review

Role of acyl-CoA:cholesterol acyltransferase in cellular cholesterol metabolism

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1. INTRODUCTION

In recent years the entry of cholesterol into cells by receptor-mediated endocytosis of plasma lipoproteins and the subsequent intracellular fate of free and esterified cholesterol has received much attention by workers interested in cholesterol metabolism and its role in pathological conditions such as coronary heart disease (1). Uptake by this route has a profound effect on the metabolic balance of cholesterol within the cell. Thus, as a result of entry of cholesterol, the intracellular rate of its synthesis is reduced and its esterification with fatty acids, catalyzed by the subject of this article acyl-CoA:cholesterol acyltransferase (ACAT), is increased.

The prototype for this cellular role for ACAT has been the cultured fibroblast, the model on which the low density lipoprotein (LDL) receptor pathway was originally based (2). Several tissues, however, have a more important role to play in the balance of cholesterol in the body and they show a more complex pattern of cholesterol metabolism than the fibroblast. In the liver, uptake of cholesterol leads to an increase in the amount of cholesteryl ester within the cell that is dependent on the activity of ACAT. This ester can be secreted by the liver in very low density lipoprotein (VLDL). Alternatively, free cholesterol may be directed towards bile acid synthesis, a process initiated by cholesterol 7α-hydroxylase which, like ACAT, is located in the endoplasmic reticulum. A similar fate may also occur with the cholesterol synthesized endogenously by the cell.

In steroid hormone-producing tissue such as the adrenal cortex, cholesteryl ester can be formed in the same way through ACAT activity. These tissues also contain an active hormone-sensitive cholesteryl ester hydrolase which releases free cholesterol from the esters stored in the cell. This sterol is then transported to the mitochondria where the first steps in the synthesis of steroid hormones occur. Here the balance of cholesteryl ester in the cell depends on the resultant of the activities of ACAT and the cholesteryl ester hydrolase.

In other tissues the flux of cholesterol is organized in different ways. In the intestinal epithelial cell, dietary cholesterol appears to be absorbed and directed towards esterification by ACAT, followed by secretion in chylomicrons in the lymph. A pancreatic esterase has also been implicated in this process (see below) but we believe that the weight of evidence at present favors the view that ACAT is the major enzyme involved. Events that may be related to the function of arterial cells and to their pathology also involve intracellular fluxes of cholesterol in which ACAT plays an important part. The non-uniform distribution of cholesterol between different cellular membranes also emphasizes the importance of the precise regulation of the intracellular flux of sterol. ACAT activity appears to respond to increasing the load of cholesterol in the cell, and the regulation of this activity is important to understand because it may reflect and contribute to the control of the state of cholesterol metabolism in the cell as a whole.

In this article we discuss the biochemical and cellular properties of ACAT in detail in liver and the intestine. We also briefly consider these topics in steroid hormone-producing tissue and in arterial tissue in an attempt to relate the function of ACAT as it is understood at present to the pattern of cholesterol metabolism in each tissue. We have covered the literature in most detail over the period since this subject was last reviewed by Spector, Mathur, and Kaduce in 1979 (3). Recent reports on ACAT in tissues not covered in detail here include lung fibroblasts (4), lung (5), lactating mammary gland (6), and arterial smooth muscle (7).

Abbreviations: ACAT, acyl-CoA:cholesterol acyltransferase; LDL, low density lipoprotein; VLDL, very low density lipoprotein; HDL, high density lipoprotein; TLC, thin-layer chromatography; HMG, 3-hydroxy-3-methylglutaryl.

2. BIOCHEMICAL STUDIES

2.1 Assay of acyl-CoA:cholesterol acyltransferase activity

The earliest assays of esterification of cholesterol by subcellular fractions used exogenous labeled cholesterol as substrate. However, as was fully discussed in the previous review (3), this method suffers from the major problem that the labeled substrate mixes to an unknown extent with the cholesterol that is present in the membranes of the microsomal fraction. Thus the specific activity and, indeed, the concentration of the substrate are not known and hence absolute values of enzyme activity cannot be calculated. The most widely used assay of ACAT requires a ^{14}C-labeled fatty acyl coenzyme A as substrate. However, many variations in procedure exist, making comparisons of the absolute activities reported from different laboratories difficult.

The assay procedure commonly used involves the incubation of up to 1 mg of microsomal protein with a fatty acyl-CoA, a thiol (dithiothreitol or reduced glutathione), and fatty acid-free bovine serum albumin. The latter component allows a higher fatty acyl-CoA concentration to be achieved than would otherwise be possible without partial solubilization of the membrane by the fatty acyl-CoA, which is a weak detergent (3, 8). The dependence of the rate of cholesteryl ester formation on the concentration of fatty acyl-CoA is complex (3, 9, 10). Increasing concentrations of fatty acyl-CoA initially promote formation of cholesteryl esters but, after a plateau region, any further increase in this substrate concentration rapidly inhibits esterification. Presumably, at this stage, the detergent properties of the CoA ester become dominant. A 2-min reaction time is usually suitable when oleoyl-CoA is used as substrate and 10 min is usual for palmitoyl-CoA, which is a slightly poorer substrate. Care should be taken with some commercial preparations of oleoyl-CoA to ensure that they are radiochemically pure.

The amounts of fatty acid-free bovine serum albumin used by workers vary from none (8) to about 1 mg/ml (10, 11). The concentration of fatty acyl-CoA achievable and hence the apparent rate of esterification depend upon the ratio of fatty acyl-CoA to bovine serum albumin. After the end of the reaction period, the products are usually extracted from the reaction mixture using chloroform–methanol 2:1 (v/v). It is advisable that an internal standard (e.g., [^{3}H]cholesteryl ester) be added to correct for losses in extraction. These esters are not commercially available but can be conveniently synthesized in the laboratory (12). The extracted lipids are separated by TLC on silica gel. An eluent such as petroleum ether–diethyl ether–acetic acid 95:5:0.5 (v/v/v) will separate the cholesteryl ester from the labeled substrate and also from triacylglycerol, a potential contaminant also synthesized by an enzyme located in the endoplasmic reticulum. For the reasons mentioned above and also because substantial variations in ACAT activity can be observed between groups of animals, it is essential that experiments measuring ACAT activity should be conducted using the appropriate internal controls.

As will be discussed below (section 2.2), there are reasons to believe that ACAT is not saturated with its substrate cholesterol in the microsomal membrane. Thus, the rates of cholesteryl ester synthesis determined by the methods just outlined in reality only estimate the rates under the conditions of the assay used and are dependent on the properties of the particular membrane preparation. They are not a true absolute activity of ACAT. One way to measure the activity of ACAT under conditions of saturation with cholesterol is to dissociate the microsomal membrane in a controlled way using a detergent. Additional substrate cholesterol can now be presented to the enzyme in a system that is less compartmentalized than the microsomal vesicles. Provided care is taken to minimize the inhibition of ACAT by detergent, which is a major problem (see below), assays can be performed which allow the traditional kinetic parameters for the dependence of ACAT activity on the concentration of cholesterol to be determined (13–15). This procedure, of course, removes the enzyme from the constraints of the membrane environment and it is this combination of membrane and enzyme that may be of greatest interest in some studies.

In studies of the formation of cholesteryl esters in isolated intact cells, a fatty acyl-CoA cannot be used as the radioactive substrate since it does not penetrate into the cell. A labeled fatty acid is routinely used in studies of this kind. This substrate readily enters the cell, is converted to its CoA ester, and can be esterified to cholesterol. Again, this procedure cannot give an absolute measure of enzyme activity since here the resultant of the activities of two enzymes and of a transport process are measured. The fatty acid itself may also be metabolized in different ways and the ester formed may be hydrolyzed in the experimental incubations. However, in cultured fibroblasts, the activities determined using oleic acid on intact cells agree well with those estimated from membrane fractions of the same cells using oleoyl-CoA as substrate (16).

2.2 Studies of ACAT activity in subcellular fractions

Most of the detailed studies of ACAT activity have used the enzyme from the microsomal fraction of rat liver. In general, the results from studies of rat liver have been applicable to other tissues and species as far as the basic properties of ACAT are concerned, but a number of important differences have recently emerged (see below).

Many studies aimed at defining the optimal conditions for the assay of ACAT activity showed that the enzyme is readily inhibited by detergents such as Triton or by

organic solvents like acetone and ethanol (3, 11, 17). The enzyme activity appears to be very sensitive to its environment in the membrane. This means that when detergents or organic solvents are used as vehicles for presenting potential activators or inhibitors to the enzyme, their effects must be taken into account in appropriate control assays.

It is well known that microsomal vesicles are a heterogeneous population. It is therefore important to know whether the distribution of ACAT activity within the microsomal fraction is uniform and how the distribution is related to other enzymes of cholesterol metabolism, cholesterol 7α-hydroxylase, and HMG-CoA reductase. A study of this problem, using sucrose density gradients to fractionate the microsomal vesicles, showed that ACAT activity was associated with RNA-rich vesicles (rough microsomes). In contrast, HMG-CoA reductase and cholesterol 7α-hydroxylase were both associated with vesicles with a low ribosomal coating (18). If labeled cholesterol was added to the whole population of vesicles and cofactors were provided for cholesterol esterification and 7α-hydroxylation, the specific activities of the two products were found to be different. This observation led to the suggestion that distinct substrate pools exist in the microsomal membrane for ACAT and for cholesterol 7α-hydroxylase. This conclusion may be of physiological significance since, at the level of the endoplasmic reticulum in liver, cholesterol can be directed towards bile and bile acid synthesis or towards secretion as ester in VLDL.

In these studies the cholesteryl ester was found to be mainly associated with the smooth microsomal fraction, separate from the esterifying enzyme. Hashimoto and Fogelman (19) made a similar observation, and also demonstrated that ACAT activity was associated with the cytoplasmic surface of the microsomal vesicle in agreement with another report (9). It was suggested that the cholesteryl ester synthesized in the rough endoplasmic reticulum was functionally transferred to the smooth membranes which acted as a trap for the esters. In the cell this process could be the first step of the packaging of the cholesteryl ester into VLDL. Studies on the transfer of cholesterol from heated smooth microsomes to RNA-rich microsomes containing ACAT activity led to the suggestion that cholesterol enhanced the measured activity both by increasing the supply of substrate and by acting as a non-substrate modulator. No suggestions were made as to the mechanism of such an activation by cholesterol (20).

