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REGULATION OF CHOLESTEROL BIOSYNTHESIS

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THE CHOLESTEROL BIOSYNTHETIC PATHWAY

Introduction[1]

Regulation of endogenous biosynthesis of cholesterol is a topic that has engaged the interest of a wide spectrum of researchers over the past 30 years. This process in all its ramifications bears on many essential aspects of cell

[1]Abbreviations:
HMG-CoA 3-Hydroxy-3-methylglutaryl Coenzyme A
IPP Isopentenyl pyrophosphate
FPP Farnesyl pyrophosphate
LDL Low-density lipoproteins
MVA Mevalonic acid
SO Squalene 2,3-oxide
SDO Squalene 2;3:22,23-dioxide
OSC Oxidosqualene cyclase.

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function and thus new advances in cell and molecular biology are being applied to the problem. Because of considerable variation in methodology among workers, the results often supply answers that vary among different groups. In addition, there is difficulty in applying data obtained in animals to man. We indicate in this review those areas where further work is required to resolve controversies. Despite these problems, remarkable advances have been made, culminating in the sequencing of the genes for HMG-CoA reductase and the LDL receptor, two key elements in determining the level of cholesterol in the organism (62). This accomplishment will provide a firm foundation for many advances in the future. Because of constraints of space, we focus on a limited number of topics, and we offer our apologies to those colleagues whose important work we were unable to include in this review.

Overview of Polyisoprenoid Synthesis and the Role of HMG-CoA Reductase

The three major constituents of cells that are of polyisoprenoid origin are the sterols, dolichols, and ubiquinones. The synthesis of these substances is closely related to growth, development, and differentiation of all cells. It is to be expected that the regulatory sites in the biosynthetic pathway common to each would be subject to several regulatory feedback loops, and these would be greatly influenced by the level of each product. In this review we focus primarily on the regulation of cholesterol biosynthesis. The other polyisoprenoid products are discussed insofar as they influence cholesterol synthesis.

There is general agreement that many factors that regulate cholesterol biosynthesis modulate the activity of the enzyme 3-hydroxy-3-methylglutaryl Coenzyme A reductase (HMG-CoA reductase), which reduces HMG-CoA to mevalonic acid. The subsequent biosynthesis of polyisoprenoids from mevalonic acid and the role that HMG-CoA reductase plays in determining the flux of isopentenyl pyrophosphate units throughout the pathway are shown in Figure 1.

Note that in addition to the three major products (the sterols, dolichols, and the polyisoprenoid side-chain of ubiquinone) there are some components minor in amount but not in importance, e.g. isopentenyl tRNA. The regulation of such a multibranched pathway is extraordinarily complex and, as might be expected, a multiplicity of factors are brought to bear on the regulation of the key regulatory enzyme, HMG-CoA reductase. Regulation at this particular step might be thought of as a coarse control, and other steps in different branches of the pathway might be tuned for finer control. The chart in Figure 1 is used as a reference to point out some of the new factors that have recently been elucidated; as they are discussed, we indicate areas in which further work is required.

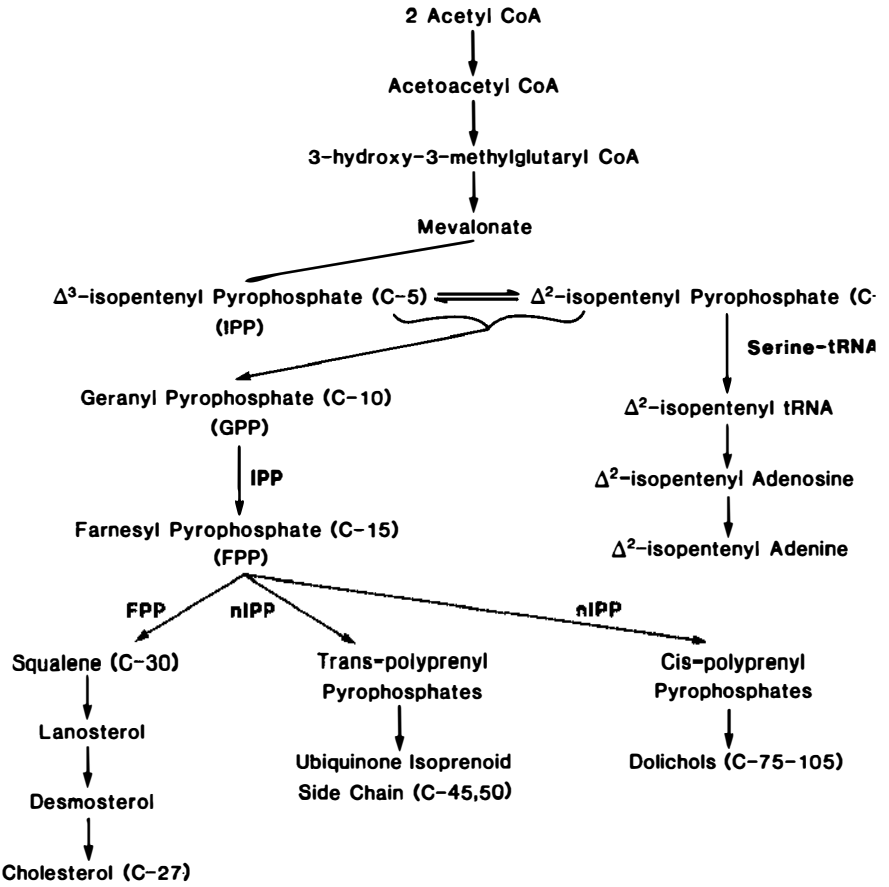

Figure 1 Pathway of isoprenoid biosynthesis. The numbers in parentheses refer to the number of ca atoms in the molecule. From (106) with permission of the publishers.

With respect to the overall polyisoprenoid pathway, it should be noted that after mevalonic acid has formed, a series of phosphorylations and decarboxylations, not shown in detail, forms the basic 5-carbon isoprenoid building block, isopentenyl pyrophospate (IPP). This latter compound polymerizes to form a 10-carbon intermediate, geranyl pyrophosphate (GPP), and a 15-carbon derivative, farnesyl pyrophosphate (FPP). FPP also represents a major branch point in the pathway: two moles may condense to form squalene with subsequent conversion to the sterols. On the other hand, IPP can continue in a sequential series of condensations with a molecule of FPP to form a longer all-*trans* polyprenyl derivative that becomes a side-chain of ubiquinone. The FPP can also be extended by sequential IPP addition in another direction to form the dolichols. The latter differ from the all-*trans* polyprenyl pyrophosphate in

that they contain isoprenoid units with *cis* double bonds instead of *trans*, the molecules are larger, and they generally contain sixteen 5-carbon isoprenoid units. In addition to an initial all-*trans* farnesyl unit, the remaining double bonds are *cis* and generally one of the terminal double bonds is saturated. FPP serves as the base on which IPP is added to lead to the formation of ubiquinones (101) and dolichols (106) as well as the synthesis of squalene; this shows that FPP is an important branch point in the synthesis of all the major products of the polyisoprenoid pathway. Figure 1 also includes isopentenyl tRNA as a product because recent work has shown that it or another product of the mevalonic pathway, i.e. isopentenyl adenosine or isopentenyl adenine, is implicated in an as yet unknown manner with the initiation of DNA synthesis in the S phase of the cell growth cycle (140).

