SENSITIVITY OF HEPATIC 3-HYDROXY-3-METHYLGLUTARYL COENZYME A REDUCTASE TO EXTERNAL STIMULI AND ITS RELATIONSHIP TO IN VIVO RATES OF CHOLESTEROL SYNTHESIS IN THE FETAL RAT

by

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B.Sc., University of British Columbia, 1985

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES

(Department of Pathology)

We accept this thesis as conforming to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

September 1991

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Date 19 September 1991
ABSTRACT

During gestation the fetus requires cholesterol for membrane accretion, steroidogenesis and lipoprotein synthesis. Very little is known, however, about the mechanisms which regulate cholesterol synthesis in the fetus.

When the bile-acid binding resin cholestyramine (CY) is fed to rats throughout gestation the activity of fetal hepatic 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase (EC 1.1.1.34, rate-limiting enzyme of cholesterol synthesis in adult rats) increases. CY is not absorbed from the gut into the circulation and therefore cannot cross the placenta. Hence, maternal CY feeding must alter fetal HMG CoA reductase activity by influencing maternal factors which are able to cross the placenta. The mechanism(s) mediating this increase in fetal hepatic HMG CoA reductase activity and whether this increase indicates an increase in hepatic cholesterol synthesis have not been previously studied. Thus, the objectives of this thesis are: (1) to determine if fetal hepatic HMG CoA reductase activity can be altered by changes in circulating hormones, cholesterol or fatty acids, and (2) to determine if changes in the activity of hepatic HMG CoA reductase can be correlated to changes in the rate of hepatic cholesterol synthesis in the near term rat.

Experiments investigating the first objective demonstrated that fetal hepatic HMG CoA reductase activity cannot be altered through changes in maternal cholesterol or glucocorticoid levels and does not appear to be related to the concentration of insulin or thyroid hormone in fetal plasma. The quantity and/or type of fatty acid delivered to the fetus, however, appears to be a powerful determinant of fetal reductase activity. Maternal CY ingestion resulted in an increase in both the active (unphosphorylated) and total activity of fetal HMG CoA reductase. Maternal lipase activities and fetal hepatic fatty acid composition of rats fed CY suggest that altered delivery of fatty acids to the fetus may mediate changes in fetal hepatic HMG CoA reductase activity. Feeding pregnant rats diets containing different amounts or types of fat produced fetal reductase activities that were more than two fold greater with 20% polyunsaturated (safflower oil) or monounsaturated (olive oil) fat diets then with the 20% saturated (palm oil) or 5% (safflower) fat diets. Thus, the quantity and/or type of fatty acid delivered to the fetus appears
to be able to influence fetal hepatic HMG CoA reductase activity.

Experiments studying the second objective demonstrated that, in contrast to adults, HMG CoA reductase activity is not correlated to in vivo rates of cholesterol synthesis in near term rat liver. Increased fetal hepatic reductase activity with CY or 20% fat feeding was not accompanied by increased rates of $[^3]$H]water incorporation into digitonin precipitable sterols in fetal rat liver during late gestation. In addition, hepatic reductase activity was found to increase during late gestation and to remain high until suckling commenced whereas rates of cholesterol synthesis peaked on gestation day 20 and then decreased, one day before birth. Thus, under the conditions studied, HMG CoA reductase activity did not indicate the in vivo rate of cholesterol synthesis in the near term rat liver.
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<tr>
<td>ACTH</td>
<td>adrenocorticotropic hormone</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>AMP</td>
<td>adenosine monophosphate</td>
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<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
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<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>°C</td>
<td>degrees celsius</td>
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<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
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<td>curie</td>
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<td>cholestyramine</td>
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<tr>
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<td>disintegrations per minute</td>
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<tr>
<td>EDTA</td>
<td>ethylenediamine tetraacetic acid</td>
</tr>
<tr>
<td>FAS</td>
<td>fatty acid synthase</td>
</tr>
<tr>
<td>FCS</td>
<td>fetal calf serum</td>
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<tr>
<td>HDL</td>
<td>high density lipoprotein</td>
</tr>
<tr>
<td>HFC</td>
<td>high saturated fat and cholesterol diet</td>
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<tr>
<td>HMG CoA</td>
<td>3-hydroxy-3-methylglutaryl coenzyme A</td>
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<tr>
<td>IDL</td>
<td>intermediate density lipoprotein</td>
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<td>LDL</td>
<td>low density lipoprotein</td>
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<td>μg</td>
<td>microgram</td>
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<tr>
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<tr>
<td>ng</td>
<td>nanogram</td>
</tr>
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</tr>
<tr>
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<td>picomole</td>
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<tr>
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<td>palm oil</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SFO</td>
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<tr>
<td>T&lt;sub&gt;4&lt;/sub&gt;</td>
<td>thyroxine</td>
</tr>
<tr>
<td>TAT</td>
<td>tyrosine aminotransferase</td>
</tr>
<tr>
<td>TG</td>
<td>triacylglycerol</td>
</tr>
<tr>
<td>t.l.c.</td>
<td>thin layer chromatography</td>
</tr>
<tr>
<td>Tris</td>
<td>tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>Tris-HCl</td>
<td>tris(hydroxymethyl)aminomethane hydrochloride</td>
</tr>
<tr>
<td>VLDL</td>
<td>very low density lipoprotein</td>
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First and foremost I would like to thank my research supervisor, Dr. Sheila Innis, for patience in guiding me through the often torturous paths of research and for giving me the chance to prove myself. I would also like to thank the members of my supervisory committee, Dr. Jiri Frohlich, Dr. Peter Leung, Dr. Haydn Pritchard, Dr. Urs Steinbrecher and Dr. David Walker for their help and guidance during the lean years when the data seemed fragmented, unclear and contradictory. Thanks also to Dr. Peter Hahn for his interest and comments. I would also like to acknowledge the assistance of Dr. David Kitts in setting up the corticosterone radioimmunoassay and Dr. Peter Jones for constructive comments on the method for precipitating cholesterol with digitonin.

I must also acknowledge the expertise of the people who cared for my numerous rats at The Research Centre. Thank you, Carol Ford, for running an efficient and helpful animal unit which kept my rats happy and healthy.

I have also been helped, in sometimes strange and bizarre ways, by the friends who have worked beside me in the crowded lab of Room 179 of The Research Centre. Especially, I thank Jennifer Hamilton, Dianne (two n’s) Arbuckle, France Rioux, Dr. Nancy Auestad, Dr. Nina Hrboticky, Janette King and Dr. Archanna Datta. Thanks also to Laurie Nicol who was not only a sympathetic ear to my thesis birth pains (he got a lot of practice in our fertile lab) but also acted as a highly qualified second pair of arms and hands in helping me move the seemingly insurmountable stack of test tubes through their various stages of analyses.

Thanks to my students at Augustana University College. While finishing this thesis I was simultaneously getting my first experience as a teacher. My students during the Fall of 1990 and the Winter of 1991 taught me how to think on my feet, speak clearly, say what I really intend to say and to have confidence in myself.

I gratefully acknowledge the financial assistance from the British Columbia and Canadian Heart Foundations. Many thanks also to the administration of Augustana University College for giving me the time required to finish writing the thesis.

Finally I would like to thank my family and many friends (especially Mark Lewis) who at times believed more strongly than I did in my ability to complete this task. Special thanks to my parents, Marie and Lawrence Haave, who allowed me to go wherever my dreaming led me. Thank you Dad for drawing figures 1.1 and 1.2.
SOLI DEO GLORIA
1 INTRODUCTION

1.1 Cholesterol transport and metabolism

Cholesterol is a hydrophobic molecule required by mammalian cells for life. Its 4-ringed structure gives it rigid planarity endowing it with the ability to alter cell membrane order and fluidity (reviewed by Bloch 1983 and McMurchie 1988). Cholesterol is also the obligate precursor of bile acids and various steroids which facilitate intestinal fat absorption and systemic hormonal communication, respectively (reviewed by Turley & Dietschy 1982). Although cholesterol is required for life, an overabundance of this molecule can produce morbidity through, for example, the development of arteriosclerosis (see review by Brown & Goldstein 1984) or cholelithiasis (Turley and Dietschy 1982). Thus, it is apparent that the body must maintain optimal homeostatic levels of cholesterol (reviewed by Dietschy 1984).

Body cholesterol homeostasis is maintained through an elaborate system of plasma transport and cellular uptake, synthesis and secretion as depicted in figure 1.1. Because of its hydrophobic nature, cholesterol must be transported among tissues in the blood as part of complex water-soluble particles known as lipoproteins (reviewed by Hoeg & Brewer 1986). Lipoproteins are comprised of cholesterol, cholesteryl ester, triacylglycerol, phospholipid and apoproteins. Lipoproteins are commonly divided into high density lipoprotein (HDL), intermediate density lipoprotein (IDL), low density lipoprotein (LDL), very low density lipoprotein (VLDL) and chylomicrons (reviewed by Gotto et al 1986).

Cholesterol carried within lipoproteins may originate from either exogenous (dietary) or endogenous sources (see review by Brown & Goldstein 1984). Exogenous cholesterol absorbed from the diet is packaged, along with triacylglycerol, into the chylomicrons which are released into the circulation via the thoracic lymph duct. The role of chylomicrons is principally to deliver fatty acids derived from dietary triacylglycerol to extraintestinal tissues (eg. muscle, adipose tissue). After entry of chylomicrons into the circulation, lipoprotein lipase located on the capillary endothelium of extrahepatic tissues hydrolyse triacylglycerol to fatty acid and glycerol. Apoprotein C from chylomicrons is transferred to HDL, and a smaller cholesterol, phospholipid-rich particle containing apoprotein B and E, known as a chylomicron remnant is left. The chylomicron remnant particle, is bound by hepatic remnant or LDL receptors via
Figure 1.1. The major transport pathways for cholesterol of exogenous and endogenous origin (modified from Brown & Goldstein 1984). Cholesterol of exogenous origin is absorbed from the diet through the intestinal wall and packaged into chylomicrons. Triacylglycerol (TG) within chylomicrons is hydrolysed by lipoprotein lipase (LPL) in the capillaries of adipose and muscle tissue. The resulting cholesterol-rich remnants are cleared by liver cells releasing cholesterol into the hepatic cholesterol pool. Hepatic cholesterol maybe secreted into the intestine as bile acid or biliary cholesterol, or packaged into very-low-density lipoprotein (VLDL) and secreted into the blood, inaugurating the endogenous pathway. VLDL TG, similar to chylomicrons, is hydrolysed within capillaries of muscle and adipose tissue by LPL producing cholesterol-rich VLDL remnants or intermediate-density lipoproteins (IDL). IDL may be cleared by the hepatic remnant receptor or the LDL receptor. Remaining plasma IDL is metabolized to LDL. LDL eventually binds to LDL receptors of liver or extrahepatic tissues. VLDL are also thought to be able to bind to the LDL and remnant receptor (Kostner 1989). The intestinal and hepatic secretion of nascent HDL and the subsequent removal of cholesterol from extrahepatic cells by high-density lipoprotein (HDL) and lecithin:cholesterol acyltransferase (LCAT) are not depicted in this figure. HDL cholesterol is able to be taken up by the liver forming a pathway described as "reverse cholesterol transport".
apoprotein E (see reviews by Dolphin 1985 and Gregg & Brewer 1988), internalized and the cholesterol of exogenous origin mixes with the hepatic cholesterol pool to become indistinguishable from cholesterol of endogenous origin (reviewed by Brown & Goldstein 1984).

Cholesterol in the liver may be secreted into the circulation as a component of triacylglycerol-rich VLDL (see review by Brown & Goldstein 1984). The triacylglycerol of VLDL, similar to that of chylomicrons, is also hydrolysed by lipoprotein lipase to leave a cholesterol-rich VLDL remnant known as IDL which contains apoproteins E, B and A, but depleted of apoprotein C (Lakshmanan et al 1981). IDL may be internalized by the liver or further metabolised to LDL (see reviews by Hoeg & Brewer 1986, Gregg & Brewer 1988). Further metabolism involves transfer of apoproteins A and E to HDL. The final LDL particle contains only one molecule of apoprotein B and binds to the LDL (apo B/E) receptor, but not to the remnant (apo E) receptor of the liver (see review by Brown & Goldstein 1984). LDL, therefore, has a longer half-life than the other cholesterol-rich apo B containing lipoproteins. High levels of plasma LDL-cholesterol have been correlated with an increased risk for developing atherosclerosis (reviewed by Brown & Goldstein 1984). In rats, however, the remnant receptor is very efficient (Oschry & Eisenberg 1982) and hence very little IDL is metabolized to LDL. Rat plasma, therefore, contains relatively low concentrations of LDL. This is one reason why this species is "less" susceptible (or more resistant) to developing arteriosclerosis than the human.

Cholesterol in the liver may also be secreted as HDL. Newly secreted HDL appears as a nascent discoidal particle containing a high proportion of protein (see review by Glickman & Sabesin 1982). HDL plays a major role in delivery of cholesterol from extrahepatic tissues to the liver and, at least in adult rats, to steroidogenic tissues such as the adrenal and ovary (see review by Eisenberg 1984). Cholesterol transferred from extrahepatic tissues to HDL is esterified in a reaction catalysed by the plasma protein lecithin:cholesterol acyl transferase (LCAT). HDL cholesteryl ester may then be transferred to other lipoproteins (LDL, VLDL) by cholesteryl ester transfer protein (CETP) for delivery to the liver in a process known as "reverse cholesterol transport" (Eisenberg 1984). In rats, although plasma LCAT activity is very high, cholesteryl ester transfer activity is absent (Oschry & Eisenberg 1982). This is probably a major
reason for the characteristic predominance of HDL cholesterol in rat plasma (Oschry & Eisenberg 1982).

1.1.1 Hepatic cholesterol metabolism

The only quantitatively significant route for excretion of cholesterol from the body is in bile, either as cholesterol itself or, following further metabolism in the liver, as bile acid (reviewed by Turley & Dietschy 1982). The enzyme cholesterol 7α-hydroxylase, catalyses the obligatory first reaction in the conversion of cholesterol to bile acid and is found only in the liver (Jelinek et al 1990). Thus, the liver plays a fundamental role in the maintenance of body cholesterol homeostasis as it is involved in secretion and uptake of cholesterol from plasma, de novo cholesterol synthesis, and biliary excretion (see review by Hoeg & Brewer 1986). The uptake of plasma cholesterol into the hepatocyte leads to alterations in pathways of cholesterol metabolism (figure 1.2), so that intracellular cholesterol homeostasis can be maintained (see review by Brown & Goldstein 1984). Briefly, the uptake of cholesterol by the liver increases the size of the intracellular cholesterol pool and inhibits further de novo synthesis of cholesterol (Hoeg & Brewer 1986). This is achieved by decreased catalytic activity of 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase (EC 1.1.1.34) via inactivation, decreased synthesis and/or increased degradation of HMG CoA reductase (see review by Rudney & Sexton 1986). The increase in hepatocyte cholesterol also promotes increased activity of acyl:cholesteryl acyl transferase (ACAT) leading to formation of cholesteryl esters and effectively removing free cholesterol (Brown & Goldstein 1984). This protects the hepatocyte from accumulating free cholesterol which can be potentially toxic to the cell (see review by Goldstein & Brown 1990). Uptake of plasma cholesterol within LDL also inhibits synthesis of LDL (apo B/E) receptors (Brown & Goldstein 1984) effectively reducing uptake of cholesterol from the plasma pool. Hepatic internalization of plasma cholesterol, however, does not inhibit hepatic remnant receptor activity (Cooper & Yu 1978, also see reviews by Gotto et al 1986 and Hoeg & Brewer 1986).

Studies in a number of laboratory animals, such as the rat, guinea pig, hamster and rabbit have shown that when cholesterol balance is perturbed, cholesterol synthesis is modified before LDL receptor number (reviewed by Dietschy 1984). Thus, animals such as the rat, which have a high cholesterol synthetic
Figure 1.2. Hepatic cholesterol metabolism (modified from Brown & Goldstein 1984). Low-density lipoprotein (LDL) is taken up from the circulation into a hepatocyte by receptor-mediated endocytosis. The resulting release of unesterified cholesterol from the lysosome increases the intracellular supply of cholesterol. The increase in cholesterol supply (A) inhibits activity of HMG CoA reductase (rate-limiting enzyme of cholesterol synthesis), (B) increases the activity of acylcholsterol acyl transferase (ACAT - rate-limiting enzyme of cholesterol esterification) and (C) inhibits synthesis of LDL receptor through suppression of receptor gene transcription into mRNA. Thus, the cellular level of cholesterol is self-limiting. Remnant particles containing apo B_{100} and apo E (i.e. chylomicron remnants) and IDL with apo B_{48} and apo E are believed to be cleared via an apo E receptor as described in figure 1. Cholesterol thus delivered is effective in regulating cholesterol metabolism/homeostasis in an identical manner to that described for cholesterol derived from LDL.
activity maintain cholesterol homeostasis primarily through changes in hepatic cholesterol synthesis. In contrast, animals such as the rabbit or guinea pig which have a low rate of cholesterogenesis, and therefore a limited ability to significantly alter cholesterol synthesis, appear to maintain cholesterol homeostasis primarily through changes in expression of hepatic LDL receptors (Dietschy 1984).

Cholesteryl esters produced by the action of ACAT may be assembled, with other lipids and proteins, into VLDL for secretion, or stored within the hepatocyte as cholesteryl ester droplets. Later, cholesteryl esters may be returned to the free cholesterol pool by cholesteryl ester esterase hydrolysis, then used for membrane biogenesis, lipoprotein synthesis or secreted into the bile as biliary cholesterol or, after further metabolism, as bile acid (see review by Brown & Goldstein 1984).

1.1.2 Cholesterol biosynthetic pathway

Konrad Bloch and his associates (see review by Bloch 1965) made large contributions to the elucidation of the biosynthetic pathway of cholesterol (reviewed by Spurgeon & Porter 1981, Schroepfer 1982), shown in figure 1.3. The enzymes catalysing the reactions which produce HMG CoA are located in the cytosol and use cytosolic acetyl CoA derived from the mitochondrion (see review by Fielding & Fielding 1985). The cytoplasmic location distinguishes these initial reactions from the ketogenic pathway which produces ketones (acetoacetate and \( \beta \)-hydroxybutyrate) from acetyl CoA in the mitochondrion.

HMG CoA produced by the cytosolic enzyme HMG CoA synthase is reduced to mevalonic acid by the microsomal enzyme HMG CoA reductase using NADPH. This reaction is rate-limiting in the biosynthesis of cholesterol (reviewed by Rodwell et al 1976). Mevalonic acid is metabolized to farnesyl pyrophosphate through a series of cytosolic reactions. The remaining intermediates (squalene and lanosterol) and product (cholesterol) are formed by reactions which are catalysed by enzymes located in the microsomal membranes (reviewed by Gaylor 1981).

There are two branch points in the cholesterol biosynthetic pathway whereby mevalonate may be used for products other than cholesterol. Isopentenyl pyrophosphate may be used for the synthesis of dimethyl allyl pyrophosphate which may be further metabolized to isopentenyl tRNA (see review by Rudney
Figure 1.3. The biosynthetic pathway of cholesterol and alternative pathways of mevalonate metabolism (modified from Beg et al 1987). Cholesterol biosynthesis is of the greatest quantitative importance. Farnesyl pyrophosphate is also used to farnesylate certain proteins such as Ras and lamin B which are thought to be involved with the regulation of cell growth (Goldstein & Brown 1990).
& Sexton 1986) or catabolized via dimethylacrylyl CoA and HMG CoA to acetyl CoA which can then be oxidized to CO$_2$ within the mitochondrion (see review by Fielding & Fielding 1985). The second branch point is at farnesyl pyrophosphate which may be used for the synthesis of ubiquinone, dolichol (Rudney & Sexton 1986) and certain growth regulating proteins such as Ras and lamin B (reviewed by Goldstein & Brown 1990). The quantitative importance of these alternative metabolic pathways, however, is minor compared to the utilization of mevalonate for the synthesis of cholesterol (see review by Goldstein and Brown 1990). In the liver, for example, the relative rate of incorporation of labelled mevalonate into cholesterol is 70 and 1400 fold greater than the rate of incorporation into ubiquinone and dolichol, respectively (Elmberger et al 1987) and 600 fold greater than the rate of incorporation into CO$_2$ (Marinier et al 1987).

1.1.3 Regulation of cholesterol biosynthesis

1.1.3.1 HMG CoA reductase

HMG CoA reductase catalyses the rate-limiting reaction in cholesterogenesis, and in vitro assay of its activity reflects the rate of in vivo de novo cholesterol formation under most circumstances (reviewed by Rodwell et al 1976). HMG CoA reductase is a transmembrane glycoprotein located primarily in the smooth endoplasmic reticulum (Orci et al 1984), has a half-life of less than 3 hr under unstimulated conditions (see review by Fielding & Fielding 1985) and displays a marked diurnal rhythm (Rodwell et al 1976). Its activity is thought to be influenced by changes in cholesterol supply and demand (see reviews by: Rodwell et al 1976, Smith & Gotto 1985). An increase in cholesterol demand due, for example, to feeding rats the non-absorbed bile acid-binding resin cholestyramine (CY), is accompanied by a pronounced increase in HMG CoA reductase activity (Goldfarb & Pitot 1972, Tanaka et al 1982), whilst an increase in cholesterol supply through high dietary intake suppresses activity of the reductase (Rodwell et al 1976).

In the rat, the system for removal of plasma lipoprotein remnant particles is very efficient (Oschry & Eisenberg 1982). Thus, the coordination of hepatic cholesterol synthesis with cholesterol supply in this species appears to be explained by receptor-mediated uptake of cholesterol, or a closely related sterol.
product, in remnant particles derived from chylomicrons or VLDL (Lakshmanan et al 1981, Panini et al 1984). The hepatic uptake of remnant particles is believed to depend on prior loss of apoprotein C, rather than on the hydrolysis of triacylglycerol, from the parent chylomicron or VLDL (Windler et al 1980, Lakshmanan 1981). Although hepatic reductase activity is suppressed by the cholesterol derived from remnant lipoproteins, it is stimulated by triacylglycerol concomitantly delivered. This has been explained by the stimulation of hepatic VLDL secretion (Van Zuiden et al 1983). The net response of hepatic HMG CoA reductase activity to a particular remnant particle depends, therefore, upon the relative balance between the quantity of cholesterol and triacylglycerol in the particle (Van Zuiden et al 1983).

The exact mechanisms by which reductase activity is regulated are not completely understood. It may involve immediate short-term control through changes in the degree of reductase phosphorylation (see review by Beg et al 1987), followed by long term control of enzyme synthesis mediated by direct interaction of a putative oxysterol/receptor complex with nuclear DNA (reviewed by Kandutsch & Taylor 1985). It has been hypothesized that oxygenated metabolites of cholesterol, known as oxysterols rather than cholesterol itself, are responsible for inhibition of reductase activity (Kandutsch & Taylor 1985). These oxysterols inhibit reductase activity in cultured cell lines by decreasing transcription of the reductase gene (Nakanishi et al 1988), as well as by increasing the rate of reductase protein degradation (Jingami et al 1987, Skalnik et al 1988). Decreased HMG CoA reductase mRNA levels, as mediated by 25-hydroxysterol, appears to depend upon the synthesis of new protein (Trzakos et al 1989). The effect on reductase degradation, in contrast, appears to involve the membrane-spanning region of the enzyme (Jingami et al 1987, Skalnik et al 1988). This region has been hypothesized to be involved in re-orienting the membrane bound reductase in the presence of high levels of cholesterol in the microsomal membrane such that it is more susceptible to degradation (see review by Beg et al 1987). The quantity of HMG CoA reductase mRNA is also influenced by mevalonate or nonsterol products of mevalonate (Clarke et al 1985, Nakanishi et al 1988). The available evidence suggests a multivalent control whereby sterols decrease mRNA synthesis and nonsterol mevalonate products decrease mRNA translation (Nakanishi et al 1988), both thereby leading to reduced HMG CoA reductase activity.
Studies in adult rats have correlated a decrease in HMG CoA reductase activity with an increase in microsomal membrane cholesterol and/or cholesterol/phospholipid molar ratio (Sabine & James 1976, Mitropoulos et al 1978, Mitropoulos et al 1981, Sipat & Sabine 1981, Venkatesan & Mitropoulos 1982), and increased phospholipid fatty acid saturation (Davis & Poznansky 1987). The phospholipid fatty acid composition and cholesterol content of cell membranes are determinants of the membrane fluidity (reviewed by McMurchie 1988). Since HMG CoA reductase is a membrane associated enzyme, it is reasonable that its activity may be under viscotropie regulation (Mitropoulos & Venkatesan 1977, Mitropoulos et al 1981, Sipat & Sabine 1981). Further, it has been suggested that an increase in microsomal membrane cholesterol may re-orient HMG CoA reductase such that the reductase protein is more susceptible to phosphorylation (inactivation), and degradation (reviewed by Beg et al 1987). Although the exact mechanism is unclear, it is likely that the regulation of HMG CoA reductase by cholesterol, cholesterol metabolites or its precursors involves an initial alteration in activity of existing reductase protein, which is later followed by changes in the rate of enzyme protein synthesis and/or degradation (see reviews by Sabine & James 1976, Beg et al 1987).

