initially high but declined as HMG-CoA reductase became activated. Glucagon prevented the loss of activity, whereas insulin promoted inactivation of the enzyme. Similar affects on the activity of HMG-CoA reductase kinase, and the state of phosphorylation of HMG-CoA reductase in liver have been reported after injection of rats with glucagon (Beg et al., 1979). Thus insulin and glucagon have concerted effects on the activities of both HMG-CoA reductase and HMG-CoA reductase kinase.

In liver, the binding of glucagon to specific cell-surface receptors results in the activation of adenylate cyclase and an elevation in cytoplasmic cyclic AMP concentrations. The resulting effects of the increase in cyclic AMP are thought to be a consequence of the phosphorylation of a variety of cellular proteins by an enzyme termed cyclic AMP-dependent protein kinase (EC 2.7.1.37) (Greengard, 1978). Although the activation of this protein kinase is likely to underlie the effects of glucagon on the HMG-CoA reductase phosphorylation system, both HMG-CoA reductase kinase and HMG-CoA reductase kinase appear to be distinct from cyclic AMP dependent protein kinase (Ingebretsen et al., 1981). Another mechanism by which glucagon could regulate the phosphorylation of HMG-CoA reductase and HMG-CoA reductase kinase is through the phosphorylation of inhibitor 1 by cyclic AMP dependent protein kinase (Fig. 2). In principle, this would prevent the dephosphorylation of both HMG-CoA reductase and HMG-CoA reductase kinase by protein phosphatase 1 and could account for the observed changes in the state of phosphorylation of the two enzymes. A complication with this hypothesis, however, arises from the fact that protein phosphatases 2A and 2C are also active on HMG-CoA reductase and HMG-CoA reductase kinase. Hemmings et al. (1981) studied the regulation of HMG-CoA reductase and HMG-CoA reductase kinase by protein phosphatase 1 and suggested that this would prevent the dephosphorylation of a number of important regulatory enzymes in addition to HMG-CoA reductase and HMG-CoA reductase kinase. However, the mechanism of insulin action in liver and other tissues remains a major unsolved problem. It is noteworthy, however, that insulin appears to stimulate liver protein phosphatase activity (Curnow & Larner, 1979), and it is possible that this stimulation may be involved in the regulation of HMG-CoA reductase and HMG-CoA reductase kinase by this hormone.

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The role of non-esterified cholesterol concentration in endoplasmic-reticular membranes in the regulation of hydroxymethylglutaryl-CoA reductase

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The maintenance of an optimal cholesterol concentration requires the co-ordinate regulation of a number of processes involved in the cellular metabolism of cholesterol. Recent work has demonstrated that cells in culture can express specific receptors on plasma membranes that bind lipoproteins (Goldstein & Brown, 1977). The bound lipoprotein is endocytosed, the pinosomes fuse with lysosomes and hydrolysis of cholesteryl esters results in the release of non-esterified cholesterol. This cholesterol is responsible for at least three metabolic controls over the cellular metabolism of cholesterol (Brown & Goldstein, 1976): (i) it reduces the activity of HMG-CoA* reductase, thus decreasing the rate of cellular cholesterologenesis; (ii) it stimulates the activity of ACAT, thus increasing the rate of cellular cholesterol esterification; and (iii) it inhibits the expression of specific receptors for lipoproteins. In the hepatocyte the increased influx of lipoprotein cholesterol is also responsible for an increased rate of bile acid biosynthesis through increased activity of cholesterol 7α-hydroxylase (Myant & Mitropoulos, 1977). An example of this co-ordinated control is provided by the influx to the liver of dietary cholesterol taken up by the liver in the form of the metabolites of intestinal lipoproteins (Balasubramaniam et al., 1978a). Moreover, similar co-ordinated control of hepatic metabolism is observed after a sharp increase in the rate of hepatic cholesterologenesis that follows the administration to rats of a bolus of mevalonic acid (Mitropoulos et al., 1978a).

