THE EFFECT OF EARLY DIET ON HEPATIC CHOLESTEROL METABOLISM IN PIGLETS

by

ANGELA MARIE DEVLIN

B.Sc., The University of Western Ontario, 1992

THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES

(Individual Interdisciplinary Studies Graduate Program)
[Pediatrics/Biochemistry/Physiology/Pathology]

THE UNIVERSITY OF BRITISH COLUMBIA
1998

ABSTRACT

Plasma total, low density lipoprotein (LDL) and high density lipoprotein (HDL) cholesterol concentrations increase immediately following birth. Interestingly, this increase is greater in breast-fed infants than in infants fed formula. The reason(s) why there are differences in plasma cholesterol concentrations between breast-fed and formula-fed infants is not known. However, this difference may be a consequence of the variations in lipid composition between milk and infant formula. Little is known regarding the specific effects of the lipid component(s) of infant diets on the expression of genes involved in hepatic lipid metabolism. The studies presented in this thesis determined whether the addition of cholesterol, arachidonic acid [20:4(n-6)] and docosahexaenoic acid [22:6(n-3)] to formula, and the positional distribution of fatty acids in formula triglycerides increases plasma cholesterol in formula-fed piglets to levels observed in milk-fed piglets. In study #1, piglets were fed from birth to 18 days of age with either a conventional infant formula (conventional formula) or a formula with synthesized triglycerides (TG) (synthesized TG formula). The conventional infant formula had 70% of the total 16:0, representing 23% of total fatty acids, esterified at the *sn*-1 and 3 positions of the formula triglyceride. The synthesized TG formula contained a similar percentage of 16:0, representing 23% of total fatty acids, but had 47% of the total 16:0 esterified at the centre (*sn*-2) position of the formula triglyceride. Each of the conventional and synthesized TG formulae were provided either without (<0.10 mM) or with 0.65mM cholesterol added to formula, 0.52mmol/L as unesterified cholesterol and 0.13 mmol/L as cholesterol oleate. A reference group of piglets was also fed sow milk.

In study #1, the levels of hepatic HMG-CoA reductase mRNA, 7-α-hydroxylase (C7H) mRNA, and acetyl CoA carboxylase (ACC) mRNA were higher in the formula-fed than milk-fed piglets, irrespective of the formula cholesterol content or the positional distribution of fatty acids in the formula triglyceride. This was accompanied by lower plasma total and HDL cholesterol concentrations, lower hepatic triglyceride concentrations and lower concentrations of bile acids, cholesterol and phospholipid in bile of the formula-fed than milk-fed piglets. Adding cholesterol to the formula increased hepatic cholesterol concentrations and decreased hepatic levels of fatty acid synthase (FAS) mRNA, but had no effect on the plasma cholesterol concentrations of the formula-fed piglets. Directing 16:0 to the *sn*-2 position of the formula triglyceride led to lower plasma total cholesterol and triglyceride concentrations, lower concentrations of bile acids in bile, lower hepatic levels of FAS mRNA and activity, and higher hepatic levels of ACC mRNA than in piglets fed the conventional formula.

In study #2, piglets were fed the conventional formula either without or with egg phospholipid (9.5g/L) to provide 0.8% 20:4(n-6) and 0.3% 22:6(n-3) of total fatty acids, or sow milk from birth to 15 days of age. Supplementing the conventional formula with egg phospholipid resulted in higher levels of 20:4(n-6) and 22:6(n-3) in liver and bile phospholipid, higher plasma HDL concentrations, higher bile acid and phospholipid concentrations in bile and lower hepatic ACC mRNA levels in the formula-fed piglets. The levels of 20:4(n-6) and 22:6(n-4) in liver and bile phospholipid were also higher in the piglets fed the supplemented formula than in the piglets fed milk. A significant inverse relation was found between the levels of hepatic ACC mRNA and the percentage of 20:4(n-6) in liver triglyceride and the percentage of 22:6(n-3) in liver phospholipid. Egg

phospholipid supplementation of formula had no effect on hepatic LDL receptor mRNA or hepatic FAS activity and mRNA in the formula-fed piglets. The piglets fed either the supplemented or the conventional formula had lower levels of plasma cholesterol and higher levels of hepatic HMG-CoA reductase activity and mRNA and C7H mRNA than piglets fed milk.

These studies show that early diet, that is, milk compared to formula feeding, results in lower levels of hepatic HMG-CoA reductase activity and mRNA and C7H mRNA accompanied by higher plasma cholesterol concentrations in piglets. Supplementing formula with cholesterol or the preferential esterification of 16:0 at the *sn*-2 position of the formula triglyceride did not raise plasma cholesterol concentrations and had no effect on hepatic HMG-CoA reductase activity and mRNA or C7H mRNA in formula-fed piglets. Supplementing formula with egg phospholipid, increased bile and liver phospholipid 20:4(n-6) and 22:6(n-3), decreased the levels of hepatic ACC mRNA and increased the concentrations of bile acids and phospholipid in bile. These findings suggest that milk-fed piglets have lower rates of hepatic cholesterol synthesis, lower rates of conversion of cholesterol to bile acids and the lipid present in sow milk and formula may be metabolized differently. These findings are significant in that they raise the question as to whether or not this effect of early diet will continue through to adulthood and influence metabolic response to diet fat.

TABLE OF CONTENTS

Appendix II. Results Tables Study 1

Appendix III. Results Tables Study 2

List of Tables

List of Figures

Chapter 1. Introduction

Chapter 3. Materials and Methods

Chapter 4. Results

Study #1

Study #2

Abbreviations

ACC, acetyl CoA carboxylase
ANOVA, analysis of variance
apo, apolipoprotein
ATP, adenosine triphosphate
C7H, 7-α-hydroxylase
cDNA, copy deoxyribonucleic acid
DNA, deoxyribonucleic acid
dNTPs, deoxynucleotide triphosphates
DTT, dithiothreitol
EDTA, ethylenediamine tetraacetic acid
FAS, fatty acid synthase
HCl, hydrogen chloride
HDL, high density lipoprotein
HMG-CoA, hydroxymethyl glutaryl coenzyme A
IDL, intermediate density lipoprotein
IgG, immunoglobulin G
KCl, potassium chloride
KH_2PO_4, potassium phosphate
LDL, low density lipoprotein
LPL, lipoprotein lipase
LRP, LDL receptor related protein
$MgCl_2$, magnesium chloride
mRNA, messenger ribonucleic acid
NaCl, sodium chloride
NADPH, nicotinamide diamine phosphate
PL, phospholipid
RT-PCR, reverse transcription-polymerase chain reaction
SRE, sterol regulatory element
TBE, tris borate EDTA
TG, triglyceride
T4, thyroxine
VLDL, very low density lipoprotein
16:0, palmitic acid
18:1(n-9), oleic acid
18:2(n-6), linoleic acid
18:3(n-3), linolenic acid
20:4(n-6), arachidonic acid
22:6(n-3), docosahexaenoic acid

ACKNOWLEDGEMENTS

I would like first to acknowledge and thank my thesis supervisor, Dr. Sheila Innis for providing support and encouragement throughout the duration of this thesis project. The members of my thesis committee, Dr. Derek Applegarth, Dr. Ross MacGillivray, Dr. Christopher McIntosh and Dr. Haydn Pritchard were also essential in the completion of this thesis, and I want to thank each for their expertise in the various disciplines covered by the work. I am also grateful for the technical support provided by Robert Shukin, Janette King, Roger Dyer and Laurie Nichol. My fellow Ph.D. students, Carolanne Nelson, Patty Williams, Loraina Stephen, and Dr. France Rioux and Dr. Sylvia De La Pressa are also deserving of thanks, both for their insightful discussions and support. I would like to extend a special thanks to my parents, James and Joanne Devlin who always provided support, even from a distance. Thank-you also to my sisters, Katherine and Christine and my friends Jennifer Burke, Daphne Moffat and Joanne Waxman. Finally, I would like to thank Cameron Thorn who always supported my endeavours.

1. INTRODUCTION

1.1. OVERVIEW

Plasma total, low density lipoprotein (LDL) and high density lipoprotein (HDL) cholesterol concentrations increase rapidly in human infants following birth (Ginsburg & Zetterstrom 1980, Kallio et al. 1993, Lane & McConathy 1986, Van Biervleit et al. 1986, Van Biervleit et al. 1980). Interestingly, the postnatal increase in plasma cholesterol concentration is greater in breast-fed (milk-fed) infants than in infants fed formulae (Kallio et al. 1997,Lane & McConathy 1986, Lourdes et al. 1994, Mize et al. 1995, Van Biervleit et al. 1986, Wong et al. 1993). A similar diet-related difference in plasma cholesterol has also been observed in young baboons and piglets fed milk and formula (Mott et al. 1990, Rioux & Innis 1993). Further, blood lipid concentrations of long chain polyunsaturated fatty acids, specifically, arachidonic acid [20:4(n-6)] and docosahexaenoic acid [22:6(n-3)], are higher, whereas levels of linoleic acid [18:2(n-6)] are lower, in infants fed milk than in infants fed formula not containing 20:4(n-6) and 22:6(n-3) (Innis et al 1997,1994,Makrides et al. 1994). The long-term consequence of these diet-related differences in blood fatty acid and cholesterol concentrations is not known. In the short-term, however, it implies there may be diet-related differences in the metabolic processing and handling of fatty acids and cholesterol during the suckling period among infants fed different diets.

It seems reasonable to suggest that the compositional differences, specifically the differences in lipid composition, between milk and formula (Huisman et al. 1996, Innis 1992, Jensen & Jensen 1992, Spear et al. 1992) could be responsible for the differences in plasma cholesterol and fatty acid concentrations which are present

between milk-fed and formula-fed infants. Both the dietary intake and the endogenous synthesis of cholesterol and fatty acids (triglycerides) are important determinants of circulating lipoprotein levels. Therefore, the higher plasma cholesterol concentrations and higher plasma 20:4(n-6) and 22:6(n-3) in breast-fed infants than in formula-fed infants can reasonably be suggested to be a consequence of the higher dietary intake of cholesterol, and/or 20:4(n-6) and 22:6(n-3) from milk than from formula. In addition, diet-induced alterations in the synthesis, secretion to plasma, tissue uptake (clearance), and excretion of cholesterol or its bile acid products could also be involved in contributing to the differences in plasma lipids between breast-fed and formula-fed infants.

1.2. LIPID COMPOSITION OF THE INFANT DIET

Both human milk and infant formula supply about 50% of total energy from fat. This amount of fat is high relative to an average Canadian adult diet (30-40% fat), but is important in providing for the high energy demands of early growth with a relatively energy dense food.

1.2.1. Lipid Composition of Mammalian Milk

Human milk contains approximately 4g/dL lipid, 1g/dL protein and 7.0g/dL carbohydrate (lactose and other oligosaccharides), minerals, and water-soluble vitamins (Jensen 1989, Neville and Picciano 1997). The milk-fat globule contains approximately 98% triglyceride, with the remaining 2% being mainly phospholipid and sterols. The milk fat provides about 50% of the total energy in milk. The triglyceride is contained in the core of the milk-fat globule and is surrounded by a membrane containing phospholipid, unesterified cholesterol, protein and fat-soluble vitamins. Both the

amount (percentage of total energy) and composition of fatty acids in human milk can be quite variable. Examples of factors shown to influence the lipid content and fatty acid composition of milk include the stage of lactation, that is, mature milk has a higher concentration of lipid than colostrum, time within a nursing period, with the lipid concentration increasing during a nursing, and the fat content and fatty acid composition of the maternal diet (Jensen 1989).

The most abundant saturated fatty acid in human milk is palmitic acid (16:0), which is present at about 20-25% of the total fatty acids (Innis 1992). Human milk also contains smaller amounts of other saturated fatty acids, including stearic acid (18:0) and lauric acid (12:0) + myristic acid (14:0). These fatty acids represent about 6-8% and 8-15% of the total fatty acids, respectively. About 70% of the total 16:0 is esterified at the *sn*-2 position of the triglyceride in milk (Martin et al. 1993) (**Figure 1.1**).

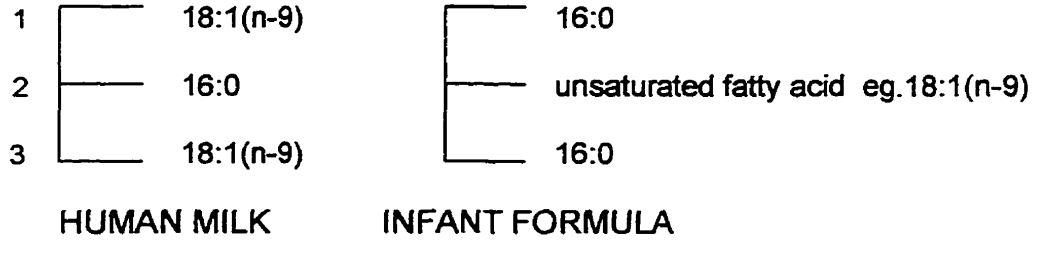


Figure 1.1. Schematic representation to illustrate most usual positional distribution of 16:0 in human milk and infant formula triglyceride.

The positioning of 16:0 in the milk triglyceride is unusual in that most vegetable and animal fats contain the majority of 16:0 esterified at the *sn*-1and 3 positions of the triglyceride (Innis et al. 1998). Oleic acid [18:1(n-9)] is present at about 30-40% of the total fatty acids, and is found primarily at the *sn*-1 and 3 positions in the milk triglyceride.

The essential fatty acids, linoleic acid [18:2(n-6)] and linolenic acid [18:3(n-3)], are also present in human milk and represent about 10-16% and 1%, respectively, of total fatty acids. Linoleic acid is usually found at the *sn*-1 and 3 positions of the milk triglyceride, whereas 18:2(n-6), in most dietary fats and oils, as well as most tissue and plasma lipids, is found esterified to the *sn*-2 position of the triglyceride (Jensen 1989). The long chain polyunsaturated fatty acids, arachidonic acid [20:4(n-6)] and docosahexaenoic acid [22:6(n-3)] are also present in human milk, and usually represent about 0.4% and 0.2%, respectively, of the total fatty acids. Over 150 different fatty acids have been identified in human milk; thus, milk contains small amounts of other saturated, monounsaturated and (n-6) and (n-3) polyunsaturated fatty acids. The unsaturated fatty acids, 16:0 and other longer chain saturated fatty acids found in milk are derived from the maternal circulation (Innis et al. 1998). The mammary gland synthesizes saturated fatty acids up to 14:0, but does not synthesize longer chain fatty acids. The concentrations of unsaturated fatty acids, 16:0 and other longer chain fatty acids are therefore dependent, in part, on the diet of the woman.

The phospholipid in human milk is found in the milk fat globule membrane. Phosphatidylethanolamine and phosphatidylcholine are the most abundant phospholipids and represent about 37% and 30%, respectively, of the total phospholipid (Jensen 1989). Human milk also contains sphingomyelin, representing about 26% of the total phospholipid, and small amounts of lysolecithin, phosphatidylinositol, phosphatidylserine and cerebrosides. The predominant fatty acids present in phosphatidylcholine and phosphatidylethanolamine are 18:0, primarily at the *sn*-1

position and 18:2(n-6), primarily at the *sn*-2 position. The milk phospholipid also contains 20:4(n-6) and 22:6(n-3) both found primarily at the *sn*-2 position.

Cholesterol is also present in human milk usually in the range of 0.5-0.8 mmol/L (Jensen 1989). Most cholesterol is present in the unesterified form and is present as a component of the milk fat globule membrane. However, small amounts of cholesterol ester may also be present together with triglyceride in the core of the milk fat globule molecule. Levels of cholesterol in milk are not reflective of maternal plasma cholesterol concentrations (Tsang et al. 1978).

The composition of sow milk is very similar to human milk. Sow milk contains approximately 5.0-6.7g/dL lipid, 5.5-6.0g/dL protein and 5.8-6.2g/dL carbohydrate (lactose and other oligosaccharides), minerals, and water-soluble vitamins (Hrboticky et al. 1990, Innis and Dyer 1997, Innis et al. 1993). Another species commonly used, as an animal model in nutrition research is the rat. The major fatty acid composition of sow milk, rat milk and human milk are given in **Table 1.1.** The predominant saturated fatty acid in sow milk and human milk is 16:0. In contrast, the predominant saturated fatty acids in rat milk are the medium chain fatty acids 8:0, 10:0 and 12:0. The absorptive process for medium chain fatty acids differs from long chain fatty acids in that they are not incorporated into chylomicrons but are absorbed directly into the portal circulation (reviewed in section 1.3.) (Bach and Babayan 1982). As a result, medium chain fatty acids are absorbed more quickly than long chain fatty acids. The high medium chain fatty acid content of rat milk relative to human milk, therefore, is a limiting factor when using the rat as an animal model to study infant lipid metabolism. As well, sow milk and human milk contain similar amounts of 18:1(n-9), whereas the amount of

18:1(n-9) present in rat milk is about a third of the amount found in sow milk and human milk. Therefore, the greater similarities in the fatty acid composition of sow milk to human milk make the piglet a more attractive animal model for the study of infant lipid nutrition.

Table 1.1. Major fatty acid composition and cholesterol content of human, sow and rat milk.

	sow milk[3]	rat milk[4]	human milk[5]
fatty acids[1]			
8:0 + 10:0	0.1-0.3	22.3	3.5
12:0 + 14:0	3.3-3.5	25.8	15.8
16:0	27.6-30.7	20.9	23.0-24.0
18:0	4.4-5.5	2.2	7.2-7.4
18:1(n-9)	32.0-40.4	12.3	31.24
18:2(n-6)	8.2-13.0	12.7	12.8-13.8
18:3(n-3)	0.6-0.8	0.9	1.1
20:4(n-6)	0.7	0.7	0.3-0.4
22:6(n-3)	0.1	0.4	0.15-0.2
cholesterol[2]	0.52	1.4	0.4

[1]Data for fatty acids is g/100g. [2]Data for cholesterol is mmol/L. [3]Adapated from Hrboticky et al. 1990, Innis and Dyer 1997, Innis et al. 1993. [4]Adapted from Staggers et al. 1981. [5]Adapted from Huisman et al. 1996, Martin et al. 1993.

1.2.2. Lipid Composition of Infant Formula

The main differences in the lipid composition of most commercially available infant formulae and milk are: the positional distribution of fatty acids in the dietary

triglyceride; the concentration of 18:2(n-6) and 18:3(n-3); the absence of the long chain polyunsaturated fatty acids, 20:4(n-6) and 22:6(n-3), and the lack of cholesterol. Most infant formulae fat blends are prepared from vegetable oils. Most infant formula contain a blend of two or more oils such as palm olein oil or coconut oil as a source of saturated fatty acids, together with one or more of soy bean oil, high oleic safflower oil, sunflower oil, or corn oil as sources of unsaturated fatty acids. The lipid in these oils is present as triglyceride. Phospholipid, usually from soybeans, is used to emulsify the lipid in order to enable combination with the other aqueous ingredients of the formula, which include protein, carbohydrate, minerals and water-soluble vitamins.

Infant formulae contain variable amounts of 16:0 depending on the saturated oil used by the manufacturer. Irrespective of the amount of 16:0 present, and in contrast to milk, only about 6% of the total 16:0 in formula is esterified to the 2 position of the formula triglyceride (Innis 1992, Jensen & Jensen 1992, Spear et al. 1992) (**Figure 1.1**). Levels of unsaturated fatty acids, on the other hand, are high in the *sn*-2 position. The positioning of fatty acids in the formula triglyceride is relevant to the pathway of absorption of the fatty acids. This is discussed more fully in section 1.3.1.

Infant formulae also contain higher concentrations of 18:2(n-6) and 18:3(n-3) than those found in most human milks. As mentioned above, infant formulae in North America contain no carbon chain (C) 20 or 22 fatty acids, for example 20:4(n-6) and 22:6(n-3), and are very low in cholesterol (<0.10mmol/L).

1.3. LIPOPROTEIN METABOLISM

1.3.1. Dietary Lipid Digestion & Absorption

The digestion of dietary lipid is quite complex relative to that of many other nutrients, in part, because of the insoluble nature of lipid in the aqueous environment of the gastrointestinal tract. Dietary lipid, primarily triglyceride (97-98%) which contains three fatty acids esterified to a glycerol backbone, and small amounts of cholesterol, phospholipid and fat-soluble vitamins are digested, and then the products are absorbed by enterocytes. Following absorption, the products of lipid digestion are reassembled and packaged into chylomicrons, and secreted into intestinal lymphatic vessels entering the circulation at the thoracic duct (reviewed by Field and Mathur 1995, Hussain et al. 1996, Thomson et al. 1993).

The digestion of dietary lipid begins in the stomach, with gastric lipase which is produced and secreted by gastric mucosal cells (Moreau et al. 1988). In some species, such as rodents, however, dietary lipid digestion begins in the mouth as a result of the presence of lingual lipase (Gargouri et al. 1989). Gastric lipase hydrolyzes the fatty acids esterified at the *sn*-3 position of the triglyceride to release as much as 15% of the total triglyceride fatty acids (Carriere et al. 1993, Carriere et al. 1997, Gargouri et al. 1989). The remainder of lipid digestion occurs in the duodenum and involves pancreatic enzymes. Pancreatic and biliary secretions are released into the duodenum in response to the stimulatory action of cholecystokinin secreted from duodenal mucosal cells. Cholesystokinin is secreted in response to the presence of lipid and peptides (amino acids) in the duodenum. Dietary triglyceride is hydrolyzed by pancreatic lipase (triacylglycerol acylhydrolase, EC 3.1.1.3) which preferentially cleaves fatty acids from

the *sn*-1 and 3 positions of the glycerol backbone to release a *sn*-2 monoglyceride and two unesterified fatty acids for absorption (Small 1991). Long chain unesterified saturated fatty acids have a melting point above body temperatures and at the alkaline pH of the intestinal lumen have the tendency to bind with divalent cations, such as calcium, forming insoluble fatty acid soaps that are unable to be absorbed and are excreted (Lein 1994). As a consequence, the positioning of 16:0 in the milk or formula triglyceride has the potential to result in differences in the extent of absorption, as well as the rate of absorption, of 16:0 between milk-fed and formula-fed infants.

Dietary cholesterol ester is hydrolyzed to release unesterified fatty acids and cholesterol by the pancreatic enzyme, cholesterol esterase (EC 3.1.1.13) (Wilson & Rudel 1994), which requires bile acids in order to be active (Jacobson et al.1990). Additional sources of cholesterol are also available for absorption. These include the cholesterol secreted into the duodenum as a constituent of bile, and that released into the lumen of the intestine as a result of intestinal epithelial cell turnover (Grundy 1983). Phospholipid of both dietary and biliary origin, as well as intestinal epithelial cell membrane phospholipid is hydrolyzed by pancreatic phospholipase A_2 (EC 3.1.1.4). This enzyme hydrolyzes the fatty acid at the *sn*-2 position to release an unesterified fatty acid and lysophospholipid (Van Deenen et al. 1963).

The constitutents of bile, primarily bile acids and phospholipid, function to solubilize the luminal lipid by forming 'mixed micelles'. Pancreatic lipase is activated at the lipid-water interface and requires colipase. Colipase functions to anchor pancreatic lipase at the lipid-water interface of the mixed micelle and protects it from the detergent actions of bile salts (Borgstrom 1975). The mixed micelles, containing the products of

digestion, now approach and cross the unstirred water layer (Dietschy et al. 1971) and enter the enterocyte. The unstirred water layer is a series of water lamellae at the interface of the water phase of the intestinal lumen and the brush border membrane of enterocytes (Wilson & Rudel 1994). The exact mechanism of transport of fatty acids across the brush border membrane of the enterocyte is not known, but has been proposed to occur via an active transport process involving a membrane bound fatty acid transport protein (Schaffer & Lodish 1994).

Following transfer across the mucosal cell membrane, a cytosolic fatty acid binding protein shuttles long chain fatty acids to the endoplasmic reticulum (Ockner & Manning 1974). Similarly, cholesterol is also carried to the endoplasmic reticulum by a sterol carrier protein. Re-esterification of *sn*-2 monoglyceride with unesterified fatty acids occurs with the formation of triglyceride (Brindley & Hubscher 1965). In addition to the triglyceride originating from the re-esterification of *sn*-2 monoglyceride with dietary triglyceride derived unesterified fatty acids, triglyceride can also be synthesized *de novo* via the 3-glycerol phosphate pathway (**Figure 1.5**). Cholesterol becomes esterified with a fatty acid to form cholesterol ester, primarily through the action of acyl Coenzyme A:cholesterol acyltransferase (EC 2.3.1.26) (Haugen & Norum 1976). Lysophospholipid is similarly reassembled to form phospholipid. The resulting products, triglyceride, cholesterol ester and phospholipid, are then packaged into chylomicrons. The nascent chylomicrons travel to the Golgi apparatus where they undergo modifications and are concentrated into secretory vesicles which are then secreted into intestinal lymphatic vessels and enter the circulation at the thoracic duct (**Figure 1.2**).

1.3.2. Exogenous Lipid Transport and Metabolism

Chylomicrons are the largest of the lipoprotein particles with a diameter of 75-450nm. They are composed of triglyceride (85-92%), phospholipid (6-12%), cholesterol (1-3%), protein (1-2%) and fat-soluble vitamins (Hussain et al. 1996). Triglyceride and cholesterol ester are located in the core of the molecule surrounded by phospholipid and free cholesterol, with the protein components, specifically, apolipoproteins (apo), found on the surface.

Apo B48 is an absolute requirement for the assembly and secretion of chylomicrons. There are two circulating forms of apo B, apo B48 and apo B100. Both apo B48 and apo B100 are products of the *apob* gene. Apo B48 is produced by the intestine of most mammals, except rodents, and apo B100 is produced by the liver (Greeve et al. 1993). Apo B48 represents the amino-terminal portion of apo B100 and is produced as a result of post transcriptional editing of the *apo b* gene transcript or mRNA (Chen et al. 1987, Powell et al. 1987). The full-length *apo b* transcript is modified only in the intestine. The cytosine in a CAA codon undergoes deamination to produce a uridine residue resulting in an in-frame stop codon and a truncated product known as apo B48. This reaction is catalyzed by an enzyme complex, the catalytic unit defined as apo b mRNA editing enzyme catalytic polypeptide (apobec-1) (Teng et al. 1993). This enzyme is a zinc-dependent, cytosine deaminase and has been shown to be a RNA-binding protein (MacGinnitie et al. 1995). In addition to apo B48, chylomicrons also contain apo AI, AIV, CII and CIII, all of which are synthesized by the intestine (Hussain et al. 1996).

Following secretion into the circulation, chylomicrons interact with other lipoproteins, mostly HDL, and acquire apo E and more apo C. Clearance of chylomicrons from the circulation is rapid, with a typical half-life of about 10 minutes. Clearance occurs in two stages, firstly, hydrolysis by lipoprotein lipase (LPL) and release of lipid components at peripheral tissues and secondly, uptake of remnant particles by the liver (Hussain et al. 1996). As chylomicrons circulate they interact with LPL which is bound by heparin- sulfate proteoglycans at the surface of capillary endothelial cells (Cheng et al. 1981). LPL hydrolyzes the triglyceride core of chylomicron particles to release unesterified fatty acids and partial glycerides, which are then available for uptake by peripheral tissues (recently reviewed by Beisiegel 1996, Goldberg 1996). As a consequence of triglyceride hydrolysis, the chylomicron particle is reduced in size and is now referred to as a remnant particle. Adipose tissue, muscle and lactating mammary glands are the primary sites of LPL synthesis. The activity of LPL in adipose tissue and skeletal muscle is enhanced during feeding as a consequence of increased circulating levels of insulin and epinephrine. During fasting, on the other hand, LPL activity is decreased
in response to the increased circulating levels of glucagon. In addition, apo CII is a necessary co-factor for LPL activity (Goldberg et al. 1990).

The chylomicron remnants are cleared, via receptor-mediated endocytosis, by the liver. The LDL receptor and LDL receptor-related protein (LRP) are responsible for the majority of hepatic uptake of chylomicron remnants. Although apo E serves as a ligand for LRP, LDL receptor uptake of chylomicron remnants may be quantitatively

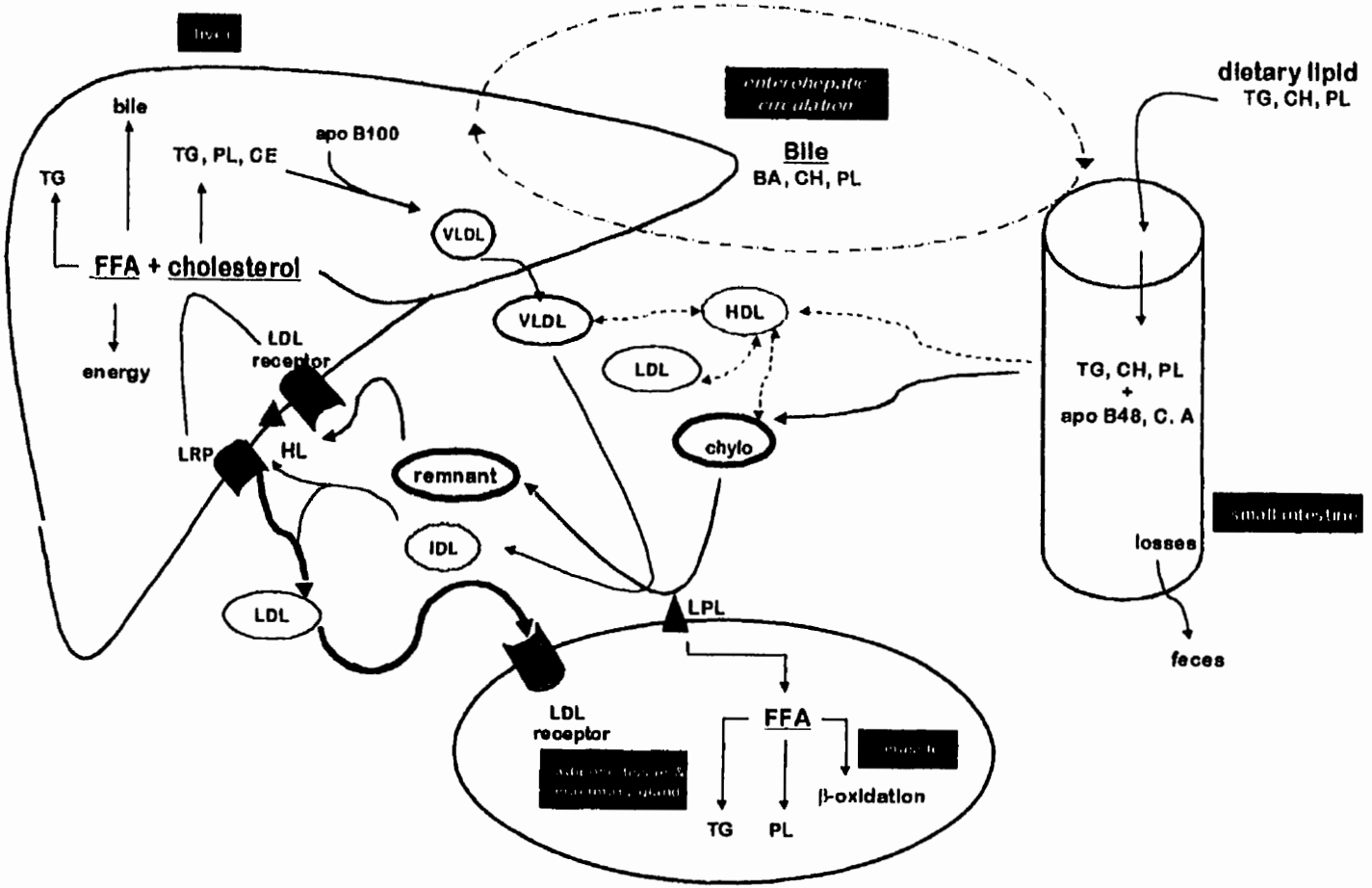


Figure 1.2. Transport of exogenous and endogenous lipid.

apo, apolipoprotein; BA, bile acids; CH, cholesterol;chylo, chylomicron; CE, cholesterol ester; FFA, free fatty acids; HDL, high density lipoprotein;HL, hepatic lipase; IDL, intermediate density lipoprotein; LDL, low density lipoprotein;LPL, lipoprotein lipase; LRP, LDL receptor-related protein; PL, phospholipid; remnant, chylomicron remnant; TG,triglyceride; VLDL, very low density lipoprotein.
— chylomicron metabolism; —— VLDL metabolism; ▬ LDL metabolism; ---- HDL metabolism.

more important (De Faria et al. 1996, Herz et al. 1995, Mortimer et al. 1995). In addition, hepatic lipase has been shown not only to hydrolyze lipid components of remnant particles, but has also been shown to bind remnant particles. This enzyme, may, therefore, facilitate binding of remnant particles to receptors located at the surface of the liver (De Faria et al. 1996). Some evidence is also available to suggest there may be an additional hepatic receptor responsible for the uptake of remnant particles (Ziere et al. 1996).

Following uptake of remnant particles into hepatocytes, the lipid components can be stored or used for the synthesis of hepatic lipoproteins. Cholesterol can also be used for synthesis of bile acids. Hepatic lipase, a lipolytic enzyme synthesized and secreted by hepatocytes and transported to the sinusoidal surface of the liver (Breedveld et al. 1997), can further hydrolyze the remaining lipid components of the remnant particles.

1.3.3. Endogenous Lipid Transport & Metabolism

VLDL functions to carry endogenously synthesized lipids to extra-hepatic tissues. VLDL are triglyceride-rich, apo B-containing lipoproteins that are produced primarily by the liver (reviewed by Yao & McLeod 1994). The amount of VLDL assembled and secreted by the liver is dependent on the availability of lipid substrates including triglyceride (Boren et al. 1993), cholesterol ester (Cianflone et al. 1990), phospholipid (Yao & Vance 1988) and the post translational modification and intracellular degradation of apo B (Yao & McLeod 1994). Triglyceride and phospholipid synthesis occurs in the smooth endoplasmic reticulum and apo B synthesis occurs in the rough endoplasmic reticulum (Alexander et al. 1976). Apo B100 is co-translationally inserted into the

endoplasmic reticular membrane, providing the foundation upon which the lipoprotein is assembled (Yao and McLeod 1994). Following lipidation, the lipoprotein is released from the membrane and the particles travel to the Golgi complex, form secretory vesicles and are then released by exocytosis from fenestra of hepatic sinusoidal endothelium. Microsomal triglyceride transfer protein is responsible for the addition of some triglyceride to the developing lipoprotein (Sharp et al. 1993).

Similar to chylomicrons, VLDL delivers fatty acids to extra-hepatic tissues. Interaction with LPL results in hydrolysis of the core triglyceride releasing free fatty acids, which can be taken up by the surrounding tissue (Beisiegel 1996, Goldberg 1996). The resulting particles, termed intermediate density lipoproteins (IDL), follow one of two fates. IDL can travel back to the liver where they are taken up by hepatocytes or evolve to become LDL particles through interaction with HDL. Interaction with HDL enriches the IDL particles with cholesterol and depletes them of apo E and C. The resulting LDL particles are rich in cholesterol and contain apo B100. Apo B100 serves as a ligand for the LDL receptor. Consequently, LDL enters cells via the LDL receptor and delivers cholesterol to hepatic and extra-hepatic tissues, excluding the brain.

1.4. Hepatic Lipid Metabolism

The major site for the synthesis and secretion of circulating plasma lipoproteins is the liver. Hepatic lipid concentrations are important determinants of hepatic lipoprotein synthesis and secretion (Boren et al. 1993, Cianflone et al. 1990, Yao & Vance 1988). Hepatic triglyceride and cholesterol concentrations determine the rate of endogenous cholesterol synthesis, the uptake of circulating lipoproteins and the secretion of

cholesterol in bile. These close inter-relationships between hepatic lipid levels and plasma lipoprotein lipid levels suggest that the higher plasma cholesterol in breast-fed than formula-fed infants may involve differences in hepatic processing of lipids, Including the synthesis, uptake (clearance), assembly into and secretion in lipoproteins and/or excretion of cholesterol in bile.

1.4.1. Cholesterol Synthesis

Cholesterol synthesis is regulated by the activity of the enzyme, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (EC 1.1.1.34) (Goldstein & Brown 1990). HMG-CoA reductase is found in the endoplasmic reticulum membrane and catalyzes the conversion of HMG-CoA to mevalonate. Mevalonate can be further metabolized to cholesterol (**Figure 1.3**).

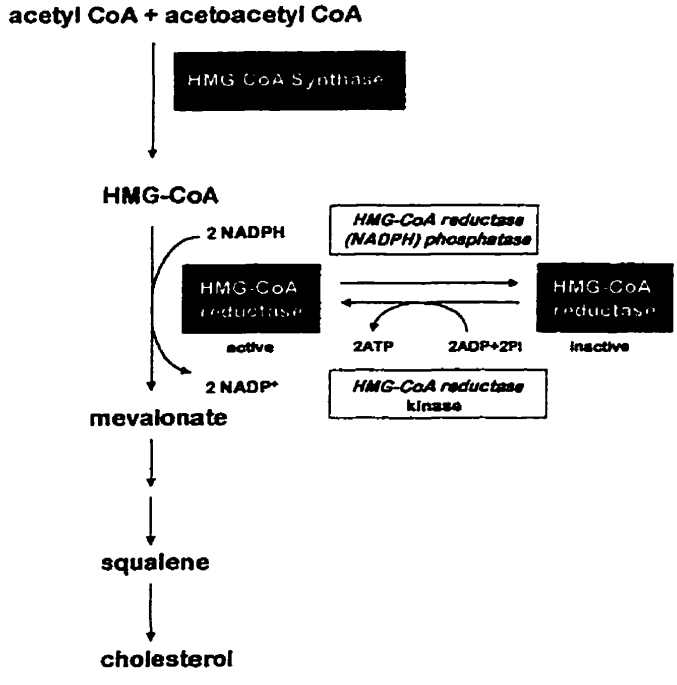


Figure 1.3. Schematic representation of major steps in the pathway of cholesterol synthesis.

Expression of HMG-CoA reductase is controlled by a negative feedback mechanism whereby an increase in the cellular sterol concentration decreases HMG-CoA reductase expression, primarily at a transcriptional level (Chin et al. 1982, Clarke et al. 1985, Liscum et al. 1983,Nakanishi et al. 1988). A sterol regulatory region (SRE) has been identified in the promoter region of the HMG-CoA reductase gene. Specific proteins binding to this region have been shown to repress transcription when cellular sterol concentrations are adequate (Osborne 1991, Osborne et al. 1988). Furthermore, mevalonate has also been shown to regulate the expression of HMG-CoA reductase at a post-transcriptional level (Nakanishi et al. 1988). Short-term regulation occurs at a post translational level. This is achieved by phosphorylation of the HMG-CoA reductase enzymes reversibly inhibiting enzyme activity (Goldstein & Brown 1990). In adult animals, high dietary intakes of saturated fat and cholesterol have been shown to decrease hepatic activity, and to a lesser degree, mRNA levels for HMG-CoA reductase (Hackman et al. 1996, Jackson et al. 1990, Rudling 1992, Spady & Cuthbert 1992, Xu et al. 1995). In some cases, this was accompanied by higher plasma cholesterol concentrations (Jackson et al. 1990, Rudling 1992, Xu et al. 1995). It seems reasonable to speculate that the difference in dietary cholesterol intake and plasma cholesterol concentrations between milk-fed and formula-fed infants is accompanied by a difference in the hepatic synthesis of cholesterol.

1.4.2. LDL Receptor Uptake of Lipoproteins

The LDL receptor is a cell surface glycoprotein responsible for receptor-mediated endocytosis of apo B100 and apo E-containing lipoproteins (Brown & Goldstein 1986). The receptor is synthesized in the endoplasmic reticulum, travels to the Golgi complex

becoming glycosylated, and then migrates to the cell surface where it binds lipoproteins. Binding of lipoproteins to the receptor leads to clustering of the receptor-lipoprotein complexes in clatharin-coated pits and the internalization of the complex via endocytosis, with subsequent formation of endosomes. Within the endosome, the lipoprotein dissociates from the receptor, and the receptor is recycled to the cell surface with the lipoprotein components, triglyceride, cholesterol ester, phospholipid and apolipoproteins, undergoing lysosomal degradation.

Expression of the LDL receptor gene is down-regulated in response to an increase in cellular cholesterol concentrations (Brown & Goldstein 1986). Regulation of expression of the LDL receptor gene is thought to occur at a transcriptional level. Depletion of cellular sterol concentrations stimulates transcription, largely as a result of the enhancing actions of a sterol-regulatory element (SRE-1) identified in the 5'-flanking region of the gene (Sudhof et al. 1987). The SRE-1 is a conditionally positive element that enhances LDL receptor gene transcription in the absence but not in the presence of cholesterol (Dawson et al. 1988, Smith et al. 1990). DNA-binding proteins that complex with SRE-1 when endoplasmic reticulum membrane cholesterol concentrations are depleted have been identified and are termed SRE binding proteins (SREBP) (Briggs et al. 1993, Wang et al. 1993a, Wang et al. 1994).

It has been estimated that about 70% of plasma LDL clearance in humans and other species occur via the LDL receptor. The liver is responsible for 80-90% of LDL-receptor mediated clearance (Dietschy & Spady 1985, Spady et al. 1985). Therefore, consideration of the effect of infant diet on the expression of hepatic LDL receptor may provide insight into the effect of early diet on cholesterol homeostasis. Previous studies

have found that dietary fat and cholesterol decrease hepatic LDL receptor expression at transcriptional, translational and post translational levels in adult animals (Horton et al. 1993, Kurushima et al. 1995, Rudling 1992, Stucchi et al. 1995).

