In presenting this thesis in partial fulfilment of the requirements for an advanced degree at the University of British Columbia, I agree that the Library shall make it freely available for reference and study. I further agree that permission for extensive copying of this thesis for scholarly purposes may be granted by the head of my department or by his or her representatives. It is understood that copying or publication of this thesis for financial gain shall not be allowed without my written permission.

Department of Obstetrics and Gynaecology

The University of British Columbia
Vancouver, Canada

Date April 10, 1990
ABSTRACT

Epidemiological studies have shown that cholesterol is a major risk factor for the development of atherosclerosis. Since the atherosclerotic plaque develops over a long period interventions early in life may be of some benefit. In addition, it has been shown that the enzymes involved in cholesterol metabolism can be manipulated in early life. Therefore, studies of the developmental patterns of the key enzymes in cholesterol metabolism are of great importance. Acyl coenzyme A: cholesterol acyltransferase (ACAT) is the primary enzyme which catalyzes the conversion of free cholesterol to cholesterol esters in cells. A better understanding of the role and control of ACAT during development is needed in order to trace the possible causes in early life that lead to atherosclerosis in the adult.

This research focused on the developmental pattern of ACAT in the rat liver, intestine, brown and white adipose tissue (BAT and WAT, respectively) and aorta. Age specific changes were observed in the rat liver, intestine and BAT. The rat liver and intestine possess significant amounts of ACAT activity throughout development and there appears to be marked variations in activity during this time. The rat BAT and WAT appear to be devoid of ACAT activity throughout
development with the exception of adult BAT. Due to the small amount of the aortic tissue samples and/or the insensitivity of the assay, no definite conclusions could be made from this aortic study.

In searching for factors that might control the ACAT enzyme the immediate effects of short-term manipulation of diet on the activity of ACAT were studied. The rats were all weaned early on day 18 to one of the following diets: Purina Rat Chow, high carbohydrate (HG), high fat (HF), or 2% cholesterol. The HF was the only diet that consistently increased hepatic ACAT activity in all the age groups. The cholesterol diets significantly increased the activity of ACAT in the 22 and 25 day old rats. The HG diet increased the activity of ACAT in the 22, 25, and 30 day old rats. No significant differences were observed between the adult control and HG diet groups. Feeding rats a HF or HG diet precipitated a dramatic drop in intestinal ACAT activity in the 22 day old animals. These effects were not observed in the older animals. The high cholesterol diet had no significant effect on the intestinal enzyme's activity in 22 day old rats. There was no significant change in the BAT and WAT ACAT activity with the experimental diets with the exception that all the experimental diets decreased ACAT activity in the adult BAT.

iii
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>ii</td>
</tr>
<tr>
<td>TABLE OF CONTENTS</td>
<td>iv</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>ix</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>x</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>xi</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>xiii</td>
</tr>
<tr>
<td>1. LITERATURE REVIEW</td>
<td>1</td>
</tr>
<tr>
<td>1.1 Introduction</td>
<td>1</td>
</tr>
<tr>
<td>1.1.1 Cholesterol and Cholesterol Ester Metabolism</td>
<td>1</td>
</tr>
<tr>
<td>1.1.2 ACAT and its Role in Atherosclerosis</td>
<td>13</td>
</tr>
<tr>
<td>1.1.3 ACAT Enzyme Properties</td>
<td>15</td>
</tr>
<tr>
<td>1.1.4 ACAT and the Liver: Role and Specific Properties</td>
<td>17</td>
</tr>
<tr>
<td>1.1.5 ACAT and the Intestine: Role and Specific Properties</td>
<td>19</td>
</tr>
<tr>
<td>1.1.6 ACAT and Adipose Tissue</td>
<td>23</td>
</tr>
<tr>
<td>1.1.7 ACAT and the Aorta: Role and Specific Properties</td>
<td>24</td>
</tr>
</tbody>
</table>
1.2 Control of ACAT

1.2.1 Introduction
1.2.2 Substrate level
1.2.3 Phosphorylation/Dephosphorylation
1.2.4 Hormonal Control
   1.2.4.1 Progesterone and Estrogen
   1.2.4.2 Thyroid Hormone
   1.2.4.3 Insulin

1.3 Exposure to Dietary Manipulation:
   Introduction
   1.3.1 Cholesterol
      1.3.1.1 Prenatal Period
      1.3.1.2 Suckling Period
         1.3.1.2.1 Dietary Intervention
      1.3.1.3 Weaning Period

1.4 Objectives

2. EXPERIMENTAL

2.1 Experimental Design and Rationale I
2.2 Experimental Design and Rationale II
2.3 Materials and Methods
   2.3.1 Materials
2.3.1.1 Animals  
2.3.1.2 Chemicals  
2.3.1.3 Equipment  
2.3.1.4 Diets  

2.3.2 Methods  
2.3.2.1 Animal Care  
2.3.2.2 Animal Breeding  
2.3.2.3 Treatment of Animals  
2.3.2.4 Blood Collection and Preparation  
2.3.2.5 Serum Triglyceride Analysis: Principle  
2.3.2.6 Total Serum Cholesterol Analysis: Principle  
2.3.2.6.1 Procedure Used  
2.3.2.7 Preparation of Tissues for Enzyme Analysis  
2.3.2.8 Protein Analysis  
2.3.2.9 Preparation of Radiolabelled Coenzyme A  
2.3.2.10 Enzyme Assay  
2.3.2.10.1 Principle of Assay  
2.3.2.10.2 Procedure Used  
2.3.2.10 Statistical Evaluation  

vi
3. RESULTS

3.1 Developmental study

3.1.1 Development of ACAT in the Liver

3.1.2 Development of ACAT in the Intestine

3.1.3 Development of ACAT in BAT

3.1.4 Development of ACAT in WAT

3.1.5 Development of ACAT in the Aorta

3.1.6 Serum Cholesterol and Triglyceride Levels During Development

3.3 Diet study

3.3.1 The Effects of Diets on Liver ACAT Activity

3.3.2 The Effects of Diets on Intestine Activity

3.3.3 The Effects of Diets on WAT ACAT Activity

3.3.4 The Effects of Diets on BAT ACAT Activity

3.3.5 The Effects of Diets on Aorta ACAT Activity

3.3.6 Serum Cholesterol and Triglyceride Levels
# LIST OF TABLES

<table>
<thead>
<tr>
<th>I.</th>
<th>List of Abbreviations.</th>
<th>xiii</th>
</tr>
</thead>
<tbody>
<tr>
<td>II.</td>
<td>Examples of Late Effects on Early Adaptation.</td>
<td>52</td>
</tr>
<tr>
<td>III.</td>
<td>Chemicals and Suppliers.</td>
<td>59</td>
</tr>
<tr>
<td>IV.</td>
<td>Suppliers Names and Addresses.</td>
<td>60</td>
</tr>
<tr>
<td>V.</td>
<td>Equipment Used in Experiments.</td>
<td>61</td>
</tr>
<tr>
<td>VI.</td>
<td>Composition of Diets.</td>
<td>63</td>
</tr>
<tr>
<td>VII.</td>
<td>Composition of High Fat and High Carbohydrate Diets.</td>
<td>63</td>
</tr>
<tr>
<td>VIII.</td>
<td>ACAT Activity in BAT and WAT.</td>
<td>78</td>
</tr>
<tr>
<td>IX.</td>
<td>ACAT Activity in the Aorta.</td>
<td>78</td>
</tr>
<tr>
<td>X.</td>
<td>The Effects of Diets on ACAT Activity in WAT.</td>
<td>87</td>
</tr>
<tr>
<td>XI.</td>
<td>The Effects of Diets on ACAT Activity in the Aorta.</td>
<td>90</td>
</tr>
<tr>
<td>XII.</td>
<td>The Effects of Diets on Serum Cholesterol and Triglyceride Levels.</td>
<td>93</td>
</tr>
</tbody>
</table>

ix
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Exogenous and Endogenous Fat Transport Pathways.</td>
<td>8</td>
</tr>
<tr>
<td>2.</td>
<td>LDL Pathway.</td>
<td>12</td>
</tr>
<tr>
<td>3.</td>
<td>Control of ACAT via Phosphorylation / Dephosphorylation.</td>
<td>32</td>
</tr>
<tr>
<td>4.</td>
<td>Ontogeny of ACAT in the Liver.</td>
<td>75</td>
</tr>
<tr>
<td>5.</td>
<td>Ontogeny of ACAT in the Intestine.</td>
<td>77</td>
</tr>
<tr>
<td>6.</td>
<td>Cholesterol and Triglyceride Levels Throughout Development.</td>
<td>81</td>
</tr>
<tr>
<td>7.</td>
<td>The Effects of Diets on ACAT Activity in the Liver.</td>
<td>84</td>
</tr>
<tr>
<td>8.</td>
<td>The Effects of Diets on ACAT Activity in the Intestine.</td>
<td>86</td>
</tr>
<tr>
<td>9.</td>
<td>The Effects of Diets on ACAT Activity in BAT.</td>
<td>89</td>
</tr>
<tr>
<td>10.</td>
<td>The Relationship Between the Ontogeny of ACAT in the Liver and Intestine and Serum Cholesterol and Triglyceride Levels.</td>
<td>102</td>
</tr>
<tr>
<td>11.</td>
<td>ACAT activity in rat fetal and placental tissues.</td>
<td>154</td>
</tr>
</tbody>
</table>
ACKNOWLEDGEMENTS

I would like to thank:

Dr. Peter Hahn for his supervision, guidance, constructive criticism, and support. My stay in his lab was truly rapsichordic.

My supervisory committee, Dr. P. Jones, Dr. J. Frohlich, and Dr. D. Seccombe for their advice and suggestions.

The Chairman of my committee, Dr. P.C.K. Leung for his support and encouragement.

Kevin Glatiotis for his patience, understanding and unrelenting assistance with my work.

My parents, Eileen and Ernie Little for their help in putting everything into perspective.
This Thesis Is Dedicated To My Family.

My Parents,
Ernie and Eileen Little.

My Brothers and Sisters,
Phil, Anne-Marie, Patricia Margaret, Miriam Francis,
David, John, Bernard, and Monica-Mary
and to
Kevin.

xii
### Table I

**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>T3</td>
<td>3,3',3'-triiodothyronine;</td>
</tr>
<tr>
<td>DHBS</td>
<td>3,5-dichloro-2-hydroxy-benzenesulfonic acid;</td>
</tr>
<tr>
<td>HMG-CoA reductase</td>
<td>3-hydroxy-3-methylglutaryl coenzyme A reductase;</td>
</tr>
<tr>
<td>ACAT</td>
<td>Acyl-coenzyme A:cholesterol acyltransferase;</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin;</td>
</tr>
<tr>
<td>BAT</td>
<td>Brown adipose tissue;</td>
</tr>
<tr>
<td>CHO</td>
<td>Carbohydrate;</td>
</tr>
<tr>
<td>CE</td>
<td>Cholesterol ester;</td>
</tr>
<tr>
<td>CEase</td>
<td>Cholesterol esterase;</td>
</tr>
<tr>
<td>CO</td>
<td>Cholesterol oxidase;</td>
</tr>
<tr>
<td>CoA</td>
<td>Coenzyme A;</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic AMP;</td>
</tr>
<tr>
<td>°C</td>
<td>Degree Celsius;</td>
</tr>
<tr>
<td>dH2O</td>
<td>Distilled water;</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithioerythritol;</td>
</tr>
<tr>
<td>FH</td>
<td>Familial hypercholesterolemia;</td>
</tr>
<tr>
<td>FA</td>
<td>Fatty acid;</td>
</tr>
<tr>
<td>FC</td>
<td>Free cholesterol;</td>
</tr>
<tr>
<td>FFA</td>
<td>Free fatty Acids;</td>
</tr>
<tr>
<td>G6PDH</td>
<td>Glucose-6-phosphate dehydrogenase;</td>
</tr>
<tr>
<td>GK</td>
<td>Glycerol kinase;</td>
</tr>
<tr>
<td>HG</td>
<td>High carbohydrate;</td>
</tr>
<tr>
<td>HDL</td>
<td>High density lipoprotein;</td>
</tr>
<tr>
<td>HFC</td>
<td>High Fat and Cholesterol;</td>
</tr>
<tr>
<td>HF</td>
<td>High fat diet;</td>
</tr>
<tr>
<td>H2O2</td>
<td>Hydrogen peroxide;</td>
</tr>
<tr>
<td>IDL</td>
<td>Intermediate density lipoprotein;</td>
</tr>
<tr>
<td>LCAT</td>
<td>Lecithin: cholesterol acyltransferase;</td>
</tr>
<tr>
<td>LDL</td>
<td>Low density lipoprotein;</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA;</td>
</tr>
<tr>
<td>mm</td>
<td>Nanometer;</td>
</tr>
<tr>
<td>PTU</td>
<td>Propylthiouracil;</td>
</tr>
<tr>
<td>2% cholesterol diet</td>
<td>Purina Rat Chow plus 2% cholesterol diet;</td>
</tr>
<tr>
<td>S.E.</td>
<td>Standard Error;</td>
</tr>
<tr>
<td>STZ-D</td>
<td>Streptozocin-induced diabetic;</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin layer chromatography;</td>
</tr>
<tr>
<td>T4</td>
<td>Thyroxine;</td>
</tr>
<tr>
<td>TG</td>
<td>Triglyceride;</td>
</tr>
<tr>
<td>VLDL</td>
<td>Very low density lipoprotein;</td>
</tr>
<tr>
<td>WAT</td>
<td>White Adipose tissue;</td>
</tr>
</tbody>
</table>
LITERATURE REVIEW

1.1 INTRODUCTION

1.1.1 Cholesterol and Cholesterol Ester Metabolism

The uptake of cholesterol ester into cells by receptor-mediated endocytosis and its subsequent de-esterification and re-esterification by intracellular enzymes has generated much interest particularly because of the link between these processes and pathological conditions such as atherosclerosis (Brown and Goldstein, 1984). The control of the enzymes governing cholesterol anabolism and catabolism is integral to the maintenance of whole body cholesterol homeostasis.

Cholesterol is an essential component of cell membranes serving a vital function in altering membrane fluidity and it is an important precursor for the biosynthesis of steroid hormones and bile acids. The ability of the body to absorb and synthesize cholesterol vary markedly in different species. In comparison to the human, small mammals such as the rat and hamster synthesize large amounts of cholesterol (Dietschy, 1984). The human synthesizes approximately 10 mg of cholesterol per day per kg body weight (while on a low cholesterol diet) in comparison to the rat and hamster who
synthesize approximately 40 mg per day per kg body weight and 100 mg per day per kg body weight, respectively (Hashimoto, et al., 1974; Dietschy, 1984). Traditionally, the liver has been regarded as the major tissue responsible for the maintenance of cholesterol homeostasis. In the past the liver was estimated to synthesize over 80% of the endogenous cholesterol. However, more recent literature suggests that the liver is responsible for 51, 40, 27, 18, and 16% of total body cholesterol synthesis in the rat, squirrel monkey, hamster, rabbit, and guinea pig, respectively (Dietschy et al., 1983). Other tissues such as the adrenal, ovary, small intestine, stomach, spleen, and heart appear to play a more substantial sterol synthetic role than was previously believed (Dietschy et al., 1983). Due to this major contribution of extrahepatic tissues to cholesterol metabolism it is important that some of these additional tissues be included in a study about cholesterol metabolism during development.

Cholesteryl esters serve two crucial functions in mammalian cells. They provide an intracellular storage form of cholesterol and a means by which cholesterol is transported in the blood plasma (Spector, et al., 1979). A storage form of cholesterol provides a safeguard for cells that utilize a large quantity of cholesterol for
biosynthetic purposes. Free cholesterol resides to a large extent within the phospholipid bilayer where it modulates the fluidity of the membrane. Any alteration in the fluidity of the membrane via changes in the cholesterol content can have detrimental effects on the permeability and properties of the membrane bound enzymes and transport systems (Spector, et al., 1979). Hence a specialized storage pool of cholesterol in the form of cholesterol ester is present within most mammalian cells which abolishes the need to extract free cholesterol from the fragile membrane systems.

The primary enzyme responsible for the esterification of cholesterol in plasma is Lecithin: cholesterol acyltransferase (LCAT) which mediates the transfer of fatty acids between lecithin and cholesterol (Dobiasova, 1983; Frohlich and McLeod, 1987). LCAT is a key player in cholesterol and triglyceride (TG) homeostasis (Frohlich and McLeod, 1987).