In summary the IPP unit can be directed to at least four products, and controlling the flow of the IPP units to these important cellular materials is now a major area of research. In addition to the FPP branch point, HMG-CoA reductase represents one of the first major, committed steps; consequently it has been the object of a great deal of research (for review, see 115, 125).

In recent landmark work from the Brown and Goldstein laboratory, the gene of HMG-CoA reductase from a Chinese hamster ovary cell line was isolated and sequenced. This work, as well as detailing the amino acid sequence, revealed many features of the structure of HMG-CoA reductase (88, 119). The molecule consists of a polypeptide of 887 amino acids. Of particular interest are the amino acid sequence encompassing the membrane-spanning region and the role that anchorage in the endoplasmic reticulum membrane plays in regulation (53). Recently the complete amino acid sequence of the human enzyme has been deduced from full-length cDNA clones (90). It is 888 amino acids long and displays a high degree of conserved sequences with the hamster enzyme. The conserved domains are in the membrane-spanning region and the catalytic site.

Endogenous and Exogenous Factors Regulating HMG-CoA Reductase Activity

In cholesterol synthesis, it seems clear that cholesterol, introduced to the cell as cholesterol esters transported on low-density lipoproteins (LDL), modulates sterol synthesis by exerting a feedback regulation on the concentration of LDL membrane receptors and on the level of HMG-CoA reductase. There is also evidence that the enzymes HMG-CoA synthase (26) and acetoacetyl CoA synthase are coordinately linked to the modulation of sterol synthesis. Also, under certain conditions HMG-CoA and/or acetoacetyl CoA synthase activity can take over the rate-limiting function (5, 152). These aspects are omitted from the discussion because of space restraints. For an excellent review of this area the reader is referred to the discussion by Bergstrom et al (5). Our focus is primarily on HMG-CoA reductase.

The nature of the down regulation of reductase by LDL has been much studied (12, 61, 62). Studies of tissue cultures have demonstrated lipoprotein receptors on the surface membranes of hepatic and extrahepatic cells including fibroblasts, smooth muscle cells, and lymphocytes (61). Once LDL is bound to the receptor, it is internalized by endocytosis. As a result of lysosomal fusion with the endocytotic vesicle, the LDL protein component is degraded to amino acids and cholesterol esters are hydrolyzed. The free cholesterol is available for transfer into the cytosolic compartment possibly on one of several identified sterol carrier proteins (8, 51). The presence of increased amounts of free cholesterol within the cell results in the down regulation of HMG-CoA reductase activity by a mechanism as yet not completely understood.

Studies of cultured Chinese hamster ovary (CHO) cells (18), UT-1 cells (48), avian myleoblasts (145), and rat hepatocytes (41) indicate that cholesterol derived from LDL causes a decrease in the amount of HMG-CoA reductase enzyme by accelerating its degradation, as well as by reducing its rate of synthesis. Recently, Chin et al (23) reported the successful transfection of UT-2 cells, a mutant CHO cell line that lacks HMG-CoA reductase, with a pRed-227 plasmid that contains a cDNA for hamster HMG-CoA reductase. In these transfected cells, cholesterol derived from LDL did not repress the synthesis of HMG-CoA reductase but did cause a 50–60% decrease in enzyme activity. These results clearly demonstrated the significant effect of cholesterol on enzyme degradation independent of its effect on enzyme synthesis.

The mechanism by which cholesterol stimulates the degradation of HMG-CoA reductase protein has not been established. One controversial mechanism that has been proposed involves phosphorylation of the enzyme (83). Another possible mechanism involves an indirect effect of cholesterol on the physical properties of the membrane in which HMG-CoA reductase is embedded. By changing the fluidity of the membrane surrounding the enzyme, the reductase protein may be more susceptible to degradation by a cytosolic protease. Alternatively, Chin et al (22) suggested that cholesterol binding in the membranous domain of the reductase may cause the enzyme to cluster and eventually bud off into the cytosol where it is eventually degraded within lysosomes.

The Brown and Goldstein group has shown that, in a cell line induced to produce large amounts of HMG-CoA reductase by continually growing CHO cells in the presence of the competitive inhibitor compactin, LDL decreases reductase activity primarily by reducing the level of mRNA for the reductase, although increased degradation of the enzyme is also an important factor (22). Brown and Goldstein presented evidence that the signal for this increased degradation is mediated via localization of cholesterol in the membrane domain of the reductase (53). More recent work from this group (103) has shown that sequences responsible for both promotion and inhibition of transcription are distributed over 500 base pairs extending 300 base pairs upstream of the reductase transcription initiation sites. This portion of the 5' end of the reduc-

tase gene contains sequences responsible for cholesterol-mediated inhibition of transcription. Kandutsch and collaborators (14, 15, 21, 52, 78) have provided definitive evidence that an oxysterol derivative of cholesterol may be the active inhibitor rather than cholesterol. 25-Hydroxycholesterol has been used as a typical inhibitory oxysterol to study the effect of these derivatives on cholesterogenesis. Recently, Saucier et al (125a) demonstrated its presence in cultured cells.

Kandutsch's group (14) presented evidence, based on enucleated cells, that 25-hydroxycholesterol affects reductase by attaching to a protein (79) that interacts with the genetic machinery either at the transcription or translational level. The nature of the interaction is not known but the result is that the synthesis of the enzyme can be inhibited (48, 139). In addition, there is evidence that the degradation of the reductase is also increased. Edwards and colleagues (145) have shown that this is the major effect in avian myeloblasts. In a CHO cell line (UT-1) 25-OH cholesterol affects both processes but the major effect is a suppression of synthesis (48). Recently, Chang et al (17, 18) showed that 25-hydroxycholesterol or LDL inactivated the reductase in CHO cells by accelerating the reductase degradation rate. It is interesting to note that this acceleration required the synthesis of mediator proteins with a rapid turnover rate. Other studies have investigated the enhanced level of reductase resulting from the combined effects of feeding mevinolin (an inhibitor analogous to compactin) and cholestyramine to rats. The data show an increase in the mRNA level that is lowered when the animals are fed cholesterol (40, 89).