1.4.3. Bile Acid Metabolism

In the liver, cholesterol can be converted to bile acids and secreted in bile, esterified and stored, incorporated into lipoproteins and secreted, or used for membrane synthesis. Conversion of cholesterol to bile acids occurs only in the liver, and the rate of this conversion is controlled by the activity of 7-α-hydroxylase (C7H) (**Figure 1.4**).

Bile acids are released into the small intestine in response to ingestion of fat. The majority of bile acids, about 97-99%, are reabsorbed and travel back to the liver via the portal system, with a small proportion of the total pool excreted in feces. The conversion of cholesterol to bile acids is the only significant and regulated pathway by which cholesterol can be excreted from the body.

The enterohepatic circulation of bile acids is driven by both chemical (transport) and mechanical (muscular) forces (Piccoli et al. 1993). Bile acids are reabsorbed by both passive and active transport mechanisms in the distal small intestine. A brush border membrane bound transport protein, ileal Na^+/bile acid cotransporter (IBAT), has been isolated from rat and hamster enterocytes (Schneider et al. 1995, Wong et al. 1994) and is believed to be involved in the active transport of bile acids across the brush border membrane of enterocytes. An intestinal bile acid binding protein (I-BABP), has also been isolated in several species. This cytosolic ligand-binding protein has been shown to function in the intracellular transport of bile acids within enterocytes (Amano et al. 1992, Gantz et al. 1989, Gong et al. 1994, Lin et al. 1991,Walz et al.

1988). Following intestinal absorption, bile acids enter the portal circulation and complex with either albumin or lipoproteins (Kramer et al. 1979). Bile acids return to the liver and are cleared from the circulation through the actions of a membrane-bound

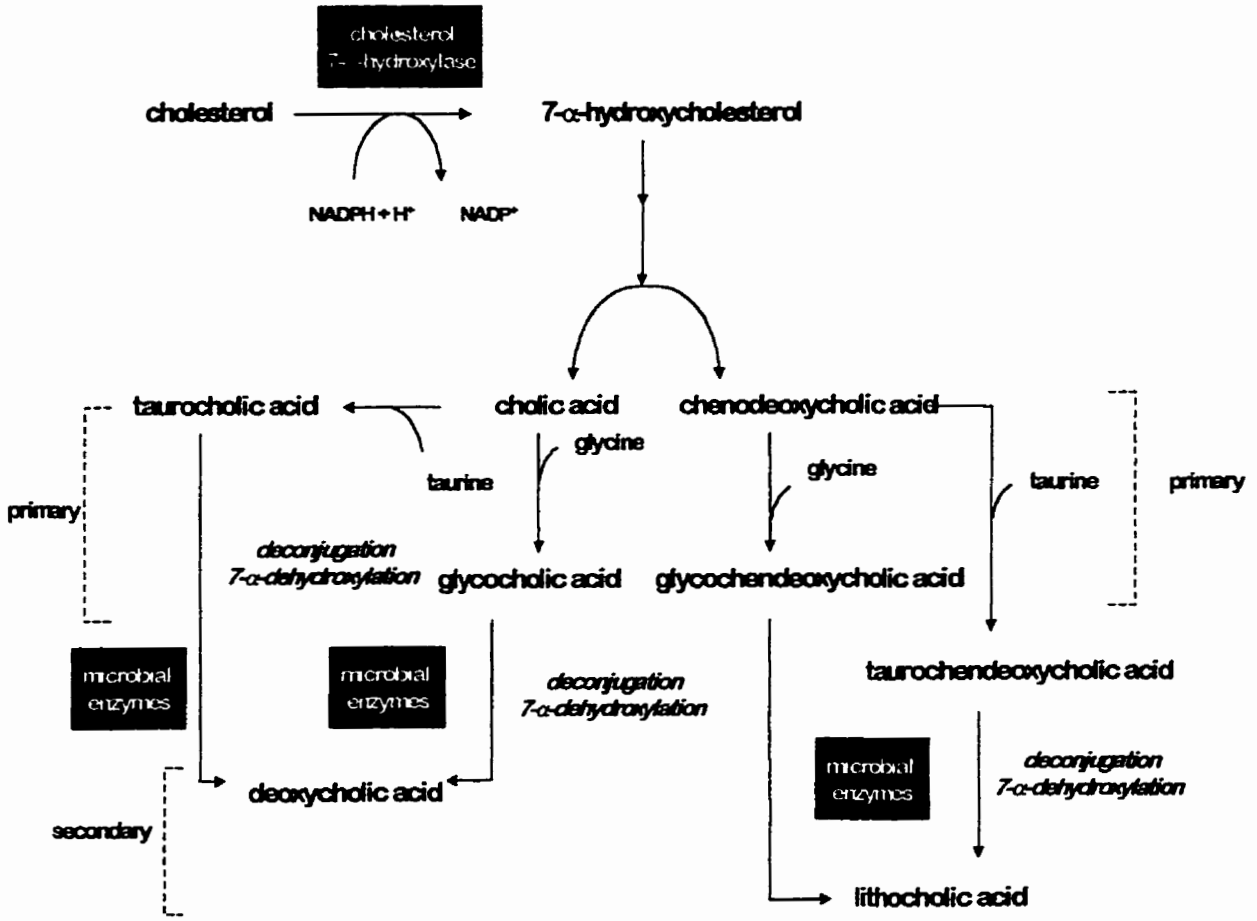


Figure 1.4. Schematic representation of major steps in the pathway of bile acid synthesis.

Na+/bile acid cotransporter (Ananthanarayanan et al. 1994, Hagenbuch et al. 1991). Hepatic clearance of bile acids is high, therefore, the concentration of bile acids in the systemic circulation is relatively low. Furthermore, reabsorption of bile acids by the

intestine is also high, with less than 1-3% of the bile acids secreted by the liver excreted in the stools. The amount of bile acids returned to the liver and the availability of free cholesterol in the liver partially determines the rate of further bile acid synthesis (Heuman et al. 1991). Cholesterol is also secreted into bile and can either be reabsorbed into the portal circulation or excreted in feces, with this representing the only route by which cholesterol can be excreted from the body.

It is generally thought that the expression of C7H is upregulated in response to increases in hepatic cholesterol concentrations. A species-specific response to dietary cholesterol has been found for hepatic expression of C7H (Dueland et al. 1993, Ramirez et al. 1994, Rudel et al. 1994, Shefer et al. 1992, Spady & Cuthbert 1992, Xu et al. 1995). It has been observed that rodents respond to diets containing cholesterol by increasing hepatic levels of C7H mRNA and enzyme activity (Dueland et al. 1993, Ramirez et al. 1994, Shefer et al. 1992, Spady & Cuthbert 1992). However, studies conducted in rabbits and monkeys have found that cholesterol feeding results in decreased levels of hepatic C7H mRNA and activity (Rudel et al. 1994, Xu et al. 1995). These results from studies with rabbits and monkeys led to the hypothesis that hepatic C7H expression may be decreased during high cholesterol intakes in an attempt to lower the amount of bile acids available for cholesterol absorption. This response may be a consequence of the increased cholesterol delivered to the liver during high cholesterol intakes.

In addition to cholesterol, dietary fatty acids have also been found to influence hepatic expression of C7H. Dietary long chain polyunsaturated fatty acids, specifically, those of the (n-3) series have been shown to increase biliary cholesterol, bile acids and

phospholipid secretion in adult rats (Smit 1994). It is, therefore, reasonable to question whether or not differences in the production of bile acids are involved in the differences in plasma cholesterol concentrations between milk-fed and formula-fed infants.

1.4.4. Triglyceride and Fatty Acid Synthesis

Triglyceride is the major lipid component of VLDL produced by the liver. Liver triglyceride can originate from the uptake and re-assembly of fatty acids and glycerol

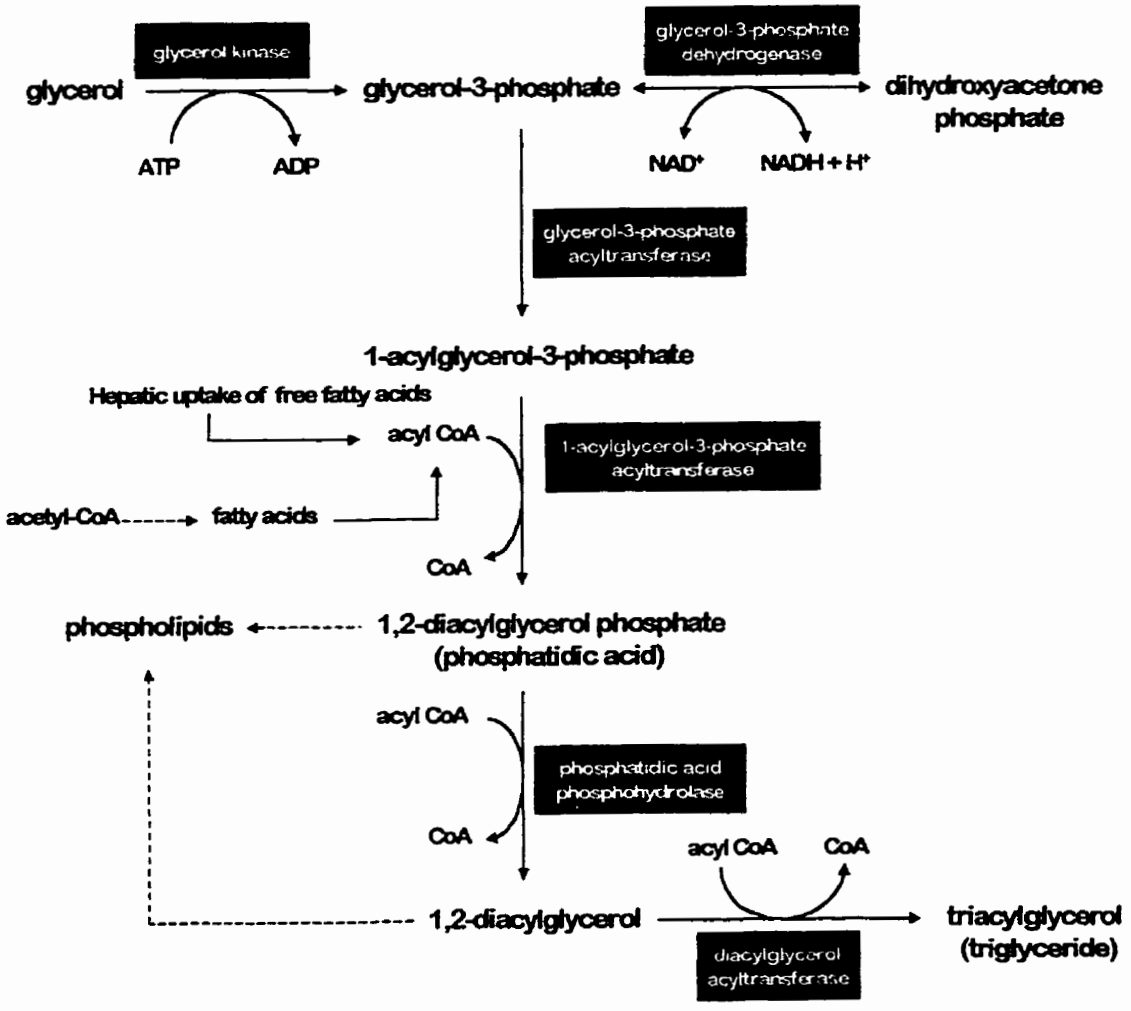


Figure 1.5. Schematic representation of the pathway of triglyceride synthesis.
------ Intermediary steps not shown.

following hepatic lipase hydrolysis of triglyceride in lipoproteins and uptake of albumin bound unesterified fatty acids. In addition, hepatic triglyceride can originate from the endogenous synthesis of fatty acids and subsequent assembly into triglyceride. The pathway involved in the synthesis of triglyceride is illustrated in **Figure 1.5**.

The availability of fatty acids for triglyceride synthesis is considered to be a rate limiting factor in the amount of triglyceride produced by the liver. The fatty acids available for hepatic triglyceride synthesis can originate from either endogenous synthesis or from uptake of unesterified fatty acids from the circulation. Decreased hepatic fatty acid synthesis has been shown to decrease hepatic triglyceride synthesis and decrease hepatic VLDL secretion (Arbeeny et al. 1992, Stals et al. 1994).

Hepatic fatty acid synthesis is regulated by two enzymes, acetyl CoA carboxylase (ACC), the enzyme catalyzing the rate limiting step of fatty acid synthesis, and fatty acid synthase (FAS) (Clarke et al. 1990, Clarke & Jump 1993, Jump et al. 1994, Katsurada et al. 1990). The pathway of hepatic fatty acid synthesis is illustrated in **Figure 1.6**. The majority of studies previously investigating the specific effects of dietary lipid on hepatic fatty acid synthesis have focused on the expression of FAS. Dietary polyunsaturated fatty acids have been shown to decrease the levels of hepatic FAS mRNA and activity (Clarke et al. 1990, Clarke & Jump 1993). The exact mechanism by which polyunsaturated fatty acids decrease the expression of FAS has not been fully elucidated. It is thought, however, that fatty acids directly interact with nuclear factors that behave as repressor proteins, and subsequently bind to repressor elements, resulting in an inhibition of gene transcription (Clarke and Jump 1996).

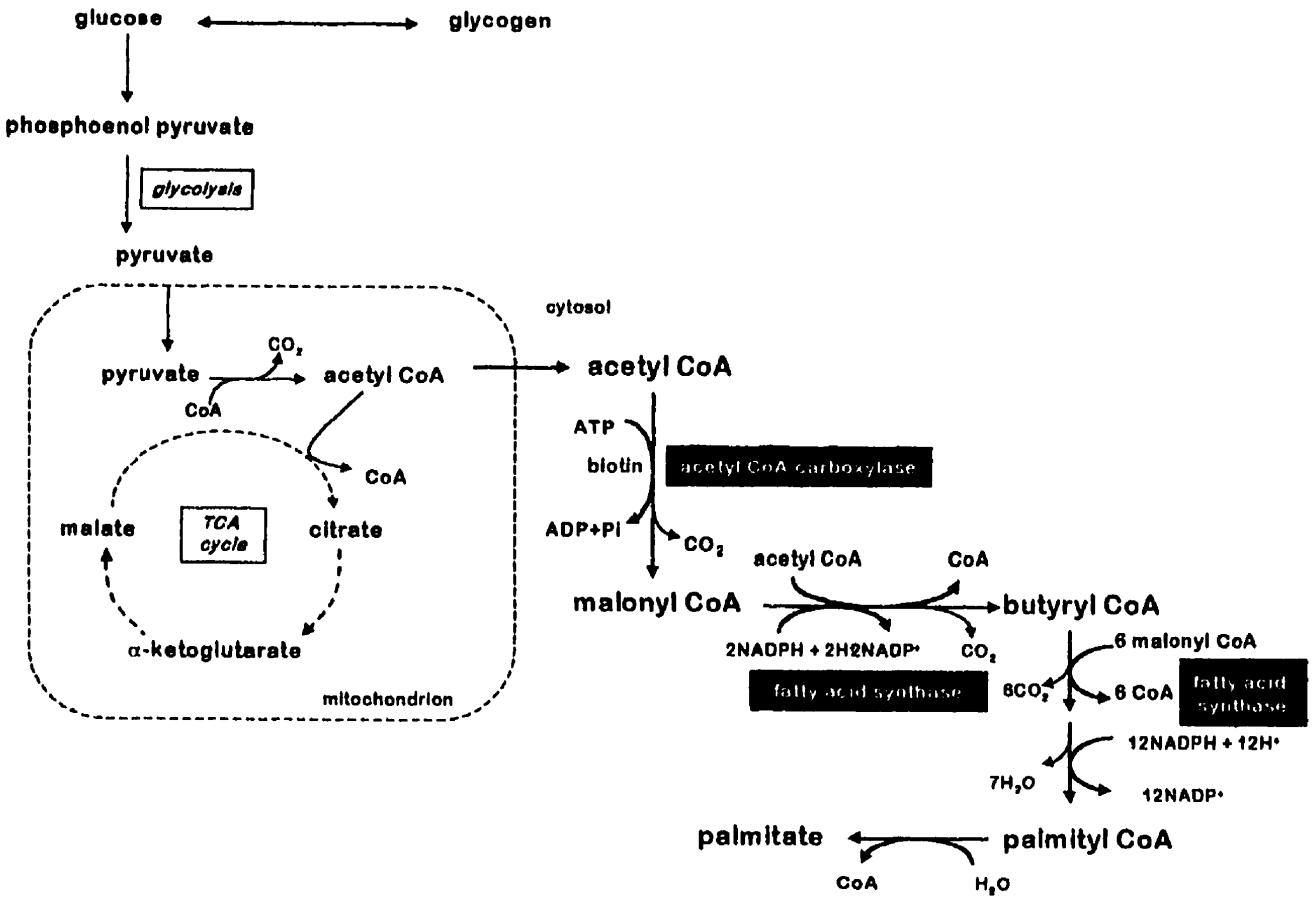


Figure 1.6. Schematic representation of the synthesis of palmitic acid.
Overall reaction:

$$\text{acetyl CoA} + 7\text{malonyl CoA} + 14\text{NADPH} + 14\text{H}^+ \longrightarrow \text{palmitate} + 7\text{CO}_2 + 14\text{NADP}^+ + 8\text{CoA} + 6\text{H}_2\text{O}$$

In addition to dietary polyunsaturated fatty acids, dietary cholesterol has also been shown to influence hepatic fatty acid metabolism. Diets containing cholesterol have been shown to increase hepatic synthesis of fatty acids, triglyceride and VLDL in hamsters (Fungwe et al. 1994a). In contrast, *in vitro* studies have identified a sterol regulatory element in the promoter of both the ACC and FAS genes (Bennet et al. 1995, Lopez et al. 1996) that function to enhance transcription when cellular cholesterol concentrations are depleted (Kawabe et al. 1996). Therefore, the presence of long chain polyunsaturated fatty acids and cholesterol in milk, but the absence of both from most infant formulae, raises the question of whether or not there are differences in hepatic fatty acid synthesis between breast-fed and formula-fed infants.

1.5. DIETARY FAT & LIPID METABOLISM

1.5.1. Dietary Fat & Lipid Metabolism

Dietary cholesterol and saturated fatty acids have both been shown to be hypercholesterolemic and to induce changes in hepatic cholesterol metabolism in adult humans and animals (**Figure 1.7**) (Bertolotti et al. 1995, Bonanome & Grundy 1988, Kurushima et al. 1995, Mattson & Grundy 1985, Spady & Cuthbert 1992). Diets high in saturated fat and cholesterol inhibit expression of HMG-CoA reductase (Hackman et al. 1996, Jackson et al. 1990, Rudling 1992, Spady & Cuthbert 1992, Xu et al. 1995) and LDL receptor (Horton et al. 1993, Kurushima et al. 1995, Rudling 1992, Stucchi et al. 1995), however, the effect on the expression of C7H is species-specific (Dueland et al. 1993, Ramirez et al. 1994, Rudel et al. 1994, Shefer et al. 1992, Spady & Cuthbert 1992, Xu et al. 1995).

Whether or not the positional distribution of fatty acids in dietary triglyceride influences the hepatic expression of HMG-CoA reductase, LDL receptor or C7H has not been previously determined.

Dietary fat and cholesterol have also been found to influence hepatic synthesis of fatty acids and triglyceride. Dietary cholesterol has been shown to enhance the synthesis of fatty acids, triglyceride and VLDL in rodents as measured by $[^3H]_20$ incorporation into fatty acids (Fungwe et al. 1992, 1993, 1994a,b). Findings of studies in HepG2 cells, however, suggest that high intakes of cholesterol can inhibit the hepatic expression of FAS and ACC (Bennet et al. 1995, Kawabe et al. 1996, Lopez et al. 1996). More relevant to the infant diet,

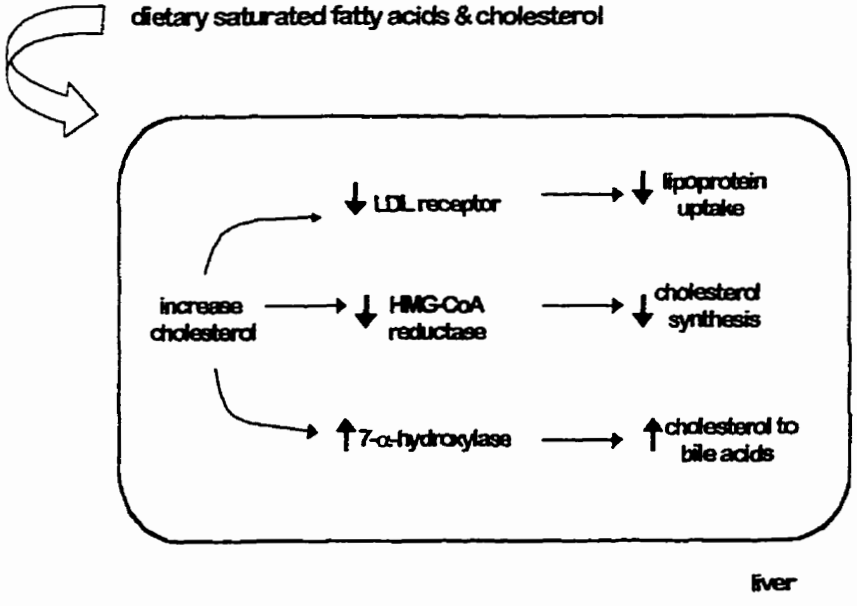


Figure 1.7 Schematic representation of the influence of dietary fat on hepatic lipid metabolism.

FAS activity was found to be lower in rat pups fed rat milk or a milk substitute containing cholesterol than rats fed a milk substitute not containing cholesterol (Auestad et al. 1988). In reference to the effects of dietary fatty acids on FAS expression, 18:3(n-3) has been shown to be more efficacious at inhibiting hepatic FAS expression than 18:2(n-6) (Clarke et al. 1990, Clarke & Jump 1993). As well, the desaturation products of 18:2(n-6), for example 20:4(n-6), seem also more efficacious at inhibiting FAS expression than the carbon chain (C) 18 precusors (Clarke & Clarke 1982, Clarke & Jump 1993). Dietary polyunsaturated fatty acids also influence hepatic sterol metabolism. Diets supplemented with fish oils containing eicosapentaenoic acid [20:5(n-3)] and 22:6(n-3) have been found to increase biliary bile acid, cholesterol and phospholipid secretion in adult animals (Smit et al. 1994). Fish oil supplementation has also been shown to decrease hepatic HMG-CoA reductase activity (Froyland et al. 1996,Smit et al. 1991) and 20:4(n-6) supplementation has been shown to decrease hepatic LDL receptor activity and mRNA levels (Rumsey et al. 1995,Scorci-Thomas et al. 1992).

It is reasonable to suggest, therefore, that the differences in lipid composition between milk and formula may be responsible for the higher plasma cholesterol concentrations in breast-fed than formula-fed infants. As well, it seems reasonable to hypothesize that the differences in lipid composition between milk and formula alter hepatic lipid metabolism in addition to plasma cholesterol levels.

1.5.2. Early Diet and Cholesterol Metabolism

Most studies on the ontogenesis of hepatic cholesterol metabolism have been conducted in rodents. The activity and mRNA levels of both HMG-CoA reductase and C7H are low during the suckling period, and increase at the time of weaning in rats fed by their natural mothers (milk-diet) (Ness et al. 1979, Ness 1994, Smith et al. 1995). The low expression of HMG-CoA reductase during milk-feeding suggests low levels of cholesterol synthesis, presumably as a result of the high fat content of the milk diet. Consistent with these studies in rats, previous studies in piglets have found lower activity of HMG-CoA reductase in piglets fed milk than in those fed formula (Rioux & Innis 1993). Studies in human infants have found lower rates of cholesterol synthesis, estimated from incorporation of deuterated water into cholesterol, in infants who were breast-fed than in infants fed formula (Lourdes et al. 1994). These studies suggest that the high fat milk diet does regulate cholesterol metabolism in young infants. Further evidence to support this suggestion comes from studies that found higher LDL receptor mRNA levels in milk-fed compared to formula-fed baboons (Mott et al. 1993). In contrast, studies using ligand blotting techniques found no differences in the level of LDL receptor protein between milk and formula-fed piglets (Rioux & Innis 1993). The effects of specific milk lipid components, such as cholesterol, fatty acid (specifically 16:0) positional distribution in formula triglyceride and the (n-6) and (n-3) polyunsaturated fatty acids, 20:4(n-6) and 22:6(n-3), on hepatic cholesterol synthesis and hepatic LDL receptor clearance of plasma lipoproteins has not been fully explored. Thus, more studies are needed to determine how

and at what level the infant milk and formula diet regulate hepatic cholesterol synthesis, hepatic LDL receptor clearance of plasma lipoproteins and whether or not this is related to the difference in plasma cholesterol between milk-fed and formula-fed infants.

Some evidence is also available to suggest that differences in bile acid metabolism are present between milk-fed and formula-fed infants. Studies in baboons fed a cholesterol containing diet found differences in bile acid composition of bile in baboons who had been fed formula during infancy than in baboons fed milk during infancy (Jackson et al. 1993, Mott et al. 1990, Mott et al. 1991). More specifically, the baboons fed formula as infants had a larger cholic acid pool size and smaller cholic acid fractional turnover rate than baboons fed milk but no differences in bile acid synthetic rates, as determined by the product of the pool size and fractional turnover rate, were observed (Jackson et al. 1993). The effect of diet on C7H expression, as an indicator or bile acid synthesis, during early milk and formula feeding is not known.

Similarly, little is known about the effect of the high fat infant diet on hepatic fatty acid and triglyceride synthesis. The important metabolic role of (n-6) and (n-3) series fatty acids during development, and the differences in tissue concentration of (n-6) and (n-3) fatty acids between milk and formula-fed infants and animals suggest that the infant diet may also influence hepatic lipogenesis.

1.5. OVERALL OBJECTIVES

I. To determine the effect of dietary cholesterol, the positional distribution of fatty acids, specifically palmitic acid (16:0), in dietary triglyceride and dietary arachidonic acid [20:4(n-6)] and docosahexaenoic acid [22:6(n-3)] on plasma cholesterol concentrations and hepatic lipid metabolism in formula-fed piglets.

II. To determine if differences in hepatic lipid metabolism accompany the differences in plasma cholesterol concentrations that result from formula compared to milk feeding in piglets.

2. HYPOTHESES & SPECIFIC AIMS

2.1. STUDY #1

2.1.1. Hypotheses

1. The addition of cholesterol to formula and the preferential positioning of 16:0 at the *sn*-2 position of formula triglyceride, similar to that in milk, results in higher plasma cholesterol concentrations in formula-fed piglets.

2. The higher plasma concentrations of cholesterol in milk-fed than in formula-fed piglets is accompanied by lower hepatic activity and mRNA for HMG-CoA reductase, lower levels of hepatic LDL receptor mRNA and higher levels of hepatic C7H mRNA.

3. The addition of cholesterol to formula and the preferential positioning of 16:0 at the *sn*-2 position of formula triglyceride will decrease the levels of hepatic HMG-CoA reductase mRNA and LDL receptor mRNA, and increase the levels of hepatic C7H mRNA in formula-fed piglets.

4. The addition of cholesterol to formula will lower the levels of hepatic activity and mRNA for fatty acid synthase and mRNA for acetyl CoA carboxylase in formula-fed piglets.

2.1.2. Specific Aims

To determine in exclusively formula-fed piglets the effect of:

a. adding cholesterol to formula

b. increasing the proportion of 16:0 at the *sn*-2 position of the formula triglyceride

c. both adding cholesterol and increasing the proportion of 16:0 at the *sn*-2 position of the formula triglyceride

on:

- plasma total, HDL and apo B-containing lipoprotein cholesterol and triglyceride concentrations
- hepatic triglyceride concentrations
- hepatic total cholesterol concentrations
- bile acid, cholesterol and phospholipid concentrations in bile
- hepatic HMG-CoA reductase, LDL receptor and C7H mRNA hepatic mRNA
- hepatic ACC mRNA levels
- hepatic FAS mRNA and activity levels

2.2. STUDY #2

2.2.1. Hypotheses

1. A dietary intake of the long chain fatty acids, 20:4(n-6) and 22:6(n-3), which are present in milk, will increase plasma cholesterol concentrations in formula-fed piglets.

2. A dietary intake of the long chain fatty acids, 20:4(n-6) and 22:6(n-3), which are present in milk, will decrease hepatic activity and mRNA for fatty acid synthase in formula-fed piglets.

3. A dietary intake of the long chain fatty acids, 20:4(n-6) and 22:6(n-3), which are present in milk, will decrease hepatic activity and mRNA for HMG-CoA reductase in formula-fed piglets.

4. A dietary intake of the long chain fatty acids, 20:4(n-6) and 22:6(n-3), which are present in milk, will decrease levels of hepatic C7H mRNA in formula-fed piglets.

2.2.2. Specific Aims

To determine in exclusively formula-fed piglets the effect of adding 20:4(n-6) and 22:6(n-3), at concentrations similar to those found in milk, to formula on:

- plasma total, HDL and apo B-containing lipoprotein cholesterol and triglyceride concentrations
- hepatic triglyceride concentrations
- hepatic total cholesterol concentrations
- bile acid, cholesterol and phospholipid concentrations in bile
- hepatic HMG-CoA reductase mRNA and activity levels
- hepatic LDL receptor and C7H mRNA levels
- hepatic ACC mRNA levels
- hepatic FAS mRNA and activity levels

2. 3. Experimental Approach

Piglets were used in these studies because of their suitability as an animal model for the study of infant lipid nutrition and metabolism (Innis 1993). The digestion and absorption of fat and lipoprotein metabolism in pigs are very similar to humans (Chapman 1980). The composition of sow milk is also similar to human milk (Hrboticky et al. 1990, Huisman et al. 1996, Innis and Dyer 1997, Innis et al. 1993, Martin et al. 1993), as discussed in section 1.2.1..

The evolutionary pattern of lipoproteins during the suckling period in piglets is very similar to that observed for humans (Hollanders et al. 1985). As in human infants (Ginsburg & Zetterstrom 1980, Kallio et al. 1993, Lane & McConathy 1986, Van Biervleit et al. 1986, Van Biervleit et al. 1980), serum total, HDL and LDL cholesterol concentrations rise immediately following birth and remain high for the duration of the suckling period (Hollanders et al. 1985). As well, the majority of circulating cholesterol is carried in LDL particles, similar to humans (Chapman 1980, Ginsburg & Zetterstrom 1980, Van Biervleit et al. 1980). Porcine LDL particles, however, are more heterogeneous with the particles distributed within the density range of 1.019-1.090 g/ml, whereas human LDL particles are distributed within the density range of 1.019-1.060 (Chapman 1980). Porcine HDL particles are distributed within a density range of 1.090-1.21 g/ml, whereas human HDL particles are distributed within the density range of 1.063-1.21 g/ml. However, the primary difference between human and porcine lipoprotein metabolism is in the composition of HDL particles and metabolism. In humans, HDL particles are heterogeneous, classified as HDL_3, HDL_{2a} and HDL_{2b}

(Reviewed by Fielding and Fielding 1995) containing either one of (or both) apo AI and apo AII. However, porcine HDL particles are more homogeneous, resembling human HDL_3 particles (Pussinen et al. 1995), and contain apo AI but not apo AII (Jackson et al. 1973). Furthermore, pigs have no functional cholesterol ester transfer protein (Barter et al. 1981, Ha et al. 1982), but do have functioning phospholipid transfer protein that is thought to be responsible for the phospholipid transfer in pig plasma (Pussinen et al. 1997, Speijer et al. 1991).

3. MATERIALS AND METHODS

3.1. ANIMALS AND DIETS

3.1.1. Housing and Feeding Protocol

Male Yorkshire piglets of normal gestation (116-118d) with a birth weight greater than 1kg were obtained from Peter Hill Holdings (Langley, British Columbia, Canada) within 12hr of birth. At this time, day 0, piglets were randomly assigned to be fed one of the formulae. A further group of piglets were identified within 12hr of birth and left with the sow to be milk-fed. Littermates were never assigned to the same diet group. The nursing sows were fed a typical 16% protein lactation feed with canola oil as the source of fat. The sow milk-fed (milk) piglets received only sow milk for the duration of the piglet feeding period.

The piglets assigned to receive formula were brought to the British Columbia Research Institute For Child and Family Health and housed in the animal containment unit. The piglets were housed together in bins with 3-4 piglets per bin, with all bins contained in a single room equipped with temperature and light controls. For the first 9 days, piglets were bottle-fed by hand, to appetite, every 1.5hrs and then, for the remainder of the study, every 3hrs from 0600 hr to midnight. The formula was supplemented on days 1-5, 6-9 and 10-12 with 1.25%, 0.75% and 0.25% (wt/vol), respectively, bovine colostrum-derived immunoglobulins (LaBelle Associates, Inc. Bellingham, Washington), containing primarily secretory IgG, to provide passive immunity, respectively. During the first 9 days, feeds were also supplemented with Pedialyte (Ross, Columbus, OH), an oral electrolyte solution, to maintain hydration as required. Water bottles were also attached to cage sides. Heating was provided to the

piglets with spot heat lamps attached above each cage to achieve a temperature of about 27°C. The spot heat lamps also provided the lighting in the room to mimic the light levels in the farm facilities where the sow-milk fed piglets were reared.

All the procedures involving the piglets were approved by the Animal Care Committee of the University of British Columbia and conformed to the guidelines of the Canadian Council on Animal Care.

3.1.2. Diets

In study #1, the piglets allocated to receive formula were assigned to one of four formula groups. The formulae were similar in nutrient composition, but differed in the positional distribution of fatty acids in the formula triglyceride and in cholesterol content (**Table 3.1**). A conventional formula was made with a fat blend of 48% palm-olein oil, 26% soybean oil, 14% high oleic acid sunflower oil and 12% coconut oil. This provided, in total fatty acids, 23% 16:0, with 93.7% of the total 16:0 esterified to the *sn*-1 and 3 positions of the triglyceride. This formula was similar in composition to typical, commercially available term infant formula with palm-olein oil as the source of saturated fatty acids. A synthesized triglyceride (TG) formula was made with synthesized triglycerides (Betapol®, Loders Croklaan, Wormerveer, The Netherlands). This formula also provided 22-23% of total fatty acids as16:0, but 47% of the total 16:0 was esterified to the *sn*-2 position of the triglyceride. The levels of other saturated fatty acids, 18:1(n-9), 18:2(n-6) and 18:3(n-3) were also similar in the synthesized TG formula to that in the conventional formula, although the enrichment of 18:1(n-9), 18:2(n-6) and 18:3(n-3) at the *sn*-2 position of the triglyceride varied inversely with the enrichment of 16:0. The conventional and synthesized TG formulae contained no carbon chain (C) 20 or 22 (n-6)

or (n-3) fatty acids. Each formula was made without (-) and with (+) added cholesterol (0.52 mmol/L unesterified cholesterol + 0.13mmol/L cholesteryl oleate). The amount of cholesterol added and the proportion as unesterified cholesterol were based on the cholesterol content and relative amounts of unesterified cholesterol in human and pig milk (Jensen 1989, Jensen and Jensen 1992, Jones et al. 1990).

In study #2, the piglets designated to receive formula were assigned to one of two formula groups. One group of piglets was fed the conventional formula with cholesterol. The other group of piglets was fed the conventional formula with cholesterol, but also supplemented with egg phospholipid (+egg PL) to provide 20:4(n-6) and 22:6(n-3) to give a final concentration of 0.8% 20:4(n-6) and 0.25% 22:6(n-3) in the formula fatty acids. The formulae used in both studies #1 and #2 were prepared by Ross Laboratories, Columbus, Ohio as liquid, ready-to-feed formula.

The sow milk had similar levels of 18:1(n-9) (37.5%), but had lower 18:2(n-6) (11.1%) and 18:3(n-3) (1.1%) than any of the formula. About 60% of the 16:0 in the milk was esterified to the *sn*-2 position of the milk triglyceride. The sow milk also contained 0.52 mmol/L cholesterol and 0.7% and 0.1% fatty acids as 20:4(n-6) and 22:6(n-3), respectively. The fatty acid composition of the formulae and sow milk were determined by gas liquid chromatography following precipitation of fatty acid methyl esters using the method of Lepage and Roy (1986).

3.2. TISSUE COLLECTION AND ANALYSIS

3.2.1. Tissue Collection

At 18 days of age (study #1), or at 15 days of age (study #2) and between 0900hr and 1030hr, the piglets were fed 60ml of formula. Then, 3 hr later, the piglets

were anaesthetized with ketamine:rompun, 37.5:3.75 mg/kg (MTC Pharmaceuticals, Cambridge, ON; Bayvet Division, Chenango, Ltd., Etobicoke, ON, respectively) by intramuscular injection. This protocol was used in order to control the time post-feed and amount of feed consumed prior to blood collection. The piglets were not fasted prior to study because under normal circumstances milk-fed piglets and human infants are never in a fasted state. Similar control of the volume of milk consumed at the last feed, however, to that of the formula-fed piglets was not possible for the milk-fed piglets. Blood samples were drawn by cardiac puncture using syringes rinsed with 0.4mM EDTA. The animals were sacrificed by intracardiac injection of 1M KCl. Plasma was prepared by centrifugation and with the exception of aliquot samples for HDL cholesterol, the samples were frozen at -80°C until further biochemical analyses. After laparotomy, the liver was removed, bile was drawn from the gallbadder and the gallbladder removed. The bile was immediately frozen in liquid nitrogen and stored at -80° until further lipid analysis. The liver was blotted dry, visible connective tissue removed, and the organ then weighed. One gram samples of liver were taken for immediate preparation of microsomal and cytosolic fractions. Additional 1g samples were also taken and immediately frozen in liquid nitrogen and stored at -80°C until preparation of RNA. An additional portion of liver was taken, homogenized in saline and aliquots immediately frozen in liquid nitrogen and stored at -80°C for later tissue lipid analysis.

3.2.2. Plasma Biochemical Analyses

The plasma total cholesterol and triglyceride concentrations were determined using enzymatic colorimetric kits from Diagnostic Chemicals Ltd. (Charlottetown, PEI).

Table 3.1. Major fatty acid composition and cholesterol content of formula[4] and sow milk.

	Formula					
	synthesized TG[4]		conventional[4]		conventional-egg PL[5]	sow milk
	(-)	(+)	(-)	(+)		
fatty acids[1]						
12:0 + 14:0	8.9	8.4	7.9	7.6	6.6	3.8
16:0	23.3	23.4	24.1	24.0	24.7	30.5
18:0	4.4	4.4	5.2	5.1	6.6	9.4
18:1(n-9)	38.5	39.1	38.4	38.6	37.0	37.5
18:2(n-6)	20.3	20.4	20.6	20.7	20.0	11.1
18:3(n-3)	2.1	2.1	2.1	2.1	1.8	1.1
20:4(n-6)	0.0	0.0	0.0	0.0	0.8	0.7
22:6(n-3)	0.0	0.0	0.0	0.0	0.25	0.1
cholesterol[2] (mmol/L)	<0.10	0.65	<0.10	0.65	0.65	0.52
position of 16:0[3]						
sn-2	47.0	47.0	6.3	6.3	6.3	60.4
sn-1,3	53.0	53.0	93.7	93.7	93.7	39.6

[1]Data for fatty acids in g/100g. [2]Data for cholesterol in mmol/L, formula or milk. [3]Percent of total 16:0 in *sn*-2 or *sn*-1,3 position of the formula or milk triglyceride. [4]The formulae contained (per L) 67g total fat, 58g protein, 60g carbohydrate and 4427 kJ. [5]Formulae provided with (+) and without (-) cholesterol. [6]Formula supplemented with 9.5g/L egg phospholipid which contained in total fatty acids 16:0, 29.3%; 18:0, 15.7%; 18:1(n-9), 27.5%; 18:2(n-6), 15.3%; 18:3(n-3), 0.1; 20:4(n-6), 5.6%; 22:6(n-3), 1.8%.

The total cholesterol assay kit was based on the method of Allain et al. 1974. This method was designed based on the principle that cholesterol esters are hydrolyzed to unesterified cholesterol by cholesterol esterase. The unesterified cholesterol is then oxidized by cholesterol oxidase to cholesten-3-one and hydrogen peroxide. The hydrogen peroxide that is produced will react with 4-aminoantipyrine and phenol in the presence of peroxidase to yield a chromagen with maximum absorbance at 505nm. Therefore, the intensity of the colour is directly proportional to the amount of total cholesterol in the sample. The kit provided 4-aminoantipyrine, peroxidase, cholesterol esterase and cholesterol oxidase in one reagent and a phenol reagent. The amount of total cholesterol in piglet plasma was determined using 25μl of plasma. Regression analysis was used to generate a standard curve of absorbance values at 505nm determined from various concentrations of a cholesterol standard. The equation generated from this was used to calculate the concentrations of total cholesterol in piglet plasma from the absorbance values at 505nm. The intra- and inter-assay variation was determined to be less than 5%.

The triglyceride assay kit was based on the method of McGowan et al. 1983. This method is based on the principle that triglyceride is hydrolyzed to glycerol and unesterified fatty acids by lipase. In the presence of ATP and glycerol kinases the glycerol is converted to glycerol-1-phosphate. The glycerol-1-phosphate is oxidized by glycerol phosphate oxidase to yield hydrogen peroxide. The condensation of hydrogen peroxide with 3,5-dicholoro-2-hydroxy-benzenesulfonic acid and 4-aminoantipyrine in the presence of peroxidase produces a red colored quinoneimine dye which absorbs at 515nm. The kit provided a reagent with ATP, 3,5-dicholoro-2-hydroxy-benzenesulfonic

acid, 4-aminoantipyrine, peroxidase, glycerol phosphate oxidase, glycerol kinase and lipase, a buffer and a triglyceride glycerol standard. The amount of triglyceride in piglet plasma was determined using 10μl of plasma. Regression analysis was used to generate a standard curve of absorbance values at 515nm determined from various concentrations of the triglyceride glycerol standard. The equation generated was used to calculate the concentrations of triglyceride in piglet plasma from the absorbance values at 515nm.

