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University of Alberta

Effects of palmitic acid on endogenous cholesterol synthesis in humans

by

Stephanie Lynne Cook 

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of Masters of Science

in

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Dr. M. T. Clandinin (Supervisor)

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Date: February 5, 1997

Abstract

This study was undertaken to establish the effect of palmitic acid on serum lipoproteins and endogenous cholesterol synthesis in humans. Four diets were formulated to provide combinations of two levels of palmitic acid in relation to two levels of linoleic acid. Subjects received each of the diet treatments for 21 days. On day 21 a fasting blood sample was drawn for lipoprotein determination and measurement of background deuterium levels. A priming dose of deuterium was consumed and a second blood sample obtained 24 hours after the first. Isotope ratio mass spectrometry was used to determine the incorporation of deuterium into the newly synthesized cholesterol molecule. Serum total and LDL-cholesterol were not significantly affected by high levels of palmitic acid when diets also contained high levels of linoleic acid. Fractional synthetic rates did not differ significantly among diet treatments, suggesting no relationship between the endogenous synthesis of cholesterol and palmitic acid content.

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SFA	saturated fatty acids
PUFA	polyunsaturated fatty acids
MUFA	monounsaturated fatty acids
HDL	high density lipoproteins
LDL	low density lipoproteins
VLDL	very low density lipoproteins
IDL	intermediate density lipoproteins
apoE	apolipoprotein E
apoB	apolipoprotein B
LCAT	lecithin:cholesterol acyltransferase
CETP	cholesteryl ester transfer protein
FH	familial hypercholesterolemia
Lp (a)	lipoprotein a
mRNA	messenger ribonucleic acid
DNA	deoxyribonucleic acid
DPS	digitonin-preceptible sterols
[14-C]	14 carbon
IRMS	isotope ratio mass spectrometry
NADPH	reduced nicotinamide-adenine dinucleotide phophate
FSR	fractional synthetic rate
HMG-CoA	3-hydroxy-3-methylglutaryl-coenzyme A
D_2O	deuterium oxide
C16:0	palmitic acid
C18:2	linoleic acid
acetyl CoA	acetyl coenzyme A

Chapter I

INTRODUCTION

Over the past few decades a large and substantial body of evidence from laboratory, epidemiological and clinical studies implicates elevated serum cholesterol in the etiology of coronary heart disease. Common to the majority of this research is one pervading link; that diet is a significant determinant of an individuals serum cholesterol levels. Many nutritional factors have been implicated in the development of hypercholesterolemia. However, no one nutrient has received as much attention as dietary fat. Despite this, relatively little conclusive evidence exists that provides the mechanisms by which specific dietary fats exert a hypercholesterolemic effect, nor is it clear why specific fatty acids, within lipid classes, exert very different effects on plasma lipoproteins. It is therefore the intent of this chapter to review the evidence that links diet fat to coronary heart disease with emphasis on diet fat composition and serum cholesterol levels.

Dietary Fat and Hypercholesterolemia

Structural Classes of Dietary Fat

The first indication that the type of dietary fat fed may impact cholesterol levels was nearly forty years ago when Kinsell and his colleagues discovered that feeding a chemically constant formula diet composed exclusively of protein and vegetable fat led to prompt and significant reductions in total serum cholesterol(1). These effects were not noted to occur when similar amounts of animal fat were fed and were reversed when animal fat was substituted for vegetable fat. Observations such as this are noteworthy in that, although Kinsell recognized that this may be due to the absence of cholesterol in plant fats, it was hypothesized that vegetable fats may contain some component which influences sterol and phospholipid metabolism. Prior to this, it had been a generally accepted fact that animal and vegetable fats were essentially interchangeable insofar as their effects upon serum lipids were concerned.

This landmark study was followed over the next few years by more and more evidence that not all dietary fats are alike. Although lacking experimental data, Sinclair suggested that the precise chemical composition of ingested fatty acids, and primarily the degree of saturation, was significant in the relationship to serum cholesterol(2). Ahrens et al. in a series of studies, determined that when formula feeds were provided containing either corn or coconut oil, the level of total plasma cholesterol was 40% higher on the coconut oil diet than the corn oil diet(3, 4). These researchers, reluctant to conclude that the depression of serum lipids during the corn oil feed was due to the absence of cholesterol, determined that ingestion of cholesterol with lard did not produce higher levels of serum lipids than did lard alone. Following this, Ahrens et al. provided evidence that serum levels of both cholesterol and phospholipids varied inversely with the iodine number of various dietary fats, with serum cholesterol increasing as the degree of saturation increased(5). The results of this study were supported when it was determined that elimination of double bonds by hydrogenation mitigated the lipid lowering action of unsaturated fatty acids(6).

The highest lipid levels encountered thus far were produced by feeding butter or coconut oil; two fats which are not only highly saturated but also contain a high proportion of shorter chain acids. Considerable insight was gained when experiments were conducted which contrasted butter with cocoa-butter(7). Although nearly identical in terms of degree of saturation, the saturated fatty acids (SFA) of cocoa-butter are predominately C16 and C18 whereas that of butter are C4 to C14 acids. Serum cholesterol and phospholipid levels were significantly higher during the butter-feeding periods than during that of the cocoa-butter. Although not confirmed by more rigorous methods, the lack of change in body weight was considered indicative of equal absorption of both fats. Consequently, Ahrens et al. interpreted these results as evidence that SFA of short and intermediate chain length cause higher serum lipid levels than do the longer-chain SFA. Similar feeding studies followed that lend support to this postulation (8, 9).

At approximately the same time as these important laboratory trials were conducted, epidemiological evidence began to appear linking diets of North Americans and Western Europe to higher serum cholesterol levels. It was Keys et al. who identified that the most

striking difference between these diets was the higher content of animal fat consumed in the Western world(10). Keys cautioned, however, that liberal use of vegetable fat in otherwise low-fat diets may raise serum cholesterol, and reinforced the view that effects of dietary fat on blood cholesterol must be made in terms of chemical composition rather than origin. As a result, Keys et al. undertook a series of studies to obtain information about the effects of fatty acids in a mixed diet(11, 12). By varying both quantity and quality of dietary fat, Keys et al. developed a predictive regression equation associating changes in serum cholesterol to the dietary content of saturated and polyunsaturated fatty acids (PUFA)(10). According to Keys, SFA raise serum cholesterol concentrations about twice as much as PUFA lower them. It was concluded that monounsaturated fatty acids (MUFA) have no significant relationship to serum cholesterol levels. In the years to come, this equation would be utilized extensively to predict the serum cholesterol response to specific fatty acids.

By the mid 1960's a substantial body of evidence had accumulated which linked dietary fat to atherosclerotic heart disease. As a result, health professionals and the media began recommending North Americans to alter the composition of their dietary fat intake. According to a number of scientists, however, these recommendations were premature, in that quantitative data was lacking that clearly defined causal effects of specific dietary fats. To begin, Hegsted et al. reported that numerous factors other than dietary fat, such as phytosterols, pectin and dietary carbohydrate, influenced serum cholesterol levels(13). In 1965, Hegsted et al. fed a total of 36 different test diets using five dietary saturated fats varying in chain length, to institutionalized men over a 2 year period(13). Through regression analyses, these researchers determined that changes in SFA alone could account for only 72% of the total variation in serum cholesterol observed. When individual fatty acids were assessed, 67% of the total variance in serum cholesterol could be explained by changes in myristic acid (14:0) alone; indicating that this saturated fat may be the most important determinant of serum cholesterol levels. PUFA lowered serum cholesterol and no specific effects could be detected for stearic, lauric, or the shorter chain saturated acids. This was the first indication that particular fatty acids, rather than structural classes of the fats, could influence serum cholesterol levels. Based

on this data, Hegsted concluded that the most effective diets for lowering serum cholesterol should provide a small portion of myristic and palmitic acid and a high proportion of PUFA. These authors suggested that the proportion of fatty acids in dietary fat, rather than the total amount of dietary fat, may be an essential factor in the development of atherosclerosis. Consistent with other researchers at the time was the finding that MUFA had little effect on serum cholesterol levels, except for their ability to dilute the total fatty acid pool.

In 1971, results of one of the largest epidemiological studies ever initiated began to be published. The Framingham Heart Study, which had begun over forty years before, had identified a strong positive correlation between serum cholesterol levels and rates of coronary heart disease across ranges of cholesterol measurements(14). Clinical trials in humans identified that lowering levels of serum cholesterol with diet or drugs decreases the subsequent incidence of coronary heart disease(15). As a result, research on precise mechanisms by which dietary constituents exert a hypercholesterolemic effect became of paramount importance. Central to the research at this time was the discovery that specific dietary fats may differentially affect not only total serum cholesterol but also serum lipoprotein profiles. The bulk of research that would follow focused on clarifying these effects of dietary fat on lipid profiles.

Vega et al. demonstrated that a diet rich in PUFA lowered the cholesterol content of all lipoproteins (VLDL, LDL, and HDL), when compared to a diet rich in SFA(16). Likewise, in 1986, the Lipid Research Clinics Coronary Primary Prevention Trials discovered that a decreased intake of saturated fat lowered both total serum cholesterol and LDL-cholesterol in hypercholesterolemic men(17). Subsequently, other researchers provided additional evidence to support the finding that specific fatty acids act independently to influence lipoprotein levels. For example, Reiser et al. concluded that beef tallow (a source of both stearic and oleic acid) did not affect LDL-cholesterol whereas stearic acid, a SFA, may impact serum cholesterol levels in a manner similar to that of monounsaturated fats(18). Further evidence to support this conclusion was provided by Bonanome et al. who contrasted the metabolic effects of stearic acid, palmitic acid (16:0) and oleic acid (18:1). When compared to the high palmitic acid diet,

total plasma cholesterol and LDL-cholesterol were significantly lower during consumption of the high stearic acid diet. Interestingly, these metabolic effects of stearic acid were found to be very similar to that of oleic acid. The oleic acid content of plasma triglycerides and cholesterol esters also increased significantly during the high stearic acid period, suggesting that stearic acid may be rapidly converted to the monounsaturate, oleic acid.

Although scientists generally agreed that SFA were more likely to exert a hypercholesterolemic effect than PUFA, the role of MUFA did not become apparent until the early 1980's. A number of previous studies identified MUFA as being as effective as polyunsaturates in their cholesterol-lowering effect. However, Reeves et al. suggested that MUFA may exert their cholesterol lowering effects through a completely different mechanism(20). This hypothesis was supported by Grundy et al. who showed that a 40% fat diet containing 27% monounsaturated fat was as effective as a 20% mixed fat diet in lowering plasma LDL-cholesterol when compared to a 40% fat diet rich in saturates(21). Moreover, unlike the low fat diet, the diet rich in monounsaturated fat did not decrease levels of HDL-cholesterol. Similarly, other researchers found that MUFA were as effective as PUFA in lowering LDL-cholesterol levels in normolipidemic subjects without the concomitant reduction in HDL-cholesterol characteristic of diets rich in polyunsaturates(22, 23). However, this effect of monounsaturated fat remains controversial as subsequent researchers have failed to obtain similar results(24). Although total serum cholesterol and LDL-cholesterol were similarly lowered by MUFA-rich diets and PUFA-rich diets, neither regime produced an appreciable effect on HDL-cholesterol levels. More recently, Wahrburg et al. investigated the effects of two diets rich in either MUFA or PUFA on serum lipid profiles in normolipidemic subjects(25). Both diets led to a significant reduction in total LDL and HDL-cholesterol concentrations. Still others have shown that large amounts of MUFA may, in fact, raise some plasma lipids in humans (26). Clearly, more research designed to determine the precise effects of MUFA on plasma cholesterol levels is necessary.

Cholesterol and Plasma Lipoproteins

Plasma lipoproteins are complexes of lipids and protein that circulate in the bloodstream in order to transport triglycerides, cholesterol esters, free cholesterol and apolipoproteins(27). These spherical particles have an outer coat consisting of amphiphilic phospholipids, apolipoproteins and a core of varying proportions of cholesteryl ester and triglycerides. Excessive amounts of one such lipoprotein, low density lipoproteins (LDL), has been strongly associated with the incidence of atherosclerosis. In contrast, a second class of lipoproteins, high density lipoproteins (HDL), are associated with decreased risk. Transport of lipoproteins in plasma is dependent on a group of proteins and enzymes known collectively as the lipoprotein transport proteins(28). Over the past ten years, genes for all the lipoproteins have been isolated, sequenced, and mapped in the human genome. This information has allowed for an understanding of mechanisms by which cholesterol is transported and metabolized, as well as providing insight on the pathogenesis of lipoprotein disorders and atherogenesis. It is the intent of this section to review the present knowledge relating to cholesterol transport, as well as abnormalities of lipoprotein metabolism.

Cholesterol

Cholesterol is a fatty, wax-like substance essential for the normal structure and function of cell membranes in every tissue of the body. Biological functions of membranes are compromised if cells contain too little or too much cholesterol, hence the homeostasis of cholesterol is tightly regulated(29). Mammalian cells have the capacity to synthesize their own cholesterol requirement, as well as extract and deliver cholesterol to plasma. Despite this ability to locally produce cholesterol, most cholesterol utilized by peripheral tissues originates from the liver(29). In plasma, cholesterol circulates as a component of lipoproteins; primarily in the form of cholesteryl esters. Free cholesterol equilibrates freely between lipid surfaces and as a result its level in plasma membranes depends on the cholesterol content of plasma and lymph. This continuous transport is

fundamental to maintenance of homeostasis of cell membranes in tissues throughout the body.

Exogenous Cholesterol Transport

Approximately half of the dietary cholesterol consumed will be absorbed into intestinal cells and incorporated into triglyceride-rich chylomicrons(29). These chylomicrons are then released into the lymphatic system and eventually enter the plasma. Once reaching plasma, chylomicrons are acted on by lipoprotein lipase which hydrolyzes most of the triglyceride and releases free fatty acids for uptake by tissues. The loss of triglyceride converts chylomicrons to chylomicron remnants which deliver dietary cholesterol to the liver. In order for the liver to receive this cholesterol, apolipoprotein E (apoE) on the surface of the chylomicron remnant must bind to the hepatocyte surface. This process is sufficiently rapid that, even in the postprandial state, little cholesterol normally remains in the plasma.

Endogenous Cholesterol Synthesis

Whole-body cholesterol homeostasis is largely regulated by the liver. Hepatic cholesterol, whether of dietary origin or synthesized by the hepatocytes, is incorporated into very low density lipoproteins (VLDL)(29). This lipoprotein contains a large structural apolipoprotein, known as apolipoprotein B (apoB). Most of the cholesterol contained in these lipoproteins is present in its esterified form. This increases the mass of the lipoproteins, because the ester is nondiffusable and consequently unable to permeate the membrane(28). The primary function of VLDL is to transport hepatic triglycerides. However, once in plasma, VLDL are acted upon by lipoprotein lipase which hydrolyzes the triglyceride to liberate free fatty acids to tissue. Concomitant to this process is the acquisition of cholesterol esters and apoE from HDL by the cholesteryl ester transfer process(28). As a result, the products of VLDL are cholesteryl ester and apoE-rich remnants known as intermediate density lipoproteins (IDL). These lipoproteins are rapidly taken up by the liver or further catabolized into LDL. Although the precise mechanism by which this conversion occurs is uncertain, the catabolism of IDL to LDL

involves the hydrolysis of triglycerides as well as a loss of apoE to HDL(29). The result is the formation of a cholesterol-rich protein (LDL) that is catabolized very slowly in human plasma.

Removal of LDL from the plasma is dependent upon LDL-receptors; cell-surface receptors reliant upon the apoB portion of LDL which serves as a ligand(30). Once recognition has occurred, the LDL particle can be internalized and its cholesterol content delivered to peripheral cells for cell metabolism and suppression of endogenous cholesterol synthesis. Although extrahepatic tissues contain LDL-receptors, primary uptake of plasma LDL occurs in the liver. Changes in cholesterol demand are reflected in the liver by changes in the expression and activity of receptors; if the level of cell cholesterol is high the receptor is down-regulated; if low, the receptor is up-regulated(30). ApoB is degraded before the entry of cholesterol, and has been determined to provide no intracellular signal to changes in cholesterol demand. Consequently, the amount of cholesterol molecules delivered to the cell per binding of LDL does not affect receptor activity. Rather, modification of cholesterol clearance appears to be solely related to the number and activity of the LDL-receptors, which is primarily regulated by the amount of intracellular cholesterol(30).