There are two known enzymes which catalyze the conversion of cholesterol to cholesterol esters (CE) intracellularly: cholesterol ester hydrolase and acyl-coenzyme A: cholesterol acyl-transferase (ACAT).
Cholesterol ester hydrolase catalyzes the reaction:

\[ \text{Cholesterol} + \text{fatty acid} \rightleftharpoons \text{cholesterol ester} + \text{H}_2\text{O} \]

This reversible reaction does not require ATP or coenzyme A. Cholesterol ester hydrolase esterifies the free fatty acid without need for prior chemical activation. There are two forms of this enzyme distinguished from each other by different pH optima (Spector, 1979).

It is the prevailing opinion of most researchers that ACAT is the major enzyme responsible for the intracellular esterification of cholesterol. ACAT catalyzes the reaction:

\[ \text{Cholesterol} + \text{Acyl-CoA} \rightarrow \text{Cholesterol Ester} + \text{CoA} \]

In contrast to the cholesterol ester hydrolase pathway, the non-reversible ACAT reaction requires the prior activation of the fatty acid; thus, it requires both coenzyme A (CoA) and ATP (Spector, 1979). Additional properties of the ACAT enzyme and its reaction are dealt with in section 1.1.3.

Due to the insolubility of most lipids in aqueous media, these substances are transported by a group of globular lipid-protein complexes called lipoproteins. The lipoproteins each consist of a core of neutral lipid
(primarily TG and CE) surrounded by a phospholipid and protein bilayer. Chylomicrons are primarily responsible for the import of exogenous TG and cholesterol. Very low density lipoproteins (VLDL) principally transport TG originating from the liver. Low density lipoprotein (LDL) and high density lipoproteins (HDL) are concerned with the transport of endogenous cholesterol (Brown and Goldstein, 1977). In humans, LDL is the carrier of most of the cholesterol in plasma and approximately three-fourths of this cholesterol is in the esterified form (Brown and Goldstein, 1976).

The LDL pathway regulates the synthesis, uptake, and disposal of cholesterol. Much attention has been focused on this pathway particularly because of the well known and accepted correlation between elevated plasma LDL levels and the associated risk of coronary artery disease. A defect in this pathway, such as that occurring in the genetic disease familial hypercholesterolemia (FH), causes elevated cholesterol levels and the precocious occurrence of atherosclerosis (Brown and Goldstein, 1983). The normal process of atherosclerosis begins early in life with an accumulation of cholesterol and cholesterol esters within the intima and media of the artery wall (Brown and Goldstein, 1977). At birth human infants possess a large
number of LDL receptors and they have LDL concentrations similar to other animal species. In industrialized societies, the human LDL level rises three to four-fold with increasing age; the increase noted as early as in childhood (Brown and Goldstein, 1984).

The lipid transport system can be divided into two pathways: an exogenous route for TG and cholesterol absorbed from the small intestine and an endogenous route for cholesterol and TG entering the bloodstream from the liver and other non-intestinal tissues (Brown and Goldstein, 1983; Figure 1). The exogenous pathway begins in the small intestine where dietary cholesterol and other lipids are passively absorbed across the brush border of the small intestine. Within the intestinal cell the cholesterol is esterified through the action of ACAT and packaged in chylomicrons for entry into the lymph (Meddings et al., 1987). The chylomicrons eventually deliver the triacylglycerol component to adipose tissue for storage and the muscle for beta-oxidation to supply energy. The ester bond of the triacylglycerol is cleaved by the action of lipoprotein lipase, an extracellular enzyme most active in the cardiac and skeletal muscle, adipose tissue, and the lactating mammary gland. The remaining cholesterol-ester rich chylomicron remnants are removed from the circulation
Figure 1. Exogenous and endogenous pathway of fat transport. Dietary cholesterol in the form of cholesterol ester is absorbed through the wall of the small intestine and is transported along with triglycerides in the chylomicron particle. Lipoprotein lipase in the adipose tissue and muscle hydrolyzes the triglycerides liberating fatty acids. The cholesterol-rich remnants bind specific receptors on the hepatic membrane. The cholesterol can be secreted into the intestine as such or in the form of bile acid or it can be transported with triglyceride in VLDL, thus initiating the endogenous pathway. The triglyceride portion of the particle is again removed in the adipose tissue and the muscle. The cholesterol-rich intermediate density lipoprotein (IDL) either binds to the hepatic LDL receptors or it is converted in the circulation to LDL (adapted from Brown and Goldstein, 1984).
Figure 1. Exogenous and Endogenous pathway
by specific hepatic receptors. Once in the liver cell, the cholesterol is secreted into the intestine as such or as bile acids or it is packaged along with triacylglycerol in VLDL initiating the endogenous pathway.

Once again the triacylglycerol component is removed resulting in a cholesterol-rich intermediate density lipoprotein (IDL). A portion of the IDL binds to LDL receptors and is rapidly engulfed by the liver cell. The remaining IDL particle stays within the circulation until its apoprotein E dissociates and the particle is converted to LDL. HDL, originating from the liver, is secreted into the plasma as a nascent discoid-shaped particle almost completely devoid of cholesterol ester. These nascent particles are converted to spherical lipoproteins by the accumulation of cholesterol esters. LCAT catalyzes this reaction in the plasma. The cholesterol esters are then transferred from HDL to VLDL or LDL (Brown and Goldstein, 1984).

The circulating plasma LDL is removed from the plasma by two distinct mechanisms. Approximately 20% to 40% of LDL clearance involves non-specific endocytosis and is referred to as receptor-independent LDL uptake (Meddings, et al., 1987). The uptake of 60% to 80% of the lipoprotein-bound cholesterol and cholesterol esters occurs through the
process of receptor-mediated endocytosis (Brown and Goldstein, 1983; Meddings, et al., 1987; Figure 2). LDL is bound by a receptor on the surface of the cell in a coated clathrin pit. This membrane involutes and pinches off thus engulfing the LDL and the receptor. Once inside the cell the receptor and the LDL dissociate. The receptor is recycled back to the cell surface and the LDL is delivered to a lysosome. Apoprotein B-100 is broken down into amino acids and the cholesterol ester bond is cleaved to yield unesterified cholesterol and fatty acids. When an adequate amount of cholesterol has accumulated through this pathway to meet the biosynthetic requirements of the cell three metabolic processes result. First, the oversupply of cholesterol inhibits 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase), the rate limiting enzyme in cholesterol synthesis. Second, the transcription of the receptor gene into messenger RNA (mRNA) is suppressed thus inhibiting the synthesis of additional LDL receptors. Third, and most significant for this thesis, the ACAT enzyme is activated promoting the re-esterification of cholesterol to cholesterol ester for storage purposes (Brown and Goldstein, 1984).
Figure 2  The LDL pathway. The cholesterol containing LDL particle is bound to specific receptors located in coated clathrin pits and internalized via receptor-mediated endocytosis. As a direct result of cholesterol uptake HMG-CoA reductase, the rate limiting enzyme in cholesterol synthesis is inhibited, the receptors are down-regulated and the ACAT enzyme is activated promoting esterification of cholesterol (adapted from Brown and Goldstein, 1984).
1.1.2 ACAT and its Role in Atherosclerosis

Atherosclerosis is the primary cause of death in the western civilization (Ross, 1988). There were approximately 12,000 atherosclerosis related deaths in Canada in 1986. (Statistics Canada, 1988). The development of atherosclerosis is progressive, beginning in early childhood with the accumulation of fatty streaks and manifesting the sequelae of this disease in adulthood with advanced aortic fibrous plaques (Ross, 1988). The development of the atherosclerotic plaque is characterized by an accumulation of free cholesterol and cholesterol esters in the intima and media of the arterial wall. The scavenger cells within the plaque become so dense with cholesteryl esters that they take on a foamy appearance, hence the term foam cell. The cholesteryl ester-laden LDL's are delivered to the macrophages of the damaged smooth muscle cell and are subsequently hydrolyzed by acid cholesteryl esterase (ACE) (Brown and Goldstein, 1983a; Sprinkle et al., 1987). In addition to the exogenous LDL cholesterol, the arterial tissue is capable of synthesizing cholesterol de novo. When the macrophage takes up more cholesterol than it can use or export the excess is esterified as is the case in other tissues. Although, CEase and ACAT are both capable of performing this function, it is now believed that ACAT plays
the predominant role. Neutral cholesteryl esterase (NCE) within the cell can hydrolyze these cholesteryl esters (Sprinkle et al., 1987). There is some dispute as to whether or not LCAT is present in arterial tissues. Abdulla et al. (1968) noted an increase in LCAT in the human atherosclerotic artery. Even though the LCAT may be present in arterial tissue it appears that ACAT plays a more substantial role in atheroma formation because the increase in activity of ACAT in the atheroma lesion was greater than the increase in activity of LCAT (Abdulla et al., 1968; Hashimoto et al., 1974).

The atherosclerotic plaque is a complicated structure. The intima lesions consist of proliferated smooth muscle cells, macrophages, lymphocytes and a large amount of extracellular material including collagen, fibrin, glycosaminoglycans, and cholesterol. In the advanced atherosclerotic state the lesion may include large areas of calcification, thrombosis, and extracellular depots of connective tissue and cholesterol. These deposits protrude into the lumen compromising the normal flow of blood (Brown and Goldstein, 1983a; Ross, 1988).

It is well documented that arteries undergoing atherogenic change characteristically show increased esterification of cholesterol and an accumulation of
cholesterol esters (St. Clair, 1976). Hashimoto et al. and others have reported that the activity of ACAT is increased up to 50-fold in rabbit atherosclerotic arteries compared with non-diseased arteries (Hashimoto et al., 1974; St Clair et al., 1970; Gallo et al., 1977; Helgerud et al., 1982). Cholesterol oleate is the major lipid associated with diseased arteries. Sprinkle and co-workers (1987) conducted an enzymatic developmental study on rabbits and they found convincing results which suggest that ACAT may play a significant role in an accumulation of cholesteryl esters during the early phases of lesion development. These same investigators suggest that the noted increase in ACAT activity in the rabbits fed cholesterol is due to enzyme induction, membrane fluidity changes, or increased substrate concentration at the enzyme site.

1.1.3 ACAT Enzyme Properties

Acyl-coenzyme A: cholesterol acyltransferase is a membrane bound microsomal enzyme that is responsible for the intracellular esterification of cholesterol (Erickson and Cooper, 1980; Helgerud et al., 1982) to long-chain fatty-acyl cholesterol esters (Gavey et al., 1983). There are a number of tissues from different species possessing ACAT activity including human, rat, guinea pig, and char liver.
(Erickson and Cooper, 1980; Gavey et al., 1983; Mukherjee, et al., 1958; Drevon, 1978; Dannevig and Norum, 1983) and intestine (Norum et al., 1979; Suckling et al., 1983; Dannevig and Norum, 1983), rabbit, pigeon and monkey arteries (Hashimoto et, al., 1974; Brecher and Chobian, 1974) and mouse Ehrlich ascites tumor cells (Brenneman, 1977). ACAT has also been isolated in the adrenals, ovaries, rat skin (Norum, 1974), and human skin fibroblasts (Spector et al., 1979).

It has been found that oleoyl-CoA is the best substrate for the rat liver ACAT enzyme, with the rates of synthesis from acyl CoA decreasing from cholesteryl oleate > palmitate > stearate > linoleate (Goodman et al., 1964). This appears to be the case in several other tissues and it is the consensus of most investigators that cholesteryl oleate is the predominant ester formed via intracellular cholesterol ester synthesis (Spector et al., 1979). Goodman and colleagues also observed that the highest ACAT activities are found in the mitochondrial and microsomal fractions (1964). Hepatic ACAT in vitro is inhibited by fatty acids in concentrations above $1 \times 10^{-5}$M while this inhibition is reversed by the addition of serum albumin. The hepatic ACAT enzyme is sensitive to detergents; taurocholate and glycocholate inhibit ACAT (Goodman et al.,
1964; Spector et al., 1979). The activity of ACAT is dependent on acyl CoA concentrations. The pH optimum of ACAT from different species range from 7.2 to 7.8 (Spector et al., 1979; Norum et al., 1981; Helgerud, 1981).

1.1.4 ACAT and the Liver: Role and Specific Properties

The liver is the major site of cholesterol synthesis and the predominant route for cholesterol catabolism. It is generally accepted that hepatic ACAT plays an integral role in intracellular cholesterol homeostasis. Its principal role is the intracellular esterification of cholesterol. By doing so ACAT may also mediate cholesterol homeostasis in several other capacities. It is speculated that it might participate in directing the pool of free cholesterol into the different excretory pathways: biliary cholesterol, bile acid synthesis, and VLDL (Nervi et al., 1984). ACAT might maintain membrane integrity and function by esterifying the excess cholesterol. An alteration in the cholesterol:phospholipid ratio invokes a change in membrane permeability and membrane enzyme activities (Erickson et al., 1984). In the rat, a great percentage of VLDL is of hepatic origin and it is speculated that the cholesterol ester portion of this particle is assembled by the ACAT reaction (Erickson and Cooper, 1980). The contribution of VLDL to the total plasma
cholesterol pool is small in humans. Thus, it is unlikely that ACAT participates to any great extent in the control of plasma cholesterol (Erickson and Cooper, 1980), since LCAT is the major enzyme responsible for the synthesis of plasma cholesterol esters (Glomset, 1968).

ACAT activity has been found in the liver of many species. In decreasing order the relative activities are rat, pig, dog, monkey, rabbit, calf, and guinea pig (Spector et al., 1979; Stokke, 1974). Although originally it was thought that ACAT was absent in the human liver (Stokke, 1974), more recent literature states that it is indeed present (Erickson and Cooper, 1980; Balasubramaniam et al., 1978; Angelin et al., 1987). The human enzyme appears to exhibit activities similar to those found in the rabbit (Erickson and Cooper, 1980). A circadian rhythm of low amplitude has been detected for rat hepatic ACAT activity and it parallels the activity of HMG-CoA reductase and thus cholesterol biosynthesis (Erickson et al., 1984).

ACAT activity in rat liver microsomes is associated with the RNA-rich vesicles; ACAT is localized in membranes originating from the rough endoplasmic reticulum (Balasubramaniam et al., 1978b). In contrast, both HMG-CoA reductase and cholesterol 7-alpha hydroxylase are confined to endoplasmic reticulum membranes with low ribosomal
coating (Balasubramaniam et al., 1978b). Balasubramaniam and co-workers have shown that when labelled cholesterol is added to hepatic cells with the addition of cofactors for both ACAT and cholesterol 7-alpha hydroxylase, the specific activities of the two enzymes were found to be different (1978b). This suggested that there might be at least two distinct cholesterol substrate pools in the endoplasmic reticulum; one for the ACAT reaction and one pool that acts as a substrate for cholesterol 7-alpha hydroxylase (Balasubramaniam et al., 1978b; Spector et al., 1979).

1.1.5 ACAT and the Intestine: Role and Specific Properties

Numerous in vitro studies have demonstrated that the properties of the intestinal ACAT enzyme are in many ways very similar to those of the hepatic enzyme. Only those characteristics which are unique to the intestine will be discussed in this section.

The small intestinal mucosa is considered a key player in cholesterol metabolism because it is the site for uptake of the sterols present in the intestinal contents and because it is one of the most active tissues responsible for endogenous cholesterol production (Stange and Dietschy, 1985; Turley and Dietschy, 1981). In the intestinal
epithelial cell intracellular esterification is considered a regulatory step in the absorption of cholesterol. Eighty to ninety percent of the cholesterol transferred into the lymph is esterified with fatty acids from both dietary and endogenous sources (Treadwell and Vahouny, 1968). In the past it has been suggested that regulation of cholesterol absorption in the gut and the retention of cholesterol in intestinal cells as cholesterol esters is under the control of cholesterol ester hydrolase of pancreatic origin (Angel and Farkas, 1974; Gallo et al., 1977). However, more recent literature suggests that this regulation is under the control of ACAT (Helgerud et al., 1982; Norum et al., 1983). If this is the case, ACAT is indeed an important regulatory enzyme given the unequivocal evidence that dietary cholesterol plays a causal role in the pathogenesis of coronary artery disease in man (Kannel, 1988). What role ACAT plays in the intestine during development is largely unknown. Norum and co-workers (1983) suggest that the function of ACAT in cholesterol absorption in the adult rat intestine is in esterifying cholesterol so that cholesterol esters can be exported by the enterocyte into the lymph.