HDL total cholesterol was determined in plasma using the enzymatic kit for measurement of total cholesterol, following precipitation of apo B lipoproteins with heparin-manganese chloride (Gidez et al. 1982) within 6 hr of blood collection. The amount of cholesterol associated with apo B-containing lipoproteins (chylomicron+VLDL+LDL) was calculated as the difference between the total and HDL cholesterol concentrations.

Plasma unesterified fatty acid concentration was determined using an enzymatic kit from Wako Chemicals USA (Richmond, Virginia). This method was designed based on the principles that unesterified fatty acid and Coenzyme A (CoA) when treated with acyl-CoA synthetase form acyl-CoA. The acyl-CoA is then oxidized by acyl-CoA oxidase to 2,3-*trans*-enoyl-CoA and hydrogen peroxide. The hydrogen peroxide that is produced will react with 4-aminoantipyrine and 3-methyl-N-ethyl-N-(β-hydroxyethyl)-aniline in the presence of peroxidase to yield a purple chromagen with maximum absorbance at 550nm. Therefore, the intensity of the colour is directly proportional to the amount of unesterified fatty acid in the sample. The kit provided one reagent containing 4-aminoantipyrine, CoA, acyl-CoA synthetase and ascorbate oxidase; and a

second reagent containing peroxidase, acyl-CoA oxidase and 3-methyl-N-ethyl-N-(β-hydroxyethyl)-aniline. The amount of unesterified fatty acid in piglet plasma was determined using 50μl of plasma. Regression analysis was used to generate a standard curve of absorbance values at 550nm determined from various concentrations of an unesterified fatty acid standard. The equation generated from this was used to calculate the concentrations of unesterified fatty acid in piglet plasma from the absorbance values at 505nm. The intra and inter assay variability as determined by the manufacturer was less than 10%.

Plasma thyroid hormone concentrations were determined using Gammacoat™ [^{125}I] radioimmunoassay kits from INCSTAR Corporation (Stillwater, MN). Measurement of total triiodothyronine (T_3) was based on the principle that T3 present in samples competes with an [^{125}I]-labelled T_3 tracer for a limited number of binding sites on a rabbit anti-human T_3 antibody immobilized on the lower inner wall of the Gammacoat™ tube. The amount of [^{125}I]-labelled T_3 tracer bound to the tube as determined using a Gamma Counter, is inversely proportional to the concentration of T_3 in the sample. A standard curve is prepared from serum standards and sample values determined from the standard curve by interpolation. The assay for free T_3 follows a similar procedure except an [^{125}I] free T_3 tracer is used. The assay for total and free T_4 uses mouse monoclonal anti-human T_4 antibody coated tubes and an [^{125}I]-labelled T_4 tracer and an [^{125}I]-labelled free T_4 tracer. The sensitivity of the total T3, free T3, total T4 and free T4 radioimmunoassay as determined by the manufacturer is 0.09ng/ml, 0.02 pg/ml, 0.98μg/dL and 0.07ng/dL, respectively. The intra and inter assay variability as determined by the manufacturer was less than 10%.

Plasma insulin concentrations were determined using a radioimmunoassay from Immunocorp (Montreal, PQ). Measurement of insulin was based on the principle that insulin present in samples competes with an [^{125}I]-labelled insulin for a limited number of binding sites on an anti-insulin antibody immobilized on the lower inner wall of tubes provided by the kit. The amount of [^{125}I]-labelled insulin bound to the tube as determined using a Gamma Counter, is inversely proportional to the concentration of insulin in the sample. A standard curve was prepared from serum standards and the sample values were determined from the standard curve by interpolation. The sensitivity of the assay as determined by the manufacturer when defined by the amount of insulin standard that will reduce the maximal binding of the tracer by 5%, is 26.7pmol/L. The intra and inter assay variability as determined by the manufacturer was less than 10%.

The plasma glucagon (pancreatic) concentrations were determined using a radioimmunoassay from ICN Biochemicals Inc. (Costa Mesa, CA). Measurement of glucagon was based on the principle that glucagon present in samples competes with an [^{125}I]-labelled porcine pancreatic glucagon for a limited number of binding sites on a rabbit anti-porcine pancreatic glucagon antibody. The procedure involves incubating the standards, controls and samples with the rabbit anti-porcine pancreatic glucagon antibody followed by addition of the [^{125}I]-labelled porcine pancreatic glucagon. Polyethylene glycol and a goat anti-rabbit gamma globulin antibody are then added to precipitate the antigen-antibody complex. The amount of [^{125}I]-labelled pancreatic glucagon present in the precipitate as determined using a Gamma Counter, is inversely proportional to the concentration of pancreatic glucagon in the sample. A standard

curve was prepared from serum standards and the sample values were determined from the standard curve by interpolation. The cross-reactivity of the rabbit anti-porcine pancreatic glucagon with porcine pancreatic glucagon, enteroglucagon and human insulin was 100%, 0.0013% and 0.0005%, respectively.

3.3.3. Liver and Bile Lipid Analyses

Total lipids were extracted from liver according to the method of Folch et al. (1957). Bile lipid was extracted using chloroform:methanol (1:2, vol/vol), and exposed to fluorescent light overnight to permit photodegradation of biliary pigments, prior to quantifying cholesterol, total bile acids (Mashige et al. 1981) and phospholipid (Chen et al. 1956). Liver lipid fractions were separated by thin layer chromatography (Hrboticky et al. 1990). The bands were visualized with 2'7'-dicholorofluorescein (Supelco Inc., Bellefonte, PA) and then scraped and eluted from the silica with chloroform:methanol (2:1, vol/vol). Liver and bile total cholesterol concentrations and liver triglyceride concentrations were determined using the same method described for plasma after reconstitution in isopropanol. Liver and bile phospholipid was determined by assay of lipid phosphorus (Chen et al. 1956, Gurantz et al.1981, respectively). Protein was determined by the method of Lowry et al. 1952.

Liver and bile fatty acid methyl esters were prepared by transmethylation using methanolic HCl (1:5, vol/vol) at 100°C for 5 min. Methyl esters were partitioned twice with 3ml of saline and 4ml pentane. The pooled pentane layers were dried under N_2. The liver phospholipid, triglyceride and cholesterol ester and bile phospholipid fatty acid methyl esters were separated and quantified by gas liquid chromatography (Varian 6000, Varian Canada Ltd., Georgetown, ON) with flame-ionization detection by using a

30m x 0.25m ID nonbonded, glass capillary SP 2330 column (Supelco, Inc. Bellefonte, PA). Helium was the carrier gas at a column flow of 1ml/min. and an inlet pressure of 103.35 kPa. Fatty acid methyl esters were identified by comparison with retention times of authentic standards and quantified on the basis of 17:0, added in known quantity, as an internal standard.

3.3.4. RT-PCR Quantification of mRNA

The RT-PCR method used to determine the levels of LDL receptor, HMG-CoA reductase, C7H, ACC and FAS mRNA was developed based on methodology described by Chelley and Kahn (1994). This procedure involved reverse transcribing a known quantity of total RNA to generate a pool of cDNA, representing the RNA in the original sample. PCR was used to amplify the cDNA corresponding to the mRNA of interest. The expression of β-actin for each sample was also determined and this was used as an internal control for the efficiency of each RT-PCR reaction. The intra and inter assay variability between RT-PCR reactions was determined on random samples of liver RNA from formula-fed piglets. Both were determined to be less than 10%. The findings are presented in the Results section, **Table 4.2** and **Figure 4.3** and **4.5**.

Total RNA was isolated from liver tissue using guanidinium isothiocyanate followed by cesium chloride density gradient centrifugation (Chirgwin et al. 1979), in study #1 and by using TRIZOL® (Life Technologies Inc., Burlington, ON), in study #2. The TRIZOL® reagent is a phenol and guanidinium isothiocyanate solution and follows a modified protocol of the gaunidinium-phenol-chloroform extraction procedure for RNA isolation described by Chomczynski and Sacchi (1987). Following extraction of total RNA, the RNA preparations were treated with 10 units of RQ1 RNase-free DNase (Promega, Madison

Wisconsin) to ensure no genomic DNA contamination. The RNA was quantified by determining the optical density reading at a wavelength of 260nm using a spectrophotometer (Du 640, Beckman Instruments Inc. Fulerton, CA).

Total RNA (2.5μg) was used for first strand cDNA synthesis using M-MLV reverse transcriptase (Gibco BRL, Burlington, Canada) in 50mM Tris-HCL, 75mM KCl, 10mM DTT, 3mM $MgCl_2$, 2mM dNTPs (Gibco BRL, Burlington, Canada) and 10 units recombinant RNase inhibitor (Clontech, Palo Alto, California) using 250pmol random hexamers (Gibco BRL, Burlington, Canada) as a primer for synthesis in a total volume of 25μl. The reaction time was 10 min at 20°C followed by 60 min at 37°C. The resulting cDNA pool for each RNA sample was divided into aliquots (3μl) with one aliquot used in each PCR reaction. Separate PCR reactions were run for LDL receptor, HMG-CoA reductase, C7H, ACC, FAS and β-actin.

The PCR reaction contained 20mM Tris-HCl, 50mM KCl, 1.5mM $MgCl_2$, 0.8mM dNTP (Gibco BRL, Burlington, Canada), 12pmol of each gene specific primer and 2.5 units of *Taq* DNA Polymerase (Gibco BRL, Burlington, Canada). The PCR reactions for FAS and C7H also contained 2.5% dimethyl sulfoxide (Filichkin and Gelvin 1992, Varadaraj and Skinner 1994). The primer sequences used to amplify a fragment of each gene are given in **Table 3.2.** The primers designed to amplify a fragment of the ACC cDNA were based on the human cDNA sequence characterized from HepG2 cells which codes for the 265kDa enzyme and were designed to amplify a fragment between nucleotide 1920 and nucleotide 2217 (Abu-Elheiga et al. 1995). The primers designed to amplify a fragment of β-actin cDNA, FAS cDNA, LDL receptor cDNA, HMG-CoA reductase cDNA

and C7H cDNA were based on the published human sequences for each gene (Ng et al. 1985, Jayakumar et al. 1995. Yamamoto et al. 1984, Luskey et al. 1985, Nishimoto et al. 1993, respectively). The PCR amplification cycle was 94°C for 30 sec followed by 65° for 1.5 min for HMG-CoA reductase, LDL receptor and β-actin; and 94°C for 30 sec. followed by 60°C for 1.5min. for ACC, C7H and FAS. The number of cycles used for the PCR reactions were gene specific and were determined to be in the exponential phase of the amplification process (Chelley and Kahn 1994) (**Figure 3.1**.).

Table 3. 2. Primer sequences used for PCR.

gene	primer sequence	PCR fragment	ref.
acetyl-CoA carboxylase			
ACC1 (forward)	5'-AAGCTTTCAAATGAACAGAATTGA-3'	297bp	Abu-Elheiga et al. 1995
ACC2 (reverse)	5'-GAGCCATTCATGATCACCACATAG-3'		
β-actin			
AC-2 (forward)	5'-TGATCCACATCTGCTGGAAGGTGG-3'	524bp	Ng et al. 1985
AC-3 (reverse)	5'-GGACCTGACTGACTACCTCATGAA-3'		
fatty acid synthase			
FAS1 (forward)	5'-AAGAAGGATCACAGGGACAACC-3'	323bp	Jayakumar et al. 1995
FAS2 (reverse)	5'-CCACACTATGCTCAGGTAGCCA-3'		
HMG-CoA reductase			
HMG-L (forward)	5'-ATTATGTGCTGCTTTGGCTGCATG-3'	267bp	Luskey et al. 1985
HMG-R (reverse)	5'-TTGAGGAGAAGGATCAGCTATCCA-3'		
7-α-hydroxylase			
7AH-L (forward)	5'-AATCCTCTTGAGTTCCTCAGAGC-3'	206bp	Nishimoto et al. 1993
7AH-R (reverse)	5'-CCATCCATCGGGTCAATGCTTCT-3'		
LDL receptor			
LDL-2 (forward)	5'-GACAACCCCGTCTATCAGAAGACC-3'	98bp	Yamamoto et al. 1984
LDL P (reverse)	5'-GACCATCTGTCTCGAGGGGTAGG-3'		

Figure 3.1. Representative photograph of RT-PCR products obtained to ascertain the number of PCR cycles that will be used in the RT-PCR assay. This photograph shows the final PCR products obtain for HMG-CoA reductase. Lane 1, Ladder (50bp DNA Ladder, Gibco BRL, Burlington, Canada); lane 2, 22 cycles; lane 3, 25 cycles; lane 4, 27 cycles; lane 5, 30 cycles; lane 6, 32 cycles; lane 7, 35 cycles; lane 8, 37 cycles; lane 9, 40 cycles; lane 10, 42 cycles.

The number of PCR cycles completed for each gene was: LDL receptor 35 cycles, HMG-CoA reductase 35 cycles, C7H 35 cycles, FAS 35 cycles, ACC 35 cycles, and β-actin 25 cycles.

The PCR reactions (10µl each) were resolved on a 1.5% agarose-1XTBE gel and stained with ethidium bromide. A photograph was taken with Kodak TRI-X pan film. The negatives were scanned with a video densitometer (Model 620, Bio-Rad, Mississauga, Ontario, Canada) to determine the relative intensity of the bands and this was expressed per µg RNA used in the original RT reaction.

3.3.5. Enzyme Assays

Liver microsomes and cytosolic fractions were prepared according to the method of Hackman et al. (1996) with some modifications. Liver samples (1g) were immediately homogenized in 9ml of ice-cold buffer containing 0.3M sucrose, 50mM NaCl and 10mM EDTA at a pH 7.4 using a Potter-Elvejhem tissue homogenizer then centrifuged, 8500*g*

for 15 min at 4°C. The supernatant was removed and centrifuged at 100,000*g*, 60 min at 4°C. The top layer of fat was removed and the remaining supernatant, the cytosolic fraction, was used for determination of FAS activity (Kumar and Dodds 1981). The resulting microsomal pellet was resuspended in 3.5 ml of original homogenization buffer, 10mM DTT plus 2mg/ml aprotinin (proteinase inhibitor) and then stored at -80°C until further analysis.

FAS activity was determined in cytosolic fractions using a spectrophotometric assay according to the method of Kumar and Dodds (1981). This method involved measuring the rate of NADPH oxidation determined by following the change in the absorbance of NADPH at 340nm. Briefly, aliquots of isolated hepatic cytosolic fractions containing 5mg of protein in 1M KH_2PO_4 pH 7.0 and 14.97mM DTT were incubated for 15 min. at 37°C. The aliquots were divided in three and to each was added NADPH and acetyl CoA at final concentrations of 1mM and 0.33mM, respectively, at 30°C. The change in absorbance at 340nm was then measured at 30°C for 2-3 min to determine the background rate of NADPH oxidation for each sample. Following this, malonyl CoA was added to each sample to yield a final concentration of 4.2mM at 30°C. The change in absorbance at 340nm was then measured at 30°C for 2-3 min. A standard curve was prepared from known concentrations of NADPH. The net change in absorbance at 340nm was calculated and for each sample the amount of NADPH oxidized determined from the standard curve by interpolation. The final activity was calculated as nmol of NADPH oxidized per min. per mg protein.

HMG-CoA reductase activity was determined using the method of Hackman et al. (1996), with some modifications. Microsomal fractions containing 1mg of protein were

incubated for 60min. at 37°C with the substrate cofactor mix containing, at a final concentration, 0.2M KH_2PO_4, 40mM glucose-6-phosphate, 5mM NADP, 20mM EDTA and 10mM DTT at a pH of 7.4. Following this, 1 unit of glucose-6-phosphate dehydrogenase (type XV from Baker's Yeast, Sigma Chemicals, St. Louis MO), 25µl of 176µM HMG-CoA (Sigma Chemicals) and 3.7 KBq of [^{14}C]HMG-CoA (Dupont NEN Products, Boston, MA) was added, to a final total volume of 210µl, and the reaction allowed to proceed for 15 min, at 37°C. The reactions were stopped by addition of 25 µl of 6M HCl, and then incubated at 37°C for 30 min to allow conversion of mevalonate to mevalonolactone. The [^{14}C]mevalonolactone was separated from the remaining [^{14}C]HMG-CoA by extraction with toluene. Ten ml of toluene and 0.5 g of sodium sulphite were added, the reaction shaken for 5 min, and then placed at room temperature for 30 min. The amount of [^{14}C]mevalonolactone resulting from the reaction was determined by liquid scintillation counting (LS 6000IC Beckman, Fullerton, CA).

3.4. STATISTICAL ANALYSIS

3.4.1. Study #1

Analysis to determine the effect of formula-feeding in comparison to milk-feeding from birth to 18 days of age was performed using one-way analysis of variance (ANOVA). Two-way ANOVA was used to examine the effect of the formula cholesterol content and the positional distribution of 16:0 in the formula triglyceride. Formal tests of differences among groups utilized Fisher's Least Square Difference and were based on least squares means and standard errors calculated from the ANOVA. All calculations were performed using the Statistical Package for the Social Sciences (SPSS), standard version 7.5.1

(SPSS Inc, Chicago, Illinois). Differences were considered statistically significant if the *F* statistic *P* value was <0.05.

3.4.2. Study #2

Analysis to determine the effect of supplementing formula with egg phospholipid, providing 20:4(n-6) and 22:6(n-3), was performed with one-way analysis of variance (ANOVA). If no statistically significant effects of feeding the standard-AA+DHA formula compared to the standard formula were found, the results of the two groups of formula-fed piglets were combined and then compared to the reference group of sow milk-fed piglets. Differences were considered statistically significant if the *F* statistic *P* value was <0.05.

Linear regression was used to explore the potential relation between the percentage of 20:4(n-6) and 22:6(n-3) in hepatic phospholipid and triglyceride and the levels of FAS mRNA and ACC mRNA. Where significant relations were found, the precision with which analysis of the percentage of 20:4(n-6) and 22:6(n-3) in liver triglyceride and phospholipid could be used to predict the hepatic levels of ACC mRNA and FAS mRNA and activity was determined by calculation of the 95% prediction limits. All calculations were performed using the Statistical Package for the Social Sciences (SPSS) for Windows, standard version 7.5.1 (SPSS Inc, Chicago, Illinois).

4. RESULTS

4.1. STUDY#1-PART I

4.1.1. Body and liver weights and Plasma lipid and hormone concentrations.

The body and liver weights of the piglets at the end of the feeding period are shown in **Figure 4.1 (Appendix I).** There were no differences in either body weight or liver weight among diet groups.

Piglets fed the conventional formula, with most of the 16:0 esterified to the triglyceride *sn*-1 and 3 positions, however, had significantly higher plasma cholesterol and triglyceride concentrations than piglets fed the synthesized TG formula (**Figure 4.2, Appendix II**). The inclusion of cholesterol in the conventional and synthesized TG formula was associated with higher mean plasma total and HDL cholesterol and triglyceride concentrations than in piglets fed the conventional or synthesized TG formula without added cholesterol. However, the differences, representing an increase of about 6% and 2% for total cholesterol, 2% and 6% for HDL cholesterol and 19% and 3% for triglycerides, respectively, were not of statistical significance. The piglets fed formula, regardless of the formula cholesterol content or the positional distribution of 16:0 in the formula triglyceride, had significantly lower ($P<0.05$) plasma concentrations of total and HDL cholesterol and triglyceride than the piglets fed sow milk. The piglets fed formula also had lower concentrations of apo B-containing lipoprotein cholesterol than the piglets fed sow milk. Statistical analysis of differences in apo B were not conducted because apo B cholesterol levels were calculated from the difference between the values for total cholesterol and the values for HDL cholesterol, and not directly measured.

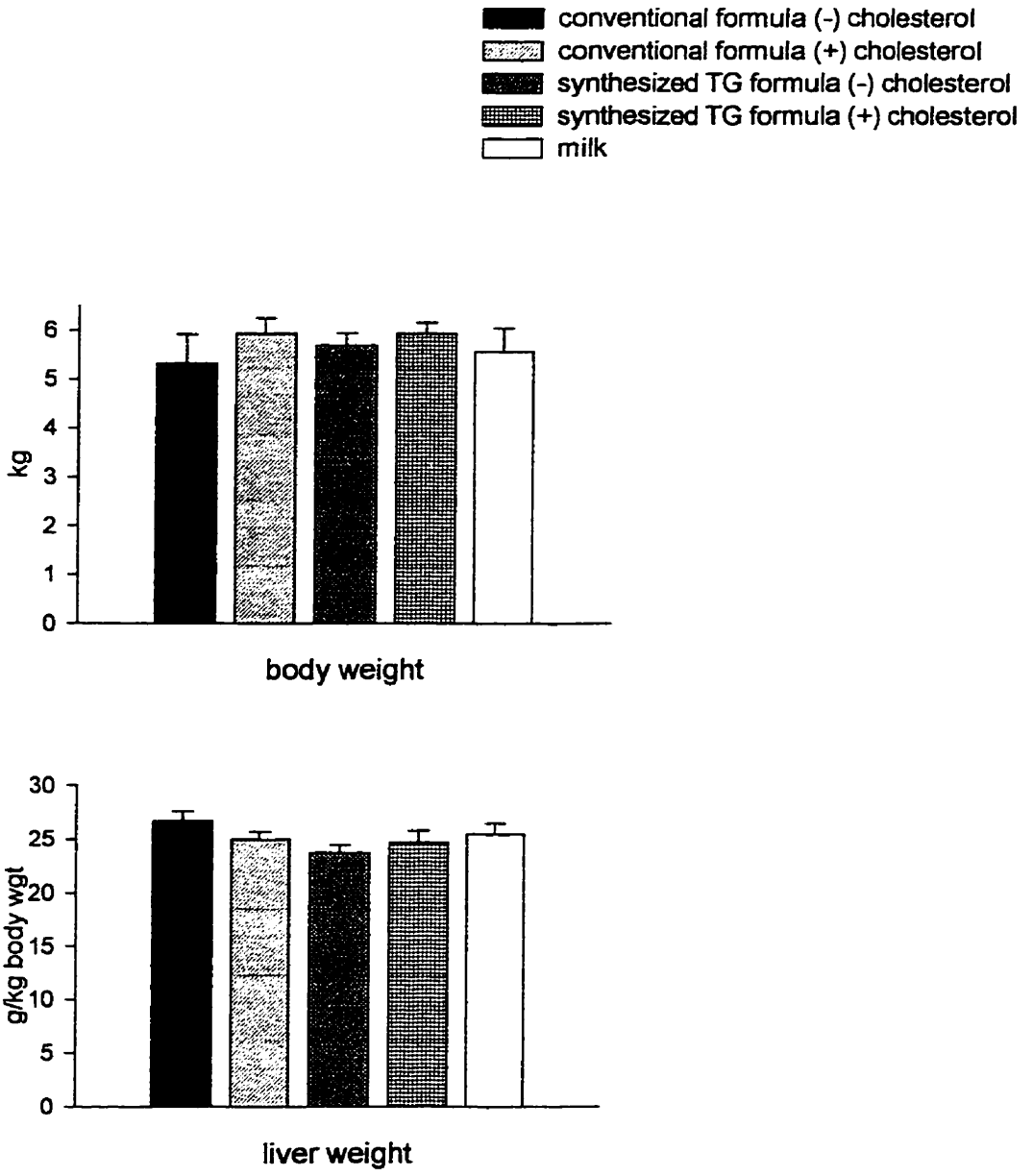


Figure 4.1. Body and liver weights of piglets fed formula or milk to 18 days of age. Data presented as mean + SEM, n=6 for each diet group.

.

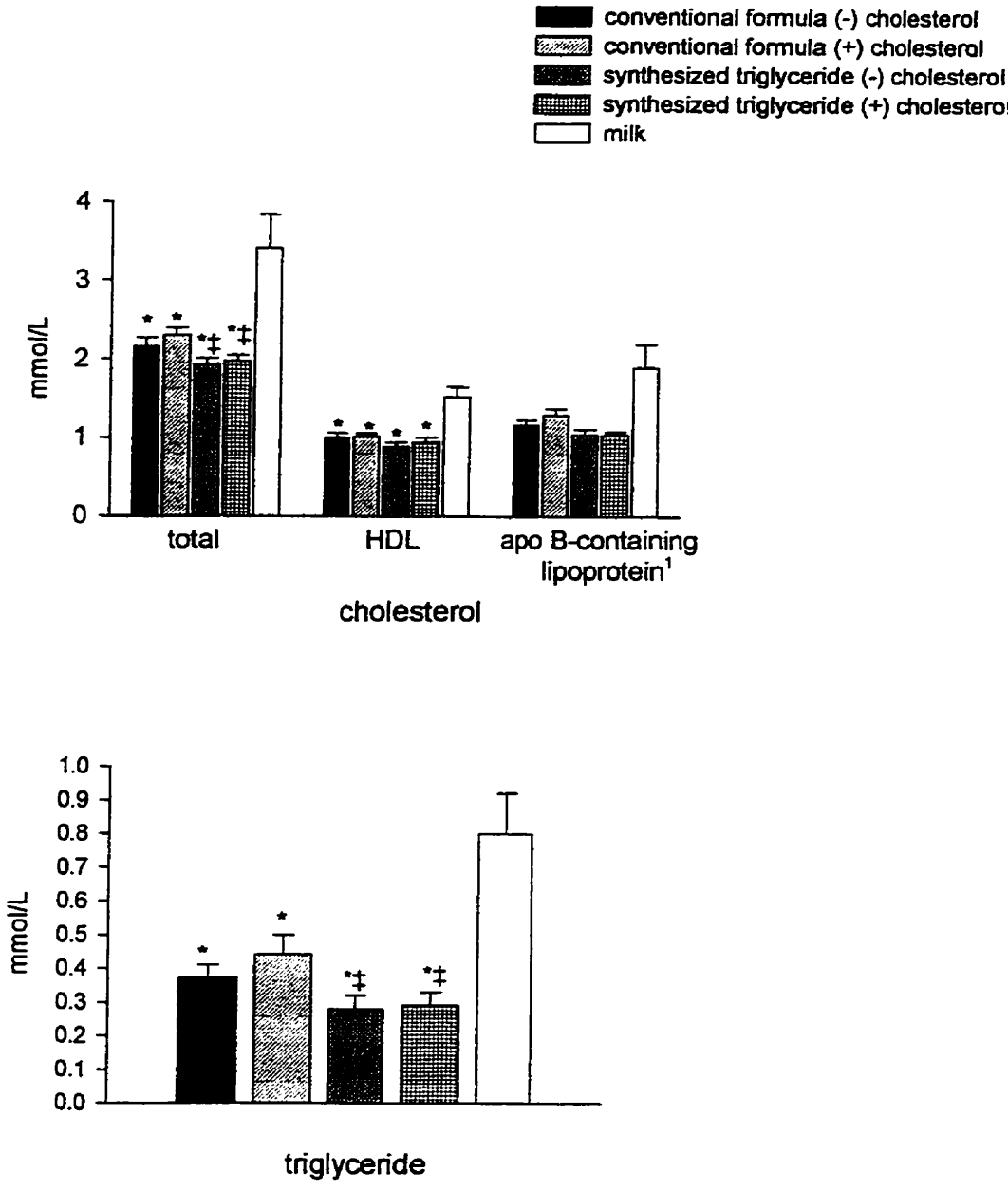


Figure 4.2. Concentrations of cholesterol and triglyceride in plasma of piglets fed formula or milk to 18 days of age.
Data presented as means + SEM, n=6 for all diet groups. *Values for piglets fed formula significantly different from value for piglets fed milk ($P<0.05$). ‡Significant effect of the positional distribution of 16:0 in the formula triglyceride ($P<0.002$). No significant effect of addition of cholesterol to the formula was found. [1]Not analyzed statistically. Analysis was conducted on non-fasted blood samples.

Neither the positional distribution of 16:0 in the triglyceride nor the cholesterol content of the formula had any significant effect on the plasma thyroid hormones or unesterified fatty acid concentrations in the formula-fed piglets (**Table 4.1.**). However, plasma free T4 levels, were significantly higher, and glucagon and unesterified fatty acid concentrations were lower ($P<0.005$) in the piglets fed formula than in the piglets fed milk. Further, the plasma levels of total T3, free T3, glucose and insulin were consistently lower, and the levels of total T4 were consistently higher, although not significantly higher, in piglets fed formula than piglets fed milk.

4.1.2. Hepatic and bile lipid composition.

The addition of cholesterol to the formula was associated with significantly ($P<0.05$) higher hepatic cholesterol concentrations but had no significant effect on the hepatic triglyceride concentrations of the formula-fed piglets (**Figure 4.3, Appendix III**). The positional distribution of 16:0 in the formula triglyceride, on the other hand, had no significant effect on hepatic triglyceride or cholesterol concentrations. The piglets fed either the conventional or synthesized TG formula without cholesterol had significantly lower ($P<0.05$) hepatic cholesterol concentrations than the piglets fed milk. The piglets fed formula, regardless of cholesterol concentrations or the positional distribution of 16:0 in the formula triglyceride, had significantly lower ($P<0.0001$) hepatic triglyceride concentrations than the piglets fed milk.

The positional distribution of 16:0 in the formula triglyceride did have a statistically significant effect on bile acid concentrations in bile with piglets fed the synthesized TG formula having a significantly lower ($P<0.01$) concentration of bile acids in bile than piglets

Table 4.1. Plasma thyroid hormone, insulin, glucagon, glucose, free fatty acids & lactate levels in piglets fed formula & milk for 18 days[1].

	Formula				Sow milk
	conventional[2]		synthesized TG[2]		
	(-)	(+)	(-)	(+)	
thyroid hormones					
total T3 (nmol/L)	1.28±0.2	1.84±0.1	1.66±0.2	1.53±0.1	1.87±0.4
free T3 (pmol/L)	0.89±0.2	1.34±0.3	1.40±0.2	1.09±0.2	1.83±0.5
total T4 (nmol/L)	58.0±7.7	69.7±7.8	71.4±3.8	66.8±8.8	56.7±3.5
free T4 (pmol/L)	26.1±2.5*	36.6±3.3*	41.4±2.0*	35.0±4.0*	19.8±1.9
insulin (pmol/L)	45.0±7.1	55.7±11	41.9±4.2	53.3±13	65.1±4.8
glucagon (pmol/L)	162±64.7*[a]	249±53.2*[b]	344±40.1*[b]	324±106*[a]	419±52.2
glucose (mmol/L)	5.0±0.3	5.4±0.6	5.7±0.5	5.6±0.3	7.0±0.4
free fatty acid (mEQ/L)	0.11±0.04*	0.15±0.04*	0.08±0.0*	0.13±0.0*	0.41±0.04
lactate (mmol/L)	2.5±0.4	3.1±0.3	2.2±0.4	2.6±0.4	3.0±0.3

[1]Data presented as mean ± SEM, n=6, except for glucagon where,[a] n=3 and [b] n=5. [2]Formulae provided with (+) or without (-) added cholesterol. *Significantly different from values for piglets fed milk ($P<0.05$). No statistically significant effect of the addition of cholesterol or the positional distribution of 16:0 in the formula triglyceride was found.

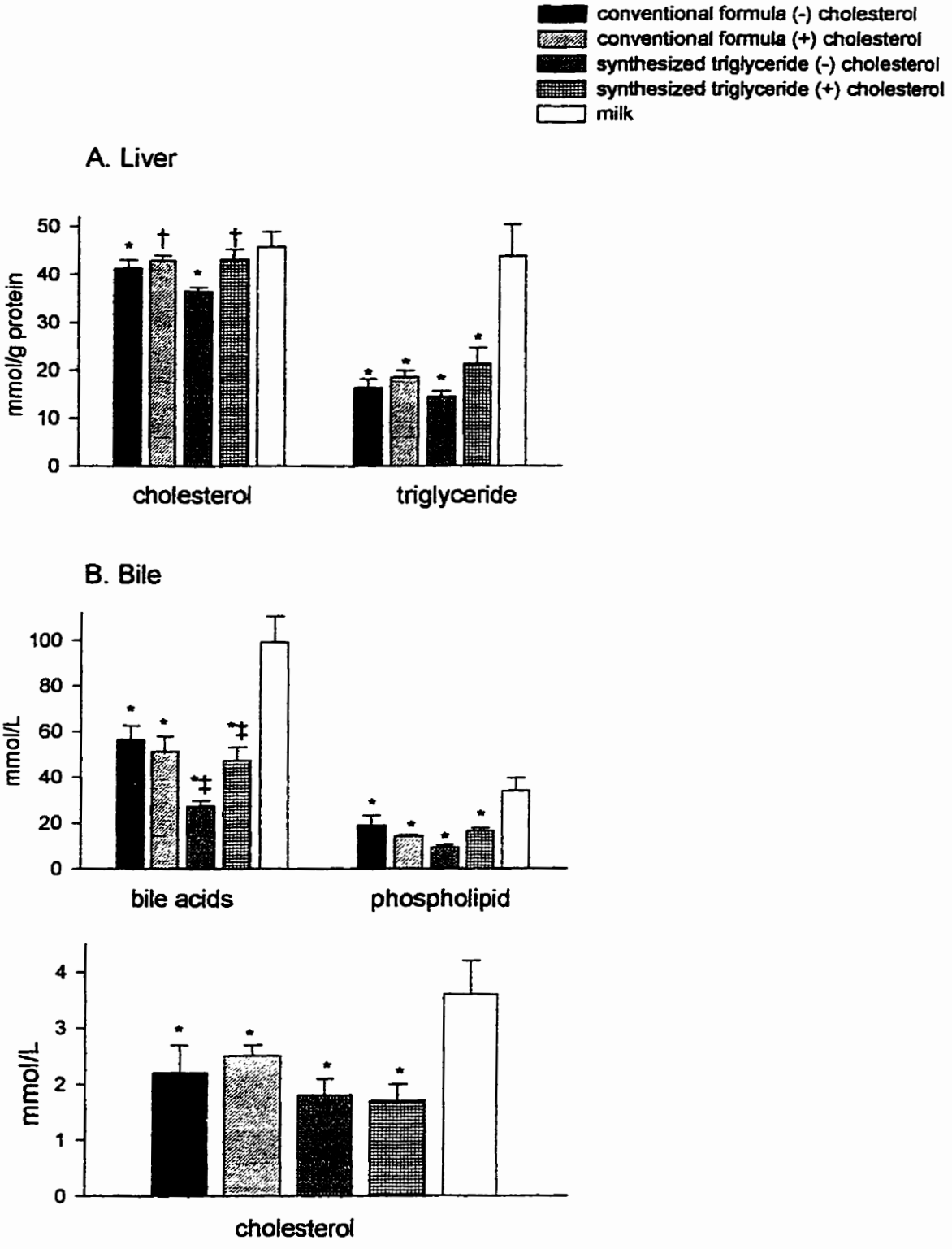


Figure 4.3. Concentrations of liver lipids and bile lipids and bile acids in piglets fed formula or milk to 18 days of age.
Data presented as mean + SEM, n=6 for each diet group.*Values for piglets fed formula significantly different from value for milk-fed piglets ($P<0.05$). †Significant effect of adding cholesterol to the formula ($P<0.05$).‡ Significant effect of the positional distribution of 16:0 in the formula triglyceride ($P<0.01$).

fed the conventional formula (**Figure 4.3, Appendix III**). The addition of cholesterol to the formula, however, had no effect on the bile cholesterol or bile acid concentrations. Interestingly, the addition of cholesterol to the conventional formula was associated with a 25% decrease in bile phospholipid concentrations, whereas, the addition of cholesterol to the synthesized TG formula was associated with a 69% increase in bile phospholipid concentrations. A significant interaction ($P<0.03$) between the positional distribution of fatty acids in the formula triglyceride and the cholesterol content of formula was observed for bile phospholipid concentrations. However, when analyzed separately, these differences were not statistically significant. However, the piglets fed formula, regardless of cholesterol content or the positional distribution of 16:0 in the formula triglyceride, had significantly lower ($P<0.05$) concentrations of bile acids, total cholesterol and phospholipid in bile than piglets fed milk.

4.1.3. Hepatic LDL receptor, HMG-CoA reductase and C7H RNA.

The integrity of the total RNA isolated from the piglet liver is shown in **Figure 4.4**. A RT-PCR assay was developed and used to determine mRNA levels for HMG-CoA reductase, LDL receptor and C7H because of the greater degree of sensitivity and accuracy afforded with this method compared to Northern Blot Hybridization (Chelley and Kahn 1994, Foley et al. 1993). The inter and intra assay variability of each RT-PCR reaction were determined to be less than 10% (**Table 4.2. and Figure 4.5.**). Examples showing the final products obtained for each of the RT-PCR reactions for each of the genes studied are illustrated in **Figure 4.6.**. Neither the addition of cholesterol to the formula nor the positional distribution of 16:0 in the formula triglyceride had any statistically significant effect on LDL receptor, HMG-CoA reductase or C7H mRNA levels

in the formula-fed piglets (**Figure 4.7, Appendix IV**). The levels of HMG-CoA reductase mRNA and C7H mRNA in the formula-fed piglets, in contrast, were significantly ($P<0.05$) higher than in the milk-fed piglets. LDL receptor mRNA levels were similar in the milk-fed and formula-fed piglets.

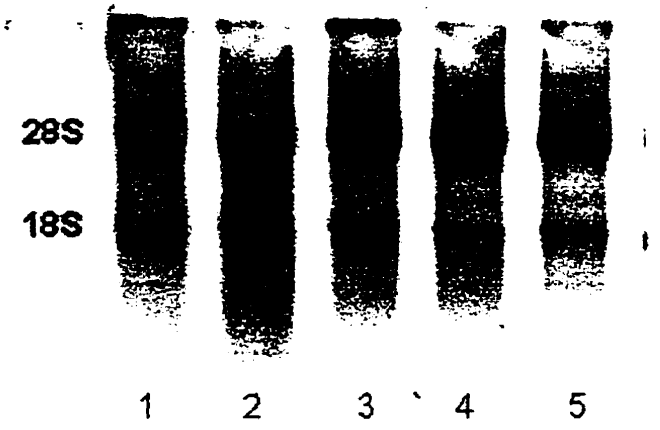


Figure 4.4. A representative photograph illustrating the integrity of total RNA isolated from liver of piglets fed formula or milk for 18 days. Five micrograms of total RNA was resolved on 1XTBE gel and stained with ethidium bromide. The ribosomal bands are marked. Lane 1, milk; lane 2, conventional formula (-) cholesterol; lane 3, conventional formula (+) cholesterol; lane 4, synthesized TG formula (-) cholesterol; lane 5, synthesized TG formula (+) cholesterol.

Table 4.2. RT-PCR Intra- & Interassay Variability

	intra-assay variability		**inter assay variability**	
gene	mean[1] ± SEM	percentage[2]	mean[1] ± S.D.	percentage[3]
β-actin				
HMG-CoA reductase	241.8 ± 4.2	1.7%	244.2 ± 2.4	1.0%
LDL Receptor	242.7 ± 7.4	3.1%	229.4 ± 15.2	6.6%
C7H	237.7 ± 5.3	2.2%	238.8 ± 12.1	5.1%

[1]Values given in absorbance units per μg of RNA. [2]Percentage = (SEM / mean)*100.
[3]Percentage = (S.D. / mean)*100.

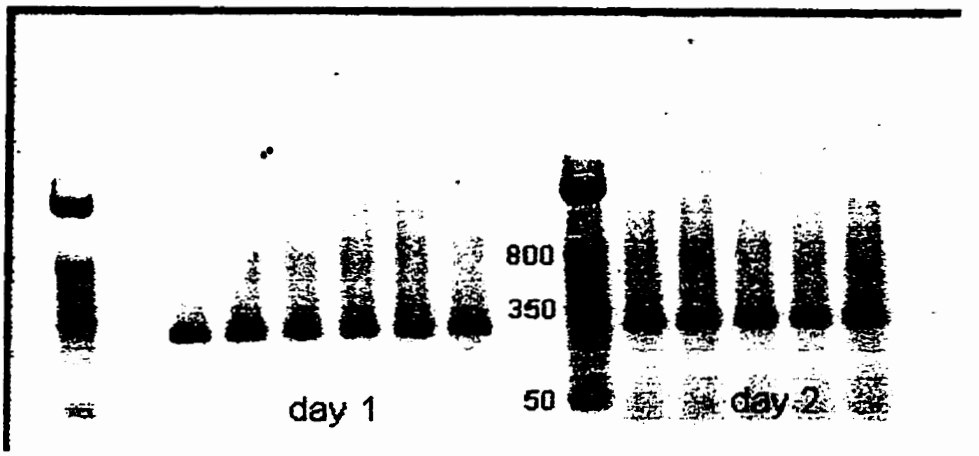


Figure 4.5. Representative photograph showing examples of the RT-PCR products obtained to calculate the inter and intra-assay variability for HMG-CoA reductase. Ten μl of the PCR reaction were resolved on a 1.5% 1 X TBE gel stained with ethidium bromide.

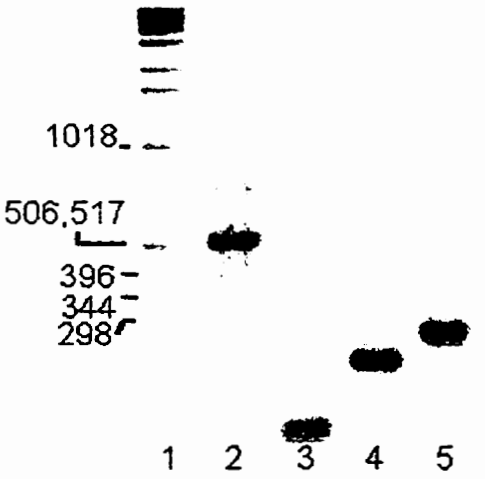


Figure 4.6. Representative photograph showing examples of the RT-PCR products obtained for each gene. Ten μl of the PCR reaction were resolved on a 1.5% 1 X TBE gel stained with ethidium bromide. Lane 1, ladder; lane 2, β-actin; lane 3, LDL receptor; lane 4, HMG-CoA reductase; lane 5, C7H.

