[*The study of regulation of biogenesis of cholesterol, an isoprenoid compound, has assumed importance in view of its implication in atherosclerosis and gallstones. Although "feedback-type inhibition" of HMGCoA reductase, the rate-limiting key enzyme in isoprene pathway is known to occur in cholesterol-fed animals for three decades, it has not been possible to demonstrate inhibition of the enzyme by the end-products or other effectors. This article summarizes the current work on the inhibition of HMGCoA reductase by ATP.Mg (ADP.Mg), by cholesterol derivatives and by Fe^{++}, all of which require cytosolic protein factors. An integrated picture is emerging on the regulation of cholesterol biogenesis with a heat-stable activator protein and a heat-labile inhibitor protein, present in the cytosol, functioning in association with a variety of effectors. The protein factors have added a new dimension in the study of control of cholesterol levels.—Ed.*]

PROTEIN FACTORS IN THE REGULATION OF CHOLESTEROL BIOGENESIS

T. RAMASARMA

Department of Biochemistry, Indian Institute of Science, Bangalore 560 012, *India*

CHOLESTEROL is an ubiquitous membrane component in mammalian tissues. Metabolically, cholesterol provides the nucleus for bile acids and steroid hormones. Cholesterol, a polyprenoid, is continuously synthesized in all the tissues. In man its synthesis is about 1 g per day, 80% of it being accounted in the liver. Hepatic cholesterol is packed into lipoproteins and is transferred to the blood to maintain a concentration of about 160 mg/100 ml in the normal individuals. Of this about 2/3 occurs in particles (about 250 Å diameter), called low-density lipoproteins (LDL). The view that circulating cholesterol is destined for use by other tissues persists, despite the overwhelming evidence of independent capacity for its synthesis in all tissues. On the other hand, increased serum cholesterol (as LDL) is undoubtedly correlated with increased incidence of cholesterol deposits in arteries leading to atherosclerotic deaths. Increased cholesterol in bile secretion leads to the development of cholesterol gallstones. These pathological implications gave cholesterol notoriety and also spurred studies on its absorption, biogenesis and degradation. Research during the last three decades was concentrated on the effects of diets, hormones and drugs, with the major aim of regulating hepatic biogenesis and reducing blood cholesterol.

Early Studies on Regulation of Cholesterol Biosynthesis

In 1950 Taylor and Gould[1] using radioactive carbon tracers, just then introduced into biological research, first reported that dietary cholesterol caused a decrease in hepatic cholesterol synthesis. Two years later Tomkins and Chaikoff[2] found a similar large depression

of cholesterol synthesis in fasted animals that was reversed on refeeding with any calorie source. A large increase in biogenesis of cholesterol was found by Kandutsch and Saucier[3] to occur at midnight compared to noon, as a part of circadian rhythm. These early studies on large, rapid changes in cholesterol biogenesis sparked enormous interest around the world and led to the finding that the hepatic microsomal enzyme, 3-hydroxy-3-methylglutaryl CoA (HMGCoA) reductase, responsible for the synthesis of the key intermediate in the isoprene pathway, mevalonate, *is the rate-limiting step and the target of regulation*[3–9]

Metabolic regulation occurs by "feedback mechanism" in which the end-product inhibits the unique, committed step early in sequence of the reactions in the pathway. With cholesterol, most of these essential conditions are fulfilled—the end-product, cholesterol, given in the diet is absorbed and accumulated in the most significant tissue of its synthesis, the liver, resulting in decreased HMGCoA reductase activity, the rate-limiting, unique step in the isoprene pathway. Yet it is undecided whether this effect is due to "feedback inhibition" of the enzyme activity or "repression" of the enzyme–protein synthesis (see Ramasarma[10] for a review of the early work). In addition to cholesterol, ubiquinone, dolichol and isopentenyl adenosine (in t-RNA) are the end-products of the isoprene pathway. The regulatory steps of this branched pathway with multiple end-products are shown in Fig. 1, based on the information available in the animal systems. The primary control appears to be at the step of HMGCoA reductase but it was not possible to show inhibition of this enzyme by adding emulsions of cholesterol[4,6] or other end-products to microsomes. But Higgins and Rudney[11] made an important observation that dietary cholesterol effected HMGCoA reductase in two stages—80% decrease of activity, but not of immunoprecipitable protein, within the first 6 hr and a decrease of both after 24 hr. An inhibition

