review

Regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase by reversible phosphorylation–dephosphorylation

Peter J. Kennelly and Victor W. Rodwell

Department of Biochemistry, Purdue University, West Lafayette, IN 47907

I. PHYSICAL PROPERTIES AND BIOSYNTHETIC ROLE OF 3-HYDROXY-3-METHYLGLUTARYL COENZYME A REDUCTASE

3-Hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase, E.C. 1.1.1.34) catalyzes the reduction of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) to mevalonate by two equivalents of NADPH. It is one of only three types of enzymes known to catalyze a four-electron, pyridine nucleotide-linked oxidoreduction —the others being histidinol dehydrogenase and the nucleoside diphosphate sugar dehydrogenases (1). In mammalian cells, HMG-CoA reductase is integrally associated with the endoplasmic reticulum (2, 3) with its active site facing the cytosol (4). The enzyme is made up of a single, glycosylated (2, 3) polypeptide with M_r of 90,000–100,000 (5–8). Whether it exists as a monomer or as an oligomer in vivo is not yet known.

The amino acid sequence of HMG-CoA reductase, deduced from the nucleotide sequence of an mRNA clone obtained from a Chinese hamster ovary cell line which overproduces the enzyme, indicates a polypeptide of 887 amino acids (5). The N-terminal third of this polypeptide (residues 1–267) is extremely rich in hydrophobic amino acids and is therefore thought to anchor HMG-CoA reductase to the microsomal membrane (5). The enzyme's active site is presumed to be located in the more hydrophilic portion of the enzyme nearer to the C-terminus (5). The amino terminus of HMG-CoA reductase lacks a recognizable "signal" sequence of the type frequently observed on transmembrane and secreted proteins (5). Membrane insertion is a cotranslational event which appears to require a signal recognition particle (2), although no cleavage of the N-terminus occurs (2, 5).

Microsomal membranes subjected to a slow freeze-thaw cycle release their HMG-CoA reductase activity with high efficiency (9, 10). This release results from the

freeze-fracture of the lysosomes that contaminate most microsomal preparations. The freed lysosomal proteases liberate from the microsomal membrane a soluble, catalytically active (but extensively proteolytically degraded) fragment of the enzyme (**Fig. 1**) (11) which has a molecular weight of 50,000–56,000 (as compared to 90,000–100,000 for intact HMG-CoA reductase). This active fragment, which has been purified to homogeneity in a number of laboratories (for a review, see 12), was for many years thought to be the intact, native enzyme. Recently, the undegraded native form of HMG-CoA reductase has been solubilized in the presence of inhibitors of proteolysis using nonionic detergents (8, 13).

In mammals, the production of mevalonate by HMG-CoA reductase is the third step in the lengthy pathway that leads from acetyl-CoA to sterols and other isoprenoids (**Fig. 2**). In liver and intestine, the richest tissue sources of HMG-CoA reductase, the major carbon flux through the sterol/polyisoprenoid biosynthetic pathway is directed into cholesterol. Under essentially all experimental conditions examined thus far, the activity of HMG-CoA reductase is rate-limiting for sterol biosynthesis from acetyl-CoA (14–16). Moreover, manipulation of the activity of HMG-CoA reductase through alterations of its quantity and/or of its catalytic efficiency appears to be the most important mechanism by which cells regulate sterol production (14, 16). Because of its preeminent role in the control of cholesterol synthesis and the often serious physiological consequences associated with high levels of cholesterol in the circulation, considerable effort has been directed towards delineating both the factors that regulate HMG-CoA reductase activity and the molecular mechanisms by which they act. These studies have focused principally on HMG-CoA reductase in liver and intestine, the tissues responsible for two-thirds to three-fourths of the body's cholesterol biosynthesis (17). Mechanisms implicated in the regulation of HMG-CoA reductase include the manipulation of enzyme quantity through transcriptional (18, 19) and post-transcriptional processes (20–22) and enzyme degradation (23–26), as well as the alteration of enzyme catalytic activity by membrane composition and fluidity (27–30), by thiols (31, 32), by microtubules (33, 34), or by cytosolic lipid inhibitors and their binding proteins (35–37). We will review here another regulatory mechanism, the reversible inactivation and reactivation of HMG-CoA reductase via its covalent phosphorylation and dephosphorylation. Additional information on this topic is available in recent reviews authored by Beg and Brewer (38), Gibson et al. (39, 40), Ingebritsen (41), and Ness (42).

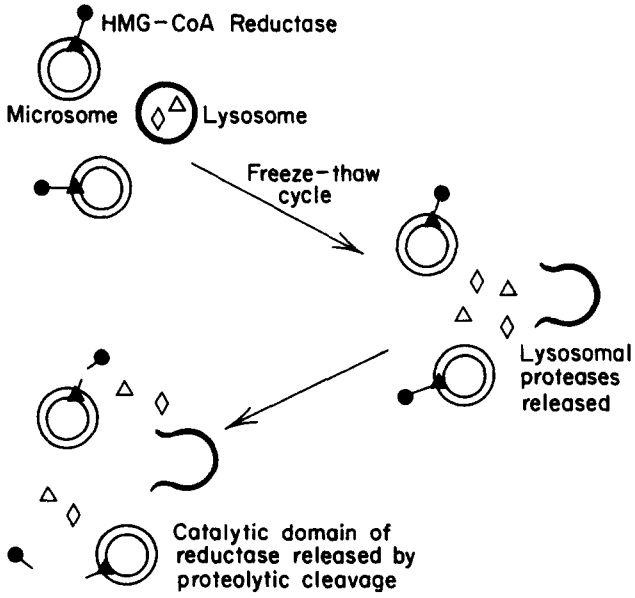

Fig. 1. Freeze-thaw solubilization of HMG-CoA reductase relies on the action of lysosomal proteases. Symbols include: ◊ and △, lysosomal proteases; ▲●, microsomally bound HMG-CoA reductase; ▲/, presumed membrane anchor region of HMG-CoA reductase; /●, catalytically active domain of HMG-CoA reductase.

II. HMG-CoA REDUCTASE IS REVERSIBLY INACTIVATED BY COVALENT PHOSPHORYLATION AND REACTIVATED BY DEPHOSPHORYLATION

HMG-CoA reductase exists in interconvertible active and inactive forms

In 1973 Beg, Allman, and Gibson (43) reported that the HMG-CoA reductase activity of washed rat liver microsomes was dramatically reduced by prior treatment with Mg^{2+}, ATP, and a fraction from rat liver cytosol. The degree of reduction achieved was time-dependent and the lost activity was restored by treatment with a second cytosolic fraction. These investigators therefore proposed that HMG-CoA reductase might undergo interconversion between two forms of differing specific activity, and suggested protein phosphorylation as a potential mechanism (43).

Evidence supporting the concept that HMG-CoA reductase exists in active and inactive (or less active) forms was soon forthcoming. Goodwin and Margolis (44) observed that incubation of the post-mitochondrial supernatant of rat liver homogenates at 37°C prior to assay increased the rate of [^{14}C]acetate incorporation into sterols 8- to 20-fold without affecting the rate of [^{14}C]mevalonate incorporation into sterols. They therefore suggested that one or more of the enzymes catalyzing the synthesis of mevalonate from acetyl-CoA had undergone activation. Higgins and Rudney (45) used antibody to HMG-CoA reductase to probe the molecular mechanism by which dietary cholesterol lowers HMG-CoA reductase activity.

