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Studies of the effect of 3β-hydroxy-5α-cholest-8(14)-en-15-one on microsomal acyl coenzyme A:cholesterol acyltransferase activity

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Rice University, 1989
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STUDIES OF THE EFFECT OF 3β-HYDROXY-5α-CHOLEST-8(14)-EN-15-ONE ON MICROSONAL ACYL COENZYME A:CHOLESTEROL ACYLTRANSFERASE ACTIVITY

by

DOLORES H. NEEDLEMAN

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE DOCTOR OF PHILOSOPHY

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STUDIES OF THE EFFECT OF 3β-HYDROXY-5α-CHOLEST-8(14)-EN-15-ONE ON MICROSOMAL ACYL COENZYME A:CHOLESTEROL ACYLTRANSFERASE ACTIVITY

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ABSTRACT

3β-Hydroxy-5α-cholest-8(14)-en-15-one (15-ketosterol) is a hypocholesterolemic agent which affects cholesterol metabolism at several levels, including sterol biosynthesis and cholesterol absorption. This dissertation describes studies on the effect of 15-ketosterol on intestinal acyl CoA:cholesterol acyltransferase (ACAT) activity, an enzyme involved in the esterification and absorption of dietary cholesterol.

Addition of 15-ketosterol to jejunal microsomes decreased the level of cholesterol esterified by ACAT. Incubation of 15-ketosterol with rat jejunal microsomes reduced the [1-14C]oleoyl CoA-dependent esterification of microsomal cholesterol (50% inhibition: 3.0 μM). This reduction in cholesterol esterification was accompanied by the formation of [14C]labeled material which, upon analysis by thin layer chromatography, comigrated with 15-ketosteryl oleate. The esterification of 15-ketosterol was confirmed by incubating [2,4-3H]15-ketosterol with unlabeled oleoyl CoA and jejunal microsomes. Analyses using either normal phase thin layer chromatography or reverse phase high pressure liquid chromatography detected the formation of [3H] material comigrating with 15-ketosteryl oleate.

Oral administration of 15-ketosterol to rats lowered ACAT activity relative to pair-fed controls. ACAT activity in rat jejunal microsomes was lowered 82% (P<0.02) and 77% (p<0.001) in animals fed a chow diet containing 0.05% and
0.10% 15-ketosterol, respectively. Analysis of the cholesterol and cholesterol ester content of rat jejunal microsomes showed that oral administration of either 0.10% or 0.125% 15-ketosterol did not affect the concentrations of these compounds. Additional studies showed that reduction of rat jejunal ACAT activity was dependent upon both the duration of oral administration of 15-ketosterol and the concentration of the compound in the diet. Reduction of ACAT activity was observed as early as 3 hours after ingestion of the first meal containing 0.10% 15-ketosterol. Significant reductions in microsomal ACAT activity were observed at concentrations as low as 0.05% 15-ketosterol in the diet. Little or no incorporation of [1-14C]oleoyl CoA into material comigrating with 15-ketosteryl oleate was observed.

In additional studies, incubation of 10 μM (25R)-3β,26-dihydroxy-5α-cholest-8(14)-en-15-one, a metabolite of 15-ketosterol, with rat jejunal microsomes resulted in a 55% reduction in the oleoyl CoA-dependent esterification of microsomal cholesterol.
I dedicate this dissertation to my husband, David Needleman, who gave me his unfailing love and support, and to my parents, Dolores Van Dyke Elliott and William Elliott, who believed in the value of education and taught me to strive for excellence.
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### Table of Abbreviations

The following abbreviations are used throughout the text of this dissertation.

- ACAT = acyl coenzyme A:cholesterol acyltransferase
- BSA = bovine serum albumin
- BSTFA = bis(trimethyl)trifluoracetamide
- C = centigrade
- Ci = Curie(s)
- cm = centimeter
- CoA = coenzyme A
- DEP = diethyl pyrocarbonate
- DMSO = dimethylsulfoxide
- DOC = deoxycholate
- dpm = disintegrations per minute
- DTT = dithiothreitol
- EDTA = ethylenediamine tetracetic acid
- GLC = gas-liquid chromatography
- gm = gram(s)
- HDL = high density lipoprotein
- HPLC = high pressure liquid chromatography
- HMG-CoA = 3-hydroxy-3-methylglutaryl coenzyme A
- 15-ketosterol = 3β-hydroxy-5α-cholest-8(14)-en-15-one
- LDL = low density lipoprotein
- M = molar
- m = meter(s)
- mCi = millicurie(s)
- mg = miligrams(s)
min = minute(s)
mL = milliliter(s)
mmol = millimole(s)
μCi = microcurie(s)
μg = microgram(s)
μm = micrometer(s)
μmol = micromole(s)
μM = micromolar
NADPH = reduced nicotinamide adenine dinucleotide phosphate
NMR = nuclear magnetic resonance
nmol = nanomole(s)
PCEH = pancreatic carboxyl ester hydrolase
PPO = 2,5-diphenyl-1,3-oxazole
pmol = picomole(s)
p.s.i. = pounds per square inch
radio-TLC = thin layer radiochromatography
rpm = revolutions per minute
S.E.M. = standard error of the mean
SDS = sodium dodecyl sulfate
TLC = thin layer chromatography
TMS = trimethylsilyl
x g = times gravity
VLDL = very low density lipoprotein
v/w = volume per weight
v/v = volume per volume
°C = degrees Celsius
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Chapter 1

Introduction
In mammalian tissues, cholesterol is the predominant member of the sterol family. It plays an essential role in the modulation of membrane fluidity and serves as a precursor for the biosynthesis of steroid hormones and bile acids. Cholesterol is also a major constituent of atherosclerotic lesions. In recent years, the correlation between high blood cholesterol levels, atherosclerosis, and cardiovascular disease has been firmly established\(^1\). It is this importance of cholesterol to both health and disease that has stimulated intensive research into the metabolism and transport of this compound.

Recent work has indicated that the synthesis, uptake, and degradation of lipoprotein particles, particularly low density lipoprotein particles (LDL), play a major role in the regulation of cholesterol transport and metabolism. Brown and Goldstein have proposed a mechanism for the uptake of cholesterol by mammalian cells which involves the binding of LDL particles to a cell surface receptor, the endocytosis of the LDL-receptor complex, the degradation of the LDL particle with the release of amino acids, fatty acids, and cholesterol, and the recycling of the LDL receptor to the cell surface\(^1\)\(^-\)\(^3\). These authors suggest that cholesterol liberated from LDL controls cellular cholesterol metabolism via three processes\(^2\)\(^-\)\(^3\). First, an accumulation of cholesterol reduces \textit{de novo} production of sterol by inhibiting the synthesis of \textit{3}-hydroxy-\textit{3}-methylglutaryl coenzyme A (HMG-CoA) reductase, the major regulatory enzyme of sterol biosynthesis. Second, the incoming LDL-derived cholesterol increases the amount of cholesteryl esters by activating acyl CoA:cholesterol acyltransferase (ACAT) activity, an enzyme important to the intracellular metabolism of cholesterol. Finally, the accumulation of cholesterol decreases the synthesis of new LDL receptors. There is evidence that the reduction of LDL receptors through either the feedback inhibition of receptor synthesis or the hereditary
lack of functional receptors, as in familial hypercholesterolemia, contributes to high blood cholesterol levels\textsuperscript{1,2}.

The feedback control by LDL-derived cholesterol is important to the LDL receptor hypothesis of the regulation of cholesterol metabolism. However, there is evidence that cholesterol may not be the active species involved in this LDL-mediated regulation. It appears that oxygenated derivatives of cholesterol may serve in this capacity. Kandutsch and Chen have shown that there is no effect on the level of HMG-CoA reductase activity in cultured fetal mouse cells when carefully purified cholesterol is added to the serum free, chemically defined growth medium\textsuperscript{4}. Instead, HMG-CoA reductase activity is reduced when autoxidation products of cholesterol are incubated with fetal mouse cells\textsuperscript{4}. Other studies have shown that incubation of two autoxidation products of cholesterol, 7-ketocholesterol and 25-hydroxycholesterol with cultured cells results in a reduction in HMG-CoA reductase activity and a stimulation of cholesterol ester formation from labeled oleate\textsuperscript{4,5}. Thus, 7-ketocholesterol, 25-hydroxycholesterol, and LDL particles appear to alter in vitro cholesterol metabolism in a similar manner. Since the standard technique for the isolation of LDL renders the particles sensitive to oxidation, it is possible that oxygenated sterols are responsible for the observed regulation of sterol metabolism by LDL\textsuperscript{4}.

A large number of oxygenated sterols have been evaluated for their effects on sterol metabolism\textsuperscript{4-17}. Some of these sterols are known autoxidation products of cholesterol, while others are known precursors or metabolites of cholesterol. Many oxygenated sterols have been found to be potent inhibitors of sterol biosynthesis and HMG-CoA reductase activity in cultured mammalian cells\textsuperscript{4-17}. In addition, it has been demonstrated that there is a good correlation
between the actions of various oxygenated sterols on HMG-CoA reductase activity in cultured cells and the affinity of these same sterols for a cytosolic oxysterol binding protein\textsuperscript{15,18}.

Recently, the detection of oxygenated sterols in both cultured cells and plasma has been reported. Saucier and coworkers have reported that cultured Chinese hamster lung (Dede) cells contained 24(S),25-epoxycholesterol and 25-hydroxycholesterol in cellular concentrations within the range required to repress HMG-CoA reductase activity\textsuperscript{19}. Saucier and coworkers have also reported that addition to the culture medium of a concentration of mevalonate high enough to repress HMG-CoA reductase activity by 90% resulted in the biosynthesis of two new regulatory oxygenated sterols, 32-oxolanosterol and 32-hydroxylanosterol\textsuperscript{17}. In addition, it has been reported that oxygenated sterols were present in human plasma (Kudo et al.\textsuperscript{20} and the references cited therein). In one study, Kudo et al. added highly purified $[\text{14C}]$cholesterol to plasma in order to permit the detection and quantitation of oxygenated sterols formed by autoxidation of cholesterol during processing of the samples\textsuperscript{20}. Special attempts to suppress autoxidation of cholesterol included the use of an all-glass closed system (under argon) for saponification and extraction followed by rapid removal of cholesterol from the polar sterols by reverse phase high pressure liquid chromatography. Under these conditions, Kudo and coworkers reported that chromatographic analyses of the $[\text{3H}]$acetate derivatives of the polar sterols detected the presence of 3\(\beta\),26-dihydroxy-cholester-5-ene, (24S)-3\(\beta\),24-dihydroxy-cholester-5-ene, and 3\(\beta\),7\(\alpha\)-dihydroxy-cholester-5-ene in human plasma. These combined results lend support to the hypothesis that oxygenated sterols are natural regulators of cholesterol metabolism\textsuperscript{4}. 
One oxygenated sterol, 3β-hydroxy-5α-cholest-8(14)-en-15-one (15-ketosterol, figure 1), has been extensively studied. This compound is a potent hypocholesterolemic agent which affects cholesterol metabolism at several levels. Studies have shown that 15-ketosterol is a potent inhibitor of both HMG-CoA reductase activity and sterol biosynthesis. 15-Ketosterol has also been demonstrated to reduce intestinal absorption of exogenous cholesterol. In addition, the presence of 15-ketosterol has been detected in rat skin. Thus, this compound may serve as a natural regulator of cholesterol metabolism. In view of the observation that this naturally occurring compound affects the two ultimate sources of cholesterol in the body, namely biosynthesis and absorption, further investigations into the actions of 15-ketosterol on sterol metabolism have been done. The following dissertation summarizes studies which examine the action of 15-ketosterol on ACAT activity, an enzyme important to the absorption of dietary cholesterol as well as to the general cellular metabolism of cholesterol.

Overview of cholesterol absorption

The absorption of dietary sterol is one factor that has a major influence on both cholesterol metabolism and transport. There is ample evidence to suggest that plasma cholesterol levels are determined, in part, by the intestinal absorption of dietary sterol. As a consequence, it is essential to understand the underlying mechanism of cholesterol absorption. Unfortunately, the absorption of dietary cholesterol is an exceedingly complex process and not well understood.

It is known that cholesterol absorption in mammals occurs in the jejunum of the small intestine. This is a region of the intestinal tract which possesses
Figure 1: Structure of 3β-hydroxy-5α-cholest-8(14)-ene-15-one
an extremely large absorptive surface. The size of the surface area is primarily
due to the projection of villi into the lumen of the small intestine. In several
species, including man, the villi project from additional folds, known as the
plicae circulares. The villi are covered with a single layer of highly polarized
enterocytes which are capped with brush border membranes. The functional
orientation of enterocytes is such that the brush border membranes are
exposed to the contents of the lumen and provide the actual absorptive surface.

It is generally accepted that absorption of cholesterol by enterocytes
occurs from an aqueous micellar phase in which sterol is solubilized in an
emulsion of bile salt, phospholipid, fatty acids, and monoacylglycerols.
According to this concept, the rate limiting step in sterol uptake is the diffusion of
the micelles across the unstirred water layer surrounding the villi. The micelle
functions to provide a constant supply of unesterified cholesterol to the brush border membrane.

Regulation of diffusion across the unstirred water layer is complex. There
is evidence that diffusion is controlled by both the composition and the size of
the micelle. However, studies of intestinal contents, after centrifugation at
100,000 x g for 15 hours, have indicated that no more than 10% of the total
cholesterol is in the clear micellar supernatant. Light microscopy and
polarized light microscopy of intestinal contents have shown that several other
phases including an oil phase, a viscous isotrophic phase, and fat droplets
coexist with the micelle.

The contribution of nonmicellar phases to cholesterol absorption has
been studied by Reynier and coworkers using supersaturated solutions
composed of cholesterol, oleate monoolein, and taurocholate. Using both
microscopic and polarized light microscopic techniques, these supersaturated
solutions were shown to be composed of a viscous isotropic phase, a paracrystalline phase, and a micellar phase. The authors reported that the uptake of [4-\(^{14}\)C] cholesterol by rat intestinal sacs increased linearly with the [4-\(^{14}\)C] cholesterol content of either micellar solutions or supersaturated solutions. They suggested that, in the presence of such solutions, cholesterol uptake occurred from both micellar and nonmicellar phases.

Little is known about the events occurring at the surface of the brush border membrane during cholesterol absorption. Transfer of cholesterol across the brush border membrane is thought to be a passive process which involves exchange diffusion\(^{30,32}\). In this model, an entering molecule of cholesterol displaces another molecule of cholesterol from the opposite side of the bilayer, and forces it into the cytoplasm. The model is supported by the observations that cholesterol does not accumulate in the brush border membrane, and that pinocytosis of micelles does not occur\(^{30}\). The results of recent studies have suggested that the uptake of cholesterol is selective and is not necessarily a passive process.

Child and Kuksis have studied the rates of uptake of 7-dehydrocholesterol, 7-dehydrocampesterol, and 7-dehydrositosterol by isolated brush border membrane vesicles\(^{30}\). Micellar solutions composed of the test sterols, sodium taurocholate, egg phosphatidylcholine, and egg phosphatidyl-ethanolamine were prepared, and the uptake of the sterols by the brush border membrane vesicles was followed through HPLC analyses of lipid extracts of the vesicles. In these studies, the authors reported that uptake of 7-dehydrocholesterol was 4 times greater than uptake of 7-dehydrositosterol, and that uptake of 7-dehydrocampesterol was 2.5 times greater than uptake of 7-
dehydrositosterol. Based upon these results, the authors suggest that uptake of cholesterol by brush border membranes is a selective process.

Additional studies of the uptake of cholesterol by loops of rat intestine have indicated that there may be a saturable component of the sterol uptake process that is influenced by metabolic inhibitors. In these studies, the addition of inhibitors such as sodium azide, potassium cyanide, 2,4-dinitrophenol, or ouabain, reduced the uptake of cholesterol to 40-60% of the control values. On the basis of these results, the authors suggest that the initial uptake of cholesterol occurs by an active transport system. Others have suggested that the influence of metabolic inhibitors on cholesterol uptake is the result of either the loss of membrane integrity or the dissipation of a chemical gradient that acts to draw cholesterol into the intestinal epithelial cells.

Once cholesterol has entered the enterocyte, it is rapidly distributed throughout the intracellular membranes. The movement to the endoplasmic reticulum is significant, since it is considered to be the site of esterification and chylomicron assembly. The method of movement of cholesterol from the brush border membrane to the endoplasmic reticulum is unknown. Green has suggested three possible mechanisms: spontaneous transfer between membranes via the aqueous phase, transfer facilitated by soluble carrier proteins, and transfer modulated by membrane vesicles. Spontaneous exchange between membranes has been demonstrated between liposomes, between liposomes and erythrocyte ghosts, between crude preparations of endoplasmic reticulum and liposomes, between mitochondria and liposomes, and between plasma membranes and liposomes. Spontaneous exchange between membranes involves the transfer of cholesterol from a membrane with a higher cholesterol/phospholipid ratio to one with a lower
cholesterol/phospholipid ratio\(^1\). It is known that the brush border cholesterol/phospholipid ratio is 0.9, while the cholesterol/phospholipid ratio of endoplasmic reticulum membranes is as low as 0.1\(^1\). The difference in these ratios might facilitate the transfer of cholesterol between intracellular membranes in enterocytes. In addition, a Sterol Carrier Protein\(_2\)-like activity has been reported to be present in enterocytes\(^{30,34}\). The addition of this Sterol Carrier Protein\(_2\)-like activity to incubations of liposomes and rat jejunal microsomes has also been reported to stimulate levels of jejunal ACAT activity\(^{34}\). These latter results suggest that facilitated transfer of cholesterol within the enterocyte is possible.

The processes by which absorbed, dietary cholesterol is incorporated into chylomicrons and secreted into the lacteals of the villi are largely unknown. Active synthesis of proteins appears to be necessary for normal chylomicron secretion. It is reported that addition of inhibitors of protein synthesis, such as puromycin, to incubations of isolated villus cells inhibits the secretion of chylomicrons\(^{30}\). There is also evidence to suggest that the microtubular network of the enterocyte is involved in chylomicron secretion. Reaven and Reaven quantitated the microtubular and the lipid droplet content of the apical, golgi, and basal regions of rat jejunal enterocytes\(^{30}\). They reported that oral administration of colchicine or vinblastine to post-absorptive rats lowered the number of microtubules and increased the amount of lipid present in the apical region of enterocyte.

Esterfication of dietary cholesterol has also been demonstrated to be important to absorption\(^{30,32}\). It is not known whether the esterification of cholesterol occurs before, during, or after the assembly of chylomicrons. However, Hashimoto and Fogelman reported that rat hepatic ACAT activity was
primarily located on rough microsomes, and that cholesteryl esters formed by the action of hepatic ACAT were transferred to smooth microsomes. Based upon these results, the authors suggested that the smooth endoplasmic reticulum served as a trap for cholesteryl esters. It is conceivable that movement of cholesteryl esters to the smooth endoplasmic reticulum plays some role in the incorporation of cholesteryl esters into lipoprotein particles such as chylomicrons.

Two enzymes are implicated in the esterification of dietary cholesterol. One of these activities is pancreatic carboxyl ester hydrolase (PCEH), a bile acid activated enzyme which reversibly esterifies cholesterol and hydrolyzes cholesteryl esters. PCEH is secreted into the lumen of the small intestine upon entry of chyme into the duodenum. After its entry into the lumen of the small intestine, PCEH primarily serves as a hydrolytic enzyme, and acts on a variety of substrates including cholesteryl esters, vitamin E acetate, vitamin A palmitate, vitamin D₃ acetate, glycerides, phospholipids, and lysophospholipids. Several laboratories have reported that this enzyme is taken up by the villus enterocyte, where it functions to catalyze the esterification of cholesterol. The second enzyme is ACAT, a long chain acyl CoA-dependent activity (figure 2).

ACAT is a ubiquitous enzyme which has also been implicated in the regulation of general intracellular cholesterol metabolism. It is a membrane bound enzyme which is located on the rough endoplasmic reticulum of cells. ACAT is present in a wide variety of cell types including human fibroblasts, rat hepatoma cells, rat hepatocytes, mouse peritoneal macrophages, Ehrlich ascites cells, and Chinese hamster ovary (CHO) cells. ACAT activity is also present in the intestines of rat, rabbit, guinea pig, and human; in the livers
Figure 2: The esterification reaction catalyzed by ACAT
of guinea pig, pig, human, and monkey; in the arteries of rabbit, monkey, and pigeon, and in rat ovary. The specificity of ACAT for acyl CoA molecules has been reported for most of these systems. Based upon these results, Spector and coworkers have suggested that oleoyl CoA is the preferred substrate for ACAT.

While investigations into PCEH activity and function utilize purified enzymatic preparations, the study of ACAT activity is largely confined to the use of a microsomal assay system. Unfortunately, work with a microsomal assay system is complicated by the presence of several acyl CoA-dependent enzymes. The presence of these other enzymatic activities must be recognized and controlled. Methods for the solubilization and reconstitution of ACAT have only been reported for the rat liver, pig liver, and Ehrlich ascites cell, and CHO cell enzymes. Of these methods, a partial purification (5.6 to 6.7 fold) was reported only for the pig liver enzyme.

The regulation of ACAT has been extensively studied. It has been reported that the level of ACAT activity in microsomes is affected by a variety of factors. For instance, with the exception of the bovine adrenal enzyme, ACAT activity is not saturated with respect to cholesterol. Factors which increase the supply of cholesterol, such as cholesterol feeding or addition of cholesterol to microsomal assay systems, stimulate ACAT activity. The level of ACAT activity in microsomes has also been reported to be affected by other factors including the fatty acid composition of the microsomal membrane (liver, intestine), the addition of progesterone to incubations of either whole cells or microsomes (liver, bovine adrenal), the addition of 25-hydroxycholesterol to incubations of whole cells (hepatocyte, fibroblast, CHO cells), the addition of a
Sterol Carrier Protein2-like activity to incubations of rat jejunal microsomes and liposomes34,37-39,50, thyroxine administration to rats51, diurnal variation (rat liver, rat intestine)52,53, and fasting (rat liver, rat intestine)53. It has also been reported that the level of ACAT activity is altered by assay conditions which promote phosphorylation, such as the addition of cytosol, 4 mM MgCl2, 4 mM ATP, and 40 mM NaF to incubations of microsomes (rat liver, rat intestine, bovine adrenal cells)37-39,43.

However, the regulation of ACAT by phosphorylation has recently been questioned. Mitropolous and Venkatesan reported that incubations of rat liver microsomes with cytosol, 4 mM MgCl2, 2 mM ATP, and 50 mM NaF, increased the levels of ACAT activity54. These authors reported that the same incubation conditions also increased the net transfer of cholesterol from liposomes to rat liver microsomes. Based upon these results, Mitropoulos and Venkatesan concluded that the increase in the level of rat liver ACAT activity upon incubation of microsomes with cytosol, MgCl2, ATP, and NaF, could be interpreted either in terms of phosphorylation or in terms of modulation of the supply of cholesterol. In addition, Einarsson and coworkers reported that the levels of ACAT activity in human liver microsomes are not affected by the addition of 5 mM MgCl2, 2 mM ATP, and 50 mM NaF to the assay system55.

The relative importance of PCEH and ACAT to the esterification of absorbed cholesterol

The relative importance of PCEH and ACAT in cholesterol esterification and absorption is not well understood. Several groups have reported that PCEH serves as the primary cholesterol esterifying enzyme in enterocytes. However, other groups have reported that microsomal ACAT serves as the
primary cholesterol esterifying enzyme in enterocytes. As a consequence, this subject remains a topic of controversy.

A role for PCEH in esterification of absorbed cholesterol was first inferred from reports of the presence of a bile salt-dependent cholesterol esterase in the cytosol of intestinal epithelial cells\textsuperscript{30,36}. The origin and function of this cytosolic cholesterol esterase was investigated using both depancreatized rats and rats with diverted pancreatic flow\textsuperscript{30,36,56}. The authors reported that the level of radiolabeled cholesterol esterified by the cytosolic cholesterol esterase was reduced in depancreatized rats and in rats with diverted bile flow. This decline in enzyme activity was associated with decreases in lymphatic output of radiolabeled cholesterol. These results led to the hypothesis that PCEH was taken up from the lumen of the small intestine by the enterocyte, where it catalyzed the esterification of cholesterol. However, subsequent investigations of the role of PCEH have provided conflicting results.

In one experiment the role of rat PCEH and rat jejunal ACAT in the absorption of radiolabeled cholesterol was studied in lymph duct-cannulated rats which had normal levels of both enzymes and in lymph duct-cannulated rats which were deficient in PCEH activity\textsuperscript{57}. The deficiency in PCEH activity was accomplished by either diverting pancreatic flow (through cannulation of the common bile and pancreatic duct), or by treating the pancreatic fluid with antibodies directed against purified rat PCEH and perfusing the treated pancreatic fluid into rats. Gallo and coworkers reported that a deficiency in PCEH activity resulted in an 83% decline in the level of [4-\textsuperscript{14}C]cholesterol absorption. This decline in cholesterol absorption was correlated with a 75% decline in the level of [4-\textsuperscript{14}C]cholesterol esterified by a cholesterol esterase activity present in the cytosol of isolated enterocytes. The authors also reported
that the amount of [14C]cholesteryl esters secreted into lymph was unaffected by diversion of pancreatic flow or by immunoprecipitation of PCEH. Gallo and coworkers suggested that the residual cytosolic cholesterol esterase activity was sufficient to account for the amount of cholesteryl esters secreted into lymph. Based upon these results, the authors concluded that cholesterol esterase played an essential role in the esterification of absorbed cholesterol.

In another study, rabbit polyclonal antibodies directed against purified PCEH were used to determine the localization of PCEH in rat small intestine58. Small rings of jejunum were isolated from both normal rats and pancreatic duct (common duct)-cannulated rats. The rings were rinsed briefly with physiological saline prior to fixation with paraformaldehyde, and were analyzed using the unlabeled antibody in combination with horseradish peroxidase-antihorseradish peroxidase complex. Gallo and coworkers reported the detection of a uniform distribution of specific reaction product within the absorptive cells of control rats. Reaction product was also detected in the lamina propria and the submucosa of jejunal segments taken from control animals. The authors also reported that no reaction product was detected in intestinal slices taken from pancreatic duct-cannulated rats. Based upon these results, Gallo and coworkers concluded that PCEH was taken up by rat villus enterocytes.

Contrasting results regarding the role of PCEH in the esterification of absorbed cholesterol have been reported30,59. Watt and Simmonds examined the absorption and lymphatic secretion of radiolabeled cholesterol after duodenal infusion of a micellar solution composed of bile salts, labeled cholesterol, and glycerolipids30. They reported that the level of cholesterol absorption in rats, possessing both bile fistulae and diverted pancreatic flow
(pancreatic duct cannulations), was not significantly different from the level of cholesterol absorption in rats with normal pancreatic flow. They also reported that there was no correlation between the levels of cytosolic cholesterol esterase activity and the levels of cholesteryl ester secretion into lymph. In addition, Field measured the activity of the cytosolic cholesterol ester hydrolase activity in rabbit intestinal cells after subcellular fractionation. He reported that washing or wiping intestinal segments free of adherent mucous before scraping the segments and subsequently homogenizing the isolated enterocytes greatly reduced the levels of the cytosolic cholesterol esterase. Based upon these results, the author suggested that the presence of PCEH in intestinal subcellular fractions was the result of contamination of the preparation with pancreatic fluid.

However, in a recent study, Lechene and coworkers examined carefully washed human enterocytes for the presence of a bile salt-dependent cholesterol ester hydrolase. In this study, fragments of human jejunum were washed with ice-cold saline, and were opened longitudinally. The mucosa was gently scraped off with a microscope slide, and the cells were suspended in a buffer solution. Subcellular fractionation was performed after the cell suspension was washed 5 times by cycles of centrifugation and resuspension in fresh buffer. The authors reported the presence of a bile salt-dependent cholesterol ester hydrolase in the cytosol of human enterocytes. Using antibodies directed against purified human PCEH, purified human pancreatic lipase, and purified human chymotrypsinogen, Lechene and coworkers studied the distribution of pancreatic enzymes in slices of human jejunum. They reported that a positive immunoreaction with antibodies directed against PCEH was detected in endocytotic vesicles within enterocytes and in intracellular
spaces. No immunoreactivity was obtained when antibodies against human human pancreatic lipase and purified human chymotrypsinogen were used. The authors suggested that their results supported that concept that PCEH was taken up by jejunal enterocytes.

It is clear that investigations into the role of PCEH in the esterification of absorbed cholesterol have produced confusing and often contradictory results. As a consequence, the question concerning the exact function(s) of PCEH is unresolved.

Adding to this confusion is a large body of evidence which strongly suggests that ACAT plays a major role in cholesterol absorption. It has been shown that microsomal ACAT activity predominately esterifies cholesterol delivered to the enterocyte from dietary, biliary, or lipoprotein sources rather than cholesterol synthesized within the enterocyte\textsuperscript{37,39,46}. Several studies have reported that there is a gradient of intestinal ACAT activity which follows the gradient of cholesterol absorption. In rat, rabbit, and man, the gradient of microsomal ACAT activity extends the length of the small intestine, with the highest activity found in the jejunum, and the lowest activity in the duodenum and ileum\textsuperscript{37,39,45,61,62}. Along the villus/crypt axis of the intestinal mucosa, ACAT activity, as measured by the oleoyl CoA-dependent esterification of endogenous cholesterol, has been reported to be greatest in unfractionated homogenates of villus cells isolated from the jejunums of chow-fed rabbits and the jejunums of chow-fed rats\textsuperscript{45,62}. Other investigators have reported that ACAT activity, as measured by the oleoyl CoA-dependent esterification of exogenous cholesterol (334 \textmu M), is greatest in unfractionated homogenates of crypt cells isolated from the jejunums of chow-fed rats\textsuperscript{63}. 
Several laboratories have reported that intestinal ACAT activity is not saturated with respect to cholesterol, and that the enzyme responds to changes in cholesterol levels. ACAT activity rises with the addition of cholesterol to the microsomal assay system, with the addition of cholesterol to the diet, and with the addition of cholesterol to cultured cells isolated from cholesterol-fed rabbits. This increase in jejunal ACAT activity has been shown to be greatest in villus enterocytes. Field and coworkers have reported that the addition of 1% cholesterol, 10% corn oil to the diet of rabbits preferentially increases the level of ACAT activity in unfractionated homogenates of villus cells. Stange and coworkers have reported that addition of 0.5% cholesterol to the diets of rats also preferentially increases the level of ACAT activity in unfractionated homogenates of villus cells. Norum and coworkers have reported that the ratio of ACAT activity in villus/crypt cells shifts in favor of villus cells upon cholesterol feeding.

Recently, specific inhibitors of ACAT activity have been used to assess the role of this enzyme in cholesterol absorption. One compound N-(1-oxo-9-octadecenyl)-DL-tryptophan (Z) ethyl ester (57-118, Sandoz) was shown to be a competitive inhibitor, with respect to oleoyl CoA, of the ACAT activity present in rabbit jejunal microsomes. In a study in which 57-118 (200 mg/kg) was administered by gavage to cholesterol-fed rabbits (1% cholesterol in chow diet), a 59% reduction in microsomal ACAT activity was observed. The activity of cholesterol esterase was also measured using the supernatant fraction obtained upon isolation of jejunal microsomes by centrifugation at 100,000 x g. The authors reported that the level of esterification of [14C]cholesterol by cholesterol esterase was not affected by administration of 57-118. In additional experiments, the administration of 57-118 (200 mg/kg) was correlated to a 65%
decrease in cholesterol absorption as determined by the ratio of \([3H]\) cholesterol (intravenous injection) to \([14C]\) cholesterol (oral administration) in whole blood\(^{66}\). Finally, the presence of 57-118 was determined by HPLC analysis of the total lipid extract from isolated subcellular fractions of washed enterocytes. Heider and coworkers reported that after oral administration of 57-118, the compound was present in rabbit jejunal microsomes in concentrations equivalent to those required to obtain inhibition of ACAT activity \textit{in vitro}.

A related ACAT inhibitor, 3-(decyldimethylsilyl)-N-[2-(4-methylphenyl)-1-phenylethyl] propanamide) or 58-035 (Sandoz), was also used to examine the role of intestinal ACAT in cholesterol absorption\(^{67,68}\). In one set of studies, 58-035 was administered to chow-fed rats fitted with mesenteric lymph cannulae\(^{67}\). Two experimental approaches were used. In one protocol, 58-035 was administered to rats as 2 intragastric doses (20 mg/kg/dose) separated by an 8 hour interval. Two hours after administration of the second dose of 58-035, a lipid emulsion containing \([3H]\)cholesterol was given as an intragastric dose. In the second case, 58-035 was administered to rats as a prolonged duodenal infusion (17.5 mg over 8 hours). Gastric infusion of \([3H]\)cholesterol was started two hours after the initiation of duodenal infusion of 58-035, and continued for 6 hours. In both approaches, lymph was collected into a preweighed vessel for a period of 1 hour prior to the administration of the \([3H]\)cholesterol. After initiation of the \([3H]\)cholesterol meal, lymph was collected into a preweighed vessel which was then changed every two hours for a period of 6 to 8 hours. In both cases, a pronounced decrease in the absorption of cholesterol was observed. This decreased absorption was characterized by a reduction in the amount of total \([3H]\)cholesterol (free cholesterol + cholesteryl esters), as measured by mass and by radioactivity, secreted into lymph. A decline in the \([3H]\) cholesteryl
ester content of whole lymph, lymph chylomicrons, and lymph very low density lipoproteins (VLDL) was detected. The reduction of cholesterol absorption was greatest 2 to 3 hours after gastric administration of 58-035. However, under these experimental conditions, the effect of 58-035 was short-lived, and cholesterol absorption returned to the levels of control animals 5 to 6 hours after gastric administration of the drug. This decrease in cholesterol absorption was correlated with a 79% decline in the ACAT activity of jejunal microsomes. In addition, the authors reported that the presence of 57-118 was detected in the lipid extracts of jejunal microsomes isolated from treated animals, but did not provide the details. Lymphatic triacylglyceride secretion, the level of PCEH activity in isolated pancreas, and the level of cholesterol ester hydrolase activity in the cytosol of jejunal enterocytes remained unaffected by administration of 58-035.

In addition to these compounds, Lederle ACAT inhibitor 1 (N'-(2,4-difluorophenyl)-N-[4-(2,2-dimethylpropyl)phenyl]-methyl]-N-heptylurea) has been shown to be a potent noncompetitive inhibitor, with respect to oleoyl CoA, of the ACAT activity present in rabbit jejunal microsomes, in rat liver microsomes, in rat adrenal microsomes, and in cultures of monkey thoracic aorta smooth muscle cells. The authors reported that this inhibition of ACAT activity was specific, since other esterifying enzymes, such as monkey serum lecithin:cholesterol acyltransferase (LCAT), porcine PCEH, and the acyl CoA: retinol acyltransferase (ARAT) present in rabbit jejunal microsomes, were unaffected by addition of Lederle inhibitor 1 to the assay systems. Lederle ACAT inhibitor 1 was also reported to produce a dose-dependent reduction of [14C]cholesterol absorption when orally administered to rats fed a chow diet supplemented with 1% cholesterol-0.5% cholic acid (w/w) for a period of 2
The reduction in cholesterol absorption was characterized by a dose-dependent inhibition of the increases in liver and serum cholesterol concentration, and by an increase in the fecal excretion of neutral [14C]labeled sterol.

The combined results from the use of these three compounds indicated that intestinal ACAT played a major role in cholesterol absorption. However, one contrasting set of investigations showed no reduction in cholesterol absorption upon administration of either 58-035 or Lederle ACAT inhibitor 1 68. The experimental design differed from the previous experiments at several key points. In this study, 58-035 was administered to rats as a single gastric dose (20 mg/kg) 3 hours prior to the gastric infusion of [14C]cholesterol. Lymph was then collected for 6 hours, and the samples were subsequently pooled (0-6 hours) for analysis. No change in the [14C]cholesterol or [14C]cholesteryl ester content of the pooled lymph was observed. Yet, ACAT activity in mucosal homogenate was reduced by 45% (6 hours after gastric dose). The same authors reported that administration of Lederle ACAT inhibitor 1 to chow-fed rats (0.003% by weight for 2 weeks) had no effect on either ACAT activity in mucosal homogenates or absorption of [14C]cholesterol. The lack of inhibition of ACAT activity suggested that, under these conditions, Lederle ACAT inhibitor 1 was not absorbed by the enterocytes. Unfortunately, the intracellular levels of Lederle inhibitor 1 were not quantitated.

Investigations into the function of PCEH and ACAT in the absorption and esterification of dietary cholesterol have provided evidence to support a role for each of these enzymes. Several groups have reported experimental results which indicate that PCEH is taken up by villus enterocytes where it functions as a cytosolic cholesterol esterifying activity30,36,56-58,60. Lack of PCEH activity
has been reported to reduce the esterification and lymphatic absorption of exogenous cholesterol. Other groups have reported that intestinal ACAT activity is important to the esterification and absorption of exogenous cholesterol. Inhibition of ACAT activity has also been reported to lower the esterification and absorption of exogenous cholesterol. In an attempt to provide an explanation for conflicting results, two hypotheses have been proposed in which PCEH and ACAT function in concert.

One hypothesis proposes that PCEH serves to promote cholesterol uptake by enterocytes. In this model, dietary cholesterol is esterified by PCEH present in the lumen of the small intestine. The resulting cholesteryl esters then cross the brush border membrane where intracellular PCEH then hydrolyzes the cholesteryl esters to release free cholesterol. ACAT then acts to esterify the absorbed, dietary cholesterol. The resulting cholesteryl esters are then incorporated into chylomicrons, and secreted into lymph.

A second hypothesis proposes that, under conditions of low dietary cholesterol, intracellular PCEH functions as the primary esterifying activity. When the content of dietary cholesterol is high, ACAT serves as the principal esterifying enzyme. It is clear that much more work needs to be done in order to evaluate the relative importance of these two enzymes to the esterification and absorption of dietary cholesterol.

Factors influencing cholesterol absorption

The level of cholesterol absorption is affected by a wide variety of factors. These include the growth and differentiation of the small intestine, gastrointestinal motility, pancreatic secretions, bile, diet, and pharmaceutical agents. These factors can be divided into two groups, those which
affect nutrient absorption in general, and those which specifically affect cholesterol absorption. The effect(s) of any one these factors on either nutrient absorption in general or cholesterol absorption in particular is often difficult to determine, since there is a complex relationship between these factors, especially between the physiological variables.

**General influences**

The renewal of the enteric epithelium is a physiological parameter that has a major influence on nutrient absorption. The capacity of the small intestine to absorb nutrients directly depends upon the structural and functional integrity of the mucosa\(^\text{72}\). Epithelial renewal is extremely rapid, with a turnover rate on the order of 2-3 days in rat and 5-6 days in man\(^\text{72}\). Detailed cell cycle analyses have established the pattern of intestinal renewal. Within the mucosa, cell division and proliferation is confined to stem cells located in the crypts of Lieberkuhn\(^\text{31}\). The degree of cell differentiation and maturation increases with the relative distance from the stem cells\(^\text{31}\). Less mature enterocytes are found near the base of the villus, while more mature enterocytes are found close to the tips of the villus\(^\text{30}\). Several absorptive enzymes such as sucrase, maltase, alkaline phosphatase, and ACAT are reported to follow a gradient of activity along the villus/crypt axis of the mucosa that is correlated to the degree of enterocyte maturation\(^\text{45,62,63,72}\).