The activity of HMG CoA reductase may also be modulated by changes in the enzyme phosphorylation state (see reviews by Beg et al 1987 and Zammit & Easom 1987). Phosphorylation of reductase protein involves 3 separate and specific kinase systems, including reductase kinase, protein kinase C and a Ca\(^{2+}\), calmodulin-dependent kinase (reviewed by Beg et al 1987). Inactivation by phosphorylation is possibly mediated through changes in HMG CoA reductase conformation (Miller et al 1989). Phosphorylation-dephosphorylation is rapid and reversible, and is thus viewed as a method of short, rather than long, term regulation (see reviews by Zammit & Easom 1987 and Beg et al 1987). However, phosphorylation of HMG CoA reductase may affect its long-term regulation by increasing its rate of degradation (Marrero et al 1986, Miller et al 1989, Parker et al 1989).

HMG CoA reductase is known to be sensitive to several hormones (reviewed by Edwards et al 1983). In most cases the relevance of hormonal effects in vitro or in vivo to normal physiology is unclear. The hepatic HMG CoA reductase activity of diabetic rats is low and can be increased by intravenous
infusion or pharmacological administration of insulin (Huber et al 1973, Edwards et al 1983, Easom & Zammit 1985). Pharmacological injection of insulin to normal rats also increases reductase activity (Huber et al 1973). In addition, physiological concentrations of insulin are able to increase reductase activity in hepatocytes prepared from normal rats. This latter effect seems to be mediated by increased expression of reductase protein (Stange et al 1982, Gibbons et al 1984).

Glucagon administration to normal rats prevents the normal diurnal rise, and inhibits an insulin-induced increase, in HMG CoA reductase activity (Edwards et al 1983). Cultured rat hepatocytes have also been shown to have reduced reductase activity after addition of glucagon at physiological concentrations (Stange et al 1982, Gibbons et al 1984).

Thyroid hormone increases HMG CoA reductase activity in cultured hepatocytes when added at physiological levels (Stange et al 1982), and maintains reductase activity at the normal diurnal maximum in normal rats (Edwards et al 1983). These effects may be due, in part, to decreased degradation of reductase mRNA (Simonet & Ness 1988). A decrease in hepatic reductase activity in propylthiouracil (PTU) treated (hypothyroid) rats was increased by pharmacological administration of thyroxine to give circulating thyroxine levels 4-fold above normal (Field et al 1986). These effects of thyroid hormone on HMG CoA reductase activity may be related to increased biliary bile acid (Edwards et al 1983) or cholesterol (Field et al 1986) secretion, or hepatic lipoprotein uptake (Krul & Dolphin 1982, Dolphin & Forsyth 1983).

In vitro and in vivo studies have reported induction of reductase activity by pharmacological doses of catecholamines (Edwards et al 1983). HMG CoA reductase in rabbit liver has also been shown to be increased by noradrenalin (Devery et al 1986). These findings may have physiological significance as cholesterogenesis has been shown to be increased in stressed, fasted rats (Edwards et al 1983).

The effects of glucocorticoids on hepatic HMG CoA reductase activity is controversial. For example, adrenalectomy has been shown to abolish or not affect the diurnal cycle of HMG CoA reductase activity (Edwards et al 1983). Similarly, pharmacological doses of glucocorticoids have been shown to have no effect or to return reductase activity to normal in adrenalectomized rats (Edwards et al 1983).
**Table 1.1**

*Factors affecting HMG CoA reductase activity.*

<table>
<thead>
<tr>
<th>Factor</th>
<th>Effect on reductase activity</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>microsomal cholesterol</td>
<td>decrease</td>
<td>Mitropoulos et al 1978, 1981</td>
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<tr>
<td></td>
<td></td>
<td>Sabine &amp; James 1976</td>
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<tr>
<td></td>
<td></td>
<td>Sipat &amp; Sabine 1981</td>
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<td></td>
<td></td>
<td>Venkatesan &amp; Mitropoulos 1982</td>
</tr>
<tr>
<td>microsomal FA&lt;sup&gt;1&lt;/sup&gt; saturation</td>
<td>decrease</td>
<td>Davis &amp; Poznansky 1987</td>
</tr>
<tr>
<td>phosphorylation</td>
<td>decrease</td>
<td>Beg et al 1987&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td>of reductase protein</td>
<td></td>
<td>Zammit &amp; Easom 1987&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td>diabetes</td>
<td>decrease</td>
<td>Edwards et al 1983&lt;sup&gt;2&lt;/sup&gt;</td>
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<td></td>
<td></td>
<td>Huber et al 1973</td>
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<td></td>
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<td>Easom &amp; Zammit 1985</td>
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<tr>
<td>insulin</td>
<td>increase</td>
<td>Huber et al 1973</td>
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<td>Gibbons et al 1984</td>
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<tr>
<td></td>
<td></td>
<td>Stange et al 1982</td>
</tr>
<tr>
<td>glucagon</td>
<td>prevents normal diurnal increase &amp; insulin induced increase</td>
<td>Edwards et al 1983&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>decrease in cultured rat hepatocytes</td>
<td>Gibbons et al 1984</td>
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<td></td>
<td></td>
<td>Stange et al 1982</td>
</tr>
<tr>
<td>thyroid hormone</td>
<td>maintains at normal diurnal max.</td>
<td>Edwards et al 1983&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>increases in cultured hepatocytes</td>
<td>Stange et al 1982</td>
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<tr>
<td>catecholamines</td>
<td>increases</td>
<td>Edwards et al 1983&lt;sup&gt;2&lt;/sup&gt;</td>
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<td></td>
<td></td>
<td>Devery et al 1986</td>
</tr>
<tr>
<td>adrenalectomy</td>
<td>no effect or abolishes diurnal cycle</td>
<td>Edwards et al 1983&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td>glucocorticoids</td>
<td>no effect or counteracts adrenalectomy</td>
<td>Edwards et al 1983&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>increases in cultured rat hepatocytes</td>
<td>Stange et al 1982</td>
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<tr>
<td></td>
<td></td>
<td>Pullinger &amp; Gibbons 1983</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lin &amp; Snodgrass 1982</td>
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<sup>1</sup>Fatty acid (FA).

<sup>2</sup>Review article.
Administration of dexamethasone or hydrocortisone to hypophysectomized or adrenalectomized rats, however, blocked the increase in mRNA and enzyme activity that occurred with thyroid hormone treatment (Simonet & Ness 1989). This suggests that glucocorticoids may elicit different effects on HMG CoA reductase activity depending on the dose and the presence of other hormones (Edwards et al 1983). Addition of synthetic glucocorticoids (triamcinolone or dexamethasone) to rat hepatocytes led to increased activity of HMG CoA reductase (Stange et al 1982, Lin & Snodgrass 1982, Pullinger & Gibbons 1983). Cholesterol synthesis in these same studies, however, was increased (Lin & Snodgrass 1982) or decreased (Pullinger & Gibbons 1983) by the addition of dexamethasone.

Hormonal regulation of HMG CoA reductase activity is thought to occur through both immediate (short-term) and delayed (long-term) mechanisms. Short-term regulation has been shown to occur immediately after pharmacological administration of insulin or glucagon to rats and appears to involve modulation of the phosphorylation state of existing enzyme (see reviews by Beg et al 1987 and Zammit & Easom 1987). Long-term regulation, however, is delayed by approximately 4 hrs and involves an alteration in the rate of enzyme synthesis or turnover (see reviews by Edwards et al 1986, Beg et al 1987 and Zammit & Easom 1987).

1.1.3.2 Regulation of cholesterol biosynthesis by alternative mechanisms

Although HMG CoA reductase activity reflects the rate of cholesterol synthesis under most experimental conditions (reviewed by Rodwell et al 1976), several situations have been described in which other enzymes may become rate-limiting. HMG CoA synthase activity, and consequently rates of cholesterol synthesis, are inhibited by glucocorticoids or their pharmacological analogs in hepatocytes (Pullinger & Gibbons 1983) and in a variety of other cells (Ramachandran et al 1978, Johnston et al 1979) in culture. In this instance HMG CoA reductase activity is actually increased due to limited substrate availability.

Rates of cholesterol synthesis are decreased in vitro (Bruscalupi et al 1985) and in vivo (Trentalance et al 1984) in the regenerating liver. The high reductase activity in this situation has been speculated to
occur in order to maintain synthesis of mevalonate products (isopentenyl tRNA, ubiquinone, dolichol), other than cholesterol (Trenatalance et al 1984), which are required for cellular proliferation (Quesney-Hueneus et al 1979). Under these circumstances the activity of squalene synthase (see review by Brown & Goldstein 1980) or squalene epoxidase (Eilenberg & Shechter 1987), rather than HMG CoA reductase, may limit rates of cholesterol synthesis, thereby diverting the products of mevalonate to synthesis of isopentynyl tRNA, dolichol and/or ubiquinone. Inhibition at the level of squalene synthesis, resulting in decreased cholesterol and increased ubiquinone formation, has been demonstrated in vitamin A deficient rat liver and during cold exposure (see review by Pennoch & Threlfall 1983).

The presence of an endogenous competitive inhibitor of HMG CoA reductase activity may also lead to a disparity between cholesterol synthesis and reductase activity (Bensch et al 1978, Brown et al 1978). Here, rates of cholesterol synthesis are low due to inhibition of HMG CoA reductase, but the reductase activity when assayed in isolated microsomes is increased. This is explained by dilution or loss of inhibitor(s) during the microsomal preparation (Bensch et al 1978, Brown et al 1978), compensatory increased reductase protein synthesis (Kitka et al 1980, Ryan et al 1981, Chin et al 1982) and/or decreased degradation (Sinensky & Logel 1983) in response to decreased synthesis of putative mevalonate or cholesterol metabolites (Pullinger & Gibbons 1983).

1.2 Fetal cholesterol metabolism

Cholesterol in fetal rat plasma, as in the adult, is carried within lipoproteins. LDL is the predominant lipoprotein in fetal rat plasma (Schlag & Winkler 1978, Argiles & Herrera 1981, Johansson 1983, Erickson et al 1988, Perrin Ansart et al 1988) and is present at approximately 2- and 10-fold greater concentrations than HDL and VLDL, respectively (Schlag & Winkler 1978, Erickson et al 1988). In contrast, it is well known that in the adult rat most of the plasma cholesterol is associated with HDL, and LDL cholesterol is very low (Oschry & Eisenberg 1982, and reviewed by Chapman 1986). Immediately following parturition, LDL-cholesterol levels decrease from the high level found in fetal plasma (Erickson et al 1988), possibly due to the loss of the fetal placental membranes (Plonne et al 1990) as well as changes
in hepatic metabolism (Perrin Ansart et al 1988). Cholesterol levels in chylomicrons, VLDL, LDL and HDL begin to increase with suckling. Two weeks after birth and coincident with the start of natural weaning, levels of plasma LDL begin to decrease, and the lipoprotein profile begins to resemble that found in the adult rat (Johansson 1983).

The low LDL and high HDL cholesterol levels characteristic of adult rat plasma are believed to be largely due to the absence of cholesteryl ester transfer protein activity and an efficient system for the removal of remnant particles (Oschry & Eisenberg 1982). In the fetus, however, the activity of the LDL (apo B/E) receptor may be lower than in the suckling or adult rat (Erickson et al 1988); cholesteryl ester transfer protein activity, however, is low as in neonatal (ie. one day old newborns) and adult rats (Perrin Ansart et al 1988). The low LCAT activity of fetal plasma, which does not increase until after suckling commences seems likely to be causally related to the low HDL-cholesterol in fetal plasma (Erickson et al 1988).

Chylomicrons and their remnant particles are absent in fetal plasma, probably due to the lack of oral fat consumption by the fetus and subsequent intestinal fat absorption and secretion in lipoprotein (Mak & Trier 1979). The low levels of VLDL and absence of VLDL remnants in fetal plasma is not understood but has been hypothesized to be due to rapid catabolism of VLDL to LDL (see review by Coleman 1989). Others, however, have suggested direct secretion of LDL rather than VLDL by the placenta, fetal placental membranes or liver (Belknap & Dietschy 1988, Perrin Ansart et al 1988, Coleman 1989, Plonne et al 1990).

1.2.1 Sources of fetal cholesterol

Clearly, cholesterol in fetal tissues and plasma may originate from fetal de novo synthesis or via placental transport from the maternal compartment. Information from studies of radiolabelled cholesterol transfer have indicated that placental transfer of cholesterol is low probably accounting for only 10 - 20% of cholesterol in the late gestation fetus (Goldwater & Stetton 1947, Chevallier 1964, Calandra et al 1975). However, the changing pool size and substrate specific activities caused by the lack of steady-state conditions in the rapidly growing fetus and mother (Popjak 1954) require cautious interpretation of these
studies.

Low placental transfer of cholesterol has been inferred indirectly by studies of fetal cholesterol biosynthesis in cholesterol fed pregnant rats (Calandra et al 1975, Feingold et al 1983, Belknap & Dietschy 1988). These studies found a higher cholesterol synthetic rate in the fetus and fetal liver than in maternal or nonpregnant rat liver, which was not reduced by maternal cholesterol feeding. This finding infers that fetal tissue cholesterol originates mainly from endogenous fetal de novo synthesis rather than from placental transfer (Calandra et al 1975, Feingold et al 1983, Belknap & Dietschy 1988). Little is known, however, about the regulation of the apparent high rates of fetal hepatic cholesterol synthesis during late gestation.

1.2.2 Fetal HMG CoA reductase

The total (phosphorylated plus unphosphorylated) activity of rat liver HMG CoA reductase increases throughout the last third of gestation to reach levels approximately 2-fold higher than adult male values, then declines precipitously at birth and remains low until weaning when a final increase in activity occurs (McNamara et al 1972, Carlson et al 1978, Ness et al 1979, Leoni et al 1984, Weiss et al 1988). Active (unphosphorylated) reductase activity (Leoni et al 1984) and steady-state levels of HMG CoA reductase mRNA (Levin et al 1989) also follow a similar developmental pattern. Surprisingly, the developmental profile described for ACAT activity found in fetal and neonatal rats resembles this pattern (Bruscalupi et al 1986). In contrast, bile acid synthesis from cholesterol and the activity of 7α-hydroxylase (the first enzyme of bile acid synthesis) is low during late gestation and increases during suckling (Little et al 1976, Hahn & Innis 1984).

The low activity of HMG CoA reductase activity during suckling and high activity following weaning reflects similar developmental changes in the rate of hepatic cholesterol synthesis (Ballard & Hanson 1967, Taylor et al 1967). The regulatory physiological/biochemical events are unclear but seem likely to include the change in the major dietary substrate from fat to carbohydrate (McNamara et al 1972, Ferre et al 1986). Such a dietary regulation in the young postnatal animal may reasonably be proposed to be mediated like
that in the adult (Rodwell et al 1976). Recent studies have indicated, however, that the postnatal changes in reductase activity at weaning may involve an increase in total reductase activity relative to enzyme mass, i.e. catalytic efficiency (Weiss et al 1988). This appears consistent with earlier work by McNamara et al (1972) which found that the rise in reductase activity at weaning was not inhibited by cycloheximide. Although it seems that the regulation of HMG CoA reductase activity during development may involve mechanisms other than enzyme synthesis and/or degradation little information is available on this topic. Some evidence exists to suggest that fetal hepatic HMG CoA reductase may not be susceptible to the same regulatory mechanisms that are known to control the enzyme in adult and postnatal rat liver. For example, addition of cholesterol to fetal, unlike newborn and adult, hepatocytes does not inhibit HMG CoA reductase activity (Leoni et al 1986). Similar loss of cholesterol feedback control of HMG CoA reductase activity has been shown in hepatoma (Siperstein et al 1971).

It has been postulated that active control of developing fetal liver cholesterol synthesis by adrenal hormones may coordinate the increasing substrate demand for fetal adrenal steroidogenesis with the rate of cholesterol production (Simpson et al 1985). The complex hormonal shifts which occur during perinatal (i.e. approximately one day before and after birth) development are believed to control the development of several hepatic enzyme systems (reviewed by Bohme et al 1983). Thus, it is conceivable that hormonal changes may be of importance in mediating the changes in HMG CoA reductase activity which occur during perinatal and neonatal development. Active, but not total, HMG CoA reductase activity in rat hepatocytes has been shown to be responsive to insulin and glucagon by the 18th day of gestation (Leoni et al 1985). Immediately before and after birth, however, the fetal reductase becomes insensitive to insulin (Leoni et al 1985), possibly due to the increase in fetal plasma corticosterone levels which occurs during late gestation (Holt & Oliver 1968, Cohen 1973). Studies with human fetal tissue, however, have led to the hypothesis that, because it preferentially utilizes plasma LDL cholesterol for steroidogenesis, the fetal adrenal is a major determinant of circulating LDL cholesterol levels (Carr et al 1980, Carr & Simpson 1982, see also review by Carr & Simpson 1981). Although addition of dexamethasone to human fetal hepatocytes has been shown to increase HMG CoA reductase activity (Carr & Simpson 1984), direct evidence of an
interrelationship between adrenal cholesterol utilization, plasma cholesterol and the regulation of hepatic HMG CoA reductase is not available. The source of cholesterol for steroidogenesis in the fetal rat adrenal is unknown. In the adult rat, however, adrenal HMG CoA reductase activity is low and plasma HDL cholesterol is the major source of substrate (Gwynne et al 1985). If the adrenal of fetal rats likewise prefers HDL cholesterol, this may be partially responsible for the low levels of plasma HDL cholesterol in the fetus relative to the adult rat.

1.2.3 Cholesterol biosynthesis in the fetus

Cholesterol synthesis, like HMG CoA reductase activities, in fetal rat hepatocytes has been shown to be sensitive to insulin as well as glucagon 4 - 5 days before, but not immediately before or after birth (Leoni et al 1985). Cholesterol synthesis in human fetal hepatocytes (early to midgestation) has been shown to be stimulated by glucocorticoids, estrogens, progesterone (Carr & Simpson 1984) and HDL, inhibited by LDL, IDL, and VLDL (Carr & Simpson 1984a), and not influenced by insulin or glucagon (Carr & Simpson 1984).

Information on the developmental changes in hepatic cholesterol synthesis is at present conflicting. The high rate of fetal cholesterol synthesis has been reported to decline before (Ballard & Hanson 1967, Pillay & Bailey 1982) and after birth (Carroll 1964, Leoni et al 1984). The description of a prenatal (i.e. before birth) decline during late gestation in hepatic cholesterol synthesis (Ballard & Hanson 1967, Pillay & Bailey 1982) is distinctly different from the high HMG CoA reductase activity (McNamara et al 1972, Leoni et al 1984) assayed in fetal liver at this time. Hence, any changes in fetal hepatic HMG CoA reductase activity (Naseem et al 1980, Innis 1985) may not necessarily indicate altered rates of cholesterol synthesis.
1.3 Significance

The fetus is a rapidly developing organism (Greengard et al 1972) and as such requires cholesterol for membrane accretion (Belknap & Dietschy 1988). Cholesterol is also required during development as substrate for the synthesis of plasma lipoproteins which may be involved in the supply of essential fatty acid to extrahepatic tissues (Nouvelot et al 1986) and may also be used to deliver cholesterol to the fetal adrenal and/or placenta for steroidogenesis (Carr et al 1980, and reviewed in Carr & Simpson 1981). The resulting steroids (eg. glucocorticoids) have been implicated in the regulation of maturation of certain metabolic enzyme systems (see review by Bohme et al 1983). Thus, stimuli which influence the synthesis of cholesterol may affect fetal development.

Atherosclerosis begins to develop during early life but usually does not manifest itself clinically until much later as coronary heart disease, cerebral or peripheral vascular disease, or abdominal aneurysms (see review by McGill 1988). These diseases are major causes of death in North American and European countries (reviewed by Ball 1987). Levels of LDL and HDL cholesterol are thought to be positively and negatively related, respectively, to the risk for developing atherosclerosis (McGill 1988), and are influenced by hepatic cholesterol metabolism (see review by Brown & Goldstein 1984). In the rat (Hahn & Walker 1979, Naseem et al 1980, Naseem et al 1980a, O'Brien et al 1983, Coates et al 1983, Innis 1983, Hahn 1984, Innis 1985, Brown et al 1990), and other animals such as the Carneau pigeon, guinea pig and non-human primate (Li et al 1979, Li et al 1980, Hassan et al 1981, Hassan et al 1982, Mott et al 1982, Subbiah et al 1983, Mott et al 1985, Lewis et al 1988, Aubert et al 1988, Subbiah et al 1989, Mott et al 1990), changes in perinatal diet appear to have lasting effects on cholesterol metabolism. Studies in the rat have been interpreted to suggest that HMG CoA reductase in this species is most susceptible to long-term alteration during prenatal life (Naseem et al 1980). The mechanism of this phenomenon, however, is unknown (reviewed by Innis 1985). Modulation of cholesterol synthesis by external stimuli during the fetal period, however, may influence cholesterol metabolism in adulthood (Innis 1983, 1985, 1989). By investigating the factors which may influence hepatic reductase activity and their relationship to cholesterol synthesis in the fetal rat, these studies may help to determine the mechanisms
by which the long-term homeostatic regulation of cholesterol metabolism may be affected during early life.

1.4 Rationale

Previous studies have demonstrated increased HMG CoA reductase activity in near-term fetus of pregnant rats fed CY (Innis 1983). CY binds bile acids in the gut thereby increasing their fecal excretion resulting in increased hepatic bile acid and cholesterol synthesis and uptake of plasma cholesterol (reviewed by Packard & Shepard 1982). CY is a high molecular weight polystyrene resin which is not absorbed into the circulation from the gut (Gallo & Sheffner 1965) and hence will not cross the placenta and enter the fetal compartment. Hence, any alteration of fetal reductase activity by maternal CY feeding must be mediated by maternal factors which are able to cross the placenta. The resin has a well characterized effect on cholesterol synthesis (Jeske & Dietschy 1980) and bile acid metabolism (Botham et al 1981, Packard & Shepherd 1982, Innis 1984) and has also been reported to increase rat plasma corticosterone (Hassan et al 1984). High fat and cholesterol diets, also appear to lead to increased plasma corticosterone (Brindley et al 1981) and to accumulation of adrenal cholesterol (Civen et al 1984) in male rats. Corticosterone has been shown to cross the placenta (Zarrow et al 1970). Although data on the effects of glucocorticoids are not consistent, glucocorticoid stimulation of cholesterol synthesis in rat (Lin & Snodgrass 1982, Stange et al 1982) and human fetal hepatocytes (Carr & Simpson 1984) has been reported. The perinatal changes in hepatic HMG CoA reductase activity (McNamara et al 1972) and corticosterone (Holt & Oliver 1968, Cohen 1973, Henning 1978), the principle rat glucocorticoid, have a similar temporal pattern suggesting a potential relationship between hepatic cholesterol synthesis and utilization for adrenal steroidogenesis. On the other hand, glucocorticoids are known to induce maturation of several fetal enzymes (see review by Bohme et al 1983) and may have direct effects on hepatic cholesterol metabolism (see review by Edwards et al 1983). Cystathionase (EC 4.2.1.15), which catalyses the cleavage of cystathionine to cysteine in the transsulfation pathway from methionine, and tyrosine aminotransferase (TAT, EC 2.6.1.5), which catalyses the transamination of tyrosine, are not directly related to cholesterol metabolism. Cystathionase (Heinonen 1975), but not TAT (Serini et al 1959, Franz & Knox 1967, Perry et al 1983), is able to be induced by
glucocorticoids in fetal rat liver. Corticosterone crosses the rat placenta readily (Dupuoy et al 1975). Thus, it is conceivable that changes in this hormone, rather than in fat or cholesterol, may be involved in mediating the alteration in fetal enzymes.