Agents already known to increase cholesteryl ester synthesis in intact cells (e.g., in cultured fibroblasts, ref. 2) were readily shown to have a similar effect on the microsomal ACAT activity. Thus progesterone was found to be an inhibitor (10, 21) and 25-hydroxycholesterol activated ACAT (10, 21, 22). Studies on the concentration dependence of the inhibition of ACAT activity by progesterone have led to the suggestion that this steroid interacts with the enzyme at a site other than the catalytic site in such a way that an increase in the size of the pool of substrate cholesterol available to the enzyme is prevented (23). The inhibitory effect of progesterone has been shown to be reversible (21). On the other hand, 25-hydroxycholesterol can be esterified by the enzyme (progesterone, with no 3β-hydroxyl group, cannot), but the mechanism by which this hydroxy sterol activates liver ACAT has not been defined. It may be related to the promotion of access of free cholesterol to the enzyme (cf. studies on the intestinal enzyme). The effect of 25-hydroxycholesterol appears to be independent of other in vitro manipulations that increase the rate of cholesteryl ester synthesis by microsomal preparations (11, 24). These include addition of substrate in the form of liposomes or in organic solvent and activation by ATP and other factors, as will be discussed below. Since 25-hydroxycholesterol may be formed in the liver in vivo, the speculation has been made that this sterol is a biological regulator of ACAT as well as, possibly, HMG-CoA reductase (25, 26). The evidence for this hypothesis is however, at least in the case of ACAT, not substantial. Other hydroxy sterols have, in contrast, been shown to inhibit ACAT, but their actions on other enzymes of sterol metabolism make them unsuitable as experimental tools (10, 27).

The use of the detergent-based assay of ACAT activity allowed experiments on the substrate specificity of the enzyme towards sterols to be examined in a way similar to earlier studies on membrane proteins that have cholesterol as their substrate (28, 29). As had been observed previously for the rat liver microsomal cholesterol 7α-hydroxylase, the sterol side chain was found to be important in the interaction between the enzyme and the substrate. A sterol of the molecular length of cholesterol proved to be optimal (28). It is interesting to note that this dependence on the structure of the sterol is also found in biophysical studies on the interaction of cholesterol and phospholipids (30).

It was apparent from many of the earlier studies of microsomal ACAT activity that the amount of cholesteryl ester formed in assays could be increased by the addition of cholesterol from a detergent or organic solvent vehicle (10, 17). The concept arose that the microsomal pool of cholesterol that surrounds ACAT does not saturate the enzyme. More recently these experiments have been confirmed using cholesterol-rich liposomes to donate cholesterol to the microsomal vesicles. This method of delivery of cholesterol has the advantage that the inhibitory effect of detergents and organic solvents and also their effect on the membrane structure as a whole is avoided. ACAT activity has been increased by adding cholesterol in this way to liver microsomes from a variety of animals, including the rat (24, 31, 32), rabbit, guinea pig (Suckling, K. E. and J. M. Dietschy, unpublished observations), and monkey (33). Cholesterol-rich lipopro-

teins can also be used to deliver additional cholesterol to microsomal vesicles in vitro (34).

These experiments involving cholesterol enrichment of microsomes can be performed in two ways. Either the microsomes and the cholesterol donor are incubated together and the ACAT activity is determined directly in the incubation mixture (24), or the microsomes can be separated from the cholesterol-enriching medium by centrifugation after a suitable period of preincubation. Assays are then performed on the washed microsomal fraction (31, 32). In experiments of the latter design, it can be shown that cholesterol is transferred to the microsomal vesicles as the first and rate-determining step and this transfer leads to the observed increase in enzyme activity (34).

In a detailed study of the mechanism of the cholesterol transfer from liposomes and its subsequent esterification, Mitropoulos et al. (31) and Synouri-Vrettakou and Mitropoulos (32) observed that when rat liver microsomes were preincubated in the absence of a thiol (i.e., not the usual ACAT assay conditions in which a thiol is invariably present), a time- and temperature-dependent increase in the ACAT activity resulted. This increase was enhanced in a concentration-dependent way by including cholesterol-rich liposomes in the preincubation buffer. However, the increase was prevented by the presence of dithiothreitol. The authors calculated the rate at which labeled cholesterol from the donor liposomes equilibrated with the enzyme substrate pool in the microsomal vesicles and concluded that the observed increase in the amount of cholesteryl ester formed was due to transfer of cholesterol to the substrate pool of ACAT from both the donor liposomes and from other microsomal vesicles.

The transfer of cholesterol from liposomes to microsomal vesicles can be facilitated by lipid exchange proteins. These proteins, which are found widely in blood plasma but also inside cells, have varying specificities towards lipids. The nonspecific phospholipid exchange protein from rat liver is effective (35–37), as is the sterol carrier protein known as SCP_2 (38). Since cholesterol is known to be distributed in a specific way in cell membranes, substantially found associated with plasma membranes of cells, it is possible that such facilitated exchange of cholesterol between intracellular membrane compartments may have physiological significance.

From the preceding discussion of in vitro experiments it is apparent that several factors may contribute to the regulation of the activity of ACAT in isolated microsomal vesicles. Of these the most widely discussed has been the supply of substrate cholesterol, but in addition the effects of cholesterol on the enzyme itself by a possible allosteric mechanism and the opposing effects of 25-hydroxycholesterol and progesterone have been considered. Another candidate for a regulatory mechanism becomes apparent when we consider recent studies of the regulation of HMG-CoA reductase, another enzyme of the endoplasmic reticulum which, in certain situations, appears to be regulated in the opposite sense to ACAT (39). Extensive studies have shown that HMG-CoA reductase can be inhibited by a specific kinase as a short-term regulatory mechanism. The complementary activation can be catalyzed by one of a number of phosphoprotein phosphatases that are present in the liver cytoplasm (40). In steroid hormone-producing tissue, the enzyme that catalyzes the reverse of the ACAT reaction, cholesterol ester hydrolase, is activated by phosphorylation in response to the triggering of steroidogenesis in the cell by a trophic hormone such as ACTH in the adrenal cortex (41).

Three groups examined the possibility that ACAT from rat liver might be activated or inhibited by an ATP-dependent process (24, 42, 43). Although there were some differences in the detailed procedures adopted, the basic conclusion of all three groups was that in vitro ACAT activity could be increased by incubating microsomes with ATP, $MgCl_2$, NaF, and a protein fraction from the 100,000 *g* supernatant of the liver cell. Some apparent protein kinase activity was also present in the microsomes themselves, but the maximum activation effect was dependent on the presence of the supernatant fraction. The protein responsible for the activation was partially purified (43).

ACAT activity could be inhibited by incubation with $MgCl_2$ and a protein fraction from the 100,000 *g* supernatant. The activation and inhibition was reversible and could be repeated through several cycles of modification of activity. All three groups interpreted these observations as being consistent with the activation of ACAT by a protein kinase which would be opposite in effect to the known inhibition that took place with HMG-CoA reductase. It was also shown that the activation due to ATP and the other components was independent of the effect of adding extra substrate cholesterol to the microsomal vesicles from cholesterol-rich liposomes (24) and also of the activating effect of 25-hydroxycholesterol (11). Thus these three mechanisms for the activation of ACAT activity in microsomal preparations in vitro can act independently of each other and in an additive manner. The external stimulus for the activation by ATP is unknown: it may occur by a cAMP-mediated mechanism (42). However one report did not confirm this observation (24).

More recent reports have looked at the in vitro activation phenomena from different angles. In one (44), conditions were described in which ACAT activity was inhibited by an ATP-dependent process in vitro. The assay used the detergent-based method (section 2.1) and very long incubation times. Clearly these studies were carried out on a different system, detergent-treated membranes, from that described by the original reports which all concerned intact microsomes.

In another study the basic findings of activation of

ACAT by an ATP-dependent process were confirmed (45), but different conclusions on the mechanism by which the activation is achieved were reached on the basis of the kinetics of transfer of cholesterol to microsomal vesicles containing ACAT. These workers suggested that the events in the in vitro system may not involve a phosphorylation of the protein but instead affect the rate at which cholesterol can be supplied to the enzyme. As with all potential regulatory systems involving covalent modification, conclusive evidence can only be obtained using purified enzyme and a demonstration that phosphorylation occurs at a specific site.

A fascinating possibility was raised by the further observation made at about the same time that another liver microsomal enzyme, cholesterol 7α-hydroxylase, could be activated in vitro by an ATP-dependent mechanism (46, 47). Thus the three microsomal enzymes of importance in the direction of cholesterol metabolism in the liver, HMG-CoA reductase, cholesterol 7α-hydroxylase, and ACAT may be regulated together (24, 48). Under certain physiological conditions, for example during rapid uptake of cholesterol into the liver from chylomicron remnants, such a regulatory system would be consistent with most of the observations made in in vitro studies on the activities of these enzymes; although it should be noted that the effect of dietary cholesterol on the activity of cholesterol 7α-hydroxylase and on the rate of the synthesis of bile acids as a whole is a matter of some dispute (49, 50). There are also conditions and tissues where the rate of cholesterol synthesis (equivalent to the activity of HMG-CoA reductase in these discussions) appears to be regulated independently of ACAT activity (see below). Clearly, the question of the coordinated metabolism of cholesterol in cells and how this is translated into the activities of the individual enzymes involved is one that requires immediate attention.

2.3 Solubilization and reconstitution of ACAT activity — the effect of the membrane environment

Many of the questions raised by the experiments already described, as well as more detailed molecular questions about the mechanism and regulation of ACAT, could be approached if the enzyme could be solubilized and purified from the microsomal membrane and reconstituted in a membrane of defined lipid composition. This classical approach of membrane biochemistry has not been an easy one for ACAT. ACAT activity seems to be highly labile and sensitive to detergents and many of the conventional procedures have not been successful. In all of the methods used for reconstitution of solubilized ACAT activity, the key step appears to be efficient removal of detergent in order to achieve good yields and activity.

Two approaches have been followed in the published reports. Initially, the basic strategy adopted by Kaduce, Schmidt, and Spector (51) in their studies on ACAT from Ehrlich ascites cells was followed (12, 52). The proteins of the microsomal membranes were released using detergents and, in the first case using rat liver microsomes, the solubilized proteins were partially fractionated with polyethylene glycol, a procedure widely used in the purification of cytochromes P-450 from microsomal membranes (12). The detergent used for solubilization (Triton X-100) was then replaced with octyl glucoside and reconstitution was performed in the presence of liposomes of defined lipid composition. Dilution of the mixture and centrifugation yielded a microsome-like pellet with high ACAT activity. In the second procedure, using pig liver microsomes, the solubilized proteins were fractionated by a chromatographic procedure before reconstitution was carried out by dialyzing the detergent, deoxycholate, to low concentration in the presence of the desired liposomes. The resulting dialyzate contained ACAT activity (52).

In many respects both preparations showed similar properties. If the concentration of cholesterol in the liposomes used for reconstitution was increased, the resulting reconstituted system showed increased ACAT activity, in agreement with other in vitro studies. A level of cholesterol about 3–4 times greater than normally found in rat liver microsomes was necessary to achieve the highest activity. As with the preparation from Ehrlich cells, the ACAT activity was found to drop when the cholesterol in the membranes exceeded a certain level in the rat liver system. Presumably, here the activating effect of increased substrate supply is overcome by the well-known effect of cholesterol on the packing of lipids which causes the membrane to become more rigid. This change in activity correlated with the ordering of the lipids in the liposomes used for reconstitution as determined by a fatty acid spin probe (12). The drop in activity occurred at a defined cholesterol concentration and was independent of the type of phospholipid used in the liposomes, within certain limits. The importance of the fluidity of the membrane was emphasized by the observation that, if reconstitution was carried out using liposomes containing phospholipids with saturated fatty acyl chains, the observed ACAT activity was very low. Effects of the saturation of fatty acyl chains were also observed in a detergent-solubilized preparation from Ehrlich cells (53). In both cases liposomes prepared from egg yolk phosphatidylcholine gave rise to more ACAT activity than those prepared from dipalmitoyl or dimyristoyl phosphatidylcholine.