The ability of the renewal process to alter the level of nutrient absorption is exemplified by the adaptation of the gastrointestinal tract in response to partial enterectomy, surgical transection, irradiation, coeliac disease, infective enteritis, diabetes mellitus, and hyperthyroidism\(^\text{72}\). This adaptive response is characterized by increased villus height and crypt depth, increased mucosal mass, dilation and lengthening of the gut, shortening of the cell cycle,
accelerated cell production, and accelerated cell maturation. The functional result of the adaptive response is a compensation for loss of absorptive capacity.

The regulation of mucosal growth, differentiation, and adaptation is exceedingly complex and not well understood. There is evidence to indicate that the renewal of the enteric epithelium is subject to regulation by hormones, such as thyroxin and growth hormone, as well as by peptides released either from endocrine cells or neurons located within the walls of the gastrointestinal tract. Gut peptides such as gastrin, secretin, cholecystokinin (CCK), and enteroglucagon are reported to enhance epithelial cell renewal, and mediate the adaptive response. The developmental expression of the brush border enzymes of suckling rats are reported to be dependent upon glucocorticoids. In addition, the circadian cycle of cell proliferation is reported to both influence the effects of hormones like insulin, glucagon, somatostatin, and gastrin, and be influenced by hormones such as epidermal growth factor.

Gastrointestinal motility, pancreatic function, and biliary secretions are also factors which are critical to efficient nutrient absorption. It is known that there is a strong interaction between oral intake of food, the secretion of gastrointestinal peptides, and the stimulation of gastrointestinal motility, pancreatic secretions, and biliary secretions. Recent evidence indicates that each food constituent initiates a set of characteristic signals which results in specific physiological and biochemical responses. Differences in signaled responses are observed between large and small molecules, between dietary fat and dietary carbohydrates, and between short chain and long chain fatty acids. As an example, gastrointestinal motility is mediated by several intestinal peptides including, CCK, gastrin, glucagon, motilin, neurotensin, pancreatic...
polypeptide, secretin, somatostatin, and substance \textit{P} \textsuperscript{30}. Ingestion of dietary fat has been shown to both delay gastric emptying and promote the release of two of these peptides, neurotensin and pancreatic polypeptide \textsuperscript{30}. Evidence indicates that biliary secretions are stimulated by dietary long-chain triacylglycerides, while pancreatic secretions are increased by the intraduodenal infusion of sodium oleate \textsuperscript{30}.

\textit{Specific influences}

A wide variety of factors have been reported to specifically influence the level of cholesterol absorption. Many of these factors are dietary in their origin. For instance, addition of fibers, such as pectin, alfalfa, or cellulose, to the diet are reported to reduce the level of cholesterol absorption in rats \textsuperscript{30}. Soy protein, casein, and even calcium gluconate are reported to reduce the levels of cholesterol absorption \textsuperscript{30}. In addition, ginseng saponins and cabbage are reported to be effective in increasing cholesterol absorption in rodents \textsuperscript{30}.

Plant sterols and other sterol derivatives also affect the levels of cholesterol absorption. Chronic administration of sitosterol is reported to decrease the absorption of dietary cholesterol in rat, rabbit, mouse, and man \textsuperscript{30}. Administration of other sterols such as stigmasterol, sitostanol, and shellfish sterols are reported to lower cholesterol absorption in rodents \textsuperscript{30}.

Many of these sterols can serve as substrates for absorption in the intestine, although absorption of cholesterol is predominant. Several studies have reported the relative absorption ratios of two or more radiolabeled cholesterol analogs. In these experiments, the absorption of one sterol serves as an "internal standard" for the absorption of the other sterol. The ratios of the absorption of several sterol pairs have been reported \textsuperscript{30}. These studies indicate that absorption in intact animals is extremely sensitive to side chain alkylation.
and to the reduction of the Δ5 double bond. Child has assembled the reported absorption ratios of several sterol pairs and used this information to rank the rates of intestinal absorption of sterol analogs. Child suggests that the relative rates of absorption of several common sterols, in descending order, are as follows: cholesterol = vitamin D$_3$ > 7-dehydro cholesterol > cholestanol > campesterol > sitosterol = stigmasterol = ergosterol > sitostanol.

The precise manner by which plant sterols and other sterol derivatives lower the absorption of dietary cholesterol is largely unknown. There are several steps by which plant sterols could interfere with the selective absorption of cholesterol. These include competition for incorporation into bile salt micelles, entry into the brush border membrane, intracellular esterification, and assembly into chylomicrons. There is evidence to indicate that sitosterol decreases the maximum solubilization of cholesterol into micelles. However, most sterols do not solubilize into micelles as readily as cholesterol. It is unlikely that interference with the incorporation of cholesterol into bile salt micelles is the sole mechanism of action. There is evidence that sitosterol does not cross the interfacial barrier between aqueous and lipid phases as readily as cholesterol. This observation has led to the suggestion that plant sterols bind to the brush border membrane, and interfere with the transfer of cholesterol from the micelles to the enterocyte. This idea is supported by the observation that many plant sterols lower cholesterol absorption only under conditions in which micelles are saturated with plant sterols. Other studies have shown that there is no correlation between the ability of phytosterols to bind to the brush border membrane and the action of these compounds on cholesterol absorption.
Results from several studies indicate that plant sterols and other sterol derivatives block the intracellular esterification of absorbed, dietary cholesterol. Tavani and coworkers studied intestinal ACAT activity in jejunal microsomes, and showed that esterification of exogenous cholesterol (10μg) by ACAT was reduced 61% and 63% when sitosterol (10μg) or stigmasterol (10μg) were added to the assay system. In addition, these authors have examined the ability of ACAT to esterify plant sterols. Their results showed that sitosterol, stigmasterol, and 3-epicholesterol were esterified at 5% the rate of cholesterol, suggesting that the inability of phytosterols to serve as efficient substrates for ACAT was at least part of the reason for their low levels of intestinal absorption.

Since bile salts are important to normal lipid absorption, they also have a major influence on the absorption of dietary cholesterol. Recent work has shown that the level of cholesterol absorption is dependent upon the composition of bile. There is evidence to indicate that intestinal perfusion of deoxycholate into patients with gallstones reduces the absorption of cholesterol by 50%. Ursodeoxycholate reportedly reduces cholesterol uptake in mice by 50%, but does not decrease cholesterol absorption in humans or rats. Certain bile acids appear to be more important in the absorption of dietary sterol than others. Several studies have reported that addition of cholic acid or chenodeoxycholic acid to the standard diet of rat, mouse, or patients with gallstones, has no effect on cholesterol absorption.

There are also several other compounds which specifically affect cholesterol absorption. Long term feeding (3-4 weeks) of the detergent pluronic L-81 has been shown to reduce the absorption of cholesterol from a perfused emulsion. Although the initial uptake of cholesterol was not affected by pluronic L-81, the mucosal content of cholesterol was greatly increased. These
results suggest that pluronic L-81 inhibits the assembly or secretion of chylomicrons. The bile acid sequestering agents are also of interest, since they are used in the treatment of hypercholesterolemia\textsuperscript{78}. Cholestyramine and colestipol-hydrochloride consistently reduce the uptake and lymphatic delivery of dietary cholesterol\textsuperscript{30,78}. In patients on a low cholesterol diet, chronic administration of either cholestyramine (16-24 gm/day) or colestipol-hydrochloride (20-25 gm/day) results in a 20% decline in human blood cholesterol levels. The antibiotic, neomycin (1-2 gm/day), has also been shown to inhibit cholesterol absorption as well as reduce blood cholesterol by 20%\textsuperscript{78}. In addition to these compounds, there is some evidence to suggest that gossypol acetic acid reduces plasma cholesterol levels in monkeys by acting at the level of cholesterol absorption\textsuperscript{30,79}.

Although many agents have been shown to lower cholesterol absorption, few of these compounds effectively reduce blood cholesterol levels. Certainly, one way to lower cholesterol absorption and blood cholesterol is to follow a diet that is lower in cholesterol and fat\textsuperscript{1,78}. However, the response of blood cholesterol levels to changes in diet is variable\textsuperscript{1,78}. Some individuals show appreciable changes in plasma cholesterol and LDL-cholesterol when dietary intake of cholesterol is changed, while others show little or no response to changes in diet. This variability in response is due to the complex interaction of cholesterol metabolic pathways with genetic and dietary factors. For instance, patients with familial hypercholesterolemia usually obtain no more than a 10-15% reduction in blood cholesterol levels as a consequence of changes in dietary habits\textsuperscript{78}. In addition, reductions in the level of cholesterol absorption are reported to stimulate hepatic and intestinal cholesterol synthesis and increase the levels of hepatic and intestinal HMG-CoA reductase activities\textsuperscript{30,80}. 
As a consequence, the effect of large reductions in dietary cholesterol and subsequent cholesterol absorption may be offset by increased sterol synthesis.

As a consequence of the variable effect of dietary manipulation, the use of pharmaceutical agents to lower blood cholesterol levels is often required. Several of these drugs lower blood cholesterol levels by acting at the level of cholesterol absorption. For instance, administration of sitosterol (5-15 gm/day) has been reported to reduce blood cholesterol\(^7\). However, the use of sitosterol has also been reported to cause nausea and diarrhea\(^7\). The bile acid sequesterents are effective in reducing both cholesterol absorption and blood cholesterol levels. Yet, administration of cholestyramine or colestipol-hydrochloride causes abdominal pain, constipation, nausea, heartburn, bloating, and interference with the absorption of certain drugs, thyroid preparations, and fat soluble vitamins\(^7\). Neomycin also possesses severe side effects including abdominal pain, nausea, diarrhea, toxicity to the auditory nerve, and renal dysfunction\(^7\).

Hypocholesterolemic compounds capable of acting on other aspects of cholesterol metabolism have been developed. For example, mevinolin and compactin are competitive inhibitors of HMG-CoA reductase activity\(^1.7,81-83\). Nicotinic acid decreases synthesis and secretion of VLDL, and inhibits the mobilization of free fatty acids\(^7\). Fibrin acid derivatives such as clofibrate, gemfibrozol, bezafibrate, and fenofibrate have been reported to affect both cholesterol and triacylglyceride metabolism\(^30,7,84\). For instance, clofibrate is reported to increase lipoprotein lipase activity, increase the levels of HDL-C, and reduce hepatic synthesis of VLDL\(^7\). Fenofibrate is reported to inhibit cholesterol synthesis, lower HMG-CoA reductase activity in rat liver microsomes, increase aortic cholesterol esterase activity in rabbits, increase
hepatic uptake of cholesterol, increase the catabolism of LDL, increase LCAT activity, inhibit fatty acid synthesis in rat liver, inhibit triacylglyceride synthesis in rat liver, inhibit VLDL synthesis in rats, and increase muscle and adipose tissue lipoprotein lipase activity.

15-Ketosterol, a potent regulator of cholesterol metabolism

In recent years an oxygenated sterol, 15-ketosterol, has been shown to be a potent regulator of cholesterol metabolism which effectively produces a significant reduction in serum cholesterol levels upon oral administration to rats, mice, baboons, and rhesus monkeys. Studies of 15-ketosterol have shown that the compound affects cholesterol metabolism at several levels.

Experiments with cultured mammalian cells have demonstrated that 15-ketosterol inhibits sterol biosynthesis. This inhibition of sterol biosynthesis is associated with lowered activities of three early enzymes in the biosynthetic pathway, namely HMG-CoA reductase, cytosolic acetoacetyl thiolase, and HMG-CoA synthase. Oral administration of 15-ketosterol to rats (0.10% in chow diet for 10 days) also results in decreased levels of intestinal HMG-CoA reductase activity.

Experiments have shown that 15-ketosterol serves as an efficient precursor of cholesterol. This conversion to cholesterol is observed upon incubation of radiolabeled 15-ketosterol with rat liver homogenates, and after oral and intravenous administration of radiolabeled 15-ketosterol to rats and baboons.

Oral administration of 15-ketosterol alters the lipoprotein profiles of rhesus monkeys and baboons. In rhesus monkeys, LDL and the percentage of total cholesterol associated with these lipoprotein particles is
decreased. High density lipoprotein (HDL) and the percentage of total cholesterol associated with these lipoprotein particles is increased. At the same time, there is a shift in the HDL profile to one in which the HDL₂ species is predominate.

Dietary administration of 15-ketosterol to rats results in a marked inhibition of the absorption of exogenous cholesterol²⁷,²⁸. In one set of experiments, [4-¹⁴C]cholesterol was administered as an intragastric bolus to lymph-duct cannulated rats that had been maintained on a diet supplemented with 15-ketosterol (0.05% in a chow diet for 10 days). The results showed that oral administration of 15-ketosterol was associated with a 64 % decline in the absorption of [4-¹⁴C]cholesterol²⁸. In another set of experiments, the effect of dietary administration of 15-ketosterol (0.10% in a chow diet) on the fate of [4-¹⁴C]cholesterol and [2,4-³H]15-ketosterol was studied after intragastric administration of the labeled sterols to rats²⁷. In these studies, oral administration of 15-ketosterol was associated with decreased absorption of labeled cholesterol, as indicated by decreased levels of ¹⁴C in the various tissue and organs and increased levels of ¹⁴C in the feces of 15-ketosterol-treated rats. Studies of the tissue distribution of ³H label after oral administration of [2,4-³H]15-ketosterol to rats demonstrated both a rapid conversion of [2,4-³H]15-ketosterol to [³H]cholesterol and [³H]cholesterol esters and a decrease in the levels of ³H recovered in the tissues and organs of 15-ketosterol-treated rats²⁷. These latter results suggested a low absorption of 15-ketosterol and/or a more rapid clearance and biliary excretion of 15-ketosterol and its metabolites. Additional experiments with mesenteric lymph-duct cannulated rats have shown that after intragastric administration of [2,4-³H]15-ketosterol, substantial amounts of 15-ketosterol are rapidly absorbed into
lymph. Most of the $^3$H in lymph is associated with chylomicrons, and almost all the $^3$H in chylomicrons is in the form of 15-ketosteryl esters$^{92}$.

In order to more fully examine the role of esterification in the reduction of cholesterol absorption by 15-ketosterol, the effect of the compound on PCEH and jejunal ACAT activity has been examined. The effect of 15-ketosteryl oleate on the activities of partially purified porcine PCEH and highly purified rat PCEH has been studied using substrates in the form of mixed micelles of 15-ketosteryl [1-$^{14}$C]oleate or cholesteryl [1-$^{14}$C]oleate, taurocholate, and phosphatidylcholine$^{95}$. Under these assay conditions, 15-ketosteryl oleate is an efficient substrate for both porcine and rat PCEH, and is a weak competitive inhibitor of cholesteryl oleate hydrolysis. In other studies, incubation of 15-ketosterol with either rat jejunal or rat hepatic microsomes significantly lowers the levels of oleoyl CoA-dependent esterification of cholesterol by ACAT$^{96}$. This reduction in cholesterol esterification is accompanied by the formation of material with the chromatographic mobility of 15-ketosteryl oleate$^{96}$. The effect of 15-ketosterol on microsomal ACAT activity is of particular interest, since it indicates that administration of the compound affects the level of intracellular cholesterol esterification as well as the levels of cholesterol synthesis, cholesterol absorption, and lipoprotein transport.

In view of the relative importance of ACAT to both intracellular cholesterol metabolism and cholesterol absorption, the effect of 15-ketosterol on microsomal ACAT activity was more fully characterized. The following dissertation describes studies in which two animal models, the rat and the Rhesus monkey, were used to further examine the effect of 15-ketosterol on ACAT activity. These experiments fell into two classes. One set of studies was designed to investigate the effect of 15-ketosterol and related compounds on
the ACAT activity of jejunal microsomes. The other set of experiments was designed to examine the effect of oral administration of 15-ketosterol on the ACAT activity of hepatic and jejunal microsomes.
Chapter 2

Materials and Methods
Chemicals

All chemicals were of analytical reagent grade unless otherwise stated.

The following substances were obtained from Mallinckrodt, Inc., (St. Louis, MO or Paris, KY): potassium hydroxide, anhydrous potassium dihydrogen phosphate, anhydrous dipotassium hydrogen phosphate, sodium chloride, potassium chloride, sodium hydroxide, sodium carbonate, sucrose, sodium fluoride, acetic anhydride, sulfuric acid, glacial acetic acid, silicic acid (100 mesh powder), and cupric sulfate pentahydrate.

The following substances were obtained from Sigma Chemical Company (St. Louis, MO): Tris (HCl), ethylenediamine tetraacetic acid (EDTA; disodium salt, 99.5 % pure), Folin-Ciocalteu reagent (2.0 N phenol), sodium dodecyl sulfate (SDS), deoxycholate (DOC; sodium salt), dithiothreitol (DTT), bovine serum albumin-fatty acid deficient (BSA), oleic acid (95 % pure), oleoyl coenzyme A (95 % pure), cholesteryl oleate (95 % pure), Triton WR 1339 (tyloxapol), and dimethylsulfoxide (DMSO).

The following substance was obtained from United States Chemical Corporation (Cleveland, OH): cholesterol (U.S.P. grade). Dr. Alemka Kisic purified this compound by way of its dibromide derivative97. The purified cholesterol was determined by TLC to be > 99% pure.

The following substance was obtained from New-Chek-Prep (Elysion, NY): triolein (99 % pure).

The following substances were obtained from Fisher Scientific (Fair Lawn, NJ): sodium potassium tartrate tetrahydrate, Ottawa sand (standard, 20-30 mesh).

The following substance was obtained from MCB Manufacturing Chemists, Inc. (Cincinnati, OH): trichloroacetic acid (TCA).
The following substance was obtained from Clarkson Chemicals, Inc. (Williamsport, PA): Unisil (activated silicic acid, acid washed, 100-200 mesh).

The following substances were obtained from Petrach systems (Bristol, PA): bis(trimethylsilyl)trifluoracetamide (BSTFA), triacontane (C₃₀ alkane).

The following substance was obtained from Johns-Manville Products Corporation (Lompoc, CA): Hyflo Super-Cel (a celite diatomite filter aid).

The following substance was obtained from Beckman Instruments, Inc. (Fullerton, CA): 2,5-diphenyl-1,3-oxazole (PPO primary fluor).

The following substance was obtained from Applied Scientific Laboratories, Inc. (State Collage, PA): trimethylchlorosilane.

3β-Hydroxy-5α-cholest-8(14)-en-15-one (> 99 % pure), the oleate ester of 3β-hydroxy-5α-cholest-8(14)-en-15-one (>99 % pure), (25 R)-3β,26-
dihydroxy-5α-cholest-8(14)-en-15-one (> 99 % pure), 5α-cholest-8(14)-en-
3,15-dione (> 99 % pure), and 3α-hydroxy-5α-cholest-8(14)-en-15-one (> 99 %
pure) were prepared and purified as described previously⁸⁻⁹⁻¹⁰⁻¹⁰¹.

Gases

All gases were obtained from Matheson Gas Products (a division of Will Ross, Inc., Houston, TX).

Solvents

All solvents were of high pressure liquid chromatographic (HPLC) grade and were purchased from either Mallinckrodt, Inc. (St. Louis, MO or Paris, KY) or Burdick and Jackson (Muskigon, MI). Ether (U.S.P.) was used for anesthesia. Solvents were mixed in a volume to volume ratio unless otherwise stated. Solvents used for HPLC analyses were filtered through a 0.4 μm Nylon 66
membrane (Rainin Instrument Co., Inc, Emeryville, CA), and were degassed prior to use.

Radioactive compounds

[1-14C]Oleoyl coenzyme A was obtained from two sources, Amersham Radiochemicals (56 µCi/µmol; Arlington Heights, IL) and New England Nuclear (58.7 µCi/µMol; Boston, MA). The labeled oleoyl CoA was subsequently diluted to a specific activity of 11 dpm/pmol and a concentration of 0.50 mM (radioactivity: 96% to > 98%).

Sodium [1-14C]oleate was obtained from Amersham Radiochemicals (56 µCi/µmol, 97 % pure). The labeled sodium oleate was subsequently diluted to a specific activity of 11 dpm/mol and a concentration of 0.50 mM.

[1-14C]Cholesterol (55 µCi/µmol) was obtained from Amersham Radiochemicals, and was purified by Dr. Alemka Kisic using silicic acid column chromatography (> 99 % pure).

[3H]Cholesteryl oleate was either synthesized by Dr. Larry Miller from [7n-3H]cholesterol as described previously or was purchased from New England Nuclear ([cholesteryl 1,2,6,7-3H (N)]-oleate; 65.8 Ci/mmol)96. In either case, the [3H]cholesteryl oleate was diluted to a specific activity of 20 mCi/mmol, and was purified by silicic acid column chromatography (98 to > 99% pure).

[2,4-3H]3β-Hydroxy-5α-cholest-8(14)-en-15-one (> 99 % pure) was synthesized by Dr. Ker-Shi Wang according to previously described methods and had a specific activity of 91.1 mCi/mmol96.

[4-14C]3β,26-Dihydroxy-5α-cholest-8(14)-en-15-one (> 99 % pure) was prepared according to previously described methods and had a specific activity of 107 µCi/mg99,100.
Measurement of radioactivity

Radioactivity was measured in a Beckman LS 9000, Beckman LS 9800, or a Packard 4640 liquid scintillation counter. Aliquots of nonpolar samples, such as cholesteryl oleate, triacylglycerides, and 15-ketosteryl oleate, were assayed in scintillation fluid (10 ml) composed of PPO (0.4 %) in toluene. Radioactivity in more polar samples, such as the origins of TLC plates, were assayed using a scintillation fluid (10 ml) composed of PPO (0.4 %) in a mixture of 2:1 toluene-ethanol.

Silanization of glassware

The glassware used to prepare sterol stock solutions were rinsed successively with methanol and toluene, and then were filled with a solution of 5 % trimethylchlorosilane in toluene. After 20 minutes, the solution was removed, and the glassware were rinsed successively with toluene and methanol, and then allowed to dry.

Preparation of sterol solutions

In experiments which required the addition of oxygenated sterols to the ACAT assay system, stock solutions of the compounds were prepared using DMSO as the solvent. The compounds were weighed on an analytical balance, placed in a volumetric flask, and dissolved in sufficient DMSO to give the desired concentration. Dilutions of the these solutions were made to obtain the range of concentrations desired for an experiment. All sterol solutions were prepared on the day of the experiment.
Preparation of trimethylsilyl ether (TMS) derivatives of sterols

Sterol samples were dissolved in a 1:1 mixture of ethyl acetate and BSTFA. An aliquot of triacontane (C₃₀) standard (0.400 mg/ml in ethyl acetate) sufficient to give a final concentration of 20 ng/μl was added to each sample. The samples were capped, and the solutions were heated at 70 °C for 45 minutes. After cooling to room temperature, 1 μl aliquots of each sample were injected into the gas chromatograph.

Saponification conditions

The dried samples were dissolved in 10 % ethanolic KOH, flushed with nitrogen, capped, and heated for 50 minutes in an 80 °C water bath. One part water and one part hexane were added, and the samples were thoroughly mixed. The hexane layer was washed with 1/2 part water, and then evaporated to dryness under nitrogen. The saponified samples were stored at -20 °C until use.

Liebermann Burchard colorimetric assay for cholesterol and cholesteryl esters

The levels of cholesterol and cholesteryl palmitate in fractions obtained from silicic acid-Super Cel column chromatography were quantitated using a modification of the method of Abell et al.¹⁰² Duplicate aliquots (500 μl) of cholesterol samples were evaporated to dryness under nitrogen. Duplicate aliquots (500 μl) of cholesteryl palmitate samples were evaporated to dryness, and redissolved in toluene (100 μl). An aliquot (1.80 ml) of the Liebermann Burchard reagent was then added to every tube. Absorbances at 620 nm were recorded after 30 minutes using a Coleman Junior 2A model linear absorbance spectrophotometer.
The Liebermann Burchard reagent was prepared on the day of the assay. A mixture of acetic anhydride and concentrated sulfuric acid (20:1 v/v) was cooled on ice in a stoppered flask for 9 minutes. Glacial acetic acid (10 parts) was added to the mixture, and the reagent was allowed to warm to room temperature.

Ultraviolet spectrophotometric assay for 15-ketosterol and 15-ketosteryl esters

All ultraviolet assays were performed using matched quartz cuvettes with a one centimeter light path. Duplicate aliquots (100 μl) of 15-ketosterol or 15-ketosteryl palmitate were evaporated to dryness under nitrogen in 13 x 100 mm tubes. Isopropyl alcohol (spectral grade, 2.50 ml) was added to each sample, and the absorption was read at 259 nm.

Chromatography

A. Silicic acid column chromatography

[3H]Cholesteryl oleate was purified by silicic acid column chromatography. A glass column (1 cm x 50 cm) was plugged at the stopcock end with glasswool which was topped with sand (Ottawa standard, 20-30 mesh) to a depth of ~ 0.5 cm. The column was packed with a slurry of Unisil and toluene. Samples of [3H]cholesteryl oleate were dissolved in the eluting solvent (1 ml toluene), and applied to the column. The column was rinsed three times with toluene (1 ml), and the compound was eluted with toluene. Approximately 30-60 fractions were collected, 1 fraction every 2 minutes. Aliquots (50 μl) were taken for determination of radioactivity. The fractions which contained the peak of [3H] radioactivity were assayed for purity by using radio-TLC with either benzene or 9:1 hexane-ethyl acetate as the solvent system. The fractions which
contained the highest purity of [³H]cholesteryl oleate were pooled, and the concentration was adjusted to ~20,000 dpm/25 µl.

B. Silicic acid-Super Cel column chromatography

Silicic acid-Super Cel column chromatography was performed using modifications of the method of Clayton et al. Silicic acid and Hyflo Super-Cel were mixed in a 1:1 ratio (w/w) and were prepared as a slurry in toluene. The column was plugged at the stopcock end with glasswool which was topped with sand (Ottawa standard, 20-30 mesh) to a depth of ~ 0.5 cm. The slurry was poured into the column (1 x 100 cm) and packed under nitrogen pressure (5-10 p.s.i.). After addition of authentic standards of cholesterol, cholesteryl palmitate, 15-ketosterol, and 15-ketosteryl palmitate, the sample was applied to the column in a small volume of toluene (1.0 ml), followed by 3 rinses of equal volume. Fractions were collected every 32 minutes. Cholesteryl esters, 15-ketosteryl esters, and cholesterol were eluted from the column using toluene (fractions 1-140). After the elution of these compounds, the solvent was changed to 9:1 toluene-ether (fractions 141-265). After elution of 15-ketosterol, the solvent was changed to 2:1 chloroform-methanol (fractions 266-300) and finally to methanol (fractions 301-325). Aliquots (300 µl) were taken for determination of radioactivity. Toluene scintillation fluid was used for nonpolar samples, while toluene-ethanol scintillation fluid was used for fractions containing chloroform-methanol or methanol. Colorimetric assays of cholesterol and cholesteryl palmitate were carried out using the Liebermann-Burchard method as described. 15-Ketosterol and 15-ketosteryl palmitate were assayed by following their absorbance at 259 nm using spectral grade isopropyl alcohol as the solvent.
C. Thin-layer chromatography

Thin-layer chromatography (TLC) analyses were performed using precoated plates of silica gel G (0.25mm; Analtech, Inc., Newark, DE). Compounds on the plate were localized by exposure to iodine vapor or by heating the plate in an oven at 130 °C after spraying with a molybdic acid solution. This solution was prepared as follows. Ammonium molybdate (20 gm) was dissolved in concentrated sulfuric acid (25 ml) by heating. The solution was allowed to cool to room temperature and was diluted to 400 ml with water.

D. Thin-layer radiochromatographic analyses

Thin-layer radiochromatographic (radio-TLC) analyses were performed by measuring the radioactivity on plates of silica gel G. Nonradioactive chromatographic standards and radioactive samples were applied to separate lanes. The plates were developed in an appropriate solvent system. Small zones, 1 cm in width, were scraped quantitatively from the sample lane into counting vials. The appropriate scintillation fluid was added, and the radioactivity was measured as described below. The chromatographic standards were localized as previously described. The mobilities of the radioactive components were compared to those of authentic standards.

E. Gas-liquid chromatography

Quantitation of sterol samples by gas-liquid chromatography (GLC) was done in one of two ways. Samples from the initial rat feeding studies were quantitated using a Hewlett-Packard model 5730 A gas chromatograph equipped with a falling needle injector and a 0.1 μm DB-5 capillary column (0.3 mm x 15 m; J and W Scientific, Rancho Cordova, CA). TMS derivatives of the samples were injected into the instrument as 1 μl aliquots and were eluted using nitrogen as the carrier gas (30 ml/min) and a temperature gradient
programmed from 200 °C to 290 °C at a rate of 8 °C/min. The temperatures of the injection port and the flame ionization detector (FID) were 300 °C.

In subsequent studies, sterol samples were analyzed using a Perkin-Elmer Sigma 2000 gas chromatograph equipped with a 0.1 μm DB-5 capillary column (0.3 mm x 15 m). Samples were injected as 1 μl aliquots in a splitless fashion and were eluted using nitrogen as the carrier gas (40 ml/min) and a temperature gradient programmed from 150 °C to 280 °C at a rate of 7.5 °C/min. The temperature of the FID detector was maintained at 290 °C while the temperature of the injection port was programmed to rise from an initial value of 50 °C to 280 °C in ~ 10 sec.

All GLC determinations were made in duplicate using triacontane (C$_{30}$) as an internal standard. Quantitation of the samples was achieved using standard curves constructed for the TMS derivatives of the 15-ketosterol and of cholesterol.

F. High pressure liquid chromatography

High pressure liquid chromatography (HPLC) was performed on a Waters 510 instrument using a reverse phase 5 μm C$_{18}$ Microsorb column (4.6 mm x 25 mm; Rainin, Emeryville, CA) coupled to an RP 18 NewGuard cartridge (3.2 mm x 1.5 cm; BrownLee Labs Inc., Santa Clara, CA) and gradient elution with 2 solvent mixtures. Solvent mixture A was composed of isopropanol/methanol/water (5:4:1), and solvent mixture B was composed of isopropanol/methanol (4:1). Samples were dissolved in chloroform (100 μl), and were injected as 50 μl aliquots into the instrument. Initially a mixture of solvent A and solvent B (3:2) was pumped under isocratic conditions for 15 minutes. The mobile phase was then linearly programmed to a ratio of solvent A to solvent B of 1:3 for the second 15 minutes and then maintained at this ratio
thereafter. The flow rate was increased in a linear fashion for the first 5 minutes from 1.0 ml/min to 1.25 ml/min. In the next 10 minutes, the flow rate was increased in a linear fashion from 1.25 ml/min to 1.45 ml/min. Thereafter, the flow rate was maintained at 1.45 ml/min. The gradients and flow rates were programmed using either a Waters 680 automated gradient controller or a Waters 600 multisolvant delivery system (Waters Associates, Milford, MA). Fractions (77 drops/fraction) were collected. Absorbance was monitored at 210 mg and/or at 259 nm using either a Waters model 481 spectrophotometer or a Shimadzu U.V. spectrophotometric detector (Shimadzu Corporation, Kyoto, Japan).

Animals

Male Sprague-Dawley rats were purchased from Harlan Sprague-Dawley, Inc. (Houston, TX). The rats were housed two per cage in metal cages, and were maintained on a strict light/dark cycle (12 hours light). They were permitted a period of adaptation to their environment during which they were fed ground rat chow ad libitum for 4-5 days. In those experiments which used a controlled feeding protocol, an additional 2-3 weeks of adaptation were allotted in order to condition the rats to a controlled feeding schedule in which food was available only during the first 4 hours of the dark cycle. After the period of adaptation was completed, the rats were selected, matched, and sorted into dietary groups on the basis of body weight and serum cholesterol levels. The animals were then placed in individual cages and allowed an additional 1-2 days of adjustment prior to the initiation of the experiment. Animals used in studies of hepatic ACAT activity were housed in metabolic cages. In all other cases, the rats were housed in metal cages.
Rhesus monkeys (*Macaca mulatta*) were used in a collaborative study with members of the Arteriosclerosis Research Center, Bowman Gray School of Medicine of Wake Forest University, NC. This study involved 10 male monkeys and 8 female monkeys. The animals were placed in individual cages under controlled environmental conditions (24 °C; light-dark cycle, light 6:00 AM to 6:00 PM).

**Diet**

Purina Formulab Chow #5008 Diet was purchased from Ralston Purina Company (Checkerboard Square, St. Louis, MO) and was fed to all rats used in these studies. This diet is made from ground corn, soybean meal, fish meal, meat and bone meal, dried milk products, wheat germ meal, dried beet pulp, ground oat groats, wheat middlings, dehydrated alfalfa meal, animal fat, brewer's yeast, cane molasses, and vitamin and salt supplements. The diet was estimated to contain ~ 0.030 % cholesterol\(^{105}\). The approximate composition is listed as 23.5 % protein, 6.5 % fat, 49.4 % carbohydrates, and 6.8 % salts. 15-Ketosterol was incorporated into the ground diet by mixing an aliquot of the compound with a small quantity (~ 10 g) of basal diet using a ceramic mortar and pestle. This quantity was then diluted with ground chow to the desired concentration of 15-ketosterol and was thoroughly mixed by shaking in a sealed container for 1 hour.

All rhesus monkeys were maintained on an experimental diet containing 0.30 mg of cholesterol/kcal of diet (0.123% by weight). Highly purified cholesterol comprised 83.2% of the cholesterol in the diet. The remainder of the cholesterol was provided in the form of butter (6.5%), beef tallow (5.1%), lard (4.3%), and egg yolk replacement (0.8%). The diet was composed of casein,
lactalbumin, wheat flour, dextrin, sucrose, applesauce, lard, butter, beef tallow, egg yolk replacement mixture (22% casein, 50% lard, 16% soybean lecithin, 2.5% sucrose, 1.6% Hegsted's salt mixture, and water), safflower oil, vitamin mixture, Ausman-Hayes mineral mixture, vitamin D₃ in corn oil, sodium chloride, and cholesterol (table 2-1). This diet provided 43, 18, and 39% of calories from fat, protein, and carbohydrate respectively.

The animals were maintained on the high cholesterol diet for a period of at least 14 months. They were fed the diet at a level of 120 cal/kg body weight/day. The absolute amount of diet provided was adjusted frequently (at least once/month). Unless stated otherwise, half of the food was available for a period of 1 hour at 800 hours, and the other half was given at 1400 hours. The second meal was left with the animal overnight. However, the food was usually consumed within one hour.

Isolation of rat sera

Rat serum samples were collected at the beginning and end of all feeding studies. At the start of an experiment, blood (~0.5 ml) was taken from the tail vein of rats which had been lightly sedated with ether. Upon termination of a study, blood (~0.50 ml) was collected from the necks of decapitated rats. Sera was prepared by allowing the blood samples to clot at room temperature for 1 to 2 hours. The serum was separated from the coagulated cells by two different procedures. For the initial feeding studies, Sure-Sep Junior serum plasma separators (General Diagnostics, Morris Plains, NJ) were placed on the top of the sample tubes, and the tubes were centrifuged at 1050 x g for 20 minutes at 23 °C in a Dynac table-top centrifuge (Clay Adams, Parsippany, NJ). The serum was removed using a pasteur pipette. For studies in which a
Table 1: Composition of diet consumed by rhesus monkeys

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>gm/100 diet (gm)</th>
<th>Protein (gm)</th>
<th>Fat (gm)</th>
<th>Carbohydrate (gm)</th>
<th>Cal/100 gm of diet</th>
<th>Cholesterol (mg)</th>
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</thead>
<tbody>
<tr>
<td>Casein</td>
<td>8.0</td>
<td>8.0</td>
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<td>--</td>
<td>32.0</td>
<td>--</td>
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<td>8.0</td>
<td>--</td>
<td>--</td>
<td>32.0</td>
<td>--</td>
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<td>0.36</td>
<td>30.6</td>
<td>140.7</td>
<td>--</td>
</tr>
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<td>--</td>
<td>--</td>
<td>6.0</td>
<td>24.0</td>
<td>--</td>
</tr>
<tr>
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<td>--</td>
<td>--</td>
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<td>1.08</td>
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<td>--</td>
</tr>
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<td>--</td>
<td>6.0</td>
<td>--</td>
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<td>6.0</td>
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<td>--</td>
<td>63.0</td>
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Total  100.115 20.46 21.77 44.77 456.81 138.17

* The egg yolk replacement mixture consisted of casein (22%), lard (50%), soybean lecithin (16%), sucrose (2.5%), Hegsted's salt mixture (1.6%), and water (7.9%).
controlled feeding schedule was employed, serum was separated by centrifugation at 14,000 rpm for 2 minutes at 23 °C in an Eppendorf centrifuge 5415 (Brinkman Instruments, Inc., Westbury, NY) and was removed using a pasteur pipette.

**Analysis of serum cholesterol**

Serum cholesterol levels were assayed by a modification of the Cholesterol Auto-Test (CHOD-PAP Enzymatic Method, catalog # 148393; Bio-Dynamics; BMC Division, Boehringer Mannheim, Indianapolis, IN). The assay reagent was prepared by mixing together 250 ml of component 1 (buffer/4-aminophenzone solution), 8 ml of component 2 (cholesterol esterase/horseradish peroxidase solution), 4 ml of component 3 (cholesterol oxidase solution), and 5 ml of component 4 (phenol solution). The volume was adjusted to 1 liter by the addition of water. Duplicate aliquots (50 µl) were taken from each serum sample, and diluted with water to 0.20 ml. Assay reagent (1.8 ml) was added to each sample, and the solutions were incubated in a water bath at 37 °C for 30 minutes. Absorbance was recorded at 500 nm using a Beckman DB-GT grating spectrophotometer. Determinations of cholesterol concentrations were achieved using a standard curve.

**Preparation of hepatic microsomes**

Rat hepatic microsomes were prepared according to the method of Erickson and coworkers. The liver was excised, rinsed with ice cold physiological saline, patted dry, and weighed. All subsequent steps were performed at 4 °C. The liver was placed in a beaker with 5 x volume/weight preparation buffer (0.10 M Tris, 0.25 M sucrose, 1.0 mM EDTA, pH 7.4) and was
thoroughly minced. The mixture was homogenized in a Potter-Elvejham glass/teflon homogenizer (four strokes at moderate speed). The homogenate was centrifuged at 12,000 x g for 10 minutes (Dupont-Sorvall high speed centrifuge, SA 600 rotor, 10,700 rpm). The floating fatty layer was removed, and the supernatant was centrifuged at 105,000 x g for 1 hr (Beckman L265B or L550 ultracentrifuge, TI 42.1 rotor, 37,000 rpm). The resulting pellet was resuspended in 1/2 the original buffer volume and was centrifuged a second time at 105,000 x g for 1 hr. The second pellet was resuspended in the preparation buffer to a protein concentration of 10-13 mg/ml and was frozen at -70 °C until use. Protein determinations were done using the biuret protein assay as described below. 

*Preparation of intestinal microsomes*

Rat intestinal microsomes were prepared by a modification of the method of Suckling and coworkers. The small intestine was excised, and the intestinal contents were removed by thorough rinsing with ice-cold physiological saline. The organ was patted dry, weighed, and the proximal half of the intestine minus the first 5 cm was cut open longitudinally. This region of the small intestine corresponded to the rodent jejunum. All subsequent steps were performed at 4 °C. The epithelial cells were removed by scraping with a microscope slide. The isolated cell mass was disrupted by vortexing in 25 ml of preparation buffer (0.10 M sucrose, 0.050 M potassium phosphate, 0.050 M potassium chloride, 3.0 mM freshly added DTT, pH 7.2). The dispersed cells were washed three times by resuspension and centrifugation at 500 x g for 10 minutes (Dupont-Sorvall high speed centrifuge, SS 34 rotor). Each pellet of washed cells was resuspended in 10 ml of preparation buffer, treated with solid...
NaF to give a final concentration of 150 mM and then sonicated for 2 minutes at 4 °C (20 kHz, power setting 5.5; Model w375 sonicator, Heat Systems and Ultrasonics, Inc.). The resulting homogenate was centrifuged for 10 minutes at 700 x g. The supernatant from this step was decanted and centrifuged at 8500 x g for 10 minutes. The resulting supernatant was then centrifuged at 100,000 x g for 1 hour. The isolated microsomes were washed by resuspension in the preparation buffer followed by a second centrifugation step at 100,000 x g. The final microsomal pellet was resuspended in 1.0 ml of potassium phosphate buffer (0.10 M, pH 7.4) and frozen at -70 °C until use. The protein concentration was determined using the method of Peterson as described below.

