review

Multivalent feedback regulation of HMG CoA reductase, a control mechanism coordinating isoprenoid synthesis and cell growth

Michael S. Brown[1] **and Joseph L. Goldstein**

Departments of Molecular Genetics and Internal Medicine, University of Texas Health Science Center at Dallas, Dallas, TX 75235

Abstract The availability of compactin (ML-236B), a potent competitive inhibitor of 3-hydroxy-3-methylglutaryl Coenzyme A reductase, has permitted the demonstration of a hitherto unsuspected aspect of mevalonate metabolism and isoprenoid synthesis in cultured mammalian cells. 3-Hydroxy-3-methylglutaryl Coenzyme A reductase, the enzyme that synthesizes mevalonate, appears to be regulated through a multivalent feedback mechanism. Full suppression of the reductase requires the presence of at least two regulators: 1) cholesterol, which is normally derived exogenously from plasma low density lipoprotein (LDL), and 2) a nonsterol product, which is normally synthesized endogenously from mevalonate. Evidence indicates that both of these regulators of the reductase may be essential for the growth of mammalian cells in culture. The multivalent feedback regulation of 3-hydroxy-3-methylglutaryl Coenzyme A reductase, together with secondary regulatory changes in other enzymes of the sterol synthetic pathway, coordinates the branched pathway of mevalonate metabolism so as to assure a constant supply of cholesterol and nonsterol products. These new findings have important implications for the understanding of isoprenoid metabolism and its relation to cell growth.—**Brown, M. S., and J. L. Goldstein.** Multivalent feedback regulation of HMG CoA reductase, a control mechanism coordinating isoprenoid synthesis and cell growth. *J. Lipid Res.* 1980. **21:** 505–517.

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Mevalonate, a key intermediate in the biosynthesis of cholesterol, is formed by the enzyme 3-hydroxy-3-methylglutaryl Coenzyme A reductase (HMG CoA reductase). In cultured mammalian cells such as human fibroblasts, the activity of HMG CoA reductase, and hence the formation of mevalonate, is controlled through a feedback mechanism mediated by cholesterol that enters cells bound to a plasma lipoprotein, low density lipoprotein (LDL) (1, 2). In the absence of plasma LDL, cells in culture synthesize their own cholesterol, maintaining high levels of HMG CoA reductase. When LDL is added to the culture medium, the LDL-derived cholesterol reduces the activity of HMG CoA reductase, thereby turning off the cell's cholesterol synthesis (1, 2). Recent experiments show that this well-understood control mechanism constitutes only one part of the HMG CoA reductase story. Cultured cells are now known to use mevalonate for synthesis of several isoprenoid compounds in addition to cholesterol. Like cholesterol, these nonsterol compounds are essential for cell growth; they also participate with cholesterol in a type of multivalent feedback regulation of HMG CoA reductase. This article reviews recent evidence supporting the concept that HMG CoA reductase is not suppressed fully in fibroblasts unless the cells have adequate levels of both cholesterol and a nonsterol product derived from the metabolism of mevalonate.

New insights into the branched pathway of mevalonate metabolism in cultured cells have been made possible through the use of compactin, a fungal metabolite that is also known as ML236B.[2] In 1976, Endo, Kuroda, and Tanzawa (3) isolated compactin and made the fundamental observation that this com-

Abbreviations: CHO, Chinese hamster ovary; HMG CoA, 3-hydroxy-3-methylglutaryl Coenzyme A; LDL, low density lipoprotein.

[1] Address correspondence to Dr. Michael S. Brown, Department of Molecular Genetics, University of Texas Health Science Center at Dallas, 5323 Harry Hines Blvd., Dallas, TX 75235.

[2] The two compounds, compactin and ML-236B, are identical fungal metabolites isolated from strains of *Penicillium brevicompactum* (44) and *Penicillium citrinum* (3), respectively. Previous studies have shown that these two metabolites have identical biological activity in inhibiting HMG CoA reductase activity (4). For simplicity, we have used the single term compactin to refer to both metabolites.

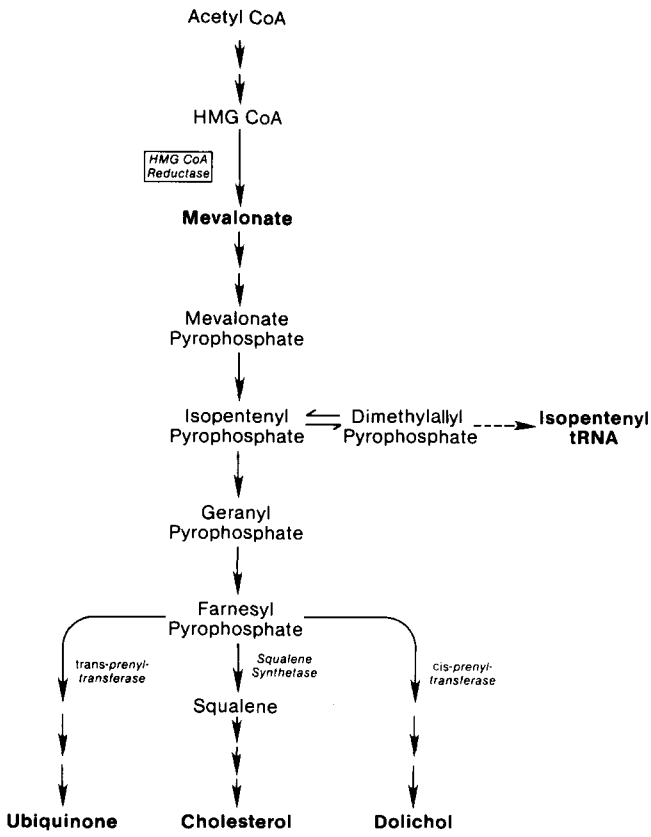

Fig. 1 The branched pathway of mevalonate metabolism in mammalian cells.

pound is an extremely potent competitive inhibitor of HMG CoA reductase. Incubation of cultured cells with compactin blocks mevalonate production (4, 5) and converts the cells into mevalonate auxotrophs. This property of compactin allows it to be used as a tool to amplify regulatory mechanisms that otherwise are difficult to study experimentally. Analysis of the adaptive responses of cells to compactin-induced mevalonate deprivation quickly disclosed that cultured cells require mevalonate, not only for cholesterol production but also for synthesis of one or more nonsterol compounds that are essential both for regulation of HMG CoA reductase and for cell growth (4–8).

Branched pathway of mevalonate metabolism

Fig. 1 shows the branched pathway of mevalonate metabolism as it was originally elucidated for mammalian liver through the classic studies of Bloch, Lynen, Popjak, Cornforth, Porter, Olsen, Rudney, Hemming and others (reviewed in refs. 9–13). A similar, if not identical, pathway is now known to occur in cultured cells. In fibroblasts and smooth muscle cells, radiolabeled mevalonate is converted to three known end-products: cholesterol, dolichol, and the polyisoprene side chain of ubiquinone (6, 14, 15). Each of these compounds contains a polyisoprene structure derived from repeated polymerizations of the basic 5-carbon building block, isopentenyl pyrophosphate. The latter compound is derived from the 6-carbon precursor, mevalonate. In the initial polymerization reaction, isopentenyl pyrophosphate is coupled with its isomer, dimethylallyl pyrophosphate, in a so-called "head-to-tail" condensation to form the 10-carbon intermediate, geranyl pyrophosphate. This compound then reacts with another molecule of isopentenyl pyrophosphate in another "head-to-tail" condensation to form the key 15-carbon intermediate, farnesyl pyrophosphate (9).

