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Study of avian Acyl-CoA Cholesterol Acyltransferase

by

Donghui Cheng

A dissertation submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Biochemistry

Major Professor: Carl L. Tipton

Iowa State University

Ames, Iowa

1997

Graduate College

Iowa State University

This is to certify that the Doctoral dissertation of

Donghui Cheng

has met the dissertation requirements of Iowa State University

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Major Professor

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For the Major Program

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For the Graduate College

TABLE OF CONTENTS

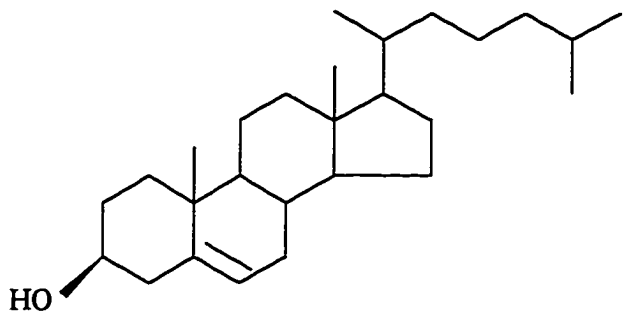
LIST OF ABBREVIATIONS

acyl-CoA cholesterol acyltransferase	ACAT
N-acetylleucylleucylnorleucinal	ALLN
1,4-butanedithiol	BdiT
cholesteryl ester	CE
diethyl pyrocarbonate	DEPC
dihydrosphingosine	DHS
1,2-dioleoylglycerol	DOG
2,2'-dithiodipyridine	DTDP
5,5'-dithiobis(2-nitrobenzoic acid)	DTNB
DL-dithiothreitol	DTT
ethyl alcohol	EtOH
20(R)-20,25-dihydroperoxy-cholest-5-ene-3β-ol	HP-B
iodoacetamide	IAM
lecithin cholesterol acyltransferase	LCAT
methyl-β-cyclodextrin	MβCD
2-mercaptoethanol	2-Mer
methyl alcohol	MeOH
N-butylmaleimide	NBM
N-ethylmaleimide	NEM

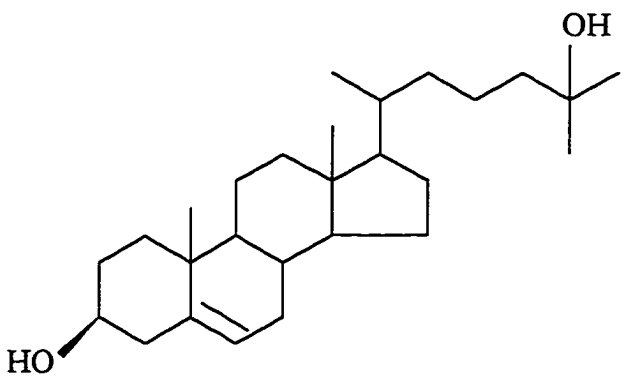
25-hydroxycholesterol	25-OH
phosphatidylcholine	PC
phosphatidylethanolamine	PE
phospholipids	PL
lysophospholipids	lysoPL
p-hydroxymercuribenzoate, Na salt	PMB
phosphatidylserine	PS
specific activity	SA
sodium dodecyl sulfate	SDS
sphingomyelinase	SMase
triacylglycerol	TAG

LIST OF STRUCTURES

cholesterol



25-hydroxycholesterol



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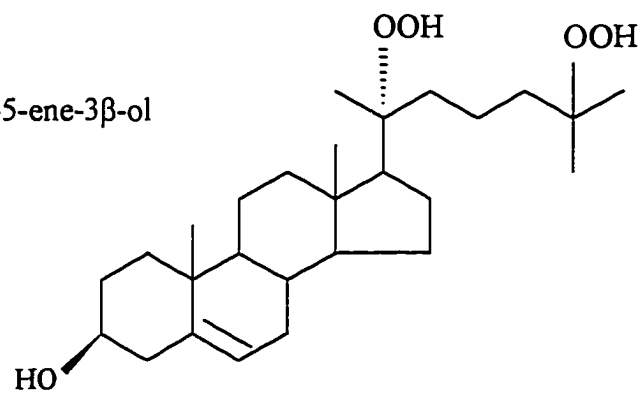


CHAPTER I. INTRODUCTION

Acyl-CoA Cholesterol Acyltransferase (ACAT) (EC.2.3.1.26) catalyzes intracellular esterification of cholesterol with long chain fatty acyl Coenzyme A. ACAT is an integral membrane protein and resides in the cytoplasmic side of the rough endoplasmic reticulum [1]. ACAT activity has been observed in many different kinds of tissues and plays a very important role in the maintenance of intracellular cholesterol homeostasis. In the liver, the production and secretion of lipoproteins, which deliver various lipid molecules to peripheral tissues, require a lipid core of triacylglycerols and cholesteryl esters. Dietary absorption of cholesterol requires the function of intestinal ACAT, which controls the rate-limiting step of absorption [2]. Overaccumulation of cholesteryl esters in macrophages and smooth muscle cells in large arteries leads to formation of fatty streaks and atherosclerosis.

Despite its biological significance, ACAT has not been isolated and studied in purity. M. Shih has reported the observation of a sigmoidal relationship between the enzymatic activity and cholesterol mole fraction in the liposomes when she studied mouse liver microsomal ACAT [3]. In her work, the enzyme was extracted with 1.5% deoxycholate-1 N KCl and reconstituted into cholesterol-PC liposomes. In 1993, T.Y. Chang and coworkers made a major breakthrough in ACAT research and cloned a human macrophage enzyme that had ACAT activity [4]. Expression of the cDNA in insect Sf9 cells, which lack intrinsic ACAT activity and have very low intracellular cholesterol, allowed Chang et al [5] to study the enzyme at the molecular level. By using the formation of ^{3}H-25-hydroxycholesteryl oleate as the way to measure ACAT activity, they found that

the human enzyme is activated by cholesterol through a cooperative mechanism [5]. Thus, in mammalian cells, cholesterol serves as an allosteric activator as well as a substrate of ACAT.

A high profile of cholesterol esterification was observed in the yolk sac membrane of fertilized chicken eggs by J.H. Shand et al [6] during their study of lipid metabolism in chick embryos. The importance of cholesteryl esters in the formation and secretion of lipoproteins in the yolk sac membrane is understood as part of the supply of nutrients to serve as structural components and energy source for the developing embryos [7]. More than 5 fold higher ACAT activity was observed at day 16 in the yolk sac membrane compared with that in the liver or intestine [6].

Cyclodextrins are a family of cyclic molecules composed of different number of glucose units. Among the commercially available cyclodextrins, β-cyclodextrin and its derivatives have been found to be the most efficient molecules to extract cholesterol from lipid bilayers [8,9]. A complex of cholesterol with methyl-β-cyclodextrin can be used as an efficient delivery tool to provide cells or other biological systems with cholesterol [10].

In the present work, microsomes of the yolk sac membrane from chicken eggs 16 days after fertilization were used to characterize ACAT. Because of its vital role in embryo development and the much higher specific activity of ACAT, the yolk sac membrane provided a better system for the study of the enzyme, and any information learned from the avian enzyme could be useful in the understanding of its mammalian counterpart. The property of the yolk sac membrane enzyme was compared to the mammalian equivalent with regard to chemical modification and regulation of activity by (oxy)sterols. Methyl-β-

cyclodextrin was used to deplete microsomes of cholesterol and was found to be very important in enhancing substrate access to the enzyme.

CHAPTER II. LITERATURE REVIEW

A. Biological Functions of ACAT

Cholesterol is an essential structural component of mammalian plasma membrane. It induces ordered and tighter packing of the lipid bilayer and reduces the permeability of the membrane to small molecules [11]. As the precursor of steroid hormones and bile acids, cholesterol plays an important part in steroidogenesis and dietary fat absorption. Recently, the importance of cholesterol in embryo development was illustrated by the finding of covalent modification of the secreted signaling molecule Hedgehog by cholesterol and the modification was vital for the normal function of the key patterning molecule during embryogenesis [12]. However, the amount of free cholesterol inside the cell has to be regulated within certain levels, and a higher concentration of free cholesterol is detrimental to normal cellular functions. Culturing macrophages in the presence of acetyl-LDL or ACAT inhibition by specific ACAT inhibitors resulted in elevations in intracellular free cholesterol, which in turn caused cellular necrosis and cell death [13,14]. Enrichment of cholesterol in whole cells or membrane preparations inhibited several membrane-bound enzymes, including (Na^+ + K^+)-ATPase, calcium pump, alkaline phosphatase, and carnitine palmitoyltransferase [15-18]. Alteration in cellular cholesterol content affected the development and function of tight junctions in epithelial barriers [19]. The cell, therefore, employs very sophisticated mechanisms to keep the amount of free cholesterol within a certain range.

The two rate-limiting enzymes in *de novo* biosynthesis of cholesterol, 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthase and HMG-CoA reductase are subjects of sterol-mediated feedback control [20,21]. The cellular content of free cholesterol regulates the amounts of the enzymes at the transcriptional level through sterol regulatory element binding proteins, and the activity of the reductase is controlled at the post-transcriptional level as well, through protein synthesis and degradation. Cholesterol derived exogenously through the low density lipoprotein (LDL) pathway [22] suppresses the activities of the synthase and reductase and is subjected to sterol-mediated feedback control via the LDL receptor at the transcriptional level in a similar fashion as the other two enzymes [20,21].

ACAT functions in converting excess free cholesterol to cholesterol esters and, together with HMG-CoA synthase, HMG-CoA reductase, and the LDL receptor, maintaining a near constant and low intracellular concentration of free cholesterol. When the cellular cholesterol content rises, through either stimulated *de novo* synthesis or increased uptake of exogenous cholesterol, ACAT activity is elevated and cholesterol ester synthesis is increased. Cholesterol loading of cultured cells, both primary and cell lines, stimulates ACAT activity in many different cell types [23]. Dietary feeding of whole animals with cholesterol increases ACAT activity in the livers and intestines of many different animals, including rat, rabbit, hamster, and guinea pig (reviewed by Suckling and Stange [1]). As the storage form of free cholesterol, cholesteryl esters are not inert but undergo a constant cycle of hydrolysis, catalyzed by cholesteryl ester hydrolase, and re-esterification, catalyzed by ACAT, and depending on the cellular requirement of free cholesterol, both enzymes can be activated or inhibited through mechanisms that are still

not quite clear and are under active investigation. There is a cytosolic protein inhibitor of the neutral cholesteryl ester hydrolase and the inhibitor activity is regulated by physiological, hormonal, and nutritional factors [24,25]. Understanding of the mechanisms controlling ACAT activity has been accelerated by the cloning of the human cDNA [4] and will be discussed in detail in a section on regulation of ACAT activity. Accumulation of cholesteryl esters inside the cell leads to the formation of cytosolic lipid droplets.

Besides the important function in regulating cholesterol homeostasis inside the cell, ACAT plays a special role in the production and secretion of lipoproteins in the liver. Whole animal experiments showed that a diet of high cholesterol content increased hepatic esterified cholesterol 4.6 fold and LDL-cholesterol 7 fold in adult male monkeys [26] and feeding miniature pigs for 21 days with a diet containing an ACAT inhibitor inhibited hepatic microsomal ACAT and decreased VLDL and LDL apoB pool sizes [27]. The decreases in VLDL and LDL apoB pool sizes were due to decreases in the rates of lipoprotein production and secretion. When the livers were isolated from African green monkeys and perfused with a medium containing ACAT inhibitors, significant reductions in the rates of secretion of cholesteryl ester and apoB were observed and the correlation between the percent inhibition of cholesteryl ester secretion and of apoB secretion was positive ($r = 0.84$) [28]. An experiment *in vitro* with the human hepatoma cell line (HepG2) showed that a specific ACAT inhibitor inhibited cholesteryl ester formation and apoB secretion concomitantly, and activation of ACAT by 25-hydroxycholesterol elevated cholesteryl ester content and lipoprotein secretion [29]. A coordinated regulation of cholesteryl ester synthesis and lipoprotein assembly and secretion in the liver is the working

model currently proposed [28]. ACAT inhibitors have been sought enthusiastically by the pharmaceutical industry, aiming at reducing the secretion of lipoproteins and cholesteryl esters into the blood stream.

Bile salts, derived from cholesterol in the liver, function as detergents to facilitate the digestion and absorption of dietary lipids in the intestine. The secretion of bile salts in the liver is found to be inversely correlated with the formation of cholesteryl esters and hepatic ACAT activity, as demonstrated by an ACAT inhibition experiment in male rats [1]. Thus, in the liver, the flow of cholesterol, either to bile salt production or cholesteryl ester formation, is tightly controlled by physiological requirements through the rate-limiting enzymes cholesterol-7α-hydroxylase and ACAT.

The requirement of apoB-containing lipoproteins in embryonic development was demonstrated by an apoB knockout experiment in which mouse embryos with homozygously disrupted apoB gene were found to be not viable and most of them were resorbed during early stages of gestation [30]. ApoB-containing lipoproteins secreted from the yolk sac membrane provide essential lipid nutrients to serve as structural components and energy source for the developing embryos in avian as well as mammalian systems [7,31]. Experiments with chicken embryos indicated that cholesteryl esters were synthesized extensively, about 80% of the free cholesterol was converted to cholesteryl esters, in the yolk sac membrane and the esters were very important in the assembly and stability of lipoproteins secreted into embryonic circulation [7]. Esterification of cholesterol, which exists primarily as the free sterol in the yolk, facilitates the absorption

of fatty acids as well as cholesterol and contributes importantly to the absorption and transportation of lipid from yolk to embryos [7].

For intact animals, cholesterol can be derived from two different sources: *de novo* biosynthesis and dietary absorption. For an average-sized human, dietary uptake of cholesterol is between 300 and 500 mg daily and endogenous biosynthesis gives rise to 700 to 900 mg per day [2]. Studies showed that the site of cholesterol absorption in the intestine correlates with the longitudinal distribution of ACAT activity [1]. To be absorbed into the body, dietary cholesteryl esters have to be hydrolyzed, by pancreatic cholesteryl ester hydrolase, to the free sterol before being taken up by the villous cells. ACAT facilitates cholesterol absorption by catalyzing the formation of cholesteryl esters which are incorporated into chylomicrons prior to entering lymphatics. In fact, the rate-limiting step of cholesterol absorption lies at the activity of intestinal ACAT and inhibition of this enzyme reduces dietary uptake of the sterol and increases secretion into feces [1,2]. Experiments *in vivo* indicated that reduction in cholesterol absorption by inhibition of intestinal ACAT has a far-reaching effect on lowering plasma total cholesterol [1,2].

Steroid hormone-producing tissues meet their cholesterol need primarily through uptake of plasma lipoproteins; *de novo* synthesis of cholesterol only plays a minor part [1]. After getting into the cell, lipoproteins are delivered and hydrolyzed in lysosomes. Free cholesterol released from lysosomes is converted into esters by ACAT, and cholesteryl esters are stored and used for future steroid hormone synthesis. With changes in the rates of exogenous absorption and steroidogenesis, the content of cellular cholesteryl esters can fluctuate 20 fold without affecting the level of free cholesterol [32].

The early stage of atherosclerosis is characterized by the formation in the subintimal space of fatty streaks which consist of lipid, mainly cholesteryl esters, deposits along blood vessel walls. The uptake of modified LDL through the scavenger receptor by macrophages is not subjected to feedback control, as is the LDL pathway, and leads to massive accumulation of cholesteryl esters and foam cell formation. Inhibition of aortic ACAT activity can reduce cholesteryl ester synthesis and enhance reverse cholesterol transport to the liver for clearance, through high density lipoprotein and lecithin cholesterol acyltransferase. Regression of fatty lesions has been observed in animals treated with cholesterol-lowering drugs or low cholesterol diet [33]. There has been an extensive effort from pharmaceutical companies to search for ACAT inhibitors and a variety of compounds, ranging from tranquilizers and antihypertensives to antifungals and steroids [2], have been found to inhibit ACAT activity to various degrees both *in vitro* and *in vivo*. Presumably, ACAT inhibitors could interfere with dietary cholesterol uptake in intestine, synthesis and secretion of cholesteryl ester and lipoprotein in the liver, or accumulation of cholesteryl esters along blood vessel walls, or all of them. The study of ACAT structure and understanding of enzyme catalysis and regulation will surely be very useful in helping to design and produce ACAT inhibitors to prevent and cure atherosclerosis in the future.

B. Structure and Molecular and Biochemical Studies of ACAT

Because of extreme sensitivity towards detergents and the minute quantity in tissues of this enzyme, ACAT has not been isolated and purified to homogeneity, only partial

purification of the protein was achieved with the use deoxycholate for solubilization followed by ammonium acetate precipitation and Sepharose 4B column chromatography [34]. Most of the studies of this protein use microsomal preparations from tissues of different sources. A major breakthrough was made when Chang and his coworkers cloned and expressed a human macrophage cDNA, the protein product of which has ACAT activity, in mutant Chinese Hamster Ovary (CHO) cells [4]. Those mutant cells lack intrinsic ACAT activity. The cDNA has 1650 base pairs and codes for an integral membrane protein of 550 amino acids and 64, 805 dalton. In the primary sequence, 5 segments have high hydrophobicity and two of them are potential transmembrane α helices. The deduced protein bears high sequence homology with human acyl-CoA synthetase and firefly luciferase, both of which function in acyl thiolation and acyl transfer. Previous experiments using radiation inactivation estimated the functional size of rat liver ACAT to be 170-180 kDa [35] and 213 $\pm$ 35 kDa [36]. In the deduced protein sequence of the human ACAT, there is a varied version of the leucine heptad motif which is a signal for protein dimerization. It is possible that the protein oligomerizes under physiological conditions and might function as an oligomer. There are 9 cysteines in the polypeptide, none of them in close vicinity to the others in the primary sequence. Purification of ACAT has been attempted for many years in Chang's lab and in others, success, however, has not been achieved, even with the cloning of the human cDNA and all the structural information learned thereafter, and the extensive exploitation of the powerful techniques of molecular biology [23]. Failure to transform bacterial cells with ACAT-containing vectors is probably part of the reason for unsuccessful trials [4], unique properties and structural features of the

enzyme and the particular microenvironment necessary around it must be major factors hampering the achievement of a homogenous protein. Study of ACAT is, by now, still restricted to microsomal preparations or detergent solubilized extracts, and immunological analysis of the protein can only be done using antibodies raised against short synthetic peptides deduced from the cDNA sequence or a chimeric protein consisting of part of the predicted ACAT sequence fused to bacterial glutathione S-transferase [5,37].

At about the same time when Chang et al made their success in cloning human ACAT [4], another group claimed identification of a porcine liver protein that had cholesterol esterification activity and found the sequence of the protein to be identical to that of liver carboxylesterase [38]. Using molecular biology techniques, they were able to identify a human liver cDNA clone and expressed it in CHO cells. The expression of the clone led to a 20 fold increase in cellular cholesteryl ester synthesis and a near 3 fold elevation in ACAT activity. The nucleic acid sequence of the clone was found to be identical to that of human liver carboxylesterase. Having apparent ACAT activity, the sequence of this enzyme bears no homology with that reported by Chang et al [4] and the discrepancy awaits further investigation.

By use of the human cDNA as a probe and fluorescence in situ hybridization technique, Chang and his colleagues located the ACAT gene of their clone to human chromosome 1, band q25 [39]. By a similar approach, Chang et al isolated a cDNA of ACAT from mouse liver and found 87% identity between the deduced protein sequences of the human and mouse counterparts [40]. The predicted protein from mouse liver has 540 amino acids, at least two potential transmembrane α helices, a leucine heptad motif,

and sequences homologous to those found in proteins functioning in acyl thiolation and acyl transfer [40].

Two yeast genes encoding for ACAT-related enzymes were discovered [41], and the predicted protein sequences were 23% identical to the human protein [4]. The yeast proteins are predicted to have at least two transmembrane domains and a leucine zipper motif, similar to the human protein. Both of the genes had to be disabled to have a complete abolishment of sterol esterification in the yeast [41].

A rabbit liver ACAT cDNA was cloned and found to be more than 90% homologous to the human equivalent [42]. More than six mRNA bands, ranging from 1.7 to 6.2 kb, were shown on the Northern blot using a liver cDNA-derived RNA as the probe and mRNAs from different rabbit tissues. The possibility that tissues have different mRNAs, probably due to alternative splicing, and therefore, different isoforms of ACAT was implied.

ACAT-knockout mice were generated using human ACAT cDNA [4] as a probe and gene targeting in mouse embryonic stem cells [43]. Decreased cholesterol esterification was observed in adrenal glands, fibroblasts, and macrophages of the mutant mice, but a similar level of cholesterol esterification activity as in wild-type mice and substantial amounts of cholesteryl esters were observed in the liver. ACAT gene disruption did not affect dietary uptake of cholesterol and the mutant mice had a healthy phenotype. The result suggests that in mouse other undiscovered cholesterol esterification enzymes, the genes of which are different from the gene targeted in the gene-disruption experiment, may exist and function redundantly in the liver and intestine.

Biochemical studies of ACAT were performed using microsomal preparations from different organs of white New Zealand male rabbits [44]. Inhibition experiments using diethyl pyrocarbonate and acetic anhydride implicated a histidine residue in the active site and possible participation of the histidine in catalysis [44]. Based on the sensitivity of the histidine toward DEPC and acetic anhydride, two distinct subtypes of ACAT were identified among the 14 tissues tested. The aortic enzyme, representing one subtype, had apparent Ki's of 40 μM and 500 μM for DEPC and acetic anhydride, respectively, while the liver ACAT, representing the other subtype, had Ki's of 1500 μM and 5 mM, respectively.

At least two sulfhydryls were identified in microsomal ACAT from white New Zealand male rabbits and modification of them with thiol-reactive agents affected the enzymatic activity [45]. Experiments with p-hydroxymercuribenzoate and protection against PMB modification by oleoyl-CoA indicated that one sulfhydryl was necessary for catalysis, and the other was near an inhibitory CoA binding site [45].

One serine residue was shown to be essential for the catalysis of cholesterol esterification in chinese hamster ovary cells [46]. SRD-4 cells are mutant CHO cells that lack intrinsic ACAT activity. Wild-type and mutant ACAT cDNAs were cloned and sequence comparison indicated a single point mutation in the mutant ACAT, codon 265 is changed from Ser to Leu. The single mutation results in an inactive enzyme.

In plasma, cholesterol esterification is catalyzed by lecithin cholesterol acyltransferase (LCAT), an enzyme that associates with high density lipoprotein and uses phosphatidylcholine as the acyl donor. The protein has been purified to homogeneity and

structural and mechanistic information is known in detail. The typical active-site triad composed of a serine, histidine, and aspartate, has been identified to function in catalysis of cholesteryl ester formation [47]. Previous studies of LCAT had suggested the involvement of two cysteine residues in catalysis [48], site-directed mutagenesis of the two cysteines, however, gave a fully active enzyme [49,50]. It turns out that the cysteine residues do not participate in the catalytic activity, but their location in the vicinity of the active site poses a steric hindrance to substrate binding upon modification of the sulfhydryls by thiol-blocking agents [49,50]. Considering the similar enzymatic function of ACAT and LCAT, the two enzymes may share many common mechanistic features, and the knowledge learned about LCAT may be useful in the exploration of structure and catalytic mechanism of ACAT.

C. Regulation of ACAT Activity

Studies of ACAT in rat liver microsomes have indicated that the optimal condition for cholesterol esterification is around 37^0C and neutral pH [51]. The rat liver enzyme has a preference for oleoyl CoA as the acyl donor, but it can utilize other acyl CoAs as well [52]. ACAT activity exhibits a cycle of diurnal rhythm with maximal and minimal activities at mid-light and mid-dark phases, respectively [53]. Other enzymes involved in cholesterol metabolism, i.e., HMG-CoA reductase, cholesteryl ester hydrolase, and the LDL receptor also show cycles of diurnal variation, with different maximum and minimum activity phases. The concentration of cholesteryl esters parallels the change in the enzyme

activity, but on the contrary, the cellular free cholesterol level stays about the same during the entire cycle [53].

The possibility that ACAT activity is regulated through reversible phosphorylation was examined in the macrophage cell line J774.2 and male Wistar rat liver [54]. Experimental evidence did not support the hypothesis that ACAT activity was modulated through phosphorylation-dephosphorylation, even though other enzymes involved in cholesterol metabolism, e.g. HMG-CoA reductase, are regulated this way. Sequence examination of human macrophage ACAT cDNA can not identify any consensus phosphorylation site in the protein [4], consistent with the biochemical study [54].

A short-lived protein factor that inhibits ACAT activity endogenously inside the cell was first proposed by Chang et al when they studied cholesterol esterification in Chinese Hamster Ovary cells [55,56]. When the cells were treated with the protein synthesis inhibitor cycloheximide, a 8 fold increase in ACAT activity was observed and the increase reached a maximum in 6-8 hours. When the cells were provided with cholesterol exogenously through LDL or endogenously through *de novo* synthesis, cholesteryl ester synthesis was elevated and the cycloheximide sensitive activation of ACAT was abolished. The possible existence of the short-lived protein inhibitor of ACAT in the cell was confirmed by experiments conducted by I. Tabas and G. Boykow with mouse peritoneal macrophages [57]. In the experiment, treatment of the macrophages with cycloheximide led to a 10 fold activation of ACAT within 4 hours and withdrawal of cycloheximide led to return of the activity to control level in 4 hours. When mouse peritoneal macrophages or CHO cells were treated with two structurally different cysteine protease inhibitors,

ALLN or epoxysuccinylleucylamido-3-methylbutane ethyl ester, the sterol-stimulated cholesterol esterification was diminished [58]. Based on previous observation and other experimental evidence, Tabas et al proposed a proteolysis/inhibitor pathway that, together with the controls in HMG-CoA synthase, HMG-CoA reductase, and the LDL receptor, regulates the level of free cholesterol and cholesterol esterification in the cell [58]. In the pathway, expansion of the cellular sterol pool, through either endogenous or exogenous supply of cholesterol, activates a set of cysteine proteases which then cleave and inactivate the intrinsic ACAT inhibitor. The destruction of the inhibitor allows a full activation of ACAT, resulting in an increased synthesis of cholesterol esters. The net result of this is a reduced level of cellular free cholesterol, preventing any toxic effect of free cholesterol to the cell [58].

Many studies have shown that under physiological conditions, ACAT is not saturated with cholesterol and addition of cholesterol directly or through lipoproteins or liposomes to whole cells or microsomes can activate ACAT substantially [1,59]. Cholesterol oxidase susceptibility indicates that about 90% of cellular free cholesterol is associated with the plasma membrane with the rest distributed in intracellular membrane systems [60]. Transfer of the sterol from other cellular fractions to the ACAT substrate pool, therefore, can be a limiting factor [1]. Lipid molecules such as progesterone and sphingomyelin have been shown to affect cholesterol esterification by affecting accessibility of the sterol to the enzyme substrate pool [61-69].