In experiments in which a controlled feeding schedule was used, slight modifications of this protocol were introduced in order to shorten the preparation time. The following changes in the method were made. NaF (150 mM) was incorporated into the preparation buffer. The isolated mucosal cells were washed twice by resuspension and centrifugation at 1000 x g for 5 minutes. The sonicated homogenate was centrifuged for 10 minutes at 700 x g. The supernatant from this step was decanted and centrifuged at 8500 x g for 10 minutes. The resulting supernatant was then centrifuged at 100,000 x g for 12 minutes (TLA 100.2 rotor, 55,000 rpm, Beckman TL-100 ultracentrifuge). The unwashed microsomal pellet was resuspended in 500 μl of potassium phosphate buffer (0.10 M, pH 7.4) and frozen at -70 °C until use.

Rhesus monkey microsomes were prepared from the mid-jejunum of all monkeys. The jejunum was isolated from the animal upon necropsy. The organ was filled with saline and then clamped shut. It was then inverted three times, and then the saline solution was drained from the organ. Fresh saline
solution was poured into the jejunum, and the process was repeated. Three cycles of washing were done in this manner. After the organ had been drained, patted dry, and weighed, ~ 20 cm of mid-jejunum was isolated for preparation of microsomes.

The following modifications to the method of preparation were used. NaF (150 mM) was incorporated into the preparation buffer. The isolated mucosal cells were washed 3 times by resuspension in the preparation buffer (25 ml) and centrifugation at 1000 x g for 5 minutes. Each pellet of washed cells was divided into half, and each portion was resuspended in preparation buffer (15 ml). The cells were disrupted by sonicating for two 15 second bursts at 75 watts (s-75 probe; Branson Sonifiers, Inc.), and the homogenates from the two halves were pooled. The pooled homogenate was centrifuged for 10 minutes at 700 x g. The supernatant from this step was decanted and centrifuged at 8500 x g for 10 minutes. The supernatant from the 8500 x g spin was decanted and transported on ice to the Bowman Gray School of Medicine (Wake Forest University, NC). The microsomes were then pelleted by centrifugation at 100,000 x g for 20 minutes (37,000 rpm, Ty 70.1 rotor, Beckman L550 ultracentrifuge). The isolated microsomes were suspended in 10 ml of 0.10 M potassium phosphate buffer, pH 7.4, and frozen at -70 °C until shipment by Federal Express. The samples were shipped on dry ice to Houston and were promptly placed at -70 °C upon arrival.

Colorimetric assay of protein

The protein concentrations of hepatic microsomes were determined by the method of Gornall et al.\textsuperscript{106}. Duplicate aliquots of hepatic microsomes were diluted to 0.50 ml with water. Biuret reagent (2.0 ml) was added to each
sample, the solutions were mixed, and the absorbance at 550 nm was recorded after 30 minutes using a Beckman DB-GT grating spectrophotometer. Quantitation of the protein concentrations was achieved using a standard curve constructed from several concentrations of BSA.

Biuret reagent was prepared by dissolving cupric sulfate pentahydrate (1.5 gm) and sodium potassium tartrate tetrahydrate (6.0 gm) in 500 ml of glass distilled water (recently boiled). A 10% solution of sodium hydroxide (300 ml, freshly prepared, carbonate free) was added to this solution with constant swirling. The reagent solution was diluted to 1 liter with glass distilled water (recently boiled).

The protein concentrations of intestinal microsomes were determined by the method of Peterson107. Duplicate aliquots of the protein samples were diluted to 1 ml with water. DOC (0.15%,100 μl) was added, and the solutions were mixed. After 10 minutes, TCA (72%,100 μl) was added to the samples, and the precipitated protein was pelleted by centrifugation at 3000 x g for 15 minutes at room temperature (Beckman table top centrifuge). The resulting supernatant was removed, and the pellets were dissolved in 1.0 ml of water. An aliquot (1 ml) of reagent A (2.5% SDS, 0.2 N NaOH, 0.025% copper sulfate, 0.050% potassium tartrate, and 2.5% sodium carbonate; prepared on day of assay) was added to each sample, and the solutions were mixed. After 10 minutes, an aliquot (500 μl) of reagent B (Folin-Ciocalteu reagent; diluted 1/5 on day of assay) was added, and the solutions were mixed. After 30 minutes, absorbances were recorded at 750 nm using a Beckman DB-GT grating spectrophotometer. Quantitation of the protein concentrations was achieved using a standard curve constructed from several concentrations of BSA.
Assay of hepatic ACAT activity

Hepatic ACAT activity in liver microsomes was assayed using modifications of the conditions described by Erickson et al. This method is based upon the determination of oleoyl CoA-dependent esterification of microsomal cholesterol. The assay mixture consisted of rat liver microsomal protein (400 µg) in Tris (HCl) buffer (0.10 M, pH 7.5) containing sucrose (0.25 M), EDTA (1.0 mM), and BSA (1.0 mg/ml) in a final volume of 0.50 ml. The mixture was incubated in a shaking water bath at 37 °C for 6 minutes. The reaction was initiated by the addition of [1-14C]oleoyl CoA (11 dpm/pmol, 10 nmol) to give a final concentration of 20 µM. The assay was terminated after a 2 minute incubation at 37 °C by the addition of 2:1 chloroform-methanol (5 ml). An internal standard of [3H]cholesteryl oleate (10,000 dpm/25 µl toluene) was added followed by the addition of 2:1 chloroform-methanol (10 ml) and acidified water which had been saturated with chloroform (2.5 ml, pH 3). The solutions were thoroughly mixed and were stored overnight at 4 °C. The aqueous phase and the denatured protein at the interphase were removed by aspiration. The organic phase was evaporated to dryness at 40 °C under a stream of nitrogen. The residue was dissolved in chloroform (125 µl) and was spotted onto a silica gel G plate.

The TLC plates were developed in 9:1 hexane-ethyl acetate. This solvent system allowed the resolution of cholesteryl oleate from 15-ketosteryl oleate and triacylglycerides. Under these conditions, 15-ketosteryl oleate and triacylglycerides comigrated on the plates. The radioactive products were localized by radio-TLC and by use of a molybdate spray. The following Rf values were observed: oleic acid, 0.00; triacylglycerides/15-ketosteryl oleate,
0.43; cholesteryl oleate, 0.58. The components on the plates were subsequently scraped and analyzed for radioactivity.

All ACAT assays included the addition of control tubes which used microsomal protein that had been denatured with 2:1 chloroform-methanol prior to incubation. The control assays, designated zero time assays, provided the [\(^{14}\text{C}\)] background values for the non-enzymatic formation of \([^{14}\text{C}]\)oleic acid from \([^{14}\text{C}]\) oleoyl CoA. These background levels represented 10% or less of radioactivity with the chromatographic mobility of oleic acid. The background values were used in the calculation of enzymatic activities.

**Assay of intestinal ACAT activity**

Intestinal ACAT activity was assayed under incubation conditions similar to those described by Helgerud et al.\(^{53}\) and Norum et al.\(^{108}\). This method measured the oleoyl CoA-dependent esterification of microsomal cholesterol. The assay mixture consisted of either rat microsomal protein (20 µg) or Rhesus monkey microsomal protein (40 µg) in potassium phosphate buffer (0.10 M, pH 7.4) containing BSA (6.0 mg/ml) and DTT (1.0 mg/ml) in a final volume of 0.50 ml. The assay tubes were incubated in a shaking water bath (37 °C) for 6 minutes. In experiments which included the addition of exogenous sterol to the assay mixture, the incubation time was extended to 10 minutes. In either case, the reaction was initiated by the addition of [1-\(^{14}\text{C}\)]oleoyl CoA (11 dpm/pmol, 20 nmol) to give a final concentration of 40 µM. The assay was terminated after a 2 minute incubation at 37 °C by the addition of 2:1 chloroform-methanol (5 ml). Zero time assays were included as previously described. An internal standard of \([^{3}\text{H}]\)cholesteryl oleate (10,000 dpm/25 µl toluene) was added, followed by cholesteryl oleate (80 µg), 15-ketosteryl oleate (40 µg), and triolein (40 µg). The
unlabeled standards were dissolved in toluene and were added to the tubes as 10 μl aliquots. An additional 10 ml of chloroform-methanol and 2.5 ml of acidified water (pH 3) which had been saturated with chloroform were added. The solutions were thoroughly mixed and were stored overnight at 4 °C. The aqueous phase and the denatured protein at the interphase were removed by aspiration. The organic phase was evaporated to dryness at 40 °C under a stream of nitrogen. The residue was dissolved in chloroform (125 μl) and spotted onto a silica gel G plate.

The TLC plates were developed using either 9:1 hexane-ethyl acetate or benzene containing methanol (0 to 0.4%). The use of hexane-ethyl acetate permitted the resolution of bands of radioactivity comigrating with cholesteryl oleate, 15-ketosteryl oleate/triacylglycerides, and oleic acid. In addition to the resolution of radioactivity comigrating with cholesteryl oleate and oleic acid, the benzene system permitted the separation of 15-ketosteryl oleate from triacylglycerides. Methanol was added to benzene when the resolution between 15-ketosteryl oleate and triolein became poor. This tended to occur on cold, dry days. When the hexane-ethyl acetate solvent system was employed, the radioactive products were localized by radio-TLC and by use of a molybdate spray as previously described. When the TLC plates were developed in benzene, the components on the plates were localized by exposure to iodine, and the following Rf values were observed: oleic acid, 0.00; 15-ketosteryl oleate, 0.34; triacylglycerides, 0.43; cholesteryl oleate, 0.69. All components were subsequently analyzed for radioactivity as described below.

In selected cases, intestinal ACAT activity was assayed using modifications of the method of Billheimer. This method involves the addition of exogenous cholesterol to the assay mixture. The cholesterol substrate was
prepared by injecting a solution of cholesterol (0.3 mg in 30 μl acetone) into a hot (60 °C), rapidly vortexing solution of Triton WR-1339 (0.10 M potassium phosphate buffer, pH 7.4). The assay mixture consisted of rat microsomal protein (20 μg) in potassium phosphate buffer (0.10 M, pH 7.4) containing BSA (6.0 mg/ml), DTT (0.10 mg/ml), cholesterol (10.0 μg), and Triton WR-1339 (0.30 %) in a final volume of 0.50 ml. The assay tubes were incubated for 30 minutes in a shaking water bath at 37 °C. The reactions were initiated with the addition of [1-14C]oleoyl CoA (11 dpm/pmol, 20 nmol) to give a final concentration of 40 μM. The assay was terminated 10 minutes later with the addition of 2:1 chloroform-methanol (5 ml). Zero time assays were included as previously described. An internal standard of [3H]cholesterol oleate (20,000 dpm) was added, followed by the addition of cholesterol oleate (100 μg), 15-ketosterol oleate (100 μg), triolein (100 μg), 2:1 chloroform-methanol (10 ml), and 0.88 % KCl (2.5 ml). The unlabeled standards were added as previously described. The solutions were thoroughly mixed, and were stored overnight at 4 °C. The extracted lipids were subjected to TLC as previously described using benzene as the solvent system.

**Computer programs**

The following programs were used for the calculation and graphing of results: Excel (Microsoft Corporation, Redmond, WA), Jazz (Lotus Development Corporation, Cambridge, MA), Statview 512+(Brainpower Inc., Calabasa, CA), Graphit (Richard Light, Rice University), and Graphit Data Entry (William K. Wilson, Rice University).
Statistics

Data are presented as mean values ± standard errors of the mean (S.E.M.). Results were analyzed using either Student's t test, paired Student's t test, or a 2 factor analysis of variance (ANOVA). Data analysis was accomplished using the Statview 512 program.
Chapter 3

The Effect of 3β-Hydroxy-5α-cholest-8(14)-en-15-one on Rat Intestinal ACAT Activity in Vitro
Introduction

The effect of 15-ketosterol on microsomal ACAT activity was first examined using rat hepatic microsomes. The addition of 15-ketosterol to incubations of hepatic microsomes resulted in a reduction in the oleoyl CoA-dependent esterification of microsomal cholesterol. This decline in cholesterol esterification was accompanied by the formation of material with the chromatographic mobility of 15-ketosteryl oleate. These studies were subsequently expanded to include intestinal ACAT activity.

Analogous studies using rat intestinal microsomes had obtained similar results. Addition of 15-ketosterol to incubations of jejunal microsomes resulted in a decrease in the oleoyl CoA-dependent esterification of microsomal cholesterol which was accompanied by the formation of material with the chromatographic mobility of 15-ketosteryl oleate.

These initial investigations into the effect of 15-ketosterol on cholesterol esterification in rat intestinal microsomes were done using slight modifications of the method of Suckling and coworkers. This method assayed the [1-14C] oleoyl CoA-dependent esterification of microsomal cholesterol in the following manner. The modified procedure used an assay mixture which consisted of rat microsomal protein (250 μg) in potassium phosphate buffer (0.10 M, pH 7.4) containing BSA (1.0 mg/ml) and DTT (1.0 mg/ml) in a final volume of 0.50 ml. The assay tubes were incubated in a shaking water bath (37 °C) for 6 minutes. The reactions were initiated by the addition of [1-14C]oleoyl CoA (11 dpm/pmol, 10 nmol) to give a final concentration of 20 μM. The assays were terminated after a 2 minute incubation at 37 °C by the addition of 2:1 chloroform-methanol (5 ml). The [14C] labeled lipid products were extracted and were analyzed by normal phase TLC using hexane-ethyl acetate (9:1) as described in chapter 2.
Since the assay protocol used microsomes, which contain several oleoyl CoA-dependent enzymatic activities, other radiolabeled products besides cholesteryl oleate were generated. This solvent system allowed for the separation and quantitation of bands of labeled components comigrating with cholesteryl oleate, 15-ketosteryl oleate/triacylglycerides, and oleic acid.

This modified protocol differed from the method of Suckling and coworkers in the following ways. In the assay buffer, DTT (1.0 mg/ml) was substituted for glutathione (1.0 mg/ml). A lower microsomal protein concentration was used to assay enzyme activity (125 µg instead of 250 µg). Finally, the enzymatic reactions were initiated with 10 nmol of radiolabeled oleoyl CoA (11 dpm/pmol) instead of 20 nmol [1-14C]oleoyl CoA (5.5 dpm/pmol).

Although the modified assay protocol was sufficient for the preliminary investigations into the effect of 15-ketosterol on intestinal ACAT activity, the method was not adequate for subsequent studies of enzyme activity. The methodology, upon which the modified assay was based, was designed to estimate cholesterol esterification in microsomes. As a consequence, the rates of cholesterol esterification with respect to parameters such as protein concentration were not reported. It was determined that under the modified assay conditions, cholesterol esterification and microsomal protein concentration were inversely related over the range of 50-150 µg protein/assay (figure 3). This relationship was probably the result of the fact that there were several oleoyl CoA-dependent enzymatic activities competing for the substrate. As the amount of protein in the assay increased, the consumption of oleoyl CoA also rose (figure 4), and ACAT could not efficiently compete for the limited substrate supply.
Figure 3: Formation of $[^{14}\text{C}]$lipid products with the chromatographic mobilities of cholesteryl oleate, triacylglycerides, and oleic acid upon incubation of rat jejunal microsomes with radiolabeled oleoyl CoA plotted as a function of microsomal protein concentration. Panel A = cholesteryl oleate. Panel B = triacylglycerides. Panel C = oleic acid. Enzymatic reactions were initiated with 10 nmol $[^{14}\text{C}]$ oleoyl CoA and were terminated after a 2 minute incubation at 37 °C. Error bars represent the S.E.M. of replicate assays (N = 4).
Figure 4: Percentage of $[^{14}\text{C}]$oleoyl CoA converted to radiolabeled lipid products during incubation with rat jejunal microsomes plotted as a function of microsomal protein concentration. Total consumption of this substrate was calculated as the sum of the $[^{14}\text{C}]$ radioactivity (dpm) incorporated into cholesteryl oleate, triacylglycerides, and oleic acid divided by the amount of $[1^{14}\text{C}]$oleoyl CoA (dpm) added to the assay mixture. These values were then expressed as percentages. Enzymatic reactions were initiated with 10 nmol $[1^{14}\text{C}]$oleoyl CoA (11dpm/pmol) and were terminated after a 2 min. incubation at 37 °C. Error bars represent the S.E.M. of N = 4 replicates.
Therefore, the amount of cholesterol esterification declined as the protein concentration in the assay rose.

This problem in substrate supply was further compounded by the fact that the protocol did not saturate the system with respect to oleoyl CoA. This assay system, like many of the earlier methods, showed a biphasic substrate course for oleoyl CoA\(^{37-39}\). Cholesterol esterification increased with the concentration of oleoyl CoA up to a certain point. After this peak of activity, cholesterol esterification rapidly declined as the concentration of oleoyl CoA rose. This has been interpreted in terms of the amphipathic nature of oleoyl CoA\(^{37-39}\). As the level of substrate in the assay reached critical micelle concentration, oleoyl CoA formed detergent micelles, and solubilized the microsomal membranes.

Solubilization of ACAT resulted in a loss of activity\(^{37-39}\).

Addition of BSA to the assay system was previously shown to ameliorate the inhibition of ACAT by high concentrations of oleoyl CoA\(^{37-39}\). BSA acted by binding to free oleoyl CoA and effectively raising the critical micelle concentration. Those assay systems which were reported to have classical saturation kinetics (apparent K\(_m\): 4-25 µM) used relatively high BSA/oleoyl CoA ratios (> 0.15 mg BSA/nmol oleoyl CoA\(^{37-39,44,45,61}\). Those assay systems which were reported to have a biphasic substrate course used relatively low BSA/oleoyl CoA ratios (< 0.067 mg BSA/nmol oleoyl CoA\(^{37-39,43,63}\). In view of this information, the intestinal ACAT assay conditions were extensively investigated. This chapter summarizes these studies. This work included the optimization of the intestinal ACAT assay conditions and the use of this new assay system to evaluate the effect of 15-ketosterol on \textit{in vitro} ACAT activity.
Optimization of Rat Jejunal ACAT Assay Conditions

The first parameter that was examined was the BSA/oleoyl CoA ratio. In this experiment, microsomal protein (20 μg) was added to tubes containing phosphate buffer (0.10 M, pH 7.4), DTT (1.0 mg/ml), and varying amounts of BSA (0, 0.250, 0.50, 1.00, 2.00, and 3.00 mg) in a final assay volume of 0.50 ml. The tubes were incubated for 6 minutes at 37 °C, and the reactions were initiated by the addition of 10 nmol [1-14C]oleoyl CoA (11 dpm/pmol). The reactions were terminated 2 minutes later with the addition of 2:1 chloroform-methanol (5 ml), and radiolabeled lipid products with the chromatographic mobilities of cholesteryl oleate, triacylglycerides, and oleic acid were analyzed as described earlier. The assays were done using replicates of four. Zero time control assays were included as previously described (Chapter 2).

The results from this experiment are presented in figure 5. Cholesterol esterification was calculated in terms of specific activity (pmol cholesteryl oleate formed/min/mg protein), and the results were plotted as a function of the BSA/oleoyl CoA ratio (figure 5, panel A). The results showed that cholesteryl oleate synthesis was maximized between 0.05 and 0.15 mg BSA/nmol oleoyl CoA. Triacylglyceride synthesis and oleic acid formation were calculated in terms of total dpm/assay, and the results were analyzed in the same manner (figure 5, panels B and C). Both triacylglyceride synthesis and oleic acid formation were inhibited as the BSA/oleoyl CoA ratio increased. These two enzymatic processes were significantly decreased at ratios greater than 0.050 and 0.10 mg BSA/nmol oleoyl CoA respectively.

In addition, the total consumption of substrate was monitored in order to determine which assay conditions saturated the microsomal enzymes with respect to oleoyl CoA. Substrate consumption was calculated as the sum of the
Figure 5: Formation of radiolabeled lipid products with the chromatographic mobilities of cholesteryl oleate, triacylglycerides, and oleic acid upon incubation of rat jejunal microsomes with [1-\(^{14}\)C]oleoyl CoA plotted as a function of the ratio of BSA to oleoyl CoA in the ACAT assay mixture. Panel A = cholesteryl oleate. Panel B = triacylglycerides. Panel C = oleic acid. The enzymatic reactions were initiated with 10 nmol of [1-\(^{14}\)C]oleoyl CoA and were terminated after a 2 minute incubation at 37 °C. Microsomal protein concentration was 20 µg/assay. Error bars represent the S.E.M. of replicate assays (N = 4).
$^{14}$C radioactivity (dpm) incorporated into cholesteryl oleate, triacylglycerides, and oleic acid divided by the amount of [1-$^{14}$C]oleoyl CoA (dpm) added to the assay mixture. These values were expressed as the percentage of oleoyl CoA consumed and were plotted as a function of the BSA/oleoyl CoA ratio (figure 6). Utilization of oleoyl CoA declined as the ratio increased. Consumption of oleoyl CoA was lowest when the ratio was 0.3 mg/nmol.

In view of these results, the BSA/oleoyl CoA ratio was then set to 0.15 mg/nmol. This ratio was chosen since it maximized cholesteryl oleate formation while it lowered triacylglyceride synthesis, oleic acid formation, and substrate consumption.

The concentration of [1-$^{14}$C]oleoyl CoA added to the assay was the next parameter examined. Microsomal protein (20 µg) was added to an assay buffer containing BSA, DTT (1.0 mg/ml) and potassium phosphate (0.10 M, pH 7.4). The tubes (n =4 replicates) were incubated for 6 minutes at 37 °C, and the reactions were initiated with the addition of varying amounts of [1-$^{14}$C]oleoyl CoA (11 dpm/pmol) as described (Chapter 2). The BSA/oleoyl CoA ratio was held constant at 0.15 mg/nmol. Zero time control assays were included as previously described. The reactions were terminated 2 minutes later, and radioactive products with the chromatographic mobilities of cholesteryl oleate, triacylglycerides, and oleic acid were analyzed as previously described (figure 7). Under these conditions, cholesterol esterification showed saturation kinetics, and reached a maximum at 20 µM oleoyl CoA. Triacylglyceride synthesis reached a maximum at 5 µM oleoyl CoA. Oleic acid formation increased with the concentration of oleoyl CoA but did not reach saturation. Total consumption of oleoyl CoA was calculated as previously described. The results were plotted as a function of substrate concentration (figure 8).
Figure 6: Percentage of $[^{14}\text{C}]$oleoyl CoA converted to radiolabeled lipid products plotted as a function of the ratio of BSA to oleoyl CoA in the ACAT assay mixture. Total consumption of oleoyl CoA was calculated as described. Enzymatic reactions were terminated after a 2 minute incubation at 37 °C. The microsomal protein concentration was 20 µg/assay. Error bars represent the S.E.M. of replicate assays (N = 4).
Figure 7: Formation of $[^{14}C]$ lipid products with the chromatographic mobilities of cholesteryl oleate, triacylglycerides, and oleic acid plotted as a function of the concentration of radiolabeled oleoyl CoA present in the ACAT assay mixture. Panel A= cholesteryl oleate. Panel B= triacylglycerides. Panel C= oleic acid. Enzymatic reactions were initiated with varying amounts of $[^{14}C]$ oleoyl CoA and were terminated after a 2 minute incubation at 37 °C. The BSA/oleoyl CoA ratio was held constant at 0.15 mg/nmol, and the microsomal protein concentration was 20 µg/assay. Error bars represent the S.E.M. of replicate assays (N = 4).
The results showed that consumption of substrate reached a minimum at 40 μM and stabilized at ~ 3%.

Based upon these results, all subsequent enzyme assays were initiated with 20 nmol oleoyl CoA (40 μM final concentration). This concentration saturated the system with respect to cholesteryl oleate and triacylglyceride synthesis, while providing for moderate levels of oleic acid formation.

Time was the next parameter investigated. In this experiment, microsomal protein (15 μg) was added to an assay buffer containing BSA (6.0 mg/ml), DTT (1.0 mg/ml), and phosphate buffer (0.10 M, pH 7.4) in a final volume of 0.50 ml. The tubes (n = 4 replicates) were incubated for 6 minutes at 37°C, and the reactions were initiated with the addition of 20 nmol of [1-14C] oleoyl CoA (11 dpm/pmol). The reactions were terminated at various times, and radioactive products with the chromatographic mobilities of cholesteryl oleate, triacylglycerides, and oleic acid were analyzed as previously described (figures 9,10). Zero time control assays were included as previously described (Chapter 2). Under these conditions, cholesterol esterification and consumption of oleoyl CoA were linear up to 4 minutes (R²=0.96), while triacylglyceride synthesis and oleic acid formation were linear up to 8 minutes (R²=0.95 and 0.96 respectively). In order to remain within the linear regions of these curves, the assay time was fixed at 2 minutes.

The level of microsomal protein in the assay was then optimized. Varying amounts of microsomal protein were added to an assay buffer consisting of BSA (6.0 mg/ml), DTT (1.0 mg/ml), and potassium phosphate (0.10 M, pH 7.4). The tubes (n = 4 replicates) were incubated for 6 minutes at 37°C, and the reactions were initiated with the addition of 20 nmol of [1-14C]oleoyl CoA (11 dpm/pmol). The reactions were terminated after 2 minutes, and
Figure 8: Percentage of $[1^{-14}C]$oleoyl CoA converted to radiolabeled lipid products upon incubation with jejunal microsomes plotted as a function of the concentration of $[1^{-14}C]$oleoyl CoA in the ACAT assay mixture. Total consumption of substrate was calculated as previously described. The enzymatic reactions were initiated with varying amounts of $[1^{-14}C]$oleoyl CoA (11 dpm/pmol) and were terminated after a 2 minute incubation at 37 °C. The BSA/oleoyl CoA ratio was 0.15 mg/nmol, and the microsomal protein concentration was 20 µg/assay. Error bars represent the S.E.M. replicate assays ($N = 4$).
Figure 9: Formation of $[^{14}\text{C}]$ lipid products with the chromatographic mobilities of cholesteryl oleate, triacylglycerides, and oleic acid upon incubation of rat jejunal microsomes with radiolabeled oleoyl CoA plotted as a function of incubation time. Panel A = cholesteryl oleate. Panel B = triacylglycerides. Panel C = oleic acid. Enzymatic reactions were initiated with 20 nmol of $[^{14}\text{C}]$oleoyl CoA and were incubated at 37 °C for varying lengths of time. The BSA/oleoyl CoA ratio was 0.15 mg/nmol, and the microsomal protein concentration was 20 μg/assay. The error bars represent the S.E.M. of replicate assays (N = 4).
Figure 10: Percentage conversion of [1-14C]oleoyl CoA to radiolabeled lipid products upon incubation with intestinal microsomes plotted as a function of incubation time. Total consumption of this substrate was calculated as previously described. Enzymatic reactions were initiated with the addition of 20 nmol [1-14C]oleoyl CoA (11 dpm/pmol) and were incubated at 37 °C for varying lengths of time. The BSA/oleoyl CoA ratio was 0.15 mg/nmol, and the protein concentration was 20 μg/assay. The error bars represent the S.E.M. of replicate assays (N = 4).
radioactive products with the chromatographic mobilities of cholesteryl oleate, triacylglycerides, and oleic acid were analyzed as previously described (figures 11,12). Zero time control assays were included as described (Chapter 2). Cholesteryl oleate synthesis was linear with protein up to 40 µg ($R^2=0.90$), while triacylglyceride synthesis and oleic acid formation were linear with respect to protein up to 100 µg ($R^2=0.96$ and 0.83, respectively). Consumption of oleoyl CoA was calculated as previously described and was plotted as a function of protein concentration. The results showed that consumption of substrate was linear with respect to protein up to 60 µg/assay ($R^2=0.97$). In order to remain in the linear regions of these curves, microsomal protein concentration was fixed to 20 µg/assay.

In summary, the assay conditions for rat jejunal ACAT activity were redesigned, and the improved intestinal ACAT assay protocol was as follows. Rat microsomal protein (20 µg) was added to an assay mixture consisting of BSA (6.0 mg/ml), DTT (1.0 mg/ml), and potassium phosphate buffer (0.10 M, pH 7.4) in a final volume of 0.50 ml. The assays were initiated with 20 nmol of [1-14C]oleoyl CoA (11 dpm/pmol), and the reactions were terminated after 2 minutes with the addition of 2:1 chloroform-methanol (5 ml). Under these optimized assay conditions, the activity of rat jejunal ACAT was measured as ~900 pmol cholesteryl oleate formed/min/mg protein. This level of enzyme activity was much higher than the ~73 pmol cholesteryl oleate formed/min/mg protein obtained under the old assay conditions. With respect to protein concentration, BSA concentration, and oleoyl CoA concentration, the optimized assay conditions closely resembled the rat jejunal ACAT assay conditions described by Helgerud et al.53 and Norum et al.108. Under their assay conditions, Helgerud and coworkers reported significant variation in ACAT
activity between preparations of microsomes\textsuperscript{108}. This biological variation was also observed and was most distinct between preparations of microsomes isolated from individual rats.
Figure 11: Formation of radiolabeled lipid products with chromatographic mobilities of cholesteryl oleate, triacylglycerides, and oleic acid upon incubation of rat jejunal microsomes with [1-14C]oleoyl CoA plotted as a function of the concentration of microsomal protein in the assay mixture. Panel A = cholesteryl oleate. Panel B = triacylglycerides. Panel C = oleic acid. The enzymatic reactions were initiated with 20 nmol [1-14C]oleoyl CoA and were terminated after a 2 minute incubation at 37 °C. The BSA/oleoyl CoA ratio was 0.15 mg/nmol. Error bars represent the S.E.M. of replicate assays (N = 4).
Figure 12: Percentage of $[^{14}\text{C}]$oleoyl CoA converted to lipid products upon incubation with jejunal microsomes plotted as a function of the amount of microsomal protein present in the ACAT assay mixture. Total consumption of this substrate was calculated as previously described. Enzymatic reactions were initiated with 20 nmol of $[^{14}\text{C}]$oleoyl CoA (11 dpm/pmol) and were terminated after a 2 minute incubation at $37\, ^\circ\text{C}$. The BSA/oleoyl CoA ratio was 0.15 mg/nmol. Error bars represent the S.E.M. replicate assays ($N = 4$).
Comparison of Substrate Specificity: Oleoyl CoA vs Oleic Acid

There are two cholesterol esterifying activities which have been reported to exist in mammalian intestines. The first is ACAT, a fatty acyl CoA-dependent microsomal enzyme\textsuperscript{37-39}. The second is pancreatic carboxyl ester hydrolase, a bile acid activated, fatty acid-dependent enzyme which is part of the exocrine secretions of the pancreas\textsuperscript{36}.

The improved assay protocol was designed to measure ACAT activity as expressed by the [1-\textsuperscript{14}C]oleoyl CoA-dependent esterification of microsomal cholesterol. However, this assay system generated [1-\textsuperscript{14}C]oleic acid as one of the lipid products. There was concern that this oleic acid could have been used to synthesize [\textsuperscript{14}C]cholesteryl oleate, and thus have interfered with the measurement of ACAT activity.

In order to address this question, the revised assay system was used to examine the ability of oleoyl CoA and oleic acid to esterify cholesterol. In this experiment, microsomal protein (20 \textmu g) was added to the assay buffer, and the mixture was incubated for 6 minutes at 37 °C. In one set of 4 replicate tubes, the assays were initiated with 20 nmol of [1-\textsuperscript{14}C]oleoyl CoA (11 dpm/pmol), and in another set of replicate tubes, the assays were initiated with 20 nmol [1-\textsuperscript{14}C]oleic acid (11 dpm/pmol). All reactions were terminated after a 2 minute incubation at 37 °C with the addition of 2:1 chloroform-methanol, and the radiolabeled lipid products were analyzed as previously described.

Under these assay conditions, there was essentially no incorporation of [1-\textsuperscript{14}C]oleic acid into cholesteryl oleate. Addition of [1-\textsuperscript{14}C]oleoyl CoA to incubations of rat jejunal microsomes resulted in 489 ± 38 pmol cholesteryl oleate formed/min/mg protein, while addition of [1-\textsuperscript{14}C]oleic acid to intestinal microsomes resulted in 21 ± 5 pmol cholesteryl oleate formed/min/mg protein.
The Effect of 15-Ketosterol upon Addition to Incubations of Rat Jejunal Microsomes

Once the rat intestinal ACAT assay system had been optimized, the effect of 15-ketosterol on ACAT activity was re-examined. In this experiment, microsomal protein (20 μg) was added to assay mixtures containing BSA (6.0 mg/ml), DTT (1.0 mg/ml), various concentrations of 15-ketosterol, and potassium phosphate buffer (0.10 M, pH 7.4) in a final volume of 0.50 ml. 15-Ketosterol was added to the assay system as 5 μl aliquots of DMSO solutions. There were 3 replicate tubes per concentration of 15-ketosterol. The assay tubes were incubated in a shaking water bath (37 °C) for 10 minutes. The assays were initiated with the addition of 20 nmol [1-14C]oleoyl CoA (11 dpm/pmol), and the reactions were terminated after a 2 minute incubation at 37 °C. Zero time assays and incubations with and without 5 μl DMSO were included as controls. The [1-14C] lipid products were extracted, and subsequently analyzed by normal phase TLC using benzene as the eluting solvent. This system permitted the separation and quantification of bands of labeled components with the chromatographic mobilities of cholesteryl oleate, 15-ketosteryl oleate, triacylglycerides, and oleic acid. Cholesterol esterification and 15-ketosteryl oleate formation were calculated in terms of specific activity (pmol/min/mg protein), while triacylglyceride synthesis and oleic acid formation were calculated in terms of total dpm/assay. The results were then plotted as a function of 15-ketosterol concentration (figure 13).

Addition of 15-ketosterol to incubations of intestinal microsomes resulted in a concentration-dependent inhibition of oleoyl CoA-dependent cholesterol esterification. A 50% reduction in the levels of cholesterol esterification was obtained upon addition of 3 μM 15-ketosterol to the assay system. This
Figure 13: Formation of radiolabeled lipid products with the chromatographic mobilities of cholesteryl oleate, 15-ketosteryl oleate, triacylglycerides, and oleic acid upon incubation of rat jejunal microsomes with 15-ketosterol and [1-14C]oleoyl CoA. Panel A=cholesteryl oleate (closed circles) and 15-ketosteryl oleate (open circles). Panel B= triacylglycerides. Panel C= oleic acid. Enzymatic reactions were initiated with 20 nmol [1-14C]oleoyl CoA and were terminated after a 2 minute incubation at 37°C. Error bars represent the S.E.M. of triplicate assays.
reduction in the esterification of microsomal cholesterol was accompanied by
the formation of [1-14C]labeled material which migrated with authentic
standards of 15-ketosteryl oleate (figure 13, panel A). The formation of
radiolabeled material with the chromatographic mobilities of either
triacylglycerides or oleic acid was not significantly affected by the addition of 15-
ketosterol to the assay system (figure 13, panels B and C).

The esterification of 15-ketosterol was also examined using a second
type of experiment. In this case, [2,4-3H]15-ketosterol was added to incubations
of intestinal microsomes, and the enzymatic reactions were initiated with
unlabeled oleoyl CoA. The formation of [2,4-3H]15-ketosteryl oleate was then
followed by normal phase TLC. The experimental protocol was as follows.
Microsomal protein (20 μg) was added to assay mixtures containing BSA (6.0
mg/ml), DTT (1.0 mg/ml), various concentrations of [2,4-3H]15-ketosterol, and
potassium phosphate buffer (0.10 M, pH 7.4) in a final volume of 0.50 ml. [2,4-
3H]15-Ketosterol was added to the assay system as 5 μl aliquots of DMSO
solutions. There were 3 replicate tubes per concentration of [2,4-3H]15-
ketosterol. The assay tubes were incubated for 10 minutes at 37 °C. The
assays were initiated with the addition of 20 nmol oleoyl CoA (0.50 mM), and
the reactions were terminated after a 2 minute incubation at 37 °C. Zero time
assays and incubations with and without 5 μl DMSO were included as controls.
The [2,4-3H]lipid products were extracted, and subsequently analyzed by
normal phase TLC using benzene as the eluting solvent. This system allowed
for the separation and quantification of bands of labeled components with the
chromatographic mobilities of cholesteryl oleate, 15-ketosteryl oleate,
triacylglycerides, and oleic acid. Under these conditions, [2,4-3H]15-ketosterol
comigrated with unlabeled oleic acid. [2,4-3H]15-Ketosteryl oleate synthesis
was calculated in terms of specific activity (pmol/min/mg protein) and these results were plotted as a function of [2,4-\(^3\)H] 15-ketosterol concentration (figure 14).

Addition of [2,4-\(^3\)H] 15-ketosterol to the intestinal ACAT assay system resulted in the formation of radiolabeled material which comigrated with authentic standards of 15-ketosteryl oleate. The amount of radioactivity migrating as 15-ketosteryl oleate rose as the concentration of [2,4-\(^3\)H] 15-ketosterol in the assay increased. No other bands of [\(^3\)H] radioactivity besides that of [2,4-\(^3\)H] 15-ketosterol and [\(^3\)H] 15-ketosteryl oleate were detected.

In order to further establish that 15-ketosterol was a substrate for intestinal ACAT, the [\(^3\)H] labeled lipid products of microsomes incubated with [2,4-\(^3\)H] 15-ketosterol were analyzed by reverse phase HPLC. This chromatographic method allowed for the separation and analysis of free cholesterol, free 15-ketosterol, the linolenate, arachidonate, linoleate, elaidate, oleate, and stearate esters of 15-ketosterol, triacylglycerides, and the linolenate, arachidonate, linoleate, oleate, and stearate esters of cholesterol.

In this experiment, microsomal protein (80 \(\mu\)g) was added to assay mixtures which contained [2,4-\(^3\)H] 15-ketosterol (20.0 \(\mu\)M final concentration, 11 dpm/pmol) in addition to the usual ingredients. The final assay volume was 2.0 ml. The tubes were incubated for 10 minutes at 37 °C prior to initiation of the reactions with 80 nmol of unlabeled oleoyl CoA. The reactions were terminated after a 4 minute incubation at 37 °C as previously described (Chapter 2). These assays were designed to generate the amount of labeled mass equivalent to 4 replicate assays. Zero time incubations were included as a control. The [\(^3\)H] labeled lipid products were extracted as described (Chapter 2). The samples
Figure 14: Formation of $[^3\text{H}]$ material with the chromatographic mobility of 15-ketosterol oleate. Rat jejunal microsomes were incubated with $[2,4-^{3}\text{H}]$15-ketosterol. The enzymatic reactions were initiated with the addition of 20 nmol oleoyl CoA and were terminated after a 2 minute incubation at 37 °C. Error bars represent the S.E.M. of triplicate assays.
were then subjected to reverse phase HPLC (Chapter 2). The fractions from these chromatographic analyses were assayed for radioactivity.

The chromatograms are presented in figure 15. The results showed that addition of [2,4-3H]15-ketosterol to incubations of rat jejunal microsomes produced [3H]labeled material which eluted with authentic standards of 15-ketosteryl oleate.