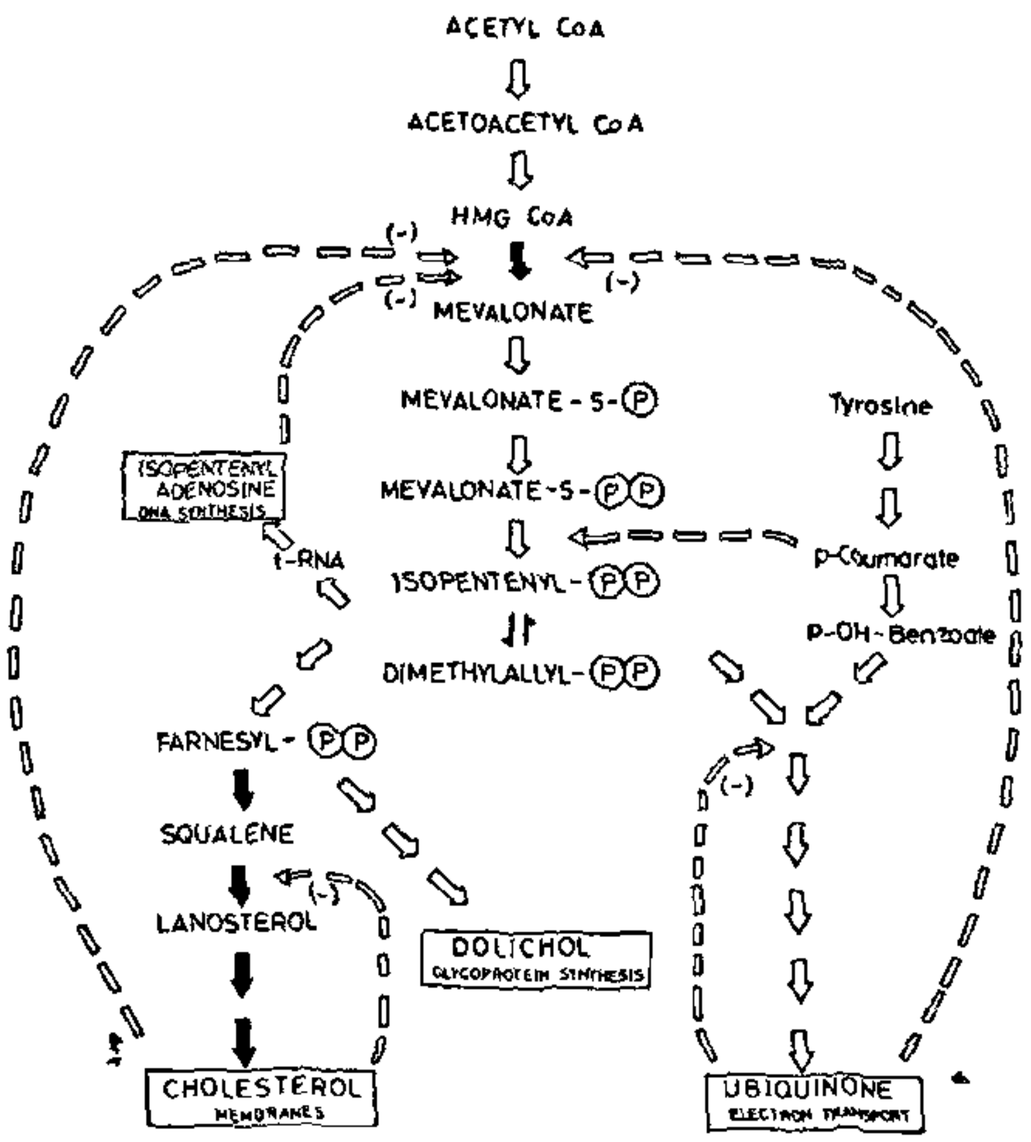

FIG. 1. Isoprene pathway in mammalian tissues. About 30 steps are involved in cholesterol synthesis from acetyl CoA of which 2/3 occur in microsomes including HMGCoA reductase and reactions after farnesyl-PP (shown by solid arrows). The end-products and their major functions are shown in blocks. Thin broken arrows denote control of the steps by products, with the sign (—) indicating decrease. In the absence of clear demonstration, branching for ubiquinone is shown at isopentenyl-PP. This is adapted from Ramasarma[10] and newer information on dolichol and isopentenyl adenosine is included.

of the enzyme activity is certainly indicated by this, and, a demonstration of physiologically meaningful effector(s), capable of showing inhibition of microsomal HMGCoA reductase activity *in vitro*, is necessary.