According to current evidence, farnesyl pyrophosphate constitutes the major branch-point in polyisoprene biosynthesis (10, 11). This compound can participate in three different enzymatic reactions. First, two molecules of farnesyl pyrophosphate can condense into "head-to-head" fashion to form squalene, a 30-carbon compound that is the first committed intermediate in the sterol synthetic pathway (12). Second, the farnesyl pyrophosphate chain can be extended by repeated "head-to-tail" condensations with isopentenyl pyrophosphate groups or short-chain polyprenols to form long-chain polyprenols. In these long-chain polyprenols all of the double bonds are in the *trans* configuration. When the chain length reaches 10 isoprene units in human cells, the polyprenyl group is transferred to an aromatic ring derived from tyrosine to initiate the synthesis of ubiquinone (13).

In a third set of reactions, farnesyl pyrophosphate can be converted into the long-chain polyisoprenyl alcohol, dolichol, through repeated additions of up to 16 additional isopentenyl residues (11). The unique feature of the latter condensation reactions is that *cis* double bonds are produced in the polyisoprene chain. Thus, in dolichol the first three double bonds, which are derived from farnesyl pyrophosphate, are *trans*, whereas the remaining double bonds are in the *cis* configuration. The *cis*-additions of isopentenyl pyrophosphate appear to be specific reactions that are unique to the dolichol synthetic pathway, and the enzyme that makes the first *cis* addition of an isopentenyl pyrophosphate group to farnesyl pyrophosphate can be considered to catalyze the first unique (committed) reaction in dolichol synthesis (11).

A fourth product of mevalonate metabolism, isopentenyl adenosine, is a constituent of transfer RNA. In bacteria, yeast, and mammalian liver, isopentenyl adenosine is synthesized from mevalonate via transfer of the dimethylallyl group from dimethylallyl pyrophosphate to the N^6 position of specific adenosine moieties of certain preformed transfer RNA molecules (16). Although such synthesis has not yet been demonstrated for cultured mammalian cells, it is reasonable to assume that it occurs since transfer RNA

containing isopentenyl adenosine seems to be a ubiquitous constituent of mammalian tissues, having been demonstrated in calf liver, chick embryo, and human liver (16).

From the scheme of Fig. 1, it can be seen that the production of mevalonate must be tightly controlled in order to yield sufficient isopentenyl pyrophosphate and farnesyl pyrophosphate to satisfy cellular requirements for all four of the mevalonate end-products. Moreover, the control mechanism must be sufficiently complex to allow constant synthesis of the three nonsterol end-products at a time when LDL-cholesterol is added or removed from the culture medium, causing the synthetic rates of mevalonate and cholesterol to vary by more than 50-fold (2, 6). As discussed below, this coordinated control appears to be achieved through a type of cumulative feedback regulation of HMG CoA reductase that is mediated by LDL-derived cholesterol together with one or more of the other end-products of mevalonate metabolism. The initial evidence for this regulation was obtained in studies of cultured human fibroblasts (4, 6).

Role of LDL-derived cholesterol and nonsterol products of mevalonate metabolism in the multivalent feedback regulation of HMG CoA reductase in human fibroblasts

All animal cells require cholesterol as a structural component of their plasma membranes. Yet under the usual conditions of growth in cell culture, human fibroblasts and many other cell types express a low HMG CoA reductase activity and produce mevalonate and cholesterol at minimal rates (1, 2, 17). This occurs because the cells use a surface receptor to supply themselves with cholesterol from the LDL that is present in the serum of the culture medium (2, 18). Binding of LDL leads to cellular uptake of the lipoprotein through adsorptive endocytosis with subsequent delivery to lysosomes. The cholesterol liberated from the lysosomal hydrolysis of LDL fulfills the cell's requirement for membrane synthesis. It also suppresses HMG CoA reductase activity, apparently by suppressing the synthesis of enzyme molecules (19).

When fibroblasts are transferred to medium from which the LDL has been removed, the cells develop a marked increase in HMG CoA reductase activity, which is paralleled by a rise in cholesterol synthesis. The subsequent addition of LDL to the culture medium suppresses HMG CoA reductase activity and mevalonate production by as much as 98%, but suppression never becomes 100%; thus, small levels of HMG CoA reductase activity are always detectable in cell homogenates even when cells are grown in the presence of maximal levels of LDL (4, 6, 19).

Studies with compactin first revealed that the low levels of HMG CoA reductase activity that persist in the presence of LDL are important for cellular metabolism (4). When compactin was added to cells grown in the presence of LDL, the small amount of residual mevalonate production was inhibited. The cells responded to this mevalonate deprivation by developing a 5- to 10-fold increase in the amount of HMG CoA reductase enzyme. The enzyme was not active within the cell because it was inhibited by the compactin. However, the induced enzyme could be detected by assays of its activity in cell-free homogenates under conditions in which the reversible compactin inhibition was overcome by dilution (4).

The increased amount of HMG CoA reductase enzyme in compactin-grown cells appears to be produced as a result of the cellular deprivation of one or more of the nonsterol products that is normally synthesized from mevalonate. This conclusion was suggested by the observation that the induced HMG CoA reductase could not be fully suppressed even when the cells were given high levels of LDL (4). The added LDL was effective in delivering cholesterol to the compactin-treated cells; indeed, it produced a marked increase in the cellular content of free and esterified cholesterol (4). However, despite an abundance of intracellular cholesterol, the amount of HMG CoA reductase remained relatively high. In the presence of compactin the enzyme could be fully suppressed only when the cells were given a small amount of mevalonate in addition to the LDL-cholesterol (4).

The experiments of Figs. 2 and 3 illustrate the multivalent feedback suppression of HMG CoA reductase by LDL and mevalonate in fibroblasts. In the first experiment (**Fig. 2**), fibroblasts had been grown in the absence of LDL and incubated for 24 hr with compactin so that HMG CoA reductase activity, as measured in cell-free extracts, was high (300 pmol of mevalonate formed per min per mg of cell protein). At zero time, a saturating level of LDL was added to the culture medium. After 24 hr, HMG CoA reductase activity had declined by about 85% to 50 pmol·min^{-1}·mg protein^{-1}, but it did not decline any further over the next 24 hr (closed circles, Fig. 2), even though the cholesterol content of the cells had increased markedly. Although the LDL-derived cholesterol could not further suppress HMG CoA reductase, the addition of mevalonate at 24 hr produced a rapid suppression of the remaining enzymatic activity (open circles, Fig. 2).