In summary, the optimized intestinal ACAT assay system was used to re-evaluate the effect of 15-ketosterol on ACAT activity. Addition of 15-ketosterol to the assay system resulted in an inhibition of oleoyl CoA-dependent esterification of microsomal cholesterol. This reduction in cholesterol esterification was specific since neither oleoyl CoA-dependent triacylglyceride synthesis nor oleoyl CoA-dependent oleic acid formation were significantly affected by 15-ketosterol. Furthermore, the decline in cholesterol esterification was accompanied by the formation of material which comigrated with authentic standards of 15-ketosteryl oleate. The synthesis of 15-ketosterol oleate was also examined by incubating intestinal microsomes with [2,4-3H]15-ketosterol, and unlabeled oleoyl CoA. Only material migrating as [2,4-3H]15-ketosterol and [2,4-3H]15-ketosteryl oleate were detected (normal phase TLC and reverse phase HPLC). The results from these combined experiments showed that 15-ketosterol was an alternate substrate for rat intestinal ACAT. Furthermore, this compound was capable of inhibiting cholesterol esterification by this enzyme.
Figure 15: Reverse phase HPLC of products from ACAT assay incubations. Jejunal microsomes were incubated with 20 μM [2,4-3H]15-ketosterol as described in text. Panel A = zero time control incubations in which exogenous 15-ketosterol was added to denatured microsomal protein. Panel B = addition of exogenous 15-ketosterol to active microsomes. Peak 1 = retention time of free 15-ketosterol. Peak 2 = retention time of 15-ketosteryl oleate.
Chapter 4

The Effect of 3\(\beta\)-Hydroxy-5\(\alpha\)-cholest-8(14)-en-15-one on Rat Intestinal ACAT Activity \textit{In Vivo}
Introduction

The experiments described in the previous chapter were performed in order to determine the effect of direct addition of 15-ketosterol on ACAT activity in jejunal microsomes. The following experiments were designed to investigate the effect of oral administration of 15-ketosterol on microsomal ACAT activity. Male Sprague-Dawley rats were used in a series of feeding studies which correlated changes in microsomal ACAT activity with the effects of the compound on other variables such as serum cholesterol levels, food consumption, body weight, and intestinal weight. The experiments were also designed to correlate the changes in these variables to dosage of 15-ketosterol and to duration of administration of the compound.

Preliminary Studies of the Effect of Dietary 15-Ketosterol on Hepatic and Intestinal ACAT Activity in Rats

The results obtained from previous studies demonstrated that direct addition of 15-ketosterol to incubations of either rat hepatic microsomes or rat jejunal microsomes resulted in a decline in the levels of microsomal cholesterol esterified by ACAT\textsuperscript{96}. This reduction in cholesterol esterification was accompanied by the formation of material with the chromatographic mobility of 15-ketosteryl oleate\textsuperscript{96}. In view of these results, investigations into the effects of oral administration of 15-ketosterol on rat microsomal ACAT activity were launched. These experiments were designed to meet two main goals: 1) to determine the effect of oral administration of 15-ketosterol on ACAT activity in hepatic and jejunal microsomes; 2) to confirm previous work regarding the compound's effect on food consumption, weight gain, and serum cholesterol.
The following experiments used the same general protocol. Male Sprague-Dawley rats were maintained on a light-dark cycle (6:00 AM to 6:00 PM, light) and fed the basal diet (Purina Formulab 5008) for 1 week prior to the initiation of an experiment. Experimental (N=8), pair-fed control (N=8), and ad libitum rats (N=8) were selected from a pool of 36 animals and were matched for body weight and serum cholesterol levels. These selected animals (150-170 gm) were placed in individual cages and were given 1 day of adjustment prior to the initiation of the study. The experimental (treated) rats had free access to 30 gm of ground basal diet containing 15-ketosterol. Pair-fed controls were fed the basal chow, but only in the amount the matched experimental counterpart consumed the previous day. On the first day of the study, the pair-fed animals were provided with 30 gm of the basal diet. Ad libitum rats were provided with 30 gm of the basal diet each day. Food consumption and body weight were recorded between 2:00 and 3:00 PM each day for a period of 7 days. At the end of this time, the rats were killed by decapitation (7:30-9:30 AM), and microsomes were prepared from the liver and jejunum of the individual rats.

Hepatic ACAT activity and intestinal ACAT activity were assayed as previously described (Chapter 2). These assay procedures involved the addition of [1-14C]oleoyl CoA to incubations of microsomal protein, and thus depended upon endogenous cholesterol for the generation of radiolabeled cholesteryl oleate. The [14C]labeled lipid products were separated by TLC using hexane-ethyl acetate (9:1). Radiolabeled material with the chromatographic mobility of cholesteryl oleate was quantitated as previously described (chapter 2). ACAT activity was calculated in terms of specific activity (pmol cholesteryl oleate formed/min/mg protein).
Food consumption and body weight

Previous work had shown that oral administration of 15-ketosterol significantly altered food consumption and weight gain in rats. In order to account for the effects of these alterations on ACAT activity, the food consumption and weight gain of experimental and control rats were closely monitored.

The effect of oral administration of 15-ketosterol on the food consumption of rats was studied at concentrations of 0.10% and 0.05% 15-ketosterol in the basal diet. The average daily food consumption of treated, pair-fed, and ad libitum animals was subsequently plotted (figure 16), and the results were analyzed by paired t test. (table 2).

Oral administration of 0.10% 15-ketosterol resulted in a large reduction in the food consumption of treated animals (figure 16, panel A). A significant decrease in the mean food consumption of treated animals was first observed on the second day of the study (treated vs pair fed: 38%, p<0.001; treated vs ad libitum: 54%, p<0.001). The reduction in the food consumption of treated animals continued for the remainder of the experiment (treated vs ad libitum, table 2). With the exception of day 2, no significant changes between the food consumption of treated rats and their pair-fed controls were observed (table 2).

The effect of oral administration of 15-ketosterol on food consumption was sharply reduced when the concentration of the compound was lowered to 0.05% 15-ketosterol in the diet (figure 16, panel B). A significant decrease in the food consumption of treated animals was observed on day 3 (14.7%, treated vs ad libitum, 0.02<p<0.05), day 5 (11.1%, treated vs ad libitum, 0.01<p<0.02), and day 6 (8.6%, treated vs ad libitum, 0.01<p<0.02). No significant changes
Figure 16: Average daily food consumption of experimental (closed circles), pair-fed (open circles), and ad libitum (open triangles) rats. The panels correspond to the concentration of 15-ketosterol fed to the rats. Panel A = 0.10% 15-ketosterol-fed rats and their corresponding controls. Panel B = 0.050% 15-ketosterol-fed rats and their respective controls. Error bars represent the S.E.M. of N=8 rats/group.
Table 2: Analysis of the effect of oral administration of 15-ketosterol (treated) on food consumption (paired t test)

<table>
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<tr>
<th>Comparison</th>
<th>Day 1</th>
<th>Day 2</th>
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<th>Day 4</th>
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* ad lib = ad libitum  
* ns = not significant  
* N = 8 rats/group

Table 3: Analysis of the effect of oral administration of 15-ketosterol on weight gain (paired t test)

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* ad lib = ad libitum  
* ns = not significant  
* N = 8 rats/group
between the food consumption of treated animals and pair-fed controls were observed (table 2).

The effect of oral administration of 15-ketosterol on weight gain was also studied at concentrations of 0.10% and 0.05% 15-ketosterol in the diet. The average daily changes in the body weights of treated, pair-fed, and ad libitum rats were plotted (figure 17), and the results were analyzed by paired t test (table 3).

Administration of 0.10% 15-ketosterol suppressed weight gain in treated rats (figure 17, panel A). At the onset of the experiment, treated, pair-fed, and ad libitum rats weighed 165 ± 1.2 gm, 167 ± 3 gm, and 167 ± 2 gm respectively. After 7 days, these same groups weighed 159 ± 3.2 gm, 158 ± 4 gm, and 210 ± 4 gm respectively. The declines in the weight gain of treated and pair-fed animals were first observed on the second day of the experiment (treated vs ad libitum, p<0.001; pair-fed vs ad libitum, p<0.001). The suppression in the weight gain of both treated and pair-fed rats continued for the duration of the study (treated vs ad libitum and pair-fed vs ad libitum, table 3). No significant differences between the weight gain of treated and pair-fed rats were observed.

These experimental results were subsequently expanded to examine the effect of oral administration of 0.05% 15-ketosterol on weight gain in rats (figure 17, panel B). The results showed that administration of 0.05% 15-ketosterol at this concentration had no effect on the body weights of treated rats. At the onset of the experiment, treated, pair-fed, and ad libitum rats weighed 169 ± 4 gm, 168 ± 3 gm, and 167 ± 2 gm, respectively. After 7 days, these same groups weighed 191 ± 4 gm, 194 ± 3 gm, and 200 ± 3 gm, respectively. No significant changes in weight gain between treated, pair-fed, and ad libitum rats were observed (table 3).
Figure 17: Average daily change in body weight of experimental (closed circles), pair-fed (open circles), and ad libitum (open triangles) rats. The panels correspond to the concentration of 15-ketosterol present in the diet. Panel A = 0.10% 15-ketosterol-fed rats and their corresponding controls. Panel B = 0.050% 15-ketosterol-fed rats and their respective controls. Error bars represent the S.E.M. of N = 8 rats/group.
The combined results regarding the effect of oral administration of 15-ketosterol on food consumption and weight gain in rats were in excellent agreement with results obtained from previous studies\textsuperscript{21,28}. In order to further examine the reproducibility of these observations, the data from several feeding studies were analyzed by ANOVA\textsuperscript{110}. The effects of administration of 0.10% 15-ketosterol were analyzed using data obtained in the described experiment plus 5 other analogous feeding studies. In a similar fashion, the effects of administration of 0.05% 15-ketosterol were analyzed using data taken from the described experiment, plus 2 other analogous feeding studies. In all cases, food consumption was calculated in terms of the total amount of food consumed by an individual rat during an experiment. These values were then used in the analysis of food consumption. Body weight was analyzed using the individual body weights measured on day 7.

The results obtained from these statistical analyses showed that there were significant changes in food consumption and body weight upon oral administration of 15-ketosterol. The decline in total food consumption upon administration of 0.10% 15-ketosterol was significant (mean squares and population variance of treated vs mean squares and population variance of \textit{ad libitum}, $p<0.0001$). In a similar fashion, the reduction in body weight upon administration of 0.10% 15-ketosterol was significant (mean squares and population variance of treated vs mean squares and population variance of \textit{ad libitum}, $p=0.0001$). The effect of 15-ketosterol administration on total food consumption was sharply reduced when the dosage was reduced to 0.05%. However, the smaller changes in the total food consumption of treated rats were still significant (mean squares and population variance of treated vs mean squares and population variance of \textit{ad libitum}, $p=0.008$). Administration of
0.05% 15-ketosterol had no significant effects on body weight. In addition, these analyses showed that pair-fed rats closely matched their treated counterparts with respect to total food consumption and body weight, since no significant differences between these two groups of animals were observed.

**Serum cholesterol level**

The effect of oral administration of 15-ketosterol on serum cholesterol levels was studied at dosages of 0.10% and 0.05% 15-ketosterol (figure 18). After 7 days on a chow diet containing 0.10% 15-ketosterol, the average serum cholesterol concentration of treated rats was lowered 52.8% with respect to pair-fed control animals (panel A: paired t test, p<0.001), and 61.2% relative to *ad libitum* controls (panel A: paired t test, p<0.001). Under these conditions, the average serum cholesterol levels of pair-fed rats were reduced 17.9% relative to *ad libitum* controls (panel A: paired t test, p<0.001). The hypocholesterolemic effect was greatly reduced when the dosage was lowered to 0.05% 15-ketosterol. At this dosage, the average serum cholesterol levels of treated animals were lowered 9.4% with respect to pair-fed controls (panel B: paired t test, p<0.001), and 21.4% relative to *ad libitum* controls (panel B: paired t test, p<0.001). Under these experimental conditions, the average serum cholesterol levels of pair-fed rats were lowered 14.2% relative to *ad libitum* controls (panel B: paired t test, p<0.001). These combined results were in agreement with previous work.

The reproducibility of the effect of oral administration of 15-ketosterol on serum cholesterol levels in rats was examined by ANOVA using data from three studies which examined the effect of oral administration of 0.10% 15-ketosterol on serum cholesterol levels. The results from these studies had demonstrated a 40.0%, 56.7%, and 52.8% decrease in the serum cholesterol levels of treated
Figure 18: Average serum cholesterol levels of experimental (filled columns), pair-fed (open columns), and ad libitum (hatched columns) rats. Panel A = 0.10% 15-ketosterol-fed rats and their controls. Panel B = 0.050% 15-ketosterol-fed rats and their corresponding controls. All rats were maintained on their respective diets for a period of 7 days. Error bars represent the S.E.M. of N = 8 rats/group.
rats relative to pair-fed controls. These studies also showed that the serum cholesterol levels of treated animals relative to ad libitum controls were reduced by 44.1%, 66.3%, and 61.2%. The serum cholesterol levels of pair-fed rats were reduced by 6.9%, 22.1%, and 17.9%, with respect to ad libitum animals. Upon statistical analysis by ANOVA, these combined results were found to be significant (mean squares and population variance of treated vs mean squares and population variance of pair-fed, p=0.0001; mean squares and population variance of treated vs mean squares and population variance of ad libitum, p=0.0001; mean squares and population variance of pair-fed vs mean squares and population variance of ad libitum, p=0.0002).

**Hepatic ACAT activity**

The effect of oral administration of 0.10% 15-ketosterol on the levels of ACAT activity in rat liver microsomes was examined in 3 separate feeding studies. Food consumption was monitored throughout each study in order to confirm both the decline in food consumption upon administration of the compound and the effectiveness of pair-feeding in matching the food consumption of treated rats (figure 19). The results from experiments 1, 2, and 3 demonstrated that administration of 0.10% 15-ketosterol lowered food consumption in treated rats relative to ad libitum controls. After 7 days of treatment, the food consumption of treated rats from experiments 1, 2, and 3 were lowered 23% (p<0.001), 44% (p<0.001), and 47% (p<0.001) respectively (paired t test: day 7, treated vs ad libitum). With the exception of the first day of the second experiment, no significant differences between the food consumption of treated rats and their pair-fed controls were observed (paired t test: treated vs pair-fed, 0.02<p<0.05). Thus, the results confirmed the
Figure 19: Average daily food consumption of 0.10% 15-ketosterol-treated (closed circles), pair-fed (open circles), and ad libitum (open triangles) rats. The panels represent the data collected from 3 replicate experiments. Panel A = experiment 1, panel B = experiment 2, panel C = experiment 3. Error bars represent the S.E.M. of N = 8 rats/group.
effectiveness of pair-feeding in matching the food consumption of treated animals.

Upon termination of an experiment, liver microsomes were isolated from each rat, and the levels of hepatic ACAT activity were subsequently assayed (table 4). The results were analyzed by paired t test. In the first experiment, administration of 0.10% 15-ketosterol did not significantly affect the oleoyl CoA-dependent esterification of microsomal cholesterol relative to either pair-fed or ad libitum controls. No significant differences in the levels of cholesterol esterification between pair-fed and ad libitum animals were detected. The results from the second experiment also showed that administration of 0.10% 15-ketosterol did not significantly alter the levels of oleoyl CoA-dependent cholesterol esterification relative to either pair-fed or ad libitum controls. In addition, no significant differences in the level of cholesterol esterification between pair-fed and ad libitum rats were observed. However, a third experiment showed that administration of 0.10% 15-ketosterol significantly lowered the levels of oleoyl CoA-dependent cholesterol esterification by 26% (treated vs pair fed, 0.01<p<0.02). No significant changes in the levels of cholesterol esterification were observed between treated and ad libitum rats, or between pair-fed and ad libitum animals.

Although the results from two experiments had found no significant differences in the levels of microsomal ACAT activity between treated rats and pair-fed controls, the absolute values for the microsomal ACAT activity of treated rats were lower than their respective pair-fed controls in all 3 cases. In order to further examine the significance of this decline, the data from these three studies were analyzed by ANOVA. The results showed that the observed decline in cholesterol esterification by microsomal ACAT upon oral
Table 4: The effect of dietary 15-ketosterol (0.10% in a chow diet for 7 days) on ACAT activity in rat hepatic microsomes

<table>
<thead>
<tr>
<th>Dietary group</th>
<th>[4-14C]cholesteryl oleate (pmol/min/mg ± S.E.M.*)</th>
<th>Comparison</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exp. 1-experimental</td>
<td>346 ± 60</td>
<td>treated vs pair-fed</td>
<td>0.50&lt;p&lt;0.10</td>
</tr>
<tr>
<td>pair-fed</td>
<td>390 ± 40</td>
<td>treated vs ad libitum</td>
<td>0.40&lt;p&lt;0.50</td>
</tr>
<tr>
<td>ad libitum</td>
<td>289 ± 35</td>
<td>pair fed vs ad libitum</td>
<td>0.50&lt;p&lt;0.10</td>
</tr>
<tr>
<td>Exp. 2-experimental</td>
<td>628 ± 72</td>
<td>treated vs pair-fed</td>
<td>0.05&lt;p&lt;0.10</td>
</tr>
<tr>
<td>pair-fed</td>
<td>935 ± 110</td>
<td>treated vs ad libitum</td>
<td>0.05&lt;p&lt;0.10</td>
</tr>
<tr>
<td>ad libitum</td>
<td>879 ± 124</td>
<td>pair-fed vs ad libitum</td>
<td>0.70&lt;p&lt;0.80</td>
</tr>
<tr>
<td>Exp. 3-experimental</td>
<td>762 ± 67</td>
<td>treated vs pair-fed</td>
<td>0.01&lt;p&lt;0.02</td>
</tr>
<tr>
<td>pair-fed</td>
<td>1031 ± 70</td>
<td>treated vs ad libitum</td>
<td>0.60&lt;p&lt;0.70</td>
</tr>
<tr>
<td>ad libitum</td>
<td>803 ± 59</td>
<td>pair-fed vs ad libitum</td>
<td>0.02&lt;p&lt;0.05</td>
</tr>
</tbody>
</table>

* S.E.M. of n = 8 rats per group
administration of 0.10% 15-ketosterol was significant (mean squares and population variance of treated vs mean squares and population variance of pair-fed, \( p=0.0012 \)). No significant changes in the levels of cholesterol esterification were detected between treated and ad libitum rats or between pair-fed and ad libitum animals.

**Intestinal ACAT activity**

The effect of oral administration of 15-ketosterol on ACAT activity in rat jejunal microsomes was also studied at dosages of 0.10% and 0.05% 15-ketosterol. Food consumption was closely monitored in order to confirm the effectiveness of pair-feeding in matching the food consumption of treated rats (figure 16). These results have been previously discussed (see food consumption and weight body weight). In general, no significant differences between the food consumption of treated and pair-fed rats were observed. Upon termination of an experiment, jejunal microsomes were isolated from each rat, and the levels of intestinal ACAT activity were subsequently assayed (figure 20). The results were analyzed by paired t test.

Oral administration of 0.10% 15-ketosterol resulted in a 77% reduction in the levels of microsomal cholesterol esterified by jejunal ACAT (figure 20 panel A: treated vs pair-fed, \( p<0.001 \)). After 7 days on their respective diets, the rates of oleoyl CoA-dependent cholesterol esterification in microsomes from treated and pair-fed were 167 ± 40 pmol/min/mg and 741 ± 40 pmol/min/mg respectively.

Administration of 0.05% 15-ketosterol resulted in a 82% decline in the levels of microsomal cholesterol esterified by jejunal ACAT (figure 20 panel B: treated vs pair-fed, 0.01<\( p<0.02 \)). After 7 days on their respective diets, the rates of oleoyl CoA-dependent cholesterol esterification in microsomes from treated and pair-fed rats were 52 ± 18 pmol/min/mg and 285 ± 118 pmol/min/mg respectively.
Figure 20: Average cholesterol esterification in jejunal microsomes of experimental (filled columns) and pair-fed (open columns) rats. Panel A = 0.10% 15-ketosterol-fed rats and their controls. Panel B = 0.050% 15-ketosterol-fed rats and their respective controls. All rats were maintained on their respective diets for a period of 7 days. Error bars represent the S.E.M. of N = 8 rats/group.
respectively. The combined results from these two studies indicated that the ACAT activity of jejunal microsomes was strongly reduced by oral administration of the 15-ketosterol.

Since the 15-ketosterol has been previously shown to lower the activity of intestinal HMG-CoA reductase in the small intestine, there was concern that the observed effect on intestinal ACAT activity might be a reflection of a decreased concentration of cholesterol in the microsomal membranes. In order to address this question, the levels of free cholesterol and cholesteryl esters in rat jejunal microsomes were investigated. In addition, the level of free 15-ketosterol in jejunal microsomes isolated from treated rats was determined.

The levels of these compounds were assayed in the following manner. Microsomal samples (~ 0.75 ml) from 0.10% 15-ketosterol fed rats (N = 8) and pair-fed controls (N = 8) were obtained. \([^{3}H]\) Cholesteryl oleate (2 x 10^4 dpm, 0.4 \(\mu\)g) and \([4^{-14}C]\) cholesterol (4 x 10^4 dpm, 0.1 ng) were added to each microsomal suspension as internal standards. The samples were extracted according to Folch et al. The resulting lipids were dissolved in toluene (1 ml), applied to silica gel mini-columns (Spice cartridge mini-columns; 15 x 15 mm; Analtech, Newark, De), and eluted with 9:1 toluene-ether (4.5 ml). The eluted samples were subjected to reverse phase HPLC for the separation of cholesterol, 15-ketosterol, and cholesteryl esters. Free cholesterol, and free 15-ketosterol were quantitated in the form of their TMS derivatives, by capillary gas liquid chromatography (GLC). The cholesteryl esters were saponified using 10% ethanolic KOH. The resulting free cholesterol was then quantitated in the form of its TMS derivative by GLC.

These GLC analyses were made using a model 5730A Hewlett-Packard unit equipped with a 0.1 \(\mu\)m capillary DB-5 column (0.3 mm x 15 m, J.
and W. Scientific, Rancho Cordova, CA) using temperature programming from 200 °C to 290 °C at a rate of 8 °C/min. Sample injection was accomplished using a falling needle injector. All GLC determinations were made in duplicate using n-triacontane (C30) as an internal standard. Quantitation of the samples was achieved using standard curves constructed for TMS derivatives of 15-ketosterol and cholesterol.

The results from these analyses are presented in table 5. No changes in the levels of microsomal cholesterol or cholesteryl esters were detected (paired t test: treated vs pair fed; cholesterol-0.40<p<0.50, cholesterol esters-0.40<p<0.50). The level of the 15-ketosterol in microsomes isolated from experimental animals was low (0.43 ± 0.10 μg/mg protein or ~0.6% that of cholesterol).

Summary

Preliminary studies on the effect of dietary 15-ketosterol on food consumption, body weight, serum cholesterol, hepatic ACAT activity, and jejunal ACAT activity were done. The results showed that the changes in food consumption, body weight, and serum cholesterol levels upon oral administration of 0.10% and 0.05% 15-ketosterol were both significant and reproducible effects. ACAT activity in hepatic microsomes was only slightly reduced after 7 days on a chow diet containing 0.10% 15-ketosterol. ACAT activity in jejunal microsomes was greatly reduced by dosages of 0.10% and 0.05% 15-ketosterol. Analysis of the sterol content of jejunal microsomes showed that, at a dosage of 0.10% 15-ketosterol, there were no changes in the levels of cholesterol or cholesteryl esters. Furthermore, the mean level of microsomal 15-ketosterol in experimental animals was low with respect to
Table 5: Effect of dietary 15-ketosterol (0.10% in a chow diet for 7 days) on the levels of free cholesterol, cholesteryl esters, and free 15-ketosterol in rat jejunal microsomes

<table>
<thead>
<tr>
<th>Sterol</th>
<th>Sterol concentration (μg/mg protein ± S.E.M.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Experimental</td>
</tr>
<tr>
<td>Free cholesterol</td>
<td>74.1 ± 11.6 (n = 7)</td>
</tr>
<tr>
<td>Chol. of Chol. esters</td>
<td>4.7 ± 3.0 (n = 7)</td>
</tr>
<tr>
<td>Free 15-ketosterol</td>
<td>0.43 ± 0.10 (n = 7)</td>
</tr>
</tbody>
</table>

*Chol. = Cholesterol  ** Less than 0.002 μg/mg protein
cholesterol content. The results from these sterol analyses indicated that the reduction of ACAT activity in jejunal microsomes upon oral administration of 15-ketosterol was not due to a decrease in the microsomal cholesterol content.

These studies showed that dietary 15-ketosterol had a profound effect on the levels of intestinal ACAT activity, serum cholesterol levels, food consumption, and body weights of rats. In view of these results, the effects of this compound on these variables were further investigated. The following experiments were designed to examine how dosage and duration of treatment affected these variables.
The Effects of Oral Administration of Various Concentrations of 15-Ketosterol on the Levels of ACAT Activity in Rat Jejunal Microsomes

In the previous section, oral administration of 15-ketosterol was shown to reduce ACAT activity in jejunal microsomes. However, this effect has been studied at only two dosages, 0.05% and 0.10% 15-ketosterol in the diet. The following dose-response experiment was designed to examine the effects of several concentrations of 15-ketosterol on ACAT activity in jejunal microsomes. In addition, the effects of several concentrations of 15-ketosterol on serum cholesterol levels, food consumption, body weight, and intestinal weight were examined.

The experiment was done in the following manner. Male Sprague-Dawley rats (N=77) were selected from a pool of 94 animals that had been adapted to a strict controlled feeding schedule (8:00 AM to 12:00 PM) and a reverse light cycle (6:00 AM to 6:00 PM, dark) for two weeks. These 77 rats were matched with respect to fasting serum cholesterol levels and body weight, and divided into 11 groups of N=7 rats (table 6). The animals were placed into individual metal cages and allowed two days to recover from the disruption of their schedule. 15-Ketosterol was mixed into the basal chow at dosages of 0.025%, 0.050%, 0.075%, 0.100%, and 0.125%. Each dosage of 15-ketosterol was administered to one group of experimental animals (30 gm diet/rat/day). Each group of experimental rats was matched to a group of pair-fed controls which were fed the basal diet, but only in the amount consumed by their treated counterparts the previous day. However, on the first day of the study, the pair-fed rats were provided with 30 gm of the basal diet/rat. One group of rats received the basal diet containing no 15-ketosterol. This group of rats received 30 gm/rat/day of the basal diet and was designated ad libitum control group.
Table 6: Initial serum cholesterol levels (mg/dl) and body weights (gm) of treated, pair-fed, and ad libitum rats

<table>
<thead>
<tr>
<th>Dosage</th>
<th>Mean serum cholesterol ± S.E.M.</th>
<th>Mean body weight ± S.E.M.</th>
</tr>
</thead>
<tbody>
<tr>
<td>15-ketosterol</td>
<td>Treated</td>
<td>Pair-fed</td>
</tr>
<tr>
<td>0.00%</td>
<td>--------</td>
<td>--------</td>
</tr>
<tr>
<td>0.025%</td>
<td>90.8 ± 5.6</td>
<td>90.9 ± 7.6</td>
</tr>
<tr>
<td>0.050%</td>
<td>91.1 ± 6.6</td>
<td>90.0 ± 6.5</td>
</tr>
<tr>
<td>0.075%</td>
<td>90.4 ± 5.6</td>
<td>90.5 ± 6.0</td>
</tr>
<tr>
<td>0.10%</td>
<td>90.5 ± 5.5</td>
<td>90.9 ± 5.2</td>
</tr>
<tr>
<td>0.125%</td>
<td>92 ± 6.55</td>
<td>89.9 ± 6.2</td>
</tr>
</tbody>
</table>

S.E.M. = standard error of the mean of N = 7 rats/group
All animals were maintained on their controlled feeding schedule. The beginning of the experiment was staggered over 2 days, with 4 rats from each dietary group starting on one day, and the remaining rats starting their dietary regimens the next day. Food consumption and body weight were monitored daily. The animals were killed by decapitation in a staggered fashion over two days, such that all rats had been on their dietary regimens for 9 days. The sacrifices were initiated 3 hours after the completion of the 4 hour controlled feeding period. The total time required for sacrifice and removal of the intestinal mucosa was 2 hours on the first day and 1.5 hours on the second day. Jejunal microsomes were prepared from the scraped mucosa. The time required for preparation of jejunal microsomes was 3 hours.

This sacrifice time was selected because of results obtained from a pilot study in which the variation in ACAT activity in 15-ketosterol-fed rats (0.10% in chow diet for 10 days) over a 24 hour period of time was examined (unpublished data). The results from this pilot study showed that ACAT activity in the jejunal microsomes of 15-ketosterol-fed rats was reduced immediately after ingestion of a meal as well as 2.4 hours, 4.8 hours, and 7.2 hours after ingestion of a meal.

*Food consumption and weight gain*

Helgerud and coworkers have reported that the level of rat jejunal ACAT activity varies relative to the time of food intake\(^5^3\). In order to minimize the effects of this variable, a controlled feeding protocol was used. The animals were adapted to the controlled feeding schedule for a period of two weeks prior to the study. Food consumption and body weight were monitored during the period of adaptation (figure 21). At the start of the adaptation period, the rats consumed 5.6 ± 0.5 gm/rat/day (N=94 rats), and the average body weight was
Figure 21: Average daily food consumption (panel A) and average daily weight gain (panel B) of rats during the period of adjustment to the controlled feeding schedule. Male Sprague-Dawley rats were maintained on a strict reverse light cycle (6:00 AM to 6:00 PM-dark) and were adapted to a 4 hour controlled feeding schedule in which food was available from 8:00 AM to 12:00 PM. The error bars represent the S.E.M. of N = 94 rats.
147 ± 1.1 gm (N=94 rats). Food consumption rose until day 6 (panel A), and then stabilized at ~ 12.5 gm/rat/day. Body weight rose steadily throughout the adaptation period (panel B). By the end of the adaptation period, the average body weight was 194 ± 2 gm (N=94 rats).

The effect of various concentrations of 15-ketosterol on food consumption was studied by monitoring the average daily food intake of treated and control rats. The data were plotted as a function of the concentration of 15-ketosterol in the diet and were initially analyzed by paired t test (figure 22). The results showed that the amount of food consumed was affected by the level of 15-ketosterol in the diet.

The addition of 0.025% 15-ketosterol to the basal diet did not significantly affect the food consumption of rats relative to pair-fed or ad libitum controls (figure 22, panel A). At the onset of the experiment the average food consumption of treated, pair-fed, and ad libitum rats was 14.6 ± 0.6 gm, 12.6 ± 0.8 gm, and 13.7 ± 0.6 gm, respectively. After 9 days, the average food consumption of these same groups had risen to 17.9 ± 0.5 gm, 17.6 ± 0.7 gm and 15.9 ± 0.6 gm, respectively (treated day 1 vs treated day 9, p<0.001; pair-fed day 1 vs pair-fed day 9, p<0.001 ad libitum day 1 vs ad libitum day 9, 0.02<p<0.05). With the exception of day 8 (treated vs pair-fed, 0.01<p<0.02), no significant changes in food consumption between treated, pair-fed and ad libitum rats were observed.

A transient decrease in food consumption was observed at a level of 0.050% 15-ketosterol in the diet (figure 22, panel B). At the onset of the study, the average food consumption of treated, pair-fed, and ad libitum rats was 12.9 ± 0.8 gm, 14.2 ± 0.8 gm, and 13.7 ± 0.6 gm, respectively. After 9 days on their respective diets, the food consumption of treated, pair-fed, and ad libitum rats
Figure 22: Average daily food consumption of treated (closed circles), pair-fed (open circles), and ad libitum (open squares) rats maintained on a controlled feeding schedule for 9 days. The panels are ranked in order of increasing dosage of 15-ketosterol in the diet of the treated rats. Panel A = 0.025% 15-ketosterol-fed rats and their corresponding controls. Panel B = 0.050% 15-ketosterol-fed rats and their controls. Panel C = 0.075% 15-ketosterol-fed rats and their controls. Panel D = 0.10% 15-ketosterol-fed rats and their controls. Panel E = 0.125% 15-ketosterol-fed rats and their controls. Error bars represent the S.E.M. of N = 7 rats/group.
was 14.6 ± 0.8 gm, 14.5 ± 1 gm, and 15.9 ± 0.6 gm, respectively. Only ad libitum rats experienced a significant change in the level of food consumption relative to initial values (ad libitum day 1 vs ad libitum day 9, 0.02<p<0.05). No significant differences between the food consumption of treated, pair-fed, or ad libitum rats were observed on days 1-4. Significant declines in food consumption were observed on day 5 (treated vs ad libitum, 0.02<p<0.05; pair-fed vs ad libitum, 0.02<p<0.05), day 6 (treated vs ad libitum, 0.02<p<0.05; pair-fed vs ad libitum, p<0.001), and day 7 (treated vs ad libitum, 0.01<p<0.02; pair-fed vs ad libitum, p<0.001). No significant declines in food consumption were observed on days 8 and 9. In addition, no significant changes between the food consumption of treated and pair-fed rats were detected.

At a level of 0.075% 15-ketosterol in the diet the following observations were made (figure 22, panel C). At the start of the experiment, the average food consumption of treated, pair-fed, and ad libitum rats was 12.2 ±0.6 gm, 12.4 ± 0.9 gm, and 13.7 ± 0.5 gm, respectively. By the end of the study, the average food consumption of these same groups was 13.0 ±0.7 gm, 13.2 ± 0.4 gm, and 15.9 ±0.6 gm, respectively. Only ad libitum rats experienced a significant change in the level of food consumption relative to initial values (ad libitum day 1 vs ad libitum day 9, 0.02<p<0.05). No significant changes between the food consumption of treated, pair-fed, and ad libitum rats were observed during the first two days of the study. Significant declines in the average food consumption of treated and pair-fed animals relative to ad libitum controls were observed on day 3 (pair-fed vs ad libitum, 0.001<p<0.01), day 4 (treated vs ad libitum, p<0.001), day 5 (treated vs ad libitum, 0.01<p<0.02; pair-fed vs ad libitum, 0.01<p<0.02), day 6 (treated vs ad libitum, 0.01<p<0.02; pair-fed vs ad libitum, 0.01<p<0.02), day 7 (pair-fed vs ad libitum, 0.001<p<0.01), day 8 (treated vs ad
libitum, $p<0.001$; pair-fed vs ad libitum, $p<0.001$), and day 9 (treated vs ad libitum, $p<0.001$; pair-fed vs ad libitum, $p<0.001$). No significant changes between the average food consumption of treated and pair-fed rats were found.

As the concentration of 15-ketosterol was increased to 0.10% in the diet, the following changes in food consumption were observed (figure 22, panel D). At the onset of the study, the food consumption of treated, pair-fed, and ad libitum animals was $12.1 \pm 0.7$ gm, $14.1 \pm 0.5$ gm, and $13.7 \pm 0.5$ gm, respectively. Food consumption in treated rats then declined as the days progressed, and reached a nadir of $4.6 \pm 0.6$ gm on day 3. By the end of the study, the food consumption of treated, pair-fed, and ad libitum rats was $12.6 \pm 0.7$ gm, $12.7 \pm 1.0$ gm, and $15.9 \pm 0.6$ gm, respectively. Only ad libitum rats experienced a rise in the level of food consumption relative to initial values (ad libitum day 1 vs ad libitum day 9, $0.02<p<0.05$). Significant declines in the food consumption of treated rats were first observed on day 2 (treated vs ad libitum, $0.001<p<0.01$). Thereafter, significant changes in food consumption were observed on day 3 (treated vs ad libitum, $p<0.001$; treated vs pair-fed, $p<0.001$; pair-fed vs ad libitum, $0.02<p<0.05$), day 4 (treated vs ad libitum, $p<0.001$; pair-fed vs ad libitum, $p<0.001$), day 5 (treated vs ad libitum, $p<0.001$; pair-fed vs ad libitum, $p<0.001$), day 6 (treated vs ad libitum, $p<0.001$; treated vs pair-fed, $p<0.001$; pair-fed vs ad libitum, $p<0.001$), day 7 (treated vs ad libitum, $p<0.001$; pair-fed vs ad libitum, $p<0.001$), day 8 (treated vs ad libitum, $0.01<p<0.02$; treated vs pair-fed, $p<0.001$; pair-fed vs ad libitum, $p<0.001$), and day 9 (treated vs ad libitum, $0.001<p<0.01$; pair-fed vs ad libitum, $0.01<p<0.02$).

At a level of 0.125% 15-ketosterol in the diet, the suppression of food consumption was greatest (figure 22, panel E). At the start of the experiment, the food consumption of treated, pair-fed, and ad libitum rats was $14.8 \pm 0.9$ gm,
10.8 ± 1.6 gm, and 13.7 ± 0.5 gm, respectively. By day 9, the food consumption of these same groups was 8.8 ± 0.4 gm, 8.7 ± 0.6 gm, and 15.9 ± 0.6 gm, respectively. The food consumption of treated and pair-fed rats on day 9 had significantly declined relative to the initial values (treated day 1 vs treated day 9, p<0.001; pair-fed day 1 vs pair-fed day 9, p<0.001). Only ad libitum rats experienced a rise in the level of food consumption relative to initial values (ad libitum day 1 vs ad libitum day 9, 0.02<p<0.05). Significant changes in the food intake of treated, pair-fed, and ad libitum groups were first observed on day 3 (treated vs pair-fed, p<0.001; treated vs ad libitum, p<0.001; pair-fed vs ad libitum, 0.02<p<0.05). Significant declines in the food consumption of treated and pair-fed rats relative to ad libitum animals were observed on day 4 (treated vs ad libitum, p<0.001; pair-fed vs ad libitum, p<0.001), day 5 (treated vs ad libitum, p<0.001; pair-fed vs ad libitum, p<0.001), day 6 (treated vs ad libitum, p<0.001; pair-fed vs ad libitum, p<0.001), day 7 (treated vs ad libitum, p<0.001; pair-fed vs ad libitum, p<0.001), day 8 (treated vs ad libitum, p<0.001; pair-fed vs ad libitum, p<0.001), and day 9 (treated vs ad libitum, p<0.001; pair-fed vs ad libitum, p<0.001). With the exception of day 3, no significant changes between the food consumption of treated rats and pair-fed controls were observed.

In order to further quantitate the suppression of food consumption upon oral administration of 15-ketosterol, the total food consumption (days 1-9) of each animal was calculated, and the data were analyzed by ANOVA and by linear regression (figure 23). These analyses showed the total food consumption of treated and pair-fed rats was significantly affected by the level of 15-ketosterol in the diet (ANOVA: means squares of total food consumption of treated rats at each dosage vs population variance of treated rats, p=0.0001; mean squares of total food consumption of pair-fed rats at each dosage vs
Figure 23: The relationship of total food consumption (days 1-9) to dosage of 15-ketosterol in the diet. Treated and control rats were fed their respective diets for 9 days and were maintained on a controlled feeding schedule during that time. Total food consumption from 1-9 days was calculated for each rat, and the data were analyzed by linear regression. Panel A = the regression analysis of the treated animals. Panel B = the regression analysis of the pair-fed controls. Zero percentage 15-ketosterol corresponds to the ad libitum control. Error bars represent the S.E.M. of N = 7 rats/dosage.
population variance of pair-fed rats, p=0.0001). Furthermore, the decline in food consumption upon oral administration of 15-ketosterol was linear over the range of concentrations tested (figure 21: treated rats, y = -547x + 145, R^2 = 0.83; pair-fed rats, y = -449x + 137, R^2 = 0.81).

The effect of oral administration of 15-ketosterol on body weight was analyzed in a similar fashion. The average daily change in body weight was calculated, and the data were plotted as a function of the concentration of 15-ketosterol in the diet (figure 24). The data were initially analyzed by paired t test. The results showed that the body weights of rats were affected by the level of 15-ketosterol in the diet.