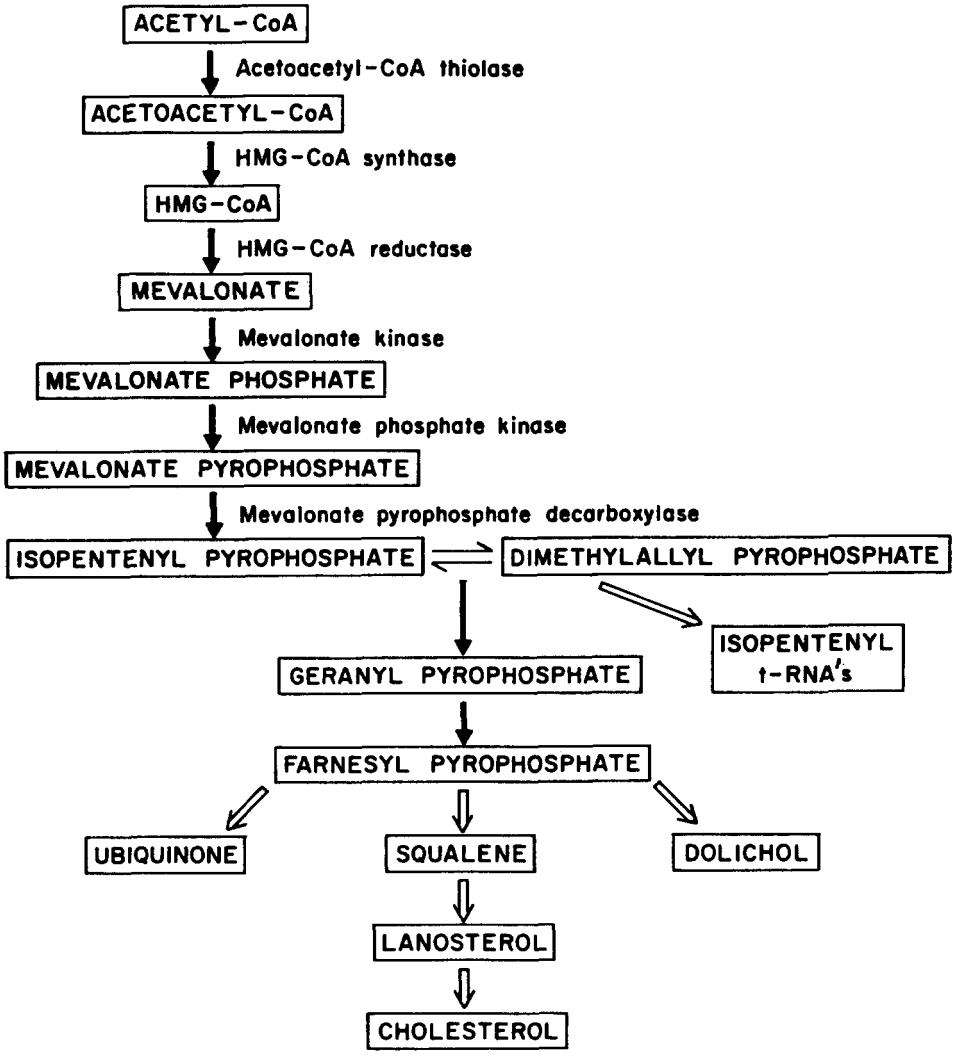

Fig. 2. The sterol/isoprenoid biosynthetic pathway.

They observed an initial decline in the activity of HMG-CoA reductase which was not accompanied by a decrease in immunotitratable enzyme molecules. This decrease in activity was followed at a significantly later time by a decrease in enzyme quantity. The mechanism by which cholesterol feeding caused the initial enzyme inactivation was not, however, apparent.

The MgATP-dependent decrease in HMG-CoA reductase activity that followed treatment with cytosolic or endogenous microsomal factors (46–53) and the activation (or reactivation) that followed treatment with a cytosolic factor (50–58) have since been confirmed in numerous laboratories. The phenomenon has been observed in a variety of tissues, including brain (53), liver (50, 51, 55), leukocytes (59), and intestine (49, 52, 60), and in a variety of animal species including pigs, dogs, rodents, chickens, frogs, fish (57), insects (61), and humans (59, 62). The effect of MgATP treatment on the kinetic parameters of HMG-CoA reductase, a decrease in V_{max} with no change in the K_m values for HMG-CoA or NADPH, coupled with the inability of dialysis or dilution to restore the lost activity, suggested direct enzyme inactivation rather than production of an inhibitory substance or stimulation of a reaction which competed for substrate(s) (47, 51). The failure of β,γ-methylene ATP (an ATP analog incapable of donating its terminal phosphoryl group) to inactivate HMG-CoA reductase was consistent with this observation (47).

Nordstrom, Rodwell, and Mitschelen (51) demonstrated that the reversible inactivation–reactivation of microsomal HMG-CoA reductase could be repeated for several cycles. They partially purified the cytosolic activating and inactivating factors responsible and identified them as catalytic proteins (51). These proteins inactivated and reactivated freeze-thaw solubilized, 1400-fold purified HMG-CoA reductase, demonstrating that this process was not dependent upon the presence of the microsomal membrane (51).

Several laboratories noted that NaF, a potent inhibitor of protein phosphatases, blocked the activation of HMG-CoA reductase, suggesting the participation of a protein phosphatase (51, 52, 55, 56, 58). To determine whether a phosphatase was indeed responsible, Ingebritsen and co-workers (50, 63) used a purified cytosolic phosphorylase *a* phosphatase to reactivate MgATP-inactivated HMG-CoA reductase. Thus, it appeared that the interconversion of HMG-CoA reductase between active and inactive forms was dependent upon a phosphorylation–dephosphorylation mechanism.

The interconversion of HMG-CoA reductase between active and inactive forms is accomplished by covalent phosphorylation and dephosphorylation of the enzyme

While the ATP-dependence of the inactivation and the fluoride inhibition of the (re)activation of HMG-CoA reductase suggested that the enzyme was regulated by phosphorylation–dephosphorylation, other evidence raised doubts concerning this interpretation. The requirement for both ATP and ADP for inactivation (47, 51) had no precedent among known phosphorylated enzymes. In addition, while freeze–thaw solubilized HMG-CoA reductase could be inactivated, this had proved suprisingly difficult as compared to the microsomally bound enzyme (47, 51).[1] Several laboratories therefore asked whether the MgATP-dependent inactivation of HMG-CoA reductase was accompanied by the covalent phosphorylation of the enzyme.

While the first attempt to incorporate radioactivity from [γ-^{32}P]- or [6-^{3}H]-labeled ATP into HMG-CoA reductase (48) failed due to an underestimation of the specific activity of the enzyme (64), Beg, Stonik, and Brewer (65), Keith and co-workers (64, 66) and Gil et al. (67) subsequently isolated ^{32}P-labeled HMG-CoA reductase solubilized via the freeze–thaw method following inactivation of the microsomally bound enzyme with [γ-^{32}P]ATP.[2] Since radioactivity comigrated with HMG-CoA reductase activity on polyacrylamide (64, 67) and isoelectric focusing gels (66), and with HMG-CoA reductase protein on SDS-polyacrylamide gels (64–66), the phosphate appeared to be covalently bound. The ^{32}P-labeled enzyme was also precipitated by antibodies raised against the proteolytically solubilized, fragment form of HMG-CoA reductase (64, 65). Finally, Keith, Kennelly, and Rodwell (66) definitively established the covalent nature of the phosphorylation of HMG-CoA reductase by isolating [^{32}P]phosphoserine from acid hydrolysates of the ^{32}P-labeled active fragment form of the enzyme.[3]

While the above data documented that HMG-CoA reductase could be covalently phosphorylated, the association of this process with enzyme inactivation remained less clear. As electrophoretically pure (albeit proteolytically solubilized) HMG-CoA reductase and purified HMG-CoA reductase inactivators (kinases) became available, it was possible to clearly and unambiguously demonstrate the concomitant inactivation and phosphorylation of the enzyme. Beg, Stonik, and Brewer (68) and Ferrer and Hegardt (69) observed parallel inactivation of, and ^{32}P-incorporation into, electrophoretically pure HMG-CoA reductase by electrophoretically pure HMG-CoA reductase kinase(s) from rat liver microsomes. Harwood, Brandt, and Rodwell (70) obtained analogous data using a partially purified HMG-CoA reductase kinase from rat liver cytosol and observed that this cytosolic HMG-CoA reductase kinase required ADP as an allosteric activator. This explained the observations of Brown, Brunschede, and Goldstein (47) and of Nordstrom, Rodwell, and Mitschelen (51) that both ATP and ADP are required for inactivation of HMG-CoA reductase.

The availability of ^{32}P-labeled HMG-CoA reductase also permitted documentation of the concomitant reactivation and dephosphorylation of previously inactivated HMG-CoA reductase. Using either partially (65, 67, 71–73) or fully (69, 74) purified HMG-CoA reductase "activators" (phosphatases), three separate laboratories observed that activation of ^{32}P-labeled HMG-CoA reductase was accompanied by concomitant release of [^{32}P]phosphate from the enzyme. The HMG-CoA reductase "activators" also dephosphorylated known phosphoproteins such as phosphorylase *a* (71, 73), acetyl-CoA carboxylase (71), and glycogen synthase (73), lending further support to the concept that the activation of HMG-CoA reductase is the direct result of its dephosphorylation. Thus, examination of both the inactivation and reactivation of HMG-CoA reductase clearly demonstrated that these processes were accompanied by, and the consequence of, the covalent phosphorylation and subsequent dephosphorylation of the enzyme, respectively.