Despite the ability of all cells to synthesize their cholesterol requirement, only hepatic cells have the ability to catabolize it. As a result, metabolic turnover results in the accumulation of cholesterol in cells which must be eliminated. Reverse cholesterol transport is the process whereby cells deliver cholesterol to plasma HDL for removal(31). During this acquisition process, HDL is formed in different sizes, ranging from HDL4, the smallest and most dense, to HDL1, the largest and least dense. HDL is produced by the liver and consists primarily of phospholipids and apoA1, the principle HDL apolipoprotein. Circulating HDL obtains esterified cholesterol and converts it to unesterified cholesterol esters by the plasma enzyme lecithin:cholesterol acyltransferase (LCAT), an enzyme system which utilizes apoA1 as a cofactor. The majority of this cholesterol ester is than transferred via the cholesteryl ester transfer protein (CETP) to other lipoproteins and subsequently delivered to the liver. A smaller amount of

cholesterol is also delivered directly to the liver by HDL, a process dependent on the binding of this lipoprotein to an HDL-receptor(31).

Lipoprotein Abnormalities

Studies which have investigated the molecular biology of atherosclerosis have determined its pathogenesis to be dependent on lipoprotein abnormalities(27). Although significant heterogeneity exists among patients suffering from coronary artery disease, investigations of survivors from myocardial infarction reveals that between 50-80% present with one of four primary lipid abnormality(32). These commonly encountered abnormalities are: an increase in LDL-cholesterol levels, a decrease in HDL-cholesterol levels, an increased concentration of chylomicron remnants and IDL, and the presence of an increased concentration of abnormal lipoproteins (Lp a) in plasma.

The first and most extensively studied abnormality of lipid metabolism was the result of increased concentrations of plasma LDL. In 1985, Michael Brown and Joseph Goldstein were awarded the Nobel Prize in Medicine for their investigation of disorders of LDL metabolism. As mentioned, LDL is a cholesterol-rich particle containing apoB on its surface. ApoB is recognized by hepatic and extrahepatic LDL-receptors prior to its incorporation into the cell(32). Normally, 70% of circulating LDL will be cleared by LDL-receptors, primarily by hepatocytes. Although abnormalities in the genes involved in VLDL secretion or processing as well as variations in other apolipoproteins can result in elevated concentrations of circulating LDL, the most frequently encountered abnormalities occur in the apoB and LDL-receptor genes(33). A single gene located on chromosome 2p24 codes for both apoB-100 and apoB-48(32). ApoB-100 is produced exclusively in the liver, with the LDL-receptor binding portion residing in the carboxy-terminal half of the protein. In the autosomal dominant disorder called heterozygous hypobetalipoproteinemia, having a frequency of about 1:1000, a mutation in the apoB gene results in premature termination of the protein. As a result, little or no plasma apoB is produced(34). Familial defective apoB-100 is the second type of autosomal dominant disorder of the apoB gene. This disorder occurs at a rate of about 1 in every 500 and is the result of a mutant gene that causes a single amino acid deviation: the replacement of

glutamine for arginine in the LDL-receptor binding region. The resultant protein contains only 2-4% of the normal binding activity leading to significantly elevated LDL concentrations(35).

In addition to these disorders of the apoB gene, mutations in the genes that code for the LDL-receptor itself can produce dramatic elevations of LDL-cholesterol(36). The human LDL-receptor gene is located on chromosome 19q13, and codes for a polypeptide of 866 amino acids in length. Mutations in this gene are responsible for familial hypercholesterolemia (FH). Heterozygous FH is also inherited as an autosomal dominant trait, occurring at a rate of about 1 per every 500 in most populations. This disease is characterized by a markedly elevated LDL concentration (up to 140 mg/dL higher than normal); the direct result of defective catabolism of both the LDL and VLDL remnants(37). The specific defect resides in the inability of LDL-receptors to uptake and degrade LDL and apolipoproteins B and E(37). Despite this common defect, there is considerable heterogeneity in the progression and severity of the resultant atherosclerosis, primarily as a result of other risk factors and the particular mutation in the LDL-receptor gene. Normally, the LDL-receptor is synthesized in the endoplasmic reticulum and transported to the Golgi apparatus for glycosylation. The receptors are than transported to the cell surface, where they cluster in specific regions. In these receptor-enriched regions, internalization then takes place through endocytosis. Consequently, a mutation in the gene responsible for any of these processes can lead to an alteration in LDL metabolism(36). Studies designed to elucidate defects in the LDL-receptor protein have lead to the classification of mutations in the gene according to their effects on receptor phenotype in cultured skin fibroblasts from FH patients(38). A Type I mutation is characterized by a lack of mRNA or detectable proteins, Type II by a defect in processing and transport of the proteins, Type III by a failure of LDL to bind normally, Type IV by an inability to internalize the receptor-ligand complex and finally, Type V by a failure of receptors to recycle at the cell surface. Despite this classification, over 100 different mutations have been identified, making the prospect of a simple DNA-based diagnostic test for FH unlikely in the immediate future. Fortunately, homozygous FH

patients are rare, but easily detectable because of rapid and severe atherogenesis leading to fatal coronary disease at an early age(39).

Defects in metabolism of HDL are significantly more rare and varied than are those of LDL, however a number of specific defects have been identified(32). Defective HDL production can result from mutations in the apoA1 gene, leading to an inability to synthesize the requisite apolipoproteins(40). Individuals who are homozygous for such a mutation are prone to premature atherosclerosis. Interestingly, a structural mutation of this gene, referred to as A-1milano, produces low levels of HDL but does not appear to increase risk of coronary artery disease(41). Deficiencies of LCAT or CETP also lead to deviations in HDL levels because of defects in the formation of the cholesterol ester and transfer, respectively. In addition, gene mutations that result in an inability to process chylomicrons and VLDL lead to very low HDL levels. Fortunately, disorders of HDL metabolism occur at a rate less than 1 per every 10 000, and do not impact the degree of coronary artery disease to the same extent as disorders of LDL metabolism(40).

Clearance of the triglyceride-rich lipoproteins, chylomicrons and VLDL is dependent upon hepatic receptors that recognize apoE on their surfaces(42). Defective clearance of apoE-containing lipoproteins is a relatively frequent occurrence because of significant structural variations in the apoE gene. Approximately 1% of the population exhibits a defective apoE allele. Although the majority do not exhibit fasting hyperlipidemia, they can be shown to have difficulties clearing chylomicron remnants from their bloodstream. Approximately 2% of these individuals have type III hyperlipoproteinemia or dysbetalipoproteinemia, two conditions that are associated with premature atherosclerosis(43).

Lp (a) is an altered form of LDL with a large glycoprotein (apo-a) bonded to the apoB portion of LDL(33). Lp(a) is present in varying concentrations in all individuals, with higher levels being a risk factor for premature atherosclerosis. Although the precise mechanism by which this altered lipoprotein exerts its atherogenic effect is unknown, this protein closely resembles plasminogen and may therefore interfere with both LDL and plasminogen metabolism(44). Like most other disorders of lipid metabolism, presence of high concentrations of this mutant lipoprotein appears to follow a similar familial pattern.

Dietary Influences on Plasma Cholesterol and Lipoproteins

Although the mechanisms involved in the transport of cholesterol have been determined, relatively little conclusive evidence exists regarding the effect of diet on serum cholesterol concentrations and lipoproteins. Recently, groups of researchers have suggested that the relationship between cholesterol and coronary heart disease is tenuous at best and that efforts to lower dietary saturated fat and cholesterol do little to prevent atherogenesis. Statements such as these reflect the uncertainty and continued controversy over the effect of specific fatty acids on circulating lipids. It is therefore the intent of this section to review the present knowledge regarding dietary influences on serum lipids and lipoproteins.

Over 80 years ago Anitschkow and Chalatow showed that feeding cholesterol to rabbits caused atherosclerosis. Over the next 40 years, researchers would determine that diets rich in cholesterol would cause severe hypercholesterolemia and atherosclerosis in a variety of animal species, including non-human primates(44, 45). These observations formed the basis for numerous subsequent research efforts, designed to elucidate the precise mechanisms whereby cholesterol influences humans. However, a critical role of dietary cholesterol in the causation of coronary heart disease is not universally accepted, based on numerous reports that humans respond less to dietary cholesterol than do most other species(46, 47). Because cholesterol homeostasis is precisely controlled in the body by feed-back regulation, a number of researchers have even suggested that dietary cholesterol has no influence at all on serum cholesterol levels in humans(48). Although data to support this position is variable, the majority of recent research efforts have identified that serum cholesterol concentrations in humans respond more to the major dietary nutrients than to dietary cholesterol itself; with the polyunsaturated to saturated ratio being the key determinant of serum cholesterol levels(49).

Saturated Fatty Acids

The action of dietary SFA as a lipid class to raise total cholesterol levels, and specifically LDL-cholesterol levels, has been well established(49, 50, 51). Although mechanisms whereby SFA raise LDL levels are not completely understood, it has been suggested that they interfere with LDL-receptor mediated clearance of LDL. Evidence to support this theory points mainly to the LDL fraction as saturated fats do not raise triglyceride levels as would be expected if the synthesis of VLDL, the precursor of LDL, were stimulated(51). Studies of LDL apolipoprotein B-100 have suggested that diets high in saturated fats appear to suppress the LDL-receptor mediated clearance of LDL but it is unlikely that this is the only mechanism involved(44). This is because suppression of LDL-receptors does not follow the theory of down-regulation of the LDL-receptor with increasing intracellular cholesterol levels. It has been hypothesized that SFA suppress LDL-receptor synthesis by redistributing cholesterol among various cellular compartments to favor inhibition of receptor synthesis(33). Saturated fats, then would be expected to decrease the content of mRNA for LDL-receptors. Interestingly, decreased LDL-receptor mRNA abundance has been reported when saturated fats were fed to some animal species. Loscalzo et al. provided an alternate mechanism by which SFA raise cholesterol(52). These researchers showed that in vitro enrichment of cell membrane phospholipids with SFA interferes with normal function of LDL-receptors within the membrane. However, in vivo studies have, to date, failed to support this theory. The precise effects of SFA are complicated by overwhelming evidence revealing that all saturates do not affect lipoprotein profiles equally. Thus, each type of SFA must be considered separately, and independently of cholesterol, to provide a more accurate representation of the overall effects of saturated fats.

Lauric Acid

SFA are frequently divided into two broad categories: long-chain and medium-chain. The long-chain saturated fatty acids include stearic acid (18:0), palmitic acid (16:0) and myristic acid (14:0), whereas saturates containing 8 and 10 carbons belong to the medium-chain group. Long-chain fatty acids are absorbed as triglycerides and

incorporated into chylomicrons whereas medium-chain fatty acids are absorbed directly into the portal circulation as free fatty acids(53). In contrast, lauric acid (12:0) is considered an intermediate-length fatty acid as it appears to enter circulation partially as chylomicron triglycerides and partially as free fatty acids(53).

Lauric acid is found in significant concentrations in two tropical oils; coconut and palm-kernel(53). Consequently, lauric acid is commonly incorporated into a variety of commercially prepared foods. Despite its relatively wide use, the influence of lauric acid on serum cholesterol concentrations is uncertain. Early researchers reported that lauric acid exerts a hypercholesterolemic effect similar to that of palmitic acid, whereas Hegsted et al. reported only a very small effect(54, 55). More recently, Denke and Grundy incorporated lauric acid into a synthetic fat to determine its effect on plasma cholesterol(56). This fat was compared with palm oil (identical to the synthetic fat except for the substitution of lauric for palmitic acid) and with safflower oil (primarily oleic acid). The oil enriched with lauric acid raised LDL-cholesterol concentrations when compared to oleic acid, but increased LDL concentration only about two-thirds as much as did palmitic acid. Although data concerning this fatty acid is limiting, it appears that lauric acid must be presently considered as a cholesterol-raising fatty acid with an effect closer to that of palmitic than oleic acid.

Myristic Acid

Myristic acid is a SFA found in butter fat, and smaller quantities in coconut and palm-kernel oils, comprising only a small amount of the total saturated fat intake of the North American diet(53). Like lauric acid, the precise effects of this saturate on plasma cholesterol concentration is uncertain. Keys et al. and Hegsted et al. both identified myristic acid as a cholesterol-raising fat, but the former equated the effects of myristic to palmitic acid whereas the latter proposed that myristic acid raise raises the total serum cholesterol even more than palmitic acid(54, 55). To date, few researchers have directly investigated the actions of myristic acid on cholesterol levels in humans. Hayes and Khosla accumulated data from feeding 16 different cholesterol-free fat blends to cebus monkeys and generated multiple regression equations similar to those developed by Keys

and Hegsted, to predict the effect of individual acids on the plasma cholesterol response(57). These researchers determined that the percent of energy contributed by myristic and linoleic acid explained almost 92% of the variation in plasma cholesterol. In the absence of cholesterol, only natural fats containing myristic acid appeared hypercholesterolemic. However, when cholesterol was added, palmitic acid also raised serum cholesterol levels. The relative potency between these two saturates in the presence of cholesterol was similar to that originally reported by Hegsted(13). Although data from human trials is lacking, it seems unlikely that the response to specific fatty acids would vary significantly from other primates.

Stearic Acid

Stearic acid contributes substantially to the SFA content of beef fat and other animal fats, constituting 7-10% of total fatty acid intake in the North American diet(53). Early studies of Ahrens et al., Keys et al., and Hegsted et al., suggested that stearic acid, in contrast with other SFA, did not raise serum cholesterol concentrations(7, 10, 13). Since this time, confidence in this conclusion has grown as a result of numerous human studies involving a variety of different sources of stearate, as well as a variety of measurement techniques, that have produced similar results(58). More specifically, stearic acid does not elevate LDL-cholesterol concentrations, rather it exerts a neutral effect on lipoproteins similar to that of oleic acid(59). The reason for this is not entirely clear, however, a number of mechanisms have been proposed. Kritchevsky suggested that absorption of stearic acid from the gut may be reduced as a result of the position of its naturally occurring triglycerides(60). However, subsequent researchers have failed to support this hypothesis(61). Recently, Emken utilized stable-isotope-tracer methodology to determine the absorption of stearic and palmitic acid in human subjects(62). The results show that absorption of both these fatty acids is very similar and concluded that differences in absorption alone are not adequate to explain differences in the cholesterolemic effects for these two saturates. Another possible reason for the lack of LDL-raising is that stearic acid is rapidly desaturated to oleic acid at the hepatic level(53). Studies in animals and humans have confirmed that much of stearic acid is

rapidly converted to oleic acid as only a single desaturation at w9 position is required. In contrast, myristic and palmitic acid must be elongated first before desaturation can occur; allowing these fatty acids to accumulate in tissues and exert an LDL-raising effect(63). Evidence for other mechanisms such as preferential incorporation of stearic acid into phospholipids rather than cholesteryl ester or triglycerides, or its resistance to oxidation as part of the LDL-cholesterol particle, have been hypothesized but data investigating these effects is limited(63). Despite this, the pervading view is that saturated fats are nutritionally undesirable and outweigh the neutral effects of stearic acid on plasma lipids. Clearly, the general recommendations to reduce intake of saturated fats as a class must be reconsidered.

Palmitic Acid

The principal dietary SFA is palmitic acid; comprising approximately 60% of the total saturated fatty acid intake of North Americans(53). Palmitic acid is the major saturate in animal fats, occurring in large proportions in both meat and dairy products. A substantial fraction of palmitic acid is also present in varying proportions in many plant oils; comprising about 45% of the fatty acids in palm oil and approximately 25% of cottonseed oil(64). The earlier studies by Keys and Hegsted involving palmitic acid identified this saturate as exerting a significant hypercholesterolemic effect(10, 13). However, these findings have been recently questioned by a number of researchers, such that the effects of palmitic acid remain controversial.

Tholstrup et al., in a controlled metabolic feeding study, investigated the effects of three diets differing only in the major fatty acid supplied (stearic, palmitic or myristic plus lauric)(65). These researchers concluded that cholesterol-raising properties of saturated fats could be attributed solely to lauric, myristic and palmitic acid; with palmitic acid impacting cholesterol approximately mid-way between these other saturates. These results parallel those suggested by both Keys and Hegsted nearly 30 years earlier. Interestingly, however, when Keys' equation is modified to treat palmitic acid as neutral (similar to stearic acid), the equation is a better predictor of the observed changes in serum cholesterol levels. This observation has been supported by Hayes who also

hypothesized that 16:0 is a neutral fatty acid(66). In a study which exchanged 5% of energy from 12:0 plus 14:0 for 16:0 in healthy young men consuming a low cholesterol diet, the dietary combination of 12:0 plus 14:0 produced a significantly higher serum cholesterol than did 16:0.