The experiment of **Fig. 3** demonstrates that extremely small amounts of mevalonate (in the range of 10^{-4} M) are sufficient to suppress the reductase in the presence of compactin plus LDL. In this experiment, cells were grown for 3 days in the absence of

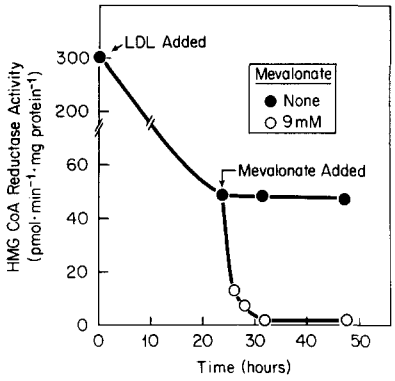

Fig. 2. Multivalent feedback regulation of HMG CoA reductase activity in human fibroblasts by LDL and mevalonate. Monolayers of human fibroblasts (1×10^5 cells/60-mm dish) were grown in medium containing 10% fetal calf serum as previously described (4). On day 5 of cell growth, each monolayer was switched to growth medium containing 10% human lipoprotein-deficient serum. On day 6, each monolayer received medium containing 10% human lipoprotein-deficient serum and 2.5 μM compactin. On day 7 (zero time), duplicate dishes were harvested for measurement of HMG CoA reductase activity (4), and each of the remaining dishes received LDL at a final concentration of 50 μg protein/ml. Twenty four hours later, the cells were divided into two groups, one receiving no mevalonolactone (●) and the other receiving mevalonolactone at a final concentration of 9 mM (○). Duplicate dishes of cells were harvested at the indicated time for measurement of HMG CoA reductase activity (4).

compactin until they reached near-confluence, after which they received 1 μM compactin. This concentration of compactin was chosen because it inhibits [^{14}C]acetate incorporation into cholesterol by 90 to 95% but leaves sufficient functional reductase activity to support cell survival. When the cells were incubated with 1 μM compactin in the *absence* of LDL for 4 days (open circles), they responded by developing an extremely high level of HMG CoA reductase activity as measured in vitro (1200 pmol·min^{-1}·mg protein^{-1}). Inclusion of mevalonate in the culture medium at concentrations up to 1 mM had only a slight suppressive effect on the enzyme. However, when the mevalonate concentration in the culture medium was raised to 5 mM, the amount of measurable HMG CoA reductase was reduced by 93% to 82 pmol·min^{-1}·mg protein^{-1} (open circles, Fig. 3). The adaptive response was strikingly different when the cells were grown with 1 μM compactin in the *presence* of a saturating level of LDL. These cells had a partially suppressed HMG CoA reductase activity of 200 pmol·min^{-1}·mg protein^{-1} (closed circles, Fig. 3). Under these conditions the addition of a very low concentration of mevalonate (50 μM) suppressed the reductase by an additional 87%, to 26 pmol·min^{-1}·mg protein^{-1}. Higher concentrations of mevalonate suppressed the enzyme even further to 2 pmol·min^{-1}·mg protein^{-1}.

The results of the above experiments have been interpreted in the following way: cells require at least two products from mevalonate in order for HMG CoA reductase to be suppressed. One of these products, cholesterol, is required in large amounts. It can be supplied either exogenously from LDL or endogenously from mevalonate (19, 20). The other mevalonate-derived product is required in much smaller amounts. When cells are grown with compactin in the *absence* of LDL, they require high concentrations of mevalonate in order to synthesize cholesterol plus the trace product. HMG CoA reductase is not suppressed until both requirements are met, and thus a large amount of mevalonate is required for complete enzyme suppression. This interpretation is supported by the additional observation that the same high concentrations of mevalonate that are required to suppress HMG CoA reductase under these conditions (i.e., 5 to 10 mM) are also required to suppress LDL receptor activity and stimulate cholesterol ester synthesis, two reactions that occur only after the cholesterol requirement of cells is satisfied.[3] When fibroblasts are grown with compactin *plus* LDL, their cholesterol requirement is satisfied by the LDL-derived cholesterol as evidenced by the fact that cholesterol esterification is activated (4). Nevertheless, HMG CoA reductase remains relatively high because the other mevalonate-derived substance is lacking. Under these conditions the provision of a small amount of exogenous mevalonate is sufficient to permit synthesis of the putative nonsterol mevalonate-derived product and thereby completely suppress HMG CoA reductase.

Similar multivalent regulation of HMG CoA reductase by LDL-cholesterol and mevalonate can be

[3] Faust, J. R., J. L. Goldstein, and M. S. Brown. Unpublished observations.

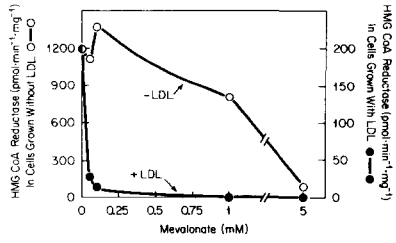

Fig. 3. Mevalonate-mediated suppression of HMG CoA reductase activity in human fibroblasts grown in the presence of compactin with or without LDL. Monolayers of human fibroblasts (1×10^5 cells/60-mm dish) were grown in medium containing 10% fetal calf serum as previously described (4). On day 3 of cell growth, each monolayer received 2 ml of growth medium containing 15% fetal calf lipoprotein-deficient serum, 1 μM compactin, either no LDL (○) or 50 μg protein/ml of LDL (●), and the indicated concentration of mevalonolactone. On day 5, each dish received 2 ml of fresh medium of the same composition. On day 7, duplicate dishes of cells were harvested for measurement of HMG CoA reductase activity (4).

demonstrated in the absence of compactin. However, the data are not so striking because in the absence of compactin the cells are always synthesizing mevalonate and thus HMG CoA reductase is always partially suppressed. Nevertheless, **Table 1** shows that low concentrations of exogenous mevalonate fail to suppress HMG CoA reductase when cells are grown in the *absence* of LDL (column a). Significant suppression is only achieved at mevalonate concentrations in the range of 2.4 mM. Near-saturating and saturating levels of LDL (20 and 50 μg protein/ml), suppress HMG CoA reductase by a maximum of 96% and 98% to 12 and 6.1 pmol·min^{-1}·mg protein^{-1}, respectively (columns b and c). These activities can then be further suppressed by the addition of small amounts of exogenous mevalonate (50 μM). This experiment supports the conclusion originally drawn from the compactin studies: the small amount of HMG CoA reductase that remains active in the presence of LDL is important for the synthesis of substances other than cholesterol and the reductase is fully suppressed only when the requirements for both cholesterol and the other mevalonate-derived product are satisfied. In subsequent studies of this regulatory mechanism, cultured cells have been incubated with compactin in order to enhance the amount of reductase that is not suppressed by LDL-cholesterol.

What is the mechanism for mevalonate-mediated suppression of HMG CoA reductase?