Norum and co-workers have found that the activity of ACAT is greater in the villous cells than in the crypt cells of the intestinal epithelium (1983). These findings seem
logical given the specific functions of each of these cells. Absorptive villous cells evolve from division, differentiation, and migration of the stem cells at the base of the intestinal villous. The mature villous cells, in contrast to the immature crypt cells, house an assortment of enzymes and transport proteins associated with digestion and uptake of nutrients. Hence, the ACAT enzyme would serve a useful purpose here in esterifying cholesterol to ready it for transport into the lymph. On the other hand, the crypt cells continually utilize cholesterol for membrane synthesis associated with cell differentiation and proliferation, thus cholesterol esterification is an unwanted process (Norum et al., 1983).

Rat intestinal ACAT activity displays a diurnal variation related to the animal's feeding schedule: increase in ACAT activity with fasting and a decrease with feeding (Helgerud, 1982).

ACAT activity is the highest in the proximal intestine and lowest in the distal portion in rat (Haugen and Norum, 1976) and humans (Helgerud, 1981). In rabbits, the mid-gut exhibits the highest ACAT activity. The suggestion that ACAT plays a role in the absorption of cholesterol is given further credence by the fact that the cluster of cholesterol absorptive sites are predominantly located in
the proximal intestine (Sylen and Nordstrom, 1970); Suckling and Stange, 1985). It should also be noted that compounds such as Sandoz 58-035 \([3-(decyldimethyl-silyl)N[2-(4-methylphenyl)1-phenylethyl propanamide]\), when administered in vivo, provoke a dramatic decrease in the activity of ACAT which is accompanied by a decrease in absorption of cholesterol and a decrease in cholesteryl esters entering the lymph (Suckling and Stange, 1985). Cholesterol esterase (CEase) seems to predominantly work to hydrolyze and take up cholesterol; re-esterification is thought to occur via the ACAT reaction. Therefore, it appears that CEase and ACAT work in tandem: CEase aids in the uptake and hydrolysis of cholesterol esters and ACAT re-esterifies the cholesterol for transport into the lymphatic system.

Norum et al., (1981) summarize the additional roles of ACAT in the intestine in three points. First, ACAT may act as a protective enzyme, protecting the cell from excessive accumulation of cholesterol by esterifying it. Second, ACAT promotes storage of cholesterol for future use in membrane or lipoprotein synthesis. Lastly, ACAT may affect lipoprotein metabolism.
1.1.6 ACAT and Adipose Tissue

Adipose tissue contains one of the largest pools of exchangeable cholesterol and it is a major site for cholesterol storage (Angel and Farkas, 1974). Fat tissue contains more cholesterol than most other organs or membranes when it is expressed per unit of protein (Farkas et al., 1973; Pittman et al., 1975). A high level of cholesterol esterase (CEase) has been reported in rats and humans (Pittman et al., 1975).

ACAT activity has been reported to be low or absent in adult rat adipose tissue concomitantly with little cholesteryl ester stores (Angel and Farkas, 1974). This seems peculiar since in other tissues a considerable part of cholesterol is stored as acylesters. In addition, cholesterol is reported to be released from the adipose tissue when catecholamines are administered (Farkas et al., 1973) and during starvation of adult rats (Angel and Farkas, 1974). No data are available for infant or fetal animals. White adipose tissue may play a special role in the maintenance of total plasma cholesterol, since HMG-CoA reductase activity in WAT of obese mice was found to be high, even though the hepatic enzyme showed low activity (Hahn, 1980). Whether or not ACAT is present in adipose tissue during development is unknown. Brown adipose tissue
differs in metabolic development from WAT. The primary role of BAT is to produce heat by oxidizing fatty acids. Brown fat is found in all hibernators and newborn babies, rats, and mice (Johansson, 1959). It contributes about 25% of the total lipids to the body in the suckling rat; the only tissue that produces more is the skin (Hahn, 1986). Although the developmental pattern of HMG-CoA reductase in BAT has been determined, (HMG-CoA reductase is high in the fetal BAT and decreases to the 14th postnatal day; Hahn and Smale, 1982), there has been no developmental studies pertaining to the role of ACAT in adipose tissue.

1.1.7 ACAT and the Aorta: Role and Specific Properties

ACAT activity has been found in the arteries of the rat, rabbit, pigeon, squirrel monkey, rhesus monkey, dog, swine, and humans (Suckling and Stange, 1985). The distribution of ACAT in aortic tissue is similar to that in the liver (Goodman et al., 1964; Suckling and Stange, 1985). However the concentration of ACAT in this tissue is lower than is found in the hepatic tissue. In many ways the arterial enzyme is similar to the intestinal and hepatic enzymes. The enzyme prefers fatty acyl CoA as a cofactor, it is inhibited by detergents, and it has a pH optimum of 7.4 (Hashimoto et al., 1974; Suckling and Stange, 1985).
Oleic acid is synthesized in the atherosclerotic artery. Due to the aortic ACAT's preference for oleoyl CoA it seems likely that this locally synthesized fatty acid is used by ACAT. The intracellular cholesterol esters are predominantly cholesteryl oleate whereas the LDL derived cholesteryl esters that filter into the intima and media is mostly cholesteryl linoleate (Suckling and Stange, 1985).

It has been suggested that ACAT operates within the cell to protect the cell from excess cholesterol accumulation. This is a controversial issue since it is not known whether the increase in ACAT activity in the atherosclerotic artery represents an etiological event, an exacerbating factor, or a passive consequence of an increased availability of free cholesterol (Gillies, et al., 1986). Spector et al. (1979) suggests that the accumulation of cholesteryl esters is a result of an increased availability of cholesterol and that the ACAT reaction provides a mechanism for the removal of a potentially harmful excess of cholesterol. Other investigators have shown that ACAT inhibitors reduce the extent of the atheromatous lesion suggesting that this increased esterification by ACAT serves as a biochemical trapping mechanism for cholesterol entering the aortic wall (Bell and Schaub, 1986; Gillies et al., 1986). Both of these
investigators have used adult rabbit aortae and arteries and again no studies have been completed looking at these parameters during development. Since the atherosclerotic plaque develops over a long period of time it is important that the ontogeny of ACAT, one of the key enzymes in cholesterol metabolism, be clearly established. In addition, it has been shown that some enzymes in cholesterol metabolism can be manipulated in early life by early intervention therefore knowledge of the developmental pattern of the ACAT enzyme is of interest (Rymaszewski et al., 1985; Subbiah and Hassan, 1982).

1.1.8 Summary

The LDL pathway regulates the synthesis, uptake, and disposal of cholesterol. The uptake of cholesteryl esters via receptor mediated endocytosis and an accumulation of excess cholesterol mediates three important processes. The excess cholesterol inhibits HMG-CoA reductase and the synthesis of additional LDL receptors, and it activates the ACAT enzyme, promoting esterification of cholesterol for storage. As mentioned, the ACAT enzyme has a number of important properties that distinguish it from the other cholesterol esterifying enzymes. The properties of the ACAT
enzyme have been well characterized however the developmental profile of this enzyme has not been elucidated.
1.2 CONTROL OF ACAT

1.2.1 Introduction

The unesterified cholesterol within the cell exists primarily within the membrane where it functions to modulate the fluidity of the phospholipid bilayer thus allowing the proper functioning of the membrane bound enzymes and transport systems (Spector et al., 1979). It is generally accepted that the maintenance of the concentration of the unesterified cholesterol pool is crucial. By esterifying cholesterol and providing a storage pool of cholesterol, ACAT participates in the control of free cholesterol within the cell.

The control of the ACAT enzyme has often been linked to the control of cholesterol 7-alpha hydroxylase and HMG-CoA reductase. Usually when the activity of HMG-CoA reductase is high ACAT activity is low and vice versa. The mechanisms directing the activity of these enzymes are not well understood. It is thought that long term regulation is governed by enzyme protein synthesis and degradation (Beirne et al., 1977; Mitropoulos et al, 1978). These three enzymes appear to be reciprocally regulated in the short term by phosphorylation/dephosphorylation and substrate supply.
There are a number of factors which appear to specifically regulate ACAT activity in the short term. Those that will be dealt with in this section include the availability of cholesterol, phosphorylation and dephosphorylation, and hormones including progesterone, estrogen, thyroid hormone, and insulin. The last segment deals with the effects of dietary manipulation on cholesterol metabolism.

1.2.2 Substrate Level

The most obvious factor that controls ACAT activity is the substrate supply or the availability of cholesterol. Many investigators have shown that altering the level of cholesterol in the liver in vivo causes a change in the activity of ACAT. In rats, if the hepatic cholesterol concentration is increased by cholesterol or cholate feeding, an atherogenic diet, fasting, or the administration of mevalonic acid, ACAT activity increases (Erickson et al., 1980). Similarly, the ACAT activity in the rat intestinal mucosa appears to be susceptible to the change in cholesterol content because an increase in ACAT activity was noted when rats were fasted and a decrease in activity occurred when rats were fed a control HG diet (Helgerud et al., 1982). This might be due to the alteration in the unesterified cholesterol content in the microsomes which was
high after fasting and low after feeding (Helgerud et al., 1982). Others have suggested that as opposed to ACAT being regulated by the substrate supply, cholesterol or one of its substrates may regulate ACAT activity by interactions with one or more of the enzyme's regulatory sites or by changing the fluidity of the endoplasmic reticular membrane (Helgerud et al., 1982; Suckling et al., 1982).

On the other hand if the substrate supply of cholesterol is the major regulator of ACAT activity then it would be expected that reducing the absorption of cholesterol by administering cholestyramine would subsequently inhibit the ACAT enzyme. This appears to be the case in the rabbit intestine (Field and Salome, 1982) but not so in the rat (Stange et al., 1983). Although cholestyramine appeared to have no effect on rat intestinal ACAT, it stimulated cholesterol synthesis and increased the cholesterol content of the enterocyte. Stange and co-workers (1983) propose an explanation for this in their substrate pool theory which suggests that there are several different functional pools of cholesterol that meet the different metabolic needs of the intestinal cell. The pool that supplies the substrate for the ACAT reaction is different from the newly synthesized (or HMG-CoA reductase supplied) pool. Thus, in this case HMG-CoA reductase and
ACAT appear to be regulated by different mechanisms a phenomena which does not support earlier reports of reciprocal regulation of these two enzymes.

1.2.3 Phosphorylation/Dephosphorylation

In addition to substrate-supplied regulation of the ACAT enzyme discussed in the previous section it appears that ACAT may also be regulated in the short term by phosphorylation and dephosphorylation. It is thought that activation of rat hepatic and intestinal ACAT by phosphorylation occurs through a ATP-dependent protein kinase pathway and that protein phosphatase inactivates this enzyme via dephosphorylation (Gavey et al., 1983; Suckling et al., 1983b; Scallen and Sanghvi, 1983; Figure 3). The second messenger for this system is unknown, but it might be cAMP-mediated (Basheeruddin et al., 1982; Suckling and Stange, 1985). It should be noted that others have found no evidence for this system of control in human liver microsomal preparations (Einarsson et al., 1989). This same group of investigators reported that HMG-CoA reductase of human liver origin was inactivated/activated by dephosphorylation/ phosphorylation.

Scallen and Sanghvi (1983) propose that the three enzymes in hepatic cholesterol metabolism, HMG-CoA
Figure 3. Schematic diagram of the proposed short term control of ACAT by phosphorylation and dephosphorylation. (Adapted from Gavey et al., 1983).

ACAT (Phosphorylated / Active)

Protein phosphatase

↓

Protein kinase

ACAT (Dephosphorylated / Inactive)
reductase, 7 alpha-hydroxylase, and ACAT are reciprocally regulated by phosphorylation/ dephosphorylation. The enzyme involved in synthesis of cholesterol i.e. HMG-CoA reductase is inactivated by phosphorylation whereas the enzymes involved in utilization of the cholesterol, ACAT and 7 alpha-hydroxylase are activated by phosphorylation. Therefore, this coordinated regulation of the three key enzymes in cholesterol metabolism provides a short term mechanism for the control of intracellular unesterified cholesterol homeostasis.

Although there is substantial in vitro evidence that ACAT and HMG-CoA reductase are coordinately controlled this way there are conditions and tissues in which these two enzymes appear to be regulated independently. For instance, ACAT activity is increased in culture hepatocytes by the addition of 25-hydroxycholesterol and mevalonic acid but this effect is absent after an 18 hour incubation period. In contrast, the inhibition by these treatments on HMG-CoA reductase persisted for at least 22 hours, thus it is possible that the mechanism of control is different for these two enzymes (Suckling and Stange, 1985). Other treatments such as feeding cholestyramine or a fat-free diet to rats increases HMG-CoA reductase but has no apparent impact on ACAT (Suckling and Stange, 1985; Innis, 1986).
This suggests that other mechanisms apart from or in addition to phosphorylation/dephosphorylation might be involved in the control of the HMG-CoA reductase and ACAT enzymes. However, a definite answer to this phosphorylation/ dephosphorylation debate will have to await further experiments using purified enzyme.

1.2.4 Hormonal Control

Hormonal regulation of ACAT activity has not been well established except for the effects of estrogen and progesterone. However, one can speculate that hormones such as the thyroid hormone and insulin participate in cholesterol metabolism during development.

1.2.4.1 Progesterone and Estrogen

A number of in vitro experiments have demonstrated that ethinyl estradiol and progesterone affect the hepatic activity of ACAT. Ethinyl estradiol increases ACAT activity in the rat possibly by increasing the substrate supply to the liver via increasing the number of lipoprotein receptors (Del Pozo et al., 1983; Chao et al., 1979; Suckling and Stange, 1985). Progesterone inhibits hepatic ACAT activity in addition to increasing the biliary cholesterol output in male rats (Erickson and Cooper 1980; Del Pozo et al., 1983).
This inhibition was reversed by feeding the rats a cholesterol diet or by injection of ethinyl estradiol. If this progesterone-related decrease in enzyme activity occurs in vivo one could postulate that this may be a contributing factor to the sex-related differences in cholesterol and lipoprotein metabolism (Erickson and Cooper, 1980).

1.2.4.2 Thyroid Hormone

It is well known that thyroid hormones are essential for normal growth and tissue differentiation (Nathanielsz, 1976). Alterations in thyroid status causes changes in plasma lipids and lipoproteins. There is an inverse relationship between thyroid activity and plasma cholesterol levels. Thus, hyperthyroidism as a pathological condition in a patient or in the hyperthyroid-induced experimental animal is associated with hypocholesterolemia and the reverse is true for hypothyroidism (Heimberg et al., 1985). The etiology of hypercholesterolemia associated with decreased levels of thyroid hormones may result from increased hepatic output of the VLDL or decreased uptake of LDL (Heimberg et al., 1985). Cholesterol synthesis in rat liver is reduced to one-fifth the level of control animals after thyroidectomy. This reduction of cholesterolgenesis and the inhibition on HMG-CoA reductase can be counteracted
by the administration of thyroid extract or triiodothyronine (Goldfarb, 1980). The control of the thyroid gland on cholesterol metabolism is likely through the effects these hormones have on cholesterol 7 alpha-hydroxylase rather than a direct effect on HMG-CoA reductase (Hahn and Innis, 1984; Heimberg et al., 1985).

It is possible that maternal thyroid hormones do cross the placenta since T4 given to pregnant rats was shown to have a direct effect on the enzyme phosphoenolpyruvate carboxykinase in rat fetuses (Hahn and Hassanali, 1982). Hahn and others (1977) have shown that plasma cholesterol levels are reduced in suckling animals when T4 is given. Given the apparent effects of thyroid hormones on HMG-CoA reductase it is probable that these hormones also mediate, to some extent, the ACAT reaction during development. The only experiments linking thyroid hormones and ACAT activity are those by Hahn (1986b). It was found that infant rat intestinal ACAT activity increased almost 5-fold when PTU was added to the mother's drinking water. HMG-CoA reductase activity was only slightly decreased in this case. However, it has been shown by others that the administration of thyroxine causes a decrease in exogenous and endogenous cholesterol absorption and thyroidectomy enhances the
absorption and transport of cholesterol (Ponz de Leon et al., 1984). Given the proposed role ACAT plays in the absorption of cholesterol one could speculate that the thyroid hormones may participate in the control of the ACAT enzyme during development.