HMG-CoA reductase is phosphorylated at multiple sites

Once it became firmly established that HMG-CoA reductase was inactivated by phosphorylation, efforts were directed toward the determination of the number, structure, and function of its phosphorylation sites. As previously noted, Keith at al. (66) had determined that

[1] At that time, freeze-thaw solubilized fragment HMG-CoA reductase was thought to represent the intact, native protein.

[2] The studies referred to in this paragraph all utilized the catalytically active fragment of HMG-CoA reductase solubilized by the action of endogenous proteases.

[3] The proposed amino acid sequence of HMG-CoA reductase (5) contains 70 seryl residues, about half of which are present in the hydrophilic, C-terminal half of the sequence.

the proteolytically solubilized fragment form of HMG-CoA reductase was covalently phosphorylated exclusively at seryl residues. Tryptic digestion of ^{32}P-labeled fragment HMG-CoA reductase inactivated with endogenous microsomal HMG-CoA reductase kinases yielded two phosphorylated peptides, suggesting that the enzyme contains at least two structurally distinct types of phosphorylation sites (66, 75).[4]

Recent evidence suggests that these structurally distinguishable sites might also be functionally distinguishable. Treatment of freeze–thaw solubilized fragment HMG-CoA reductase with a purified microsomal HMG-CoA reductase kinase yielded ^{32}P-labeled HMG-CoA reductase phosphorylated at only one site, as determined by analysis of the tryptic phosphopeptides (69). However, phosphorylation at this apparently single site was sufficient to inactivate the enzyme (69). In addition, when partially inactivated fragment HMG-CoA reductase phosphorylated with ^{32}P at multiple sites was treated with either of two partially purified high molecular weight, cytosolic HMG-CoA reductase phosphatases, full reactivation of the enzyme followed release of only approximately one-half of the bound ^{32}P-phosphate (71). Although further ^{32}P release occurred, this subsequent release was not accompanied by any further increases in HMG-CoA reductase activity. HMG-CoA reductase thus appears to be phosphorylatable at multiple sites, only a portion of which function in the direct catalytic inactivation of the enzyme. The function(s) of the remaining phosphorylation site(s) is presently unclear. Possibilities include controlling the susceptibility of the "inactivation" site to phosphorylation or dephosphorylation, or altering the rate at which HMG-CoA reductase is proteolytically degraded (76).

III. MODULATION OF HMG-CoA REDUCTASE ACTIVITY BY REVERSIBLE PHOSPHORYLATION-DEPHOSPHORYLATION IN VIVO IS A PHYSIOLOGICALLY IMPORTANT MECHANISM OF REGULATORY CONTROL

As it became evident that the activity of HMG-CoA reductase could be modulated via covalent phosphorylation–dephosphorylation in vitro, it was asked whether this represented a physiologically operative means of regulatory control. The appropriate use of protein kinase and protein phosphatase inhibitors and of HMG-CoA reductase phosphatases permits measurements of both the quantity of HMG-CoA reductase molecules present and the proportion of these that are in the active, dephosphorylated form (51, 57, 77). This latter quantity is referred to as the modulation state (or its reciprocal, the phosphorylation state) of HMG-CoA reductase (51, 57, 77). Ingebristen and co-workers (77) used this methodology to ask whether insulin and glucagon, hormones known to regulate sterol biosynthesis, do so by altering the modulation state of HMG-CoA reductase in vivo. They observed that treatment of rat hepatocytes with these hormones or with cyclic AMP (the second messenger of glucagon) rapidly altered the fraction of HMG-CoA reductase present in the active (dephosphorylated) form (77). These alterations occurred within 30 min—long before changes in the total quantity of HMG-CoA reductase could be detected—and were accompanied by parallel changes in the rate of sterol biosynthesis. The rate of cholesterol biosynthesis was thus controlled by the modulation state of HMG-CoA reductase. We have confirmed these observations and have shown, in addition, that cyclic GMP is capable of regulating rat hepatocyte cholesterol biosynthesis via changes in HMG-CoA reductase modulation state. Moreover, we have observed that the modulation state of HMG-CoA reductase responds to physiological concentrations of these effectors within as little as 10–15 min of their administration (77a).

Panini and Rudney (60) observed that exposure of rat ileal epithelial cells to bicarbonate ions decreased the fraction of HMG-CoA reductase present in the active (dephosphorylated) form. This decrease became apparent within 30 min, was complete within about 60 min, and was reversed by removal of bicarbonate. As was previously shown in rat hepatocytes, the change in the modulation state of HMG-CoA reductase was reflected in a parallel change in the rate of sterol biosynthesis.

Changes in the modulation state of HMG-CoA reductase in vivo were also demonstrated in intact animals. Erickson et al. (56) Beg, Stonik, and Brewer (78), and Arebalo and co-workers (79, 80) observed that intragastric administration of mevalonolactone to rats produces a rapid decrease in the proportion of their hepatic HMG-CoA reductase in the active (dephosphorylated) form. Arebalo and coworkers (80, 81) also asked whether the rapid inactivation of HMG-CoA reductase observed in rats fed cholesterol (45, 82) was a consequence of increased enzyme phosphorylation. They observed that within 20 min after feeding rats cholesterol the fraction of their hepatic HMG-CoA reductase in the active (dephosphorylated) form decreased (80, 81). Holloway et al. (83)

[4] It is sometimes observed that purification of freeze-thaw solubilized HMG-CoA reductase can yield two polypeptides that, as judged by SDS-polyacrylamide gel electrophoresis, differ in molecular mass by 1,000–2,000 Daltons. Detection of multiple tryptic phosphopeptides might therefore reflect heterogeneity of the HMG-CoA reductase preparation from which they were derived. For example, if a single phosphorylation site was located near the C- or N-terminus, multiple phosphopeptides might reflect the presence of multiple C- or N-termini rather than multiple phosphorylation sites. While this possibility cannot be excluded from consideration, the authors consider it unlikely. The tryptic phosphopeptides are small; only a single phosphopeptide is produced when the enzyme is digested with cyanogen bromide (75), and no heterogeneity of the radioactive protein was detectable when the ^{32}P-labeled HMG-CoA reductase used in these studies was analyzed by SDS-gel electrophoresis (66, 75).

reported that feeding rats diets containing either excessive or deficient quantities of ascorbic acid for 9 weeks altered the amount of active (dephosphorylated) hepatic HMG-CoA reductase without any change in total enzyme activity. Such diets elevated body cholesterol levels consequent to depressed bile acid biosynthesis. Stacpoole and co-workers (84) administered dichloroacetate to rats, a drug that reduces serum cholesterol levels in human subjects. This decreased hepatic HMG-CoA reductase activity and increased the fraction of the enzyme in the inactive (phosphorylated) form within 1 hr after administration (84). Finally, Kelley and Story (85) observed that the modulation state of rat hepatic HMG-CoA reductase was sensitive to the feeding regimen and to the composition of the diet.

Leoni et al. (86) examined changes both in the rate of cholesterol biosynthesis and in the modulation state of HMG-CoA reductase in rat hepatocytes isolated at various stages of pre- and postnatal life. The modulation state of the enzyme, as well as total quantity, underwent large variations during development. Significantly, the rate of sterol biosynthesis correlated best with the HMG-CoA reductase present in the active (dephosphorylated) form, indicating that during early developmental stages the modulation of HMG-CoA reductase activity plays a major role in the control of cholesterol biosynthesis.

Most cancerous and precancerous cells exhibit defective or impaired regulation of cholesterol biosynthesis characterized by high levels of HMG-CoA reductase activity and lack of responsiveness to normal feedback regulation by cholesterol (for a review see 87). Feingold et al. (88) observed that in several rats with Morris hepatomas the fraction of their hepatic HMG-CoA reductase present in the active (dephosphorylated) form (53–75%) greatly exceeded that of normal cells (10–20%), indicating that the rise in HMG-CoA reductase activity in cancerous cells results, at least in part, from changes in its phosphorylation state.