Studies which have identified palmitic acid as a cholesterol raising saturate have focused on its effects in the LDL fraction. It has been suggested that palmitic acid may suppress expression of the LDL-receptors, or accelerate VLDL secretion from the liver to elevate plasma LDL. However, evidence has accumulated to suggest that palmitic acid may also enhance HDL production. Lindsey et al. examined the qualitative effects of specific fatty acids on plasma lipoprotein metabolism by feeding six, low-fat, cholesterol-free diets to hamsters for four weeks(67). The fat blends differed only in their source of fat; coconut oil, palm oil, soybean oil, safflower oil, butter, corn oil, and canola oil. In three of the diets, the PUFA/MUFA/SFA ratio was held constant while the 12:0, 14:0 and 16:0 were varied. Replacing 12:0 plus 14:0 from coconut oil with palm oil induced a significant increase in HDL-cholesterol and a slight decrease in LDL-cholesterol. More recently, Khan et al. fed each of palm oil, cottonseed oil and sunflower oil to fifty one healthy volunteers to determine their effect on lipoprotein levels(68). Serum total cholesterol was increased by each experimental fat. In addition, HDL-cholesterol was shown to have increased by 14.6% with the palm oil treatment whereas the sunflower oil treatment resulted in only a 2.3% increase and the cottonseed oil treatment actually decreased HDL-cholesterol values. Based on this, it has been suggested that if palmitic acid raises serum cholesterol levels it may exert this effect by increasing the concentration of that lipoprotein fraction known to favorably impact atherogenesis(67, 68).

The controversial role of dietary palmitic acid is further compounded by other researchers that have failed to demonstrate elevated plasma cholesterol following palmitic acid consumption. Ng et al. compared the effects of palmitic acid and oleic acid in normocholesterolemic subjects(69). Prior to being assigned to either a palm oil rich diet or an olive oil rich diet, subjects were challenged with a diet high in coconut oil. The test diet, as expected, significantly raised all serum lipoproteins. However, exchanging 7% of

energy between palm oil and olive oil produced identical lipoprotein profiles. These researchers concluded that in healthy humans exchanging palmitic acid for oleic acid, within the range of these fatty acids are normally present in a typical diet, will not affect serum cholesterol concentration.

Trans Fatty Acids

Recently, a group of unsaturated fatty acids, the *trans* fatty acids, have become the source of much controversy in North America as a result of their potential health hazards. Chemically, the *trans* fatty acids differ from the more typical *cis* configuration in that the carbon atoms forming the double bond are located on opposite sides of the acyl chain(70). As a result, the acyl change is more linear and rigid, with a higher melting point(70). *Trans* fatty acids occur naturally in dairy and other animal fats, in some plants and are produced commercially by the hydrogenation process. Current intakes of *trans* fatty acids by North Americans are estimated to be approximately 2-4% of energy intake, with the predominant source being hydrogenated vegetable oils(71).

A number of studies have identified a relationship between *trans* fatty acids and plasma lipid and lipoprotein concentrations(70,72,73,74). The collective results of the majority of these studies support the conclusion that when *trans* fatty acids are compared to saturated fats, plasma lipid concentrations tend to be lower. However, when compared to liquid vegetable oils, regardless of source (corn, soy, peanut, or safflower oil) the addition of *trans* fatty acids tended to raise blood cholesterol levels. In 1992, Zock et al. further determined that the substitution of a *trans* fatty acid for a *cis* fatty acids resulted in an increase in both the total and LDL-cholesterol and a decrease in HDL cholesterol concentrations(75). Since this time these results have been confirmed by others(76,77). Although little is known regarding the mechanisms by which *trans* fatty acids influence plasma cholesterol and lipoprotein levels, it has been shown that *trans* fatty acids have the ability to alter the acitivity of a number of enzyme systems of lipid metabolism(75,76). Based on this evidence, it seems reasonable to conclude that *trans* fatty acids should be included in the list of cholesterol-raising fatty acids, with an effect approximately mid-way between that of linoleic acid and lauric or myrsitic acid.

Mechanisms for the Effects of Diet Fat on Plasma Cholesterol Levels

Although the response of plasma cholesterol levels to intake of various dietary fats has been examined, the mechanism by which these changes are induced has not been well-characterized. A variety of possible theories have been proposed to explain the mechanism whereby unsaturated fats lower plasma cholesterol and specific saturated fats raise it. Unfortunately, no single mechanism has been shown to apply in all cases despite years of investigations by several groups of researchers. The intent of this section is to review these proposed mechanisms and attempt to resolve discrepancies in findings and interpretations of results from a number of research efforts. Clearly, establishing the mechanism whereby specific fatty acids exert a hypercholesterolemic effect will have important implications for prevention and treatment of hyperlipidemia.

Fecal Sterol Excretion

Early studies designed to elucidate the mechanism whereby PUFA lower serum cholesterol focused on fecal excretion of either neutral sterols or bile acids. It has been proposed that if unsaturated fats cause a sustained inhibition in absorption of cholesterol by the intestinal tract, a decreased excretion of both endogenous and exogenous neutral steroids will result with a concomitant reduction in plasma cholesterol. In 1965, Avigan and Steinberg fed high-fat liquid diets containing either coconut oil (SFA), corn oil or safflower oil (PUFA) to six hypercholesterolemic subjects(78). By feeding labeled cholesterol (cholesterol-4-^{14}C), these researchers measured specific radioactivity of plasma cholesterol, as well as fecal excretion of sterol and bile acids. Although all the subjects exhibited lowered plasma cholesterol levels when fed the PUFA diet, there was no consistent relationship between plasma cholesterol and fecal sterol excretion. Similarly, Spritz et al. presented findings that significant changes in excretion of total fecal steroids did not occur when plasma cholesterol concentrations were altered by exchanges of unsaturated and saturated dietary fats(79).

These findings stand in conflict with several other groups of researchers that have shown increases in cholesterol excretion during ingestion of unsaturated fat. For

example, Moore et al. reported increased fecal excretion of neutral sterols and bile acids in young men fed a high PUFA diet when compared to a high SFA diet(80). Connor et al. fed formula diets containing either highly saturated fat (cocoa butter) or polyunsaturated fat (corn oil) to healthy male subjects(81). Both neutral sterols and bile acid excretion were increased by consuming dietary polyunsaturated fat. These early studies were unable to clearly establish a link between dietary fat saturation and changes in fecal excretion.

Several plausible explanations have been proposed to explain the highly variable results of these studies. Individuals may respond very differently to dietary fats and patients with different types of lipid abnormalities may not respond by the same mechanism to diets containing unsaturated fats. Rate of cholesterol absorption may change with age, and vary between sexes(82). Changes in cholesterol excretion may initially change with an alteration of diet, but when studies are extended, a new steady state of fecal excretion may result. Many of the studies did not control for other dietary constituents that may effect absorption of cholesterol(82). Finally, earlier studies may have had a variety of technical and analytical deficiencies leading to inaccurate measurement of the excretion products of cholesterol.

Results of more recent studies have provided more consistent and reliable evidence that fecal excretion of steroids is not dependent upon dietary fat saturation. Grundy and Ahrens conducted cholesterol balance studies in eleven patients with various types of hyperlipidemias for periods of up to 128 days(83). Using combinations of saturated and unsaturated fats in liquid diets, these researchers identified no changes in neutral steroid excretion during unsaturated fat feeding and no increase in fecal steroids of endogenous origin. Furthermore, in the five subjects where reliable measurements of exogenous cholesterol absorption could be made, substitution of unsaturated for saturated fat failed to produced a significant decrease in dietary cholesterol absorption. To determine the accuracy of the methodology employed, these researchers conducted a series of studies investigating various disorders known to effect cholesterol absorption (i.e. biliary obstruction, cholestyramine therapy, abetalipoproteinemia.) While the mechanisms by which cholesterol absorption is reduced differed in each of these

conditions, the results revealed consistent and significant reductions in cholesterol absorption. Similarly, using similar methodologies, Shepherd reported that a 40% safflower fat diet significantly reduced LDL-cholesterol but had no effect on fecal neutral or acidic steroid excretion(84). Clearly, studies based on fecal sterol excretion have failed to provide a simple explanation to account for the plasma cholesterol-lowering action of polyunsaturated fats.

Cholesterol Absorption

The hypothesis that diets rich in PUFA result in a decreased absorption of exogenous cholesterol was first suggested by Woods et al.(85). These researchers followed normolipidemic subjects through a six-month transition from a predominately SFA diet to a diet rich in PUFA and concluded that PUFA decreased absorption of cholesterol. Unfortunately, subsequent studies attempting to reproduce these results have failed. Grundy and Ahrens measured changes in cholesterol absorption in five hyperlipidemic subjects consuming liquid diets varied in fat source and containing moderate amounts of cholesterol(83). Three of the five subjects showed no difference in absorption, whereas absorption of exogenous cholesterol increased while on the PUFA-rich diet in the other two subjects. Nestel et al. conducted cholesterol balance studies on eight healthy men fed either PUFA-rich or SFA-rich diet and concluded that absorption of exogenous cholesterol was similar among both groups(86). More recently, McNamara et al. examined the effects of dietary fat and cholesterol on cholesterol homeostasis in fifty normolipidemic men(87). Diets provided 35% of calories as either saturated fat or polyunsaturated fat, first low, then high in dietary cholesterol. By measuring cholesterol absorption and synthesis in each diet period, these researchers concluded that the type of fat is highly correlated with plasma lipid levels but fat quality does not alter the absorption of exogenous cholesterol. Since this time a variety of researchers have attempted to associate dietary fat quality to absorption of exogenous cholesterol. Although some variability in results have been reported, a substantial body of evidence exists to suggest that dietary fat does not influence the absorption of dietary cholesterol and dietary cholesterol has little, if any, effect on circulating cholesterol levels.

Changes in Plasma Lipoprotein Composition

A number of researchers have attempted to link the lowering of plasma cholesterol by diets containing PUFA to specific changes in the lipoprotein molecule. Spritz and Mishkel hypothesized that when unsaturated fatty acids replace SFA in circulating lipids an alteration in the plasma lipoproteins occurs which causes a decrease in serum cholesterol levels(88). To test this hypothesis, these researchers fed liquid diets containing 40% of calories as either saturated or unsaturated fat to twelve subjects. Changes in lipid concentrations occurred in the low but not the high density lipoproteins with little or no effect on the protein moiety of the lipoproteins. Consequently, during PUFA feeding, the cholesterol and phospholipid to protein ratio of LDL fell significantly. These researchers further suggested that unsaturated fatty acids occupied a greater area than saturated acids, thus diets high in PUFA result in fewer lipid molecules carried per lipoprotein. Others have identified dietary fat to have a profound effect on the composition of lipoproteins. For example, Morrisett et al. reported that PUFA-rich diets increased the linoleate content of LDL and reduced the content of palmitate and oleate(89). As a result, thermotropic properties were altered which increased fluidity of these lipoproteins and may have altered the function of membrane proteins; including the LDL-receptor. Similarly Shepherd et al. examined the effects of dietary saturated and polyunsaturated fat on the chemical composition of LDL(84). These researchers identified that high PUFA diets markedly decrease the percentage of cholesterol and increase phospholipid content of the LDL molecule. Furthermore, polyunsaturated fat feeding increased the fractional clearance of LDL from plasma, a phenomena attributed to these alterations in the LDL molecule. This theory is supported by Spritz et al. who identified that changes in composition of the LDL phospholipids influences catabolic rate of this lipoprotein(88). Although numerous studies have identified dietary fat as a key determinant of composition and, consequently, function of plasma lipoproteins, the question of whether these changes are profound enough to affect overall cholesterol balance remains controversial.

Lipoprotein Receptors

As reviewed previously, the concentration of LDL-cholesterol in plasma is determined by production rate and rate of removal of LDL-cholesterol from circulation by receptor-dependent transport. In genetic abnormalities of LDL metabolism, mutations which affect either apolipoprotein B or the LDL-receptor can affect either number or efficacy of the LDL-receptors, resulting in profound alterations in serum cholesterol. It has been postulated that the composition of dietary fat intake may, similarly, affect the number or activity of hepatic LDL-receptors. Fernandez and McNamara, fed diets differing only in their source of dietary fat to guinea pigs, and identified that corn oil containing diets increased the number of LDL-receptors as compared to either olive oil or lard-containing diets(90). Fox et al. reported that diets rich in SFA reduced LDL-receptor mRNA levels in the baboon, whereas PUFA-rich diets increase these levels(91). More recently, Dietschy et al. investigated the effects of specific fatty acids on receptor-dependent transport(92). These researchers discovered that SFA with 14 and 16 carbons (myristic and palmitic acid) suppressed receptor-dependent LDL-cholesterol transport in the liver, increased LDL-cholesterol production rate and thereby raised plasma LDL concentrations. Conversely, linoleic acid restored receptor activity, lowered the production rate and thereby decreased plasma LDL-cholesterol concentrations. These researchers concluded that the ingestion of triacylglycerols without added dietary cholesterol had relatively little effect on LDL-receptor activity or LDL-cholesterol production rates. The addition of cholesterol to a predominately saturated fatty acid diet resulted in a significant loss of receptor activity and an increase in LDL-cholesterol production rate. In contrast, adding cholesterol to a predominately unsaturated fatty acid diet partially restored receptor activity which was lost with the saturated diet. These results lended support to the hypothesis that dietary fat quality affects LDL-receptor activity and that a complex interaction among cholesterol, dietary lipid classes and receptor-mediated lipid transport exists.

Endogenous Cholesterol Synthesis

The ability of dietary fat saturation to enhance endogenous cholesterol synthesis represents the final theoretical mechanism proported to explain the hypercholesterolemic effects of SFA. Cholesterol enters the body pool from only two sources, absorption from the diet (approximately 250-500mg/day in humans), or synthesis within various tissues of the body (700-900mg/day)(44). The rate of cholesterol synthesis varies considerably, both among different tissues and within a given tissue under differing conditions. Despite this large variability, the liver and intestine are the primary organs of cholesterol synthesis(44). The balance of cholesterol across the body is critical to ensure adequate supplies of cholesterol to all major organ systems. Thus, an elaborate series of regulatory and transport mechanisms have evolved to ensure maintenance of cholesterol homeostasis. Clearly, an understanding of cholesterol homeostasis necessitates information on the quantitative importance of various tissues and organ systems involved in cholesterol synthesis and clearance.

A considerable number of studies have been conducted in order to provide information about cholesterol metabolism in normal and hyperlipidemic humans. In 1973, Goodman et al. proposed a three-pool model that describes long-term turnover of serum cholesterol in humans(93). This model provides estimates of the amounts of exchangeable cholesterol in the pools of the body by dividing the body into three compartments. The first compartment (pool 1) consists of cholesterol in rapid equilibrium with plasma cholesterol and includes plasma, red blood cells, liver cholesterol, and much of the cholesterol in the viscera (intestine, pancreas, spleen, kidney and lung). The second compartment (pool 2) consists of that cholesterol which equilibrates at an intermediate rate with plasma cholesterol such as some of the cholesterol contained in the visceral and peripheral tissues. Most of the cholesterol in the peripheral tissues, however, equilibrate slowly with serum cholesterol and comprise pool 3. The addition of a fourth pool has been suggested to represent the nonexchangeable cholesterol (i.e. skin, bone and CNS) but is not widely recognized. The vast majority of cholesterol catabolism and excretion is suggested to occur in pool 1 as there is no independent exit of cholesterol via other body pools. In order to confirm such a theory,

Dell et al. compared cholesterol pool size estimates from kinetic analysis of plasma turnover with total body cholesterol content in baboons(94). Carcass analysis allowed for the calculation of synthesis occurring in pools 2 and 3. These researchers determined that the central pool is responsible for up to 89% of total cholesterol production rate in the baboon. This finding suggests that such a model is a useful and predictive simplification of whole body cholesterol turnover. Because the cholesterol contained in this pool equilibrates rapidly with serum cholesterol, it is reasonable to speculate that any changes in serum cholesterol levels following alterations in dietary intake are likely the result of changes in the synthesis of cholesterol in central pool tissues.