1.2.4.3 Insulin

Insulin is regarded as a prime candidate for the control of cholesterol metabolism for a number of reasons. Diabetes mellitus is often accompanied by high levels of LDL and VLDL and low levels of HDL which predisposes the individual to precocious atherosclerosis. Diabetes as well as the streptozocin-induced diabetes are frequently associated with hypercholesterolemia. The rise in plasma cholesterol levels noted in the streptozocin-induced diabetic (STZ-D) rats is accentuated when the rats are fed an atherogenic diet (Jiao et al., 1988; Goldfarb, 1980). In addition, cholesterol synthesis is reported to be increased two to three times normal rates in the intestine of diabetic animals and humans (Jiao et al., 1989).

Diabetes also causes changes in the key enzymes in cholesterol metabolism. HMG-CoA reductase and cholesterol 7 alpha-hydroxylase are both increased substantially in the STZ-D rat intestine (Goldfarb, 1980). Jiao et al. (1988)
reported that microsomal ACAT activity in intestinal epithelial cells is higher in STZ-D rats than in normal rats. This would infer that cholesterol esterification is increased by the diabetic state and that insulin might inhibit cholesterol esterification via the ACAT reaction. It is interesting to note that Jiao and co-workers (1988) reported that the insulin action on the regulation of ACAT is opposite in the liver and the intestine; ACAT activity is decreased in the liver whereas it is increased in the intestine. These investigators suggest that the insulin deficiency in diabetes could be a major factor in the enhanced ACAT activity in the intestine. The exact mechanism whereby insulin deficiency regulates ACAT activity is difficult to assess without the complete purification of the enzyme protein, however it is proposed that this regulation occurs through protein synthesis, phosphorylation/ dephosphorylation or by the alteration in membrane fluidity (Jiao et al., 1988).
1.3 Exposure to Dietary Manipulation: Introduction

It has been shown that environmental factors acting on the individual early in life may alter the later development of that individual (Hahn, 1989; Hahn, 1987; Hahn and Koldovsky, 1966; Hassan and Subbiah, 1989; Reiser et al., 1977). Thus any adaptive response in adult life may be conditioned by the first adaptation to an external stimulus early in life. This section shall deal with the control exerted by nutritional factors during early life on the development of cholesterol metabolism.

Mammalian development has a prenatal, a suckling and a weaning stage, distinguished by the mode of food intake and the composition of this food. Obviously, the prenatal diet is supplied via the placenta, and any change in the composition of the diet must be through the mother. Immediately after birth, the diet is mostly or exclusively milk, depending on the degree of maturity of the species at birth. For instance, the rat, mouse, and hamster are born very immature and are thus completely dependent on maternal milk, whereas the more mature human neonate and the guinea pig neonate can be partly dependent on and completely independent of maternal milk, respectively. The diet composition of the neonate can be manipulated easily in the case of animals capable of eating independently of the
mother, and less easily in those dependent on mother’s milk. Finally, the weaning period starts when the infant commences to eat other food instead of or in addition to milk. In some species, this coincides with the suckling period, e.g. in guinea pigs.

1.3.1 Cholesterol

1.3.1.1 Prenatal Period

The mammalian fetus receives its nutrients via placental transfer. Glucose is the primary component of the fetal "diet." There appears to be maternal-fetal transfer of cholesterol in some species (Pitkin et al., 1972), but it is also synthesized de novo in a number of fetal tissues, including the human adrenal, liver, testes, brain, and ovary (Carr and Simpson, 1982). The rate of cholesterol synthesis in the fetus as judged by the activity of the rate limiting enzyme in cholesterol synthesis, 3-hydroxy-3-methyl glutaryl coenzyme A (HMG-CoA) reductase, increases in the liver just prior to birth to rates two to three times higher than those in the adult (McNamara et al., 1972). Concomitantly, the fetal plasma level of cholesterol is low during this interval and does not depend on the maternal level (Shafrir and Khassis, 1982). The hepatic production of bile acids is the major pathway of cholesterol catabolism. Cholesterol 7
alpha-hydroxylase, the major regulatory enzyme in bile acid synthesis, is low in the fetus (Hahn and Innis, 1984), possibly reflecting the immaturity of the systems of bile acid synthesis, hepatic uptake, and secretion and ileal reabsorption (Innis, 1985). It has been suggested that bile acids of maternal origin indirectly affect fetal cholesterol metabolism by crossing the placenta and inhibiting fetal hepatic HMG-CoA reductase (Hahn, 1987). Much attention has been focused on the potential effects of early nutrition on the development of cholesterol metabolism in the adult. However, little information has been collected on the fetal and neonatal outcome of manipulation of the maternal diet. It appears that hepatic HMG-CoA reductase activity in fetal rats is altered by the quantity of fat and the fatty acid composition of the maternal diet (Haave et al., 1989).

Feeding cholestyramine, a non-absorbable resin that binds bile acids, to pregnant rats results in a 50% increase in fetal hepatic HMG-CoA reductase activity with no apparent change in the maternal or fetal plasma lipid levels (Innis, 1988). In contrast, pregnant rats fed a high fat and cholesterol (HFC) diet had significantly increased plasma cholesterol levels, but the HMG-CoA reductase activity in the fetus was not affected (Innis, 1988). It should be noted that others have found that feeding cholestyramine or
cholesterol to pregnant rats has no effect on fetal cholesterol synthesis (Miguel and Abraham, 1976). Hassan and co-workers have shown that cholestyramine fed to pregnant rats results in a 2.6-fold increase in fetal cholesterol 7 alpha-hydroxylase activity compared to the control values (1985).

1.3.1.2 Suckling Period

The suckling period, which constitutes the second phase of development, begins at birth with a dramatic change in supply, rate, and composition of food intake. The transition from a prenatal high carbohydrate "diet" to a neonatal high fat milk diet is preceded by a period of starvation which may last from 10 minutes to several hours. As mentioned, the maturity of the species at birth dictates the dependency on the mother for milk.

The cholesterol content of the milk also displays inter-species variation. Rat milk contains approximately 120 mg/dl (Carroll, 1964), while human breast milk contains 20-24 mg/dl (Hahn, 1982). Blood cholesterol levels dramatically increase from low fetal levels to high postnatal values in most mammals (Carroll and Hamilton, 1973), while HMG-CoA reductase activity in the liver, intestinal mucosa and brown adipose tissue (BAT) decrease
after birth (McNamara et al., 1972; Hahn and Smale, 1982; Kroeger and Hahn, 1983; Hahn and Walker, 1979). This has also been confirmed by more direct methods, i.e. the incorporation of tritiated water into cholesterol (Belknap and Dietschy, 1988; Stange and Dietschy, 1984), the technique considered to be the most reliable way to study lipid metabolism (Jeske and Dietschy, 1980; Dietschy and Spady, 1984). Using the same method, it was also shown that cholesterol synthesis is low in all tissues of infant rats except in the brain (Belknap and Dietschy, 1988; Hahn, 1986). It has been suggested that the postnatal rise in total cholesterol is linked to the cholesterol content of the milk (Friedman and Byers, 1961) and of the diet (Mott et al., 1978; Whatley et al., 1981). However, it was found that the postnatal rise in cholesterol occurs in two steps. The first (immediately after birth) occurs independent of diet. The second increase seems to depend on diet, since the rise did not occur in infants fed only tea with sugar. Thus, even though plasma cholesterol levels seem to depend to some degree on the consumption of milk, this is not the sole regulator (Hahn and Koldovsky, 1966). Undoubtedly, other factors are involved. There appears to be little difference between the rate of cholesterol synthesis in
various tissues during this time, with the exception of the high rate of synthesis noted in the brain (Hahn, 1986).

Previous investigators have proposed that the decrease in HMG-CoA reductase during the suckling period is due to a "factor" in the milk (McNamara et al, 1972; Boguslawski and Wrobel, 1974). To date no such factor has been isolated. However, it is well established that there is an inverse relationship between blood levels of total cholesterol and hepatic HMG-CoA reductase, thus it is more probable that cholesterol or one of its metabolites, or VLDL, is responsible (Park and Subbiah, 1988). The low activity of HMG-CoA reductase during the suckling phase could be attributed to a decrease in the synthesis of the enzyme and an increase in phosphorylation (inactivation) of the enzyme protein, particularly in the infant rat intestinal mucosa (Hahn, 1987). Cholesterol 7 alpha-hydroxylase activity is very low during the suckling period (Hahn and Innis, 1984). This low rate of turnover of bile acids (Hahn, 1986) is indicative of the considerable demand for cholesterol for growth and development during the suckling period, even though one might think that bile acid synthesis should be high given the increased consumption of cholesterol. To date there are no studies that have looked at the ontogeny of ACAT in the rat.
1.3.1.2.1 Dietary Intervention

In the past few years, there has been a renewed interest in the effects of dietary manipulation on cholesterol metabolism during the suckling period. Just as the placenta regulates the passage of nutrients from the mother to the fetus, the mammary glands maintain that function postnatally. Given that studies of breast milk have shown that approximately two thirds of milk cholesterol is derived from the maternal blood (Pitkin et al, 1972), it is not surprising that the maternal diet influences cholesterol metabolism in the suckling animal.

It is, however, not always easy to come to the right conclusion concerning the role of diet in development. An example is the often cited and refuted paper by Reiser and Sidelman (1972), which concluded that an early nutritional change in the cholesterol content of milk has an effect on the plasma cholesterol levels of the offspring that persists into adulthood. This conclusion was based on the cholesterol determination in the milk of only one mother rat.

Nevertheless, milk undoubtedly contains substances that affect the immediate and probably also the later metabolic pattern of the individual. Thus, the offspring of mother
rats drinking milk or water from the 14th day of pregnancy up to the 30th day after delivery showed the effect of the mother's diet (Kritchevsky et al., 1983). That is, the hepatic cholesterol 7 alpha-hydroxylase was three times more active in the offspring of milk-fed mothers than in those whose mothers received water throughout. The main conclusion to be drawn from this experiment is that it is the intra-uterine period that is more important for future development (Kritchevsky et al., 1983). This, however, is not borne out by other data, which indicate that postnatal changes in diet composition play an equally important role.

Overfeeding neonatal rats by reducing the litter size to 3 from 14 results in an elevation of total blood cholesterol, thought to be due to the concomitant rise in insulin levels. HMG-CoA reductase activity is decreased in this circumstance, and the rate of growth is increased (Hahn and Walker, 1979). Feeding a HFC diet to pregnant rats decreased the activity of HMG-CoA reductase in suckling rats up to the 14th postnatal day, after which no effect was found (Innis, 1985). The decrease in enzyme activity is in agreement with other studies of 21-day-old rats born to dams fed a HFC diet and suckled by dams fed a control diet (Naseem et al., 1980a; Naseem et al., 1980b).
Early dietary intervention with cholestryramine appears to affect the key enzymes in cholesterol metabolism. HMG-CoA reductase activity was increased on postnatal days 8 and 14 in pups born to dams fed cholestryramine and cross fostered to dams fed a control diet (Innis, 1988). Cholestryramine given to nursing dams had a transient effect on the suckling pups' hepatic HMG-CoA reductase activity, since an increase was seen only to the 14th postnatal day (Innis, 1988; Hassan and Subbiah, 1989). HMG-CoA reductase activity was found to be enhanced three to five times in the livers of 17-day-old rats artificially reared on a low cholesterol diet compared to pups in normal cholesterol and mother-reared groups (Auestad et al., 1988).

1.3.1.3 Weaning Period

The suckling phase gradually merges into the weaning period, when solid food increasingly replaces the milk diet. In the rat, the dietary transition period commences around the 16th or 17th postnatal day, with the completion of weaning occurring around postnatal day 30 (Krecek and Kreckova, 1957; Henning, 1981). The rate of cholesterol synthesis increases in most tissues at weaning (except the brain), with the liver and the intestinal mucosa becoming the main cholesterol producers (Hahn, 1986). The activity
of hepatic HMG-CoA reductase increases rapidly at weaning if a high carbohydrate (HG) diet is fed, but remains low if the rat is weaned to a diet high in fat (HF) (McNamara et al., 1972; Hahn et al, 1978). This phase of development is also marked by a decrease in blood cholesterol (McNamara et al, 1972; Hahn, 1989). As rat milk is thought to contain an inhibitor of HMG-CoA reductase activity (Hahn, 1987), the dramatic rise in HMG-CoA reductase is the result of a diet-related removal of the inhibition of the reductase activity and a net synthesis in the reductase protein. Rat hepatic 7 alpha-hydroxylase activity is low in the suckling period and rises at the time of weaning (Hahn and Innis, 1984). The factors controlling this rise are unknown. The high reductase activity associated with lower 7 alpha-hydroxylase activity might be a manifestation of the great demand for cholesterol during this active period of growth and development (Naseem et al, 1980b). The ontogeny of ACAT reveals a dramatic and sudden increase in intestinal activity at weaning. Hepatic activity decreases at this time (Little and Hahn, 1989). The sudden rise in ACAT activity in the intestinal mucosa may be a reflection of the increased rate of cholesterol synthesis occurring at weaning. It is probable that the developmental changes
noted in the key enzymes in cholesterol metabolism are altered to some extent by the change in diet.

A considerable amount of research has been completed in the area of weaning and nutrition. The effects of premature weaning are long-lasting and often persist into adult life. Premature weaning of female rats results in a 50% increase in serum cholesterol compared to rats weaned at the usual time and subsequently exposed to a high cholesterol diet in adult life (Hahn et al, 1978). Subbiah and others have shown that premature weaning of guinea pigs causes a slight decrease in hepatic cholesterol 7 alpha-hydroxylase that persists to 6 weeks of age and is not raised by adding cholesterol to the diet (1985). A decrease in plasma cholesterol levels coupled with a decrease in glucagon and an increase in insulin levels occurs when rats are weaned to a HG diet (Hahn et al., 1980).

Intrauterine or postnatal exposure to a HFC diet results in a 75% decrease in HMG-CoA reductase and a 164% increase in 7 alpha-hydroxylase activity. Weanlings nursed by dams fed a HFC diet had a 66% decrease in HMG-CoA reductase and a 150% increase in cholesterol 7 alpha-hydroxylase activity compared to those nursed by mothers on a normal lab diet (Naseem et al., 1980b).
Cholestyramine included in the diets of post-weanling male rabbits immediately reduced arterial ACAT activity. This decrease persisted for an additional 9 weeks while the animals were fed a normal HG diet (Rymaszewski et al., 1986). Feeding cholestyramine-enriched diets to male pups and their mothers from postnatal day 14 to 21 had no effect on the plasma cholesterol levels but significantly increased reductase activity temporarily (Innis, 1988). The fact that cholestyramine lacks the ability to have any lasting effects on HMG-CoA reductase, supported by the results of other studies that demonstrate the persistent effects of early dietary manipulation on HMG-CoA reductase, suggests that there might be a "critical period" in development when the adjustment of cholesterol metabolism can have permanent effects on metabolism later in life (Innis, 1988; Naseem, 1980a; Subbiah et al., 1985). To my knowledge the effects of a dietary manipulation (high cholesterol, HF, or HG diet) on the ACAT enzyme has not been looked at previously.