It has been observed that large amounts of mevalonolactone given to rats greatly reduce reductase levels. Edwards and collaborators reported (41) that mevalonolactone inhibits the rate of synthesis and enhances the rate of degradation of the reductase in rat hepatocytes. The level of mRNA was also reduced by the mevalonolactone treatment (24). These observations were recently extended by Popjak et al (114), who showed that mevalonate gives rise to a product that decreases the level of reductase-specific mRNA. On the other hand, Peffley & Sinensky (110) reported evidence that mevalonate inhibits the reductase in a mutant cell Mev-1 auxotrophic for mevalonate via a mechanism that strongly suggests translational control rather than reduction in transcription. Mevalonate also plays a role in the initiation of DNA synthesis in the cell cycle. This effect may be exerted via isopentenyl adenine. This topic has been reviewed by Siperstein (140) and indicates that a nonsterol product of mevalonate metabolism may also be involved in regulation of sterol biosynthesis.

A large number of cytosolic protein factors have also been shown to affect the activity of the reductase. These are the sterol carrier proteins (SCP_1 and SCP_2), fatty-acid-binding protein, and Z protein (146). These proteins may be a related family that can influence the effect of lipids on the activity of HMG-CoA reductase and other enzymes in the polyisoprenoid pathway. They could act by

influencing the binding of the hydroxysterol metabolites of cholesterol or they might bind lipid inhibitors, e.g. fatty acyl CoA's, which our group found to be powerful inhibitors of the reductase (86). The same considerations apply to the hydroxylated methyl sterols, intermediates between lanosterol and cholesterol. The reader is referred to the excellent reviews of Schroepfer (127, 128) for an extensive analysis of these effects.

There are several cascade-type reactions that bear directly upon the formation of an active and an inactive form of the HMG-CoA reductase enyzme. The control mechanism involves regulation by a cycle of phosphorylation and dephosphorylation via protein kinases, which in turn are also regulated by kinases and phosphatases. The cycle, as finally worked out by Gibson, Beg, and Rodwell and coworkers (4, 68, 73, 80, 81), closely resembles the amplification cascade of phosphorylation and dephosphorylation that regulates glycogen synthesis and breakdown. Gibson and coworkers (108) recently showed a connection between phosphorylation of the enzyme and its increased susceptibility to degradation. This observation has great potential significance in explaining the role of phosphorylation and dephosphorylation in regulating overall enzyme activity and the relatively short half-life of the enzyme. However, the regulatory importance of this phosphorylation-dephosphorylation cycle is still a major research area. The pro and con arguments were objectively discussed in a recent review by Kennelly & Rodwell (83).

Post-mevalonate Regulation of Polyisoprenoid and Cholesterol Synthesis

This area has seen much activity within the last few years because the production and metabolism of mevalonic acid must be carefully regulated in order to provide the isopentenyl pyrophosphate and farnesyl pyrophosphate needed for the synthesis of sterols, dolichols, and ubiquinones. Furthermore, the rate of sterol synthesis is several hundred-fold greater in most instances than the rate of synthesis of the other components. Additionally, the requirement for these other isoprenoid products will vary as the cell goes through its growth and developmental cycles. One may then anticipate that some of the pathways will be shut down and others will be accelerated as the cell adjusts pathways to maximize the production of the isoprenoid products most in need. In addition to regulation at the HMG-CoA reductase step, in the cholesterol pathway regulation also occurs beyond mevalonic acid. The early studies of Gould & Swyryd (63), and more recently Faust et al (47) and Nambudiri et al (98), showed that when HMG-CoA reductase is bypassed by the addition of mevalonic acid (MVA), cholesterol added in the form of LDL suppressed the incorporation of MVA into sterols. Gould & Swyryd showed that this inhibitory step was beyond the farnesyl pyrophosphate (FPP) step; Faust et al (46) indicated that the squalene synthetase reaction was a site at which the LDL exerts an inhibitory effect.

The other two major products of the isoprenoid pathway, the dolichols and the ubiquinones, share the same biosynthetic steps until the FPP branch point is reached. Attempts have been made to study whether procedures that inhibit sterol biosynthesis have the same effect on the dolichol and ubiquinone pathways. Mills & Adamany (96) showed that 25-hydroxycholesterol, a powerful inhibitor of reductase, inhibited dolichol synthesis in smooth muscle cells. James & Kandutsch (74), working with L-cells in tissue culture, observed that dolichol synthesis was closely related to the level of HMG-CoA reductase, but in some situations, large fluctuations in sterol synthesis occurred with little change in dolichol synthesis. Keller et al (82) claimed that they could not observe a correlation between the activity of reductase and dolichol synthesis in liver. James & Kandutsch (75) showed that dolichol synthesis was affected by treatments known to alter reductase activity and, furthermore, dolichol synthesis was decreased by dietary cholesterol and by fasting, and was increased by feeding cholestyramine. A plot of the rates of dolichol vs cholesterol synthesis suggested that, after the formation of the isoprenoid units, the dolichol pathways were saturated at a lower concentration of isoprene units than is required for cholesterol synthesis. A similar observation was made by Gold & Olson (57) in an early study with respect to ubiquinone synthesis in liver.

Kandutsch extended these studies with a thorough analysis of what occurs in differentiating tissues (for review, see 76). In developing brain, the situation appears to be quite different in that dolichol synthesis is independent of sterol synthesis. A similar situation pertains to other differentiating processes, i.e. spermatogenesis and erythroid cell development. Other studies by Carson & Lennarz (13), using compactin to inhibit HMG-CoA reductase activity, showed that development in sea urchin embryos could be restored by the addition of dolichol. They also showed that there was a period during development when the embryos were sensitive to compactin, i.e. when the synthesis of large amounts of dolichol were needed. These results generally support those of Kandutsch and his colleagues showing that when dolichols are needed specifically for development, then the synthesis of dolichol synthesis can be quite sensitive to inhibitors of HMG-CoA reductase.

The foregoing results indicate that under some conditions, specific inhibition of reductase activity affects both sterol and dolichol synthesis in a parallel manner. On the other hand, there is evidence to indicate that differentiating cells produce differential inhibitions of isoprenoid compound synthesis. Some products of the polyisoprenoid pathway are synthesized relatively normally while sterol synthesis is inhibited (76).

A consensus seems to be forming that HMG-CoA reductase will be subject to inhibitory signals by LDL cholesterol and other nonsteroid products of the polyisoprenoid pathway, either acting singly or in concert, depending upon the needs of the cell. The latter are in general closely related to the stages of cell

development. Brown & Goldstein (12), in a review, outlined these possibilities and present the hypothesis that HMG-CoA reductase is never completely suppressed unless cholesterol and one or more nonsterol products are present. This feedback regulation hypothesis for reductase is analogous to those that have been worked out in bacterial systems and termed "multivalent" feedback regulation.