Detection of changes in the modulation state of HMG-CoA reductase requires care in the selection of the experimental time frame and of assay techniques. Many investigators asked whether the diurnal variation in HMG-CoA reductase activity reflects a diurnal variation in the modulation state of the enzyme. Only recently have these efforts met with success. By using a rapid sampling technique involving cold-clamping of liver tissue, Easom and Zammit (89) observed that the modulation state of HMG-CoA reductase varies in parallel with the diurnal variation of enzyme activity and quantity.

In all of the above experiments, changes in the proportion of HMG-CoA reductase in the active form in vivo were interpreted as reflecting changes in its degree of phosphorylation, as was the case in vitro. Beg et al. (68) directly demonstrated changes in the amount of phosphate bound to HMG-CoA reductase in vivo following exposure to the hormone glucagon. They observed that rats fed [^{32}P]phosphate incorporated ^{32}P radioactivity into immunoprecipitable hepatic HMG-CoA reductase. Furthermore, when these rats were injected with glucagon, which decreases the proportion of HMG-CoA reductase in an active form in rat hepatocytes (77), the amount of ^{32}P radioactivity bound to HMG-CoA reductase increased as expected (68). This demonstrated not only that covalently phosphorylated HMG-CoA reductase exists in vivo, but that its in vivo phosphorylation state responds to effectors in the direction predicted.

The above studies, which derive from numerous independent laboratories, indicate that modulation of HMG-CoA reductase activity by phosphorylation-dephosphorylation constitutes a physiologically important mechanism of regulatory control. Phosphorylation-dephosphorylation produces rapid changes in response to appropriate dietary, hormonal, or pharmacological effectors, usually within 10 to 30 min. These changes in phosphorylation state are often transient. Ingebritsen et al. (77), Erickson et al. (56), Arebalo and co-workers (79–81), and our own laboratory have noted that the modulation state of HMG-CoA reductase returned to near control values within 1 to 2 hr following an initial stimulus. Subsequently, a reduction in the quantity of HMG-CoA reductase appeared to supersede phosphorylation-dephosphorylation as the major mechanism of regulatory control. Phosphorylation-dephosphorylation of HMG-CoA reductase allows cells to rapidly adjust their rate of cholesterol biosynthesis, often prior to a subsequent adjustment in the quantity of enzyme protein present. The latter represents the major mechanism of long-term regulatory control (14). Once this long-term adjustment becomes manifest, the phosphorylation state of reductase apparently returns to a "ready" position to await the arrival of further stimuli.

IV. ARGUMENTS AGAINST THE REGULATION OF HMG-CoA REDUCTASE BY PHOSPHORYLATION-DEPHOSPHORYLATION

The proposition that HMG-CoA reductase is subject to reversible, covalent phosphorylation-dephosphorylation, and that this process performs an important regulatory function in vivo, has been the subject of continuing controversy. Despite the accumulation of a large body of supporting evidence, the physiological importance of the reversible, covalent phosphorylation of HMG-CoA reductase is still questioned in some quarters. We will, therefore, review the four main arguments that have been raised against the phosphorylation-based regulation of HMG-CoA reductase and detail the relevant evidence concerning them.

"HMG-CoA reductase inactivator" is actually mevalonate kinase, not a protein kinase

Most assays of HMG-CoA reductase, and hence of the modulation of its activity by phosphorylation-dephosphorylation, measure mevalonate formation. In 1982, Ness and co-workers (90) purported to account for the apparent "MgATP-dependent inactivation" of HMG-CoA reductase as the phosphorylation of mevalonate by mevalonate kinase. They reported that the apparent MgATP-dependent decrease in mevalonate production was solely due to the subsequent conversion of mevalonate to mevalonate-5-phosphate in the presence of the added MgATP. They therefore suggested that the MgATP-dependent inactivation of HMG-CoA reductase was attributable to the presence of mevalonate kinase, rather than to covalent phosphorylation of the enzyme (42, 90).

While mevalonate kinase can indeed, under appropriate conditions, mimic the activity of HMG-CoA reductase kinase ("HMG-CoA reductase inactivator"), these two phenomena have proved to be both distinct and readily distinguishable. Nordstrom, Rodwell, and Mitschelen (51) and Harwood and Rodwell (91) reported that no mevalonate-5-phosphate was formed following the inactivation of HMG-CoA reductase with MgATP and an HMG-CoA reductase kinase from rat liver cytosol, and Ferrer and Hegardt (69) reported that purified microsomal HMG-CoA reductase kinase was devoid of mevalonate kinase activity. In addition, the MgATP-dependent inactivation of HMG-CoA reductase has been demonstrated in the presence of mevalonate trapping pools (52) or of trace quantities of a radioactively labeled mevalonate internal standard (51, 70, 92). In neither case was mevalonate-utilizing activity detected. In our own laboratory, the reversible modulation of HMG-CoA reductase activity has also been observed using a spectrophotometric assay that monitors the utilization of NADPH rather than the production of mevalonate — a process with which the presence of mevalonate kinase cannot interfere[5] (D. Sherban, unpublished observations). Moreover, the techniques used by Ness and co-workers (90, 93) significantly differed from those utilized by other investigators. Most investigators employ two separate incubations for assay of HMG-CoA reductase kinase activity (91, 92). During the first, HMG-CoA reductase is inactivated, in the absence of substrates, with HMG-CoA reductase kinase. In the second, the residual HMG-CoA reductase activity is then measured. It has been consistently observed that the degree of inactivation of HMG-CoA reductase depends directly upon the duration of the first incubation, during which no mevalonate is present or can be formed (51, 70, 92). The second incubation is then performed in the presence of the kinase inhibitor EDTA. Harwood and Rodwell (91) demonstrated that the high EDTA concentrations generally used to assay HMG-CoA reductase activity, and hence its inactivation and reactivation, effectively inhibit mevalonate kinase and preclude it from interfering with the assay of HMG-CoA reductase. Significantly, Ness and co-workers (90, 93) attempted to simultaneously inactivate and assay HMG-CoA reductase in a single incubation in the absence of added EDTA, conditions under which any mevalonate kinase present would be active and have access to the pool of mevalonate formed by HMG-CoA reductase. Lastly, both Harwood and Rodwell (91) and Beg and Stonik (94) demonstrated that mevalonate kinase and HMG-CoA reductase kinase are physically distinct enzymes separable by either ion-exchange or size-exclusion chromatography.

Thus, even ignoring the experiments that demonstrate the incorporation of covalently bound [^{32}P]phosphate into HMG-CoA reductase protein with parallel enzyme inactivation, the MgATP-dependent inactivation of HMG-CoA reductase clearly cannot be attributed to the presence of mevalonate kinase. However, care must be taken to ensure that interference from this source does not occur. This can be accomplished by use of spectrophotometric assay techniques or by inclusion of sufficiently high qualities of EDTA during the assay of HMG-CoA reductase activity following its prior inactivation.

HMG-CoA reductase is refractory to inactivation/phosphorylation following solubilization from the microsomal membrane

While the activity of microsomal HMG-CoA reductase is readily reduced by treatment with MgATP and HMG-CoA reductase kinase, early attempts to inactivate HMG-CoA reductase following its freeze-thaw solubilization met with difficulty and, in some instances, failure. Recently, however, several investigators have successfully inactivated freeze-thaw solubilized HMG-CoA reductase (47, 51, 68-70). In certain of these studies ^{32}P-labeled ATP was used to observe the concomitant incorporation of ^{32}P into HMG-CoA reductase protein (68-70). However, in many instances the investigators were unable to obtain rates or extents of inactivation of solubilized HMG-CoA reductase comparable to those achieved with the microsomally bound form of the enzyme (47, 51, 70). Other investigators reported an inability to detect either inactivation of, or ^{32}P incorporation into, solubilized HMG-CoA reductase (93, 95). Harwood and co-workers (70) noted that, while rapid and complete inactivation of the fragment form of HMG-CoA reductase could be achieved, this required far higher concentrations of HMG-CoA reductase kinase than those required to inactivate the microsomally bound form of the enzyme. Under the conditions employed, the activity of HMG-CoA reductase kinase toward the fragment form of the

[5]Mevalonate kinase might actually stimulate, rather than inhibit, HMG-CoA reductase activity by removing the mevalonate produced.

enzyme was estimated to be only 5% that displayed toward the microsomal form as a result of a lower affinity of HMG-CoA reductase kinase for the proteolytically solubilized form of the enzyme (70).

