EFFECT OF HUMAN APOLIPOPROTEIN E PHENOTYPE ON ENDOGENOUS CHOLESTEROL SYNTHESIS AS MEASURED BY DEUTERIUM INCORPORATION

By

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ABSTRACT

Apolipoprotein (apo) E polymorphism may influence an individual's risk of developing ischemic heart disease through its effect on plasma cholesterol levels. Compared to individuals homozygous for the ε-3 allele (E3/3 phenotype), presence of the ε-2 allele (E2/2 or E3/2 phenotype) leads to lower, and the ε-4 allele (E4/4 or E4/3 phenotype) to higher, plasma cholesterol levels. The mechanisms responsible for these differences have not been completely identified, but are thought to be a result of the different binding properties of the apo E isoproteins. The purpose of this research project was to determine whether the rate of endogenous cholesterol production in healthy normolipidemic males is related to apo E phenotype. The study was of seven days duration. Subjects were selected from a group of 113 volunteers who were screened for apo E phenotype and plasma cholesterol and triglyceride levels. Subjects (E2/(2 or 3) group = 9, E4/(4 or 3) group = 9) consumed a standardized Western diet for five days and fasted on Day 6. Subjects drank 0.7 g deuterium oxide (D_2O)/kg body water at 0700 hr on Day 5, followed by dilute deuterium labelled drinking water to maintain constant enrichment levels on Days 5 and 6. The fractional synthetic rate (FSR) of cholesterol was determined over four consecutive 12-hour intervals on Days 5 and 6 by measurement of the incorporation of the stable isotope-labelled precursor, D_2O, from body water into free plasma cholesterol. Deuterium enrichment of plasma cholesterol and plasma water was determined by isotope ratio mass spectrometry. E2/- subjects had significantly lower (mean ± SEM) cholesterol FSR (0.070 ± 0.002/day) than E4/- subjects (0.097 ± 0.002/day) during the feeding period ($p < 0.05$). Cholesterol FSR was significantly reduced in all subjects during the fasting period ($p < 0.001$), although there were no differences between phenotype groups (E2/- = -0.002 ± 0.001/day; E4/- = 0.003 ± 0.001/day). Mean nocturnal cholesterol FSR following both the feeding and fasting periods was greater than daytime FSR ($p < 0.05$). These findings suggest that regulation of endogenous cholesterol synthesis may contribute to plasma cholesterol variations in men with different apo E phenotypes.

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I. INTRODUCTION

Ischemic heart disease (IHD) is the leading cause of death in North America (1). Major coronary risk factors include high blood pressure, cigarette smoking, elevated lipid and lipoprotein levels, diabetes mellitus, and increased age (2). In particular, the relationship between plasma cholesterol level and IHD is well established (2, 3). This relationship is influenced by a person's genetic background, such as the presence of an inherited lipoprotein disorder, and by modifiable environmental factors, such as diet.

Recently, much attention has been given to the development of dietary guidelines for the public that may reduce the risk of IHD. Dietary recommendations include guidelines for fat quality and quantity, and cholesterol and energy intake (4, 5). The modifications potentially improve a person's blood lipid profile, specifically by lowering their plasma total and low density lipoprotein (LDL) cholesterol concentrations. Human metabolic studies, however, have demonstrated a high degree of variation in the responsiveness of individuals to dietary manipulations (6, 7). Genetic factors may be an important cause of this variation.

One genetic factor, apolipoprotein (apo) E phenotype, may contribute to as much as 7% of the variation in plasma lipid levels in a given population (8 - 10). This effect of apo E could be an important influence on an individual's IHD risk. Although there is no conclusive proof that apo E phenotype is directly related to IHD, recent studies indicate that individuals with a particular apo E phenotype suffer from the effects of IHD at an earlier age than those of a different phenotype (11, 12).

The mechanisms by which apo E phenotype influences plasma cholesterol levels have not yet been clearly identified. The cholesterol level differences may be due in part to the receptor binding

abilities of the apo E proteins (13, 14), the catabolic rate of the apo E-containing lipoproteins (15, 16), and the regulation of cholesterol absorption by the apo E protein (17). One additional mechanism which may be capable of causing cholesterol level variations is the ability of the apo E protein to influence the regulation of endogenous cholesterol production.

The aims of this research project were: i) to determine rates of cholesterol synthesis in men with different apo E phenotypes and ii) to identify whether apo E-related differences in cholesterol synthetic rate vary in response to the short-term removal of dietary influences on cholesterol synthesis regulation. These aims were addressed by determination of fractional cholesterol synthetic rates in two groups of individuals with different phenotypes for apo E under conditions of feeding and fasting. Volunteers from Vancouver, B. C. were screened for determination of their plasma cholesterol and triglyceride levels, and their apo E phenotype. Nineteen subjects of the appropriate phenotype were chosen for participation in the cholesterol synthesis measurement study. Subjects were placed on an experimental diet for four days to standardize their nutrient and caloric intake. Cholesterol synthetic rate measurements were then carried out during a 48-hour period consisting of first a day of feeding, and then a day of fasting. Cholesterol synthetic rate was determined by measurement of the incorporation of the stable isotope-labelled precursor, deuterium oxide, from body water into free plasma cholesterol. Knowledge of cholesterol synthesis regulation in these individuals will improve our understanding of the manner by which apo E modulates plasma cholesterol levels.

II. REVIEW OF THE LITERATURE

II.1. Location and Structure of Apo E. Apo E is a glycoprotein consisting of 299 amino acids with a molecular weight of 34,000 daltons (18). Apo E is synthesized primarily in the liver and in several peripheral tissues not involved in lipoprotein synthesis including the brain, spleen and kidney (19, 20). Apo E is found in human plasma associated with chylomicrons and their remnants, with very low density lipoproteins (VLDL), and with a cholesterol-enriched subfraction of high density lipoproteins (HDL) usually designated as HDL_1, HDL_C or "HDL-with-apo E" (21).

The structure of apo E is genetically determined. Population and family studies indicate that apo E exhibits genetic polymorphism with three common alleles for the protein occurring at a single genetic locus (22, 23, 24). The alleles, designated ε-2, ε-3, and ε-4, code for three structurally different isoforms: E2, E3, and E4 respectively. Two of the three alleles are inherited in a co-dominant fashion yielding six possible phenotypes: three homozygous, designated E2/2, E3/3, and E4/4, and three heterozygous, designated E3/2, E4/3, and E4/2.