The addition of 0.025% 15-ketosterol to the diet did not significantly affect the average daily change in the body weights of treated, pair-fed, or ad libitum animals (figure 24, panel A). At the start of the experiment, the average body weights of treated, pair-fed, and ad libitum rats were 196 ± 12 gm, 195 ± 7 gm, and 194 ± 15 gm, respectively. By the end of the study, the body weights of these same groups had risen to 248 ± 3 gm, 237 ± 3 gm, and 234 ± 6 gm, respectively (treated day 0 vs treated day 9, p<0.001; pair-fed day 0 vs pair-fed day 9, p<0.001; ad libitum day 0 vs ad libitum day 9, 0.02<p<0.05). Treated, pair-fed, and ad libitum rats gained weight for the first few days. Significant changes in the body weights of these animals, relative to the initial values, were observed by third day of the study (treated day 0 vs treated day 3, p<0.001; pair-fed day 0 vs pair-fed day 3, p<0.001; ad libitum day 0 vs ad libitum day 3, p<0.001). Under these experimental conditions, the body weights of all three groups then stabilized, and no significant changes in the body weights of treated, pair-fed, and ad libitum animals were observed between days 3 and 7. At this point, all three groups began a second period of weight gain, and
Figure 24: Average daily change in body weight of treated (closed circles), pair-fed (open circles), and ad libitum (open squares) rats maintained on a controlled feeding schedule for 9 days. The panels are ranked in order of increasing dosage of 15-ketosterol in the diets of treated rats. Panel A = 0.025% 15-ketosterol-fed rats and their controls. Panel B = 0.050% 15-ketosterol-fed rats and their corresponding controls. Panel C = 0.075% 15-ketosterol-fed rats and their controls. Panel D = 0.10% 15-ketosterol-fed rats and their controls. Panel E = 0.125% 15-ketosterol-fed rats and their controls. The error bars represent the S.E.M. of N = 7 rats/group.
significant changes in the body weights of treated, pair-fed, and ad libitum rats relative to day 7 values were detected (treated day 7 vs treated day 9, p<0.001; pair-fed day 7 vs pair-fed day 9, p<0.001; ad libitum day 7 vs ad libitum day 9, p<0.001).

Addition of 0.050% 15-ketosterol to the diet did not significantly affect the average daily change in the body weights of treated, pair-fed, or ad libitum animals (figure 24, panel B). At the start of the experiment, the average body weights of treated, pair-fed, and ad libitum rats were 194 ± 15 gm, 195 ± 15 gm, and 194 ± 15 gm, respectively. By the end of the study, these same groups weighed 227 ± 6 gm, 225 ± 7 gm, and 234 ± 6 gm, respectively. Only ad libitum animals experienced a significant gain in body weight relative to initial values (ad libitum day 0 vs ad libitum day 9, 0.02<p<0.05). Ad libitum rats gained weight for the first few days. Significant changes in the body weights of these animals, relative to the initial values, were observed by third day of the study (ad libitum day 0 vs ad libitum day 3, p<0.001). Under these experimental conditions, the body weights of ad libitum rats then stabilized, and no significant changes in the body weights were observed between days 3 and 7. At this point, the ad libitum rats began a second period of weight gain, and significant changes in the body weights relative to day 7 values were detected (ad libitum day 7 vs ad libitum day 9, p<0.001).

The addition of 0.075% 15-ketosterol to the basal diet produced significant reductions in the body weights of treated and pair-fed animals relative to ad libitum controls (figure 24, panel C). At the start of the study, the average body weights of treated, pair-fed, and ad libitum rats were 196 ± 11 gm, 194 ± 14 gm, and 194 ± 15 gm, respectively. By the end of the study, these same groups weighed 218 ± 4 gm, 209 ± 3 gm, and 234 ± 6 gm, respectively. Only ad
libitum animals experienced a significant change in body weight relative to initial values (ad libitum day 0 vs ad libitum day 9, 0.02<p<0.05). No significant changes between the body weights of treated, pair-fed, and ad libitum rats were observed on days 1-4. Significant declines in the percentage change in body weights of treated and pair-fed rats relative to ad libitum controls were observed on days 5 (pair-fed vs ad libitum, 0.02<p<0.05), day 6 (pair-fed vs ad libitum, p<0.001), day 7 (pair-fed vs ad libitum, 0.001<p<0.01), day 8 (treated vs ad libitum, 0.01<p<0.02; pair-fed vs ad libitum, p<0.001), and day 9 (pair-fed vs ad libitum, 0.001<p<0.01).

As the concentration of 15-ketosterol was increased to 0.10% in the diet, the following changes in body weight were observed (figure 24, panel D). At the start of the experiment, the average body weights of treated, pair-fed, and ad libitum rats were 196 ± 9 gm, 194 ± 15 gm, and 194 ± 15 gm, respectively. By the end of the study, these same groups weighed 199 ± 6 gm, 192 ± 5 gm, and 234 ± 6 gm, respectively. Only ad libitum animals experienced a significant change in body weights relative to initial values (ad libitum day 0 vs ad libitum day 9, 0.02<p<0.05). No significant differences between treated, pair-fed, and ad libitum rats were observed on the days 1-2. Significant declines in the percentage change in body weights of treated and pair-fed rats relative to ad libitum controls were observed on day 3 (treated vs ad libitum, 0.001<p<0.01), day 4 (treated vs ad libitum, 0.01<p<0.2; pair-fed vs ad libitum, p<0.001), day 5 (treated vs ad libitum, 0.001<p<0.01; pair-fed vs ad libitum, p<0.001), day 6 (treated vs ad libitum, p<0.001; pair-fed vs ad libitum, p<0.001), day 7 (treated vs ad libitum, 0.001<p<0.01; pair-fed vs ad libitum, 0.001<p<0.01), day 8 (treated vs ad libitum, p<0.001; pair-fed vs ad libitum, p<0.001) and day 9
(treated vs ad libitum, p<0.001; pair-fed vs ad libitum, p<0.001). No significant changes between treated and pair-fed rats were detected.

At a level of 0.125% 15-ketosterol in the diet, the following was observed (figure 24, panel E). At the start of the study, the average body weights of treated, pair-fed, and ad libitum rats were 197 ± 3 gm, 195 ± 3 gm, and 194 ± 15 gm, respectively. By the end of the study, these same groups weighed 188 ± 4 gm, 184 ± 4 gm, and 234 ± 6 gm, respectively. Only ad libitum animals experienced a significant change in body weights relative to initial values (ad libitum day 0 vs ad libitum day 9, 0.02<p<0.05). No significant differences between treated, pair-fed, and ad libitum rats were observed on the days 1-2. Significant declines in the percentage change in body weights of treated and pair-fed rats relative to ad libitum controls were observed on day 3 (pair-fed vs ad libitum, p<0.001), day 4 (treated vs ad libitum, 0.001<p<0.01; pair-fed vs ad libitum, p<0.001), day 5 (treated vs ad libitum, p<0.001; pair-fed vs ad libitum, p<0.001), day 6 (treated vs ad libitum, 0.001<p<0.01; pair-fed vs ad libitum, p<0.001), day 7 (treated vs ad libitum, p<0.001; pair-fed vs ad libitum, p<0.001), day 8 (treated vs ad libitum, p<0.001; pair-fed vs ad libitum, p<0.001), and day 9(treated vs ad libitum, p<0.001; pair-fed vs ad libitum, p<0.001). No significant changes between treated and pair-fed rats were detected.

In order to further quantitate the decline in body weight upon oral administration of 15-ketosterol, the body weights of treated, pair-fed, and ad libitum rats after 9 days on their respective dietary regimens were analyzed by ANOVA and by linear regression (figure 25). These analyses showed that the body weights of treated and pair-fed rats were significantly affected by the concentration of 15-ketosterol in the diet (ANOVA: means squares of body
Figure 25: Relationship of body weight to dosage of 15-ketosterol in the diet. Treated and control rats were fed their respective diets for 9 days and were maintained on a controlled feeding schedule during that time. The body weights measured upon termination of the experiment were analyzed by linear regression. Panel A = the regression analysis of the treated animals. Panel B = the regression analysis of the pair-fed controls. Zero percentage 15-ketosterol corresponds to weights of the ad libitum controls. Error bars represent the S.E.M. of N = 7 rats/dosage.
weights of treated rats at each dosage vs population variance of treated rats, p=0.0001; mean squares of body weights of pair-fed rats at each dosage vs population variance of pair-fed rats, p=0.0001). Furthermore, these reductions in body weight upon oral administration of 15-ketosterol were linear over the range of concentrations tested (figure 23; experimental, y = -437x + 247, R²=0.60; pair fed, y = -461x + 242, R²=0.67).

**Serum cholesterol levels**

The effect of oral administration of various concentrations of 15-ketosterol on serum cholesterol levels was examined by plotting the average values of treated and controls groups as a function of the dosage of 15-ketosterol (figure 26). These data were then analyzed by paired t test, by ANOVA, and by linear regression. Under these experimental conditions, the mean values for the cholesterol levels of treated, pair-fed, and ad libitum rats were higher than the initial values and the values observed in previous studies (table 6, figure 18). However, the results showed that oral administration of 15-ketosterol reduced the serum cholesterol levels of treated rats relative to either pair-fed or ad libitum controls, although the magnitude of the decreases were lower than results obtained from previous studies (ANOVA: mean squares and population variance of cholesterol levels of treated rats vs mean squares and population variance of cholesterol levels of pair-fed rats, p=0.0001; mean squares and population variance of cholesterol levels of treated rats vs mean squares and population variance of cholesterol levels of ad libitum rats, p=0.0001). These changes in serum cholesterol levels upon oral administration of the 15-ketosterol were linear over the range of concentrations tested (R²=0.82). Significant declines in the serum cholesterol levels of treated animals were observed at concentrations of 0.075% 15-ketosterol (paired t test: treated vs pair
Figure 26: Mean serum cholesterol levels of experimental (filled columns), pair-fed (open columns) and ad libitum (hatched columns) rats plotted as a function of dosage of 15-ketosterol in the diet of the experimental rats. Error bars represent the S.E.M. of N = 7 rats.
fed, p<0.001; treated vs ad libitum, p<0.001), 0.10% 15-ketosterol (paired t test: treated vs pair fed, p<0.001; treated vs ad libitum, p<0.001), and 0.125% 15-ketosterol (paired t test: treated vs pair fed, p<0.001; treated vs ad libitum, p<0.001). No significant differences between the serum cholesterol levels of pair-fed and ad libitum rats were detected.

**Intestinal weight**

Previous work had established that administration of 0.10% 15-ketosterol to rats for 8 days resulted in an enlargement of the proximal half of the small intestine\(^{112}\). The intestinal enlargement strongly resembled the adaptation of the intestines in human and rat upon intestinal bypass surgery\(^{72}\). This enlargement was not observed upon oral administration of 15-ketosterol to baboons\(^{112}\).

The effect of different levels of 15-ketosterol on the enlargement of the small intestine was examined by monitoring the wet weight of the proximal half of the small intestine after the intestine had been flushed with saline and patted dry (figure 27). These weights were plotted as a function of the concentration of 15-ketosterol in the diet, and the data were analyzed by paired t test, by ANOVA, and by linear regression.

Intestinal enlargement, as measured by an increase in wet weight, was observed in treated rats relative to ad libitum controls (ANOVA: mean squares and population variance of weight of treated rats vs mean squares and population variance of weight of ad libitum rats, p=0.0001). The degree of intestinal enlargement in experimental animals was dependent upon the concentration of 15-ketosterol in the diet (ANOVA: mean squares of weights of treated rats at 0-0.125% 15-ketosterol vs population variance of treated rats at 0-0.125% 15-ketosterol, p=0.0001). In addition, the degree of intestinal
Figure 27: Average intestinal weight of experimental (filled columns), pair-fed (open columns), and ad libitum (hatched columns) rats plotted as a function of the dosage of 15-ketosterol in the diet of the experimental rats. Error bars represent the S.E.M. of N = 7 rats.
enlargement upon oral administration of 15-ketosterol was linear over the range of concentrations tested ($R^2=0.85$). Significant changes in the intestinal weights of treated rats relative to *ad libitum* controls were observed at concentrations of 0.050% 15-ketosterol (paired t test: treated vs *ad libitum*, p<0.001), 0.075% 15-ketosterol (paired t test: treated vs *ad libitum*, p<0.001), 0.10% 15-ketosterol (paired t test: treated vs *ad libitum*, p<0.001), and 0.125% 15-ketosterol (paired t test: treated vs *ad libitum*, p<0.001).

Food restriction, as represented by pair-feeding, resulted in significant decreases in intestinal wet weight relative to *ad libitum* controls (ANOVA: mean squares and population variance of weight of pair-fed rats vs mean squares and population variance of weight of *ad libitum* rats, p=0.0001). The magnitude of the decline in intestinal wet weight was dependent upon the severity of the food restriction (ANOVA: mean squares of weights of pair-fed rats at 0-0.125% 15-ketosterol vs population variance of pair-fed rats at 0-0.125% 15-ketosterol, p=0.0001). In addition, the magnitude of the declines in the intestinal weights of pair-fed rats was linear over the range of concentrations tested ($R^2=0.86$). Significant changes in the intestinal weights of pair-fed rats relative to *ad libitum* controls were observed at concentrations of 0.050% 15-ketosterol (paired t test: pair-fed vs *ad libitum*, p<0.001), 0.075% 15-ketosterol (paired t test: pair-fed vs *ad libitum*, p<0.001), 0.10% 15-ketosterol (paired t test: pair-fed vs *ad libitum*, p<0.001), and 0.125% 15-ketosterol (paired t test: pair fed vs *ad libitum*, p<0.001).

**Intestinal enzyme activity**

Intestinal ACAT activity was measured using aliquots of jejunal microsomes (Chapter 2). This assay procedure involved the incorporation of [1-14C]oleoyl CoA into [1-14C]cholesteryl oleate and thus depended upon
endogenous cholesterol for the generation of radiolabeled cholesteryl oleate. The use of benzene as a solvent for normal phase TLC permitted the separation and quantification of radioactive components with the chromatographic mobilities of cholesteryl oleate, triolein, 15-ketosteryl oleate, and oleic acid.

Cholesterol esterification by intestinal ACAT was calculated in terms of specific activity (pmol cholesteryl oleate formed/min/mg protein), and the average specific activities of experimental and control rats were plotted as a function of the concentration of 15-ketosterol in the diet (figure 28). The data was analyzed by paired t test and by ANOVA.

The results showed that ACAT activity in the intestinal microsomes of treated rats was reduced by oral administration of 15-ketosterol (ANOVA: mean squares and population variance of treated rats vs mean squares and population variance of pair-fed rats, p=0.0003; mean squares and population variance of treated rats vs mean squares and population variance of ad libitum rats, p=0.0001). Significant declines in the levels of cholesterol esterification in jejunal microsomes were observed upon oral administration of 0.050% 15-ketosterol (paired t test: treated vs pair fed, 0.02<p<0.05), 0.075% 15-ketosterol (paired t test: treated vs pair fed, 0.02<p<0.05; treated vs ad libitum, 0.02<p<0.05), 0.10% 15-ketosterol (paired t test: treated vs pair fed, 0.02<p<0.05; treated vs ad libitum, 0.02<p<0.05), and 0.125% 15-ketosterol (paired t test: treated vs pair fed, 0.01<p<0.02; treated vs ad libitum, 0.02<p<0.05). Under these conditions, no significant changes in the microsomal ACAT activities of pair-fed and ad libitum rats were detected.

Although it had been established that 0.10% 15-ketosterol did not change the concentration of microsomal cholesterol, there was a possibility that a dosage of 0.125% 15-ketosterol did alter microsomal cholesterol
Figure 28: Average levels of cholesterol esterification in jejunal microsomes isolated from experimental (filled columns), pair-fed (open columns), and ad libitum (hatched column) rats plotted as a function of the dosage of 15-ketosterol in the diet of the experimental rats. Error bars represent the S.E.M. of N = 7 rats.
concentration. In order to address this question, the concentrations of free cholesterol in experimental and control animals were determined.

This experiment was done in the following manner. Aliquots of jejunal microsomes isolated from 0.125% 15-ketosterol fed rats (N = 7), pair-fed controls (N = 6), and ad libitum (N = 7) were obtained. [4-14C]Cholesterol (4 x 10^4 dpm, 0.10 ng) was added to each microsomal suspension as an internal standards. The samples were extracted according to Folch et al.111. The level of free cholesterol was then quantitated, after TMS derivitization, by GLC. The sterol samples were analyzed using a Perkin-Elmer Sigma 2000 gas chromatograph equipped with 0.1 μm DB-5 column (30 m). Samples were injected as 1μl aliquots in a splitless fashion and were eluted using nitrogen (40 ml/min) as the carrier gas and a temperature gradient programmed from 150 °C to 280 °C at a rate of 7.5 °C/min. The temperature of the FID detector was maintained at 290 °C, while the temperature of the injection port was programmed to rise from an initial value of 50 °C to 280 °C in ~ 10 sec. All GLC determinations were done in duplicate using triacontane (C30) as an internal standard.

The data are presented in table 7. Under these experimental conditions the levels of microsomal cholesterol in treated, pair-fed, and ad libitum rats were higher than the results described earlier in this chapter. However, the results from these determinations showed that a dosage of 0.125% 15-ketosterol did not change the levels of microsomal cholesterol (paired t test: treated vs pair fed, p>0.90; treated vs ad libitum, p>0.90; pair-fed vs ad libitum, p>0.90).

The amount of radioactivity incorporated into material with the chromatographic mobilities of triacylglycerides and oleic acid was calculated in
terms of total dpm/assay, and the average formation of these compounds was plotted as a function of dosage (figures 29, 30). The data was analyzed by paired t test and by ANOVA. The results showed that the levels of triacylglyceride synthesis and oleic acid formation in treated rats were not significantly different from those levels observed in pair-fed or ad libitum controls. Furthermore, no significant differences in the levels of triacylglyceride formation or oleic acid synthesis between pair-fed and ad libitum rats were detected. These data suggested that the reduction of intestinal ACAT activity was specific, since other oleoyl CoA-dependent enzymatic processes were not affected by dietary 15-ketosterol.

The amount of radioactivity with the chromatographic mobility of 15-ketosteryl oleate was calculated in terms of total dpm/assay, and the average levels of radioactivity were plotted as a function of dosage (figure 31). The data showed that there was very little, if any, radioactivity incorporated into material with the chromatographic mobility of 15-ketosteryl oleate. Statistical analyses were done using both the paired t test and ANOVA. No significant differences between treated, pair-fed, or ad libitum rats were found. The level of radioactivity in this region did not significantly change with respect to the concentration of 15-ketosterol in the diet.
Table 7: Effect of dietary administration of 15-ketosterol (0.125% in diet; under controlled feeding schedule for 9 days) on the level of free cholesterol in rat jejunal microsomes

<table>
<thead>
<tr>
<th>Dietary group</th>
<th>Free cholesterol concentration</th>
<th>µg/mg protein ± S.E.M.</th>
</tr>
</thead>
<tbody>
<tr>
<td>15-ketosterol</td>
<td>113 ± 9.5 (n = 7)</td>
<td></td>
</tr>
<tr>
<td>Pair-fed control</td>
<td>106 ± 10.6 (n = 6)</td>
<td></td>
</tr>
<tr>
<td>Ad libitum</td>
<td>115 ± 12.3 (n = 7)</td>
<td></td>
</tr>
</tbody>
</table>
Figure 29: Average formation in jejunal microsomes isolated from experimental (filled columns), pair-fed (open columns), and ad libitum (hatched columns) rats of material with the chromatographic mobility of triacylglycerides plotted as a function of dosage of 15-ketosterol in the diet of experimental rats. Error bars represent the S.E.M. of N = 7 rats.
Figure 30: Average formation in jejunal microsomes isolated from experimental (filled columns), pair-fed (open columns), and ad libitum (hatched column) rats of radiolabeled material with the chromatographic mobility of oleic acid plotted as a function of the dosage of 15-ketosterol in the diet of the experimental rats. Error bars represent the S.E.M. of N = 7 rats.
Figure 31: Average formation in jejunal microsomes isolated from treated (filled columns), pair-fed (open columns) and ad libitum (hatched column) rats of radiolabeled material with the chromatographic mobility of 15-ketosteryl oleate plotted as a function of the dosage of 15-ketosterol in the diet of treated rats. Error bars represent the S.E.M. of N = 7 rats.
Summary

This feeding study was designed to examine the effect of various concentrations of 15-ketosterol on food consumption, body weight, serum cholesterol levels, intestinal enlargement, and intestinal ACAT activity. The results showed that food consumption, body weight, and serum cholesterol levels were suppressed by oral administration of 15-ketosterol in a concentration-dependent fashion. The degree of intestinal enlargement, as measured by intestinal weight, was also dependent on the concentration of 15-ketosterol in the diet. The levels of cholesterol esterification by microsomal ACAT were reduced by oral administration of 15-ketosterol. Analysis of the levels of microsomal cholesterol showed that administration of 0.125% 15-ketosterol had no effect on the cholesterol content of treated animals. In addition, the reduction of ACAT activity appeared to be selective, since oleoyl-CoA dependent formation of material with the chromatographic mobilities of triacylglycerides and oleic acid were unaffected by oral administration of 15-ketosterol. Finally, extremely low levels of radioactivity with the chromatographic mobility of 15-ketosterol oleate were detected, and no consistent effect of administration of 15-ketosterol was observed.
Intestinal ACAT Activity in 15-Ketosterol-Fed Rats: In Vivo Time Course

In order to determine the relationship between the duration of oral administration of the 15-ketosterol and the effect of the compound on ACAT activity in intestinal microsomes, the following feeding study was designed. In addition, the relationship between duration of oral administration of 15-ketosterol and the effects of the sterol on food consumption, body weight, serum cholesterol level, and intestinal weight were studied.

The experimental design was as follows. Male rats (N=84) were selected from a pool of 100 animals that had been adapted to a strict controlled feeding schedule (8:00 AM to 12:00 PM) and a reverse light cycle (6:00 AM to 6:00 PM, dark) for 15 days. These 84 rats were matched with respect to serum cholesterol levels and body weights, and were divided into 3 groups (28 rats/group). The animals were then allowed 2 days to recover from the disruption of their schedule prior to the initiation of the study. One group of rats, designated experimental (treated), was fed a chow diet supplemented with 0.10% 15-ketosterol (30 gm of diet/rat/day). The experimental animals were matched to pair-fed controls. The pair-fed rats were fed the basal diet, but only in the amounts consumed by their treated counterparts the previous day. On the first day of the study, the pair-fed rats were provided with 30 gm/rat of the basal diet. The third group of rats received 30 gm/rat/day of the basal diet and was designated the ad libitum control group. The controlled feeding schedule was maintained throughout the study. The rats were fed their respective dietary regimens for varying lengths of time. Food consumption and body weight were monitored daily. The animals were killed at selected times by light sedation with 5 mg of injected ketamine followed by decapitation 2 minutes after injection. Sacrifices were initiated 3 hours after the completion of the 4 hour
controlled feeding period. One set of treated (N = 4), pair-fed (N = 4), and ad libitum rats (N = 4) were terminated on day 0 (3 hours after completion of the first feeding period), day 1, day 2, day 4, day 6, and day 10. The total time required for termination and removal of the intestinal mucosa was 0.5 hours/time point. The time required for preparation of jejunal microsomes was 2 hours/time point.

Food consumption and body weight

Helgerud and coworkers have reported that the level of rat jejunal ACAT activity varies relative to the time of food intake. In order to minimize the effects of this variable, a controlled feeding protocol was used. The animals were adapted to the controlled feeding schedule for a period of 15 days prior to the study. Food consumption and body weight were monitored during the period of adaptation (figure 32). At the start of the adaptation period, the average food consumption was 7.0 ± 0.5 gm/rat/day (N = 100), and the average body weight was 118 ± 1 gm/rat/day (N = 100). Food consumption (panel A), and body weight (panel B) rose steadily throughout the period of adaptation. By the end of the adaptation period, the average food consumption was 17.6 gm/rat/day (N = 100), and the average body weight was 186 ± 2 gm (N = 100).

The effect of oral administration of 15-ketosterol on food consumption was also examined. The average daily food consumption of treated and control rats was plotted, and the data were analyzed by paired t test (figure 33). The results showed that the average daily food consumption of treated and pair-fed rats declined relative to ad libitum controls. At the start of the study, the food consumption of treated, pair-fed, and ad libitum rats was 12.8 ± 0.6 gm, 13.5 ± 0.5 gm, and 13.0 ± 0.4 gm, respectively. By the end of the experiment, the food intake of these same groups was 12.4 ± 0.8 gm, 11.5 ± 1.0 gm, and 16.6 ± 0.7
Figure 32: Average daily food consumption (panel A) and average daily weight gain (panel B) of rats during the period of adjustment to the controlled feeding schedule. Male Sprague-Dawley rats were maintained on a strict reverse light cycle (6:00 AM to 6:00 PM-dark) and were adapted to a 4 hour controlled feeding schedule in which food was available from 8:00 AM to 12:00 PM. Error bars represent the S.E.M. of N = 100 rats.
Figure 33: The average daily food consumption of experimental and control groups. Closed circles = 0.10% 15-ketosterol fed. Open circles = pair-fed controls. Open diamonds = ad libitum controls. Error bars represent the SEM per group. Day 0, N=28 rats/group; day 1, N=20 rats/group; days 2, N=16 rats/group; days 3-4, N=12 rats/group; days 5-6, N=8 rats/group; day 7-10, N=4 rats/group.
gm respectively. Only the food consumption of the ad libitum rats had significantly changed relative to initial values (paired t test: ad libitum day 0 vs ad libitum day 10, p<0.001). Significant declines in the food consumption of treated and pair-fed rats were observed on day 2 (paired t test: treated vs ad libitum, 0.001<p<0.01; pair-fed vs ad libitum, p<0.001), day 3 (paired t test: treated vs ad libitum, p<0.001; pair-fed vs ad libitum, p<0.001), day 4 (paired t test: treated vs ad libitum, p<0.001; pair-fed vs ad libitum, p<0.001), day 5 (paired t test: treated vs ad libitum, p<0.001; pair-fed vs ad libitum, p<0.001), day 6 (paired t test: treated vs ad libitum, 0.001<p<0.01; pair-fed vs ad libitum, 0.001<p<0.01), day 7 (paired t test: treated vs ad libitum, 0.01<p<0.02; pair-fed vs ad libitum, p<0.001), day 9 (paired t test: treated vs ad libitum, p<0.001; pair-fed vs ad libitum, p<0.001), and day 10 (paired t test: treated vs ad libitum, 0.001<p<0.01; pair-fed vs ad libitum, 0.001<p<0.01). No changes between the food consumption of treated and pair-fed rats were observed except on day 2 (paired t test: treated vs pair-fed, p<0.001), and day 7 (paired t test: treated vs pair-fed, 0.001<p<0.01).

The effect of oral administration of 15-ketosterol on body weight was studied in a similar manner. The average daily change in body weight was calculated, and the data were subsequently plotted (figure 34). The data were analyzed by paired t test. The results showed that the percentage change in body weights of treated and pair-fed rats was reduced relative to ad libitum animals. At the onset of the experiment, the average body weights of treated, pair-fed, and ad libitum rats were 192 ± 2 gm, 195 ± 2 gm, and 194 ± 2 gm, respectively. By the end of the study, the average body weights of treated, pair-fed, and ad libitum were 185 ± 6 gm, 184 ± 10 gm, 232 ± 8 gm, respectively. The ad libitum rats were the only group to gain body weight relative to the initial
Figure 34: Average daily change in body weight of experimental (closed circles), pair-fed (open circles), and ad libitum (open diamonds) rats. Error bars represent the S.E.M. per group. Day 1, N = 20 rats/group; day 2, N = 16 rats/group; days 3-4, N = 12 rats/group; days 5-6, N = 8 rats/group; days 7-10, N = 4 rats/group.
values (ad libitum day 0 vs ad libitum day 10, p<0.001). Significant declines in the percentage change in the body weights of treated and pair-fed rats were observed relative to ad libitum animals on day 2 (treated vs ad libitum, p<0.001; pair-fed vs ad libitum, p<0.001), day 3 (treated vs ad libitum, p<0.001; pair-fed vs ad libitum, p<0.001), day 4 (treated vs ad libitum, p<0.001; pair-fed vs ad libitum, p<0.001), day 5 (treated vs ad libitum, p<0.001; pair-fed vs ad libitum, p<0.001), day 6 (treated vs ad libitum, p<0.001; pair-fed vs ad libitum, p<0.001), day 7 (treated vs ad libitum, p<0.001; pair-fed vs ad libitum, p<0.001), day 8 (treated vs ad libitum, p<0.001; pair-fed vs ad libitum, p<0.001), day 9 (treated vs ad libitum, p<0.001; pair-fed vs ad libitum, p<0.001), and day 10 (treated vs ad libitum, p<0.001; pair-fed vs ad libitum, p<0.001). No significant changes between treated and pair-fed rats were observed.

**Serum cholesterol levels**

The effect of the duration of oral administration of 0.10% 15-ketosterol on serum cholesterol levels were examined by monitoring the serum cholesterol levels of treated and control groups (table 8) and analyzing the data by paired t test and by ANOVA. The results showed that administration of 0.10% 15-ketosterol reduced the serum cholesterol levels of treated rats relative to either pair-fed or ad libitum controls (ANOVA: mean squares and population variance of treated rats vs mean squares and population variance of pair-fed rats, p=0.0001; mean squares and population variance of treated rats vs mean squares and population variance of ad libitum rats, p=0.0001). The magnitude of the reduction in the serum cholesterol levels of treated rats was dependent upon the duration of oral administration of 15-ketosterol (ANOVA: mean squares of treated rat on days 0-10 vs population variance of treated rats on days 0-10, p=0.0001). No significant changes in the serum cholesterol levels of
Table 8: The effect of duration of 15-ketosterol administration (0.10% in a chow diet) on the average serum cholesterol levels of treated, pair-fed, and ad libitum rats.

<table>
<thead>
<tr>
<th>Duration of dietary regimen</th>
<th>Average Serum Cholesterol (mg/dl) ± S.E.M. (N=7 rats/group)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>15-ketosterol-fed</td>
</tr>
<tr>
<td>Day 0*</td>
<td>104.5 ± 1.4</td>
</tr>
<tr>
<td>Day 1</td>
<td>85.2 ± 0.5</td>
</tr>
<tr>
<td>Day 2</td>
<td>83.2 ± 2.8</td>
</tr>
<tr>
<td>Day 4</td>
<td>67.6 ± 7.4</td>
</tr>
<tr>
<td>Day 6</td>
<td>58.2 ± 3.8</td>
</tr>
<tr>
<td>Day 10</td>
<td>61.3 ± 4.8</td>
</tr>
</tbody>
</table>

* 3 hours after completion of first meal of the experiment.
treated, pair-fed, and ad libitum animals were observed between 0 and 2 days of administration of 15-ketosterol. Significant declines in the serum cholesterol levels of treated rats relative to either pair-fed or ad libitum rats were detected on day 4 (paired t test: treated vs pair-fed, -19.3%, p<0.001; treated vs ad libitum, -19.3%, p<0.001), day 6 (paired t test: treated vs pair-fed, -32.7%, p<0.001; treated vs ad libitum, -32.9%, p<0.001), and day 10 (paired t test: treated vs pair-fed, -34.4%, p<0.001; treated vs ad libitum, -33.2%, p<0.001).

Under these experimental conditions, the magnitudes of the declines in serum cholesterol levels of treated rats were lower than results obtained from previous studies (figure 18). No significant changes between the serum cholesterol levels of pair-fed and ad libitum rats were detected. In addition, there were no significant day to day changes in the serum cholesterol levels of pair-fed rats or ad libitum rats.

*Intestinal weights*

The effect of the duration of oral administration of 15-ketosterol on intestinal weight was monitored by measuring the weight of the proximal half of the small intestine (table 9). The data were subsequently analyzed by paired t test and by ANOVA.

Intestinal enlargement, as measured by an increase in the weight of the proximal half of the small intestine, was observed in treated rats relative to ad libitum controls (ANOVA: mean squares and population variance of treated rats vs mean squares and population variance of ad libitum rats, p=0.0001). The magnitude of the weight of the small intestines of treated rats was dependent upon the duration of administration of 0.10% 15-ketosterol (ANOVA: mean squares of treated rats on days 0-10 vs population variance of treated rats on days 0-10, p=0.0001). No significant changes in the weight of the small
Table 9: The average weights of the proximal half of the small intestines of treated (0.10% 15-ketosterol in a chow diet), pair-fed, and ad libitum rats - Effect of the duration of dietary regimens

<table>
<thead>
<tr>
<th>Duration of dietary regimen</th>
<th>15-ketosterol-fed</th>
<th>Pair-fed</th>
<th>Ad libitum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 0*</td>
<td>3.0 ± 0.2</td>
<td>3.5 ± 0.3</td>
<td>3.2 ± 0.3</td>
</tr>
<tr>
<td>Day 1</td>
<td>3.1 ± 0.1</td>
<td>3.5 ± 0.1</td>
<td>3.3 ± 0.1</td>
</tr>
<tr>
<td>Day 2</td>
<td>3.5 ± 0.1</td>
<td>3.4 ± 0.1</td>
<td>3.3 ± 0.1</td>
</tr>
<tr>
<td>Day 4</td>
<td>4.7 ± 0.3</td>
<td>2.5 ± 0.1</td>
<td>3.3 ± 0.1</td>
</tr>
<tr>
<td>Day 6</td>
<td>5.4 ± 0.1</td>
<td>2.7 ± 0.1</td>
<td>3.5 ± 0.1</td>
</tr>
<tr>
<td>Day 10</td>
<td>6.0 ± 0.4</td>
<td>2.6 ± 0.2</td>
<td>3.4 ± 0.2</td>
</tr>
</tbody>
</table>

* 3 hours after completion of first meal of the experiment.
intestines of treated, pair-fed, and *ad libitum* rats were detected on days 0-2. Significant changes in the intestinal weights of treated rats relative to *ad libitum* controls were observed on day 4 (paired t test: treated vs *ad libitum*, *p*<0.001), day 6 (paired t test: treated vs *ad libitum*, *p*<0.001), and day 10 (paired t test: treated vs *ad libitum*, *p*<0.001).

Food restriction, as represented by pair-feeding, resulted in significant decreases in the intestinal weight of pair-fed rats relative to *ad libitum* controls (ANOVA: mean squares and population variance of pair-fed rats vs mean squares and population variance of *ad libitum* rats, *p*=0.0024). The magnitude of the intestinal weights of pair-fed animals was dependent upon the duration of paired feeding (ANOVA: mean squares of pair-fed rats on days 0-10 vs population variance of treated rats on days 0-10, *p*=0.029). No significant changes in the weight of the small intestines of treated, pair-fed, and *ad libitum* rats were detected on days 0-2. Significant changes in the intestinal weights of pair-fed rats relative to *ad libitum* controls were observed on day 4 (paired t test: pair-fed vs *ad libitum*, *p*<0.001), day 6 (paired t test: pair-fed vs *ad libitum*, *p*<0.001, and day 10 (paired t test: pair-fed vs *ad libitum*, *p*<0.001).

**Intestinal enzyme activity**

Intestinal ACAT activity was measured using aliquots of jejunal microsomes (Chapter 2). This assay procedure involved the incorporation of [1-14C]oleoyl CoA into [1-14C]cholesteryl oleate, and thus depended upon endogenous cholesterol for the generation of radiolabeled cholesteryl oleate. The use of benzene as a solvent for normal phase TLC permitted the separation and quantification of radioactive components with the chromatographic mobilities of cholesteryl oleate, triolein, 15-ketosteryl oleate, and oleic acid.
Cholesterol esterification by intestinal ACAT was calculated in terms of specific activity (pmol cholesteryl oleate formed/min/mg protein), and the average specific activities of treated, pair-fed, and ad libitum rats were plotted as a function of the duration of 15-ketosterol administration (figure 35). Intestinal ACAT activity was also calculated as the percentage the activities of pair-fed rats and as the percentage of ad libitum animals. These values were also plotted as a function of the duration of 15-ketosterol administration (figure 36). The data were subsequently analyzed by paired t test and by ANOVA.

The results showed that the ACAT activity in the intestinal microsomes of treated rats was reduced relative to pair-fed and ad libitum controls (ANOVA: mean squares and population variance of treated rats vs mean squares and population variance of pair-fed rats, p=0.0001; mean squares and population variance of treated rats vs mean squares and population variance of ad libitum rats, p=0.0001). The magnitude of the reduction in the intestinal ACAT activity of treated rats was dependent upon the duration of oral administration of 15-ketosterol (ANOVA: mean squares of treated rats as a percentage of pair-fed from 0-10 days vs population variance of treated rats as a percentage of pair-fed, p=0.0001; mean squares of treated rats as a percentage of ad libitum from 0-10 days vs population variance of treated rats as a percentage of ad libitum, p=0.0001). The onset of the reduction of ACAT activity in jejunal microsomes of treated rats occurred prior to the onset of both the changes in the serum cholesterol levels and the changes in intestinal weights of treated rats. As shown above, no significant changes in the serum cholesterol levels or intestinal weights of treated, pair-fed, and ad libitum rats were detected between 0-2 days of oral administration of 0.10% 15-ketosterol. Significant reductions in the ACAT activity of jejunal microsomes of treated rats relative to pair-fed and
Figure 35: Average levels of cholesterol esterification in jejunal microsomes isolated from experimental and control rats plotted as a function time on the dietary regimen. Hatched bars = ad libitum controls, black bars = 0.10% 15-ketosterol-fed rats, open bars = pair-fed controls. Error bars represent the S.E.M. of N = 4 rats/group.
Figure 36: Cholesterol esterification in intestinal microsomes of experimental rats represented as a percentage of control activity and plotted as a function of time on diet. Open bars = % pair-fed controls, hatched bars = % ad libitum controls. Error bars represent the S.E.M. of N = 4 rats/group.
ad libitum controls were observed on day 0 (paired t test: treated vs pair-fed, p<0.001), day 1 (paired t test: treated vs ad libitum, p<0.001), day 2 (paired t test: treated vs pair-fed, p<0.001; treated vs ad libitum, p<0.001), day 4 (paired t test: treated vs pair-fed, p<0.001; treated vs ad libitum, p<0.001), day 6 (paired t test: treated vs pair-fed, p<0.001; treated vs ad libitum, p<0.001), day 10 (paired t test: treated vs pair-fed, p<0.001; treated vs ad libitum, p<0.001). No significant changes between the microsomal ACAT activities of pair-fed and ad libitum rats were detected except on day 4 (paired t test: pair-fed vs ad libitum, p<0.001). In addition, no significant day to day changes in the microsomal ACAT activities of treated or ad libitum rats were detected.