Progesterone was shown to inhibit microsomal ACAT activity from rat liver, and the inhibition was not due to a direct effect on enzyme catalysis [61]. Fluorescent staining

of fibroblasts revealed extensive accumulation of free cholesterol in lysosomes when the cells were cultured in the presence of LDL and progesterone for 24 hours [62]. Reduced cholesteryl ester synthesis was observed along with the lysosomal sterol accumulation. Removal of progesterone from the cell culture reduced the amount of accumulated free cholesterol in lysosomes and increased ACAT activity and cholesterol ester synthesis [62]. The result indicates that progesterone affects ACAT activity indirectly, by sequestering the sterol in lysosomes, therefore, limiting the enzyme access to the substrate [62].

Distribution of sphingomyelin in the cell is very similar to that of cholesterol, with the majority of the lipid molecule located in the plasma membrane and the minority in intracellular membranes [63]. Despite the distinct structural differences, sphingomyelin and cholesterol are shown to interact strongly with each other in membranes and concentrations of the two lipids are related closely and positively. The hydrogen bond between the NH group of sphingomyelin and the hydroxy group of cholesterol and van der Waals interactions between these two allow a tight lateral packing between the two molecules and high cohesion energy in membranes containing sphingomyelin and cholesterol [64,65]. When fibroblasts or macrophages were treated with SMase, hydrolysis of sphingomyelin was accompanied by a loss of cholesterol in the plasma membrane with a concomitant increase in cellular esterified cholesterol and ACAT activity [63,66,67]. SMase treatment of fibroblasts inhibited endogenous biosynthesis of cholesterol and HMG-CoA reductase activity as examined in the cells [63]. Incubation of macrophages in the presence of acetyl-LDL and an ACAT inhibitor led to a rise in cellular free cholesterol, and at the same time, cellular sphingomyelin content [67]. A whole-animal feeding experiment showed that a

high cholesterol diet increased concentrations of cholesterol in the liver, whole plasma, and VLDL, and the increases were accompanied by an elevation in VLDL-sphingomyelin level and a decrease in catabolism of sphingomyelin in the liver [68]. It is very suggestive that the cell tends to accommodate changes in cellular free cholesterol content by modulating the concentration of sphingomyelin [63,67]. Regulation of dietary cholesterol uptake by the content of sphingomyelin in the apical membrane of intestine cells was implicated in an experiment with the human intestinal cell line CaCo-2 [69]. Treatment of the cells with SMase resulted in a 50% reduction in absorption of cholesterol from bile salt micelles and active neutral SMase activity was detected in membranes prepared from the cells and from human pancreatic juice [69]. All together, it is possible to conclude that the amount of cellular sphingomyelin, mainly plasma membrane sphingomyelin, could affect the cell's ability to accommodate cellular free cholesterol, and a decrease in the level of sphingomyelin can stimulate cholesteryl ester synthesis and inhibit cholesterol biosynthesis. The effect of this sphingolipid on cholesterol esterification is very likely through an effect on cellular free cholesterol, thus affecting the substrate pool size of ACAT.

The fact that cholesterol can activate ACAT has been reported by many investigators for a long time, but the mechanism of activation was not known. There have been suggestions that the sterol stimulated ACAT activity through mechanisms of both a direct effect on substrate supply and a non-substrate modulation [1], but direct experimental evidence had been lacking due to the extreme difficulty of purifying the protein. The cloning of the human macrophage ACAT cDNA by Chang et al [4] has allowed the use of molecular biology as well as traditional biochemical techniques in exploration of the

structure and regulation of ACAT and cholesterol esterification in cells, tissues, and whole animals, and possible mechanisms for sterol-mediated activation of ACAT have been examined at the transcriptional, translational, and post-translational levels.

When rabbits (male New Zealand White) were fed a high-fat/high-cholesterol diet for 8 weeks, the concentration of esterified cholesterol in the liver increased 164 fold and the hepatic ACAT activity increased 17 fold [42]. Analysis of changes in mRNA level in the liver revealed a 2 fold elevation in ACAT mRNA mass. When the same kind of rabbits were fed with the same diet for 3 weeks, a 10 fold increase in liver cholesteryl ester content was observed with no significant changes in ACAT mRNA mass being detected [70]. *In vitro* experiments with primary hepatocytes showed a 6-10 fold increase in cellular cholesteryl ester level upon treatment of the cells with β-VLDL or mevalonolactone for 3 days, but no significant changes were observed in ACAT mRNA level and cellular free cholesterol content [70]. Inhibition of ACAT resulted in a reduced cholesterol ester synthesis with no detectable change in ACAT mRNA level [70]. Similarly, no differences were observed in the amount of ACAT mRNA in HepG2 cells under conditions of either cholesterol loading or cholesterol depletion, even though the whole-cell and microsomal ACAT activities were elevated or inhibited under these same conditions [71]. The above results suggest that regulation of the enzyme activity at the gene transcriptional level is probably not the major mechanism the cell uses to control cholesteryl ester synthesis [42,70,71]. Activation of ACAT upon cholesterol loading is more likely modulated by post-transcriptional steps, possibly by a direct and immediate effect of substrate availability. The modest change in the ACAT mRNA mass observed in the long term feeding

experiment implies that transcriptional activation of the enzyme activity may just be an adjustment to the chronic effect of cholesterol feeding, requiring a certain threshold of cholesterol level in the cell to activate gene transcription [42,70]. On the other hand, the atherogenic feeding of the rabbits generated a disease-like state in the liver, that resulted in the entering of macrophages into the liver. The increase in ACAT mRNA level may simply reflect the increase in the amount of total mRNAs [42,70].

A chimeric polypeptide, consisting of glutathione S-transferase fused with the first 131 amino acids of the human ACAT, was used to raise specific polyclonal antibodies against ACAT in rabbits [37]. When human fibroblasts, HepG2 cells, or CHO cells were cultured in the presence of LDL or 25-hydroxycholesterol, a substantial amount of cholesterol esterification was observed. Western blot analysis of ACAT concentration under these treatments revealed no significant changes in the protein mass, indicating that activation of cholesterol ester synthesis by the sterol does not require an increase in the enzyme mass. The implication of the result is that the sterol-mediated activation of ACAT is probably not being controlled at the translational level [37].

Functional expression of high ACAT activity in insect Sf9 cells, which do not have intrinsic ACAT activity and have a very low endogenous cholesterol level, allowed a detailed catalytic analysis of ACAT in the presence of cholesterol [4,5]. After being solubilized and reconstituted into liposomes, ACAT activity was assayed with increasing concentrations of cholesterol, and the activity was plotted and shown to change as a function of cholesterol concentration in a sigmoidal pattern [5]. Using ^{3}H-25-hydroxycholesterol as the substrate, the formation of ^{3}H-25-hydroxycholesteryl oleate was

shown to be activated by addition of cholesterol in a sigmoidal pattern [5]. Since only the protein-coding sequence of ACAT was used in the expression of the enzyme, any inhibitory factors of ACAT that may exist endogenously in the cell could not account for the activation [5]. The experiments demonstrate clearly that cholesterol serves not only as a substrate of the enzyme, but interacts cooperatively with ACAT and acts as an allosteric activator [5]. Summarizing all the experiments on the gene transcription, translation, and post-translation studies, it is reasonable to conclude that, in mammalian cells, the primary mechanism of the sterol-mediated activation of ACAT lies at a post-translational step through a dual function of cholesterol, acting as both a substrate and an allosteric regulator of the enzyme.

In mammalian systems, 25-hydroxycholesterol among other oxysterols has been found to form endogenously in animals and cultured cells, and can be esterified, or metabolized to bile salts and steroid hormones [72-74]. Not only is the existence of 25-hydroxycholesterol physiologically relevant, it has been shown to affect cholesterol metabolism by affecting rate-limiting enzymes. 25-Hydroxycholesterol interacts with sterol regulatory element binding proteins, which are gene transcriptional activators of cholesterol biosynthesis and the LDL receptor, and suppresses endogenous synthesis of cholesterol and exogenous uptake from LDL [46]. It also stimulates cholesteryl ester formation in cells or microsomal preparations by activating ACAT in mammalian systems [5,46,72,74]. Experiments with cell cultures showed that addition of 25-hydroxycholesterol to cells did not cause changes in ACAT mRNA and protein mass even though it increased the enzyme activity [37,71]. In rat liver microsomes, 25-hydroxycholesterol activated ACAT and

cholesterol esterification, and the activation seemed not to be mediated by an effect on the rate and extent of cholesterol transfer between liposomes and microsomes [72]. In both intact cells and cell extracts, the oxysterol was shown to activate ACAT, but the amplitude of activation diminished with the addition of increasing concentrations of cholesterol [5]. It seemed that the effect on ACAT activation by the oxysterol is more on accessibility of cholesterol to the enzyme [5]. More studies are needed to clear the discrepancy and explore the mechanism of activation. Taken together, it is probable that 25-hydroxycholesterol acts as a physiological regulator of cholesterol ester synthesis and it exerts its effect on post-translational steps.

CHAPTER III. MATERIALS AND METHODS

A. Materials

Chicken eggs, 16-days after fertilization, were obtained from Hy-Vac (Adel, Iowa). Mouse livers of female mice (Strain FVB) were kindly provided by Dr. Chris Tuggle's lab (Animal Science, Iowa State University). Protease inhibitors (aprotinin, pepstatin, leupeptin, antipain, benzamidine, and ALLN) were from Sigma Chemical Co. (St.Louis, MO). 2-Mercaptoethanol, DL-dithiothreitol, iodoacetamide, *N*-ethylmaleimide, *p*-hydroxymercuri-benzoate sodium salt, MES, HEPES, 2,2'-dithiodipyridine, 5,5'-dithiobis(2-nitrobenzoic acid), sodium dodecyl sulfate, cholic acid sodium salt, Triton X-100, 4-aminoantipyrine, sodium 3,5-dichloro-2-hydroxybenzene sulfonate, methyl-β-cyclodextrin, cholesterol, 25-hydroxycholesterol, oleoyl coenzyme A, sphingomyelin, L-α-phosphatidyl-L-serine, 1,2-dipalmitoyl-DL-α-phosphatidylethanolamine, dioleoyl-L-α-phosphatidylcholine, L-α-lysophosphatidylcholine, triolein, dioleoylglycerol, cholesteryl oleate, oleic acid, linolenic acid, long chain ceramide (bovine brain), dihydrosphingosine, sphingomyelinase, horseradish peroxidase, and cholesterol oxidase were all from Sigma. Phosphatidylcholine, 20 mg/ml in chloroform, was isolated from chicken eggs following a procedure by White et al [75]. 5-Cholesten-3β,25-diol 3-oleate was purchased from Steraloids Inc. (Wilton, NH). 20(R)-20,25-Dihydroperoxy-cholest-5-ene-3β-ol was prepared as described by Tipton et al [76]. *p*-Methylaminophenol sulfate, 1,4-butanedithiol, diethyl pyrocarbonate, and *N*-butylmaleimide were purchased from Aldrich Chemical Co.

(Milwaukee, WI). THAM, Na_2^+ EDTA, sucrose, sodium bisulfite, sodium sulfite, perchloric acid, cupric sulfate, and 7 ml polyethylene scintillation vials, were obtained from Fisher Scientific (Fair Lawn, NJ). CHAPS and bovine serum albumin, fatty acid poor, were purchased from Calbiochem Co. (La Jolla, CA). Scintillation fluid, Ecolume, was obtained from ICN Pharmaceuticals Inc. (Costa Mesa, CA). 1-^{14}C-Oleoyl CoA was from DuPont NEN (Boston, MA). [1α,2α(n)-^{3}H]Cholesteryl oleate was from Amersham Life Science Inc. (Arlington, IL). Silica gel (40-140 mesh) was a product of J.T. Baker Inc. (Phillipsburg, NY). Plates for thin layer chromatography, silica gel 60 precoated, 20 X 20 cm, were from Merck (Darmstadt, Germany). Pre-packed columns of Sephadex G-25 were from Pharmacia Biotech Inc. (Piscataway, NJ). Centricon 10 cartridges (molecular weight cutoff: 10,000) were from Amicon, Inc. (Beverly, MA).

Sorvall RC-5B centrifuge (DuPont Instruments), L5-75 Ultracentrifuge (Beckman), Airfuge® Ultracentrifuge (Bechman), and UV160U spectrophotometer (Shimadzu) were used in the study. Packard Tri-carb 1600TR liquid scintillation analyzer with a dual radioisotope counting program was used for radioactivity counting. LiposoFast extrusion device from Avestin, Inc. (Ottawa, Canada) was employed for liposome preparation.

B. Methods

1. Preparation of yolk sac membrane microsomes from fertilized chicken eggs The procedure described by J.H. Shand et al [6] was followed. Yolk sac membranes were isolated from 16- or 15-day old chick embryos, which were reported to have maximal

ACAT activity [6]. The membrane was washed in ice-cold NaCl (0.9%) 4 or 5 times to remove excess yolk. The following steps were performed at 4°C. After homogenation of the yolk sac membrane by a mechanical homogenizer in about 3 volumes of medium I (0.25 M sucrose, 1 mM EDTA, 10 mM Tris-Cl, pH 7.4), the membrane homogenate was centrifuged at 10,200 g for 15 min. Most of the time, the lipid associated with the membrane was so abundant that a yellow fat layer formed at the top of the homogenate. When this occurred, the underlying solution was removed and centrifuged a second time to remove the remaining fat. The reddish cloudy solution was then centrifuged at 106,500 g for 30 min. The pellet was suspended in medium II (50 mM MES, 50 mM Tris-Cl, pH 7.2) and centrifuged at 106,500 g for another 30 min. The new pellet was resuspended in medium II. The microsomes had protein concentrations of 20 to 50 μg/μl and were stored in aliquots at -70°C. In the study of the effect of protease inhibitors on ACAT activity upon storage, the microsomes were prepared in medium II plus 1 μg/ml each aprotinin-pepstatin-leupeptin-antipain and 17 μg/ml benzamidine.

When the effect of ALLN on ACAT activity during storage at 4°C was studied, 450 μl microsomes or microsomes plus protease inhibitors were mixed with 50 μl 10 μg/ml ALLN, dissolved in medium II, to make a final ALLN concentration of 1 μg/ml. The mixture was stored at 4°C for 10 days and assayed for ACAT activity on the day it was prepared and every other day thereafter. In a study of the effect of DTT on ACAT activity during storage at 4°C, a similar procedure was followed except the final DTT concentration was 1 mM.

Protein concentration was determined by the Lowry method [77].

2. Preparation of mouse liver microsomes Mice were sacrificed by cervical dislocation and livers were isolated on ice. The livers were homogenized in about 3 volumes of medium I and centrifuged at 10,000 g at 4°C for 30 min. The supernatant was centrifuged at 106,255 at 4°C for one hour. The pellet was washed in medium II plus protease inhibitors (1 μg/ml each aprotinin-pepstatin-leupeptin-antipain and 17 μg/ml benzamidine) and centrifuged again. The new pellet was resuspended in medium II-protease inhibitors and stored at -70°C in aliquot.

3. Solubilization of microsomal ACAT Zwitterionic detergent CHAPS was used to extract ACAT from microsomes. Typically, the solubilization mixture contained 0.5% CHAPS and 0.5 M NaCl in medium II, and the protein to CHAPS ratio was 1 to 1 (w/w). The mixture was shaken slowly at 4°C for 30 min and centrifuged at 106,500 g at 4°C for one hour. ACAT activity and protein content of the extract were determined for each preparation. To determine ACAT activity, one volume of the extract was preincubated at room temperature (r.t.) for 30 min with nine volumes of liposomes of 0.30 mole fraction of cholesterol in egg PC followed by the regular ACAT assay procedure.

4. ACAT assay The assay for ACAT followed exclusively M. Shih's work [3]. One hundred and sixty microliters of microsomes with 15 to 250 μg protein were preincubated at 37°C for 10 min. To start the esterification reaction, 40 μl of ^{14}C-oleoyl CoA solution (250 μM ^{14}C-oleoyl CoA-0.5 mg/40 μl BSA, 25 dpm/pmole) was added to the assay mixture. After 5 min, 2 ml of hexane/2-propanol (3/2, v/v) was added to terminate the reaction. Ten μl of cholesteryl-^{3}H-oleate (about 20,000 dpm) was introduced to the mixture as an internal standard. The organic layer was separated and dried under a

stream of N_2. The solid residue was redissolved in 200 µl hexane/diethyl ether (98/2, v/v) and applied onto a column of 0.7 g Silica gel (40-140 mesh) which had been prewashed with 3 ml hexane/diethyl ether. The cholesteryl oleate was eluted from the column with 6 ml hexane/diethyl ether and collected in a 7 ml scintillation vial. After evaporation of the solvent, 200 µl of the same solvent was added to the vial followed by 5 ml Ecolumn counting fluid. The amount of cholesteryl-^{14}C-oleate formed was determined by dual radioisotope counting.

25-Hydroxycholesterol can be esterified by ACAT under the assay condition [5,72,74], but the ester with oleate (5-cholesten-3β,25-diol 3-oleate) does not elute out of the silica column with the elution solvent used in the assay, as demonstrated by the result of TLC developed in 90:10:1 (v/v/v) petroleum ether:dithyl ether:acetic acid. Thus, in the ACAT assay in the presence of 50 µM 25-OH, the radioactivity eluted from the column was associated only with cholesteryl oleate.

When exogenous cholesterol was needed, microsomes were incubated first with liposomes of cholesterol-phosphatidylcholine at room temperature for 30 min followed by the regular assay procedure.

5. DTDP treatment of ACAT extract For this particular experiment, ACAT extract was prepared in 100 mM HEPES, pH 8.0, a proper condition for DTDP treatment. ACAT extract was incubated with 10 mM IAM-HEPES at r.t. for 20 min in the dark followed by different concentrations of DTDP/EtOH at 30^{0}C for 75 min. The concentration of EtOH was 1% (v/v). To remove 2-thiopyridine and unreacted IAM and DTDP, a pre-packed column of Sephadex G-25 was used and 0.5% CHAPS-0.5 M NaCl-medium II was the

elution buffer. The eluant was concentrated using a Centricon 10 cartridge (molecular weight cutoff: 10,000). The amount of free thiol was determined by DTNB method [78] and 1% SDS was necessary to get clear solutions.

6. Treatment of ACAT with chemical reagents An aliquot of 9.7 mg of PMB was mixed with 13.454 ml of medium II and stirred vigorously for a few hours. The suspension was allowed to stay undisturbed overnight. The clear supernatant was transferred to another container, and the concentration of PMB was determined spectrophotometrically by using a molar extinction coefficient of 4400 $M^{-1}cm^{-1}$ [79]. The PMB solution was stored in the dark and at room temperature. The incubation of microsomal ACAT with PMB was 10 min at 37°C.

Stock solutions of IAM, NEM, NBM, and 2-Mer were prepared in medium II. Due to limited solubility in aqueous buffer, BdiT, DEPC, and HP-B were dissolved in EtOH. In that case, the final concentration of EtOH was 1% (v/v) and EtOH treated microsomes were used as the control microsomes. The concentration of HP-B was determined using Xylenol Orange method and H_2O_2 as the standard [80]. Treatment of microsomes with IAM, NEM, and NBM was 20 min at r.t. in the dark and treatment with 2-Mer and BdiT was 30 min at 37°C. Treatment with DEPC was 10 min at 37°C. Proper controls were prepared under exact time and temperature conditions except buffer replaced the reagents.

To measure the amount of thiol remaining after treatment of microsomes with thiol-reactive agents, the microsomes were centrifuged at 100,000 g at 4°C for 30 min, and washed twice before being resuspended in medium II. The amount of thiol was determined by the DTNB method [78] with 1% SDS.

7. Oxidative modification of microsomes Five μl of microsomes with 170 μg protein was diluted into 155 μl H_2O_2-medium II solutions of different concentrations. The mixture was incubated at r.t. for 60 min followed by the regular ACAT assay. The concentration of the original stock solution of H_2O_2 was determined spectrophotometrically using a molar extinction coefficient of 43.6 M^{-1} cm^{-1} at 240 nm [81].

Forty μl of microsomes with 1360 μg protein was diluted into 600 μl $CuSO_4$-medium II solution and the final $CuSO_4$ concentration in the mixture was 5 μM. The mixture was shaken at r.t. overnight. Eighty μl of $CuSO_4$-treated microsomes was mixed with 80 μl 2 mM EDTA-medium II and shaken at r.t. for 30 min followed by the regular ACAT assay. Two controls were made in this experiment. One control, labeled as overnight control, was prepared along with the $CuSO_4$ treatment and used to assess the effect of 1 mM EDTA. The other control, labeled as zero-time control, was prepared by thawing an aliquot of microsomes and assayed for ACAT activity, along with the overnight control and $CuSO_4$ treated microsomes.

When the effect of minor membrane lipid molecules was studied, 5 μl of microsomes with 170 μg protein was mixed with 153.4 μl medium II and 1.6 μl EtOH solutions of linolenic acid, or lysoPC, or DOG, or bovine brain ceramides, or DHS. The final EtOH concentration was 1% (v/v) and the final concentrations of linolenic, DOG, or DHS were 100 nM while those of lysoPC and ceramides were 0.0025% (w/v). The mixture was shaken at r.t. for 60 min prior to the regular ACAT assay.

8. Quantitation of synthesis of cholesteryl ester, triacylglycerol and phospholipids Five μl of microsomes with 220 μg protein was incubated with 155 μl medium II or

MβCD-medium II at r.t. for 30 min. The concentration of MβCD in the treatment was 1 mM. The mixture was incubated at 37°C for 10 min prior to the addition of 40 μl ^{14}C-oleoyl CoA. Two ml 2/1 (v/v) chloroform/MeOH was added to the mixture after a 5 min reaction time at 37°C followed by the addition of 10 μl ^{3}H-cholesteryl oleate. The top aqueous layer was extracted one more time with 2/1 chloroform/MeOH and the organic extracts were combined and dried under N_2. The solid residue was redissolved in 50 μl 2/1 (v/v) chloroform/MeOH and applied onto a TLC plate which had been pre-washed in MeOH for 5 min and air-dried completely. For the quantitation of neutral lipids, the plate was developed in petroleum ether:diethyl ether:acetic acid, 80:20:0.5 (v/v/v) [82] and for complex lipids in chloroform:methanol:water:acetic acid, 70:30:4:2 (v/v/v/v) [83]. Compared with standards, the spots corresponding to CE, TAG or PL were scraped into a scintillation vial and counted for radioactivity.

9. Cholesterol depletion experiment One ml of microsomes was mixed with 5 ml methyl-β-cyclodextrin solution and stirred slowly at 4°C for one hour. The amount of protein in the microsomes was 44 mg. The mixture was centrifuged at 106,500 g at 4°C for 30 min. The pellet was suspended in 1.3 ml medium II and subjected to the same treatment with methyl-β-cyclodextrin (MβCD) two more times. One wash with medium II after the third depletion was needed to get rid of MβCD. Gentle homogenization was sometimes needed during resuspension and the final microsomes were prepared in a small volume of medium II to have a concentrated protein solution. The amount of ACAT activity, protein content, cholesterol and phospholipid concentration were measured after each step of depletion.

10. Preparation of liposomes The liposomes were prepared following the procedure of R.C. MacDonald et al [84]. Cholesterol was recrystallized twice from methanol and dissolved in chloroform. A solution of phosphatidylcholine, 20 mg/ml in chloroform, was mixed with different amounts of cholesterol/chloroform so that certain mole ratios of cholesterol to PC were obtained. The combined solution was dried first under a stream of N_2 and then under vacuum at 40-45^{0}C for 30 min. The waxy residue was suspended in medium II and the final PC concentration was 10 mg/ml. The mixture was then frozen and thawed 10 times, using a dry ice-ethanol bath and r.t. water bath, respectively. The multilamellar vesicles were extruded 19 times through 2 polycarbonate membranes (100 nm pore size) mounted on an extrusion device. The unilamellar liposomes were stored under N_2 at 4^{0}C. The concentrations of cholesterol and PC were determined for each liposome preparation.

11. Determination of cholesterol concentration The procedure described by Omodeo Sale et al [85] was followed with moderate modification. Cholesterol was extracted three times from microsomes or liposomes using 3/2 (v/v) hexane/2-propanol. The solvent was evaporated under N_2, and the solid residue was redissolved in ethanol. Reagent A contained 822 μM 4-aminoantipyrine, 1.9 mM Na 3,5-dichloro-2-hydroxybenzene sulfonate, 200 IU/L peroxidase and 50 mM phosphate buffer (3 mM Na cholate-0.5% Triton X-100, pH 7.0) and was prepared on the day of the assay. To a 200 μl sample or standard containing 5 to 20 μg cholesterol in ethanol, 1 ml reagent A was added. After a 10 min incubation at 37^{0}C, absorbance at 505 nm was measured and taken as blank. Ten μl 25 IU/ml cholesterol oxidase dissolved in 50 mM phosphate buffer was added to the sample

prior to a second incubation at 37°C for 15 min. The absorbance at 505 nm was measured again, and the difference between the two readings was used to calculate the amount of cholesterol.

12. Determination of phospholipid concentration Phospholipid concentration was determined according to W.D.Harris et al's work [86], and the assumption was made that the majority of phospholipid in the microsomes or liposomes had one mole of phosphorus per mole of phospholipid. An aliquot of microsomes or liposomes in a final volume of 120 µl was mixed with 1 ml 70% perchloric acid. For the supernatant in the cholesterol depletion experiment, a larger volume of sample, e.g. 500 µl, was used to ensure a large enough sample of phosphorus and the sample was first evaporated to dryness and then redissolved in 120 µl water. After digestion, 200 µl of digest was diluted with 1.7 ml H_2O followed by 200 µl 5% ammonium molybdate and 400 µl Elon reducing reagent. Absorbance at 795 nm was measured 10 min later.

13. Thin-layer chromatography of yolk sac membrane microsomes and cholesterol depletion supernatant Lipids in microsomes or the cholesterol depletion supernatant were extracted by 2/1 (v/v) chloroform/methanol. After evaporation of the extraction solvent, the solid residue was redissolved in 2/1 chloroform/MeOH to make a solution of 20 mg/ml. All the lipid standards were dissolved in chloroform, hexane, or ethanol depending on their solubility in different solvent systems. The TLC plate was washed in methanol for 5 min and air-dried completely. After application of sample and standards, the plate was developed twice, up to the middle of the plate, in 120:60:10 (v/v/v) chloroform:methanol:water, and the third time, all the way to the top of the plate, in

80:20:1.5 (v/v/v) hexane:diethyl ether:acetic acid according to a procedure in Lipid Analysis [87]. The spots were visualized by I_2 vapor.

CHAPTER IV. RESULTS AND DISCUSSION

A. Results

1. Comparison of ACAT of avian and mammalian sources

Studies of structure and function of ACAT from different sources, mainly mammalian tissues, have been done using chemical reagents. In this work, a few thiol-reactive agents and DEPC, a histidine-reactive chemical, were used to study ACAT from avian yolk sac membrane.