Fig. 4 shows the time course of the decline in HMG CoA reductase activity after the addition of mevalonate to fibroblasts previously incubated with compactin plus LDL. The enzyme activity decayed with first-order kinetics with a half-time of about 70 min. Evidence to date suggests that this loss in activity is due to the irreversible inactivation or degradation of the enzyme.[3] HMG CoA reductase in cultured human fibroblasts, as in liver (21, 22), is susceptible to reversible inactivation through phosphorylation catalyzed by a cytosolic enzyme that uses ATP (23). This inactivation can be reversed in liver or in fibroblasts by treatment of the enzyme with alkaline phosphatase (24).[3] However, to date we have not been able to demonstrate reactivation (by alkaline phosphatase treatment) of the HMG CoA reductase activity that is lost in cells cultured with mevalonate. At the present time, it is not known whether mevalonate enhances the rate of irreversible degradation of HMG CoA reductase, inhibits its synthesis, or both. When added to cell-free extracts of fibroblasts, mevalonate does not inhibit the activity of HMG CoA reductase (4), nor does it accelerate its inactivation by the phosphorylating enzyme.[3]

TABLE 1. Suppression of HMG CoA reductase by mevalonate in human fibroblasts grown in the absence of compactin

Concentration of Mevalonate in Medium	HMG CoA Reductase Activity		
	−LDL (a)	+LDL, 20 μg protein/ml (b)	+LDL, 50 μg protein/ml (c)
mM	*pmol·min^{-1}·mg protein^{-1}*		
0	285	12.0	6.1
0.05	268	6.0	2.8
0.2	218	6.5	2.6
2.4	147	4.8	2.6
9.6	22	3.4	3.2

Monolayers of human fibroblasts (7.5 × 10^5 cells/60-mm dish) were grown in medium containing 10% fetal calf serum as previously described (4). On day 4 of cell growth, each monolayer was switched to growth medium containing 10% human lipoprotein-deficient serum, the indicated concentration of LDL, and the indicated concentration of mevalonolactone. On day 5, after incubation for 24 hr at 37°C duplicate dishes of cells were harvested for measurement of HMG CoA reductase activity (4).

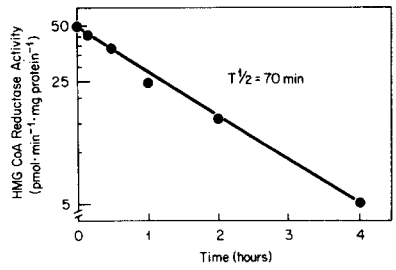

Fig. 4. Time course of the mevalonate-mediated suppression of HMG CoA reductase activity in human fibroblasts grown in the presence of compactin and LDL. Monolayers of fibroblasts (1 × 10^5 cells/60-mm dish) were grown in medium containing 10% fetal calf serum as previously described (4). On day 5 of cell growth, the cells were switched to medium containing 10% lipoprotein-deficient serum. On day 6, each cell monolayer received growth medium containing 10% human lipoprotein-deficient serum, 2.5 μM compactin, and 50 μg protein/ml of LDL. On day 7, at zero time each dish received 9 mM mevalonolactone. After incubation at 37°C for the indicated time, duplicate dishes of cells were harvested for measurement of HMG CoA reductase activity (4).

Search for the nonsterol regulator of HMG CoA reductase: direct addition of mevalonate end-products

The regulator that acts cumulatively with cholesterol to suppress HMG CoA reductase could be mevalonate or any of the products derived from its metabolism. It is unlikely, however, that the putative regulator is squalene or any distal intermediate in the sterol synthetic pathway. This conclusion is based on the observation that the addition of squalene at concentrations up to 1.6 mM did not suppress HMG CoA reductase in fibroblasts grown in compactin plus LDL under conditions in which 0.2 mM mevalonate gave complete suppression (**Fig. 5A**). On the other hand, 0.8 mM squalene produced an 80% suppression of HMG CoA reductase in fibroblasts grown in the

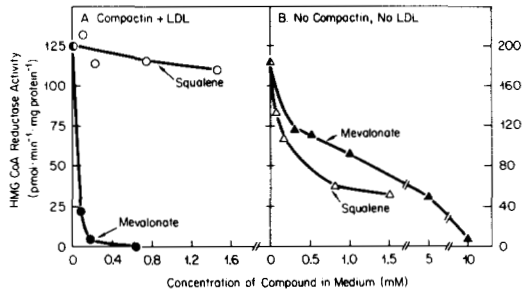

Fig. 5. Suppression of HMG CoA reductase activity by mevalonate and squalene in human fibroblasts grown in the presence of compactin plus LDL (A) or in the absence of compactin and LDL (B). Monolayers of human fibroblasts (8×10^4 cells/60-mm dish) were grown in medium containing 10% fetal calf serum as previously described (4). Panel A: On day 6 of cell growth, each monolayer received 2 ml of growth medium containing 10% human lipoprotein-deficient serum, 2.5 μM compactin, 25 μg protein/ml of LDL, 10 μl of ethanol, and the indicated concentration of sodium mevalonate (●) or squalene (○). After incubation for 24 hr at 37°C, duplicate dishes were harvested for measurement of HMG CoA reductase activity (4). Panel B: On day 5 of cell growth, each monolayer was switched to growth medium containing 10% human lipoprotein-deficient serum. On day 7, each monolayer received medium containing 10% human lipoprotein-deficient serum, 10 μl of ethanol, and the indicated concentration of sodium mevalonate (▲) or squalene (△). After incubation for 24 hr at 37°C, duplicate dishes were harvested for measurement of HMG CoA reductase activity (4).

absence of both compactin and LDL (Fig. 5B). In fact, squalene was even more active than mevalonate in these cells. These data suggest that squalene can generate enough cholesterol to fulfill the cholesterol requirement of cells, but it cannot fulfill the requirement for the additional substance derived from mevalonate. Similar observations were made with lanosterol.[3]

The addition to the culture medium of two of the other end-products of mevalonate metabolism, ubiquinone and dolichol (added in dimethylsulfoxide or ethanol), also failed to suppress HMG CoA reductase in fibroblasts grown in compactin plus LDL.[3] The interpretation of this experiment must be qualified since no independent evidence was obtained to prove that these apolar compounds entered the cell in a metabolically active form.

The other product derived from mevalonate, the isopentenyl adenosine of transfer RNA, has also been difficult to study. Free isopentenyl adenosine, like many adenosine derivatives, is a potent toxin for mammalian cells (25). When added to cultured human fibroblasts at concentrations as low as 40 μM, isopentenyl adenosine causes an immediate cessation of protein synthesis and a rapid decline in the activity of HMG CoA reductase.[3] The significance of this latter observation is unclear because of the general toxicity of isopentenyl adenosine, which may never exist in a free state within cells under physiological conditions.

The three nonsterol end-products—ubiquinone, dolichol, and isopentenyl adenosine—have also been incubated with the HMG CoA reductase enzyme in cell-free extracts. So far no direct inhibition of the enzyme activity has been demonstrated.[3] Moreover, these compounds do not appear to influence the phosphorylation reaction by which fibroblast HMG CoA reductase can be inactivated in vitro.[3]

Inasmuch as farnesyl pyrophosphate is located at the major branch point of the mevalonate pathway (Fig. 1), it is attractive to speculate that this intermediate plays a direct role in the regulation of HMG CoA reductase. Indeed, the activity of the reductase appears to be tuned to assuring a sufficient supply of farnesyl pyrophosphate to support synthesis of its three end-products. To date, however, studies of the influence of farnesyl pyrophosphate on the reductase in vitro or in vivo have not been reported.