In the past, investigators have been concerned with the ability of environmental factors to control and alter genetically regulated development. This section has reviewed a number of animal studies which suggest that dietary manipulation of cholesterol metabolism during the three phases of development can have persistent and
permanent effects on metabolism. Since development is a non-reversible process, there appears to be a critical period during which changes in the diet can have lasting consequences.
### Table II

Examples of late Effects of Early Adaptation

<table>
<thead>
<tr>
<th>EFFECTOR</th>
<th>RESULT IN ADULT</th>
</tr>
</thead>
<tbody>
<tr>
<td>PW to HG Diet</td>
<td>More prone to hypercholesterolemia</td>
</tr>
<tr>
<td>PW TO HF Diet</td>
<td>Prevents hypercholesterolemia</td>
</tr>
<tr>
<td>Overnutrition *</td>
<td>Elevated plasma cholesterol and insulin</td>
</tr>
<tr>
<td>Undernutrition *</td>
<td>Obesity</td>
</tr>
<tr>
<td>Insulin *</td>
<td>Obesity and hypercholesterolemia</td>
</tr>
</tbody>
</table>

* * Between days 3 and 10 after birth
1.4 Objectives

Very little information is available concerning the developmental profile of the ACAT enzyme. A better understanding of the role and control of ACAT during development is needed in order to trace the possible causes early in life that lead to atherosclerosis in the adult. The specific objective of this study was to determine changes in ACAT activity throughout development in rat liver, intestine, aorta, and adipose tissue (BAT and WAT). To further the understanding of the control of ACAT the second study aimed to determine the effects of dietary manipulation on the activity of the ACAT enzyme. The third objective was to demonstrate whether or not there is any relationship between serum cholesterol levels and hepatic and intestinal ACAT activity.
2. EXPERIMENTAL

2.1 EXPERIMENTAL RATIONALE AND DESIGN I

ACAT is responsible for the intracellular esterification of free cholesterol to long-chain fatty-acyl cholesterol esters. Very little information is available concerning ACAT activity during development. Of the three enzymes thought to be rate limiting in cholesterol metabolism, HMG-CoA reductase and 7 alpha-hydroxylase show very low activities in the liver of suckling rats (Hahn and Walker, 1979) and HMG-CoA reductase is low in the gut of infant rats compared to prenatal activity levels (Hahn and Smale, 1981). There is no information regarding the developmental aspects of ACAT in the liver and intestine. Due to the major contribution of extrahepatic tissues to cholesterol metabolism it is important that some of these additional tissues be included in a study about cholesterol metabolism during development.

Accordingly, the first study was designed to establish the ontogeny of ACAT activity in rat liver, intestine, aorta, and adipose tissue (BAT and WAT). The serum cholesterol levels were monitored throughout development in order to determine if a relationship exists between the ACAT enzyme activity and serum cholesterol levels.
To determine the ontogeny of ACAT (study I) the rats were killed on postnatal days 10, 14, 18, 21, 22, 30, and 60. Fetal tissues were obtained on day 21 of gestation by caesarean section. Newborn rats were taken immediately upon delivery (no suckling) and 24 hours postnatally (suckling ad libitum) before tissues were removed following decapitation. The proximal jejunal section of the small intestine was used in this study because Haugen and Norum have shown that ACAT activity is the highest in this portion of the gut (1976). All rats were weaned on postnatal day 21 to Purina Rat Chow. ACAT activity was measured by the rate of incorporation of $[1^{-14}C]$ oleoyl coenzyme A into cholesterol esters according to a method developed by Helgerud et. al (1981).
2.2 EXPERIMENTAL RATIONALE AND DESIGN II

It has been shown that environmental factors acting on the individual early in life may alter the later development of that individual (Hahn, 1989; Hahn, 1987; Hahn and Koldovsky, 1966; Hassan and Subbiah, 1989; Reiser, 1977). Animal studies have shown that dietary manipulation of cholesterol metabolism during an animal's early development can have persistent and permanent effects on that organism later in life.

High plasma cholesterol is a known risk factor for atherosclerosis. This disease is the major cause of death and disability in the industrialized nations. Due to the unequivocal link between dietary cholesterol and coronary artery disease and the fact that ACAT plays a major role in overall cholesterol homeostasis, the corollary of dietary manipulation on ACAT is integral to the complete understanding of the regulation of cholesterol metabolism throughout development. The effect of exposure to high dietary cholesterol, fat, and carbohydrate upon ACAT activity throughout development is unknown. Therefore, the second study was designed to determine whether or not a change in the rat's diet has any effect on the activity of the ACAT enzyme.
Investigated in the second part of this study were the changes in the activity of ACAT in the liver, intestine, adipose tissue, and aorta associated with specific modifications in the diet. The dietary change included weaning rats early on day 18 to one of the following diets: 2% cholesterol, high fat, and high carbohydrate. The rats used in this experiment were 22, 25, 30 and 60 days old. The experimental protocol was the same as that used in study I.
2.3 MATERIALS AND METHODS

2.3.1 Materials

2.3.1.1 Animals

Wistar strain (our own breed and from the University of British Columbia, Animal Care) were used in all experiments. A total of 306 and 111 Wistar rats were used in the developmental (study I) and diet (study II) experiments, respectively.

2.3.1.2 Chemicals

A complete list of the chemicals used in this study and the respective suppliers is given in Table III AND IV.

2.3.1.3 Equipment

A complete list of the equipment used in the experiments is outlined in Table V.
<table>
<thead>
<tr>
<th>Chemicals and Suppliers</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Chemicals</strong></td>
</tr>
<tr>
<td>Chloroform</td>
</tr>
<tr>
<td>Cupric Sulfate</td>
</tr>
<tr>
<td>Diethyl Ether</td>
</tr>
<tr>
<td>Dithioerythritol</td>
</tr>
<tr>
<td>Ethanol</td>
</tr>
<tr>
<td>Glacial Acetic Acid</td>
</tr>
<tr>
<td>Methanol</td>
</tr>
<tr>
<td>Petroleum Ether</td>
</tr>
<tr>
<td>Phenol Reagent</td>
</tr>
<tr>
<td>Potassium Chloride</td>
</tr>
<tr>
<td>Potassium Phospate</td>
</tr>
<tr>
<td>Potassium Sodium Tartrate Crystal</td>
</tr>
<tr>
<td>Sodium Carbonate</td>
</tr>
<tr>
<td>Sodium Chloride</td>
</tr>
<tr>
<td>Sucrose</td>
</tr>
<tr>
<td><strong>Proteins and Enzymes</strong></td>
</tr>
<tr>
<td>Albumin Bovine</td>
</tr>
<tr>
<td>Oleoyl Coenzyme A</td>
</tr>
<tr>
<td><strong>Radiochemicals</strong></td>
</tr>
<tr>
<td>Oleoyl Coenzyme A</td>
</tr>
<tr>
<td>[Oleoyl-1-14C]</td>
</tr>
<tr>
<td>(Specific Activity = 53.5mCi/mmol)</td>
</tr>
<tr>
<td><strong>Diagnostic Kits</strong></td>
</tr>
<tr>
<td>Cholesterol and Triglyceride</td>
</tr>
<tr>
<td><strong>Chromatographic and Scintillation Supplies</strong></td>
</tr>
<tr>
<td>Iodine</td>
</tr>
<tr>
<td>Lipid Standards</td>
</tr>
<tr>
<td>Silica Gel TLC glass plates</td>
</tr>
<tr>
<td><strong>Diet Supplies</strong></td>
</tr>
<tr>
<td>Agar</td>
</tr>
<tr>
<td>Casein</td>
</tr>
<tr>
<td>Cholesterol</td>
</tr>
<tr>
<td>Choline bitartrate</td>
</tr>
<tr>
<td>Corn oil</td>
</tr>
<tr>
<td>Dextrose</td>
</tr>
<tr>
<td>dl-methionine</td>
</tr>
<tr>
<td>Mineral Mix (AIN)</td>
</tr>
<tr>
<td>Vitamin Mix (AIN)</td>
</tr>
<tr>
<td>NAME</td>
</tr>
<tr>
<td>------------------</td>
</tr>
<tr>
<td>Amersham</td>
</tr>
<tr>
<td>BDH</td>
</tr>
<tr>
<td>Biopacific Scientific</td>
</tr>
<tr>
<td>Chemonics Scientific</td>
</tr>
<tr>
<td>E.M. Science</td>
</tr>
<tr>
<td>Fisher</td>
</tr>
<tr>
<td>J.T. Baker</td>
</tr>
<tr>
<td>Medigas</td>
</tr>
<tr>
<td>NEN</td>
</tr>
<tr>
<td>Sigma</td>
</tr>
<tr>
<td>USBC</td>
</tr>
</tbody>
</table>

Table IV

Suppliers names and addresses
<table>
<thead>
<tr>
<th>Equipment</th>
<th>TYPE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Balance</td>
<td>Mettler AE100</td>
</tr>
<tr>
<td>Balance</td>
<td>Mettler PE300</td>
</tr>
<tr>
<td>Centrifuge</td>
<td>Beckman model J6B</td>
</tr>
<tr>
<td>Hand held homogenizer</td>
<td>Wheaton 2ml</td>
</tr>
<tr>
<td>IEC centrifuge</td>
<td>IEC</td>
</tr>
<tr>
<td>N+ Analytical Evaporator</td>
<td>Meyer N-Evap III</td>
</tr>
<tr>
<td>Polytron homogenizer</td>
<td>Brinkman Instruments</td>
</tr>
<tr>
<td>Scintillation counter</td>
<td>Beckman LS9000</td>
</tr>
<tr>
<td>Spectrophotometer</td>
<td>Gilford Stasar II</td>
</tr>
<tr>
<td>Ultracentrifuge</td>
<td>Beckman model L7-55</td>
</tr>
<tr>
<td>Ultracentrifuge</td>
<td>Beckman model LB-55</td>
</tr>
<tr>
<td>Vertis homogenizer</td>
<td>Vertis-23</td>
</tr>
<tr>
<td>Vortex-type mixer</td>
<td>Fisher Vortex-Genie</td>
</tr>
<tr>
<td>Waterbath</td>
<td>Dubnoff Metabolic Shaking Incubator</td>
</tr>
</tbody>
</table>
2.3.1.4 Diets

Male and female white Wistar rats used in the developmental experiments (experiment I) were all weaned on postnatal day 21 and fed Purina Rat Chow and water *ad libitum*. Newborn rats were taken immediately at birth and thus they were not allowed access to their mother's milk. The pups taken 24 hours postnatally were allowed to suckle *ad libitum*. Suckling rats were kept in the same cage as their mothers; therefore, they had free access to their mother's milk, food, and water.

In experiment II (effect of dietary manipulation on ACAT activity) male and female rats aged 22, 25, 30, and 60 days were fed diets of HF, HG, cholesterol-enriched Purina Rat Chow (2% cholesterol), or normal Purina Rat Chow. Rats were prematurely weaned on postnatal day 18. The adult rats (day 60) were weaned on postnatal day 18 to Purina Rat Chow and subsequently fed the experimental diet for at least 5 days prior to the removal of the tissues. The composition of the individual diets are given in Tables VI, VII, and Appendix B.
Table VI

Approximate composition of diets given as a percentage of protein, fat, carbohydrate (CHO), and other nutrients (minerals, vitamins, elements, fibre)

<table>
<thead>
<tr>
<th>DIETS</th>
<th>Protein</th>
<th>Fat</th>
<th>CHO</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>Purina Rat Chow</td>
<td>23.0</td>
<td>4.5</td>
<td>65.0</td>
<td>7.5</td>
</tr>
<tr>
<td>High Carbohydrate</td>
<td>23.0</td>
<td>2.0</td>
<td>69.0</td>
<td>6.0</td>
</tr>
<tr>
<td>High Fat</td>
<td>34.0</td>
<td>46.0</td>
<td>11.0</td>
<td>9.0</td>
</tr>
<tr>
<td>Cholesterol (2%)</td>
<td>22.0</td>
<td>6.5</td>
<td>64.0</td>
<td>7.0</td>
</tr>
</tbody>
</table>

Table VII

Composition of high fat and high carbohydrate diets.

<table>
<thead>
<tr>
<th>NUTRIENTS</th>
<th>High Fat (g)</th>
<th>High Carbohydrate (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>200.0</td>
<td>200.0</td>
</tr>
<tr>
<td>DL-methionine</td>
<td>3.0</td>
<td>3.0</td>
</tr>
<tr>
<td>Dextrose</td>
<td>66.7</td>
<td>600.0</td>
</tr>
<tr>
<td>Corn oil</td>
<td>268.0</td>
<td>20.0</td>
</tr>
<tr>
<td>Mineral Mix (AIN)</td>
<td>35.0</td>
<td>35.0</td>
</tr>
<tr>
<td>Vitamin Mix (AIN)</td>
<td>10.0</td>
<td>10.0</td>
</tr>
<tr>
<td>Choline bitartrate</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Water and Agar</td>
<td>650ml of 1.0% Agar</td>
<td>285ml of 1.0% Agar</td>
</tr>
</tbody>
</table>
2.3.2 Methods

2.3.2.1 Animal Care

The animals were housed in a temperature controlled (23-25°C) animal unit with food (Purina Rat Chow or experimental diets) and water available ad libitum. Lighting was automatically regulated providing a 1600-0400 light and 0400-1600 dark cycle.

2.3.2.2 Animal Breeding

The rats used for experiments performed on postnatal days 10 to 60 were harem-bred by placing one male and two females together in an animal cage for a period of approximately 10 days. After the 10-day breeding period the females were removed and housed together until a few days before the estimated time of delivery when they were separated into cages of their own. On postnatal day 2, the litters were sexed and cross-fostered with litters born on the same day; the litter ratio was ten pups per dam.

The prenatal, neonatal, and diet studies required dated pregnancies, thus two female rats were placed in a cage with one male and day 1 of pregnancy was determined by the presence of spermatozoa in vaginal smears. The females were
then placed alone in a cage for the remainder of gestation. Additional dated pregnant rats were obtained from the University of British Columbia animal care unit. Male and female rats were used for all experiments.

2.3.2.3 Treatment of Animals

Experiments were performed between 0730 and 1000 hours. All rats (except the fetal groups) were killed by a sharp blow to the head followed by decapitation. The dams were anesthetized (sodium pentothal 40 mg/kg) and the fetuses were removed by rapid hysterectomy and killed by decapitation.

2.3.2.4 Blood Collection and Preparation

Blood samples were collected immediately following decapitation and then placed on ice. The blood from fetal, neonatal, and the infant rats were pooled in order to collect 30 ul of serum for subsequent assays. The samples were centrifuged at 4000 rpm (Beckman model J6B centrifuge) for 15 minutes in order to separate the serum from the cells. The serum portion was then removed and stored in Eppendorf tubes at -20°C until serum cholesterol and triglyceride analysis was performed.
2.3.2.5 Serum Triglyceride Analysis: Principle

Serum triglycerides are hydrolyzed to glycerol and free fatty acids (FFA). The glycerol is converted to glycerol-1-phosphate when ATP and glycerol kinase (GK) are added in vitro. The glycerol-1-phosphate is then oxidized to hydrogen peroxide (H$_2$O$_2$) by glycerol phosphate oxidase. In the presence of peroxidase the H$_2$O$_2$ condenses with 3,5-dichloro-2-hydroxy-benzenesulfonic acid (DHBS) to yield a red colored quinoneimine dye. The intensity of the color is directly proportional to the concentration of triglycerides in the sample.

2.3.2.6 Serum Cholesterol Analysis: Principle

Cholesterol esters are hydrolyzed to free cholesterol by cholesterol esterase (CE). In the presence of cholesterol oxidase (CO) free cholesterol is oxidized to cholesten-3-one and H$_2$O$_2$. When peroxidase is added the H$_2$O$_2$ couples with 4-aminoantipyrine and phenol to yield quinoneimine and water. The intensity of the color in the sample is directly proportional to the concentration of total cholesterol (Diagnostic Chemicals Manual, 1987).
2.3.2.6.1 Procedure Used

Blood for serum cholesterol and triglyceride analysis was obtained immediately following decapitation. Total serum cholesterol and triglyceride assay kits (Biopacific Diagnostic) were used for in vitro quantitative determination of total serum cholesterol and triglycerides.

2.3.2.7 Preparation of Tissue for Enzyme Assay

The rat tissues (liver, BAT, WAT, and aorta) were rapidly excised and chilled on ice. The duodenal segment of the bowel was removed and discarded. The next 20-cm was removed and washed with 0.9% ice cold saline containing 1mM of Dithioerythritol (DTT). This segment was opened longitudinally and the mucosa was scraped with a glass slide to a consistent depth and placed in 1ml of 0.2M sucrose solution. The intact segment distal to the duodenum in the fetal and neonatal rats was taken and homogenized without prior scraping. Mucosal samples from the infant and young adult rats were pooled together. The mucosa/sucrose solution was gently mixed and centrifuged at 2500 x g for 10 minutes at 4°C (Beckman model J6B centrifuge) to remove the unbroken cells and debris. The mucosal pellet was then suspended in 1 ml of the sucrose buffer and homogenized with a motor-driven vertis homogenizer with 4 continuous passes.
with the pestle. The glass tubes were placed on ice during homogenization. The homogenate was centrifuged at 6800 x g for 15 minutes at 4 °C in an IEC centrifuge with an International IEC rotor (cat.# 874) in order to sediment the cellular debris, mitochondria, nuclei, and most of the lysosomes (Norum et al., 1979). The 'microsomal fraction' was obtained by centrifuging the supernatant at 140,000 x g for 30 min at 4 °C in a Beckman L7-55 or a L8-55 ultracentrifuge using a Type 50.3 or a Type 25 Ti rotor. The pellet was suspended in 0.2M potassium phosphate (KH₂PO₄) buffer (pH 7.4) and manually homogenized using a Wheaton hand-held tight-fitting homogenizer. The homogenate was then stored at -80 °C. It has been found that the ACAT enzyme is stable at this temperature for long periods of time (up to 1 1/2 years) (Helgerud et al., 1981).