Inhibition of HMGCoA Reductase by ATP. Mg

An important observation on regulation *in vitro* of HMGCoA reductase by ATP. Mg was made by Beg *et al.*[12]. When microsomes were preincubated with ATP. Mg, pronounced inhibition of HMGCoA reductase activity was found. The temptation to bring the concept of interconvertible forms of the enzyme through phosphorylation is understandable once an effect with ATP. Mg was

obtained. It will then be possible to explain the hormonal responses. Typical of the hormone effects is the example of insulin which in normal animals had no effect on hepatocyte HMGCoA reductase activity but reversed its depression obtained on treatment with streptozotocin[13]. Treatment with nonadrenaline increased HMGCoA reductase activity in normal, cholesterol-fed or starved animals, but this action was direct at the cytosolic level and not mediated through adrenergic receptors of the plasma membrane[14]. But there are no compelling reasons to believe that these or any of the hormonal effects on HMGCoA reductase are through adenyl cyclase–cyclic AMP system.

Extensive data have been obtained on the inhibitory effect of ATP. Mg on HMGCoA reductase fitted with a phosphorylation mechanism. This scheme is diagrammatically shown in Fig. 2 and is based on the following observations: ATP and Mg were both required and EDTA prevented the inhibition; removal of residual ATP. Mg after preincubation by dialysis of microsomes did not restore the activity; washing microsomes repeatedly decreased progressively the sensitivity to ATP. Mg inhibition[12,15]; ATP. Mg inhibition required another protein present in microsomes as well as cytosol (m.w. 2,00,000 daltons), possibly a protein kinase[15,16]; under conditions of inactivation, the enzyme was found, labelled with ^{32}P-γ-ATP after precipitating with specific antibodies developed by Rudney's group[17]; reactivation was obtained when incubated with another cytosolic protein (m.w. 20-30,000 daltons) or a phosphatase[15,18]. All these were sufficient evidences for phosphorylation of the enzyme by a protein kinase leading to inactivation and dephosphorylation by a phosphatase to restore the activity. Further evidence on another reversible phosphorylation of the inactivator-protein—a purported protein kinase[18]—was also obtained. Thus the analogy with the well-established phosphorylase system is complete and therefore HMGCoA reductase has now been included in the list of