Changes in the quantity or type of fat ingested by adult rats influence hepatic HMG CoA reductase activity (Craig et al 1972, Goldfarb & Pitot 1972, Ide et al 1978, Mitropoulis et al 1980, Jenke et al 1982) and cholesterol synthesis (Avigan & Steinberg 1958, Linazasoro et al 1958, Diller & Harvey 1964, Bortz 1967, Serdarevich & Carroll 1972). Maternal fatty acids are known to cross the placenta and have been estimated to contribute up to 50% of the fatty acids present in the late gestation rat fetus (reviewed by Coleman 1986). Further, maternal diet has been shown to influence the fatty acid composition of the fetal tissues (Chaikoff & Robinson 1933, Winkler et al 1979, Samulski & Walker 1982, Stammers et al 1983, Clarke et al 1988). Also, the quantity and/or type of fat fed to animals during development can influence cholesterol metabolism in adulthood (Mott et al 1982, Coates et al 1983, O'Brien et al 1983, Mott et al 1985, Lewis et al 1988). It is conceivable, therefore, that fetal hepatic HMG CoA reductase activity, and hence possibly cholesterol metabolism, may be influenced by the type and/or quantity of fat ingested by the pregnant rat. Any alterations in fetal hepatic HMG CoA reductase activity by maternal dietary fat may conceivably be related to the mechanism of imprinting cholesterol metabolism.

Finally, the developmental profile of cholesterol synthesis and HMG CoA reductase activity is not clear. Cholesterol synthesis and HMG CoA reductase activity have been shown to be high before birth. The available information, however, indicates that HMG CoA reductase activity decreases after birth (McNamara et al 1972, Leoni et al 1984). Cholesterol synthesis, on the other hand, has been reported to decline both before (Ballard & Hanson 1967, Pillay & Bailey 1982) and after birth (Carroll 1964, Leoni et al 1984). Hence, the developmental profile of perinatal cholesterol synthesis needs to be clarified. Whether or not HMG CoA reductase activity is correlated to rates of cholesterol synthesis in fetal rat liver is, therefore, uncertain.
1.5 Thesis objectives

The objectives of this thesis are:

1) to determine if fetal hepatic 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase (EC 1.1.1.34) activity can be altered by changes in circulating hormones, diet cholesterol, or the and/or quantity of dietary fat,

2) to determine if changes in the activity of fetal hepatic HMG CoA reductase can be correlated to changes in the rate of hepatic cholesterol synthesis.

1.6 Specific aims of thesis

The first experiment examines the effects of administering cortisol or adrenocorticotropin (ACTH) or feeding diets containing cholestyramine (CY), high fat and cholesterol or various quantities or types of fat to pregnant rats on hepatic lipid metabolism in the pregnant rat and her fetuses. Specifically, the activity of the enzymes HMG CoA reductase, cystathionase, tyrosine aminotransferase (TAT), fatty acid synthase (FAS) and tissue lipases was assayed. The concentration of tissue and plasma lipids and plasma hormones were also determined.

The developmental profile of fetal hepatic microsomal HMG CoA reductase activity and its relationship to the microsomal and plasma lipids and fatty acids is investigated in the second experiment.

The third experiment determined cholesterol synthesis \textit{in vivo} from the rates of hepatic $[^3\text{H}]$water incorporation into digitonin precipitable sterols. The results from these studies are compared to the developmental pattern of HMG CoA reductase obtained in experiment 2. Rates of fetal hepatic cholesterol synthesis were also determined in pregnant rats fed semisynthetic diets containing 20 or 5% (wt/wt) safflower oil or a commercial control diet with or without the addition of 5% (wt/wt) CY. These results are compared to fetal reductase activities obtained with the same diets in experiment 1.
2 MATERIALS AND METHODS

2.1 Chemicals and radioisotopes

DL-[methyl-\(^{3}\)H]HMG CoA (10.9 Ci/mmol), [2-\(^{14}\)C]mevalonic acid (50.1 mCi/mmol), [9,10-\(^{3}\)H]triolein (20 Ci/mmol), [1-\(^{14}\)C]oleic acid (56 Ci/mmol), [7-\(^{3}\)H(N)]cholesterol (11.4 Ci/mmol) and \([^{3}\)H]OH (1 Ci/g) were purchased from Dupont (Canada) Inc., Missisauga, Ontario. The \(^{125}\)I-corticosterone used as tracer and antisera specific to rat corticosterone (11,21-dihydroxypregnene-3,20-dione) were purchased from Radioassay Systems Labs. Inc., Carson, California. The cross-reactivity of the antisera for other steroids was less than 0.1%. Radioimmunoassay kits for assay of thyroxine binding globulin (TBG) and thyroxine (T\(_4\)), and enzymatic kits for triacylglycerol and cholesterol determinations were obtained from Biopacific Diagnostic Inc., West Vancouver, British Columbia. Enzymatic reagents for assay of unesterified cholesterol were from Boehringer Mannheim Canada Ltd., Dorval, Quebec. The insulin radioimmunoassay kit was from Amersham Corp., Oakville, Ontario. Cholestyramine (Questran\(^\text{R}\)) is a product of Bristol Myers Canada, Bellevue, Ontario. Adrenocorticotropic (ACTHAR\(^\text{R}\)) and hydrocortisone 21-hemisuccinate (cortisol, 11a, 17a, 21-trihydroxy-4-pregnene-3,20-dione) were from Armour Pharmaceutical Co., Kankakee, Illinois, and Sigma Chemical Co., St. Louis, Missouri, respectively. Ingredients for the semisynthetic diets were from the following sources: United States Biochemical Corp., Cleveland, Ohio (safflower, olive and palm oil and vitamin-free casein); Teklad Test Diets, Madison, Wisconsin (sucrose, starch, non-nutritive cellulose, lard and AOAC vitamin mix); General Biochemicals, Chagrin Falls, Ohio (Bernhart-Tomarelli mineral mix). All other chemicals were reagent grade and acquired from BDH Chemicals Canada Ltd., Vancouver, British Columbia or Sigma Chemical Co., St. Louis, Missouri.

2.2 Equipment

All procedures requiring centrifugation at < 10 000g were performed in a low speed centrifuge with a swinging basket rotor from Beckman Instruments, Inc. or from the International Equipment Company (IEC) model numbers J-6B and DPR 6000, respectively. Centrifugations at 10 000g were carried out in a
high speed centrifuge from IEC using IEC rotor # 874. Centrifugation at 100 000g was performed in ultracentrifuges from Beckman Instruments, Inc. (model numbers L8-55 and L7-55) using a Beckman rotor type 50.3 Ti. Supplies for gas liquid chromatography (GLC) were from Supelco Canada Ltd., Oakville, Ontario and NuChek Prep., Elyssian, Missouri. Fatty acid methyl esters were separated and quantified utilizing a Varian 6000 GLC equipped for capillary column analysis with flame ionization detection and a Varian Vista 402 data system. An SP 2300 fused silica (Chromasorb WAW) column (30 m x 0.2 mm) was used. All \[^{125}\text{I}\] compounds were counted in a LKB-Wallac 1272 Clinigamma gamma-counter purchased from Fischer Scientific, Vancouver, British Columbia. \[^{3}\text{H}\] and \[^{14}\text{C}\] content was determined by liquid scintillation spectroscopy using aqueous counting scintillant from Amersham Corp., Oakville, Ontario and a LS 9000 beta-counter from Beckman Instruments (Canada), Inc., Ontario.

2.3 Housing and breeding of rats

Female rats (220-280 g) of the Wistar strain (University of British Columbia, Animal Care) were housed in an environmentally controlled animal unit with a regulated dark (0400-1600 h)-light (1600-0400 h) cycle. Laboratory rat chow (Purina Rodent Laboratory Chow #5001, Purina Mills, Inc., St. Louis, Missouri) and water were available ad libitum unless specifically indicated as otherwise. The animals were bred overnight and day 0 of gestation was dated the following morning if spermatozoa were present in vaginal smears. All studies were conducted without prior fasting in order to eliminate potential changes in metabolite or hormone transfer to the fetus and at 5 - 6 hours into the 12 hour dark cycle. This time coincides with the peak activity of hepatic HMG CoA reductase in the normal adult rat (Easom & Zammit 1984). The rats were studied on gestation days 16, 18, 19, 20, 21 (normal term, 22 days), on postnatal days 0 (before and after suckling), 1 and 2, and in adulthood at an age of 4 - 6 months (mean \( \pm \) S.E.M., males: 535 \( \pm \) 22 g, females: 262 \( \pm \) 4 g body wt) as indicated in each experiment. Pregnant and adult rats were anaesthetized (experiment I & II: 40 mg sodium pentothal/Kg body wt; experiment III: diethyl ether). Blood samples were drawn from the abdominal aorta. In pregnant rats, the uterus was tied and the fetuses delivered by rapid hysterectomy and decapitated. For study of neonates, neonatal rats were killed by
decapitation at the times indicated in the individual experiment. Fetal and newborn blood was collected from the neck after decapitation. Fetal, placental and neonatal tissue within each litter was always pooled for analyses. All procedures were reviewed and approved by the University of British Columbia Animal Care Committee and conformed to the guidelines of the Canadian Council on Animal Care.

2.4 Diets and treatments

2.4.1 High fat and cholesterol diet (experiment I)

Rats were fed a standard chow diet containing 4% (by wt) fat and minimal (270 ppm) cholesterol, or a diet in which carbohydrate was isocalorically substituted to give a total of 20% (by wt) fat (15% (by wt) lard plus 5% (by wt) corn oil) and 5% (by wt) cholesterol (Innis & Clandinin 1980) from gestation day 0 until sacrifice. The composition of the diets fed is detailed in tables 2.1 and 2.2.

2.4.2 Cortisol and ACTH treatment (experiment I)

A preliminary study was undertaken to determine the diurnal cycle of plasma corticosterone (appendix figures 6.1 and 6.2). The results were similar to data published for female rats (Critchlow et al 1963). The preliminary studies also showed that cortisol administration suppressed endogenous corticosterone for 12 h, whilst ACTH caused a 3-4 fold increase in plasma corticosterone which lasted approximately 1 h, following administration. Rats were, therefore, given cortisol (5 mg at 1600h) or ACTH (2 µg at 800 and 2000h) in 0.9% NaCl by subcutaneous injection each day from day 9-19 of gestation. Controls recieved no hormone treatment. The doses of cortisol and ACTH administered were pharmacological. Saline injections had no significant effect on any of the parameters assayed and, were, therefore, not routinely given to the control animals. Hormones were not given and the rats were not fasted on the day of sacrifice.

2.4.3 Cholestyramine treatment (experiment I & III)

Pregnant rats were fed the standard laboratory diet or the same diet with CY added at a level of
5% (by wt diet) throughout gestation. This was achieved by grinding rat chow into a fine powder and uniformly mixing in CY. A small amount of water was added to the ground diets at the time of feeding in order to avoid potential inhalation of the CY powdered diet by the rats. The small amount of sucrose added in the resin was not balanced by addition of extra sucrose to the standard diet.

2.4.4 Diets containing safflower, olive or palm oil (experiment I & III)

The semipurified diets all contained the same essential nutrient density per 1000 Kcal of diet. The composition of the diet and fatty acid composition of the oils used, are shown in Tables 2.1, 2.2 and 2.3. The diet containing high fat had 20% (by wt) safflower, olive or palm oil. The low fat had 5% by wt safflower oil. Additional sucrose was substituted isocalorically for 15% wt/wt fat (table 2.2) to maintain essential nutrient density of the 5% fat diet. These diets were fed throughout gestation.

| Table 2.1 |
| Ingredients contained in all semisynthetic diets. |
| (per 4498 Kcal) |
| choline chloride | 1 g |
| L-methionine | 3 g |
| vitamin mix | 10 g |
| mineral mix | 50 g |
| cellulose | 50 g |
| vitamin free casein | 250 g |
| SeO² | 84 µg |
| MnCl₂·4H₂O | 280 mg |
Table 2.2

Variable components of the semisynthetic diets.

<table>
<thead>
<tr>
<th>(g/4498 kcal diet)</th>
<th>high fat</th>
<th>low fat</th>
</tr>
</thead>
<tbody>
<tr>
<td>HFC</td>
<td>SFO(^2)</td>
<td>OO(^3)</td>
</tr>
<tr>
<td>sucrose</td>
<td>424</td>
<td>424</td>
</tr>
<tr>
<td>safflower</td>
<td>-</td>
<td>200</td>
</tr>
<tr>
<td>olive</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>palm</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>corn</td>
<td>50</td>
<td>-</td>
</tr>
<tr>
<td>lard</td>
<td>155</td>
<td>-</td>
</tr>
<tr>
<td>cholesterol</td>
<td>50</td>
<td>-</td>
</tr>
</tbody>
</table>

\(^1\)High saturated fat and cholesterol diet (HFC).
\(^2\)Safflower oil diet (SFO).
\(^3\)Olive oil diet (OO).
\(^4\)Palm oil diet (PO).

2.5 Preparation of tissues

2.5.1 Plasma (experiment I & II)

Adult blood was collected into a syringe containing disodium ethylenediaminetetra-acetate (EDTA - 1 mg/mL) as the anticoagulant. Fetal and neonatal blood was collected with pasteur pipettes coated with EDTA. Collected blood was centrifuged 3000g x 15 min at 4 C. Separated plasma was stored frozen at -80°C until analyzed.
### Table 2.3

**Major fatty acid components of the dietary oils**

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Palm oil</th>
<th>Olive oil</th>
<th>Safflower oil</th>
</tr>
</thead>
<tbody>
<tr>
<td>% total</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14:0(^2)</td>
<td>1.4</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>16:0</td>
<td>47.3</td>
<td>12.2</td>
<td>8.9</td>
</tr>
<tr>
<td>18:0</td>
<td>4.7</td>
<td>3.3</td>
<td>3.3</td>
</tr>
<tr>
<td>18:1</td>
<td>34.8</td>
<td>72.8</td>
<td>8.8</td>
</tr>
<tr>
<td>18:2 n-6</td>
<td>9.7</td>
<td>8.2</td>
<td>77.2</td>
</tr>
<tr>
<td>18:3 n-3</td>
<td>0.3</td>
<td>0.4</td>
<td>0.1</td>
</tr>
</tbody>
</table>

\(^1\)The fatty acid composition of the oils was determined by gas liquid chromatography of the methyl esters as described in Materials and Methods.  
\(^2\)Number of carbons: number of double bonds n-number of carbons between first double bond and the methyl end of the fatty acid.

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2.5.2 Liver microsomes and cytosol (experiment I & II)

The livers were rapidly removed, blotted, rinsed and homogenized (0-4°C) in 3 volumes 0.225 M sucrose with 0.025 M tris HCl (pH 7.8) and 10 mM glutathione. Homogenization was with 3 strokes of a Potter-Elvejhem homogenizer. Aliquots equivalent to 0.5 g of female liver and 1 g of pooled fetal liver were taken for preparation of microsomes and cytosol. The liver homogenate was centrifuged at 10 000g for 20 min (4°C) and the resulting supernatant centrifuged at 100 000g for 1 h (4°C). The 100 000g supernatants were used as the source of cytosolic enzymes. The microsomal pellets were resuspended in 0.1 M sucrose containing 0.05 M KCl, 0.04 M KH\(_2\)PO\(_4\), 0.03 M EDTA (pH 7.2) and 0.02 M dithiothreitol. Prepared microsomes and cytosol were then stored at -80°C until analyzed for enzyme activities. Microsomes were prepared with and without 50 mM NaF in all homogenization buffers and assay systems.
NaF was used to prevent conversion of inactive HMG CoA reductase to its unphosphorylated (active) form (Hunter & Rodwell 1980). Total adrenal HMG CoA reductase was assayed in whole adrenal homogenates from control and cortisol treated pregnant rats and in pooled fetal adrenal homogenates. The adrenal homogenates prepared from three nonpregnant female rats were also studied for reference.

2.5.3 Post-mitochondrial supernatant (experiment I)

Post-mitochondrial supernatants were prepared at 0-4°C for analysis of lipase activity. Maternal adipose and skeletal muscle (soleus, extensor digitorum longus) were homogenized using a polytron homogenizer. Maternal and fetal liver and fetal muscle were homogenized using a Potter-Elvejhem homogenizer in 4 volumes of 0.25 M sucrose-1 mM EDTA in the presence of heparin (6 U/mL). The homogenate was then centrifuged at 10 000g for 15 min and the fat free post-mitochondrial supernatant under the floating fat layer was collected and stored at -80°C until used for the assay of lipase activity.

2.6 Enzyme assays

2.6.1 HMG CoA reductase (experiment I & II)

Microsomal HMG CoA reductase (EC 1.1.1.34) activity was assayed as the rate (pmol/min) of formation of [3H]mevalonate from [3H]HMG CoA (Goodwin & Margolis 1976).

\[
\text{HMG CoA} + \text{NADPH} \xrightarrow{\text{HMG CoA reductase}} \text{mevalonate} + \text{NADP} + \text{HCl} \\
\text{NADP} + \text{glucose-6-phosphate} \xrightarrow{\text{glucose-6-phosphate dehydrogenase}} \text{NADPH} + 6\text{-phosphogluconate}
\]
Briefly, 0.1 mL of microsomal suspension (0.1 - 0.5 mg protein) was aliquoted into 20 mL screw-cap tubes containing 0.05 mL of resuspension buffer (0.1 M sucrose containing 0.05 M KCl, 0.04 M KH₂PO₄ and 0.03 M EDTA at pH 7.2) and incubated in a 37°C oscillating water bath. After pre-incubating for 20-30 min the reaction was started by the addition of 0.05 mL of a substrate cofactor mix (SCM: 0.8 mM HMG CoA, 120 mM glucose-6-phosphate, 8 mM NADP, 7 international units (IU) glucose-6-phosphate dehydrogenase and 1.5 μCi/mL DL-[methyl-³H]HMG CoA). The reaction was stopped 20 min later by the addition of 0.05 mL 12 N HCL. The mixture was then incubated for a further 30 min, removed from the water bath and approximately 0.3 g sodium sulfite added. Mevalonolactone was extracted from the reacted mixture with the addition of 20 mL toluene followed by 2-3 min of vigorous shaking on an Eberbach shaker. After allowing the test-tubes to stand at room temperature for 30 min, 10 mL of the toluene extract was aliquoted into scintillation vials. ACS was then added and the toluene counted for [³H] content. Blanks were run in a similar manner with the exception that the HCl was added before the SCM. Recovery was determined from the percentage of [¹⁴C]mevalonic acid recovered in toluene extracts as mevalonolactone. Active (unphosphorylated) reductase activity was determined by preparing and assaying microsomes for reductase activity in the presence of 50 mM NaF. NaF inhibits the activity of phosphatases present within the hepatocyte and hence prevents activation of phosphorylated HMG CoA reductase.

2.6.2 Cystathionase (experiment I)

Cytosolic liver fractions were assayed for cystathionase (EC 4.4.1.1) activity (Gaull et al 1969) by the spectrophotometric measurement of cysteine (Gaitonde 1967).

\[
\text{cystathionine} + \text{pyridoxal phosphate} \xrightarrow{\text{cystathionase}} \text{cysteine} + \alpha\text{-ketobutyrate} + \text{pyridoxamine phosphate}
\]

Briefly, 0.1 mL of cytosolic protein (0.2 - 2.5 mg) was added to 0.25 mL of 1.0 mM pyridoxal phosphate in buffer (100 mM Tris-HCl at pH 8.4) and preincubated at 37°C for 5 min. The reaction was started with
the addition of 0.25 mL of 16 mM cystathionine in buffer. After an incubation period of 30 min, the reaction was stopped with the addition of 0.1 mL 3.5 N perchloric acid. Blank reactions were performed by addition of perchloric acid to the sample prior to the addition of cystathionine. The reacted mixture was centrifuged (3000 g for 30 min at 4 °C), 0.1 mL of the supernatant added to 0.5 mL acid ninhydrine (250 mg/6 mL acetic acid + 4 mL 12 N HCl), the test-tube covered and then incubated for 10 min in a boiling water bath. The tube was then cooled in ice-cold water and 5 mL of 95% ethanol immediately added. The absorbance of the final mixture was then read in a spectrophotometer at 560 nm. A standard curve was constructed with known concentrations of cysteine. Activity was calculated as the rate (nmol/min) of formation of cysteine from cystathionine per mg of cytosolic protein.

2.6.3 Tyrosine aminotransferase (experiment I)

Tyrosine aminotransferase (TAT, EC 2.6.1.5) activity in the cytosolic fraction of liver was determined spectrophotometrically by assay of the rate (nmol/min) of formation of p-hydroxyphenylpyruvate (pHPP) from L-tyrosine and α-ketoglutarate (α-KG) (Diamondstone et al 1966).

\[
\begin{align*}
\text{L-tyrosine} + \alpha\text{-KG} & \xrightarrow{TAT} \text{pHPP} \\
& \text{pyridoxal phosphate} \quad \text{L-glutamate} \quad \text{NaOH} \quad \text{p-hydroxybenzaldehyde}
\end{align*}
\]

All reagents were dissolved in 0.2 M potassium phosphate buffer at pH 7. Sample (0.03 - 0.07 mg cytosolic protein in 0.1 mL buffer) was added to 0.8 mL tyrosine (6.86 mM) and 0.025 mL pyridoxal phosphate (1.2 mM). After preincubation at 37 °C, the reaction was started by the addition of 0.025 mL α-ketoglutarate (0.3 M). The reaction was stopped 20 min later by the addition of 0.05 mL NaOH (10 N). Sample blanks were prepared by the addition of NaOH before the addition of α-ketoglutarate. Reacted samples and blanks were allowed to stand at room temperature for 30 min. The absorbance was then determined in
a spectrophotometer at 331 nm. Test tubes containing known concentrations of \( p \)-hydroxyphenylpyruvate were run with the assay and used to construct a standard curve. The activity of TAT was calculated as the rate (nmol/min) of formation of \( p \)-hydroxyphenylpyruvate from tyrosine per mg of cytosolic protein.

2.6.4 Fatty acid synthase (experiment I)

The activity of fatty acid synthase (FAS, EC 2.3.1.85) was determined using a spectrophotometric method (reviewed by Nepokroeff et al 1975).

\[
\text{Malonyl CoA} + \text{Acetyl CoA} + \text{NADPH} \xrightarrow{\text{FAS}} \text{Butyryl CoA} + \text{NADP} + \text{CO}_2 + \text{H}_2\text{O}
\]

Cytosolic protein (0.6 - 1.2 mg cytosolic protein in 0.075 mL) was added to 1.05 mL H\(_2\)O, 0.9 mL 1 M KH\(_2\)PO\(_4\) at pH 7, and 0.1 mL of 15 mM dithiothreitol and preincubated at 37°C. 15 min later, 0.1 mL of acetyl CoA (0.33 mM) and 0.1 mL of NADPH (1 mM) were added to 0.775 mL of the preincubated mix. The rate of nonspecific oxidation of NADPH was recorded as the change in absorbance at 340 nm at 37°C for 2 - 3 minutes. Malonyl CoA (0.025 mL at 4.2 mM) was then added, the media-sample mixed and the rate of NADPH oxidation during fatty acid synthesis was recorded at 340 nm for 2 - 3 minutes. Absorbance readings were converted to nmol NADPH with the use of a standard curve constructed from NADPH. The activity of fatty acid synthase was calculated as the rate (nmol/min) of NADPH oxidized after addition of malonyl CoA minus the rate (nmol/min) of nonspecific NADPH oxidation per mg cytosolic protein.