The regulation in vivo of HMG-CoA reductase seems to involve changes in the concentration of enzyme protein and changes in the activation state of the enzyme (Edwards et al., 1980; Arebalo et al., 1982). The role of non-esterified cholesterol (Faust et al., 1982) or of certain oxygenated sterols (Kandutsch et al., 1978; Faust et al., 1982) as the repressors of enzyme synthesis has been suggested. At the post-translational level, modulation of the activation state of the enzyme has been attributed to a cycle of phosphorylation/dephosphorylation (Gibson & Ingebretsen, 1978) or to a mechanism that involves interaction of non-esterified cholesterol with the enzyme confined to endoplasmic-reticular membranes (Sabine & James, 1983).
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now appreciated that HMG-CoA reductase and ACAT are confined to different regions of the membrane (Balasubramaniam et al., 1978b; Mitropoulos et al., 1978c).

Modulation in vitro of non-esterified cholesterol concentration in liver microsomal fraction and effects on HMG-CoA reductase and ACAT activities

The pre-incubation of rat liver microsomal fraction can result in transfer of microsomal cholesterol to endoplasmic reticulum vesicles and in changes in the activities of HMG-CoA reductase and ACAT. This is consistent with the characteristics of Arrhenius plots of HMG-CoA reductase in treated preparations that suggest increased concentration of cholesterol in the environment of the enzyme (Venkatesan & Mitropoulos, 1982). Moreover, diversion of this transfer of cholesterol to phosphatidylcholine liposomes reverses the effect on ACAT activity, and under these conditions a reciprocal relation is observed between ACAT activity and the rate of transfer of microsomal cholesterol to the liposomal vesicles. The cholesterol donor for this intervesicular transfer is probably plasma membranes. Thus the increase in ACAT activity and the decrease in HMG-CoA reductase activity can be augmented in the presence of a plasma-membrane added to the pre-incubation mixture (Fig. 1).

The preincubation of liver microsomal fraction in the presence of a preparation of human plasma and the re-isolation of the treated microsomal vesicles resulted in a time- and plasma concentration-dependent increase in the concentration of non-esterified cholesterol, a decrease in the activity of HMG-CoA reductase and an increase in the activity of ACAT (Mitropoulos et al., 1981). The changes in the characteristics of Arrhenius plots of HMG-CoA reductase activity in the treated preparations are consistent with an increase in the concentration of cholesterol in the vesicles that contain the enzyme. Thus plasma-treated preparations with high non-esterified cholesterol content showed constant activation energy between 37 and 20°C, whereas the enzyme in the non-treated microsomal fraction or the lipoprotein-deficient-plasma-treated preparation showed breaks in activation energy at about 29°C (Venkatesan & Mitropoulos, 1982). We have attributed the increase in ACAT activity in the plasma-treated preparations to an expansion of the size of the cholesterol pool available to the enzyme due to the transfer of non-esterified cholesterol to vesicles that contain the enzyme (Mitropoulos et al., 1981). The kinetics of transfer of cholesterol to the microsomal vesicles and to the ACAT substrate pool can best be studied using artificial membrane vesicles. Thus the pre-incubation of the microsomal fraction with phosphatidylcholine/cholesterol liposomes and re-isolation of the treated microsomal vesicles can provide the rate of transfer of the substrates of liposomal cholesterol with the ACAT substrate pool (Symouri-Vrettakou & Mitropoulos, 1983). Transfer of liposomal cholesterol to microvesicular vesicles follows first-order kinetics with respect to the donor concentration, but the rate constant is decreased with increasing concentration of liposomal cholesterol (Table 1). However, the rate of increase in ACAT activity and the rate of equilibrium of liposomal cholesterol with its substrate pool increased with increasing concentration of liposomal cholesterol transferred to the microvesicular vesicles. The transfer of liposomal cholesterol to the ACAT substrate pool is not direct in the sense that it is channeled to the vesicles that contain ACAT through a larger pool of microvesicular cholesterol, probably through plasma membranes.