As shown in Figure 1, 500 μM DEPC inhibited ACAT activity 90%: 396 ± 10.6 (zero DEPC) vs 41 ± 0.8 (500 μM DEPC) pmole/min/mg. Higher concentrations of DEPC, e.g. 2 mM, did not result in a further decrease in the enzyme activity and the addition of 1% (v/v) EtOH to microsomes had little effect: 360 ± 1.1 (microsomes) vs 396 ± 10.6 (microsomes + EtOH) pmole/min/mg.

Figure 2 shows the result of treating microsomes with PMB of different concentrations. The insert is a plot of ACAT SA against PMB concentration from zero to 20 μM. ACAT activity increased when the concentration of PMB increased from zero to 20 μM: 437 ± 3.8 (zero PMB) (100%) vs 526 ± 16.2 (20 μM) (120%) pmole/min/mg, and then dropped dramatically: 437 ± 3.8 (zero μM) (100%) vs 7.8 ± 0.4 (100 μM) (2%) pmole/min/mg.

Treatments of microsomes with IAM, or NEM, or NBM were repeated several times during the course of the study. The result of one of those experiments is plotted in Figure

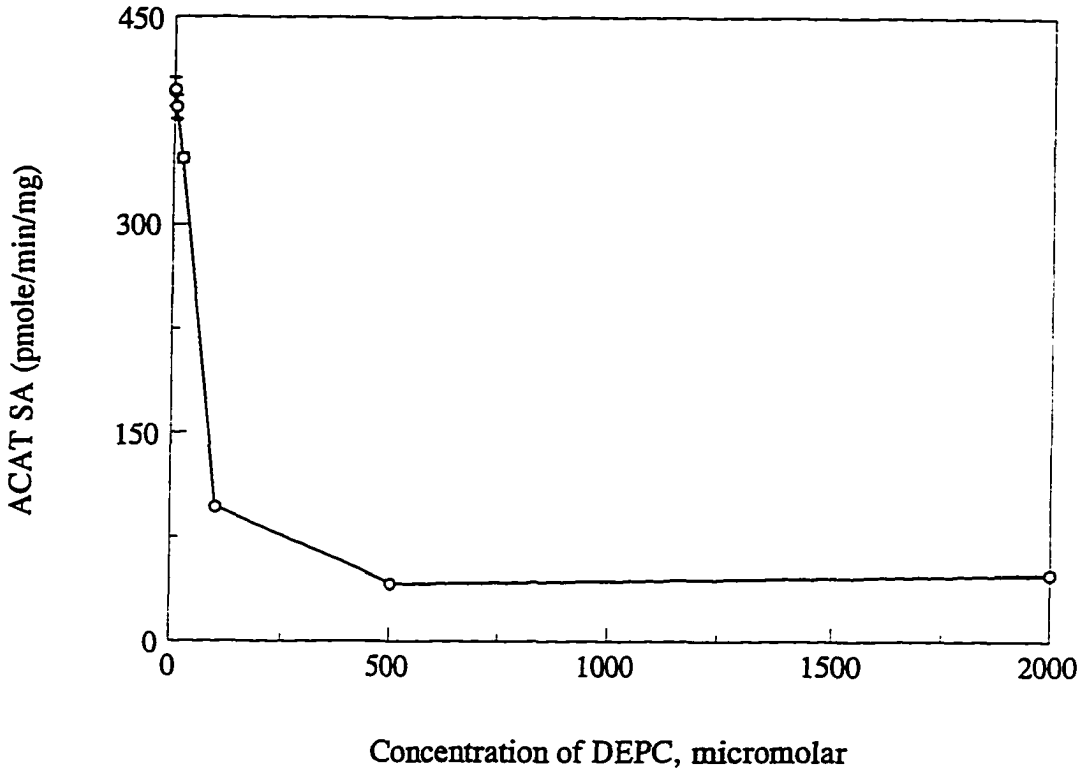


Figure 1. Effect of DEPC on ACAT activity. Five µl of microsomes was incubated at 37°C for 10 min with 155 µl medium II containing different concentrations of DEPC. The stock solution of DEPC was prepared in EtOH and EtOH treated microsomes were the control. The final concentration of EtOH was 1% v/v. Each value is a mean of duplicates with a bar showing the range; where no bar is shown, it is smaller than the symbol.

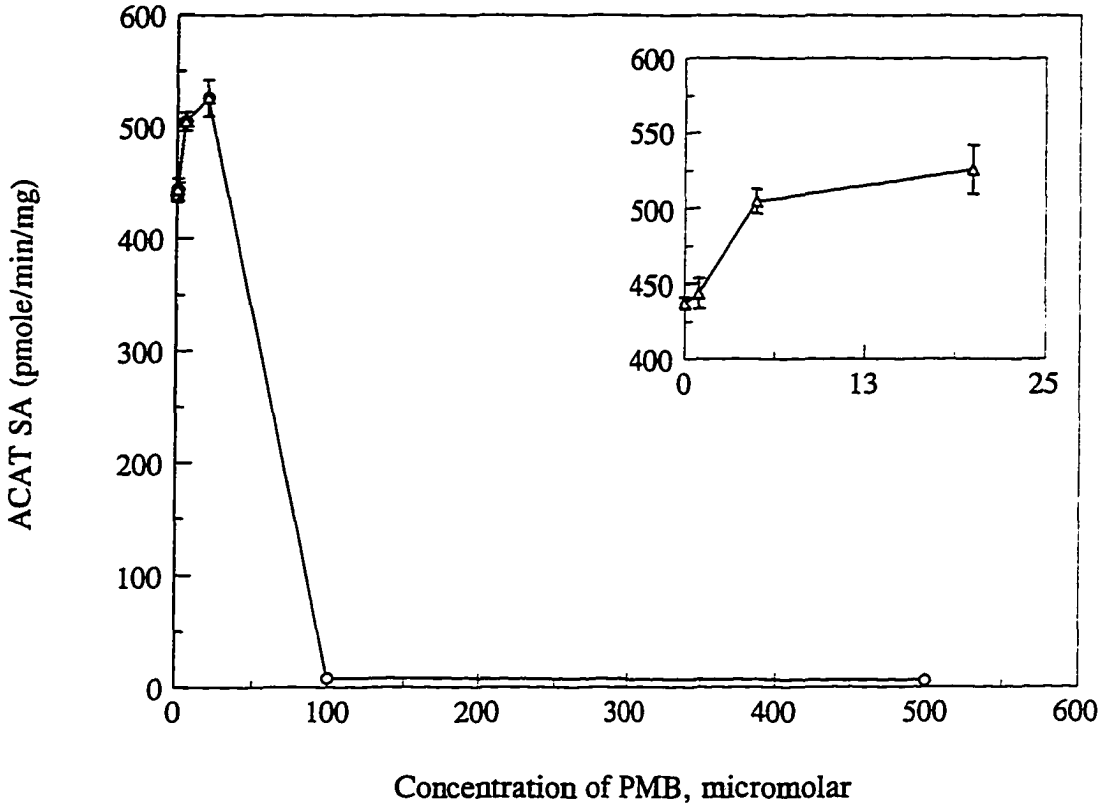


Figure 2. Effect of PMB on ACAT activity. Five µl of microsomes was incubated at 37°C for 10 min with 155 µl medium II containing different concentrations of PMB. The insert was a plot of ACAT SA against PMB concentration from 0 to 20 µM. Each value is a mean of duplicates with a bar showing the range; where no bar is shown, it is smaller than the symbol.

3. Incubation of microsomes with IAM resulted in an increase in ACAT activity: 135 ± 3.2 (zero IAM) (100%), 168 ± 1.0 (1 mM IAM) (124%), and 204 ± 2.8 (20 mM IAM) (151%) pmole/min/mg (Figure 3, IAM treatment). An increase in ACAT activity was consistently observed with 20 mM IAM incubation, even though the magnitude changed a little each time, ranging from 113% to 152% of the control. Treatment of microsomes with 1 mM NEM increased the enzyme activity to a small extent: 120 ± 1.0 (zero NEM) (100%) vs 139 ± 2.0 (1 mM NEM) (116%) pmole/min/mg. Treatment of microsomes with 20 mM NEM produced more variable results, 78% of the original ACAT activity remained in the experiment shown in Figure 3 (NEM treatment), while 101% and 113% of the original activity were obtained in another two experiments (data shown in Figure 4). Incubation of microsomes with a more hydrophobic maleimide derivative, NBM, resulted in an increase in the enzyme activity first: 426 ± 4.1 (zero NBM) (100%) vs 518 ± 10.7 (1 mM NBM) (122%) pmole/min/mg, and then a dramatic decrease: 426 ± 4.1 (zero NBM) (100%) vs 4.9 ± 0.4 (20 mM NBM) (1%) pmole/min/mg (NBM treatment). The same result, 1% of the original activity in the treatment with 20 mM NBM, was obtained in another experiment (see Figure 4). The difference in ACAT activity in control microsomes in the experiments shown in Figure 3 was because of the use of different batches of microsome preparations.

Microsomes were treated sequentially with 20 mM IAM or NEM followed by 500 μM PMB or 20 mM NBM, shown in Figure 4 (A or B). Treatment of microsomes with 500 μM PMB resulted in a 98% inhibition of the enzyme activity (Figure 4A): 7.8 ± 0.1 (500 μM PMB, bar b) vs 484 ± 3.4 (control, bar a) pmole/min/mg. Treatment of

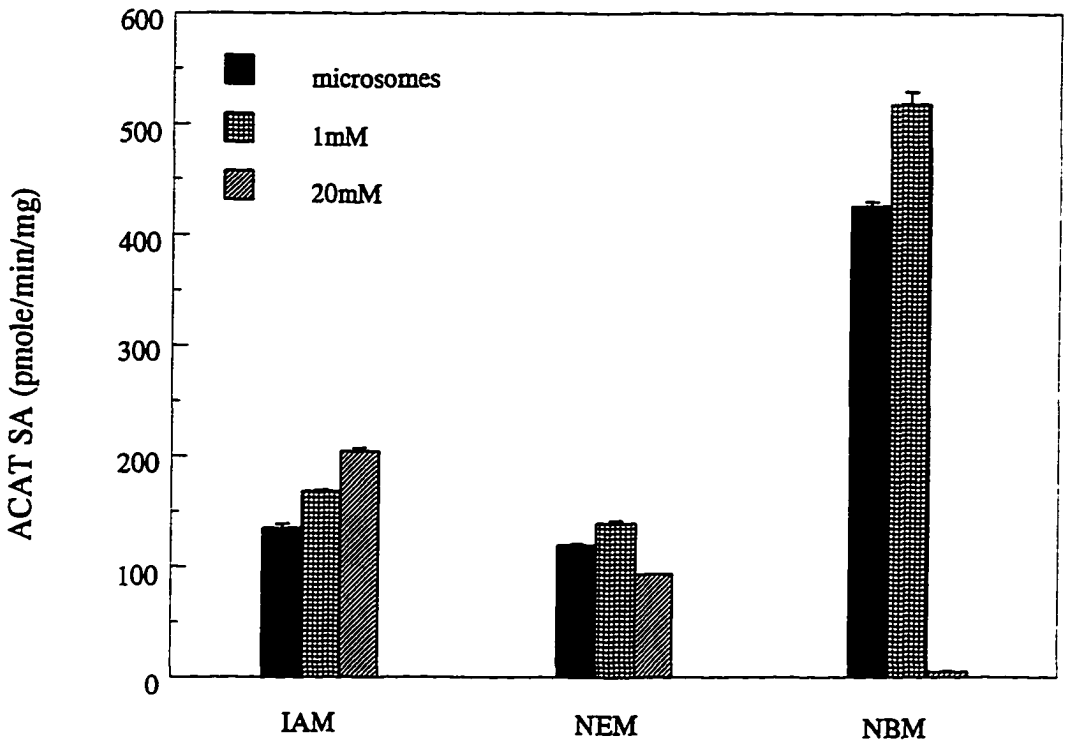


Figure 3. Effect of thiol-reactive agents on ACAT activity. Five μl of microsomes was incubated at r.t. for 20 min in the dark with 155 μl medium II containing IAM, or NEM, or NBM followed by the regular enzyme assay. Each value is a mean of duplicates with the range indicated by error bars.

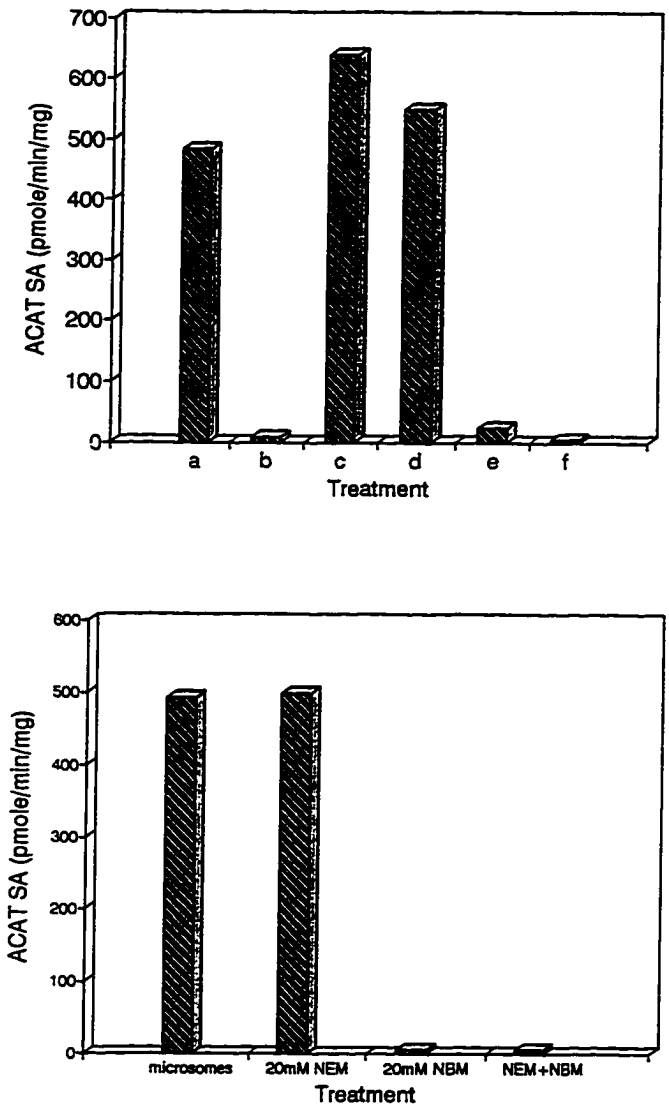


Figure 4. Effect of sequential treatment of microsomes with thiol-reactive agents on ACAT activity. In Figure A, 5 µl of microsomes was mixed with 40 µl medium II containing IAM or NEM and incubated at r.t. for 20 min in the dark. An aliquot of 115 µl PMB solution was added to the mixture prior to the second incubation at 37^{0}C for 10 min. Bar a represents the control, bar b represents the treatment with 500 µM PMB, bar c represents the treatment with 20 mM IAM, bar d represents the treatment with 20 mM NEM, bar e represents the treatment with 20 mM IAM + 500 µM PMB, and bar f represents the treatment with 20 NEM + 500 µM PMB. In Figure B, similar treatment of microsomes with 20 mM NEM and 20 mM NBM was performed except the condition for treatment with NBM was at r.t. for 20 min in the dark. Each value is a mean of duplicates and in all the cases, the error range was ≤ 12% of the mean.

microsomes with 20 mM IAM or NEM resulted in an increase of 32% or 13% of the control activity: 639 ± 3.2 (20 mM IAM, bar c) or 549 ± 9.4 (20 mM NEM, bar d) pmole/min/mg. Pretreatments of microsomes with 20 mM IAM or NEM did not protect the enzyme from the inactivation caused by incubation with 500 μM PMB. There was 5% enzyme activity remaining after IAM + PMB treatment: 23 ± 1.0 (bar e) pmole/min/mg , and 1% remaining after NEM + PMB treatment: 4.7 ± 0.1 (bar f) pmole/min/mg. A similar experiment, shown in Figure 4B, was performed with a pretreatment of microsomes with 20 mM NEM followed by 20 mM NBM, at r.t. for 20 min in the dark. Incubation of microsomes with 20 mM NEM had no effect on ACAT activity: 499 ± 6.3 (20 mM NEM) vs 493 ± 13.3 (control) pmole/min/mg. Treatment of microsomes with 20 mM NBM caused a complete inactivation of the enzyme: 4.9 ± 0.1 (20 mM NBM) pmole/min/mg, and preincubation with 20 mM NEM did not prevent the inactivation: 4.7 ± 0.6 (20 mM NEM + 20 mM NBM) pmole/min/mg.

The thiol content in microsomes with or without the treatment of thiol-reactive agents was measured (column 2 in Table 1) and the percentage of reagent-reactive SH was calculated (column 3 in Table 1). PMB at 20 μM modified 6% of the total microsomal thiols and caused a 20% increase in ACAT activity. PMB at 100 μM modified 29% of the thiols and inactivated the enzyme completely (2% of the control activity). Treatment of microsomes with 20 mM IAM modified 18% of the total thiols and increased ACAT activity from 13% to 52% (varied from experiment to experiment). NEM at 20 mM reacted with 45% and 44% of total microsomal thiols and had an effect on activity that was more variable: from 78% to 113% of the control. Treatment of microsomes with 20 mM

Table 1. Thiol content in microsomes. Forty μl of microsomes with 1520 μg protein was incubated with 160 μl thiol-reactive agents. Microsomes were centrifuged at 100,000 g at 4°C for 30 min and washed twice. The thiol content is expressed as nmol per mg protein.

	SH remaining after modification	Percent of SH reacted with the reagent
microsomes[a]	57.4 ± 0.6	—
20 μM PMB	54.2 ± 0.1	6
100 μM PMB	40.8 ± 1.0	29
20 mM IAM	47.3 ± 0.2	18
microsomes[b]	61.4 ± 0.5	—
20 mM NEM	34.0 ± 1.3	45
20 mM NBM	23.5 ± 0.5	62
microsomes[c]	58.2 ± 0	—
20 mM NEM	32.4 ± 0.1	44
20 mM NBM	22.8 ± 0.7	61

[a], [b], [c] are different experiments with the same batch of microsomal preparation.

NBM inhibited ACAT completely (1% of the control) and the thiols that were modified were 62% and 61%.

Solubilization of ACAT was achieved by using the zwitterionic detergent CHAPS. The ACAT SA in microsomes and extracts and resuspended pellets of different CHAPS concentrations is shown in Figure 5. The CHAPS to protein ratio in 0.5%, 1%, 1.5%, 2% CHAPS solubilization mixtures was 1:1, 2:1, 3:1, 4:1 by weight, respectively. Solubilization of ACAT using 0.5% CHAPS with a detergent to protein ratio of 1:1 by weight gave the highest activity in the extract, and 0.5% CHAPS was used in the following solubilization experiments.

Microsomes were solubilized with 0.5% CHAPS-medium II or 0.5% CHAPS + 0.5 M NaCl-medium II and the pellet was resuspended in 0.5% CHAPS-medium II, shown in Figure 6A. The addition of 0.5 M NaCl to the solubilization mixture caused an increase in ACAT activity in the extract and in the ratio to that in the pellet: without NaCl, ACAT SA was 109 ± 0.4 pmole/min/mg in the extract and 311 ± 4.2 pmole/min/mg in the pellet (panel a), with NaCl, ACAT SA was 1671 ± 41.3 and 251 ± 3.8 pmole/min/mg, respectively (panel b). In Figure 6B, microsomes were solubilized with 0.5% CHAPS + 0.5 M NaCl-medium II and the pellet was resuspended in either 0.5% CHAPS-medium II (panel c) or 0.5% CHAPS + 0.5 M NaCl-medium II (panel d). The presence of 0.5 M NaCl in resuspension buffer increased the enzyme activity in the resuspended pellet. Results of Figure 22A and B were from two different batches of microsomal preparation.

When microsomes were solubilized with 0.5% CHAPS-0.5 M NaCl-100 mM HEPES, pH 8.0, the extract was treated with 10 mM IAM followed by different

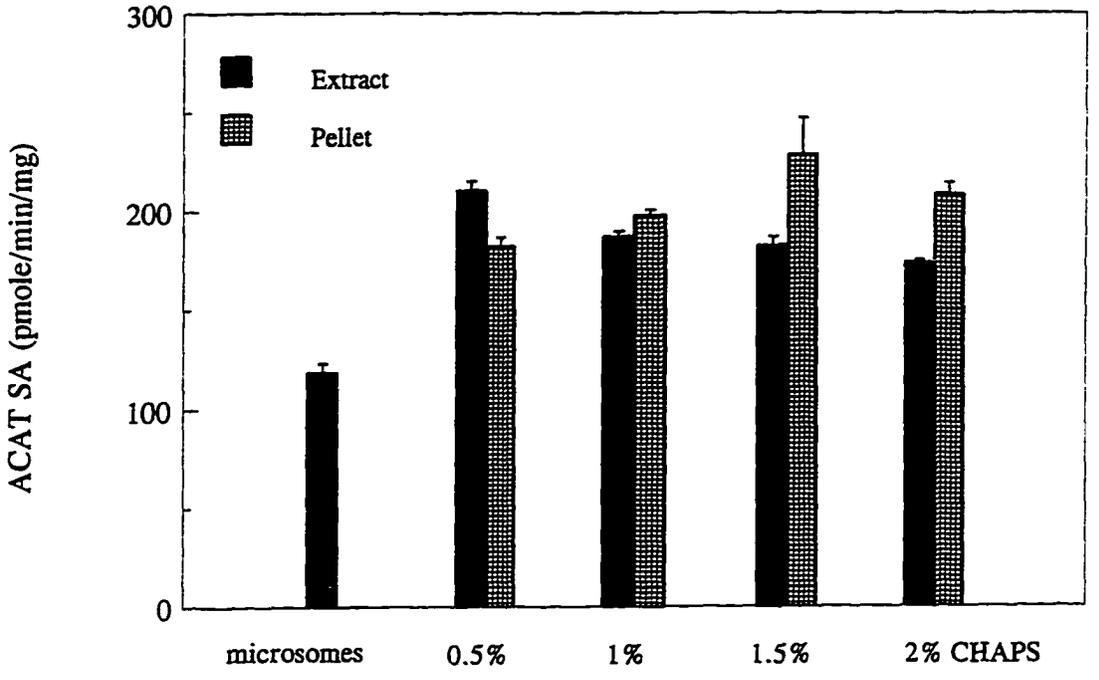


Figure 5. Solubilization of ACAT of yolk sac membrane by CHAPS. Eight-four μl of microsomes with 3 mg protein was mixed with 516 μl medium II of different concentrations of CHAPS. After centrifugation, the pellet was resuspended in medium II with various CHAPS concentrations. Each value is a mean of duplicates with the range indicated by error bars.

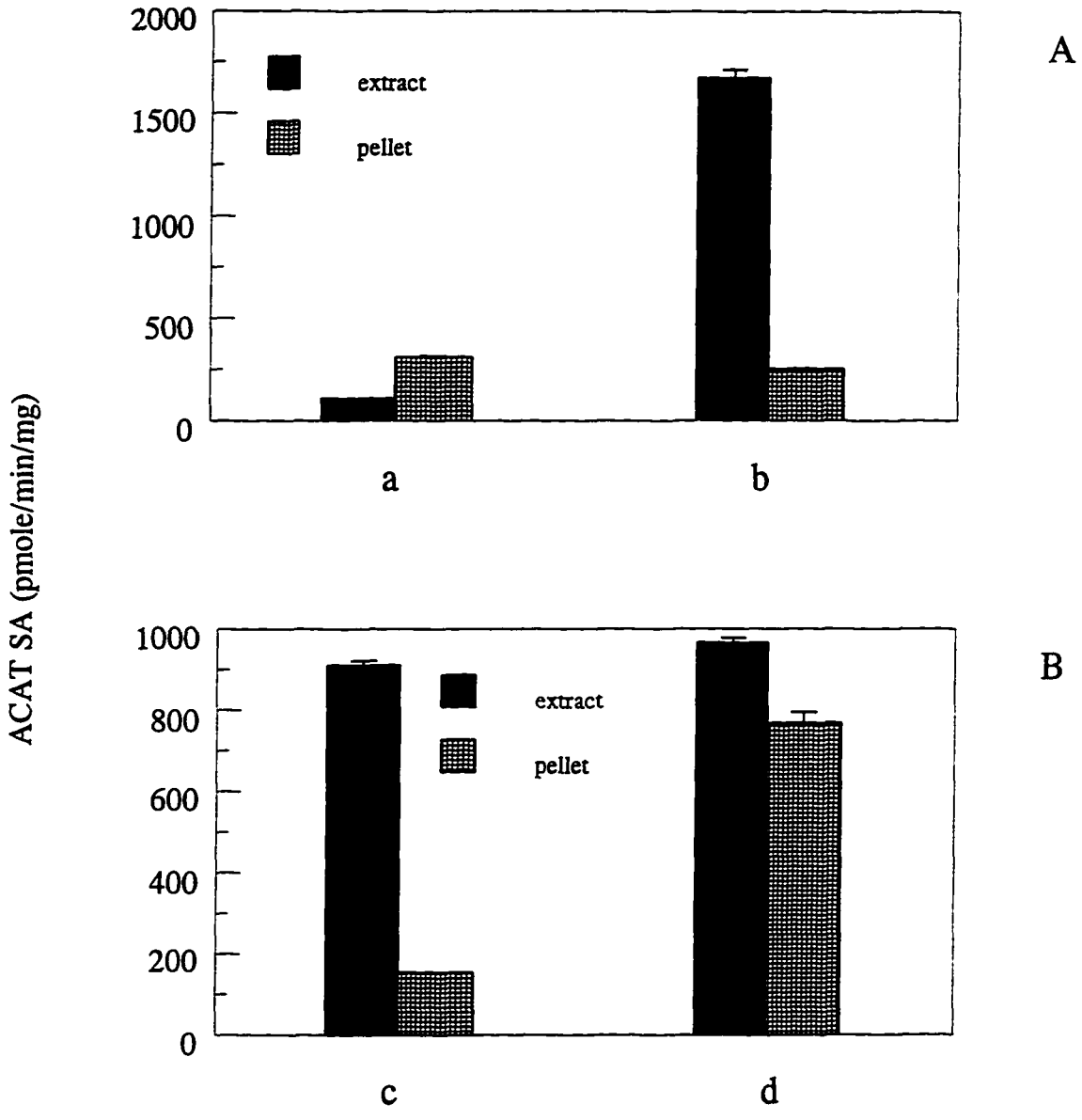


Figure 6. Solubilization of ACAT of yolk sac membrane by CHAPS. In Figure 6A, microsomes were solubilized with 0.5% CHAPS-medium II, panel a, or 0.5% CHAPS + 0.5 M NaCl-medium II, panel b, and the pellet was resuspended in 0.5% CHAPS-medium II. In Figure 6B, microsomes were solubilized with 0.5% CHAPS + 0.5 M NaCl-medium II and the pellet was resuspended in either 0.5% CHAPS-medium II, panel c, or 0.5% CHAPS + 0.5 M NaCl-medium II, panel d. Each value is a mean of duplicates with the range indicated by error bars.

concentrations of DTDP, shown in Figure 7. As the thiol content in the extract decreased, in the presence of increasing concentration of DTDP, ACAT SA decreased. At 2 mM DTDP, the thiol content in the extract was 80% of the original: 20.0 ± 0.05 (2 mM DTDP) vs 25.0 ± 0.05 (zero DTDP) nmole/mg protein and the enzyme activity was 22% of the original: 106 ± 0.5 (2 mM DTDP) vs 493 ± 28.6 (zero DTDP) pmole/min/mg.