Several investigations have attempted to determine which factors regulate the flow of isopentenyl pyrophosphate to the three major polyisoprenoid entities: sterols, dolichols, and ubiquinone. Faust et al (47) carried out studies with human fibroblasts on the effect of inhibitors of HMG-CoA reductase, e.g. LDL and compactin, on sterol and ubiquinone biosynthesis. They found that adding these substances to the medium of a confluent cell culture inhibited the incorporation of mevalonic acid into cholesterol while the incorporation to ubiquinone was greatly stimulated. They reasoned that in the presence of LDL or compactin, MVA incorporation to cholesterol was inhibited because LDL cholesterol was inhibiting some post-MVA step. They reasoned further that the inhibition was probably at the FPP-to-squalene step, and this allowed IPP to be shunted to ubiquinone synthesis. This was supported by the observation of Faust et al (46) indicating that LDL inhibited mevalonate incorporation into cholesterol at the squalene synthetase level.

Studies by Nambudiri et al (98) showed that the same data could be interpreted in another way. Under the conditions in which MVA incorporation into ubiquinone increased in the presence of compactin or LDL, the incorporation of 4-hydroxybenzoic acid (the aromatic precursor of ubiquinone) was actually inhibited, and the inhibition could only be restored by increasing the concentration of MVA. This showed that the increase of MVA incorporation into ubiquinone in the presence of compactin was apparently due to an increased specific activity of the precursor, and not an increase in flux. Nambudiri et al (98) offered the explanation that when inhibitors of HMG-CoA reductase were present, the synthesis of MVA was inhibited and consequently the pool of endogenous MVA was very small. Thus, an exogenous labeled MVA molecule would not be appreciably diluted, and the specific activity of the pool would be increased. The consequent rise in MVA incorporation into ubiquinone would reflect enhanced specific radioactivity but not increased synthesis. This explanation was supported by similar results when acetate was the tracer (118). The final conclusion was that in confluent fibroblasts, both ubiquinone and cholesterol synthesis are regulated at the same step, i.e. HMG-CoA reductase, and that the diversion of isoprenoid units to ubiquinone synthesis when sterol synthesis is inhibited was not the cause of the apparent increase in ubiquinone synthesis (124). Studies by Wilton (153) of the effect of cholesterol on the synthesis of both ubiquinone and tetrahymenol in *Tetrahymena pyriformis* confirmed the interpretations of Nambudiri et al (98).

Volpe & Obert (149) added an inhibitor of cholesterol biosynthesis, 3β(2-diethylaminoethoxy)androst-5-ene-17-one hydrochloride (U-18666A) (113), to cultured glial and neuroblastoma cell lines. They observed that nanomolar quantities of U-18666A caused a marked inhibition of total sterol synthesis from acetate or mevalonate within three hours. There was no effect on fatty acid synthesis. They located the site of inhibition as the reduction of desmosterol to cholesterol, confirming earlier observations by Cenedella and collaborators (7, 16) on the site of U-18666A inhibition. Volpe & Obert (149) also noted that exposure of C-6 glial cells to the drug caused a marked stimulation of the incorporation of [^{14}C]acetate and of [^{3}H]mevalonate into ubiquinone, yet caused no inhibition of HMG-CoA reductase. These results supported the interpretation of the data of Faust et al (47), who postulated that the flow of isopentenyl pyrophosphate units was directed toward ubiquinone when the pathway to cholesterol was blocked. These data seemed to contradict the hypothesis of Nambudiri et al (98); however, further work on this point has confirmed the hypothesis of Nambudiri et al (98) and is discussed in the following section.

Oxysterols as Regulators of Cholesterol Synthesis

Sexton et al (131) re-examined the effect of U-18666A in a line of intestinal epithelial cells (IEC-6) and in human skin fibroblasts (GM 0043). They confirmed the observations of Volpe & Obert (149) that cholesterol synthesis from mevalonic acid was inhibited and reductase activity was unaffected. However, the increased incorporation of mevalonic acid into ubiquinone was artifactual and was due to the generation of a contaminant by the drug. Sexton et al identified the substance as squalene 2,3:22,23-dioxide (SDO) (131). Previous work (19, 27, 49, 50, 99, 102) had shown that squalene 2,3-oxide (SO) and SDO accumulate in the presence of inhibitors of oxidosqualene cyclase (OSC). It became clear that U-18666A was a powerful inhibitor of OSC in the cell lines used by Sexton et al. Nelson et al (99) studied the conversion of SDO to polar sterols, extending the previous studies of Corey et al (27) and Shishibori et al (138). The latter authors observed that SDO was converted to 24,25-oxidolanosterol by preparations of OSC from liver. Nelson et al (99) found SDO conversion to 24,25-oxidolanosterol only under anaerobic conditions. Under aerobic conditions, the rat liver homogenate converted SDO to a new product, 24,25-epoxycholesterol. No evidence of 25-hydroxycholesterol could be found, although 25-hydroxylanosterol could be converted to 25-hydroxycholesterol. This work has been extended by Spencer et al (141a), who showed that 24(S),25-epoxycholesterol repressed HMG-CoA reductase activity in cultured cells.

Sexton et al found that SDO accumulated in the presence of U-18666A was metabolized to compounds more polar than cholesterol when the drug was removed from the culture medium (131). The relationship of these changes to

regulation of HMG-CoA reductase was investigated further by the same laboratory (105). It was noted that U-18666A caused a small inhibition of reductase activity, which was greatly enhanced when the drug was withdrawn. The assumption was that the SDO accumulated in the presence of the drug was converted to inhibitory oxysterols when the drug was removed. A further surprising observation was the fact that U-18666A exerted a biphasic effect on reductase activity, i.e. it was inhibitory at low concentrations of U-18666A and noninhibitory or stimulatory at higher concentrations. SDO was also shown to be a potent inhibitor of reductase activity and this inhibition could be abolished by U-18666A. These observations could be rationalized by the assumption that SDO, because it was converted to an inhibitory oxysterol by the action of oxidosqualene cyclase, was a powerful inhibitor of reductase. This would be the situation at low concentrations of U-18666A where partial inhibition of OSC would allow SDO to be formed. At high concentrations, OSC activity was completely shut down; thus no cyclized derivatives of SDO could be formed and the inhibition was abolished. These observations emphasized the existence of an alternate pathway for formation of oxysterols which branched off from the normal pathway at the level of squalene 2,3-oxide via squalene oxidocyclase. This is shown in Figure 2.