The amino acid sequences of the three isoforms differ by the presence of one or two cysteine residue substitutions for arginine at site numbers 112 and 158 (Table I) (18, 25). The most common form, apo E3, contains a cysteine residue at site #112, and an arginine residue at site #158. The least common forms, apo E2 and E4, contain two cysteine and two arginine residues, respectively. The amino acid substitutions cause each isoform to have a different isoelectric point allowing separation of the proteins by isoelectric focusing using a pH gradient between pH4 and pH6. Several minor apo E isoforms develop as a result of post-translational additions of sialic acid residues to the parent isoforms (21, 24). These proteins migrate further towards the anode on an electrophoresis gel than their parent protein. Other rare forms have been identified (26).

Table I. Comparison of the structures of apolipoprotein E isoforms (adapted from Reference 21).

	Apo E Isoforms		
	E2	E3	E4
Relative Charge	0	+1	+2
Residue #112	Cys*	Cys	Arg
Residue #158	Cys	Arg	Arg

*cys = cysteine; arg = arginine

II.2. Relative Phenotype and Allele Frequency of Apo E. The frequency of occurrence of the six different apo E phenotypes has been measured in various populations. Apo ε-3 is by far the most commonly occurring allele and individuals with the apo E3/3 phenotype make up the greatest proportion of the general population (Table II) (27). The ε-2 and ε-4 allele frequencies are more variable although the E4/3 phenotype frequency is usually greater than E3/2. Homozygous E4/4 and E2/2 phenotypes make up the smallest population proportion, having a usual frequency of 1 - 5%. No differences in allele or phenotype distribution based on age or sex have been reported (10).

The distribution of the relative allele frequencies varies significantly in populations of different ethnic or cultural backgrounds (Table III). Comparison of average allele frequencies in Caucasian population samples (8, 9, 26, 28, 29) with those from Japanese (30, 31) and Chinese (32) samples reveals a significantly lower ε-3, and higher ε-4, allele frequency in the Caucasian samples. The relative frequency of the ε-2 allele is also significantly higher in the Caucasian populations compared with the Japanese. Davignon and co-workers (10) have recently analyzed data from a

Table II. Apolipoprotein E genotypes and phenotypes and their prevalence in the general population (adapted from Reference 27).

Genotype	Phenotype	Prevalence (%)
ε-2/ε-2	E 2/2	1
ε-3/ε-2	E 3/2	12
ε-3/ε-3	E 3/3	55
ε-4/ε-3	E 4/3	26
ε-4/ε-4	E 4/4	3
ε-4/ε-2	E 4/2	3

Table III. Relative allele frequencies for apolipoprotein E in populations of different ethnic backgrounds (adapted from Reference 10).

		Relative Allele Frequencies		
Sample Population (ref)	n	ε–2	ε–3	ε–4
Chinese				
Beijing, China (32)	95	0.053	0.883	0.064
Japanese				
Asahikawa, Japan (30)	576	0.037	0.846	0.117
Hiroshima & Nagasaki, Japan (31)	110	0.023	0.891	0.086
Caucasian				
Framingham, USA (26)	1209	0.072	0.786	0.140
Ottawa, Canada (8)	102	0.078	0.770	0.152
Nancy, France (9)	223	0.130	0.742	0.128
Helsinki, Finland (29)	615	0.041	0.733	0.227
Christchurch, New Zealand (28)	426	0.120	0.720	0.160

large number of population samples and have concluded that the "genetic distance" between ethnic groups resulting from the polymorphism of the apo E gene is caused primarily by ε-3 allele frequency variation. On the contrary, one recent report from Japan indicates that there is no difference in allele frequency distribution between Caucasians and Japanese (33).

Allele frequencies among Caucasian populations are similar, with a few notable exceptions. The population sample from Helsinki, Finland (29) has a significantly higher relative frequency of the ε-4 allele compared to all other Caucasian groups studied (10). Relative frequency of the ε-2 allele is greatest in samples from Nancy, France (9) and Christchurch, New Zealand (28), being three times greater than that of the ε-2 frequency in the Finnish population.

These allele frequency distributions may contribute to the variation in IHD incidence between these populations. The higher ε-3 allele frequency in Japanese and Chinese populations may contribute to the lower rates of IHD observed in these populations (10). In the Finnish population, the high relative frequency of the ε-4 allele may contribute to this population's susceptibility to IHD (10, 17, 29).

II.3. Role of Apo E in Cholesterol Metabolism. Apo E is involved in a number of important pathways of cholesterol metabolism because of its association with plasma lipoproteins and its ability to interact with cellular lipoprotein receptors. Apo E mediates the uptake of dietary cholesterol by the liver through its association with chylomicrons and chylomicron remnants. Apo E on cholesterol-enriched chylomicron remnants binds with high affinity to specific hepatic receptors and is responsible for remnant clearance (34). These remnant receptors, also named "apo E receptors", differ from the classic LDL (apo B/E) receptors in that their synthesis is not regulated by cholesterol influx as are the LDL receptors (34, 35). The presence of apo E on triglyceride-rich VLDL may be important for the transport of endogenous cholesterol back to the liver through its suspected role in the regulation of the normal conversion of VLDL to intermediate density

lipoproteins (IDL) and then to LDL (29). Following hydrolysis of VLDL triglyceride by lipoprotein lipase, some VLDL cholesterol may also be returned directly to the liver by way of either the remnant (apo E) or the LDL receptor (10). LDL (apo B/E) receptors on extrahepatic and hepatic tissues interact not only with apo B-containing LDL, but with apo E containing lipoproteins as well (34). Apo E on HDL_1 plays a role in reverse-cholesterol transport, the process by which storage cholesterol esters are transported from peripheral cells to the liver for excretion. Hepatic uptake of HDL cholesterol ester may occur either by transfer of the esters to lower density lipoproteins which are then incorporated into the liver, or by direct hepatic uptake of the HDL_1 particle by apo E mediated pathways (36).

The structural differences between apo E isoforms are thought to alter their function in cholesterol metabolism. Metabolic studies conducted on isolated apo E isoproteins conclude that apo E3 is the normal form and that apo E2 and E4 have properties that cause them to be metabolically abnormal. Numerous *in vitro* binding studies have demonstrated the ability of the apo E isoforms to bind to specific cell surface receptors. Apo E2 binding activity has been shown to be defective compared to apo E3 or E4 in the presence of human fibroblasts and ^{125}I-labelled LDL (13, 14). Apo E3 and E4 successfully compete with labelled LDL for fibroblast binding while a greater proportion of ^{125}I-LDL binds to the cells when incubated with apo E2. The defective binding is thought to result from the cysteine substitution on the protein (13). It is interesting to note that no difference was observed in the binding ability of apo E4 compared to E3, possibly because the substitution of arginine for cysteine is outside the receptor binding region on the protein (37).