The disparity in the facility with which microsomal and solubilized HMG-CoA reductase are inactivated suggested to some that the MgATP-dependent inactivation of HMG-CoA reductase was the result of the formation of a microsomally associated, MgATP-dependent inhibitor, the presence of mevalonate kinase, or an MgATP-stimulated HMG-CoA and/or NADPH-utilizing activity, rather than a phosphorylation-dependent inactivation of the enzyme (42). It was argued that any [^{32}P]phosphate incorporation seen might represent a nonspecific, nonregulatory phosphorylation event (42, 93, 95). The explanation for this difficulty came to light with the discovery by Ness, Way, and Wickham (11) that the solubilized HMG-CoA reductase used for the above experiments was a proteolytic fragment (M_r 50,000–55,000) of the native enzyme (M_r 90,000–100,000). Recent experiments with the solubilized native form of HMG-CoA reductase show that it can be rapidly and completely (90%+) inactivated by an ADP-dependent, cytosolic HMG-CoA reductase kinase with a facility approaching that displayed toward the microsomal enzyme (13). The proteolytically solubilized fragment form of HMG-CoA reductase thus may lack structural moieties present on the intact native enzyme important for its recognition by, or its binding to, HMG-CoA reductase kinase.

The phosphorylation state of HMG-CoA reductase in vivo does not change in response to long-term dietary or pharmacological manipulations

In many studies in which rats or cultured cells have been subjected to a particular dietary manipulation, drug, etc. for hours or days, no change in the phosphorylation state of HMG-CoA reductase was detected (14, 96–98). While changes in HMG-CoA reductase activity were observed, these were adequately accounted for by alterations in the quantity of HMG-CoA reductase protein present. Some investigators have interpreted this as evidence that changes in the phosphorylation state of HMG-CoA reductase do not occur in vivo, and that phosphorylation–dephosphorylation of the enzyme is a phenomenon of no physiological import. This conclusion is not justified when all of the evidence is considered.

Numerous studies using intact animals or cultured cells have shown that the modulation state of HMG-CoA reductase changes rapidly in response to dietary or pharmacological factors (see part III, above). Moreover, when both the rate of sterol biosynthesis and the modulation state of HMG-CoA reductase were examined, both changed in parallel (60, 77, 86), indicating that the rate of sterol biosynthesis was regulated by phosphorylation-induced changes in the activity of HMG-CoA reductase. In addition, these experiments have consistently revealed that, in order to measure changes in the modulation state of HMG-CoA reductase, due consideration must be given to the selection of both experimental techniques (51, 57, 89) and time frame (56, 77, 79–81). For example, besides being rapid, changes in activity resulting from phosphorylation–dephosphorylation are oftentimes transient, rarely persisting for over an hour. This transience probably accounts for the majority of cases in which changes in reductase modulation state were not detected. Lengthy treatments allow the phosphorylation state of HMG-CoA reductase to revert to its original value and be superseded by the relatively slow-acting, long-term control mechanism of regulation of enzyme quantity.

This is not to say that changes in the modulation state of HMG-CoA reductase are always transient. During pre- and postnatal development, the modulation state of HMG-CoA reductase appears to regulate cholesterol biosynthesis (86), and modulation of HMG-CoA reductase activity by phosphorylation–dephosphorylation may function in controlling the diurnal cycle of sterol biosynthesis (89). In Morris hepatoma cells (88) and rats chronically fed diets containing excessive or deficient quantities of ascorbic acid (83), changes in HMG-CoA reductase modulation state were persistent over time. However, present evidence points to long-term changes in HMG-CoA reductase modulation state as being the exception, rather than the rule. Phosphorylation–dephosphorylation appears to act in concert with other, more slowly responding mechanisms to provide cells with a rapid and verstaile mechanism of broad dynamic range for virtually instantaneous control of sterol biosynthesis. Phosphorylation–dephosphorylation provides for an immediate, rapid response to many physiological stimuli, but generally persists only long enough to allow the long-term adaptive response, a change in the actual quantity of HMG-CoA reductase, to occur. At this point, the modulation state of HMG-CoA reductase reverts to a "ready" position to await new stimuli. Failure to observe changes in the modulation state of HMG-CoA reductase were, we suggest, probably the result of examining an inappropriate time period or, perhaps more rarely, an inappropriate stimulatory effector.

Cells have no need for rapid-acting, phosphorylation-based regulation of HMG-CoA reductase

It has been suggested that regulation of sterol biosynthesis via the phosphorylation and dephosphorylation of HMG-CoA reductase must not occur because there is no clear reason why it should be needed. Rather, it is argued, regulation of HMG-CoA reductase occurs exclusively via

changes in the quantity of enzyme protein (14, 42). While this teleological argument is difficult to address experimentally, several important observations do bear on this issue. Although changes in HMG-CoA reductase protein levels appear to represent the predominant mechanism for long-term adjustment of sterol biosynthetic rates, such changes require time periods on the order of hours to make themselves felt. Modulation of HMG-CoA reductase activity by phosphorylation–dephosphorylation permits cells to respond within minutes to physiological effectors. Therein lies the apparent raison d'etre. It is also significant that two other regulatory enzymes that participate in cellular cholesterol homeostasis, acyl-CoA:cholesterol O-acetyltransferase and cholesterol-7-α-hydroxylase, appear to be regulated by phosphorylation–dephosphorylation mechanisms (99). This suggests that coordinate regulation of these enzymes by phosphorylation–dephosphorylation may play an important role in maintaining appropriate levels of unesterified cholesterol (99). While we cannot, at present, categorically state why HMG-CoA reductase is regulated by phosphorylation–dephosphorylation, it is apparent that this does in fact occur. Further research will, in time, provide the definitive answer to this important question.

We acknowledge the assistance of the following persons in the preparation of this review: Michael J. Beach, David M. Gibson, John F. Gill, Jr., H. James Harwood, Jr., and Gunter B. Kohlhaw. Journal paper number 10,080 from the Purdue Agricultural Experiment Station. Supported by grants from the USPHS (HL 19223) and from the American Heart Association, Indiana Affiliate, and by an NSF Predoctoral Fellowship (8166394) awarded to Peter J. Kennelly.

Manuscript received 19 October 1984.