The incorporation of labeled oleoyl CoA into material with the chromatographic mobilities of triacylglycerides and oleic acid was examined by calculating the total dpm generated/assay and plotting the average formation of these compounds as a function of duration of 15-ketosterol administration (figures 37, 38). The data were analyzed by paired t test and by ANOVA. The results showed that oleoyl CoA-dependent formation of triacylglycerides was not significantly affected by oral administration 15-ketosterol, except on day 6 (paired t test: treated vs ad libitum, p<0.001). No significant changes in the oleoyl CoA-dependent synthesis of triacylglycerides were detected between pair-fed and ad libitum rats, except on day 4 (paired t test: pair-fed vs ad libitum, p<0.001). However, the formation of material with the chromatographic mobility of triacylglycerides in treated, pair-fed, and ad libitum rats did vary relative to duration of the dietary regimens (ANOVA: mean squares of treated rats from 0-10 days vs population variance of treated rats from 0-10 days, p=0.0001; mean squares of pair-fed rats from 0-10 days vs population variance of pair-fed rats from 0-10 day, p=0.0001; mean squares of ad libitum rats from 0-10 days vs
Figure 37: Average formation in jejunal microsomes isolated from experimental and control rats of radiolabeled material with the chromatographic mobility of triacylglycerides plotted as a function of time on the dietary regimen. Open bars = pair-fed controls, filled bars = 0.10% 15-ketosterol-fed rats, hatched bars = ad libitum controls. Error bars represent the S.E.M. of N = 4 rats/group.
Figure 38: Average formation in jejunal microsomes isolated from experimental and control rats of radiolabeled material with the chromatographic mobility of oleic acid plotted as a function of time on the dietary regimens. Open bars = pair-fed controls, filled bars = 0.10% 15-ketosterol-fed rats, hatched bars = ad libitum controls. Error bars represent the S.E.M. of N = 4 rats/group.
population variance of ad libitum rats from 0-10 days, p=0.0001). The results also showed that oleoyl CoA-dependent formation of material with the chromatographic mobility of oleic acid was not affected by oral administration of 15-ketosterol. No significant changes in the synthesis of oleic acid were detected between pair-fed and ad libitum rats. However, the formation of material with the chromatographic mobility of oleic acid in treated, pair-fed, and ad libitum rats did vary relative to duration of the dietary regimens (ANOVA: mean squares of treated rats from 0-10 days vs population variance of treated rats from 0-10 days, p=0.0001; mean squares of pair-fed rats from 0-10 days vs population variance of pair-fed rats from 0-10 day, p=0.0001; mean squares of ad libitum rats from 0-10 days vs population variance of ad libitum rats from 0-10 days, p=0.001).

The formation of material with the chromatographic mobility of 15-ketosteryl oleate in experimental and control groups was expressed as the average dpm generated/assay and was plotted as a function of time (figure 39). The amount of radioactivity incorporated into this region was extremely low. Moreover, no consistent effects of oral administration of 15-ketosterol or duration of the compound's administration were detected. These data were consistent with the presence of low levels of 15-ketosterol in jejunal microsomes of treated animals.

Additional experiments were done in which cholesterol was added to incubations of intestinal microsomes, one matched set of experimental, pair-fed, and ad libitum rats per time point. Cholesterol was suspended in a solution of Triton WR-1339 and was added to incubations of jejunal microsomes such that the final concentrations was 100 μM. This concentration was chosen because it had been shown to saturate microsomal ACAT activity\textsuperscript{109}. Enzyme activity was
Figure 39: Average formation in jejunal microsomes isolated from experimental and control rats of radiolabeled material with the chromatographic mobility of 15-ketosteryl oleate plotted as a function of time on a dietary regimen. Open bars = pair-fed controls, filled bars = 0.10% 15-ketosterol-fed rats, hatched bars = ad libitum controls. Error bars represent the S.E.M. of N = 4 rats/group.
measured according to the method of Billheimer\textsuperscript{109}.

Cholesterol esterification by ACAT was calculated in terms of specific activity (pmol cholesteryl oleate formed/min/mg protein), and the activities of both control and exogenous cholesterol incubations were compared (table 10). Addition of 100 $\mu$M cholesterol to incubations of jejunal microsomes isolated from \textit{ad libitum} rats resulted in $535\% \pm 100\%$ stimulation in the oleoyl CoA-dependent esterification of microsomal cholesterol relative to control values. Addition of 100 $\mu$M cholesterol to incubations of jejunal microsomes isolated from pair-fed rats resulted in $393\% \pm 82\%$ stimulation in the oleoyl CoA-dependent esterification of microsomal cholesterol relative to control values. Addition of 100 $\mu$M cholesterol to incubations of jejunal microsomes isolated from treated rats resulted in $626\% \pm 138\%$ stimulation in the oleoyl CoA-dependent esterification of microsomal cholesterol relative to control values. No significant differences in the levels of stimulation of cholesterol esterification between treated, pair-fed, and \textit{ad libitum} rats were detected. Under conditions of 100 $\mu$M exogenous cholesterol, the level of cholesterol esterification in the jejunal microsomes of treated rats was still reduced relative to pair-fed and \textit{ad libitum} controls (t test: treated vs pair-fed, $p<0.001$; treated vs \textit{ad libitum}, 0.01$p<0.02$). Under these same conditions, no significant changes in the level of cholesterol esterification between pair-fed and \textit{ad libitum} rats were detected.
Table 10: The effect of exogenous cholesterol (100 μM) on the level of ACAT activity in jejunal microsomes isolated from treated (0.10% 15-ketosterol), pair-fed and ad libitum rats

<table>
<thead>
<tr>
<th>Duration of Treatment</th>
<th>Treatment group</th>
<th>Cholesterol esterification ± S.E.M.</th>
<th>Percentage Change</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Triton control</td>
<td>100 μM Cholesterol</td>
</tr>
<tr>
<td>Day 0</td>
<td>15-ketosterol</td>
<td>44 ± 0</td>
<td>195 ± 2</td>
</tr>
<tr>
<td></td>
<td>pair-fed</td>
<td>145 ± 27</td>
<td>387 ± 12</td>
</tr>
<tr>
<td></td>
<td>ad libitum</td>
<td>86 ± 5</td>
<td>282 ± 8</td>
</tr>
<tr>
<td>Day 1</td>
<td>15-ketosterol</td>
<td>15 ± 2</td>
<td>68 ± 10</td>
</tr>
<tr>
<td></td>
<td>pair-fed</td>
<td>52 ± 7</td>
<td>204 ± 13</td>
</tr>
<tr>
<td></td>
<td>ad libitum</td>
<td>214 ± 2</td>
<td>863 ± 0</td>
</tr>
<tr>
<td>Day 2</td>
<td>15-ketosterol</td>
<td>27 ± 1</td>
<td>115 ± 7</td>
</tr>
<tr>
<td></td>
<td>pair-fed</td>
<td>40 ± 1</td>
<td>174 ± 6</td>
</tr>
<tr>
<td></td>
<td>ad libitum</td>
<td>45 ± 6</td>
<td>244 ± 4</td>
</tr>
<tr>
<td>Day 4</td>
<td>15-ketosterol</td>
<td>9 ± 3</td>
<td>102 ± 7</td>
</tr>
<tr>
<td></td>
<td>pair-fed</td>
<td>42 ± 2</td>
<td>225 ± 13</td>
</tr>
<tr>
<td></td>
<td>ad libitum</td>
<td>33 ± 2</td>
<td>358 ± 6</td>
</tr>
<tr>
<td>Day 6</td>
<td>15-ketosterol</td>
<td>10 ± 4</td>
<td>108 ± 1</td>
</tr>
<tr>
<td></td>
<td>pair-fed</td>
<td>50 ± 6</td>
<td>254 ± 21</td>
</tr>
<tr>
<td></td>
<td>ad libitum</td>
<td>47 ± 3</td>
<td>299 ± 43</td>
</tr>
<tr>
<td>Day 10</td>
<td>15-ketosterol</td>
<td>15 ± 4</td>
<td>113 ± 11</td>
</tr>
<tr>
<td></td>
<td>pair-fed</td>
<td>29 ± 1</td>
<td>274 ± 1</td>
</tr>
<tr>
<td></td>
<td>ad libitum</td>
<td>27 ± 0.5</td>
<td>224 ± 5</td>
</tr>
</tbody>
</table>
Summary

The in vivo time course study was designed to determine the relationship between duration of oral administration of 0.10% 15-ketosterol and the effect of this compound on ACAT activity in rat jejunal microsomes, food consumption, body weight, serum cholesterol level, and the weight of the small intestine. The results showed that the magnitude of both the reduction in serum cholesterol levels and the enlargement of the small intestine in treated rats was dependent upon the duration of oral administration of 15-ketosterol. Significant changes in either serum cholesterol levels or intestinal weight were not observed on days 1 and 2 of administration of 15-ketosterol, but were detected from 4-10 days of administration of the compound. The results also showed that the magnitude of the reduction in the levels of cholesterol esterification by microsomal ACAT was dependent upon the duration of oral administration of 15-ketosterol. Significant reductions in the levels cholesterol esterification by microsomal ACAT were detected as early as 3 hours after completion of the first meal containing 15-ketosterol. It is interesting to note that the reduction in cholesterol esterification by microsomal ACAT occurred prior to changes in serum cholesterol levels and intestinal weight. As seen in previous studies, the reduction of cholesterol esterification by microsomal ACAT was not accompanied by changes in the synthesis of material with the chromatographic mobilities of triacylglycerides or oleic acid. The levels of radioactivity with the chromatographic mobility of 15-ketosteryl oleate was extremely low in treated, pair-fed, and ad libitum animals, and no consistent effect of either administration of 15-ketosterol or duration of 15-ketosterol administration was detected.

Additional studies in which 100 μM cholesterol suspended in a solution of Triton WR-1339 was added to incubations of jejunal microsomes were done.
Addition of cholesterol in this manner consistently resulted in a stimulation of the synthesis of material with the chromatographic mobility of cholesteryl oleate. No significant differences in the levels of stimulation of cholesterol esterification between treated, pair-fed, and ad libitum rats were detected. The data indicated that the ACAT enzyme present in the jejunal microsomes of treated rats was capable of responding to the addition of cholesterol in a quantitatively similar way as the ACAT enzyme present in the microsomes of pair-fed and ad libitum animals. However, even after addition of cholesterol, the level of cholesterol esterification in the jejunal microsomes of treated rats was still reduced relative to pair-fed and ad libitum controls. These latter results suggested the reduction in the levels of cholesterol esterification by microsomal ACAT upon oral administration of 15-ketosterol were not due changes in the availability of cholesterol, since under conditions in which ACAT activity was saturated with respect to cholesterol, the levels ACAT activity in the jejunal microsomes of treated rats were still reduced relative to control animals.
Chapter 5

The Effect of 5α-Cholest-8(14)-en-3β-ol-15-one on Intestinal ACAT Activity in Rhesus Monkeys
Introduction

The rat is an animal model that is frequently used in the study of cholesterol metabolism and atherosclerosis. There are several factors that make the rat a useful model system. It is easy to obtain large, homogenous populations of animals for study. The rat is easy to maintain, and experimental manipulations involving surgery and/or diet can be accomplished with a great rate of success.

However, the rat possesses characteristics which make it a less than ideal model system. This animal is resistant to atherosclerosis and heart disease. Even under conditions of high dietary cholesterol, the rat does not readily form atherosclerotic lesions. In addition, the architecture of certain rat and human organ systems is not very similar. For instance, the rodent digestive system differs from the human intestinal tract in several ways. The rat possesses a large cecum and a shorter colon. This rodent's intestinal tract does not possess a gall bladder, and the mucosal surface of the small intestine lacks plicae. As a consequence, it is necessary to use animal models in addition to the rat in order to more fully examine various aspects of cholesterol biochemistry.

The nonhuman primates, such as baboon and rhesus monkey, represent an animal model which permits experimentation under conditions which more closely approximate human physiology. Primates, both human and nonhuman, share many biochemical and physiological properties. The architecture of organ systems such as the digestive tract are strikingly similar between humans and nonhuman primates. When exposed to diets high in fat and cholesterol, nonhuman primates, such as rhesus monkeys, undergo several metabolic changes that are similar to humans, including a rise in blood
cholesterol levels, an increase in LDL levels, and the development of atherosclerotic lesions\textsuperscript{113}.

Our laboratory has chosen to use the rhesus monkey in order to extend its investigations into the effect of 15-ketosterol on cholesterol metabolism. Previous work had demonstrated that the sterol was a potent hypocholesterolemic agent in these animals\textsuperscript{23}. Oral administration of the compound at 75 mg/kg body weight/day lowered total LDL and LDL cholesterol as well as raised total HDL and HDL cholesterol. This chapter describes preliminary investigations into the effect of the compound on rhesus intestinal ACAT activity. This work includes the adaptation of the rat microsomal assay system in order to measure intestinal ACAT activity in rhesus monkeys as well as the evaluation of the effect of the compound intestinal ACAT activity.

**Experimental conditions**

Jejunal microsomes were prepared from 17 animals that were used in collaborative investigations between our laboratory and laboratories at the Arteriosclerosis Research Center (Bowman Gray School of Medicine, Wake Forest University, North Carolina). This feeding study was designed as follows.

Initially 10 male monkeys and 8 female monkeys were maintained on an experimental diet containing 0.30 mg cholesterol/kcal of diet for a period of at least 14 months (see chapter 2 for details). The animals were matched on the basis of lipoprotein cholesterol levels and were divided into two groups, experimental and control. At the end of the pretreatment period, the mean body weights of the control male monkeys (6.51 ± 0.21 kg, N = 5) and the experimental male monkeys (7.10 ± 0.4 kg, N = 5) did not differ significantly (t test: p>0.2). Similarly, the mean body weights of female control animals (4.74 ±
0.19 kg, N = 4) and the female experimental animals (4.54 ± 0.30 kg, N = 3) did not differ significantly (t test: p > 0.5).

15-Ketosterol was administered to each experimental animal during the treatment period at a level of 75 mg/kg of body weight/day. The compound was administered daily at ~730 hours as a single dose in the form of a feedball composed of a mixture of the diet (30 gm) and the appropriate amount of 15-ketosterol. The sterol was ground in a mechanical pill grinder and was mixed into the feedball. This procedure did not change the purity of the sterol as judged by TLC (silica gel G, hexane/ethyl acetate 7:3) or by reverse phase HPLC (C18 Rainin column, isopropanol, methanol, water, 5:4:1). During the same treatment period, the control animals received a feedball which did not contain 15-ketosterol. On day #245 of the treatment period, one female experimental monkey (#805) died with a confirmed diagnosis of shigellosis.

After 25.5 months of treatment, the experiment was terminated, and the animals were necropsied. The mean ages of the experimental and control animals at the time of necropsy did not differ (experimentals 89.4 ± 3.5 months, control 88.2 ± 3.0 months; t test, p > 0.7). The necropsy procedure was done as follows. Two animals were necropsied per day for a period of two weeks (one at 10:00 am and the other at 1:00 pm). The dose was given 4 hours before sacrifice. After the drug ball was consumed, food was offered for a period of 1 hour. Just prior to sacrifice, the animals were given ketamine sedation and were brought to the necropsy room. The animals were anesthetized and were then exsanguinated. Approximately 45 minutes into the necropsy, ~ 20 cm of mid-jejunum was obtained and jejunal microsomes were prepared from this sample.
The effect of oral administration of 15-ketosterol on body weight was examined. The results showed that oral administration of 15-ketosterol did not significantly affect the body weights of treated animals (t test: treated vs control, 0.2<p<0.3). After 25.5 months of treatment, the mean body weight of experimental (N = 8) and control (N = 9) monkeys were 8.17 ± 0.93 kg and 7.07 ± 0.4 kg respectively. The average change in mean body weight in the individual animals from the end of the pretreatment period and after 25.5 months of treatment was not significantly different (treated, +26.7 ± 4.3 %; controls, +23.3 ± 4.8%; t test, 0.6<p<0.7). In addition, the corresponding values for the weights of male and female animals were not significantly different.

The effect of oral administration of 15-ketosterol on food consumption was also studied. Analysis of the food consumption on the day of necropsy showed that the total food consumption (30 gm feedball plus meal) of experimental and control animals was not significantly different (t test, 0.2<p<0.3). The total food consumption of experimental and control monkeys on the day of necropsy was 107 ± 21.6 gm and 76.1 ± 10.9 gm respectively.

The microsomal samples obtained from monkeys involved in this feeding study were used to measure jejunal ACAT activity. There were two major goals to the following experiments. The first goal was to determine if the microsomal ACAT activity of a control monkey was affected by direct addition of 15-ketosterol. The second goal was to determine if oral administration of 15-ketosterol could lower ACAT activity in jejunal microsomes isolated from treated rhesus monkeys.
Adaptation of Rat Microsomal Assay Conditions to Measure Rhesus Monkey Jejunal ACAT Activity

In order to determine the effects of 15-ketosterol on rhesus intestinal ACAT activity, it was necessary to establish assay conditions. Since no rhesus monkey assay protocols had been described, the assay method used to measure the human intestinal enzyme was examined. The human ACAT assay method was very similar to the rat intestinal assay conditions used in the studies described in chapters 3 and 4. The human assay method differed from the rat assay protocol in that 0.20 M potassium phosphate buffer was used instead of 0.10 M potassium phosphate buffer, and that 150 μg of microsomal protein was used instead of 20 μg microsomal protein. Although the human assay method used 2.5 mg BSA/assay and 16-17 nmol [1-14C]oleoyl CoA/assay instead of the 3 mg BSA/assay and 20 nmol [1-14C]oleoyl CoA/assay used in the rat assay system, the ratio of BSA to oleoyl CoA was the same in both assay systems (0.15 mg/nmol). As a consequence, the possibility of using the rat intestinal assay protocol to measure ACAT activity in rhesus monkey jejunal microsomes was investigated. Aliquots of jejunal microsomes from one control animal (786) were used in the following studies.

In one experiment, oleoyl CoA-dependent esterification of microsomal cholesterol was assayed as a function of the concentration of rhesus monkey microsomal protein. Varying amounts of jejunal microsomal protein were added to an assay buffer consisting of BSA (6.0 mg/ml), DTT (1.0 mg/ml), and potassium phosphate (0.10 M, pH 7.4). There were 3 replicate tubes per protein concentration. The tubes were incubated for 6 minutes in a shaking water bath (37 °C), and the reactions were initiated with the addition of 20 nmol of [1-14C]oleoyl CoA (11 dpm/pmol). The final assay volume was 0.50 ml. The
reactions were terminated after a 2 minute incubation at 37 °C by the addition of 2:1 chloroform-methanol (5 ml). Zero time assays were included as previously described (Chapter 2). An internal standard of [3H]cholesteryl oleate (10,000 dpm/25 μl toluene) was added, followed by cholesteryl oleate (80 μg), 15-ketosteryl oleate (40 μg), and triolein (40 μg). The unlabeled standards were dissolved in toluene and were added to the tubes as 10 μl aliquots. An additional 10 ml of chloroform-methanol and 2.5 ml of acidified water (pH 3) which had been saturated with chloroform were added. The solutions were thoroughly mixed and were stored overnight at 4 °C. The aqueous phase and the denatured protein at the interphase were removed by aspiration. The organic phase was evaporated to dryness at 40 °C under a stream of nitrogen. The residue was dissolved in chloroform (125 μl), spotted onto a silica gel G plate, and the plate was developed using benzene.

The use of benzene as the TLC solvent system permitted the separation and quantitation of bands of radiolabeled components with the chromatographic mobilities of cholesteryl oleate, triacylglycerides, and oleic acid. Cholesteryl oleate synthesis was calculated in terms of specific activity (pmol cholesteryl oleate formed/min/mg microsomal protein). The formations of material with the chromatographic mobilities of triacylglycerides and oleic acid were calculated in terms of total dpm generated/assay. The levels of cholesteryl oleate synthesis, triacylglyceride formation, and oleic acid synthesis were plotted as functions of the microsomal protein concentration (figure 40).

The results showed that the rat jejunal assay conditions could be used to measure jejunal ACAT activity in rhesus monkeys. Cholesterol esterification (panel A) was linear with respect to protein concentration over the measured range of 0-80 μg/assay (R² = 0.92). Triacylglyceride formation (panel B) was
Figure 40: Formation of radiolabeled lipid products with the chromatographic mobilities of cholesteryl oleate, triacylglycerides, and oleic acid plotted as a function of the amount of microsomal protein present in the ACAT assay mixture. Panel A = cholesteryl oleate. Panel B = triacylglycerides. Panel C = oleic acid. Enzymatic reactions were initiated with 20 nmol of [1-14C]oleoyl CoA and were terminated after a 2 minute incubation at 37 °C. Error bars represent the S.E.M. of triplicate assays.
also linear with respect to protein concentration over the measured range of 0-80 µg/assay ($R^2 = 0.93$). Oleic acid synthesis (panel C) was linear with respect to protein concentration over the measured range of 0-80 µg/assay ($R^2 = 0.97$). In order to remain within the linear region of these curves, rhesus monkey microsomal protein concentration was fixed to 40 µg/assay.

In order to determine if an assay using rhesus monkey microsomes was linear with respect to assay time, the following experiment was done. Rhesus monkey microsomal protein (40 µg) was added to an assay tube containing BSA (6.0 mg/ml), DTT (1.0 mg/ml) and potassium phosphate buffer (0.10 M, pH 7.4). The tubes were incubated as described, and the reactions were initiated with the addition of [1-14C]oleoyl CoA (11 dpm/pmol, 20 nmol). The final volume was 0.50 ml. The reactions were terminated at various times with the addition of 2:1 chloroform-methanol (5 ml). There were 3 replicate assays per time point. The radioactive products were extracted as described above and were separated by TLC using benzene as the solvent. Cholesteryl oleate synthesis was calculated in terms of specific activity (pmol cholesteryl oleate formed/min/mg microsomal protein). The formations of material with the chromatographic mobilities of triacylglycerides and oleic acid were calculated in terms of total dpm generated/assay. The levels of cholesteryl oleate synthesis, triacylglyceride formation, and oleic acid synthesis were plotted as functions of assay time (figure 41).

The results showed that product formation using these assay conditions was linear with respect to time. Cholesterol esterification (panel A) was linear over the measured range of 0-6 minutes ($R^2 = 0.96$). Oleic acid formation (panel C) was also linear over the measured range of 0-6 minutes ($R^2 = 0.94$). Triacylglyceride synthesis (panel B) was linear over the range of 0-4 minutes.
Figure 41: Formation of radiolabeled lipid products with the chromatographic mobilities of cholesteryl oleate, triacylglycerides, and oleic acid plotted as a function of incubation time. Panel A = cholesteryl oleate. Panel B = triacylglycerides. Panel C = oleic acid. Enzymatic reactions were initiated with 20 nmol [1-14C]oleoyl CoA and were incubated at 37 °C for varying lengths of time. The microsomal protein concentration was 40 µg. Error bars represent the S.E.M. of triplicate assays.
(R² = 0.93). In view of these results, the length of time for the assay of rhesus monkey intestinal ACAT activity was fixed at 2 minutes.

In summary, the results from these studies indicated that the assay conditions used to measure rat jejunal ACAT activity could easily be adapted to measure the analogous enzyme in rhesus monkey jejunal microsomes.
Addition of 15-Ketosterol to Incubations of Rhesus Monkey Jejunal Microsomes

The effect of direct addition of 15-ketosterol to incubations of rhesus monkey intestinal microsomes was studied. Aliquots of jejunal microsomes obtained from one control animal (884) were used in the following study. Microsomal protein (40 μg) was added to assay mixtures containing BSA (6.0 mg/ml), DTT (1.0 mg/ml), various concentrations of 15-ketosterol, and potassium phosphate buffer (0.10 M, pH 7.4) in a final volume of 0.50 ml. 15-Ketosterol was added to the assay system as 5 μl aliquots of DMSO solutions. There were 3 replicate tubes per concentration of 15-ketosterol. The assay tubes were incubated for 10 minutes in a shaking water bath (37 °C). The assays were initiated with the addition of 20 nmol [1-14C]oleoyl CoA (11 dpm/pmol), and the reactions were terminated after a 2 minute incubation at 37 °C by the addition of 2:1 chloroform-methanol (5 ml). Zero time assays and incubations with and without 5 μl DMSO (solvent control) were included as controls. The [14C]lipid products were extracted and subsequently analyzed by normal phase TLC using benzene as the eluting solvent. This system permitted the separation and quantification of bands of labeled components with the chromatographic mobilities of cholesteryl oleate, 15-ketosteryl oleate, triacylglycerides, and oleic acid. Cholesterol esterification was calculated in terms of specific activity (pmol cholesteryl oleate formed/min/mg protein), while triacylglyceride synthesis, 15-ketosteryl oleate formation, and oleic acid synthesis were calculated in terms of total dpm/assay. The results were then plotted as a function of 15-ketosterol concentration (figure 42).

Addition of 15-ketosterol to incubations of jejunal microsomes resulted in an inhibition of oleoyl CoA-dependent cholesterol esterification (panel A).
Figure 42: Average formation in jejunal microsomes isolated from control monkey 884 of material with the chromatographic mobilities of cholesteryl oleate, 15-ketosteryl oleate, triacylglycerides, and oleic acid. Jejunal microsomes (40 μg protein) were incubated with 15-ketosterol and $[^{14}C]$oleoyl CoA. Panel A = cholesteryl oleate. Panel B = 15-ketosteryl oleate. Panel C = triacylglycerides (closed circles) and oleic acid (open circles). Enzymatic reactions were initiated with 20 nmol $[^{14}C]$oleoyl CoA and were terminated after a 2 minutes incubation at 37 °C. Error bars represent the S.E.M. of triplicate assays.
significant decline in cholesterol esterification by microsomal ACAT occurred upon the addition of as little as 1 μM 15-ketosterol. The addition of 4.5 μM 15-ketosterol was calculated to produce a 50% decline in the levels of cholesterol esterification. This reduction in the esterification of microsomal cholesterol was accompanied by the formation of [1-14C]labeled material which migrated with authentic standards of 15-ketosteryl oleate (Panel B). Neither triacylglyceride synthesis nor oleic acid formation were significantly affected by the addition of 15-ketosterol to the assay system (panel C).

The esterification of 15-ketosterol by rhesus monkey ACAT was also examined using radiolabeled 15-ketosterol. In this case, [2,4-3H]15-ketosterol was added to incubations of jejunal microsomes (control monkey 882), and the enzymatic reactions were initiated with unlabeled oleoyl CoA. The formation of [2,4-3H]15-ketosteryl oleate was then followed by normal phase TLC.

The experimental protocol was as follows. Microsomal protein (40 μg) was added to assay mixtures containing BSA (6.0 mg/ml), DTT (1.0 mg/ml), various concentrations of [2,4-3H]15-ketosterol, and potassium phosphate buffer (0.10 M, pH 7.4) in a final volume of 0.50 ml. [2,4-3H]15-Ketosterol was added to the assay system as 5 μl aliquots of DMSO solutions. There were 3 replicate tubes per concentration of [2,4-3H]15-ketosterol. The assay tubes were incubated for 10 minutes in a shaking water bath (37 °C). The assays were initiated with the addition of 20 nmol oleoyl CoA (0.50 mM), and the reactions were terminated after a 2 minute incubation at 37 °C by the addition of 2:1 chloroform-methanol (5 ml). Zero time assays and incubations with and without 5 μl DMSO (solvent control) were included as controls. The [3H]lipid products were extracted and were subsequently analyzed by normal phase TLC using benzene as the eluting solvent. Under these conditions, [2,4-3H]15-
ketosterol comigrated with unlabeled oleic acid ($R_f = 0.00$). The formation of radiolabeled material with the chromatographic mobility of 15-ketosteryl oleate was calculated in terms of specific activity (pmol 15-ketosteryl oleate formed/min/mg protein), and these results were plotted as a function of [2,4-$^3$H]15-ketosterol concentration (figure 43).

Addition of [2,4-$^3$H]15-ketosterol to the rhesus monkey microsomes resulted in the formation of radiolabeled material which comigrated with authentic standards of 15-ketosterol oleate. The amount of radioactivity migrating as 15-ketosteryl oleate rose as the concentration of [2,4-$^3$H]15-ketosterol in the assay increased. No other bands of [$^3$H] radioactivity besides that of [2,4-$^3$H]15-ketosterol and [2,4-$^3$H]15-ketosteryl oleate were detected.

In summary, jejunal microsomes isolated from one rhesus monkey were used to evaluate the effect of direct addition of 15-ketosterol intestinal ACAT activity. Addition of 15-ketosterol to the assay system resulted in an inhibition of oleoyl CoA-dependent esterification of microsomal cholesterol. This reduction in cholesterol esterification was specific since neither oleoyl CoA-dependent triacylglyceride synthesis nor oleic acid-formation were significantly affected by 15-ketosterol. Furthermore, the decline in cholesterol esterification was accompanied by the formation of material which comigrated with an authentic standard of 15-ketosteryl oleate. The synthesis of 15-ketosterol oleate was also examined by incubating jejunal microsomes with [2,4-$^3$H]15-ketosterol and unlabeled oleoyl CoA. Only material migrating as [2,4-$^3$H]15-ketosterol and [2,4-$^3$H]15-ketosteryl oleate were detected by normal phase TLC. The results from these combined experiments indicated that 15-ketosterol lowered the levels of cholesterol esterification by the ACAT activity present in jejunal microsomes and served as an alternate substrate for ACAT.
Figure 43: Average formation in jejunal microsomes isolated from monkey 882 of material with the chromatographic mobility of 15-ketosteryl oleate. Jejunal microsomes (40 μg) were incubated with [2,4-3H]15-ketosterol and oleoyl CoA. Enzymatic reactions were initiated with 20 nmol oleoyl CoA and were terminated after a 2 minute incubation at 37 °C. Error bars represent the S.E.M. of triplicate assays.
The Effect of Oral Administration of 15-ketosterol on Jejunal ACAT Activity in Rhesus Monkey Microsomes.

The effect of oral administration 15-ketosterol (75 mg/kg body weight/day as a single oral dose for 25.5 months) on intestinal ACAT activity in jejunal microsomes isolated from rhesus monkeys was examined by measuring enzyme activity in 8 animals that had been consuming a high fat and high cholesterol diet. These values were compared to the intestinal ACAT activities of 9 control monkeys that had been consuming only the diet high in fat and cholesterol.

In all cases, rhesus monkey ACAT activity was assayed in the following manner. Microsomal protein (40 μg) was added to assay mixtures containing BSA (6.0 mg/ml), DTT (1.0 mg/ml), and potassium phosphate buffer (0.10 M, pH 7.4) in a final volume of 0.50 ml. The assay tubes (N=3 replicate tubes/monkey) were incubated for 6 minutes in a shaking water bath (37 °C). The assays were initiated with the addition of 20 nmol [1-14C]oleoyl CoA (11 dpm/pmol), and the reactions were terminated after a 2 minute incubation at 37 °C by the addition of 2:1 chloroform-methanol (5 ml). Zero time assays were included as controls. The [14C]lipid products were extracted and subsequently analyzed by normal phase TLC using benzene as the eluting solvent. The level of cholesterol esterification was calculated in terms of specific activity (pmol cholesteryl oleate formed/min/mg protein), while the formation of material with the chromatographic mobilities of triacylglycerides, 15-ketosteryl oleate, and oleic acid were calculated in terms of total dpm/assay. The results are presented in table 11.
Table 11: Effect of oral administration of 15-ketosterol (75 mg/kg body weight/day for 25.5 months) on oleoyl CoA-dependent formation of material with the chromatographic mobilities of cholesteryl oleate, 15-ketosteryl oleate, triacylglycerides, and oleic acid in the jejunal microsomes isolated from rhesus monkeys.

<table>
<thead>
<tr>
<th>Formation of Radiolabeled components</th>
<th>Experimental ± S.E.M. (N=8)</th>
<th>Control ± S.E.M. (N=9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesteryl oleate (pmol/min/mg)</td>
<td>433 ± 55</td>
<td>636 ± 70</td>
</tr>
<tr>
<td>15-Ketosteryl oleate (total dpm/assay)</td>
<td>61 ± 19</td>
<td>59 ± 29</td>
</tr>
<tr>
<td>Triacylglycerides (total dpm/assay)</td>
<td>2070 ± 491</td>
<td>2700 ± 1160</td>
</tr>
<tr>
<td>Oleic acid (total dpm/assay)</td>
<td>4340 ± 294</td>
<td>4860 ± 411</td>
</tr>
</tbody>
</table>
The data showed that oral administration of 15-ketosterol to rhesus monkeys consuming a diet high in fat and cholesterol significantly reduced the levels of ACAT activity in jejunal microsomes. Cholesterol esterification by intestinal ACAT was reduced 32% in jejunal microsomes isolated from experimental animals (t test: 0.02<p<0.05). This effect appeared to be specific since neither triacylglyceride synthesis nor oleic acid formation were significantly changed. The levels of radioactivity with the chromatographic mobility of 15-ketosteryl oleate were extremely low and were not significantly affected by oral administration of 15-ketosterol. No significant differences between male and female animals were detected. In addition, the levels of ACAT activity in jejunal microsomes isolated from both treated and control monkeys were higher than the levels reported for the human intestinal enzyme (human enzyme, ~210 pmol/min/mg protein)$^{61}$.

The coefficient of variation (standard deviation/N times 100) of experimental and controls animals was calculated in order to compare the variability of the replicate assays. In experimental monkeys, the average coefficients of variation of the formation of material with the chromatographic mobilities of cholesteryl oleate, triacylglycerides, oleic acid, and 15-ketosteryl oleate were 11.3 ± 2.5, 8.5 ± 2.1, 9.0 ± 1.0, and 45.9 ± 19.3, respectively. In control animals, the average coefficients of variation of the formation of material with the chromatographic mobilities of cholesteryl oleate, triacylglycerides, oleic acid, and 15-ketosteryl oleate were 9.6 ± 2.2, 6.9 ± 1.4, 11.2 ± 2.2, and 49.8 ± 6.0, respectively. No significant differences between experimental and control animals were detected.

The interpretation of these combined results required that the effect of oral administration of 15-ketosterol on the level of cholesterol in the microsomal
membranes of treated animals be determined. Since the compound has been shown to inhibit rat intestinal HMG-CoA reductase activity, there was concern that the observed effect of 15-ketosterol on microsomal ACAT activity was a reflection of the concentration of cholesterol in the microsomal membranes. In order to address this question, the levels of free cholesterol and cholesteryl esters were determined. In addition, the level of free 15-ketosterol in jejunal microsomes isolated from experimental and control monkeys was determined.

The levels of these compounds were assayed in the following manner. Aliquots (1.00 ml) of suspensions of jejunal microsomes from each of the 17 monkeys were obtained. [3H]Cholesteryl oleate (2 x 10^4 dpm, 0.4 μg) and [14C]cholesterol (2 x 10^4 dpm, 0.1 ng) were added to each microsomal suspension as internal standards. The samples were extracted according to Folch et al. The eluted samples were subjected to reverse phase HPLC for the separation of cholesterol, 15-ketosterol, and cholesteryl esters. The cholesterol esters were saponified using 10% ethanolic KOH. The levels of free cholesterol, cholesterol of cholesteryl esters, and free 15-ketosterol were quantitated, in the form of their TMS derivatives, by capillary GLC. The sterol samples were analyzed using a Perkin-Elmer Sigma 2000 gas chromatograph equipped with 0.1 μm DB-5 capillary column (30 m). Samples were injected as 1 μl aliquots in a splitless fashion and were eluted using nitrogen as the carrier gas (40 ml/min) and a temperature gradient programmed from 150 °C to 280 °C at a rate of 7.5 °C/min. The temperature of the FID detector was maintained at 290 °C while the temperature of the injection port was programmed to rise from an initial value of 50 °C to 280 °C in ~ 10 sec. All GLC determinations were made in duplicate using triacontane (C_{30}) as an internal standard. Quantitation of the samples was achieved using standard curves constructed for TMS
derivatives of the 15-ketosterol and of cholesterol. The results from these analyses are presented in table 12.

The data showed that oral administration of 15-ketosterol to rhesus monkeys at a dosage of 75mg/kg body weight/day for 25.5 months did not significantly change the levels of free cholesterol or cholesterol of cholesteryl esters in intestinal microsomes (t test: 0.30<p<0.40 and 0.40<p<0.50 respectively). These results suggest that the reduction of ACAT activity was not due to a decrease in the concentrations of cholesterol or cholesteryl esters in the jejunal microsomes isolated from treated animals.

Analysis of the 15-ketosterol content of these intestinal microsomes showed that the concentration of this compound was extremely low (0.50 % of free cholesterol). This result was consistent with the lack of formation of material with the chromatographic mobility of 15-ketosteryl oleate upon assay of these same microsomal samples.

However, the low levels of free 15-ketosterol in the microsomal samples led to concern that the compound was being degraded during analysis. In order to determine that the low level of 15-ketosterol was not due to degradation, 15-ketosterol (lot 310, 1.0 mg) was added to an aliquot of a suspension of jejunal microsomes isolated from one control animal (882). [3H]Cholesteryl oleate (2 x 10^4 dpm, 0.4 μg) and [14C]cholesterol (2 x 10^4 dpm, 0.1 ng) were added to the microsomal suspension as internal standards. The sample was extracted according to Folch et al.111. The eluted sample was subjected to reverse phase HPLC for the separation of cholesterol, 15-ketosterol, and cholesterol esters104. The concentration of 15-ketosterol in the sample was determined using GLC as described above.
Table 12: Effect of oral administration of 15-ketosterol (75 mg/kg body weight/day for 25.5 months) on the levels of free cholesterol, cholesterol of cholesteryl esters, and free 15-ketosterol in jejunal microsomes isolated from rhesus monkeys

<table>
<thead>
<tr>
<th>Sterol</th>
<th>Sterol concentration (μg/mg protein ± S.E.M.)</th>
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<tbody>
<tr>
<td></td>
<td>Experimental*</td>
</tr>
<tr>
<td>free cholesterol</td>
<td>45.8 ± 8.9</td>
</tr>
<tr>
<td>cholesterol of cholesteryl esters</td>
<td>5.0 ± 1.0</td>
</tr>
<tr>
<td>free 15-ketosterol</td>
<td>0.23 ± 0.18</td>
</tr>
</tbody>
</table>

* N= 8 monkeys  ** N = 9 monkeys
The recovery under these conditions was 85%. Of the 1.23 mg 15-ketosterol added to the microsomal suspension, 1.06 mg of 15-ketosterol was recovered. The results suggested that under these experimental conditions, the low levels of 15-ketosterol in the microsomal samples from the jejunums of treated animals were probably not due to degradation of the compound during analysis.

In summary, the rat microsomal ACAT assay procedure was adapted to measure intestinal ACAT activity in rhesus monkeys. This assay protocol was then employed to examine the effect of 15-ketosterol on intestinal ACAT activity in rhesus monkeys. Addition of 15-ketosterol to incubations of rhesus monkey jejunal microsomes resulted in a reduction in the oleoyl CoA-dependent synthesis of material with the chromatographic mobility of cholesteryl oleate. This reduction in cholesteryl oleate synthesis was accompanied by the formation of material with the chromatographic mobility of 15-ketosteryl oleate. In addition, the formation of material with the chromatographic mobilities of triacylglycerides and oleic acid were not affected by direct addition of 15-ketosterol to incubations of jejunal microsomes. Oral administration of 15-ketosterol (75 mg/kg body weight/day for 25.5 months) to rhesus monkeys consuming a diet high in fat and cholesterol resulted in a 32% reduction of cholesterol esterification by intestinal ACAT in jejunal microsomes isolated from treated animals. This reduction appeared to be specific, since the formation of material with the chromatographic mobilities of triacylglycerides and oleic acid were not affected. Extremely low levels of material with the chromatographic mobility of 15-ketosteryl oleate were detected. Analysis of the sterol content of the jejunal microsomes isolated from the monkeys used in this feeding study, showed that there were no changes in the levels of free cholesterol or
cholesterol esters and revealed that the concentration of 15-ketosterol was very low compared to that of cholesterol.
Chapter 6

The Effects of Analogs and/or Metabolites of 3β-Hydroxy-5α-cholest-8(14)-en-15-one on Rat Intestinal ACAT Activity In Vitro
It is well established that 15-ketosterol affects cholesterol metabolism at several levels. The compound has been shown to reduce serum cholesterol levels, serve as a precursor of cholesterol, inhibit absorption of exogenous cholesterol and decrease the levels ACAT activity in jejunal microsomes\textsuperscript{21-28}. In experiments, in which 15-ketosterol was added to incubations of jejunal microsomes, the compound was shown to inhibit esterification of microsomal cholesterol. This inhibition was accompanied by the formation of material with the chromatographic mobility of 15-ketosteryl oleate. In other studies, in which 15-ketosterol was fed to rats (0.1\% in chow diet for 7 days) reduction in the levels of cholesterol esterification in incubations of jejunal microsomes was observed. Under these conditions, no significant formation of material with the chromatographic mobility of 15-ketosteryl oleate was detected. This latter result was consistent with the presence of extremely low levels of 15-ketosterol in microsomes isolated from animals fed 15-ketosterol. These results suggested that the mechanism(s) involved in the reduction of ACAT activity was more complicated than previously thought. As a consequence, it was of interest to evaluate the effects of metabolites of 15-ketosterol on the levels of ACAT activity in jejunal microsomes.