Kinetic *in vivo* investigations reveal that the catabolic rate of apo E4-containing chylomicrons and VLDL is greater than the rate of those with apo E2 or E3 protein (15). The absence of cysteine residues on apo E4 may increase the catabolic rate by preventing the formation of disulfide bonds between apo E4 and other proteins. Apo E resides primarily on HDL during the fasting state. Following a meal, apo E shifts from HDL onto post-prandial chylomicron remnants. The shifting

of apo E4 may occur more rapidly than shifting of E3 or E2 protein because E4 is not as tightly bound to HDL, resulting in the higher rate of catabolism of E4-containing chylomicrons (37).

II.4. Influence of Apo E on Plasma Cholesterol Concentration. Much of the interest surrounding determination of apo E phenotypes results from the discovery that individuals with particular lipoprotein disorders often have identical apo E phenotypes. Over 90% of individuals reported in the literature as suffering from the genetic disorder Type III hyperlipoproteinemia (or familial dysbetalipoproteinemia), have been found to be of the apo E2/2 phenotype (38). These patients have elevated triglyceride levels and are prone to the development of premature atherosclerotic vascular disease (22). However, presence of the E2/2 phenotype is not sufficient to cause type III hyperlipoproteinemia, as only 4% of E2/2s develop the disorder (21, 39). Secondary factors such as the presence of other genes for familial hyperlipidemia are necessary in order for the lipoprotein disorder to occur (10, 28). Among individuals with type V hyperlipoproteinemia, prevalence of the apo ε-4 allele has been found to be markedly increased in some studies (33, 40).

Consistent reports of apo E-related variations in plasma cholesterol levels in different populations have led to the current focus on the metabolic consequences of apo E polymorphism. Individuals who are homozygous for the ε-2 allele, and who do not have type III hyperlipoproteinemia, often have significantly lower plasma total and LDL cholesterol levels than individuals of the E3/3 phenotype (8, 9, 26, 29-31, 38). In contrast, individuals carrying the ε-4 allele usually have higher plasma cholesterol levels than E3/3s. Presence of a single ε-3 allele (E4/3 or E3/2 phenotypes) appears to moderate the effect of the rare alleles such that the usual ranking of cholesterol levels is: E2/2 < E3/2 < E3/3 < E4/ (3 or 4). The average effect of the apo E alleles on plasma cholesterol levels has been calculated for various populations (8). The ε-2 allele has a "cholesterol-lowering" effect that is two to three times greater than the "cholesterol-raising" effect of the ε-4 allele, relative to the mean plasma cholesterol concentration of individuals with the E3/3 phenotype (9, 10).

The significance of the apo E allele effect on plasma cholesterol differs between groups of various ethnic backgrounds. This may be due to the presence of other influential factors within a given population. The ε-4 allele has been found to have a significant cholesterol raising effect in the Finnish population (29), however, this effect is absent in some Japanese populations (30). These findings suggest the presence of a combined environmental-genetic effect influencing the plasma lipids of the Finnish population (41).

The role of apo E in maintaining cholesterol homeostasis is not completely understood. A defect in the ability of the apo E2 protein to bind to its receptor on the liver would be expected to cause an increase in plasma cholesterol levels through the accumulation of chylomicron and VLDL remnants, and HDL_1 in plasma which is contrary to observations that E2 individuals have lower plasma cholesterol levels. A proposed mechanism for this apparent discrepancy is that the reduced cellular concentrations of cholesterol in the liver, caused by accumulation of apo E-containing lipoproteins in plasma, results in up-regulation of the LDL receptor system. Production of LDL receptors increases, followed by enhanced uptake of plasma LDL (38). A delay in the apo E-dependent conversion of VLDL to LDL may also reduce the synthetic rate of LDL and result in reduced levels of LDL cholesterol in the plasma of E2/2 or E3/2 individuals (29, 38)

The effect of the ε-4 allele on plasma cholesterol levels is less well understood. The rapid catabolism of E4-containing lipoproteins may result in increased intracellular cholesterol concentrations and a down-regulation of the LDL receptor system. This in turn would cause an accumulation of LDL in plasma, and the increased levels of plasma cholesterol seen in E4 subjects (38).

The binding and catabolic properties of the apo E isoproteins have been shown to influence the rate of clearance of exogenous fat. Using the vitamin A-fat loading test it was demonstrated that

individuals of the E3/2 phenotype have delayed clearance of dietary fat while E4/3 and E4/4 individuals have increased clearance (37). Apo E polymorphism may therefore have an indirect effect on plasma cholesterol levels through its influence on various stages of lipid and lipoprotein metabolism.

II.5. Potential Relationship between Apo E Polymorphism and Cholesterol Synthesis. The ability of the LDL receptor system to maintain cholesterol homeostasis in response to changing levels of cholesterol within the cell includes regulation of the activity of 3-hydroxy-3-methylglutaryl coenzyme A (HMG Co A) reductase, the rate-limiting enzyme in cholesterol synthesis (35). Up- or down-regulation of the system would therefore be expected to affect the rate of endogenous cholesterol production. Variations in cholesterol synthetic rates in individuals with different apo E phenotypes may contribute to the apo E-related differences in plasma cholesterol levels.

The measurement of cholesterol synthesis in individuals with different apo E phenotypes has been reported by only one researcher (17). Cholesterol synthesis and absorption measurements were performed on 39 men selected on the basis of their plasma cholesterol levels. Four individuals were found to have one or two ε-2 alleles, 22 were homozygous E3/3, and 13 were either apo E4/4 or E4/3. Cholesterol synthesis was determined by a sterol balance study and by measurement of the serum level of lathosterol, a cholesterol precursor. Serum sterol measurements indicated that men in the E2 group synthesized significantly more cholesterol than those in the E4 group.

The increased synthetic rate of cholesterol in men with the ε-2 allele is not consistent with the observations that plasma cholesterol levels are significantly lower in this group of individuals compared to those with the ε-4 allele. The proposed up-regulation of the LDL receptor system occurring as a result of defective E2 binding may indeed result in a temporary increase of endogenous cholesterol synthesis. However, total daily production may actually be lower in individuals with the ε-2 allele and higher in those with the ε-4 allele, thus contributing to the

observed plasma cholesterol differences. The indirect approach of using serum lathosterol measurements from a single time point to indicate cholesterol synthesis rate emphasizes the need for additional research into the factors controlling cholesterol synthesis and plasma cholesterol levels in individuals with different apo E phenotypes.