To compare reactivity of mouse liver microsomal ACAT towards thiol-reactive agents with the yolk sac enzyme, mouse liver microsomes were prepared and treated with 20 mM IAM or NEM, or NBM, 10 μM, 1 mM, and 20 mM, shown in Figure 8. Treatment of mouse liver microsomes with 20 mM IAM caused a little reduction in ACAT activity: 754 ± 1.0 (control) (100%) vs 687 ± 14.0 (IAM) (91%) pmole/min/mg, and treatment with 20 mM NEM caused a substantial inactivation: 754 ± 1.0 (control) (100%) vs 164 ± 3.0 (NEM) (22%) pmole/min/mg. NBM at 10 μM had no effect on ACAT activity, and 1 mM and 20 mM NBM inhibited the enzyme 47% and 99% (402 ± 0.6 and 6 ± 0.1 pmole/min/mg), respectively.

Microsomes of yolk sac membrane or mouse liver were treated with 500 μM PMB at 37^{0}C for 10 min followed by either 5 mM 2-Mer, Figure 9A, or BdiT, Figure 9B, at 37^{0}C for 30 min. In the experiment shown in Figure 9A, PMB of 500 μM completely inactivated ACAT of either yolk sac membrane or mouse liver: 2% or 1% of the corresponding controls. Reagent 2-Mer of 5 mM had no or little effect on ACAT of either source: 103% or 94% of the controls. For ACAT of yolk sac membrane, 5 mM 2-Mer recovered from the PMB treatment 87% of the original activity, while for the enzyme of mouse liver, 2-Mer had little effect on recovery: only 6% of the original was detected after

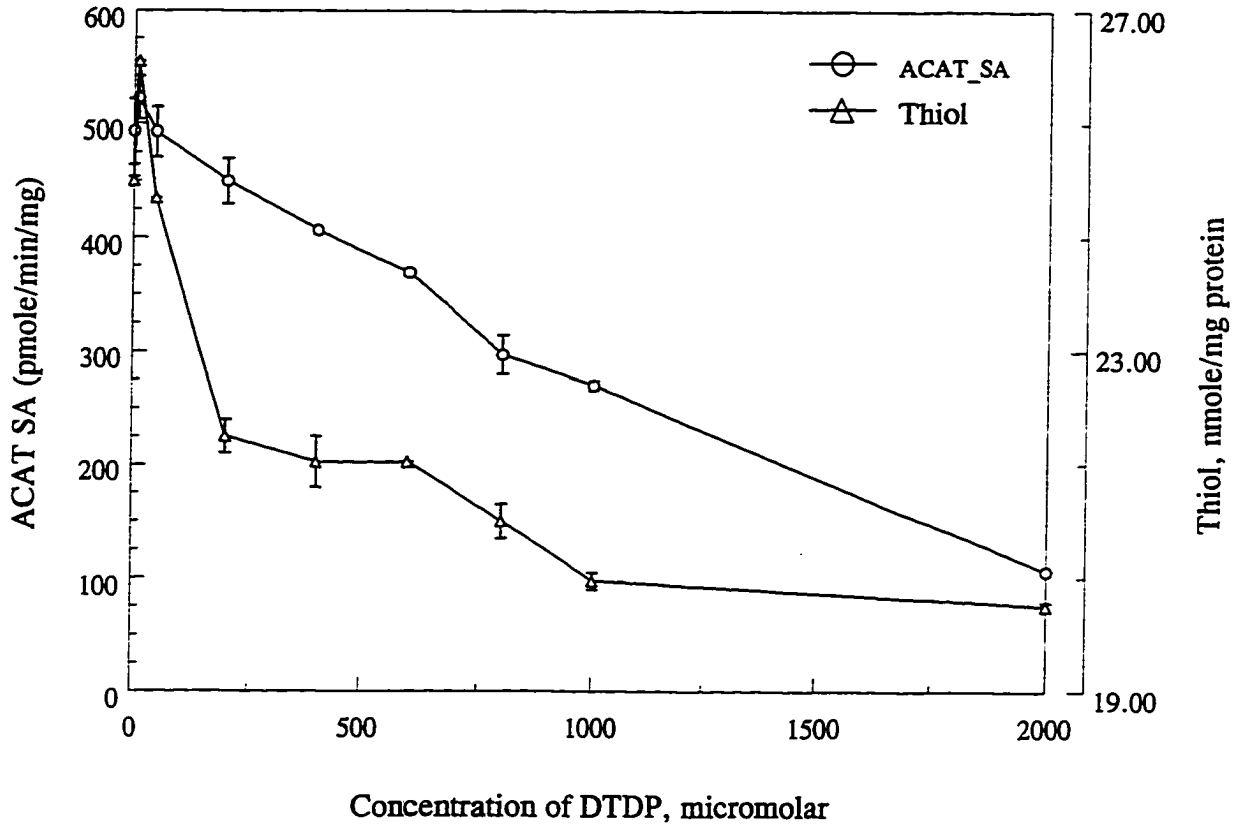


Figure 7. Effect of DTDP on ACAT activity and thiol content in the extract. An aliquot of 980 µl extract with 2352 µl protein was treated with 10 mM IAM followed by different concentrations of DTDP. Stock solution of DTDP was prepared in EtOH and EtOH treated extract was the control. The ACAT SA, represented by the line with circles, is scaled on the 1st Y axis and the thiol content, represented by the line with triangles, is scaled on the 2nd Y axis. Each value is a mean of duplicates with a bar showing the range; where no bar is shown, it is smaller than the symbol.

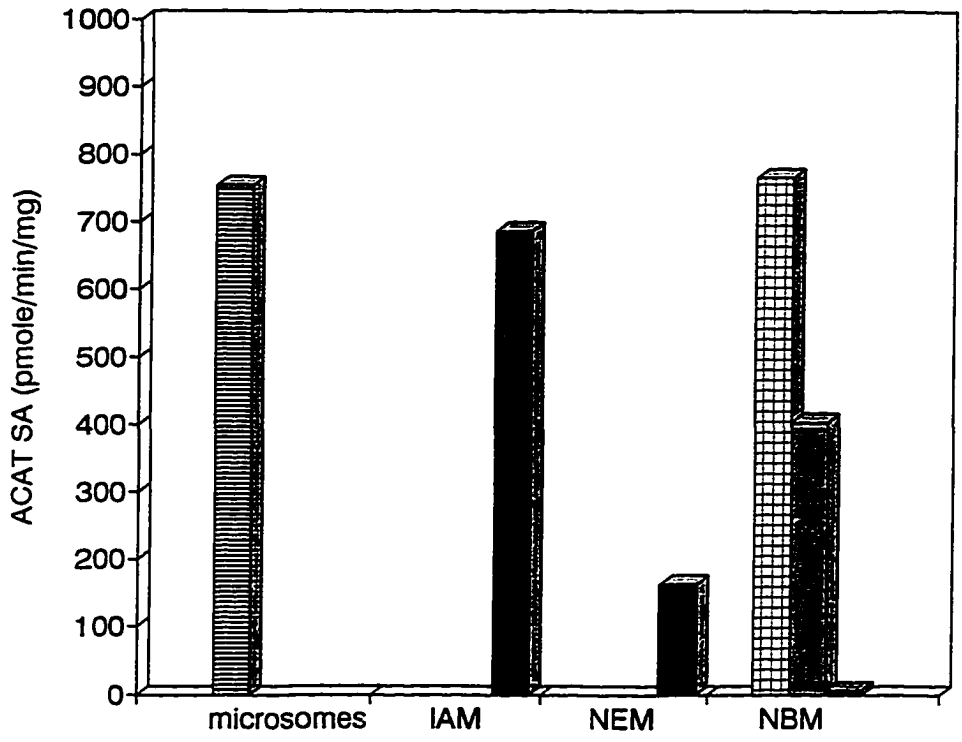


Figure 8. Effect of thiol-reactive agents on ACAT of mouse liver microsomes. Five µl of microsomes was incubated at r.t. for 20 min in the dark with 155 µl medium II containing IAM, or NEM, or NBM followed by the regular enzyme assay. The bar with - represents the value of microsomes, the crossed bar represents the value of 10 µM NBM treated microsomes, the hatched bar represents the value of 1 mM NBM treated microsomes, and the filled bars represent the values of 20 mM reagent treated microsomes. Each value is a mean of duplicates. In all cases, the range is ≤ 2% of the mean.

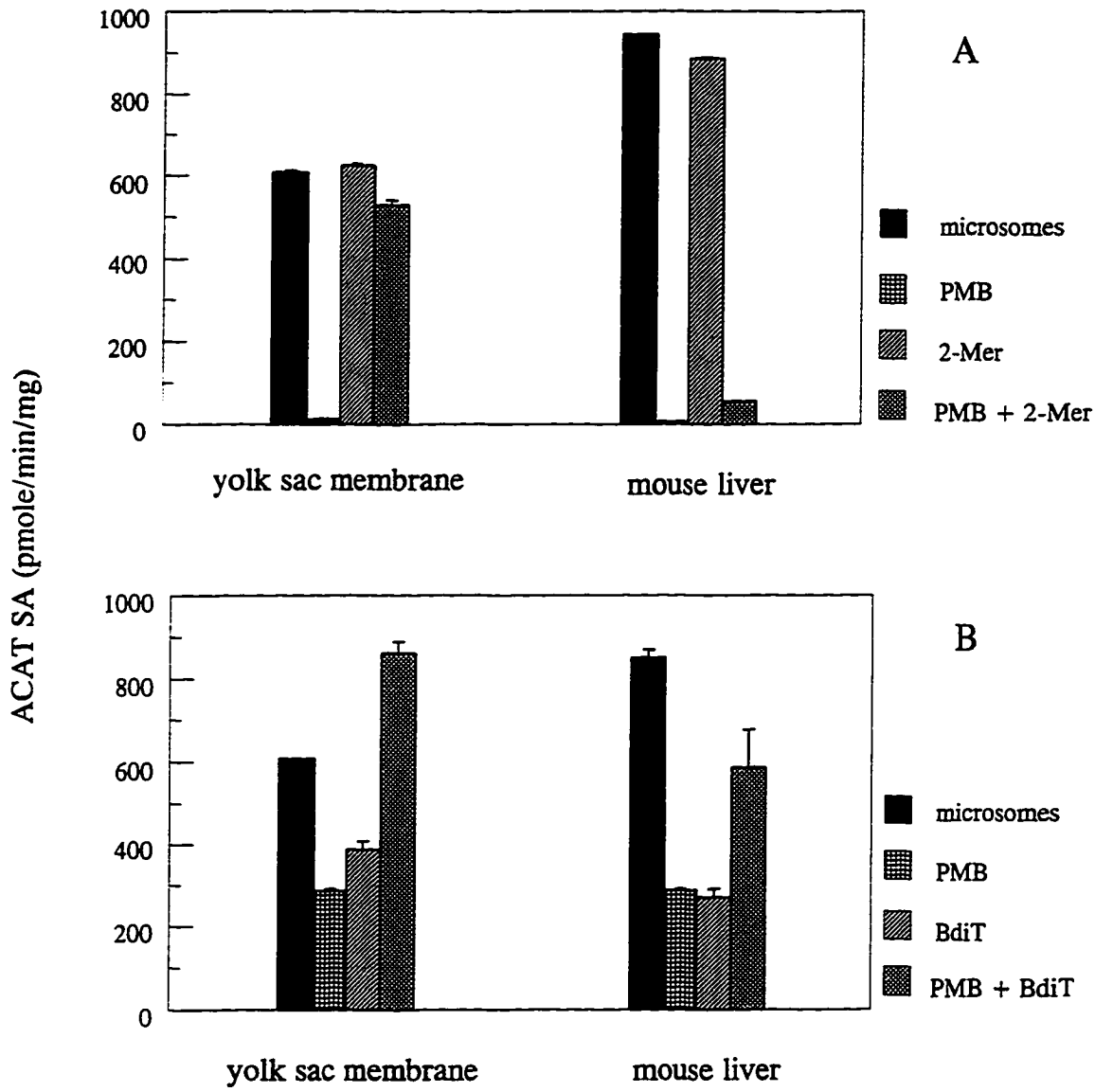


Figure 9. Effect of thiol-reactive agents on ACAT of yolk sac membrane or mouse liver. Microsomes were incubated with 500 μM PMB at 37°C for 10 min followed by 5 mM 2-Mer, Figure 9A, or BdiT, Figure 9B, at 37°C for 30 min. Each value is a mean of duplicates with the range indicated by error bars.

the sequential incubation. In Figure 9B, PMB at 500 μM inhibited the enzyme 53% or 66% of the controls and 5 mM BdiT inhibited 36% or 68%. Sequential incubation of microsomes with PMB and BdiT activated ACAT of yolk sac membrane: 608 ± 0.5 (control) (100%) vs 860 ± 28.6 (PMB + BdiT) (141%) pmole/min/mg, and the same treatment recovered 69% of the original activity in mouse liver: 850 ± 18.0 (control) vs 585 ± 90.5 (PMB + BdiT) pmole/min/mg. Experiments shown in Figure 9A and B used different batches of microsomes of either source and different stock solutions of PMB.

Microsomes of yolk sac membrane or mouse liver were treated sequentially with cholesterol hydroperoxide (HP-B) and DTT, Table 2. The data of mouse liver are from Dr. M. Shih's dissertation [3]. ACAT of the avian source lost 22% of the control activity by oxidation of HP-B and DTT could recover completely the lost activity. On the other hand, HP-B was much more effective on inactivation of the mammalian enzyme and DTT could not reverse the inhibitory effect by HP-B oxidation.

2. Effect of cholesterol redistribution on ACAT activity

ACAT of yolk sac membrane was found to be quite tolerant of repeated freezing and thawing: SA of 753 ± 4.4 pmole/min/mg after the 1st cycle vs 842 ± 16.0 after the 6th cycle, an increase of 12%. Freezing and thawing is known to be destructive of protein structure and enzymes tend to become inactive after repeated freezing and thawing. To further investigate this phenomenon, microsomes were thawed and stored at 4^0C for 10 days. The ACAT activity was measured on the day the membrane preparation was thawed (day 0) and every other day thereafter. The experiment was carried out 3 times; the 1st experiment started the next day after the microsomes were prepared, the 2nd experiment

Table 2. Effect of cholesterol hydroperoxides on ACAT activity. Microsomes were incubated with HP-B at 37°C for 15 min before being treated with DTT at 37°C for 30 min followed by the regular ACAT assay. HP-B was prepared in EtOH solution and 1% (v/v) EtOH was the Control.

	ACAT SA pmole/min/mg	Percent of control
Yolk sac membrane microsomes:		
Control	231 ± 14.2	100
1.2 mM DTT	245 ± 17.9	106
138 μM HP-B	180 ± 30.6	78
138 μM HP-B + 1.2 mM DTT	226 ± 6.8	98
Mouse liver microsomes[a]:		
Control	—	—
1.3 mM DTT	1203	100
100 μM HP-B	112	9
100 μM HP-B + 1.3 mM DTT	171	14

[a] Data are from M. Shih's Ph. D. dissertation [3].

was after 3.5 weeks storage at -70°C, and the 3rd after 7 weeks storage at -70°C. The results of these 3 experiments are plotted in Figure 10 as ACAT SA vs days of storage at 4°C.

As one could see from Figure 10, storage of microsomes at -70°C slowly inactivated ACAT. When the microsomes were just made and stored at -70°C overnight, the ACAT SA was 504 ± 6.4 pmole/min/mg (100%) (value at day 0). After 3.5 weeks storage at -70°C, the SA decreased to 466 ± 7.9 pmole/min/mg (92%). After 7 weeks at -70°C, the SA decreased to 395 ± 11.2 pmole/min/mg (78%). During storage at 4°C, the activity first increased, then slowly declined.

To study the possible effects of proteases during storage and thawing, the same time course experiment as in Figure 10, was carried out, but with a mixture of protease inhibitors (1 μg/ml each aprotinin-pepstatin-leupeptin-antipain and 17 μg/ml benzamidine) included in the microsome preparation. Figure 11 shows the ACAT SA vs days of storage at 4°C in the absence (no inh) or presence (inh) of protease inhibitors. Figure 11A shows the result of the experiment conducted the day after the microsomes were prepared and stored at -70°C, while Figure 11B and 11C shows the results of experiments done 3.5 weeks and 7 weeks later, respectively.

As shown in Figure 11, the inclusion of protease inhibitors in microsome preparation results in a more sustained ACAT activity, compared to that in the absence of inhibitors. Even at the end of 10 days, the microsomes still had quite active ACAT: 673 ± 1.9 (inh) vs 104 ± 0.9 (no inh) pmole/min/mg (Figure 11A). However, it seems that any proteases that were present may have been inactivated during the storage at -70°C, and the effect of

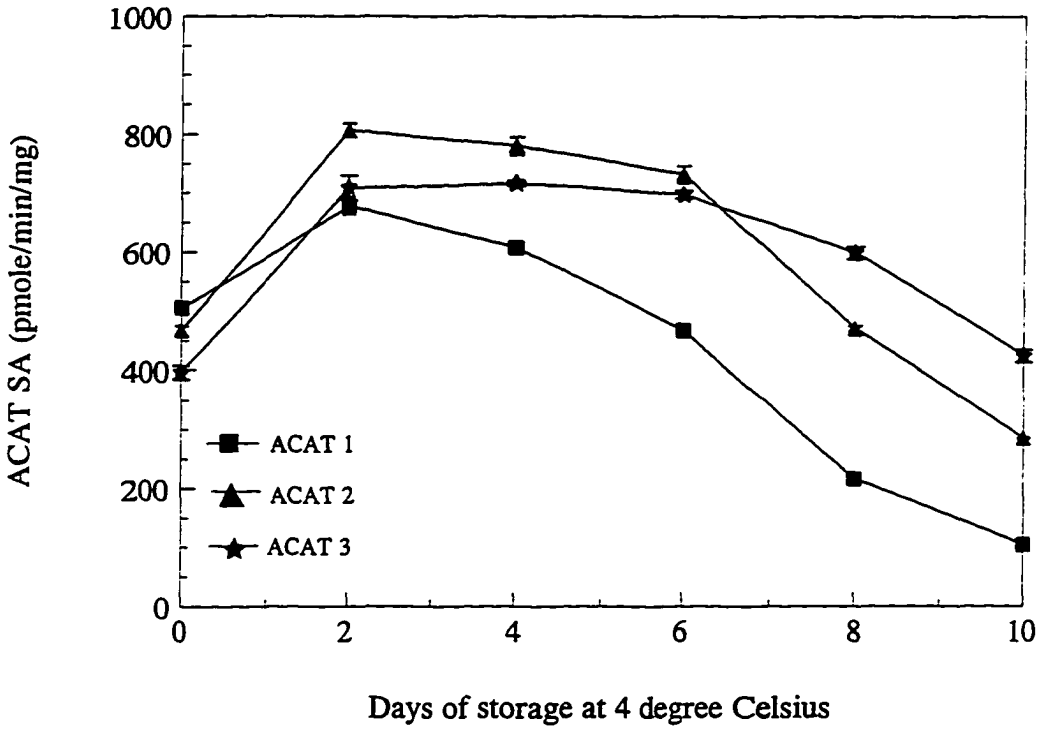
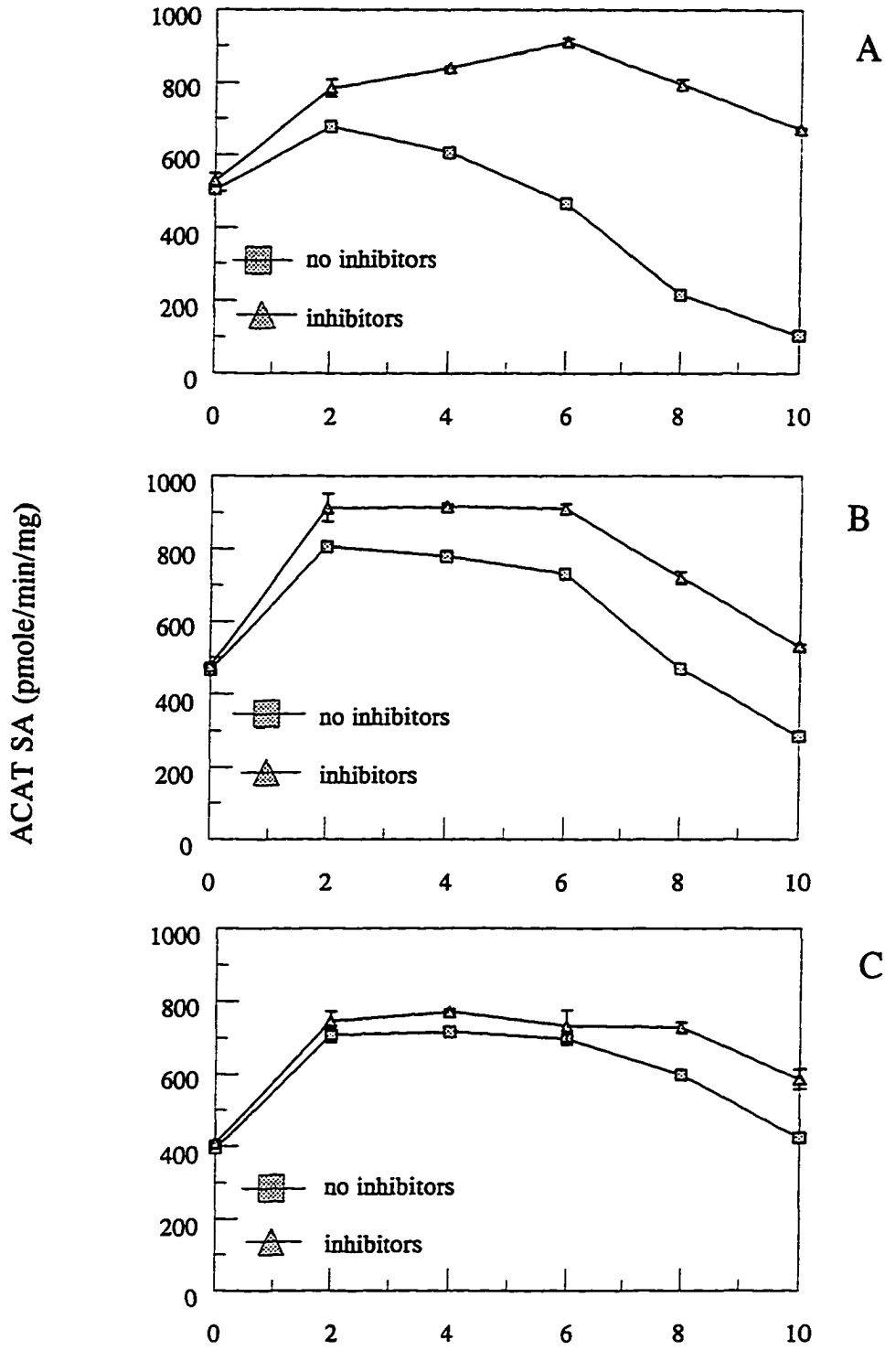


Figure 10. Effect of storage at 4°C on ACAT activity. The microsomes were taken out of -70°C freezer and thawed and stored at 4°C. ACAT activity was measured on the day they were thawed (day 0) and every other day thereafter. The line with ■ represents the experiment carried out the next day after the microsomes were prepared, and the lines with ▲ and ★ represent experiments done 3.5 weeks and 7 weeks later, respectively. Each value is a mean of duplicates with a bar showing the range; where no bar is shown, it is smaller than the symbol.

Figure 11. Effect of storage at 4^0C on ACAT activity in the absence or presence of protease inhibitors. The microsomes with or without protease inhibitors were taken out of -70^0C freezer and thawed and stored at 4^0C. ACAT activity was measured the day after the microsomes were thawed and every other day thereafter. Data of Figure 11A were collected the day after the microsomes were prepared, while those of Figure 11B and 11C were collected 3.5 weeks and 7 weeks later, respectively. Each value is a mean of duplicates with a bar showing the range; where no bar is shown, it is smaller than the symbol.


A
B
C
1000
800
600
400
200
0
0
2
4
6
8
10
no inhibitors
inhibitors
ACAT SA (pmole/min/mg)
Days of storage at 4 degree Celsius

protease inhibitors on ACAT SA diminished dramatically. By the 7th week (Figure 11C), ACAT was almost as active in the absence of inhibitors as in the presence of inhibitors.

One of the interesting features observed in Figure 10 is that the ACAT SA goes up the first 2 days at 4^0C prior to a decrease. In the 1st experiment, the enzyme specific activity on day 2 was 135% of that on day 0, while in the 2nd experiment, it was 173%. The biggest increase, to 179%, was seen in the 3rd experiment. To test the possible function of cysteine proteases during 4^0C storage period, a time course experiment similar to that shown in Figure 11 was carried out with the addition to microsomes of ALLN, a neutral cysteine protease inhibitor. The result of this experiment is shown in Figure 12. The addition of ALLN to microsomes had little effect on ACAT activity during storage at 4^0C, in the absence or presence of the mixture of protease inhibitors.

The same time course experiment as shown in Figure 11 was repeated, with the addition of DTT to the microsomes on the day they were thawed. As shown in Figure 13, DTT had only a small effect on ACAT stability during storage at 4^0C.