There appear to be some differences between the rat liver and pig liver preparations in their preferences for the phospholipid head group. In the pig liver system, liposomes composed of phosphatidylethanolamine or phosphatidylcholine gave good activity but phosphatidylinositol and phosphatidylserine inhibited the enzyme. In the rat liver system, the highest activities were observed when a mixture of phosphatidylcholine and phosphatidyl-

serine and cholesterol were used to form the liposomes used for reconstitution. In this lipid mixture the liposomes bore a net negative charge due to the phosphatidylserine, which also increased the activity over that produced by phosphatidylcholine and cholesterol alone. If the liposomes were made positively charged by including stearylamine, the resulting reconstituted system had very low activity. These varied observations may reflect the different environments taken up by ACAT when it is reconstituted by the two procedures as well as the different lipid mixtures used.

The deoxycholate-dialysis procedure was also applied to microsomes prepared from Chinese hamster ovary cells (54). The activation of ACAT caused by LDL, which can be observed in intact cells, was abolished in the reconstituted enzyme. This is consistent with the idea that the effect of LDL cholesterol on ACAT activity requires the presence of the whole LDL pathway for the internalization and utilization of the lipoprotein. This is clearly impossible in an isolated system. Experiments with this preparation also supported the idea that observed ACAT activity depends upon the size of the pool of cholesterol available to it.

A particularly striking demonstration of the importance of the transfer of cholesterol to vesicles containing reconstituted rat liver ACAT was made by incubating vesicles containing ACAT activity made from liposomes containing no cholesterol with cholesterol-rich liposomes (12). The rate of cholesteryl ester formation which was observed was linear over a period of about an hour and was thought to be the result of cholesterol being transferred from the cholesterol-rich vesicles to those containing the reconstituted enzyme.

The second approach to studying the effect of membrane lipids in modulating the activity of ACAT is to alter the phospholipid composition of the microsomal membrane directly without solubilization using a phospholipid-exchange protein (55). In this way changes in the fatty acid composition of the phospholipids can be made without altering the phospholipid:cholesterol ratio of the membrane. Membranes enriched in dipalmitoyl phosphatidylcholine by this method gave a lower ACAT activity than membranes into which the unsaturated dioleoyl phosphatidylcholine had been exchanged. Enrichment with 1-palmitoyl-2-linoleoyl phosphatidylcholine increased the ACAT activity by 20% over control but, in contrast, dilinoleoyl phosphatidylcholine enrichment led to an almost total loss of activity. This was surprising since measurements of the ordering of a fatty acid spin label in the bulk membrane phase enriched with either dilinoleoyl or dioleoyl phosphatidylcholine gave the same result. Thus the bulk membrane environment reported by the spin probe was not the only factor that affected the observed ACAT activity: some specificity towards the fatty acyl groups in the phospholipids may also exist. Such a microenvironmental effect would not be detectable by the spin label. Similar results with dilinoleoyl phosphatidylcholine were also reported by Hashimoto and Dayton (56) and both groups found that the phospholipid-exchange protein was not essential to produce this effect. Exchange occurred but at a slower rate in the absence of the exchange protein. The dilinoleolyl phosphatidylcholine apparently did not cause any detectable changes in the overall structure of the microsomes.

From most of the experiments described in this section one can draw the general conclusion that in vitro the measured activity of ACAT is affected by the supply of substrate cholesterol to the enzyme through the membrane. The ordering of the membrane ('fluidity') also plays a role, but this may be moderated by some specificity for the fatty acyl groups in the phospholipids. Dietary experiments to be described in a following section are also consistent with this view.

2.4 In vitro studies in the small intestine

In many respects, in vitro studies have shown intestinal ACAT activity to be similar to hepatic activity. Thus similar assay conditions can be used: oleate and palmitate are the preferred fatty acyl substrates. The apparent activity can be stimulated by preincubating the enzyme preparation with cholesterol-rich liposomes (22, 57, 58) or lipoproteins (59) rich in free cholesterol, particularly those from patients with LCAT deficiency. These data suggest that, under routine assay conditions with either a mucosal homogenate or microsomes as enzyme source, ACAT is not saturated with substrate from endogenous membrane-bound cholesterol. Most likely the added cholesterol directly serves as substrate for the enzyme after equilibration with the membrane, since exogenous labeled cholesterol is also readily esterified after preincubation with intestinal microsomes (60, 61).

Interestingly, the relative increase induced in ACAT activity by cholesterol-rich liposomes may be modified by treating the donor animals with different diets (57). With a given liposome preparation ACAT activity was stimulated 3.0-fold in control animals, while after cholesterol feeding this factor decreased to 2.2-fold (57). On the other hand, when intestinal cholesterol and bile acid uptake was reduced by feeding a diet containing cholestyramine, the same liposomes prompted a 4.2-fold increase in enzyme activity (57). From this evidence it appears that one possible in vivo mechanism of enzyme regulation is altering the availability of free cholesterol as substrate leading to a reciprocal variation of the in vitro effect of exogenous cholesterol.

As discussed in detail above (section 2.1), the hepatic enzyme is very sensitive to detergents. Little work has been done on the mucosal enzyme in this respect, although, in contrast to pancreatic esterase, ACAT is inhibited by taurocholate (59). This effect appears to be

due to a detergent action of the bile acid rather than a more specific interaction. As in the liver, progesterone inhibited ACAT activity in mucosal microsomes (22). A similar effect was observed in vitro with ethinyl estradiol. When this compound is administered to rats by injection it leads to an increase in hepatic ACAT activity (section 3.2). Thus its effect when added to membranes is probably distinct from its pharmacological effect in the intact animal.

Recent studies by Field and Mathur (22) have demonstrated that mucosal ACAT activity may be increased by 25-hydroxycholesterol. This effect was inversely related to the microsomal cholesterol content, suggesting that the hydroxysterol improved the availability of the substrate to the enzyme. This effect was independent of the esterification of 25-hydroxycholesterol itself.

It should also be noted that intestinal ACAT may be inhibited by agents such as 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) which block thiol groups necessary for enzyme action. This property is also common to other acyltransferases and may explain the protective effect of thiols on enzyme activity.

More exciting was the development of a series of inhibitors of intestinal ACAT which may also be used in vivo to inhibit cholesterol absorption (7, 62). One of these substances, N-(1-oxo-9-octadecenyl)-DL-tryptophan(Z) ethyl ester or Sandoz compound 57-118, drastically and specifically inhibits ACAT at micromolar concentrations without affecting acyl-CoA:retinol acyltransferase or other mucosal esterifying enzymes (62). β-Sitosterol, which differs from cholesterol only by an additional ethyl group at C-24, is a comparatively poor substrate for oleoyl-CoA-dependent esterification and, in particular, does not compete with cholesterol (58). Thus, the esterification rate for the plant sterol was only 1/60 of that of cholesterol and enrichment of the micosomal membranes with β-sitosterol did not interfere with ACAT activity as long as the membrane cholesterol content was unaltered. In addition to the substrate-controlled regulation of ACAT activity discussed above, recent in vitro work has also suggested that, similar to intestinal HMG-CoA reductase (63–66), mucosal ACAT may be regulated by a phosphorylation-dephosphorylation mechanism (24). Initially it was observed that ACAT activity was increased by a simple preincubation of a cell homogenate in phosphate buffer (57). Since reductase activity showed a similar response (63), experiments were carried out to see whether ACAT activity could be affected by an ATP-dependent mechanism. A stimulation of activity by 2 mM ATP was observed in the presence of sodium fluoride, indicating that in the 8,500-*g* supernatant fraction used there is both an ATP-dependent (activating) kinase and an NaF-sensitive (inactivating) protein phosphatase (24). On the other hand, the ACAT activated by ATP could be partly inactivated by 2 mM $MgCl_2$, probably through the action of a Mg^{2+}-dependent phosphatase. This reversible activation-inactivation could be shown to be independent of the substrate effect using cholesterol-rich liposomes (24).

Thus, in most respects the intestinal ACAT is similar to the hepatic enzyme. There is no evidence to prove the identity of both activities and, in other cells (section 5), major differences have been observed.

3. REGULATION OF CHOLESTEROL ESTERIFICATION IN THE LIVER

3.1 Studies in isolated and cultured hepatocytes

An obvious model system for a broader study of the role of ACAT in the liver is the hepatocyte. Parallel studies to those which demonstrated the importance of ACAT in the LDL pathway can be carried out and, in addition, the specific roles of the liver in cholesterol metabolism and those of bile production and VLDL secretion can be examined. In this respect the hepatocyte is a much more complex model cell than the 'classical' fibroblast. Like the intestinal epithelial cells discussed in this article, the hepatocyte has a functional polarity, with the sinusoidal face reflecting the uptake and secretion of lipoproteins and the bile canalicular face the secretion of free cholesterol and bile salts into bile. This polarity is, of course, functionally lost when the hepatocytes are released from the tissue during preparation.

Measurement of ACAT activity in intact cells requires, as has already been discussed, the use of a labeled fatty acid rather than a fatty acyl-CoA, and a number of studies of ACAT activity in hepatocytes have used this approach. Also, in some cases, the change in the amount of cholesteryl ester in the cell when subject to certain treatments has been used as an index of ACAT activity (67). Many of the studies of ACAT activity in isolated hepatocytes have been aimed at understanding the role of this enzyme in VLDL synthesis and secretion. Fewer attempts to integrate this with the corresponding questions in bile acid synthesis have been reported.

25-Hydroxycholesterol, which stimulates ACAT activity in microsomal preparations, was also shown to activate cholesteryl ester formation in isolated hepatocytes. Further activation was obtained when the cells were additionally incubated with mevalonic acid (68). This treatment presumably leads to an increase in the supply of substrate cholesterol to ACAT. Mevalonic acid also stimulated the formation of cholesteryl esters assayed using ^3H_2O as labeled precursor in freshly isolated hepatocytes (69). The VLDL secreted by cells in which cholesterol esterification was stimulated in this way contained elevated amounts of cholesteryl esters over the unstimulated controls. Thus, ACAT may be important in controlling the ester content of secreted lipoprotein (compare the

intestine). It was later shown that the neutral lipid composition of the hepatocyte directs the composition of the core lipids of the VLDL particle (70): cholesteryl ester-rich cells secreted cholesteryl ester-rich VLDL and triacylglycerol-rich cells secreted triacylglycerol-rich VLDL.

Pullinger and Gibbons (71) showed that incubation of hepatocytes with oleic acid increased the synthesis of cholesteryl ester and this resulted in an increase in the amounts of cholesteryl ester present in the core of the VLDL. Inhibition of the rate of endogenous synthesis of cholesterol did not significantly alter the rate of secretion of cholesterol in VLDL. Presumably the intracellular stores of cholesterol available to ACAT were high enough to prevent any reduction in the amount of cholesteryl ester available for secretion in lipoprotein.