2.6.5 Lipases (experiment I)

Lipase activity was assayed as the release of \([9,10-^3\text{H}]\)oleate from a substrate emulsion containing 0.1 mCi \([^3\text{H}]\)triolein and 36 mg cold triolein (Hietanen & Greenwood 1977) with fetal calf serum (FCS) as the source of apoprotein (apo) CII.

\[
\text{triolein} \xrightarrow{\text{lipase}} \text{glycerol} + \text{fatty acid}
\]
The substrate emulsion was prepared by adding 0.1 mCi of [9,10-3H]triolein to 36 mg unlabelled triolein and 1.5 mg lyssolecithin. After evaporation of organic solvent, 2.55 mL of 0.2 M Tris-HCl at pH 8 and 0.45 mL of 1% (w/v) BSA (3 mL total volume) were added and the mixture sonicated in an ice bath at 60 W for 4 min at 30 sec intervals. NaCl (178 mg) was added to 1.5 mL of the substrate emulsion, the other half of the emulsion containing no NaCl.

The reaction tubes contained 0.1 - 1.5 mg postmitochondrial supernatant protein in 0.1 mL buffer and 0.05 mL FCS and were preincubated at 37°C for 30 min. The reaction was started with the addition of 0.05 mL substrate emulsion (+/- NaCl) and incubated for 30 min at 37°C. The reaction was stopped and free fatty acids extracted by the addition of 3.25 mL chloroform:methanol:heptane (23:25:18, v/v/v). The free fatty acid extract was washed with 1.05 mL KHCO₃ (0.1 M) at pH 10.5. An aliquot of the upper organic phase was pipetted into a liquid scintillation vial, evaporated, ACS added and then counted for [3H] content.

Lipoprotein lipase activity was corrected for nonspecific lipase activity by taking into account any lipase activity that occurred in the presence of 1 M NaCl. Blanks were run in the absence of postmitochondrial protein (0.1 mL H₂O replacing protein volume). Efficiency of fatty acid extraction was determined with [14C]oleic acid extraction from the substrate emulsion and accounted for in all calculations. Lipase activities were defined as: lipoprotein lipase (EC 3.1.1.34), lipase activity requiring FCS for maximal activity and inhibited by 1 M NaCl; hepatic triacylglycerol lipase, lipase activity occurring in the presence of 1 M NaCl and with no FCS (reviewed by Nilsson-Ehle et al 1980); placental lipase, lipase fully active at 1 M NaCl in the presence of FCS. Enzyme activities were calculated as the rate (nmol/min) of oleate released per mg protein.

2.7 Plasma hormone assays (experiment 1)

Plasma insulin, free and total T₄ levels were determined by radioimmunoassay (RIA) utilizing competitive binding principles and commercial RIA kits containing ¹²⁵Iodinated hormones. The level of unbound T₄, which is considered to represent the biologically active hormone, was calculated from the
thyroid binding globulin uptake and T₄ assays as documented in the kits. Corticosterone was determined by competitive binding assay essentially as described (Henning 1980). Intraassay variation was determined by carrying out multiple determinations on the same sample within the same assay and was found to be less than 3%. A known rat plasma sample was determined with each assay to control for potential interassay variation. Maternal insulin was not routinely determined due to high standard deviations resulting from the lack of maternal fasting.

2.8 Determination of tissue lipid composition (experiment I, II & III)

Plasma total cholesterol levels were quantified with a colorimetric method (Zak et al 1954) in experiment I, and with an enzymatic kit in experiments II and III. Plasma triacylglycerol, and free cholesterol were assayed using enzymatic kits with glycerol and cholesterol, respectively, as standards. Fetal hepatic triacylglycerol and cholesterol levels were assayed similarly in an aliquot of total liver lipid extract (Folch et al 1957) after resuspension of the dried lipid in 2-propanol. The standards in this case were similarly prepared, and for the assay of triacylglycerol was triolein rather than glycerol. Phospholipid phosphorous was assayed after digestion of an aliquot of the total liver lipid extract with 70% perchloric acid (Chen et al 1956).

Triacylglycerols and phospholipids were separated from each other and other lipid classes by thin-layer chromatography on silica gel 60 t.l.c. plates with petroleum ether/diethyl ether/acetic acid (85:15:3, v/v/v) as the solvent system. Fatty acid methyl esters were prepared by transmethylation with 14% (v/v) BF₃ in methanol (Morrison & Smith 1961) and separated by GLC. Helium was used as the carrier gas (1 mL/min) and the inlet splitter was set at 100:1 (Innis 1986). The GLC oven temperature was programmed from 80°C and was raised after 2 min to 170°C at 20°C/min, held for 19.5 min, then raised to 190°C at 20°C/min, held for 18 min and then finally raised to 240°C at 20°C/min and held at this temperature for a further 15 min. The injector and detector temperatures were set at 240 and 260 °C, respectively.
2.9 Measurement of \textit{in vivo} rates of lipogenesis (experiment III)

On the day of the experiment, at 0900 h (5 hr into a 12 hr dark cycle), 100 mCi \( ^3 \text{H} \) water in 0.5 mL saline was injected into the maternal or adult male tail vein. The procedure was conducted under light diethyl ether anaesthesia, the rats allowed to regain consciousness and then maintained in a fume hood without food or water. Exactly one hour later, they were again anaesthetized with diethyl ether (Jeske and Dietschy 1980) and the uterus of the pregnant rat quickly removed. The pregnant and adult male rats were exsanguinated from the abdominal aorta (Jeske and Dietschy 1980). For practical reasons, neonatal rats were injected intra-peritoneally with 2 mCi of \( ^3 \text{H} \) water. This quantity of \( ^3 \text{H} \) water was calculated on a body weight basis and resulted in a specific activity in the neonatal plasma similar to that in the fetuses. Blood and liver were collected one hour after injection of \( ^3 \text{H} \) water, and neonatal and fetal tissue pooled for each litter. The livers were immediately frozen in liquid nitrogen. Plasma was separated by centrifugation and stored at -80\(^\circ\)C as described previously (Innis 1983).

2.9.1 Tissue lipid extraction and saponification

A portion (approximately 1 g) of liver was homogenized in 2 mL saline and an aliquot used for determination of protein concentration (Lowry et al 1951). The remaining tissue homogenate was extracted for lipid (Folch et al 1957), the organic extract dried under \( \text{N}_2 \) and saponified overnight in 3 mL alcoholic KOH (6 mL 50\% KOH in 94 mL ethanol) at 80\(^\circ\)C (Andersen and Dietschy 1978). The following morning, the lipid samples were allowed to cool to room temperature and adjusted to 50\% (vol/vol) ethanol by the addition of water. Nonsaponifiable lipids were extracted 3 times with 3 mL petroleum ether. The remaining lower phase (50\% ethanol) was acidified to pH < 2 with HCl and the saponifiable lipid extracted 3 times with 3 mL petroleum ether (Weiss & Dietschy 1969). The saponifiable fraction was washed with 50\% (v/v) ethanol, evaporated under \( \text{N}_2 \), stored overnight \textit{in vacuo} over Drierite\textsuperscript{TM} and counted for \( ^3 \text{H} \) content the following morning.
2.9.2 Digitonin precipitation of nonsaponifiable lipid

The nonsaponifiable lipid extract was precipitated with digitonin (Sperry 1963) after being washed with 50% (v/v) ethanol and then dried under N₂. Pyridine was used to split the digitonide into digitonin and free sterol (Jeske and Dietschy 1980). The digitonin precipitable sterols (DPS) were extracted with diethyl ether and dried under N₂. DPS were counted for [³H] after storage overnight in vacuo over H₂SO₄.

2.9.3 Calculation of rates of lipid synthesis

As preparation for these studies, preliminary work was done to determine the rate of equilibration of [³H]water between the maternal and fetal circulation. This was done by injecting [³H]water into the maternal tail vein of pregnant rats and sacrificing them at various times between 0 and 60 min post-injection and collecting maternal and fetal blood for determination of plasma water radioactivity. The resulting equilibrium curves were then used to determine the correction factor required to convert the fetal water specific radioactivity at 60 min to the average specific radioactivity over the entire 60 min period. The results obtained confirmed data recently published by Belknap and Dietschy (1988) which showed that the specific activity of maternal plasma decreased whereas that of fetal plasma increased with time. This is explained by equilibration of the maternal plasma and intracellular water with other pools of bulk water in the body such as the bile, intestinal secretions, cerebrospinal fluid, urine (reviewed by Dietschy & Spady 1984) and also the fetuses. Hence, the specific activity of the maternal or fetal plasma water determined after 1 hr is multiplied by a factor (k) to give the mean specific activity of plasma water during the 60 min experiment. The mean specific activity of plasma water was calculated as:

$$\text{mean specific activity} = \frac{k \times \text{(dpm } ^3\text{H/ml plasma})}{(\text{nmol } \text{H}_2\text{O/ml } \text{H}_2\text{O})(y \text{ mL } \text{H}_2\text{O/ml plasma})}\)$$

where k=0.91 for fetal plasma and 1.09 for maternal plasma, the factor y=0.96 for fetal plasma and 0.92 for maternal plasma reflects the proportion of water in plasma (Dietschy and Spady 1984, Belknap and Dietschy 1988). Preliminary studies found that the change in plasma water specific activity from 0 to 60 min after [³H]water injection was similar in adult rats injected intra-peritoneally and those injected using
a tail vein. The rate of $^3$H$_2$O equilibration throughout the neonatal body water compartment was assumed to be similar to $^3$H$_2$O equilibration within adult rats. Therefore, the correction factor (k) of 1.09 used in the calculations for the adult was used in the calculation of mean specific body water activity in the neonates. Using this and the $[^3]$H recovered as liver cholesterol or saponifiable lipid, rates of lipid synthesis were then calculated as nanomoles of $[^3]$H water incorporated into DPS or saponifiable lipid per hour (Jeske and Dietschy 1980, Belknap and Dietschy 1988) per mg protein. A known amount of $[^3]$H cholesterol was added to unlabelled samples, extracted and precipitated with each experiment to correct for losses in the recovery of DPS. Recovery of $[^3]$H cholesterol for the separate experiments averaged 86.4 ± 1.3 % (mean ± SEM, n=11). Recovery of $[^3]$H linoleate exceeded 96%. Contamination of the saponifiable and DPS fraction with $[^3]$H water was < 1%, as determined by adding a known amount of $[^3]$H water to unlabelled liver tissue and determination of radioactivity in extracted lipid.

2.10 Statistical analysis

The data are given as means ± S.E.M.. Analyses for statistically significant differences between the groups fed the diets with and without CY, or between the rats fed a control diet and those fed a diet containing high fat and cholesterol were done using Students’ t-test. Significant effects of cortisol or ACTH were determined using analysis of variance and group comparisons with Tukey’s test. Comparisons in enzyme activity or lipid composition among the rats fed 20% fat were made by analysis of variance with significant differences being determined with Newman Keul’s test in the Number Cruncher Statistical System, version 5.1 (Kaysville, Utah). Significant differences between rats fed 20% and 5% safflower oil were determined with Student’s t-test.

Statistical differences during perinatal development of hepatic HMG CoA reductase activity, lipid synthesis, lipid content and specific microsomal phospholipid fatty acids and plasma lipids were determined using analysis of variance followed by Duncan’s multiple range test. In all cases the results are expressed as means ± S.E.M.. In instances where the S.E.M. is small, the values given in figures may be too small to be indicated as error bars in the figure and therefore not shown.
3 RESULTS

3.1 Experiment I: Sensitivity of fetal hepatic HMG CoA reductase activity to external stimuli

3.1.1 Effect of feeding pregnant rats diets containing high fat and cholesterol

3.1.1.1 Pregnancy outcome

The inclusion of high levels of saturated fat (20% wt/wt) and cholesterol (5% wt/wt) in the diet of the pregnant rat had no adverse effect on maternal or fetal weight (table 3.1).

3.1.1.2 Plasma lipid and hormone levels

The high saturated fat and cholesterol diet resulted in an 8 fold increase in maternal plasma cholesterol as well as a significant elevation of the maternal plasma corticosterone (table 3.1). The diet

| Table 3.1 |
The effect of a high saturated fat and cholesterol diet during pregnancy on body and liver weights and on maternal and fetal plasma lipid and hormone levels.1 |

<table>
<thead>
<tr>
<th></th>
<th>Maternal</th>
<th>HFC2</th>
<th>Fetal</th>
<th>HFC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body weight, g</td>
<td>319 ± 11</td>
<td>327 ± 13</td>
<td>3.4 ± 0.3</td>
<td>3.8 ± 0.3</td>
</tr>
<tr>
<td>Liver weight, g/100g</td>
<td>3.6 ± 0.5</td>
<td>4.0 ± 0.1</td>
<td>6.7 ± 0.2</td>
<td>6.4 ± 0.4</td>
</tr>
<tr>
<td>Cholesterol, mg/dL</td>
<td>63 ± 7</td>
<td>521 ± 81+</td>
<td>63 ± 5</td>
<td>77 ± 6</td>
</tr>
<tr>
<td>Triacylglycerol, mg/dL</td>
<td>175 ± 12</td>
<td>264 ± 51</td>
<td>73 ± 5</td>
<td>69 ± 9</td>
</tr>
<tr>
<td>T4, μg/dL</td>
<td>1.8 ± 0.2</td>
<td>1.7 ± 0.3</td>
<td>0.9 ± 0.0</td>
<td>0.9 ± 0.0</td>
</tr>
<tr>
<td>Free T4, ng/dL</td>
<td>1.3 ± 0.0</td>
<td>1.6 ± 0.2</td>
<td>0.7 ± 0.0</td>
<td>0.7 ± 0.0</td>
</tr>
<tr>
<td>Insulin, μunits/mL</td>
<td>N.D.3</td>
<td>N.D.</td>
<td>61 ± 4</td>
<td>77 ± 12</td>
</tr>
<tr>
<td>Corticosterone, μg/dL</td>
<td>50 ± 3</td>
<td>82 ± 8+</td>
<td>28 ± 5</td>
<td>19 ± 3</td>
</tr>
</tbody>
</table>

1The studies were conducted on the 20th day of gestation. Liver and plasma samples from each litter were pooled prior to assay. Values are mean ± S.E.M., n = 6 litters or pregnant rats per diet. Mean differences were determined using Students' t-test, *p < 0.01.

2High saturated fat and cholesterol diet (HFC).

3Not determined (N.D.)
had no significant effect on triacylglycerol or \( T_4 \) levels in maternal or fetal plasma. The fetal plasma cholesterol, insulin and corticosterone levels, however, were also not significantly affected by the HFC diet (table 3.1).

### 3.1.1.3 Enzyme activities

Despite the diet-induced maternal hypercholesterolemia the fetal hepatic reductase activities were not significantly altered (table 3.2). Fetal hepatic cystathionase and TAT activity were also not affected by the HFC diet (table 3.2). In contrast, the activities of HMG CoA reductase, TAT and cystathionase were significantly lower in the maternal liver of rats fed HFC rather than the control diet.

<table>
<thead>
<tr>
<th></th>
<th>Maternal</th>
<th>Fetal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>control</td>
<td>HFC(^2)</td>
</tr>
<tr>
<td>HMG CoA reductase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>total, pmol/min/mg protein</td>
<td>920 ± 76</td>
<td>78 ± 15(^+)</td>
</tr>
<tr>
<td>active, pmol/min/mg protein</td>
<td>266 ± 29</td>
<td>47 ± 5(^+)</td>
</tr>
<tr>
<td>% active</td>
<td>30 ± 4</td>
<td>70 ± 8(^+)</td>
</tr>
<tr>
<td>FAS(^3), nmol/min/mg protein</td>
<td>14.2 ± 1.2</td>
<td>17.7 ± 3.2</td>
</tr>
<tr>
<td>TAT(^4), nmol/min/mg protein</td>
<td>57 ± 5</td>
<td>32 ± 6(^*)</td>
</tr>
<tr>
<td>Cystathionase, nmol/min/mg prot.</td>
<td>21.5 ± 0.8</td>
<td>14.5 ± 1.2(^*)</td>
</tr>
</tbody>
</table>

\(^1\)The studies were conducted on the 20th day of gestation. Liver and plasma samples from each litter were pooled prior to assay. HMG CoA reductase, total and active, represents enzyme activities following microsomal preparation and assay in the absence and presence, respectively, of 50 mM NaF. Values are mean ± S.E.M., \( n = 6 \) litters or pregnant dams per diet. Mean differences were determined using Student's t-test, \( ^+ p < 0.05, ^* p < 0.01. \)

\(^2\)High saturated fat and cholesterol diet (HFC)

\(^3\)Fatty acid synthase (FAS)

\(^4\)Tyrosine aminotransferase (TAT)
3.1.2 Effect of administering cortisol or ACTH to pregnant rats

3.1.2.1 Pregnancy outcome

The administration of large doses of cortisol resulted in a significantly higher maternal weight gain during pregnancy than found in the control pregnant rats (table 3.3). The hormone had no significant effect on the maternal or fetal liver, adrenal, placenta or fetal body weights. The placenta/fetal body weight ratio, however, was significantly lower when compared to the control group (table 3.3). The administration of ACTH had no significant effect on any of the maternal or fetal organ or body weights (table 3.3). The ACTH dose in these studies is approximately 7 fold lower than that associated with increased adrenal weight and altered morphology and function of the adrenal zona glomerulosa in the adult rat (Lehoux et al 1985).

Table 3.3

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Cortisol</th>
<th>ACTH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pregnant rat</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>body weight gain, g</td>
<td>94 ± 6</td>
<td>125 ± 6+</td>
<td>102 ± 5</td>
</tr>
<tr>
<td>liver weight, mg/g body wt</td>
<td>37.0 ± 0.7</td>
<td>37.0 ± 1.2</td>
<td>39.5 ± 1.0</td>
</tr>
<tr>
<td>adrenal weight, µg/g body wt</td>
<td>201 ± 12</td>
<td>205 ± 9</td>
<td>225 ± 15</td>
</tr>
<tr>
<td>Fetuses</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body weight, g</td>
<td>3.7 ± 0.2</td>
<td>4.2 ± 0.3</td>
<td>3.2 ± 0.3</td>
</tr>
<tr>
<td>liver weight, mg/g body wt</td>
<td>7.5 ± 3.4</td>
<td>7.4 ± 1.9</td>
<td>7.9 ± 2.3</td>
</tr>
<tr>
<td>adrenal weight, µg/g body wt</td>
<td>662 ± 36</td>
<td>794 ± 62</td>
<td>834 ± 117</td>
</tr>
<tr>
<td>Placenta, g/organ</td>
<td>6.2 ± 0.3</td>
<td>7.1 ± 0.3</td>
<td>5.9 ± 0.4</td>
</tr>
<tr>
<td>Placenta, mg/g fetus</td>
<td>180 ± 12</td>
<td>135 ± 10+</td>
<td>192 ± 17</td>
</tr>
</tbody>
</table>

Cortisol or ACTH was injected subcutaneously into pregnant rats from day 9 to 19 of gestation. Maternal and fetal tissue were collected on the 20th day of gestation. The data are given as means ± S.E.M., control n = 11, cortisol n = 11, ACTH n = 8. Levels of significance were calculated for the control versus treated rats using analysis of variance and pair-wise comparisons using Tukey's test, *p < 0.05, +p < 0.01.
3.1.2.2 Plasma lipid and hormone levels

Cortisol, but not ACTH, significantly increased the plasma cholesterol and triacylglycerol level in pregnant rats (table 3.4). In the preliminary studies, the circulating corticosterone level was decreased 50% and increased 300% by cortisol and ACTH, 30 min after their respective administration. The effect of cortisol lasted approximately 12 h, and that of ACTH about 1 h following administration. The results in table 3.4 were obtained from rats at the peak diurnal activity of HMG CoA reductase (Shapiro & Rodwell 1972), which was about 17 h and 13 h after the last administration of cortisol or ACTH, respectively. Endogenous corticosterone production was still inhibited, as evidenced by the reduced plasma corticosterone level in the pregnant rats treated with cortisol. The plasma corticosterone of pregnant rats treated with ACTH, on the other hand, was similar to the control values at this time (table 3.4). The administration of cortisol or ACTH had no effect on the maternal plasma thyroxine, although plasma free thyroxine was significantly decreased by both hormones.

The fetuses of rats given cortisol had increased plasma levels of cholesterol, thyroxine and free thyroxine, but similar triacylglycerol, corticosterone and insulin to fetuses from the control rats (table 3.4). Fetal plasma cholesterol and insulin were increased whereas free thyroxine was decreased in the ACTH treated group. Fetal plasma triacylglycerol, corticosterone, and thyroxine were not significantly altered by ACTH.

3.1.2.3 Enzyme activities

The administration of cortisol resulted in a significantly reduced activity of the total HMG CoA reductase, but had no effect on the active (unphosphorylated) reductase or FAS activity (table 3.5) in the maternal liver. The fetuses of these rats had similar total and active reductase and FAS activities to that of fetuses in the control group.

The hepatic total HMG CoA reductase and FAS activities of the pregnant rats treated with ACTH were similar to the activities in the control rats (table 3.5). The maternal active reductase activity, however, was increased due to ACTH. Maternal ACTH administration had no effect on fetal hepatic HMG CoA
reductase activity. Fetal hepatic FAS activity, however, was increased by ACTH treatment.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Cortisol</th>
<th>ACTH</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pregnant rat</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cholesterol, mg/dL</td>
<td>63 ± 2.7</td>
<td>94 ± 7.4</td>
<td>78 ± 7.2</td>
</tr>
<tr>
<td>triacylglycerol, mg/dL</td>
<td>209 ± 28</td>
<td>361 ± 45</td>
<td>286 ± 47</td>
</tr>
<tr>
<td>corticosterone, µg/dL</td>
<td>43 ± 7.2</td>
<td>18 ± 4.0</td>
<td>27 ± 5.5</td>
</tr>
<tr>
<td>thyroxine, µg/dL</td>
<td>1.7 ± 0.2</td>
<td>1.5 ± 0.0</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td>free thyroxine, ng/dL</td>
<td>1.1 ± 0.2</td>
<td>0.7 ± 0.1</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td><strong>Fetuses</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cholesterol, mg/dL</td>
<td>60 ± 2.4</td>
<td>76 ± 4.7</td>
<td>82 ± 7.0</td>
</tr>
<tr>
<td>triacylglycerol, mg/dL</td>
<td>64 ± 2.9</td>
<td>68 ± 5.9</td>
<td>67 ± 4.7</td>
</tr>
<tr>
<td>corticosterone, µg/dL</td>
<td>15 ± 2.8</td>
<td>15 ± 3.2</td>
<td>22 ± 3.4</td>
</tr>
<tr>
<td>thyroxine, µg/dL</td>
<td>0.9 ± 0.0</td>
<td>1.0 ± 0.0</td>
<td>0.9 ± 0.0</td>
</tr>
<tr>
<td>free thyroxine, ng/dL</td>
<td>0.7 ± 0.0</td>
<td>0.5 ± 0.0</td>
<td>0.5 ± 0.0</td>
</tr>
<tr>
<td>insulin, µunits/mL</td>
<td>71 ± 6</td>
<td>90 ± 6</td>
<td>115 ± 23</td>
</tr>
</tbody>
</table>

1Cortisol or ACTH were administered from day 9 to 19 of gestation and maternal and fetal tissues collected on gestation day 20. Fetal plasma for each litter was pooled prior to assay. The data are given as means ± S.E.M., control n = 11, cortisol n = 11, ACTH n = 8. Levels of significance were calculated for the control versus hormone treated rats using analysis of variance and pair-wise comparisons using Tukey's test, *p < 0.05, +p < 0.01.
Table 3.5
Effect of cortisol or ACTH administration on hepatic HMG CoA reductase and fatty acid synthase activity in the pregnant rat and her fetuses.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Cortisol</th>
<th>ACTH</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pregnant rat</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HMG CoA reductase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>total, pmol/min/mg</td>
<td>855 ± 89</td>
<td>402 ± 68 *</td>
<td>1178 ± 131</td>
</tr>
<tr>
<td>active, pmol/min/mg</td>
<td>236 ± 28</td>
<td>301 ± 70</td>
<td>657 ± 118 *</td>
</tr>
<tr>
<td>FAS, pmol/min/mg</td>
<td>15.3 ± 1.5</td>
<td>12.1 ± 1.4</td>
<td>19.0 ± 2.6</td>
</tr>
<tr>
<td>cystathionase, nmol/min/mg</td>
<td>21.5 ± 0.8</td>
<td>16.9 ± 1.0 *</td>
<td>13.7 ± 1.0 *</td>
</tr>
<tr>
<td>TAT, nmol/min/mg</td>
<td>57.3 ± 4.8</td>
<td>45.2 ± 6.6</td>
<td>84.9 ± 10.8 *</td>
</tr>
<tr>
<td><strong>Fetuses</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HMG CoA reductase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>total, pmol/min/mg</td>
<td>608 ± 102</td>
<td>521 ± 68</td>
<td>784 ± 94</td>
</tr>
<tr>
<td>active, pmol/min/mg</td>
<td>157 ± 24</td>
<td>211 ± 54</td>
<td>288 ± 17</td>
</tr>
<tr>
<td>FAS, pmol/min/mg</td>
<td>7.7 ± 0.2</td>
<td>7.0 ± 0.9</td>
<td>13.5 ± 2.0 *</td>
</tr>
<tr>
<td>cystathionase, nmol/min/mg</td>
<td>16.2 ± 1.2</td>
<td>11.1 ± 1.0 *</td>
<td>9.6 ± 1.6 *</td>
</tr>
<tr>
<td>TAT, nmol/min/mg</td>
<td>6.3 ± 1.4</td>
<td>2.7 ± 0.4 *</td>
<td>4.7 ± 0.8</td>
</tr>
</tbody>
</table>

\*Cortisol or ACTH were administered subcutaneously to pregnant rats from gestation day 9 to 19 and maternal and fetal tissues collected on gestation day 20. Fetal tissue for each litter was pooled prior to assay. The data are given as means ± S.E.M., control n = 11, cortisol n = 11, ACTH n = 8. Levels of significance were calculated for the control versus the hormone treated rats using analysis of variance and pair-wise comparisons using Tukey's test, \*p < 0.05, \+p < 0.01.