Changes in serum cholesterol levels which accompany alterations in dietary fat content suggest that changes in endogenous cholesterol synthesis may occur. Unfortunately, due to the lack of a suitable biosynthetic precursor, direct in vivo measurements comparing endogenous cholesterol synthesis in humans following consumption of diets differing in fat quality are lacking. Such a relationship, however, has been studied in a number of animal models. Fernandez et al. examined the effect of dietary fat quality on rates of endogenous cholesterol synthesis in guinea pigs by determining incorporation of labeled water (3H) into digitonin-preceptible sterols (DPS)(90). Through measurements of sterol balance, these investigators determined whole-body cholesterol synthesis rates to be unaffected by type of dietary fat fed, but labeled incorporation into DPS revealed that the rate of cholesterol synthesis in animals fed a predominately PUFA diet was significantly lower than that of animals fed either a MUFA or SFA-based diet. In a subsequent study utilizing a pig model, these researchers further determined that hepatic cholesterogenesis is decreased following consumption of diets rich in MUFA. Unfortunately, other investigators have failed to conclusively support these results. Triscari et al. concluded that cholesterol synthesis is not regulated by dietary lipid in rats(95), whereas Mercer et al. identified that hepatic cholesterogenesis decreased in gerbils following consumption of safflower oil when compared to lard(96). Further complicating this relationship was the finding that increased rates of hepatic cholesterol synthesis occurred in hamsters following consumption of safflower oil when compared to either olive oil or coconut oil(97). Such discrepancies in results reinforce

the complex nature of endogenous cholesterol synthesis and lead to speculation regarding significant variation both between and among species.

It is a well established fact that feeding of cholesterol to many animal species, particularly non-human primates, reveals significant variability in plasma cholesterol response(98, 99, 100, 101). Within a given species of primates, some animals are hyperresponders, and develop hypercholesterolemia when dietary cholesterol is increased. Hyporesponders however, show much less of an increment to plasma lipid levels when dietary cholesterol is increased. It has been suggested that these responses are genetically determined although the mechanisms responsible remain unclear(102). In many species of laboratory animals, changes in serum cholesterol are elicited through variations in dietary cholesterol intake whereas contribution of other nutrients (i.e. SFA) seem less important(102).

In humans, high cholesterol diets do not produce the great range in serum cholesterol concentrations observed in non-human primates. Rather, quality of dietary fat fed appears to be a better predictor of serum cholesterol levels. Nevertheless, numerous studies have demonstrated a large patient-to-patient variability both in response to dietary cholesterol as well as to alterations in dietary fat quality(103, 104). Grundy et al. examined the effects of individual variation in response to SFA and PUFA, concluding that plasma cholesterol response to SFA in humans is highly variable but a more consistent predictor of plasma lipid than is dietary cholesterol(103). More recently Glatz et al. fed diets differing only in their cholesterol content to volunteers(104). These researchers concluded that subgroups of both hyperresponders and hyporesponders exist within the larger population with 19% and 0% increases in mean serum cholesterol, respectively. To date, numerous studies in free-living subjects have confirmed that the majority of a population have precise feedback mechanisms which prevents increases in plasma cholesterol levels secondary to dietary changes. McNamara et al. compared a low cholesterol diet (approximately 250mg/day) versus a high cholesterol diet (approximately 800mg/day) during intakes of 35% of calories from a predominately PUFA or SFA diet, in 50 subjects(87). These investigators concluded that the majority of subjects had precise feedback control mechanisms that were able to compensate for a large increase in

dietary cholesterol without affecting plasma cholesterol levels. In those subjects where plasma cholesterol rose significantly (hyperresponders), a lack of sufficient feedback suppression of endogenous cholesterol synthesis appeared to be the cause. Results such as this indicate that the key determinants of an individual's plasma cholesterol are sensitivity to dietary fat quality and the degree of precision of the feedback control response to dietary cholesterol. It appears that, like non-human primates, plasma cholesterol response to dietary factors in humans is at least in-part individually determined. Whether the pattern of this response is genetically determined, however, remains to be elucidated.

In summary, neither changes in fecal sterol excretion or exogenous cholesterol absorption can be definitively linked to increased plasma cholesterol concentrations observed following modifications in dietary fat saturation. Alterations in lipoprotein composition and uptake may be partially responsible for the observed changes in serum levels, but are unlikely to drastically affect overall cholesterol balance. Endogenous cholesterol synthesis, however, does appear to be affected by the quality of the dietary fat fed and may therefore be the key determinant of plasma cholesterol levels in most humans. Although such a relationship has been defined in animal models, data involving humans has not been thoroughly investigated. Furthermore, investigations to date have provided saturated fats from the whole lipid class rather than single fatty acid sources. Because specific fatty acids are known to possess highly variable effects on plasma cholesterol, studies investigating such a relationship between individual fatty acids and endogenous cholesterol synthesis are of particular relevance. If future studies can confirm that endogenous cholesterol response to specific SFA is highly variable, the results could have profound clinical and economic significance.

Measurement of Endogenous Cholesterol Synthesis

Rates of cholesterol synthesis can be assessed by a variety of in vitro and in vivo techniques including measurements of the activity of the rate limiting enzyme in

cholesterol biosynthesis and rates of incorporation of various [14C] substrates into cholesterol. Although both of these techniques provide a reliable measure of the relative rates of cholesterol synthesis in a given organ, neither technique provides data from which absolute rates of cholesterol synthesis can be calculated(105, 106). Techniques which employ [^{14}C] substrates requires that labeled substrates be metabolized to [^{14}C] acetyl CoA. Labeled acetyl CoA pools will, however, be diluted by acetyl CoA entering from the pool of unlabeled substrates. Consequently, the calculated rates of cholesterol synthesis are frequently underestimated.

More recent studies have identified that use of [3H] water for measuring rates of cholesterol synthesis is superior to the [14C] substrate method(107). Since the specific activity of cell water is constant in all tissues in the body, a reliable determination of cholesterol synthesis can be made with this method. Recent advances in isotope ratio mass spectrometry (IRMS) have further improved quantitation of endogenous rates of cholesterol synthesis. This technique allows for the use of small amounts of deuterium in metabolic studies which previously required radioactive substances with undesirable side effects and limitations in their uses(108). Because deuterium is a naturally occurring substances in humans, a safe and accurate measurement of deuterium incorporation from body water into the newly synthesized cholesterol molecule can be made.

Measurement of Cholesterol Synthesis Using Deuterated Water

The deuterium-uptake procedure for measurement of human cholesterol synthesis offers accurate estimations of cholesterogenesis without the limitations inherent in other techniques. The intent of this section is to review theoretical and practical considerations associated with deuterium and discuss some of the assumptions inherent in its use.

Although the use of deuterium as a tracer dates back to the 1930's it was not used to study human lipid metabolism until 1953(109). In the following years, the deuterium-uptake technique was refined, primarily as a result of improved precision in measurement using isotope ratio mass spectrometry(105).

The deuterium-uptake method is based on the same principles as that of tritiated water (^{3}H), in that tracer water equilibrates between the intracellular site of synthesis and

extracellular fluids such as urine or plasma. Extracellular fluids can therefore be used to determine the degree of labeling. This technique relies on three fundamental assumptions(106). Firstly, it is assumed that a constant fraction of deuterium atoms in free cholesterol is synthesized *de novo* originates from plasma water. A second assumption is that cholesterol rapidly exchanges between sites of synthesis and the rest of the central pool (i.e. plasma, liver and intestine). Consequently, the level of deuterium in plasma provides a measure of precursor pool enrichment. The third assumption is that deuterium uptake into plasma free cholesterol represents synthesis in the central pool only, and does not account for any influx of cholesterol from other pools. However, because most sterol is produced in the liver and intestine and because the rate of inter-pool cholesterol exchange is slow, this assumption is justified.

Fractional Uptake Rate of Label

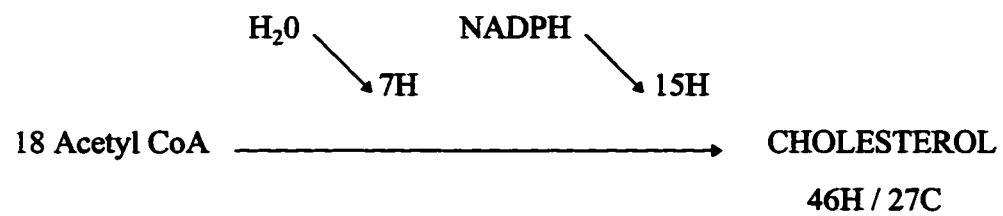
To accurately measure endogenous cholesterol synthesis using deuterium uptake, the ratio of incorporation of deuterium versus hydrogen into cholesterol must be examined. Because not all the precursors along the cholesterol biosynthetic pathway incorporate label at a rate proportional to that of body water, the fraction of each of the 46 protons incorporated per each of the newly synthesized cholesterol molecules must be known(105). Essentially, the hydrogens which become part of the completed cholesterol molecule originates from one of three sources. Seven are obtained directly from body water and therefore reflect the exact tracer concentration. Fifteen originate from reduced NADPH and the remaining 24 from cytosolic acetyl CoA(105). Previous studies have identified that protons (either hydrogen or deuterium) derived from H_20 or NADPH equilibrate with body water(101). However, those derived from acetyl CoA are unlabelled, except in prolonged measurement periods that allow for recycling of acetate into acetyl CoA pools. The ratio of 0.81 labeled protons per carbon atom (or 22 deuterium atoms for every 27 carbon atoms) has become generally accepted to represent the small degree of recycling(111).

Although assumptions concerning enrichment of protons from water and acetate are well established and defined, there is concern regarding those protons derived from

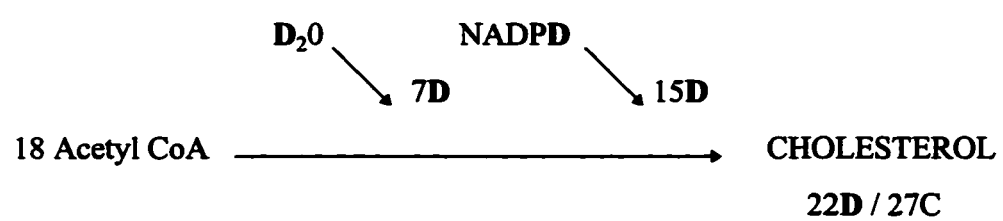
NADPH(105). Of the three sources of protons, enrichment from this source is the most uncertain. Protons used in cholesterol biosynthesis originate from two sources; the pentose phosphate pathway and the malic enzyme system. The former does not exchange protons with cellular water and therefore does not contribute to enrichment during cholesterogenesis. However, the malic enzyme system may exchange NADPH with protons from cellular water(111). The small variation observed in the deuterium/carbon ratio from a variety of both *in vitro* and *in vivo* studies suggests alterations in NADPH equilibrium.

Fig. I-I. **Cholesterol Synthesis:**

Under Normal Conditions:



In the Presence of Deuterium Oxide (D_20):



The origin of hydrogen and deuterium atoms during *de novo* synthesis of cholesterol.

Factors Influencing Endogenous Cholesterol Synthesis

The literature regarding metabolic control of sterol synthesis in experimental animals is well described and characterized. It is accepted that synthesis of cholesterol varies

widely both between and among species, as well as being affected by a number of factors. For example, body composition, feeding state, composition of diet as well as time of day all impact on the rate of cholesterol synthesis(106). Unfortunately, much of this data has been derived from studies incorporating invasive techniques and the use of radiolabeled substrates; thus information regarding the processes that control and impact cholesterogenesis in humans is significantly more limited. Despite this, improvements in available techniques suitable for human studies have advanced the literature that describes the processes and influencing factors that impact on cholesterol flux.

Feeding State

It has been reported in a variety of animal models that fractional synthetic rate (FSR; the fraction of polymer derived from endogenous synthesis during the labeling experiment) of cholesterol is influenced by feeding state. The deuterium uptake methodology was used to determine the FSR of plasma cholesterol in humans during periods of fasting and feeding (106). Five normal subjects consumed a typical North American diet for six days, and then fasted for the seventh day. On the morning of day seven a priming dose of deuterium was consumed and blood samples collected at regular intervals. Using isotope ratio mass spectrometry, enrichment of plasma cholesterol was determined and calculated as the fractional precursor deuterium enrichment over time. The results identified that the FSR for the period following fasting was significantly lower than that following feeding, demonstrating that, as in animal models, control of cholesterol synthesis in humans is sensitive to the absorptive state.

Diurnal Rhythm in Cholesterol Synthesis

A greater incorporation of deuterium into cholesterol at night as compared to the daytime period was first identified using indirect markers (112). Since this time, a diurnal pattern of sterol synthesis has been confirmed by others (107, 113). In one study, five normal subjects consumed three self-selected meals/day over a 48h study period(107). Following a priming dose of deuterium, 4h blood samples were collected and deuterium uptake into cholesterol identified. The results revealed distinct differences

in D_2O uptake over each 24h period, with peak synthesis occurring shortly after 0600 and the lowest levels occurring in late afternoon and early evening. This data suggests that whole body cholesterol synthesis is not constant throughout the day but varies in a predictable manner. Why cholesterol synthesis is greatest at this time is unclear, however it may be in response to a decrease in endogenous cholesterol input. Further, lower FSR values in the afternoon may be reflective of an increased content of unlabeled (dietary) cholesterol. Although data from animal models has been controversial, it has been suggested that incoming dietary cholesterol inhibits cholesterol synthesis. In fact, conversion of HMG-CoA to mevalonic acid has been shown to be a major site of inhibition by cholesterol feeding (114).

Procedural Concerns

Deuterium can be measured in any body water pool, however circulatory cholesterol provides the most attractive source because it is both readily accessible and is part of the rapid turnover pool that comprises hepatic and intestinal sterol. Erythrocytes are frequently used to assess sterol synthetic rate since these cells are unable to synthesize cholesterol and therefore any sterol at this site would be obtained from plasma. However, the rate of equilibration between plasma and erythrocytes is unknown and has been shown to result in underestimations of sterol synthesis(105). Measurements in plasma have been shown to produce more reliable and consistent results(115). Within plasma, deuterium uptake occurs in both the free and ester cholesterol pools, with incorporation into the free compartment exceeding that of the ester by twofold(105). This rapid uptake into free sterol indicates its role as the primary compartment to receive newly synthesized cholesterol, confirming the appropriateness of plasma as an acceptable substance for analysis. Relatively large initial plasma volumes are necessary for cholesterol deuterium enrichment analyses. This is because a minimum of 1 mg free cholesterol is required to produce the 1 μL of combustion water necessary for IRMS analysis. Consequently, a minimum of 2 mL plasma is required per replicate(105).

Modeling Assumptions

The theoretical cholesterol deuterium-enrichment plateau is considered to be about one-half that of the body water enrichment (0.478 x deuterium enrichment of body water)(105). This model assumes enrichment increases to a value at the maximum theoretical plasma cholesterol enrichment, which is represented by plasma water enrichment and can be corrected using the appropriate deuterium/carbon enrichment ratio. Although data has identified a relatively long period of time is required for cholesterol enrichment to attain plateau, the initial 6-24 h interval of D_2O uptake is representative of overall incorporation and is the optimal period for measuring synthesis(105). Periods shorter than 6h will not be accurate as D_2O may not have equilibrated across body-water compartments and periods of time beyond 24h may be influenced by both recycling and mixing of labeled sterol into other pools(105).

Chapter II

EXPERIMENT 1

Effect of High vs Low Palmitic Acid at High vs Low P/S Ratios

Rationale

Today's health professionals have accepted the current notion that saturated fats are detrimental to human health. Palmitic acid is the most abundant saturated fatty acid in the food supply. Despite its ubiquitous appearance in the North American diet, the effects of palmitic acid on plasma lipoprotein profiles have been variable. Consequently, studies designed to determine the precise effects of palmitic acid on lipoprotein levels are of importance. This experiment was conducted to determine the effects of two levels of palmitic acid, high (8% of energy) and low (2% of energy) at high and low polyunsaturated fat to saturated fat ratios (P/S) on lipoprotein profiles and endogenous cholesterol synthesis. If palmitic acid is shown to have no effect on plasma cholesterol levels, than it is difficult to implicate this saturate in the etiology of coronary artery disease.

Hypotheses

It is hypothesized that in healthy subjects the intake of palmitic acid at both high and low levels of intake will not increase plasma total and LDL-cholesterol concentrations, nor will it increase rates of endogenous cholesterol synthesis.

Experimental Methods

Subjects

A total of six males and six females with no history of medical abnormalities were recruited from advertisements posted at the University of Alberta campus. Upon interviewing, volunteers were informed of the purpose of the study, expected responsibility and the dietary regime. An in-depth questionnaire was completed by the

subjects to screen for potential medical problems that could affect lipoprotein levels as well as to characterize their activity level and sleeping patterns. Subjects were non-smokers and were not taking any medications or vitamin supplements during the study period. The protocol was approved by the Ethics Review Committee at the University of Alberta. Subjects gave informed consent prior to the investigation.