In a further study, the regulation of ACAT and HMG-CoA reductase in cultured hepatocytes was compared (72). The importance of this comparison has been discussed already. This study confirmed that the synthesis of cholesteryl ester was increased by 25-hydroxycholesterol and mevalonic acid. Cholesterol derived from human HDL as a model extracellular source of sterol also activated the synthesis of cholesteryl ester, but with all these treatments the synthesis of triacylglycerol was not affected. The effects of 25-hydroxycholesterol and mevalonic acid were additive, suggesting that they act by a different mechanism (cf. the in vitro studies discussed earlier). These two treatments caused an inhibition of the activity of HMG-CoA reductase, but the characteristics of the inhibition were different from those of the complementary activation of ACAT. In particular, the effect of 25-hydroxycholesterol on intact cells (unlike the effect on microsomal fractions) was rapidly lost, being completely absent after 18 hr incubation. The effect of 25-hydroxycholesterol on HMG-CoA reductase continues for at least 22 hr, so it is likely that the mechanism of the action of this hydroxysterol on these two enzyme activities is different. It is possible that the effect on ACAT is mediated mainly through the membrane and that the effect on HMG-CoA reductase also involves an element of protein synthesis.

Mevalonic acid was shown to increase the level of intracellular cholesteryl ester in hepatocytes but, when intracellular synthesis of cholesterol was inhibited with mevinolin, no change was observed (73). Thus these cells responded to an increase in substrate supply from intracellular synthesis, but otherwise the levels of cholesterol were maintained from previously available sources in the culture medium. The authors suggested that the intracellular hepatic cholesteryl ester pool might slowly turn over to supply cholesterol for bile acid synthesis. However, it is not clear if the level of hepatic neutral cholesterol esterase activity is sufficient to supply the necessary amount of sterol (74). This comment is based on studies in which hepatocytes were prepared from rats that had been kept in a steady state of [^{3}H]cholesterol. These cells released cholesterol as ester into the culture medium and the secretion was inhibited by cycloheximide, colchicine, or EDTA. Cholesterol feeding increased the amount of cholesterol that was secreted. Free cholesterol from HDL was exchangeable with that of the cells and could be esterified. However, no hydrolysis of intracellular cholesteryl ester was observed, suggesting that the turnover of cholesteryl ester occurs mainly by secretion of the esters in VLDL (74).

Another inhibitor of ACAT, Sandoz compound 58-035 (3-[decyldimethylsilyl]-N-[2-(methylphenyl)-1-phenylethyl]-propanamide), was shown to be efficient in inhibiting enzyme activity in Fu5AH rat hepatoma cells and also in arterial smooth muscle cells in culture. The effect was also observed in microsomal fractions (7).

3.2 Studies in the intact animal

The last major approach to understanding the role of ACAT activity in the liver that we shall discuss is the measurement of enzyme activity in microsomal fractions prepared from the livers of animals that have been subjected to various preliminary manipulations, usually dietary or pharmacological in nature. Since many of these regimens are also known to affect HMG-CoA reductase activity, it is possible to compare the regulation of both activities under the same conditions in the same experiments.

Both HMG-CoA reductase and cholesterol 7α-hydroxylase are known to exhibit a marked diurnal variation in their activity (75, 76) and it is of obvious interest to know whether ACAT activity varies in line with these two enzyme activities. In one study (10), this was shown to be the case. ACAT activity at the mid-dark time point in rat liver microsomes (the peak of activity for both HMG-CoA reductase and cholesterol 7α-hydroxylase) was 1.4 times higher than at the mid-light point. However, such an effect was not observed in two other studies (17; Suckling, K. E. and J. M. Dietschy, unpublished results). It is possible that other effects such as the supply of substrate cholesterol, which has been implicated many times as contributing to the measured ACAT activity, masked any diurnal variation of activity in these studies.

In many published studies, ACAT activity and the activity of HMG-CoA reductase or the rate of synthesis of cholesterol, an almost equivalent parameter, have been measured together. It is convenient to classify the results of experiments of this type in the form in **Table 1.** In the table we can see that a group of treatments that include feeding a diet enriched in cholesterol or cholate or an 'atherogenic diet,' infusion of mevalonic acid or cholesterol-rich chylomicrons intravenously, injection of estrogen, and fasting all increase the ACAT activity and inhibit the rate of cholesterol synthesis. Several of these treat-

TABLE 1. Regulation of cholesterol metabolism in the liver

Treatment/Diet	Species	ACAT Activity	Cholesterol Synthesis: HMG-CoA Reductase	LDL Uptake	Bile Acid Synthesis: Cholesterol 7α-Hydroxylase
Cholesterol feeding	Rat	↑ 10,49	↓ 10	→ 150	? 49,50,76
	Rabbit	↑ [a]	↓ 155	↓ 149	→ 154
	Guinea pig	↑ 80,81	↓ 157,158		
	Hamster	↑ [a]	↓ [a],156	↓ [b]	
Cholate feeding	Rat	↑ 10	↓ 10	→ 151	→50,73↓159
Estrogen	Rat	↑ [a],49	↓ [a]	↑ 77	↓ 160
Fat-rich diet	Rat	↑ 10,83,84,94	↓ 10		↓ 76
Fasting	Rat	↑ 10	↓ 10	↑ [b]	↓ 76
Mevalonic acid i.v.	Rat	↑ 10,81	↓ 10,67	→ [b]	
i.g.	Rat	↑ 81,85			
Cholesterol-rich chylomicrons	Rat	↑ 85	↓ 152,153		
Cholestyramine	Rat	→ 10	↑ 10	→ 151	↑ 76,162
	Hamster	→ [a]	↑ [a],161	↑ [b]	
Fat-free diet	Rat	→ [a]	↓ [a]		
	Hamster	→ [a]	↑ [a]		
Triton WR-1399 injection	Rat	→ 10	↑ 10		
Progesterone	Rat	↓ 49			
Diurnal variation	Rat	? [a],10,17	✓75		✓76,162

The numbers refer to references in the bibliography.
[a]Suckling, K. E., S. Turley, and J. M. Dietschy, unpublished results.
[b]Spady, D. K., E. F. Stange, and J. M. Dietschy, unpublished results.

ments presumably cause an increase in the supply of substrate cholesterol to the endoplasmic reticulum of the liver, either by local synthesis (mevalonic acid) or through the circulation by increasing the available cholesterol in plasma for uptake by the liver (infusion of cholesterol-rich chylomicrons and cholesterol feeding and possibly also cholate feeding and the fat-rich diet). Ethinyl estradiol may increase the supply of cholesterol to the liver by raising the numbers of lipoprotein receptors (77). Fasting is known to increase the activity of LDL receptors (78; Spady, D. K., E. F. Stange, and J. M. Dietschy, unpublished results). Mevalonic acid administered intravenously or intragastrically may also increase the level of hepatic cholesterol. The suggestion of enhanced synthesis of cholesterol in peripheral tissues and subsequent transport from thence to the liver appears unlikely (79).

Certain treatments shown in the table affect HMG-CoA reductase activity in both directions, but not ACAT. These include a synthetic fat-free diet, feeding a diet containing the bile-salt sequestrant, cholestyramine, and injection of Triton WR 1399. Thus cases exist where ACAT activity and HMG-CoA reductase activity are regulated independently. It may be that the situations under which these two enzyme activities are regulated in parallel may be limited and that many apparent similarities in mechanism may conceal some fundamental differences. In fact, Erickson and her colleagues (10) showed that there is no quantitative correlation between ACAT and HMG-CoA reductase activities in rat liver microsomes prepared from animals subjected to a wide range of treatments. A positive correlation was found with the ACAT activity and the microsomal contents of free and also esterified cholesterol. Thus dietary experiments support the notion that ACAT activity is limited by the supply of cholesterol and that it is also reflected in the amount of cholesteryl ester found in the liver, especially in the microsomal fraction.

Progesterone, which inhibits ACAT, causes an increase in biliary cholesterol output when injected into male rats (49). The inhibition due to progesterone could be reversed by feeding the animals diets containing cholesterol or by injection of ethinyl estradiol. Both of these treatments led, as expected, to an increase in ACAT activity, and the interesting observation was made that the rate of biliary cholesterol secretion correlated inversely with the microsomal level of cholesteryl ester and the ACAT activity. This work suggests that the esterification of cholesterol entering the liver and the secretion of cholesterol in the bile are closely coupled processes. This raises important questions concerning the way in which the movement of cholesterol is organized in the liver cell.

A number of related experiments have been carried out in other laboratory animals. ACAT activity is increased by cholesterol feeding in the rabbit, guinea pig, and hamster (79, 80; Suckling, K. E. and J. M. Dietschy, unpublished results). Interestingly, in the rabbit, the liver

microsomal ACAT activity correlates positively with the plasma cholesterol over a range from normal up to several times normal (Suckling, K. E. and J. M. Dietschy, unpublished results). In the hamster, cholestyramine feeding increases HMG-CoA reductase activity but has no effect on ACAT activity, just as in the rat. In liver samples from a cholesterol-fed cynomolgus monkey, the ACAT activity is also increased over controls and this increase correlates with the microsomal cholesterol content (35).

Interestingly, the rate of cholesteryl ester synthesis estimated under similar conditions in the microsomal fraction of the livers of a range of species is approximately related to the rate of synthesis of cholesterol in these livers. In the rat, the rate of ester synthesis and of sterol synthesis are relatively high; the values in most of the other species for both activities are much lower. Thus the rat liver appears to be more active in cholesterol metabolism than that from other animals. This observation may be related to the fact that in the rat, unlike most other species, LDL is a minor lipoprotein and most of the cholesterol transported in the plasma is carried in a fraction of the HDL (82).

A more specific type of dietary experiment is to vary the fatty acids included in a semi-synthetic diet, thereby inducing changes in the fatty acids present in the cell membranes. In turn, this might cause an alteration in the physical state of the membrane, perhaps related to those discussed earlier. Mitropoulos, Venkatesan, and Balasubramaniam (83) fed rats diets enriched in either saturated or unsaturated fats and compared the kinetic behavior of the ACAT activity in microsomes prepared from the livers of these animals. The lipid composition of these membranes showed that the phospholipids had been enriched in the fatty acids that were fed (83). Enrichment in unsaturated fatty acids led to inactivation of HMG-CoA reductase, but ACAT was activated 1.5- to 2-fold over the enzyme from animals fed the saturated fat-enriched diet. Addition of cholesterol to the diet led to a further increase in ACAT activity, as would be expected. The changes in the kinetic parameters of ACAT observed in these experiments were thought to be consistent with the feeding of the unsaturated fat-rich diet, causing an increase in the substrate cholesterol available to the enzyme.

In a similar study, Spector, Kaduce, and Dane (84) showed that the ACAT activity of microsomes prepared from the livers of rats fed a diet enriched in unsaturated fat was 70–90% higher than the activity found in microsomes from rats fed a diet enriched in saturated fat. Both groups found that the saturation of the fat in the diet affected ACAT in the same way. However, other microsomal enzyme activities were affected in different ways, with no consistent trend being apparent. It seems likely from these experiments and from those discussed in the previous section that ACAT activity is stimulated by the presence of certain phospholipids containing unsaturated fatty acids and that a specific interaction may possibly exist.