Another set of reactions that may play a role in this regulation are those that constitute the "*trans*-methylglutaconyl shunt pathway" described by Edmond and Popjak in extrahepatic tissues (26). By this sequence of reactions, isopentenyl pyrophosphate can be recycled back to HMG CoA and eventually to acetyl CoA. This pathway has not been studied in cultured cells; if it does function in such cells, the various intermediates in this pathway might play a part in the multivalent feedback regulation of HMG CoA reductase.

Synthesis of nonsterol regulators of HMG CoA reductase: the flux diversion hypothesis

The studies described above demonstrate that HMG CoA reductase is not fully suppressed in fibroblasts unless one or more nonsterol regulators is present along with cholesterol. But maintenance of high reductase activity alone would not be sufficient to assure synthesis of the nonsterol products in the presence of LDL. When LDL is absent, the vast bulk of synthesized mevalonate is incorporated into cholesterol. When HMG CoA reductase is suppressed partially by LDL, cells must have some way of diverting the small amounts of synthesized mevalonate preferentially into the crucial nonsterol products.

Faust, Goldstein, and Brown (6) established a model system to test this flux diversion hypothesis. Fibroblasts were incubated in the presence of compactin *plus* LDL and then the cells were given trace amounts of high specific activity [^{3}H]mevalonate (6). The authors hypothesized that under these conditions of mevalonate deprivation and cholesterol excess the cells might preferentially divert the exogenous [^{3}H]-mevalonate into the most crucial nonsterol end-products. The results of these experiments supported

TABLE 2. Incorporation of [^{3}H]mevalonate into lipids in human fibroblasts incubated in the presence of compactin with and without LDL

Lipoproteins in Growth Medium	[^{3}H]Mevalonate Incorporated into				
	Squalene (a)	Lanosterol (b)	Cholesterol (c)	Total Sterol Branch (a) + (b) + (c)	Ubiquinone
	pmol/mg protein				
None	4.0	3.0	54	61	2.3
LDL	5.0	4.0	3.0	12	4.7
% Change[a]	+25%	+33%	−95%	−80%	+100%

[a] Percent change due to presence of LDL in culture medium.

Monolayers of human fibroblasts (8×10^4 cells/60-mm dish) were grown in medium containing 10% fetal calf serum as previously described (4). On day 5 of cell growth, each monolayer was switched to growth medium containing 10% human lipoprotein-deficient serum, 1 μM compactin, and either no LDL or 25 μg protein/ml of LDL. After incubation at 37°C for 24 hr, each dish received a tracer concentration (8 μM) of [^{3}H]mevalonolactone (875 cpm/pmol). After incubation for 24 hr, the cells were harvested, and the cellular content of the indicated [^{3}H]lipid was measured as previously described (6). Each value represents the average of duplicate incubations.

the flux diversion hypothesis. Instead of incorporating the mevalonate primarily into cholesterol, as occurs under normal conditions, fibroblasts grown in compactin *plus* LDL incorporated the mevalonate preferentially into another lipid with the chromatographic properties of ubiquinone (6). Ubiquinone is only one of the nonsterol end-products of mevalonate metabolism in cultured cells. Nevertheless, Faust et al. (6) used this compound as a model that might reveal general features of the control mechanisms for nonsterol pathways of mevalonate metabolism in cultured cells.

Table 2 shows the results of a representative experiment in which tracer amounts of [^{3}H]mevalonate (8 μM) were given to intact fibroblasts incubated with compactin (6). Cells incubated with compactin in the *absence* of LDL incorporated 54 pmol of [^{3}H]mevalonate into cholesterol per mg of cell protein; only 1/20th as much [^{3}H]mevalonate was incorporated into ubiquinone. On the other hand, when cells were incubated with compactin in the *presence* of LDL, the incorporation of [^{3}H]mevalonate into cholesterol fell by 95% and the incorporation into ubiquinone increased by 100%. As a result of this reciprocal change, cells incubated with trace amounts of [^{3}H]-mevalonate in the presence of LDL *plus* compactin incorporated 1.5 times more [^{3}H]mevalonate into ubiquinone than into cholesterol (Table 2).

This study provided the initial evidence that cultured cells could regulate cholesterol synthesis by altering the activity of enzymes distal to mevalonate formation. Moreover, the reciprocal changes in ubiquinone and cholesterol synthesis suggested that the enzyme(s) suppressed by LDL-cholesterol must have been located in the sterol pathway distal to the last common step in cholesterol and ubiquinone metabolism, i.e., distal to farnesyl pyrophosphate. This observation focused attention on the first such enzyme, i.e., squalene synthetase (See Fig. 1) and raised the possibility that changes in squalene synthetase activity may be linked to the multivalent feedback regulation of HMG CoA reductase.

Direct measurements of squalene synthetase activity in cell-free extracts of human fibroblasts confirmed the prediction of the intact cell studies. Addition of LDL to fibroblasts suppressed squalene synthetase activity by about 90% (27). The rate of decline in activity was relatively slow with the half-time being about 15 hr. This is much less rapid than the 2-hr half-time for LDL-mediated suppression of HMG CoA reductase in fibroblasts (19, 27). Conversely, when LDL was removed from fibroblasts, squalene synthetase activity increased relatively slowly: it rose 8-fold over 30 hr. The increase was blocked by the protein synthesis inhibitor cycloheximide,[3] suggesting that changes in enzyme level may be due to changes in the synthetic rate of the enzyme protein.

The suppression of squalene synthetase activity by LDL provides one mechanism for diversion of mevalonate metabolites away from the cholesterol pathway. In addition, diversion of metabolites into ubiquinone is facilitated by the apparent high affinity for farnesyl pyrophosphate of the initial enzymes in the ubiquinone pathway relative to those of the cholesterol pathway. This high affinity was apparent in studies in which fibroblasts were incubated with compactin *plus* LDL and were then given increasing doses of [^{3}H]mevalonate (6). Mevalonate incorporation into ubiquinone rose in a hyperbolic fashion with increasing mevalonate concentrations. Full saturation

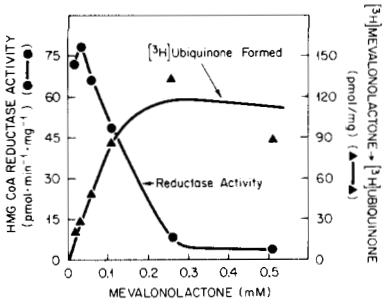

Fig. 6. Incorporation of [^{3}H]mevalonate into [^{3}H]ubiquinone-10 (▲) and activity of HMG CoA reductase (●) in human fibroblasts incubated with compactin *plus* LDL and increasing concentrations of mevalonate. Monolayers of fibroblasts (8×10^4 cells/60-mm dish) were prepared as previously described (6). On day 5 of cell growth, each dish received 2 ml of medium containing 10% human lipoprotein-deficient serum, 1.1 μM compactin, and 25 μg protein/ml of LDL. After incubation at 37°C for 24 hr, the dishes were divided into two groups. Each dish in the first group received the indicated concentration of unlabeled mevalonolactone. Each dish in the second group received the indicated concentration of [^{3}H]mevalonolactone. After incubation for 24 hr, the first group of dishes was harvested for measurement of HMG CoA reductase activity (●) and the second group of dishes was harvested for measurement of the cellular content of [^{3}H]ubiquinone-10 (▲). (Data reprinted from ref. 6).