Procedure for the liver, adipose tissue, and aorta were as stated for the intestinal mucosa excluding suspension of the tissue in the sucrose buffer and the initial centrifugation step (2500 x g for 10 minutes); this step was omitted. The liver tissues were homogenized as explained for the intestinal mucosa. The adipose tissue and aortic samples were homogenized in .02M sucrose buffer using a Brinkman Polytron homogenizer. The aorta and BAT samples
were always pooled together (2-9 tissues per sample). Subsequent steps were as stated for the intestinal mucosa.

2.3.2.8 Protein Analysis

The protein content of the samples was analyzed according to Lowry et al. (1951) using bovine serum albumin (BSA) as a standard (Gilford Stasar II spectrophotometer at 660 nm).

2.3.2.9 Preparation of Radiolabelled Oleoyl Coenzyme A

Oleoyl-1-\textsuperscript{14}C (53.5 mCi/mmol) was purchased from NEN and stored at -20 °C. Prior to its use in the ACAT assay, the radioisotope was reconstituted in 3.3 ml of 0.01 M sodium acetate and ethanol buffer (pH 6.0) and 1.7 ml of unlabelled oleoyl CoA containing 5ml of the sodium acetate and ethanol buffer and 5mg of oleoyl CoA. The oleoyl-1-\textsuperscript{14}C preparation was kept in approximately 1ml aliquots and the unused portions of the labelled substrate and buffer was stored at -20 °C for use at a later date.

2.3.2.10 Enzyme Assay

2.3.2.10.1 Principle of Assay

The ACAT enzyme has been extensively studied since its discovery by Mukherjee and co-workers in 1958. The early
experiments investigating the esterification of cholesterol by subcellular fractions used exogenous labelled cholesterol as a substrate. However, this method evoked questions concerning the heterogeneity of the labelled substrate. (Spector, 1979). The most popular ACAT assay employed today uses $^{14}$C-labelled fatty acyl-CoA as a substrate. It has been found that oleoyl-CoA is the best substrate for the ACAT assay, with the rates of synthesis from acyl CoA being cholesteryl oleate > palmitate > stearate > linoleate (Goodman et al., 1964). In addition, cholesterol esters formed within the cell are synthesized primarily from cholesteryl oleate (Spector et al., 1979). There appears to be no value in adding any exogenous cholesterol to the system when an acyl CoA is used (Spector et al., 1979). The highest ACAT activity in rat and guinea pig liver homogenates is found in the microsomal fraction (Helgerud et al., 1981; Stokke and Norum, 1970; Beck and Drevon, 1978).

Although many variations of the ACAT assay exist the procedures all have a number of universal characteristics. Customarily, about 1 mg of microsomal protein is incubated with a fatty acyl-CoA, a thiol, and fatty acid free BSA. The BSA serves as a protective mechanism thus preventing the solubilization of the membrane by fatty acyl-CoA when high concentrations of the acyl-CoA are used. The rate of the
cholesterol ester formation is dependent upon the concentration of the substrate added to the system. Moderate but increasing concentrations of fatty acyl-CoA will stimulate the formation of cholesterol esters, however, after a plateau region is achieved additional substrate causes inhibition. This is probably due to the detergent properties of the fatty acyl-CoA. It has been established that a 2 minute reaction time is preferred when using oleoyl CoA as a substrate and 10 minutes must be allowed for when using palmitoyl-CoA. Upon completion of the reaction period the reaction is generally halted by adding chloroform-methanol. The extracted lipids, triglyceride (TG), free cholesterol (FC), cholesterol ester (CE), fatty acids (FA) are then separated by thin layer chromatography (TLC). A solution of petroleum ether, diethyl ether, and acetic acid is generally used as an eluent.

2.3.2.10.2 Procedure Used

The assay of ACAT activity was performed as described by Helgerud, Saarem, and Norum (1981) with a few modifications. The activity of this enzyme was determined by the formation of labelled cholesterol esters from \([1-^{14}C]\) oleoyl CoA and endogenous cholesterol. The final incubation mixture contained 120-150 ug microsomal protein, 100 ul of
5% BSA in 0.2 M potassium phosphate (KH$_2$PO$_4$) buffer (pH 7.4), and 200 - 300 ul of KH$_2$PO$_4$ buffer to make the final volume to 450 ul. Incubations were carried out in glass tubes shaken continuously in a shaking water bath for 5 minutes at 37 °C. The reaction was started by the addition of [1-$_{14}$C] oleoyl CoA. A standard reaction time of 2 minutes was used. The reaction was stopped by adding 10 ml of chloroform-methanol (2:1 v/v) and 0.5 ml of dH$_2$O. The reaction mixture was then spun at 2000 rpm for 5 minutes 4 °C (Beckman model J6B centrifuge) to separate the phases. The top water phase was then removed and 2ml of 0.88% potassium chloride (KCl) was added to wash the extract before spinning at 2000 rpm for 10 minutes. The chloroform phase was then transferred to glass vials and dried under nitrogen. The remaining lipid extract was resuspended in 100ul of chloroform and applied to silica gel G thin layer chromatography (TLC) plates. The plates were developed with petroleum ether: diethyl ether: acetic acid (83:17:3 v/v) and the band of cholesterol ester was located by visualizing with iodine vapor. The cholesterol ester zone was scraped off the plate with a small putty knife and placed in scintillation vials. The radioactivity was measured by liquid scintillation with 2 ml scintillation fluid in a Beckman LS9000 Liquid Scintillation System.
Esterification rates were calculated as pmol of cholesteryl [1-\textsuperscript{14}C] oleate formed per mg microsomal protein per minute.

2.3.2.11 Statistical Analysis

Data are given as means ± standard error of 6 or more determinations. A one way analysis of variance (ANOVA) model was used to test the hypothesis of equality of ACAT activity throughout development (developmental study) and the equality of responses to different dietary regimes (diet study). Once the null hypothesis was rejected the Tukey-Kramer multiple comparisons test was performed to establish which means were significantly different from each other. This was done for both studies. The computerized statistical package SYSTAT was used.
3. RESULTS

3.1. Developmental Study

Developmental changes in the activity of the ACAT enzyme as measured by the rate of esterification of (1-\(14\)C) oleoyl CoA to cholesterol in rat liver and intestine are shown in Figure 4 and 5 and the results for the adipose tissue (BAT and WAT) and aorta appear in Tables VIII and IX. ACAT activity is expressed as \(\text{pmol}\cdot\text{mg protein}^{-1}\cdot\text{min}^{-1}\), mean ± the standard error (S.E.).

3.1.1 Development of ACAT in Liver

Activity was low in fetal tissue (age = -1) and decreased to a nadir at birth (age = 0). Activity progressively increased during the suckling period to postnatal day 14 (161.30 ± 19.438 pmol\cdot mg protein\(^{-1}\cdot min\(^{-1}\)) and then declined to less than half of this value on postnatal days 18, 21, 22, 25, and 30. The rats were weaned on day 21. The highest values were found in the adult animal (166.68 ± 15.30 pmol\cdot mg protein\(^{-1}\cdot min\(^{-1}\)) (Figure 4).
Figure 4. Ontogeny of ACAT in Liver. ACAT activity (mean ± S.E. of 6 or more rats) expressed as pmol/mg Pr/min as a function of age. The fetal and neonatal values were obtained on day 21 gestation (−1), at birth (0), and 24 hours postnatal (1), respectively. Numbers next to the data points denote age in days.

P < .001 vs. −1 (Fetus) for all groups except days 0, 1, 22, 25;
P < .002 vs. 14 day for all age groups except day 60;
P < 0.05 vs 25 day for days 10, 14, 18, and 60;
P < .001 vs. 60 day for all age groups except day 14.
3.1.2 Development of ACAT in Intestinal Mucosa

ACAT activity decreased significantly from a moderately high fetal value (70.93 ± 5.326 pmol.mg protein⁻¹.min⁻¹) to virtually no activity on day 14 (Figure 2). It then increased reaching maximal values on day 22 (151.65 ± 12.298 pmol.mg protein⁻¹.min⁻¹) before decreasing again to day 60 (9.43 ± 3.677 pmol.mg protein⁻¹.min⁻¹).

3.1.3 Development of ACAT in BAT

ACAT activity was low in BAT throughout development (Table VIII). During the post-weaning period the ACAT activity increased from 6.47 ± 1.663 pmol.mg protein⁻¹.min⁻¹ (postnatal day 30) to 20.28 ± 4.093 pmol.mg protein⁻¹.min⁻¹ (postnatal day 60).

3.1.4 Development of ACAT in WAT

WAT contained insignificant amounts cholesterol-esterifying activity throughout development compared to the activity in the liver and intestinal mucosa during the same period (Table VIII). The results for WAT ACAT activity from postnatal day 10 to 60 were all less than 3.50 pmol.mg protein⁻¹.min⁻¹.
Figure 5. Ontogeny of ACAT activity in Intestinal Mucosa. ACAT activity (mean ± S.E. of 6 or more rats) expressed as pmol/mg Pr/minute as a function of age. The fetal and neonatal values were obtained on day 21 gestation (−1), at birth (0), and 24 hours postnatal (1), respectively. 

P < .001 vs. 22 day for all groups; 
P < .05 vs. −1 (Fetus) for all groups except days 0 and 25; 
P < .05 vs. 14 day old for days −1, 0, 22, 25.
Table VIII

ACAT activity (mean ± S.E. of 5 or more rats) expressed as pmol/mgPr/min throughout development in rat adipose tissue.

<table>
<thead>
<tr>
<th>Age (Days)</th>
<th>BAT</th>
<th>WAT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ACAT Activity</td>
<td>Standard Error</td>
</tr>
<tr>
<td>Fetus</td>
<td>3.59 (a)</td>
<td>1.20</td>
</tr>
<tr>
<td>0</td>
<td>0.15 (a)</td>
<td>0.06</td>
</tr>
<tr>
<td>1</td>
<td>1.48 (a)</td>
<td>0.86</td>
</tr>
<tr>
<td>10</td>
<td>1.30 (a)</td>
<td>0.53</td>
</tr>
<tr>
<td>14</td>
<td>0.12 (a)</td>
<td>0.07</td>
</tr>
<tr>
<td>21</td>
<td>0.40 (a)</td>
<td>0.15</td>
</tr>
<tr>
<td>22</td>
<td>5.89 (a)</td>
<td>1.90</td>
</tr>
<tr>
<td>25</td>
<td>0.32 (a)</td>
<td>0.25</td>
</tr>
<tr>
<td>30</td>
<td>6.48 (a)</td>
<td>1.70</td>
</tr>
<tr>
<td>60</td>
<td>20.28</td>
<td>4.10</td>
</tr>
</tbody>
</table>

(a) P < .001 vs. 60 day old
(b) P < .05 vs. 14 day old

Table IX

ACAT activity (mean ± S.E. of 3 or more samples) expressed as pmol/mgPr/min throughout development in rat aorta. Five or more rat aortae were pooled together to make up 1 sample.

<table>
<thead>
<tr>
<th>Age (Days)</th>
<th>AORTA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ACAT Activity</td>
</tr>
<tr>
<td>Fetus</td>
<td>n/a</td>
</tr>
<tr>
<td>0</td>
<td>n/a</td>
</tr>
<tr>
<td>1</td>
<td>n/a</td>
</tr>
<tr>
<td>10</td>
<td>0.74</td>
</tr>
<tr>
<td>14</td>
<td>7.65</td>
</tr>
<tr>
<td>21</td>
<td>2.75</td>
</tr>
<tr>
<td>25</td>
<td>10.69</td>
</tr>
<tr>
<td>30</td>
<td>7.80</td>
</tr>
<tr>
<td>60</td>
<td>9.63</td>
</tr>
</tbody>
</table>
3.1.5 Development of ACAT in Aorta

Activity of ACAT in the rat aorta was found to be negligible in comparison to the other tissues. However, relatively speaking, the activity increased from $0.743 \pm 0.32$ pmol.mg protein$^{-1}$ .min$^{-1}$ at day 10 to reach a peak on days 21 and 25 ($10.87 \pm 10.617$ pmol.mg protein$^{-1}$ .min$^{-1}$) (Table IX). The cholesterol esterifying activity in the aorta demonstrated large variations within the age groups.

3.1.6 Serum Cholesterol and Triglyceride During Development

Further studies were done to evaluate the relationship of ACAT activity to serum cholesterol concentrations. Serum total cholesterol levels (Figure 6) increased from low levels in the fetus ($58.65 \pm 4.85$ mg/dl) to high levels on the 10th postnatal day ($171.90 \pm 2.89$ mg/dl). It rapidly decreased with increasing age during the mid-suckling period reaching a low the day immediately following weaning (day 22 = $85.72 \pm 6.42$ mg/dl). Cholesterol levels then increased on days 25 and 30 (second peak on day 30 = 135.53 mg/dl) and then it plummeted on day 60 down to 72.75 mg/dl.

Serum triglyceride (TG) concentrations (Figure 6) revealed a similar two-peak developmental curve. Fetal TG
levels were high (152.75 mg/dl) but values decreased to 118.40 mg/dl at birth. The serum TG levels were slightly increased in animals who were allowed to suckle for up to 24 hours. Peak concentrations were obtained on day 10 (172.45 mg/dl). The serum TG levels then decreased on days 14 (141.50 mg/dl) and 21 (119.50 mg/dl). The levels increased again on day 22 (149.59 mg/dl) and stayed virtually the same through to postnatal day 30 before decreasing to 117.94 mg/dl on day 60.
Figure 6. Plasma cholesterol and triglyceride levels (mg/dl) throughout development. The ages -1, 0, and 1 refer to the fetus (day 21 gestation), and neonates (at birth and 24 hours old). Mean ± S.E. of 2 or more determinations. (P < .05).
3.2 Diet Study

In study II, the rats were weaned early at postnatal day 18 to one of the following experimental diets: HG, HF, or 2% cholesterol. With the exception of adult rats, the experimental dietary regime was maintained until the rats were killed. The adult rats were fed the experimental diets for at least 5 days before they were killed.

3.2.1 Liver

The high fat diet was the only diet that consistently increased hepatic ACAT activity throughout development (P < .001; Figure 7). In general all three diets caused an increase in the enzyme's activity. The exception was in the adult animal where it was found that the 2% cholesterol diet significantly decreased ACAT activity and the HG diet had no effect. There was a significant increase in the 22 and 25 day old hepatic ACAT activity in all the experimental groups (P < 0.001). The HF and HG diets significantly increased ACAT activity in the 30 day old rats. Both the HF and HG diets caused marked variations in ACAT activity throughout the period of development that was studied (across age groups). The 2% cholesterol diet caused a significant change in activity of ACAT in the 30 and 60 day old animals compared to the 22 day old animals fed the control diet.
However, there was no difference between the 22 day old control activity and the 25 day old animals fed 2% cholesterol.
Figure 7. The effects of diets on liver ACAT activity (mean ± S.E. of 4 or more rats) expressed as pmol/mg Pr/min as a function of age.

- Day 22 P < .001 vs. Control;
- Day 25 P < .001 vs. Control;
- Day 30 P < .002 vs. Control;
- Day 60 P < .02 vs. Control.
3.2.2 Intestine

The most striking feature of the results of dietary manipulation is the dramatic decrease in activity in the HF and HG diet groups at day 22 (Figure 8). The 2% cholesterol diet significantly increased the ACAT activity in rats aged 25 and 30 but no effect was noted on days 22 and 60. The HG diet decreased the enzyme activity in all the age groups except the 30 day old rats.

3.2.3 WAT

As mentioned before it was found that the WAT contained insignificant amounts of ACAT activity throughout development. The effects of the diets during the post-weaning period were marginal in this tissue with one exception: HG diet increased ACAT activity more than 4-fold (Table X).
**Figure 8.** The effects of diets on Intestinal Mucosa. ACAT activity (mean ± S.E. of 4 or more samples) expressed as pmol/mg Pr/min as a function of age.