The observations from in vitro experiments that ACAT activity may be regulated by phosphorylation–dephosphorylation (24, 42, 43) allowed a closer examination of the effects of increasing the load of cholesterol on the liver in the rat in vivo (85). By infusion of either mevalonic acid or cholesterol-rich chylomicrons, the ACAT activity in rat liver was increased within 1 hr of commencement of the experiment. The ACAT activity of microsomes from infused animals or control animals could be increased or decreased in vitro to identical maximum or minimum levels using the conditions defined by earlier work (24). Thus the expressed level of ACAT activity was altered by the infusion of substances that increased the supply of cholesterol to the liver. This was interpreted as being consistent with the increased supply of cholesterol to the endoplasmic reticulum, causing an increase in the active phosphorylated form of ACAT during the first hour of the experiment. It was thought likely that after this time the synthesis of new enzyme was also of importance.

4. REGULATION OF CHOLESTEROL ESTERIFICATION IN THE INTESTINE

Studies of the intestinal enzyme in vitro, discussed in section 2.4, showed that the regulation of its activity was similar in many respects to that of liver. In vivo studies have been concerned not only with the properties of ACAT itself but also with the relationship of this activity to that of the pancreatic esterase, which has also been thought to have a role in esterification of cholesterol in intestinal cells. Since the available evidence for the physiological role of this latter enzyme has been reviewed elsewhere (86), the present survey will focus on the properties of ACAT, although this topic is still not without controversy (87).

Following the initial description of coenzyme A-dependent esterification of cholesterol by rat intestinal mucosa by Haugen and Norum (60), a multitude of articles appeared presenting evidence for this process in the gut mucosa of a variety of species including guinea pig (61, 80, 88), rabbit (22, 58, 89, 90), char (91), man (92, 93), and rat (94, 95).

In rat (60) and man (93) there appears to be a gradient of ACAT-specific activity from the proximal intestine to the distal end with the highest activity being found in the proximal jejunum and the lowest in the duodenum and ileum. However, when the enzyme activity was determined in isolated epithelial cells from proximal and distal small bowel (57, 94), there were only minor differences. In the rabbit, the highest specific activity of ACAT was observed in the mid-gut, exceeding the activity in the

duodenum and ileum by 2-fold (90). It is striking that this longitudinal distribution of ACAT activity reflects the predominant sites of cholesterol absorption in the gut (95), whereas the rates of cholesterol synthesis are highest in both the proximal and distal ends of the intestine (96, 97). Since LDL uptake is uniform along the length of the intestine (96), the data are consistent with a role for ACAT in cholesterol absorption rather than in esterification of endogenous cholesterol.

On the other hand, along the vertical axis of the mucosa, ACAT activity is found predominantly in the villous cells of the rat (57, 94). In one differing study, the specific activities in crypt and villous microsomes were comparable, but due to a several-fold higher protein recovery, the crypt cells were thought to contain most of the ACAT activity (87). This applies to both the total and to the specific enzyme activity, with greater than 90% of the total esterifying activity of the epithelial cells found in the villous cell fractions. This activity in the villous cells exceeded that in the crypt cells by 2.7 times in the jejunum and 1.3 times in the ileum. Comparable results were obtained whether the mucosal cells had been obtained by EDTA treatment or by vibration (57, 94). In the chow-fed rabbit, however, there was a slight gradient of the specific activity of ACAT favoring the crypt cells. This was reversed after cholesterol feeding (90). Again, at least in the rat, there is an apparent inverse distribution of ACAT activity as compared with sterol synthesis as well as LDL uptake with respect to the villous/crypt axis (57, 96).

Intestinal ACAT activity, much like that of the hepatic enzyme, is subject to regulation by various interventions that are summarized in **Table 2.** For instance, further evidence for the concept that ACAT is involved in cholesterol absorption is based on the fact that the enzyme activity is enhanced after feeding a diet rich in cholesterol to rats (57, 94), guinea pigs (61, 80), or rabbits (90). In the rat, this leads to an increase in ACAT activity predominantly in the jejunum, as the main site of cholesterol absorption (95). In the rabbit, the effect is probably more uniform along the length of the gut. Probably depending upon the assay conditions and the preparation of the enzyme used (cell homogenate or microsomes), this result of cholesterol feeding is seen exclusively in the absorptive cells (94) or also in the crypt cells (57, 94), which are probably also exposed to luminal cholesterol although to a lesser degree. Under these circumstances, the most likely mechanism of stimulation of enzyme activity is an increase in substrate supply to an enzyme that is not saturated. This is reflected in the cholesterol content both of the whole cells and of isolated microsomes (57, 94). As discussed above (section 2.4), the decrease in the effect of adding liposomal cholesterol to a cell homogenate in vitro is consistent with this view (57), as is the decrease of the stimulatory action of added cholesterol in the medium of cultured intestine from cholesterol-fed rabbits (90) compared to biopsies from chow-fed animals. Apparently this increase was not blocked by the inhibitor of protein synthesis, cycloheximide (90), suggesting that the existing enzyme had sufficient capacity to accept the additional substrate. Taken together, the evidence both from the short-term culture and from the long-term feeding experiment is consistent with a substrate effect, which is usually accompanied by an enrichment of cellular free and esterified cholesterol pools to varying degrees in different

TABLE 2. Regulation of cholesterol metabolism in the intestine

Treatment/Diet	Species	ACAT Activity	Sterol Synthesis: HMG-CoA Reductase Activity	LDL Uptake
Cholesterol feeding	Rat	↑ 57,94	→ 57,164	→ 96
	Guinea pig	↑ 61	↓ 157,158	
	Rabbit	↑ 90	↓ 97,155	
Cholesterol/taurocholate	Rat	↑ 101	↓ 101,164	
Hyperlipoproteinemic parabiont rat	Rat	↑ 101	↓ 101	↑ 101
25-Hydroxycholesterol	Rabbit	↑ 22	↓ 22	
Mevalonate	Rat			↓ [a]
	Rabbit		↓ 166	
Cholestyramine feeding	Rat	→ 57	↑ 57	→ 96
	Rabbit	↓ 98		
Sitosterol feeding	Rat		↑ 164	
	Rabbit	↓ 58		
Fasting	Rat	↑ 91	↓ 163	→ [a]
Corn oil feeding	Rat	→ 57	↑ 57	→ 96
Safflower oil feeding	Rabbit	↑ 98		
Diurnal cycling (end of light period)	Rat	↑ 103	↓ 165	

[a]Spady, D. K., E. F. Stange, and J. M. Dietschy, unpublished results.

species. However, it is entirely possible that there are additional mechanisms, such as enzyme phosphorylation or protein synthesis, which have not yet been studied under in vivo conditions in the intestine.

The opposite type of intervention, reducing the absorption of cholesterol by giving cholestyramine or surformer, did not significantly affect ACAT activity in the rat intestine, although both interventions dramatically enhanced cholesterol synthesis and increased the cholesterol content of the enterocyte (57). In the rabbit, cholestyramine significantly decreased the enzyme activity (98). The microsomal cholesterol content was also diminished which suggested that this species responds with an inadequate increase in synthesis of cholesterol. Alternatively, the data from both the rabbit and the rat may be explained by a pool model where locally synthesized cholesterol does not equilibrate with microsomal cholesterol and is a poor substrate for ACAT (57). Similarly, sitosterol, which is known to inhibit cholesterol absorption and to stimulate synthesis of sterol in the gut, reduced the microsomal cholesterol content and, concomitantly, ACAT activity on rabbit intestine in vivo (58).

The role of ACAT in the esterification of locally synthesized cholesterol in the mucosa is controversial. While labeled mevalonolactone was recovered in the cholesteryl ester fraction in a dose-dependent fashion when incubated with isolated enterocytes, the incorporation of [^{3}H]oleic acid was not increased (99). This paradox may be explained by a rapid metabolism of mevalonate to fatty acids (99), which are subsequently esterified with cholesterol and appear as label in the cholesteryl ester band on thin-layer chromatography. Indeed, this process has recently been demonstrated in cultured mucosa (100). It was clearly shown that, even in a situation with a dramatically stimulated synthesis of cholesterol from mevalonate, less than 8% of the label in the total cholesterol was found in the sterol component of the cholesteryl ester. This result is in contrast to reports of studies with cultured hepatocytes (72) and indicates that, unlike hepatic ACAT, the intestinal enzyme esterifies predominantly absorbed, exogenous cholesterol from dietary or biliary sources, rather than from locally formed sterol. There is also recent evidence to suggest that intestinal ACAT responds to increased uptake of plasma lipoproteins in a system of parabiotic rats, where one of the pair is fed a diet rich in cholesterol and the other is rendered hyperlipoproteinemic through cross-circulation, but is fed a normal diet (101).

The effect of dietary fat on ACAT activity in the intestine depends both on the load of fat administered and on the type of fat used. A rapid intraduodenal perfusion of cholesterol-free lipid in the rat prompted a rapid drop in the mucosal free cholesterol content, ACAT activity, and secretion of cholesteryl ester into the mesenteric lymph in chylomicrons (102). Similarly, oleic acid present in the medium of organ-cultured small intestine suppressed ACAT activity (90). A diet containing 10% corn oil given to rats for 3 days did not significantly change ACAT activity (57), while a diet containing 20% sunflower oil actually led to a stimulation of enzyme activity (94). A similar effect has been demonstrated in the guinea pig fed a diet containing 10% cottonseed oil (61). When safflower oil and coconut oil at a 10% level in the diet were compared in the rabbit (98), the unsaturated fat led to an increase in ACAT activity over the saturated fat without altering the microsomal cholesterol content. Thus the fatty acid composition of the microsomal membrane appears to influence enzyme activity, possibly by altering the access of substrate to enzyme (98, see also section 2.3).

During the diurnal cycle, the maximum of intestinal ACAT activity was observed at the end of the light period (103). Feeding suppressed and fasting stimulated ACAT activity independently of the assay system and substrate used (103). These changes were apparently paralleled by corresponding changes in the amount of unesterified cholesterol in the microsomes, which was slightly higher under fasting conditions (103). It is noteworthy that, also under these various manipulations as well as cholesterol feeding, ACAT activity reflects the lymphatic secretion of esterified cholesterol and the mucosal cholesterol content (61, 80, 97).

In a more clinical perspective it was shown that ACAT activity is normal in the small intestine of pancreatectomized patients but it is low in cases with mucosal atrophy due to gluten enteropathy, inflammation, or malabsorption (92). The latter finding, of course, is not surprising since such atrophic mucosa is devoid of absorptive cells which contain the bulk of intestinal ACAT activity (57, 94).