was achieved when the extracellular mevalonate concentration was only about 0.2 mM (6). In contrast, the sterol branch of the pathway showed a much lower apparent affinity; the incorporation of [^{3}H]-mevalonate into cholesterol increased linearly with increasing mevalonate concentrations up to at least 1 mM (6). Because of the relatively high affinity of the ubiquinone branch of the pathway, mevalonate incorporation into ubiquinone exceeded that into cholesterol when the mevalonate concentration was low (ref. 6 and see Table 2). However, at high mevalonate concentrations the radioactivity incorporated into cholesterol was much higher than into ubiquinone (6). The flux diversion hypothesis proposed for ubiquinone synthesis is likely to apply to the other nonsterol mevalonate end-products as well. For example, a high affinity mechanism similar to the ubiquinone mechanism has been reported to preserve the synthesis of dolichol when HMG CoA reductase activity is suppressed in cultured cells (14, 15).

Considered together, the above data indicate that two mechanisms preserve nonsterol isoprenoid synthesis when HMG CoA reductase activity is reduced by LDL: 1) squalene synthetase is suppressed, limiting the incorporation of farnesyl pyrophosphate into cholesterol and allowing the intracellular concentration of farnesyl pyrophosphate to be maintained at a finite level; and 2) the high affinities of the initial enzymes in the nonsterol branches divert these small amounts of farnesyl pyrophosphate into the two nonsterol pathways.

The mechanisms that divert mevalonate into nonsterol pathways and the mechanisms that suppress HMG CoA reductase activity in LDL-grown cells appear to be coordinated, in that full suppression of HMG CoA reductase does not occur unless the mevalonate requirements for nonsterol pathways are satisfied. This coordination was demonstrated directly in an experiment in which fibroblasts were grown in the presence of LDL *plus* compactin and then incubated with increasing amounts of [^{3}H]mevalonate (**Fig. 6**). In one set of cells the incorporation into ubiquinone was measured, and in the other set the activity of HMG CoA reductase was determined. Complete suppression of HMG CoA reductase occurred only when the mevalonate concentration was high enough to saturate the ubiquinone synthesis pathway (Fig. 6).

In addition to squalene synthetase, more distal enzymes in the sterol branch of the mevalonate pathway appear to be suppressed by LDL.[4] Thus, in the experiment shown in Table 2 the amount of [^{3}H]mevalonate found in squalene and lanosterol, the two major intermediates in the sterol pathway, actually increased in the presence of LDL despite the lowered total flux through this branch (total sterol branch, Table 2). This finding suggests that the activities of squalene oxidocyclase and lanosterol demethylase must also have been suppressed by LDL. When unphysiologically high concentrations of [^{3}H]-mevalonate are added to intact cells in the presence of LDL, these two enzymes are easily saturated and the accumulations of squalene and lanosterol are even more striking.[3] Similar accumulations of squalene have been reported to occur in rat kidney slices (28) and in cultured renal carcinoma cells incubated with exogenous mevalonate in the presence of lipoproteins (29). In addition, cultured hepatoma cells accumulate radiolabeled lanosterol when incubated with [^{14}C]-mevalonate under conditions in which HMG CoA reductase is suppressed by 25-hydroxycholesterol (30 and see below).

Another aspect of the experiment of Table 2 deserves comment. In the presence of LDL, the total flux of [^{3}H]mevalonate into the total measured

[4] It is also likely that at least one enzyme prior to HMG CoA reductase is also suppressed by LDL. Studies by Lane and coworkers showed that cytosolic HMG CoA synthase in liver is suppressed by the feeding of cholesterol (45). This enzyme is also suppressed by LDL-cholesterol in rat adrenal gland (46). Finally, evidence for the LDL-mediated suppression of HMG CoA synthase in cultured HeLa cells has also been presented (47). In all three systems, the changes in HMG CoA synthase are delayed and of lesser magnitude than the changes in HMG CoA reductase; thus HMG CoA reductase is still felt to represent the rate-limiting step in mevalonate synthesis.

metabolites, i.e., into the total sterol branch *plus* ubiquinone, declined by 47 pmol/mg protein. The fate of the excess [^{3}H]mevalonate is not known. Some of it may be incorporated into the other nonsterol end-products that were not measured, but such incorporation is unlikely to account for such a large amount. Alternatively, some of the [^{3}H]mevalonate may be recycled back to HMG CoA via the "*trans*-methylglutaconyl shunt." Finally, it is possible that the total utilization of mevalonate actually declines, i.e., that mevalonate kinase or one of the other enzymes between mevalonate and farnesyl pyrophosphate is partially suppressed by LDL. Further studies will be needed to resolve this question.

It should be pointed out that even though squalene synthetase is suppressed about 90% by LDL-cholesterol, the enzyme is still present in large excess in the cell (27). Because of this excess, the intracellular farnesyl pyrophosphate concentration is never high enough to saturate the enzyme, even when the total amount of enzyme is reduced. In this regard, Faust et al. (6) calculated that in the presence of compactin *plus* LDL an extracellular concentration of 0.15 mM mevalonate provides an intracellular mevalonate concentration that is equal to the concentration that the cells would ordinarily maintain in the absence of compactin. Yet, fibroblasts incubated with compactin *plus* LDL incorporate [^{3}H]mevalonate into the total sterol pathway (i.e., squalene plus lanosterol plus cholesterol) at rates that increase linearly with increasing [^{3}H]mevalonate concentrations up to 20 mM[3]. Thus, at the usual intracellular concentrations of mevalonate and farnesyl pyrophosphate (equivalent to those achieved with 0.15 mM extracellular mevalonate), the squalene synthetase enzyme operates well below saturation. Since the maximal velocity of the enzyme is never reached in the cell, the suppression of squalene synthetase by LDL does not function to limit the maximal possible rate of sterol synthesis. Even when the enzyme is suppressed, squalene synthesis can be driven to high levels simply by overloading the cells with mevalonate. However, suppression of squalene synthetase establishes a condition in which at any given rate of mevalonate synthesis the intracellular level of farnesyl pyrophosphate is maintained at a level that is higher than it would be if squalene synthetase were not suppressed.

Is the nonsterol regulator of HMG CoA reductase required for cell growth?

At the same time that the studies of mevalonate metabolism in fibroblasts were pointing to the existence of a nonsterol regulator of HMG CoA reductase, studies in other laboratories were providing evidence that cultured cells require, in addition to cholesterol, a nonsterol metabolite of mevalonate to support cell growth. Kaneko, Hazama-Shimada, and Endo (5) first reported that high levels of compactin inhibited the growth of mouse L cells and human fibroblasts, even in medium containing high levels of LDL. Growth could be restored by the addition of small amounts of mevalonate (0.3 mM) in the presence of lipoprotein cholesterol. These workers postulated that the cells might require small amounts of active HMG CoA reductase in order to synthesize a mevalonate-derived substance other than a sterol that is required for growth.