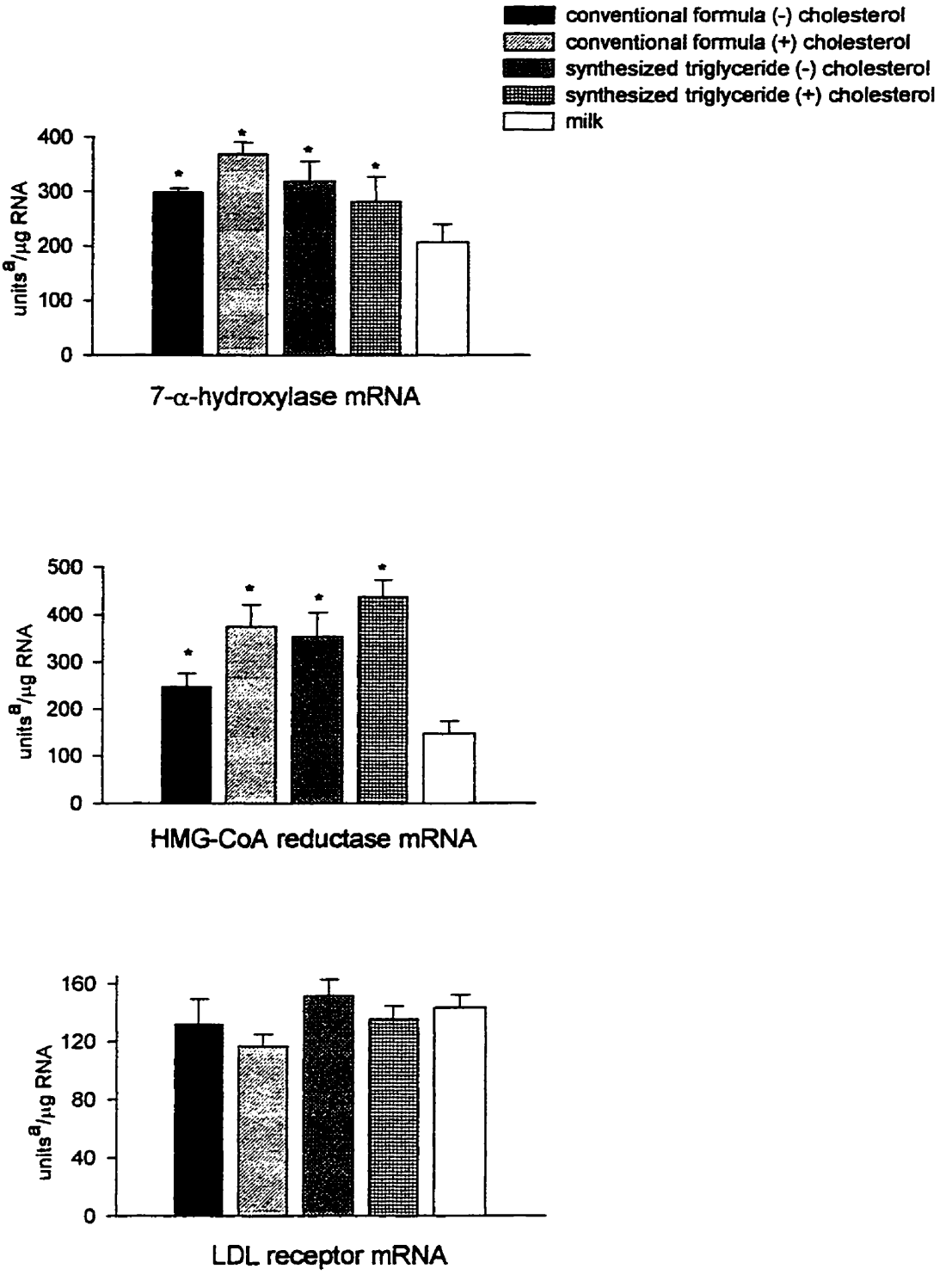


Figure 4.7. Hepatic 7-α-hydroxylase, HMG-CoA reductase and LDL receptor mRNA levels. Data presented as mean + SEM, n=6. [a]Densitometric units per mg RNA. *Values for piglets fed formula significantly different from piglets fed milk ($P<0.01$).

4.2. STUDY #1 – Part II.

In the first part of this study, the piglets fed formula, regardless of cholesterol content or the positional distribution of 16:0 in the formula triglyceride had significantly lower plasma and hepatic triglyceride concentrations than piglets fed milk. This prompted an investigation as to whether or not there are differences in hepatic fatty acid synthesis between milk and formula-fed piglets. The levels of hepatic FAS mRNA and activity and ACC mRNA were determined as indicators of fatty acid synthesis. The levels of mRNA were determined using RT-PCR, as in part I. The intra- and interassay variability are given in **Table 4.3.**. Examples showing the final products obtained for each RT-PCR reaction for FAS and ACC are illustrated in **Figure 4.8.**.

Table 4.3. RT-PCR Intra and Inter Assay Variability

	intra-assay variability		inter assay variability	
gene	mean[1] ± SEM	percentage[2]	mean[1] ± S.D.	percentage[3]
FAS	174.6 ± 7.4	4.2%	180.4 ± 12.5	6.9%
ACC	301.2 ± 20.6	6.8%	291.8 ± 22.4	7.7%

[1]Values given in absorbance units per μg of RNA. [2]Percentage = (SEM / mean)*100.
[3]Percentage = (S.D. / mean)*100.

The addition of cholesterol to the formulae led to significantly lower ($P<0.01$) levels of hepatic FAS mRNA, but had no significant effect on hepatic ACC mRNA than in piglets fed the formulae without cholesterol (**Figure 4.9, Appendix IV**). Piglets fed the formula with 16:0 at the *sn*-2 position of the formula triglyceride had significantly higher ($p<0.0001$) levels of hepatic ACC mRNA and lower ($P<0.03$) levels of hepatic FAS mRNA and activity than piglets fed the conventional formula with the majority of 16:0 at the *sn*-1,3 position. Piglets fed formula, regardless of the cholesterol content or the

positional distribution of 16:0 in the formula triglyceride had significantly higher levels of hepatic ACC mRNA than piglets fed milk. There were no significant differences in hepatic FAS mRNA and activity levels between piglets fed milk and piglets fed any of the formula.

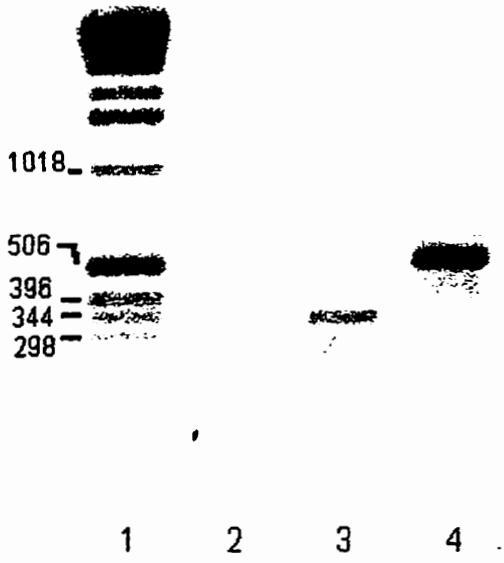


Figure 4.8. Representative photograph showing examples of the RT-PCR products obtained for each gene. Ten μl of the PCR reaction were resolved on a 1.5% 1 X TBE gel stained with ethidium bromide. Lane 1, ladder; lane 2, actin; lane 3, ACC; lane 4, FAS.

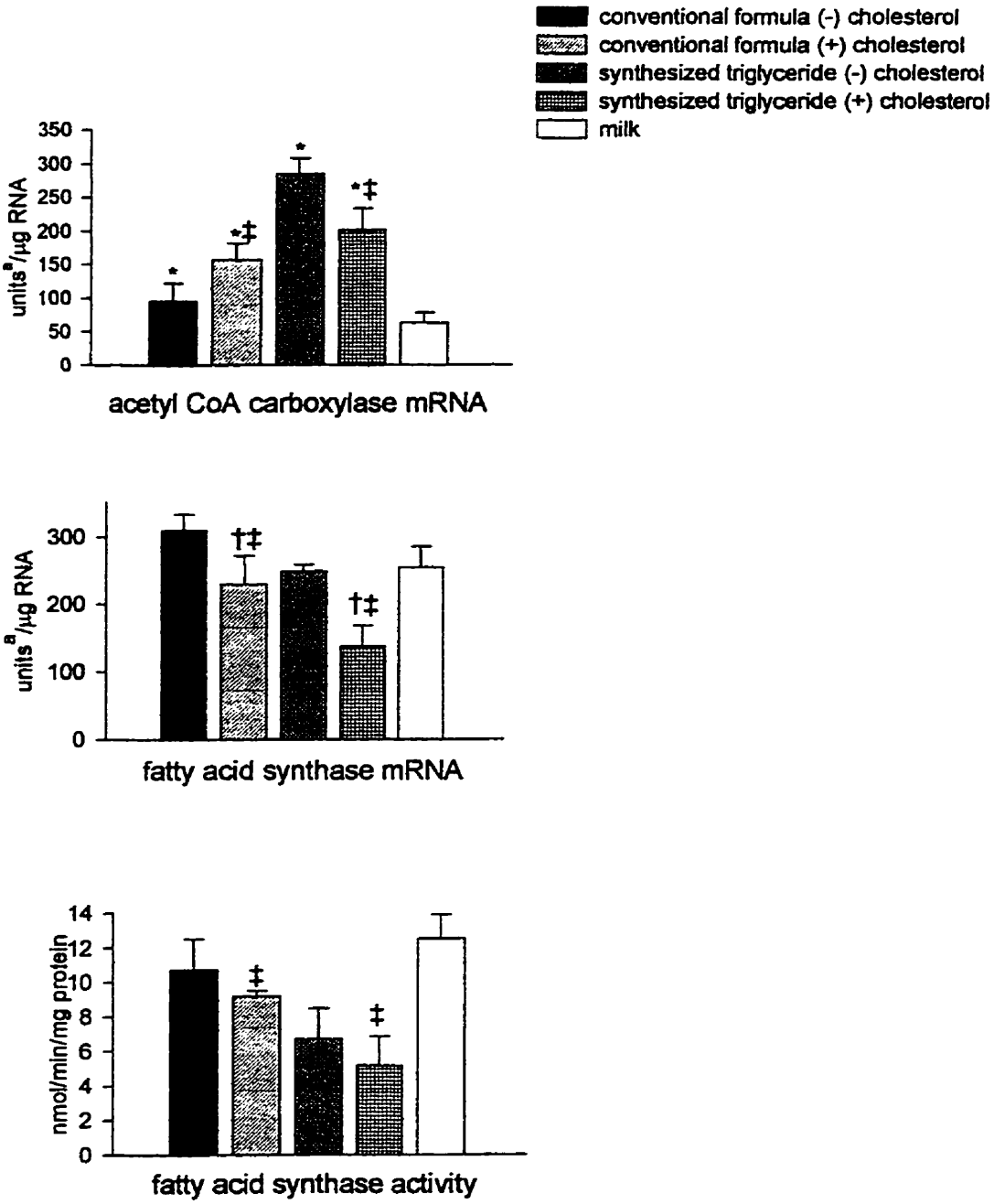


Figure 4.9. Hepatic acetyl CoA carboxylase mRNA and fatty acid synthase mRNA and activity. Data presented as mean + SEM, n=6 per diet group except for FAS activity, n=3. [a]Densitometric units per mg RNA. *Values for piglets fed formula significantly different from values for piglets fed milk ($P<0.05$). † Significant effect of adding cholesterol to the formula ($P<0.01$). ‡ Significant effect of the positional distribution of 16:0 in the formula triglyceride ($P<0.01$).

4. 3. STUDY #2

Study #2 determined the effect of including the long chain polyunsaturated fatty acids, 20:4(n-6) and 22:6(n-3), in formula in order to investigate the potential effect of these fatty acids, which are present in milk but absent from infant formula, on hepatic lipid metabolism. The conventional formula was supplemented with egg phospholipid (9.5g/L) to provide 0.8% 20:4(n-6) and 0.25% 22:6(n-3), compared to the levels of 0.7% and 0.1%, respectively, present in sow milk fatty acids (**Table 3.1**). Egg phospholipid was used as a source of 20:4(n-6) and 22:6(n-3) because it is currently being added to experimental infant formula and is undergoing clinical trials (Austead et al. 1996, Carlson et al. 1996). Originally the study was designed such that there were 6 piglets assigned to each of the formula groups, conventional and conventional-egg PL. However, three of the piglets assigned to the conventional formula group contracted severe diarrhea during the feeding period. At the end of the feeding period, the weights of these piglets were at least 1kg lower than the weights of healthy piglets fed the same diet. Thus, the 3 piglets that had suffered illness and growth failure were not included in the study. As a result, the statistical power of this study is low, and this must be considered when interpreting the results.

4. 3. 1. Body and liver weights, and plasma lipid concentrations.

No differences in body or liver weights were observed between piglets fed the conventional formula and piglets fed the conventional formula supplemented with egg phospholipid and piglets fed sow milk from birth for 15 days (**Figure 4.10, Appendix V**).

Piglets fed the conventional formula supplemented with egg phospholipid had significantly ($P<0.05$) higher plasma HDL cholesterol concentrations and higher plasma

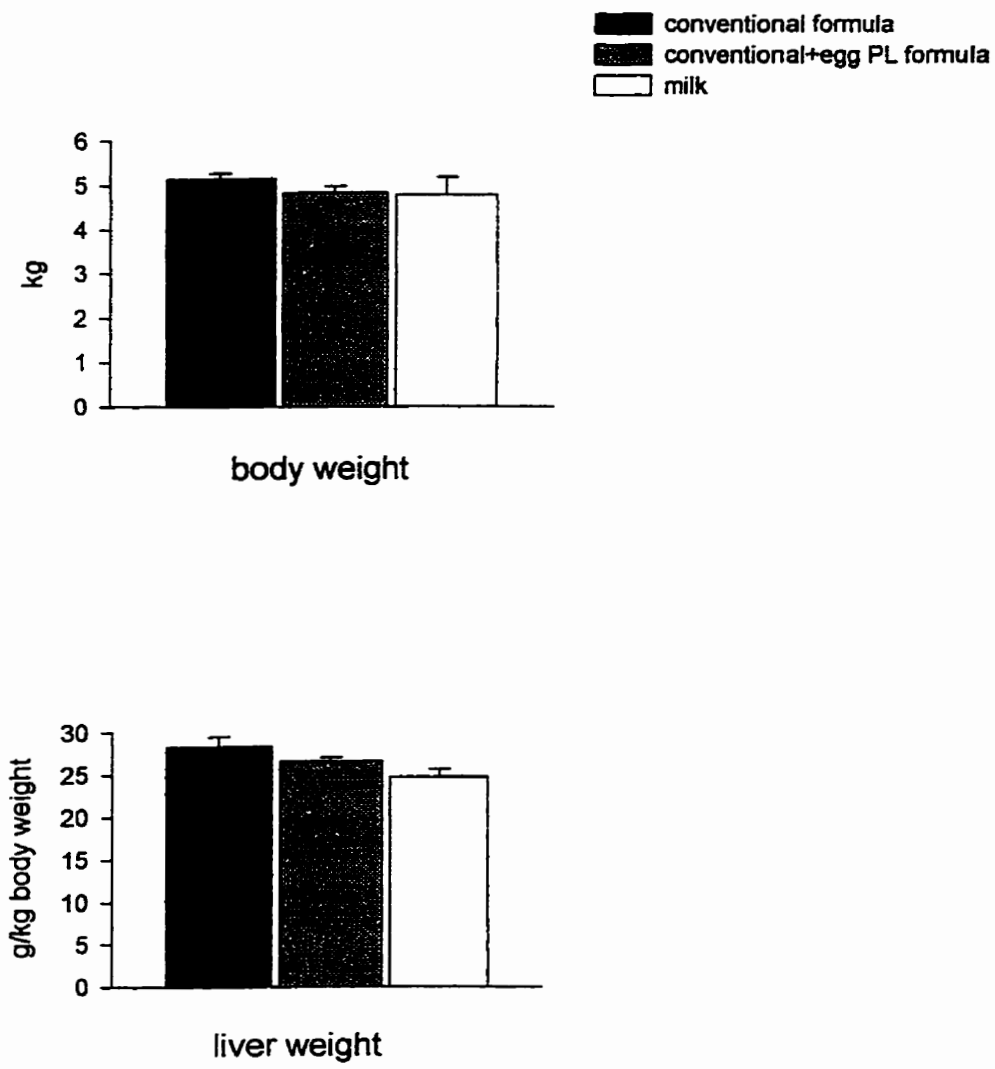


Figure 4.10. Liver & Body Weights. Data presented as mean + SEM, for conventional formula, n=3; conventional+egg PL formula, n=6; milk, n=7.

triglyceride concentrations than piglets fed milk (**Figure 4.11, Appendix VI**). The groups of piglets fed either the conventional formula or the conventional formula supplemented with egg phospholipid had significantly ($P<0.001$) lower levels of plasma total and HDL cholesterol concentrations than piglets fed milk (**Figure 4.11.**). The piglets fed either the conventional formula or the conventional formula supplemented with egg phospholipid also had lower apo B-containing lipoprotein cholesterol concentrations. However, no statistical analysis was conducted on the values obtained for apo B-containing lipoprotein cholesterol concentrations because it was calculated from the difference between the values for total cholesterol and the values for HDL cholesterol.

4. 3 .2. Hepatic lipid concentrations & fatty acids.

Supplementation of the conventional formula with egg phospholipid had no statistically significant effect on the concentration of hepatic phospholipid, triglyceride or cholesterol in the liver of formula-fed piglets (**Figure 4.12, Appendix VII**). The piglets fed the supplemented formula, however, had significantly higher concentrations of hepatic phospholipid, despite no differences in triglyceride or cholesterol concentrations, than piglets fed milk.

The piglets fed the formula supplemented with egg phospholipid had significantly higher levels of 20:4(n-6) and 22:6(n-3) in hepatic phospholipid and higher levels of 18:3(n-3) and 20:4(n-6) in hepatic triglyceride than piglets fed the conventional formula (**Figure 4.13, Appendix VII**). The piglets fed the supplemented formula also had significantly lower levels of 18:0 and 18:2(n-6) in hepatic phospholipid and lower levels of 18:1(n-9) in hepatic triglyceride than piglets fed the conventional formula. The groups of piglets fed

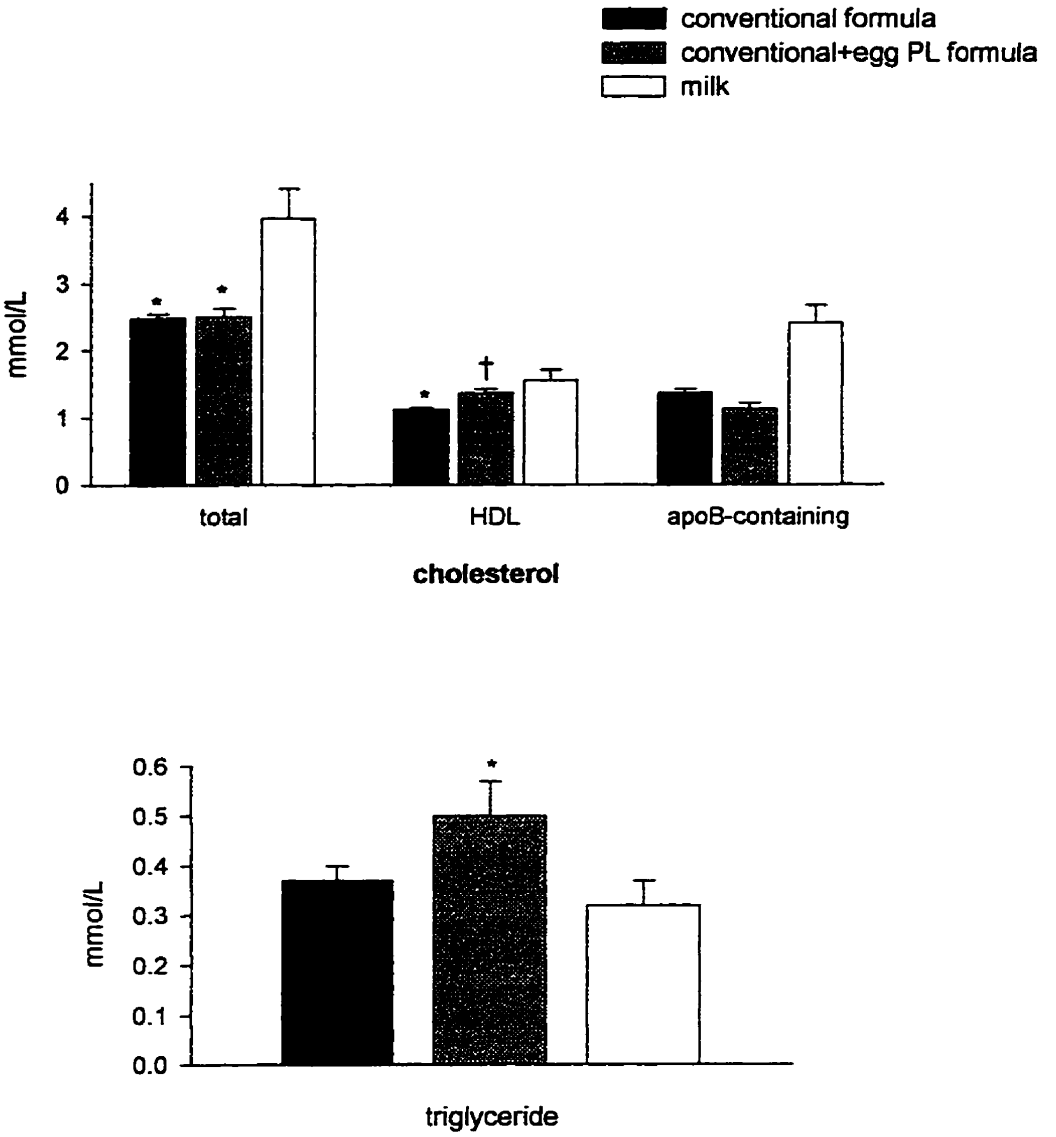


Figure 4.11. Plasma lipid concentrations.
Data presented as mean + SEM, conventional formula, n=3; conventional+egg PL formula, n=6; milk, n=7. *Significantly different from values for piglets fed milk ($P<0.05$). † Significant effect of supplementing the conventional formula with egg phospholipid ($P<0.01$).

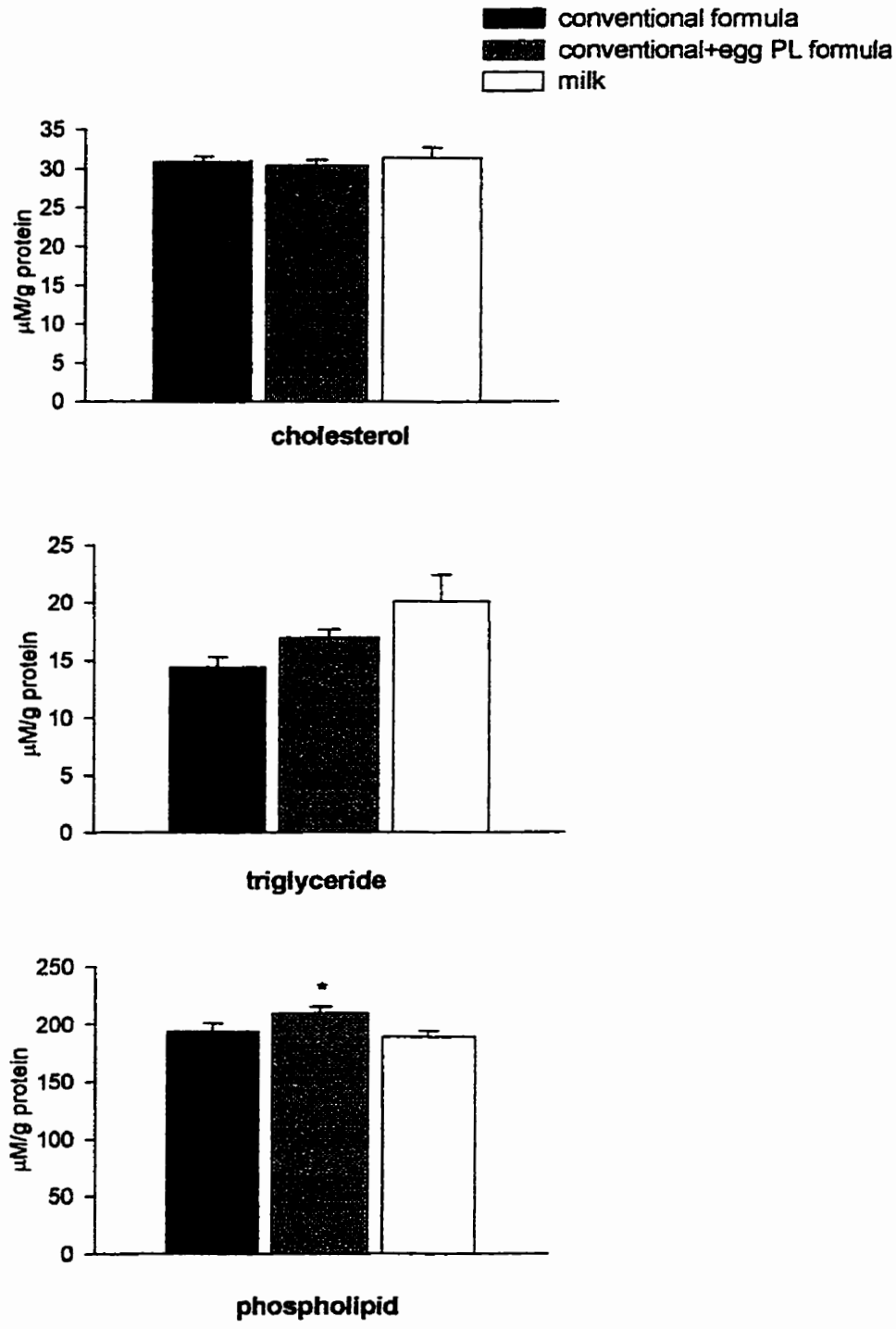


Figure 4.12. Hepatic lipid concentrations. Data presented as mean + SEM, n=3 conventional formula; n=6 conventional+egg PLformula; n=7 milk. *Significantly different from values for milk-fed piglets (P<0.05).

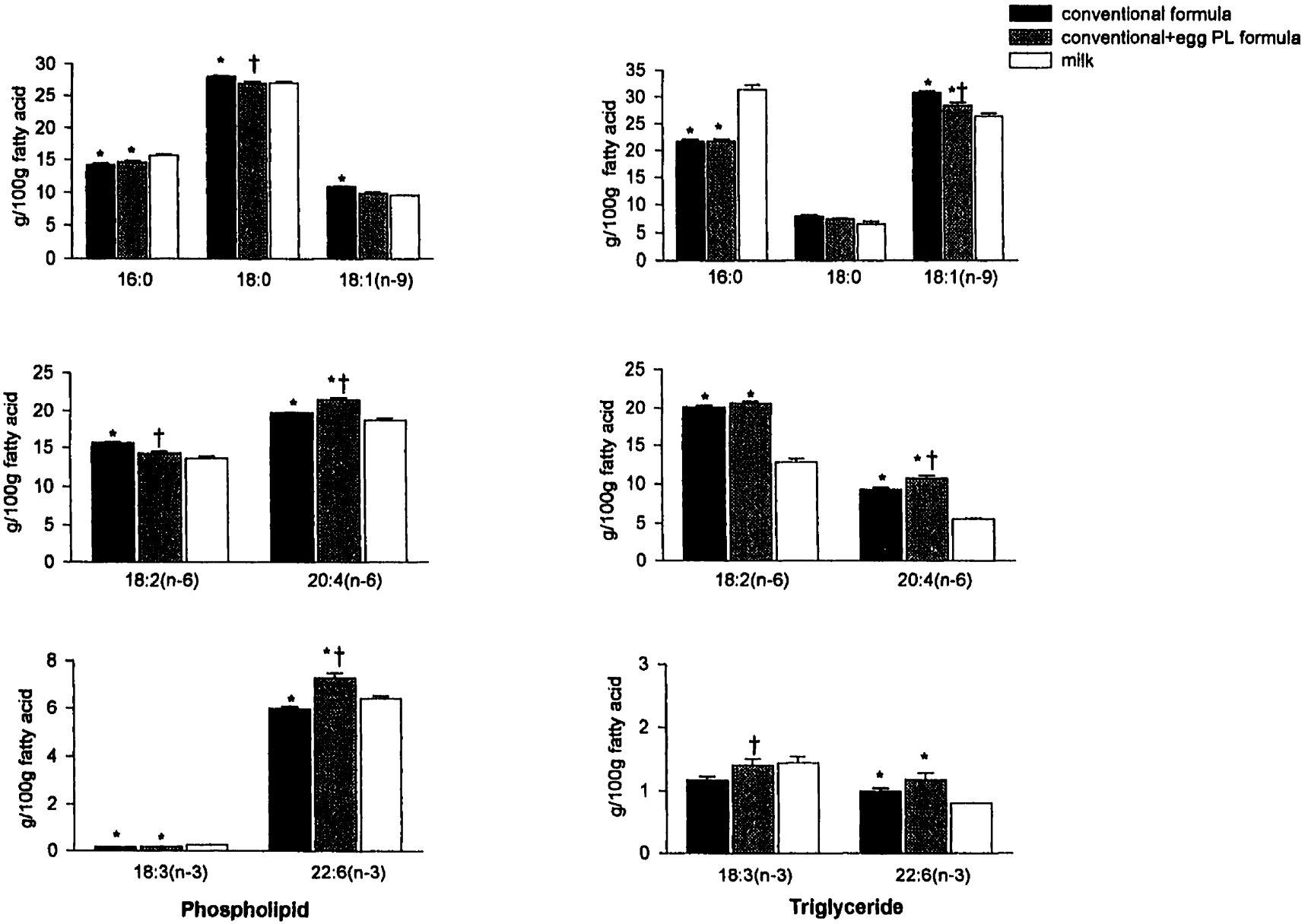


Figure 4.13. Major fatty acids in hepatic phospholipid and triglyceride. Data presented as mean + SEM, n=3 conventional formula; n=6 conventional+egg PL; n=7 milk. * Significantly different from values for milk-fed piglets ($P<0.05$). † Significant effect of supplementing the conventional formula with egg phospholipid ($P<0.05$).

the conventional formula or the conventional formula supplemented with egg phospholipid both had significantly lower levels of 16:0 in hepatic phospholipid and triglyceride, lower 18:3(n-3) in hepatic phospholipid, higher 20:4(n-6) and 22:6(n-3) in hepatic phospholipid and triglyceride, and higher 18:1(n-9) and 18:2(n-6) in hepatic triglyceride than piglets fed sow milk. In contrast to piglets fed the formula supplemented with egg phospholipid, piglets fed the conventional formula had significantly lower levels of 22:6(n-3) in hepatic phospholipid than piglets fed sow milk. Piglets fed the formula supplemented with egg phospholipid, but not piglets fed the conventional formula, on the other hand, had significantly higher 20:4(n-6) and 22:6(n-3) in hepatic phospholipid and triglyceride than the group of piglets fed sow milk.

4. 3. 3. Bile lipid concentrations and phospholipid fatty acids.

Supplementing the formula with egg phospholipid tended to increase concentrations of bile acids and phospholipids in bile of formula-fed piglets, however, this was not statistically significant (P=0.09 and P=0.10, respectively) (**Table 4.4.**). When compared to the reference group of piglets fed sow milk, piglets fed the conventional formula had significantly lower bile concentrations of bile acids and phospholipid. In contrast, there were no statistically significant differences in bile phospholipid, bile acid or cholesterol concentrations between piglets fed the formula supplemented with egg phospholipid and piglets fed sow milk.

Piglets fed the formula supplemented with egg phospholipid had significantly higher levels of 20:4(n-6) and 22:6(n-3), but lower levels of 18:1(n-9) in bile phospholipid than piglets fed the conventional formula (**Table 4.4**). The piglets fed formula, either with or without egg phospholipid had significantly lower levels of 16:0 and 18:3(n-3) in bile

phospholipid than the piglets fed milk. Piglets fed the conventional formula also had significantly higher levels of 18:1(n-9) and 18:2(n-6) than piglets fed milk. The piglets fed the formula supplemented with egg phospholipid, but not the piglets fed the conventional formula, had significantly higher levels of 20:4(n-6) and 22:6(n-3) in bile phospholipid than piglets fed milk.

Table 4.4. Bile lipid composition.

	Diet		
	conventional formula	conventional-egg PL formula	milk
bile acids (mmol/L)	45.1±3.7*	64.7±6.9	70.9±4.6
cholesterol (mmol/L)	2.7±0.4	2.9±0.3	3.2±0.3
phospholipid (mmol/L)	10.1±1.2*	16.0±2.2	16.4±1.6
major fatty acids (g/100g)			
16:0	27.6±0.3*	28.5±0.3*	29.9±0.4
18:0	13.9±0.5	13.6±0.4	12.7±0.4
18:1(n-9)	18.2±0.2*	15.9±0.4†	15.4±0.2
18:2(n-6)	23.9±0.3*	22.6±0.5	21.5±0.5
18:3(n-3)	0.3±0.0*	0.3±0.0*	0.5±0.1
20:4(n-6)	9.4±0.4	11.9±0.7*†	8.7±0.4
22:6(n-3)	2.8±0.1	3.7±0.2*†	3.0±0.1

Values presented as means ± SEM, n=3 standard formula; n=6, standard-AA+DHA formula; n=7, milk. *Significantly different from values for milk-fed piglets ($P<0.05$). †Significant effect of supplementing the formula with egg phospholipid 20:4(n-6) and 22:6(n-3), ($P<0.05$).

4. 3. 4. Hepatic LDL receptor, HMG-CoA reductase and C7H.

The integrity of the total RNA isolated from the piglet liver is shown in **Figure 4.14**. The same RT-PCR assay employed in study #1 was used to determine mRNA levels for HMG-CoA reductase, LDL receptor and C7H. Supplementing the formula with egg phospholipid had no apparent significant effect on the levels of LDL receptor mRNA or C7H mRNA (**Figure 4.15, Appendix VIII**) or HMG-CoA reductase mRNA (**Figure 4.16, Appendix VIII**). Levels of HMG-CoA reductase activity were higher although not significantly, in piglets fed the formula supplemented with egg phospholipid (**Figure 4.16, Appendix VIII**). However, the number of animals in each diet group was small, and therefore these findings are considered preliminary. No significant differences in the levels of C7H and LDL receptor mRNA were found between piglets fed either of the two formulae and the piglets fed milk (**Figure 4.15, Appendix VIII**).

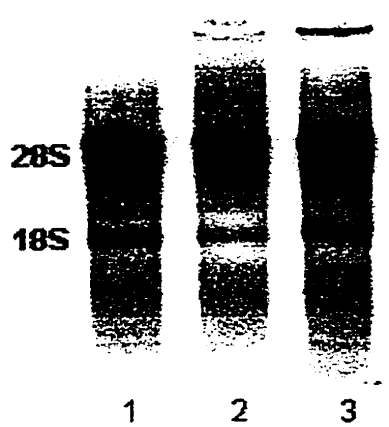


Figure 4.14. A representative photograph illustrating the integrity of total RNA isolated from liver. Five micrograms of total RNA was resolved on 1XTBE gel and stained with ethidium bromide. The ribosomal bands are marked. Lane 1, milk; lane 2, conventional formula; lane 3, conventional +egg PL formula.

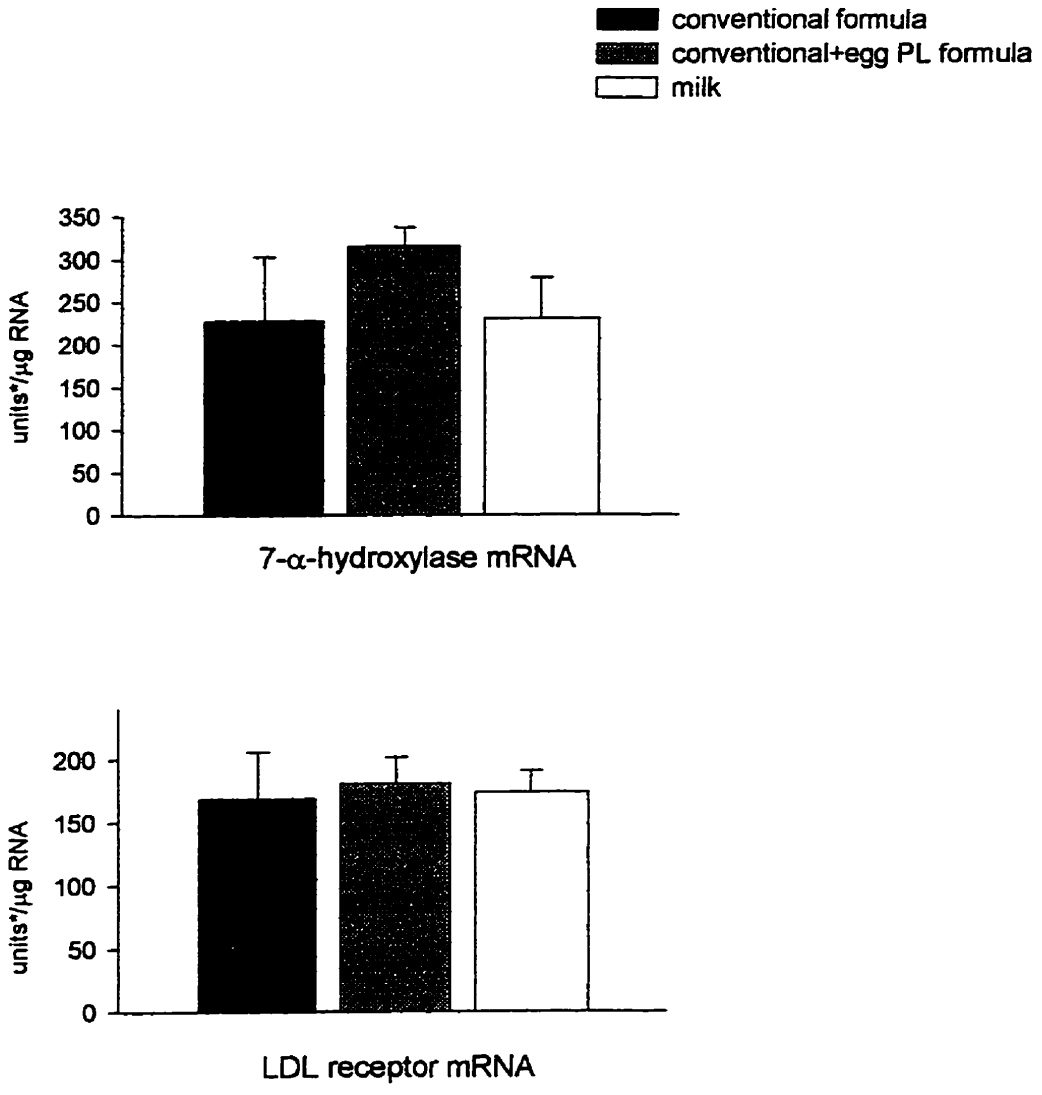


Figure 4.15. Hepatic 7-α-hydroxylase and LDL receptor mRNA. Data presented as mean + SEM, n=3 conventional formula; n=6 conventional+egg PL formula; n=7milk. *Densitometric units per µg RNA.