\*Fatty acid synthase (FAS).
\*Tyrosine aminotransferase (TAT).
Adrenal total HMG CoA reductase activity was determined in a subgroup of rats (table 3.6). Fetal adrenal reductase activity was greater than activity found in maternal or adult adrenals. Cortisol treatment, however, reduced the activity in the fetal adrenal.

3.1.3 Effect of maternal cholestyramine feeding

3.1.3.1 Pregnancy outcome

The inclusion of 5% (wt/wt) CY in the diet throughout gestation had no significant effect on maternal weight gain, litter size or weight or placental weight.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Cortisol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pmol/min/mg homogenate protein</td>
<td></td>
</tr>
<tr>
<td>Pregnant rats</td>
<td>16.9 ± 2.8</td>
<td>13.2 ± 0.5</td>
</tr>
<tr>
<td>Fetuses</td>
<td>30.7 ± 2.3</td>
<td>21.0 ± 0.9 *</td>
</tr>
<tr>
<td>Adult females</td>
<td>16.9 ± 5.2</td>
<td>N.D.²</td>
</tr>
<tr>
<td>Adult males</td>
<td>16.9 ± 2.0</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

¹Cortisol was administered to pregnant rats from day 9 to 19 of gestation. Adrenalectomy was performed on gestation day 18. Maternal and fetal tissue was collected on the 20th day of gestation. Tissue from nonpregnant rats was collected after 10 days of cortisol administration or 3 days after adrenalectomy. Fetal tissue was pooled prior to assay. The data are given as means ± S.E.M. for 7 pregnant controls, 4 pregnant rats treated with cortisol, 3 adult females and 4 adult males. Levels of significance were calculated for control versus treatments using Students' t-test, *p < 0.01.

²Not determined (N.D.).
3.1.3.2 Tissue lipid and plasma hormone levels

Cholestyramine did not have any significant effect on the concentration of cholesterol, triacylglycerol, thyroxine, insulin or corticosterone in the maternal or fetal plasma (table 3.7). The quantification of liver and placental cholesterol and triacylglycerol showed no significant effect of CY feeding in the maternal or fetal tissues studied (data not shown).

3.1.3.3 Enzyme activities

Cholestyramine treatment resulted in a significant increase in the maternal hepatic total and active HMG CoA reductase activity, and in the percent total enzyme present in the phosphorylated form (table 3.8). The activity of FAS was also significantly higher in the maternal liver of rats fed CY than in rats fed

---

<table>
<thead>
<tr>
<th></th>
<th>Maternal</th>
<th>Fetal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>control</td>
<td>CY^2</td>
</tr>
<tr>
<td>Cholesterol, mg/dL</td>
<td>67 ± 6</td>
<td>67 ± 5</td>
</tr>
<tr>
<td>Triacylglycerol, mg/dL</td>
<td>225 ± 66</td>
<td>249 ± 65</td>
</tr>
<tr>
<td>T₄, µg/dL</td>
<td>1.8 ± 0.2</td>
<td>1.4 ± 0.2</td>
</tr>
<tr>
<td>Free T₄, ng/dL</td>
<td>1.3 ± 0.1</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td>Insulin, µunits/mL</td>
<td>N.D.³</td>
<td>N.D.</td>
</tr>
<tr>
<td>Corticosterone, µg/dL</td>
<td>41 ± 3</td>
<td>33 ± 5</td>
</tr>
</tbody>
</table>

Table 3.7
The effect of cholestyramine treatment on maternal and fetal plasma lipid and hormone levels¹.

¹The studies were conducted on the 20th day of gestation. Liver and plasma samples from each litter were pooled prior to assay. Values are mean ± S.E.M., control n = 9, CY n = 7. There were no mean differences between diets as determined with Students' t-test at p < 0.05.
²5% cholestyramine diet (CY).
³Not determined (N.D.)
the control diet. Cholestyramine had no effect on the activities of cystathionase or TAT in the maternal liver.

<table>
<thead>
<tr>
<th>Liver</th>
<th>Maternal</th>
<th>Fetal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>control</td>
<td>CY^2</td>
</tr>
<tr>
<td>HMG CoA reductase^3</td>
<td>920 ± 76</td>
<td>2402 ± 69^+</td>
</tr>
<tr>
<td>total, pmol/min/mg</td>
<td>266 ± 29</td>
<td>1234 ± 200^+</td>
</tr>
<tr>
<td>active, pmol/min/mg</td>
<td>30 ± 4</td>
<td>52 ± 8^+</td>
</tr>
<tr>
<td>% active</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FAS^4, nmol/min/mg protein</td>
<td>14.2 ± 1.2</td>
<td>27.2 ± 2.5^+</td>
</tr>
<tr>
<td>TAT^5, nmol/min/mg protein</td>
<td>57 ± 4</td>
<td>61 ± 4</td>
</tr>
<tr>
<td>Cystathionase, nmol/min/mg</td>
<td>1290 ± 35</td>
<td>1261 ± 126</td>
</tr>
<tr>
<td>Adrenal</td>
<td>HMG CoA reductase</td>
<td>16.9 ± 2.8</td>
</tr>
<tr>
<td>total, pmol/min/mg</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

^1The studies were conducted on the 20th day of gestation. Liver and plasma samples from each litter were pooled prior to assay. Values are mean ± S.E.M., control n = 9, CY n = 7. Mean differences were determined using Students' t-test, *p < 0.05, ^+p < 0.01.

^2HMG CoA reductase, total and active, represents enzyme activities following enzyme preparation and assay in the absence and presence, respectively, of 50 mM NaF.

^35% cholestyramine diet (CY).

^4Fatty acid synthase (FAS).

^5Tyrosine aminotransferase (TAT).
As in the maternal liver, CY treatment significantly increased the fetal hepatic total and active HMG CoA reductase activity, as well as the proportion of enzyme in the active form (table 3.8). CY treatment had no effect on fetal TAT activity but increased and reduced fetal hepatic FAS and cystathionase activities, respectively. Fetal adrenal total reductase activity was also decreased by CY treatment.

The activity of maternal adipose lipoprotein lipase, hepatic triacylglycerol lipase and placental lipase was reduced in the rats fed CY (table 3.9). The activities of skeletal muscle lipoprotein lipase in the pregnant rat and of hepatic triacylglycerol lipase and skeletal muscle lipoprotein lipase in the fetus were not significantly altered by CY.

<table>
<thead>
<tr>
<th>Table 3.9</th>
<th>Effect of cholestyramine treatment on lipase activities in the late gestation rat1.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>Maternal</td>
<td></td>
</tr>
<tr>
<td>adipose lipoprotein lipase</td>
<td>12.1 ± 0.91</td>
</tr>
<tr>
<td>skeletal muscle lipoprotein lipase</td>
<td>0.78 ± 0.18</td>
</tr>
<tr>
<td>hepatic lipase</td>
<td>0.56 ± 0.08</td>
</tr>
<tr>
<td>Placental lipase</td>
<td>0.69 ± 0.09</td>
</tr>
<tr>
<td>Fetal</td>
<td></td>
</tr>
<tr>
<td>skeletal muscle lipoprotein lipase</td>
<td>1.46 ± 0.23</td>
</tr>
<tr>
<td>hepatic lipase</td>
<td>0.63 ± 0.10</td>
</tr>
</tbody>
</table>

1The studies were conducted on the 20th day of gestation with the fetal tissue and placentas for each litter pooled prior to assay. Values are means ± S.E.M., n = 5 for each diet. Significant differences were determined using Students' t-test, *p < 0.05, +p < 0.01.

2Cholestyramine (CY).
3.1.3.4 Fatty acid compositions

The fatty acid compositional analyses of fetal liver total lipid demonstrated an increase in the monoenoic fatty acids 16:1 and 18:1 and reduced 18:2n-6, 20:3n-6 and 22:5n-3 due to CY (table 3.10).

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>CY²</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Saturated</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14:0³</td>
<td>0.9 ± 0.0</td>
<td>1.0 ± 0.0*</td>
</tr>
<tr>
<td>16:0</td>
<td>24.0 ± 0.4</td>
<td>24.0 ± 0.1</td>
</tr>
<tr>
<td>18:0</td>
<td>14.6 ± 0.4</td>
<td>14.6 ± 0.3</td>
</tr>
<tr>
<td><strong>total</strong></td>
<td>39.4 ± 0.4</td>
<td>39.7 ± 0.4</td>
</tr>
<tr>
<td><strong>Monounsaturated</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16:1</td>
<td>4.0 ± 0.1</td>
<td>5.3 ± 0.2*</td>
</tr>
<tr>
<td>18:1</td>
<td>21.7 ± 0.1</td>
<td>25.9 ± 0.6*</td>
</tr>
<tr>
<td><strong>total</strong></td>
<td>26.1 ± 0.2</td>
<td>31.5 ± 0.6*</td>
</tr>
<tr>
<td><strong>Polyunsaturated</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:2n6</td>
<td>8.2 ± 0.6</td>
<td>5.8 ± 0.3*</td>
</tr>
<tr>
<td>18:3n3 (plus 20:0)</td>
<td>0.2 ± 0.0</td>
<td>0.1 ± 0.0</td>
</tr>
<tr>
<td>20:3n6</td>
<td>0.8 ± 0.0</td>
<td>0.7 ± 0.0*</td>
</tr>
<tr>
<td>20:4n6</td>
<td>10.5 ± 0.6</td>
<td>8.4 ± 1.1</td>
</tr>
<tr>
<td>20:5n3</td>
<td>0.7 ± 0.1</td>
<td>0.6 ± 0.0</td>
</tr>
<tr>
<td>22:4n6</td>
<td>0.5 ± 0.0</td>
<td>0.5 ± 0.0</td>
</tr>
<tr>
<td>22:5n6</td>
<td>0.1 ± 0.0</td>
<td>0.1 ± 0.0</td>
</tr>
<tr>
<td>22:6n3</td>
<td>0.4 ± 0.0</td>
<td>0.3 ± 0.0*</td>
</tr>
<tr>
<td><strong>total</strong></td>
<td>12.0 ± 0.4</td>
<td>11.0 ± 0.4</td>
</tr>
<tr>
<td><strong>total</strong></td>
<td>34.5 ± 0.4</td>
<td>28.8 ± 0.8*</td>
</tr>
</tbody>
</table>

1Fetal liver total lipid fatty acids were analysed on the 20th day of gestation with the liver for each litter pooled prior to analyses. The fatty acids are designated by their carbon length: number of double bonds. Data for the major fatty acid components are given as means ± S.E.M., n = 5 litters for each diet. Significant differences between the diets were determined using Students’ t-test, *p < 0.05, †p < 0.01.

²Cholestyramine (CY).

³Number of carbons: number of double bonds n number of carbons between the first double bond and the end of the fatty acid.
Cholestyramine resulted in reduced 18:0 and 22:5n-6, and an increased 18:1 in maternal plasma triacylglycerols and non-esterified fatty acids. Levels of other fatty acids in the maternal plasma were not altered by CY (data not shown). Maternal CY feeding also resulted in increased 14:0 and reduced 18:2n-6

Table 3.11
Effect of cholestyramine treatment on the major components of fetal plasma triacylglycerol and non-esterified fatty acids in the late gestation rat 1.

<table>
<thead>
<tr>
<th></th>
<th>Triacylglycerol</th>
<th>Non-esterified fatty acids</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>CY 2</td>
</tr>
<tr>
<td></td>
<td>%total</td>
<td>CY 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saturated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14:0 3</td>
<td>1.2 ± 0.1</td>
<td>1.6 ± 0.2*</td>
</tr>
<tr>
<td>16:0</td>
<td>27.6 ± 1.0</td>
<td>30.6 ± 1.0</td>
</tr>
<tr>
<td>18:0</td>
<td>9.3 ± 0.8</td>
<td>8.7 ± 0.8</td>
</tr>
<tr>
<td>total</td>
<td>38.6 ± 1.6</td>
<td>41.7 ± 0.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Monounsaturated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16:1</td>
<td>2.2 ± 0.4</td>
<td>2.1 ± 0.2</td>
</tr>
<tr>
<td>18:1</td>
<td>38.8 ± 0.3</td>
<td>41.2 ± 1.3</td>
</tr>
<tr>
<td>total</td>
<td>41.2 ± 0.3</td>
<td>43.6 ± 1.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Polyunsaturated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:2n6</td>
<td>8.1 ± 0.3</td>
<td>5.3 ± 0.4*</td>
</tr>
<tr>
<td>18:3n3 (plus 20:0)</td>
<td>0.6 ± 0.0</td>
<td>0.6 ± 0.0</td>
</tr>
<tr>
<td>20:3n6</td>
<td>0.5 ± 0.1</td>
<td>0.6 ± 0.2</td>
</tr>
<tr>
<td>20:4n6</td>
<td>2.6 ± 0.3</td>
<td>2.8 ± 0.5</td>
</tr>
<tr>
<td>20:5n3</td>
<td>0.5 ± 0.2</td>
<td>0.3 ± 0.2</td>
</tr>
<tr>
<td>22:4n6</td>
<td>0.8 ± 0.2</td>
<td>0.6 ± 0.2</td>
</tr>
<tr>
<td>22:5n6</td>
<td>1.1 ± 0.3</td>
<td>1.4 ± 0.4</td>
</tr>
<tr>
<td>22:5n3</td>
<td>0.7 ± 0.2</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>22:6n3</td>
<td>5.1 ± 0.3</td>
<td>2.8 ± 0.3*</td>
</tr>
<tr>
<td>total</td>
<td>20.1 ± 1.4</td>
<td>14.7 ± 1.1*</td>
</tr>
</tbody>
</table>

1 Fetal plasma fatty acids were analysed on the 20th day of gestation with the plasma for each litter pooled prior to analysis. The fatty acids are designated by their carbon length: number of double bonds. Data for the major fatty acid components are given as means ± S.E.M., n = 5 litters for each diet. Significant differences between the diets were determined using Students’ t-test, *p < 0.05, †p < 0.01.

2 Cholestyramine (CY).

3 Number of carbons: number of double bonds n number of carbons between the first double bond and the end of the fatty acid.
and 22:6n-3 in the fetal plasma triacylglycerols, with the only change in the fetal unesterified fatty acids being an increased 20:5n-3 (table 3.11).

3.1.4 Effect of feeding pregnant rats diets containing safflower, olive or palm oil

3.1.4.1 Pregnancy outcome

There were no differences in maternal weight gain, maternal liver/body weight, fetal weight or fetal liver/body weight due to type or quantity of oil in the experimental diets (data not shown). Pregnant rats fed 5% safflower oil, however, gained less weight from day 0 to day 21 of gestation than rats fed 20% safflower oil (131 ± 7, 155 ± 8 g, p < 0.05). Only 4 out of 21 rats with sperm positive vaginal smears and assigned to the palm oil diet were confirmed pregnant. This is in contrast to 6 out of 10 sperm positive rats in each of the other 3 diets which did conceive. The reason for this is unknown. The litter size and fetuses were apparently normal in the litters of rats fed the diet containing palm oil.

3.1.4.2 Tissue lipid levels

The concentrations of fetal hepatic total and free cholesterol, triacylglycerol and phospholipid and plasma total cholesterol and triacylglycerol were not affected by the quantity or type of fat ingested (data not shown). The plasma free cholesterol levels of fetus from dams fed 20% safflower oil was higher than of fetus from dams fed 5% safflower oil (0.84 ± 0.12, 0.48 ± 0.06 mmol/L, respectively). The type of oil fed, however, did not affect the plasma free cholesterol concentration. Values for the 20% palm and olive oil diets were 0.64 ± 0.07 and 0.88 ± 0.07 mmol/L, respectively.

3.1.4.3 HMG CoA reductase activity

Increasing the maternal diet fat content from 5 to 20% safflower oil increased the total and active HMG CoA reductase activity in fetal liver (table 3.12). The 20% olive oil diet was associated with higher fetal hepatic HMG CoA reductase activity in the active (unphosphorylated) state relative to the 20% safflower and palm oil diets. Fetal livers of rats fed 20% palm oil had significantly lower total HMG CoA
reductase activities than fetal livers of rats fed 20% safflower or olive oil.

3.1.4.4 Fatty acid compositions

The effect of oil type and quantity in the maternal diet on the fatty acid composition of the fetal liver phospholipids and triacylglycerols is shown in table 3.13. The distribution of saturated fatty acid was not influenced in either the triacylglycerols or phospholipids by the maternal diet fat. In comparison to fetuses of rats fed 20% olive or palm oil, fetuses of rats fed 20% safflower oil had lower 18:1 and higher 18:2, 20:4, 22:4 and 22:5 n-6 in their phospholipids and triacylglycerols. Fetuses from rats fed olive oil and palm oil were similar except for higher 18:1, 20:5n-3 and 22:6n-3 in the phospholipids and higher 22:6n-3 in the triacylglycerols of the olive oil group. Increasing the level of safflower oil in the maternal diet from 5 to 20% resulted in a decrease in 18:1 and an increase in 18:2 and 22:4 n-6 in both the phospholipids and triacylglycerols but had no significant effect on the level of any other fatty acid.

<table>
<thead>
<tr>
<th>Maternal diet</th>
<th>HMG CoA reductase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
</tr>
<tr>
<td></td>
<td>Active</td>
</tr>
<tr>
<td></td>
<td>pmol/min/mg protein</td>
</tr>
<tr>
<td>20% Palm oil</td>
<td>275 ± 46&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>20% Olive oil</td>
<td>749 ± 63&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>20% Safflower oil</td>
<td>624 ± 52&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>5% Safflower oil</td>
<td>260 ± 56&lt;sup&gt;x&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values reported are means ± SEM for analyses of n = 4 litters for the palm oil and n = 6 litters for each of the olive and safflower oil groups. Means in a column for the 20% oil diets with a different superscript a or b, or for the 20% and 5% safflower oil diet with the superscript x are significantly different, p < 0.05.
<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Phospholipids</th>
<th>Triacylglycerols</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>20% Palm oil</td>
<td>20% Olive oil</td>
</tr>
<tr>
<td>14:0 ( ^d )</td>
<td>0.6 ± 0.0</td>
<td>0.6 ± 0.0</td>
</tr>
<tr>
<td>16:0</td>
<td>21.7 ± 0.5</td>
<td>20.0 ± 0.4</td>
</tr>
<tr>
<td>18:0</td>
<td>17.0 ± 0.4</td>
<td>17.1 ± 0.2</td>
</tr>
<tr>
<td>18:1</td>
<td>17.9 ± 0.4 ( ^b )</td>
<td>20.2 ± 0.6 ( ^a )</td>
</tr>
<tr>
<td>18:2 n-6</td>
<td>6.5 ± 0.3 ( ^b )</td>
<td>5.2 ± 0.3 ( ^a )</td>
</tr>
<tr>
<td>20:4 n-6</td>
<td>19.6 ± 0.4 ( ^b )</td>
<td>18.3 ± 0.5 ( ^a )</td>
</tr>
<tr>
<td>20:5 n-3</td>
<td>0.1 ± 0.0 ( ^b )</td>
<td>0.3 ± 0.0 ( ^b )</td>
</tr>
<tr>
<td>22:4 n-6</td>
<td>0.6 ± 0.1 ( ^a )</td>
<td>0.7 ± 0.0 ( ^a )</td>
</tr>
<tr>
<td>22:5 n-6</td>
<td>3.2 ± 0.2 ( ^b )</td>
<td>2.2 ± 0.2 ( ^b )</td>
</tr>
<tr>
<td>22:5 n-3</td>
<td>0.0 ± 0.0 ( ^b )</td>
<td>0.1 ± 0.0 ( ^b )</td>
</tr>
<tr>
<td>22:6 n-3</td>
<td>9.2 ± 0.5 ( ^a )</td>
<td>12.5 ± 0.6 ( ^b )</td>
</tr>
</tbody>
</table>

1Values reported are means ± SEM for analyses of n=4 litters for the palm oil and n=6 litters for each of the olive and safflower oil groups. Means in a column for the 20% oil diets with a different superscript a or b, or for the 20% and 5% safflower oil diet with the superscript x are significantly different, p < 0.05.

2Number of carbons: number of double bonds n- number of carbons between first double bond and the methyl end of the fatty acid.
3.2 Experiment II: Perinatal development of fetal hepatic HMG CoA reductase activity

3.2.1 HMG CoA reductase activities

The activity of fetal hepatic HMG CoA reductase increased throughout gestation (figure 3.1). Before, and immediately after birth, the total (unphosphorylated + phosphorylated) reductase activity was 6-fold higher than the activity in the adult liver (91 ± 24 pmol/min/mg protein). With the commencement of suckling, however, reductase activity decreased precipitously to reach rates at 48 hrs after birth which were similar to that of the adult male. The developmental change in the activity of the active (unphosphorylated) reductase was similar, but of smaller magnitude. The latter increased significantly from 20 to 21 days of gestation (52 ± 1, 83 ± 9 pmol/min/mg protein, n=7 and n=4 litters, respectively, p < 0.05), and was significantly lower immediately after birth (65 ± 6 pmol/min/mg protein, p < 0.05).