Quesney-Huneeus, Wiley, and Siperstein (7) extended these observations by studying baby hamster kidney (BHK) cells whose growth was synchronized by a double thymidine block. When the cells were incubated in the presence of compactin, they failed to enter the S-phase of growth as evidenced by an inhibition of the incorporation of [^{3}H]thymidine into DNA. The addition of cholesterol in the form of lipoproteins failed to overcome this compactin-mediated inhibition of DNA synthesis. On the other hand, when the cells were given 0.4 mM mevalonate in the presence of lipoproteins, DNA synthesis was restored within minutes. These experiments were interpreted to indicate that the BHK cells require a mevalonate-derived substance other than cholesterol in order to enter the S-phase of growth (7). Similar findings have been obtained by Habenicht, Glomset, and Ross (8). Using cultured monkey arterial smooth muscle cells and Swiss 3T3 cells, these investigators also demonstrated that compactin inhibited DNA synthesis and cell growth and that their restoration required the addition of mevalonate.

When the results of the growth studies are considered together with the studies of enzyme regulation discussed earlier, it is apparent that HMG CoA reductase activity is not suppressed in cultured cells unless the cells have sufficient mevalonate to synthesize a nonsterol substance that is required for cell growth. This hypothesis is supported by growth experiments with Chinese hamster ovary (CHO) cells (31). **Fig. 7** shows such an experiment. CHO cells were grown for 7 days in the presence of 0, 2 μM, or 40 μM compactin, and the petri dishes were stained to visualize the colonies. At 2 μM compactin, the activity of each HMG CoA reductase enzyme molecule is inhibited by 98% (31), but the cells are able to overcome this inhibition partially by developing an increased number of HMG CoA reductase molecules. At 40 μM compactin, HMG CoA reductase is totally inhibited and no compensation is possible. In the *presence* of either 2 μM or 40 μM compactin and in the

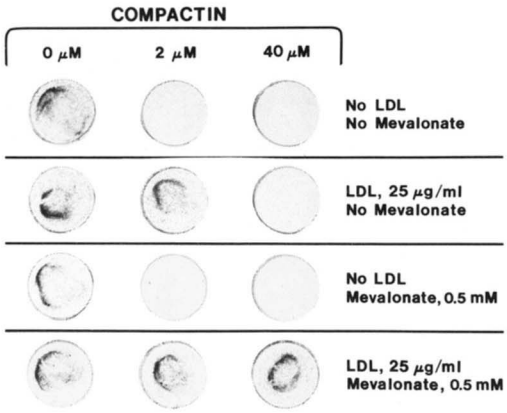

Fig. 7. Photograph of stained dishes of Chinese hamster ovary (CHO) cells, illustrating the inhibition of growth by compactin and its restoration by LDL plus mevalonate. Cells were plated (day 0) at a concentration of 2000 cells per 60-mm petri dish in 3 ml of growth medium (31) containing 10% newborn calf lipoprotein-deficient serum and the indicated concentration of compactin, LDL, and sodium mevalonate. On day 7, the cell monolayers were fixed with methanol and stained with 0.1% crystal violet. The concentration of LDL is given in terms of its protein content.

absence of both LDL and mevalonate, the cells failed to grow (first row, Fig. 7). At 2 μM compactin, LDL restored growth, apparently because the cells had developed sufficiently high levels of HMG CoA reductase to permit synthesis of the small amounts of essential nonsterol substances that are required for growth when the cholesterol requirement has been met (second row, Fig. 7). At 40 μM compactin, neither LDL alone nor a small amount of mevalonate (0.5 mM) supported growth (second and third rows, Fig. 7). However, the combination of LDL and mevalonate restored full growth of the CHO cells, even in the presence of 40 μM compactin (fourth row, Fig. 7). In additional experiments, we observed that squalene failed to restore growth to CHO cells incubated in the presence of compactin plus LDL. Thus, the same combination of substances (LDL and a small amount of mevalonate) are required for two events: suppression of HMG CoA reductase activity and stimulation of cell growth. This correlation suggests that the mevalonate-derived substance that acts cumulatively with LDL-cholesterol to support cell growth may be closely related to the mevalonate-derived substance that acts cumulatively with LDL-cholesterol to suppress HMG CoA reductase. It is not yet known whether a single substance fulfills both functions, or whether two or more mevalonate-derived substances are required.

Relation between the effects of compactin and oxygenated sterols on HMG CoA reductase

The studies of Kandutsch and Chen (32) demonstrated that oxygenated sterols, when dissolved in ethanol and added to cultured cells, are potent suppressors of HMG CoA reductase activity. These sterols, which include 25-hydroxycholesterol and 7-ketocholesterol, are much more potent than ethanol-dissolved cholesterol and are about as effective as cholesterol contained within LDL (33, 34). Inasmuch as these oxygenated sterols suppress HMG CoA reductase activity, cultured human fibroblasts and other cells will not grow in their presence unless they are given exogenous cholesterol, either contained within LDL or as free cholesterol dissolved in ethanol (33–35). Alternatively, growth can be supported by large amounts of exogenous mevalonate (33–35).

The growth inhibitory effects of 25-hydroxycholesterol and other oxygenated sterols resemble in some ways those observed with compactin. However, there is one important difference. In its effects on cholesterol metabolism, 25-hydroxycholesterol resembles LDL-derived cholesterol: it suppresses only the fraction of HMG CoA reductase that can be suppressed by LDL-cholesterol. As noted above, LDL-derived cholesterol has four important regulatory actions on cholesterol metabolism in human fibroblasts and other cultured cells: 1) it suppresses HMG CoA reductase activity; 2) it stimulates the formation of cholesteryl esters by activating the microsomal enzyme acyl-CoA cholesterol: acyltransferase; 3) it suppresses the synthesis of LDL receptors; and 4) it suppresses squalene synthetase activity (18, 27). Oxygenated sterols such as 25-hydroxycholesterol and 7-ketocholesterol reproduce each of these effects of LDL-derived cholesterol (27, 33, 36, 37), suggesting that these oxygenated sterols act as cholesterol analogues and bind to the putative intracellular sterol receptor(s) that regulate these various events.