According to this scheme, addition of a preformed oxysterol beyond the OSC step should inhibit reductase and not be affected by U-18666A. This was observed with oxysterol inhibitors such as 25-hydroxycholesterol (105) and epoxylanosterol (24,25-epoxy-5α-lanost-8-ene-3β-ol) and 25-hydroxylanosterol (5α-lanost-8-ene-3β,25-diol) (104). These compounds did not affect general metabolism of cells, as evidenced by the maintenance of normal fatty acid metabolism and overall protein synthesis. Recent observations by Rachal et al (116), Watson & Scallen (126, 150) and Spencer et al (141a) confirm the role of SDO derivatives described above (150). In addition to oxygenated lanosterol analogs derived from SDO, it should be noted that

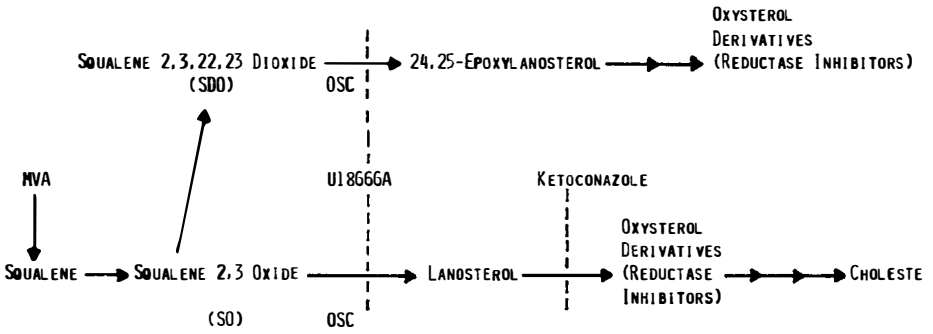

Figure 2 Alternate pathway for oxysterol formation.

lanosterol derivatives bearing an additional oxygen function at carbon 32 have been shown to inhibit reductase activity in cell cultures (52, 78). These compounds are normal intermediates in the conversion of lanosterol to cholesterol. However, situations where these oxysterols might accumulate endogenously have not been established. Possible pathways of formation and proposed regulatory roles of oxysterols in sterol biogenesis are extensively reviewed by Schroepfer (127, 128).

Our laboratory has also investigated the role of lanosterol and its oxygenated metabolites with the drug ketoconazole. This drug is an orally active imidazole derivative and is used clinically as an antifungal agent (10). It inhibits the conversion of lanosterol to ergosterol in fungi in nanomolar concentrations, whereas micromolar concentrations are required in mammalian tissues (69, 148). This action is exerted by inhibition of a cytochrome P-450 system involved in lanosterol demethylation (134). Gupta et al (67) worked with an extrahepatic nonsteroidogenic cell line in order to minimize the presence of alternate pathways related to bile acid and/or steroid hormone synthesis because these could influence the levels of oxysterols. They observed that incubation of intestinal mucosal cells with 0.15–1.5 μM ketoconazole resulted in a concentration-dependent inhibition of reductase activity. However, as the drug concentration approached 15 μM, the reductase activity paradoxically returned to control values; at 30 μM ketoconazole, a stimulation of enzyme activity was observed. The drug had no effect on reductase activity in homogenates of IEC-6 cells. Ketoconazole (0.15–30 μM) caused a concentration-dependent inhibition of the incorporation of [^{3}H]mevalonolactone into cholesterol with a concomitant accumulation of radioactivity in lanosterol (methyl sterols) and 24,25-epoxylanosterol. Treatment of cells with ketoconazole (60 μM) and [^{3}H]mevalonolactone followed by removal of the drug and radio label resulted in an inhibition of reductase activity and a redistribution of radioactivity from methyl sterols and epoxylanosterol to cholesterol and polar sterols. When 24,25-epoxylanosterol was added directly to the cells, an inhibition of reductase activity was observed, but the inhibition could be attenuated by ketoconazole. This observation suggested that 24,25-epoxylanosterol per se was not an inhibitory oxysterol but an intermediate that could be metabolized to a suppressor via a ketoconazole-sensitive pathway. In this connection, the observations of Imai et al are of interest (72). They showed that intravenous injection of SDO and 24,25-epoxylanosterol caused cell death, inflammation, and repair in rabbit aortas and pulmonary arteries within 10 days of administration. They suggested that oxygenated sterols may play the primary role in arterial cell wall injury and lesion development.

From the above discussion, it is evident that a large body of data is accumulating to support the hypothesis of Kandutsch et al (78) that oxysterols generated endogenously can play a major role in the regulation of cholesterol

synthesis via modulation of HMG-CoA reductase activity. Furthermore, the evidence indicates that these oxysterols can arise from two pathways, as shown in Figure 2: (*a*) from diversion of squalene 2,3-oxide to SDO and subsequent cyclization to oxylanosterol derivatives, and (*b*) directly from intermediates arising in the conversion of lanosterol to cholesterol. U-18666A is an inhibitor of pathway (*a*) while ketoconazole is an inhibitor of pathway (*b*). These drugs should be useful in detailing the role of these pathways in the generation of regulatory oxysterols. Further work in this area will be focused on the identity of the regulatory oxysterols and their mechanism of action.

Until recently there was no direct evidence to show that 25-hydroxycholesterol is formed in the cell. Saucier et al (125a) have now identified 24(S),25-epoxycholesterol and 25-hydroxycholesterol in cultured fibroblasts. The endogenous concentration of these oxysterols appears to be within the range required for regulation of HMG-CoA reductase. Other cholesterol derivatives with a hydroxyl group in the side-chain have been detected in extrahepatic tissues. L. L. Smith and collaborators found that 24-hydroxycholesterol [(24S)-cholest-s-en-3β-24-diol] can be synthesized from cholesterol in bovine and rat brain (31, 87). 26-Hydroxycholesterol, which is a primary intermediate in bile acid metabolism, has been detected in various lipoprotein fractions in serum (77). However, there is some question whether the concentrations present in serum would be sufficient to influence sterol synthesis (17). Since the major portion of cholesterol coming to a cell is in the form of cholesterol ester carried on low-density lipoproteins (LDL), the question arose whether the down regulation of HMG-CoA by LDL cholesterol might involve formation of an oxysterol. Panini et al (105) observed that U-18666A abolished the effect of LDL in down regulating HMG-CoA reductase, and they raised the possibility that the effect of LDL might involve the formation of a regulatory oxysterol derived from the SDO pathway.