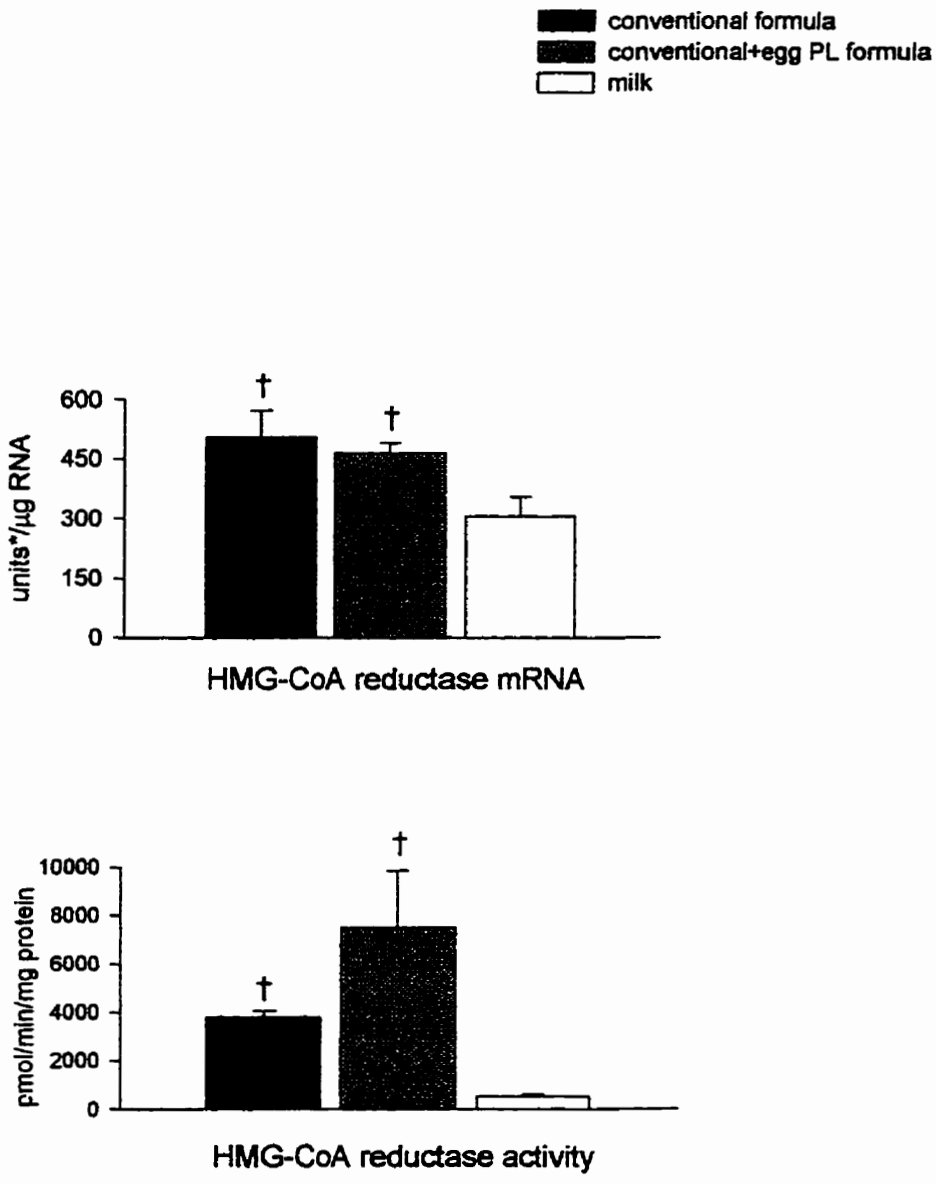


Figure 4.16. Hepatic HMG-CoA reductase mRNA and activity. Data presented as mean + SEM. HMG-CoA reductase mRNA, n=3 conventional formula; n=6 conventional+egg PL formula; n=7 milk. HMG-CoA reductase activity, n=3 conventional formula; n=2 conventional+egg PL formula; n=4 milk. *Densitometric units per µg RNA. † Significantly different from values for milk-fed piglets ($P<0.05$).

The piglets fed either the formula supplemented with egg phospholipid or the conventional formula had significantly higher levels of HMG-CoA reductase mRNA and activity than the piglets fed milk (**Figure 4.16, Appendix VIII**). This difference observed between formula and milk-fed piglets was greater for HMG-CoA reductase activity, about 10-fold, than the difference in the levels for HMG-CoA reductase mRNA, about 0.5-fold.

4. 3. 5. Hepatic ACC & FAS.

Piglets fed the formula supplemented with egg phospholipid had lower ($P = 0.08$), but not significantly, levels of hepatic ACC mRNA than the piglets fed the conventional formula and significantly lower levels of hepatic ACC mRNA than piglets fed milk (**Figure 4.17, Appendix VIII**). No significant differences in the levels of hepatic ACC mRNA were observed between the piglets fed the conventional formula and piglets fed milk. No statistically significant differences in the levels of FAS mRNA or FAS activity were found between the piglets fed the conventional or supplemented formulae and the piglets fed milk.

Regression analysis was used to investigate potential relations between the hepatic levels of fatty acids and ACC and FAS mRNA. Levels of individual fatty acids in hepatic phospholipid and triglyceride were plotted against the levels of hepatic ACC and FAS mRNA for individual piglets. A statistically significant inverse relation was present between the level of hepatic ACC mRNA and the percentage of 20:4(n-6) in hepatic triglyceride ($r=0.74$, $P<0.01$) and an inverse relation was found between the level of hepatic ACC mRNA and 22:6(n-3) in hepatic phospholipid (**Figure 4.18**). No statistically significant relations were found between the levels of 18:2(n-6) or 18:3(n-3) in hepatic phospholipid or triglyceride and the levels of hepatic ACC mRNA, or between 18:2(n-6), 18:3(n-3), 20:4(n-6) or 22:6(n-3) and FAS mRNA or activity levels.

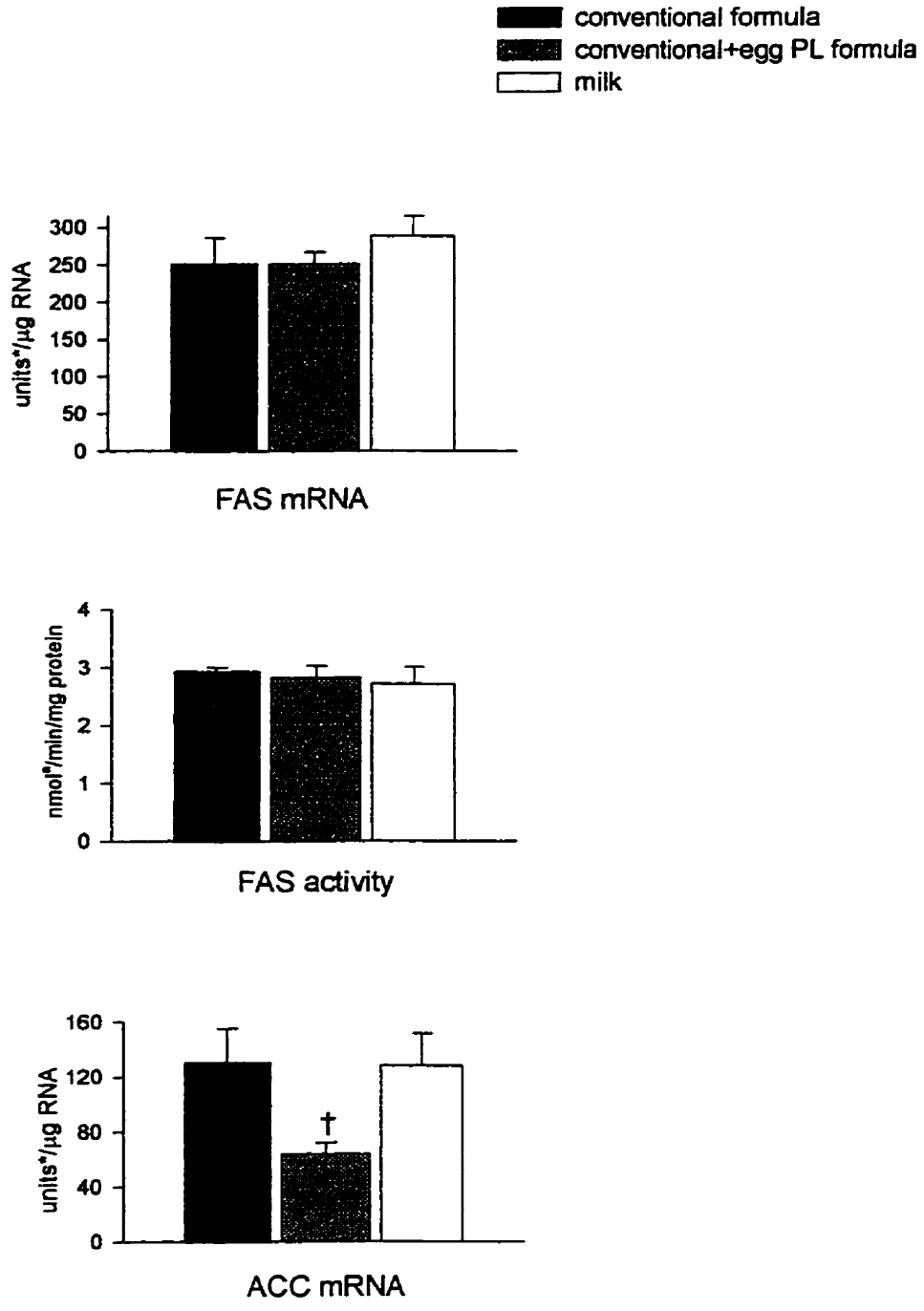


Figure 4.17. Hepatic ACC mRNA and FAS mRNA and activity. Data presented as mean + SEM, n=9 conventional formula; n=6 conventional+egg PL formula; n=7 milk. *Densitometric units per µg RNA. [a]nmol NADPH. †Significantly different from values for milk-fed piglets ($P<0.05$).

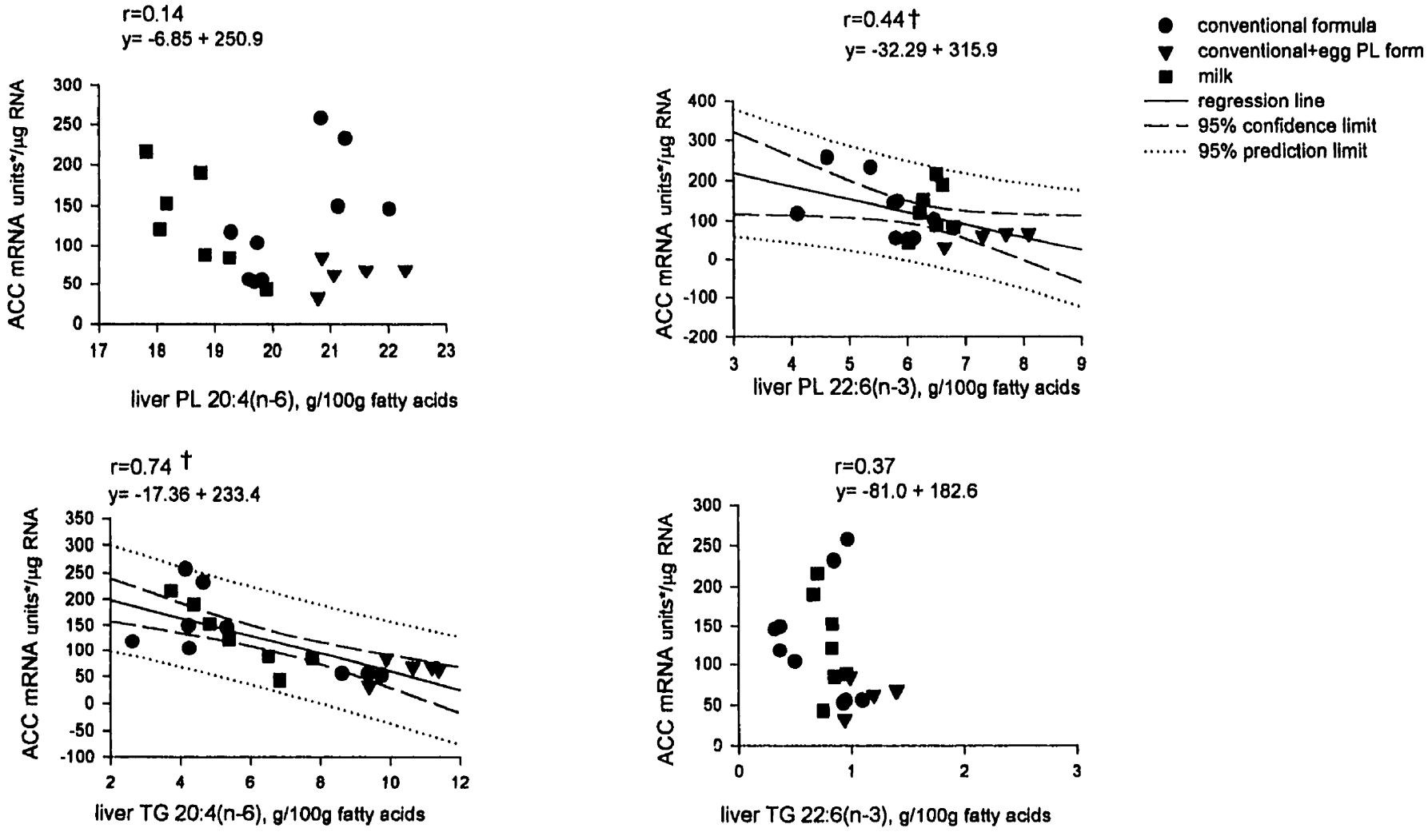


Figure 4.18. Regression analysis to explore the relation between the percentage of 20:4(n-6) and 22:6(n-3) in hepatic phospholipid and triglyceride with the levels of hepatic ACC mRNA. The results shown are 20:4(n-6) and 22:6(n-3) and ACC mRNA for individual piglets. *Densitometric units per mg RNA. The plots illustrate the 95% prediction limit (dotted line) and the 95% confidence interval (dashed line), r=correlation coefficient, † $P<0.05$.

5. DISCUSSION

5.1. STUDY #1

5.1.1. The effect of the cholesterol content of formula on plasma cholesterol.

It is unclear why the addition of cholesterol to the formula did not raise plasma cholesterol concentrations in the formula-fed piglets. Previous studies in piglets also found no effect of adding cholesterol to formula on plasma cholesterol concentrations, when the cholesterol supplied was primarily as cholesterol ester (Rioux and Innis 1993). This raised the possibility of poor availability for absorption, because the cholesterol in milk is primarily in the unesterified form. Consequently, in study #1, cholesterol was added to the formula predominantly in the unesterified form (0.52 mmol/L unesterified + 0.13mmol/L cholesteryl oleate), similar to the proportions in milk (Jensen 1989). One study has reported an increase in plasma cholesterol in infants fed formula supplemented with egg lipid containing cholesterol (Decsi et al. 1997). However, the higher plasma cholesterol in the infants fed the formula supplemented with egg lipid containing cholesterol was observed at five days of age, but not at any other ages through to 4 months of age. As well, in this study the egg lipid provided cholesterol as part of a biological membrane, that is, it was surrounded by a bilayer of phospholipid, more closely resembling the configuration of cholesterol in mammalian milk. Furthermore, recent studies in infants from birth to 6 months of age have reported that addition of cholesterol to formula does not increase serum cholesterol concentrations to the levels observed in breast-fed infants (Alasmi et al. 1996,Katoku et al. 1996). One possible explanation for the lack of an effect of the addition of cholesterol to formula on plasma cholesterol concentrations is relatively poor absorption of the cholesterol added to formula when compared to milk. Only a few

studies have addressed the possibility that differences in cholesterol absorption do exist between milk-fed and formula-fed infants. A study conducted in low-birth weight pre-term infants found higher fecal cholesterol excretion in infants fed formula supplemented with cholesterol than human infants fed milk containing a similar amount of cholesterol, or formula containing minimal amounts of cholesterol (Boehm et al. 1995). This suggests that cholesterol added to formula may not be absorbed as efficiently as cholesterol in milk, or that there may be differences in the enterohepatic circulation between milk-fed and formula-fed infants. However, studies with infant baboons found that the addition of cholesterol to formula resulted in a significant increase in plasma cholesterol concentrations (Mott et al. 1978).

The potential differences in cholesterol absorption from milk and formula could be a result of differences in the form of dietary cholesterol found naturally in milk and that added to formula. The cholesterol in human and sow milk is part of the milk-fat globule membrane, which also contains a bilayer of phospholipid. This phospholipid membrane may increase the solubility of cholesterol in the intestinal lumen and subsequently, absorption. In study #1, cholesterol was added to the formula as 80% unesterified with 20% cholesteryl oleate, with the formula fats (oils) emulsified with soy lecithin, suggesting that the cholesterol added was present in a micelle type form. Furthermore, the soy phospholipid used as an emulsifying agent in formula is phosphatidylcholine. In contrast, the milk-fat globule membrane phospholipid is comprised of phosphatidylethanolamine, phosphatidylcholine and sphingomyelin at about 37%, 30% and 26%, respectively (Jensen 1989). Whether the different species of phospholipid present in milk from that in formula facilitates the absorption of cholesterol from milk, leading to higher plasma cholesterol

concentrations in the milk-fed than formula-fed infant and animal is not known. Of possible relevance, studies conducted in CaCo-2 cells found that decreasing the sphingomyelin content of the cell membranes lowered the uptake of micelles containing cholesterol (Chen et al. 1992, Chen et al. 1993). Receiving dietary sphinogmyelin, as the milk-fed piglets did, could possibly influence cholesterol absorption through effects on enterocyte membrane composition. However, in contrast to this explanation, cholesterol absorption was not found to be different between infant baboons fed milk or baboons fed formula containing a similar amount of cholesterol (Mott et al. 1978).

Another explanation for the possible difference in cholesterol absorption from milk compared to formula may be diet-induced differences in the expression of proteins involved in cholesterol absorption. The exact mechanism by which cholesterol passes from the intestinal lumen into enterocytes is not known, but it is believed to occur as a result of passive diffusion down a concentration gradient and/or via a protein mediator (reviewed by Wilson and Rudel 1994). Three proteins have been proposed to have a role in the absorption of cholesterol, cholesterol ester hydrolase (Gallo et al. 1984,Howles et al. 1996,Lopez-Candales et al. 1993,Shamir et al. 1995), phospholipase A_2 (Mackay et al. 1997) and a brush border membrane lipid exchange protein (Lipka et al. 1995). Evidence for the role of cholesterol ester hydrolase in the absorption of cholesterol esters has been well demonstrated. It has been hypothesized that as a result of the action of cholesterol ester hydrolase on cholesterol esters, the concentration of unesterified cholesterol increases in the intestinal lumen creating a concentration gradient promoting the flux of unesterified cholesterol across the enterocyte cell membrane (Shamir et al. 1995). Cholesterol ester hydrolase has also been shown to enhance the movement of

unesterified cholesterol into cells and to promote the formation of intracellular cholesterol ester (Lopez-Candales et al. 1993). A positive correlation between the level of activity of cholesterol esterase and the amount of [^{3}H]-cholesteryl oleate absorbed has been found in rabbits (Lopez-Candales et al. 1996). As well, higher absorption of cholesterol esters has been reported for wild type mice than in homozygous cholesterol esterase knockout mice (Howles et al. 1996). Human milk cholesterol is primarily in the unesterified form. Whether or not the absorption of unesterified cholesterol is dependent on cholesterol esterase activity is controversial. Evidence disputing the role for cholesterol esterase in cholesterol absorption has come from the lack of a difference in the absorption of unesterified cholesterol between the homozygous cholesterol esterase knockout mice and the wild type mice (Howles et al. 1996). If the absorption of unesterified cholesterol is dependent on cholesterol esterase, however, there could be differences in the amount and activity of this enzyme secreted into the intestinal lumen as a result of milk compared to formula feeding.

There is also a mammary form of cholesterol esterase, referred to more commonly as bile salt-stimulated lipase, which is present in the milk of several species and which constitutes 0.5-1.0% of the total milk protein (Wang et al. 1993). *In vitro* studies have shown that this enzyme is capable of hydrolyzing a variety of esters, including triglycerides and cholesterol esters (Bernback et al. 1990,Hernell et al. 1994). Cloning of the genes for the pancreatic cholesterol esterase and mammary bile salt-stimulated lipase has shown that these proteins are products of the same gene (Nilsson et al. 1990). However, whether or not the bile salt-stimulated lipase found in milk can remain active in the intestinal lumen and function in the hydrolysis of milk lipids is as yet unclear. More

relevant to the studies in this thesis, it is not known whether or not sow milk contains bile salt-stimulated lipase (Hernell et al. 1994). Possibly, if bile salt-stimulated lipase is present in sow milk and is active in the intestinal lumen, this would result in enhanced absorption of cholesterol from milk than from formula and would explain the lack of an increase in plasma cholesterol concentrations in piglets fed formula with cholesterol added.

Another protein, which has been proposed to have a role in the absorption of unesterified cholesterol is phospholipase A_2. Studies in CaCo-2 cells have found that phospholipase A_2 hydrolysis of phospholipid, present in micelles containing egg phosphatidylcholine and unesterified cholesterol, was necessary for the uptake of unesterified cholesterol by CaCo-2 cells (Mackay et al. 1997). There is also evidence of a lipid-binding protein present in the brush border membrane of enterocytes which may also play a role in the absorption of dietary cholesterol (Boffelli et al. 1997, Lipka et al. 1995). Possibly, some constituent(s) of milk which are absent from formula may influence the expression of cholesterol ester hydrolase, phospholipase A_2 or brush border membrane lipid binding protein in a manner promoting higher absorption of cholesterol from milk than from formula. As well, the physical nature of cholesterol in milk, within the milk-fat globule membrane, may facilitate the interaction of proteins involved in cholesterol absorption with milk cholesterol and result in enhanced cholesterol absorption from milk than from formula.

5.1.2. Serum hormone and unesterified fatty acid concentrations in formula-fed and milk-fed piglets.

The levels of serum total T3 and T4 in the milk-fed and formula-fed piglets were similar to levels reported previously for piglets at 14 days of age (Harrison et al. 1996). However, the levels of serum free T3 were 75% lower than previously reported for 14 day

old piglets. Neither the addition of cholesterol nor the positional distribution of fatty acids in triglycerides in formula had any significant effect on serum free T3, or total T3 or total T4. Serum free T4, however, was significantly higher in the formula-fed piglets than in the milk-fed piglets. Studies in adult animals have provided evidence that feeding state may influence serum thyroid hormone concentrations (Dauncey et al. 1993,Takeuchi et al. 1995). Serum levels of total T3 and T4 have been shown to rise immediately following a meal in pigs (Dauncey et al. 1993). As well, studies with rats have reported lower serum T3 levels in rats fed a diet containing lard than in rats fed diets containing high oleic safflower oil, safflower oil or linseed oil (Takeuchi et al. 1995). Thus, the information available suggests that both feeding state and dietary lipid composition can have an effect on serum levels of T3 and T4. In the study here with piglets, the time post-feed that the blood was collected may have differed and differences in some fatty acids, although not in total fat, or saturated/unsaturated fatty acid balance, were present between the milk and formula. The time post-feed and amount of the last feed prior to blood samples from the milk-fed piglets was not rigorously controlled. This was not possible because they were nursed by sows. The sow-fed piglets were taken from the sow approximately 3 hr. prior to blood sampling, as for the formula-fed piglets, but it is not known if or how much they ate at 6:00-7:00 am, the time when the formula-fed piglets were fed. The time and volume of formula (60ml) of the last feed given to the formula-fed piglets, on the other hand, was rigorously controlled. The finding of similar serum insulin concentrations between the milk-fed and formula-fed piglets, however, suggests that there were no appreciable differences in the time post-feed of blood sampling between the milk-fed and formula-fed piglets. Thus, it is unlikely that the differences in serum free T4 between the milk-fed and formula-

fed piglets was a result of differences in feeding state or differences in the fat composition between milk and formula.

An alternative explanation for the difference in serum free T4 levels between milk and formula-fed piglets is the presence of thyroid hormones in milk (Grosvenor 1993, Koldovsky and Thornburg 1987) but not in formula. Definitive data to show whether or not the thyroid hormones in milk are absorbed and are active systemically are not available. Studies in infant baboons have reported significantly higher serum total T3 and free T3 levels in baboons fed formula than in baboons fed milk (Lewis et al. 1993). Furthermore, studies in human premature infants have found higher total T4 levels in infants fed milk than in formula-fed infants (Oberkotter et al. 1985). Thus, the available information suggests that the thyroid hormones in milk are absorbed and influence serum thyroid hormone levels by down-regulating the endogenous production of thyroid hormones. This seems like a more probable explanation for the higher serum free T4 levels in the formula-fed than milk-fed piglets.

There were no differences in serum insulin concentrations between the milk-fed and formula-fed piglets. Of note, the serum insulin concentrations found in both the milk-fed and formula-fed piglets are similar to values of (mean) 45.7 pmol/L (Zijlstra et al. 1996) and 48 pmol/L (McCracken et al. 1995) reported by others for milk-fed piglets at 18 days of age. Thus, the available information on serum insulin levels and the findings of study #1 here, that serum insulin concentrations are not different between milk-fed and formula-fed piglets suggests that it is unlikely that there were differences in the feeding state of the animals at the time of blood collection. Again, the suggestion that there were no differences in feeding state at the time of blood collection supports the hypothesis that the

thyroid hormones in milk are responsible for the differences in serum free T4 concentrations observed between the milk-fed and formula-fed piglets. Significantly higher levels of glucagon in serum of piglets fed milk than in piglets fed formula were found and this was not influenced by the cholesterol content or the positional distribution of fatty acids in triglycerides in the formula. The serum glucagon concentrations, however, in both the milk-fed and formula-fed piglets were higher than previously reported values of (mean) 35.5 pmol/L in 18 day-old formula-fed piglets fasted for 12 hrs. (Zijlstra et al.1996), and 44.5pmol/L in 18 day-old milk-fed ad libitum piglets (McCracken et al. 1995). The reason for the approximately 10-fold higher values (419pmol/L) found in the piglets in the study here is not known. The studies of McCracken et al. (1995) and Zijlstra et al. (1996) with 18 day-old milk-fed piglets used a radio immunoassay, similar to that used in the study here, to determine serum values of insulin and glucagon. Again, as has been suggested as a possible explanation for the differences in serum free T4 concentrations, the higher serum glucagon concentrations in the milk-fed than formula-fed piglets could be explained by differences in the feeding state of the piglets at the time of the blood collection. The absence of a difference in serum insulin concentrations between the milk-fed and formula-fed piglets, however, suggest that there are no differences in feeding state between the milk-fed and formula-fed piglets. Possibly, the differences in serum glucagon concentrations between the milk-fed and formula-fed piglets are a consequence of the hormones present in milk, similar to what has been suggested to explain the differences in serum free T4 concentrations between the milk-fed and formula-fed piglets.

The reason for the significantly higher plasma unesterified fatty acid concentrations in the piglets fed milk than in the piglets fed formula is not known. During fasting,

glucagon stimulates hydrolysis of adipose tissue triglyceride, through the actions of hormone-sensitive lipase, releasing unesterified fatty acids into the blood providing an energy source for other tissues. Therefore, the findings of higher plasma unesterified fatty acids in milk-fed than formula-fed piglets are consistent with the higher concentrations of serum glucagon in the piglets fed milk than in those fed formula. Alternatively, differences in the regulation of lipoprotein lipase hydrolysis of lipoprotein triglyceride could also be involved in the differences in unesterified fatty acid concentrations between the milk-fed and formula-fed piglets. Possibly, higher lipoprotein lipase activity at peripheral tissues in the milk-fed piglets could result in greater amounts of triglyceride hydrolysis, with subsequent release of some unesterified fatty acids to plasma, or similar hydrolysis with lower tissue uptake of unesterified fatty acids by the peripheral tissues. It seems unlikely however, because of the higher glucagon concentrations, that the milk-fed piglets would have higher levels of lipoprotein lipase activity than the animals fed formula. There is still the possibility, however, that there are differences in the uptake of unesterified fatty acids at either peripheral tissues or by the liver. However, the most probable explanation, again, is higher levels of hormone-sensitive lipase activity as a result of higher levels of glucagon in the piglets fed milk than in those fed formula, therefore, explaining the higher levels of circulating unesterified fatty acids.

5.1.3. Effect of the cholesterol content of formula and the positional distribution of fatty acids in the formula triglyceride on hepatic LDL receptor mRNA.

Despite the presence of large differences in plasma cholesterol concentrations, no differences were found in the levels of hepatic LDL receptor mRNA, as measured using a RT-PCR assay, between the piglets fed milk and the piglets fed formula. Significantly

higher levels of plasma cholesterol were found in the milk-fed than formula-fed piglets, regardless of the cholesterol content of the formula or the positional distribution of fatty acids in the formula triglyceride. About 70% of plasma LDL clearance occurs via LDL receptors, with the liver responsible for about 80-90% of this clearance (Dietschy and Spady 1985, Spady et al. 1985). Previous studies in adult animals have found decreased levels of hepatic LDL receptor mRNA and protein and increased plasma cholesterol concentrations (Horton et al 1993, Mustad et al. 1996, Rudling 1992) as a result of feeding diets containing cholesterol and saturated fatty acids. These previous findings had suggested that differences in the levels of hepatic LDL receptor mRNA could be involved in the differences in plasma cholesterol concentrations between milk-fed and formula-fed piglets.

The regulation of LDL receptor gene expression by sterols is thought to occur at a transcriptional level, whereby a decrease in cellular cholesterol concentrations results in increased transcription (Sudhof et al. 1987). Large differences in hepatic cholesterol concentrations, however, were not found between the piglets fed milk and the piglets fed formula, with or without cholesterol, even though the plasma cholesterol concentrations were 28-70% higher in the milk-fed piglets than in the piglets fed formula. Possibly, the absence of a sustained and different effect of the milk and formula diets on liver cholesterol content may explain the lack of an effect on hepatic levels of LDL receptor mRNA.

There is also the possibility that differences in the expression of hepatic LDL receptor are present between the milk-fed and formula-fed piglets, but that the differences are not evident in measures of changes in hepatic LDL receptor mRNA. As already

mentioned, previous studies have found decreased levels of hepatic LDL receptor mRNA and increased plasma cholesterol concentrations in adult hamsters, pigs and mice as a result of feeding diets containing cholesterol and either hydrogenated coconut oil (14:0), palm oil (16:0) and cocoa butter (18:0) as the source of saturated fatty acids, respectively, (Horton et al 1993, Mustad et al. 1996, Rudling 1992) compared to animals fed a diet containing little fat, 4%, or unsaturated fatty acids. Studies in monkeys have found that diets containing cholesterol and either 16:0 or 12:0+14:0, as the source of fatty acids, rather than monkeys fed a diet containing cholesterol and polyunsaturated fatty acids had decreased hepatic LDL receptor clearance (activity) of LDL (Stucchi et al. 1995). Similarily, studies in adult hamsters found diets containing cholesterol and 14:0, as the source of saturated fatty acids, decreased hepatic LDL receptor activity (Daumerie et al. 1992). The studies in monkeys (Stucchi et al. 1995) and hamsters (Daumerie et al. 1992), however, only measured the effect of diet on hepatic LDL receptor clearance (activity) and did not measure levels of hepatic LDL receptor mRNA. It is not known whether or not diet induced changes in hepatic LDL receptor activity are accompanied by changes in LDL receptor mRNA. One study however, has reported decreased levels of hepatic LDL receptor mRNA, protein and activity as a result of feeding a diet high in hydrogenated coconut oil (14:0) and cholesterol rather than diets high in polyunsaturated fatty acids and cholesterol in hamsters (Horton et al. 1993). Alternatively, *in vitro* studies with HepG2 cells found that 16:0 decreased LDL receptor activity but did not change levels of LDL receptor protein, mRNA, or rates of transcription of the LDL receptor gene (Srivastava et al. 1995). Both the milk and formula diets provided the same percentage of energy from fat, 50%, a similar percentage of 16:0 but differed only in cholesterol content.

Most of the previously reported studies (Daumerie et al. 1992, Horton et al. 1993, Mustad et al. 1996, Rudling et al. 1992, Stucchi et al. 1995) have compared the effects of diets high in saturated fatty acids to animals fed either a diet high in unsaturated fatty acids or to animals fed a control diet containing little fat, 4%, on the expression of the LDL receptor. Possibly, the absence of a difference in the hepatic levels of LDL receptor mRNA between the milk-fed and formula-fed piglets was found because there were no differences in the levels of saturated fatty acids between milk and formula, despite a difference in cholesterol content. The difference in the cholesterol content between milk and formula, in the absence of a difference in the saturated fatty acid content, may not be large enough to induce a change in the expression of the LDL receptor.

Another potential point to consider is that hepatic LDL receptor clearance of apo B-containing lipoproteins could be low during the suckling period relative to clearance levels observed in adult animals. Previously reported studies in adult animals fed diets containing 30-40% energy from fat and 14-20% 16:0 found lower levels of LDL receptor protein and activity compared to animals fed diets containing the same amount of fat but with little 16:0 (1-9%) and high levels of polyunsaturated fatty acids (Mustard et al. 1996, Stucchi et al. 1995). The findings of the previously reported studies therefore suggest that low clearance during the suckling period is quite probable and could be occurring in response to the 50% fat diet that contains 25% 16:0, characteristic of either milk or formula. Therefore, low hepatic expression of the LDL receptor gene during the suckling period in both the milk-fed and formula-fed animal could limit any further diet-induced negative feedback on hepatic LDL receptor expression as a result of cholesterol feeding, thus explaining the results of study #1. If this is the case, it would also explain the

postnatal increase in plasma cholesterol concentrations in response to both the high fat-milk and formula diets observed in human infants (Kallio et al. 1997, Lane and McConathy 1986, Lourdes et al. 1994, Mise et al. 1995, Van Biervleit et al. 1986, Wong et al. 1993). Further support for the absence of any significant diet-induced difference in LDL receptor as a result of feeding formula rather than milk can be drawn from the findings of previous studies of Rioux and Innis (1993). These studies found no differences in hepatic LDL receptor protein mass when measured using a ligand blotting technique between formula-fed and milk-fed piglets (Rioux and Innis 1993). There remains, however, the possibility that differences in LDL receptor activity may occur in the infant as a consequence of diet-induced regulation of LDL receptor gene expression at a post-translational level. It is probably more likely, however, that LDL receptor clearance of circulating apo B-containing lipoproteins is low during the suckling period. This interpretation, therefore, rejects the hypothesis that differences in the expression of LDL receptor are present between milk-fed and formula-fed piglets, although the possibility of differences not detected through measures of levels of hepatic LDL receptor mRNA only are recognized.

The findings that the addition of cholesterol to the formula had no effect on the levels of hepatic LDL receptor mRNA are in contrast to the expected effects of increased dietary cholesterol intake. This result, however, fits well with the findings that the addition of cholesterol to the formula had little effect on the concentration of cholesterol in plasma. The addition of cholesterol to the formula, however, did increase hepatic cholesterol concentrations in the formula-fed piglets similar to levels observed in the milk-fed piglets. As discussed previously, changes in LDL receptor transcription occur in response to changes in endoplasmic reticular membrane cholesterol concentrations (Briggs et al.

1993, Dawson et al. 1988, Smith et al. 1990, Sudhof et al. 1987, Wang et al. 1994). This study did not include measures of cholesterol concentrations in different hepatic pools such as the endoplasmic reticular membranes. The absence of a difference in hepatic LDL receptor mRNA among the formula-fed and milk-fed piglets suggests that neither formula compared to milk feeding nor the addition of cholesterol to the formula resulted in changes in endoplasmic reticular membrane free cholesterol levels of any significant magnitude to alter LDL receptor mRNA levels. This interpretation again, however, may be valid only if LDL receptor expression is sufficiently high during the suckling period that such an effect can be manifested.

5.1.4. Effect of the cholesterol content of formula and the positional distribution of fatty acids in the formula triglyceride on hepatic HMG-CoA reductase mRNA.

The results of this study clearly show that hepatic HMG-CoA reductase mRNA levels were higher in formula-fed piglets, regardless of the formula cholesterol content or the positional distribution of fatty acids in the formula triglyceride, than in piglets fed milk. This result is consistent with those of previous studies which show higher hepatic HMG-CoA reductase activity in piglets fed formula than in piglets fed milk (Jones et al. 1990,Rioux and Innis 1993). Also consistent with the results here, studies in human infants have found higher cholesterol fractional synthetic rates in formula-fed than in breast-fed infants (Lourdes et al. 1994).

Previous studies have reported that diets either high in cholesterol, high in saturated fat or containing both cholesterol and saturated fat showed decreased levels of hepatic HMG-CoA reductase activity and mRNA when compared to diets containing no cholesterol or saturated fat (Hackman et al. 1996, Jackson et al. 1990, Rudling 1992,

Spady and Cuthbert 1992, Xu et al. 1995). Based on this information, it is reasonable to hypothesize that piglets fed milk or formula with cholesterol would have lower levels of hepatic HMG-CoA reductase mRNA than piglets fed formula not containing cholesterol. The results, however, showed lower levels of hepatic HMG-CoA reductase mRNA in piglets fed milk and no differences between piglets fed formula with and without cholesterol, consistent with published information on HMG-CoA reductase activity and cholesterol synthesis in piglets and infants (Jones et al. 1990, Lourdes et al. 1993, Rioux and Innis 1993). As already discussed in section 5.1.1, the addition of cholesterol to the formula did not raise plasma cholesterol concentrations in the formula-fed piglets. One possible interpretation is low absorption of the cholesterol added to formula. Clearly, the lack of a difference in the levels of hepatic HMG-CoA reductase mRNA between the piglets fed formula with and without cholesterol could also be explained by low absorption of the cholesterol added to the formula.

As with LDL receptor gene expression, regulation of HMG-CoA reductase expression is thought to be sensitive and tightly regulated by changes in the endoplasmic reticular membrane cholesterol concentrations (Chin et al. 1982,Clarke et al. 1985,Liscum et al. 1983,Nakanishi et al. 1988). Large differences in hepatic total cholesterol concentrations were not found between piglets fed milk and those fed formula, even though differences in the levels of hepatic HMG-CoA reductase mRNA were present. Previous studies have found that both HMG-CoA reductase and C7H activity increase when microsomal cholesterol concentrations are depleted (Straka et al. 1990, Davis and Poznansky 1987). This study did not include measures of cholesterol concentrations in different hepatic pools, such as the endoplasmic reticular membranes.

Therefore, it is possible that differences in levels of hepatic HMG-CoA reductase mRNA between the milk and formula-fed animals might involve differences in endoplasmic reticular membrane cholesterol concentrations. Clearly, the higher plasma cholesterol in the milk-fed piglets than piglets fed formula with or without cholesterol suggests higher absorption of cholesterol in the milk-fed animal. Possibly, the lower levels of hepatic HMG-CoA reductase mRNA in the milk-fed than formula-fed piglets are a result of the higher absorption of cholesterol and subsequent higher endoplasmic reticular membrane cholesterol concentrations.

There is still the possibility that some component, other than cholesterol or the positional distribution of fatty acids in the triglyceride, present in milk but absent from formula, influences both plasma cholesterol and the levels of hepatic HMG-CoA reductase mRNA in the milk-fed animal. Such factors could include nutritional and non-nutritional components potentially involved in the poor absorption (if present) of the cholesterol added to formula when compared to the absorption of the cholesterol in milk. As previously discussed in section 5.1.2, it is not known whether or not the hormones present in milk are active in the intestinal lumen of the milk-fed animal.

Previous studies in adult animals have shown that high saturated fat and cholesterol intakes decrease both cholesterol synthesis and LDL receptor clearance of plasma LDL in a coordinated fashion when compared to animals fed diets either high in unsaturated fatty acids or not containing cholesterol (Horton et al. 1993, Kurishima et al. 1995, Spady and Cuthbert 1992, Stucchi et al. 1995). In study #1, no differences in hepatic levels of LDL receptor mRNA were found despite differences in hepatic levels of HMG-CoA reductase mRNA in formula-fed than milk-fed piglets. Another study has also

observed discordant regulation of LDL receptor and HMG-CoA reductase genes in the liver, specifically, decreased cholesterol synthesis, but no change in LDL receptor activity, in rats fed a diet supplemented with cholesterol and saturated fat rather than a diet not containing fat (Bertolotti et al. 1995). The study designed employed by Bertolotti et al. (1995) differs from the previously mentioned studies (Horton et al. 1993, Kurishima et al. 1995, Spady and Cuthbert 1992, Stucchi et al. 1995) demonstrating co-ordinate regulation of LDL receptor clearance of plasma LDL and cholesterol synthesis in that comparisons were made in the same rats. The previously mentioned studies (Horton et al. 1993, Kurishima et al. 1995, Spady and Cuthbert 1992, Stucchi et al. 1995) involved comparing the effects of different diet treatments between different animals assigned to receive different diets which is a stronger design because it is harder to find cross-sectional differences due to inter-animal variability. The experimental design employed in the experiments in study #1 are also different from all previously reported studies. The piglets were fed diets that provided the same amount of energy as fat, and the same amount and source of saturated fatty acids (16:0), differing only in the cholesterol content and the positional distribution of fatty acids in the dietary triglyceride. The findings of higher hepatic HMG-CoA reductase mRNA in the formula-fed than milk-fed piglets but no differences in hepatic LDL receptor are probably a result of the low levels of expression of the LDL receptor during the suckling period, as discussed in section 5.1.3., and is therefore a result of the age of the animals and type of diets (milk and formula) used in the study.

5.1.5. Effect of the cholesterol content of formula and the fatty acid positional distribution in formula triglyceride on hepatic C7H mRNA and bile composition.

The lower levels of hepatic C7H mRNA in the milk-fed than formula-fed piglets were accompanied by higher cholesterol, phospholipid and bile acid concentrations in bile. Of further relevance, previous studies have reported a 25% lower rate of bile acid synthesis, and lower bile acid excretion in baboons that had been breast-fed rather than fed formula with or without cholesterol, as infants (Jackson et al.1993). It is possible therefore, that milk-feeding results in lower levels of bile acid synthesis and possibly lower levels of C7H, the enzyme catalyzing the rate limiting step in the conversion of cholesterol to bile acids.

One possible explanation for the significantly higher level of hepatic C7H mRNA in the formula-fed than milk-fed piglets may be the lower concentration of bile acids in bile of the piglets fed formula than piglets fed milk. The concentration of bile acids may be relevant because bile acids are known to regulate C7H expression, primarily at a transcriptional level via a negative feedback mechanism (Crestani et al. 1994,Ramirez et al. 1994). Thus, low bile acid can be expected to be associated with higher hepatic C7H. In this regard, a bile acid-responsive element has been identified in the promoter region of the C7H gene (Chiang et al. 1994). Interestingly, bile acids not only regulate C7H expression, but have also been shown to coordinately decrease hepatic rates of transcription and hepatic levels of mRNA for HMG-CoA reductase (Pandak et al.1994, Spady and Cuthbert 1992,Xu et al. 1992). This may explain the coordinately lower levels of hepatic C7H mRNA and HMG-CoA reductase mRNA in milk-fed than formula-fed piglets. Thus, a primary reason for the differences in the levels of HMG-CoA reductase

mRNA between piglets fed milk and formula may be related to differences in the concentration of bile acids in bile rather than to the differences in plasma cholesterol. This increase in the concentration of bile acids in bile of milk-fed piglets may be a consequence of greater reabsorption of bile acids and return of bile acids to the liver that would result in a coordinate decrease in the expression of C7H and HMG-CoA reductase.