Finally, it should be stressed that inhibition of intestinal ACAT activity by compound 58-035 causes a pronounced malabsorption of cholesterol when administered in vivo, both in the cholesterol-fed rabbit (64) and in the lymph-fistula rat with a cholesterol bolus or infusion into the stomach or duodenum (104). In the rat, a 62% inhibition of mucosal ACAT was followed by a 50% reduction in the lymphatic secretion of [^{3}H]cholesteryl ester after a gastric bolus of labeled cholesterol was administered. Notably the secretion of triacylglycerol or unesterified cholesterol was unaffected by the drug. During a continuous duodenal infusion in these animals with normal pancreatic function and unimpaired mucosal cholesterol esterase activity, the secretion of absorbed cholesteryl ester mass could be virtually abolished. Interestingly, the esterification and lymphatic output of exogenous cholesterol was decreased considerably more than the output of cholesteryl ester from endogenous sources, such as synthesis and bile (104). From these results it seems reasonably well founded that the intestinal ACAT activity is actively involved in the esterification and thus in the absorption process of cho-

lesterol. Nevertheless, it cannot be excluded that additional mechanisms may also be important (105–107), although current evidence suggests that the cholesterol esterase of pancreatic origin is functional at the hydrolysis and uptake step (108) rather than during intracellular esterification (109), and may be a contaminant of luminal origin in intestinal cell preparations (89).

5. CHOLESTEROL ESTERIFICATION IN STEROID HORMONE-PRODUCING TISSUE

Steroid hormone-producing tissue, like liver, presents a complex pattern of cholesterol metabolism. Most of the tissues to be described here have been shown to take up cholesterol from plasma lipoproteins and to use this sterol for steroidogenesis. It is generally thought that cholesterol derived from this source is the primary substrate for long-term steroid hormone synthesis (110), but in the acute phase of stimulation of the tissue with the trophic hormone, the often substantial intracellular cholesterol pool must play a part (41, 111). The importance of the intracellular pool of cholesteryl ester varies between steroid hormone-producing tissues. The rat adrenal cortex contains large amounts of cholesteryl ester. At the other end of the scale, we have the Leydig cell from the testis which contains relatively little ester (112). The immediate fate of the cholesterol taken up from lipoproteins after hydrolysis in the lysosomes is unknown. This may depend upon the metabolic state of the tissue.

The cholesterol from the ester pool is made available to the cell by a soluble cholesteryl ester hydrolase which, unlike liver but similar to some arterial and nervous tissue, is hormone-sensitive. Stimulation of the cell by a trophic hormone leads to activation of the cholesteryl ester hydrolase through a cyclic AMP-dependent protein kinase (41, 112). Thus the ratio of free to esterified cholesterol is determined by a balance between the activities of ACAT and cholesteryl ester hydrolase.

Studies on hepatic ACAT activity have implicated a number of regulatory mechanisms (sections 2 and 3). Bovine adrenal cortical microsomes were examined to determine whether these effects could be reproduced in this tissue (11). Unlike the situation in the liver, the synthesis of cholesteryl esters was not increased by 25-hydroxycholesterol or by cholesterol added in organic solvent. This suggested that the environment of the enzyme in the microsomal membrane differed from that in the liver. Since cholesteryl ester hydrolase is activated by phosphorylation in the adrenal, it would seem illogical for ACAT to be activated in the same way as in the liver, by an ATP-dependent mechanism. It was found that conditions that favored activation of hepatic ACAT (ATP/$MgCl_2$, NaF) led to an inhibition of bovine adrenal cortical microsomal ACAT activity. Activation could also be achieved in an inverse way to the liver. As with the liver, both of these in vitro manipulations were reversible (11).

Many of the studies of the cholesteryl ester pool of steroid hormone-producing tissue have involved the measurement of the amount of cholesteryl ester in the cells, or the formation of esters has been estimated from the rate of incorporation of labeled fatty acid. Because the steroid hormone-producing tissue contains an active cholesteryl ester hydrolase, whose activities may vary several-fold depending on the conditions of the experiment, such measurements do not give an accurate estimate of ACAT activity. Rather the value obtained is the resultant of cholesteryl ester hydrolase and ACAT activities, depending also, in the case where a labeled fatty acid is used, on the size of the intracellular pool of cholesteryl ester. However, indications of the rate of cholesteryl ester formation can be inferred from these studies if care is taken not to over-interpret them. Experiments of this kind, relating to the uptake and use of lipoproteins for the synthesis of steroid hormones, have been reviewed in detail recently (110). The systems that have been studied include bovine adrenal cortical cells, mouse adrenal tumor Y1 cells, human choriocarcinoma cells, and rat ovarian cells. In general, the synthesis of cholesteryl esters in these cells is stimulated if the cells are first cultured in a lipoprotein-deficient medium and then have cholesterol made available to them from lipoproteins. The appropriate trophic hormone, which would stimulate the production of steroid hormone, tends to decrease the synthesis of cholesteryl esters. If, however, the oxidation of cholesterol to pregnenolone in the mitochondria is inhibited by the use of the specific inhibitor of cholesterol side chain cleavage P-450, aminoglutethimide, cholesteryl ester formation is again stimulated. Presumably the cholesterol not deposited in the mitochondrial membrane becomes available to ACAT and is esterified (see also below).

It is important to bear in mind both the method of preparation of the cells and the time period of the experiment when comparing different studies of this type in isolated cells. For example, in short-term studies of cholesteryl ester formation in monolayers of bovine adrenal cortical cells, the rate of ester formation was found to depend on the supply of cholesterol (113). Cholesterol could be supplied from endogenous synthesis from mevalonic acid, which caused an increase in the rate of ester formation. More interestingly, in the presence of ACTH the rate of cholesteryl ester formation from [^{14}C]oleic acid was found to increase after between 1 and 2 hr of stimulation. This is the most rapid demonstration of free cholesterol being made available to ACAT in steroid hormone-producing cells. Presumably the free cholesterol that was released from the cholesteryl ester store is not transferred to the mitochondira with complete efficiency at this stage and so finds its way to

ACAT in the endoplasmic reticulum. The effect disappeared after 2 hr of stimulation, by which time the steroidogenic system had reached full efficiency. This view was confirmed by the demonstration that at all time points cycloheximide, which inhibits the transport of cholesterol to the inner mitochondrial membrane where $P\text{-}450_{scc}$ is located, completely inhibited the synthesis of cortisol by the cells but markedly stimulated the production of cholesteryl ester. As observed in earlier studies, cholesterol that cannot be oxidized to hormone appears to be esterified, perhaps in order to keep the amount of free cholesterol in the cell down to an optimal level. All these levels of ACAT activity were invariably inhibited by the drug 58-035, which did not inhibit any major enzyme of cholesterol metabolism in the bovine adrenal cortical cells. As in bovine adrenal cortical microsomes, 25-hydroxycholesterol had no effect on the rate of cholesteryl ester synthesis in these cells. The mechanism and reason for this difference from most other tissues needs investigating.

Progesterone was found to inhibit ACAT activity in human choriocarcinoma cells and it was suggested that this may have a physiological function (114). However the levels of progesterone found in vivo in rat ovaries were not enough to be able to inhibit ACAT (115).

Other recent studies in a Leydig cell tumor line known as MA-10 have confirmed the general picture just outlined (112). In these cells the regulation of the use of endogenous cholesterol from intracellular stores was shown to be independent of the classical lipoprotein-uptake pathway, which also could operate under appropriate conditions. Thus, in steroid hormone-producing tissue we have the interaction of at least two different mechanisms of cholesterol supply, one hormone-sensitive tending towards hydrolysis of cholesteryl ester stores, and one responding to the absence of cholesterol reflected in the lipoprotein receptors on the cell surface and tending towards promoting esterification of the cholesterol entering the cell.

Studies have also been reported on ACAT activity in steroid hormone-producing tissue based on dietary and other manipulations on the whole animal. In these cases the microsomal fraction was usually prepared from the tissue, so that the ACAT activities measured do not have the added complication of the cholesteryl ester hydrolase activity found in studies in isolated cells.

The importance of the plasma lipoproteins as a source of cholesterol for steroidogenesis and of substrate for ACAT was demonstrated in the rat adrenal (116). The levels of circulating lipoproteins were decreased using the drug 4-aminopyrazolopyrimidine (4-APP). The ACAT activity in microsomes isolated from the adrenal glands of rats given this drug was very low. This was restored to control levels by the infusion of LDL (116). LDL is not the major carrier of cholesterol in the rat blood but under the pathological conditions induced by 4-APP it is likely that LDL is just as satisfactory as the more readily utilized rat HDL.

The plasma levels of corticosterone, the major glucocorticoid of the rat, and of the corresponding trophic hormone, ACTH, are known to show a diurnal variation. The activity of cholesteryl ester hydrolase in rat adrenals probably parallels this (41, 117, 118; there are some differences in the literature on this point). ACAT activity appears to vary inversely with cholesteryl ester hydrolase in these experiments (117–120) whether changes are induced acutely by stress or through the diurnal variation. These results agree with the picture of the inverse regulation of cholesterol esterification and ester hydrolysis that can be deduced from the in vitro experiments described above.

A good model system for ovarian tissue is the immature rat ovary stimulated with hCG. If female rats are so treated, the cholesteryl ester hydrolase activity in the ovary rises within 2–3 days (15, 115). At this stage the ACAT activity has also slightly risen. After 6–7 days, the peak of output of steroid hormone is reached and both activities of cholesteryl ester synthesis and hydrolysis are also at a peak level. This effect, which apparently is in contrast to that in the rat adrenal, shows the response of the cells as they develop into pseudo-mature steroidogenic cells. In these studies evidence was presented to show that the regulation of ACAT and HMG-CoA reductase was probably related to the intracellular levels of cholesterol and that the regulation of cholesteryl ester hydrolase was achieved through a different mechanism. Experiments similar to those described above using aminoglutethimide to stimulate ACAT activity by increasing intracellular substrate supply and 4-APP to decrease it by limiting supply from extracellular sources were performed. The analogue to ACTH in the ovary, LH, stimulated cholesteryl ester hydrolase and also inhibited ACAT activity. Thus, in the mature cell the pattern of activity was identical to that of the adrenal.

From these examples the main trends of ACAT activity in steroid hormone-producing tissues can be discerned. Most of the differences between the studies reported result from the way in which the experiments were designed, whether they were intended to demonstrate the existence of an LDL pathway in the cells, for example, or to determine the acute or long-term response of the tissue to a trophic hormone. Thus the supply of cholesterol to ACAT induced by one of several means, from lipoprotein or by directing the cholesterol to the endoplasmic reticulum using inhibitors of the further stages of cholesterol metabolism, leads to stimulation of ACAT activity. This may be reduced when the cells are stimulated with their trophic hormone. ACAT activity may also be inversely related to that of cholesteryl ester hydrolase. There is some evidence for this, and some existing evidence that has not been

interpreted in this way may also be consistent with this view. As with the liver, defined enzymological studies on ACAT remain to be done.

6. ACAT ACTIVITY IN ARTERIAL TISSUE

Characteristically, the development of atherosclerotic disease is associated with the accumulation of cholesterol and cholesteryl ester in the intima and media of certain arteries. The cholesteryl ester deposits may be intra- or extracellular and may differ with respect to their fatty acid moiety (121). The origin of the lipid may be from the plasma through infiltration of plasma lipoproteins, in particular LDL. Alternatively, a part of the cholesteryl ester from the lipoprotein may not simply be deposited in the intima, but rather taken up by various cellular elements, such as smooth muscle cells or macrophages, and subsequently be hydrolyzed and partially re-esterified intracellularly. In addition the arterial tissue, like most other organs in the mammalian body, is able to synthesize cholesterol, and it is likely that a portion of this newly formed cholesterol is also esterified. These various mechanisms in one of the key processes of atherogenesis and their quantitative significance are discussed in some detail elsewhere and the reader is referred to previous reviews on the subject (121–124).