Suppression of reductase activity resulting from the receptor-mediated internalization of LDL involves several steps (2): binding of LDL to specific cell membrane receptors, internalization and lysomal degradation of the protein, and hydrolysis of the cholesterol esters. Ketoconazole and U-18666A do not affect any of these steps (105, 132). Furthermore, although these drugs are inhibitors of acyl CoA cholesterol:acyl transferase (ACAT), the decreased cholesterol ester formation was found to be unrelated to the phenomenon of LDL-induced suppression of HMG-CoA reductase activity since other ACAT inhibitors do not prevent LDL suppression of reductase (132). The foregoing studies with ketoconazole suggest that hydroxylation involving cytochrome P-450–linked oxidases may play a role in the endogenous generation of regulatory oxysterols directly from the cholesterol molecule, which in turn regulate cholesterol formation. In support of this assumption, Sexton et al found in preliminary experiments that substances preventing LDL action in

down regulating reductase are also cytochrome P-450 inhibitors (130). More intensive investigation is required into the potential importance of this class of oxidases in regulating cholesterol synthesis.

DIETARY FACTORS

Rates of Cholesterogenesis in Various Tissues

Cholesterol levels in all cells represent a balance between synthesis (or uptake of preformed cholesterol) and degradation. The same factors are involved in an assessment of the pool of cholesterol in the whole animal. These were discussed in an excellent review by Turley & Dietschy (147). Large variations are observed in the ability of animals to absorb dietary cholesterol and accomplish endogenous synthesis. Each stage in the absorption process is beset with difficulties in rate measurement (because of variations in enzyme activity of the key enzymes in the absorptive process) as well as controversy about the actual measurement of the biosynthesis of the cholesterol molecule as a whole. Thus, it is not surprising that some of the measurements reported in the literature are still subject to controversy. Nonetheless, the data obtained by Dietschy illustrates the remarkable differences in the absorption rate in animals and man. It appears that man can absorb two to four milligrams of cholesterol per day per kilogram of body weight, whereas other species (e.g. canines and rodents) can absorb 35 to 50 times this amount. Thus, the limited absorptive capacity of man may be a major protective factor in minimizing the pathological effects of excessive dietary cholesterol.

There are also wide species variations in the synthetic rate of cholesterol in whole animals. This can vary from 118 milligrams per day per kilogram of body weight in the rat to nine milligrams per day per kilogram of body weight in man. The data on synthesis and uptake suggest that endogenous synthesis in man plays a major role in the adjustment of the body pool of cholesterol. Hence, its regulation may be a very significant factor in the formation of the body pool of cholesterol in man.

The extent of feedback repression of cholesterol biosynthesis by dietary cholesterol in human liver is open. The variation in the effects of dietary cholesterol on plasma cholesterol (147) in man suggests that variable feedback control occurs in liver. The question requires further extensive investigation. There is evidence from studies with rats (34), squirrel monkey (35), and man (6, 32) that cholesterol feeding markedly inhibits hepatic cholesterogenesis. In rats, the degree of inhibition of cholesterogenesis is directly proportional to the chylomicron remnants and LDL concentration in the blood (85, 151).

Dietschy and collaborators (33, 147) have used [^{3}H]water to great advantage to compare in vivo and in vitro overall rates of cholesterol synthesis in a variety of tissues in different species. Rat liver shows the highest rate of cholesterol

synthesis in vitro, while in man the rate is one sixth that of the rat. The rates in guinea pig and hamster are lower than in man. Measurements in vivo of rates of cholesterol synthesis with [^{3}H]water in different tissues of the rat and squirrel monkey again show that the liver accounted for a large percentage of the cholesterol synthesis by the body. However, in some species, extrahepatic tissues also play a major role. In the rat and the squirrel monkey, the liver accounted for 51% and 41% of the rate of cholesterol synthesis in the whole body. Among the extrahepatic tissues, skin muscle and the small intestine accounted for the majority of the remainder.

Effects of Dietary Fats on Hepatic Cholesterogenesis

Although a large number of studies have been performed on this topic, no clear-cut explanation has been put forward to rationalize the disagreement in results. In an early study, Avigan & Steinberg (2) found that feeding of polyunsaturated fats to rats significantly stimulated the rate of hepatic cholesterol synthesis. Subsequent work by Serdarevich & Carroll (129) confirmed these observations by showing that rats fed corn oil showed higher hepatic cholesterogenesis than a comparable group fed butter. Goldfarb & Pitot (60) showed that the stimulatory effect of dietary corn oil on cholesterogenesis was due to an increase in the activity of HMG-CoA reductase. A number of workers (28, 71, 71a, 109) have reported on the general stimulatory effects of dietary lipids on cholesterogenesis in rats, especially in animals that have been fasted and refed. Ide and coworkers (71, 71a, 71b) performed a detailed study on the effect of dietary fats of various degrees of unsaturation on HMG-CoA reductase activity and cholesterogenesis in normal and fasted-refed rats. In normal rats fed the experimental diet for a 2-week period, they observed that HMG-CoA reductase activity rose with chain length of the fat but declined with the degree of unsaturation (71). In rats fasted for 2 days and refed for 3 days, safflower oil (105) decreased reductase and cholesterogenic activities more than saturated fat. Mitropoulos et al (96a) also observed that unsaturated fat (safflower oil) fed to rats for 12 hours reduced the activity of reductase in liver microsomes compared to saturated fat (tristearin). Concomitantly there was a rise in acyl CoA : cholesterol acyl transferase activity in the animals fed unsaturated fats.

There have been several explanations offered for each type of result. For example, the stimulation in hepatic cholesterogenesis observed with unsaturated fats is explained on the basis of the observation that diets rich in unsaturated fats increased bile acid synthesis (91) and thus cholesterol catabolism leading to increased activity of HMG-CoA reductase. Another explanation, suggested by Paul et al (109), is that the microscopic fluidity of high-density lipoproteins responsible for cholesterol efflux from cells is increased following dietary uptake of unsaturated fats (137). This increased fluidity may

allow more cholesterol to be solubilized by the high-density lipoprotein molecules, thus allowing greater cellular efflux of cholesterol and reducing end-product inhibition effects on HMG-CoA reductase. A similar explanation based on the removal of cholesterol from the cell being incorporated into a lipoprotein has been proposed by Goh & Heimberg (54, 55). These investigators showed that the perfusion of rat livers with oleic acid increased the efflux of cholesterol from the cells, increased cholesterol synthesis, and markedly stimulated the activity of HMG-CoA reductase. The authors suggested that the uptake of oleic acid led to an increase in very low-density lipoprotein (VLDL) synthesis, which required the incorporation of cholesterol before secretion into the blood. As a result of enhanced lipoprotein synthesis and secretion, hepatic cholesterol stores would be decreased, thus releasing the end-product inhibition of HMG-CoA reductase activity (56), and thus increasing endogenous cholesterol synthesis.