3.2.2 Tissue lipid levels

The fetal liver cholesterol concentration shown in figure 3.2 remained constant from gestation day 19 (43.2 ± 2.5 nmol/mg protein, n=6) to birth (39.9 ± 1.4 nmol/mg protein, n=3). The concentration of cholesterol in the adult liver was 33.3 ± 1.7 nmol/mg protein. The concentration of triacylglycerol in the fetal liver also remained constant during late gestation. By 6 hrs after birth, however, cholesterol and triacylglycerol had increased 1.5- (65.5 ± 4.9, n=4) and 6.5-fold (110 ± 10, n=3), respectively over the levels found in the unfed newborn (cholesterol, 39.9 ± 1.4 and triacylglycerol, 14.5 ± 0.8 nmol/mg protein, n=3, p < 0.05). The high hepatic cholesterol concentration attained by 48 hrs after birth was due to increased cholesteryl ester. The hepatic phospholipid concentration changed little during late gestation, but did increase significantly after birth.

Cholesterol and triacylglycerol concentrations in fetal plasma increased from post-conception day 16 to 19, then decreased until nursing commenced (figure 3.2). Not unexpectedly, suckling resulted in a rapid rise in plasma triacylglycerol, but a more modest rise in cholesterol.

The cholesterol concentration of fetal rat hepatic microsomes increased from day 16 to 20 of gestation and remained higher throughout the first 48 hrs of life than that in adult liver microsomes (figure
Figure 3.1. Developmental profile of total ( ■ — ■ ) and active (unphosphorylated, ○ • • • ○ ) microsomal HMG CoA reductase activity. Liver was pooled for each litter before being analysed. Neonates < 2 hours old had not suckled. Adult samples were single, non-littermate livers. HMG CoA reductase activity was determined as described in Materials and Methods. Each point is the mean ± SEM, n ≥ 4 litters or adult rats. Statistical differences were determined with Analysis of Variance followed by Duncan's Multiple Range Test. Data points within a plot which contain a similar lower case letter in the superscript are not significantly different (p < 0.05).
Figure 3.2. Development of hepatic and plasma total cholesterol and triacylglycerol and hepatic free cholesterol and phospholipid level. Plasma and liver were pooled for each litter before analysis. Neonates < 2 hours old had not suckled. Adult samples were single, non-littermate livers. Each point is the mean ± SEM, n ≥ 4 litters or adult rats. See figure 3.1 for additional notes.
3.3). Fetal hepatic microsomal phospholipid also increased from gestation day 16 to term, but in contrast to cholesterol, was markedly lower than in the adult. As a result, the cholesterol/phospholipid ratio of the microsomal lipid of the developing rat was very high compared to that of the adult.

3.2.3 Fatty acid compositions

The major fatty acids of the perinatal and adult hepatic microsomal phospholipid are shown in table 3.14. The most prominent changes shown are a decrease in the % 18:2n-6 and an increase in the % 20:4n-6 and 22:6n-3 during the last 5 days before birth. With the exception of 20:4n-6 which decreased, these fatty acids showed little change during the first 48 hrs after birth. The hepatic microsomal phospholipid unsaturation index (U.I.) increased throughout perinatal development in an apparent inverse relationship to the microsomal cholesterol/phospholipid molar ratio (figure 3.2). The U.I. values were lower at post-conception day 16-20 than in the adult, but increased throughout the first 36 hrs after birth to values equal or greater than in the adult.

3.3 Experiment III: Rates of fetal hepatic cholesterol synthesis in vivo in situations of altered HMG CoA reductase activity

3.3.1 Cholestyramine treatment

Rates of cholesterol and fatty acid synthesis in 20th day gestation fetal liver were not affected by maternal ingestion of CY (table 3.15). The maternal hepatic cholesterol synthetic rate, in contrast, was markedly increased with CY feeding although fatty acid synthesis remained unchanged (table 3.15).

3.3.2 Feeding 5 versus 20% safflower oil

Neither fetal hepatic cholesterol nor fatty acid synthesis was affected by the quantity of fat in the maternal diet (table 3.16). Similarly, no effect on maternal hepatic cholesterol synthesis was observed. The rate of fatty acid synthesis in the maternal liver, however, was higher in rats when 5% rather than 20% fat was fed. The cholesterol and triacylglycerol levels in maternal and fetal liver and plasma were not
Figure 3.3. The microsomal cholesterol (● --- ●) and phospholipid (○ ⋅ ⋅ ⋅ ○) concentration, phospholipid fatty acid unsaturation index (* --- *) and the cholesterol/phospholipid molar ratio (□ ⋅ ⋅ ⋅ □) of perinatal and adult rat liver. Adult samples were single, non-littermate males. Liver was pooled for each litter before prepared and analysed. Neonates < 2 hours old had not suckled. Microsomes were assayed for cholesterol and phospholipid as described in Materials and Methods. The unsaturation index was calculated as described in Innis & Clandinin 1981. Results are given as mean ± SEM, n ≥ 4 litters or adult rats. See figure 3.1 for additional notes.
Table 3.14
Major fatty acids of perinatal and adult rat microsomal phospholipid.

<table>
<thead>
<tr>
<th>Fatty Acids</th>
<th>Rat, age</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Days, post-conception</td>
<td>Hours, postnatal</td>
<td>_PEER</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>18</td>
<td>20</td>
<td>21</td>
<td>&lt;2</td>
<td>6</td>
<td>12</td>
<td>36</td>
<td>48f</td>
</tr>
<tr>
<td>16:0 (^{3})</td>
<td>26.7 ± 2.4 (^{bc})</td>
<td>24.4 ± 0.7 (^{c})</td>
<td>23.0 ± 0.4 (^{bc})</td>
<td>16.4 ± 0.3 (^{*})</td>
<td>21.9 ± 0.9 (^{bc})</td>
<td>22.2 ± 1.9 (^{ab})</td>
<td>19.1 ± 1.7 (^{ab})</td>
<td>21.8 ± 0.1 (^{b})</td>
<td>22.9</td>
</tr>
<tr>
<td>18:0</td>
<td>13.8 ± 0.4 (^{*})</td>
<td>16.2 ± 0.3 (^{b})</td>
<td>16.7 ± 0.3 (^{b})</td>
<td>19.1 ± 0.6 (^{c})</td>
<td>17.0 ± 0.2 (^{b})</td>
<td>17.5 ± 0.7 (^{bc})</td>
<td>18.5 ± 0.5 (^{e})</td>
<td>19.0 ± 0.4 (^{c})</td>
<td>19.1</td>
</tr>
<tr>
<td>18:1</td>
<td>24.4 ± 0.8 (^{f})</td>
<td>20.4 ± 1.5 (^{c})</td>
<td>18.2 ± 0.6 (^{c})</td>
<td>15.3 ± 0.5 (^{b})</td>
<td>15.6 ± 0.9 (^{b})</td>
<td>15.2 ± 0.4 (^{b})</td>
<td>13.5 ± 1.5 (^{c})</td>
<td>8.5 ± 0.7 (^{c})</td>
<td>14.6</td>
</tr>
<tr>
<td>18:2n-6</td>
<td>9.7 ± 2.4 (^{f})</td>
<td>7.7 ± 0.7 (^{c})</td>
<td>7.7 ± 1.0 (^{b})</td>
<td>6.2 ± 0.8 (^{ab})</td>
<td>5.7 ± 0.6 (^{b})</td>
<td>6.8 ± 0.6 (^{b})</td>
<td>5.8 ± 0.4 (^{b})</td>
<td>4.6 ± 0.3 (^{c})</td>
<td>5.6</td>
</tr>
<tr>
<td>20:4n-6</td>
<td>5.4 ± 1.6 (^{*})</td>
<td>12.5 ± 1.2 (^{bc})</td>
<td>12.3 ± 0.6 (^{b})</td>
<td>15.7 ± 0.8 (^{ed})</td>
<td>14.4 ± 0.8 (^{bc})</td>
<td>13.4 ± 0.1 (^{b})</td>
<td>17.9 ± 1.7 (^{bc})</td>
<td>20.6 ± 0.5 (^{c})</td>
<td>13.2</td>
</tr>
<tr>
<td>22:6n-3</td>
<td>2.4 ± 0.6 (^*)</td>
<td>9.2 ± 1.2 (^{c})</td>
<td>11.8 ± 0.6 (^{bc})</td>
<td>17.9 ± 1.0 (^{c})</td>
<td>15.1 ± 0.5 (^{c})</td>
<td>15.6 ± 0.9 (^{c})</td>
<td>14.4 ± 1.3 (^{e})</td>
<td>17.6 ± 0.5 (^{c})</td>
<td>10.8</td>
</tr>
<tr>
<td>U.I.</td>
<td>105 ± 6 (^{*})</td>
<td>160 ± 10 (^{b})</td>
<td>175 ± 6 (^{c})</td>
<td>222 ± 4 (^{f})</td>
<td>199 ± 5 (^{e})</td>
<td>198 ± 6 (^{c})</td>
<td>209 ± 11 (^{c})</td>
<td>227 ± 4 (^{f})</td>
<td>172</td>
</tr>
</tbody>
</table>

\(^{1}\)Livers were pooled for each fetal, newborn or suckling litter. Adult samples were single, non-littermate male livers. Neonates < 2 hr had not suckled. Values given are means ± S.E.M. for the major fatty acids (number of carbon atoms: number of double bonds), n > 4 litters or adult rats. The unsaturation index (U.I.) includes all fatty acids of carbon chain length 8-24 identified and was calculated as previously published (Innis and Clandinin 1981). Statistical differences were determined with analysis of variance followed by Duncan's multiple range test. Values within a line with a different superscript are significantly different, p < 0.05;

\(^{2}\)For technical reasons samples from pups 48 hrs old were damaged leaving one sample analysable.

\(^{3}\)Chain length: number of double bonds, n- number carbon units between methyl end of fatty acid and first double bond.
### Table 3.15
**Effect of cholestyramine on hepatic rates of cholesterol and fatty acid synthesis**.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>CY</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmol $^3$H$_2$O incorporated/h/mg homogenate protein</td>
<td></td>
</tr>
<tr>
<td><strong>Mother</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cholesterol</td>
<td>16.2 ± 2.3</td>
<td>34.2 ± 4.1**</td>
</tr>
<tr>
<td>fatty acid</td>
<td>43.1 ± 3.0</td>
<td>53.1 ± 5.4</td>
</tr>
<tr>
<td><strong>Fetus</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cholesterol</td>
<td>34.4 ± 3.4</td>
<td>33.6 ± 0.6</td>
</tr>
<tr>
<td>fatty acid</td>
<td>92.8 ± 8.6</td>
<td>89.2 ± 10.8</td>
</tr>
</tbody>
</table>

$^1$Pregnant rats were fed throughout gestation, a control chow diet with or without the addition of 5% cholestyramine. Tissues were collected on the 20th day of gestation and analysed as described in Methods and Materials. Values are means ± S.E.M., for 6 rats per diet. Mean differences were determined using Students' t-test, ** $p < 0.01$.

$^2$Cholestyramine diet (CY).

### Table 3.16
**Effect of quantity of safflower oil in the maternal diet on fetal and maternal hepatic rates of cholesterol and fatty acid synthesis on the 21st day of gestation**.

<table>
<thead>
<tr>
<th></th>
<th>5% safflower</th>
<th>20% safflower</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmol $^3$H$_2$O/h/mg protein</td>
<td></td>
</tr>
<tr>
<td><strong>Mother</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cholesterol</td>
<td>6.70 ± 0.47</td>
<td>7.82 ± 0.47</td>
</tr>
<tr>
<td>fatty acid</td>
<td>54.3 ± 6.1</td>
<td>33.5 ± 3.6</td>
</tr>
<tr>
<td><strong>Fetus</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cholesterol</td>
<td>13.2 ± 1.6</td>
<td>15.7 ± 1.8</td>
</tr>
<tr>
<td>fatty acid</td>
<td>33.2 ± 3.9</td>
<td>40.2 ± 4.6</td>
</tr>
</tbody>
</table>

$^1$Pregnant rats were fed, throughout gestation, diets of the same caloric density containing 5% or 20% safflower oil or a control chow diet. Fetal tissue was collected on gestation day 21 and pooled within a litter prior to being analysed as described in Methods and Materials. Values are means ± S.E.M., n = 5 pregnant dams or litters per diet. Mean differences were determined using Students' t-test, $p < 0.05$ for differences between the safflower-containing diets.
significantly altered by the quantity of safflower oil fed (data not shown).

3.3.3 Perinatal development of hepatic cholesterol and fatty acid synthesis

Rates of cholesterol and fatty acid synthesis, assayed as the rate of $[^3]H$water incorporated into lipid/h/mg protein, increased in fetal rat liver from day 18 to 20 post-conception to reach values approximately 4- and 6-fold greater, respectively, than in the adult male (figure 3.4). Rates of cholesterol synthesis, however, unlike reductase activity, then decreased significantly from day 20 to 21 of gestation (34.5 ± 3.5, 20.9 ± 1.7 nmol/h/mg, respectively, p < 0.05, n = 6 litters each). Fatty acid synthetic rates similarly showed a significant decrease from gestation day 20 (92.8 ± 8.6 nmol/h/mg, n=6 litters) to day 21 (57.5 ± 5.2 nmol/h/mg, n = 6 litters, p < 0.05). Fatty acid synthesis decreased again from the last day of gestation to 20.2 ± 0.7 nmol/h/mg (n = 3, p < 0.05) in newborn rats. Although the rate of cholesterol synthesis continued to decrease from birth to 48 h, the rate of fatty acid synthesis was similar among the unfed and 48 hr old newborn and adult rats.
Figure 3.4. Developmental profile of hepatic cholesterol (● — ●) and fatty acid (△ — △) synthesis. Liver was pooled for each litter before being analysed. Neonates < 2 hours old had not suckled. Adult samples were single, non-littermate livers. Rates of $^3$H$_2$O incorporation into lipid were determined as described in Materials and Methods. Each point is the mean ± SEM, n ≥ 3 litters or adult rats. See figure 3.1 for additional notes.
4 DISCUSSION

4.1 Sensitivity of fetal hepatic HMG CoA reductase activity to external stimuli

4.1.1 High fat and cholesterol diet

The inclusion of high levels of saturated fat and cholesterol in the diet of pregnant rats had no effect on maternal or fetal weight gain. It did, however, lead to marked hypercholesterolemia and increased corticosterone levels in the maternal circulation, but had no effect on plasma triacylglycerol or T4 levels. The elevation of corticosterone due to high fat feeding is similar to previous data for male rats (Brindley et al 1981). A diet-induced hypercholesterolemia may result in increased cholesterol uptake and storage by the rat adrenal gland (Civen et al 1984, Mikami et al 1984) through a mechanism not involving increased adrenocorticotropin (Mikami et al 1984).

The high saturated fat and cholesterol diet (HFC) resulted in higher plasma cholesterol in the dam, but had no effect on the fetal plasma cholesterol or triacylglycerol levels. Similarly, but in contrast to the maternal circulation, the diet had no effect on the fetal plasma corticosterone levels. T4 and insulin were similarly not affected by maternal high fat and cholesterol feeding. Corticosterone is able to cross the placenta (Dupuoy et al 1975), and the fetal hypothalamic-pituitary axis is sensitive to maternal hormone transfer and responds with the appropriate adult tropic hormone release (Wood & Rudolph 1984). It seems possible, therefore, that increased corticosterone transfer could result in suppression of fetal corticosterone synthesis to maintain appropriate circulating levels.

The data on maternal hepatic enzyme activities following high fat and cholesterol feeding demonstrate the well-known suppression of HMG CoA reductase activity by these diets (see review by Rodwell et al 1976). The marked decrease in total reductase activity with the HFC diet suggests that the difference in cholesterogenesis was principally due to reduction of enzyme quantity rather than activation state. FAS activity was not decreased in the maternal liver of rats fed HFC in apparent contrast to the known effects of high fat diets on hepatic triacylglycerol synthesis (Brindley et al 1981). Whether this can be explained as a different response to dietary fat by the pregnant female compared to the male rat, or is due to the addition of high cholesterol to the diet is unknown.
Despite diet-induced maternal hypercholesterolemia, no significant alteration of fetal hepatic reductase activities were observed which confirms earlier work by Miguel and Abraham (1976). They reported that 25% fat plus 5% cholesterol in the maternal diet had no effect on the incorporation of $[^{14}\text{C}]$ or $[^{3}\text{H}]$ substrates into cholesterol by slices of fetal liver. Feingold et al (1983) found no effect of 2% maternal dietary cholesterol on fetal rat hepatic cholesterogenesis. In contrast, Naseem et al (1980, 1980a) found evidence of lasting reduced HMG CoA reductase activity in the progeny of rats fed 40% fat plus cholesterol diets during pregnancy. This could possibly be related to an increased placental and reduced fetal weight (unpublished observations, Innis), fetal ketonemia, and altered placental amino acid and glucose transfer (Wapnir et al 1982) found with similar very high fat diets, rather than regulation of fetal reductase activity via placental fat or cholesterol transfer.

Glucocorticoids have a well-known ability to induce the activity of TAT and FAS in adult rat liver (Lau & Roncari 1983, Iynedjian et al 1985) and cystathionase in fetal liver (Heinonen 1975). No relationship between the elevated corticosterone level and an induction of fetal hepatic TAT or cystathionase was found. In contrast, the activities of both TAT and cystathionase were lower in the maternal liver of rats fed high fat and cholesterol. The significance of these findings are unknown. The data, however, suggest that the metabolic effects of HFC diets may extend beyond the pathways of cholesterol metabolism in the adult rat liver.

In summary, the present work suggests that dietary fat and cholesterol, at levels compatible with normal fetal growth, but which are sufficient to produce marked maternal hypercholesterolemia and suppression of maternal hepatic enzyme activities, have no effect on cholesterol metabolism in the fetal compartment.

4.1.2 ACTH/cortisol administration to the pregnant dam

Little is known about the regulation of fetal hepatic cholesterol synthesis during development, or its relationship to the utilization of cholesterol by the fetal adrenal gland. Carr and Simpson have shown that the human fetal adrenal gland preferentially utilizes plasma lipoprotein cholesterol for adrenal
steroidogenesis and hypothesized that the rate of adrenal steroidogenesis is an important determinant of the plasma cholesterol level (Carr et al 1980, Carr & Simpson 1982, see also review in Carr & Simpson 1981). Similar studies of adrenal utilization of plasma cholesterol have not been reported for the developing rat, although the developmental pattern of hepatic HMG CoA reductase activity in this species is known (McNamara et al 1972). The rate of adrenal de novo cholesterol synthesis in adult rats, however, is very low and most of the cholesterol utilized in steroid hormone synthesis is acquired by uptake of plasma HDL cholesterol (Gwynne et al 1985).

Glucocorticoids cross the rat placenta freely (Zarrow et al 1970) and, since negative feedback regulation of the pituitary-adrenal axis is functional in the near-term fetus (Yamamoto et al 1983), an increased transfer of glucocorticoids from the maternal circulation may be expected to inhibit fetal adrenal glucocorticoid synthesis. Although this has not been studied directly, evidence of fetal adrenal compensation and increased transfer of glucocorticoids from the fetal to the maternal compartment following adrenalectomy of pregnant rats has been published (Dupouy et al 1975). The studies in this thesis show that maternal cortisol administration increased the fetal plasma cholesterol and reduced adrenal HMG CoA reductase activity without causing a change in the fetal hepatic reductase activity. These findings are similar to reports demonstrating increased plasma cholesterol levels in infants born to mothers treated with glucocorticoids (Anderson & Friis-Hansen 1977). Thus, our results for the fetal rat suggest, as hypothesized for the human fetus (Carr et al 1980), that the rate of adrenal steroidogenesis is an important determinant of the plasma cholesterol level. Alternatively, cortisol may have resulted in higher fetal plasma cholesterol levels by reducing receptor-mediated clearance of plasma LDL cholesterol (Henze et al 1983, Johnston et al 1983). The finding that the activity of the major hepatic regulatory enzyme in cholesterogenesis was unaltered by exogenous glucocorticoid indicates that the accompanying elevation of plasma cholesterol is unlikely to be explained by increased hepatic synthesis of cholesterol.

The elevation of plasma cholesterol and reduction of hepatic total HMG CoA reductase activity in the pregnant rats given cortisol is similar to previous reports for suckling rats treated with high dose glucocorticoids (Hahn & Smale 1982). Studies using a 100-fold lower dose of glucocorticoid found no effect
on the peak total (phosphorylated + unphosphorylated) activity of HMG CoA reductase in adult rat liver (Lin & Snodgrass 1982). The studies in this thesis also examined the active (unphosphorylated) reductase and found no effect of cortisol administration. The elevation of plasma cholesterol in the adult female given glucocorticoid, therefore, cannot be explained by increased hepatic cholesterol synthesis. Whether or not it was related to reduced adrenal plasma lipoprotein cholesterol utilization for glucocorticoid synthesis due to the high exogenous dose of cortisol is reasonable, but speculative. Glucocorticoid interference of LDL clearance may occur (Henze et al 1983) as a result of reduced recycling of LDL receptor protein (Johnston et al 1983). Reduced lipoprotein cholesterol clearance, therefore, must also be considered as a possible explanation for the hypercholesterolemia caused by this hormone.

The effect of ACTH administration on hepatic enzymes involved in cholesterol and fatty acid synthesis has not been reported previously. ACTH is known to enhance adrenal uptake of plasma cholesterol in the rat (Gwynne et al 1985). Thus, the increased hepatic HMG CoA reductase activity in pregnant rats given ACTH may infer coordination between increased adrenal cholesterol utilization and hepatic active reductase activity. ACTH is not known to cross the rat placenta (Dupouy et al 1980). The lack of effect of this peptide on the fetal reductase may, therefore, not be unexpected. Glucocorticoids are known to increase triacylglycerol synthesis (reviewed by Brindley 1981). Hence, the increase in FAS activity in fetal liver of rats treated with ACTH may be explained by increased glucocorticoid synthesis in the maternal adrenal with subsequent transfer of corticosterone across the placenta and stimulation of fatty acid synthesis in response to increased triacylglycerol synthesis in the fetal liver. Alternatively, higher fetal hepatic FAS activity may be a result of the higher plasma levels of insulin in fetuses from rats treated with ACTH as insulin is known to increase hepatic FAS activity (reviewed by Wakil et al 1983). Although maternal FAS activity followed a similar trend, the increase in maternal FAS activity was not statistically significant.

Interpretation of in vivo administration of large doses of a hormone on particular metabolic pathways must consider possible confounding effects due to accompanying changes in other hormones. Although insulin and thyroxine are known to increase hepatic HMG CoA reductase activity (see review by Edwards et al 1983), no association between changes in these hormones due to cortisol or ACTH administration and
the reductase activities was found in this study.

In summary, administration of glucocorticoid to pregnant rats resulted in higher plasma cholesterol levels, lower fetal adrenal reductase activity but did not affect the activity of fetal hepatic HMG CoA reductase. These results do show, however, that the level of cholesterol in fetal plasma is influenced by maternal high dose glucocorticoid administration.

4.1.3 Cholestyramine treatment of the pregnant dam

This report confirms an earlier study (Innis 1983) demonstrating increased total HMG CoA reductase activity in fetal liver due to maternal CY feeding. The data have been extended to show that the activity of the reductase insensitive to NaF, believed to represent functionally active enzyme (see review by Scallen & Sanghvi 1983), and FAS, were increased by resin-induced alterations in maternal metabolism. Total reductase activities in these studies were higher than previously noted (Innis 1983). No explanation can be found, other than the use of a new animal unit and animal stock. Although the assays do not give definitive evidence of increased cholesterol formation, HMG CoA reductase activity is known to reflect cholesterogenesis under a wide range of conditions (reviewed by Rodwell et al 1976) and during prenatal life (Leoni et al 1984). These studies also support earlier evidence (Leoni et al 1984, 1985) that the fetal reductase is present as both a NaF sensitive and insensitive enzyme. This implies the capacity for short-term regulation of cholesterogenesis through modulation of the active pool of reductase enzyme (Scallen & Sanghvi 1983).