REFERENCES

1. Feingold, D. S., and J. S. Franzen. 1981. Pyridine nucleotide-linked four-electron transfer dehydrogenases. Trends Biochem. Sci. **6:** 103–105.
2. Brown, D. A., and R. D. Simoni. 1984. Biogenesis of 3-hydroxy-3-methylglutaryl-coenzyme A reductase, an integral glycoprotein of the endoplasmic reticulum. *Proc. Natl. Acad. Sci. USA.* **81:** 1674–1678.
3. Liscum, L., R. D. Cummings, R. G. W. Anderson, G. N. DeMartino, J. L. Goldstein, and M. S. Brown. 1983. 3-Hydroxy-3-methylglutaryl-CoA reductase: a transmembrane glycoprotein of the endoplasmic reticulum with N-linked "high mannose" oligosaccharides. *Proc. Natl. Acad. Sci. USA.* **80:** 7165–7169.
4. Phillips, C. E., and G. C. Ness. 1984. Topography of rat liver microsomal 3-hydroxy-3-methylglutaryl coenzyme A reductase. *Biochem. Biophys. Res. Commun.* **119:** 772–778.
5. Chin, D. J., G. Gil, D. W. Russell, L. Liscum, K. L. Luskey, S. K. Basu, H. Okayama, P. Berg, J. L. Goldstein, and M. S. Brown. 1984. Nucleotide sequence of 3-hydroxy-3-methylglutaryl coenzyme A reductase, a glycoprotein of endoplasmic reticulum. *Nature.* **308:** 613–617.
6. Chin, D. J., K. L. Luskey, J. R. Faust, R. J. MacDonald, M. S. Brown, and J. R. Goldstein. 1982. Molecular cloning of 3-hydroxy-3-methylglutaryl coenzyme A reductase and evidence for regulation of its mRNA. *Proc. Natl. Acad. Sci. USA.* **79:** 7704–7708.
7. Edwards, P. A., S-F. Lan, R. D. Tanaka, and A. M. Fogelman. 1983. Mevalonolactone inhibits the rate of synthesis and enhances the rate of degradation of 3-hydroxy-3-methylglutaryl coenzyme A reductase in rat hepatocytes. *J. Biol. Chem.* **258:** 7272–7275.
8. Hardeman, E. C., H-S. Jenke, and R. D. Simoni. 1983. Overproduction of a M_r 92,000 protomer of 3-hydroxy-3-methylglutaryl-coenzyme A reductase in compactin-resistant C100 cells. *Proc. Natl. Acad. Sci. USA.* **80:** 1516–1520.
9. Brown, M. S., S. E. Dana, J. M. Dietschy, and M. D. Siperstein. 1973. 3-Hydroxy-3-methylglutaryl coenzyme A reductase. Solubilization and purification of a cold-sensitive microsomal enzyme. *J. Biol. Chem.* **248:** 4731–4738.
10. Heller, R. A., and R. G. Gould. 1973. Solubilization and partial purification of hepatic 3-hydroxy-3-methylglutaryl coenzyme A reductase. *Biochem. Biophys, Res. Commun.* **50:** 859–865.
11. Ness, G. C., S. C. Way, and P. S. Wickham. 1981. Proteinase involvement in the solubilization of 3-hydroxy-3-methylglutaryl coenzyme A reductase. *Biochem. Biophys. Res. Commun.* **102:** 81–85.
12. Kleinsek, D. A., R. E. Dugan, T. A. Baker, and J. W. Porter. 1981. 3-Hydroxy-3-methylglutaryl-CoA reductase from rat liver. *Methods Enzymol.* **71:** 462–479.
13. Kennelly, P. J., K. G. Brandt, and V. W. Rodwell. 1983. 3-Hydroxy-3-methylglutaryl-CoA reductase: solubilization in the presence of proteolytic inhibitors, partial purification, and reversible phosphorylation–dephosphorylation. *Biochemistry.* **22:** 2784–2788.
14. Brown, M. S., J. L. Goldstein, and J. M. Dietschy. 1979. Active and inactive forms of 3-hydroxy-3-methylglutaryl coenzyme A reductase in the liver of the rat. Comparison with the rate of cholesterol synthesis in different physiological states. *J. Biol. Chem.* **254:** 5144–5149.
15. Dietschy, J. M., and M. S. Brown. 1974. Effect of alterations of the specific activity of the intracellular acetyl CoA pool on the apparent rates of hepatic cholesterogenesis. *J. Lipid Res.* **15:** 508–516.
16. Rodwell, V. W., J. L. Nordstrom, and J. J. Mitschelen. 1976. Regulation of HMG-CoA reductase. *Adv. Lipid Res.* **14:** 1–74.
17. Turley, S. D., J. M. Andersen, and J. M. Dietschy. 1981. Rates of sterol synthesis and uptake in the major organs of the rat in vivo. *J. Lipid Res.* **22:** 551–569.
18. Clarke, C. F., P. A. Edwards, S-F. Lan, R. D. Tanaka, and A. M. Fogelman. 1983. Regulation of 3-hydroxy-3-methylglutaryl-coenzyme A reductase mRNA levels in rat liver. *Proc. Natl. Acad. Sci. USA.* **80:** 3305–3308.
19. Liscum, L., K. L. Luskey, D. J. Chin, Y. K. Ho, J. L. Goldstein, and M. S. Brown. 1983. Regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase and its mRNA in rat liver as studied with a monoclonal antibody and a cDNA probe. *J. Biol. Chem.* **258:** 8450–8455.
20. Jakoi, L., and S. H. Quarfordt. 1974. The induction of hepatic cholesterol synthesis in the rat by lecithin mesophase infusions. *J. Biol. Chem.* **249:** 5840–5844.
21. Kirsten, E. S., and J. A. Watson. 1974. Regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase in hepatoma tissue culture by serum lipoproteins. *J. Biol. Chem.* **249:** 6104–6109.

22. Koizumi, J., H. Mabuchi, and R. Takeda. 1982. A possible translational control of 3-hydroxy-3-methylglutaryl coenzyme A reductase induction by ML-236B (compactin) in isolated rat hepatocytes. *Biochem. Biophys. Res. Commun.* **108:** 240–246.
23. Chang, T-Y., J. S. Limanek, and C. C. Y. Chang. 1981. Evidence indicating that inactivation of 3-hydroxy-3-methylglutaryl coenzyme A reductase by low density lipoprotein or by 25-hydroxycholesterol requires mediator protein(s) with rapid turnover rate. *J. Biol. Chem.* **256:** 6174–6180.
24. Faust, J. R., K. L. Luskey, D. J. Chin, J. L. Goldstein, and M. S. Brown. 1982. Regulation of synthesis and degradation of 3-hydroxy-3-methylglutaryl-coenzyme A reductase by low density lipoprotein and 25-hydroxycholesterol in UT-1 cells. *Proc. Natl. Acad. Sci. USA.* **79:** 5205–5209.
25. Kaneko, I., Y. Hazama-Shimada, M. Kuroda, and A. Endo. 1977. Inhibition of 3-hydroxy-3-methylglutaryl coenzyme A reductase in cultured L cells by the hypocholesterolemic drug clofibrate. *Biochem. Biophys. Res. Commun.* **76:** 1207–1213.
26. Sinensky, M., and J. Logel. 1983. Inhibition of degradation of 3-hydroxy-3-methylglutaryl coenzyme A reductase by mevinolin. *J. Biol. Chem.* **258:** 8547–8549.
27. Finkel, R. S., and J. J. Volpe. 1979. A potential role for phospholipids in the regulation of 3-hydroxy-3-methylglutaryl-coenzyme A reductase in cultured C-6 glial cells. Effects of N,N-dimethylethanolamine. *Biochim. Biophys. Acta.* **572:** 461–471.
28. Mitropoulos, K. A., S. Venkatesan, B. E. A. Reeves, and S. Balasubramaniam. 1981. Modulation of 3-hydroxy-3-methylglutaryl-CoA reductase and of acyl-CoA-cholesterol acyltransferase by the transfer of non-esterified cholesterol to rat liver microsomal vesicles. *Biochem. J.* **194:** 265–271.
29. Richert, L., M. Castagna, J-P. Beck, S. Rong, B. Luu, and G. Ourisson. 1984. Growth-rate-related and hydroxysterol-induced changes in membrane fluidity of cultured hepatoma cells: correlation with 3-hydroxy-3-methylglutaryl-CoA reductase activity. *Biochem. Biophys. Res. Commun.* **120:** 192–198.
30. Sipat, A. B., and J. R. Sabine. 1981. Membrane-mediated control of hepatic β-hydroxy-β-methylglutaryl-coenzyme A reductase. *Biochem. J.* **194:** 889–893.
31. Dotan, I., and I. Shechter. 1982. Thiol-disulfide-dependent interconversion of active and latent forms of rat hepatic 3-hydroxy-3-methylglutaryl-coenzyme A reductase. *Biochim. Biophys. Acta.* **713:** 427–434.
32. Roitelman, J., and I. Shechter. 1984. Regulation of rat liver 3-hydroxy-3-methylglutaryl coenzyme A reductase. Evidence for thiol-dependent allosteric modulation of enzyme activity. *J. Biol. Chem.* **259:** 870–877.
33. Volpe, J. J. 1979. Microtubules and the regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase. *J. Biol. Chem.* **254:** 2568–2571.
34. Volpe, J. J., and K. A. Obert. 1981. Cytoskeletal structures and 3-hydroxy-3-methylglutaryl coenzyme A reductase in C-6 glial cells. *J. Biol. Chem.* **256:** 2016–2021.
35. Grinstead, G. F., J. M. Trzaskos, J. T. Billheimer, and J. L. Gaylor. 1983. Cytosolic modulators of activities of microsomal enzymes of cholesterol biosynthesis. Effects of acyl-CoA inhibition of cytosolic z-protein. *Biochim. Biophys. Acta.* **751:** 41–51.
36. Lehrer, G., S. R. Panini, D. H. Rogers, and H. Rudney. 1981. Modulation of rat liver 3-hydroxy-3-methylglutaryl coenzyme A reductase by lipid inhibitors, substrates and cytosolic factors. *J. Biol. Chem.* **256:** 5612–5619.
37. Menon, A. S., S. U. Devi, and T. Ramasarma. 1982. Inhibition of 3-hydroxy-3-methylglutaryl coenzyme A reductase by cytosolic proteins—real or artifact. *Biochem. Biophys. Res. Commun.* **109:** 619–625.
38. Beg, Z. H., and H. B. Brewer, Jr. 1982. Modulation of rat liver 3-hydroxy-3-methylglutaryl-CoA reductase activity by reversible phosphorylation. *Federation Proc.* **41:** 2634–2638.
39. Gibson, D. M., and T. S. Ingebritsen. 1978. Minireview. Reversible modulation of liver hydroxymethylglutaryl CoA reductase. *Life Sci.* **23:** 2649–2664.
40. Gibson, D. M., and R. A. Parker. 1985. Control of HMG-CoA reductase by reversible phosphorylation. *In* The Enzymes: Enzyme Control by Phosphorylation. E. G. Krebs, editor. Academic Press, New York. In press.
41. Ingebritsen, T. S. 1983. Molecular control of HMG-CoA reductase: regulation by phosphorylation–dephosphorylation. *In* 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase. J. R. Sabine, editor. CRC Press, Boca Raton, FL. 129–152.
42. Ness, G. C. 1983. Regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase. *Mol. Cell. Biochem.* **53/54:** 299–305.
43. Beg, Z. H., D. W. Allman, and D. M. Gibson. 1973. Modulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity with cAMP and with protein fractions of rat liver cytosol. *Biochem. Biophys. Res. Commun.* **54:** 1362–1369.
44. Goodwin, C. D., and S. Margolis. 1973. Specific activation of *in vitro* cholesterol biosynthesis by preincubation of rat liver homogenates. *J. Biol. Chem.* **248:** 7610–7613.
45. Higgins, M., and H. Rudney. 1973. Regulation of rat liver β-hydroxy-β-methylglutaryl-CoA reductase activity by cholesterol. *Nature (New Biology).* **246:** 60–61.
46. Bové, J., and F. G. Hegardt. 1978. Reversible modulation of rat liver 3-hydroxy-3-methylglutaryl coenzyme A reductase. Evidence for an enzyme-catalyzed phosphorylation–dephosphorylation system. *FEBS Lett.* **90:** 198–202.
47. Brown, M. S., G. Y. Brunschede, and J. L. Goldstein. 1975. Inactivation of 3-hydroxy-3-methylglutaryl coenzyme A reductase *in vitro.* An adenine nucleotide-dependent reaction catalyzed by a factor in human fibroblasts. *J. Biol. Chem.* **250:** 2502–2509.
48. Chow, J. C., M. J. P. Higgins, and H. Rudney. 1975. The inhibitory effect of ATP on HMG-CoA reductase. *Biochem. Biophys. Res. Commun.* **63:** 1077–1084.
49. Gebhard, R. L., B. G. Stone, and W. F. Prigge. 1985. 3-Hydroxy-3-methylglutaryl coenzyme A reductase activity in the human gastrointestinal tract. *J. Lipid Res.* **26:** 47–53.
50. Ingebritsen, T. S., H-S. Lee, R. A. Parker, and D. M. Gibson. 1978. Reversible modulation of the activities of both rat liver microsomal hydroxymethylglutaryl coenzyme A reductase and its inactivating enzyme. Evidence for regulation by phosphorylation–dephosphorylation. *Biochem. Biophys. Res. Commun.* **81:** 1268–1277.
51. Nordstrom, J. L., V. W. Rodwell, and J. J. Mitschelen. 1977. Interconversion of active and inactive forms of rat liver hydroxymethylglutaryl-CoA reductase. *J. Biol. Chem.* **252:** 8924–8934.
52. Oku, H., T. Ide, and M. Sugano. 1984. Reversible inactivation–reactivation of 3-hydroxy-3-methylglutaryl coenzyme A reductase of rat intestine. *J. Lipid Res.* **25:** 254–261.
53. Shah, S. S. 1981. Modulation *in vitro* of 3-hydroxy-3-methylglutaryl coenzyme A reductase in brain microsomes: evidence for the phosphorylation and dephosphorylation associated with inactivation and activation of the enzyme. *Arch. Biochem. Biophys.* **211:** 439–446.