The reason(s) why the formula-fed piglets had lower bile acid, cholesterol and phospholipid concentrations in bile than piglets fed milk is not readily apparent. One possibility, however, is that there may be increased intestinal reabsorption of bile acids and cholesterol, that are secreted in the intestine, in the enterohepatic circulation, such that little bile acids and/or cholesterol is excreted in the feces of milk-fed piglets. Lower fecal sterol loss and thus increased return of bile acids to the liver would be expected to result in feedback inhibition of the conversion of cholesterol to bile acids, and consequently, decreased cholesterol synthesis. This, therefore, could provide a reasonable potential explanation for the findings here of lower levels of hepatic C7H mRNA and HMG-CoA reductase mRNA in the milk-fed than formula-fed piglets. At present, however, there is little definitive information on the effects of early diet, that is milk or formula-feeding, on the enterohepatic circulation of bile acids, including the reabsorption of bile acids and cholesterol from bile.

Another possible explanation for the higher cholesterol and bile acid concentrations in bile of the piglets fed milk than in those fed formula is that the milk-fed piglets could have lower rates of bile secretion. For example, differences in the rates of gall bladder contraction and secretion of bile into the duodenum could be present due to the actions of some other component found in milk, such as growth factors or hormones that are not

present in formula. Thyroid hormones are present in both human and sow milks, but it is not known whether these hormones can be absorbed and whether they can have any metabolic effect. As discussed in section 5.1.2, previous studies have reported higher serum total T3 and free T3 in baboons fed formula then in baboons fed milk (Lewis et al. 1993) and higher serum free T4 levels were found in formula-fed than milk-fed piglets. Of relevance to the findings of higher levels of C7H mRNA in the formula-fed than milk-fed piglets, previous studies have shown thyroid hormone to stimulate expression of C7H (Crestani et al. 1994,Ness et al. 1994, Ness et al. 1995). It seems possible then, that the higher levels of serum free T4 in formula-fed than milk-fed piglets could also be involved in the differences in C7H mRNA levels, and possibly be involved in the lower concentrations of bile acids, cholesterol and phospholipid in bile of piglets fed formula.

Previous studies examining the effect of dietary cholesterol on C7H expression have found the responses to be species-specific. Dietary cholesterol has been shown to increase both levels of hepatic activity and mRNA for C7H in rodents (Dueland et al. 1993,Shefer et al. 1992). The opposite effect, however, has been found in rabbits and monkeys, whereby dietary cholesterol has been shown to decrease hepatic levels of C7H activity and mRNA (Rudel 1994, Xu et al. 1995). The response of hepatic C7H to diets containing cholesterol has not been previously investigated in pigs. It is difficult to conclude from the findings of this study how C7H expression in piglets responds to cholesterol. This difficulty can primarily be attributed to the question as to whether or not the cholesterol added to the formula was absorbed or if the effects of milk were specific to cholesterol. As discussed in section 5.1.1., it is reasonable to question if cholesterol added to formula was absorbed as well as the cholesterol in sow milk. The major reasons

for this suggestion is the lack of response in plasma cholesterol levels in the piglets fed the formula containing cholesterol. Assuming this to be correct, piglets fed milk in study #1 responded to the dietary cholesterol intake from sow milk by lowering hepatic levels of HMG-CoA reductase mRNA and C7H mRNA, accompanied by a post natal increase in plasma cholesterol. Conversely, it is also possible that there may be no change in the levels of HMG-CoA reductase mRNA and C7H mRNA in the milk-fed piglets. Formula feeding increases the levels of HMG-CoA reductase mRNA because of the absence of cholesterol in the diet or the low absorption of the cholesterol added to formula. Therefore, the formula-fed piglets respond by increasing the synthesis of cholesterol and increasing the levels of C7H mRNA to convert the cholesterol to bile acids required for absorption of dietary fat.

The positional distribution of fatty acids in the formula triglyceride had no effect on the levels of hepatic HMG-CoA reductase mRNA or C7H mRNA in the formula-fed piglets. Plasma cholesterol concentrations and the concentration of bile acids in bile were lower in piglets fed the synthesized TG formula than in piglets fed the conventional formula. The mechanism by which the positional distribution of fatty acids in the formula triglyceride influences plasma cholesterol concentrations and bile acid concentrations in bile in formula-fed piglets is not known. There are a few reports, however, to show that the positional distribution of fatty acids in plasma triglycerides can influence the rate of lipoprotein triglyceride hydrolysis via lipoprotein lipase, and possibly the rate and composition of remnant particles cleared by the liver (Mortimer et al. 1992,Regrave et al. 1988). Also, a positive relationship between the amount of 16:0 in the 2 position of dietary triglycerides and plasma cholesterol esters containing 16:0 has also been reported (Innis

and Dyer 1997). Possibly, the lower plasma cholesterol concentrations in the piglets fed the synthesized TG formula compared to piglets fed the conventional formula maybe related to diet-induced changes in lipoprotein fatty acid composition and/or the positional distribution of fatty acids in the triglycerides of circulating lipoproteins. The reason for the lower concentration of bile acids in bile of piglets fed the synthesized TG formula than in piglets fed the conventional formula is possibly also related to the differences in the positional distribution of fatty acids in dietary triglycerides. If 16:0 is found at the 2 position of the dietary triglyceride, it is absorbed as a 2-monoglyceride whereas if the 16:0 is found at the 1 and 3 positions the 16:0 is absorbed as a unesterified fatty acid. The unesterified 16:0 is not soluble in the environment of the intestinal lumen primarily because the melting point of 16:0 is above body temperature. Therefore, the piglets fed the synthesized TG formula may not require the same amount of bile acids as the piglets fed the conventional formula because the 16:0 is at the 2 position of the formula triglyceride and is not as dependent on bile acids for absorption.

5.1.6. The effect of the cholesterol content of formula and the positional distribution of fatty acids in the formula triglyceride on hepatic FAS and ACC.

The lower plasma and hepatic triglyceride concentrations in piglets fed formula than in the piglets fed milk were not accompanied by differences in hepatic levels of FAS mRNA or FAS activity. Studies in rats have shown that dietary polyunsaturated fatty acids inhibit hepatic fatty acid synthesis, specifically through an effect on the levels of FAS mRNA (Clarke and Jump 1993, Clarke et al. 1990, Jump et al. 1994). *In vitro* studies with HepG2 cells have also shown polyunsaturated fatty acids to lower the levels of FAS mRNA (Armstrong et al. 1991, Jump et al. 1994). The formula fed to

piglets contained about two-fold higher amounts of 18:2(n-6) than the sow milk. As a result of this, it is reasonable to expect that piglets fed the formula would have lower levels of hepatic FAS mRNA and activity than those fed milk. The sow milk, however, also contained the longer chain polyunsaturated fatty acids, 20:4(n-6) and 22:6(n-3), which were not present in the formula used in this study. Of relevance to this, previous studies have shown that the inhibitory effect of polyunsaturated fatty acids on hepatic FAS expression is fatty acid specific with both series-specific (i.e. n-6 compared to n-3 series) and chain length-specific inhibitory effects (Clarke and Clarke 1982,Clarke and Jump 1993). Specifically, recent studies have indicated that the (n-3) series fatty acids are more potent at lowering the levels of hepatic FAS mRNA and activity than the (n-6) series fatty acids (Clarke and Jump 1993). As well, products of the desaturation and elongation of 18:2(n-6), for example, 20:4(n-6), seem to be more potent at inhibiting hepatic FAS expression than the C18 precursors (Clarke and Clarke 1982,Clarke and Jump 1993). This suggests that despite the high level of total polyunsaturated fatty acids, that is 18:2(n-6) and 18:3(n-3), in the formula, the presence of the longer chain (n-6) and (n-3) fatty acids, such as 20:4(n-6) and 22:6(n-3), in milk may also influence any potential differences in hepatic FAS mRNA and activity between the milk-fed and formula-fed piglets. Therefore, the higher levels of polyunsaturated fatty acids in formula than milk and the presence of 20:4(n-6) and 22:6(n-3) in milk account for the absence of a significant difference in the levels of FAS mRNA and activity between the milk-fed and formula-fed piglets.

Dietary cholesterol has previously been shown to increase fatty acid and triglyceride synthesis, as determined by $^{3}H_2O$ incorporation into fatty acids, in both adult

hamsters and rats (Fungwe et al. 1992, 1993, 1994a, 1994b). Furthermore, recent studies have identified a sterol regulatory element in the promoter region of both the FAS and ACC genes (Bennet et al. 1995,Lopez et al.1996). This regulatory element functions to stimulate transcription of the FAS and ACC genes when cellular cholesterol concentrations are depleted (Kawabe et al. 1996,Lopez et al. 1996). Interestingly, the addition of cholesterol to the formula decreased hepatic FAS mRNA, although not activity of FAS. The addition of cholesterol to the formula also increased hepatic cholesterol concentrations in the formula-fed piglets. Thus, it seems possible that higher levels of FAS mRNA observed in piglets fed the formula supplemented with cholesterol may be related to higher liver cholesterol concentrations. As mentioned in section 5.1.1., the results of the study here give reason to question the extent of absorption of cholesterol from the supplemented formula. The absence of an increase in plasma cholesterol concentrations, or a decrease in hepatic HMG-CoA reductase mRNA levels in response to cholesterol supplementation in the formula-fed piglets suggests the cholesterol may not have been well absorbed. It is possible, however, that a sufficient proportion of the cholesterol added to formula was absorbed by the formula-fed piglets to explain the increase in hepatic total cholesterol concentrations and the lower levels of hepatic FAS mRNA in the piglets fed the formula supplemented with cholesterol.

Hepatic ACC mRNA levels were influenced by both the cholesterol content and the positional distribution of fatty acids in the triglycerides in formula. The addition of cholesterol to the conventional formula, which contained the majority of 16:0 esterified at the *sn*-1 and 3 positions of the triglyceride, was associated with higher hepatic ACC

mRNA levels in piglets fed this formula. In contrast, the addition of cholesterol to the formula with synthesized TG, in which more 16:0 was esterified at the *sn*-2 position of the formula triglyceride, was associated with a lower level of hepatic ACC mRNA in piglets fed this formula. All of the formula-fed piglets, regardless of the formula cholesterol content or the positional distribution of fatty acids in the formula triglyceride, had higher levels of hepatic ACC mRNA were higher than the piglets fed milk. Possibly, additional factors, such as higher plasma cholesterol concentrations in milk-fed piglets may be involved in the higher levels of hepatic ACC mRNA in the formula-fed than milk-fed piglets.

5.2. STUDY#2

5.2.1. The effect of supplementing formula with egg phospholipid on hepatic LDL receptor, HMG-CoA reductase and C7H.

The addition of egg phospholipid, providing both 20:4(n-6) and 22:6(n-3), to the conventional formula containing cholesterol had no significant effect on the levels of hepatic LDL receptor mRNA in the formula-fed piglets. Previous studies with HepG2 cells have found that 20:4(n-6) increases LDL receptor activity (Rumsey et al. 1995). Other studies with HepG2 cells however, have found that 22:6(n-3) decreases LDL receptor mRNA and activity (Scorci-Thomas et al. 1992). Information on the effects of supplementing diets with both 20:4(n-6) and 22:6(n-3), on the levels of hepatic LDL receptor mRNA have not been reported previously.

As discussed in section 5.1.3, changes in LDL receptor expression in response to dietary fat have been observed at transcriptional, translational and post translational levels (Srivastava et al. 1995,Stucchi et al. 1995), and usually involve changes in cellular cholesterol concentrations (Rumsey et al. 1995). Although no differences in LDL receptor mRNA were found in the studies here, there remains the possibility of differences in the levels of LDL receptor protein and activity between the piglets fed the conventional formula and those fed the same formula supplemented with egg phospholipid. The addition of egg phospholipid to the formula, however, had no effect on the plasma cholesterol concentrations or hepatic total cholesterol concentrations of the formula-fed piglets. Further, similar to the findings of study #1, study #2 also found no difference in hepatic cholesterol concentrations between piglets fed formula and piglets fed milk, even though plasma cholesterol concentrations were again lower in the

formula-fed than milk-fed piglets. Again, as discussed previously in section 5.1.3, it is possible that the hepatic clearance of apo B-containing lipoproteins may be low during the suckling period, and as a result, the already low LDL receptor shows no response to changes in dietary 20:4(n-6) and 22:6(n-3) at this time.

Piglets fed the conventional formula containing cholesterol, either with or without egg phospholipid, had higher levels of HMG-CoA reductase mRNA and activity than in piglets fed milk, and the addition of egg phospholipid to the formula, providing 20:4(n-6) and 22:6(n-3), had no effect on hepatic HMG-CoA reductase or plasma cholesterol concentrations in the formula-fed piglets. Previous studies in adult animals have found that dietary fish oil, containing 22:6(n-3) and 20:5(n-3), or 22:6(n-3), lowers hepatic HMG-CoA reductase activity (Field et al. 1987,Froyland et al. 1996,Smit et al. 1991). These reports suggest that the 22:6(n-3) in milk could be partially responsible for the lower levels of hepatic HMG-CoA reductase mRNA in the milk-fed piglets than in piglets fed formula without 22:6(n-3). The addition of egg phospholipid, with 22:6(n-3), as well as 20:4(n-6), to formula, however, had no effect on hepatic HMG-CoA reductase or plasma cholesterol concentrations in the formula-fed piglets. These results, together with those of study #1 that found consistently lower plasma cholesterol concentrations and higher HMG-CoA reductase due to formula-feeding, suggest that the lower levels of plasma cholesterol due to formula feeding may be a primary reason for the higher levels of HMG-CoA reductase mRNA in the formula-fed than milk-fed piglets. The findings of study #2 clearly suggest that differences in 20:4(n-6) and 22:6(n-3) between typical formula and milk do not explain the differences in plasma cholesterol and HMG-CoA reductase between the formula-fed and milk-fed piglets. The possibility of a type-2

statistical error in this study, however, because of the small number of animals in the group fed the formula without egg phospholipid needs to be considered.

The findings of no difference in the hepatic cholesterol concentrations, but a higher hepatic level of HMG-CoA reductase activity and mRNA in the formula-fed than milk-fed piglets in both study #1 and #2 suggests that regulation of the expression of HMG-CoA reductase may involve some mechanism, other than sterol regulation of transcription. Interestingly the results obtained in study #2, suggest a greater degree of difference between milk-fed and formula-fed piglets in the hepatic levels of HMG-CoA reductase activity than hepatic levels of HMG-CoA reductase mRNA in the formula-fed compared to milk-fed piglets. Both studies #1 and #2 did not include measures of the rate of transcription of the HMG-CoA reductase gene. Previous studies in rat liver nuclei, however, have shown that changes in rates of HMG-CoA reductase gene transcription can account for changes in HMG-CoA mRNA in response to drugs (Clarke et al. 1985). These studies here also determined levels of mRNA using total RNA and random hexamers, as primers, in the initial RT-PCR reaction. Therefore, because of this it is not possible to deduce whether or not milk or formula feeding had any effects on mRNA stability, nuclear splicing and shuttling of mRNA to the cytosol. Information on the regulation of hepatic HMG-CoA reductase expression at post-transcriptional and post translational levels has been published (Choi et al. 1993, Field et al. 1991). It seems reasonable to suggest that the differences in hepatic HMG-CoA reductase activity and hepatic HMG-CoA reductase mRNA between milk-fed and formula-fed piglets may involve effects at both transcriptional, post transcriptional and post translational levels. The results of study #1show that piglets fed formula had lower

levels of bile acids in bile, and higher levels of hepatic C7H mRNA than piglets fed milk, regardless of cholesterol content or the positional distribution of fatty acids in the formula triglyceride. Consistent with these findings, the piglets fed formula in study #2 had higher hepatic levels of C7H mRNA than piglets fed milk. The presence of 20:4(n-6) and 22:6(n-3) in the formula, as for cholesterol had no effect on C7H mRNA in the liver of the formula-fed piglets. In study #2, however, the difference in hepatic C7H mRNA between the milk-fed and formula-fed piglets was higher but not statistically significantly higher ($P<0.07$). This is probably explained by the small number of piglets (n=3) in the group fed the conventional formula without egg phospholipid. The results, however suggest that egg phospholipid, when added to formula to provide 20:4(n-6) and 22:6(n-3), does not influence hepatic levels of C7H mRNA ($P<0.2$). Published information for studies in adult animals concerning the effect of dietary long chain fatty acids on hepatic C7H expression and bile acid synthesis are inconsistent. Studies in rats have found no effect of fish oils, providing both 20:5(n-3) and 22:6(n-3), on hepatic levels of C7H activity (Smit et al. 1991). However, increased bile acid production in rats fed a diet supplemented with fish oil (Smit et al. 1994), and decreased hepatic levels of C7H activity in rats fed diets supplemented with fish oils (Al-Shurbaji et al. 1991) has also been reported.

5.2.2. The effect of supplementing formula with egg phospholipid on bile phospholipid and liver phospholipid and triglyceride fatty acids.

Supplementing the formula with egg phospholipid, providing both 20:4(n-6) and 22:6(n-3), increased 20:4(n-6) and 22:6(n-3), predominantly at the expense of 18:1(n-9), in bile phospholipid, as well as liver phospholipid. Similar findings of higher 22:6(n-3)

plus 20:5(n-3) in bile phospholipid have been found in studies with animals fed fish oil (triglyceride) supplements, providing, 22:6(n-3) plus 20:5(n-3) (Berr et al. 1992,Booker et al. 1990,Smit et al. 1994). Other studies in rats have found that large amounts of soybean phospholipid, providing high levels of 18:2(n-6), increase the levels of 18:2(n-6) in bile phospholipid (Rioux et al. 1994). The findings of these previously reported studies (Berr et al. 1992, Booker et al. 1990, Rioux et al. 1994, Smit et al. 1994) suggest that a dietary source of long chain polyunsaturated fatty acids, either from triglyceride or phospholipid, influences the fatty acid composition of bile phospholipid.

Interestingly, supplementing the formula with egg phospholipid led to higher levels of 20:4(n-6) and 22:6(n-3) in bile phospholipid than in piglets fed milk, despite similar intakes of 20:4(n-6) and 22:6(n-3) between the piglets fed the supplemented formula and the group fed milk. The reason for the apparent higher amount of 20:4(n-6) and 22:6(n-3) in bile of the piglets fed the supplemented formula than in those fed milk is not readily apparent. Conversion of 18:2(n-6) and 18:3(n-3) to 20:4(n-6) and 22:6(n-3), respectively, which were provided at higher levels in the formula than milk, could explain the higher liver and bile levels of 20:4(n-6) and 22:6(n-3) in the formula-fed than milk-fed piglets. This explanation, however, seems unlikely, since desaturation and elongation of 18:2(n-6) and 18:3(n-3) is usually considered to be inhibited by long chain (n-6) and (n-3) fatty acids (Sprecher et al. 1994). Another possible explanation is that the 20:4(n-6) and 22:6(n-3) added to formula as egg phospholipid is metabolized differently from the 20:4(n-6) and 22:6(n-3) naturally provided by milk. Little is as yet known about the digestion of milk 20:4(n-6) and 22:6(n-3) in the human, piglet or other species. It is generally assumed that on a quantitative basis most of the 20:4(n-6) and

22:6(n-3) in milk is present in the triglycerides, rather than phospholipid. Since significant amounts of 20:4(n-6) and 22:6(n-3), however, are found esterified at the 3-position of the triglyceride glycerol (Martin et al. 1993), it is possible that hydrolysis by pancreatic lipase and, consequently absorption is incomplete (Chen et al. 1990). This could explain the higher levels of 20:4(n-6) and 22:6(n-3) in bile phospholipids of the piglets fed the formula supplemented with egg phospholipid than in piglets fed milk. The digestion and absorption of dietary phospholipid is also poorly understood, and has not been fully elucidated. It may also be possible that fatty acids found in dietary phospholipid are preferentially found in bile phospholipid compared to fatty acids provided by dietary triglyceride. This suggests, therefore, that the 20:4(n-6) and 22:6(n-3) provided as egg phospholipid to the formula-fed piglets is directed towards bile phospholipid to a greater extent than the 20:4(n-6) and 22:6(n-3) found in sow milk triglyceride.

The findings of study #1, in agreement with previous studies (Rioux and Innis 1993), found that piglets fed milk have higher bile concentrations of bile acids, cholesterol and phospholipid than piglets fed formula without 20:4(n-6) and 22:6(n-3). In study #2, piglets fed the formula with 20:4(n-6) and 22:6(n-3) from egg phospholipid had increased concentrations of bile acids, cholesterol and phospholipid in bile, suggesting that dietary 20:4(n-6) and/or 22:6(n-3), at least from phospholipid, may influence bile lipid metabolism. Studies in rats showing increased secretion of cholesterol, bile acids and phospholipid in bile as a result of feeding either fish oils, providing 20:5(n-3) and 22:6(n-3) (Chautan et al. 1990,Smit et al. 1991,Smit et al. 1994), or feeding soybean phospholipid, providing 18:2(n-6) in phospholipid (Peled and

Gilat 1994,Rioux et al. 1994), further support the hypothesis that dietary polyunsaturated fatty acids can influence bile lipid metabolism. The findings of study #2 of higher bile phospholipid in piglets fed the supplemented formula, suggests that the dietary intake of phospholipid may be directly related to phospholipid secretion in bile. Another study with a small number of adult human subjects (n=5), however, found no effect of a dietary phospholipid (12g-3 times per day) supplement on bile lipid concentrations (Pakula et al. 1996). The study by Pakula et al. (1996), however, found a significant increase in bile phospholipid 18:2(n-6), which represented about 54% of the fatty acids in the phospholipid supplement, suggesting dietary phospholipid fatty acid composition does influence bile phospholipid fatty acid composition. The results of study #2, similarly show that the dietary phospholipid fatty acids, in this case 20:4(n-6) and 22:6(n-3) from egg phospholipid, are reflected in bile. Together these results suggest the possibility that dietary phospholipid contributes directly to the enterohepatic circulation of phospholipid.

5.2.3. The effect of supplementing formula with egg phospholipid on hepatic FAS.

Supplementing the formula with 20:4(n-6) and 22:6(n-3) from egg phospholipid had no effect on the hepatic levels of FAS activity and mRNA in the formula-fed piglets. Further, similar to the findings of study #1, no differences in hepatic levels of FAS mRNA or activity were found between the formula-fed and milk-fed piglets. Studies with adult rats fed long chain polyunsaturated fatty acids either from egg phospholipid (Ide and Marata 1994), fish oil (Clarke and Jump 1993,Clarke et al. 1990,Geelen et al. 1995,Jump et al. 1994) or 20:5(n-3) and 22:6(n-3) ethyl esters (Jump et al. 1994) have found decreased levels of hepatic FAS activity and mRNA. These previously reported studies (Clarke and

Jump 1993,Clarke et al. 1990,Geelen et al. 1995, Ide and Marata 1994, Jump et al. 1994) however, differ from study #2 in that the lipid source of long chain polyunsaturated fatty acids was the only source of fat in the diet. The relatively high proportions of total energy from fat and the considerable amount of long chain saturated fatty acids, specifically 16:0, provided by the formula or milk may explain the absence of an inhibitory effect of 20:4(n-6) and 22:6(n-3) on hepatic FAS expression in this study. Another possible reason for the lack of an effect on hepatic FAS mRNA and activity in study #2 may be the relatively low amount of 20:4(n-6) and 22:6(n-3) added to the formula and the low levels naturally present in milk. In this study, 0.4% of total energy was provided by 20:4(n-6) and 0.1% of total energy by 22:6(n-3).

Despite no differences in FAS expression between piglets fed the unsupplemented formula and those fed the formula with egg phospholipid providing 20:4(n-6) and 22:6(n-3), significantly higher levels of liver phospholipid 20:4(n-6) and 22:6(n-3) were observed. This suggests that changes in membrane phospholipid fatty acid composition are not necessarily involved in the regulation of expression of FAS. This suggestion is consistent with recent data to indicate polyunsaturated fatty acids inhibit gene expression through direct actions on nuclear proteins and subsequent regulation at transcriptional levels (Keller et al. 1993,Ren et al. 1996). It could be that the amount of 20:4(n-6) and 22:6(n-3) provided by the diets in this study was insufficient to induce changes in pools of 20:4(n-6) and 22:6(n-3) that interact directly with nuclear proteins, and as a result no changes in FAS expression occurred. An alternative explanation, however, could be that the regulatory effect of 20:4(n-6) and 22:6(n-3) on FAS mRNA and activity could be modulated

by the high percentage of energy derived from fat (50%) and/or the high percentage of long chain saturated fatty acids provided by both the formula and milk diets.

Another possible explanation for the absence of a difference in FAS mRNA and activity levels between piglets fed the milk and formula with and without 20:4(n-6) and 22:6(n-3) may be the presence of cholesterol in all the diets. As discussed in section 5.1.6., dietary cholesterol has been shown to stimulate hepatic fatty acid synthesis in adult rats (Fungwe et al. 1994,Lei et al. 1995). It could be that the stimulatory effect of dietary cholesterol on fatty acid synthesis is more important than the inhibitory effect of dietary long chain polyunsaturated fatty acids. Studies with HepG2 cells, however, have identified a sterol-regulatory region in the promoter of the FAS gene that functions to stimulate transcription when cellular sterol concentrations are depleted (Bennett et al. 1995,Kawabe et al. 1996). Furthermore, lower FAS activity has been found in neonatal rat pups fed milk substitutes containing cholesterol than rat pups fed a milk substitute not containing cholesterol (Auestad et al. 1988). The absence of a difference in hepatic cholesterol concentrations between piglets fed formula and piglets fed milk in study #2, therefore fits well with the findings of similar levels of FAS mRNA and activity. Therefore, an additional reduction in the levels of FAS mRNA and activity that would be expected by the addition of long chain polyunsaturated fatty acids to the formula may not be possible. Some support for this interpretation comes from the results of study #1 to show that the addition of cholesterol to the formula decreased hepatic levels of FAS mRNA. Therefore, the findings of similar levels of hepatic FAS mRNA and activity among the piglets, despite differences in the dietary intake of 20:4(n-6) and 22:6(n-3), may be explained by the similar, and relatively high intakes of cholesterol in all the piglets.

5.2.4. The effect of supplementing formula with egg phospholipid on hepatic ACC mRNA.

Supplementing formula with egg phospholipid providing 20:4(n-6) and 22:6(n-3) led to lower levels of hepatic ACC mRNA in the formula-fed piglets than in piglets fed the unsupplemented formula, or sow milk. Further, a significant inverse relation was found between the levels of 20:4(n-6) and 22:6(n-3) in liver lipids and the hepatic levels of ACC mRNA. These findings suggest that dietary 20:4(n-6) and 22:6(n-3) may influence the regulation of expression of ACC at a transcriptional level in developing piglet liver. This suggestion is consistent with the findings of previous studies that show lower levels of hepatic ACC activity and rates of transcription for the ACC gene in adult animals fed diets supplemented with polyunsaturated fatty acids (Clarke and Clarke 1982,Geelen et al. 1995,Katsurada et al. 1990,Toussant et al. 1981).

The piglets fed the formula supplemented with egg phospholipid received a similar amount of 20:4(n-6), but about two-fold higher amounts of 22:6(n-3) than the piglets fed milk. This difference in dietary intake of 22:6(n-3), together with the significantly higher levels of 22:6(n-3) in hepatic triglyceride and phospholipid in piglets fed the formula supplemented with egg phospholipid than in piglets fed sow milk could be involved in the lower levels of hepatic ACC mRNA in piglets fed the supplemented formula than in piglets fed milk. Previous studies have only investigated the effects of diets high in 18:2(n-6) on the expression of ACC and have not addressed the effects of long chain n-6 and n-3 polyunsaturated fatty acids (Clarke and Clarke 1982,Geelen et al. 1995,Katsurada et al. 1990,Toussant et al. 1981). It may be that the more potent inhibitory effect of n-3 fatty acids, compared with n-6 fatty acids as previously reported in adult rats, on the hepatic

expression of FAS (Clarke and Clarke 1982,Clarke and Jump 1993), is also the same for ACC. As well, the expression of hepatic ACC and FAS in piglets during the suckling period may not be responsive to the same regulatory factors.

No difference in the hepatic levels of ACC mRNA between piglets fed the formula without egg phospholipid and piglets fed milk was found in the studies reported here. A significant inverse relation between ACC mRNA and 20:4(n-6) in liver triglyceride, however, was also found in this study. The similar levels of 20:4(n-6) in hepatic triglyceride of piglets fed milk and of piglets fed the conventional formula with egg phospholipid could explain the absence of a difference in hepatic ACC mRNA between these two groups of piglets. The results of similar levels of 20:4(n-6) further support the role of 20:4(n-6) in the regulation of hepatic ACC.

5.3. Summary of findings.

The results of study #1 show that formula feeding alters the levels of hepatic HMG-CoA reductase and C7H mRNA, and plasma cholesterol concentrations in piglets when compared to natural milk feeding. Neither the addition of cholesterol to formula nor changing the positional distribution of fatty acids in the formula triglyceride raised the plasma cholesterol concentrations or changed the hepatic levels of HMG-CoA reductase or C7H mRNA in the formula-fed piglets in a direction resembling that of piglets fed milk. The most reasonable explanation for the higher levels of hepatic HMG-CoA reductase and C7H mRNA in piglets fed formula than in piglets fed milk is probably related to lower availability of exogenous cholesterol for the liver, secondary to the lower plasma cholesterol concentrations in the formula-fed piglets. Thus, the piglets fed milk had higher plasma cholesterol concentrations and, consequently, lower levels of hepatic HMG-CoA

reductase mRNA than the piglets fed formula. The absence of an increase in plasma cholesterol concentrations, and consequently of a decrease in hepatic levels of HMG-CoA reductase and C7H mRNA in piglets fed the formula supplemented with cholesterol may be explained by differences in the absorption of cholesterol from milk and formula. Alternatively, other effects of milk on the secretion of cholesterol, specifically into bile as either cholesterol or bile acids, might explain the results.

The results of study #2 are the first to show that supplementing formula with egg phospholipid providing 20:4(n-6) and 22:6(n-3) alters bile phospholipid fatty acid composition and bile lipid concentrations. Despite the differences in bile lipid metabolism, no effect was observed on hepatic levels of C7H or HMG CoA reductase mRNA. This suggests, as discussed above, that the higher levels of C7H and HMG-CoA reductase mRNA in formula-fed compared to milk-fed piglets is probably related to the lower plasma cholesterol concentrations in the formula-fed than milk-fed piglets. The higher 20:4(n-6) and 22:6(n-3) in bile lipids of piglets fed the formula supplemented with egg phospholipid than in piglets fed the unsupplemented formula or piglets fed milk suggest that dietary long chain fatty acids, at least when fed as phospholipid, may be used for bile phospholipid synthesis or may influence the enterohepatic circulation of bile phospholipid. This also suggests that the 20:4(n-6) and 22:6(n-3) added to formula as egg phospholipid may be metabolized differently than the 20:4(n-6) and 22:6(n-3) naturally present in milk, largely as triglycerides.

Supplementing the formula with egg phospholipid also led to lower hepatic levels of ACC mRNA, but had no effect on the levels of FAS mRNA or activity, and increased 20:4(n-6) and 22:6(n-3) in hepatic phospholipid and triglyceride. The significant inverse

relation found between the levels of hepatic ACC mRNA and 20:4(n-6) in hepatic triglyceride, and between 22:6(n-3) in hepatic phospholipid is consistent with published data to indicate long chain n-6 and n-3 fatty acids maybe involved in the regulation of ACC. The effects of dietary 20:4(n-6) and 22:6(n-3) when supplied as egg phospholipid appears to be more efficacious in increasing liver phospholipid and triglyceride levels of 20:4(n-6) and 22:6(n-3) than when supplied as sow milk. These findings again suggest that the 20:4(n-6) and 22:6(n-3) when added to formula as egg phospholipid is metabolized differently from the 20:4(n-6) and 22:6(n-3) naturally present in sow milk.

6. CONCLUSIONS

6.1. Study #1

6.1.1 The addition of cholesterol (0.52mmol/L unesterified + 0.13mmol/L cholesteryl oleate) to formula does not raise plasma cholesterol to levels observed in milk-fed piglets.

6.1.2 Directing 16:0 to the centre position of the formula triglyceride does not raise plasma cholesterol to levels observed in milk-fed piglets.

6.1.3. The higher concentrations of plasma cholesterol in milk-fed than formula-fed piglets are accompanied by higher concentrations of bile acids, cholesterol and phospholipid in bile and lower hepatic levels of C7H mRNA and HMG-CoA reductase mRNA.

6.1.4. The hepatic levels of LDL receptor mRNA are not influenced by early diet in piglets.

6.1.5. The addition of cholesterol to formula increases hepatic total cholesterol concentrations, decreases hepatic levels of FAS mRNA and activity and has no effect on hepatic levels of LDL receptor mRNA, C7H mRNA or HMG-CoA reductase mRNA.

6.1.6. Directing 16:0 to the centre position of the formula triglyceride lowers hepatic levels of FAS activity and mRNA in formula-fed piglets.

6.1.7. The higher levels of hepatic triglyceride in milk-fed than formula-fed piglets are not accompanied by higher hepatic levels of FAS activity and mRNA.

6.2. Study #2

6.2.1. Supplementing formula with egg phospholipid providing 20:4(n-6) and 22:6(n-3) does not affect the concentrations of plasma total cholesterol in piglets.

6.2.2. Supplementing formula with egg phospholipid providing 20:4(n-6) and 22:6(n-3) does not affect hepatic levels of FAS activity and mRNA, HMG-CoA reductase activity and mRNA, C7H mRNA or LDL receptor mRNA.

6.2.3. Supplementing formula with egg phospholipid providing 20:4(n-6) and 22:6(n-3) increases the levels of bile acids, cholesterol and phospholipid in bile. This was not accompanied by statistically significant differences in the levels of mRNA for C7H, HMG-CoA reductase or LDL receptor.

6.2.4. Milk-fed piglets have lower levels of hepatic HMG-CoA reductase activity and mRNA and lower levels of hepatic C7H mRNA than formula-fed piglets.

6.2.5. Supplementing formula with egg phospholipid providing 20:4(n-6) and 22:6(n-3) increases the concentrations of 20:4(n-6) and 22:6(n-3) in bile and liver phospholipids and is accompanied by lower hepatic levels of ACC mRNA.

6.2.6. Hepatic levels of ACC mRNA are negatively correlated with the levels of 20:4(n-6) in hepatic triglyceride and the levels of 22:6(n-3) in hepatic phospholipid. The correlation between hepatic ACC mRNA and hepatic

phospholipid 20:4(n-6) is stronger than for hepatic ACC mRNA and hepatic triglyceride 22:6(n-3).

6.3. Overall Conclusions

6.3.1. Early diet, milk or formula feeding, does influence the hepatic levels of HMG-CoA reductase mRNA and activity and hepatic levels of C7H mRNA.

6.3.2. The positional distribution of fatty acids in the dietary triglyceride is probably not responsible for the higher levels of plasma cholesterol in the milk than formula-fed piglets.

6.3.3. The long chain polyunsaturated fatty acids, 20:4(n-6) and 22:6(n-3) when added to formula as a component of egg phospholipid are metabolized differently by the liver than the 20:4(n-6) and 22:6(n-3) naturally found in milk.

7. LIMITATIONS OF STUDY

The experimental protocols employed in these studies could only be feasibly conducted using an animal model. Several reasons, including nutritional and physiological similarities to the human infant, and practical concerns support the choice of the piglet as an animal model. Caution, must be exercised however, with interpretation of these results with regard to the human infant. Clearly, the potential for species-related differences in metabolic response to dietary cholesterol and saturated fatty acids, and in the pathways of milk and formula fat digestion in the newborn period are important.

The statistical power of study #2 was low. Due to illness, the number of piglets fed conventional formula which were considered healthy and of appropriate body weight at the end of the experimental feeding period was 3, instead of 6. Three animals in this group, who experienced early infection, were withdrawn. There were 6 piglets in the group fed the conventional formula supplemented with egg phospholipid and 7 piglets in the milk-fed group. The piglets were randomized to receive either the supplemented or unsupplemented formula at the beginning of the study. Simply adding more piglets to the conventional formula group at the end of the study period to increase the number of piglets in this group is problematic and statistically invalid because the randomization is lost. The randomization should control within feeding block variation and there may also be seasonal variations between groups of piglets born in different months. Again, within a randomized study this is balanced. The possibility of a type 2 error, that is, failure to detect significant diet effects due to

inadequate sample size, has to be recognized when considering the results. Ideally, the study should be repeated with a more appropriate sample size.

The feeding state of milk-fed piglets at the time of blood sampling was difficult to control. At the end of the experimental feeding period, tissue samples were obtained from the formula-fed piglets exactly 3 hrs. post-prandial. In the milk-fed piglets, however, the time between blood and tissue collection and the actual last feed and the volume of the feed was difficult to control because the animals nursed with their natural mothers. The sow milk-fed animals were taken from the sow about 3-4 hrs. prior to blood and tissue collection. An approach used in infant studies to determine milk intake is test weighing before and after nursing. However, this approach can estimate the amount suckled but cannot force the time or amount which an *ad libitum* nursing piglet (or human) feeds. Expression of sow milk and bottle-feeding was also an impractical approach because of the difficulty of obtaining significant volumes of milk from the sows. It is also difficult to get a previously nursed animal to take bottle-feeds. The potential differences in test-feeding state between the milk-fed and formula-fed piglets may be important, and must be considered in interpreting reasons for differences between milk-fed and formula-fed animals, for example with regards to plasma glucagon and unesterified fatty acids.

Egg phospholipid was used as a source of 20:4(n-6) and 22:6(n-3) in study #2 because it provides both 20:4(n-6) and 22:6(n-3) and clinical studies have been conducted to investigate the effect of egg phospholipid in formula in human infants (Aeustad et al. 1996, Carlson et al. 1996). A major problem with

the interpretation of the results of study #2 is that it is not known whether or not the observed effects of the supplemented formula are a result of adding phospholipid, the long chain fatty acids or an interaction of the two. Furthermore, since egg phospholipid provides both 20:4(n-6) and 22:6(n-3), the observed effects could be the result of the 20:4(n-6) or 22:6(n-3), or again a consequence of the presence of both 20:4(n-6) and 22:6(n-3) in the supplement.

8. SIGNIFICANCE OF FINDINGS

The results of the studies reported here clearly show that early diet has an influence on hepatic cholesterol metabolism, plasma cholesterol concentrations and most importantly, hepatic gene expression. The significance of these findings is three-fold. Firstly, mimicking the dietary fat composition of infant formula does not produce similar metabolic responses as to that found in the milk-fed animal. Secondly, these findings raise the question of whether or not the observed effect of early diet will continue through to adulthood and set the stage for the metabolic response to dietary fat in adulthood. Thirdly, this profound effect of early diet on lipid metabolism in these studies raises questions regarding the appropriate levels of total fat intake and what type of fat is required by the infant for adequate growth and development while minimizing risk for developmental problems and chronic disease, such as cardiovascular disease.

The findings of this study are significant in that they show that the addition of cholesterol to formula and directing 16:0 to the centre position of the formula triglyceride failed to increase plasma cholesterol concentrations to levels observed in the milk-fed piglets. Higher plasma cholesterol concentrations in milk-fed than formula-fed infants have been well documented in both human infants and experimental animals (Kallio et al. 1997,Lane & McConathy 1986, Lourdes et al. 1994, Mize et al. 1995, Mott et al. 1990, Rioux & Innis 1993, Van Biervleit et al. 1986, Wong et al. 1993). In studies #1 and #2, what is most notable is the finding that the lower plasma cholesterol concentrations in the formula-fed than milk-fed piglets was accompanied by higher hepatic levels of

HMG-CoA reductase and C7H in the formula-fed than milk-fed piglets. It seems reasonable to question whether the effect of early diet on hepatic cholesterol metabolism observed in these studies reported here, could also be present during adulthood and influence response to dietary fat challenge. Previous studies with baboons did find that early diet does imprint the response of juvenile and adult animals to high fat and cholesterol diets (Mott et al. 1995, Mott et al. 1985, Mott et al. 1990). As well, a retrospective study found that adult men who had been fed milk as infants had higher serum cholesterol concentrations and a greater incidence of cardiovascular disease than those who had been fed formula (Fall et al. 1992). Consequently, additional prospective studies with piglets and in humans would be useful to draw more substantial conclusions on whether early diet can imprint adult metabolic response to dietary fat.