Demographic parameters of study participants is shown (Table II-I). Caloric requirements were determined to maintain the subject's current weight using the Mayo Clinic Nomogram, incorporating the subjects height, weight, age and sex(125). An activity coefficient between 1.7 and 2.0 was also incorporated to account for individual variance in activity levels. Subjects were weighed daily before breakfast to verify maintenance of stable body weight.

Table II-I. Demographics of Subjects

Subject	Gender	Age *years*	Height *cm*	Mean Weight *kg*	Energy Intake *MJ/day*
1	M	28	180.0	84.6 ± 0.8	12.6
2	M	31	179.0	77.0 ± 0.2	12.2
3	F	22	154.9	50.0 ± 0.5	8.8
4	M	27	180.0	83.8 ± 0.5	12.6
5	F	35	165.1	63.6 ± 0.3	9.2
6	F	24	181.6	64.3 ± 0.5	10.5
7	M	21	177.8	73.3 ± 0.8	14.3
8	M	22	191.8	82.4 ± 0.5	15.5
9	F	21	167.6	52.9 ± 0.6	9.7
10	F	21	162.6	66.4 ± 1.8	9.7
11	M	22	167.6	77.3 ± 1.5	14.7
12	F	22	154.9	56.6 ± 0.7	8.8
mean ± SD		24.7 ± 4.6	171.9 ± 11.5	69.3 ± 12.2	11.6 ± 2.4

Diets

The study consisted of two diet treatments of 21 days each. Each diet treatment was comprised of a three-day rotational menu partitioned into three isocaloric meals. Diets were formulated based on normal foods to provide low levels of palmitic acid (C16:0) (2% total energy) or high levels of palmitic acid (8% total energy). In the low C16:0 diet the majority of the palmitic acid was replaced with polyunsaturated fat such

that these diets contained a high P/S ratio (2.09) with 6% of calories obtained from PUFA whereas the diet high in palmitic acid contained low levels of PUFA (2% total energy) with a corresponding P/S ratio of 0.29. Both diet treatments contained 30% energy from fat. Equal amounts of fat and C16:0 were incorporated into each of the three meals of each diet treatment to ensure an equal distribution of these nutrients throughout the day. Diets were formulated using USDA handbooks, and Canola producers information packets to contain an average of 30.6 ± 1.6% energy as fat, 55.3 ± 1.8% energy as carbohydrate and 15.4 ± 0.8% energy as protein(126, 127). Diets were analyzed to verify the fatty acid composition actually fed. Dietary cholesterol content ranged from 131 to 385 mg daily, and was independent of C16:0 content. Meals for each diet treatment were provided by the Metabolic Research kitchen for consumption on site (breakfast and lunch) or packaged for take-out (supper). Meals were consumed at regular intervals; 0800-0900h, 1130-1300h, and 1730-1900h for breakfast, lunch and supper respectively depending on the individual subjects schedules. Supplementary foods were not permitted during the study except for clear tea, decaffeinated coffee or other beverages devoid of energy/caffeine.

Protocol

Subjects were randomly assigned to one of the diet treatments and stabilized for 21 days, then placed on the other diet for the same period of time. Prior to the testing days, subjects were instructed to perform the same amount of exercise and eat no later than 1800h on the eve of all test days. Subjects remained on their assigned diet for day 21 of the study period. Between 0715 and 0830h on day 21 of each diet phase, a fasting blood sample was obtained by venipuncture (30 mL) and subjects consumed a priming dose of 0.5g D_2O/kg estimated total body water (99.8 atom percent excess, ICN Biomedicals, Montreal, Canada) prior to breakfast. Total body water was estimated as 60% of body weight (taken as an average over each of the 21 day feeding periods). A 2 litre bottle of water containing 1.0g D_2O/kg estimated total body water was provided for consumption over the next 24 hour period to maintain plasma deuterium enrichment at plateau and to compensate for unlabeled water obtained in the diet. At 24 hours after the

first sample, a second fasting blood sample (30 mL) was drawn into vacutainer tubes containing disodium EDTA and SST tubes. Plasma was obtained by centrifugation at 3000 rpm for 15 min and frozen at -20°C. Serum (5 mL) was sent to the University of Alberta Hospitals for total cholesterol, LDL-C, and HDL-C determination. Total cholesterol content was determined enzymatically(110). HDL was determined following precipitation of other lipoproteins using dextran sulphate magnesium as described(119). LDL cholesterol levels were calculated by subtracting HDL cholesterol from the cholesterol level of 1.006 $g \cdot ml^{-1}$ infranatant fraction.

Fat analysis of Diets

Diets were analyzed for total fat and fatty acid composition to compare with theoretical values. Duplicate meals for each day of the 3 day menu of each diet treatment were prepared for analysis. Meals were homogenized in a Sybron/Brinkman polytron (Model PT/10/35) with the addition of water to a smooth paste. After freezing at -30 °C overnight, meals were freeze dried (Virtis 50-SRC, Gardiner, NY) at -60°C for approximately one week, until free of moisture. Weight of the freeze dried meal was recorded. A 2 g sample of each meal was weighed out in duplicate and transferred to a 150 mL beaker. One mL of tripentadeconoate (99%, Sigma) in chloroform (15.0 mg/mL) was added as an internal standard. Fat was extracted (122) by adding 40 mL chloroform:methanol 2:1 (v/v) and mixing with a magnetic stirrer for 5-10 minutes. Solvent was decanted into a separatory funnel through Whatman #1 filter paper and another 40 mL chloroform:methanol 2:1 (v/v) was added to repeat the extraction step. The beaker was rinsed with another 10 mL chloroform:methanol 2:1 (v/v) and all particles were transferred into the filter paper. After adding 20 mL water to the separatory funnel, the contents were shaken and allowed to separate overnight in a cool room. The bottom layer was collected into a pre-weighed 50 mL culture tube. A 0.2 mL aliquot was taken in duplicate for fatty acid analysis and the remaining sample was dried under nitrogen until achieving a constant weight. This weight allowed for the calculation of total fat in the 2 g meal sample, which was then used to determine total fat per meal.

The 0.2 mL aliquots were dried under nitrogen prior to saponification and transesterification. Saponification (123) was carried out by adding 1.5 mL 0.5N methanolic KOH and heating in a 110°C sandbath for 1 hour. After cooling, 1 mL water was added, samples were vortexed and allowed to separate into two layers overnight. The top layer of hexane was pipeted into a microvial and dried under nitrogen. Fatty acid methyl esters were analyzed by gas liquid chromatography (Vista 6010 GLC and Vista 402 data system; Varian Instruments, Georgetown, Ontario). Fatty acid methyl esters were identified by comparison of retention data with that of authentic standards and quantitated by peak area comparison with internal standards.

Determination of Deuterium Enrichment

Deuterium enrichment was measured in plasma water, plasma cholesterol and plasma cholesteryl ester. To extract the free and esterified cholesterol, 2 mL of plasma at each time point was combined with 4 mL of methanol and heated at 55°C for 15 min. Hexane:chloroform solution (12 mL) was added and shaken mechanically for 10 min. Water (1 mL) was added and the mixture shaken again. Centrifugation at 1500 g for 15 min. was followed by removal of the upper hexane:chloroform phase. This process was repeated, upper phases combined, and solvent removed under nitrogen. Extracts were redissolved in 200 μL chloroform and spotted onto thin-layer silica G plates (Analtech Inc., Newark, DE). G plates were run through a tank of distilled hexane and heat activated for 1 hour before spotting. Solvent tanks were lined with Whatman #1 filter paper and saturated with the solvent system for 1 hour before plates were placed in the tank. Samples were spotted on G plates, developed using petroleum ether-diethyl ether-acetic acid (80:20:1, v/v/v) and air dried. Plates were processed until the solvent system front was approximately 3 cm from the top of the plates (25-30 minutes). Lipid fractions were identified by comparison with a standard (Supelco, Bellefonte, PA), and viewed under UV light. Free and esterified cholesterol bands were scraped from the plates and eluted from the silica scrapings three times using hexane-chloroform-diethyl ether 5:2:1 (v/v/v), and dried under nitrogen. Cholesteryl ester fractions were saponified in 0.5N KOH in methanol for two hours at 110° C in a sand bath and the resultant free cholesterol

was purified by thin layer chromatography as before. The saponification step was repeated once more with the cholesteryl ester band and the free cholesterol pooled with the previous pellet. After two saponifications, no further cholesteryl ester could be converted to free cholesterol. The dried cholesterol sample was transferred to a 8 x 25 mm quartz tube, using three washes of chloroform. Cupric oxide wire (0.5 g) and a 2.5 cm-length 1 mm silver foil was added to the tube. The tube was placed inside a 15 x 9 mm Pyrex tube (Corning Glass Works, Corning, NY) and sealed at one end. Combustion tubes were evacuated to less than 50 mtorr before being sealed with a hot flame. Tubes were placed in an oven at 510° C for four hours to combust the cholesterol to carbon dioxide and water. After cooling in the furnace overnight, the tubes were attached to a vacuum manifold by means of flexible tubing between Cajon fittings (Swagelok Canada Ltd., Niagra Falls, Ontario) and the seal was broken by flexing the tube. The combustion product water was transferred by vacuum distillation into a second Pyrex tube containing 60 mg of zinc reagent.

Samples of day 22 plasma (enriched plasma), intended for plasma water enrichment measurement were diluted twenty-fold with 5% bovine serum albumin solution to lower the deuterium enrichment to within the analytical range of the instrument. Baseline plasma samples were not diluted. Plasma water samples (10 μL) were distilled into Pyrex tubes containing zinc. The water samples from combustion of cholesterol were reduced by zinc to hydrogen gas by placing the reaction tubes in a heating block at 470° C for 30 min(123). The reaction tubes could be attached directly to the mass spectrometer without further purification. The deuterium enrichment was measured by use of a Finnigan MAT 251 isotope ratio mass spectrometer (Bremen, Germany) against hydrogen prepared from a water standard. The mass three abundance was corrected for H_3^+ contribution. Multiple analyses of hydrogen produced from the reduction of a laboratory water standard demonstrated the analytical precision (coefficient of variation) of this instrument at <1%. All samples were analyzed in duplicate.

Cholesterol fractional synthesis rates (FSR) were determined from the initial incorporation rate of deuterium-labeled cholesterol into the rapid exchangeable cholesterol pool, relative to the initial precursor enrichment as determined using the body

water deuterium level(105). Maximum attainable enrichment was calculated as the body water pool enrichment corrected for the fraction of protons in *de novo* synthesized cholesterol that derive from water, relative to non-water sources using the equation:

$$FSR\ (d^{-1}) = \frac{\delta\ \text{cholesterol}\ (‰)}{\delta\ \text{plasma water}\ (‰) \times 0.478}$$

where δ cholesterol is the change in cholesterol deuterium enrichment over the 24-hour measurement interval and δ plasma water is deuterium enrichment achieved at plateau, expressed as parts per thousand (‰), relative to the standard mean ocean water value(105, 106, 107). Isotopic enrichments are expressed in per mil using the δ notation (‰) defined as:

$$\delta\ (‰) = [(R\ \text{sample}/R\ \text{standard}) - 1] \times 1000$$

where R is the ratio of the heavy to light isotope(106, 107). Because not all the various precursors along the cholesterol biosynthetic pathway incorporate label at a level proportional to body water, the constant of 0.478 must be used. This constant represents the number of hydrogen atoms per cholesterol molecule that are predicted to be replaced by deuterium (i.e. the hydrogen to deuterium ratio)(106). From these calculations, fractional synthetic rates are derived which provide an estimate of the amount of cholesterol synthesized de novo over the 24 hour measurement period.

Statistical Analysis

To assess the effect of diet on lipoprotein cholesterol and fractional synthetic rates analysis of variance was used (SAS Inc., Cary NC, USA). Significant differences between diet treatments were determined by a Duncan's multiple range test (Steel and Torrie, 1980). Statistical significance was set at $p<0.05$.

Results And Discussion

Subjects

Subject compliance in consuming all food provided from the metabolic kitchen was high, as evident from self reports of subjects as well as observation in the dining facilities. Positive feedback from subjects regarding food quality was received on a daily basis. Weight changes were minimal throughout the study period; not exceeding 1.3% of total body weight during each diet treatment. Slight fluctuations in activity levels in two subjects was accounted for by adjusting the energy intake to ensure maintenance of stable body weight.

Lipid Analysis of Diets

Lipid composition of the diets consumed is shown (Table II-II). There were no significant differences between diets in regard to total fat intake (32.2 ± 2.2% and 31.9 ± 1.9% for the low and high C16:0 diets respectively). The analyzed C16:0 content of the diet varied little from the formulated values; containing 2.1 ± 0.1% and 8.1 ± 1.0% energy on the low and high diets respectively. The linoleic acid (C18:2) content of the diets was 6.0 ± 1.0% on the low C16:0 diet and 2.0 ± 0.5% on the high C16:0 diet.

Table II-II. Fatty Acid Composition of Diets

Fatty Acid *% energy*	Total Fat	C16:0	C18:0	C18:1	C18:2n6	C18:3n3	C20:5n3
Low C16:0	32.2 ± 2.2	2.1 ± 0.1	1.1 ± 0.1	14.4 ± 1.2	6.0 ± 1.0	1.69 ± 0.15	0.03 ± 0.0
High C16:0	31.9 ± 1.9	8.1 ± 1.0	1.8 ± 0.2	7.5 ± 1.7	2.0 ± 0.5	0.21 ± 0.06	0.00 ± 0.0

Values represent means ± SD of percent energy based on two replicates for each day of the menu cycle for each diet treatment.

Lipoprotein Cholesterol Determination

Plasma lipid level response to diet treatment are reported in Table II-III and shown in Fig. II-I.

Table II-III. Lipoprotein Levels

Diet	total cholesterol *mmol/L*	LDL-cholesterol *mmol/L*	HDL-cholesterol *mmol/L*
Low C16:0	3.55 ± 0.10^{b}	1.95 ± 0.09^{b}	1.19 ± 0.02^{b}
High C16:0	4.28 ± 0.10^{a}	2.54 ± 0.09^{a}	1.30 ± 0.02^{a}

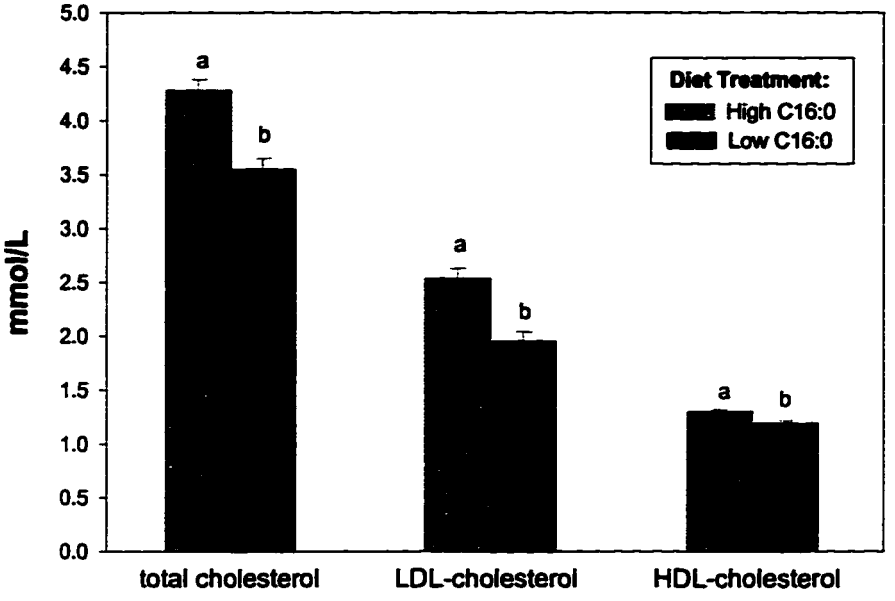
Values are adjusted means ± pooled SEM (n=12). Within lipoprotein subfractions, values that do not share a common letter are significantly different ($p<0.05$)

Total Cholesterol. Normal levels of cholesterol for persons within the age range of the subjects studied is 3.20 - 4.60 mmol/L. Comparison of the subjects response to diets low in C16:0 to those high in C16:0 resulted in a total cholesterol increase from 3.55 to 4.28 mmol/L respectively ($p<0.001$). Although this change in the diet raised total cholesterol levels significantly, the values remained within the normal range for this study group.