Microsomes were treated with 5 μM $CuSO_4$ or H_2O_2 of different concentrations to see whether oxidation of lipids had any effect on ACAT. Figure 14A shows the result of treating microsomes at r.t. for 60 min with increasing concentration of H_2O_2, and it was clear that even 1 mM H_2O_2 had no effect on ACAT SA, 512 ± 11.4 (1 mM H_2O_2) vs 499 ± 1.8 (control) pmole/min/mg. Figure 14B shows the result of treating microsomes with 5 μM $CuSO_4$ at r.t. overnight. To avoid any interference of $CuSO_4$ with the ACAT assay, 1 mM EDTA was added to the $CuSO_4$ treated microsomes 30 min before the assay. As shown in Figure 14B, overnight incubation of the control microsomes resulted in an

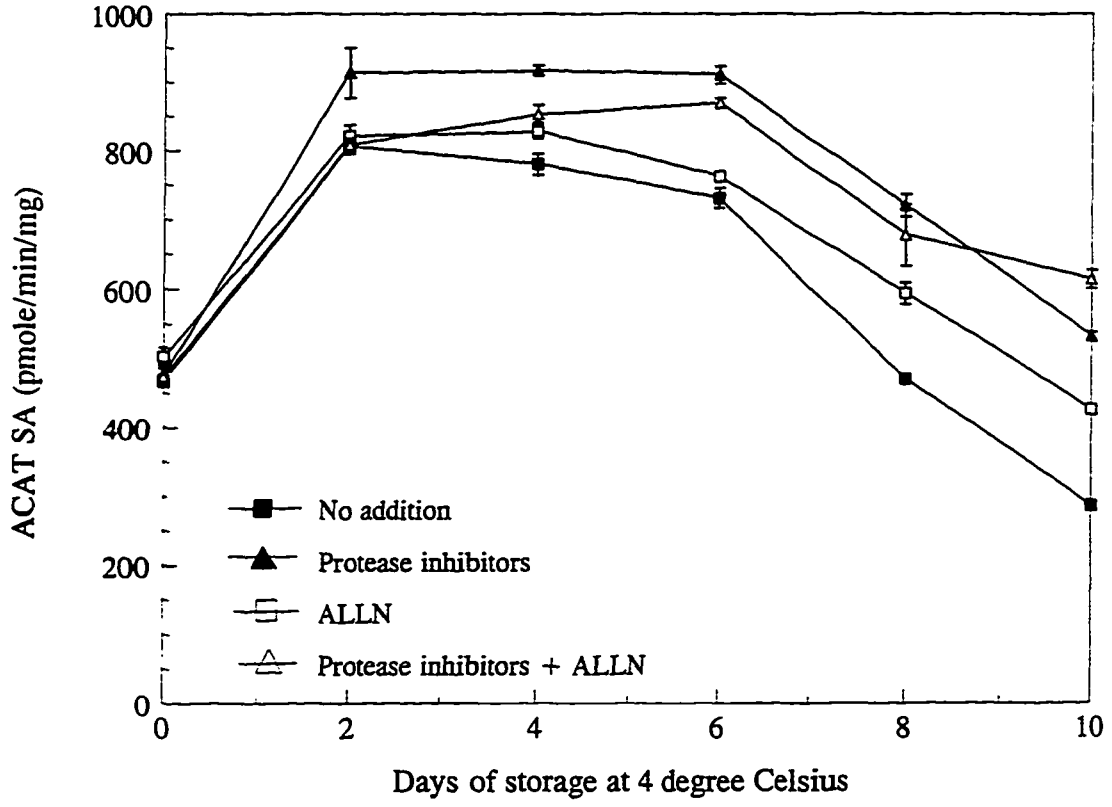


Figure 12. Effect of ALLN on ACAT activity during storage at 4°C. Microsomes or microsomes + protease inhibitors were taken out of the -70°C freezer and thawed. An aliquot of 450 µl microsomes was mixed with 50 µl 10 µg/ml ALLN and stored at 4°C for 10 days. ACAT activity was measured the day the mixture was prepared and every other day thereafter. The lines with the solid symbols were the results of experiments without ALLN while the lines with open symbols were results with ALLN. Each value is a mean of duplicates with a bar showing the range; where no bar is shown, it is smaller than the symbol.

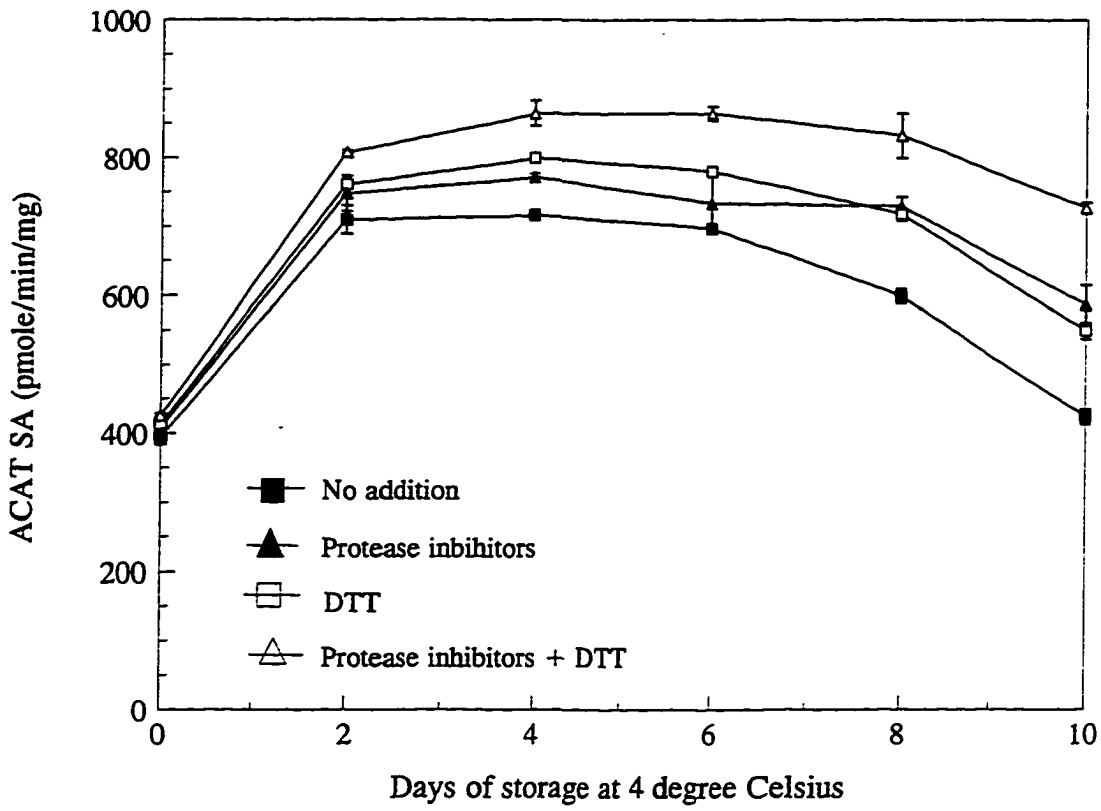


Figure 13. Effect of DTT on ACAT activity during storage at 4°C. Microsomes or microsomes + protease inhibitors were taken out of the -70°C freezer and thawed. An aliquot of 450 µl microsomes was mixed with 50 µl 10 µg/ml DTT and stored at 4°C for 10 days. ACAT activity was measured the day the mixture was prepared and every other day thereafter. The lines with the solid symbols were the results of experiments without DTT while the lines with open symbols were the results with DTT. Each value is a mean of duplicates with a bar showing the range; where no bar is shown, it is smaller than the symbol.

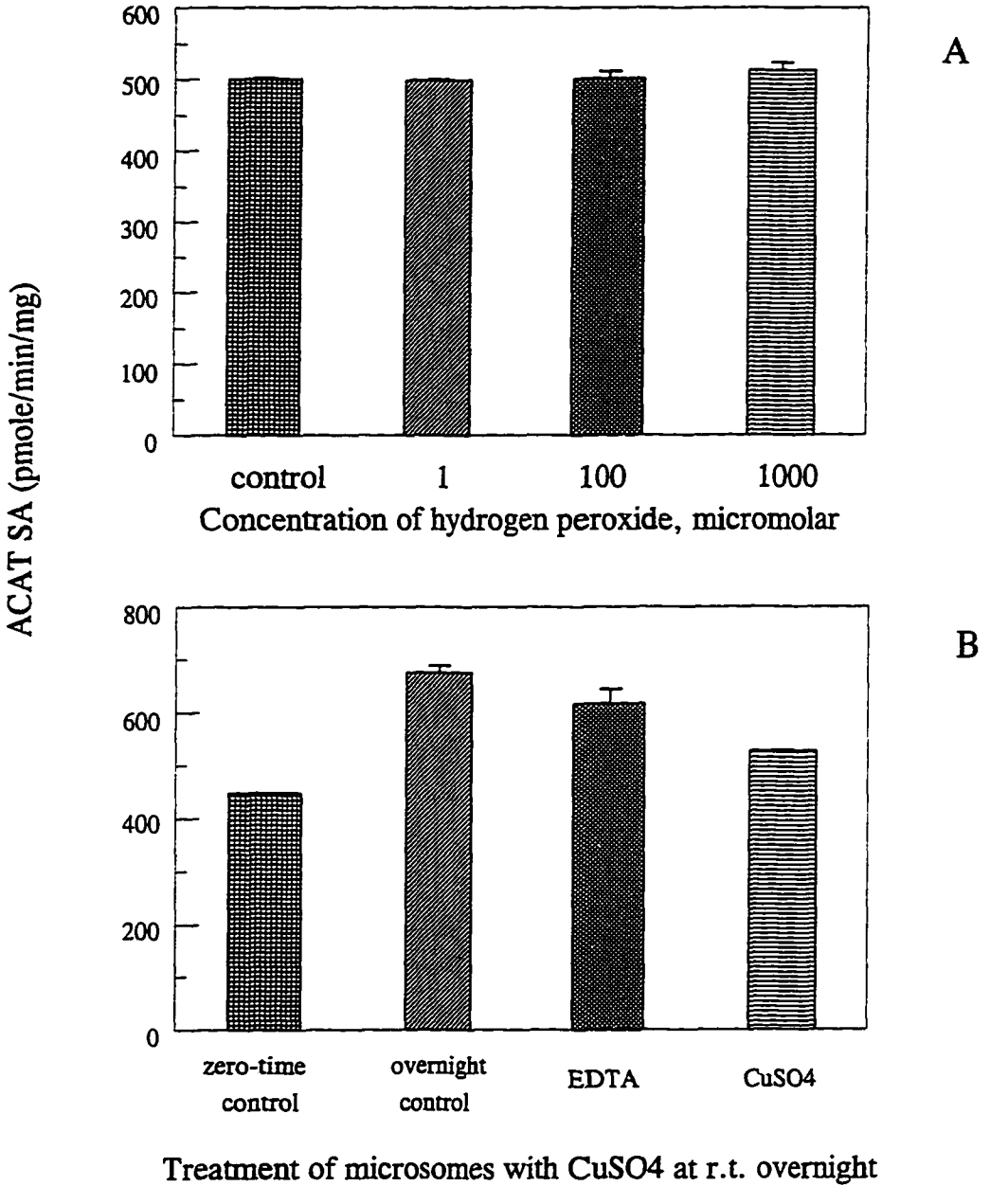


Figure 14. Effect of oxidation of microsomes on ACAT activity. Figure 14A shows the treatment of microsomes with H_2O_2 at r.t. for 60 min and Figure 14B shows the treatment of microsomes with 5 μM $CuSO_4$ at r.t. overnight. In Figure 14B, bar a represents the zero-time control, bar b represents the overnight-control, bar c represents the overnight-control with 1 mM EDTA added 30 min before the assay, bar d represents the $CuSO_4$ treatment with 1 mM EDTA added 30 min before the assay. The bar represents ACAT SA and is a mean of duplicates. The error range is represented by a bar; where no error bar is shown, it is too small to be shown.

increase in ACAT SA, 675 ± 14.6 (overnight, bar b) vs 447 ± 0.4 (zero-time, bar a) pmole/min/mg, while 5 μM $CuSO_4$ treatment led to a decrease in the enzyme activity, 526 ± 1.6 ($CuSO_4$ treated, bar d) vs 617 ± 27.5 (overnight + EDTA, bar c) pmole/min/mg.

To test the possibility that the activity of ACAT was influenced by the formation of minor lipid molecules during 4^0C storage period, microsomes were incubated at r.t. for 60 min with 100 nM linolenic acid, 0.0025% (w/v) lysoPC, or 100 nM 1,2-dioleoylglycerol, or 0.0025% (w/v) bovine brain ceramides, or 100 nM dihydrosphingosine. The stock solutions of those lipid molecules were prepared in EtOH and the final concentration of EtOH in these treatments was 1% (v/v). The result of this experiment is shown in Table 3. The addition of 1% (v/v) EtOH to microsomes had negligible effect on ACAT activity. The addition to microsomes of 100 nM linolenic acid, or 100 nM DOG, or 0.0025% (w/v) ceramides, or 100 nM DHS had little or no effect on ACAT activity. The decrease in ACAT SA seen in the treatment with 0.0025% (w/v) lysoPC could be due to the reacylation of lysoPC, competing for acyl-CoA used for the ACAT assay.

Microsomes were incubated with MβCD and the formation of cholesteryl ester, triacylglycerol, and phospholipids in the microsomes was measured. First, to find out if MβCD had an effect on ACAT activity, and the optimal concentration of the cyclodextrin, microsomes were treated with MβCD at r.t. for 30 min followed by the regular ACAT assay. As shown in Figure 15, incubation of microsomes with MβCD stimulated the enzyme activity with an optimal concentration between 1 and 5 mM; ACAT SA: 1088 ± 33.7 (1 mM) vs 278 ± 4.6 (0 mM) pmole/min/mg, a 3.9 fold increase. Then, the synthesis of CE, TAG, and PL in the presence of 1 mM MβCD was measured, and the result is

Table 3. Effect of minor membrane lipids on ACAT activity. Microsomes were incubated with lipids at r.t. for 60 min with shaking followed by the regular ACAT assay. EtOH was used to prepare stock solutions of the lipids and the final concentration of EtOH in these treatments was 1% (v/v).

Treament	ACAT SA pmole/min/mg	Percent of control
Microsomes	552 ± 3.2	—
1% (v/v) EtOH	564 ± 1.5	100
100 nM linolenic acid	563 ± 11.1	100
0.0025% (w/v) lysoPC	368 ± 4.9	65
100 nM DOG	566 ± 6.8	100
Microsomes	507 ± 2.9	—
1% (v/v) EtOH	527 ± 17.2	100
0.0025% (w/v) ceramides	595 ± 11.8	113
100 nM DHS	556 ± 8.0	106

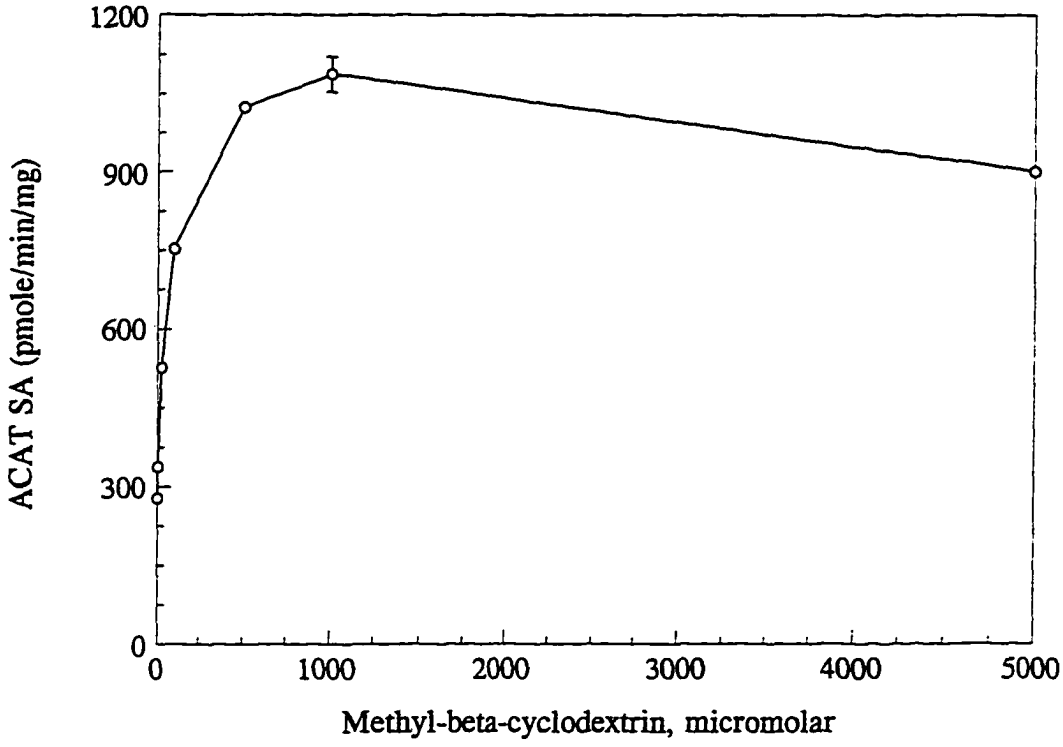


Figure 15. Effect of MβCD on ACAT activity. Five μl of microsomes with 220 μg protein was incubated with 155 μl MβCD-medium II at r.t. for 30 min followed by the regular ACAT assay. Each value is a mean of duplicates with a bar showing the range; where no bar is shown, it is smaller than the symbol.

plotted in Figure 16. The synthesis of CE was elevated 3.8 fold in the presence of MβCD: 1782 ± 5.4 vs 471 ± 24 pmole/min/mg, whereas those of TAG and PL did not change significantly: TAG: 64.7 ± 2.2 (1 mM MβCD, 70%) vs 92.6 ± 12 (control, 100%) dpm/min/μg, PL: 76.2 ± 1.2 (1 mM MβCD, 132%) vs 57.5 ± 9.6 (control, 100%) dpm/min/μg.

3. Regulation of ACAT activity by (oxy)sterols

In an attempt to investigate the function of cholesterol and 25-hydroxycholesterol in regulation of ACAT of yolk sac membrane, an experiment was conducted in which cholesterol of microsomes was depleted by incubation of microsomes with MβCD and the depleted microsomes were assayed for ACAT activity after being incubated with cholesterol-PC liposomes of different molar ratios. The experiment was carried out as follows.

First, microsomes were treated with 10 mM MβCD 3 times and the amount of cholesterol was measured after each step of depletion. As shown in Figure 17 (1st panel of bars), there was 41% of the original cholesterol left after the 1st depletion, and 22% and 23% left after 2nd and 3rd depletions, respectively. Since there were losses of proteins in the process of cholesterol depletion, the ratio of cholesterol to protein in the microsomes was measured and plotted in Figure 18 (1st panel of bars). If the amount of cholesterol in microsomes was expressed as a ratio to microsomal proteins, treatment with 10 mM MβCD was rather inefficient in removing cholesterol from the membrane; there was 82% cholesterol left after the 1st depletion, and 77% and 43% after the 2nd and 3rd, respectively (Figure 18).

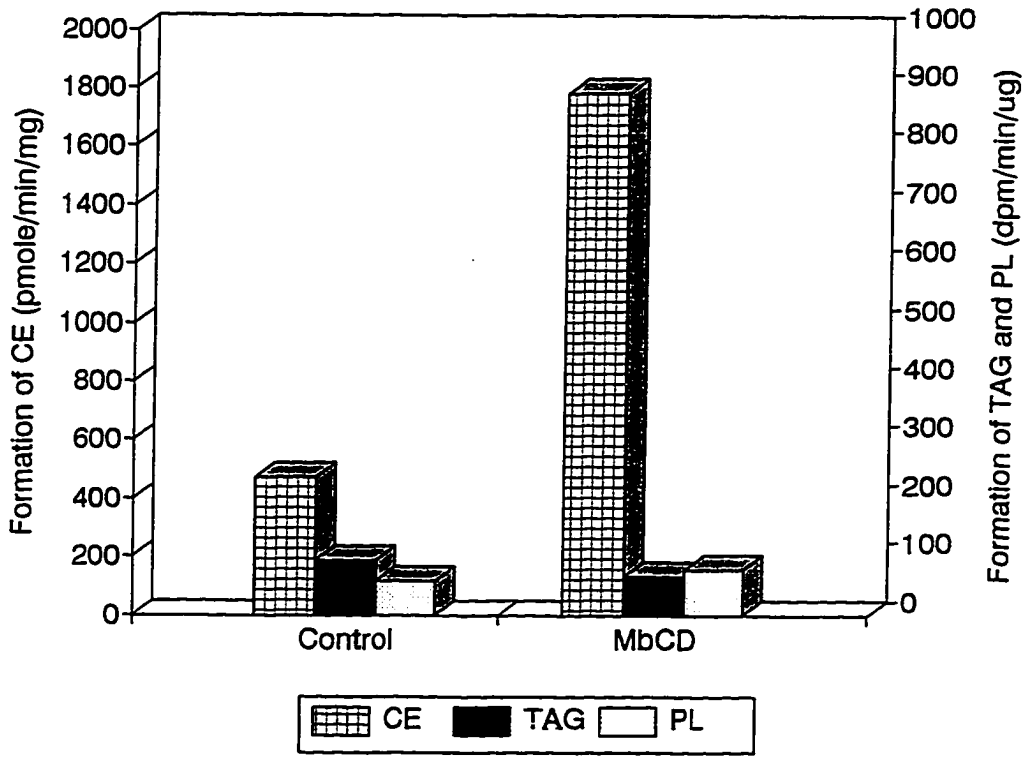


Figure 16. Effect of MβCD on the synthesis of cholesteryl ester, triacylglycerol, and phospholipids. Microsome preparation with 220 μg protein were incubated with 1 mM MβCD at r.t. for 30 min. The formation of CE, TAG, and PL was quantitated by TLC method as described in the MATERIALS AND METHODS. The formation of CE is represented by bars with crosses and the values are scaled on the first Y axis. The formation of TAG and PL is represented by solid and dot bars, respectively, and the values are scaled on the 2nd Y axis. In all cases, the range is less than 17% of the mean.

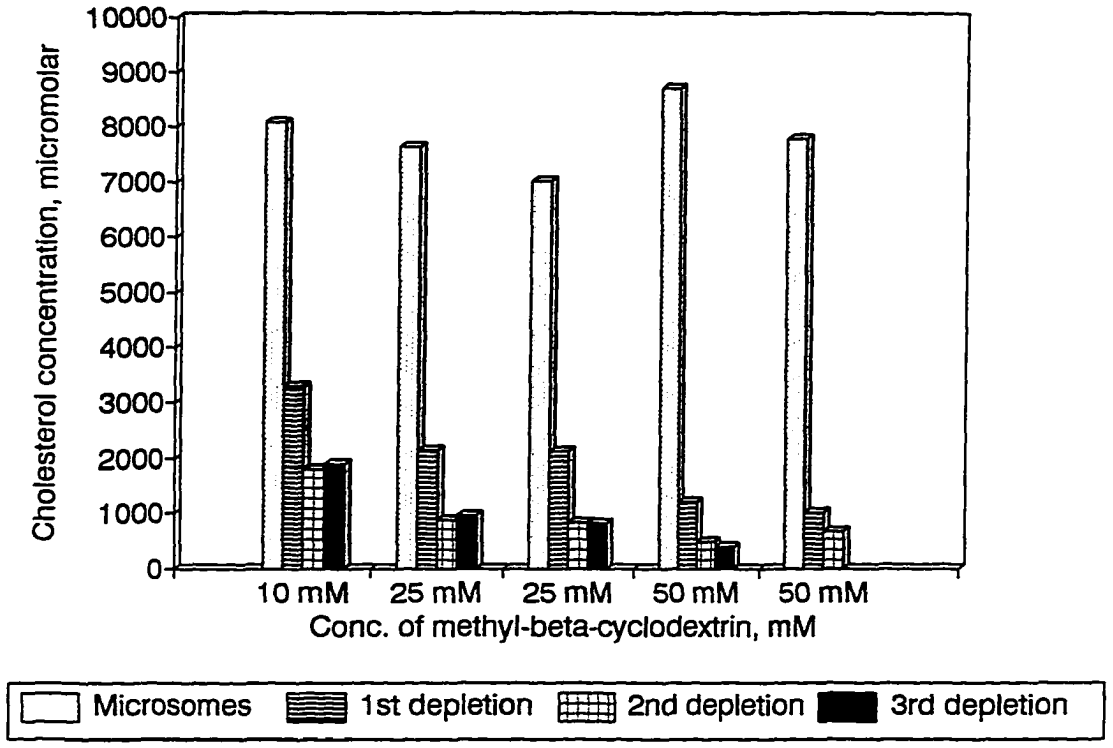


Figure 17. Depletion of cholesterol by MβCD. Microsomal cholesterol of yolk sac membrane was depleted with different concentrations of MβCD under slightly different conditions (see METHODS and RESULT). The Y axis represents concentration of cholesterol in μM. In all cases, the value is a mean of duplicates with an error range ≤ 6% of the mean.

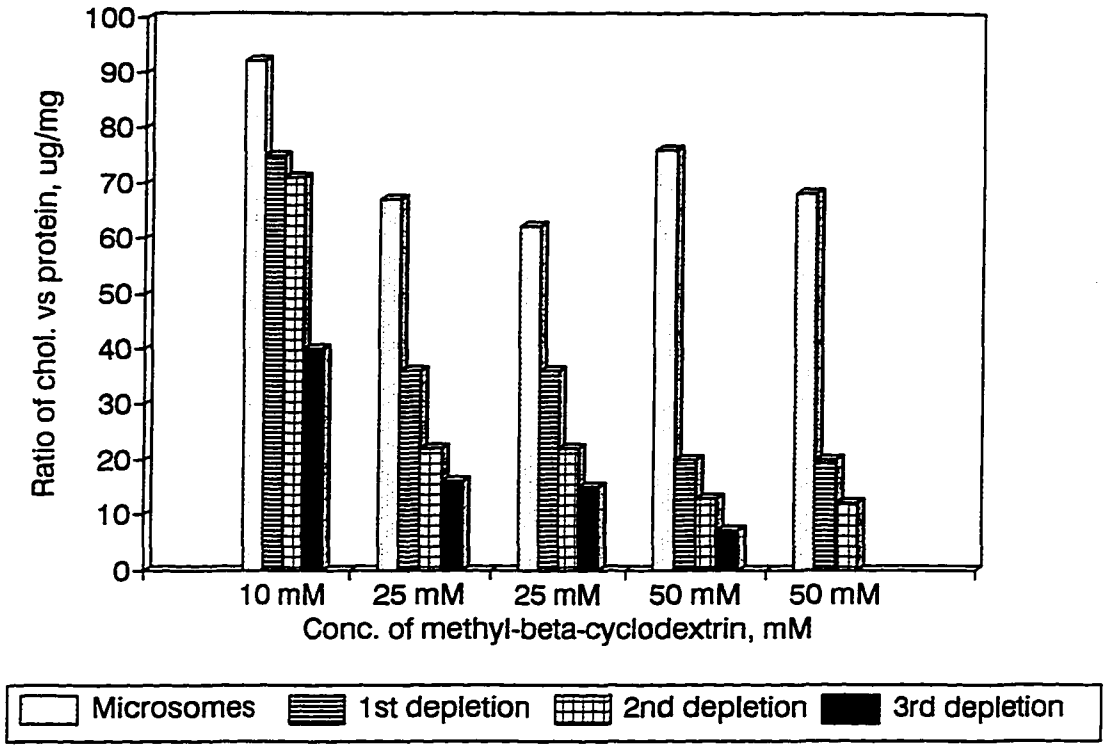


Figure 18. Depletion of cholesterol by MβCD. Microsomal cholesterol of yolk sac membrane was depleted with different concentrations of MβCD under slightly different conditions (see METHODS and RESULT). The Y axis represents ratio of cholesterol to microsomal protein, μg/mg. In all cases, the value is a mean of duplicates with an error range ≤ 10% of the mean.