The findings of studies #1 and #2 are also significant in that they provide evidence that early diet has profound effects on hepatic lipid metabolism during infancy. Lower levels of plasma cholesterol were found in the formula-fed than milk-fed piglets despite similar amounts of total energy and similar amounts of total fat (representing 50% of energy), cholesterol and saturated fatty acids provided by both the formula and milk diets. At present, controversy exists regarding the appropriate age at which attempts should be made to lower dietary fat intakes in children because of the association between high dietary fat intakes, primarily cholesterol and saturated fatty acids, with increased blood cholesterol levels, a risk factor for cardiovascular disease. The main controversy is around the energy dense nature of dietary fat and the importance of energy

intake and dietary fat for normal growth. Support for a lowering of dietary fat intake during infancy and childhood comes from autopsy studies in which it was found that fatty streak development is present early in life (Newman et al. 1986,Pesonen et al. 1990). To date, few studies have been conducted to investigate the effects of a low fat diet during childhood and these studies have found no deleterious effects on growth but a lowering of serum total and LDL cholesterol concentrations (Boulton and Magarey 1995, DISC Collaborative Research Group 1995). Only one prospective study in young infants aged 7-13 months has been conducted. This study found that serum cholesterol concentrations did not change significantly between the ages of 7-13 months in infants whose guardians received nutritional counseling to lower dietary fat intakes (<30-35% energy and < 200 mg cholesterol) (Lapinleimu et al. 1995). In contrast, an increase in serum cholesterol concentrations was found in infants whose guardians did not receive dietary advice. No differences in growth were observed between these two experimental groups. However, the study was only conducted with formula-fed infants and there were significant differences in total energy intakes between the two experimental groups. The intake of fat by both groups was less than 30% and there was only a small difference in fat intake between groups, 26% and 28% in the intervention and control groups, respectively. Therefore, additional research is required to determine in both breast-fed and formula-fed infants, the effects of consuming a diet deriving less than 30% of energy from fat compared with infants consuming a diet deriving greater than 30% of energy from fat. These findings, together with findings from

additional studies investigating whether or not early diet can imprint metabolic response to dietary fat intake in adulthood will aid in 'fine-tuning' recommendations regarding appropriate levels and type of fat intake during infancy. As well, genetic factors and the polymorphic nature of genes encoding proteins involved in lipid metabolism (reviewed by Humphries 1993,Humphries et al. 1995) must also be considered. Recently, a study in Finnish infants found that the degree of difference in serum cholesterol concentrations between breast-fed and formula-fed infants was related to the apo E phenotype of the infant (Kallio et al. 1997). In infants expressing the apo E3/E4 and E4/E4 phenotypes, the differences in serum cholesterol concentrations between breast-fed and formula-fed infants were much greater than the differences observed for infants with either the apo E3/E3 phenotype or apo E2/E2, E2/E3 or E2/E4 phenotypes.

9. FUTURE DIRECTIONS

The findings of the studies reported here that adding cholesterol to the formula, primarily in the unesterified form and at similar amounts to the levels found in milk did not raise plasma cholesterol concentrations suggests that there may be differences in the absorption of cholesterol between milk-fed and formula-fed piglets. Future studies are therefore needed to consider if differences in fat and cholesterol absorption exist between milk and formula-fed piglets and how this might relate to diet-related differences in hepatic lipid metabolism during development. These studies might include:

1. Labeling the cholesterol added to formula.

If the cholesterol added to formula, similar to the formula in study #1, was labeled either using ^{14}C or the stable isotope ^{13}C, could be traced and estimates of its metabolism more accurately determined. These studies would determine fecal cholesterol excretion and as well, the amount of labeled cholesterol in circulating lipoproteins, in the cholesterol and bile acids in bile and in intestinal hepatic cells.

2. It would also be useful to determine if differences in the expression of proteins involved in cholesterol absorption exist between milk and formula-fed piglets.

These studies would involve sampling duodenal pancreatic secretions and assaying for cholesterol esterase activity; investigating whether or not diet-induced differences exist in pancreatic levels of cholesterol esterase mRNA and protein; and determining differences in the expression of apo B48 in intestinal cells.

Studies #1 and #2 found differences in the levels of hepatic C7H mRNA and differences in the concentration of bile acids, cholesterol and phospholipid in bile between milk-fed and formula-fed piglets. The enterohepatic circulation of bile acids involves intestinal reabsorption, secretion into the portal circulation and uptake by the liver. Passive, nonionic reabsorption of glycine-conjugated dihydroxy bile acids occurs in the jejunum and of unconjugated bile acids in the colon (Piccoli et al. 1993). Glycine- and taurine-conjugated bile acids are reabsorbed via an active transport mechanism in the terminal ileum. This process involves a Na+-dependent bile acid cotransporter localized to the brush border membrane of ileal enterocytes (Kramer et al. 1993,Schneider et al. 1994,Wong et al. 1994), which transports bile acids into enterocytes, and a bile acid binding protein (Fujita et al. 1990,Gantz et al. 1989,Lin et al. 1991,Sacchettini et al. 1990,Walz et al. 1988), which transports bile acids intracellularly. Following enterocyte absorption, bile acids are secreted into the portal circulation via a Na+-independent organic ion exchange system. The bile acids are then taken up by hepatocytes via a Na+-dependent cotransporter (Ananthanarayanan et al. 1994,Hagenbuch et al. 1991). Little is known regarding the ontogenesis of the enterohepatic circulation of bile acids and what effect early diet may have on this process. Studies with rodents have suggested that the ileal bile acid reabsorption during the suckling period is by a passive process and active ileal transport does not commence until the age of weaning (Barnard et al. 1985,Sacchettini et al.1990). Future studies might consider the following:

1. Investigating the hepatic uptake of bile acids and whether or not this process is influenced by early diet.

These studies would involve comparing the effect of milk and formula-feeding in piglets on the expression of the hepatic Na+-dependent cotransporter (Ananthanarayanan et al. 1994, Hagenbuch et al. 1991).

2. Determining whether or not the absence of intestinal active transport of bile acids during the suckling period is a species-related phenomena.

These studies would determine if the genes for the ileal Na+-dependent bile acid cotransporter and the bile acid binding protein are expressed during the suckling period in milk-fed piglets.

3. Determining if early diet influences the age at which active ileal transport of bile acids commences and whether or not early diet has an effect on this process.

These studies would determine if formula-feeding influences the expression of the ileal Na+-dependent bile acid cotransporter and the bile acid binding protein.

The studies reported here are one of the first to demonstrate that early formula-feeding, using formula similar to formula designed for human infants, results in a difference in the hepatic levels of mRNA for HMG-CoA reductase and C7H when compared to milk-feeding in piglets. The findings reported here, combined with the findings of previously reported studies that cholesterol synthesis is higher in the formula-fed than milk-fed infants (Lourdes et al. 1994) and the activity for HMG-CoA reductase is higher in formula-fed than milk-fed piglets (Rioux and Innis 1993), provides good circumstantial evidence that formula-feeding increases cholesterol synthesis. It would then be important to determine if such differences do indeed occur in infants and if these differences

continue through to adulthood. As already mentioned, previous studies have suggested a long term effect of infant diet on adult cholesterol metabolism (Fall et al. 1992, Mott et al. 1995, Mott et al. 1985, Mott et al. 1990). Based on the findings of these previous studies and the findings reported here, the following studies would seem worthwhile:

1. Determining if the early diet-related differences in hepatic gene expression persist beyond infancy into adulthood in piglets.

The initial part of this study would follow a similar experimental protocol as that followed in Study #1. At 18 days, however, blood samples and a liver biopsy would be taken, and the animals then weaned to a suitable weaning diet (for piglets) and continued on this diet until 60 days of age. At 60 days, the piglets would be challenged with a diet high in fat and cholesterol for 1 year. Following this blood and tissue samples would be collected for determination of plasma and liver lipid content and the expression of hepatic HMG-CoA reductase, C7H and LDL receptor. The aorta would also be removed and fatty streak development measured using histochemical techniques.

2. Completing a prospective study of infants in the Lower Mainland to investigate the effects early diet on cholesterol metabolism.

The initial phase of this study would involve determining the effects of formula and milk-feeding on plasma lipid concentrations, apo E phenotype, and the levels of HMG-CoA reductase and LDL receptor mRNA in blood leukocytes as indicators of hepatic levels of these proteins (Powell and Kroon 1994). The second stage of this study would involve a follow-up of these infants at ages 1-3 years and 6-8 years. At

these times, plasma lipid concentrations and the levels of HMG-CoA reductase and LDL receptor mRNA in blood leukocytes would be determined to investigate if any delayed effect of early diet is present at these time points.

The profound differences in plasma cholesterol concentrations and levels of hepatic HMG-CoA reductase and C7H mRNA despite adding cholesterol to formula suggest that there could also be some other 'non-nutritive' factor present in milk but absent from formula that could influence lipid metabolism. Studies that would investigate this possibility include:

1. Determining the activity of milk-borne peptides and hormones in the intestine of milk-fed piglets .

These studies would initially involve catheterizing animals such that samples from the small intestine (duodenum) could be taken after an animal ingested milk.

2. Comparing the effect of any of the active peptides and hormones found in milk on intestinal gene expression of proteins involved in lipid metabolism.

These studies would involve both animal and cell culture experiments. CaCo-2 cells would be used in the cell culture experiments. The cells would be grown to confluence and then the various hormones, previously determined to be active, would be added to the media. The effect of the presence of these hormones on cellular lipid metabolism and secretion of lipoproteins would be determined. In the animal studies, animals would be fed milk, formula, or formula supplemented with one of the previously determined active hormones present in milk. The

effect of these diets on the expression of intestinal proteins involved in lipid metabolism would be determined.

The second study supplemented the formula with egg phospholipid to provide 20:4(n-6) and 22:6(n-3) primarily because this has been used in studies with human infants fed formula (Auestad et al. 1997, Carlson et al. 1996). However, it is difficult to determine whether the effects on lipid metabolism observed in study #2 were a consequence of supplementing the formula with phospholipid or 20:4(n-6) and 22(n-3) or an interaction of the two. More specific studies investigating the metabolic consequences of supplementing formula with 20:4(n-6) and 22:6(n-3) from different sources would be useful. These studies would involve:

1. Comparing the effects of formula supplemented with egg phospholipid, with soybean phospholipid, or a triglyceride source of 20:4(n-6); and a triglyceride source of 22:6(n-3).

These studies would follow a similar experimental protocol to that of study #2 except with a larger number of animals and more experimental feeding groups. The experimental feeding groups would include piglets assigned to receive milk, conventional formula, formula supplemented with egg phospholipid, formula supplemented with soybean phospholipid, formula supplemented with triglycerides high in 20:4(n-6) or formula supplemented with triglycerides high in 22:6(n-3).

10. REFERENCES

Abu-Elheiga, L., Jayakumar, A., Baldini, A., Chirala, S.S. and Wakil, S.J. (1995) Human acetyl-CoA carboxylase: Characterization, molecular cloning, and evidence for two isoforms. *Proc. Natl. Acad. Sci.* 92: 4011-4015.

Al-Shurbaji, A., Larsson-Backstrom, C., Berglund, L., Eggertsen, G., Bjorkhem, I. (1991) Effect of n-3 fatty acids on the key enzymes involved in cholesterol and triglyceride turnover in rat liver. *Lipids* 26:385-389.

Alasmi, A., Thorkelson, T., Krug-Wispe, S., Bayley, T.M., Jones, P.J.H., and Tsang, R.S. (1996) Cholesterol supplementation effect on cholesterol synthesis rates (FSR) in infants. *Pediatric Res.* 39: 116A.

Alexander, C.A., Hamilton, R.L. and Havel, R.J. (1976) *J. Cell. Biol.* 69:241-263.

Allain, C.C., Poon, L., Chan, S.G., Richmond, W., and Fu, P. (1974) Enzymatic determination of total serum cholesterol. *Clin. Chem.* 20: 470-.

Amano, O., Kanda, T., Ono, T. and Iseki, S. (1992) Immunocytochemical localization of rat intestinal 15kDa protein, a member of cytoplasmic fatty acid-binding proteins. *Anatom. Rec.* 234: 215-222.

Ananthanarayanan, M., Ng, O.C., Boyer, J.L. and Suchy, F.J. (1994) Characterization of cloned rat liver Na+/bile acid cotransporter using peptide and fusion protein antibodies. *Am. J. Physiol.* 267: G637-G643.

Arbeeny, C.M., Meyers, D.S., Bergquist, K.E. and Gregg, R.E. (1992) Inhibition of fatty acid synthesis decreases very low density lipoprotein secretion in the hamster. *J. Lipid Res.* 33: 843-851.

Armstrong, M.E., Blake, W.L. and Clarke, S.D. (1991) Arachidonic acid suppression of fatty acid synthase gene expression in cultured rat hepatocytes. *Biochem. Biophys. Res. Comm.* 177: 1056-1061.

Auestad, N., Korsak, R.A., Morrown, J.W., West, D.B., Bergstrom, J.D. and Edmond, J. (1988) Lipogenic potential in liver of the preweanling rat: influence of dietary cholesterol. *FASEB J.* 2:3108-3112.

Auestad, N., Montalto, M.B., Hall, R.T., Fitzgeral, K.M., Wheeler, R.E., Connor, W.E., Neuringer, M., Connor, S.L., Taylor, J.A. and Hartmann, E.E. (1997) Visual acuity, erythrocyte fatty acid composition, and growth in term infants fed formulas with long chain polyunsaturated fatty acids for one year. Ross Pediatric Lipid Study. *Ped. Res.* 41: 1-10.

Bach, A.C. and Babayan V.K. (1982) Medium-chain triglycerides: an update. *Am. J. Clin. Nutr.* 36: 950-962.

Barnard, J.A., Ghishan, F.K. and Wilson, F.A. (1985) Ontogenesis of taurocholate transport by rat ileal brush border membrane vesicles. *J Clin Invest* 75: 869-973.

Barter, P.J., Ha, Y.C., and Calvert, G.D. (1981) Studies of esterified cholesterol in subfractions of plasma high density lipoproteins. *Atherosclerosis* 38: 165-175.

Beisiegel, U. (1996) New aspects on the role of plasma lipases in lipoprotein catabolism and atherosclerosis. *Atherosicerosis* 124: 1-8.

Bennet, M.K., Lopez, J.M., Sanchez, H.B. and Osborne, T.F. (1995) Sterol regulation of fatty acid synthase promoter. *J. Biol. Chem.* 270: 25578-25583.

Bernback, S., Blackberg, L. and Hernell, O. (1990) The complete digestion of human milk triacylglycerol in vitro requires gastric lipase, pancreatic colipase-dependent lipase, and bile salt-stimulated lipase. *J. Clin. Invest.* 85: 1221-1226.

Berr, F., Goetz, A., Schreiber, E., Paumgartner, G. (1993) Effect of dietary n-3 versus n-6 polyunsaturated fatty acids on hepatic excretion of cholesterol in the hamster. *J. Lipid Res.* 34:1275-1284

Bertolotti, M., Spady, D.K. and Dietshcy, J.M. (1995) Regulation of hepatic cholesterol metabolism in the rat in vivo: effect of synthetic fat-free diet on sterol synthesis and low density lipoprotein transport. *Biochim Biophys Acta* 1255: 293-300.

Boehm, G., Moro, G.M. Muller, H., Raffler, G. and Minoli, I. (1995) Fecal cholesterol excretion in preterm infants fed breast milk or formula with different cholesterol contents. *Acta Paediatr.* 84: 240-244.

Boffelli, D., Compassi, S., Werder, M., Weber, F.E., Phillips, M.C., Schulthess, G. and Hauser, H. (1997) The uptake of cholesterol at the small-intestinal brush border membrane is inhibited by apolipoproteins. *FEBS Letters* 411: 7-11.

Bonanome, A. and Grundy, S.M. (1988) Effect of dietary stearic acid on plasma cholesterol and lipoprotein levels. *N. Engl. J. Med.* 318: 1244-1248.

Booker, M.L., Scott, T.E., LaMorte, W.W. (1990) Effects of dietary fish oil on biliary phospholipids and prostaglandin synthesis in the cholesterol-fed prairie dog. *Lipids* 25:27-32

Boren, J., Rustaeus, S., Wettesen, M., Andersson, M., Wiklund, A. and Olofsson, S.O. (1993) Influence of triacylglycerol biosynthesis rate on the assembly of apo B100-containing lipoproteins in HepG2 cells. *Arterioscler. Thromb.* 13: 1743-1754.

Borgstrom, B. (1975) On the interactions between pancreatic lipase and colipase and the substrate and the importance of bile salts. *J. Lipid Res.* 16:411-417.

Boulton, T.J.C. ad Magarey, A.M. (1995) Effects of differences in dietary fat on growth, energy and nutrient intake from infancy to eight years of age. *Acta Paediatr.* 84: 146-150.

Breedveld, B., Schoonderwoerd, K., Verhoeven, A.J.M., Willemsen, R. and Jansen, H. (1997) Hepatic lipase is localized at the parenchymal cell microvilli in rat liver. *Biochem. J.* 321: 425-430.

Briggs, M.R., Yokoyama, C., Wang, X., Brown, M.S. and Goldstein, J.L. (1993) Nuclear protein that binds sterol regulatory element of low density lipoprotein receptor promoter. *J. Biol. Chem.* 268: 14490-14496.

Brindley, D.N. and Hubscher, G. (1965) *Biochim. Biophys. Acta* 106:495-509.

Brown, M.S. and Goldstein, J.L. (1986) A receptor-mediated pathway for cholesterol homeostasis. *Science* 232: 34-47.

Carlson, S.E., Ford, A.J., Werkman, S.H., Peeples, J.M. and Koo, W.W. (1996) Visual acuity and fatty acid status of term infants fed human milk and formulas with and without docosahexaenoate and arachidonate from egg yolk lecithin. *Ped. Res. 39: 882-888.*

Carriere, F., Barrowman, J.A., Verger, R. and Laugier, R. (1993) Secretion and contribution to lipolysis of gastric and pancreatic lipases during a test meal in humans. *Gastroenterology* 105: 876-888.

Carriere, F., Rogalska, E., Cudrey, C., Ferrato, F., Laugier, R. and Verger, R. (1997) In vivo and in vitro studies on the stereoselective hydrolysis of tri- and diglycerides by gastric and pancreatic lipase. *Bioorganic Medicinal Chem* 5: 429-435.

Chapman, M.J. (1980) Animal lipoproteins: chemistry, structure, and comparative aspects. J. Lipid Res. 21: 789-853.

Chautan, M., Chanussot, F., Portugal, H., Pauli, A.-M., Lafont, H. (1990) Effects of salmon oil and corn oil on plasma lipid levels and hepatobiliary cholesterol metabolism in rats. *Biochim. Biophys. Acta* 1046: 40-45.

Chelly, J. and Kahn, A. (1994) RT-PCR and mRNA quantitation. In: Mullis, K.B., Ferre, R. and Gibbs, R.A. (eds) *Polymerase Chain Reaction.* Birkhuser, Boston, MA. p.97-109.

Chen, S.-H., Habib, G., Yang, C.-Y., Gu, Z.-W., Lee, B.R., Weng, S.-A., Silbermann, S.R., Cai, S.-J., Deslypere, J.P., Rosseneu, M., Gotto, A.M., Li, W.-H. and Chan, L.

(1987) Apolipoprotein B-48 is the product of a messenger RNA with an organ-specific in-frame stop codon. *Science* 238: 363-366.

Chen, Q., Sternby, B., Atesson, B., and Nilsson, A. (1990) Effects of human pancreatic lipase-colipase and carboxyl ester lipase on eicosapentaenoic and arachidonic acid ester bonds of triacylglycerols rich in fish oil fatty acids. *Biochim. Biophys. Acta* 1044:111-117

Chen, P.S., Toribara, T.Y. and Warner, H. (1956) Microdetermination of phosphorus. *Anal. Chem.* 28: 1756-1758.

Cheng, C.F., Oosta, A., Bensadoun, A. and Rosenberg, R.D. (1981) Binding of lipoprotein lipase to endothelial cells in culture. *J. Biol. Chem.* 256: 12893-12896.

Chiang, J.Y.L. and Stroup, D. (1994) Identification and characterization of a putative bile acid-responsive element in cholesterol 7α-hydroxylase gene promoter. *J. Biol. Chem.* 269: 17502-17507.

Chin, D.J., Luskey, K.L., Faust, J.R., MacDonald, R.J., Brown, M.S. and Goldstein, J.L. (1982) Molecular cloning of 3-hydroxy-3-methylglutaryl coenzyme A reductase and evidence for regulation of its mRNA. *Proc. Natl. Acad. Sci.* 79: 7704-7708.

Chirgwin, J., Przybyla, A., MacDonald, R. and Rutter, W. (1979) Isolation of biologically active ribonucleic acid from sources enriched in ribonucleases. *Biochemistry* 18: 5294-5298.

Choi, J.W., Lundquist, E.N. and Peffley, D.M. (1993) Inhibition of protein synthesis in baby-hamster kidney cells blocks oxysterol-mediated suppression of 3-hydroxy-methylglutaryl-CoA reductase mRNA at a post-transcriptional levels. *Biochem. J.* 296: 859-866.

Chomczynski, P. and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162 156-159

Cianflone, K.M., Yasruel, Z., Rodriguez, M.A., Vas, D. and Sniderman, A.D. (1990) Regulation of apo B secretion from HepG2 cells: evidence for a critical role for cholesteryl ester synthesis in the response to a fatty acid challenge. *J. Lipid Res.* 31: 2045-2055.

Clarke, C.F., Fogelman, A.M. and Edwards, P.A. (1985) Transcriptional regulation of 3-hydroxy-3methylglutaryl coenzyme A reductase gene in rat liver. *J. Biol. Chem.* 260: 14363-14367.

Clarke, B.A. and Clarke, S.D. (1982) Supression of rat liver fatty acid synthesis by eicosa-5,8,11,14-tetraeynoic acid without a reduction in lipogenic enzymes. *J. Nutr.* 112: 1212-1219.

Clarke, S.D. and Jump, D.B. (1993) Regulation of hepatic gene expression by dietary fats: a unique role for polyunsatured fatty acids. In: Berdanier, C.D. and Hargrove, J.L. eds. *Nutrition and Gene Expression.* CRC Press, Boca Raton, FL. p.227-246.

Clarke, S.D. and Jump, D.B. (1996) Polyunsaturated fatty acid regulation of hepatic gene transcription. *J. Nutr.* 126: 1105S-1109S.

Clarke, S.D., Armstrong, M.K. and Jump, D.B. (1990) Dietary polyunsaturated fats uniquely suppress rat liver fatty acid synthase and S14 mRNA content. *J. Nutr.* 120: 225-232.

Clarke, S.D., Turini, M. and Jump, D. (1997) Polyunsaturated fatty acids regulate lipogenic and peroxisomal gene expression by independent mechanisms. *Prostaglandins, Leukotrienes Essential Fatty Acids.* 57: 65-69.

Crestani, M., Karam, W.G., and Chiang, J.Y.L. (1994) Effects of bile acids and steroid/thyroid hormones on the expression of cholesterol 7-α-hydroxylase mRNA and the CYP7 gene in HepG2 cells. *Biochem. Biophys. Res. Commun.* 198: 546-553.

Daumerie, C.M., Woollett, L.A. and Dietschy, J.M. (1992) Fatty acids regulate hepatic low density lipoprotein receptor activity through redistribution of intracellular cholesterol pools. *Proc. Natl. Acad. Sci.* 89: 10797-10801.

Dauncey, M.J. and Morovat, A. (1993) Investigation of mechanisms mediating the increase in plasma concentrations of thyroid hormones after a meal in young growing pigs. *J. Endocrinol.* 139: 131-141.

Davis, P.J. and Poznansky, M.J. (1987) Modulation of 3-hydroxy-3-methylglutaryl-CoA redcutase by changes in microsomal cholesterol content or phospholipid composition. *Proc. Natl. Acad. Sci.* 84: 118-121.

Dawson, P.A., Hofmann, S.L., Van der Westhuyzen, D.R., Sudhof, T.C., Brown, M.S. and Goldstein, J.L. (1988) Sterol-dependent repression of low density lipoprotein receptor promoter mediated by 16-base pair sequence adjacent to binding site for transcription factor Sp1. *J. Biol. Chem.* 263: 3372-3379.

Decsi, T., Fekete, M. and Koletzko, B. (1997) Plasma lipid and apolipoprotein concentrations in full term infants fed formula supplemented with long-chain polyunsaturated fatty acids and cholesterol. *Eur. J. Pediatr.* 156:397-400.

DeFaria, E., Fong, L.G., Komaromy, M. and Cooper, A.D. (1996) Relative roles of the LDL receptor, the LDL receptor-like protein, and hepatic lipase in chylomicron remnant removal by the liver. *J. Lipid Res.* 37: 197-209.

Dietschy, J.M., Sallee, V.L. and Wilson, F.A. (1971) Unstirred water layers and absorption across the intestinal mucosa. *Gastroenterology* 61: 932-934.

Dietschy, J.M. and Spady, D.K. (1985) Quantitative importance of different organs for cholesterol synthesis and low-density lipoprotein degradation. *Biochem. Soc. Trans.* 11: 639-641.

DISC Collaborative Research Group (1995) Efficacy and safety of lowering dietary intake of fat and cholesterol in children with elevated low-density lipoprotein cholesterol. The dietary intervention study in children (DISC). *J. Am. Med. Assoc.* 273: 1429-1435.

Dueland, S., Drisko, J., Graf, L., Machleder, D., Lusis, A.J. and Davis, R.A. (1993) Effect of dietary cholesterol and taurocholate on cholesterol 7α-hydroxylase and hepatic LDL receptors in inbred mice. *J. Lipid Res.* 34: 923-931.

Fall, C.H.D., Barker, D.J.P., Osmond, C., Winter, P.D., Clark, P.M.S. and Hales, C.N. (1992) Relation of infant feeding to adult serum cholesterol concentration and death from ischaemic heart disease. *Br. Med. J.* 304: 801-805.

Fernando-Warnakulasuriya, J.P.G., Staggers, J.E., and Wells, M.A. (1981) Studies on fat digestion, absorption, and transport in the suckling rat. I. Fatty acid composition and other major lipid components. J. Lipid Res. 22: 668-674.

Field, F.J. and Mathur, S.N. (1995) Intestinal lipoprotein synthesis and secretion. *Prog. Lipid Res.* 34: 185-198.

Field, F.J., Albright, E.J., and Mathur, S.N. (1987) Effect of dietary n-3 fatty acids on HMG-CoA reductase and ACAT activities in liver and intestine of the rabbit. *J. Lipid Res.* 28: 50-58

Field, F.J., Shreves, T., Fujiwara, D., Murthy, S., Albright, E. and Mathur, S.N. (1991) Regulation of gene expression and synthesis and degradation of 3-hydroxy-3-methylglutaryl coenzyme A reductase by micellar cholesterol in CaCo-2 cells. *J. Lipid Res.* 32: 1811-1821.

Fielding, C.J. and Fielding, P.E. (1995) Molecular physiology of reverse cholesterol transport. *J. Lipid Res.* 36: 211-228.

Filichkin, S.A. and Gelvin, S.B. (1992) Effect of dimethyl sulfoxide concentration on specificity of primer matching in PCR. *Biotechniques.* 12: 828-830.

Folch, J., Lees, M. and Sloan-Stanley, G.H. (1957) A simple method for total lipid extraction and purification. *J. Biol. Chem.* 226: 497-509.

Frøyland, L., Vaagenes, H., Asiedu, D.K., Garras, A., Lie, Ø., Berge, R.K. (1996) Chronic administration of eicosapentaenoic acid and docosahexaenoic acid as ethyl esters reduced plasma cholesterol and changed the fatty acid composition in rat blood and organs. *Lipids* 31:169-178.

Fujita, M., Fujii, H., Kanda, T., Sato, E., Hatakeyama, K., and Ono, T. (1995) Molecular cloning, expression, and characterization of a human intestinal 15-kDa protein. *Eur. J. Biochem.* 233: 406-413.

Fungwe, T.V., Cagen, L., Wilcox, H.G. and Heimberg, M. (1992) Regulation of hepatic secretion of very low density lipoprotein by dietary cholesterol. *J. Lipid Res.* 33: 179-191.

Fungwe, T.V., Cagen, L.M., Cook, G.A., Wilcox, H.G. and Heimberg, H. (1993) Dietary cholesterol stimulates hepatic biosynthesis of triglyceride and reduces oxidation of fatty acids in the rat. *J. Lipid Res.* 34: 933-941.

Fungwe, T.V., Cagen, L.M., Wilcox, H.G. and Heimberg, M. (1994a) Effects of dietary cholesterol on hepatic metabolism of free fatty acid and secretion of VLDL in the hamster. *Biochem. Biophys. Res. Comm.* 200: 1505-1511.

Fungwe, T.V., Fox, J.E., Cagen, L.M., Wilcox, H.G. and Heimberg, M. (1994b) Stimulation of fatty acid biosynthesis by dietary cholesterol and of cholesterol synthesis by dietary fatty acid. *J. Lipid Res.* 35: 311-318.

Gallo, L.L., Clark, S.B., Myers, S. and Vahouny, G.V. (1984) Cholesterol absorption in rat intestine: role of cholesterol esterase and acyl coenzyme A:cholesterol acyltransferase. *J. Lipid Res.* 25: 604-612.

Gantz, I., Nothwehr, S.F., Lucey, M., Sacchettini, J.C., DelValle, J., Banaszak, L.J., Naud, M., Gordon, J.L. and Yamada, T. (1989) Gastrotropin: not an enterooxyntin but a member of a family of cytoplasmic hydrophobic ligand binding proteins. *J. Biol. Chem.* 264: 20248-20254.

Gargouri, Y., Moreau, H., Verger, R. (1989) Gastric lipases: Biochemical and physiological studies. *Biochim. Biophys. Acta* 1006: 255-271.

Geelen, M.J.H., Schoots, W.J., Bijleveld, C. & Beynen, A.C. (1995) Dietary medium-chain fatty acids raise and (n-3) polyunsaturated fatty acids lower hepatic triacylglycerol synthesis in rats. *J. Nutr.* 125: 2449-2456.

Gidez, L.I., Miller, G.J., Burstein, M., Slagele, S., Eder, H.A. (1982) Separation and quantitation of subclasses of human plasma high density lipoproteins by a simple precipitation procedure. *J. Lipid Res.* 23: 1206-1223.

Ginsburg, B.-E. and Zetterstrom, R. (1980) Serum cholesterol concentrations in early infancy. *Acta Paediatr Scand* 69: 581-585.

Goldberg, I.J. (1996) Lipoprotein lipase and lipolysis: central roles in lipoprotein metabolism and atherogenesis. *J. Lipid Res.* 37: 393-707.

Goldberg, I.J., Scheraldi, C.A., Yacoub, L.K., Saxena, U. and Bisgaier, C.L. (1990) Lipoprotein apoC-II activation of lipoprotein lipase. Modulation by apoA-IV. *J. Biol. Chem.* 265: 4266-4272.

Goldstein, J.L. and Brown, M.S. (1990) Regulation of the mevalonate pathway. *Nature.* 343: 425-430.

Gong, Y.Z., Everett, E.T., Schwartz, D.A., Norris, J.A. and Wilson, F.A. (1994) Molecular cloning, tissue distribution, and expression of a 14-kDa bile acid-binding protein from rat ileal cytosol. *Proc. Natl. Acad. Sci.* 91: 4741-4745.

Greeve, J., Altkemper, I., Dieterich, J.-H., Greten, H. and Windler, E. (1993) Apolipoprotein B mRNA editing in 12 different mammaliam species: hepatic expression is reflected in low concentrations of apoB-containing plasma lipoproteins. *J. Lipid Res.* 34:1367-1383.

Grosvenor, C.E., Picciano, M.F. and Baumrucker, C.R. (1993) Hormones and growth factors in milk. *Endocrine Rev.* 14: 710-728.

Grundy, S.M. (1983) Absorption and metabolism of dietary cholesterol. *Annual Rev. Nutr.* 3: 71-96

Gurantz, D., Laker, M.F., Hofman, A.F. (1981) Enzymatic measurement of choline containing phospholipids in bile. *J. Lipid Res.* 22: 373-376.

Ha, Y.C. and Barter, P.J. (1982) Differences in plasma cholesteryl trransfer activity in sixteen vertebrate species. *Comp. Biochem. Physiol.* 71B: 265-269.

Hackman, A.M, Pond, W.G., Mersmann, H.J., Wong, W.W., Krook, L.P. and Zhang, S. (1996) Obese pigs fed a high cholesterol diet from birth to 2 months are less susceptible than lean pigs to atherosclerosis. *J. Nutr.* 126: 564-573.

Hagenbuch, B., Stieger, B., Foguet, M., Lubbert, H. and Meier, P.J. (1991) Functional expression, cloning and characterization of the hepatocyte Na^+/bile acid cotransport system. *Proc. Natl. Acad. Sci.* 88: 10629-10633.

Harrison, A.P., Tivey, D.R., Clausen, T., Duchamp, C. and Dauncey, M.J. (1996) Role of thyroid hormones in early postnatal development of skeletal muscle and its implications for undernutrition. *Brit. J. Nutr.* 76: 841-855.

Haugen, R. and Norum, K.R. (1976) Coenzyme-A-dependent esterification of cholesterol in rat intestinal mucosa. *Scand. J. Gastroenterol.* 11: 615-621.

Hernell, O. and Blackber, L. (1994) Human milk bile salt-stimulated lipase: functional and molecular aspects. *J. Pediatr.* 125: S56-S61.

Herz, J., Qui, S.Q., Oesterle, A., Desilva, H.V., Shafi, S. and Havel, R.J. (1995) *Proc. Natl. Acad. Sci.* 92: 4611-4615.

Heubi, J.E., Balistreri, W.F. and Suchy, F.J. (1982) Bile salt metabolism in the first year of life. *J. Lab. Clin. Med.* 100: 127-136.

Heuman, D.M., Mills, A.S., McCall, J., Hylemaon, P.B., Pandak, W.M. and Vlahcevic, Z.R. (1991) Conjugates of ursodeoxycholate protect against cholestasis and hepatocellular necrosis caused by more hydrophobic bile salts. *Gastroenterology* 100: 203-211.

Hollanders, B., Aude, X and Girard-Globa, A. (1985) Lipoproteins and apoproteins of fetal and newborn piglets. *Biol. Neonate* 47: 270-279.

Horton, J.D., Cuthbert, J.A. and Spady, D.K. (1993) Dietary fatty acids regulated hepatic low density lipoprotein (LDL) transport by altering LDL receptor protein and mRNA levels. *J. Clin. Invest.* 92: 743-749.

Howles, P.N., Carter, C.P. and Hui, D.Y. (1996) Dietary free and esterified cholesterol absorption in cholesterol esterase (bile salt-stimulated lipase) gene-targeted mice. *J. Biol. Chem.* 271: 7196-7202.

Hroboticky, N., MacKinnon, M.J., Puterman, M.S. and Innis, S.M. (1990) Effect of a vegetable oil formula rich in linoleic acid on tissue fatty acid accretion in the brain, liver, plasma and erythrocytes of infant piglets. *Am. J. Clin. Nutr.* 51: 173-182.

Huisman, M., Van Beusekom, C.M., Nijeboer, H.J., Muskiet, F.A.J. and Boersma, E.R. (1996) Triglyercerides, fatty acids, sterols, mono- and disaccharides and sugar alcohols in human milk and current types of infant formula milk. *Eur. J. Clin. Nutr.* 50:255-260.

Humphries, S.E. (1993) Life style, genetic factors and the risk of heart attack: The apolipoprotein B gene as an example. *Biochem. Soc. Trans.* 21: 569-582.

Humphries, S.E., Peacock, R.E. and Talmud, P.J. (1995) The genetic determinants of plasma cholesterol and response to diet. *Baill. Clin. Endocrin. Metabol.* 9: 797-823.

Hussain, M.M., Kancha, R.K., Zhou, Z., Luchoomun, J., Zu, H. and Bakillah, A. (1996) Chylomicron assembly and catabolism: role of apolipoproteins and receptors. *Biochim. Biophys. Acta* 1300: 151-170.

Innis, S.M. (1992) Human milk and formula fatty acids. *J. Ped.* 120: S56-S61.

Innis, S.M. (1993) The colostrum-deprived piglet as a model for the study of infant lipid nutrition. *J. Nutr.* 123: 386-390.

Innis, S.M., Akrabawi, S.S., Diemsen-Schade, D.A., Dobson, M.V. & Guy, D.G. (1997) Visual acuity and blood lipids in term infants fed human milk or formulae. *Lipids* 32: 63-72.

Innis. S.M. and Dyer, R.A. (1997) Dietary triacylglycerols with palmitic acid (16:0) in the 2-position increase 16:0 in the 2-position of plasma and chylomicron triacylglycerols, but reduce phospholipid arachidonic and docosahexaenoic acid, and alter cholesteryl ester metabolism in formula-fed piglets. *J. Nutr.* 127: 1311-1319.

Innis, S.M., Nelson, C.M., Rioux, M.F. & King, D.J. (1994) Development of visual acuity in relation to plasma and erythrocyte ω-6 and ω-3 fatty acids in healthy term gestation infants. *Am. J. Clin. Nutr.* 60: 347-352.

Innis, S.M., Quinlan, P.T. and Nelson, C.M. (1998) Structured triacylglycerols in infant nutrition and metabolism. In: Lipids in Infant Nutrition ed. Lein, E. AOCS Press, Champaign, Illinois.

Jackson, B., Gee, A.N., Martinez-Cayuela, M. and Suckling, K.E. (1990) The effects of feeding a saturated fat-rich diet on enzymes of cholesterol metabolism in the liver, intestine and aorta of the hamster. *Biochim. Biophys. Acta* 1045: 21-28.

Jackson, E.M., Lewis, D.S., McMahan, C.A. and Mott, G.E. (1993) Preweaning diet affects bile lipid composition and bile acid kinetics in infant baboons. *J. Nutr.* 123: 1471-1479.

Jackson, R.L., Baker, H.N., Taunton, O.S., Smith, L.C., Garner, C.W., Gotto, A.M. (1973) A comparison of the major apolipoprotein from pig and human high density lipoproteins. *J. Biol. Chem.* 248: 2639-2644.

Jacobson, P.W., Wiesenfeld, P.W. and Gallo, L.L. (1990) Sodium cholate-induced changes in the conformation and activity of rat pancreatic cholesterol esterase. *J. Biol. Chem.* 265: 515-521.

Jayakumar, A., Tai, M-H., Huang, W-Y., Al-Feel, W., Hsu, M., Abu-Elheiga, L., Chirala, S.S. and Wakil, S.J. (1995) Human fatty acid synthase: Properties and molecular cloning. *Proc. Natl. Acad. Sci.* 92: 8695-8699.

Jensen, R. and Jensen, G. (1992) Specialty lipids for infant nutrition. I. Milks and formulas. *J. Ped. Gastr. Nutr.* 15: 232-245.

Jones, P.J.H., Hrboticky, N., Hahn, P., Innis, S.M. (1990) Comparison of breast and formula feeding on intestinal and hepatic cholesterol metabolism in the neonatal pig. *Am. J. Clin. Nutr.* 51: 979-984.

Jump, D.B., Clarke, S.D., Thelen, A. and Liimatta, M. (1994) Coordinate regulation of glycolytic and lipogenic gene expression by polyunsaturated fatty acids. *J Lipid Res* 35: 1076-1084.

Kallio, M.J.T., Salmenpera, L., Siimes, M.A., Perheentupa, J. and Miettinen, T.A. (1993) Tracking of serum cholesterol and lipoprotein levels from the first year of life. *Pediatrics* 91: 949-954.

Kallio, M.J.T., Salmenpera, L., Siimes, M.A., Perheentupa, J., Gylling, H. and Miettinen, T.A. (1997) Apoprotein E phenotype determines serum cholesterol in infants during both high-cholesterol breast feeding and low-cholesterol formula feeding. *J. Lipid Res.* 38: 759-764.

Katoku, y., Yamada, M., Yonekubo, A., Kuwata, T., Kobayashi, A. and Sawa, A. (1996) Effect of the cholesterol content of a formula on the lipid compositions of plasma lipoproteins and red blood cell membranes in early infancy. *Am. J. Clin. Nutr.* 64: 871-877.

Katsurada, A., Iritani, N., Fukuda, H., Matsumura, Y., Nishimoto, N., Noguchi, T. and Tanaka, T. (1990) Effects of nutrients and hormones on transcriptional and post-transcriptional regulation of acetyl-CoA carboxylase in rat liver. *Eur. J. Biochem.* 190: 435-441.

Kawabe, Y., Sato, R., Matsumoto, A., Hondan, M., Wada, Y., Yazaki, Y., Endo, A., Takano, T., Itakura, H. & Kodama, T. (1996) Regulation of fatty acid synthase expression by cholesterol in human cultured cells. *Biochem. Biophys. Res. Comm.* 219: 515-520.

Keller, H., Dreyer, C., Medin, J., Mahfoudi, A., Ozato, K. and Wahli, W. (1993) Fatty acids and retinoids control lipid metabolism through activation of peroxisome proliferator-activated receptor-retinoid X receptor heterodimers. *Proc. Natl. Acad. Sci.* 90: 2160-2164.

Koldovsky, O. and Thornburg, W. (1987) Hormones in human milk. *J. Pediatr. Gastroenterol. Nutr.* 6: 172-196.

Kramer, W., Buscher, H.P., Gerok, W. and Kurz, G. (1979) Bile salt binding to serum components. Taurocholate incorporation into high-density lipoproteins revealed by photoaffinity labelling. *Eur. J. Biochem.* 102: 1-9.

Kramer, W., Girbig, F., Gutjahr, U., Kowaleski, S., Jouvenal, K., Muller, G., Tripier, D. and Wess, G. (1993) Intestinal bile acid absorption. *J. Biol. Chem.* 268: 18035-18046.

Kumar, S. & Dodds, P.F. (1981) Fatty acid synthase from lactating bovine mammary gland. *Methods Enzymol.* 71: 86-97.

Kurushima, H., Hayashi, K., Shingu, T., Kuga, Y., Ohtani, H., Okura, Y. (1995) Opposite effects on cholesterol metabolism and their mechanisms induced by dietary oleic acid and palmitic acid in hamsters. *Biochim. Biophys. ACTA* 1258: 251-256.

Lane, D.M. and McConathy, W.J. (1986) Changes in the serum lipids and apolipoproteins in the first four weeks of life. *Pediatr. Res.* 20: 332-337.

Lapinleimu, H., Viikari, J., Jokinen, E., Salo, P., Routi, T., Leino, A., Ronnemaa, T., Seppanen, R., Valimaki, I. And Simell, O. (1995) Prospective randomised trial in 1062 infants of diet low in saturated fat and cholesterol. *Lancet* 345: 471-476.

Lein, E.L. (1994) The role of fatty acid composition and positional distribution in fat absorption in infants. *J. Pediatr.* 125: S62-S68.

Lepage, G., and Roy, C.C. (1986) Direct transterification of all classes of lipids in a one-step reaction. *J. Lipid Res.* 27: 114-120.