This chapter describes preliminary investigations into the effects of both metabolites and analogs of 15-ketosterol on ACAT activity. These studies include the demonstration of polar metabolites of 15-ketosterol in rat bile, the identification of a novel metabolite of 15-ketosterol, 3β,26-dihydroxy-5α-cholest-8(14)-en-15-one, and the evaluation of the effect of 3β,26-dihydroxy-5α-cholest-8(14)-en-15-one and other analogs of 15-ketosterol on intestinal ACAT activity.
Metabolism of 15-Ketosterol after Intravenous Administration to Bile Duct-Cannulated Rats

The metabolic disposition of 15-ketosterol in intact animals is important to an understanding of the mechanisms of action of this hypocholesterolemic compound. In order to more fully comprehend the fate of 15-ketosterol, the metabolism of this sterol after its intravenous administration to bile duct-cannulated rats was investigated. Biliary secretions were chosen as a subject of study because the hepatic syntheses of bile acids represented the major route for degradation of cholesterol. The bile duct-cannulated rat was selected as an experimental model because it permitted the collection of bile samples at discrete time intervals. Furthermore, the collection of uncontaminated bile samples facilitated the chemical analyses of bile. The following studies were part of a collaborative effort between myself, Arthur J. Chu, Akihiro Izumi, Phuong T. Nguyen, Ker-Shi Wang, Joanna M. Little, Bette C. Sherrill, Alemka Kisic, and George J. Schroepfer, Jr.

The experiments followed the same general protocol. Sprague-Dawley rats were maintained on a light-dark cycle (light 6:00 AM to 6:00 PM) and were fed a laboratory chow diet (Purina Formulab 5008). At the start of an experiment, the rats were anesthetized with ether, and bile duct cannulations were performed. Two silk 5-0 sutures were placed underneath the bile duct approximately 1 cm distal to the duct bifurcation. The bile duct was nicked between the sutures; dry PE 10 tubing was inserted; and the tip of the cannula was adjusted so as not to enter the bifurcation. After securing the cannula with the sutures, the cannula was exteriorized through the abdominal wall for bile collection. After surgery, the animals were placed in restraining cages, and were given free access to water and to food. Approximately 1.6 hours later,
intravenous injections of \([4-^{14}C]15\)-ketosterol in a mixture of PEG 400, saline, and ethanol (10.9: 1.0: 1.0) were made into the femoral vein. In other experiments, a portion of pooled bile samples (0-2 hours) from rats given an intravenous injection of \([4-^{14}C]15\)-ketosterol were injected into the duodenum of anesthetized rats which had been cannulated ~1 hour earlier. In all cases, bile was collected into preweighed vessels every 10 minutes for 2 hours, then every 20 minutes for 3 hours, and hourly thereafter for up to 48 hours. After determination of the weight of the collected bile, aliquots were taken for assay of radioactivity. Lipids were extracted from the bile using the method of Folch and coworkers\(^{111}\). The extracted lipids were also assayed for radioactivity. Upon termination of an experiment, selected tissues and organs were analyzed for the distribution of radioactivity.

In selected cases, intestinal lymph cannulations were performed on rats. After induction of ether anesthesia, the main intestinal lymph duct was cannulated with P.E. 50 tubing and was sutured into position. The cannula was exteriorized through the abdominal wall. While under anesthesia, portions of pooled bile samples (0-2 hours) from rats given an intravenous injection of \([4-^{14}C]15\)-ketosterol were injected into the duodenum of each of 3 male rats which had received mesenteric lymph cannulations 30-55 minutes previously. The rats were placed in restraining cages and were allowed free access to water. Lymph was collected into preweighed vessels every 2 hours for 48 hours. After determination of the weight of the collected lymph, aliquots were taken for assay of radioactivity.
Results

Intravenous injection of [4-\(^{14}\)C]15-ketosterol into male bile-duct cannulated rats demonstrated a very rapid and substantial conversion of 15-ketosterol to polar biliary metabolites. Of the administered \(^{14}\)C, 86.1 ± 1.3% was recovered in bile after 38 hours. Of the total amount of \(^{14}\)C recovered in bile after 38 hours, 49.7 ± 4.1% was excreted into bile in the first 70 minutes, and 90.1 ± 1.2% was excreted within 8 hours after injection of [4-\(^{14}\)C]15-ketosterol. The lipid and aqueous phases from Folch extracts of the bile samples collected in the first two hours after intravenous administration of [4-\(^{14}\)C]15-ketosterol were analyzed for radioactivity. The results showed that 95.3 ± 0.4% of the \(^{14}\)C radioactivity was recovered in the aqueous phase, while only 1.7 ± 0.1% of the radioactivity was recovered in the lipid phase.

A portion of the lipid phase (3.68 \(\times\) 10\(^4\) dpm) of pooled bile samples (0-2 hours) was subjected to silicic acid -Super Cel column chromatography (100 x 1 cm) along with unlabeled samples of cholesterol (5.0 mg), cholesteryl palmitate (5.0 mg), 15-ketosterol (5.0 mg) and 15-ketosteryl palmitate (6.0 mg). The column was successively eluted with toluene (fractions 1-140, 0.113 ml/min), 9:1 toluene/ether (fractions 141-272, 0.106 ml/min), 2:1 chloroform/methanol (fractions 273-307, 0.125 ml/min), and methanol (fractions 308-330). The resulting chromatogram is shown in figure 44. Total recovery of radioactivity was 106%. Most (82%) of the eluted \(^{14}\)C was more polar than 15-ketosterol. Approximately 5.1% and 12.9% of the \(^{14}\)C dpm eluted with cholesterol and 15-ketosterol respectively. None of the eluted \(^{14}\)C had the mobility of cholesterol esters or 15-ketosterol esters. Since only 2.5% of the total \(^{14}\)C of this bile sample was recovered in the lipid extract and since the extraction of added labeled 15-ketosterol from bile into the lipid phase was
Figure 44: Silicic acid-Super Cel column chromatography of the lipid extract of pooled bile samples (0-2 hrs) from rats injected with \([^{14}C]\)15-ketosterol. Closed circles represent the \([^{14}C]\) dpm, while open circles represent the absorbances of the mass standards. Peak 1 = cholesteryl palmitate. Peak 2 = 15-ketosteryl palmitate. Peak 3 = cholesterol. Peak 4 = 15-ketosterol. Peaks 5-7 are comprised of unknown polar compounds.
essentially quantitative, the total amount of free \[4^{-14}C\]15-ketosterol and free \[4^{-14}C\]cholesterol in the pooled bile sample could be calculated. These results indicated that bile collected from rats injected with \[4^{-14}C\]15-ketosterol contained very little free \([14C]\)cholesterol (~0.13%) or free \([14C]\)15-ketosterol (~0.33%). It was apparent that the \([14C]\) radioactivity appearing in bile was predominately composed of metabolites which were more polar than 15-ketosterol.

Although most of the injected \(^{14}C\) radioactivity was recovered in bile, a small portion did remain in blood, organs and other tissues. In order to determine the fate of \(^{14}C\) in these compartments, the plasma, red blood cells, organs and other tissues of rats injected with \([4^{-14}C]\)15-ketosterol were also analyzed for their \(^{14}C\) content. Of the radioactivity which was not recovered in bile or other excreta after 48 hours, most (79%) was recovered in the form of cholesterol (68.2%) and cholesterol esters (10.9%). Smaller amounts of radioactivity were associated with free 15-ketosterol (5.9%) and esters of 15-ketosterol (11.2%). Only ~3% of the \(^{14}C\) in the lipid extracts from blood, organs, and other tissues (0.35% of administered \([4^{-14}C]\)15-ketosterol) had the chromatographic mobility of material more polar than 15-ketosterol.

Additional experiments were designed in order to determine if these polar biliary metabolites underwent enterohepatic circulation. In one set of experiments, 3 male rats with bile duct cannulae received \([4^{-14}C]\)15-ketosterol as described. Bile was collected for the next 44 hours. The samples from 0-2 hours were pooled and were injected into the duodenum of each of 3 rats which had been fitted with bile cannulae. Bile was collected from these animals and was analyzed for radioactivity. The results showed that over the 44 hour collection period, 57.7 ± 2.9% of the radioactivity from injected bile appeared
in subsequent bile samples, indicating that the polar metabolites of 15-
ketosterol underwent substantial enterohepatic circulation. In another set of
experiments, pooled bile samples (0-2 hours) from animals given [4-14C]15-
ketosterol were injected into the duodenum of rats fitted with lymph cannulae.
Lymph was collected for 48 hours and was subsequently assayed for
radioactivity. The results showed that 1.2 ± 0.2% of the administered 14C
radioactivity was recovered in mesenteric lymph.

In summary, the results from these experiments demonstrated that 15-
ketosterol underwent a rapid and substantial metabolism to polar biliary
metabolites after intravenous injection into rats. A large fraction of the polar
biliary metabolites underwent enterohepatic circulation. Only trace amounts of
these metabolites were absorbed through mesenteric lymph. The detection of
polar biliary metabolites of 15-ketosterol suggested the possibility that these
compounds were somehow involved in the actions of 15-ketosterol upon
administration to intact animals. As a consequence, it was of interest to
determine if polar biliary metabolites of 15-ketosterol were biologically active
compounds. Since intestinal epithelial cells were exposed to these biliary
metabolites of 15-ketosterol, the effect of these metabolites on ACAT activity in
rat jejunal microsomes was examined.
The Effect of (25 R)-3β,26-Dihydroxy-5α-cholest-8(14)-en-15-one, a Metabolite of 15-Ketosterol, on Rat Intestinal ACAT Activity In Vitro

The investigations into the effect of 15-ketosterol after intravenous administration to bile duct-cannulated rats showed that the compound was converted into polar biliary metabolites. The results of further analyses of bile obtained from rats administered [4-14C]15-ketosterol indicated that the polar metabolites were derived not only from the metabolism of 15-ketosterol to cholesterol and then to typical bile acids, but also from a pathway initiated by the hydroxylation of carbon 26 (unpublished data). The enzymatic side chain hydroxylation of 15-ketosterol was demonstrated in studies with rat liver mitochondria. The results from these experiments showed that incubation of [4-14C]15-ketosterol with rat liver mitochondria, MgCl₂, and NADPH generated [4-14C]3β,26-dihydroxy-5α-cholest-8(14)-en-15-one as a major metabolite. The compound was isolated and purified using normal phase TLC and reverse phase HPLC, and the structure was established using a combination of chromatographic, mass spectral, and nuclear magnetic resonance (NMR) techniques. (25 R)-3β,26-dihydroxy-5α-cholest-8(14)-en-15-one was then chemically synthesized, and its structure was established through X-ray crystallography as well as through a combination of chromatographic, mass spectral, and NMR techniques. The properties of the isolated compound were compared to those of the synthetic compound, and the properties of the two compounds were found to be identical.

In order to determine if 3β,26-dihydroxy-5α-cholest-8(14)-en-15-one affected the levels of microsomal ACAT activity, chemically synthesized (25 R)-3β,26-dihydroxy-5α-cholest-8(14)-en-15-one was added to incubations of rat jejunal microsomes. The experiment was designed as follows. Two sets of
titration curves were prepared, a separate series of tubes for 15-ketosterol and
(25 R)-3β,26-dihydroxy-5α-cholesta-8(14)-en-15-one. Microsomal protein (20
μg) was added to assay mixtures containing BSA (6.0 mg/ml), DTT (1.0 mg/ml),
various concentrations of the sterol of interest, and potassium phosphate buffer
(0.10 M, pH 7.4). The final assay volume was 0.50 ml. The sterols were added
to the assay system as 5 μl aliquots of DMSO solutions. Zero time assays and
incubations with and without 5 μl DMSO were included as controls. All assays
were done in triplicate. The assay tubes were incubated for 10 minutes in a
shaking water bath (37 °C). The assays were initiated with the addition of [1-
14C]oleoyl CoA (11 dpm/pmol, 20 nmol), and the reactions were terminated
after a 2 minute incubation at 37 °C by the addition of 2:1 chloroform-methanol
(5 ml). An internal standard of [3H]cholesteryl oleate (10,000 dpm/ 25 μl
toluene) was added followed by cholesteryl oleate (80 μg), 15-ketosteryl oleate
(40 μg), and triolein (40 μg). The unlabeled standards were dissolved in toluene
and were added to the tubes as 10 μl aliquots. An additional 10 ml of
chloroform-methanol and 2.5 ml of acidified water (pH 3) which had been
saturated with chloroform were added. The solutions were thoroughly mixed
and were stored overnight at 4 °C. The aqueous phase and the denatured
protein at the interphase were removed by aspiration. The organic phase of
each assay tube was evaporated to dryness at 40 °C under a stream of
nitrogen. The residue was dissolved in chloroform (125 μl), spotted onto a
silica gel G plate, and the plate was developed using benzene. Bands of
labeled components with the chromatographic mobilities of cholesteryl oleate,
15-ketosteryl oleate, triolein, and oleic acid were scraped and quantitated.
Under these TLC conditions, both 15-ketosterol and (25 R)-3β,26-dihydroxy-5α-
cholesta-8(14)-en-15-one comigrated with oleic acid (Rf = 0.00). The levels of
cholesterol esterification were calculated in terms of specific activity (pmol cholesteryl oleate formed/min/mg protein). The levels of 15-ketosteryl oleate formation, triacylglyceride synthesis, and oleic acid formation were calculated in terms of total dpm generated/assay.

Addition of 15-ketosterol to incubations of intestinal microsomes resulted in an inhibition of oleoyl CoA-dependent cholesterol esterification (figure 45). Significant declines in the formation of material with the chromatographic mobility of cholesteryl oleate were detected at a concentration as low as 1.0 μM 15-ketosterol. A 50% reduction in the levels of cholesterol esterification occurred with the addition of 3.0 μM 15-ketosterol. This reduction in cholesterol esterification was accompanied by the formation of 14C labeled material with the chromatographic mobility of 15-ketosteryl oleate. The formation of radio-labeled material with the chromatographic mobilities of triacylglycerides and oleic acid was not affected by the addition of 15-ketosterol to the assay system. These results are in excellent agreement with previous studies (Chapter 3).

Addition of (25 R)-3β,26-dihydroxy-5α-cholest-8(14)-en-15-one to the ACAT assay system also resulted in an inhibition of oleoyl CoA-dependent esterification of microsomal cholesterol. However, the sterol was not as potent as its parent compound. A 55% reduction in cholesterol esterification was observed at 10 μM (25 R)-3β,26-dihydroxy-5α-cholest-8(14)-en-15-one (figure 46). The formation of radiolabeled material with the chromatographic mobilities of triacylglycerides and oleic acid was not affected by addition of (25 R)-3β,26-dihydroxy-5α-cholest-8(14)-en-15-one to the assay system.

In order to determine if (25 R)-3β,26-dihydroxy-5α-cholest-8(14)-en-15-one was a substrate for intestinal ACAT, [4-14C]3β,26-dihydroxy-5α-cholest-8(14)-en-15-one (10 μM) was added to incubations of intestinal microsomes.
Figure 45: Average formation of radiolabeled lipid products with the chromatographic mobilities of cholesteryl oleate, 15-ketosteryl oleate, triacylglycerides, and oleic acid upon incubation of rat jejunal microsomes with 15-ketosterol and [1-14C]oleoyl CoA. Panel A = cholesteryl oleate (closed circles) and 15-ketosteryl oleate (open circles). Panel B = triacylglycerides. Panel C = oleic acid. Enzymatic reactions were initiated with 20 nmol of [1-14C]oleoyl CoA and were terminated after a 2 minute incubation at 37 °C. Error bars represent the S.E.M. of triplicate assays.
Figure 46: Average formation of radiolabeled lipid products with the chromatographic mobilities of cholesteryl oleate, triacylglycerides, and oleic acid upon incubation of rat jejunal microsomes with (25 R)-3β,26-dihydroxy-5α-cholest-8(14)-en-15-one and [1-14C]oleoyl CoA. Panel A = cholesteryl oleate. Panel B = triacylglycerides. Panel C = oleic acid. Enzymatic reactions were initiated with 20 nmol of [1-14C] oleoyl CoA and were terminated after a 2 minute incubation at 37 °C. Error bars represent the S.E.M. of triplicate assays.
The assays were run as described with the exception that the enzymatic reactions were initiated with 20 nmol of unlabeled oleoyl CoA. Under these conditions, only radioactivity corresponding to free 3β,26-dihydroxy-5α-cholest-8(14)-en-15-one was detected. These results strongly suggested that 26-OH-15-ketosterol was not a substrate for intestinal ACAT.

In summary, (25 R)-3β,26-dihydroxy-5α-cholest-8(14)-en-15-one was found to be an active metabolite of 15-ketosterol. Addition of (25 R)-3β,26-dihydroxy-5α-cholest-8(14)-en-15-one to rat jejunal microsomes resulted in a reduction of cholesterol esterification. The formation of the oleate ester of (25 R)-3β,26-dihydroxy-5α-cholest-8(14)-en-15-one was not detected. The effect of (25 R)-3β,26-dihydroxy-5α-cholest-8(14)-en-15-one appeared to be specific, since neither oleoyl CoA-dependent triacylglyceride synthesis nor oleoyl CoA-dependent oleic acid formation were affected by addition of the compound to the assay system.
The Effect of Analogs of 15-Ketosterol on Rat Intestinal ACAT Activity In Vitro

Experiments with (25 R)-3β,26-dihydroxy-5α-cholest-8(14)-en-15-one had demonstrated that metabolites of 15-ketosterol were capable of affecting intestinal ACAT activity. In order to expand upon these results, two analogs of 15-ketosterol, 3α-hydroxy-5α-cholest-8(14)-en-15-one and 5α-cholest-8(14)-en-3,15-dione were tested for their effects upon ACAT activity in incubations of rat jejunal microsomes. The effects of these two oxygenated sterols on sterol metabolism have been previously studied98.

3α-Hydroxy-5α-cholest-8(14)-en-15-one is the 3α epimer of the 15-ketosterol in which the hydroxyl group at carbon 3 is in an axial rather than an equatorial orientation to the plane of the carbon rings. Previous work has shown that 3α-hydroxy-5α-cholest-8(14)-en-15-one is an inhibitor of HMG-CoA reductase activity in cultured L cells. The concentration of 3α-hydroxy-5α-cholest-8(14)-en-15-one required for a 50% reduction in the level of HMG-CoA reductase activity is 3 μM. The effect of the compound on microsomal ACAT activity was of interest, since 3α-hydroxy derivatives were found to be minor reaction products upon incubation of [4-14C]15-ketosterol with preparations of rat liver mitochondria.

5α-Cholest-8(14)-en-3,15-dione has also been shown to be as potent an inhibitor of HMG-CoA reductase activity as 15-ketosterol. The concentration of 5α-cholest-8(14)-en-3,15-dione required for a 50% reduction in the level of HMG-CoA reductase activity is 0.4 μM. The sterol has also been shown to lower blood cholesterol in rats85. This compound may also serve as an intermediate in the biological conversion of 3β-hydroxy-5α-cholest-8(14)-en-15-one to 3α-hydroxy-5α-cholest-8(14)-en-15-one. In view of this possibility, the effect of 5α-cholest-8(14)-en-3,15-dione on microsomal ACAT was determined.
The experiments were designed as follows. A titration curve was prepared for each compound. Microsomal protein (20 μg) was added to assay mixtures containing BSA (6.0 mg/ml), DTT (1.0 mg/ml), various concentrations of the sterol of interest, and potassium phosphate buffer (0.10 M, pH 7.4). The final assay volume was 0.50 ml. The sterols were added to the assay system as 5 μl aliquots of DMSO solutions. Zero time assays and incubations with and without 5 μl carrier solvent were included as controls. Replicate assays (N=4) were done for every concentration of sterol. The assay tubes were incubated for 10 minutes in a shaking water bath (37 °C). The assays were initiated with the addition of [1-14C]oleoyl CoA (11 dpm/pmol, 20 nmol), and the reactions were terminated after a 2 minute incubation at 37 °C by the addition of 2:1 chloroform-methanol (5 ml). An internal standard of [3H]cholesteryl oleate (10,000 dpm/ 25 μl toluene) was added, followed by cholesteryl oleate (80 μg), 15-ketosteryl oleate (40 μg), and triolein (40 μg). The unlabeled standards were dissolved in toluene and were added to the tubes as 10 μl aliquots. An additional 10 ml of chloroform-methanol and 2.5 ml of acidified water (pH 3) which had been saturated with chloroform were added. The solutions were thoroughly mixed and were stored overnight at 4 °C. The aqueous phase and the denatured protein at the interphase were removed by aspiration. The organic phase of each assay tube was evaporated to dryness at 40 °C under a stream of nitrogen. The residue was dissolved in chloroform (125 μl), spotted onto a silica gel G plate, and the plate was developed using benzene. Bands of labeled components with the chromatographic mobilities of cholesteryl oleate, 15-ketosteryl oleate, triolein, and oleic acid were scraped and quantitated. The levels of cholesterol esterification were calculated in terms of specific activity (pmol cholesteryl oleate formed/min/mg protein). The levels of 15-ketosteryl
oleate formation, triacylglyceride synthesis, and oleic acid formation were calculated in terms of total dpm generated/assay.

Addition of 3α-hydroxy-5α-cholest-8(14)-en-15-one to jejunal microsomes resulted in a reduction of oleoyl CoA-dependent cholesterol esterification (figure 47). However, the sterol was not as potent as 15-ketosterol. The concentration of 3α-hydroxy-5α-cholest-8(14)-en-15-one required for a 50% reduction in the level of ACAT activity was 14 μM. Addition of the compound had no effect on the oleoyl CoA-dependent formation of material with the chromatographic mobility of triacylglycerides. However, the formation of material with the chromatographic mobility of oleic acid was mildly reduced by addition of 3α-hydroxy-5α-cholest-8(14)-en-15-one (panel C). Extremely low levels of radioactivity were incorporated into material with the chromatographic mobility of 15-ketosteryl oleate, and no consistent effect of addition of 3α-hydroxy-5α-cholest-8(14)-en-15-one was detected. No other bands of labeled components were detected upon TLC analysis. These latter results suggested that, under these conditions, the 3α-OH epimer of 15-ketosterol was not esterified by rat jejunal ACAT.

Addition of 5α-cholest-8(14)-en-3,15-dione to the ACAT assay system resulted in a reduction of ACAT activity (figure 48). This oxysterol was not as potent an inhibitor as 15-ketosterol. The concentration of 5α-cholest-8(14)-en-3,15-dione required for a 50% reduction in the level of ACAT activity was 13 μM. Addition of the compound had no effect on the oleoyl CoA-dependent formation of material with the chromatographic mobility of triacylglycerides. However, the formation of material with the chromatographic mobility of oleic acid was mildly reduced by addition of 100 μM 5α-cholest-8(14)-en-3,15-dione (panel C). In addition, extremely low levels of radioactivity were incorporated into material
Figure 47: Formation of radiolabeled lipid products with the chromatographic mobilities of cholesteryl oleate, 15-ketosteryl oleate, triacylglycerides, and oleic acid upon incubation of rat microsomes (20 µg) with 3α-hydroxy-5α-cholesterol-8(14)-ene-15-one and [1-14C]oleoyl CoA. Panel A = cholesteryl oleate. Panel B = 15-ketosteryl oleate. Panel C = triacylglycerides (closed circles) and oleic acid (open circles). Enzymatic reactions were initiated with 20 nmol [1-14C]oleoyl CoA and were terminated after a 2 minute incubation at 37 °C. Error bars represent the S.E.M. of replicate assays (N = 4).
Figure 48: Formation of radiolabeled lipid products with the chromatographic mobilities of cholesteryl oleate, 15-ketosteryl oleate, triacylglycerides, and oleic acid upon incubation of rat jejunal microsomes (20 μg) with 5α-cholest-8(14)-en-3,15-dione and [1-14C]oleoyl CoA. Panel A = cholesteryl oleate. Panel B = 15-ketosteryl oleate. Panel C = triacylglycerides (closed circles), and oleic acid (open circles). Enzymatic reactions were initiated with 20 nmol [1-14C] oleoyl CoA and were terminated after a 2 minute incubation at 37 °C. Error bars represent the S.E.M. of replicate assays (N = 4).
with the chromatographic mobility of 15-ketosteryl oleate, and no consistent
effect of addition of 5α-cholest-8(14)-en-3,15-dione was detected. This latter
result suggested that, under these conditions, 5α-cholest-8(14)-en-3,15-dione
was not converted to 15-ketosterol and subsequently esterified.

In summary, two analogs of 15-ketosterol, 3α-hydroxy-5α-cholest-8(14)-
en-15-one, and 5α-cholest-8(14)-en-3,15-dione were tested for their effects on
ACAT activity in incubations of rat jejunal microsomes. Both sterols inhibited
the oleoyl-CoA dependent esterification of microsomal cholesterol. Under the
assay conditions used, no oleoyl CoA-dependent esterification of 3α-hydroxy-
5α-cholest-8(14)-en-15-one was detected. This latter finding was consistent
with results of previous studies which reported that the 3β-hydroxy function was
essential to the esterification of plant sterols by rat intestinal ACAT77.
Chapter 7

Discussion
The regulation of ACAT activity is an important aspect of cholesterol metabolism. The oleoyl CoA-dependent esterification of cholesterol by ACAT is considered to be important to both the intracellular metabolism of cholesterol and cholesterol absorption\textsuperscript{37-39}. However, the mechanism of the regulation of ACAT activity is not well understood, and scant information is available concerning the effect of oxygenated sterols on ACAT activity.

In recent years, one oxygenated sterol, 15-ketosterol, has been shown to be a biologically active compound which reduces serum cholesterol levels, inhibits sterol biosynthesis, alters sterol transport, and lowers cholesterol absorption\textsuperscript{21-28}. As part of ongoing investigations into the mechanisms of action of 15-ketosterol, the effect of this oxygenated sterol on ACAT activity was studied. Larry Miller and John Brabson had previously demonstrated that addition of 15-ketosterol to incubations of either rat hepatic or rat jejunal microsomes reduced the oleoyl CoA-dependent esterification of microsomal cholesterol. In conjunction with this study, they observed that this decline in cholesterol esterification was accompanied by the formation of material with the chromatographic mobility of 15-ketosteryl oleate.

The effect of 15-ketosterol on microsomal ACAT activity was of interest since it indicated that administration of the compound affected the level of intracellular cholesterol esterification. These results suggested that changes in ACAT activity could be involved in the observed inhibition of exogenous [4-\textsuperscript{14}C] cholesterol absorption by oral administration of 15-ketosterol\textsuperscript{28}. In view of the results reported by Larry Miller and John Brabson, the following studies were undertaken in order to more fully characterize the effect of 15-ketosterol on microsomal ACAT activity.
The preliminary studies concerning the action of 15-ketosterol on rat jejunal ACAT activity were done using slight modifications of the method of Suckling and coworkers\textsuperscript{43}. However, in our hands, under these assay conditions, cholesterol esterification was not linear with respect to microsomal protein concentration over the range of 10 to 200 $\mu$g protein/assay. As a result, the assay protocol was investigated, and the assay method was modified in order to obtain conditions in which the oleoyl CoA-dependent formation of material with the chromatographic mobilities of cholesteryl oleate, triacylglycerides and oleic acid were linear with respect to both microsomal protein concentration and assay time.

The redesigned rat jejunal ACAT assay system was then used to confirm the results regarding the effect of 15-ketosterol on rat jejunal ACAT activity. Addition of 15-ketosterol to incubations of rat jejunal microsomes resulted in a concentration-dependent decrease in the oleoyl CoA-dependent formation of material with the chromatographic mobility of cholesteryl oleate. The concentration of 15-ketosterol required for a 50% reduction in cholesterol esterification was 3 $\mu$M. In addition, this decline in cholesterol esterification was accompanied by the formation of material with the chromatographic mobility of 15-ketosteryl oleate. The addition of 15-ketosterol to incubations of rat jejunal microsomes did not significantly affect the oleoyl CoA-dependent formation of material with the chromatographic mobilities of either triacylglycerides or oleic acid.

In order to expand upon these results, the effect of 15-ketosterol on rhesus monkey jejunal ACAT activity was examined. In these experiments, 15-ketosterol was added to incubations of rhesus monkey jejunal microsomes, and the oleoyl CoA-dependent esterification of cholesterol was assayed using slight
modifications of the optimized rat jejunal ACAT assay method. The microsomal samples were isolated from the jejunum of a monkey that was fed a diet high in fat and cholesterol for a period of 39.5 months. Addition of 15-ketosterol to incubations of rhesus monkey jejunal microsomes resulted in a concentration-dependent decrease in the oleoyl CoA-dependent formation of material with the chromatographic mobility of cholesteryl oleate. The concentration of 15-ketosterol required for a 50% reduction in cholesterol esterification was 4.5 µM. In addition, this decline in cholesterol esterification was accompanied by the formation of material with the chromatographic mobility of 15-ketosteryl oleate. The addition of 15-ketosterol to incubations of rhesus monkey jejunal microsomes did not significantly affect the oleoyl CoA-dependent formation of material with the chromatographic mobilities of either triacylglycerides or oleic acid. These results represented the first demonstration that 15-ketosterol both inhibited the esterification of microsomal cholesterol by rhesus monkey ACAT and served as an alternate substrate for this enzyme.

The effect of oral administration of 15-ketosterol on rat microsomal ACAT activity was also examined. In one set of experiments, 15-ketosterol was fed to rats at a level of 0.10% in the chow diet for a period of 7 days prior to the preparation of hepatic and jejunal microsomes from individual rats. The data indicated that the sterol slightly reduced the levels of oleoyl CoA-dependent cholesterol esterification by hepatic ACAT (26% vs pair-fed control), but greatly reduced the levels of oleoyl CoA-dependent cholesterol esterification by the intestinal enzyme (77% vs pair-fed control). In another set of experiments, 15-ketosterol was fed at a level of 0.05% in the diet for 7 days. The data indicated that oral administration of 0.05% 15-ketosterol significantly reduced the levels of oleoyl CoA-dependent esterification in jejunal microsomes by 82%. These
combined results represented the first demonstration in intact animals of lowered levels of microsomal ACAT activity upon oral administration of an oxygenated sterol. Analysis of the cholesterol and cholesteryl ester content of jejunal microsomes, isolated from rats fed 0.10% 15-ketosterol, showed that the levels of these compounds did not significantly differ between treated rats, pair-fed rats, and ad libitum controls. These latter results indicated that the observed reduction in rat jejunal ACAT activity upon oral administration of 15-ketosterol was not due to a reduction of the cholesterol concentration of the microsomes as a result of the sterol's action as an inhibitor of HMG-CoA reductase activity. In addition, analysis of the 15-ketosterol content of jejunal microsomes isolated from treated animals indicated that the levels of this compound were very low with respect to cholesterol (~0.6% of cholesterol). In view of these combined results, additional rat feeding studies were done in order to determine how the dosage of 15-ketosterol and the duration of 15-ketosterol administration affected the oleoyl CoA-dependent esterification of cholesterol in rat jejunal microsomes.

In one experiment, the effect of oral administration of 15-ketosterol on rat jejunal ACAT activity was studied with respect to the concentration of 15-ketosterol in the diet. In this study, 0.0%, 0.025%, 0.050%, 0.075%, 0.10%, and 0.125% 15-ketosterol were administered to rats for a period of 9 days prior to the preparation of jejunal microsomes from individual rats. The results showed that oral administration of 0.025% 15-ketosterol did not significantly affect the level of oleoyl CoA-dependent cholesterol esterification in microsomes isolated from treated animals (treated vs pair-fed, treated vs ad libitum). However, administration of 0.050% 15-ketosterol and higher dosages resulted in large reductions of the levels of oleoyl CoA-dependent cholesterol esterification in
jejunal microsomes isolated from treated rats (treated vs pair-fed, treated vs ad libitum). The results also showed that oleoyl CoA-dependent formation of material comigrating with triacylglycerides and oleic acid were not significantly affected by oral administration of 15-ketosterol. Analysis of the cholesterol content of jejunal microsomes showed that the level of this compound did not significantly differ between 0.125% 15-ketosterol-fed rats, pair-fed rats, and ad libitum controls. These latter results indicated that the observed reduction in rat jejunal ACAT activity upon oral administration of 0.125% 15-ketosterol was not due to a reduction of the cholesterol concentration of the microsomes as a result of the sterol's action as an inhibitor of HMG-CoA reductase activity. In addition, extremely low level of radioactivity with the chromatographic mobility of 15-ketosteryl oleate were detected, and no effect of administration of 15-ketosterol was observed.

The effect of oral administration of 15-ketosterol on other variables such as food consumption, body weight, serum cholesterol levels, and the weight of the small intestine was also studied with respect to the concentration of 15-ketosterol in the diet. The results showed that the level of food consumption was affected by the dosage of 15-ketosterol in the diet. At a level of 0.025% 15-ketosterol, no significant changes in food consumption were observed. At a level of 0.050% 15-ketosterol, small decreases in the food consumption of treated and pair-fed rats were observed on days 5-7 (treated vs ad libitum, pair-fed vs ad libitum). At dosages of 0.050% 15-ketosterol and higher, a concentration-dependent decline in total food consumption (days 1-9) was observed. Body weight was also affected by the dosage of 15-ketosterol in the diet. No significant changes between the body weights of treated, pair-fed, and ad libitum rats were detected at dosages of 0.025% and 0.050% 15-ketosterol.
in the diet. At dosages of 0.075% 15-ketosterol and higher, a concentration-dependent decline in the body weights of treated and pair-fed rats was observed (treated vs ad libitum, pair-fed vs ad libitum). In a similar manner, serum cholesterol levels were affected by the concentration of 15-ketosterol in the diet. No significant changes between serum cholesterol levels of treated, pair-fed, and ad libitum rats were detected at dosages of 0.025% and 0.050% 15-ketosterol in the diet. At dosages of 0.075% 15-ketosterol and higher, a concentration-dependent decline in the serum cholesterol levels of treated rats was observed (treated vs ad libitum, pair-fed vs ad libitum). Finally, the degree of intestinal enlargement, as reflected by increases in the weight of the proximal half of the small intestine, was also dependent upon the concentration of 15-ketosterol in the diet. No significant changes between the intestinal weights of treated, pair-fed, and ad libitum rats were detected at a dosage of 0.025% 15-ketosterol in the diet. At dosages of 0.050% 15-ketosterol and higher, a concentration-dependent increase in the intestinal weight of treated rats was observed (treated vs ad libitum). These combined results represented the first systematic demonstration of the dependency of food consumption, body weight, serum cholesterol levels, and intestinal weight upon the dosage of 15-ketosterol in the diet.

In another experiment, the effect of oral administration of 15-ketosterol on rat intestinal ACAT activity was studied with respect to the duration of 15-ketosterol administration. This study was designed to determine the relationships between duration of 15-ketosterol administration and the effect of the compound on intestinal ACAT activity, serum cholesterol level, and the weight of the small intestine. In this experiment, 0.10% 15-ketosterol was fed to rats that were previously adapted to a controlled feeding schedule. Jejunal
microsomes were prepared from animals that had been sacrificed 3 hours after completion of the first meal containing 15-ketosterol, and after 1 day, 2 days, 4 days, 6 days, and 10 days of oral administration of 15-ketosterol.

The results showed that the level of oleoyl CoA-dependent cholesterol esterification in rat jejunal microsomes was reduced by oral administration of 15-ketosterol (treated vs pair-fed, treated vs ad libitum). The magnitude of the reduction of cholesterol esterification by microsomal ACAT was dependent upon the duration of oral administration of 15-ketosterol. Significant reductions in the level of oleoyl CoA-dependent esterification of cholesterol in rat jejunal microsomes were observed as early as three hours after administration of the first meal containing 15-ketosterol (treated vs pair-fed) and were detected after 1 day, 2 days, 4 days, 6 days, and 10 days of oral administration of the sterol (treated vs pair-fed, treated vs ad libitum). These results were the first evidence that reduction of ACAT activity by 15-ketosterol occurred after ingestion of one meal containing 15-ketosterol. As seen in previous studies, oral administration of 0.10% 15-ketosterol did not affect the oleoyl CoA-dependent formation of material with the chromatographic mobilities of either triacylglycerides or oleic acid. Extremely low levels of radioactivity with the chromatographic mobility of 15-ketosteryl oleate were detected, and no consistent effects of either administration of 15-ketosterol or duration of the sterol's administration were observed. This latter result was consistent with the detection of extremely low levels of 15-ketosterol present in the microsomes of treated rats.

The effect of oral administration of 15-ketosterol on serum cholesterol levels and intestinal weights was also studied with respect to the duration of administration of the compound. The results showed that both serum cholesterol levels and the degree of intestinal enlargement, as reflected by an
increase in the weight of the proximal half of the small intestine, were affected by oral administration of 15-ketosterol. The magnitude of these effects was dependent upon the duration of 15-ketosterol administration. No significant changes in either serum cholesterol levels or intestinal weights were observed 3 hours after completion of the first meal containing 15-ketosterol, nor were significant changes in these variables detected after 1 day or 2 days of oral administration of 0.10% 15-ketosterol. Significant changes in both serum cholesterol levels and intestinal weights were observed after 4 days, 6 days, and 10 days administration of 15-ketosterol. It is interesting to note that the reduction in microsomal ACAT activity upon oral administration of 0.10% 15-ketosterol occurred prior to the observed changes in serum cholesterol levels, intestinal weight, food consumption and body weights.

Additional experiments were done in which cholesterol (100 µM) was added to incubations of intestinal microsomes, one matched set of treated, pair-fed, and ad libitum rats per time point. Addition of cholesterol in this manner resulted in a stimulation of the synthesis of material with the chromatographic mobility of cholesteryl oleate. The degree of stimulation was consistent with studies which reported that addition of 100 µM cholesterol to incubations of microsomes saturated ACAT activity with respect to cholesterol. No significant differences in the levels of stimulation of cholesterol esterification between treated, pair-fed, and ad libitum rats were detected. The data indicated that the ACAT enzyme present in the jejunal microsomes of treated rats was capable of responding to the addition of cholesterol in a quantitatively similar way as the ACAT enzyme present in the jejunal microsomes of pair-fed and ad libitum rats. However, even after addition of cholesterol, the level of cholesterol esterification in the microsomes of treated rats was still reduced relative to pair-
fed and ad libitum rats. These latter results suggested that the reduction of ACAT activity in jejunal microsomes isolated from 15-ketosterol-fed rats was not due to changes in the availability of microsomal cholesterol.