CY is a polystyrene resin which is not absorbed from the gut into the circulation (Gallo & Sheffner 1965) and therefore cannot cross the placenta and enter the fetal compartment. Hence, any changes in fetal hepatic metabolism by CY feeding must be mediated through changes in maternal factors which are able to cross the placenta. Postnatal rat hepatic HMG CoA reductase activity is increased by an increased demand for cholesterol (Rodwell et al 1976) and by several hormones including insulin, thyroid hormone and glucocorticoids (reviewed by Edwards et al 1983). Glucocorticoids are known to cross the rat placenta
(Dupuoy et al 1975) and are involved in the maturation of several hepatic enzyme systems (see reviews by Bohme et al 1983 and Mayor & Cuezva 1985). They have also been shown to increase cholesterol synthesis and HMG CoA reductase activity in human foetal hepatocytes (Carr & Simpson 1984). Potential hormone involvement in the induction of foetal HMG CoA reductase caused by CY treatment was considered by concomitant assay of TAT and cystathionase. These enzymes are not directly related to cholesterol metabolism, but have a well defined pre- and postnatal response to several hormones (Finkelstein 1962, Cake et al 1984, Iynedjian et al 1985). Fetal hepatic cystathionase is induced by glucocorticoids (Raiha et al 1971, Heinonen 1975), whereas TAT is not (Serini et al 1959, Franz & Knox 1967, Perry et al 1983). Cholestyramine treatment was not associated with increased activity of TAT or cystathionase in either maternal or fetal liver. Thus, these studies do not support a hypothesis that altered hormonal milieu was involved in CY-induction of fetal HMG CoA reductase activity. Further, the increase in FAS activity in CY treated rats and their fetuses suggests the effects of the resin extend beyond pathways of cholesterol synthesis. The coordinate increase in HMG CoA reductase and FAS activity make it logical to propose that CY treatment increased cholesterol and fatty acid synthesis for secretion in lipoproteins from both the maternal and fetal liver. In this regard, CY feeding is known to increase hepatic VLDL secretion (see review by Packard & Shepherd 1982) as well as glycerol and palmitate incorporation into triacylglycerol by male rat liver (Kempen et al 1983). It is reasonable to expect a similar effect of CY in the pregnant female. The mechanism by which perturbation of lipid metabolism in the pregnant rat by CY provoked increased HMG CoA reductase and FAS activity in the fetus, however, is unknown.

The metabolic events following treatment with CY result from irreversible binding of intraluminal bile acids to the resin with subsequent increased fecal steroid excretion (reviewed by Packard & Shepherd 1982). The demand for cholesterol to support further bile acid synthesis is met by increased hepatic cholesterogenesis, and in species such as the rabbit by increased hepatic uptake of LDL cholesterol (Chao et al 1982, Packard & Shepherd 1982). In the rat, however, plasma LDL cholesterol (Oschry & Eisenberg 1982), hepatic LDL receptor activity (Windler et al 1980) and the contribution of LDL cholesterol to biliary steroid synthesis (Bhattacharya et al 1986) is low. Not unexpectedly, but unlike many other species, CY
does not lower LDL cholesterol levels in rat plasma (Bhattacharya et al 1986). HDL is quantitatively more important than LDL cholesterol transport (Oschry & Eisenberg 1982). However, the effects of CY on HDL delivery of cholesterol to bile (Miller et al 1982) in this species is unknown.

The potential relationship of the effects of CY on fetal HMG CoA reductase to limited placental transfer of cholesterol or a steroid hormone product is speculative. Steroidogenic tissues in the rat prefer cholesterol derived from plasma HDL as substrate for steroidogenesis (Andersen & Dietschy 1978). It is possible that CY may disrupt the flow of HDL cholesterol to the maternal adrenal due to increased need for cholesterol to provide substrate for increased maternal hepatic bile acid synthesis. This could result in decreased availability of corticosterone for placental transfer to the fetus. This would cause fetal adrenal steroidogenesis to increase in order to maintain appropriate levels of glucocorticoid in the fetal plasma in a similar manner to that observed in the adrenalectomized pregnant rat (Milkovic et al 1973). If the fetal adrenal relies on cholesterol supplied from the fetal liver, this might cause fetal hepatic HMG CoA reductase activity to increase in order to meet the demand for substrate by the fetal adrenal. However, previous studies in adult rats have shown that the adult adrenal gland can synthesize its own cholesterol de novo when delivery of cholesterol from plasma lipoproteins is limiting (Balasubramaniam et al 1977). The data presented in this thesis also do not support a theory of reduced placental transport of glucocorticoid to the fetus of CY treated rats because the concentration of corticosterone in maternal plasma of CY treated rats was not significantly different from control rats and the activity of HMG CoA reductase in the fetal adrenal glands of CY treated rats was lower than in fetal adrenals from control rats. The latter possibly indicating that the demand for cholesterol as substrate for fetal adrenal steroidogenesis is decreased by CY.

Several reports have suggested an important contribution of maternal cholesterol to fetal plasma and tissue cholesterol accretion (Goldwater & Stetton 1947, Chevallier 1964, Connor & Lin 1967, Pitkin et al 1972). Hence, CY may increase fetal hepatic HMG CoA reductase activity by somehow redirecting plasma cholesterol away from the feto-placental unit toward the maternal liver to provide substrate for increased bile acid synthesis (Packard & Shepherd 1982). Other reports, however, have found maternal cholesterol
transfer to be minimal (Popjak & Beeckmans 1950, Calandra et al 1975, Feingold et al 1983, Parker et al 1983, Belknapp & Dietschy 1988) making altered placental delivery of cholesterol an unlikely mechanism by which maternal CY feeding might increase fetal reductase activity. Unesterified fatty acids, on the other hand, are known to cross the placenta in both directions, and in the rat maternal lipid transfer is appreciable in late gestation (Noble & Shand 1981). Conceivably, derangement of maternal lipid metabolism could alter fatty acid flux to the fetus. If fatty acid delivery to the fetus is compromised, the fetal liver may compensate by increasing triacylglycerol secretion into the fetal plasma as adipose tissue stores are very limited in the fetal rat (Widdowson 1950, and reviewed by Hahn 1972). Since both cholesterol and fatty acids are integral components of lipoproteins, any increase in the hepatic synthesis and secretion of one might be anticipated to cause a commensurate increase in the other.

Placental lipid transfer becomes increasingly important to fetal fatty acid accretion in the rat as gestation progresses (Hummel et al 1975, 1975a). Maternal plasma nonesterified fatty acids and triacylglycerols both contribute to placental transfer. The relative contribution of each to fetal fatty acid accretion, however, is unknown (reviewed by Coleman 1986). Lipoprotein lipase activity has been demonstrated in rat placenta (Ramirez et al 1983, Shafrir & Barash 1987), however, the enzyme appears to be different from lipoprotein lipase in other tissues in its ability to hydrolyze very low density lipoprotein, but not chylomicron, triacylglycerol (Hummel et al 1976). Placental fatty acid uptake is believed to be followed by re-esterification, then hydrolysis by a heparin-releaseable, constitutive lipase with subsequent release to the fetal circulation (Coleman 1986, Shafrir & Barash 1987). Some data has been interpreted to suggest preferential uptake and/or transfer to the fetus of specific fatty acids by the placenta (Koren & Shafrir 1964, and reviewed by Biezenski 1975). Definitive evidence for this, however, is lacking but the maternal plasma and diet fat composition are known to influence the fatty acids deposited in the fetal tissues (Stammers et al 1983).

The effect of CY during pregnancy on triacylglycerol flux was investigated by assay of triacylglycerol lipase activities and tissue and plasma fatty acid compositions. Triacylglycerol lipases catalyze the hydrolysis of triacylglycerol. Hence, when present on the capillary endothelium, they allow nearby tissues to take up
the free fatty acid released with triacylglycerol hydrolysis. Thus, the demonstration of lower maternal hepatic triacylglycerol lipase, adipose tissue lipoprotein lipase and placental lipase activities due to CY suggests that triacylglycerol hydrolysis in the maternal compartment, and the hydrolysis and release of placental triacylglycerol, was reduced. The lower n-6 and n-3 series fatty acids, specifically 18:2n-6, in the liver and plasma triacylglycerol of fetuses from these rats may also suggest decreased availability of maternal diet-derived essential fatty acids for placental transfer, or a reduced contribution of maternal lipids to fetal fatty acid accretion. These studies did not undertake quantitative determination of placental lipid transfer, and studied the effect of CY only on the 20th day of gestation. Lipoprotein lipase is known to decrease in white adipose tissue towards term gestation, without change in the activity of lipoprotein lipase in skeletal muscle (Otway & Robinson 1968, Hamosh et al 1970, Lasuncion & Herrera 1981, Champginy & Hitier 1987). The mechanisms which regulate the change in adipose tissue lipoprotein lipase are not well understood, but may be hormonal (Ramirez et al 1983). Whether or not CY treatment alters the usual gestational pattern of lipoprotein lipase activity must, therefore, be considered.

In summary, these studies have demonstrated that fetal hepatic cholesterol metabolism is not autonomous, but responds to changes in maternal cholesterol metabolism with an increase in the activity of both the total and active forms of HMG CoA reductase. Previous studies in rats fed high levels of CY have shown lowered intraluminal bile acid concentrations, altered intestinal fatty acid uptake (Thompson & Keelan 1987) and reduced essential fatty acids in hepatic microsomal phospholipids (Innis 1986). These reports suggest impairment of normal intestinal fat absorption, which together with the data on lipase activities and fetal fatty acids in this thesis support a hypothesis that CY may alter the quantity or type of fatty acid available for placental transfer when fed during gestation. Whether or not changes in placental fatty acid transfer are causally related to the increased fetal hepatic HMG CoA reductase and fatty acid synthase activities in CY-treated rats, however, has not been definitively shown.
4.1.4 Diets containing safflower, olive or palm oil

These studies have demonstrated for the first time that the activity of hepatic HMG CoA reductase in fetal rats is influenced by the quantity of fat, as well as the fatty acid composition, of the maternal diet. The relationship between the fatty acid composition of fetal liver lipids, particularly in regard to the levels of 18:1 and 18:2n-6, and the maternal diet fat confirms previous reports which have shown that the diet fat fed during gestation influences the fatty acid composition of developing fetal tissue lipids (Chaikoff & Robinson 1933, Winkler et al 1979, Samulski & Walker 1982, Stammers et al 1983, Clarke et al 1988). The finding that fetal hepatic active (unphosphorylated) and total HMG CoA reductase activity was increased by raising the quantity of safflower oil from 5% to 20% of the maternal diet is similar to data demonstrating that total hepatic reductase activity is increased by various fat-containing versus low fat (Goldfarb & Pitot 1972) or fat-free diets (Craig et al 1972, Ide et al 1978) and by increased delivery of free fatty acid to perfused livers of adult rats (Goh & Heimberg 1977). The latter effect has been hypothesized to be due to an increased requirement for cholesterol for secretion in lipoproteins (Goh & Heimberg 1973, 1977, 1979). High fat diets, however, are known to decrease VLDL secretion from adult rat liver (Gibbons 1990). The extent of the decrease is dependent upon the type of fat ingested (Gibbons 1990). The method of exogenous fat delivery from the placenta to the fetal liver, however, is unclear and may involve either secretion of placental triacylglycerol in lipoprotein and/or release of placental free fatty acid into the fetal circulation (reviewed by Coleman 1989).

The activity of the rate-limiting enzymes of cholesterol esterification and bile acid synthesis, acyl CoA:cholesterol acyl transferase and cholesterol 7α-hydroxylase, respectively, are lower in fetal than adult liver (Hahn & Innis 1984, Bruscalupi et al 1986). It seems reasonable, therefore, to assume any increase in fetal hepatic cholesterol synthesis due to increased HMG CoA reductase activity must be accompanied by increased secretion of cholesterol into the fetal plasma. The finding that fetal plasma free cholesterol was elevated concomittantly with the hepatic HMG CoA reductase activity in rats fed 20% compared to 5% safflower oil, while the liver cholesterol level was not altered, is consistent with an hypothesis that hepatic cholesterol synthesis was increased in the former case. In contrast, however, others have reported
no effect on fetal hepatic cholesterol synthesis (determined in liver slices as the rate of incorporation of radiolabelled pyruvate, acetate or $[^3]H$water into digitonin precipitable sterols) with a 15% corn oil versus a fat-free maternal diet (Miguel & Abraham 1976).

The activity of unphosphorylated HMG CoA reductase in fetal livers of rats fed 20% olive oil was higher than in fetuses of rats fed 20% safflower or palm oil. Studies in perfused adult rat liver have shown that 18:1 produces higher HMG CoA reductase activity than either 18:2 or 16:0 (Goh & Heimberg 1977). This finding has led to the speculation that changes in HMG CoA reductase activity may be related to fatty acid-associated changes in the microviscosity of microsomal membranes (Goh & Heimberg 1977). The activity of HMG CoA reductase in the unphosphorylated (active) state has also been shown to be increased by hepatic perfusion with 18:1 (Salam et al 1989). The studies in this thesis found, however, that neither the fetal liver nor plasma cholesterol levels were altered by the type of fat fed to the pregnant rat. Thus, the significance of higher unphosphorylated reductase activity in fetal livers of rats fed olive oil is at present uncertain. The results do suggest, however, that HMG CoA reductase activity in fetal rat liver is sensitive to exogenous fatty acids transferred from the maternal compartment, in a manner similar to that of the reductase in mature liver.

In summary, the results of this study demonstrate that the type and quantity of fat fed during gestation are able to influence hepatic HMG CoA reductase activity and fatty acid composition in the fetus. Total and active hepatic HMG CoA reductase activity was 2.5- and 6-fold higher, respectively in fetuses of rats fed 20% than in rats fed 5% safflower oil. Feeding pregnant rats diets containing the non-absorbed resin cholestyramine throughout gestation, in the studies described in section 3.1.3 of this thesis also increased total and active HMG CoA reductase activity in the fetal liver, but not to as great an extent (1.5- and 2.5-fold respectively relative to control diets). CY feeding, similar to high fat feeding, also altered the fetal hepatic fatty acid composition. These changes in fetal hepatic HMG CoA reductase activity due to CY are associated with long lasting effects on cholesterol metabolism in later adulthood (Innis 1989, and reviewed in Innis 1985). Recent studies by others have shown that maternal diets containing high or low quantities of fat are also able to produce long lasting effects (Brown et al 1990). Whether the alteration of fetal
hepatic HMG CoA reductase activity with maternal CY or fat feeding is causally related to long lasting effects on cholesterol metabolism or is simply an epiphenomenon is as yet unknown.

4.2 Perinatal development of HMG CoA reductase activity

The higher HMG CoA reductase activity found in fetal compared to adult liver has been reported previously (McNamara et al 1972, Carlson et al 1978, Ness et al 1979, Leoni et al 1984, Weiss et al 1988). The studies here, however, add new information with the demonstration that HMG CoA reductase activity remains high until after birth. The total enzyme activity then decreases by approximately 40% within the first 12 hrs of postnatal life during which suckling becomes established.

McNamara et al (1972) have proposed that the low HMG CoA reductase activity in the young nursing rat may be due to the presence of a cytosolic inhibitor. Cytosol, however, was not present in the microsomal preparation used in this study for in vitro assay of HMG CoA reductase activity. It seems unlikely, therefore, that a noncovalently-bound competitive inhibitor can explain the low HMG CoA reductase activity of the 1 to 2 day old rat pup.

HMG CoA reductase is a transmembrane protein of the endoplasmic reticulum (Orci et al 1984), and may thus be susceptible to regulation by its lipid environment (see review by McMurchie 1989). Some studies (Sabine & James 1976, Mitropoulos et al 1978, Mitropoulos et al 1981), but not all (Van Huesden & Writz 1984, Innis 1986), have reported reduced reductase activity in association with increased cholesterol or decreased phospholipid fatty acid unsaturation in the microsomal membrane (Davis and Poznansky 1987). The studies in this thesis found a decrease in the cholesterol/phospholipid molar ratio and an increase in fatty acid unsaturation during perinatal development. This is consistent with "fluidization" of the microsomal membrane, as assessed by fluorescence polarization (Kapitulnik 1980, Delpech et al 1984), and is thought to be determined mainly by an increase in the degree of phospholipid acyl chain unsaturation (Keranen et al 1982, Kapitulnik et al 1987) during perinatal development. The high phospholipid fatty acid unsaturation index after birth, however, is not consistent with a role of the microsomal phospholipid fatty
acids in regulating the decrease in HMG CoA reductase activity observed at this time. Similarly, the
decrease in the microsomal cholesterol/phospholipid molar ratio from gestation day 16 to 12 hours after
birth, is not readily compatible with a role of the membrane cholesterol/phospholipid ratio in mediating
the developmental changes in microsomal HMG CoA reductase activity.

Active (unphosphorylated) HMG CoA reductase activity, in contrast to total reductase activity, was
significantly lower in the unfed newborn than in the day 21 fetus (figure 3.1). Inactivation of HMG CoA
reductase by phosphorylation is thought to be under hormonal control (reviewed by Beg et al 1987). In
adult rats, HMG CoA reductase is activated by insulin and inactivated (phosphorylated) by glucagon. The
decrease in the insulin/glucagon ratio of the fetus after birth (Girard et al 1973, Mayor & Cuezva 1985)
could possibly be involved with the decrease in active HMG CoA reductase at this time. Active HMG CoA
reductase of neonatal rat hepatocytes in culture, however, appear to be insensitive to both glucagon and
insulin (Leoni et al 1985).

4.3 Relationship between HMG CoA reductase activity and rates of cholesterogenesis during
perinatal development

The previous studies demonstrated that fetal hepatic total and active HMG CoA reductase activity is
increased 1.5- and 2.5-fold, respectively, by feeding pregnant rats diets containing CY. Feeding 20% rather
than 5% safflower oil (wt/wt) was also found to increase both total and active fetal hepatic HMG CoA
reductase activity 2.5- and 6-fold, respectively. Given the role of HMG CoA reductase in regulating
cholesterol synthesis it is thus important to understand if altered fetal reductase activity reflects changes in
fetal hepatic cholesterol synthesis. The present study demonstrated that rates of fetal hepatic cholesterol
and fatty acid synthesis do not appear to be affected by either the quantity of fat or the presence of CY in
the maternal diet. The absence of an effect of maternal dietary fat on fetal cholesterol synthesis confirms
a previous report concerning 15% corn oil compared to a fat-free diet (Miguel and Abraham 1976). The
lack of an effect by dietary CY has not been previously reported.
In agreement with others (Carroll 1964, Ballard and Hanson 1967, Leoni et al 1984), these studies found higher rates of hepatic cholesterol synthesis in the fetal than adult liver. The rate of cholesterol synthesis decreased one day before term similar to the report by other investigators on the developmental change in rates of total sterol synthesis (Pillay and Bailey 1982). Another recent study has shown that rates of fetal whole body cholesterol synthesis decrease 3 days before birth (Belknap and Dietschy 1988). In experiment II, however, HMG CoA reductase activity remained high until after birth, then decreased by approximately 40% by the 12th postnatal hour when suckling was established. This indicates that the in vitro total activity of HMG CoA reductase is not correlated to the in vivo rate of cholesterol synthesis in the near term fetal rat liver. Hence, in contrast to the assumption in previous reports that HMG CoA reductase activity limits the rate of cholesterol synthesis in fetal rat liver (McNamara et al 1972, Carlson et al 1978, Ness et al 1979, Weiss et al 1988), the studies in this thesis indicate that the high HMG CoA reductase activity in near term liver may not be coupled to rates of cholesterol synthesis. This would explain the failure to increase fetal hepatic cholesterol synthesis by CY or high fat feeding, despite the apparent increase in fetal hepatic HMG CoA reductase activity in these cases. Earlier investigations which did not find a decrease in cholesterol synthesis before birth did not include sequential day by day analysis over the last 6 days of gestation (Carroll 1964, Leoni et al 1984), thereby possibly missing the peak, or time of decline, in cholesterogenesis found here on day 20 and 21, respectively.

Uncoupling of HMG CoA reductase activity from rates of cholesterol synthesis has been observed in rapidly proliferating liver (Leoni et al 1982, Trentalance et al 1984, Bruscalupi et al 1985, Marino et al 1986) and in various cultured cell lines (Johnston et al 1979, Ramachandran et al 1980). It has been proposed that, in these situations, it is the result of mevalonate being used for the synthesis of non-sterol products, such as ubiquinone, dolichol or isopentenyl tRNA (Bruscalupi et al 1985) which are required for cellular proliferation (see review by Brown and Goldstein 1980). The late gestation fetal liver is a rapidly proliferating tissue (Greengard 1972), thus shunting of mevalonate away from cholesterol synthesis seems plausible. It is unlikely, however, that mevalonate is shunted to the mitochondria for oxidation (Marinier et al 1987), as mevalonate oxidation to CO₂ is low immediately after birth (Hahn 1986) and in the
regenerating liver (Bruscalupi et al 1985).

Disparity between HMG CoA reductase activity and cholesterol synthesis has also been observed in cultured cell lines and livers of rats treated with competitive inhibitors of HMG CoA reductase (Bensch et al 1978, Brown et al 1978). In this situation, rates of cholesterol synthesis are low, due to inhibition of HMG CoA reductase, but the \textit{in vitro} reductase activity is increased. The latter may be due to dilution or loss of inhibitor in the microsomal preparation (Bensch et al 1978, Brown et al 1978), resulting in compensatory increased reductase protein synthesis (Ryan et al 1981, Chin et al 1982, Kita et al 1983) and/or decreased degradation (Sinensky and Logel 1983) in response to decreased synthesis of mevalonate or other putative metabolites which may regulate cholesterol synthesis (Pullinger & Gibbons 1983). It is possible, therefore, that the high HMG CoA reductase activity \textit{in vitro} but low rate of cholesterol synthesis \textit{in vivo} in near term fetal liver may be explained by the presence of an endogenous competitive inhibitor.

The developmental changes in fatty acid and cholesterol synthesis were similar. As reported by others, rates of fatty acid synthesis were higher in fetal than adult liver (Ballard and Hanson 1967, Taylor et al 1967), increased from post-conception day 18 to day 20 and then decreased to birth (Lorenzo et al 1981, Pillay and Bailey 1982, Benito et al 1982). Acetyl CoA carboxylase is the only lipogenic enzyme present in fetal rat liver which displays a similar developmental pattern and might, therefore, potentially regulate fetal lipogenesis (Pillay and Bailey 1982). Since acetyl CoA carboxylase activity requires citrate, situations where glucose is used for the synthesis of glycogen rather than pyruvate would be expected to decrease cytosolic citrate and thereby limit acetyl CoA carboxylase activity (see review by Goodridge 1985). Thus, it has been suggested that the decrease in lipogenesis before birth may be due to increased utilization of substrate for glycogenesis at this time (Benito et al 1982, Lorenzo et al 1983). The fatty acid and cholesterol biosynthetic pathways both use cytosolic acetyl CoA from the mitochondria (see reviews by: Goodridge 1985, Fielding & Fielding 1985). It is conceivable, therefore, that cholesterol synthesis, similar to fatty acid synthesis, may be limited by high glycogenic activity in the late fetal liver. Increased HMG CoA reductase activity is known to occur concomitant to reduced HMG CoA as a result of low HMG CoA synthase activity (Ramachandran et al 1980). Thus, it is plausible that the high activity of HMG CoA
reductase in the prenatal liver may be explained by decreased substrate availability (Pullinger & Gibbons 1983).

The results of these studies indicate that whereas the rate of hepatic cholesterol synthesis decreased, hepatic lipogenesis continued unaltered between 0 and 48 hrs after birth. This infers that low HMG CoA reductase activity, rather than limited substrate availability, led to the decrease in hepatic cholesterol synthesis 48 hours after birth. Uptake of cholesterol-rich chylomicron remnants is known to decrease reductase activity and cholesterol synthesis (Lakshmanan et al 1981, Van Zuiden et al 1983). It is reasonable, therefore, that the decrease in reductase activity and cholesterol synthesis by the 48th hour after birth is explained by uptake of exogenous cholesterol from the rat milk diet by the liver. The increase in neonatal hepatic cholesteryl ester levels found at 48 hrs after birth (figure 3.2) is presumably a consequence of this.