LDL-Cholesterol. The normal range for LDL-cholesterol is between 1.70 and 3.00 mmol/L. Similar to the total cholesterol response, the diet high in C16:0 resulted in a LDL-cholesterol level of 2.54 mmol/L as compared to the low C16:0 diet which resulted in a significantly lower LDL-cholesterol response of 1.95 mmol/L($p< 0.001$). Despite this rise in LDL-cholesterol concentrations, values remained within the normal range.

HDL-Cholesterol. The normal HDL range is between 0.90 and 1.60 mmol/L. Diets high in C16:0 resulted in increases in the HDL pool as compared to diets low in C16:0 (1.30 and 1.19 mmol/L respectively) ($p<0.01$). HDL-cholesterol values remained within the normal range throughout the study period.

Fig. II-I. Lipoprotein Cholesterol Levels



Values are adjusted means ± pooled SEM (n=12). Within each lipoprotein subfraction, values that do not share a common letter are significantly different ($p<0.05$).

Endogenous Cholesterol Synthesis

The deuterium-uptake method determines the short-term cholesterol synthesis in humans by measuring the rate of D_20 uptake from the body water pool into the newly synthesized cholesterol molecule (test day) relative to the initial precursor enrichment (background day). From this, calculations of fractional synthetic rate (FSR) can be determined. FSR is defined as the proportion of central-pool free sterol derived from synthesis and calculated according to the equation outlined previously in 'Experimental Methods' (refer to page 38).

Fractional Synthetic Rate. FSR response to diet treatment is reported in Table II-IV and shown in Fig II-II. The mean FSR for the subjects consuming the low and high C16:0 diets did not significantly differ from one another (0.0329 and 0.0301 respectively); suggesting no effect of palmitic acid on the endogenous synthesis of

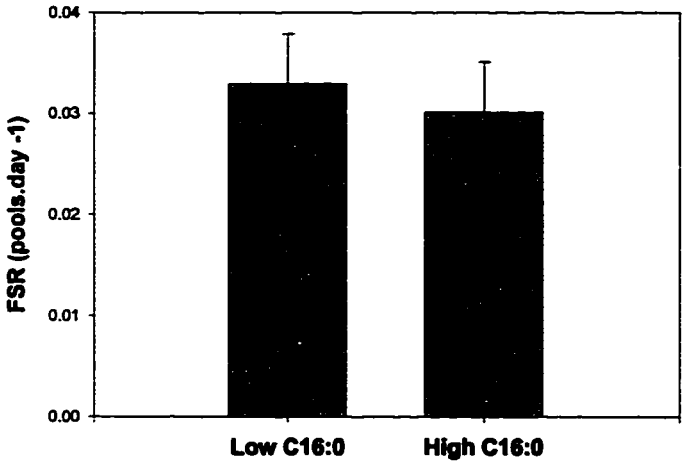
cholesterol. When fractional synthetic rates for each subject is considered (refer to Figure II-III) it is apparent that no consistent relationship exists between the level of palmitic acid and rates of fractional synthesis of cholesterol.

Table II-IV. Fractional Synthetic Rate

Diet treatment	FSR
Low C16:0	0.0329 ± 0.005
High C16:0	0.0301 ± 0.005

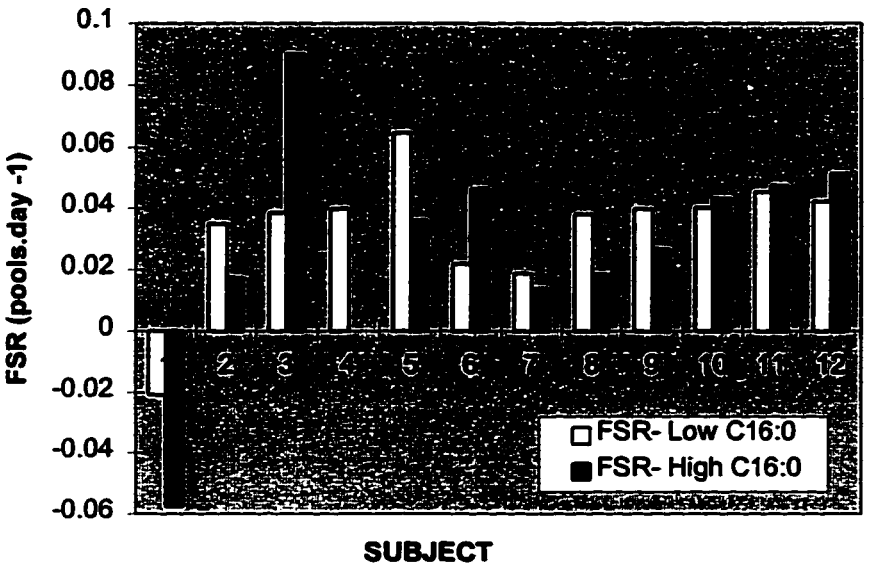
Values represent adjusted means ± pooled SEM (n=12).
Values non-significant at $p<0.05$.

Fig. II-II. Fractional Synthetic Rates by Diet Treatment



Values represent means ± pooled SEM (n=12). Values non-significant at $p<0.05$. FSR represents that fraction of cholesterol synthesized *de novo* over the 24 hour testing period.

Fig. II-III. Fractional Synthetic Rates by Subject



Discussion

In North America, palmitic acid is the predominant saturated fatty acid in the diet, contributing approximately 7-8% of the total energy intake. In this experiment, at this level of palmitic acid versus C18:2, the diet treatment exerted a significant cholesterol-raising effect. Although this was evident in each of the lipoprotein fractions, the predominant effects of palmitic acid was manifested by increases in the total and LDL-cholesterol pool. Interestingly, this increase in total cholesterol was not accompanied by a significant increase in the rate of endogenous cholesterol synthesis. This data suggests that an alternate mechanism may be responsible for the levels observed. It may be that the diet high in palmitic acid and low in PUFA resulted in alterations in fecal sterol excretion or cholesterol absorption. Although not tested in this study design, it could be hypothesized that prolonged study periods utilizing the same concentrations of palmitic acid to C18:2 could result in the attainment of a steady state, thereby blunting the observed increases in plasma cholesterol levels which occurred over the three week feeding period.

The results from this experiment identify a significant hypercholesterolemic effect when the level of palmitic acid is increased in the diet. However, concomitant to this increase was a significant reduction in the linoleic acid content of the diets. Thus, the hypercholesterolemic effect of the higher level of palmitic acid may be in response to reduction in the linoleic acid content of the diet. Further studies which provide both levels of palmitic acid at both a high and low level of linoleic acid are therefore essential to elucidate the precise effect of palmitic acid on the various lipoprotein subfractions and on rates of endogenous cholesterol synthesis. Because palmitic acid was replaced in the low C16:0 diet primarily by increases in the polyunsaturated fatty acid content of the diet, the polyunsaturated to saturated fat ratio (P/S) was reduced from 2.09 for the low C16:0 diet to 0.29 for the high C16:0 diet. Current dietary recommendations stress the importance of maintaining a P:S ratio of at least 1:1, thus, the effects of palmitic acid in relation to an acceptable intake of PUFA also warrants investigation.

Chapter III

Experiment 2

RESEARCH PLAN

Rationale

Diets containing a high proportion of saturated fatty acids elevate plasma concentrations of total and low density lipoprotein cholesterol. Because an increased LDL-cholesterol concentration represents an increased risk for coronary heart disease, recommendations have been made to reduce intake of dietary SFA and increase consumption of PUFA and MUFA. However, increased intake of PUFA have been associated with reductions in the HDL pool, and specific SFA have been shown to raise HDL levels as they expand the total cholesterol pool(116). The possibility that the intake of SFA could be suboptimal has been suggested(117). Consequently, a more appropriate balance of dietary fatty acids, incorporating specific SFA, may lower LDL levels while preventing concomitant reductions in the HDL pool.

Current literature generally agrees that saturated fatty acids are not equivalent in their cholesterol-raising potential. Historically, palmitic acid has been included as one of the cholesterol-raising SFA. However, more recent research has suggested that palmitic acid may exert a more neutral effect on plasma lipoprotein levels with minimal increases in plasma LDL-cholesterol concentrations and increases in HDL-cholesterol concentrations. Consequently, evidence to clarify the effects of palmitic acid on plasma lipoprotein levels is necessary.

If palmitic acid is hypercholesterolemic, then it must increase rates of endogenous cholesterol synthesis. To date, the effect of palmitic acid on endogenous cholesterol synthesis in humans has not been assessed. In fact, this study represents the first direct measurement in humans comparing endogenous cholesterol synthesis following consumption of diets differing in fat quality. The use of deuterium in combination with advances in isotope ratio mass spectrometry enables such measurements to be accomplished.

Palmitic acid is the most abundant saturated fat in the food supply, occurring naturally in significant quantities in both meat and dairy products. As a result, much of the palmitic acid is removed from these products in an effort to enhance their nutritional characteristics. However, if palmitic acid can be shown to have no effect on lipoprotein profiles or endogenous cholesterol synthesis, than the rationale for limiting the consumption of this fatty acid is questionable. Another rich source of palmitic acid is palm oil. Similarly, if palmitic acid can be shown to have no effect on lipoprotein levels and endogenous cholesterol synthesis, than the favorable functional characteristics of palm oil (i.e. high solid-glyceride content and resistance to oxidation) can be exploited without expecting adverse effects on serum lipoprotein levels.

Hypotheses

It is hypothesized that in healthy male subjects:

-increasing levels of C18:2 (from 2% to 10% energy) in the diet will result in a decrease in total and LDL-cholesterol levels

-at 10% energy from C18:2, an increase in palmitic acid will not increase total and LDL-cholesterol levels

-at low levels of C18:2 (2% energy) increasing the content of palmitic acid will increase LDL-cholesterol levels

-increasing C16:0 from 3% to 10% energy in the diet will increase HDL-cholesterol levels

-the intake of C16:0 at both high (10% energy) and low (3% energy) levels will not affect rates of endogenous cholesterol synthesis

Chapter IV

EXPERIMENTAL METHODS

Subjects

A total of six healthy, male volunteers aged 24.0 ± 4.7 years (range 20 - 32 years) were recruited by advertisements at the University of Alberta, Canada. Subjects were 182.4 ± 4.4 cm (mean ± SD) with a body mass index (BMI) of 23.4 ± 3.5 (Table IV-I). Energy intakes of the subjects averaged 14.7 ± 0.9 MJ/d (3500 ± 210 kcal/d). Subjects were screened by questionnaire for chronic disease, sleeping habits and exercise schedules. Subjects reported no history of significant medical or metabolic diseases, were non-smokers, were taking no medications or vitamin supplements and denied having a family history of diabetes or coronary artery disease. The protocol was approved by the Ethics Review Committee at the University of Alberta. Subjects gave informed consent prior to the investigation.

Table IV-I: Demographic Parameters of Subjects

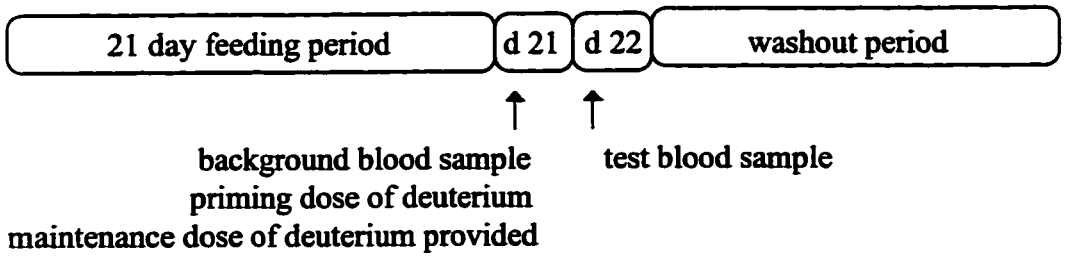
Subject	Age *years*	Height *cm*	Intake *MJ/day*	Mean Weight *kg*	BMI
1	20	175.3	14.3	66.7	21.7
2	32	180	13.4	68.0	21.0
3	26	185.4	14.3	69.2	20.1
4	21	182.9	15.1	74.3	22.2
5	25	182.9	16.0	112.9	33.8
6	20	188	15.1	75.6	21.4
mean ± SD	**24.0 ± 4.7**	**182.4 ± 4.4**	**14.7 ± 0.9**	**77.8 ± 17.6**	**23.4 ± 3.5**

Protocol and Experimental Design

The study consisted of four diet treatments of 21 days each, followed by washout periods of at least 21 days. Four of the six subjects underwent each of the four, three-week diet treatments and the remaining two subjects completed only three of the four diet treatments. Each diet treatment was comprised of a three-day rotational menu partitioned into three isocaloric meals. Diets were formulated based on normal foods to provide the following high (approximately 10-12% of MJ) or low (approximately 3% of MJ) combinations of C16:0 (palmitic acid) and C18:2 (linoleic acid): low C16:0, low C18:2; low C16:0, high C18:2; high C16:0, low C18:2 and high C16:0, high C18:2. Meals for each diet were provided by the metabolic research kitchen for consumption on site (breakfast and lunch) or packaged for take-out (supper). Meals were consumed at regular intervals; 0800-0900h, 1130-1300h, and 1730-1900h for breakfast, lunch and supper respectively depending on the individual subjects schedule. Supplementary foods were not permitted during the study except for clear tea, decaffeinated coffee or other beverages devoid of energy/caffeine. Energy intake of each subject was tailored to individual requirements based on the Harris-Benedict equation and incorporating an activity coefficient between 1.7 and 2.0 depending on the individual degree of activity. Subjects were weighed daily before breakfast to verify maintenance of stable body weight. Diets were formulated based on the Food Processor II nutrient analysis computer software program and fatty acid content from published nutrient composition tables to contain an average of 28.9 ± 1.6% energy as fat (range of 27.0-31.9% of total MJ), 16.3 ± 0.5% energy as protein (range of 15.7-16.9% of total MJ) and 56.2 ± 1.9% energy as carbohydrate (range of 53.5-57.8% of total MJ) (Table V-II). Diets were analyzed to verify the fatty acid composition actually fed. Each diet treatment was balanced for omega-3 fatty acids, cholesterol and fibre content. Between 0715 and 0830h on day 21 of each diet phase, a fasting blood sample was obtained by venipuncture (30 mL) into vacutainer tubes containing disodium EDTA (25 mL blood) and serum separator tubes (SST) (5 mL blood). Subjects then consumed a priming dose of 0.5g D_2O/kg estimated total body water (99.8 atom percent excess, ICN Biomedicals, Montreal, Canada) prior to

breakfast. Total body water was estimated as 60% of body weight (taken as the average over each of the 21 day feeding periods). A 2 litre bottle of water containing 1.0g D_2O/kg estimated total body water was provided for consumption over the next 24 hour period to maintain plasma deuterium enrichment at plateau and to compensate for unlabeled water obtained from the diet. At 24 hours after the first sample, a second fasting blood sample (25 mL) was obtained. Plasma was obtained by centrifugation at 3000 rpm and frozen at -20 °C. Serum lipoprotein cholesterol levels were obtained as described in full elsewhere (refer to 'Experimental Methods'; page 35). Serum creatinine levels were determined at the University of Alberta Hospitals using the kinetic Jaffe reaction(120, 121).

Fig. IV-I: Experimental Design of Study



Fat Analysis of Diets

Methodology for the fat analysis of diets is described in full elsewhere (refer to 'Experimental Methods'; page 36). In short, duplicate preparations of each complete meal cycle for each diet phase were homogenized in a polytron, aliquoted and stored at -30°C until analyzed for total fat and fatty acid content. Fat extraction (122) was carried out prior to saponification and transesterifiaction with KOH and boron triflouride methanol reagent (123). Fatty acid methyl esters were analyzed by gas liquid chromatography (Vista 6010 GLC and Vista 402 data system; Varian Instruments, Georgetown, Ontario) as described (123). Fatty acid methyl esters were identified by comparison of retention data with that of authentic standards and quantitated by peak area comparison with internal standards.

Determination of Deuterium Enrichment

Deuterium enrichment was measured in plasma water, plasma cholesterol and plasma cholesteryl ester as described in full elsewhere (refer to 'Experimental Methods'; page 37).