Lewis, D.S., McMahan, C.A. and Mott, G.E. (1993) Breast feeding and formula feeding affect differently plasma thyroid hormone concentrations in infant baboons. *Biol. Neonate* 63: 327-335.

Lin, M.C., Gong, Y., Geoghegan, K. and Wilson, F. (1991) Characterization of a novel 14kDa bile acid-binding protein from rat ileal cytosol. *Biochim. Biophys. Acta* 1078: 329-335.

Lipka, G., Schulthess, G., Thurnhofer, H., Wacker, H., Wehrli, E., Zeman, K., Weber, F.E. and Hauser, H. (1995) Characterization of lipid exchange proteins isolated from small intestinal brush border membrane. *J. Biol. Chem.* 270: 5917-5925.

Liscum, L., Luskey, K.L., Chin, D.L., Ho, Y.K., Goldstein, J.L. and Brown, M.S. (1983) Regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase and its mRNA in rat liver as studied with a monoclonal antibody and a cDNA probe. *J. Biol. Chem.* 258: 8450-8455.

Liu, C.-H., Huang, M.-T. and Huang, P.-C. (1995) Sources of triacylglycerol accumulation in livers of rats fed a cholesterol-supplemented diet. *Lipids* 30: 527-531.

Lopez-Candales, A., Bosner, M.S., Spilburg, C.A. and Lange, L. G. (1993) Cholesterol transport function of pancreatic cholesterol esterase: uptake and esterification in enterocytes. *Biochemistry* 32:12085-12089.

Lopez-Candales, A., Grosjlos, J., Sasser, T., Buddhiraju, C., Scherrer, D., Lange, L.G. and Kumar, V.B. (1996) Dietary induction of pancreatic cholesterol esterase: a regulatory cycle for the intestinal absorption of cholesterol. *Biochem. Cell Biol.* 74: 257-264.

Lopez, J.M., Bennett, M.K., Sanchez, H.B., Rosenfeld, J.M., and Osborne, T.F. (1996) Sterol Regulation of Acetyl Coenzyme A Carboxylase: A Mechanism for Coordinate Control of Cellular Lipid. *Proc. Natl. Acad. Sci.* 93: 1049-1053.

Lourdes, M., Cruz, A., Wong, W.W., Mimouni, F., Hachey, D.L., Setchell, K.D.R., Klein, P.D. and Tsang, R.C. (1994) Effects of infant nutrition on cholesterol synthesis rates. *Pediatr. Res.* 35: 135-140.

Lowry, O.H., Rosebrough, N.S., Farr, A.L. et al. (1952) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193: 256-275.

Luskey, K. and Stevens, B. (1985) Human 3-hydroxy-3-methylglutaryl coenzyme A reductase. Conserved domains responsible for catalytic activity and sterol regulated degradation. *J. Biol. Chem.* 260: 10271-10277.

MacGinnitie, A.J., Anant, S. and Davidson, N.O. (1995) Mutagenesis of apobec-1, the catalytic subunit of the mammalian apolipoprotein B mRNA editing enzyme, reveals distinct domains that mediate cytosine nucleoside deaminase, RNA binding, and RNA editing activity. *J. Biol. Chem.* 270: 14768-14775.

Mackay, K., Starr, J.R., Lawn, R.M. and Ellsworth, J.L. (1997) Phosphatidlylcholine hydrolysis is required for pancreatic cholesterol esterase- and phospholipase A2-facilitated cholesterol uptake into intestinal Caco-2 cells. *J. Biol. Chem.* 272: 13380-13389.

Makrides, M., Neumann, M.A., Byard, R.W., Simmer, K. & Gibson, R.A. (1994) Fatty acid composition of brain, retina, and erythrocytes in breast- and formula-fed infants. *Am. J. Clin. Nutr.* 60: 189-194.

Martin, J.-C., Bougnoux, P., Antoine, J.M., Lanson, M., and Louet, C. (1993) Triacylglycerol structure of human colostrum and mature milk. *Lipids* 28:637-643.

Mashige, F., Tanaka, N., Maki, A. et al. (1981) Direct spectrophotometry of total bile acids in serum. *Clin. Chem.* 27: 1352-1356.

Mattson, F.H. and Grundy, S.M. (1985) Comparison of effects of dietary saturated, monounsaturated, and polyunsaturated fatty acids on plasma lipids and lipoproteins in man. *J. Lipid Res.* 26: 194-202.

McCracken, B.A., Gaskins, H.R., Ruwe-Kaiser, P.J., Klasing, K.C. and Jewell, D.E. (1995) Diet-dependent and diet-independent metabolic responses underlie growth stasis of pigs at weaning. *J. Nutr.* 125: 2838-2845.

McGowan, M.W., Artiss, J.D., Strandberg, D.R. and Zak, B. (1983) A peroxidase coupled method for the colorimetric determination of serum triglycerides. *Clin. Chem.* 29: 538-542.

Mize, C.E., Uauy, R., Kramer, R., Benser, M., Allen, S. and Grundy, S. (1995) Lipoprotein cholesterol responses in healthy newborn infants defined diets from ages 1 to 12 months: comparison of diets predominant in linolenic acid versus linoleic acid, with parallel observations in infants fed a human milk-based diet. *J. Lipid Res.* 36: 1178-1167

Moreau, H., Laugier, J.F., Gargouri, Y., Ferrato, F., Verger, R. (1988) Human preduodenal lipase is entirely of gastric fundic origin. *Gastroenterology* 95: 1221-1226.

Mortimer, B.-C., Kenrick, M.A., Holthouse, D.J., Stick, D.V. and Redgrave, T.G. (1992) Plasma clearance of model lipoproteins containing saturated and polyunsaturated monoacylglycerols injected intravenously in the rat. *Biochim. Biophys. Acta* 1127: 67-73.

Mott, G.E., McMahan, C.A., and McGill, H.C. (1978) Diet and sire effects on serum cholesterol and cholesterol absorption in infant baboons (*Papio cynocephalus*). *Circulation Res.* 43:364-371.

Mott, G.E.,McMahan, C.A., Kelley, J.L., Farley, C.M. and McGill, H.C. (1982) Influence of infant and juvenile diets on serum cholesterol, lipoprotein cholesterol, and apolipoprotein concentrations in juvenile baboons (Papio sp.). *Atherosclerosis* 45: 191-202.

Mott, G.E., Jackson, E.M., McMahan, C.A., Farley, C.M. and McGill, H.C. (1985) Cholesterol metabolism in juvenile baboons. *Arteriosclerosis* 5: 347-354.

Mott, G.E., DeLallo, L, Driscoll, D.M., McMahon, C.A. and Lewis, D.S. (1993) Influence of breast and formula feeding on hepatic concentrations of apolipoprotein and low-density lipoprotein receptor mRNAs. *Biochim. Biophys. ACTA* 1169: 59-65.

Mott, G.E., Jackson, E.M., DeLallo, L., Lewis, D.S. and McMahan, C.A. (1995) Differences in cholesterol metabolism in juvenile baboons are programmed by breast-versus formula-feeding. *J. Lipid Res.* 36: 299-307.

Mott, G.E., Jackson, E.M. and McMahan, C.A. (1991) Bile composition of adult baboons is influenced by breast versus formula feeding. *J. Ped. Gastr. Nutr.* 12: 121-126.

Mott, G.E., Lewis, D.S. and McMahan, C.A. (1990) Cholesterol metabolism in adult baboons is influence by infant diet. *J. Nutr.* 120: 243-251.

Mustad, V.A., Ellsworth, J.L., Cooper, A.D. Kris-Etherton and Etherton, T.D. (1996) Dietary linoleic acid increases and palmitic acid decreases hepatic LDL receptor protein and mRNA abundance in young pigs. *J. Lipid Res.* 37: 2310-2323.

Nakanishi, M., Goldstein, J.L. and Brown, M.S. (1988) Multivalent control of 3-hydroxy-3-methylglutaryl coenzyme A reductase. *J. Biol. Chem.* 263: 8929-8937.

Ness, G.C. (1994) Developmental regulation of the expression of genes encoding proteins involved in cholesterol homeostasis. *Am. J. Med. Gen.* 50: 355-357.

Ness, G.C. and Lopez, D. (1995) Transcriptional regulation of rat hepatic low-density lipoprotein receptor and cholesterol 7α-hydroxylase by thyroid hormone. *Arch. Biochem. Biophys.* 323: 404-408.

Ness, G.C., Miller, J.P., Moffler, M.H., Woods, L.S. and Harris, H.B. (1979) Perinatal development of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity in rat lung, liver and brain. *Lipids* 14: 447-450.

Ness, G.C., Pendleton, L.C. and Zhao, Z. (1994) Thyroid hormone rapidly increases cholesterol 7α-hydroxylase mRNA levels in hypophysectomized rats. *Biochim. Biophys. Acta* 1214: 229-233.

Neville, M.C. and Picciano, M.F. (1997) Regulation of milk lipid secretion and composition. *Annu. Rev. Nutr.* 17: 159-184.

Newman, W.P., Freedman, D.S., Voors, A.W. et al. (1986) Relation of serum lipoprotein levels and systolic blood pressure to early atherosclerosis: the Bogalusa Heart Study. *N. Engl. J. Med.* 314: 138-144.

Ng, S., Gunning, P., Eddy, R., Ponte, P., Leavitt, J., Shows, T. and Kedes, T. (1985) Evolution of the functional human β-actin gene and its multi-pseudogene family: Conservation of non-coding regions and chromosomal dispersion of pseudogenes. *Mole. Cell. Biol.* 5: 2720-2732.

Nilsson, J., Blackberg, L., Carlsson, P., Enerback, S., Hernell, O. and Bjursell, G. (1990) cDNA cloning of human-milk bile-salt-stimulated lipase and envidence for its identity to pancreatic carboxylic ester hydrolase. *Eur. J. Biochem.* 192: 543-550.

Nishimoto, M., Moshiro, M. and Okuda, K. (1993) Structure of the gene encoding human liver cholesterol-7-alpha-hydroxylase. *Biochim. Biophys. ACTA* 1172: 147-150.

Oberkotter, L.V., Pereira, G.R, Paul, M.H., Ling, H., Sasanow, S. and Farber, M. (1985) Effect of breast-feeding vs formula-feeding on circulating thyroxine levels in premature infants. *J. Ped.* 106: 822-825.

Ockner, R.K. and Manning, J. (1974) Fatty acid-binding protein in small intestine. Identification, isolation and evidence for its role in intracellular fatty acid transport. *J Clin. Invest.* 54: 326-338.

Osborne, T.F. (1991) Single nucleotide resolution of sterol regulatory region in promoter for 3-hydroxy-3-methylglutaryl coenzyme A reductase. *J. Biol. Chem.* 266: 13947-13951.

Osborne, T.F., Gil, G., Goldstein, J.L. and Brown, M.S. (1988) Operator constitutive mutation of 3-hydroxy-3-methylglutaryl coenzyme A reductase promoter abolishes protein binding to sterol regulatory element. *J. Biol. Chem.* 263: 3380-3387.

Pakula, R., Konikoff, F.M., Rubin, M., Ringel, Y., Peled, Y., Tietz, A. and Gilat, T. (1996) The effects of dietary phospholipids enriched with phosphatidylethanolamine on bile and red cell membrane lipids in humans. *Lipids* 31: 295-303.

Pandak, W.M., Vlahcevic, Z.R., Heuman, D.M., Redford, K.S., Chiang, J.Y.L., and Hylemon, P.B. (1994) Effects of different bile salts on steady-state mRNA levels and transcriptional activity of cholesterol 7-α-hydroxylase. *Hepatology* 19: 941-947.

Peled, Y. and Gilat, T. (1994) Effect of dietary phospholipid and their constituents on bile composition in rats and hamsters. *Hepatology* 19: 708-713.

Pesonen, E., Norio, R., Hirvonen, J. et al. (1990) Intimal thickening in the coronary arteries of infants and children as an indicator of risk factors for coronary heart disease. *Eur. Heart J.* 11: 53-60.

Piccoli, D.A., Maller, E.S. and Watkins, J.B. Bile salts: normal metabolism and pathophysiology. In: Gracey, M. and Burke, V. (eds) *Pediatric Gastroenterology and Hepatology*. p.835-855, Blackwell Scientific Publication, London, 1993.

Powell, E.E. and Kroon, P.A. (1994) Low density lipoprotein receptor and 3-hydroxy-3-methylglutaryl coenzyme A reductase gene expression in human mononuclear leukocytes is regulated coordinately and parallels gene expression in human liver. *J. Clin. Invest.* 93: 2168-2174.

Powell, L.M., Wallis, S.C., Pease, R.J., Edwards, Y.H., Knott, T.J. and Scott, J. (1987) A novel form of tissue-specific RNA processing produces apolipoprotein B-48 in intestine. *Cell* 50: 831-840.

Pussinen, P.J., Jauhiainen, M., Metso, J., Tyynela, J. and Ehnholm, C. (1995) Pig plasma phospholipid transfer protein facilitates HDL conversion. *J. Lipid Res.* 36: 975-985

Pussinen, P.J., Olkkonen, V.M., Jauhiainen, M., Ehnholm, C. (1997) Molecular cloning and functional expression of cDNA encoding the pig plasma phospholipid transfer protein. *J. Lipid Res.* 38: 1473-1481.

Raben, D.M., Jarpe, M.B., and Leach, K.L. (1994) Nuclear lipid metabolism in NEST: Nuclear envelope signal transduction. *J. Membrane. Biol.* 142: 1-7.

Ramirez, M.I., Karaoglu, D., Haro, D., Barillas,C., Bashirzadeh, R. and Gil, G. (1994) Cholesterol and bile acids regulate cholesterol 7α-hydroxylase expression at the transcriptional levels in culture and in transgenic mice. *Mole. Cell Biol.* 14: 2809-2821.

Redgrave, T.G., Kodali, D.R. and Small, D.M. (1988) The effect of triacyl-sn-glycerol structure on the metabolism of chylomicrons and triacylglycerol-rich emulsions in the rat. *J. Biol. Chem.* 263: 5118-5123.

Ren, B., Thelen, A.P., Peters, J.M., Gonzalez, F.J., and Jump, D.B. (1997) Polyunsaturated fatty acid suppression of hepatic fatty acid synthase and S14 gene expression does not require peroxisome proliferator-activated receptor α. *J. Biol. Chem.* 272: 26827-26832.

Rioux, M.F. and Innis, S.I. (1993) Cholesterol and fatty acid metabolism in piglets fed sow milk or infant formula with or without the addition of cholesterol. *Metabolism* 42: 1552-1559.

Rioux, M.F., Perea, A., Yousef, I.M., Levy,E., Malli, L., Carrillo, M.C. and Tuchweber, B. (1994) Short-term feeding of a diet enriched in phospholipids increases bile formation and the bile acid transport maximum in rats. *Biochim. Biophys. Acta* 1214: 193-202.

Rudel, L., Deckelman, C., Wilson, M., Scobey, M. and Anderson, R. (1994) Dietary cholesterol and downregulation of cholesterol 7α-hydroxylase and cholesterol absorption in African Green monkeys. *J. Clin. Invest.* 93: 2463-2472.

Rudling, M. (1992) Hepatic mRNA levels for the LDL receptor and HMG-CoA reductase show coordinate regulation in vivo. *J. Lipid Res.* 33: 493-501.

Rumsey, S., Galeano, N.F., Lipschitz, B., Deckelbaum, R.J. (1995) Oleate and other long chain fatty acids stimulate low density lipoprotein receptor activity by enhancing acyl coenzyme A: cholesterol acyltransferase activity and altering intracellular regulatory cholesterol pools in cultured cells. *J. Biol. Chem.* 270:10008-10016

Sacchettini, J.C., Hauft, S.M., Van Camp, S.L., Cistola, D.P. and Gordon, J.L. (1990) Developmental and structural studies of an intracellular lipid binding protein expressed in the ileal epithelium. *J. Biol. Chem.* 265: 19199-19207.

Schaffer, J.E. and Lodish, H.F. (1994) Expression, cloning and characterization of a novel adipocyte long chain fatty acid transport protein. *Cell* 79: 427-436.

Schneider, B.L., Dawson, P.A., Christie, D.M., Hardikar, W., Wong, M.G. and Suchy, F.J. (1995) Cloning and molecular characterization of the ontogeny of a rat ileal sodium-dependent bile acid transporter. *J. Clin. Invest.* 95: 745-747.

Scorci-Thomas, M., Hendricks, C.L., Kearns, M.W. (1992) HepG2 cell LDL receptor activity and the accumulation of apolipoprotein B and E in response to docosahexaenoic acid and cholesterol. *J. Lipid Res.* 33:1147-1156

Shamir, R., Johnson, W.J., Zolfaghari, R., Lee, H.S. and Fisher, E.A. (1995) Role of bile salt-dependent cholesteryl ester hydrolase in the uptake of micellar cholesterol by intestinal cells. *Biochemistry* 34: 6351-6358.

Sharp, D., Blinderman, L, Combs, K.A., Kienzle, B., Ricci, B., Wager-Smith, K., Gil, C.M., Wurck, C.W., Bouma, M.-E., Rader, D.J., Aggerbeck, L.P., Gregg, R.E., Gordon, D.A. and Wetterau, J.R. (1993) Cloning and gene defects in microsomal triglcyeride transfer protein associated with abetalipoproteinemia. *Nature* 365: 65-69.

Shefer, S., Nguyen, L.B., Salen, G., Ness, G.C., Chowdhary, I.R., Lerner, S., Batta, A.K. and Tint, G.S. (1992) Differing effects of cholesterol and taurocholate on steady state hepatic HMG-CoA reductase and cholesterol 7α-hydroxylase activities and mRNA levels in the rat. *J. Lipid Res.* 33:1193-1200.

Small, D.M. (1991) The effects of glyceride structure on absorption and metabolism. *Annual Rev. Nutr.* 11:413-434.

Smit, M.J., Temmerman, A.M., Wolters, H., Kuipers, F., Beijnen, A.C., Vonk, R.J. (1991) Dietary fish oil-induced changes in intrahepatic cholesterol transport and bile acid synthesis in rats. *J. Clin. Invest.* 88:943-951

Smit MJ, Verkade HJ, Havinga R, Vonk RJ, Scherphof GL, Veld GI, Kuipers F (1994) Dietary fish oil potentates bile acid-induced cholesterol secretion into bile in rats. *J. Lipid Res.* 35:301-310

Smith, J.L., Lear, S.R. and Erickson, S.K. (1995) Developmental expression of elements of hepatic cholesterol metabolism in the rat. *J. Lipid Res.* 36: 641-652.

Smith, J.R., Osborne, T.F., Goldstein, J.L. and Brown, M.S. (1990) Identification of nucleotides responsible for enhancer activity of sterol regulatory element in low density lipoprotein receptor gene. *J. Biol. Chem.* 265: 2306-2310.

Spady, D.K. and Cuthbert, J.A. (1992) Regulation of hepatic sterol metabolism in the rat. *J. Biol. Chem.* 267: 5584-5591.

Spady, D.K, Turely, S.D. and Dietschy, J.M. (1985) Rates of low density lipoprotein uptake and cholesterol synthesis are regulated independently in the liver. *J. Lipid Res.* 26: 465-470.

Specher, H., Luthria, D., Geiger, M., Mohammed, B.S., and Reinhart, M. (1994) Intercellular communication in fatty acid metabolism. *World Rev. Nutr. Diet.* 75:1-7.

Speijer, H.J., Groener, E.M., Van Ramshorst, E. and Van Tol, A. (1991) Different locations of cholesteryl ester transfer protein and phospholipid transfer protein activities in plasma. *Atherosclerosis* 90: 159-168.

Srivastava, R.A.K., Ito, H., Hess, M., Srivastava, N., and Schonfeld, G. (1995) Regulation of low density lipoprotein receptor gene expression in HepG2 and Caco2 cells by palmitate, oleate, and 25-hydroxycholesterol. *J. Lipid Res.* 36: 1434-1446.

Stals, H.K., Top, W., Declercq, P.E. (1994) Regulation of triacylglcyerol synthesis in permeablized rat hepatocytes. *FEBS Letters* 343: 99-102.

Straka, M.S., Junker, L.H., Zacarro, L., Zogg, D.L., Dueland, S., Everson, G.T. and Davis, R.A. (1990) Substrate stimulation of 7α-hydroxylase, an enzyme located in cholesterol-poor endoplasmic reticulum. *J. Biol. Chem.* 265: 7145-7149.

Stucchi, A.F., Terpstra, A.H.M. and Nicolosi, R.J. (1995) LDL receptor activity is down-regulated similarly by a diet high in palmitic acid or high in lauric and myristic acids in cynomologous monkeys. *J. Nutr.* 125: 2055-2063.

Sudhof, T.C., Van der Westhuyzen, D.R., Goldstein, J.L., Brown, M.S. and Russell, D.W. (1987) Three direct repeats and a TATA-like sequence are required for regulated expression of the human low density lipoprotein receptor gene. *J. Biol. Chem.* 262: 10773-10779.

Takeuchi, H., Matsuo, T., Tokuyama, K. and Suzuki, M. (1995) Serum triiodothyronine concentration and Na+,K+-ATPase activity in liver and skeletal muscle are influenced by dietary fat type in rats. *J. Nutr.* 125: 2364-2369.

Teng, B., Burant, C. and Davidson, N.O. (1993) Molecular cloning of an apolipoprotein B messenger RNA editing protein. *Science* 260: 1816-1819.

Thomson, A.B.R., Schoeller, C., Keelan, M., Smith, L. and Clandinin, M.T. (1993) Lipid absorption: Passing through the unstirred layers, brush-border membrane, and beyond. *Can. J. Physiol. Pharmacol.* 71: 531-555.

Toussant, M.J., Wilson, M.D. and Clarke, S.D. (1981) Coordinate suppression of liver acetyl-CoA carboxylase and fatty acid synthetase by polyunsaturated fat. *J. Nutr.* 111: 146-153.

Van Biervleit, J.P., Rosseneu, M. and Caster, H. (1986) Influence of dietary factors on the plasma lipoprotein composition and content in neonates. *Eur. J. Pediatr.* 144: 489-493.

Van Biervleit, J., Vercaemst, R., DeKeersigieter, W., Vionaimont, N., Caster, H. and Rosseneu, M. (1980) Evolution of lipoprotein patterns in newborns. *Acta Paediatr. Scand.* 69:581-585.

Van Deenen, L.L.M., DeHass, G.H. and Heemskerk, C.H. (1963) Hydrolysis of synthetic mixed acid phosphatides by phospholipase A from human pancreas. *Biochim Biophys Acta* 67: 295-304.

Varadaraj, K. and Skinner, S.M. (1994) Denaturants or cosolvents improve the specificity of PCR amplification of a G+C-rich DNA using genetically engineered DNA polymerase. *Gene* 140: 1-5.

Walz, D.A., Wider, M.D., Snow, J.W., Dass, C. and Desiderio, D.M. (1988) The complete amino acid sequence of porcine gastrotropin, an ileal protein wich stimulates gastric acid and pepsinogen secretion. *J. Biol. Chem.* 263: 14189-14195.

Wang, C.-S. and Hartsuck, J.A. (1993) Bile salt-activated lipase. A multiple lipolytic enzyme. *Biochim. Biophys. Acta* 1166: 1-19.

Wang, X., Briggs, M.R., Hua, X., Yokoyama, C., Goldstein, J.L. and Brown, M.S. (1993) Nuclear protein that binds sterol regulatory element of low density lipoprotien receptor promoter. *J Biol Chem* 268: 14497-144504.

Wang, X., Xato, R., Brown, M.S., Hua, X., and Goldstein, J.L. (1994) SREBP-1, a membrane bound transcription factor released by sterol-regulatory proteolysis. *Cell* 77:53-62.

Wilson, M.D. and Rudel, L.L. (1994) Review of cholesterol absorption with emphasis on dietary and biliary cholesterol. *J. Lipid Res.* 35:943-953.

Wong, M.H., Ulcers, P., Cardiac, A.L. and Damson, P.A. (1994) Expression, cloning and characterization of the hamster ileal sodium-dependent bile acid transporter. *J. Biol. Chem.* 269: 1340-1347.

Wong, W.W., Hachey, D.L., Insull, W., Opekun, A.R. and Klein, P.D. (1993) Effect of dietary cholesterol on cholesterol synthesis in breast-fed and formula-fed infants. *J. Lipid Res.* 34: 1403-1411.

Xu, G., Salen, G., Batta, A.K., Shefer, S., Nguyen, L.B., Niemann, W., Chen, T.S., Arora-Mirchandani, R., Ness, G.C., and Tint, G.S. (1992) Glycocholic acid and chenodeoxycholic acid but not glycoursocholic acid inhibit bile acid synthesis in the rabbit. *Gastroenterology* 102: 1717-1723.

Xu, G., Salen, G., Shefer, S., Ness, G.C., Nguyen, L.B., Parker, T.S., Chen, T.S., Zhao, A., Donnelly, T.M. and Tint, G.S. (1995) Unexpected inhibition of cholesterol 7α-hydroxylase by cholesterol in New Zealand White and Watanabe Heritable Hyperlipidemic rabbits. *J. Clin. Invest.* 95: 1497-1504.

Yamamoto, T., Davis, C.G., Brown, M.S., Schneider, W.J., Casey, M.L., Goldstein, J.L. and Russell, D.W. (1984) The human LDL receptor: a cysteine-rich protein with multiple Alu sequences in its mRNA. *Cell* 36: 27-38.

Yao, Z. and McLeod, R.S. (1994) Synthesis and secretion of hepatic apolipoprotein B-containing lipoproteins. *Biochim. Biophys. ACTA* 1212: 152-166.

Yao, Z. and Vance, D. (1988) The active synthesis of phosphatidylcholine is required for very low density lipoprotein secretion from rat hepatocytes. *J. Biol. Chem.* 263: 2998-3004.

Ziere, G.J., Van der Kaaden, M.E., Vogelezang, C.J.M., Boers, W., Bihain, B.E., Kuiper, J., Kruijt, J.K., Van Berkel, T.J.C. (1996) Blockade of the α_2-macroglobulin receptor/low-density-lipoprotein-receptor-related protein on rat liver parenchymal cells by the 39-kDa receptor-associated protein leaves the interaction of β-migrating very-low-density lipoprotein with the lipoprotein remnant receptor unaffected. *Eur. J. Biochem.* 242:703-711.

Zijlstra, R.T., Whang, K.-Y., Easter, R.A. and Odle, J. (1996) Effect of feeding milk replacer to early-weaned pigs on growth, body composition, and small intestinal morphology, compared with suckled littermates. *J. Anim. Sci.* 74: 2948-2959.

Appendix I.

The results of Study #1 are published in:

Devlin, A.M., Innis, S.M., Shukin, R. and Rioux, M.F.(1998) Early diet influences hepatic HMG-CoA reductase and 7-alpha-hydroxylase mRNA but not LDL receptor mRNA during development. *Metabolism* 47(1): 20-26.

Devlin, A.M. & Innis, S.M. (1998) Early diet influences hepatic lipogenesis. In: *Lipids In Infant Nutrition* (Huang, V., ed.), pp. 252-267. American Oil Chemist's Society Monograph, Champaign, Illinois.

The results of Study #1 have been presented at:

Devlin, A.M. & Innis, S.M. Influence of early diet on hepatic lipogenesis. 88th American Oil Chemists' Society Annual Meeting. Seattle, Washington. May 11-14, 1997

Devlin, A.M., Rioux, M.F & Innis, S.M. The influence of diet on hepatic cholesterol metabolism during development. 39th Canadian Federation of Biological Sciences Annual Meeting, London, Ontario, Canada June 19-26 1996, 388A.

Devlin, A.M., Rioux, M.F & Innis, S.M. Alterations in hepatic cholesterol metabolism during development. Experimental Biology 96, Washington, D.C. April 14-17 1996. *FASEB J.* 10: 553A.

The results of Study #2 have been submitted to:

Devlin, A.M. & Innis, S.M. (1998) Effect of long chain fatty acids on hepatic sterol metabolism during development. *Pediatric Research* (submitted).

Devlin, A.M. & Innis, S.M. (1998) Dietary long chain fatty acids decrease hepatic acetyl CoA carboxylase but not fatty acid synthase or triglyceride in piglets. *J. Nutr.* (in press).

The results of Study #2 have been presented at:

Devlin, A.M. & Innis, S.M. Long chain polyunsaturated fatty acids alter hepatic sterol metabolism during development. Experimental Biology 98, San Francisco, C.A. April 14-17 1998.

Appendix II.I. Body and liver weights of piglets fed formula or milk to 18 days of age (Study 1).

	conventional formula		synthesized TG formula		
	(-)	(+)	(-)	(+)	sow milk
body weight (kg)	5.33±0.59	5.94±0.32	5.70±0.25	5.94±0.22	5.58±0.48
liver weight (g/kg body weight)	26.7±0.90	25.0±0.70	23.7±0.70	24.7±1.10	25.5±1.00

NOTE. Data presented as mean±SEM, n=6 for each diet group.

Appendix II.II. Concentrations of cholesterol and triglyceride in plasma of piglets fed formula or milk to 18 days of age (Study 1).

	conventional formula		synthesized TG formula		
	(-)	(+)	(-)	(+)	sow milk
cholesterol (mmol/L)					
total*‡	2.16±0.11	2.30±0.09	1.93±0.08	1.97±0.08	3.42±0.42
HDL*	1.00±0.06	1.02±0.04	0.89±0.05	0.94±0.06	1.52±0.13
apoB[1]	1.16±0.07	1.29±0.08	1.04±0.07	1.04±0.04	1.90±0.29
triglyceride *‡ (mmol/L)	0.37±0.04	0.44±0.06	0.28±0.04	0.29±0.04	0.80±0.12

NOTE. Data presented as mean±SEM, n=6 for each diet group. *Values for milk-fed piglets significantly different from values for piglets fed milk ($P<0.05$). ‡Significant effect of the positional distribution of 16:0 in the formula triglyceride ($P<0.002$). No significant effect of addition of cholesterol to formula was found. [1]Not analyzed statistically. Samples were all collected when the animals were in the non-fasted state.

Appendix II.III. Concentrations of liver lipids and bile lipids and bile acids in piglets fed formula or milk to 18 days of age (Study 1).

	conventional formula		synthesized TG formula		
	(-)	(+)	(-)	(+)	sow milk
Liver					
cholesterol*†	41.3±1.7	42.9±1.1	36.5±0.8	43.1±2.2	45.7±3.2
triglyceride*	16.3±1.9	18.6±1.3	14.4±1.2	21.3±3.4	43.8±6.6
bile					
bile acids*‡	56.3±6.1	51.4±6.4	27.2±2.5	47.4±5.6	99.3±11.2
phospholipids*	18.9±4.4	14.0±0.8	9.10±1.1	16.4±1.3	33.9±5.8
cholesterol*	2.20±0.5	2.50±0.2	1.80±0.3	1.70±0.3	3.60±0.6

NOTE. Data presented as mean±SEM, n=6 for each diet group. Values for liver cholesterol and triglyceride given in mmol/g protein. Values for bile cholesterol, bile acids, and phospholipid given in mmol/L. *Values for piglets fed formula significantly different from values for milk-fed piglets ($P<0.05$). †Significant effect of adding cholesterol to formula ($P<0.05$). ‡Significant effect of the positional distribution of 16:0 in the formula triglyceride ($P<0.01$).

Appendix II.IV. Hepatic 7-α-hydroxylase, HMG-CoA reductase, LDL recpetor, acetyl CoA carboxylase mRNA and fatty acid synthase mRNA and activity in piglets fed formula or milk to 18 days of age (Study 1).

	conventional formula		synthesized TG formula		
	(-)	(+)	(-)	(+)	sow milk
7-α-hydroxylase mRNA*	297.6±8.3	368.4±22	318.4±37	280.8±46	206.7±34
HMG-CoA reductase mRNA*	245.6±30	374.4±47	352.7±52	436.9±36	147.7±27
LDL receptor mRNA	131.8±18	116.7±8.4	151.5±11	135.6±9.1	143.7±8.4
acetyl CoA carboxylase mRNA*‡	95.9±27	156.7±25	285.4±23	201.7±32	62.2±16
fatty acid synthase mRNA†‡	309.0±24	229.0±43	248.1±10	137.0±31	253.8±32
fatty acid synthase activity‡	10.7±1.8	9.2±0.3	6.7±1.8	5.5±1.7	12.5±1.4

NOTE. Data presented as mean±SEM, n=6 for each diet group. Units for mRNA are densitometric units/μg RNA. Units for fatty acid synthase activity are nmol NADPH/min/g protein. *Values for piglets fed formula significantly different from piglets feed milk ($P<0.05$). †Significant effect of adding cholesterol to formula ($P<0.01$). ‡Significant effect of the positional distribution of 16:0 in the formula triglyceride ($P<0.01$).

Appendix III.I. Liver and body weights (Study 2).

	Diet		
	conventional formula	conventional+egg PL formula	milk
body (kg)	5.15±0.13	4.83±0.15	4.78±0.40
liver (g/kg body weight)	28.3±1.24	26.6±0.50	24.7±0.90

Values presented as means ± SEM, n=3 conventional formula; n=6, conventional+egg PL formula; n=7, milk.

Appendix III.II. Plasma Lipid Concentrations (Study 2).

	Diet		
	conventional formula	conventional+egg PL formula	milk
cholesterol			
total	2.48±0.07*	2.49±0.14*	3.96±0.44
HDL	1.12±0.02*	1.36±0.06†	1.56±0.16
apo B	1.36±0.06	1.13±0.08	2.40±0.28
triglyceride	0.37±0.03	0.50±0.07*	0.32±0.05

Values presented as means ± SEM, n=3 conventional formula; n=6, conventional+egg PL formula; n=7, milk. *Significantly different from values for milk-fed piglets ($P<0.05$). †Significant effect of supplementing the formula with egg phospholipid 20:4(n-6) and 22:6(n-3), ($P<0.01$).

Appendix III.III. Hepatic lipid concentrations and major fatty acids in hepatic phospholipid and triglyceride.

	Diet		
	conventional formula	conventional+egg PL formula	milk
Liver			
cholesterol	30.8±0.7	30.4±0.7	31.5±1.3
triglyceride	14.3±1.0	16.9±0.7	20.1±2.3
phospholipid	193±7.2	209±6.0*	188±5.4
Phospholipid Fatty Acids			
16:0	14.1±0.3*	14.6±0.2*	15.6±0.2
18:0	27.9±0.2*	26.9±0.3†	26.9±0.2
18:1(n-9)	10.8±0.1*	9.75±0.3	9.48±0.1
18:2(n-6)	15.6±0.1*	14.2±0.3†	13.6±0.3
18:3(n-3)	0.15±0.0*	0.17±0.0*	0.26±0.0
20:4(n-6)	19.7±0.1*	21.5±0.3*†	18.7±0.3
22:6(n-3)	5.97±0.1*	7.27±0.2*†	6.42±0.1
Triglyceride Fatty Acids			
16:0	21.7±0.4*	21.7±0.4*	31.4±0.9
18:0	7.87±0.2	7.37±0.2	6.52±0.5
18:1(n-9)	30.8±0.3*	28.5±0.6*†	26.5±0.5
18:2(n-6)	20.0±0.3*	20.6±0.3*	12.9±0.4
18:3(n-3)	1.16±0.1	1.40±0.1†	1.44±0.1
20:4(n-6)	9.25±0.3*	10.7±0.4*†	5.64±0.1
22:6(n-3)	0.99±0.1*	1.18±0.1*	0.80±0.0

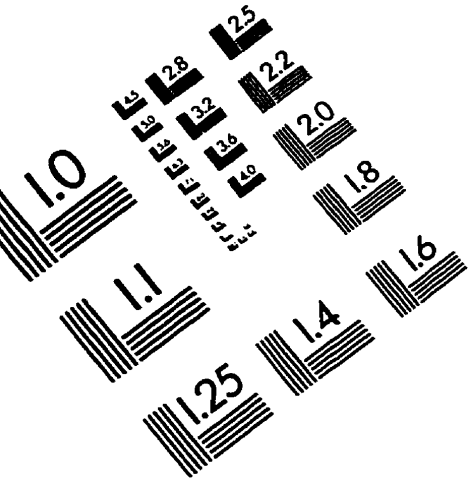
Values presented as means ± SEM, n=3 conventional formula; n=6, conventional+egg PL formula; n=7, milk. Values for liver cholesterol, triglyceride and phospholipid given as μM/g protein. Values for major fatty acids given as g/100g total fatty acids. *Significantly different from values for milk-fed piglets ($P<0.05$). †Significant effect of supplementing the formula with egg phospholipid 20:4(n-6) and 22:6(n-3), ($P<0.05$).

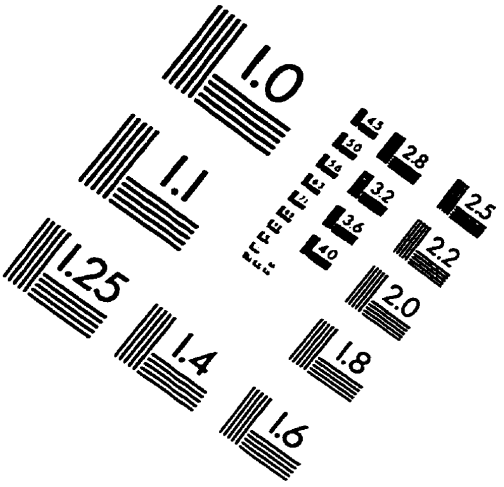
Appendix III.IV. Hepatic 7-α-hydroxylase mRNA, LDL receptor mRNA, HMG-CoA reductase mRNA and activity, acetyl CoA carboxylase mRNA and fatty acid synthase mRNA and activity.

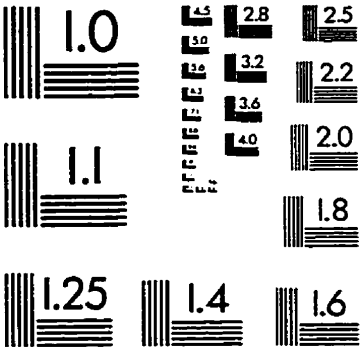
	Diet		
	conventional formula	conventional+egg PL formula	milk
7-α-hydroxylase mRNA	227.0±76	315.0±22	230.0±47
LDL receptor mRNA	168.4±38	180.3±21	173.6±18
HMG-CoA reductase mRNA	505.3±68*	465.2±26*	306.2±49
HMG-CoA reductase activity	3794±274*	7517±2346*	528.1±108
acetyl CoA carboxylase mRNA	130.2±25	63.6±8.4†	128.1±23
fatty acid synthase mRNA	251.3±35	251.0±16	288.1±27
fatty acid synthase activity	2.93±0.1	2.82±0.2	2.71±0.3

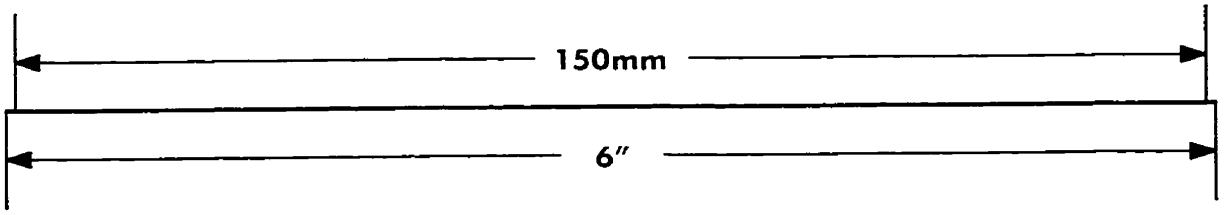
Values presented as means ± SEM, n=3 conventional formula; n=6, conventional+egg PL formula; n=7, milk. Values for mRNA given as densitometric units/μg RNA. Values for HMG-CoA reductase activity given as pmol/min/mg protein. Values for fatty acid synthase activity given as nmol NADPH/min/mg protein. *Significantly different from values for milk-fed piglets ($P<0.05$).

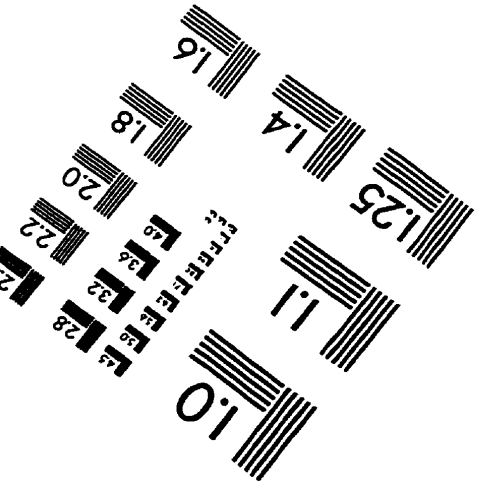
IMAGE EVALUATION
TEST TARGET (QA-3)

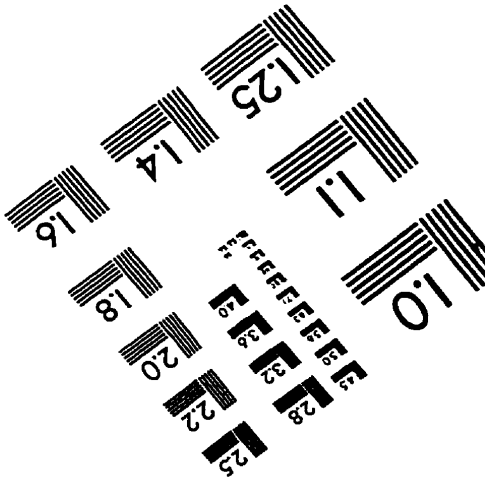












APPLIED IMAGE, Inc
1653 East Main Street
Rochester, NY 14609 USA
Phone: 716/482-0300
Fax: 716/288-5989