* Day 22 P < .001 vs. Control
* Day 25 P < .005 vs. Control
* Day 30 P < .001 vs. Control.
The effects of diets on ACAT activity (pmol/mgPr/min) in WAT ± standard error for sample size (n)

<table>
<thead>
<tr>
<th>Age (Days)</th>
<th>Control</th>
<th>High Carbohydrate</th>
<th>High Fat</th>
<th>2% Cholesterol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ACAT Activity</td>
<td>Standard Error</td>
<td>ACAT Activity</td>
<td>Standard Error</td>
</tr>
<tr>
<td>22</td>
<td>2.153</td>
<td>0.659</td>
<td>12</td>
<td>1.755</td>
</tr>
<tr>
<td>25</td>
<td>0.153</td>
<td>1.000</td>
<td>8</td>
<td>1.080</td>
</tr>
<tr>
<td>30</td>
<td>1.049</td>
<td>0.281</td>
<td>32</td>
<td>4.850</td>
</tr>
<tr>
<td>60</td>
<td>1.217</td>
<td>0.319</td>
<td>16</td>
<td>1.160</td>
</tr>
</tbody>
</table>

87
3.2.4 BAT

The most salient feature in Figure 9 is the reduced ACAT activity in adult BAT for all the diet groups, particularly the dramatic decrease noted in the adult 2% cholesterol group (P < 0.05 for all 3 diets versus the control). No significant changes were found in the 22 and 30 day old animals that were given the experimental diets. The HG and HF diets appear to cause a slight increase in the ACAT activity in the 25 day-old animals, however this increase was not significant. No change was noted with the high cholesterol diet.

3.2.5 Aorta

All three diets relatively decreased the ACAT activity in the 30 and 60 day old aortic samples (Table XI). There was not enough tissue and data from postnatal day 22 for analysis.
Figure 9. The effects of diets on BAT ACAT activity (mean ± S.E. of 4 or more samples) expressed as pmol/mg protein/minute as a function of age. (* P < .05 vs. Control)
Table XI

The effects of diets on ACAT activity (pmol/mgPr/min) in Aorta ± standard error for sample size (n)

<table>
<thead>
<tr>
<th>Age (Days)</th>
<th>DIETS</th>
<th>CONTROL</th>
<th>High Carbohydrate</th>
<th>High Fat</th>
<th>2% Cholesterol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ACAT Activity</td>
<td>Standard Error</td>
<td>(n)</td>
<td>ACAT Activity</td>
<td>Standard Error</td>
</tr>
<tr>
<td>21</td>
<td>2.748</td>
<td>0.313</td>
<td>4</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>25</td>
<td>10.687</td>
<td>10.617</td>
<td>3</td>
<td>0.000</td>
<td>3</td>
</tr>
<tr>
<td>30</td>
<td>7.801</td>
<td>4.251</td>
<td>9</td>
<td>1.500</td>
<td>4</td>
</tr>
<tr>
<td>60</td>
<td>9.633</td>
<td>5.118</td>
<td>6</td>
<td>5.960</td>
<td>4</td>
</tr>
</tbody>
</table>

90
3.2.6 Serum Cholesterol and Triglyceride Levels

The results from the analysis of the serum cholesterol and triglyceride levels appear in Table XII. From this data it is apparent that the HF and HG diets increased the serum cholesterol levels in the rats aged 21, 22, 25, and 30. The 2% cholesterol diet increased the serum cholesterol levels in the 30 and 60 day old animals.

3.4 Statistical Analysis

A one way analysis of variance (ANOVA) model was used to test the hypothesis of equality of ACAT activity throughout development (developmental study I). The one way ANOVA suggests that hypothesis is rejected at the significance level of:

- Liver \( P < .001 \) \( (F = 25.756) \)
- Intestine \( P < .001 \) \( (F = 21.462) \)
- WAT \( P < .006 \) \( (F = 3.216) \)
- BAT \( P < .000 \) \( (F = 11.194) \)
- Aorta \( P < .784 \) \( (F = 0.484) \).

In the diet study (II) significances of difference were tested using the one-way ANOVA test. The one-way ANOVA
suggest that the ACAT activity means (for control and experimental diets within one tissue) are significantly different at a significance level of:

Liver \hspace{1cm} P < .001
Intestine \hspace{1cm} P < .003.
Table XII

Plasma Cholesterol and Triglyceride Levels in animals fed Control, High Carbohydrate, High Fat and 2% Cholesterol Diets.

<table>
<thead>
<tr>
<th>Age (Days)</th>
<th>CONTROL</th>
<th>High Carbohydrate</th>
<th>High Fat</th>
<th>2% Cholesterol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TG</td>
<td>Cholesterol</td>
<td>TG</td>
<td>Cholesterol</td>
</tr>
<tr>
<td>21</td>
<td>136</td>
<td>74</td>
<td>103</td>
<td>91</td>
</tr>
<tr>
<td>22</td>
<td>155</td>
<td>72</td>
<td>104</td>
<td>86</td>
</tr>
<tr>
<td>25</td>
<td>133</td>
<td>108</td>
<td>110</td>
<td>115</td>
</tr>
<tr>
<td>30</td>
<td>94</td>
<td>93</td>
<td>61</td>
<td>124</td>
</tr>
<tr>
<td>60</td>
<td>95</td>
<td>62</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
4. DISCUSSION

4.1 Ontogeny of ACAT

4.1.1 Liver

This study examines for the first time the developmental patterns of ACAT activity in the rat liver, intestine, aorta and adipose tissue (BAT and WAT) from the late fetal stage to the adult. The major features of the ontogeny of rat hepatic ACAT activity are the very low activities noted in the fetus and newborn, the sharp rise in activity during the suckling period followed by an equally sharp decline following spontaneous weaning, and the highest hepatic activity throughout development noted in the adult animal. The fact that the developmental pattern of hepatic ACAT reveals that there is little if any cholesterol esterification mediated by the ACAT reaction prenatally (late term) and at birth and that this activity increases significantly 24 hours postnatally indicates that ACAT may belong to "neonatal" cluster of enzymes described by Greengard (1970). Greengard suggests that the severe neonatal hypoglycemia associated with birth triggers the release of glucagon which in turn stimulates the synthesis of this cluster of enzymes. The fact that Jiao and co-
workers (1988) have reported that insulin inhibits ACAT activity in adult rats further supports the idea that ACAT may be activated by glucagon in the neonatal period.

The peak activity observed on postnatal day 14 is most likely related to the cholesterol in the diet. More cholesterol is circulating in the body at the mid-suckling stage as is evident from the high serum cholesterol values. One could speculate that the ACAT enzyme is induced during the suckling period by the high fat milk diet although the effects are not apparent for some time as is the case in several other enzymes. As mentioned in the introduction, several authors have proposed that there is an inverse relationship between HMG-CoA reductase and ACAT activity. If this is the case then one would expect the activity of ACAT to be high during the suckling period because hepatic HMG-CoA reductase activity is the lowest during this time (McNamara et al. 1972). Furthermore, plasma cholesterol levels reach a peak during this time (McNamara et al. 1972). In the present study the activity of ACAT reached a peak at mid-suckling on postnatal day 14. Thus cholesterol metabolism during the suckling period in the rat liver appears to be following the same sequence as the LDL pathway proposed by Brown and Goldstein (1984; see Figure 2). The
dramatic increase noted at the mid-suckling stage could be due to diet-related induction of the enzyme.

Similarly, the decrease in hepatic ACAT activity observed between days 18 and 25 seems to be diet-related. This rapid decrease may be attributed to the infant rats nibbling on their mother's high carbohydrate chow. It has been shown that the dietary transition period commences around the 16th or 17th postnatal day in the rat (Krecek and Kreckova, 1957; Henning, 1981). The decrease in serum cholesterol levels in this study reflects the change in the rat's diet. The activity of ACAT increased almost four-fold from postnatal day 25 to 60. Why this enzyme would be so active in the adult male rats is difficult to assess. However, one can speculate that the endogenous cholesterol synthesized is not been utilized for biosynthetic purposes and thus the excess is stored as acyl esters. It is possible that this high rate of cholesterol esterification might be sex-related. Hahn (unpublished data) has observed that the ACAT activity in adult female rats were much lower than that of their male litter-mates.
4.1.2 Intestine

It is apparent from the data presented that the rat intestinal mucosa possesses significant amounts of ACAT activity at various stages of development. The key observations in the developmental pattern of ACAT in the intestine are as follows: relatively high prenatal values which decrease with age up to the 14th postnatal day; low values throughout the suckling phase; a dramatic rise in ACAT activity following complete weaning; and an equally dramatic decrease in ACAT activity to the 60th postnatal day.

One of the most intriguing observations in this study is the high ACAT activity in the fetal intestinal mucosa. Why this enzyme is so active at a time when the fetus is receiving its nutrients from the maternal blood via the umbilical veins is unknown. Hahn and Smale have also shown that the activity of HMG-CoA reductase in the fetus is high in the proximal portion of the gut but low in the distal portion (1982). It is possible that the increased esterification observed in this study is a reflection of the increased endogenous cholesterol synthesis.

In searching for factors that might control ACAT activity during prenatal life, Rymaszewski et al. found that the addition of amniotic fluid to rabbit aortic homogenates
causes a linear increase in ACAT activity (1988). Given that the fetus does swallow amniotic fluid (approximately 500 ml per day in the sheep and human; Wintour, 1986) in the latter parts of gestation it is possible that the high ACAT activity in the rat fetal intestinal mucosa is due to a factor(s) in the amniotic fluid. Cholesterol is present in human amniotic fluid (19.6 mg/dl; Natelson, 1974). Park and Subbiah have confirmed that LDL and VLDLs are present in human amniotic fluid (1987).

Hepatic and intestinal ACAT activities have been shown to be increased when the availability of cholesterol is increased (Mitropoulos et al., 1978; Helgerud et al., 1982; Suckling et al., 1982). High plasma cholesterol levels are indicative of this increased substrate supply. However, the serum cholesterol level in the fetus (58.7 mg/dl) was lower when compared to the 10 day old values (171.9 mg/dl), despite the 6-fold difference between the high fetal and low 10 day old ACAT activities. This would indicate that the substrate supply of cholesterol does not play an exclusive role in regulating ACAT activity in the fetus. Rymaszewski and co-workers (1985) suggest that the high ACAT activities observed in the fetal aorta are due either to stimulating factor(s) in the amniotic fluid or to the development of an endogenous inhibitor after birth. This might also be an
explanation for the high fetal intestinal ACAT values observed in the present study.

The intestine is an important tissue in the regulation of whole body cholesterol metabolism. It is the organ which is in direct contact with exogenous dietary cholesterol, it is the site of cholesterol absorption and one of the major cholesterol synthetic tissues in the body. The results indicated that the rat intestinal ACAT activity is low throughout the suckling phase. The developmental pattern suggests that the introduction of the high fat milk diet is affecting the enzyme's activity. Rat milk contains 47% fat, primarily as saturated fatty acids (McNamara et al., 1972; Luckey, et al., 1954) and approximately 120 mg/dl of cholesterol (Carroll, 1964). Diets rich in unsaturated fats significantly increase the intestinal ACAT activity in rabbits; however, saturated-enriched fat diets appear to have no effect on this enzyme (Field and Salome, 1982). ACAT activity has been shown to be dramatically increased by unsaturated fatty acids more so than by saturated fats (Field and Salome, 1982). It has been observed that the status of the membrane is an important modulator of ACAT activity. Perhaps the influx of saturated fats from the rat milk to the small intestine causes more rigidity within the
membrane thus altering its fluidity and the properties of the ACAT enzyme.

Berberian and others (1977) have shown that prostaglandin E2 inhibits the ACAT enzyme and thus cholesterol esterification in the rabbit aorta. The idea that prostaglandins have an effect on intestinal ACAT is pure speculation. However, it is conceivable that they exhibit some effect because prostaglandins are present in the maternal milk and there is very little synthesis in the small intestine of suckling rats (Koldovsky, 1990).

Thyroid hormone may also play a role in the enzyme activity during the suckling phase. Indirect evidence by Hahn (1986b) suggests that thyroid hormones inhibit intestinal ACAT activity during development in rats. At birth thyroid hormone levels are very low. Rat T4 and T3 concentrations increase progressively from birth achieving adult levels around postnatal days 14-16 for T4 and around postnatal day 24-26 for T3 (Sohal, 1988; Artan-Spire et al., 1983). Thus the low ACAT activity in the suckling rat intestine could be due to thyroid hormone inhibition. However, this does not explain the dramatic rise in enzyme activity 24 hours post-weaning.

The dramatic rise in the activity of the ACAT enzyme at weaning suggests that the dietary change from a high fat to
a high carbohydrate has some impact on this enzyme in the intestine. The function of this increased cholesterol esterification is not clear. However it is known that intestinal cholesterol synthesis is increased at this time (Hahn and Smale, 1982; Kroeger and Hahn, 1983). Perhaps the increased esterification is a reflection of this and a means of handling the rapid increase in cholesterol biosynthesis. Nevertheless, the high ACAT activity noted in the immediate post-weaning period suggests that this enzyme plays an important role in the gut. What exactly is this role is difficult to predict from these results however Norum and other investigators (1981) propose several functions for ACAT in the intestinal mucosa. They suggest that the high ACAT activity may be a protective mechanism in the membrane preventing excess accumulation cholesterol. As in other tissues, ACAT might be esterifying cholesterol for future hydrolysis and use in membrane and lipoprotein synthesis. ACAT may also be playing an integral role in true absorption of cholesterol from the gut.

It should be noted that the ACAT enzyme appears to have different developmental patterns and perhaps distinct functions in the liver and the intestine (Figure 10). It is possible that the intestinal ACAT enzyme is responsible for the endogenously produced cholesterol and the hepatic enzyme
THE RELATIONSHIP BETWEEN THE ONTOGENY OF ACAT IN THE RAT LIVER AND INTESTINAL MUCOSA AND PLASMA CHOLESTEROL AND TRIGLYCERIDE LEVELS.

Figure 10. Ontogeny of ACAT activity (pmol/mg protein/min) in the rat liver and intestinal mucosa compared to changes in plasma cholesterol and triglyceride levels (mg/dl) during the same period.
is accountable for the exogenous cholesterol. However an exclusive role for each is difficult to fathom since both tissues seem to respond at certain times in development to a change in the diet. It would seem more reasonable that the intestinal enzyme would be responsible for the exogenous cholesterol esterification given the proposed role of ACAT in the esterification of dietary cholesterol prior to packaging in the chylomicron. If this is the case, the low activity during the suckling phase may be attributed to the suppression of this enzyme by some factor(s) dietary or hormonal. Drevon and co-workers (1980) state that unlike adult rat hepatic ACAT the intestinal ACAT appears to predominantly esterify exogenous cholesterol from dietary or biliary sources as opposed to locally synthesized cholesterol. Whether or not this is so during development remains to be elucidated.

There appears to be no direct relationship between serum cholesterol levels and ACAT activity in the liver and intestine throughout development (Figure 10). This is not surprising because other investigators have not found any correlation between plasma cholesterol and ACAT activity in other tissues (Rymaszewski et al., 1985). The developmental
pattern of serum cholesterol reported here is compatible with earlier reports (McNamara et al., 1972; Carroll and Hamilton, 1973; Hahn, 1982; Hahn, 1978).

4.1.3 WAT

White adipose tissue contains more cholesterol per mg of protein than all other organs except skeletal muscle when expressed on a whole organ basis (Krause and Hartman, 1984). It is generally assumed that cholesterol in tissues exists as a structural component of the biological membrane, however this does not appear to be the case in adipose tissue (Angel and Farkas, 1974). In the adult most of the cholesterol is stored in the central oil droplet rather than in the membrane. It exists primarily in the unesterified form which in itself distinguishes WAT as unique amongst the tissues in the body (Krause and Hartman, 1984).

Cholesterol is reported to be released from the adipose tissue when catecholamines are administered (Farkas et al., 1973) and during starvation of adult rats (Angel and Farkas, 1974). It is thought that cholesterol is released in these situations through the lipolytic action of cyclic-AMP on cholesterol esterase. Neutral cholesterol esterase has been reported to be present in WAT and functions to hydrolyze the incoming cholesterol esters (Arnaud and Boyer,
ACAT activity has been reported to be low or absent in adult rat adipose tissue concomitantly with little cholesteryl ester stores (Angel and Farkas, 1974). This argument was strengthened by the results presented here which indicate that the activity of ACAT is undetectable not only in the adult animals but throughout development. As suggested by others it is possible that cholesterol is stored as free cholesterol in a central oil droplet of the adipocyte and is released as such upon demand (Angel and Farkas, 1974).