II.6. Measurement of Cholesterol Synthesis. A number of different methods have been employed to study cholesterol synthesis in animals and in humans. In animals, measurement of HMG Co A reductase activity *in vitro* has been used to determine rates of hepatic cholesterol synthesis (42). Rates of whole body and organ cholesterol synthesis have been determined using four different approaches: 1) sterol balance techniques, 2) kinetic analyses of the isotopic decay of radioactively labelled cholesterol, 3) measurement of serum levels of cholesterol precursors, and 4) measurement of the conversion of labelled cholesterol precursors into cholesterol.

Sterol balance methods involve feeding individuals cholesterol-free diets and collecting feces for analysis of cholesterol and its metabolites. This method assumes that a steady state of cholesterol metabolism exists and that losses of endogenous cholesterol occur only in the feces (43, 44). If a subject is placed on a cholesterol-free diet, or if the absorption of dietary cholesterol is measured and accounted for, the amount of cholesterol and cholesterol metabolites excreted should then be equivalent to the amount of cholesterol synthesized in the body (44).

Long term kinetic studies using the injection of [^{14}C]- or [^{3}H]-labelled cholesterol have been conducted on baboons (45) and on humans (46-48). Subjects are injected with labelled cholesterol and the specific radioactivity of plasma cholesterol is measured at specific time points over a period of many weeks. Analysis of the decay curve enables determination of the total body cholesterol turnover rate and the rate of cholesterol exchange between body cholesterol pools. Other parameters, such as the mass of the pools and the cholesterol production rate, can be determined by compartmental or input-output analysis of the data (46, 49). This method assumes that a steady

state of cholesterol metabolism exists and that all cholesterol catabolism is occurring within the plasma compartment (50).

Serum mevalonic acid (51, 52), plasma squalene (53), and serum methyl sterol levels (54) have been used as indirect indicators of cholesterol synthesis. The presence of these cholesterol precursors in plasma may reflect HMG Co A reductase activity since during cholesterol synthesis these compounds accumulate in liver and may leak out into plasma (44). These methods have been shown to accurately reflect the relative rate of cholesterol synthesis under controlled conditions when measured in comparison with sterol balance techniques (51, 55) or with radiolabelled mevalonic acid (53). Serum sterol measurements may be useful for comparing two types of experimental conditions but they may not always give absolute values for the total amount of cholesterol synthesized (44).

Radiolabelled precursors of cholesterol such as [^{14}C]-labelled acetate (56) and mevalonate (50) have been used to determine rates of cholesterol synthesis in isolated tissues and in the whole body of animals and humans. Measurements of the rate of formation of labelled cholesterol represents the fractional cholesterol synthetic rate. This method requires a continuous infusion of labelled precursor to maintain the specific radioactivity of the precursor pool at a constant level. This procedure may require extended measurement periods in humans. Tritiated water ([^{3}H]-H_2O) has proven to be a more useful precursor than labelled acetate or mevalonate since there is less chance that the precursor water pool will be diluted by unlabelled substrates, thus reducing its specific radioactivity (57). Rates of incorporation of tritium into newly synthesized cholesterol molecules have been determined for a number of different animal tissues (57). Unfortunately, the hazards associated with the high levels of tritium necessary in these experiments make this method unsuitable for use in human subjects.

II.7. Deuterium Incorporation Methodology. The use of a non hazardous, short-term method for determining cholesterol synthetic rates in humans eliminates the major complications encountered with conducting balance studies or using radioactive products. Use of the stable isotope-labelled precursor, deuterium oxide (D_2O), provides a practical and safe alternative to these types of studies.

Measurement of the incorporation of D_2O into newly synthesized cholesterol was first reported in the 1950s and 1960s (58, 59). Taylor and co-workers (59) studied four individuals consuming first a cholesterol-free diet and later a cholesterol-rich diet. The level of deuterium enrichment used was 5.0 g D_2O per kg estimated body water (0.5 atom % excess), a 33-fold increase over naturally occurring deuterium levels. This quantity was necessary to ensure detection of the deuterium atoms incorporated into cholesterol using the available mass spectrometric techniques. Nearly all subjects reported experiencing severe vertigo following consumption of the priming dose given to them to achieve this high level of enrichment. Even with this high level of enrichment, several days were required before acceptable levels of the isotope could be detected (59).

Since these earlier studies, increased mass spectrometer analytical sensitivity has improved the detection limits so that the required deuterium enrichment level is ten times less (60). At this lower level (0.05 atom % excess), side effects have not been observed and the deuterium enrichment of plasma cholesterol can be detected within a few hours following D_2O administration (61). Use of deuterium does not require existence of steady state metabolism because the deuterium label can be detected within a short period of time making it a promising technique for measuring short-term perturbations in cholesterol metabolism.

Additional considerations of the deuterium incorporation methodology are similar to those supporting the use of tritiated water in the determination of cholesterol synthetic rates. Since tritiated water, and presumably deuterated water, freely and rapidly penetrates all cell membranes in

the body, the plasma water deuterium enrichment would be expected to equal the level of deuterium enrichment in the intracellular water pool that is available for use in cholesterol synthesis (56). By maintaining the deuterium enrichment of the body water pool, cholesterol synthetic rates can be determined as the fractional precursor incorporation over time.

A three pool model has been developed by Goodman and co-workers (46, 47, 48) that describes the distribution of cholesterol throughout the body and the rate of cholesterol exchange between body cholesterol pools. In humans, cholesterol is synthesized primarily in the liver and in the small intestine (62). This cholesterol is thought to be part of a central pool of cholesterol that equilibrates rapidly with plasma cholesterol (46). This central pool, pool 1, also contains cholesterol from red blood cells, pancreas, spleen, kidneys, and lungs. During cholesterol synthesis, in the presence of D_2O, deuterium atoms should become incorporated onto stable, non-exchangeable sites on the sterol molecule. These newly synthesized cholesterol molecules would then exchange rapidly with free cholesterol on the various plasma lipoproteins, creating an equivalent level of deuterium enrichment throughout the plasma compartment. Measurement of the deuterium enrichment of free plasma cholesterol would therefore be expected to represent newly synthesized cholesterol from these organs (57).

Two additional smaller side pools of cholesterol are thought to exist in the body, both of which equilibrate at much slower rates with cholesterol in the central pool (46, 47). The pool with the slowest turnover rate, pool 3, consists of most of the cholesterol located in muscle and adipose tissue. The cholesterol turnover rate in pool 2 is intermediate between the rapidly exchangeable pool and pool 3. Pool 2 probably includes some of the cholesterol in the viscera in addition to some from peripheral tissues (46, 47).