Statistical Analysis

To assess the effect of diet on lipoprotein cholesterol and fractional synthetic rates a two-way analysis of variance was used (SAS Inc., Cary NC, USA.). Significant difference between palmitic and linoleic acid was determined by a Duncan's multiple range test (Steel and Torrie, 1980). Significant differences between interaction lsmeans were determined by t-tests. In the statistical analysis slight differences in the total fat content of each diet treatment was accounted for by adjusting the lipoprotein values by the ratio of dietary fat to average dietary fat intake. Two subjects did not complete the entire protocol, thus missing data was accounted for by averaging the previous values of those subjects and removing two degrees of freedom from the calculation of f-values. To test for differences in serum creatinine levels between background and test day a one-way analysis of variance was used. Statistical significance was set at $p<0.05$.

Chapter V

RESULTS

Subjects

By observation of subjects in the metabolic unit, self reports by subjects and the lack of meals returned unfinished indicated that the level of subject compliance in completing meals was high. Body weight fluctuations by each subject over each 21 day feeding period was negligible (ranging from ± 0.2 to 0.6 kg). Body weight for individuals over the entire study period varied somewhat. The group mean weight change was small (+2.29%) with the majority of weight change occurring during the washout periods (Table V-I). Mean creatinine levels were not significantly different between background and test days for each subject on each diet treatment providing a measure of similar hydration status for each test period (i.e. no dilution effect).

Table V-I: Weight Changes of Subjects

Subject	Washout 1 %	Washout 2 %	Washout 3 %	overall change %
1	+2.49	+1.90	+0.83	+5.2
2	+4.12	+0.31	+0.54	+5.0
3	+0.72	+1.64	-0.82	+1.5
4	-0.82	+0.91	-	+0.1
5	+1.87	+0.75	-	+2.6
6	-0.54	-0.11	-0.21	-0.65
net change	**+1.31**	**+0.90**	**+0.09**	**+2.29**

Values are expressed as % of the subjects weight over each of the washout periods.

Diets

Composition of the diets consumed is shown (Table V-II). The contribution of energy from protein, carbohydrate and fat was within 3-4% of energy for each of the macronutrients between diet treatments. There were no significant differences between diet treatments in regard to dietary fibre (29.9 ± 2.8g), omega-3 fatty acid (1.54 ± 0.16mg) and cholesterol (183.4 ± 26.6 mg) content. The arachidonic acid,

eicosapentaenoic acid and docosahexaenoic acid was negligible in each of the diet treatments (0.016 ± 0.006%, 0.022 ± 0.012%, and 0.030 ± 0.011% of fatty acids respectively). In diets in which the C16:0 and C18:2 content did not provide most of the required dietary fat, the remainder of the fat was provided by monounsaturated fatty acids. The analyzed fat content of meals in each diet treatment was close to the formulated values, and was consistent within each diet treatment.

Table V-II: Nutrient Composition of Diets [1]

NUTRIENT	LOW C16:0 LOW C18:2	LOW C16:0 HIGH C18:2	HIGH C16:0 LOW C18:2	HIGH C16:0 HIGH C18:2
energy (MJ)	12.9 ± 0.7	12.6 ± 1.1	12.7 ± 0.1	12.6 ± 1.0
protein (%MJ)	15.7 ± 3.1	16.9 ± 2.3	16.3 ± 0.4	16.1 ± 2.1
carbohydrate (%MJ)	56.5 ± 4.8	57.0 ± 3.3	57.8 ± 0.8	53.5 ± 2.6
total fat (%MJ)	29.2 ± 1.7 (29.3 ± 0.9)	27.6 ± 1.1 (27.0 ± 0.9)	27.8 ± 0.1 (28.0 ± 0.9)	31.1 ± 0.5 (31.9 ± 0.9)
saturated fat (%MJ)	4.8 ± 0.7 (4.3 ± 0.1)	4.7 ± 0.3 (4.0 ± 0.1)	13.9 ± 0.6 (9.8 ± 0.1)	10.2 ± 0.7 (9.8 ± 0.1)
MUFA (%MJ)	18.8 ± 0.9 (15.1 ± 0.5)	7.5 ± 0.1 (6.4 ± 0.5)	9.0 ± 0.2 (7.2 ± 0.5)	8.1 ± 0.3 (8.0 ± 0.5)
C18:2 n-6 (%MJ)	2.8 ± 0.6 (3.3 ± 0.2)	12.0 ± 1.4 (9.8 ± 0.2)	2.0 ± 0.2 (1.6 ± 0.2)	12.1 ± 0.6 (10.0 ± 0.2)
C16:0 (%MJ)	3.2 ± 0.2 (3.6 ± 0.1)	2.9 ± 0.2 (2.2 ± 0.1)	10.1 ± 0.4 (7.7 ± 0.1)	9.9 ± 0.2 (8.7 ± 0.1)
n-3 FA (%MJ)	0.5 ± 0.1 (0.2 ± 0.0)	0.5 ± 0.1 (0.3 ± 0.0)	0.5 ± 0.1 (0.2 ± 0.0)	0.40 ± 0.0 (0.1 ± 0.0)
cholesterol (mg)	200.0 ± 20.8	176.7 ± 77.5	208.0 ± 57.4	148.8 ± 22.5
dietary fibre (g)	27.1 ± 6.0	32.7 ± 8.5	28.0 ± 5.1	31.9 ± 5.3

[1] Values represent means ± SD; n=3 for each diet treatment. Diet averages are based on the average nutrients calculated from each menu cycle for a given diet. All values are derived from Food Processor II data except for C16:0 and C18:2 n-6 which are derived from published food composition tables. Values in brackets indicate values obtained by laboratory analysis of meals fed.

Lipoprotein Cholesterol Determination

Plasma lipid level response to diet treatment is reported in Table V-III and shown in Figure V-I. To account for slight differences in the total fat content of each of the diet treatments, lipoprotein values were also adjusted by the ratio of dietary fat to average dietary fat intake (Fig. V-I a, b and c.)

Table V-III: Lipoprotein Levels Normalized for Total Fat Intake[1]

	total-cholesterol	LDL-cholesterol	HDL-cholesterol
Main effects			
Low C16:0	$3.30 \pm 0.06^{*}$	$1.86 \pm 0.04^{**}$	$1.15 \pm 0.02^{***}$
High C16:0	3.61 ± 0.06	2.18 ± 0.04	1.16 ± 0.02
Low C18:2	$3.59 \pm 0.06^{\Psi}$	$2.12 \pm 0.04^{\Psi\Psi}$	$1.17 \pm 0.02^{\Psi\Psi\Psi}$
High C18:2	3.31 ± 0.06	1.92 ± 0.04	1.14 ± 0.02
Interaction effects[2]			
Low C16:0 x Low C18:2	3.17 ± 0.08^{b}	1.76 ± 0.06^{b}	1.09 ± 0.02^{b}
Low C16:0 x High C18:2	3.42 ± 0.08^{b}	1.96 ± 0.06^{b}	1.21 ± 0.02^{a}
High C16:0 x Low C18:2	4.02 ± 0.08^{a}	2.48 ± 0.06^{a}	1.24 ± 0.02^{a}
High C16:0 x High C18:2	3.20 ± 0.08^{b}	1.88 ± 0.06^{b}	1.08 ± 0.02^{b}

[1] values are adjusted means ± pooled SEM
[2] values that do not share a common letter are significantly different (NS= p>0.05)
* total cholesterol significantly different for low C16:0 vs. high C16:0, p<0.004
$^{\Psi}$ total cholesterol significantly different for low C18:2 vs. high C18:2, p<0.01
** LDL-cholesterol significantly different for low C16:0 vs. high C16:0, p<0.0001
$^{\Psi\Psi}$LDL-cholesterol significantly different for low C18:2 vs. high C18:2, p<0.01
*** HDL-cholesterol non-significant for low C16:0 vs. high C16:0
$^{\Psi\Psi\Psi}$HDL-cholesterol non-significant for low C18:2 vs. high C18;2

Total cholesterol. Normal levels of cholesterol for male subjects within the age range studied are 3.20 - 4.60 mmol/L. During the entire study period, normalized total cholesterol values were between 3.17 and 4.02 mmol/L. Feeding high C16:0 increased levels of total cholesterol from 3.30 to 3.61 mmol/L (p<0.0001) when the diet was low in C18:2. When the diet was high in C18:2 raising the level of C16:0 did not have a significant effect on total plasma cholesterol level. The effect of dietary C18:2 was also

significant, with diets high in C18:2 producing a lower total cholesterol response (3.31 mmol/L) than diets low in C18:2 (3.59 mmol/L) ($p<0.01$).

LDL cholesterol. The normal range of LDL-cholesterol is between 1.70 and 3.00 mmol/L. Throughout the study period normalized LDL-cholesterol values ranged from 1.76 to 2.48 mmol/L; within the low range of normal. Plasma LDL-cholesterol level was higher ($p<0.0001$) when subjects consumed the higher level of C16:0 (2.18 mmol/L) at the low C18:2 intake level. LDL-cholesterol levels decreased ($p<0.01$) at the high levels of C18:2 consumption (1.92 mmol/L) as compared to the lower levels of C18:2 (2.12 mmol/L). Similar to the total cholesterol response, when the diet was high in C18:2 raising the level of C16:0 did not have a significant effect on total plasma LDL-cholesterol levels.

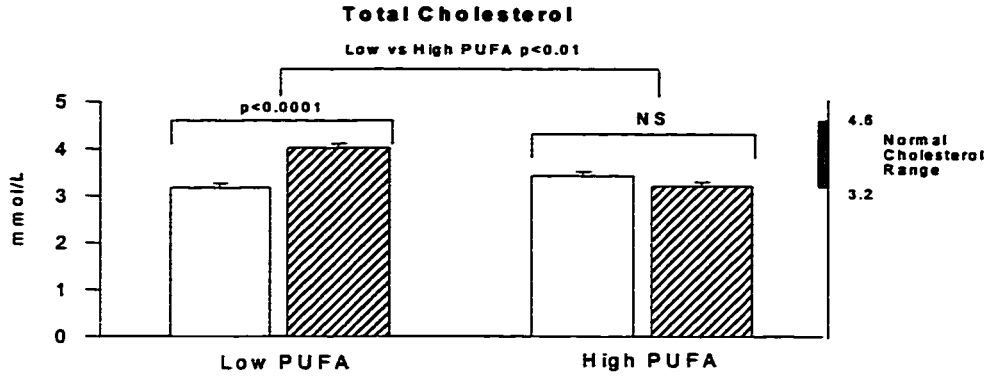
HDL cholesterol. The normal levels of HDL-cholesterol is between 0.90 and 1.60 mmol/L. Throughout the study period, normalized HDL-cholesterol values varied only minimally, ranging from 1.08 to 1.24 mmol/L. Although no significant main effects of diet on HDL-cholesterol levels were observed, at low levels of C18:2, high C16:0 raised HDL-cholesterol levels (1.09 mmol/L vs. 1.24 mmol/L) ($p<0.001$). Conversely, at high levels of C18:2, high C16:0 lead to significant reductions in HDL-cholesterol levels (1.21 mmol/L vs. 1.08 mmol/L) ($p<0.003$).

For each of the diet treatments, the mean total cholesterol, LDL-cholesterol and HDL-cholesterol for subjects was maintained within normal ranges. The highest total and LDL-cholesterol response occurred following consumption of the high C16:0, low C18:2 combination, and the lowest following the low C16:0, low C18:2 combination. However, the diet combination of high C16:0 with high C18:2 resulted in a total cholesterol response very similar to that of the low C16:0; low C18:2 diet (3.20 mmol/L vs. 3.17 mmol/L). This data suggests that in the presence of adequate C18:2, palmitic acid has no effect on lipoprotein levels. This interesting effect of palmitic acid on LDL-cholesterol levels at both low and high levels of C18:2 is apparent when the individual subjects response to diet is considered (refer to Figure V-III and V-IV). In figure V-III it is apparent that moving from a low to high level of C18:2 intake is responsible for slight reductions in LDL-cholesterol levels in the majority of subjects. In contrast, the move

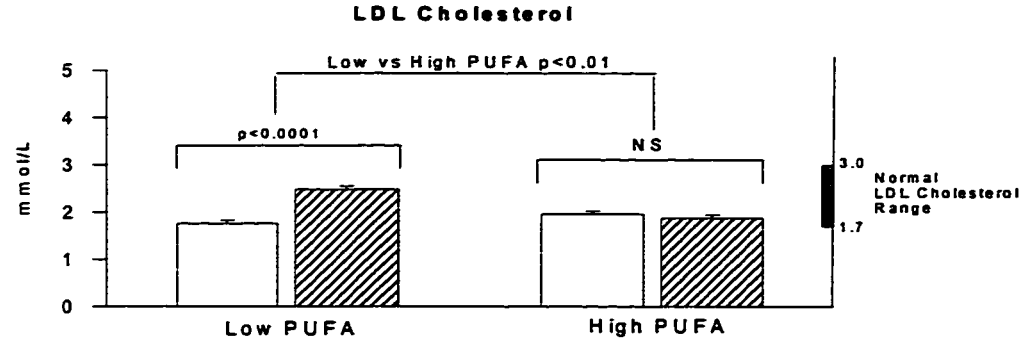
from low to high palmitic acid at low levels of C18:2 intake results in increases in LDL-cholesterol levels for all subjects. Similarly, Figure V-IV also depicts the individual LDL response to low and high C16:0 at both levels of C18:2 intake. Slight decrease in the LDL-cholesterol is apparent at the high C18:2 levels whereas significant increases in LDL levels is evident at the lower C18:2 intake. This data clearly demonstrates a protective role of C18:2 in preventing increases in the LDL-cholesterol subfraction.

Fig. V-I: Plasma Lipoprotein Cholesterol Values

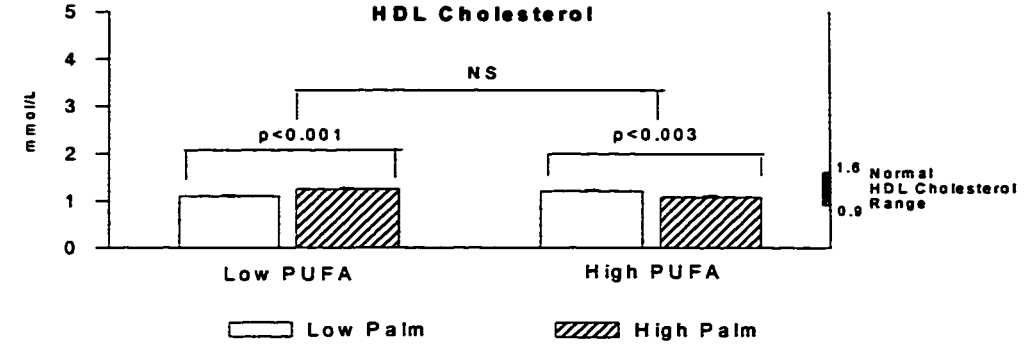
a)



b)



c)



Total cholesterol, LDL-cholesterol and HDL-cholesterol for subjects. Values represent mean ± SEM for all subjects. NS= non-significant ($p>0.05$).