Then, 25 mM MβCD was used to deplete the microsomes of cholesterol. In the experiment, microsomes were taken out of the -70^{0}C freezer and thawed and stored at 4^{0}C for 2 days before the depletion treatment to allow redistribution of cholesterol in membranes. As shown by the 2nd panel of bars in Figure 17, 28% of the original cholesterol was left in the microsomes after the 1st depletion cycle, and 11% and 13% were left after the 2nd and 3rd ones, respectively. Again, when cholesterol concentration in the microsomes was expressed as a ratio to microsomal proteins, the depletion was rather incomplete (Figure 18, the 2nd panel of bars). More than half of cholesterol (54%) stayed in the microsomes after the 1st depletion, whereas 33% and 24% remained after the 2nd and 3rd, respectively (Figure 18, the 2nd panel of bars). The experiment was repeated with one modification in the procedure. After the 2nd depletion cycle, the resuspended microsomes were stored at 4^{0}C overnight and the 3rd depletion and wash were continued the next day. As shown by the 3rd panel of bars in Figure 17 and 18, the overnight incubation made no difference in getting cholesterol out of the membrane. There was 30% (Figure 17) or 58% (Figure 18) of the original cholesterol left in the microsomes after the 1st cycle, and 12% (Figure 17) or 35% (Figure 18) left after the 2nd one, and 12% (Figure 17) or 24% (Figure 18) left after the 3rd one.

Finally, 50 mM MβCD was used and the microsomal preparation was treated 3 times consecutively. Microsomes were thawed and stored at 4^{0}C for 2 days before the experiment. After the first cycle of depletion, only 14% of the original cholesterol was left in the membrane, and after the 2nd and 3rd, 6% and 4%, respectively, remained (Figure 17, the 4th panel of bars). As shown by the 4th panel of bars in Figure 18, there was more

cholesterol left within microsomes if the relative amount of cholesterol to proteins was used to count the concentration of the sterol: 26%, 17%, and 9% of cholesterol stayed within microsomes after the 1st, 2nd, and 3rd depletion, respectively.

To find out if treating microsomes with SMase could enhance cholesterol depletion, microsomes, which had been thawed and stored at 4^0C for 2 days, were incubated with 0.12 IU/ml SMase (0.0033 IU SMase per mg microsomal proteins) at 37^0C for 30 min followed by 2 time depletion with 50 mM MβCD. As shown by the 5th panel of bars in Figure 17 and 18, incubation with SMase did not change the amount of cholesterol being depleted from microsomes. There was 13% (Figure 17) or 29% (Figure 18) of the original amount remaining in the membrane after the 1st depletion and 8% (Figure 17) or 18% (Figure 18) remaining after the 2nd.

To find out if MβCD could solubilize molecules other than cholesterol from microsomes of yolk sac membrane lipid, microsomes and supernatant of the 1st 50 mM MβCD depletion were extracted with chloroform/MeOH, 2/1 v/v, and the lipid extracts were separated by TLC. The result of the experiment was shown in Figure 19 (microsomes) and Figure 20 (supernatant). The TLC plate of microsomes showed, within the detection limit, that the microsomes had cholesteryl ester, TAG, free fatty acids, cholesterol, PE, PS, PC, and possibly SM. It was hard to pinpoint from the TLC result the existence in microsomes of diacylglycerol, ceramides, and lysophospholipids. In the supernatant, there were TAG and PC as shown in Figure 20. The existence of cholesterol in the supernatant was not detected by the TLC method, but the amount of the sterol could be shown to be 318 ± 4 μM by a more sensitive enzymatic method (METHODS).

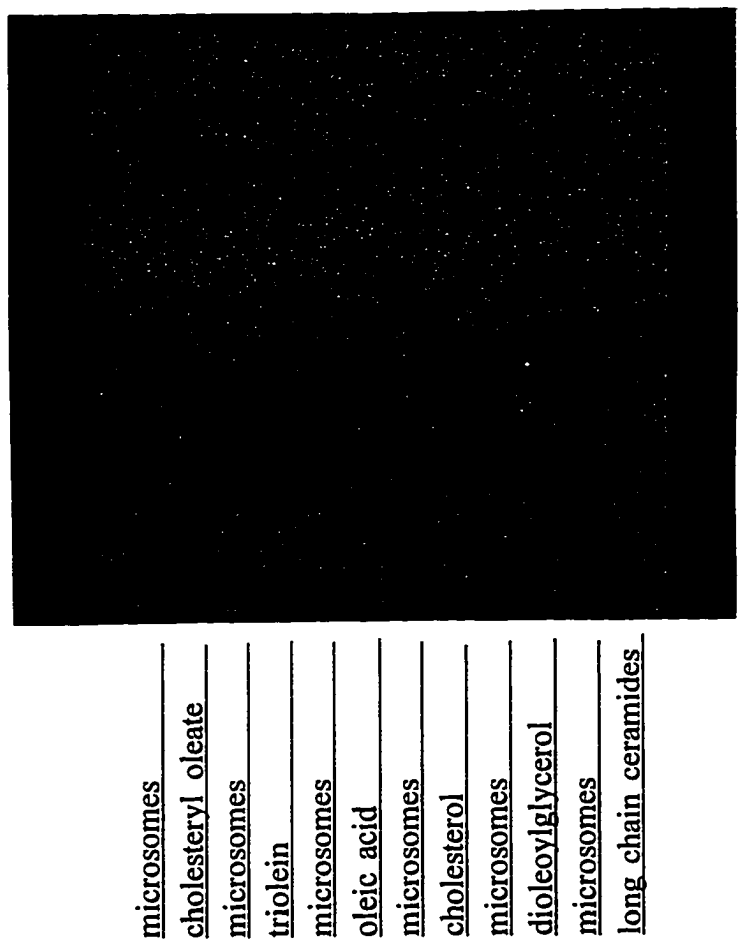
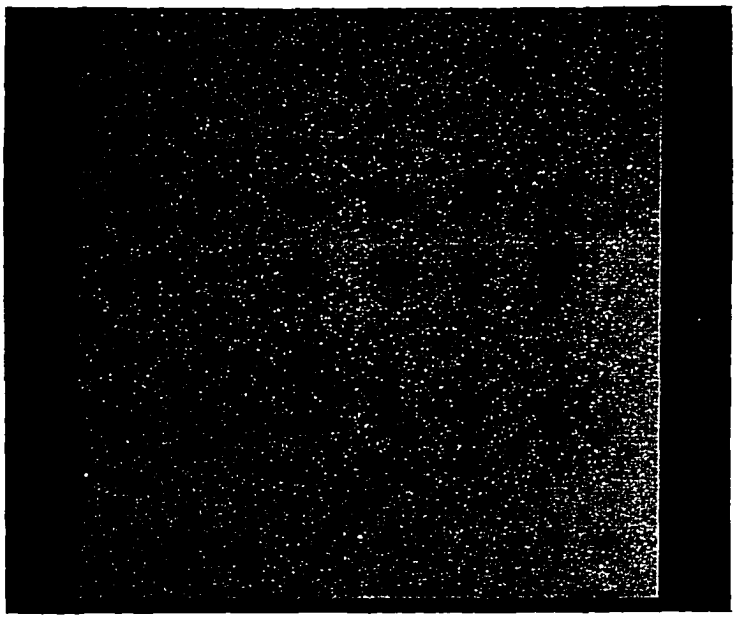


Figure 19. TLC separation of microsomal lipid components. Four hundred μg of microsomal lipid, 20 mg/ml in 2/1 (v/v) chloroform/MeOH, and 20 μg of each of lipid standards were applied onto the TLC plate in alternative lanes. After development, the plate was visualized by I_2 vapor.



microsomes
phosphatidylethanolamine
microsomes
phosphatidylserine
microsomes
phosphatidylcholine
microsomes
sphingomyelin
microsomes
lysophosphotidylcholine
microsomes

Figure 19. (continued)

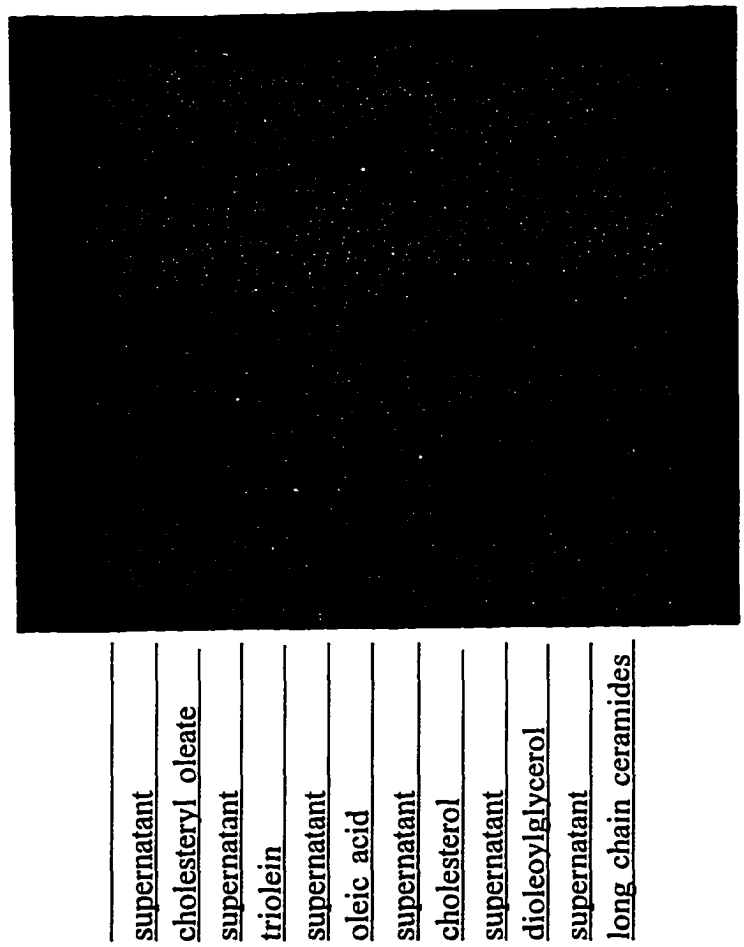


Figure 20. TLC separation of supernatant lipid components of the 1st 50 mM MβCD depletion. Six hundred μg of supernatant lipid, 20 mg/ml in 2/1 (v/v) chloroform/MeOH, and 20 μg of each of lipid standards were applied onto the TLC plate in alternative lanes. In the case of MβCD, 40 μg was applied. After development, the plate was visualized by I_2 vapor.

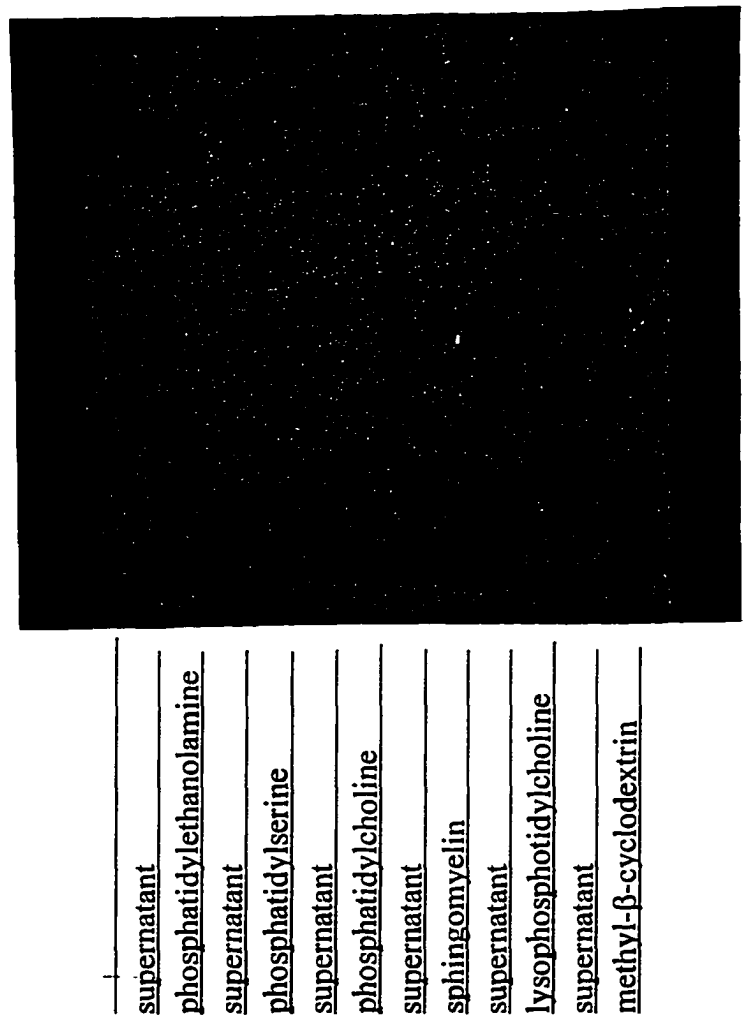


Figure 20. (continued)

When cholesterol in microsomes was depleted to a very low level, the ACAT activity in the microsomes was measured. Microsomes were taken out of the -70^{0}C freezer and stored at 4^{0}C for 2 days before the depletion experiment. Five µl treated microsomes were incubated with 155 µl cholesterol-PC liposomes of different molar ratios and the measured ACAT SA is plotted in Figure 21 against the cholesterol mole fraction in cholesterol-PC liposomes. In the experiment represented by the line with open circles, microsomes were treated with 25 mM MβCD 3 times and then incubated with liposomes at r.t. for 30 min prior to the regular ACAT assay. In the experiment represented by the line with closed triangles, microsomes were treated with 25 mM MβCD twice and stored at 4^{0}C overnight followed by the 3rd depletion and wash, and the treated microsomes were incubated with cholesterol-PC liposomes at 4^{0}C overnight prior to the regular ACAT assay. Cholesterol stimulated ACAT activity 11 fold in the 1st experiment: 278 ± 30.6 (0.38 cholesterol mole fraction) vs 25 ± 1.1 (zero cholesterol mole fraction) pmole/min/mg and 17 fold in the 2nd experiment: 331 ± 9.9 (0.38 cholesterol mole fraction) vs 19 ± 1.1 (zero cholesterol mole fraction) pmole/min/mg. Despite the substantial increase in the specific activity, the enzyme was not saturated with cholesterol at the highest cholesterol concentration used in the experiments.

Fifty mM MβCD was used to extract cholesterol 3 times from microsomes, which had been thawed and stored at 4^{0}C for 2 days. Five µl treated microsomes were incubated with 150 µl cholesterol-PC liposomes of 0.39 mole fraction in the presence of MβCD, added in a volume of 5 µl, at r.t. for 30 min followed by the regular enzyme assay. As shown in Figure 22, the addition of MβCD stimulated ACAT activity significantly, 1462

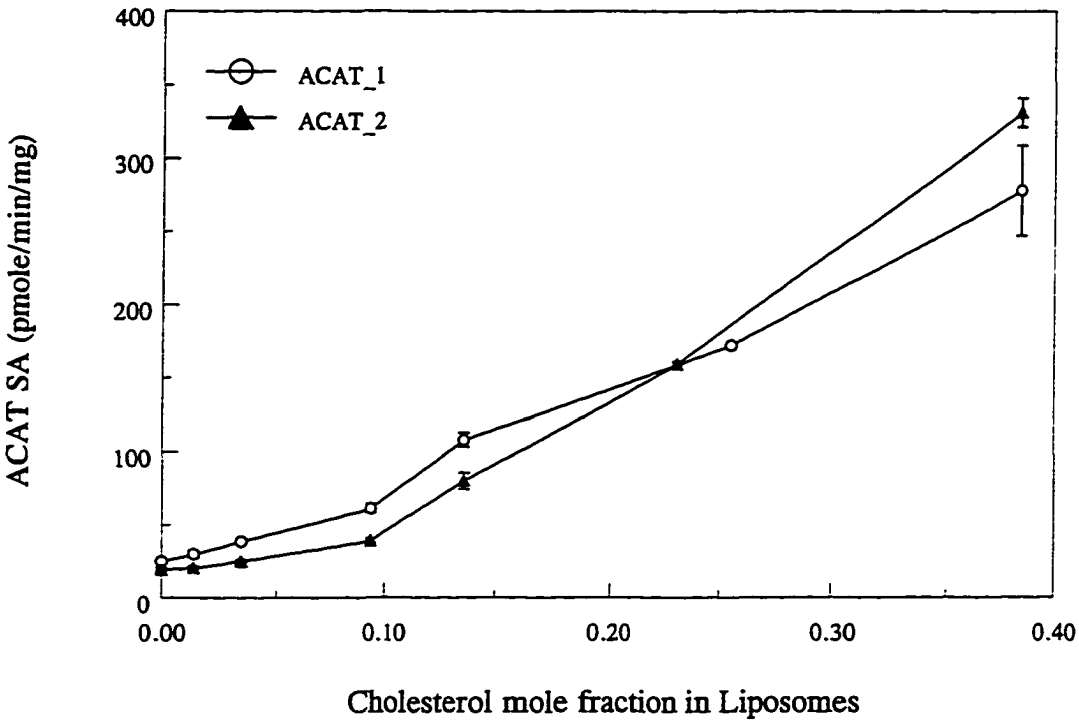


Figure 21. Effect of cholesterol mole fraction on ACAT activity. Microsomes were taken out of the -70°C freezer and stored at 4°C for 2 days before the depletion experiment. Five µl treated microsomes were incubated with 155 µl cholesterol-PC liposomes of different mole fractions prior to the regular ACAT assay. In the experiment represented by the line with open circles, microsomes were treated with 25 mM MβCD 3 times and incubated with liposomes at r.t. for 30 min. In the experiment represented by the line with closed triangles, microsomes were treated with 25 mM MβCD twice and stored at 4°C overnight followed by the 3rd treatment and wash, and the treated microsomes were incubated with liposomes at 4°C overnight. Each value is a mean of duplicates with a bar showing the range; where no bar is shown, it is smaller than the symbol.

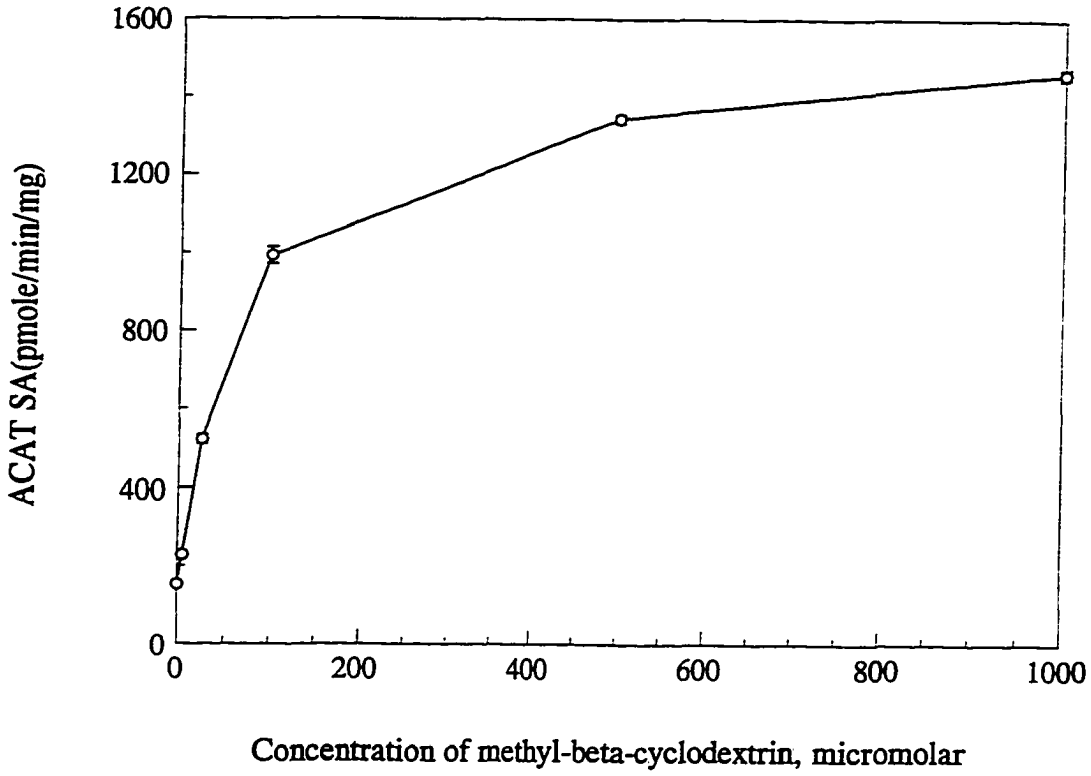


Figure 22. Effect of MβCD on ACAT activity. Microsomes were thawed and stored at 4°C for 2 days. After being depleted with 50 mM MβCD 3 times, 5 μl of the microsomes was incubated with 150 μl cholesterol-PC liposomes of 0.39 mole fraction plus different concentrations of MβCD, added in a volume of 5 μl, at r.t. for 30 min followed by the regular enzyme assay. Each value is a mean of duplicates with a bar showing the range; where no bar is shown, it is smaller than the symbol.

± 13.5 (1 mM MβCD) vs 154 ± 1.1 (zero MβCD) pmole/min/mg, a 9.5 fold increase. With the same microsomes, depleted 3 times with 50 mM MβCD, the ACAT activity was measured after the microsomes were incubated at r.t. for 30 min with cholesterol-PC liposomes of different mole fractions in the absence or presence of 1 mM MβCD, shown in Figure 23. In the absence of MβCD, cholesterol stimulated ACAT activity 14 fold: 338 ± 5.1 (0.54 cholesterol mole fraction) vs 24 ± 0.4 (zero cholesterol mole fraction) pmole/min/mg, while in the presence of MβCD, cholesterol stimulated the enzyme activity 63 fold: 1395 ± 7.3 (0.39 cholesterol mole fraction) vs 22 ± 0.1 (zero cholesterol mole fraction) pmole/min/mg. In the presence of MβCD, the ACAT activity began to decrease a little bit when the cholesterol mole fraction increased from 0.39 to 0.54: 1395 ± 7.3 (0.39 mole fraction) (100%) vs 1239 ± 12.1 (0.54 mole fraction) (89%) pmole/min/mg, whereas in the absence of the cyclodextrin, the enzyme activity increased 1.8 fold: 192 ± 5.0 (0.39 mole fraction) vs 338 ± 5.1 (0.54 mole fraction) pmole/min/mg.

It turned out that 25-OH has no effect on microsomal ACAT activity of yolk sac membrane: 371 ± 13.8 (50 μM 25-OH) vs 335 ± 2.8 (1% v/v EtOH, control) pmole/min/mg (Figure 24). MβCD at 1 mM stimulated ACAT activity 3.7 fold, the same as previously shown, whether 25-OH was present or not (Figure 24). Similarly, 25-OH has no effect on the enzymatic activity when the depleted microsomes were assayed in the presence of increasing concentration of cholesterol (Figure 25). It was noticable that the initial ACAT activity (activity assayed with zero cholesterol mole fraction in the cholesterol-PC liposomes) in the depleted microsomes (Figure 25, No addition) was higher than that previously observed (Figure 23, the line with the open circles), and the addition

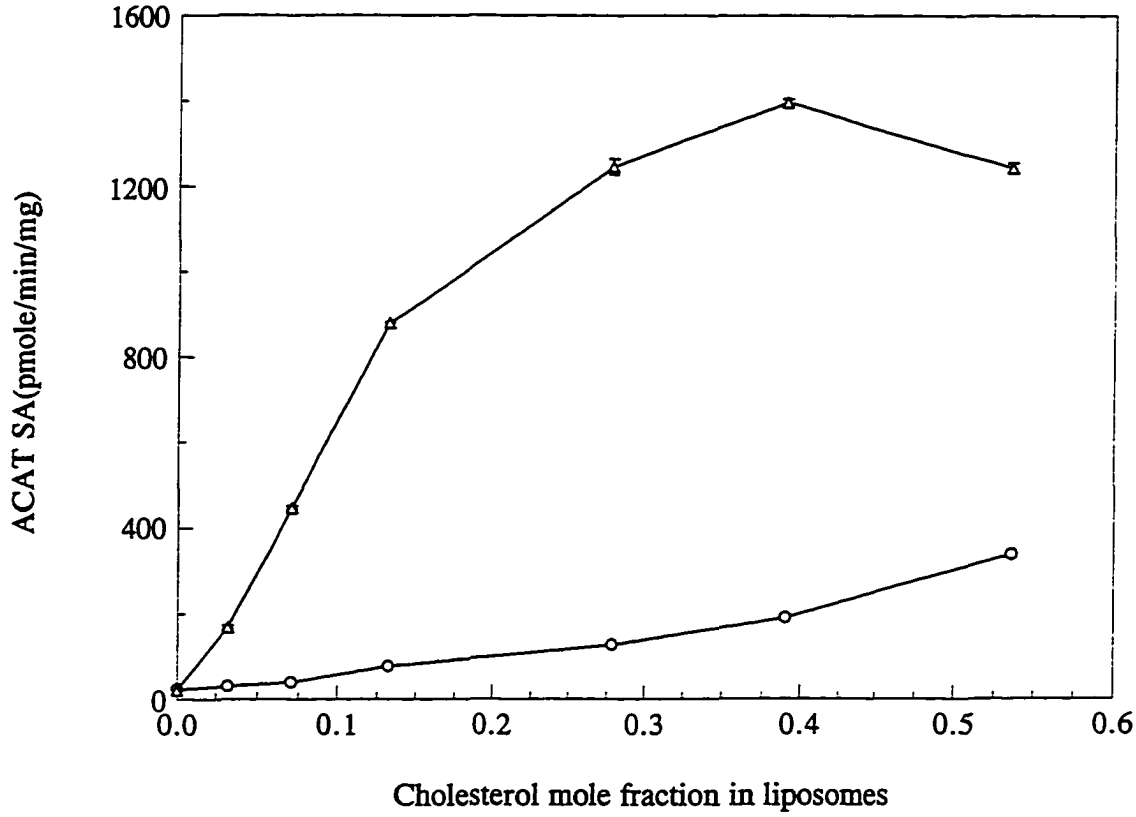


Figure 23. Effect of cholesterol mole fraction on ACAT activity in the absence or presence of 1 mM MβCD. Microsomes were thawed and stored at 4°C for 2 days. After being depleted with 50 mM MβCD 3 times, 5 μl of the microsomes was incubated at r.t. for 30 min with cholesterol-PC liposomes of different mole fractions in the absence or presence of 1 mM MβCD followed by the regular enzyme assay. The line with circles represents the assay in the absence of MβCD and the line with triangles represents the assay in the presence of 1 mM MβCD. Each value is a mean of duplicates with a bar showing the range; where no bar is shown, it is smaller than the symbol.