On the other hand, the observations of Mitropoulos et al (96a) are consistent with the presence of higher concentrations of free cholesterol in the endoplasmic reticular membrane in the environment of reductase and of acyl transferase after feeding unsaturated fats compared with saturated fat. However, Ide et al (71a,b) could not observe this correlation. The work of Van Zuiden et al (148a) provides the best simple unifying explanation of these discrepancies and suggests parameters for future investigations in this field. These workers studied the effect of removing chylomicron remnants of different composition on hepatic HMG-CoA reductase activity and hepatic VLDL secretion in perfused rat liver and in vivo. They observed that the ratio of cholesterol to triglycerides in the remnant's lipoprotein played an important role in determining the level of hepatic HMG-CoA reductase activity. Consequently a remnant lipoprotein can have two opposing effects on reductase activity. The first due to cholesterol content is inhibitory, the second due to triglyceride content is stimulatory. The net effect on regulation of reductase activity and cholesterogenesis would depend on the relative amounts of cholesterol and triglyceride in the particles. They suggested that further permutations of these effects could also be induced by the degree of unsaturation of the triglycerides and phospholipids of a lipoprotein remnant that could be manipulated by dietary means. Much more work along those lines is required to sort out these factors. For a much more comprehensive discussion of the effect of dietary manipulation of saturated and unsaturated fats administered to animals and humans on the composition and metabolism of lipoproteins, see the review by Goldberg & Schonfeld (58) in Volume 5 of the *Annual Review of Nutrition*.

Effects of Vitamin A Active Substances

There is a large body of literature on the biological roles of vitamin A active substances in a variety of cellular functions, and the subject was recently

reviewed (142, 154). Vitamin A derivatives administered to man (84, 123) and animals (1, 9, 11, 44, 155) appear to lower serum cholesterol levels. On the other hand, recent studies (5a, 156) on isoretinoin (13-*cis*-retinoic acid) administration to humans showed significant increases in mean plasma levels of cholesterol. However, many of these studies may need to be reexamined. Ott & LaChance (103a) critically analyzed factors that could influence the results of experimentation in animals. They reviewed the effect of vitamin A on cholesterol biosynthesis in the rat animal model, and cited many inconsistent and complex actions of this vitamin on cholesterol synthesis, ranging from depression to no effect to enhancement. Ott & LaChance point out that the action of this nutrient is affected by the following factors: vitamin A status of the animal, form of vitamin A used, vitamin A metabolites, animal feeding regimen, substrate flux, and supernatant protein factors.

Some studies at the cellular level with tissue extracts and cell cultures show that the direct addition of vitamin A derivatives to these preparations inhibit acetate incorporation into cholesterol. Thus Eskelson et al (45) and Erdman et al (42, 43) showed that vitamin A and its derivatives inhibited cholesterol biosynthesis from acetate/mevalonate in post-mitochondrial supernatants of perfused rat liver. These authors (43) suggested that the inhibition was localized at the 2,3-oxidosqualene cyclase step. Recently Ringler et al (120) reported that retinoic acid caused a significant decrease in the incorporation of acetate into cholesterol in hamster embryo fibroblasts.

Gupta et al (66) treated fibroblast cultures with retinoid derivatives (e.g. retinol, retinyl acetate, and retinoic acid). They observed that 5×10^{-6} M retinol and retinyl acetate caused a 50% inhibition of HMG-CoA reductase activity. Retinoic acid was less effective. These derivatives also caused an inhibition in the conversion of squalene to cholesterol, with concomitant formation of polar sterols. Thus these observations support the earlier suggestions of Erdman et al (43). Further studies should help to delineate the role that polar sterol formation may play in the expression of vitamin A effects on sterol synthesis.

Effects of Vitamin D Active Substances

A vast literature has arisen about the structure and function of vitamin D and its hydroxylated derivatives in relation to calcium and phosphorus metabolism. For recent reviews see (30, 70, 100). The model for the mechanism of action of the fat-soluble vitamin D derivatives is similar to the classic steroid hormones. Vitamin D is the steroid hormone involved in calcium homeostasis and it exerts its effects by virtue of its further metabolism to more polar metabolites that bind to receptor proteins. This complex then produces the response by interaction with the genome leading to the induction of specific proteins. The elements of the vitamin D endocrine system involve conversion of 7-dehydro-cholesterol

to vitamin D_3 by UV light or dietary ingestion of vitamin D_3. Then vitamin D_3 metabolizes to 25-hydroxy vitamin D_3 [$25(OH)D_3$], the principal form of vitamin D present in blood. This conversion takes place primarily in the liver via a cytochrome P-450 reaction. This is followed by conversion in the kidney to the two principal dihydroxylated metabolites 1,25-dihydroxy-D_3 [$1,25(OH)_2D_3$] and 24,25-dihydroxy-D_3 [$24,25(OH)_2D_3$], which are then transported to a variety of target organs. The principal targets are bone, intestine, and kidney, where major effects on calcium and phosphorus metabolism are exerted. In addition to these, a new set of targets has been discovered (30, 100).

A large number of studies have been performed to determine the biological role of $24,25\text{-}(OH)_2D_3$ and these are discussed in the reviews noted above. There is little doubt that $24,25(OH)_2D_3$ is a product of $25(OH)D_3$ metabolism as well as $1,25(OH)_2D_3$ in the kidney. In fact, it appears that $1,25(OH)_2D_3$ is the inducer of the 24-hydroxylase. Thus under normal conditions both dihydroxylated metabolites are being produced. While there is no question of the role of $1,25(OH)_2D_3$ in mediating the D_3 responses, some controversy is centered on the role of $24,25(OH)_2D_3$. DeLuca & Schnoes (30) have advanced the thesis that the $24,25(OH)_2D_3$ derivatives are not important and they are primarily precursors of a catabolic pathway for vitamin D_3 derivatives. Norman and coworkers, on the other hand, claim that both $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$ must be present to express several biological functions (70).

That the derivatives of Vitamin D_3 metabolism are related to sterol biosynthesis is suspected because the metabolic conversion of D_3 involves insertion of a hydroxyl function into a side-chain that is essentially similar to that of cholesterol. This raises the possibility that hydroxylated vitamin D_3 derivatives because of their sterol nature may affect sterol synthesis in a manner analogous to other oxysterols. These considerations were further supported by the observations of Phillipot et al (112) that $25(OH)D_3$ and $1,25(OH)_2D_3$ at 10^{-7} M are potent inhibitors of the incorporation of acetate into cholesterol. The cell lines they used were normal and leukemic guinea pig lymphocytes. They found that $1,25(OH)_2D_3$ was even more effective than 25-hydroxycholesterol as an inhibitor of cholesterol synthesis.