II.8. Calculation of the Rate of Cholesterol Synthesis. In order to calculate the absolute rate of cholesterol synthesis in human subjects, the fractional synthetic rate (FSR) of cholesterol can be

determined and related to the estimated total pool size of cholesterol. Cholesterol FSR can be calculated using the ratio of the deuterium enrichment of plasma cholesterol to the deuterium enrichment of plasma water. This product to precursor enrichment ratio is then applied to a model which predicts the proportion of deuterium atoms which may be incorporated into the cholesterol molecule (57). During synthesis of each cholesterol molecule, seven of the final 46 hydrogens are derived directly from water. An additional 15 hydrogens are incorporated during reductive steps involving NADPH. Assuming the hydrogens of NADPH were labelled with D_2O at the same level as in the body water pool, a total of 22 deuterium atoms could potentially be incorporated into each molecule of cholesterol. Dietschy and Spady (57) and others have demonstrated that the average incorporation ratio of deuterium atoms per carbon atom (D:C) into newly synthesized cholesterol is close to that predicted by this model: 22 deuterium / 27 carbon. This D:C ratio can be used in combination with the plasma product to precursor enrichment ratio to calculate cholesterol FSR.

The deuterium incorporation method has been used to calculate rates of cholesterol synthesis in humans. Cholesterol FSR was determined in five healthy males for the 12-hour nocturnal periods following a day of fasting and then a day of feeding (61). The level of deuterium enrichment maintained in each subject was approximately 0.05 atom % excess. Subjects experienced no side effects at this enrichment level. The sensitivity of the method was adequate to reveal changes in cholesterol FSR occurring during the 12-hour intervals of the 48 hour measurement period. Cholesterol FSR was significantly lower following fasting as compared to the period following feeding. However, when the results were compared to values predicted by other methods, it was felt that the model may have under-predicted the absolute amount of free cholesterol synthesized.

II.9. Assumptions of the Deuterium Incorporation Method. The deuterium incorporation model and calculation of cholesterol FSR rely on assumptions of both the behavior of deuterium within the body, and the movement of free and esterified cholesterol between body pools. One potential source of error with this method is the degree of equilibration achieved between the reductive

hydrogen of NADPH and deuterium-labelled body water (57). The measurement interval used must be long enough to ensure adequate time for this equilibration to occur, or an underestimation of the number of deuterium atoms per cholesterol molecule could result. However, if the time interval was excessively long, deuterium atoms may become incorporated into other body molecules such as glucose or free fatty acids which are precursors of acetate. This would then cause enrichment of the acetyl Co A precursor pool and would be reflected in the D:C ratio of the newly synthesized cholesterol (57). The result would be an overestimation of the actual cholesterol FSR. At the present time there is no data available on use of deuterium oxide in humans to suggest what the most appropriate time interval may be for minimizing this type of error.

Measurement of the deuterium enrichment of free plasma cholesterol is thought to represent the enrichment of cholesterol in the rapidly exchangeable pool. The deuterium incorporation method depends on the assumption that all of the *de novo* cholesterol synthesis is occurring in this central pool, rather than in either of the two side pools (46). It is also assumed that the amount of free cholesterol entering the rapidly exchangeable pool from the two smaller side pools will be minimal due to the slow turnover rates of these pools (61). If a significant amount of cholesterol were to enter the central pool from the side pools, or from de-esterification of cholesterol within the central pool, dilution of the central pool with unlabelled cholesterol could occur, resulting in an underestimation of cholesterol FSR. A further complication is that newly synthesized cholesterol may be removed from the free cholesterol pool of the plasma compartment before isotopic equilibrium is achieved, resulting in an underestimation of cholesterol production (57).

Many studies have been conducted to analyze cholesterol synthesis under different metabolic conditions in a wide variety of *in vivo* and *in vitro* experiments. Studies by Dietschy and co-workers (56, 57) using $[^3H]$-H_2O in animals have led these authors to conclude that these assumptions about cholesterol movement between the pools are valid, and that newly synthesized cholesterol is accurately represented by free plasma cholesterol.

Finally, it is recognized that exogenous cholesterol will contribute to the mass of the central pool once it has been absorbed, and may cause dilution of the labelled cholesterol. Also, the amount of cholesterol entering this pool through the diet will likely contribute to feedback regulation of endogenous cholesterol production. Differences in the amount of absorbed dietary cholesterol present may therefore contribute to variations in cholesterol FSR.

II.10. Summary. Individuals who are carrying the apo ε-2 allele often have significantly lower plasma cholesterol levels compared to those with the apo ε-4 allele. The mechanisms suggested as being responsible for these different levels include a defect in the receptor binding ability of apoprotein E2 resulting in up-regulation of the LDL receptor system, and an increased catabolic rate of apo E4-containing lipoproteins causing down-regulation of the system. These fluctuations in the LDL receptor system would be expected to alter endogenous cholesterol production through the activity of HMG Co A reductase. The proposed mechanisms have not yet been clearly demonstrated and other factors may also contribute to the plasma cholesterol variations seen in these two groups of individuals. Apo E-related alterations in endogenous cholesterol production is a likely contributor to the differences in plasma cholesterol levels.

In this study it is hypothesized that individuals carrying the ε-2 allele will synthesize cholesterol at a lower rate than will individuals carrying the ε-4 allele. Measurement of cholesterol FSR under different feeding conditions will demonstrate if the removal of influences by dietary components affects the regulation of cholesterol synthesis differently depending on the individual's phenotype. Use of deuterium oxide as a precursor for cholesterol should prove useful for measurement of cholesterol synthetic rates during 12-hour time intervals and will enable characterization of daily fluctuations in cholesterol FSR. This study will result in further development of the deuterium incorporation method by its application to a situation where a difference in cholesterol FSR may exist.

III. EXPERIMENTAL DESIGN AND METHODS

The study was conducted in two phases. Phase I, the screening phase, took place during the Fall of 1987. This involved screening potential subjects to identify normolipidemic individuals of the appropriate apo E phenotype. Phase II, the experimental phase, took place in the Spring of 1988. This phase consisted of a series of seven day experiments designed to determine the cholesterol FSR in the two groups of subjects.

III.1. Screening Procedures. One hundred and thirty one men, ages 18 - 45, were recruited for participation in the screening phase of the study. Recruitment advertisements were posted about the UBC campus. Initial contact with the volunteers was made over the telephone. A brief oral description of the study was given to each respondent at that time. Following questioning, any respondent reporting current use of cholesterol-lowering drugs or a previous diagnosis of elevated plasma cholesterol or triglyceride levels was asked not to participate. Volunteers were requested to have no food, with the exception of water, for 12 - 14 hours and to abstain from alcohol consumption for 24 - 48 hours prior to their screening appointment.