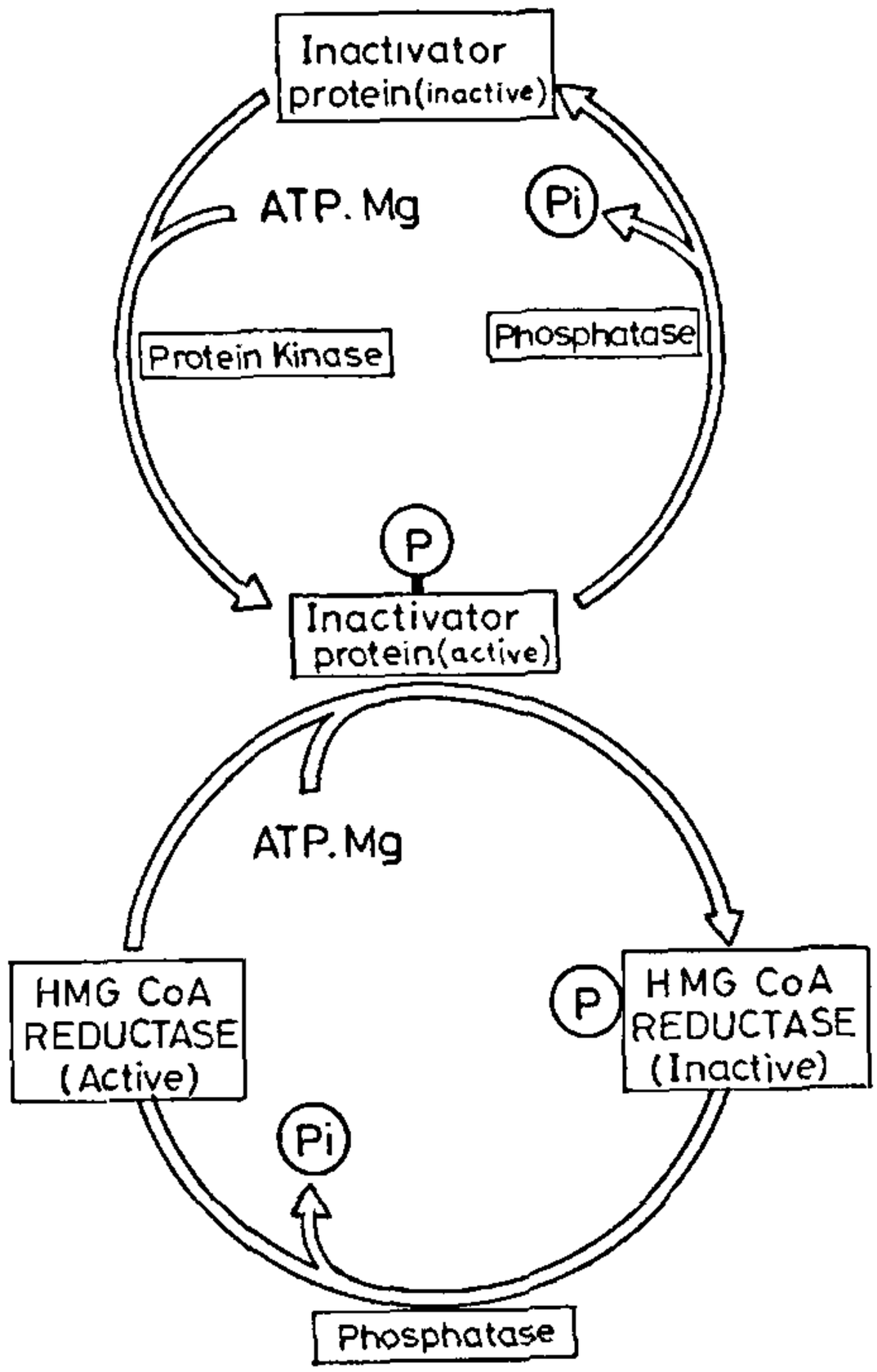

FIG. 2. Bicyclic system of reversible phosphorylattion of HMGCoA reductase and its inactivator protein (reductase kinase ?) based on the work described in Refs. 12, 15–18.

enzymes regulated by reversible phosphorylation.

Some questions remain unanswered. The inactivation of microsomal HMGCoA reductase was obtained also with ADP. Mg,[15,19,20] but not with other nucleotides. No known kinase can use ADP. Mg in place of ATP. Mg. A compromise of dual requirement of the nucleotides was suggested[15,19]. We conclusively showed that ADP itself was effective under conditions where even minor conversion to ATP was prevented[20]. In an exclusive meeting on HMGCoA reductase in the IUB Congress in Toronto (July 1979) many have agreed on the equal effectiveness of ATP and ADP but went on implicating the

"reductase kinase", despite my strong plea for restraint. Loss of activity and phosphorylation of HMGCoA reductase no doubt occurs coincidentally but this does not constitute proof that phosphorylation caused the inhibitory effect. The proportion of phosphorylated enzyme, in fact, remained constant in animals under conditions of 50-fold variation of cholesterol biogenesis casting doubt on its physiological utility[21]. The issue of identical inhibition with ADP. Mg, wherein phosphorylation is unlikely, has to be resolved. Reactivation by a cytosolic protein of the ATP. Mg-inactivated enzyme[15] may also be due to a mechanism independent of dephosphorylation. There is some recent evidence that the inhibition of purified, solubilized HMGCoA reductase by ATP. Mg in the presence of a cytosolic inactivator protein was *not* through covalent modification[22].