54. Berndt, J., and R. Gaumert. 1974. Evidence for an activating-inactivating system of 3-hydroxy-3-methylglutaryl-CoA reductase in mouse liver. *Hoppe-Seyler's Z. Physiol. Chem.* **355:** 905–910.
55. Berndt, J., F. G. Hegardt, J. Bové, R. Gaumert, J. Still, and M-T. Cardó. 1976. Activation of 3-hydroxy-3-methylglutaryl-coenzyme A reductase *in vitro*. *Hoppe-Seyler's Z. Physiol. Chem.* **357:** 1277–1282.
56. Erickson, S. K., M. A. Shrewsbury, R. G. Gould, and A. D. Cooper. 1980. Studies on the mechanisms of the rapid modulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase in intact liver by mevalonolactone and 25-hydroxycholesterol. *Biochim. Biophys. Acta.* **620:** 70–79.
57. Hunter, C. F., and V. W. Rodwell. 1980. Regulation of vertebrate liver HMG-CoA reductase via reversible modulation of its catalytic activity. *J. Lipid. Res.* **21:** 399–405.
58. Saucier, S. E., and A. A. Kandutsch. 1979. Inactive 3-hydroxy-3-methylglutaryl-coenzyme A reductase in broken cell preparations of various mammalian tissues and cell cultures. *Biochim. Biophys. Acta.* **572:** 541–556.
59. Harwood, H. J., Jr., M. Schneider, and P. W. Stacpoole. 1984. Regulation of human leukocyte microsomal HMG-CoA reductase activity by a phosphorylation and dephosphorylation mechanism. *Biochim. Biophys. Acta.* **805:** 245–251.
60. Panini, S. R., and H. Rudney. 1980. Short term reversible modulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity in isolated epithelial cells from rat ileum. Regulation of phosphorylation-dephosphorylation by bicarbonate. *J. Biol. Chem.* **255:** 11633–11636.
61. Brown, K., C. M. Havel, and J. A. Watson. 1983. Isoprene synthesis in isolated embryonic *Drosophila* cells. II. Regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity. *J. Biol. Chem.* **258:** 8512–8518.
62. Beg, Z. H., J. A. Stonik, and H. B. Brewer, Jr. 1984. Human hepatic 3-hydroxy-3-methylglutaryl coenzyme A reductase: evidence for the regulation of enzymic activity by a bicyclic phosphorylation cascade. *Biochem. Biophys. Res. Commun.* **119:** 488–498.
63. Ingebritsen, T. S., R. A. Parker, and D. M. Gibson. 1981. Regulation of liver hydroxymethylglutaryl-CoA reductase by a bicyclic phosphorylation system. *J. Biol. Chem.* **256:** 1138–1144.
64. Keith, M. L., V. W. Rodwell, D. H. Rogers, and H. Rudney. 1979. *In vitro* phosphorylation of 3-hydroxy-3-methylglutaryl coenzyme A reductase: analysis of ^{32}P-labeled, inactivated enzyme. *Biochem. Biophys. Res. Commun.* **90:** 969–975.
65. Beg, Z. H., J. A. Stonik, and H. B. Brewer, Jr. 1978. 3-Hydroxy-3-methylglutaryl coenzyme A reductase: regulation of enzymatic activity by phosphorylation and dephosphorylation. *Proc. Natl. Acad. Sci. USA.* **75:** 3678–3682.
66. Keith, M. L., P. J. Kennelly, and V. W. Rodwell. 1983. Evidence for multiple phosphorylation sites on rat liver 3-hydroxy-3-methylglutaryl coenzyme A reductase. *J. Protein Chem.* **2:** 209–220.
67. Gil, G., M. Sitges, J. Bové, and F. G. Hegardt. 1980. Phosphorylation-dephosphorylation of rat liver 3-hydroxy-3-methylglutaryl coenzyme A reductase associated with changes in activity. *FEBS Lett.* **110:** 195–199.
68. Beg, Z. H., J. A. Stonik, and H. B. Brewer, Jr. 1980. *In vitro* and *in vivo* phosphorylation of rat liver 3-hydroxy-3-methylglutaryl coenzyme A reductase and its modulation by glucagon. *J. Biol. Chem.* **255:** 8541–8545.
69. Ferrer, A., and F. G. Hegardt. 1984. Phosphorylation of 3-hydroxy-3-methylglutaryl coenzyme A reductase by microsomal 3-hydroxy-3-methylglutaryl coenzyme A reductase kinase. *Arch. Biochem. Biophys.* **230:** 227–237.
70. Harwood, H. J., Jr., K. G. Brandt, and V. W. Rodwell. 1984. Allosteric activation of rat liver cytosolic 3-hydroxy-3-methylglutaryl coenzyme A reductase kinase by nucleoside diphosphates. *J. Biol. Chem.* **259:** 2810–2815.
71. Brown, W. E., and V. W. Rodwell. 1983. Purification and properties of two native, high molecular weight hydroxymethylglutaryl-CoA reductase phosphatases. *Biochim. Biophys. Acta.* **751:** 218–229.
72. Gil, G., M. Sitges, and F. G. Hegardt. 1981. Purification and properties of rat liver hydroxymethylglutaryl coenzyme A reductase phosphatases. *Biochim. Biophys. Acta.* **663:** 211–221.
73. Sitges, M., G. Gil, and F. G. Hegardt. 1984. Partial purification from rat liver microsomes of three native protein phosphatases with activity towards HMG-CoA reductase. *J. Lipid Res.* **25:** 497–506.
74. Gil, G., M. Sitges, and F. G. Hegardt. 1981. Preparation of highly radioactive homogeneous ^{32}P-labelled hydroxymethylglutaryl coenzyme A reductase from rat liver. *Arch. Biochem. Biophys.* **210:** 224–229.
75. Font, E., M. Sitges, and F. G. Hegardt. 1982. Multiple phosphorylation of rat liver 3-hydroxy-3-methylglutaryl coenzyme A reductase. *Biochem. Biophys. Res. Commun.* **105:** 705–710.
76. Parker, R. A., S. J. Miller, and D. M. Gibson. 1984. Phosphorylation of microsomal HMG-CoA reductase increases susceptibility to proteolytic degradation *in vitro*. *Biochem. Biophys. Res. Commun.* **125:** 629–635.
77. Ingebritsen, T. S., M. J. H. Geelen, R. A. Parker, K. J. Evenson, and D. M. Gibson. 1979. Modulation of hydroxymethylglutaryl-CoA reductase activity, reductase kinase activity, and cholesterol synthesis in rat hepatocytes in response to insulin and glucagon. *J. Biol. Chem.* **254:** 9986–9989.
77a. Henneberg, R., and V. W. Rodwell. 1985. Rapid modulation of rat hepatocyte HMG-CoA reductase activity by cyclic AMP or cyclic GMP. *Physiol. Chem. Phys.* In press.
78. Beg, Z. H., J. A. Stonik, and H. B. Brewer, Jr. 1984. *In vivo* modulation of rat liver 3-hydroxy-3-methylglutaryl-coenzyme A reductase, reductase kinase, and reductase kinase kinase by mevalonolactone. *Proc. Natl. Acad. Sci. USA.* **81:** 7293–7297.
79. Arebalo, R. E., J. E. Hardgrave, B. J. Noland, and T. J. Scallen. 1980. *In vivo* regulation of rat liver 3-hydroxy-3-methylglutaryl-coenzyme A reductase: enzyme phosphorylation as an early regulatory response after intragastric administration of mevalonolactone. *Proc. Natl. Acad. Sci. USA.* **77:** 6429–6433.
80. Arebalo, R. E., C. D. Tormanen, J. E. Hardgrave, B. J. Noland, and T. J. Scallen. 1982. *In vivo* regulation of rat liver 3-hydroxy-3-methylglutaryl-coenzyme A reductase: immunotitration of the enzyme after short-term mevalonate or cholesterol feeding. *Proc. Natl. Acad. Sci. USA.* **79:** 51–55.
81. Arebalo, R. E., J. E. Hardgrave, and T. J. Scallen. 1981. The *in vivo* regulation of rat liver 3-hydroxy-3-methylglutaryl coenzyme A reductase. Phosphorylation of the enzyme as an early regulatory response following cholesterol feeding. *J. Biol. Chem.* **256:** 571–574.
82. Hardgrave, J. E., R. A. Heller, M. G. Herrera, and T. J. Scallen. 1979. Immunotitration of 3-hydroxy-3-methylglutaryl-coenzyme A reductase in various physiological states. *Proc. Natl. Acad. Sci. USA.* **76:** 3834–3838.
83. Holloway, D. E., F. J. Peterson, W. F. Prigge, and R. L. Gebhard. 1981. Influence of dietary ascorbic acid upon