In the arterial wall, intracellular cholesteryl ester metabolism is handled by a variety of enzymes such as ACAT, LCAT, cholesteryl ester hydrolase, acid cholesterol hydrolase, and cholesterol esterase (122). Again, this general subject has been previously dealt with in excellent overviews (123, 124) so that this current presentation will be limited to newer aspects of arterial ACAT and its regulation in specific cell types in cultures derived from endothelial cells, smooth muscle cells, and macrophages.

6.1 In vitro studies

A low basal ACAT activity has been described in arteries from the rat (125), rabbit (126–132), pigeon (125, 133), squirrel monkey (134), rhesus monkey (128), dog (125), cockerel (125), swine (135), and man (136). In contrast to most other tissues, very little, if any, labeled exogenous cholesterol is esterified when added to arterial microsomes, which contain the bulk of ACAT activity. As with other tissues, the activity is more easily measured using labeled fatty acid and cofactors or fatty acyl-CoA (126–128). Arterial ACAT activity is preserved by a sulfhydryl-protecting agent like dithiothreitol (126) and the enzyme is inhibited by detergents like sodium taurocholate and sodium dodecylsulfate (135). In these respects the arterial enzyme is very similar to the liver and intestinal systems.

In a similar manner, the arterial enzyme activity may be modulated by increasing the concentration of microsomal cholesterol by preincubation with cholesterol-rich lipoproteins (131) or with 20% glycerol/0.2% deoxycholate (130). Various enzyme inhibitors have been described (128) and their effect seems to be mediated in part by action on the microsomal lipid structure. To our knowledge there is at present no published evidence for an inhibitory action of one of the new ACAT inhibitors (such as 57-118) on arterial ACAT directly, except in smooth muscle cells (7).

6.2 In vivo studies

A variety of studies in different animal species has clearly shown that arterial ACAT activity is enhanced many fold when measured in animals with experimental atherosclerosis as well as in atherosclerotic human tissue (127–133, 135). There are conflicting data, however, with respect to the mechanism involved. Clearly, during experimental atherosclerosis in cholesterol-fed animals the microsomal cholesterol content is increased several fold (127, 130, 131), but it has been argued that the 2.3-fold increase observed in the rabbit should not lead to the 25-fold increase in ACAT activity observed in these studies (127). On the other hand, it is hard to predict the changes in the amount of cholesterol in the immediate microenvironment of the enzyme from the cholesterol content of the whole microsomal fraction. Other workers (122) suggested that there is both an increase in the molar ratio of free cholesterol and phospholipid and an increased amount of enzyme, since in their hands exogenous cholesterol added in vitro did not further enhance the enzyme activity in normal or atherosclerotic tissue. They concluded that ACAT under both conditions is saturated with substrate cholesterol, which is not in agreement with the studies cited above (131). Surprisingly, the cholesterol esterifying activity correlated well with the concentration of free cholesterol in the atherosclerotic microsomes but not in the normal microsomes (130). When atherosclerotic microsomes were adjusted to the cholesterol content of normal microsomes by incubation in glycerol/deoxycholate, the enzyme activity was still 4-fold greater than in normal microsomes, also suggesting that both substrate components and the amount of enzyme protein are involved in the enhancement in the rate of cholesteryl ester formation in atherosclerotic tissue (130). In contrast, the same authors report that when normal or atherosclerotic microsomes were enriched in cholesterol, their ACAT activity increased linearly over the concentration range studied and that the activity was essentially the same for both preparations when studied at the same cholesterol content (131). It seems that a final answer to this question will only be given when the enzyme is available in purified form and antibodies have been raised to allow an immunological approach for measuring the amount of ACAT enzyme.

Since oleic acid is synthesized very actively in athero-

sclerotic arteries and is the preferred fatty acyl substrate for arterial ACAT, it is likely that this locally synthesized fatty acid is used by ACAT. Thus the intracellular cholesteryl ester of foam cells is largely cholesteryl oleate, whereas the extracellular cholesteryl ester is mostly cholesteryl linoleate derived from plasma lipoproteins that have filtered into the intima/media (121).

6.3 Studies in cultured cells

The regulation of ACAT activity in cultured cells is tightly linked to the rate at which these cells take up lipoprotein cholesterol through a variety of mechanisms. The now classical fibroblast model in which ACAT activity is increased as a result of receptor-mediated endocytosis of lipoprotein appears to operate in arterial cells (137). Nevertheless, there are specific differences that characterize these events in the cultured endothelial cell, smooth muscle cell, and macrophage that will be considered in the remainder of this section.

a. Endothelial cells. Subconfluent cultures of endothelial cells exhibit principally the same LDL receptor pathway as the cultured fibroblast with high affinity binding and rapid uptake of LDL (138–144). VLDL but not HDL appear to compete for this binding site (138). There is a separate receptor for HDL (139). Native LDL caused a down-regulation of cholesterol synthesis and increased the incorporation of oleic acid into cholesteryl ester when added to proliferating subconfluent cultures, but these effects were absent in contact-inhibited cultures (140). The apparent reason for this difference is the lack of internalization of LDL in the stationary cultures, which do, however, bind LDL normally (138). In contrast, cationized LDL, which enters these cells independently of the LDL receptor, enhanced cholesteryl oleate formation both in confluent and subconfluent cultures, probably by modifying ACAT activity, which was not measured directly in these studies (140). The role of HDL in the processes is still unclear, although it has been shown to stimulate HMG-CoA reductase activity and cell proliferation (141). Another interesting observation in cultured endothelial cells is the expression of a binding site for chylomicrons (142). Unlike LDL, which is usually taken up as an intact particle, the chylomicron is only bound to the cell, and releases core triacylglycerol and cholesteryl ester which enter the cell by unknown mechanisms and are hydrolyzed in lysosomes. During this process the apoprotein of the chylomicrons remains at the cell surface. The free cholesterol made available to the cell exceeds the amount synthesized and suppresses cellular sterol formation, but the stimulation of ACAT activity that would be expected has not been demonstrated under these conditions. However, it has been shown that chylomicrons increase both the free and esterified cholesterol content of these cells (142).

b. Smooth muscle cells. Like the fibroblast, cultured smooth muscle cells exhibit a tight regulation of cellular cholesterol content by adjusting the LDL receptor-mediated uptake of cholesterol, sterol synthesis, and sterol esterification to the needs of the cell (140–142). Thus, for example, esterification of cholesterol is dramatically increased when the cells are incubated with native or cationized LDL (140). More exciting is the fact that the regulated LDL receptor can be by-passed by incubating smooth muscle cells with positively charged LDL (N,N-dimethyl-1,3-propanediamine-LDL) (143). Using this technique for overloading these cells with cholesterol, it has been shown that large deposits of cholesteryl ester are formed in the cytoplasm, thus reproducing some features of the atherosclerotic foam cells in vitro (143).

c. Macrophages. In addition to possessing the classic LDL receptor, macrophages avidly bind, internalize, and degrade acetylated LDL. These derivatives are not recognized by the fibroblast LDL receptor (144). This unique property is found only in scavenger cells like rat peritoneal macrophages, guinea pig Kupffer cells, and cultured human monocytes (144). As a consequence of the uptake of acetyl-LDL, the synthesis of cholesteryl ester is stimulated 100-fold and the cholesterol content is stimulated 38-fold (144). ACAT activity was also increased and the cells stored cholesteryl ester reversibly within cytoplasmic lipid droplets. Again, the cholesteryl ester derived from the lipoproteins was first hydrolyzed and then re-esterified, preferentially with oleate (145).

Macrophages derived from human monocytes also metabolize reductively methylated LDL independently of the LDL receptor at a rate that exceeds the rate of simple fluid endocytosis (146). In contrast to the fibroblast, which has been reported to be unresponsive to sterol taken up by the non-LDL receptor pathways (147), the macrophages exhibited a suppression of HMG-CoA reductase and a concomitant stimulation of ACAT activity (146).

One of the most interesting features of cultured macrophages is a continuous, energy-consuming cycle of esterification and hydrolysis of cholesterol (148). The half-life of the esters in these cells has been determined to be 24 hr under conditions where the cells are first incubated with acetyl-LDL and then cultured in a lipoprotein-free medium. This futile cycle is performed by a non-lysosomal cholesteryl ester hydrolase and ACAT. It may be interrupted by the presence of HDL which appears to promote removal of free cholesterol from the cell (148). It was also clearly demonstrated that when ACAT activity was inhibited by progesterone, there was less re-esterification and a more rapid drop in the cellular cholesteryl ester content. These results lead to the conclusion that the cholesteryl ester cycle is functional in states where the scavenger cells are overloaded with cholesterol, such as in familial hypercholesterolemia (137).

7. CONCLUSIONS

Several themes have recurred throughout this review and are common to the various tissues discussed: these relate to the enzymology of ACAT, the regulation of its activity, and its cellular role in the pattern of cholesterol metabolism in these cells of varying functions. The general conclusions from enzymological studies are that ACAT is a sensitive integral membrane protein, sensitive to the nature of its environment, both phospholipid and sterol. Purification has proved very difficult. A number of promising starts have been made but not brought to completion. A fuller understanding of many of the regulatory phenomena that have been discussed depends critically on success in this area. Perhaps a more indirect approach to purification using an affinity ligand or a monoclonal antibody as a probe would prove to be more successful. Even so, the purified enzyme must be reconstituted in a membrane and the problems of standardization of the assay conditions and facilitating the access of both substrates remain.

Several regulatory mechanisms have been proposed, with both long- and short-term functions, most based on the results of in vitro experiments. These include the supply of substrate, particularly cholesterol; the role of fatty acyl-CoA synthetases in regulating the supply of fatty acyl-CoA has not yet received attention. These enzymes are also integral proteins of the endoplasmic reticulum. Hydroxysterols and covalent modification may also play a role in modulating the rate of cholesterol esterification. The evidence for the latter is still in its preliminary stages and caution should be exercised in drawing up a grand scheme for the coordinate regulation of cholesterol metabolism in cells until these phenomena are established more firmly. In particular, although it is possible that, for example, in the liver, cholesterol 7α-hydroxylase, HMG-CoA reductase, and ACAT may be regulated coordinately under certain metabolic circumstances, conditions also exist where this is not the case (Tables 1 and 2). The stimuli that give rise to the changes in activity are not known. Different mechanisms probably operate in steroid hormone-producing tissue compared with liver and intestine.

All of the foregoing considerations allow us to place ACAT in its cellular role in relation to the fluxes of cholesterol that take place within the cell. In Figs. 1–4 we present in diagrammatic form the fluxes of cholesterol and its metabolites that occur in the macrophage, adrenal cortex, intestine, and liver. In each of these cells we can distinguish several functional pools of cholesterol. These pools may also have a specific cellular localization, but this is not intended to be precisely defined by the figures. Pool A is the substrate pool for ACAT. It can be seen that cholesterol derived from lipoproteins and also from newly synthesized cholesterol (from pool B) has access to this pool. Pool B represents that cholesterol that has been synthesized intracellularly before it has been dispersed far from its site of synthesis in the endoplasmic reticulum. Pool C is a general pool of cholesterol called 'metabolically active.' This represents cholesterol that is generally available to the cell for synthetic purposes or for membrane structure (pool M).