Inhibition of HMGCoA Reductase by Fe^{++} and Cytosol

Another interesting inhibitor of HMGCoA reductase became apparent in our recent studies. We noted that EDTA was needed in the homogenizing medium and not in the assay medium. In fact ATP . Mg effect was obtained only in the absence of EDTA and its addition stopped the inactivation. Peculiarly, Fe^{++} substituted for Mg^{++} in ATP inhibition[20]. It then occurred to us that Fe^{++} may be the inhibitor, with or without ATP and the essentiality of EDTA during homogenization may even be to prevent Fe^{++} from inactivating the enzyme. This hypothesis was proved true by demonstrating inhibition of HMGCoA reductase by added Fe^{++}. More important, Fe^{++}-inhibition in washed microsomes was found to be dependent on a cytosolic protein[23] having properties similar to that of the purported "reductase kinase" Irreversibility on washing or dialysis, prevention by EDTA, dependence on a protein present in unwashed microsomes and also the cytosol and the heat-lability of this protein are all features common between inhibitions by ATP. Mg and Fe^{++}.

Relationship to Lipid Peroxidation

It is instructive to note at this point that ATP and ADP, but not other nucleotides, were capable of promoting lipid peroxidation. This was traced to contamination of Fe^{++} found in the commercial samples of these two nucleotides[24, 25] Lipid peroxidation with ATP was also enhanced by Mg^{++} and could give a misleading impression of Mg-dependent phosphorylation. In fact in our experiments on HMGCoA reductase, the maximum inhibitory effect was obtained in the presence of Fe^{++}, ATP (or ADP) and the cytosolic factor[23] and these were ideal conditions for lipid peroxidation which required chelated Fe^{++}–ADP (or ATP), NADPH and microsomes[24]. Lack of effect of ATP. Mg on purified HMGCoA reductase may even be due to the absence of the membrane and the lipids needed for peroxidation. Therefore inhibition of HMGCoA reductase by ATP·Mg may be due to any one of these possibilities: lipid peroxidation aided by chelated Fe^{++}–ATP, or binding of Fe^{++} and the protein factor, or due to phosphorylation, or a combination of these. Large decrease in HMGCoA reductase activity occurs in the liver (the major cholesterol-producing as well as Fe-storage tissue) and thus Fe^{++}-inhibition becomes highly significant. The release of Fe^{++} from ferritin as a consequence of starvation or cholesterol-excess may form the basis of the rapid inactivation observed. This type of inhibition, obtained by naturally occurring cytosolic components offers a novel mechanism of regulating cholesterol biogeneis.

Activation of HMGCoA Reductase by Cytosolic Protein

Activation of HMGCoA reductase activity in microsomes by cytosol was first reported by Bucher and McGarrahan[26]. Gaylor and coworkers[27, 28] described the coordinated acti-

vation of two microsomal enzymes in cholesterol biogenesis—HMGCoA reductase and methyl sterol oxidase—by a cytosolic protein. This belongs to a set of proteins (m.w. about 13,000 daltons) variably described as Z-protein, sterol carrier protein (SCP) or fatty acid binding protein. They ligand to a variety of compounds[29]—heme, fatty acids, steroids, organic anions, bile pigments, azo-dyes and carcinogens. These proteins, constituting 3–4% of the tissue protein, are heat-stable and are inducible, *e.g.*, by clofibrate, an anti-hypercholesterolemic drug that decreases HMGCoA reductase[31] and increases ubiquinone in the liver[32]. The activator protein (m.w. 12,000 daltons) was found to be heat-stable and its effect was further increased by heme or hemoglobin, but not cytochrome *C*[28]. Also the very same protein was able to inhibit HMGCoA reductase in the presence of oxidized derivatives of cholesterol or cholesterol linoleate[27], the protein serving, a non-catalytic role by aiding the binding of the effector to the enzyme. This first report on "feedback inhibition" had not received due attention.

Integrated Regulatory Mechanism with Cytosolic Proteins

Our cytosolic protein inhibitory factor also acts in a similar manner by binding to the microsomal enzyme and the effector. In addition to Fe^{++}, other effector molecules such as steroids and ubiquinone (Menon, A.S., unpublished data) were also found to inhibit the enzyme activity in the presence of the cytosol. Inhibitory protein factors of HMGCoA reductase in serum lipoproteins, kidney and fibroblasts[19] are known. The relationship of these to our protein factor and the needed effector molecules remain to be established. The inhibitory protein factors appear to be of high molecular weight (1,00,000 daltons) and heat-labile, reminding of the properties of ATP. Mg-dependent inactivator. The activating protein of Gaylor is of small molecular weight and heat-stable