Most important, as discussed in detail above, when cells are grown in the presence of compactin, LDL is able to suppress HMG CoA reductase only partially; full suppression requires mevalonate in addition. Similarly, when human fibroblasts or Chinese hamster ovary cells are grown in the presence of compactin, 25-hydroxycholesterol is only able to suppress HMG CoA reductase partially and to the same degree as LDL-derived cholesterol. Full suppression requires the additional presence of exogenous mevalonate.[3]

Summary of the multivalent feedback regulation of HMG CoA reductase: physiologic role and possible therapeutic implications

Fig. 8 shows the broad outline of regulation of the branched pathway of mevalonate metabolism as it is beginning to emerge from the tissue culture studies

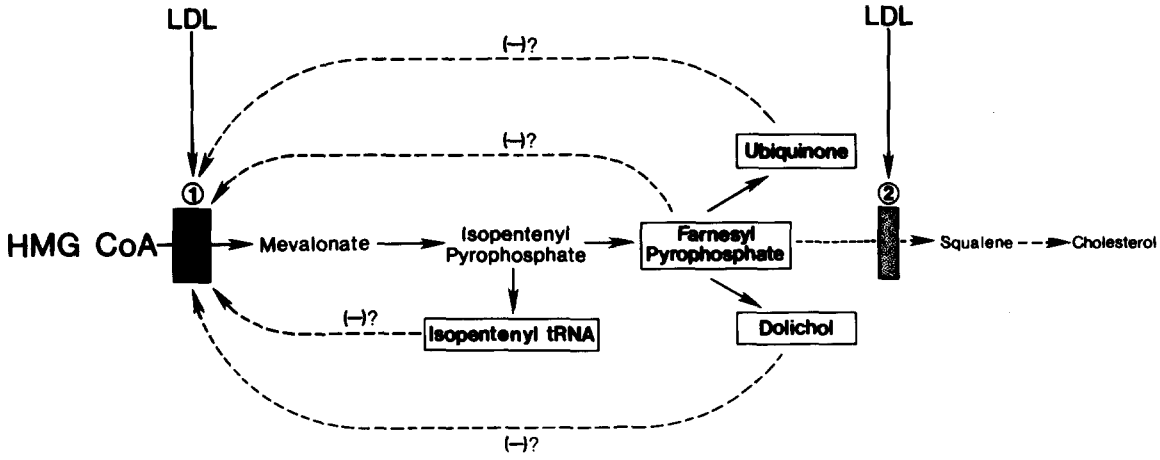

Fig. 8. Model for multivalent feedback regulation of HMG CoA reductase. The solid bar (site no. 1) indicates the primary site of action of LDL-derived cholesterol, i.e., suppression of HMG CoA reductase. The stippled bar (site no. 2) indicates a secondary site of action of LDL-derived cholesterol, i.e., suppression of squalene synthetase. The dashed lines designate the compounds that may act as nonsterol regulators of HMG CoA reductase.

that have been reviewed in this article. The major regulator, cholesterol, is ordinarily derived from the cell's environment through receptor-mediated uptake of LDL. This uptake keeps HMG CoA reductase suppressed, limiting the overall entry of metabolites into the mevalonate pathway. In a secondary regulatory action, LDL also acts to suppress squalene synthetase. This latter suppression cannot be thought of as regulating cholesterol synthesis per se because it occurs only after HMG CoA reductase has been suppressed by 98%. Rather, the suppression of squalene synthetase serves to limit the flux of farnesyl pyrophosphate into the sterol branch of the pathway. This allows the cellular concentration of farnesyl pyrophosphate to remain at a level high enough to support the synthesis of the nonsterol products, ubiquinone and dolichol, at a time when farnesyl pyrophosphate synthesis has been reduced by 98% as a consequence of the suppression of HMG CoA reductase. Diversion of farnesyl pyrophosphate into the two nonsterol products is also facilitated by the apparent high affinity for farnesyl pyrophosphate of the initial enzymes in these pathways relative to that of squalene synthetase.

When LDL is added to fibroblasts in the presence of compactin, the 98% suppression of HMG CoA reductase no longer occurs. It can be restored, however, by the addition of small amounts of mevalonate. These data indicate that full suppression of HMG CoA reductase requires at least two feedback mediators: cholesterol and a nonsterol product derived from mevalonate. This type of regulation most closely resembles a process that has been observed in several branched biosynthetic pathways in bacteria and has been called *multivalent repression* (38). In these branched biosynthetic pathways, each of the end-products can repress the synthesis of a rate-limiting enzyme only partially; when added simultaneously they repress enzyme synthesis totally. In the case of HMG CoA reductase, the mechanism by which the end-products of mevalonate metabolism lower enzyme activity is not known. Hence, we have used the mechanistically noncommittal term *multivalent feedback regulation* rather than multivalent repression.

The studies of multivalent feedback regulation of HMG CoA reductase in fibroblasts are only in their infancy. It is not yet known whether the nonsterol substance is mevalonate itself or one of its products. Some of the likely candidates for this feedback regulation are shown by the dashed lines in Fig. 8. It is quite possible that more than one of these nonsterol products plays a direct role in regulating the reductase.

The mechanism by which these substances suppress reductase is unknown. Neither mevalonate nor any of its end-products, including cholesterol, is a direct inhibitor of HMG CoA reductase, at least under the usual conditions of assay of this enzyme in vitro (4, 19, 33). The active substance appears to function by altering the amount of active enzyme in the cell. But whether this alteration involves modulation of the rate of enzyme synthesis, post-synthetic modification of the enzyme, or regulation of its degradation is unknown. It is quite possible that several of these mechanisms are operative. Moreover, the current data do not exclude the possibility that two different HMG CoA reductase enzymes are involved, one isoenzyme that is suppressed by cholesterol, and another isoenzyme that is suppressed by the putative nonsterol product (4).

A control mechanism similar to the one in fibro-

blasts may also function in mammalian liver. A number of earlier studies have shown that feeding of cholesterol to rats lowers the rate of several enzymatic steps distal to mevalonate in addition to suppressing HMG CoA reductase activity (39–41). A major site of suppression lies in the conversion of farnesyl pyrophosphate to squalene (39, 40). As in fibroblasts, this suppression may function to divert mevalonate-derived metabolites into nonsterol pathways. Indeed, studies by Olson (13) and Rao and Olson (42) have shown that ubiquinone synthesis continues uninterrupted in rat liver at a time when cholesterol feeding has drastically lowered HMG CoA reductase activity and mevalonate synthesis.

Understanding the multivalent feedback regulation of HMG CoA reductase may have at least one important therapeutic implication. When animals are treated with compactin in an attempt to inhibit cholesterol synthesis and lower plasma cholesterol levels, the effect of the drug is blunted somewhat because the animals respond by developing high levels of HMG CoA reductase in liver and other tissues (43). This increase in enzyme activity presumably occurs by a mechanism similar to the one that operates in fibroblasts. When the putative nonsterol regulator of HMG CoA reductase is identified, it may be possible to administer this compound to animals and perhaps to patients, preventing the compensatory rise in HMG CoA reductase and thus enhancing the effectiveness of compactin and other cholesterol synthesis inhibitors as well.

Note Added In Proof: Since submission of this article two developments have occurred: 1) Isopentenyl adenine has been reported to stimulate DNA synthesis after double thymidine block in baby hamster kidney cells incubated with compactin (Huneeus, V. Q., M. H. Wiley, and M. D. Siperstein, *Clinical Research 28*: 557A, 1980); and 2) Human fibroblasts have been shown to incorporate [^{3}H]mevalonate into [^{3}H]isopentenyl tRNA by a high affinity pathway that is regulated similarly to the pathway for ubiquinone synthesis (Faust, J. R., M. S. Brown, and J. L. Goldstein, *J. Biol. Chem.*, In Press, 1980).

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