Because Philippot et al (112) did not determine whether the effect was on HMG-CoA reductase, our laboratory has initiated some studies on the role of vitamin D derivatives in the regulation of cholesterol synthesis. Our preliminary observations show that HMG-CoA reductase is inhibited by vitamin D_3. Further studies on the mechanism of this effect are in progress. The role of vitamin D_3 in sterol biosynthetic processes is worthy of study in view of the fact that hydroxylated vitamin D_3 derivatives provide another model of an inhibitory sterol. In addition Peng & Taylor (111) have surveyed a large body of evidence suggesting that moderately excessive amounts of vitamin D_3 de-

rivatives exert a pathological effect on arterial tissues and thus may greatly increase the risk factor in the induction of arteriosclerosis in man and animals.

Role of Cholesterogenesis in Hepatic VLDL Production

A major unresolved question is the relation of cholesterol synthesis in the liver to the production of VLDL. VLDL is the chief lipoprotein secreted by the liver and its metabolism in the blood leads to the production of intermediate-density lipoprotein (IDL) and LDL (Apo-B-100–containing lipoproteins). The major organ for clearance of these modified plasma lipoproteins is the liver via receptors for Apo-B-100 and Apo-E. Dietschy and colleagues (141), studying the rates of cholesterol synthesis and LDL uptake in livers of rats and hamsters, found these rates were independently regulated. Furthermore it appeared that the primary response of the liver to changes in cholesterol availability was the regulation of sterol synthesis. Alterations in LDL uptake occur only when the endogenous synthetic mechanisms cannot satisfy the needs of the liver for cellular cholesterol.

Dietschy and colleagues (143) also studied the origin of cholesterol in the mesenteric lymph of the rat. Under normal conditions, it appears that approximately 60% of the cholesterol in the lymph is derived from the absorption of luminal sterol that is primarily of biliary origin. This implies that the liver was the primary site of formation of this cholesterol. Concomitant fat absorption plays a major role in determining the fate or disposition of the cholesterol synthesized in mucosal cells. In the absence of fat absorption, endogenously synthesized cholesterol was incorporated into cell membranes of the endothelium and very little appeared in the intestinal lymph. However, when fat absorption occurred, a fraction of the endogenously synthesized cholesterol was incorporated into lipoproteins and delivered by the lymph to the body pools of cholesterol.

Various situations may occur in which the output of VLDL is greatly increased. These increases can be of primary or secondary origin. Grundy (65) points out that the causes of primary overproduction of VLDL are unknown. There are a variety of reasons for secondary overproduction of VLDL, discussed in detail by Grundy in several theoretical models, that are based on defects in VLDL metabolism. These can account for the variation in observations of VLDL and LDL levels. However, as Grundy points out this is an area in which much more work is needed.

Finally, with respect to humans, a key question deals with the role that increased absorption of cholesterol plays in affecting the amounts of cholesterol secreted in VLDL and secondarily in LDL. There seems little doubt that the ingestion of large amounts of dietary cholesterol can raise the levels of plasma lipoprotein cholesterol. But this can occur in many different ways that have not been clearly delineated in man. Consequently, answers to this basic question

are very complex. Some of the factors that must be considered in the interpretation of data obtained in this area are analyzed in two recent volumes of the *Annual Review of Nutrition* (58, 64). These reviews point out the need for further specific investigation, particularly in the areas in which reported data leads to opposite conclusions.

Diurnal Variations in Hepatic Cholesterogenesis

A diurnal cycle in hepatic cholesterol synthesis has been demonstrated in rodents (38, 93, 135, 136), swine (122), and chickens (117). Similar fluctuations in cholesterol synthesis have also been demonstrated in small intestine (135) and transplantable hepatomes (59). In man, although a diurnal cycle has not been directly studied, recent analysis of plasma cholesterol precursors, mevalonic acid (107), squalene, and methyl sterols (95) showed an increase in their concentrations at night, which suggests an increase in the overall cholesterol synthetic pathway with a release of some of the pathway intermediates into the blood (94). In almost all cases, the diurnal cycle of cholesterol synthesis has been traced to a change in the activity of HMG-CoA reductase (121). Cholesterol 7α-hydroxylase (29, 97), which is involved in bile acid synthesis, and lysosomal acid cholesteryl ester hydrolase (144), involved in the hydrolysis of cholesteryl esters derived from lipoproteins, have been shown to have a circadian rhythm. More extensive studies are needed before their roles in controlling the circadian rhythm of cholesterol synthesis can be established.

McGuire et al (92) recently demonstrated that sterol carrier protein (SCP_1), a major regulatory protein of lipid metabolism and transport with rat liver, undergoes a diurnal cycle with a 7- to 10-fold increase at its peak in the dark period. Direct quantitation of SCP mRNA sequences with a cDNA probe showed that the total SCP mRNA level or its polysomal distribution did not account for the diurnal variation in SCP synthesis. Again, more studies are required to determine the significance of these observations related to the control of cholesterol synthesis through the diurnal cycle.

The diurnal cycle of cholesterol synthesis correlated with HMG-CoA reductase activity has been well documented in hepatic (3, 36) and intestinal (38, 135) tissue of the rat. Several investigators have shown that cycloheximide (37, 133) or actinomycin D (39) can abolish the cycle, which suggests that reductase mRNA production and enzyme synthesis were required for expression of the diurnal cycle. Clarke et al (25) recently used a reticulocyte lysate translation system and a reductase cDNA probe to demonstrate that during the diurnal cycle the amounts of both functional and total reductase mRNA played a major role in controlling the synthesis of HMG-CoA reductase. The mechanisms responsible for changing levels of reductase mRNA during the diurnal cycle are not well understood. Withdrawal of food, addition of cholesterol to the diet,

and fluctuations in hormones occur during a normal cyclic period; each has a significant effect on cholesterol synthesis and expression of reductase activity (121). At present, the effect of these variables on reductase mRNA transcription and translation are yet to be clearly delineated.

CONCLUSION

We have attempted to highlight some of the active areas of research in the regulation of cholesterol biosynthesis. Obviously there are more questions than there are answers at the present time, particularly when attempts are made to extrapolate the observations in tissue culture and animal systems to man. Nonetheless, spurred on by new techniques in molecular and cell biology, remarkable progress has been made. Cholesterol synthesis is closely related to many metabolic pathways and the cholesterol molecule is central to cell membrane structure and function. Thus many complex relationships remain to be understood before its role can be accurately assessed in normal and pathological cellular processes, e.g. atherosclerosis. The level of research activity in this field will remain high for many years to come.

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