Blood samples were obtained from each volunteer from 0700-1000 hrs at the Department of Laboratory Medicine, UBC Health Sciences Center Hospital. Each volunteer was in a seated position for 5 - 10 minutes before a 30 ml sample of blood was drawn from an antecubital vein into Vacutainer tubes containing powdered disodium ethylenediamine tetraacetate (EDTA) (1 mg/ml). Blood was stored at 4°C and within two hours the plasma was separated in a refrigerated centrifuge (1500 x g, 25 min), removed and stored at 4°C (63). At the time of blood sampling, volunteers completed a questionnaire on ethnic background, food allergies, food restrictions, and use of medication which might have precluded their participation in the study. Each volunteer was given a detailed written outline of the study design.

III.2. Cholesterol and Triglyceride Determinations. Plasma total cholesterol levels were determined within five days of blood collection in 0.1 ml plasma using the Liebermann-Burchard reaction (Data Medical Associates, Inc., Arlington, TX) (63, 64). An aliquot of plasma was frozen at -20°C to be used for the determination of plasma triglyceride concentration. Total plasma triglyceride level was determined in 0.02 ml plasma using an enzymatic procedure in which an esterase hydrolyzes the triglyceride to glycerol and free fatty acids. Glycerol concentration was then determined colorimetrically (Boehringer Mannheim Diagnostics, Montreal, Que.) (65). All samples for cholesterol and triglyceride determinations were performed in duplicate and standards were assayed with each batch. The coefficient of variation for the cholesterol assay was 5.1% and for the triglyceride assay it was 5.4%.

III.3. Apo E Phenotyping Procedure. Separation of VLDL from plasma, VLDL delipidation, and apo E phenotyping were carried out by the methods of Bouthillier et al (66) and Warnick et al (67). The VLDL fraction was obtained by density gradient preparative ultracentrifugation of 10 - 12 ml fresh plasma using a Beckman L3-50 ultracentrifuge with a Type 50 rotor (Beckman Instruments, Palo Alto, CA). VLDL (density < 1.006) was isolated by placing 2.5 ml of a solution containing 0.15 moles NaCl/l H_2O in a 10 ml ultraclear ultracentrifuge tube and injecting 5 - 6 ml plasma below the saline using a syringe with a wide gauge needle. A final volume of 8.5 ml was obtained by carefully pipetting additional saline onto the top. There were two tubes per volunteer. VLDL was isolated by ultracentrifugation at 16°C for 20 hr at 40,000 rpm (100,000 x g). The VLDL was removed by pipetting off the top layer. Fractions for each volunteer were pooled for a second wash under identical conditions. Washed VLDL was recovered from the top in a volume of approximately 3 ml and frozen at -20°C.

The washed fraction of VLDL contained approximately 200 μg protein, as determined by Lowry protein assay (Sigma Chemicals, St. Louis, MO) (68). The protein concentration varied with each

sample, however, preliminary tests of the phenotyping procedure indicated these variations were small and that sufficient protein was present to give clear isoform bands. The entire washed VLDL fraction was delipidated by transferring the VLDL into 10 ml freshly prepared acetone:ethanol (1:1 v/v) at -20°C. The contents were mixed and allowed to stand overnight. The protein was sedimented in a refrigerated centrifuge (1500 x g, 15 min) and the solvent removed. Protein was re-suspended in the same solvent and placed at -20°C for two hours after which the protein was re-centrifuged and the solvent removed. The protein was finally re-suspended in 5 ml cold diethyl ether, stored at -20°C for one hour, centrifuged, the solvent removed and the protein dried under a weak stream of nitrogen. The protein was stored at -20°C until being processed for isoelectric focusing.

Isoelectric focusing was performed in the Lipid Clinic Laboratory at Shaughnessy Hospital. Each run contained 11 samples and 1 control specimen. The gel was prepared by mixing 3.0 g acrylamide (BDH, Toronto, Ont.) with 80 mg N, N'-methylenebisacrylamide (Purity reagent, Bio-Rad Laboratories, Richmond, CA), 19.2 g urea (Electrophoresis purity, Bio-Rad Laboratories) and 2.0 ml carrier ampholytes (pH 4 - 6) (LKB Bromma, Fisher Scientific Instruments, Toronto, Ont.). A final volume of 40 ml was obtained by addition of distilled water. Twenty µl tetramethylenediamine (Bio-Rad Laboratories) and 80 µl freshly prepared ammonium persulfate solution (100 mg/ml) (Bio-Rad Laboratories) were added, the gel swirled and poured into cylindrical gel tubes to a height of 8 cm. Water was overlayered onto the gel solution and the gels polymerized for 30 minutes after which they were loaded into the electrophoresis cell (Model 150 A, Bio-Rad Laboratories). The lower (anode) chamber was filled with phosphoric acid (10 mmol/l) and the upper (cathode) chamber with NaOH (20 mmol/l). Gels were pre-focused for one hour at 110 volts.

Protein samples were prepared by suspending each sample in 200 µl of freshly prepared solubilization buffer containing 2.4 g urea and 16 mg dithiothreitol (Bio-Rad Laboratories) in 5.0

ml of Tris (hydroxymethyl) aminomethane-HCl buffer (10 mM, pH 8.2, reagent grade, Sigma Chemicals). Following pre-focusing, the upper buffer layer was removed and the protein loaded onto the gels. The protein was overlayered with 200 µl of a solution made by diluting 1.0 ml of solubilization buffer with an equal volume of distilled water and 20 µl ampholyte (pH 4 - 6). Gel tubes were filled with 20 mM NaOH and the upper buffer reservoir re-filled. Gels were focused for 16 hours at 250 volts followed by one hour at 450 volts. Gels were removed and placed in culture tubes containing a 0.4 g/l solution of Coomassie brilliant blue (G250, Bio-Rad Laboratories) in perchloric acid (35 g/l) for staining. After rotating for 45 - 90 min, gels were destained in 7.5% glacial acetic acid for 48 - 72 hours until the background cleared, and were then transferred to storage tubes containing fresh 7.5% glacial acetic acid.