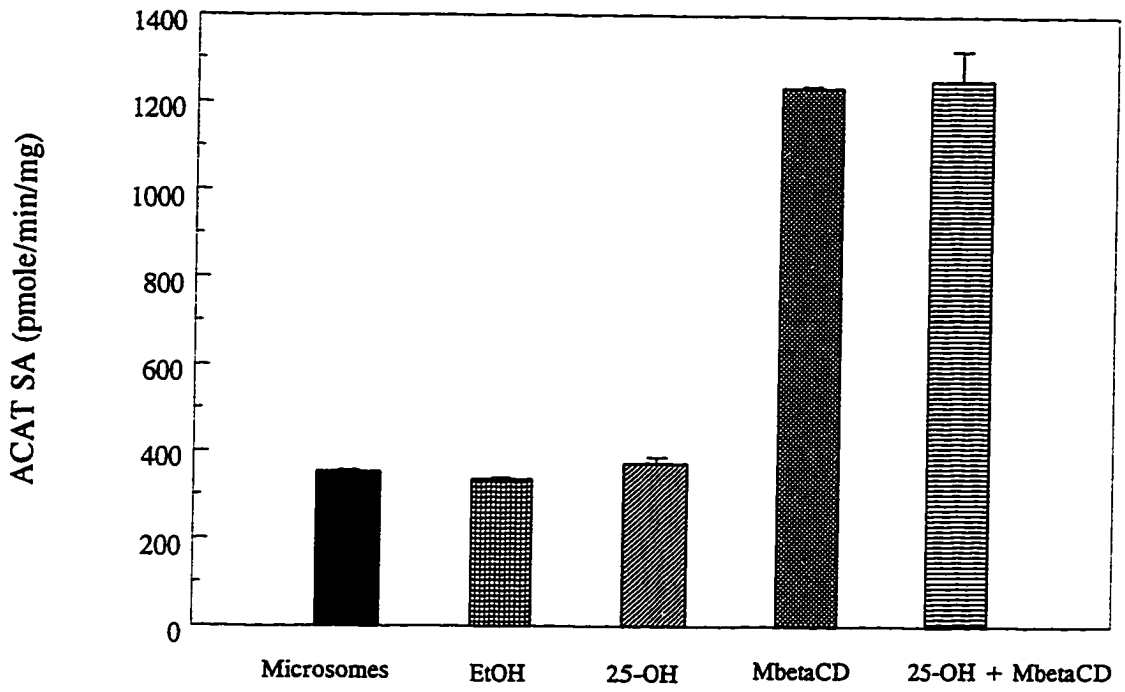


Figure 24. Effect of 25-hydroxycholesterol on microsomal ACAT activity. Five μl of microsomes was incubated at r.t. for 30 min with 50 μM 25-OH and 1 mM MβCD prior to the regular ACAT assay. Stock solution of 25-OH was prepared in EtOH and 1% v/v EtOH was the control. Except the assay of microsomes represented by the solid bar, all of the rest of assay has 1% v/v EtOH. Each value is a mean of duplicates with a bar showing the range; where no bar is shown, it is smaller than the range.

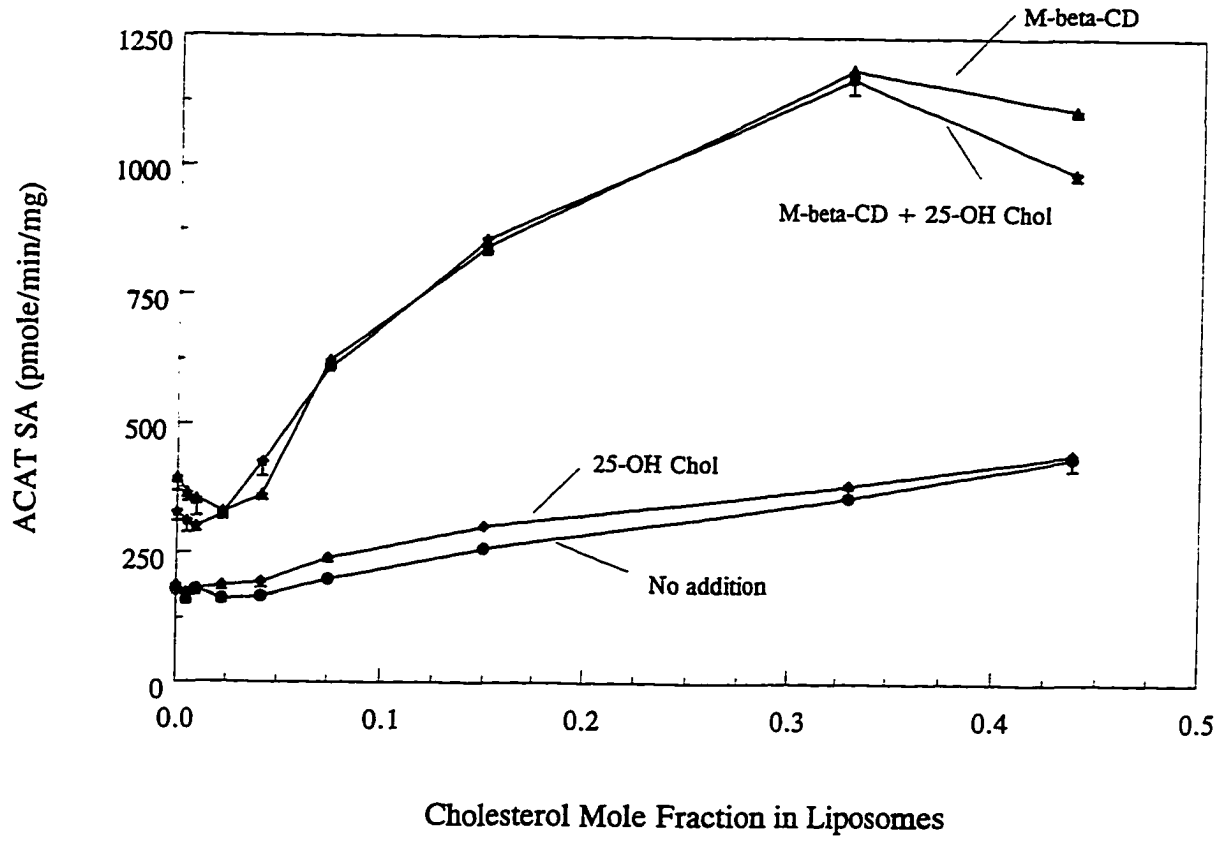


Figure 25. Effect of 25-hydroxycholesterol on ACAT in the depleted microsomes. Microsomes were depleted of cholesterol with the treatment of 50 mM MβCD for 3 times. Five μl of the depleted microsomes was incubated at r.t. for 30 min with cholesterol-PC liposomes of different mole fractions in the presence or absence of 50 μM 25-OH, or, in the presence or absence of 1 mM MβCD before the regular ACAT assay. Each value is a mean of duplicates with a bar showing the range; where no bar is shown, it is smaller than the symbol.

of 1 mM MβCD increased the initial activity further. In the experiment depicted in Figure 25, the microsomes were thawed from -70^{0}C and used right away for depletion treatment without a pre-storage of 2 days at 4^{0}C, which was done with previous experiments (Figure 17-18 and Figure 21-23). The storage at 4^{0}C was shown to lead to cholesterol redistribution and ACAT activation in microsomes, and the very low initial activities observed in the previous experiments were probably because the residual amounts of cholesterol in the depleted microsomes were not in the substrate pool and not available for esterification. Without the pre-redistribution of cholesterol at 4^{0}C for 2 days, the residual amount of cholesterol after depletion, 4% of the original sterol concentration in the microsomes, was perhaps more accessible to the enzyme, causing a higher initial activity (Figure 25).

B. Discussion

1. Comparison of ACAT from avian and mammalian sources

The function of ACAT in the yolk sac membrane is to convert cholesterol and fatty acids in the yolk to cholesteryl esters, which can be packaged into lipoproteins and secreted into circulation. The esterification facilitates the absorption of the sterol and free fatty acids and the presence of esters in lipoproteins stabilizes the assembly and structure of the particles [7]. Thus, the enzyme functions significantly in providing essential lipid nutrients to the developing embryo, not only as a source of energy, but also as essential membrane components and vitamins [6,7]. Under this particular circumstance, in which the

physiological role of the enzyme is to support and nourish development, a sustained and high activity in the yolk sac membrane is desired. On the other hand, ACAT in the liver plays more of a regulatory function, converting excess free cholesterol to esters and preventing any toxic effect of the sterol. The liver protein may be more sensitive to changes in its environment and be subject to modification and inactivation. The higher specific activity found in the yolk sac membrane [6] and better stability upon storage provided a suitable system for the study of ACAT. Compared with the enzyme in the liver, intestine, and adrenal cortex under normal physiological conditions, more than 5 fold higher ACAT activity was reported in yolk sac membranes of chicken eggs 16 days after fertilization [6]. Microsomes prepared from yolk sac membranes after storage at -70^{0}C for 6 months had active ACAT, while mouse livers stored in the same way for 3 months contained inactive enzyme when made into microsomes (data not shown). Deoxycholate extracts from mouse liver microsomes had to be used within 2 days of preparation to have active enzyme for functional analyses [3], whereas about 80% of the initial ACAT activity remained after storage at 4^{0}C for 3 weeks for CHAPS-solubilized extracts from yolk sac membrane (data not shown). The difference in the properties of the detergents themselves (deoxycholate is anionic while CHAPS is zwitterionic) may play a part in the difference in stability, but the intrinsic properties of the enzyme from different sources are probably more important in maintaining the stability. Characterization of ACAT from yolk sac membrane is, therefore, biologically significant, and the avian enzyme provides some advantage over the mammalian equivalent in the study of the protein. Similar to the mammalian enzyme [44,45], ACAT of yolk sac membrane is sensitive to inhibitions by

DEPC and PMB (Figure 1 and 2). A close examination of the profiles of DEPC and PMB inactivation revealed a closer resemblance of the avian enzyme to that of aortic subtype, with regard to the sensitivity towards chemical modification, rather than that of the liver subtype in rabbits [44,45]. For example, treatment of microsomes with 500 μM DEPC inhibited 90% of the activity for the avian enzyme (Figure 1) and fully inactivated the rabbit aortic ACAT [44], while the same treatment of rabbit liver enzyme led to a inhibition of only about 25% [44]. Complete inhibition of the liver enzyme by DEPC required 10 fold higher concentration [44]. Likewise, ACAT from yolk sac membrane (Figure 2) and rabbit aorta [45] was completely inactivated by treatment with 500 μM PMB, whereas the liver enzyme retained about 75% of its activity [45]. In the treatment of microsomes with different concentrations of PMB, the enzyme of yolk sac membrane (Figure 2, insert) and of rabbits [45] both showed an increase in the activity followed by complete inactivation. Therefore, the structure and regulation of the avian enzyme may very likely be comparable to that of mammalian origin and study of the former can provide useful information about the latter.

The focus of the present study is to characterize ACAT from yolk sac membrane of chicken eggs after fertilization and compare the properties of the avian enzyme with that from mammalian sources. Although abundant information has been learned about mammalian ACAT using molecular biology techniques, direct biochemical study of the enzyme has not been achieved with the pure protein. Studies of regulation of ACAT activity, e.g. by cholesterol supply, by 25-hydroxycholesterol, and other oxysterols, have been conducted in microsomal preparations or detergent extracts. Purification of the protein

of the avian source was attempted in the early part of this work, but instability of the enzyme activity and sensitivity towards its surrounding environment prevented successful purification. Detergent solubilization followed by ammonium sulfate precipitation and column chromatography using individual or combinations of size permeation, ion exchange, activated thiol Sepharose, and hydrophobic interaction (Phenyl Sepharose and Propyl Agarose) columns and preparative IEF failed to yield the active enzyme. In her dissertation, M. Shih [3] discussed and justified the method of preparing and assaying ACAT of mouse liver microsomes, and adaptation of her method of measuring the enzyme activity to the avian system works satisfactorily. Measurement of the ACAT activity in microsomal preparations or detergent extracts becomes the only way to characterize the enzyme in the current study.

From the cDNA sequence, the human macrophage ACAT is deduced to have 9 cysteine residues [4]. At least one cysteine should exist as thiol (SH) without engaging in a covalent linkage if no intermolecular disulfide bonds are formed between one ACAT molecule and another ACAT or other proteins. Depending on where the thiol is located on the protein, a hydrophilic environment close to the surface of the protein or hydrophobic surroundings buried inside, it may be subject to chemical modifications that may or may not affect enzyme catalysis. Modification of ACAT of yolk sac membrane by thiol-reactive agents revealed some very interesting properties of the avian enzyme. When the enzyme was treated with low concentrations of PMB, NEM, and NBM, increases in ACAT SA were observed (insert in Figure 2, Figure 3 and 4). Elevation in the activity was consistently seen in treatments of microsomes with IAM, and higher concentrations of IAM

produced bigger increases (Figure 3). High concentrations of PMB and NBM inhibited the enzyme completely (Figure 2-4). It is likely that, at low concentrations, PMB and NBM reacted with thiols that were easily accessible and quite reactive, and the modification of those sulfhydryls altered the protein conformation such that the enzyme became more active. Alternatively, some unidentified factors might exist in the lipid bilayer and their modification leads to changes in the membrane fluidity and enzyme activity. High concentrations of PMB and NBM led to modification of more SHs, reactive or less reactive, easily or not easily accessible, and inactivated the enzyme. As shown in Table 1, a very small fraction of the total microsomal thiols reacted with IAM at 20 mM, and an even smaller fraction of them reacted with PMB at 20 μM. The elevation in ACAT activity seen in Figure 2-4 can be the consequence of modification of a few thiols, exposed or peculiarly located on the protein, causing a positive alteration in protein conformation. A rise in ACAT activity prior to inactivation upon PMB modification was also observed in microsomal preparations from rabbit liver and aorta [45], probably due to the same reason as it is in the yolk sac membrane.

Another interesting feature about the avian enzyme is that the thiol(s) involved directly or indirectly in catalysis may sit in a non-polar vicinity, as suggested by the result listed in Figure 3 and 4. IAM or NEM at 20 mM did not inhibit ACAT activity, while at the same concentration, NBM, the more hydrophobic analog of NEM, inactivated the enzyme completely. The hydrophobic molecule PMB inactivated ACAT at concentrations as low as 100 μM. IAM and NEM are hydrophilic molecules, their reactivity towards exposed surface sulfhydryls is much greater than to hydrophobic or buried SH(s). On the

other hand, hydrophobic reagents like PMB and NBM penetrate better into the inside of proteins and their reactivity towards buried or hydrophobic sulfhydryls is much bigger. Failure to protect ACAT activity with preincubations of IAM or NEM against treatments of PMB or NBM indicates that the sulfhydryl(s) modified by PMB and NBM must come from cysteine(s) that were different from those that reacted with IAM and NEM. It is tempting to propose that ACAT protein has at least two categories of thiols, distinct by the environments they occupy and by the opposite effect on catalysis brought about upon their modification. One group of thiols is located in polar regions, vulnerable to modification by hydrophilic reagents and capable of inducing enzyme activity after being modified. The other group sits in non-polar environments, susceptible to chemicals that are hydrophobic and modification of them causes inhibition. The reaction ACAT catalyzes involves one amphipathic (acyl-CoA) and two hydrophobic molecules (cholesterol and cholesteryl ester), the active site of the enzyme and the area around it should be quite non-polar. The data generated from chemical modification of ACAT are in good agreement with the scheme predicted about ACAT.

Table 1 lists the amount of total thiols in yolk sac membrane microsomes and the fractions of sulfhydryls that are reactive towards sulfhydryl-modifying agents. About 40% of the total thiols are not available for modification by sulfhydryl-blocking reagents, even with NBM at 20 mM. Those thiols must be buried deep inside protein structures and can not be approached easily. Although PMB at 100 μM reacted with a much smaller fraction of sulfhydryls than NBM at 20 mM did, both of them inactivated the enzyme completely. It is probable that the structure of PMB, with a planar benzene ring, fits better the geometry

of the site(s) where thiol(s) are formed, resulting in inhibition at a much lower concentration.

Inactivation of ACAT by thiol-reactive agents does not prove the direct participation of cysteine(s) in catalysis. Loss of catalytic activities by treatments of enzymes with sulfhydryl-modifying chemicals can be the consequence of blockage of thiols functioning directly in catalysis, however, steric hindrance to substrate binding by the modification of thiols in the vicinity of the active site or conformational change induced by the attachment of modifying groups onto proteins would lead to destruction of catalytic ability, too. LCAT is a perfect example showing the influence of noncatalytic thiols on enzyme activity. In LCAT, cysteines 31 and 184 do not participate in catalysis, but their modification poses steric restraint to substrate binding because of their location near the active site [49,50]. Once the pure protein is available, the role of cysteines in ACAT catalysis can be studied and might be shown to be just like that in LCAT.

Like the mammalian enzyme activity, ACAT from yolk sac mambrane can be extracted into detergent, deoxycholate or CHAPS, solutions (data not shown). The best concentration for CHAPS was found to be 0.5% (w/v) (Figure 5). The effect of NaCl on ACAT SA in both extract and pellet (Figure 6A and B) could come from an increase in ionic strength in the presence of the salt, causing solubilization of more peripheral proteins and making the disintegration of microsomal membranes more efficient. Another probability is that the presence of the salt stabilizes the structure of ACAT in the detergent micelles, leading to a higher recovery of the activity when assayed.

ACAT SA in the CHAPS extract decreases gradually as the number of thiols being reacted with DTDP increases (Figure 7). There was not a single concentration of DTDP at which the enzyme activity dropped sharply.

Even though ACAT from yolk sac membrane and mammalian sources shares many common features with regard to inhibition by histidine- and cysteine-reactive agents, there are subtle differences in the sensitivity towards modification. It turned out that the enzyme activity in mouse liver microsomes was more vulnerable to modifications by thiol-reactive agents (Figure 8), compared with the avian enzyme (Figure 3). As shown in Figure 9A, 2-Mer has little effect on the enzyme activity from both sources, and it can recover most of the activity of yolk sac membrane (87%) from the pretreatment of microsomes with PMB, but it is incapable of rescuing the enzyme of mouse liver (6%). BdiT is a molecule of higher hydrophobicity, compared with 2-Mer, and has a pair of SHs. By itself, BdiT has an inhibitory effect on ACAT from both systems, presumably it can react with the same sulfhydryl(s) as PMB does, resulting in a loss of enzyme activity. After preincubation of microsomes with PMB, treatment with BdiT recovered the majority of mouse liver activity (69%) while stimulating the yolk sac membrane enzyme (141%). One of the possibilities for the activation of ACAT from yolk sac membrane upon the sequential treatment of PMB and BdiT is that BdiT reacts with two kinds of thiols, modification of the first leads to an activation of the enzyme, whereas modification of the second causes inhibition. The inhibition observed in BdiT alone treatment is the net outcome of the two opposite effects, and in the case of pretreating microsomes with PMB, BdiT releases the inhibitory thiol(s) from PMB blockage, so the net outcome now is a stimulated activity. In conjunction with

the result showing higher sensitivity towards NBM treatment (Figure 8), the result of the experiments shown in Figure 9 indicates that, in the mouse protein, the thiol(s) associated directly or indirectly with catalysis is located in a more hydrophobic vicinity than that in the avian enzyme, and release of the SH(s) in mouse liver microsomes requires non-polar molecules like BdiT.

The PMB-BdiT experiment (Figure 9B) was repeated several times during the investigation, a complete inhibition of ACAT by PMB could not be achieved, even though exactly the same procedures were followed in the preparation of the microsomes and PMB solutions in both PMB-2-Mer (Figure 9A) and PMB-BdiT (Figure 9B) experiments. There is no good explanation for the different efficacies of ACAT inhibition by PMB (Figure 7A and B), except the possible differences in the batches of microsomes and PMB stock solutions used.

The effect of cholesterol hydroperoxides on mouse liver ACAT was studied in detail by M. Shih [3], and HP-B was found to have a complete and irreversible inhibition on the microsomal enzyme (Table 2). Oxidation of methionine residue(s) in the mammalian enzyme was suggested [3]. The enzyme from yolk sac membrane, on the contrary, was much less sensitive to oxidation by HP-B, and the lost activity could be recovered completely by DTT.

2. Effect of cholesterol redistribution on ACAT activity

It is known that cellular membranes are not homogeneous structures of randomly distributed lipids with embedded proteins. Rather, they contain microorganizations that differ from each other by the size of domains, by lipid and protein compositions, and by

the kinetic movement of each component [88]. Cholesterol-rich and cholesterol-poor domains are shown to exist in the lateral plane of the membrane and the distribution of the sterol in the two leaflets of the bilayer and among different membrane compartments is known to be asymmetrical [88]. Transport of the sterol among different membrane structures and maintenance of the gradient of cholesterol in the cell require lipid carrier proteins and transporting vesicles. Upon breaking-up of the cell and subsequent cellular fractionation, the non-equilibrium condition of cholesterol is disrupted, and redistribution of cholesterol among membrane fragments may now be able to proceed consequently. Cholesterol redistribution in the microsomes of yolk sac membrane turned out to activate ACAT considerably and made a very interesting case in the study of the enzyme. Following is a series of experiments designed to dissect the mechanism of ACAT activation upon storage at 4^0C.

A short-lived protein inhibitor of ACAT has been reported in mammalian cells and the inhibitor is susceptible to proteolytic cleavage by cysteine proteases [55-58]. The nearly two fold increase in ACAT activity observed during the first two days of the 4^0C storage experiment could be due to the destruction of an intrinsic ACAT inhibitor, if it also exists in the microsomes of yolk sac membrane and its loss happened much faster than the inactivation of the enzyme itself, resulting in the elevation of the activity seen in Figure 10 and 11. To examine the possible action of any cysteine proteases that may exist in the microsomal preparation, ALLN, a neutral cysteine protease inhibitor, was added to the microsomes at the beginning of the experiment (Figure 12). Apparently, ALLN did not affect the activation process because the microsomes had very similar ACAT activity in the

absence or presence of ALLN. Since the time course experiments described above were carried out in air, there was a possibility of oxidative inactivation of the putative ACAT inhibitor, which might have thiol(s) that were susceptible to oxidation. From the present work and Chang's work [34], it is known that ACAT activity of any source is not influenced by the presence of DTT. As one can see from Figure 13, the inclusion of the sulfhydryl protecting agent did not change the course of activation, and it helped sustain the activity a little bit higher. Therefore, either the yolk sac membrane does not have an intrinsic protein inhibitor to regulate ACAT activity, or the putative inhibitor does not partition into the microsomes when the microsomes are prepared, or the factor is not susceptible to cleavage by cysteine proteases. In any case, the activation of ACAT observed in the 4^0C storage experiments can not be explained by the results of the experiments aimed at exploring any inhibitory factor(s) of ACAT in the yolk sac microsomes.

Another possibility for the increase in ACAT SA during storage at 4^0C is a change in the microsomal membrane structure that affected the enzyme activity. It is known that the structure and fluidity of lipid bilayers affect activity and properties of membrane-associated proteins, including enzymes, receptors, and transporters [89-92], and oxidation of the lipids, phospholipids as well as cholesterol, can change membrane fluidity and microheterogeneity [93-96]. Lipids in lipoproteins are susceptible to oxidation, mediated by either the cells in the blood vessel wall or metal ions in circulation, and oxidation of lipids leads to changes in both lipid and protein structures [97]. In the majority of literature on lipoprotein oxidation, $CuSO_4$ has been used as a catalyst for oxidation. Examination of

microsomal oxidation catalyzed by 5 μM $CuSO_4$ revealed no elevation in ACAT activity (Figure 14B) (actually, a little bit of decrease in the activity was observed), and storage of microsomes at 4^0C overnight resulted in 150% increase in ACAT activity, probably due to the same mechanism that increased the enzyme activity as depicted in Figure 10-13. Hydrogen peroxide is known to react with sulfur-containing amino acids, i.e., cysteines and methionines, in proteins, and under certain circumstances, it also reacts with tryptophans, tyrosines, and cystines [98]. Oxidation of microsomes using increasing concentrations of H_2O_2 did not affect ACAT activity (Figure 14A). Among other factors that affect membrane structure, hydrolysis of membrane complex lipids by lipases results in the formation of free fatty acids, lysophospholipids, diacylglycerols, ceramides, etc., and the formation of these minor membrane lipid molecules affects bilayer packing and microheterogeneity, which in turn affects enzymes embedded in it [99,100]. DAG is known to act as an intracellular second messenger and activate protein kinase C and the following signaling pathways [101]. The role of sphingomyelin-derived second messengers, especially ceramides, in intracellular signal transduction has been realized more recently, and their involvement in regulation of cell proliferation, differentiation, and apoptosis is becoming more clear [102-109]. These lipid molecules generated from hydrolysis of complex lipids in the plasma membrane interact with a variety of proteins and kinases and induce various processes inside the cell. Inhibition of ACAT activity by short-chain ceramides and dihydroceramide was reported in CHO cells [110]. Treatment of microsomes of yolk sac membrane with linolenic acid, DOG, ceramides, and DHS had little or no effect on ACAT activity (Table 3). In the case of treatment of microsomes with

lysoPC, the lower-than-control enzyme activity is probably due to a competition of lysoPC with cholesterol for acyl-CoA, lowering the effective concentration of the other substrate of ACAT. The effect on lipid bilayers and membrane proteins brought by oxidation of microsomes or the addition of minor membrane lipids may not be big enough to influence ACAT activity, and responses induced by second messengers may require intact cells for the whole pathway(s) to function. On the other hand, ACAT from the yolk sac membrane may not be very sensitive to any changes in the global environment of the lipid bilayer. Early studies on dietary feeding of whole animals with different compositions of fatty acids suggested that membrane fluidity and lipid composition influenced hepatic and intestinal ACAT activities [1]. The insensitivity of the avian enzyme to the treatments affecting membrane structure may reflect the subtle difference in the physiological functions of the enzyme under different circumstances. Failure to reproduce the ACAT activation seen in the experiments depicted in Figure 10-13 by disturbance of microsomal membrane structure or addition of the second messengers suggests that changes in the lipid bilayer and membrane proteins may not be the mechanism that caused the activation, and signaling effect of second messengers may require a whole network of factors that are only present in the intact cells.

There has been mounting evidence that under physiological conditions, ACAT is not saturated with substrate cholesterol and addition of exogenous cholesterol to microsomes can increase the synthesis of cholesterol esters. Without adding cholesterol exogenously, J. Corton and D. Hardie showed that redistribution of endogenous cholesterol among microsomal vesicles could increase ACAT activity and in their case, sterol carrier proteins

were the possible factors that induced and facilitated the redistribution [54]. The activation of ACAT observed in the 4°C storage experiment could be due to a redistribution of cholesterol in the microsomes during the storage period, and the redistribution led to an increase in the ACAT substrate pool, resulting in the activation seen in the first 2 days of the experiment. To test the hypothesis, any factor that can help move cholesterol among membrane vesicles would be very useful in delineating the mechanism.