Evidence for modulation in vivo of non-esterified cholesterol concentration in liver endoplasmic reticulum membranes

Starvation in rats is associated with a marked decrease in the activity of liver HMG-CoA reductase (Dietzchy & Brown, 1974) and an increase in the activity of ACAT (Erickson et al.,

1976; Mitropoulos & Venkatesan, 1977). In the case of ACAT, the fact that non-esterified cholesterol can be transferred to liver microsomal fraction from various donors and that this transfer is associated with a manifold increase in activity (Hashimoto & Dayton, 1979; Mathur et al., 1981), suggest that also at cellular level non-esterified cholesterol controls its activity. However, a relation between activity of HMG-CoA reductase of ACAT and concentration of non-esterified cholesterol in endoplasmic reticular membranes is difficult to establish, since we are unable to prepare pure populations of such membranes. Moreover it is
To see whether this condition is also associated with changes in interaction of HMG-CoA reductase with the endoplasmic reticular membrane the temperature-induced kinetics of the enzyme from rats in this condition were compared with those from rats that received food. Rats were denied food for 6 h and were killed at 07:00 h together with rats fed the standard diet or 2% cholesterol-supplemented diet for 6 h. The activity of HMG-CoA reductase was lower and ACAT was higher in the starved rats compared with these activities in rats given in the standard diet (Table 2). Both activities were similar in the starved rats and in those fed cholesterol-supplemented diet. The Arrhenius plots of the enzyme in the preparations from all three conditions showed a discontinuity in activation energy at about 28°C. Activation energies above this break were higher in the preparations from starved or cholesterol-fed rats compared with those in preparations from rats fed the standard diet.

In a different experiment rats were starved either for 12 or for 36 h and killed at 07:00 h, the same time as other rats fed the standard diet for 12 h. The activity of HMG-CoA reductase was lower and ACAT was higher in the starved rats compared with these activities in rats given the standard diet (Table 2). Arrhenius plots of HMG-CoA reductase in the preparations from rats fed the standard diet showed a discontinuity at about 28°C, whereas these plots for the enzyme from rats starved for 12 or 36 h showed a constant activation energy between 37 and 22°C. These latter plots have similar characteristics to those observed previously for the enzyme in microsomal fraction from rats fed overnight on a cholesterol-supplemented diet (Mitropoulos & Venkatesan, 1977; Venkatesan & Mitropoulos, 1982), from rats injected intravenously with a load of mevalonic acid (Mitropoulos et al., 1978a) or from rats fed overnight on a diet supplemented either with 10% safflower oil and 2% cholesterol or with 10% tristearoylglycerol and 2% cholesterol (Mitropoulos et al., 1980). All these conditions are also associated with a marked decrease in the activity of HMG-CoA reductase. The finding that changes in the interaction of the enzyme with the membrane are seen as clearly as changes in activity (Mitropoulos et al., 1978b) suggests that changes in the concentration of non-esterified cholesterol in endoplasmic reticular membrane can be the primary event in modulation of enzyme activity in vivo.

### Table 1. Effects of pre-incubation of liver microsomal fraction in the presence of [1H]cholesterol/phosphatidylcholine (1:1, mol/mol) liposomes on transfer of cholesterol and on ACAT activity

<table>
<thead>
<tr>
<th>Condition</th>
<th>Ratio of liposomal to microsomal cholesterol</th>
<th>Rate of ACAT increase x 10(-2) (activity/min)</th>
<th>Microsomal pool (pmol/min per ml)</th>
<th>ACAT substrate pool (pmol/min per ml)</th>
<th>Half-time of transfer of [1H]cholesterol to liver microsomal fraction (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.0</td>
<td>0.2</td>
<td>166</td>
<td>32</td>
<td>9.0</td>
</tr>
<tr>
<td>1</td>
<td>1.0</td>
<td>8.3</td>
<td>105</td>
<td>25</td>
<td>42</td>
</tr>
<tr>
<td>2</td>
<td>2.0</td>
<td>10.5</td>
<td>253</td>
<td>13</td>
<td>60</td>
</tr>
<tr>
<td>4</td>
<td>4.0</td>
<td>12.4</td>
<td>323</td>
<td>7</td>
<td>120</td>
</tr>
</tbody>
</table>

* Determined from the slope of the linear relation between activity and pre-incubation time.
† Calculated from the first order rate of unidirectional transfer of liposomal cholesterol.
‡ Calculated from the rate of increase in specific radioactivity of [1H]cholesterol esters formed on assay.