4.4 Concluding remarks

The major findings of this thesis are (1) fetal hepatic HMG CoA reductase total activity can be altered by maternal diet or drug treatment which does not affect fetal growth, and (2) the total activity of the reductase is not necessarily related to the rate of hepatic cholesterol synthesis in the fetal rat. Some of the questions which arise out of these studies are: What are the mechanisms by which HMG CoA reductase is altered? What is limiting the rate of cholesterol synthesis in fetal rat liver if not HMG CoA reductase activity? What causes hepatic HMG CoA reductase and cholesterogenesis to assume their characteristic developmental profile? Are there in vivo methods, other than CY and fat feeding, which influence the activity of HMG CoA reductase in fetal rat liver?

The total activity of fetal hepatic HMG CoA reductase was altered by CY and fat feeding in rats. The activity of an enzyme may be altered by changes in (1) the rate of enzyme protein synthesis and/or degradation, and (2) the catalytic efficiency of existing enzyme. A first step in exploring how CY and fat feeding altered reductase activity may be to determine if the changes in reductase activity were due to
alterations in catalytic efficiency or enzyme protein mass as determined by protein synthesis and/or
degradation. Absolute amounts of reductase protein may be measured with the use of an antibody specific
to rat HMG CoA reductase protein (Harwood et al 1987). Alterations in the absolute amount of reductase
protein may be the result of changes in enzyme synthesis and/or degradation. These two possibilities could
possibly be distinguished from each other by, for example, preparing cultured fetal hepatocytes from
pregnant rats fed a control versus an experimental diet and then measuring rates of HMG CoA reductase
protein accumulation and depletion in the presence or absence of cycloheximide (an inhibitor of eukaryotic
protein synthesis).

Rates of protein synthesis depend in part on the availability of mRNA for translation. Steady-state levels
of mRNA, similar to steady-state levels of a specific protein, may be influenced by its rate of synthesis (gene
transcription) and degradation. Absolute amounts (steady-state levels) of HMG CoA reductase mRNA in
fetal rat liver could be determined using cDNA of HMG CoA reductase radiolabelled with $^{32}$P (Levin et
al 1989). Changes in rates of transcription or mRNA degradation could be distinguished by determining
changes in HMG CoA reductase mRNA levels over time in the presence or absence of actinomycin D (an
inhibitor of gene transcription) in primary cultured fetal hepatocytes prepared from rats fed control versus
experimental diets.

Catalytic efficiency of fetal hepatic HMG CoA reductase has been expressed as the ratio of total activity
(phosphorylated + unphosphorylated) of the enzyme over the concentration of reductase protein (Weiss
et al 1988). Hence, any changes in reductase activity due to modifications in enzyme efficiency could be
determined in fetal livers from rats fed control versus experimental diets.

The studies presented in this thesis observed that changes in the in vitro activity of fetal hepatic HMG
CoA reductase resulting from maternal diet or drug treatment, or normally occurring with advancing
gestational and postnatal age were not necessarily accompanied by corresponding changes in the in vivo rate
of cholesterol synthesis. Possible explanations include the presence of a non-covalently bound inhibitor of
HMG CoA reductase, some enzyme, other than HMG CoA reductase, limits the rate of cholesterol
synthesis at these stages of development, or limited substrate availability. The most logical first step would
seem to be to confirm which enzymes are rate-limiting for cholesterol synthesis in fetal rat liver. This can be accomplished by a number of experimental methods (reviewed by Denton & Pogson 1976). Rate-limiting steps in metabolic pathways may be determined directly by measuring the mass action ratio (ie. ratio of product to substrate at physiological concentrations found in the freeze-clamped tissue) and comparing this to the apparent equilibrium constant of the specific reaction being studied (Denton & Pogson 1976). This method, however, would be a laborious approach due to the complexity of the cholesterol biosynthetic pathway and the number of intermediates which would need to be examined.

Another direct method which could be used to determine the rate-limiting step of fetal hepatic cholesterol biosynthesis is to measure the reaction rates for each reaction (Denton & Pogson 1976). Administration of a radiolabelled precursor (such as $[^3H]$water) over time such that its specific radioactivity is essentially constant would enable the identification of the intermediate which is slowest to attain a specific radioactivity similar to the radiolabelled precursor. The enzyme catalysing the formation of this intermediate would be limiting the rate of cholesterol biosynthesis. This method, however, would require the ability to quantitatively isolate each intermediate in the cholesterol biosynthetic pathway after complete and immediate cessation of all metabolic activity in the fetal liver (eg. freeze-clamping).

The most logical point for a rate-limiting reaction to occur in any metabolic pathway is near its beginning or at key branch points (Denton & Pogson 1976). This enables the cell to limit the amount of substrate entering the metabolic pathway before it becomes committed to the final product. Hence, substrate may be shunted to other processes if the need arises without substantial quantities being tied up as various intermediates. Thus, the enzymes in cholesterol biosynthesis, acetyl CoA thiolase, HMG CoA synthase, HMG CoA reductase, prenyl transferase and squalene synthase (reviewed in: Brown & Goldstein 1980, Goldstein & Brown 1990) would be logical points to determine if they are influenced in fetal liver by developmental age in the same manner as the rate of fetal hepatic cholesterol synthesis. The activity of cytosolic HMG CoA synthase during perinatal development has been determined (Shah & Bailey 1977, Caswell & Bailey 1983) and found to be low and unchanging before birth. The developmental profile of prenyl transferase and squalene synthase has been determined (Bruenger & Rilling 1986), but not before
birth. It needs to be remembered that a competitive inhibitor of these enzymes could cause apparent
dissociations between in vitro enzyme activity and in vivo rates of cholesterol synthesis, similar to that
previously discussed in relation to HMG CoA reductase. If the rate-limiting step was found to occur at
HMG CoA reductase, as in the adult liver (see review by Rodwell et al 1976), this would strongly suggest
that HMG CoA reductase activity is low in vivo due to the presence of a non-covalently bound inhibitor
which is lost during the preparation of microsomes for in vitro assay of HMG CoA reductase.

Once the rate-limiting step of cholesterol synthesis in fetal rat liver has been established it would be of
interest to investigate how this step is regulated to produce the characteristic developmental pattern of
cholesterol synthesis. It would be necessary to first determine whether the rate-limiting step is regulated
by substrate availability or by enzyme activity. This could be determined by comparing changes in the
concentration of the substrate for the rate-limiting step to changes in the rate of cholesterol biosynthesis.
Generally, parallel changes in substrate concentration and biosynthetic rates suggest that the change in flux
through the metabolic pathway is initiated by changes in substrate concentration, whereas inverse changes
indicate that the change in the rate of biosynthesis is due to a change in the activity of the enzyme catalysing
the rate-limiting step (see review by Denton & Pogson 1976).

If enzyme activity is controlling cholesterol synthesis, then the mechanism by which its activity is altered
should be determined. This could be done by using a similar experimental approach to that outlined for
determining how HMG CoA reductase activity may be altered by CY and fat feeding (ie. catalytic efficiency
vs. protein synthesis/degradation). It has been reported that the insulin/glucagon ratio increases throughout
gestation and then drops immediately before birth (reviewed by Mayor & Cuezva 1985). Thus, it would
be of interest to determine if changes in the insulin/glucagon ratio control the rise and fall of cholesterol
synthesis during the perinatal period through the regulation of the activity of the rate-limiting enzyme.

Conceivably, substrate availability, for example acetyl CoA, could control cholesterol biosynthesis in the
fetal rat liver. As a result the developmental profile of cholesterogenesis may be an indirect result of the
glucocorticoid induction of glycogenesis which occurs just before birth (Mayor & Cuezva 1985). This may
be investigated by observing either, the effect of inhibiting glycogenesis on the in vivo rate of cholesterol
synthesis, or the flow of radiolabelled carbon units from $[^{14}\text{C}]$glucose into cholesterol versus glycogen during perinatal development.

The studies in this thesis show that changes in HMG CoA reductase activity due to fat or CY feeding were accompanied by alterations in the fatty acid composition of the fetal rat liver. It is reasonable that this was related to altered placental delivery of fatty acid, but this is speculative. It is not known whether or not the fetal reductase is sensitive to the type and/or amount of carbohydrate or protein in the maternal diet, or to hormones other than cortisol or ACTH (eg. growth hormone, sex steroids). However, if HMG CoA reductase activity does not regulate rates of cholesterol synthesis in the fetal rat liver, the relevance of determining other modes of altering fetal reductase must be questioned. On the other hand, although cholesterol synthesis does not seem to be limited by the activity of fetal hepatic HMG CoA reductase, alterations in fetal reductase activity produced by perinatal CY (Innis 1983, 1989) or fat feeding (Brown et al 1990) have been observed to be associated with long-term effects on cholesterol metabolism. It still remains to be determined how these long-term effects are produced by maternal dietary fat or CY, and whether the associated changes in fetal hepatic HMG CoA reductase activity are directly responsible for these long term effects, or if they are simply an epiphenomenon secondary to more important effects in the fetus yet to be discovered.
5 REFERENCES


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neonatal life of guinea pig on its subsequent response to dietary cholesterol. *Atherosclerosis* 32: 93-98.


Figure 6.1 Diurnal rhythm of plasma corticosterone in male (● — ●) and female (△ ⋅ ⋅ ⋅ △) rats. Rats were housed as described in Materials and Methods. Blood samples were obtained from rats under light diethyl ether anaesthesia by heart puncture with sampling time being within 1 min after onset of unconsciousness.
Figure 6.2 Effects of (A) ACTH and (B) cortisol on the diurnal cycle of plasma corticosterone in female rats. Rats were given cortisol, 5 mg at 1600 h, or ACTH, 2 µg at 0800 and 2000 h, daily by subcutaneous injection. See figure 6.1 for additional notes. • — control, □ • • □ effect of ACTH, △ • • △ effect of cortisol.
Table 6.1

*Effects of diets containing cholestyramine or high saturated fat and cholesterol, and cortisol or ACTH treatment on hepatic enzyme activities in female rats.*

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>HFC</th>
<th>CY</th>
<th>Cortisol</th>
<th>ACTH</th>
</tr>
</thead>
<tbody>
<tr>
<td>liver/body, g/Kg</td>
<td>3.20 ± 0.11</td>
<td>3.66 ± 0.13*</td>
<td>3.26 ± 0.06</td>
<td>3.58 ± 0.09*</td>
<td>3.54 ± 0.15</td>
</tr>
<tr>
<td>HMG CoA reductase, pmol/min/mg protein</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>active</td>
<td>445 ± 46</td>
<td>48 ± 2*</td>
<td>2458 ± 106*</td>
<td>490 ± 46</td>
<td>786 ± 35*</td>
</tr>
<tr>
<td>total</td>
<td>1006 ± 65</td>
<td>156 ± 25*</td>
<td>4891 ± 330*</td>
<td>777 ± 128</td>
<td>858 ± 56</td>
</tr>
<tr>
<td>FAS, nmol/min/mg protein</td>
<td>13.4 ± 0.9</td>
<td>24.0 ± 2.2*</td>
<td>13.3 ± 1.1</td>
<td>12.3 ± 1.6</td>
<td>15.1 ± 1.2</td>
</tr>
<tr>
<td>TAT, nmol/min/mg protein</td>
<td>45.0 ± 7.0</td>
<td>30.0 ± 3.7</td>
<td>51.7 ± 4.5</td>
<td>20.4 ± 2.3*</td>
<td>42.9 ± 3.6</td>
</tr>
<tr>
<td>Cystathionase, nmol/min/mg protein</td>
<td>26.7 ± 1.4</td>
<td>19.1 ± 0.7*</td>
<td>27.7 ± 0.8</td>
<td>16.5 ± 2.0*</td>
<td>22.7 ± 1.2</td>
</tr>
</tbody>
</table>

Rats were fed and housed as described in Materials and Methods. Female rats were either fed diets containing high saturated fat and cholesterol (HFC) or cholestyramine (CY) for 20 days, or subcutaneously administered cortisol, 5 mg at 1600 h, or ACTH, 2 μg at 0800 and 2000 h, daily for 12 days. Hepatic activities of HMG CoA reductase, fatty acid synthase (FAS), tyrosine aminotransferase (TAT), cystathionase, lipoprotein lipase and hepatic lipase were assayed as described in Materials and Methods. Results are given as means ± SEM, n > 6 for each treatment. Significant differences were determined using analysis of variance (p < 0.05) followed by paired tests with Fisher's LSD, *p < 0.0125.
Table 6.2
Effects of cholestyramine treatment on hepatic lipids and the activity of hepatic and lipoprotein lipase in female rats.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>CY</th>
</tr>
</thead>
<tbody>
<tr>
<td>lipoprotein lipase, nmol/min/mg protein</td>
<td></td>
<td></td>
</tr>
<tr>
<td>adipose</td>
<td>15.3 ± 2.4</td>
<td>12.2 ± 1.8</td>
</tr>
<tr>
<td>muscle</td>
<td>0.34 ± 0.08</td>
<td>0.35 ± 0.08</td>
</tr>
<tr>
<td>hepatic lipase, nmol/min/mg protein</td>
<td>0.33 ± 0.02</td>
<td>0.24 ± 0.03*</td>
</tr>
<tr>
<td>liver lipids, µg/mg protein</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cholesterol</td>
<td>1.92 ± 0.10</td>
<td>1.90 ± 0.08</td>
</tr>
<tr>
<td>triacylglycerol</td>
<td>5.53 ± 0.31</td>
<td>3.42 ± 0.14**</td>
</tr>
</tbody>
</table>

Rats were fed and housed as described in Materials and Methods. Female rats were either fed control or cholestyramine (CY) diets for 20 days. Hepatic activities of lipoprotein lipase and hepatic lipase and the hepatic concentration cholesterol and triacylglycerol were assayed as described in Materials and Methods. Results are given as means ± SEM, n = 5 for each diet. Significant differences were determined using Student's t-test. *p < 0.05, **p < 0.01.
Table 6.3
Effects of diets containing cholestyramine or high saturated fat and cholesterol, and cortisol or ACTH treatment on plasma hormones and lipids in female rats.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>HFC</th>
<th>CY</th>
<th>Cortisol</th>
<th>ACTH</th>
</tr>
</thead>
<tbody>
<tr>
<td>T&lt;sub&gt;4&lt;/sub&gt;, µg/dL</td>
<td>3.08 ± 0.40</td>
<td>2.08 ± 0.25</td>
<td>3.06 ± 0.09</td>
<td>3.62 ± 0.29</td>
<td>2.89 ± 0.17</td>
</tr>
<tr>
<td>free T&lt;sub&gt;4&lt;/sub&gt;, ng/dL</td>
<td>2.53 ± 0.47</td>
<td>1.81 ± 0.21</td>
<td>2.09 ± 0.09</td>
<td>1.36 ± 0.10</td>
<td>1.41 ± 0.05</td>
</tr>
<tr>
<td>corticosterone, µg/dL</td>
<td>32.6 ± 7.5</td>
<td>102 ± 10</td>
<td>86.3 ± 2.6</td>
<td>15.5 ± 5.1</td>
<td>100 ± 7</td>
</tr>
<tr>
<td>cholesterol, mg/dL</td>
<td>61.2 ± 1.7</td>
<td>151 ± 7</td>
<td>56.4 ± 1.4</td>
<td>59.0 ± 1.8</td>
<td>64.4 ± 3.2</td>
</tr>
<tr>
<td>triacylglycerol, mg/dL</td>
<td>35.0 ± 5.3</td>
<td>55.9 ± 5.9</td>
<td>58.9 ± 4.4</td>
<td>78.3 ± 10.4</td>
<td>67.0 ± 5.0</td>
</tr>
</tbody>
</table>

1Rats were fed and housed as described in Materials and Methods. Female rats were either fed diets containing high saturated fat and cholesterol (HFC) or cholestyramine (CY) for 20 days, or subcutaneously administered cortisol, 5 mg at 1600 h, or ACTH, 2 µg at 0800 and 2000 h, daily for 12 days. Plasma levels of cholesterol, triacylglycerol, corticosterone and thyroxine (T<sub>4</sub>) were determined as described in Materials and Methods. Results are given as means ± SEM, n ≥ 6 for each treatment. Significant differences were determined using analysis of variance (p < 0.05) followed by paired tests with Fisher's LSD, *p < 0.0125.
Figure 6.3 Enzyme kinetics of fetal hepatic HMG CoA reductase. Double reciprocal plots of adult male (□---□) and fetal (● --- ●) rat hepatic HMG CoA reductase activity (pmol mevalonate formed/min/mg microsomal protein) versus substrate (HMG CoA) concentration (mM). Values represent means ± S.E.M. for 4 fetal litters and 3 adults. Fetal liver was collected on the 20th day of gestation and pooled for each litter. Adult and fetal hepatic microsomes were prepared for HMG CoA reductase assay as described in Materials and Methods. $V_{\text{max}}$ was 553 ± 41 and 594 ± 38, and $k_m$ 81.3 ± 4.6 and 81.9 ± 13.8 pmol/min/mg microsomal protein, for the fetus and adult, respectively.
### Table 6.4

**Effects of hormones on digitonin-precipitable sterols (DPS) and fatty acids (FA) synthesis and HMG CoA reductase activity in fetal hepatocytes.**

<table>
<thead>
<tr>
<th></th>
<th>Insulin, $10^{-7}$ M</th>
<th>Cortisol, $10^{-6}$ M</th>
<th>ACTH, $10^{-7}$ M</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPS, pmol/mg/h</td>
<td>828 ± 17$^*$</td>
<td>920 ± 144</td>
<td>448 ± 109</td>
<td>575 ± 104</td>
</tr>
<tr>
<td>FA, pmol/mg/h</td>
<td>1064 ± 63</td>
<td>796 ± 71</td>
<td>615 ± 73</td>
<td>788 ± 144</td>
</tr>
<tr>
<td>HMG CoA reductase</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>total, % control</td>
<td>80</td>
<td>128</td>
<td>98</td>
<td>100</td>
</tr>
<tr>
<td>active, % control</td>
<td>120</td>
<td>114</td>
<td>91</td>
<td>100</td>
</tr>
</tbody>
</table>

1Hepatocytes were incubated for 90 min with 2 mCi $^{14}$Cacetate and insulin $10^{-7}$ M, cortisol $10^{-6}$ M, ACTH $10^{-7}$ M or no hormone. Cells and media were analyzed for $^{14}$C incorporation into DPS and FA as follows. Fetal hepatocytes were prepared with a non-perfusion method based on that of Carr & Simpson (1984) and Conti Devirgiliis et al (1981) but modified to increase viability and reduce contamination with hematopoietic cells. Briefly, livers were collected aseptically from 20-30 fetuses, pooled, rinsed in 25 mL Ca$^{2+}$ and Mg$^{2+}$-free Hank's Balanced Salt Solution (HBSS) containing 0.5 mM EGTA and supplemented with 25mM glucose. The livers were minced with crossed scalpels blades to 1 mm$^3$ pieces and washed twice. The tissue was transferred to 50 mL CaCl$_2$, 25 mM glucose, 0.5 mg/mL collagenase, 0.3 mg/mL DNAse and 1 mg/mL hyaluronidase plus antibiotics (penicillin 1000 U/mL, streptomycin 10 g/mL and fungizone 2.5 g/mL). Digestion was facilitated by shaking (70 oscillations/min) in the presence of glass beads (0.16 mm diameter) at 37 C for 15 min. The media and free cells were carefully decanted, replaced with fresh media containing collagenase increased to 2 mg/mL, and the incubation repeated. The procedure was repeated twice more. Cells were immediately harvested from the spent media and washed consecutively with fetal calf serum (FCS), phosphate-buffered saline and HBSS, 5 mL each by centrifugation (400 rpm for 2.5 min). Cells released by digest 1 were primarily hematopoietic and were discarded. Cells from digests 2-4 were resuspended and pooled in a small volume of Dulbecco's Modified Eagle's Medium (DMEM), examined for viability by Trypan blue exclusion (final concentration 0.2%) and counted by the nucleii method of Sandford et al (1951). The cells were plated in DMEM supplemented with 0.4 mM ornithine, glucose and antibiotics as above, in Primeria tissue culture dishes (35 mm) at a density of 4x10$^6$ cells/g liver with >95% viability. The high glucose concentration used prevents glycogenolysis (Harris 1975) and improved viability of the fetal hepatocytes. Cell glycogen, assayed by the anthrone reaction (Seifter et al 1950) was constant at 15 $\mu$g/mg cell protein for at least 8 h under these conditions. After incubation, the media was aspirated and the cells harvested by scraping with a rubber policeman. The cells were washed, pelleted and disrupted by sonication, but not further fractionated. All assays utilized cells and media pooled from 3-6 dishes. Values are means (+ S.E.M.) for 3 identical experiments done on separate days, with 3 replicates of each treatment in each. Values for a given hormone which are significantly different by Students' t-test from the control are indicated as $^*p < 0.05$. HMG CoA reductase activity was assayed without (total) and with (active) 50 mM NaF in 6 dishes in 2 separate experiments for each hormone, and calculated as a % of the control. Values given represent the averages. The activity of the total, but not the active, reductase was consistently higher in the presence of insulin or cortisol. ACTH had no effect.
Table 6.5

Effect of maternal adrenalectomy on hepatic and adrenal HMG CoA reductase activity and plasma lipids.\(^1\)

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Adrenalectomy</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pregnant rat</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hepatic HMG CoA reductase activity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>total, pmol/min/mg protein</td>
<td>411 ± 25</td>
<td>536 ± 198</td>
</tr>
<tr>
<td>active, pmol/min/mg protein</td>
<td>112 ± 13</td>
<td>149 ± 48</td>
</tr>
<tr>
<td>plasma lipid levels</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cholesterol, mg/dL</td>
<td>77.6 ± 6.7</td>
<td>63.2 ± 3.3</td>
</tr>
<tr>
<td>triacylglycerol, mg/dL</td>
<td>160 ± 29</td>
<td>123 ± 44</td>
</tr>
<tr>
<td><strong>Fetuses</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HMG CoA reductase activity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>liver</td>
<td></td>
<td></td>
</tr>
<tr>
<td>total, pmol/min/mg protein</td>
<td>534 ± 50</td>
<td>302 ± 37(^+)</td>
</tr>
<tr>
<td>active, pmol/min/mg protein</td>
<td>65 ± 9</td>
<td>77 ± 5</td>
</tr>
<tr>
<td>adrenal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>total, pmol/min/mg</td>
<td>30.7 ± 2.3</td>
<td>31.2 ± 1.4</td>
</tr>
<tr>
<td>Plasma lipid levels</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cholesterol, mg/dL</td>
<td>80.9 ± 8.4</td>
<td>62.0 ± 2.3</td>
</tr>
<tr>
<td>triacylglycerol, mg/dL</td>
<td>57.4 ± 5.8</td>
<td>45.4 ± 6.0</td>
</tr>
</tbody>
</table>

\(^1\)Pregnant rats were adrenalectomized on the 18th day of gestation. Adrenalectomized rats were given 0.9% NaCl in their drinking water. Maternal and fetal tissues were collected on the 20th day of gestation and fetal tissue pooled prior to assay. HMG CoA reductase activity and plasma lipid levels were assayed as described in Materials and Methods. The data are given as means ± S.E.M., control \(n = 5\), adrenalectomized \(n = 4\). Levels of significance were determined between the two groups using Students’ t-test, \(+p < 0.01.\)
Figure 6.4. Cholesterol and fatty acid synthesis in the placenta and fetal adrenal and body during late gestation. Rates of lipid synthesis were determined as described in Methods and Materials and expressed as nmol $^3$H$_2$O per hour per mg of homogenate protein. All fetal and placental tissue were pooled within a litter prior to analysis. Each data point represents the mean of $n \geq 3$ litters ± SEM.
Figure 6.5. Cholesterol and fatty acid synthesis in the carcass, intestine and brain of rats during late gestational and early postnatal development. Carcass contained all tissues remaining after the brain, intestine, adrenal, liver and placental membranes were removed. Rates of lipid synthesis were determined as described in Methods and Materials and are expressed as nmol $^3$H$_2$O per hour per mg of homogenate protein. All fetal tissues were pooled within a litter prior to analysis. Each data point represents the mean of $n \geq 3$ litters or adult rats ± SEM.