Thus in the macrophage (**Fig. 1**), cholesterol is made available to the cell through modified plasma lipoproteins and this sterol becomes available to ACAT (pool A) after being transferred from the lysosomes into the general pool C. Since this tissue contains an active cholesteryl ester hydrolase, the reverse reaction returns the cholesterol into pool C and the futile cycle ensues, unless the cholesterol is removed from the cell by the presence of a suitable acceptor.

At a higher level of complexity, in the adrenal cortex (**Fig. 2**), cholesterol can also enter the cell in normal lipoproteins. This sterol may become substrate for steroidogenesis. However, the evidence suggests that these cells are capable of bringing about great changes in their intracellular fluxes of cholesterol, particularly by the activation of the cholesteryl ester hydrolase. Thus, depending upon the conditions and whether the cells are stimulated by ACTH, cholesterol for steroidogenesis may be derived from any of the inputs into pool C: that is, pool B, the cholesteryl ester pool, or exogenous cholesterol. Similarly, the ACAT substrate pool, pool A, may be supplied from pools B or C. The precise ebb and flow of the flux in these cells remains to be determined.

An additional input of cholesterol is available to the intestinal epithelial cells (**Fig. 3**). Here dietary sterol is absorbed and, according to the evidence discussed earlier, this sterol is highly directed to the ACAT substrate pool A and is then secreted into the lymph in chylomicrons. Newly synthesized cholesterol (pool B) does not appear to penetrate into pool A to any great extent. Thus these cells shown an apparently compartmentalized pattern of cholesterol metabolism.

The liver (**Fig. 4**) is the most complex cell type. We cannot consider here the question of the sources of cholesterol for bile acid synthesis but it is clear from the figure that several possibilities exist. Pool D is the substrate pool for cholesterol 7α-hydroxylase, the initial step in the oxidation of cholesterol to bile acids. It can be supplied from either pool B or C, as can the pool that supplies the free cholesterol found in bile (pool E). There is evidence to suggest that the 7α-hydroxylase pool, pool D, may itself be subdivided. There are many details in this scheme that need to be resolved before the fluxes can be defined with confidence. The main supplies of cholesterol to ACAT are probably from pool C, and the cholesteryl ester synthesized by ACAT may be stored in the cell or, more normal-

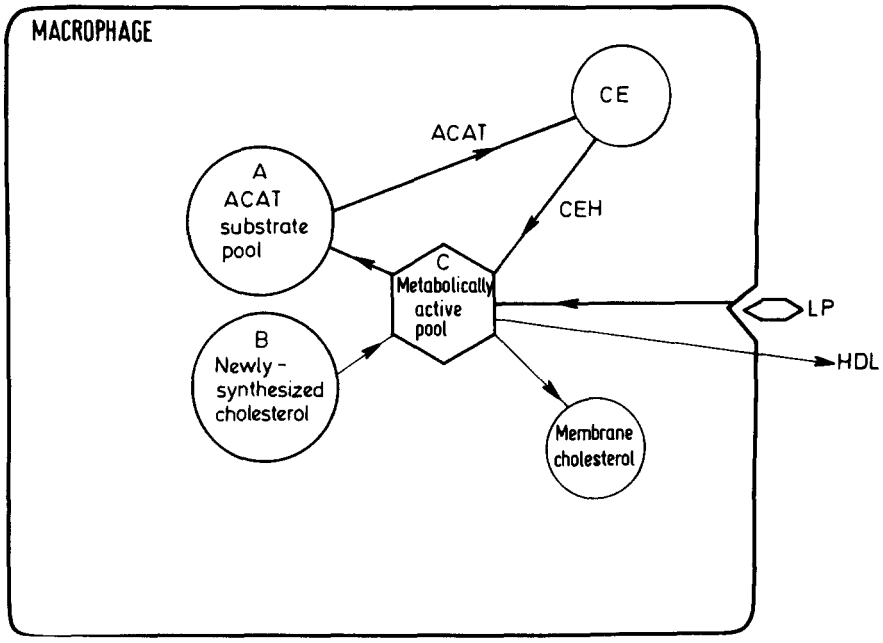

Fig. 1. Pathways of cholesterol in the macrophage.

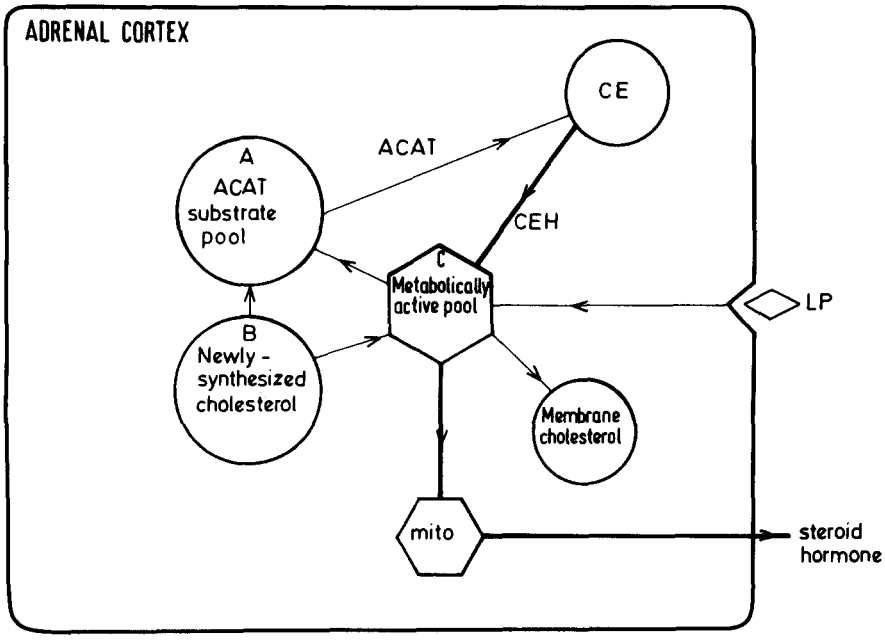

Fig. 2. Pathways of cholesterol in the adrenal cortex.

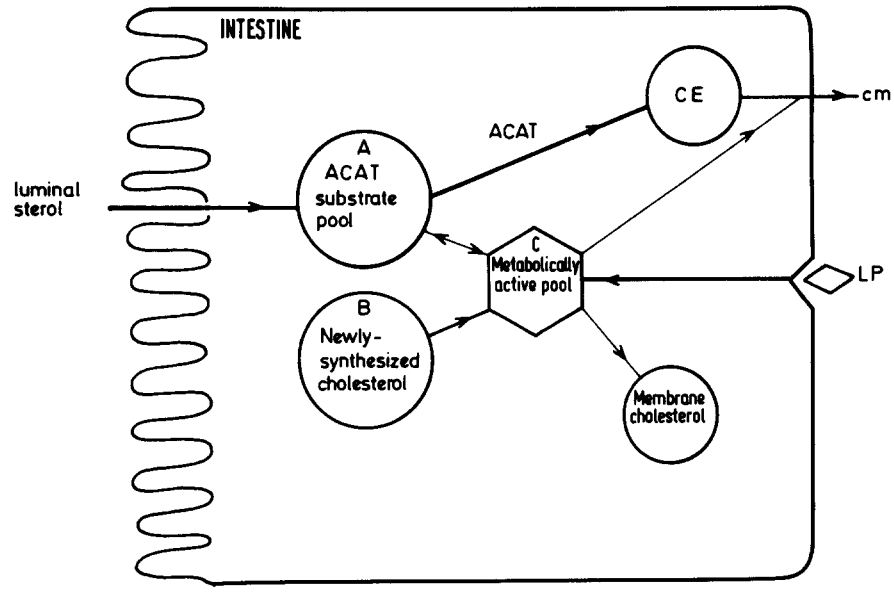

Fig. 3. Pathways of cholesterol in the intestinal epithelial cell.

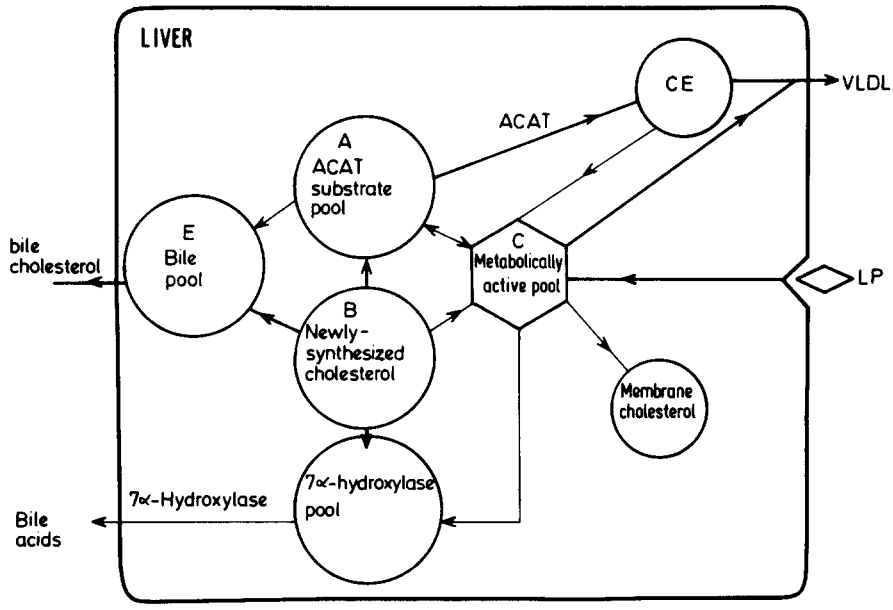

Fig. 4. Pathways of cholesterol in the liver.

ly, it may be exported as a component of VLDL. In this process the liver cell is similar to the intestinal cell.

Generalizing these observations, we can see that whilst cholesterol can move freely within cells, perhaps aided by a sterol-carrier protein, the fluxes are probably highly organized. The best example of this is the intestinal epithelial cell, but there is also some evidence from the possible coupling of ACAT activity and secretion of bile, and from the changes in flux that can be induced in the adrenal cortex, that such control probably also exists in other cells. A cholesteryl ester cycle may have a role to play in the regulation of the flux of free cholesterol in many cells. For example, by inhibition of oxidation of cholesterol in the mitochondria of the adrenal cortex, the cycle appears to be stimulated and the cell becomes more closely analogous to the macrophage. The role of ACAT may be to sense the level of free cholesterol in the cell and, by esterifying the excess, to maintain that level most suitable for the cell, as well as to provide in certain cells a store of cholesteryl ester. In this respect it is central to the metabolic fluxes of cholesterol in all cells. These fluxes and the compartmentation of cellular cholesterol that arises from distinct metabolic states and the relation of these to the function of the cell and the intracellular enzyme activities are the next major targets for research in intracellular cholesterol metabolism.

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