4.1.4 BAT

Results indicated that there is little if any ACAT activity in BAT throughout development. It was thought, perhaps naively, that because HMG-CoA reductase is active even in the fetal tissue there might be similar or inverse activities of the ACAT enzyme. This does not appear to be the case. In contrast to what was found in WAT, ACAT activity appeared to be elevated in the adult animal. Surprisingly, this post-weaning rise occurs at a time when the amount of brown fat relative to body weight is decreasing with postnatal age, thus there is little if any cell growth and little cell membrane requirement (Aherne and Hull, 1966; Tarkkonen and Julku 1968). In addition,
cholesterol is not a precursor for any known hormone synthesis in this tissue (Nedergaard 1986). It is possible that the ACAT enzyme of BAT origin is participating in the regulation of plasma cholesterol levels in the adult animal.

4.1.5 Aorta

ACAT and its relation to cholesterol ester metabolism is of particular interest because of its role in atherosclerosis. It is well documented that arteries undergoing atherogenic change characteristically show an increase in cholesterol esterifying activity by ACAT and a progressive increase in cholesterol ester content (St. Clair, 1976). The activity of ACAT has been reported to be increased up to 50-fold in atherosclerotic arteries (Hashimoto, 1974; St. Clair, 1976). It has been suggested that ACAT operates intracellularly to protect the cell from excess cholesterol accumulation. This is a controversial issue since it is not known whether the increase in ACAT activity in the atherosclerotic artery represents an exacerbating factor or a passive consequence of an increased availability of free cholesterol (Gillies, et al., 1986).

Activity of ACAT in the rat aorta was found to be negligible in comparison to the other tissues. The variability of ACAT activity in the infant rats may be due
in part to the small amount of arterial tissue. A small sample size in all of the age groups may have contributed to wide variations in activity. It is likely that the ACAT assay used is not sensitive enough for this small amount of tissue. Further studies with larger pools of tissue or with tissue from a different animal model are needed. Needless to say the results presented here do not support Sprinkle and co-worker's findings in which ACAT activity from rabbit aortae decreased as a quadratic function with age (Sprinkle et al., 1987).

4.2 Effects of Diets on ACAT Activity

In the previous study results indicated that there is a marked variation in the activity of ACAT in the liver, intestine, and BAT throughout development. An extension of this work was a dietary study which focused on the immediate effects of short-term manipulation of diet on the activity of ACAT. The rats in this study were all weaned early on day 18 to one of the following diets: Purina Rat Chow, high carbohydrate, high fat, or 2% cholesterol.
4.2.1  Liver

The argument that diets high in fat increase the activity of ACAT was strengthened by the present results. In fact the high fat diet was the only diet that consistently increased hepatic ACAT activity throughout development (Figure 7). The response of ACAT to a fat diet seems to be dependent on the type of fat used. Spector, Kaduce, and Dane (1980) fed rats diets enriched in either saturated or unsaturated fats and observed that the hepatic ACAT activity was 70-90% higher in the unsaturated fat-fed animals than the activity of those rats fed saturated fats. Presumably, the fatty acid composition of the membrane has some influence on the enzyme activity (Field and Salome, 1982) possibly by causing a more fluid endoplasmic reticular membrane. Unfortunately only corn oil (unsaturated fat) was used in this study thus a comparison between different fat diets cannot be made. Seccombe and colleagues (1987) also used corn oil in their rabbit studies. They too found an increase in ACAT activity (1.5 times) with the high fat diet. Surprisingly, an even greater increase in ACAT activity (4.5 times) was observed when carnitine was added to the fat diet.

The cholesterol diet significantly increased the ACAT activity in the 22 and 25 day old animals. This confirms
earlier reports which state that 0.1 - 5% cholesterol-rich diets increase ACAT activity 2-3 fold (Erickson et al., 1978; Erickson et al., 1980; Mitropoulos et al., 1978). The obvious mechanism would be that this increase provides additional substrate for the reaction. Suckling et al. (1982) suggest that the normal supply to the microsomal enzyme is probably suboptimal which allows the enzyme to respond to changes in cholesterol supply.

4.2.2 Intestine

The most striking feature of the results of dietary manipulation is the dramatic decrease in activity in the HF and HG diet groups at day 22 (Figure 8). The effects of dietary fat on intestinal ACAT varies with the type of fat used and the amount of fat administered (Suckling and Stange, 1985). In the present study a 45% corn oil diet was given to the rats. This precipitated a rapid drop in enzyme activity in the 22 day old rats. Others have shown that an intraduodenal perfusion of cholesterol-free lipid causes similar changes in ACAT activity plus a decrease in mucosal free cholesterol content and decreased secretion of cholesteryl ester into the mesenteric lymph (Bennett Clark,
1979; Suckling and Stange, 1985). Stange and co-workers (1983) observed no significant effects on ACAT activity when rats were fed a 10% corn oil diet for three days. The 2% cholesterol diet significantly increased the ACAT activity in 30 day old rats as seen in the liver but no effect was noted on days 22, 25, and 60. This lack of stimulation in these rats was unexpected because many investigators have reported an increase in ACAT activity with cholesterol feeding in rats (Stange et al., 1983a; Helgerud et al., 1982; Norum et al., 1983), guinea pigs (Drevon, 1978), and rabbits (Field and Salome, 1982). The HG diet decreased the enzyme activity in all the age groups except the 30 day old rats.

4.2.3 Aorta, BAT, and WAT

Examination of the effects of dietary manipulation on the aortic ACAT activity provided no conclusive results. This was likely due to a small sample size, small amounts of tissue, and an assay that was not sensitive enough for this small amount of tissue.

From the developmental study it was apparent that there was little if any ACAT activity in BAT throughout development except in the adult tissue. It was proposed that this dramatic increase in ACAT activity might be
affected by a change in diet. All three diets significantly decreased ACAT activity in the adult BAT (postnatal day 60). However such was not the case for the other age groups. There was no significant differences between the BAT in rats fed an experimental diet and the BAT of rats fed the Purina Rat Chow.

The developmental results indicated that the rat white adipose tissue is devoid of ACAT activity. Similarly, there appeared to be no diet-induction of ACAT activity. It was thought that there might be a change in activity with the 2% cholesterol diet because a direct correlation between dietary cholesterol level and adipocyte cholesterol content has been demonstrated by Angel and Farkas (1974). However, the results presented here neither confirm nor deny this correlation they merely suggest that the cholesterol accumulated is not stored in the ester form via the ACAT reaction (Little and Hahn, 1989; Little and Hahn, 1990).
5. SUMMARY AND CONCLUSIONS

5.1 Developmental Study I

The uptake of cholesterol ester into cells by receptor-mediated endocytosis and its subsequent de-esterification and re-esterification by intracellular enzymes has generated much interest particularly because of the link between these processes and pathological conditions such as atherosclerosis (Brown and Goldstein, 1984). It has been shown that the pathogenesis of the atherosclerotic plaque begins early in life although the clinical symptoms are not apparent until mid or late adulthood. Animal studies have shown that dietary manipulation of cholesterol metabolism during an animal's early development can have persistent and permanent effects. Therefore it is important that the ontogeny of ACAT, one of the key enzymes in cholesterol metabolism, be clearly established.

The primary focus of the research presented here was in establishing the ontogenic pattern of the ACAT enzyme. Using an ACAT assay developed by Helgerud, Saarem, and Norum (1981) the developmental profile of ACAT activity in the rat liver, intestine, brown and white adipose tissue, and aorta was defined. A total of 306 Wistar rats were used. The ACAT activity was measured in the late-term fetus, newborn,
and on postnatal days 1, 10, 14, 18, 21, 22, 25, 30, and 60. The findings of these studies can be summarized as follows:

i. The rat liver and intestine possess significant amounts of ACAT activity throughout development and there appears to be marked variations in activity during this time. These results suggest that the ACAT enzymes originating from these two tissues play major roles in cholesterol metabolism throughout development in the rat;

ii. The rat brown and white adipose tissues appear to be devoid of ACAT activity throughout development with the exception of adult BAT. The high ACAT activity in the adult BAT suggests that this tissue may participate in the regulation of cholesterol metabolism;

iii. Due to the small amount of the aortic tissue samples and/or the insensitivity of the assay, no definite conclusions could be made from this aortic study.
5.2 Diet Study II

In searching for factors that might control the ACAT enzyme the immediate effects of short-term manipulation of diet on the activity of ACAT was studied. A total of 111 Wistar rat was used in this study. The rats were all weaned early on day 18 to one of the following diets: Purina Rat Chow, high carbohydrate, high fat, or 2% cholesterol. The results can be summarized as follows:

i. The HF was the only diet that consistently increased hepatic ACAT activity. These results suggest that fatty acids play an important role in the control of this enzyme possibly by altering the fluidity of the reticular membrane. The cholesterol diets significantly increased the activity of ACAT in the 22 and 25 day old rats. This suggests that the substrate supply to the ACAT enzyme is also an important regulator of activity in these animals. The HG diet increased the activity of ACAT in the 22, 25, and 30 day old rats. No significant differences were observed between the adult control and HG diet groups;

ii. Feeding the rats a HF or HG precipitated a dramatic drop in intestinal ACAT activity in the 22
day old animals. These effects were not observed in the older animals suggesting that the enzyme of the 22 day old intestine is particularly sensitive to changes in the diet. The high cholesterol diet had no significant effect on the enzyme’s activity;

iii. There was no significant change in the BAT and WAT ACAT activity with the experimental diets. Examination of the effects of dietary manipulation on aortic ACAT activity provided no conclusive results.

Acyl-coenzyme A: cholesterol acyl-transferase undoubtedly plays an important role in cholesterol esterification and hence this enzyme may be involved in the pathogenesis of atherosclerosis.
5.3 Suggestions for Future Work

Although the present study established the ontogenic pattern of ACAT in the intestine and liver the role the enzyme plays in these two tissues during development is still unknown. It has been suggested that ACAT aids in the absorption of cholesterol. ACAT activity has been found to be decreased by the inhibitor Sandoz compound 58-035 (Suckling and Stange, 1985; Sampson et al., 1987). This compound also causes pronounced malabsorption of cholesterol when administered in vivo (Suckling and Stange, 1985). This compound could be used in an experiment designed to test whether or not intestinal ACAT plays a role in the absorption of exogenous cholesterol and at what period of development does this enzyme display this function. Labelled cholesterol could be given to the rats in addition to the inhibitor and the changes in the cholesterol ester content in the intestinal mucosa and the lymph could be monitored.

It is likely that there is some hormonal control of the ACAT enzyme during development. It seems suitable that further studies should incorporate the effects of such hormones as T3, T4, insulin, and glucagon on this enzyme. These hormones probably play an important role in
controlling the activity of the ACAT enzyme especially during development.

It was found in the present study that the HF and HG diets had profound effects on the ACAT activity in the 22 day old rat intestine. However, the question of whether or not these diets had any lasting effects on the enzyme was not addressed in this study. Further studies are needed in order to determine whether or not these changes in ACAT persist into adulthood even after the animal is given a recovery period to normalize on a Purina Chow diet.

To understand completely the regulation of cholesterol storage of adipose tissue, one must establish whether or not the increase in cholesterol content in the adipose tissue of cholesterol-fed rats is in the ester or free form. If there is an increase in cholesterol ester content it is probably due to the action of CEase. The data presented here suggest that ACAT activity of adipose tissue origin is not affected by an increase in dietary cholesterol or fat.

It has been found that ACAT is also present in the steroid-producing tissues. The concentration of cholesteryl esters varies among these tissues. For instance the rat adrenal cortex contains large amounts of cholesteryl ester whereas the Leydig cell from the testis appears to contain
very little cholesterol ester (Suckling and Stange, 1985). I was curious as to whether or not ACAT plays the same role in the placenta as it does in the adrenal, that is to provide a storage pool for steroid hormone synthesis.

It was found in this study that there is some ACAT activity in the rat placenta albeit in low concentrations (Appendix A). Based on the Brown and Goldstein LDL uptake model one might propose that the uptake of lipoproteins by the placental cells would stimulate the formation of cholesteryl esters. However, it is well known that cholesteryl esters are not present in the placenta to any great degree, a finding which is inconsistent with the Brown and Goldstein model (Simpson and Burkhart, 1980). There are at least two possible explanations for this. The first is that the placenta continually utilizes cholesterol for steroid hormone synthesis thus the ACAT reaction is an unwanted process. The second plausible explanation is that the stimulation of ACAT (or CEase) by the LDL uptake is overridden by an inhibitory pathway. It has been found by Simpson and Burkhart (1980) that the ACAT enzyme is inhibited by progesterone and pregnenolone in humans. It appears that cholesterol ester synthesis is inhibited when progesterone is synthesized resulting in a decrease in free
cholesterol and an inhibition of ACAT (Simpson and Burkhart, 1980). Therefore, this could account for the low concentrations of ACAT activity observed in the rat placenta. It would be worth the time to expand on these preliminary rat results and move into the human model and investigate the activity of ACAT in the human placenta at different stages of gestation.
6. REFERENCES


Balasubramaniam, S., Venkatesan S., Mitropoulos, A. and Peters, T.J. The submicrosomal localization of acyl-coenzyme A: cholesterol acyltransferase and its substrate, and


Personal communication between P. Hahn and K.A. Norum


Rymaszewski, Z., Sprinkle, D.J., Yunker, R.L., Subbiah, M.T.R. High Acyl-CoA cholesterol acyl transferase activity in fetal rabbit aorta: Evidence for the presence of


Stange, E.F. and Dietschy, J.M. Age-related decreases in tissue sterol acquisition are mediated by changes in cholesterol synthesis and not low density lipoprotein uptake in the rat. J. Lipid Res. 24, 703-713 (1984).


7.1. APPENDIX A.

ACAT ACTIVITY IN RAT FETAL AND PLACENTAL TISSUES
ACAT ACTIVITY IN RAT FETAL AND PLACENTAL TISSUES

Figure 11. ACAT activity (pmol/mg Pr/min) in the liver, intestinal mucosa, BAT, and placenta in the rat on day 21 gestation.
7.2 APPENDIX B.

COMPOSITION OF PURINA RAT CHOW AND PURINA PLUS 2% CHOLESTEROL DIET
Appendix B.

Composition of Purina Rat Chow and Purina Rat Chow plus 2% diet.

<table>
<thead>
<tr>
<th>NUTRIENTS</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ground extruded corn</td>
<td>Choline chloride</td>
</tr>
<tr>
<td>Sybean pulp</td>
<td>Folic acid</td>
</tr>
<tr>
<td>Fish meal</td>
<td>Riboflavin</td>
</tr>
<tr>
<td>Ground oats</td>
<td>Thiamin</td>
</tr>
<tr>
<td>Brewers' dried yeast</td>
<td>Niacin</td>
</tr>
<tr>
<td>Alfalfa meal</td>
<td>Pyridoxine hydrochloride</td>
</tr>
<tr>
<td>Cane molasses</td>
<td>Ferrous sulfat</td>
</tr>
<tr>
<td>Wheat germ meal</td>
<td>Vitamin A supplement</td>
</tr>
<tr>
<td>Dried whey</td>
<td>D-activated animal sterol</td>
</tr>
<tr>
<td>Meat meal</td>
<td>Vitamin E supplement</td>
</tr>
<tr>
<td>Animal fat with BHA</td>
<td>Calcium iodate</td>
</tr>
<tr>
<td>Dicalcium phosphate</td>
<td>Ferrous carbonate</td>
</tr>
<tr>
<td>Salt</td>
<td>Manganous oxide</td>
</tr>
<tr>
<td>Wheat middlings</td>
<td>Cobalt carbonate</td>
</tr>
<tr>
<td>Calcium carbonate</td>
<td>Copper sulfate</td>
</tr>
<tr>
<td>Vitamin B-12 supplement</td>
<td>Zinc sulfate</td>
</tr>
<tr>
<td>DL-methionine</td>
<td>Zinc oxide</td>
</tr>
<tr>
<td>Calcium pantothenate</td>
<td></td>
</tr>
</tbody>
</table>

156