### Table 2. Effects of starvation on the activity of ACAT (a) and HMG-CoA reductase (b) in liver microsomal fraction

In Expt. 1 each condition contained four groups of rats killed at 01:00 h. Expt. 2 each condition contained three groups of rats killed at 07:00 h. All values are means ± S.D. for the number of groups in each dietary condition.

#### 1. Standard diet

<table>
<thead>
<tr>
<th>Experimental conditions</th>
<th>ACAT activity (pmol/min per ml of mixture)</th>
<th>Activity at 37°C (pmol/min per ml)</th>
<th>Break (°C)</th>
<th>Activation energy (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Standard diet</td>
<td>74 ± 25</td>
<td>251 ± 93</td>
<td>15.9 ± 0.6</td>
<td>45.2 ± 1</td>
</tr>
<tr>
<td>Starved for 6h</td>
<td>137 ± 1*</td>
<td>81 ± 23*</td>
<td>19.3 ± 3.8</td>
<td>45.4 ± 1</td>
</tr>
<tr>
<td>Cholesterol-supplemented</td>
<td>188 ± 2*</td>
<td>60 ± 28*</td>
<td>19.1 ± 2.1</td>
<td>41.4 ± 4</td>
</tr>
<tr>
<td>2. Standard diet</td>
<td>64 ± 2</td>
<td>119 ± 22</td>
<td>18.1 ± 0.2</td>
<td>50.3 ± 4</td>
</tr>
<tr>
<td>Starved for 12h</td>
<td>232 ± 31*</td>
<td>12 ± 7*</td>
<td>23.2 ± 3</td>
<td>18.2 ± 1</td>
</tr>
<tr>
<td>Starved for 36h</td>
<td>218 ± 45*</td>
<td>4 ± 2*</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Value is significantly different from the corresponding values for groups fed on the standard diet at the P < 0.01 level.
The role of oxysterols in the regulation of cholesterol biosynthesis

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Most mammalian cells are able to satisfy their requirements for cholesterol from either external (lipoprotein) or internal (biosynthetic) sources. Although the rate of cellular cholesterol biosynthesis appears to bear an inverse relationship to the availability of external lipoprotein cholesterol, the exact nature of the regulatory molecule(s) remains to be unequivocally established. Several oxygenated derivatives of cholesterol (oxysterols) are powerful inhibitors of cholesterol biosynthesis, an effect which appears to be due primarily, but not exclusively, to a decrease in the activity of HMG-CoA reductase. One of the aims of the present paper is to examine the relationship between oxysterol structure and inhibitory potency. Various hypotheses have been proposed to explain the means by which these compounds exert their effects at the molecular level and these will also be discussed. However, the main objective of this paper is to review the evidence for the natural involvement of a component of this type in the normal regulatory sequence of events culminating in the suppression of cellular cholesterol biosynthesis.