Identification of the apo E phenotype was made using a combination of visual inspection and relative isoform band densities (66). The location of the isoform bands was determined by comparison with the control specimen. Relative band density was determined using a scanning densitometer (Appraise ver. 2.2, Beckman Instruments, Palo Alto, CA). Homozygous E4/4 individuals were distinguished from E4/3 individuals by visual identification of a strong E4 band, with E3 and E2 bands present, but of weaker intensity. The E4/3 heterozygotes had E4 and E3 bands of similar intensities. Homozygous E3/3 and E2/2 individuals were identified by the presence of a very dense band at the appropriate position. The ratio of the apo E3 to the apo E2 band was used as an additional criteria to distinguish between the E3/3, E3/2 and E2/2 phenotypes. Use of band ratios reduced the interference caused by sialylated proteins that had migrated away from the parent location. For example, sialic acid residues on E3 proteins can cause a weak band to appear at the E2 position in an individual who is an E3/3 homozygote. An E3:E2 ratio greater than 1.2 indicated a greater proportion of E3 protein, and identified the subject as having the E3/3 phenotype (66). An E3/2 phenotype was identified by an E3:E2 ratio of 0.5 - 1.1. Homozygous E2/2 subjects had an E3:E2 ratio of less than 0.4 (66).

All volunteers were sent a letter containing the results of their plasma cholesterol determination. Subjects were not informed of their apo E phenotype until after their participation in the study.

III.4. Selection of Subjects. Subjects for the study (n=19) were chosen from the volunteers who participated in the screening phase of the study (Appendix A). Subject selection was based primarily on the presence and density of an E2 or E4 band. Individuals with the densest bands were chosen if their lipid levels were within the normal range (total cholesterol = 100 - 220 mg/dl; triglyceride = 10 - 190 mg/dl). Band density was chosen as a selection criteria in order to eliminate the possibility of including a subject who was phenotyped incorrectly due to the interference of sialylated apo E proteins. All subjects were Caucasian. The study group consisted of nine individuals with the ε-2 allele (E2/2=1, E3/2=8), nine with the ε-4 allele (E4/4=1, E4/3=8), and 1 with the E4/2 phenotype. Two individuals with the E4/2 phenotype were originally selected but one withdrew on the second day of the study. The remaining E4/2 individual was not placed in either group and his data are presented separately whenever the effect of the apo E alleles is being considered. Subjects were provided with a detailed set of instructions and a description of the experimental protocol (Appendix B), and gave informed written consent prior to participating in the study (Appendix C). All procedures were approved by the UBC Clinical Screening Committee for Research and Other Studies Involving Human Subjects.

III.5. Experimental Protocol. Subjects participated in the study in groups of two or three. One week prior to the experimental period subjects reported to the testing facility in the School of Family and Nutritional Sciences where height and weight measurements while wearing light clothing and no shoes were taken, and information was obtained on each subject's usual pattern of activity. A fasting blood sample was drawn for baseline cholesterol determination. A food record sheet with detailed instructions on recording quantities of liquid and solid foods, and reporting brand names of food items (Appendix D) was provided to subjects with a set of measuring cups

and spoons, and diagrams of food models. Food records were kept during the three days preceding the experimental week.

The study was of seven days duration. On Days 1 - 5 subjects reported to the testing area at fixed times (0730, 1230, and 1730 hr) where they consumed an experimental diet designed to stabilize their nutrient and caloric intake. Subjects were free to pursue their usual activities and to go home each night. Following the evening meal on Day 5, no food was consumed with the exception of decaffeinated coffee, tea, or energy-free broth until the completion of the study on the morning of Day 7. Subjects were asked to remain in the testing facility under supervision from 0730 - 1930 hours on Day 6.

On the morning of Day 5, subjects drank a priming dose of deuterium oxide (D_2O)(99.8 atom % excess (A%E), ICN Biomedicals, Montreal, Que.) that was calculated to establish their body water deuterium enrichment at 0.07 A%E. This level of enrichment was decided upon after a preliminary study revealed that 0.05 A%E led to unacceptably high standard deviations in the mass spectrometric analyses of cholesterol deuterium enrichment. The D_2O priming dose of 0.7 g D_2O/kg total body water was calculated by assuming that body fat content was 10% of body weight, and that total body water was 73% of the fat-free mass (69). Dilute deuterium labelled drinking water was provided on Days 5 and 6 to maintain constant enrichment levels. Throughout the feeding day, drinking water containing 1.4 g D_2O/kg H_2O was consumed. This enrichment level was used to account for the expected amount of water present in the diet. During the fasting day, subjects drank water containing 0.7 g D_2O/kg H_2O. Thirty ml blood samples were obtained 30 minutes after administration of the D_2O priming dose on Day 5 and at 12-hour intervals over the next 48 hours (times 12, 24, 36, 48 hrs). Plasma was obtained by centrifugation and the samples frozen at -20°C.

III.6. Experimental Diet and Determination of Energy Requirements. The diet was designed using Canadian Nutrient File data (70) to contain 40% of calories as fat (polyunsaturated fatty acid:saturated fatty acid (P:S) = 0.4), 15% as protein, 45% as carbohydrate, and a cholesterol level of 200 mg/1000 kcal. This nutrient distribution is representative of the typical North American diet with the exception that it did not include any calories from alcohol (71). The diet was identical on each study day and was fed as three isocaloric meals. The sample menu (Table IV) contained 3000 calories and was adjusted to meet each subject's estimated energy requirements by the application of a correction factor to the weight of each food item or serving. Most of the food was purchased in advance and frozen for later use. Macaroni and cheese casseroles were prepared in bulk and frozen in 1 kg portions. Milk, yogurt, eggs and produce were purchased weekly. Meals were prepared daily by a trained food preparation technician and attention was given to ensure consistent preparation and serving procedures on each day. Meals were consumed under supervision to ensure complete consumption of the diet. Subjects were permitted to consume limited amounts of energy- and caffeine-free carbonated beverages, coffee, and tea. If requested, subjects were provided with two cans (255 ml) of energy-free carbonated beverage to take home with them at night. During the deuterium labelling period, subjects prepared their beverages (coffee, tea, and broth) with the labelled water. Carbonated beverages were unavailable during this time.

Individual energy requirements were determined by an estimate of subjects' basal metabolic rate using the Mayo Clinic Nomogram (72), multiplied by an activity coefficient of 1.7 (73) (Appendix E). Additional energy was provided to subjects reporting a high level of physical activity such as daily weight training or jogging. Estimates of these additional requirements were made using energy expenditure values for the particular activity applied to the amount of time the subject expected to be performing that activity (74, 75) (Appendix F). If the subject did not participate in the activity on a daily basis, the total additional energy requirement was calculated and divided evenly over the five day feeding period. Subjects were weighed at the beginning of each study day.