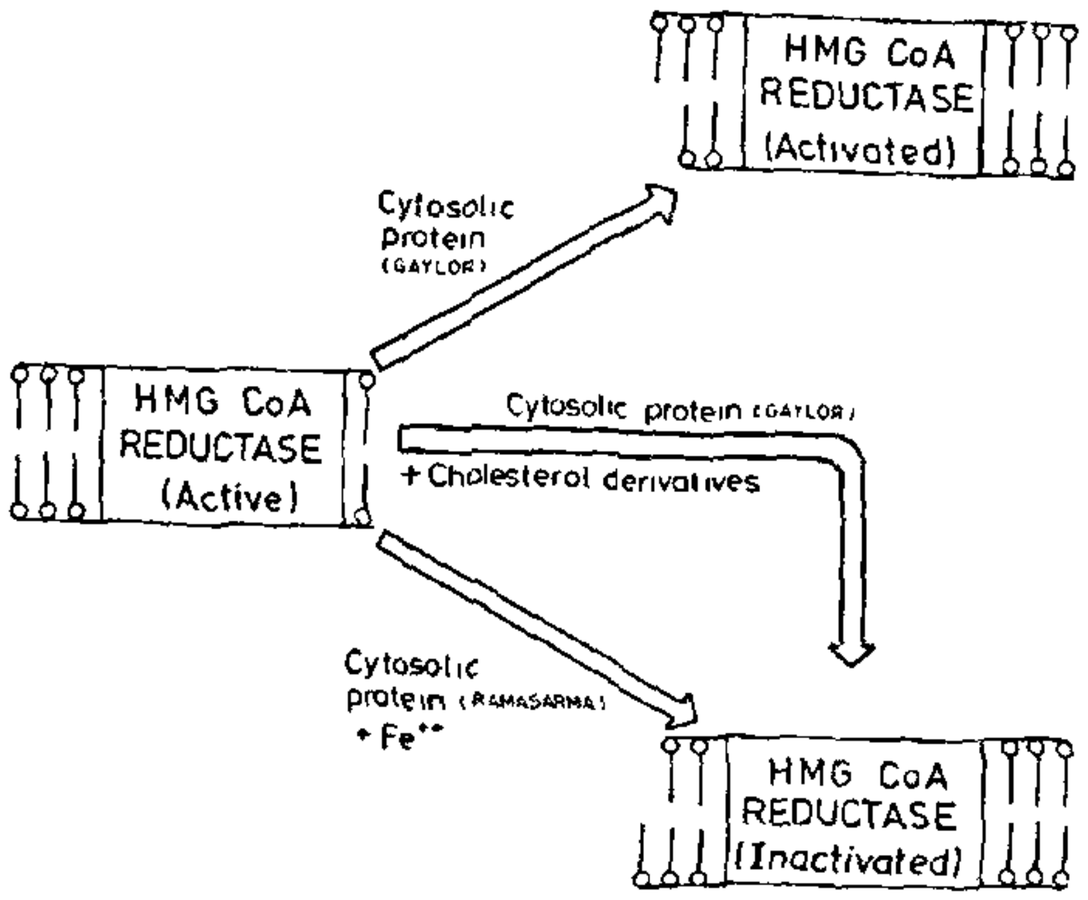

FIG. 3. Protein factors and effectors in the regulation of HMGCoA reductase. Three levels of activity are shown schematically. The cytosolic proteins are described in the references 23 and 28 and identified by the names in parenthesis. The boundary conditions may be determined by the activator protein on the one side and inactivator protein + Fe^{++} on the other.

(Fig. 3). An integrated picture is emerging on the regulation of HMGCoA reductase with a heat-stable activator protein and a heat-labile inhibitor protein, both present in the cytosol, possibly related to the lipoproteins, and functioning in association with a variety of effectors to coordinate the activities of microsomal enzymes in isoprene pathway.

It would be of interest to find out how these protein factors affect the activity of HMGCoA reductase and also how the effectors of a variety of chemical nature—metal ions, steroids, drugs and others—modify the protein factors. It is possible that the regulation of the activity seen in the animals—in the circadian rhythm, in drug treatment or in starvation—may be through changes in and/or interaction between these protein factors and effectors. The protein factors have thus added a new dimension to the research on regulation of cholesterol biogenesis.

Acknowledgments

The work in this laboratory was supported by grants from the Indian Council of Medical

Research and the Department of Atomic Energy and currently from the Department of Science and Technology.

