of activity increase of these enzymes were found to be similar in all three cell types with one exception: the HMG-CoA reductases of 25-RA and 25-RB cells never reached the highly elevated states reached by wild type cells during the entire time course (Fig. 5a). Also, the time course of activity increase of HMG-CoA reductase was shown to be slower in 25-RA and 25-RB cells than in the wild type cells. This observation was confirmed in three separate experiments. We suspect that this may be because at early time points after removal of serum lipids (≤24 h), derepression of HMG-CoA reductase activities are largely prevented by the high contents of cellular cholesterol accumulated in 25-RA and 25-RB cells (Table V). These results suggest that the derepression of HMG-CoA reductases of 25-RA and 25-RB cells may be more sensitive to inhibition by stored cellular cholesterol than are the other three cholesterogenic enzymes measured. Other possibilities are not excluded at present. We are currently pursuing this observation further in this laboratory.

After all three cell types were grown in 10% delipidated serum for 24 or 48 h, resistance to suppression by 25-OH cholesterol of the first four cholesterogenic enzymes in 25-RA and 25-RB cells were demonstrated (Fig. 6, a to d). While these data do not permit us to conclude that the degrees of resistance to suppression were different in 25-RA and 25-RB cells, it was clear that there were lower per cent suppressions of each one of the four enzyme activities in both cell types in comparison with those in wild type cells. The same results

### Table V

| Growth time | Cellular cholesterol content | Wild type | 25-RA | 25-RB |
|-------------|-----------------------------|-----------|------|------|
| Zero time   | µg.mg⁻¹ protein             | 20.2      | 70.6 | 63.8 |
| 1st day     |                            | 8.1       | 28.9 | 16.0 |
| 2nd day     |                            | 9.9       | 20.9 | 16.4 |
| 3rd day     |                            | 14.7      | 25.2 | 23.1 |

Fig. 6. Suppression of HMG-CoA reductase (a), HMG-CoA synthase (b), cytosolic acetoacetyl-CoA thiolase (c), and mevalonate kinase (d) of three cell types by varying concentrations of 25-OH cholesterol. Cells were grown as described in Fig. 5. On Day 1 (for reductase) or Day 2 (for synthase, thiolase, and kinase), the cells grown in F-12 + 10% De-S were fed 15 ml of F-12 + 10% De-S + 0.3% MeSO with or without 25-OH cholesterol. After 24 h, cells were harvested for enzyme assays. One flask of cells was used to prepare cell extract for reductase assay, while two (for 25-RA and 25-RB cells) or four (for wild type cells) flasks of cells were used to prepare cell extracts for the other three enzyme assays. Enzyme activities of control cells (untreated with 25-OH cholesterol) were found to be: (a) reductase, 0.58 (wild type), 0.32 (25-RA), and 0.42 (25-RB); (b) synthase, 5.67 (wild type), 15.3 (25-RA), and 11.2 (25-RB); (c) thiolase, 330 (wild type), 521 (25-RA), and 420 (25-RB); (d) kinase, 3.3 (wild type), 7.5 (25-RA), and 4.5 (25-RB). Values for the control cells were determined from duplicate cell extracts; variation between the duplicates was within 5% of the mean. All other experimental points represent mean of duplicate enzyme assays from a single source of cell extract. Variation between the duplicate assays were within 10% of the mean. Enzyme activities are expressed as nanomoles.min⁻¹.mg⁻¹.
have been consistently seen in two separate experiments. It is important to point out that the results presented in Fig. 6, a to d, cannot be consequences of nonspecific cytotoxic effects of 25-OH cholesterol on cell growth, since earlier experiments in this laboratory have shown that treatment of 25-OH cholesterol at 0.3 μg/ml for 24 h has minimal inhibitory effect on cell growth of all three cell types (data not shown). Moreover, in a different experiment, when we treated wild type and 25-RA cells with various concentrations of 25-OH cholesterol in the same manner as what was described in Fig. 6, a to d, but for only 8 h, we have found that there were lower per cent suppressions of each of the four enzyme activities in 25-RA cells in comparison with those found in wild type cells. We do not know whether the defects manifested in clones 25-RA and 25-RB are due to a single gene mutation or not; however, the fact that these abnormalities were found in two independently isolated clones suggests that these phenotypic expressions were caused by a single gene mutation. The data presented in this report do not permit us to determine whether these two clones possess the same biochemical lesion (with different degrees of impairment at the mutated genetic locus).

**DISCUSSION**

Data presented in this report have shown that in CHO cells the activities of the first four enzymes in the cholesterol biosynthetic pathway are all derepressible and are all regulated by LDL. We are currently examining other steps in the pathway to see if any other enzymes are also regulated by LDL. The importance of the observed activity increases of these cholesterologenic enzymes (Fig. 2, b to d) other than that of HMG-CoA reductase in contributing to the overall increase in rate of cholesterol synthesis in CHO cells remains to be demonstrated more thoroughly. The data presented in Table IV show that, under certain conditions, large increases in activities of several cholesterologenic enzymes catalyzing early steps in the pathway can cause a significant increase in the rate of cholesterol synthesis in intact CHO cells without large change in activity of HMG-CoA reductase. Two more important problems remain to be elucidated: the identity of suppressor(s) derived from LDL-bound cholesterol (or its metabolite) remains unknown; the mechanisms of suppression on individual cholesterologenic enzymes by LDL also remains unknown. However, the phenotypic characteristics of clones 25-RA and 25-RB presented in this report suggest the existence of a common cellular factor controlling the intracellular action(s) of 25-OH cholesterol on all four enzymes; the function of this factor is defective or abnormal in clones 25-RA and 25-RB. Since 25-OH cholesterol has been shown to mimic the intracellular action(s) of LDL (by Brown et al. (10) and by our own studies presented in Fig. 2, a to d), these results imply that this putative common cellular factor may also play a crucial role in controlling the intracellular action(s) of LDL on various cholesterologenic enzymes in CHO cells. Consistent with this notion is the following finding in this laboratory: in two separate experiments, the activities of the first four cholesterologenic enzymes in clones 25-RA and 25-RB were all found to be more resistant to suppression by LDL (at 15 μg and 50 μg of protein/ml) as compared to those found in wild type CHO cells. These studies are under further investigation in this laboratory. The nature of this cellular factor is unknown at present; it may be a specific segment of DNA in the chromosome (i.e., a regulatory gene), or a specific gene product carrying regulatory roles, such as an enzyme converting cholesterol (or its analogs) into specific metabolite(s), or a specific protein or enzyme needed by cholesterol (or its analogs) to generate the proper regulatory signal at the transcriptional or post-transcriptional level. Other possibilities are not excluded at present. We believe that further characterizations of clones 25-RA and 25-RB and of other isolated mutants or variants (11, 15, 29, 30, 32, 33) will help elucidate the identities and functions of this and other regulatory factors controlling cholesterol biosynthesis in animal cells. We also believe that further characterization of these mutants or variants will help elucidate the nature of a postulated common control mechanism which may regulate coordinately cellular cholesterol synthesis and unsaturated fatty acid synthesis in CHO cells (11).

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