Fig. V-II: Individual Responses in LDL-cholesterol Levels

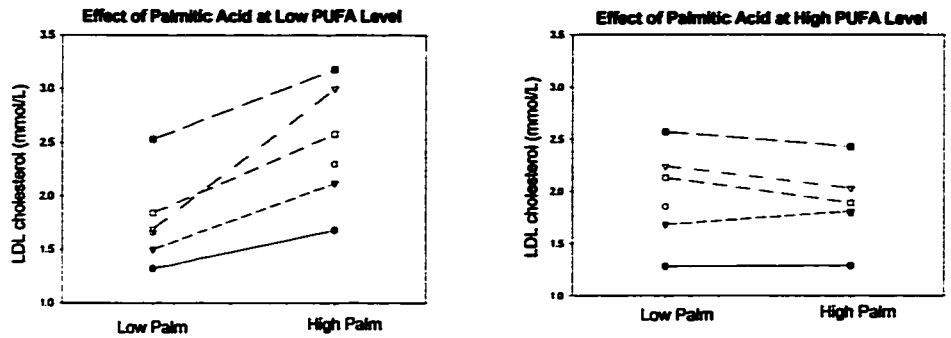
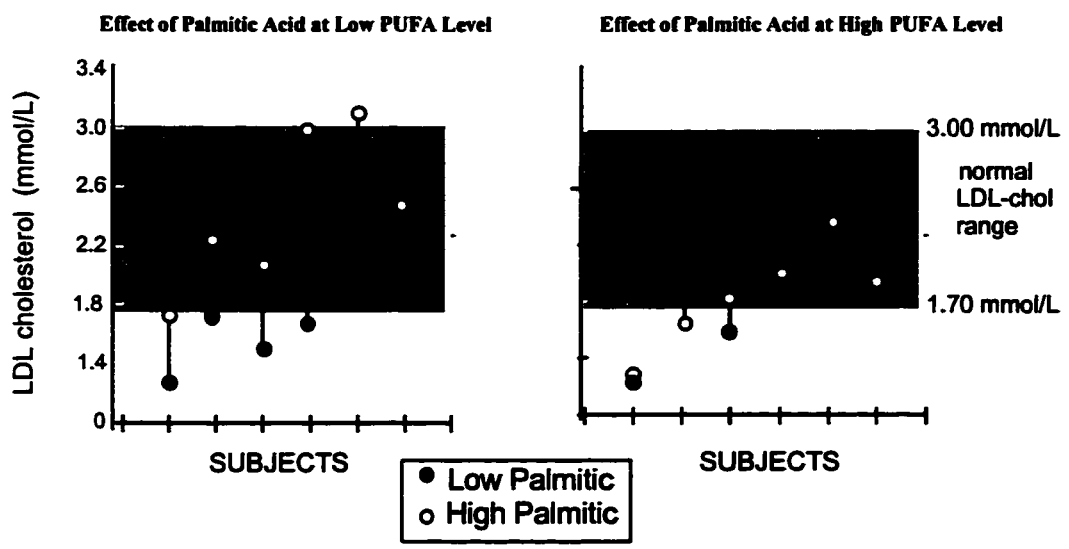


Fig. V-III: Individual Responses in LDL-cholesterol



Fractional Synthetic Rate

Effect of diet on cholesterol FSR values is reported in Table V-IV and shown in Fig. V-IV and Fig. V-V. Values for FSR were not significantly affected by either the C16:0 or C18:2 content of the diet. This data suggests that palmitic acid does not influence endogenous cholesterol synthesis for subjects who have cholesterol values within the normal range.

Table V-IV. Fractional Synthetic Rates

Diet treatment	Fractional Synthetic Rate
Low C16:0, Low C18:2 (n=6)	0.0365 ± 0.008
Low C16:0, High C18:2 (n=6)	0.0448 ± 0.008
High C16:0, Low C18:2 (n=6)	0.0338 ± 0.008
High C16:0, High C18:2 (n=4)	0.0569 ± 0.01

FSR values are means ± pooled SEM

Figure V-IV: Fractional Synthetic Rates

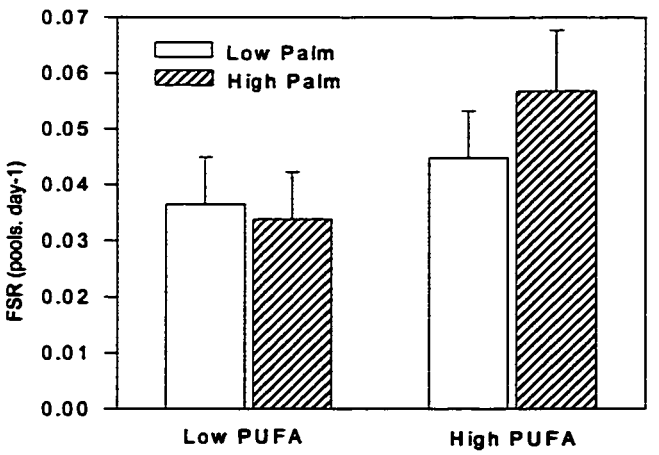
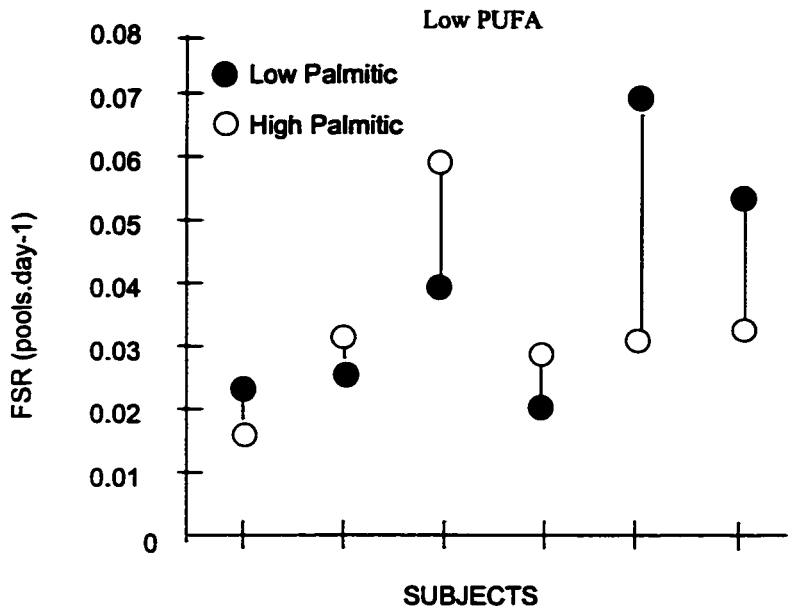


Fig V-II: FSR for total plasma cholesterol in subjects consuming the following diets: Low C16:0, Low C18:2 (n=6); Low C16:0, High C18:2 (n=6); High C16:0, Low C18:2 (n=6);High C16:0, High C18:2 (n=4). Diet fat effect non-significant at $p<0.05$. Values are means ± pooled SEM.

Fig V-V: Individual Responses in Fractional Synthetic Rates



Chapter VI

DISCUSSION

Despite numerous reports of the effects of dietary fatty acid composition on circulating lipids, the precise effects of a particular fatty acid on lipoprotein levels and endogenous cholesterol synthesis have not been defined. Palmitic acid was chosen primarily because of its ubiquitous appearance in the North American diet, as well as its controversial metabolic effects. The effect of palmitic acid was investigated in relation to linoleic acid, such that the effects of this saturated fat could be evaluated in regard to current dietary recommendations. The diets utilized are of particular relevance because those designated as high in palmitic acid contained palmitate in concentrations similar to present consumption patterns of North America and those designated high in linoleic acid contained the amount of C18:2 presently recommended.

The results from this study support the hypothesis that increasing levels of C18:2 in the diet will result in a decrease in both the total and LDL-cholesterol levels. This reduction in these lipoprotein subfractions was maintained and even enhanced in the majority of subjects as the level of palmitic acid was increased from 3% to 10% of total energy. Conversely, at low levels of dietary C18:2 (2% energy) increasing the content of palmitic acid resulted in significant increases in both total and LDL-cholesterol. This data lends support to previous research suggesting that palmitic acid is "conditionally" hypercholesterolemic(116, 128).

Many previous studies have shown that palmitic acid raises serum total and LDL-cholesterol concentrations when compared to MUFA and PUFA(69, 129). However, it has been suggested that palmitic acid may be neutral in healthy, normocholesterolemic subjects if the diet is moderate in fat, contains little cholesterol and the linoleic acid intake is adequate(69). Under these conditions, palmitic acid is expected to exert an effect on the lipoprotein subfractions similar to that of oleic acid. In the present study this reliance on adequate dietary linoleic acid was confirmed. Although not contrasted with oleic acid, a decrease in total and LDL-cholesterol was observed when the diet rich

in both palmitic acid and linoleic acid was contrasted with the other diet treatments. Some investigators, however, have reported that palmitic acid is hypercholesterolemic relative to oleic acid even when the diets were low in cholesterol and the subjects normocholesterolemic(130). Such discrepancies confirm the requirement for further studies to clarify the effects of palmitic acid under a variety of conditions.

Current dietary recommendations suggest that no more than 30% of total energy be derived from fat. Of this at least one-third, or 10% of calories is suggested to be obtained from C18:2. The diets in this study designated as high in linoleic acid contained C18:2 at levels currently recommended. Clearly, the presence of adequate linoleic acid is of primary importance and cannot be overlooked. Perhaps the addition of linoleic acid in the high C16:0, high C18:2 diets resulted in a more favorable polyunsaturated fatty acid to saturated fatty acid ratio (P/S) that ultimately lowered plasma cholesterol levels (Table V-I). For example, in the high C16:0, low C18:2 diet the P/S ratio was 0.14 and the resultant total serum cholesterol level observed was 4.02 ± 0.08 mmol/L. In contrast, the P/S ratio of the high C16:0, high C18:2 diet was 1.19 (very close to the recommended 1.0 ratio) and the corresponding total serum cholesterol value was 3.20 ± 0.08 mmol/L. This rationale supports the addition of adequate amounts of dietary saturated fat as the diet low in C16:0 and high in C18:2 (P/S = 2.55) resulted in the second highest total cholesterol response (3.42 ± 0.08 mmol/L); providing evidence that, at some point, the intake of saturated fat may become suboptimal. In fact, this dependency on an appropriate level of SFA and linoleic acid content of the diet has been reported previously. Specifically, it has been suggested that C18:2 stimulates LDL-receptor activity which is enhanced in the presence of SFA (131). This concept is based on the premise that for maximal up-regulation of LDL-receptor activity, and consequently the removal of LDL, a threshold intake of both C18:2 and SFA is required. Although previous researchers have not identified at what levels such up-regulation occurs, it is apparent from the data presented here that C18:2 at 3% energy (i.e. the high C16:0, low C18:2 diet) was not sufficient to up-regulate LDL-receptor activity. However, the C18:2 content of the high C16:0, high C18:2 diet (10% energy) was adequate to promote decreased LDL levels. Clearly, further

research is required to establish at what combination of C18:2 and saturated fat this beneficial effect occurs.

Table VI-I. Polyunsaturated Fat to Saturated Fat (P/S) Ratio of Test Diets

Diet	P/S ratio	Total Cholesterol *mmol/L*
Low C16:0, Low C18:2	0.58	3.17 ± 0.08
Low C16:0, High C18:2	2.55	3.42 ± 0.08
High C16:0, Low C18:2	0.14	4.02 ± 0.08
High C16:0, High C18:2	1.19	3.20 ± 0.08

The finding that palmitic acid does not exert a cholesterol-raising effect in the presence of adequate linoleic acid has practical applications to the food industry as well as to nutritionists. Palm oil possesses several characteristics that are desirable for its incorporation into food products. Firstly, it has a high solid-glyceride content, giving the required consistency without hydrogenation. Secondly, it is very resistant to oxidation and consequently has a long shelf-life(64). These factors, in combination with its competitive price, point to the potential for incorporating palm oil into a variety of food products. For example, margarines based on a higher level of palm stearin in combination with linoleic acid would be easily spreadable at refrigerator temperature but still retain shape at room temperature without hydrogenation. Furthermore, the findings of this study may also have important economic significance to canola oil producers. Because canola oil is a rich source of linoleic acid, its combination with palm in oils and margarines may improve physical and nutritional properties of using either alone.

In the present comparison there was not the favorable increase in HDL levels associated with diets rich in palmitic acid as observed previously. Rather, HDL concentrations appeared to be unrelated to the amount of both palmitic acid and linoleic acid, and may therefore respond more to other dietary constituents not investigated in the present study. This finding is in agreement with Temmes et al. who also failed to observe an increase in HDL-cholesterol levels (130) but is in contrast to that of Lindsey et al. and

Khan et al. who both identified significant increases in HDL-cholesterol concentrations following palmitic acid feeding(68, 69).

Although effects of dietary fats on circulating cholesterol levels have been thoroughly investigated, it is less clear as to what changes in cholesterol metabolism occur to account for these dietary effects. Numerous studies have addressed this question with the bulk of research suggesting that changes in the endogenous synthesis of cholesterol may occur(83, 85, 86, 87). Our results do not provide data to implicate changes in endogenous cholesterol synthesis as the mechanisms for altered total and LDL-cholesterol levels when the high intake of C16:0 is consumed. Rather, the endogenous rate of cholesterol synthesis was not significantly affected by either the palmitic acid or linoleic acid content of the diet, despite an affect of these nutrients on lipoprotein levels. While significant overall differences in the FSR for cholesterol were not observed, it appears that larger FSR values occurred in individual subjects who exhibited a greater change in serum cholesterol values in response to diet treatment (refer to Figure V-III and V-V). This relationship, unlike that of the LDL-cholesterol response, appears to be independent of the dietary linoleic acid level.

A number of studies have suggested that C16:0 does not raise serum total and LDL-cholesterol values to the extent predicted by the original regression equations of Keys and Hegsted(69,128,129). Although the present study provides evidence to suggest that a threshold quantity of both linoleic acid and saturated fat is responsible for the disparate effect of C16:0 on total and LDL-cholesterol levels, there is evidence to suggest that other factors may also be important. For example, a significant amount of the palmitic acid in this study design was provided by palm oil, a rich source of tocotrienols (64). Evidence suggests that these tocotrienols may have the ability to modulate cholesterol synthesis by suppressing 3-hydroxy-3-methyl glutaryl coenzyme A reductase (HMGR) activity, the rate limiting step in cholesterol biosynthesis(132). This suppression has been postulated to result from two post transcriptional processes; the controlled degradation of HMGR and modulation of the efficiency of translation of its mRNA(133). Furthermore, the reduction in cholesterol has been identified as specifically lowering the serum low density lipoprotein cholesterol and apolipoprotein B

concentration(134). However, studies which have identified this beneficial effect have provided tocotrienols to human subjects at levels of approximately 400 mg/day. The diets in the present study contained, at most, 50 mg of tocotrienols daily. Thus, it is difficult to cite this proposed action of the tocotrienols as a cholesterol-lowering mechanism in this study until further clinical trials are conducted to define the effects of more physiological quantities of these compounds.

Summary

Based on this research, the consumption of pamitic acid by normolipidemic men who typically consume a relatively low fat diet (30% energy from fat and containing the recommended intake of polyunsaturated fat) is unlikely to have an appreciable effect on lipoprotein profiles. Furthermore, it can be inferred that adequate amounts of saturated fat, and particularly palmitic acid, are necessary to obtain an appropriate balance of fatty acids required to promote a more favorable serum cholesterol response. A 1990 WHO report suggests from epidemiological data that a progressive fall in mortality due to cardiovascular disease occurs as the intake of saturated fatty acids decreases to about 10% of energy. Evidence for lowering the level of saturated fat in the diet below this level is missing leading one to question the benefit of reductions beyond this point. In fact, the findings of this research suggest the potential for a suboptimal intake of saturated fat. This finding may be of particular importance as low and even negligible levels of dietary saturated fat have become frequently recommended by health professionals to promote lowered lipid levels. Clearly, dietary recommendations have not kept pace with the rapidly expanding knowledge of the importance of the effects of individual fatty acids, and the importance of the total rather than saturated fat-derived energy intake

Future Research

The finding that palmitic acid is not hypercholesterolemic in the presence of adequate linoleic acid confirms previous researcher suggesting a "conditional" total and LDL-cholesterol response to palmitic acid. However, determination of the optimal

percent energy of linoleic acid and the effect of increasing palmitic acid to levels beyond those studied in this thesis need to be examined.

The contribution of dietary cholesterol to hypercholesterolemia has received much attention but has recently been considered to play only a minor role in the development of atherosclerosis. However, the interaction of dietary cholesterol with other nutrients and specifically fatty acids requires further study. Although palmitic acid did not exert an effect in our cholesterol moderate diets in the presence of C18:2, it is possible that a different effect could have occurred if the experimental diets contained higher levels of cholesterol. If so, it would be beneficial to determine at what cholesterol intake the protective role of linoleic acid on plasma lipoprotein levels is maintained.

The lack of change in endogenous cholesterol synthesis despite changes in lipoprotein levels provides further controversy regarding the mechanisms responsible for alterating lipoprotein levels. Clearly, changes in fecal sterol excretion, cholesterol absorption and lipoprotein receptor regulation may contribute more significantly to whole-body cholesterol homeostasis than previously anticipated and warrants further investigation.

Conclusions

The results of this study reinforce the fact that saturated fatty acids are not equally hypercholesterolemic. Unfortunately, as consumers we choose foods not nutrients and because any individual food will contain a mixture of many fatty acids, dietary recommendations reflecting this data are difficult to propose. However, because palmitic acid is an abundant fatty acid in meat and dairy products, foods items which are high in nutritive value, a reevaluation of the recommendations to limit consumption of these foods is warranted. Similarly, foods containing relatively large quantities of the saturates lauric and myristic acid which are known to be potently hypercholesterolemic must also be reevaluated. Clearly, the evaluation of foods must not be based solely on saturated fat content. Rather, careful consideration of the relative abundance of the individual fatty acids present in the total diet consumed is necessary.

Chapter VII

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