1. Taylor, C. B. and Gould, R. G., *Circulation*, 1950, **2**, 467.
2. Tomkins, G. M. and Chaikoff, I. L., *J. Biol. Chem.*, 1952, **196**, 569.
3. Kandutsch, A. and Saucier, S. E., *Ibid.*, 1969, **244**, 2299.
4. Bucher, N. L. R., Overath, P. and Lynen, F., *Biochim. Biophys. Acta*, 1960, **40**, 491.
5. Siperstein, M. D. and Fagan, V. M., *J. Biol. Chem.*, 1966, **241**, 602.
6. Linn, T. C., *Ibid.*, 1967, **242**, 990.
7. Shapiro, D. J. and Rodwell, V. W., *Biochim. Biophys. Res. Commun.*, 1969, **37**, 867.
8. White, L. W. and Rudney, H., *Biochemistry*, 1970, **9**, 2725.
9. Gregory, K. W. and Booth, R., *Biochem. J.*, 1975, **148**, 337.
10. Ramasarma, T. in *Current Topics in Cellular Regulation* (B. L. Horecker and Stadtman, E. R. eds.), 1972, **6**, 169.
11. Higgins, M. and Rudney, H., *Nature (New Biol.)* 1973, **246**, 60.
12. Beg, Z. H., Allmann, D. W. and Gibson, D. M., *Biochem. Biophys. Res. Commun.*, 1973, **54**, 1362.
13. Lakshmanan, M. R., Nepokroeff, C. M., Ness, G. C., Dugan, R. E. and Porter, J. W., *Ibid.* 1973, **50**, 704.
14. Rajan George and Ramasarma, T., *Biochem. J.*, 1977, **162**, 493.
15. Nordstrom, J. L., Rodwell, V. W. and Mitschellen, J. J., *J. Biol. Chem.*, 1977, **252**, 8924.
16. Beg, Z. H., Stonik, J. A. and Brewer, H. B., *Proc. Natl. Acad. Sci. (U.S.A.)*, 1979, **76**, 4375.
17. Keith, M. L., Rodwell, V. W., Rodgers, D. H. and Rudney, H., *Biochem. Biophys. Res. Commun.*, 1979, **90**, 969.
18. Beg, Z. H., Stonik, J. A. and Brewer, H. B., *Proc. Natl. Acad. Sci. (U.S.A.)*, 1980, **255**, 8541.
19. Brown, M. S., Brunschede, G. Y. and Goldstein, J. L., *J. Biol. Chem.*, 1975, **250**, 2502.
20. Rajan George, Satish Menon, A. and Ramasarma, T., *Indian J. Biochem. Biophys.*, 1980, **17**, 1.
21. Brown, M. S., Goldstein, J. L. and Dietschy, J. M., *J. Biol. Chem.*, 1979, **254**, 5144.
22. Ness, G. C., Spindler, C. D. and Benton, G. A., *J. Biol. Chem.*, 1980, **255**, 9013.
23. Ramasarma, T., Paton, B. and Goldfarb, S., *Biochem. Biophys. Res. Commun.*, 1981 (In press).
24. Wills, E. D., *Biochem. J.*, 1969, **113**, 325.
25. Hochstein, P., Nordenbrand, K. and Ernster, L., *Biochem. Biophys. Res. Commun.*, 1964, **14**, 323.
26. Bucher, N. L. R. and McGarrahan, K., *J. Biol. Chem.*, 1956, **222**, 1.
27. Spence, J. T. and Gaylor, J. L., *Ibid.*, 1977, **252**, 5852.
28. Billheimer, J. T. and Gaylor, J. L., *Ibid.*, 1980, **255**, 8128.
29. Ketterer, B., Tipping, E. and Hackney, J. F., *Biochem. J.*, 1976, **155**, 511.
30. Fleischner, G., Meijer, P. K. F., Levine, W. G., Gatmaitan, Z., Gluck, R. and Arias, I. M., *Biochem. Biophys. Res. Commun.*, 1975, **67**, 1401.
31. White, L. W., *J. Pharmacol. Exp. Therap.*, 1971, **178**, 361.
32. Krishnaiah, K. V., Inamdar, A. R. and Ramasarma, T., *Biochem. Biophys. Res. Commun.*, 1967, **27**, 474.