Cyclodextrins are cyclic molecules composed of different number of glucose units. With a hydrophilic surface and hydrophobic interior, cyclodextrins can form inclusion complexes with hydrophobic molecules and enhance the solubility of lipophiles in aqueous solutions [111]. Utilization of cyclodextrins in delivery of non-polar drug molecules and supplementation of natural lipid carriers in circulation and cell cultures has been very important in pharmaceutical, clinical, and basic research [112-115], and the usefulness of these lipid-solubilizing sugar oligomers in cholesterol research is becoming more significant. Studies of reverse cholesterol transport and intracellular cholesterol trafficking use cyclodextrins as effective cholesterol acceptors [116-119]. Among the commercially available cyclodextrins, β-cyclodextrin with 7 glucose units is the most effective molecule to solubilize cholesterol from lipid bilayers [8,9]. Cyclodextrins themselves are quite toxic to animals and their solubilities in aqueous solutions are limited. Derivatives of cyclodextrins have been synthesized chemically and found to be much less toxic and more soluble [113]. In their study of reverse cholesterol transport, P. Yancey et al found that the methyl derivative of β-cyclodextrin was more effective than the 2-hydroxypropyl derivative or β-cyclodextrin itself in extracting cholesterol from the plasma membrane of mouse L-cell

fibroblasts [116]. Not only are cyclodextrins used as acceptors in cholesterol depletion experiments, complexes of cholesterol with cyclodextrins can efficiently provide the sterol to membranes or cells in cholesterol repletion experiments [10,120]. Addition of MβCD to microsomes turned out to enhance ACAT activity greatly, nearly a 4 fold increase in ACAT SA (Figure 15). The formations of TAG and PLs, processes involving other ER membrane-associated enzymes in microsomal fractions and acyl-CoA as the other substrate, did not change significantly (Figure 16). It is known that the cellular membranes are heterogeneous structures of lipid and protein microdomains and cholesterol-rich and cholesterol-poor domains are distinct structural and kinetic organizations [88]. The results of these experiments indicate that MβCD can shuffle cholesterol around among microsomal fragments, from cholesterol-rich microdomains to cholesterol-poor microdomains, or from non-ACAT substrate pools to the ACAT substrate pool, and the enhanced redistribution of cholesterol resulted in increased enzyme activity (Figure 15). In agreement with the suggestion that a change in ACAT substrate pool size, not changes in microsomal membrane structure, affected ACAT activity, activities of the enzymes that are membrane-embedded and catalyze the formations of TAG and PLs did not change significantly (Figure 16). Thus, redistribution of cholesterol among microsomal vesicles during the storage period was very likely the mechanism that increased ACAT activity seen in the first two days of the experiments (Figure 10-13). During the course of this study, M. Liza et al reported an about 4 fold activation of rat liver microsomal ACAT in the presence of 6 mM 2-hydroxypropyl-β-cyclodextrin and explained the activation as a result of the facilitated redistribution of cholesterol in the presence of the cyclodextrin molecule [121]. Therefore,

cases of increases in ACAT activities by the effect of cholesterol redistribution may be quite general. The reason it has not been reported earlier could be that, most of the time, people study the enzyme from mammalian cells, and the mammalian enzyme is not very stable under storage, not stable enough to show an increase in the activity first before being inactivated. Other times, people just do not recognize the situation. In one incidence, Chang et al observed a nearly 2 fold increase in ACAT activity when they compared the cell extracts incubated for 30 min at 37^{0}C with that at 4^{0}C [5]. There was no explanation by the authors for the observed enzyme activation, but it is very plausible to suggest that a faster redistribution of cholesterol at the higher temperature was the factor leading to the increase. The property and stability of ACAT of yolk sac membrane allows the study of the minor but very interesting situation.

The activation of ACAT by redistribution of cholesterol in the absence of MβCD did not exceed two fold under any conditions studied (Figure 10-13 and Figure 14B), while in the presence of the cyclodextrin, an around four fold increase in the activity was consistently observed (Figure 15-16). Inactivation of the enzyme prior to the full activation was not the reason for the lowered activation because, as shown in Figure 11C, ACAT had been staying active for about 4 days before beginning to lose the activity. Even though any cellular cytosolic factor(s) that establishes and maintains the non-equilibium condition of cholesterol in the cell was removed or destroyed during the preparation of microsomes, some lipid or protein factors, which also help maintain microdomains of cholesterol in membranes, might exist in lipid bilayers and remain functional through the preparation. The interaction of cholesterol with those factors may restrain the movement of the sterol

from a complete random redistribution among membrane fragments. In other words, the association of cholesterol with the non-ACAT substrate pool limits the movement of the sterol, lowering the full potential of cholesterol to activate the enzyme. The formation of the inclusion complex with MβCD shifts the equilibrium towards the ACAT substrate pool, leading to a higher degree of enzyme activation. Thus, the use of the cyclodextrin in the study allows the understanding of ACAT activation upon storage and reveals the possible full potential of activation of the enzyme by endogenous cholesterol in cellular membranes.

3. Regulation of ACAT activity by (oxy)sterols

Studies on regulation of ACAT by cholesterol have been done previously on different systems and conditions, but the majority of them focused on the enzyme from mammalian sources and a lot were not in detail and complete. Taking the advantage of low concentration of intracellular cholesterol and lack of endogenous ACAT activity in insect Sf9 cells, Chang et al were able to express the human ACAT functionally and demonstrate the allosteric effect of cholesterol on the enzyme [5]. However, it appeared that the human enzyme could be saturated with cholesterol at relatively low concentration. At about 0.10 mole fraction of cholesterol, the enzyme activity reached a maxinum [5]. According to P. Yeagle's report [122], plasma membranes normally contain about 45 mol% of cholesterol and ER membranes have 10-12 mol% of the sterol. Chang et al's result implied that under normal physiological conditions, ACAT was saturated with cholesterol, which was contradictory with the majority of the literature reports. 25-Hydroxycholesterol has been shown to activate ACAT in mammalian cells, microsomal preparations, and cell extracts [3,5,46,71-74], and the activation mechanism was sought after in the works of Chang et al

[5] and Bhuvaneswaran [72]. No definite conclusion was drawn from their works. Regulation of ACAT of yolk sac membrane by cholesterol and 25-OH was explored in the current work and summaried in the following section.

To study the avian ACAT in relationship to cholesterol and oxysterols, microsomes of yolk sac membrane were depleted of cholesterol after repeated treatments with MβCD of different concentrations. As shown in Figure 17, higher concentrations of MβCD solubilized more cholesterol from the membrane. With 10 mM MβCD, a little bit less than half the amount of the initial sterol remained after 1st depletion and about one fourth remained after the 3rd (the 1st panel of bars in Figure 17), but with 50 mM MβCD, less than one sixth the amount of the initial sterol remained after the 1st depletion and only 4% remained after the 3rd (the 4th panel of bars in Figure 17). Loss of microsomal proteins, the majority of which probably were peripheral instead of integral membrane proteins, was observed in the process of cholesterol depletion (data not shown). Apparently, concentration of MβCD is an important factor determining the percentage of cholesterol being extracted, no matter how the content of cholesterol is expressed (as concentration in μM, Figure 17, or as a ratio to microsomal proteins, Figure 18). Two kinetic pools of cholesterol, a fast pool and a slow pool, were observed in the plasma membranes of several cell types when reverse cholesterol transport was studied in cell cultures [117]. The fast pool could be restored with cholesterol after a recovery time, presumably through the redistribution of the sterol in membranes. Since the redistribution of cholesterol was shown to happen among microsomal vesicles, it might be possible that more sterol could be depleted out if sufficient time was allowed for the redistribution. Overnight incubation of

the microsomes at 4°C after two cycles of depletion did not make any difference in the extent of cholesterol being extracted (the 3rd panel of bars in Figure 17 and 18). The amount of cholesterol in the plasma membrane has been shown to correlate positively with that of sphingomyelin and hydrolysis of the sphingolipid by SMase leads to a loss of cholesterol in the membrane [63-69]. Treatment of microsomes with SMase before the depletion cycles did not change the amount of cholesterol being solubilized (the 5th panel of bars in Figure 17 and 18). It appears that the extent of cholesterol solubilization depends only on the concentration of the cyclodextrin used, other factors that affect cholesterol redistribution do not play a role.

Besides cholesterol, β-cyclodextrins can solubilize cholesteryl esters, TAGs, PLs, and sphingomyelins, even though the magnitudes of extraction are much smaller [8,9]. Consistent with the literature, TAG and PC were found in the supernatant of the 1st 50 mM MβCD depletion (Figure 20).

Depletion of microsomal cholesterol with treatments of 25 mM MβCD for three times lowered cholesterol content in the microsomes to 0.042 (the line with open circles, Figure 21) and 0.026 (the line with dotted circles, Figure 21) mole fraction of total lipids (PLs plus cholesterol) in microsomes. In the ACAT assay, the depleted microsomes were mixed with liposomes, so the cholesterol concentration would be even lower, 0.0018 (the line with open circles) and 0.0015 (the line with dotted circles), because the liposomes contained no cholesterol (the first point in the plot). When the depleted microsomes were assayed for ACAT activity after incubation with cholesterol-PC liposomes of increasing cholesterol mole fractions at r.t. for 30 min (the line with open circle) or at 4°C overnight

(the line with dotted circles), the enzyme SA increased progressively, without reaching a plateau (Figure 21). At the highest concentration used, 0.38 mole fraction, and with overnight incubation with the liposomes, ACAT was not saturated with cholesterol. Normally in the cell, the ER membranes are shown to contain 10-12 mol% of cholesterol [122]. Cholesterol mole fraction in microsomes of yolk sac membrane was measured to be from 0.099 to 0.12, in very good agreement with the literature. At around 0.10 mole fraction of cholesterol, the human macrophage ACAT activity was shown to reach a plateau [5], the same was true for the mouse liver enzyme except at a higher cholesterol mole fraction (about 0.3) [3]. Perhaps, the avian enzyme had a very high Km such that it would not be saturated easily with high sterol concentrations, or, the increase in membrane rigidity or some structural changes brought by increasing concentrations of cholesterol favored the catalysis. The first possibility sounded unreasonable considering the function of the enzyme in yolk sac membrane, efficient conversion of substrate to product, meaning smaller Km, would provide cholesteryl esters for lipoprotein assembly more effectively. The second possibility might work in the case of this enzyme for its unique property and function. A third possibility for not being able to saturate the enzyme was that the transfer of cholesterol from cholesterol-PC liposomes to microsomes was slow, limiting the access of the sterol to ACAT substrate pool. When microsomal cholesterol was depleted to 0.016 mole fraction of total lipids (PLs plus cholesterol) in microsomes with treatment of 50 mM MβCD (0.0012 mole fraction in the ACAT assay when the microsomes were mixed with liposomes containing no cholesterol) (the first point in the plot, Figure 23), the enzyme was assayed in the presence of MβCD. Since MβCD is known, from above results, to be

capable of facilitating cholesterol redistribution among microsomal vesicles, it should enhance the transfer of cholesterol from liposomes, the source of substrate, to the microsomes, the site of enzyme catalysis. Indeed, an increase in MβCD concentration increased ACAT SA significantly, and the activity approached a plateau as the MβCD concentration reached 1 mM (Figure 22). When the depleted microsomes were assayed with liposomes of increasing cholesterol mole fractions in the presence of 1 mM MβCD, the enzyme activity reached a maximum around 0.4 cholesterol mole fraction (the line with circles, Figure 23). Thus, failure to saturate the enzyme with physiologically high limit of cholesterol (Figure 21 and the line with circles in Figure 23) is the result of limitation of substrate availability, and the use of MβCD accelerated transfer of cholesterol from liposomes to the enzyme substrate pool and eliminated the restraint of slow transfer of the substrate.

The shape of the ACAT activity vs cholesterol mole fraction curve is sigmoidal (Figure 23, the line with the triangles), suggesting allosteric interaction of cholesterol with ACAT. In his book, I. Segel discussed schematically enzymatic catalyses that show sigmoidal enzyme vs substrate profiles [123]. Cooperative interaction between an enzyme and its substrate shows sigmoidicity in the activity vs substrate concentration plot and is best explained mathematically by Hill equation and plot [123].

$$\frac{v}{V\text{max}} = \frac{[S]^n}{K' + [S]^n} \qquad \text{Hill equation}$$

where v is enzyme activity, V_{max} is the maximal activity, [S] is substrate concentration, K' is a constant consisting of interaction factors and the intrinsic dissociation constant, and n, Hill coefficient, is the number of substrate binding sites for each enzyme molecule.

Fitting of the data to Hill equation gave a Hill coefficient of 2.1 ± 0.4 (Figure 26). This suggests that the avian enzyme has multiple, possibly two, binding sites for cholesterol, and binding at one site enhances binding at the other site and causes a change in the conformation of the enzyme such that a more active state of the enzyme results. It is not known from current data if ACAT works as a monomer with multiple binding sites or as an oligomer of multi-subunits. The human macrophage enzyme was deduced to be 65 kDa [4], but antibodies of ACAT interacted with bands of higher molecular weights on Western blots [5,37]. Analysis of the deduced ACAT sequence revealed the existence of a dimerization motif [4], and radiation inactivation experiments indicated the functional size of rat liver ACAT to be 170-180 kDa [35] or 213 ± 35 kDa [36]. Plenty of evidence suggests that ACAT can oligomerize and may function as an oligomer, however, a definite answer can be assured only when the protein is purified and studied in homogeneity. In mammalian systems, sterol-mediated activation of ACAT is understood as being regulated not at the transcriptional and translational levels, but at posttranslational step(s) [42,37,70-71]. For the enzyme from both human macrophages [5] and mouse liver [3], cholesterol was shown to interact with ACAT cooperatively and act as an allosteric activator as well as a substrate [3,5]. In the current work, ACAT from yolk sac membrane of chicken eggs 16 days after fertilization is shown to response sigmoidally to increasing cholesterol mole concentration possibly through an allosteric effect of the sterol.

A sigmoidal profile of activity vs concentration is characteristic of cooperative substrate binding during enzyme catalysis, but cases do exist where a catalysis showing

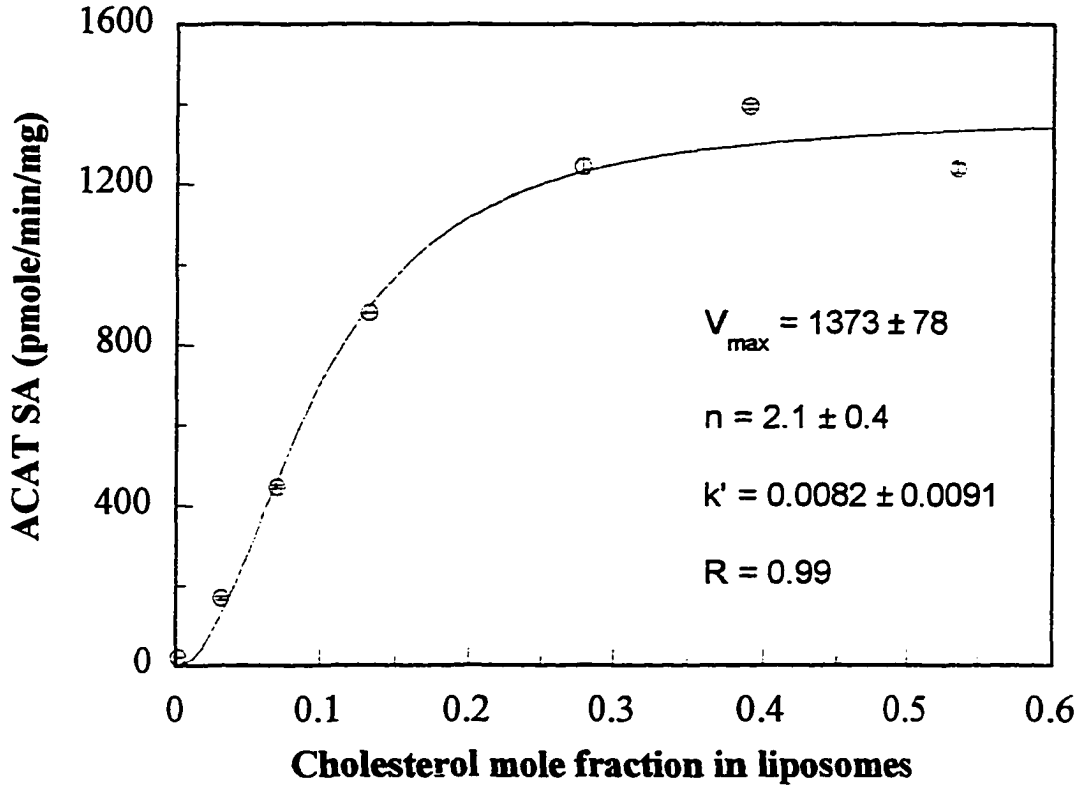
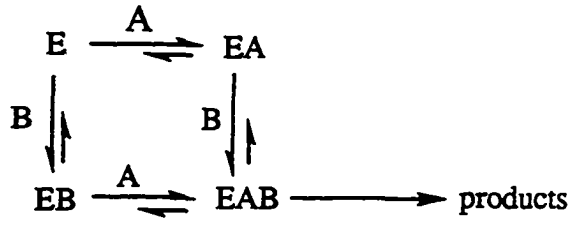


Figure 26. Fitting to Hill equation. Experimental data shown in Figure 23 (ACAT SA vs cholesterol mole fraction in the presence of 1 mM MβCD) is fitted to Hill equation. The points represent the actual values determined experimentally and the line is the result of fitting. R is the correlation coefficient and represents the relationship between the X and Y data to indicate how well the calculated curve fit the data.

sigmoidicity is not associated with substrate-enzyme cooperative interaction [123]. I. Segel discussed this kind of reactions as follows [123]:



In a Random Bi Bi reaction of enzyme E with substrates A and B, if the formation of EAB complex is much slower than the conversion of EAB to products and if one route to form EAB complex is more favored than the other, then the profile of velocity vs concentration of one substrate will be sigmoidal. In a reaction like this, sigmoidicity does not indicate allosteric interaction between an enzyme and its substrate. The mechanism of ACAT catalysis is not known, neither are the association constants of ACAT binding to cholesterol and to acyl-CoA, thus, the possibility of slow bindings of cholesterol and acyl-CoA to ACAT proceeding a fast formation of cholesteryl ester cannot be ruled out in the avian system. In any case, the sigmoidal shape of SA vs concentration of the avian enzyme suggests it may act quite similarly to its mammalian counterparts, interacting with substrate cholesterol specifically.

The significance of the usage of MβCD in the study of ACAT-cholesterol relationship was clearly demonstrated by the saturation of ACAT in the presence of the cyclodextrin compared with that in the absence (Figure 23). The ACAT assay solution was a mixture of microsomal vesicles and small-size unilamellar liposomes (in the extrusion device used to prepare liposomes, membrane pore size was 100 nm), encounter of

cholesterol in the liposomes with ACAT on the ER membrane could be achieved by two ways (cholesterol in the microsomal membrane was depleted to about one tenth of the usual content in ER membrane, so the major supply of the sterol was from liposomes, Figure 23). Cholesterol could diffuse through the aqueous medium, but desorption of the sterol from lipid bilayers and aqueous diffusion would be very slow considering the solubility of the sterol in aqueous solution. In pure water, solubility of cholesterol is 1.8 μg/ml or 4.7 μM [124]. Or, movement of the sterol between membranes could be promoted by collision or contact of ER membranes with liposomes, and the efficiency of cholesterol transfer from one bilayer to another may very well depend on the duration and distance of the contact and may not be high. Cholesterol transport inside the cell among different membrane compartments is proposed to proceed through three possible mechanisms: aqueous diffusion, vesicle-mediated transport, and soluble protein or lipid carriers. Vesicular transport and cytosolic carriers are most likely the major mechanisms [125,126]. Artificial vesicles like liposomes do not possess the property of the cellular vesicles in that the possible protein and lipid factors that mediate cellular lipid transfer between bilayers or vesicle-membrane fusion are not present, and so artificial vesicles may not function effectively in some cases of *in vitro* studies [88]. The access of cholesterol to the ACAT substrate pool had become a problem in the study of regulation of ACAT by the sterol and could only be overcome by the use a factor that can facilitate the access. In general, for processes that involve apolar molecules in aqueous media or reactions that have enzymes and substrates in different membrane compartments, transfer of non-polar molecules between membrane vesicles could be problematic and rate-limiting. The ability of

cyclodextrins to form inclusion complexes with lipophiles increases solubilities of non-polar molecules in aqueous solutions and enhances the transport and access of substrates to their enzymes. Cyclodextrins should be found more useful in situations where transfer of hydrophobic content between systems of membrane fragments is needed.

To address the question how 25-OH activates ACAT in mammalian cells, the depleted microsomes of yolk sac membrane were assayed for ACAT activity with increasing concentration of cholesterol in the absence or presence of 25-OH and/or MβCD Figure 25). If the effect of 25-OH is on the access of cholesterol to ACAT, the addition of MβCD should compromise and mask the effect of the oxysterol because both of them interact with the same activation mechanism. On the other hand, if 25-OH interacts with the enzyme directly, an increase in the activity by the addition of the oxysterol should be additive on top of the enhancement by the cyclodextrin because they affect different aspects of the enzyme catalysis. Surprisingly, 25-OH has no effect on ACAT activity in microsomes (Figure 24) and in the depleted microsomes assayed with increasing concentration of cholesterol (Figure 25). No matter what it is in mammalian cells, the mechanism of ACAT activation by 25-OH is absent in the avian system. Another cholesterol oxidation product tested in the study, HP-B, was shown to have a much smaller inhibitory effect on the avian enzyme, compared with that from mouse liver (Table 2). Apparently, ACAT of yolk sac membrane is different from its mammalian counterpart in that it is not very sensitive to influences of oxysterols. Considering the subtle differences in the function of the enzyme in the yolk sac membrane of fertilized eggs and in the liver, it is not unreasonble to see a high and sustained, rather than fluctuating and susceptible to

change, enzyme activity in such a vital and supportive tissue as the yolk sac membrane. Once the pure protein is available from both sources, a comparison of the structures will allow the identification of substrate binding sites, allosteric site(s), and possibly 25-OH interaction site, and the mechanism of oxysterol activation of ACAT can then be resolved.

Like other oxysterols, 25-OH has been shown to form endogeneously and inhibit cholesterol biosynthesis through the rate-limiting enzyme HMG-CoA reductase in both whole animals and cultured cells of mammalian origin [72-74]. However, whether the regulation of cholesterol homeostasis by oxysterols is physiologically relevant is not clear. It would be interesting to see if 25-OH has an effect on HMG-CoA reductase of yolk sac membrane and compare its sensitivity towards oxysterols with that of mammalian sources, although the *de novo* synthesis of cholesterol in the yolk sac membrane has not been reported and shown to be significant, and the activity of HMG-CoA reductase may not be high enough to be detectable. If the oxysterol has no effect on the enzyme from yolk sac membrane of chicken eggs after fertilization, it would be very interesting to see whether it has an effect on ACAT and HMG-CoA of mammalian source during embryogenesis and at the early stages of life. The formation and amounts of oxysterols have been shown to increase with pathogensis and aging [74], thus, the effect of cholesterol oxidation products on cholesterol metabolism may quite well be a chronic and adaptive result of the formation and accumulation of the oxidation products, and are not the original regulators of the metabolism. A life at its beginning, without oxidative accumulation, may lack the response to the oxysterols simply because they are physiologically irrelevant.

CHAPTER V. CONCLUSION

Recent progress in molecular cloning of human macrophage ACAT has accelerated the process of ACAT investigation, however, study of the enzyme is still limited to microsomal preparations or detergent extracts due to lack of the homogenous protein. Having higher activity and better stability compared with the mammalian counterpart, ACAT from yolk sac membrane of chicken eggs 16 days after fertilization provides a good working system for the study of the critical enzyme in cholesteryl ester synthesis, which plays a very important role in the supply of lipid molecules as structural components and energy source during embryogenesis. The avian enzyme shares many common features with the mammalian equivalent, including catalytically related histidine(s) and cysteine(s), detergent extractable activity, and possible cooperative interaction of cholesterol with the enzyme. There are differences in the properties of the enzyme, though, between the avian and mammalian sources, and the most interesting one is the absence of any effect of 25-OH on the enzyme from yolk sac membrane. This disparity may reflect the subtle difference in the function of the enzyme under different physiological circumstances, or it may be the manifestation of an important question: whether cholesterol oxidation products are the initial regulators of sterol-mediated mechanism of cholesterol metabolism, or their effects are secondary and adaptive with the original signaling. Tests of 25-OH and probably other oxysterols on cholesterol biosynthesis pathway in yolk sac membrane of chicken eggs after fertilization and in mammalian system during embryogenesis and early stages of life, and on cholesterol esterification during mammalian embryo development would be worthy of

investigation. The oxysterols may turn out not to be the original regulators, but their accumulation with time make the cell adapt their effect.

Redistribution of cholesterol among membrane fragments after the breakdown of the cell can lead to activation of ACAT since the enzyme is not saturated with the substrate under normal cellular conditions. Activation of ACAT during storage at 4^0C is found unlikely to be due to destruction of any possible inhibitor(s) of ACAT existing in microsomal preparations, neither is it due to alteration in microsomal membrane structure induced by oxidation of membrane proteins and lipids and by addition of minor membrane lipids. The likelihood of any effect induced by second messengers is small. With the application of a cyclodextrin, which has the ability to form inclusion complexes with non-polar molecules, the answer is pinpointed to be the consequence of cholesterol redistribution among lipid bilayers, leading to increased availability of substrate to the enzyme. Redistribution of cholesterol among membranes may happen quite commonly during experiments and may have caused some unexplainable phenomenon before. Lipid or protein factor(s) may exist and associate with cholesterol in the lipid bilayer, limiting complete random distribution/movement of the sterol in membranes and helping maintain microdomains of cholesterol-rich and -poor organizations. Sphingomyelin can be one of the factors that interact specifically with cholesterol, since cholesterol has been shown to have a high affinity with it.

The use of MβCD in the study led to the recognition of cholesterol redistribution and made possible saturation of ACAT with cholesterol. The finding that cyclodextrins can enhance cholesterol transfer between liposomes and microsomes and eliminate the 8-

limitation of slow movement of non-polar molecules in aqueous media should make them more useful in *in vitro* studies of apolar molecule transport between membrane vesicles.

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ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my major professor Carl L. Tipton for his advice and guidance during my study at Iowa State University. His kindness and patience eased me of the tough and down times in the research, and his open and warm attitude toward students allowed an academic environment of freedom and friendship.

Also, I would like to thank Dr. James A. Thomas and people in his lab for their intellectual suggestions and discussions. Their unconditional allowance of the usage of equipments and chemicals is deeply appreciated.

I wish to thank Department of Biochemistry & Biophysics and Iowa State University for giving me the opportunity to fulfill my dream of becoming a Doctor of Philosophy.

My sincerest gratitude and appreciation goes to my dearest husband, Weidong, for his love, support, and companionship during my six years of study. His care and understanding helped me go through the long and hard life of a graduate. Ronald, my beloved son, brings to our family so much fun and energy. They make my day-to-day life so colorful and enjoyable and give me the greatest love and joy of life.

Finally, I wish to express my deepest love and thanks to my parents. Without them and without their strong believe in me, I would not be writing any of this and become a Ph. D.