enzymes of sterol biosynthesis in the guinea pig. *Biochem. Biophys. Res. Commun.* **102:** 1283–1289.
84. Stacpoole, P. W., H. J. Harwood, Jr., C. E. Varnado, and M. Schneider. 1983. Regulation of rat liver hydroxymethylglutaryl coenzyme A reductase by a new class of noncompetitive inhibitors. Effects of dichloroacetate and related carboxylic acids on enzyme activity. *J. Clin. Invest.* **72:** 1575–1585.
85. Kelley, M. J., and J. A. Story. 1985. Effect of type of diet and of feeding status on modulation of hepatic HMG-CoA reductase in rats. *Lipids.* **20:** 53–55.
86. Leoni, S., S. Spagnuolo, L. Conti-Devirgiliis, L. Dini, M. T. Mangiantini, and A. Trentalance. 1984. Cholesterogenesis and related enzymes in isolated rat hepatocytes during pre- and postnatal life. *J. Cell. Physiol.* **118:** 62–66.
87. Gregg, R. G., and P. A. Wilce. 1983. 3-Hydroxy-3-methylglutaryl coenzyme A reductase and cancer. *In* 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase. J. R. Sabine, editor. CRC Press, Inc., Boca Raton, FL. 245–257.
88. Feingold, K. R., M. H. Wiley, A. H. Moser, and M. D. Siperstein. 1983. Altered activation state of hydroxymethylglutaryl-coenzyme A reductase in liver tumors. *Arch. Biochem. Biophys.* **226:** 231–241.
89. Easom, R. A., and V. A. Zammit. 1984. Diurnal changes in the fraction of 3-hydroxy-3-methylglutaryl-CoA reductase in the active form in rat liver microsomal fractions. *Biochem. J.* **220:** 739–745.
90. Ness, G. C., G. A. Benton, S. A. Deiter, and P. S. Wickham. 1982. Influence of mevalonate kinase on studies of the MgATP-dependent inactivator of 3-hydroxy-3-methylglutaryl coenzyme A reductase. *Arch. Biochem. Biophys.* **214:** 705–713.
91. Harwood, H. J., Jr., and V. W. Rodwell. 1982. HMG-CoA reductase kinase: measurement of activity by methods that preclude interference by inhibitors of HMG-CoA reductase activity or by mevalonate kinase. *J. Lipid Res.* **23:** 754–761.
92. Ingebritsen, T. S., and D. M. Gibson. 1981. Assay of enzymes that modulate S-3-hydroxy-3-methylglutaryl-CoA reductase by reversible phosphorylation. *Methods Enzymol.* **71:** 486–497.
93. Ness, G. C., C. D. Spindler, and G. A. Benton. 1980. Characteristics of magnesium adenosine triphosphate-dependent inactivators of 3-hydroxy-3-methylglutaryl coenzyme A reductase. *J. Biol. Chem.* **255:** 9013–9016.
94. Beg, Z. H., and J. A. Stonik. 1982. Reversible inactivation of 3-hydroxy-3-methylglutaryl coenzyme A reductase: reductase kinase and mevalonate kinase are separate enzymes. *Biochem. Biophys. Res. Commun.* **108:** 559–566.
95. Jenke, H-S., M. Löwel, and J. Berndt. 1981. *In vivo* effect of cholesterol feeding on the short term regulation of hepatic hydroxymethylglutaryl coenzyme A reductase during the diurnal cycle. *J. Biol. Chem.* **256:** 9622–9625.
96. Clegg, R. J., B. Middleton, G. D. Bell, and D. A. White. 1982. The mechanism of cyclic monoterpene inhibition of hepatic 3-hydroxy-3-methylglutaryl coenzyme A reductase *in vivo* in the rat. *J. Biol. Chem.* **257:** 2294–2299.
97. Dugan, R. E., T. A. Baker, and J. W. Porter. 1982. Regulation of short-term changes in hepatic β-hydroxy-β-methylglutaryl-CoA reductase activity. *Eur. J. Biochem.* **125:** 497–503.
98. Kleinsek, D. A., A. M. Jabalquinto, and J. W. Porter. 1980. *In vivo* and *in vitro* mechanisms regulating rat liver β-hydroxy-β-methylglutaryl coenzyme A reductase activity. *J. Biol. Chem.* **255:** 3918–3923.
99. Scallen, T. J., and A. Sanghvi. 1983. Regulation of three key enzymes in cholesterol metabolism by phosphorylation/dephosphorylation. *Proc. Natl. Acad. Sci. USA.* **80:** 2477–2480.