The effect of oral administration of 15-ketosterol on the levels of ACAT activity in rhesus monkey jejunal microsomes was also investigated. In this experiment, the compound was administered (75 mg/kg body weight/day for a period of 25.5 months) to rhesus monkeys consuming a diet high in fat and cholesterol. This treatment protocol resulted in a 32% reduction in oleoyl CoA-dependent formation of material with the chromatographic mobility of cholesteryl oleate. This reduction in the ACAT activity of treated monkeys was not accompanied by changes in the oleoyl CoA-dependent formation of material with the chromatographic mobilities of either triacylglycerides or oleic acid. These results represented the first demonstration of lowered levels of microsomal ACAT activity upon oral administration of 15-ketosterol to primates. In addition, extremely low levels of radioactivity with the chromatographic mobility of 15-ketosteryl oleate were detected, and no consistent effect of 15-ketosterol administration was observed. Analysis of the cholesterol and cholesteryl ester content of jejunal microsomes isolated from treated and control monkeys showed that there were no significant changes in the levels of free cholesterol or cholesterol esters as a result of oral administration of 15-ketosterol. These latter results indicated that the observed reduction in rhesus monkey jejunal ACAT activity upon oral administration of 15-ketosterol was not due to a reduction of the cholesterol concentration of the microsomes as a result of the compound's action as an inhibitor of HMG-CoA reductase activity. These analyses also showed that the concentration of 15-ketosterol in the microsomes of treated animals was very low compared to that of cholesterol.
(~0.5% of cholesterol). This result was consistent with the formation of extremely low levels of radioactivity comigrating with 15-ketosteryl oleate during incubations of jejunal microsomes isolated from treated monkeys.

In order to more fully study the metabolic disposition of 15-ketosterol in intact animals, the effect of intravenous administration of radiolabeled 15-ketosterol to bile duct-cannulated rats was investigated. The results from these experiments demonstrated that after intravenous injection into rats, 15-ketosterol underwent a rapid and substantial conversion to polar biliary metabolites. A large fraction of these polar biliary metabolites was subject to enterohepatic circulation. Only trace amounts were absorbed through mesenteric lymph. The results of further analyses of bile obtained from rats given intravenous injections of [4-14C]15-ketosterol indicated that the polar metabolites were derived not only from the metabolism of 15-ketosterol to cholesterol and then to typical bile acids, but also from a pathway initiated by the hydroxylation of carbon 26 (unpublished data and reference 100). Additional experiments with rat liver mitochondria demonstrated the enzymatic side chain hydroxylation of 15-ketosterol. The results from these experiments showed that incubation of [4-14C]15-ketosterol with rat liver mitochondria, MgCl₂, and NADPH generated [4-14C]3β,26-dihydroxy-5α-cholest-8(14)-en-15-one as a major metabolite.

The detection of polar biliary metabolites of 15-ketosterol, such as 3β,26-dihydroxy-5α-cholest-8(14)-en-15-one, suggested the possibility that these compounds were somehow involved in the observed effects of administration of 15-ketosterol to intact animals. (25R)-3β,26-Dihydroxy-5α-cholest-8(14)-en-15-one was previously shown to inhibit the activity of HMG-CoA reductase in cultured CHO cells¹⁰⁰,¹⁰². Since intestinal epithelial cells of 15-ketosterol-
treated animals would be exposed to polar biliary metabolites of 15-ketosterol, the effect of these metabolites, particularly 3β,26-dihydroxy-5α-cholest-8(14)-en-15-one, on the levels of ACAT activity in jejunal microsomes was examined.

*In order to determine if 3β,26-dihydroxy-5α-cholest-8(14)-en-15-one could affect intestinal ACAT activity, chemically synthesized (25R)-3β,26-dihydroxy-5α-cholest-8(14)-en-15-one was added to incubations of rat jejunal microsomes. The addition of 10 μM (25R)-3β,26-dihydroxy-5α-cholest-8(14)-en-15-one to the ACAT assay system resulted in a 55% reduction of the oleoyl CoA-dependent synthesis of material with the chromatographic mobility of cholesteryl oleate. The oleoyl CoA-dependent synthesis of material with the chromatographic mobilities of triacylglycerides and oleic acid were not affected by addition of (25R)-3β,26-dihydroxy-5α-cholest-8(14)-en-15-one to the microsomal assay system. Under these incubation conditions, the oleoyl CoA-dependent esterification of (25R)-3β,26-dihydroxy-5α-cholest-8(14)-en-15-one was not detected. In addition, extremely low levels of radioactivity with the chromatographic mobility of 15-ketosterol oleate were detected, and no consistent effect of the addition of (25R)-3β,26-dihydroxy-5α-cholest-8(14)-en-15-one to the assay system were detected. These combined results represented the first demonstration that a polar biliary metabolite of 15-ketosterol acted as an inhibitor of microsomal ACAT activity.*

*In order to more fully explore the effects of 15-oxygenated sterols on intestinal ACAT activity, two analogs of 15-ketosterol, 3α-hydroxy-5α-cholest-8(14)-en-15-one, and 5α-cholest-8(14)ene-3,15-dione were added to incubations of rat jejunal microsomes. Previous work had shown that these oxygenated sterols were inhibitors of HMG-CoA reductase activity in cultured cells. In addition to their action on sterol biosynthesis, these compounds*
possessed certain characteristics which made the study of their effects on microsomal ACAT activity of interest. For instance, 3α-hydroxy derivatives of 15-ketosterol were detected as minor reactions products upon incubation of [4-14C]15-ketosterol with rat liver mitochondria. In view of the possibility that 3α-hydroxy derivatives could serve as active biological metabolites of 15-ketosterol, the effect of 3α-hydroxy-5α-cholesta-8(14)-en-15-one, the 3α epimer of 15-ketosterol, on microsomal ACAT activity was examined. The effect of 5α-cholesta-8(14)-ene-3,15-dione on microsomal ACAT activity was studied for two reasons. First, 5α-cholesta-8(14)-ene-3,15-dione could serve as an intermediate in the biological conversion of 3β-hydroxy-5α-cholesta-8(14)-en-15-one to 3α-hydroxy-5α-cholesta-8(14)-en-15-one and may therefore be present in intact animals as a metabolite of 15-ketosterol. Second, oral administration of 5α-cholesta-8(14)-ene-3,15-dione to rats results in lowered serum cholesterol levels. This reduction could be partially attributed to a reduction in cholesterol absorption through inhibition of rat jejunal ACAT activity.

The results from these studies demonstrated that both oxygenated sterols reduced the levels of ACAT activity in rat jejunal microsomes, although none of these compounds was as potent as 15-ketosterol. As previously stated, the concentration of 15-ketosterol required for a 50% reduction in cholesterol esterification was 3 μM. In these studies, the concentrations of 3α-hydroxy-5α-cholesta-8(14)-en-15-one and 5α-cholesta-8(14)-en-3,15-dione required for a 50% reduction in cholesterol esterification were 14 μM and 13 μM, respectively.

The results described in this dissertation indicate that 15-ketosterol, the polar biliary metabolite 3β,26-dihydroxy-5α-cholesta-8(14)-en-15-one, and 15-ketosterol analogs, 3α-hydroxy-5α-cholesta-8(14)-en-15-one and 5α-cholesta-8(14)-ene-3,15-dione, are unique among inhibitors of ACAT activity. For
instance, 15-ketosterol is the only ACAT inhibitor to serve as an efficient substrate for ACAT, and is the only oxygenated sterol to lower both serum cholesterol levels and microsomal ACAT activity upon oral administration to rats and rhesus monkeys. In terms of oxygenated sterols, 15-ketosterol, 3β,26-dihydroxy-5α-cholest-8(14)-ene-3,15-dione, 3α-hydroxy-5α-cholest-8(14)-en-15-one, and 5α-cholest-8(14)-en-3,15-dione are the only known oxysterols to reduce cholesterol esterification by ACAT upon direct addition to rat jejunal microsomes. In addition, other investigations have demonstrated that 15-ketosterol is the only ACAT inhibitor which affects several levels of cholesterol metabolism including cholesterol synthesis and cholesterol absorption. Only one other oxygenated sterol, the analog of 7-ketocholesterol (SC 31769), has been reported to both inhibit HMG-CoA reductase activity upon incubation with human fibroblasts and inhibit oleoyl CoA-dependent cholesterol esterification upon direct addition to cell free extracts of human fibroblasts.

The inhibition of ACAT activity by 15-ketosterol, its metabolites, and/or analogs is significant since several groups have reported that other oxygenated sterols, such as 25-hydroxycholesterol, 7-ketocholesterol, and 6-ketocholestanol, stimulate rather than inhibit ACAT activity. For instance, Brown and coworkers examined the effect of 25-hydroxycholesterol, 7-ketocholesterol, and 6-ketocholestanol on the esterification of endogenous cholesterol by cultured human fibroblasts. They reported that incubation of fibroblasts with sodium oleate and each of these sterols resulted in increases in the intracellular content of cholesteryl esters and the suppression of HMG-CoA reductase activity. These authors also reported that the [1-14C]oleoyl CoA-dependent esterification of exogenous cholesterol was increased in microsomes isolated from fibroblasts that had been incubated with 10 μg 25-
hydroxycholesterol. Drevon, Weinstein, and Steinberg reported that incubation of cultured adult rat parenchymal liver cells with \([3H]\)oleate and 10 \(\mu g/ml\) of 25-hydroxycholesterol stimulated cholesterol esterification by 3-6 fold and had no effect on the incorporation of \([3H]\)oleate into phospholipids and triacylglycerides\(^{115}\). The authors also reported that this stimulation of cholesterol esterification reached a maximum after a 15 minute incubation of the cultured cells with 25-hydroxycholesterol. However, after 18 hours of incubation of the cultured cells with 25-hydroxycholesterol, no stimulation of cholesterol esterification was observed. Erickson and coworkers reported that oral administration of 0.1% and 0.5% 7-ketocholesterol to rats for a period of 18 hours inhibited HMG-CoA reductase activity by 60-70%, and increased the total cholesterol and microsomal cholesteryl ester content of the liver\(^{10}\). However, this effect was short-lived, and after 66 hours of oral administration of 7-ketocholesterol, no changes in these variables were observed. Field and Mathur reported that 25-hydroxycholesterol stimulated cholesterol esterification both upon incubation with \([1-14C]\)oleate and intact rabbit intestinal epithelial cells and upon incubation with \([1-14C]\)oleoyl CoA and isolated rabbit jejunal microsomes\(^{116}\). These authors also reported that the stimulation of ACAT activity by 25-hydroxycholesterol was inversely related to the microsomal cholesterol content. In addition, Miller and Melnykovych have reported that incubation of the macrophage-like P388D cells with \([3H]\)oleate and 25-hydroxycholesterol resulted in a stimulation of cholesterol esterification\(^{49}\). It is interesting to note that 25-hydroxycholesterol is not an efficient substrate for oleoyl CoA-dependent esterification by ACAT in rabbit jejunal microsomes\(^{116}\). It has been suggested that these oxygenated sterols, particularly 25-hydroxycholesterol, stimulate ACAT activity by increasing the amount of
cholesterol available for esterification rather than by directly acting upon the enzyme. It is not clear as to why direct addition of 15-ketosterol, 3β,26-dihydroxy-5α-cholest-8(14)-en-15-one, 3α-hydroxy-5α-cholest-8(14)-en-15-one and 5α-cholest-8(14)-ene-3,15-dione to microsomes act to inhibit ACAT activity, while direct addition of 25-hydroxycholesterol to microsomes acts to stimulate ACAT activity.

However, it is clear that 15-ketosterol is one of the more potent of the ACAT inhibitors. Several inhibitors of ACAT activity have been reported, and the potency of these various compounds can be compared by examining the concentration required for 50% reduction of ACAT activity (IC50). For instance, the most potent of these ACAT inhibitors are the acylamides 58-035 (Sandoz) and 57-118 (Sandoz). It was reported that the IC50 of 58-035, after direct addition to either FU5AH hepatoma cell microsomes or swine granulosa cell microsomes, was 0.1 μM. The IC50 of 57-118, after direct addition to rat jejunal microsomes, was reported to be 0.5 μM. Other groups reported that Lederle inhibitor 1 was a potent inhibitor of ACAT activity. The values of IC50 of Lederle 1 after direct addition to rat jejunal microsomes, rat liver microsomes, rat adrenal microsomes, or microsomes isolated from cultured smooth muscles cells were 0.14 μM, 0.74 μM, 1.18 μM, and 0.89 μM, respectively. In addition, Goldstein and coworkers reported that direct addition of the 7-ketocholesterol analog, SC31769, to cell free extracts of human fibroblasts inhibited the oleoyl CoA-dependent esterification of cholesterol with an IC50 of 3.6 μM. In comparison, the results described in chapter 3 and chapter 6 showed that direct addition of 15-ketosterol reduced cholesterol esterification by ACAT in both rat jejunal microsomes (IC50 = 3.0 μM) and rhesus monkey jejunal microsomes (IC50 = 4.5 μM).
There are several ACAT inhibitors which are not as potent as 15-ketosterol. For example, direct addition of progesterone to microsomes was reported to inhibit rat liver ACAT activity with an IC50 of 17 µM. Bell has reported that direct addition of the major tranquilizer, chlorpromazine, to microsomes isolated from rabbit aorta inhibited the oleoyl CoA-dependent esterification of cholesterol with an IC50 of 0.1 mM. Bell has also reported that direct addition of the local anesthetics lidocaine, tetracaine, benzocaine, and dibucaine, inhibited ACAT activity in rabbit arterial microsomes over the range of 0.25 mM to 5.0 mM. He reported that the potency of inhibition was dibucaine > benzocaine > tetracaine > lidocaine > procaine with inhibition of about 85% occurring with 0.25 mM dibucaine. In addition to these compounds, Hudson and coworkers reported that the fibrin acid derivatives, bezafibrate and clofibrate, reduced ACAT activity in microsomes isolated from rabbit peritoneal macrophages that were previously incubated with acetylated LDL. The authors reported an IC50 between 0.5 and 1.0 mM for bezafibrate and also demonstrated ~40% reduction in ACAT activity upon addition of 2.5 mM clofibrate to microsomes isolated from rabbit peritoneal macrophages.

Several ACAT inhibitors are reported to be effective upon oral administration to animals. Heider and coworkers reported that oral administration of 57-118 (200 mg/kg) to cholesterol-fed rabbits resulted in both a 59% decrease in the level of ACAT activity in jejunal microsomes and a 65% decline in absorption of exogenous cholesterol by the intestine. These authors have also reported that after oral administration of 57-118, the presence of the compound was detected in jejunal microsomes at a level of 2.24 ± 0.11 nmol/mg microsomal protein. Bennett-Clark and Tercyak have reported that either gastric administration (20 mg/kg) or duodenal infusion (17.5 mg over 8
hours) of 58-035 to lymph-duct cannulated rats results in a 79% decline in the level of ACAT activity in jejunal microsomes which was associated with 63% decrease in the lymphatic output of total cholesterol. The authors reported that after administration of 58-035, the presence of the compound was detected in jejunal microsomes, but did not provide the details. Oral administration of Lederle inhibitor 1 to cholesterol-fed rats was reported to result in a dose-dependent decrease in serum cholesterol levels (IC₅₀=5.2 mg/kg/day), and a 80% increase in fecal excretion of exogenous [4-¹⁴C] sterols (0.01% Lederle 1 in diet). The determination of the concentration of Lederle 1 in the jejunal microsomes of treated rats was not reported. In addition, oral administration of the plant sterol β-sitosterol (1% in diet) to rabbits was reported to reduce the levels ACAT activity in jejunal microsomes by 61%, and to reduce the cholesterol content of jejunal microsomes by 48%. This reduction in ACAT activity was reported to be accompanied by the detection of high concentrations of β-sitosterol (8.4 µg/mg protein) relative to cholesterol (8.4 µg/mg protein) in jejunal microsomes isolated from β-sitosterol-fed rabbits. Bell and Schaub reported that oral administration of chlorpromazine (20 mg/kg/day) to cholesterol-fed rabbits for 12 weeks resulted in a 40% reduction in total arterial cholesterol and a 47% reduction in arterial cholesteryl ester content. In addition, Stahlberg and coworkers have reported that oral administration of 0.016% ciprofibrate, 0.1% bezafibrate, or 0.3% clofibrate for two weeks reduced ACAT activity in rat liver microsomes by 50-70%. In bezafibrate-treated rats, the reduction in ACAT activity was accompanied by a 60% decrease in the concentration of cholesteryl esters in hepatic microsomes. These authors also reported that the concentration of microsomal cholesterol was not affected by oral administration of bezafibrate. Furthermore, preincubation of the microsomal
fraction with unlabeled cholesterol increased ACAT activity to the same extent in both bezafibrate-treated and untreated rats. Based upon these results, Stahlberg and coworkers suggested that bezafibrate lowered ACAT activity by either directly acting upon ACAT or by decreasing the amount of ACAT protein in microsomes rather than by affecting either the cholesterol concentration in microsomes or the accessibility of microsomal cholesterol to ACAT.

In comparison to these compounds, it is interesting to note that 15-ketosterol is the only ACAT inhibitor that is effective both under conditions of low dietary cholesterol and under conditions of high dietary cholesterol. The experiments described in Chapter 4 demonstrated that oral administration of 0.10% 15-ketosterol to chow-fed rats reduced the level of ACAT activity in jejunal microsomes by 77%. The experiments described in Chapter 5 demonstrated that oral administration of 15-ketosterol (75 mg/kg/day) to rhesus monkeys maintained on diet high in fat and cholesterol resulted in a 32% decline the levels of ACAT activity in jejunal microsomes.

The body of evidence obtained from both the results described in this dissertation and the results of cholesterol absorption studies is now sufficient to begin to develop a hypothesis regarding the action of 15-ketosterol on ACAT activity and cholesterol absorption. The effects of 15-ketosterol on this aspect of cholesterol metabolism begin upon first exposure of the small intestine to a meal containing the compound. The initial absorption of 15-ketosterol by enterocytes produces a reduction of the level of cholesterol esterification by jejunal ACAT, probably as a result of the compound's ability to serve as an alternate substrate. This reduction in cholesterol esterification may result in a reduction in the levels of cholesterol absorption. The resulting 15-ketosterol esters are packaged into chylomicrons and secreted into the lymph.
This concept is supported by the following observations. 1) Addition of 15-ketosterol to incubations of jejunal microsomes of either rats or rhesus monkeys resulted in the formation of 15-ketosteryl oleate through the action of ACAT (Chapters 3 and 5). 2) A reduction in the oleoyl CoA-dependent esterification of cholesterol in rat jejunal microsomes was observed as early as three hours after completion of the first meal containing 0.10% 15-ketosterol (Chapter 4). 3) Experiments with lymph-duct cannulated rats have shown that after intragastric administration of [2,4-\textsuperscript{3}H]15-ketosterol, substantial amounts of the labeled sterol were rapidly excreted into lymph\textsuperscript{92}. 4) Analyses of the lymph showed that most of the \textsuperscript{3}H in lymph was associated with chylomicrons, and almost all of the \textsuperscript{3}H in chylomicrons was in the form of 15-ketosteryl esters\textsuperscript{92}.

Upon entry into the circulation, the chylomicrons containing 15-ketosteryl esters are taken up by the liver, and the 15-ketosteryl esters are hydrolyzed to form free 15-ketosterol. This free 15-ketosterol is then converted into polar biliary metabolites which are subsequently secreted into the lumen of the small intestine. 15-Ketosterol and/or its polar metabolites could then act to further reduce both intestinal ACAT activity and the absorption of dietary cholesterol. The reduction of cholesterol absorption probably contributes to the hypocholesterolemic effect of 15-ketosterol.

This concept is supported by the following observations. 1) Analyses of the tissue distribution after intravenous injections of chylomicrons containing [2,4-\textsuperscript{3}H]15-ketosteryl esters to rats indicated that there was a very rapid and selective uptake of \textsuperscript{3}H by liver\textsuperscript{92}. 2) Analyses of lipid extracts of rat liver showed that this uptake was followed by a conversion of [2,4\textsuperscript{3}H]15-ketosteryl esters to free [2,4-\textsuperscript{3}H]15-ketosterol, [\textsuperscript{3}H]cholesterol, and [\textsuperscript{3}H]cholesteryl esters\textsuperscript{92}. 3) Analyses of radioactivity in lipid extracts of bile after intravenous administration
of [4-14C]15-ketosterol to bile duct-cannulated rats, demonstrated that there was a rapid and substantial conversion of [4-14C]15-ketosterol to polar biliary metabolites (Chapter 6). 4) The addition of one of these polar biliary metabolites, 3β,26-dihydroxy-5α-cholest-8(14)-en-15-one, to rat jejunal microsomes reduced the levels of cholesterol esterification by ACAT (Chapter 6). 5) Furthermore, oral administration of 15-ketosterol to either rats or rhesus monkeys resulted in a decline in the levels of ACAT activity in jejunal microsomes (Chapter 4, Chapter 5). 6) Analyses of radioactivity in lymph after gastric administration [4-14C]cholesterol to lymph duct-cannulated rats demonstrated that oral administration of 15-ketosterol (0.05% in chow diet for 10 days) resulted in a 64% decline in the absorption of [4-14C]cholesterol. 7) Additional studies have examined the effect of dietary administration of 15-ketosterol (0.10% in diet) on the fate of [4-14C]cholesterol and [2,4-3H]15-ketosterol after intragastric administration of the labeled sterols to rats. In these experiments, oral administration of 15-ketosterol was associated with decreased absorption of [4-14C]cholesterol and [2,4-3H]15-ketosterol. The distribution of the 3H label in tissue also indicated a rapid conversion of [2,4-3H]15-ketosterol to [3H]cholesterol and [3H]cholesteryl esters.

An alternate pathway for the intestinal uptake and esterification of 15-ketosterol involves the action of PCEH, an enzyme which is implicated in the absorption of dietary cholesterol. Investigations have shown that 15-ketosteryl [1-14C]oleate is an efficient substrate for partially purified porcine PCEH and purified rat PCEH. It is possible that absorbed 15-ketosterol is esterified by PCEH in the same manner as absorbed, dietary cholesterol is reported to be esterified by PCEH. The resulting competition between 15-ketosterol and cholesterol for esterification by PCEH could further contribute
to the observed decrease in cholesterol absorption upon oral administration of 15-ketosterol to rats.

The results of the studies described in this dissertation have demonstrated that jejunal ACAT activity was affected by oral administration of 15-ketosterol. Several experiments were designed to explore the mechanism behind the observed reduction in ACAT activity. Analyses of the cholesterol content of rat and rhesus monkey microsomes showed that the reduction in ACAT activity was not due to a decrease in the cholesterol content as a result of 15-ketosterol and/or its metabolites action(s) as inhibitors of HMG-CoA reductase activity. Other experiments were done in which exogenous cholesterol was added to the microsomes in order to saturate ACAT activity with respect to cholesterol. The data from these studies indicated that under conditions of high concentrations of exogenous cholesterol, the levels of cholesterol esterification in treated rats remained lower than their respective controls. These results suggested that the reduction in ACAT activity upon oral administration of 15-ketosterol was not due to changes in the availability of cholesterol for esterification by ACAT. In addition, analysis of the 15-ketosterol content of microsomes detected extremely low levels of the compound in treated animals. These results were consistent with the detection of extremely low levels of radioactivity comigrating as 15-ketosteryl oleate upon incubation of microsomes isolated from treated animals. These latter observations could suggest that the reduction in ACAT activity upon oral administration of 15-ketosterol was not due to the action of the compound as an alternate substrate for ACAT. However, it is possible that the flux of 15-ketosterol through the enterocyte is so fast that high steady state concentrations of 15-ketosterol, 15-ketoteryl esters, or its metabolites do not accumulate in microsomes. Studies with lymph duct-
cannulated rats have shown that intragastric administration of [2,4-3H]15-ketosterol results in a very rapid secretion of [2,4-3H]15-ketosteryl esters into lymph. Additional studies in which [2,4-3H]15-ketosterol and [4-14C]cholesterol were administered to lymph duct-cannulated rats the appearance of 3H in lymph preceded the appearance of 14C in lymph. These latter results suggested that the flux of absorbed 15-ketosterol through the enterocyte is faster than the flux of absorbed cholesterol. Therefore, a reduction in cholesterol esterification by microsomal ACAT as a result of the action of 15-ketosterol as an alternate substrate cannot be eliminated as a mechanism of action.

It is important to note that there are other mechanisms by which 15-ketosterol and/or its metabolites could lower ACAT activity. It is conceivable that 15-ketosterol and/or its metabolites could affect the phosphorylation state of the ACAT. It has been reported that the level of ACAT activity is altered by assay conditions which promote phosphorylation, such as the addition of cytosol, 4 mM MgCl2, 4 mM ATP, and 40 mM NaF to incubations of rat liver microsomes, rat intestinal microsomes, and microsomes isolated from cultures of bovine adrenal cells. However, this regulation by phosphorylation has recently been questioned. Mitropoulos and Venkatesan have also reported that incubation of rat liver microsomes with cytosol, 4 mM MgCl2, 2 mM ATP, and 50 mM NaF, increases the levels of ACAT activity. These authors have also reported that the same incubation conditions increases the net transfer of cholesterol from liposomes to rat liver microsomes. Based upon these results, Mitropoulos and Venkatesan have concluded that the increase in the level of rat liver ACAT activity upon incubation of microsomes with cytosol, MgCl2, ATP, and NaF, could be interpreted either in terms of phosphorylation or in terms of
modulation of the supply of cholesterol. In addition, Einarsson and coworkers have reported that the levels of ACAT activity in human liver microsomes are not affected by the addition of 5 mM MgCl₂, 2 mM ATP, and 50 mM NaF to the assay system⁵⁵.

15-Ketosterol and/or its metabolites could affect the levels of synthesis and/or degradation of the ACAT protein. Unfortunately, there is no information regarding the synthetic and degradative rates of the ACAT protein. However, on the basis of differential reactivities towards chemical modification by diethyl pyrocarbonate (DEP) and acetic anhydride, Kinnunen and coworkers have reported the existence two subtypes of ACAT, a DEP-resistant ACAT activity present in rabbit liver, pancreas, intestine, and adrenals and a DEP-sensitive ACAT activity present in rabbit kidney, lung, testis, heart, spleen, brain, aorta, and stomach¹²⁶. These authors have also reported that cholesterol feeding results in the conversion of the DEP-resistant subtype present in both rabbit intestine and pancreas to the DEP-sensitive subtype. Since this work was done using a microsomal system, it was difficult to determine if the protein modification protocols labeled ACAT or a regulatory protein associated with ACAT. Nevertheless, the results reported by Kinnunen and coworkers suggest the possibility that 15-ketosterol and/or its metabolites could affect either the expression of ACAT subtypes or the expression of a regulatory protein associated with ACAT.

In addition, 15-ketosterol and/or its metabolites could affect ACAT activity by binding to regulatory sites on the enzyme. Since intestinal ACAT activity has not been successfully purified or even solubilized and reconstituted into a liposomal system, relative little is known about either the kinetic properties or the regulatory properties of the enzyme. Adding to this situation is
the possibility that the incorporation of 15-ketosterol and/or its metabolites into the microsomal membranes of enterocytes could be sufficient to alter the physical structure of the bilayer. ACAT is an enzyme noted for its sensitivity to its lipid environment\textsuperscript{37-39}.

15-Ketosterol and/or its metabolites could also affect jejunal ACAT activity through their incorporation into bile salt micelles. The incorporation of these compounds into micelles could be sufficient to disrupt the incorporation of dietary cholesterol into bile salt micelles, thus leading to a decreased flux of lumenal cholesterol into the enterocytes of intact rats. It has been shown that agents which interfere with the formation of bile salt micelles, such as the bile acid sequesterant cholestyramine, reduce the level of microsomal ACAT activity in enterocytes\textsuperscript{30,37-39}.

In order to determine which of these possible mechanisms is important to the action of 15-ketosterol on microsomal ACAT activity, additional work must be done. These future studies will require the development of a reconstituted liposomal enzyme system. A liposomal system would allow comparisons of the apparent $K_m$ of 15-ketosterol to the apparent $K_m$ of cholesterol under controlled conditions, and would allow a better determination of the effects of the lipid composition of the bilayer on jejunal ACAT activity. In addition, a purified or even partially purified preparation of jejunal ACAT would permit the generation of antibodies directed against ACAT activity. As a result, questions regarding the effect of oral administration of 15-ketosterol on the levels of intracellular ACAT protein could begin to be addressed. Unfortunately, in contrast to the hepatic enzyme, jejunal ACAT activity has resisted attempts to solubilize and reconstitute its activity. Several groups have reported that the enzyme is extremely sensitive to detergents and quickly loses activity upon
solubilization\textsuperscript{37-39}. As a consequence, extensive screening of solubilization conditions will have to be done in order develop a method for the reconstitution of jejunal ACAT activity into a liposomal system.

The results described in this dissertation in conjunction with results from other studies have demonstrated that 15-ketosterol lowers \textit{in vitro} jejunal ACAT activity and inhibits cholesterol synthesis in cultured cells. It would be of considerable interest to determine the relative contribution of the inhibition of cholesterol absorption and the inhibition of cholesterol synthesis to the overall hypocholesterolemic effect of 15-ketosterol in intact animals. Several methods have been described which used a combination of isotopic, chromatographic, and sterol balance techniques to determine the contribution of absorption and synthesis to the overall metabolism of cholesterol\textsuperscript{127-134}. However, the execution of these methods is not trivial, and the interpretation of the results is far from simple and must take into consideration both the advantages and the limitations of each method.

A number of approaches for the assessment of cholesterol absorption have been described\textsuperscript{127-132}. For example, in one method, a single intravenous dose of radioactive cholesterol is administered at the beginning of a study, and the amount of dietary cholesterol, the level of plasma radioactivity, the level of fecal radioactivity, and the fecal sterol content are closely monitored for several days\textsuperscript{127}. The level of cholesterol absorption is then calculated using the sterol balance technique. In this procedure, daily absorption of exogenous cholesterol (mg/day) = daily cholesterol uptake (mg/day) - daily unabsorbed dietary cholesterol (mg/day), where daily unabsorbed dietary cholesterol (mg/day) = daily fecal total neutral sterols (mg/day) - daily fecal endogenous neutral sterols (mg/day, radioactive cholesterol and its metabolites). This
method of determining the level of cholesterol absorption is used for measuring short-term cholesterol absorption. However, this first method does not take into account the loss of nonabsorbed cholesterol through either bacterial degradation of the sterol molecule, incomplete colonic emptying, or incomplete fecal collection. Another method for the measurement of cholesterol absorption involves continuous oral labeling with radioactive cholesterol and is also used for short-term determinations of the levels of cholesterol absorption. The amount of cholesterol absorbed daily is calculated as the difference between cholesterol intake and unabsorbed dietary cholesterol. Unabsorbed dietary cholesterol is determined using the following pair of simultaneous equations: \( Z = X + Y \), where \( Z \) = total fecal neutral sterols, \( X \) = fecal neutral sterols of endogenous origin, and \( Y \) = unabsorbed dietary cholesterol; and \( Z \cdot SA_Z = X \cdot SA_X = Y \cdot SA_Y \), where \( SA_Z \) = specific activity of total fecal sterols, \( SA_X \) = specific activity of plasma cholesterol, and \( SA_Y \) = specific activity of dietary cholesterol. These equations are then solved for \( Y \). This second method may be used any time after 4 days of oral administration of radioactive cholesterol. Like the first protocol, this second method does not account for losses of nonabsorbed cholesterol. A third protocol also makes use of constant labeling with radioactive cholesterol and can be used to obtain data regarding long term levels of cholesterol absorption. However, the attainment of the isotopic steady state is required before an assessment of the level of cholesterol absorption can be made. Unfortunately, the isotopic steady state is often difficult to attain. In this third method, daily absorption of dietary cholesterol (mg/day) = daily cholesterol turnover (mg/day) \( \cdot \) the fraction of plasma cholesterol derived from dietary cholesterol. The daily cholesterol turnover can be determined by either using sterol balance methods or from
specific activity time curves of plasma cholesterol. These time curves are calculated using complex computer models which assume that body cholesterol exists either in one pool\textsuperscript{127,129,130}, two pools consisting of a fast turnover pool (blood cholesterol, hepatic cholesterol, and intestinal cholesterol) and a slow turnover pool (peripheral cholesterol)\textsuperscript{127,130}, or three pools consisting of a fast turnover pool (blood cholesterol, hepatic cholesterol, and intestinal cholesterol), an intermediate turnover pool (some peripheral tissues), and a slow turnover pool (arterial, adipose, connective, and muscle tissue)\textsuperscript{131}. Unfortunately, this third method does not account for losses of nonabsorbed cholesterol. A fourth method for determining the level of cholesterol absorption involves the oral administration of a single dose of radioactive cholesterol and calculates the percentage of the dose not recovered in the feces\textsuperscript{127,132}. Unlike the other methods, the percentage absorption is calculated only after the losses from sources other than absorption, such as bacterial degradation, incomplete colonic emptying, and/or incomplete fecal collection, are corrected for using orally administered radioactive sitosterol as an internal standard. The following equation is then used: percentage of dietary cholesterol absorbed = \[1- \frac{(\text{radioactivity of fecal cholesterol/\text{radioactivity of fecal sitosterol}) \times (\text{radioactivity of administered sitosterol/\text{radioactivity of administered cholesterol}})}{\times 100}.

Several methods to determine the levels of whole body cholesterol synthesis have also been described. For example, Grundy and Ahrens have estimated the daily synthesis of cholesterol in man by measuring the excretion of radiolabeled cholesterol and its conversion products during periods of controlled sterol intake (sterol balance) and daily intravenous injections of radiolabeled cholesterol\textsuperscript{130}. This method requires that all sterol metabolism be under isotopic steady state conditions prior to the calculation of sterol synthesis.
from turnover rates generated using the 1 pool or 2 pool computer models described above. This technique has the advantage that the levels of cholesterol absorption can also be derived from the data. A kinetic method for the determination of the levels of whole body cholesterol synthesis has also been described\textsuperscript{133}. In this method, a radiolabeled precursor to cholesterol, such as tritiated water or [2-\textsuperscript{14}C]mevalonate, is given as a single intravenous dose at the start of the study. Blood, urine, and fecal samples are then collected for a period of eight weeks, and lipid analyses are performed on these samples. The flux rates of cholesterol between a two pool model is then calculated from these data. Although this procedure permits a more direct estimate of whole body cholesterol synthesis, it has the disadvantage that the levels of cholesterol absorption cannot be calculated from the data. In addition to these two methods, the incorporation of [2-\textsuperscript{14}C]acetate into cholesterol from freshly isolated peripheral mononuclear leukocytes has been reported to reflect the levels of whole body cholesterol synthesis\textsuperscript{134}. This third technique can easily be used in conjunction with methods to measure the levels of cholesterol absorption in the whole animal. However, care has to be taken when interpreting the results obtained from this method since tissue specific responses to inhibitors of cholesterol synthesis may not be reflected in mononuclear leukocytes.

Several studies have been reported which used different combinations of protocols to measure cholesterol absorption and synthesis\textsuperscript{129,130,133,134}. Unfortunately, the implementation of the methods for the assessment of cholesterol absorption and cholesterol synthesis is not trivial and varying results have been obtained. For example, depending upon the experimental methods and the dietary conditions, the level of cholesterol absorption has been
calculated to be as low as 108 mg/day and as high as 1 gm/day\textsuperscript{129,130}. Depending upon the experimental conditions, the level of cholesterol absorption has been reported to either show saturation kinetics or to demonstrate no saturation kinetics\textsuperscript{127,132}. Furthermore, cholesterol synthesis has been reported to be as low as 37 mg/day and as high as 1407 mg/day depending upon both the procedure used and the dietary conditions\textsuperscript{130,133}.

In addition to the considerations described above, studies designed to examine the relative contribution of inhibition of cholesterol absorption and inhibition of cholesterol synthesis to the hypocholesterolemic effect of 15-ketosterol would have to take into account additional factors. First, 15-ketosterol is converted to cholesterol in the tissues of rats and baboons\textsuperscript{25,26,87,89,94}. As a consequence, any experiments which use dual isotope or sterol balance techniques would have to account for this third source of cholesterol. Second, there is evidence that changes in the extent of cholesterol absorption can significantly affect the level of hepatic cholesterol synthesis. For example, a decrease in the level of cholesterol absorption would result in decreased delivery of cholesterol to the liver and an elevation of the levels of hepatic HMG-CoA reductase activity. Evidence for this effect has been reported upon use of hypocholesterolemic agents, such as diosgenin, which act at the level of cholesterol absorption. Diosgenin is reported to lower the serum cholesterol levels of cholesterol-fed rabbits and chickens\textsuperscript{135} and to decrease the levels of hepatic cholesterol in cholesterol-fed rats\textsuperscript{136,137}. This compound is also reported to be a weak inhibitor of sterol biosynthesis in mouse L cells and is reported to be over 150 times less active than 25-hydroxycholesterol\textsuperscript{138}. Diosgenin is also reported to have little or no effect on the levels of HMG-CoA reductase activity in these cells\textsuperscript{138}. However, administration of diosgenin at a
level of 1% in a chow diet is reported to inhibit the intestinal absorption of cholesterol (-38% and -46% as measured by 2 different methods) and to markedly increase the levels of hepatic HMG-CoA reductase activity (+161%);\textsuperscript{137}. In contrast, 15-ketosterol has been shown to be a more potent inhibitor of cholesterol absorption (-64% upon oral administration of 0.05% 15-ketosterol to rats)\textsuperscript{28} and to inhibit cholesterol synthesis in both cultured L cells and cultured CHO cells (IC\textsubscript{50} = 0.1\mu M)\textsuperscript{8}. In view of these effects, oral administration of 15-ketosterol would be expected to result in a suppression of the elevation of hepatic HMG-CoA reductase activity induced by the inhibition of cholesterol absorption. This effect was observed by Miller and coworkers who reported the results from three separate experiments in which 0.1% 15-ketosterol was fed to rats for a period of 8 days. At this dosage, the inhibition of cholesterol absorption by 15-ketosterol was expected to be at least equal to or even greater than the reduction in cholesterol absorption observed at 0.05% 15-ketosterol. The authors reported that oral administration of 0.1% 15-ketosterol produced only a moderate rise (+55%, +50%, +49%) in hepatic HMG-CoA reductase activity relative to pair-fed controls. Furthermore, in two experiments, no significant change in hepatic HMG-CoA reductase activity relative to ad libitum rats was observed while in the third study, only a 30% rise in enzyme activity was detected relative to ad libitum rats. In addition to these considerations, 15-ketosterol has been shown to be converted into at least one biologically active metabolite. It is clear that the difficulties involved in the experimental techniques in conjunction with the additional considerations produced by oral administration of 15-ketosterol would make the interpretation of experiments designed to determine the relative contribution of the inhibition
of cholesterol absorption and the inhibition of cholesterol synthesis to the overall hypocholesterolemic effect of 15-ketosterol extremely difficult.

Conclusions

In summary, the work described in this dissertation examined the effect of administration of 15-ketosterol on microsomal ACAT activity. The results from these investigations showed that 15-ketosterol reduced rat and rhesus monkey ACAT activity upon direct addition to jejunal microsomes. The decrease in intestinal ACAT activity upon direct addition of 15-ketosterol to microsomes was due to the ability of the sterol to serve as an alternate substrate for the enzyme.

Oral administration of 15-ketosterol to both rats and rhesus monkeys was also shown to reduce the level of ACAT activity in jejunal microsomes. Additional studies demonstrated that the reduction of rat intestinal ACAT activity was dependent upon the duration of oral administration of 15-ketosterol and upon the concentration of the compound in the diet. Other experiments demonstrated the conversion of 15-ketosterol into polar biliary metabolites, and showed that one of these polar metabolites reduced ACAT activity upon direct addition to jejunal microsomes. The results obtained from these investigations, in conjunction with data from other studies, provided the basis for a hypothesis concerning the mechanism behind the 15-ketosterol-induced reductions of cholesterol absorption. It is likely that the action of 15-ketosterol upon both cholesterol synthesis and cholesterol absorption is responsible for the hypocholesterolemic effect of this compound.
REFERENCES


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