Relationship between oxysterol structure and inhibitory activity

Purified, non-lipoprotein cholesterol is a very poor inhibitor of cholesterol synthesis and of HMG-CoA reductase when added to the culture medium of several different types of cell in vitro (Bell et al., 1976; Kandutsch et al., 1978; Krieger et al., 1978; Peng et al., 1979; Drevon et al., 1980). It is probable that the inhibitory effects of cholesterol described in previous reports (e.g. Rothblat & Buchko, 1971) resulted from steroidal contaminants of cholesterol rather than from cholesterol itself (Kandutsch et al., 1978). These inhibitors have been identified as oxygenated cholesterol derivatives such as 7-oxocholesterol, 7α- and 7β-hydroxycholesterol and 25-hydroxycholesterol, all of which arise spontaneously from cholesterol by air oxidation. The ready susceptibility of cholesterol to such free-radical-induced air oxidation is such that it is difficult, during longer incubation periods, to completely exclude their non-enzymic contaminants of cholesterol rather than from cholesterol itself (Kandutsch et al., 1978). However, introduction of a second oxygen function into the molecule in addition to that at C-3 usually gives rise to a large increase in inhibitory activity. The position of the second oxygen function is of some importance in determining the inhibitory power of the derivative and Table 1 shows the activities of oxysterols classified according to substituent position. Although each sterol type shows a rather wide range of activity, selection of the most potent sterol in each group shows that, in general, the greater the molecular distance between C-3 and the second oxygen group, the greater the inhibitory activity. An intact (i.e. an iso-octyl) side-chain is also required for full activity, a gradual decrease in the length of the side-chain resulting in a gradual diminution in biological effectiveness (Kandutsch & Chen, 1974). Within some groups of steroids there appears to be an inverse relationship between inhibitory effectiveness and the extent to which the oxygen function is sterically hindered (Gibbons, 1983). For example, as the 15-hydroxy group becomes increasingly shielded by the presence of more bulky substituents at C-14, so the potency of the resultant steroid decreases (Schroepfer et al., 1979). In addition, introduction of axial hydroxy groups at the 3α-, 6β-, 7α- or 15β-positions produces steroids which are less inhibitory than those in which each hydroxy group is in the corresponding, less hindered equatorial conformation. Oxygen functions in the conformationally flexible positions such as those in Ring D and in the side-chain also appear to produce more inhibitory steroids and it has previously been suggested that the biological potency of steroid hormones is dependent upon conformationally flexible functional groups which permit effective hydrogen bonding or hydrophilic interactions with receptor molecules (Romers et al., 1974; Duax et al., 1980).

Molecular mechanism of oxysterol action

The sequence of events initiated by the entry of an oxysterol into (or formation within) the cell and culminating, amongst other effects, in the suppression of HMG-CoA reductase, is largely unknown. However, there is evidence that, as for some steroid hormones, there is a specific cytosolic receptor protein for oxysterols (Kandutsch & Shown, 1981). In general, the affinity with which this protein binds a particular oxysterol is directly related to the effectiveness of the steroid in inhibiting HMG-CoA reductase.

Much of the effort in this field has been confined to attempts to determine whether oxysterols decrease HMG-CoA reductase activity by decreasing the steady-state concentration of the enzyme (quantitative effects) or by decreasing the efficiency of pre-existing enzyme (qualitative effects). There is currently no general agreement as to which of these mechanisms is correct. However, any proposal involving modification of pre-existing enzyme would have to explain why the oxysterol-mediated suppression of enzyme activity requires not only the intact, integrated cell structure (Kandutsch & Chen, 1975; Erickson et al., 1978; Cavenee et al., 1981) but also continuing protein

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**Table 1. Effectiveness of oxysterols in the inhibition of HMG-CoA reductase in L-cells (all sterols contain an oxygen function at C-3)**

<table>
<thead>
<tr>
<th>Position of additional oxygen function</th>
<th>Range of activity (number of required % inhibition of 5α-cholestane)</th>
<th>Most powerful inhibitor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Concentration of steroid (μmol) tested</td>
<td>5α-Cholestane 3β,4β-diol</td>
</tr>
<tr>
<td>1</td>
<td>1.5–9.0</td>
<td>4 3β-Hydroxy-5α-cholestan-6-one</td>
</tr>
<tr>
<td>7</td>
<td>2.0–10.0</td>
<td>4 3β-Hydroxy-5α-cholestan-7-one</td>
</tr>
<tr>
<td>9</td>
<td>1.0–3.0</td>
<td>2 5α-Lanostane 3β,9α-diol</td>
</tr>
<tr>
<td>15</td>
<td>0.3–8.8</td>
<td>20 Several</td>
</tr>
<tr>
<td>32</td>
<td>0.5–3.3</td>
<td>10 14β-Hydroxyethylcholesterol-6-ene 3β-ol</td>
</tr>
<tr>
<td>20–25</td>
<td>0.05–3.5</td>
<td>5 25-Hydroxycholesterol</td>
</tr>
</tbody>
</table>

ABBREVIATIONS: HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; LDL, low-density lipoprotein.