Table IV. Sample diet fed to subjects on each study day.*

	Food Item	Quantity	
BREAKFAST:	whole wheat bread	60	grams
	2% milk	250	
	soft sunflower margarine	15	
	Kellogg's bran flakes	50	
	seedless raisins	20	
	apple juice	110	
	omelette:		
	brick cheese	45	
	egg whites	2	whites
	egg yolk	0.5	yolk
	butter	6.5	grams
	soft sunflower margarine	4.5	
LUNCH:	whole wheat bread	30	grams
	soft sunflower margarine	10	
	dip:		
	1.55% yogurt	30	
	Miracle Whip	15	
	macaroni and cheese:		
	cooked macaroni	160	
	evaporated 2% milk	100	
	cheddar cheese	50	
	soft sunflower margarine	8	
	egg	0.5	egg
	chopped onion	60	ml
	carrot sticks	60	grams
	celery sticks	40	
	canned crushed pineapple	150	
SUPPER:	whole wheat bread	30	grams
	soft sunflower margarine	10	
	cooked chicken thigh (no skin)	75	
	hollandaise sauce:	1	
	egg yolk	0.38	yolk
	lemon juice	3.75	ml
	butter	10	grams
	cooked rice	150	
	frozen peas	75	
	frozen carrots	75	
	vanilla ice milk	100	
	Hershey's chocolate syrup	35	
	Cool Whip	15	

*Diet contains 3000 kcal and was modified to meet each subjects' estimated energy requirements (see Methods).

III.7. Choice of Nutrition Assessment Tool and Analysis of Food Records. Three day food records were chosen as the most appropriate nutrition assessment tool for obtaining information on the usual consumption of major nutrients and energy by each subject group. Seven day records would have been preferred, since a more precise estimate of the major nutrients can be obtained from six days of record keeping (76). However, it was felt that three day records would cause less interference of the subjects' eating habits, and that individual recording accuracy would be higher during the shorter time interval. After returning their food records, subjects were questioned further to improve the accuracy of their recording. Additional information was requested on portion sizes, brand names, use of food extras such as sugar or dressings, and subjects were asked whether the recording days were typical of the subjects' perceived usual intake. Subjects were asked to submit recipes for non-standard menu items.

Food records were analyzed for major nutrient content using the Nutricom program containing Canadian Nutrient File data (Smart Engineering Ltd., North Vancouver, B.C.). Food items and quantities were entered using Canadian Nutrient File food codes. Consistent selections were chosen for items that were of questionable source or content , e.g. a stick margarine containing "unspecified vegetable oils" was always selected from the 16 possible margarines when a subject failed to report the type of margarine used. Missing values for cholesterol were noted, and appropriate corrections made using values obtained from nutrient composition tables (77). The percentage of calories derived from fat, carbohydrate, protein, and alcohol were determined from the computerized information and by calculating the alcohol-related calories for each day, and taking an average value for the three days. A common correction factor was applied to each of the values to arrive at a final total of 100%.

III.8. Laboratory and Analytical Procedures. Lipids from each time point were extracted in triplicate (61). Methanol (8 ml) was added to 4 ml plasma, heated at 55°C (15 min), 24 ml hexane-chloroform (4:1 v/v) was added and the mixture was shaken. Water (2 ml) was added, the

mixture shaken (10 min), and after centrifugation (1500 x g, 15 min) the upper chloroform layer containing the lipid fraction was removed. Additional hexane-chloroform (24 ml) was added to the methanol phase, followed by shaking and centrifugation. The chloroform layer was removed and combined with the first. This was dried at 55°C under nitrogen to evaporate the solvent, leaving the lipid extract. Lipid extracts were separated by thin layer chromatography. Thin layer silica gel plates (20 x 20 cm, 250 μm, Whatman Inc., Clifton, N.J.) were activated for 30 minutes at 100°C and the extract, dissolved in chloroform, was streaked onto the plate. Plates were developed in petroleum ether/diethyl ether/acetic acid (135:15:1.5 v/v/v) for 75 minutes, dried in air, and re-developed for an additional 60 minutes to ensure separation of free cholesterol from the mono- and diglyceride bands. Lipid fractions were visualized in iodine vapor against appropriate standards (Supelco, Toronto, Ont.). The free cholesterol band was scraped from the plate and the cholesterol removed by two additions of hexane/chloroform/ether (5:2:1 v/v/v) followed by centrifugation to remove all silica. The cholesterol-containing solvent was transferred to a 7-dram vial and the solvent removed under nitrogen.

Four recovery studies were conducted on the combined extraction-chromatography procedure to determine the net loss of cholesterol from the plasma samples during these procedures. A [^{14}C]-labelled cholesterol standard was introduced into duplicate 4 ml plasma samples and the lipid extraction and chromatography carried out as described. Scintillation counting of the final recovered product revealed a mean loss of 24.8% of the cholesterol standard. This loss was considered acceptable because the FSR calculations depended upon the isotopic enrichment of the cholesterol sample rather than the absolute amount of cholesterol used in the analyses.

Extracts containing approximately 2 mg free cholesterol were transferred in chloroform into Vycor combustion tubes (18 cm x 6 mm, Corning Glass Works, Corning, N.Y.) containing 0.5 g finely ground cupric oxide (BDH, Toronto, Ont.) and a piece of pure silver wire (2.5 cm x 1 mm). Chloroform was removed under vacuum by freezing the tube in liquid nitrogen followed by

gradual heating until the solvent boiled off and pressure returned to baseline. Tubes were sealed under vacuum using a hydrogen torch. The samples were then transported to the Stable Isotope Laboratory at the Clinical Nutrition Research Center, Chicago, IL for mass spectrometric analyses.

Cholesterol was combusted within the Vycor tubes at 750°C for two hours and slowly cooled to room temperature. The water produced during combustion was vacuum distilled into a second Vycor tube containing 80 mg zinc reagent (Biogeochemical Laboratories, Indiana University, Bloomington, IN). Plasma samples, with the exception of the baseline samples, were diluted six-fold with a 5% bovine serum albumin (Sigma, St. Louis, MO) solution to reduce the deuterium enrichment level to within range of the standards. Baseline plasma was not diluted for analysis. Duplicate 2 µl plasma samples were vacuum distilled into zinc-containing reduction tubes. During all transfers, care was taken to avoid contamination from hydrogen in the atmosphere or vacuum lines and to minimize fractionation of the hydrogen isotopes. Deuterium-enriched water from cholesterol and plasma was reduced to hydrogen-deuterium gas by placing the reduction tubes in a 480°C oven for 30 minutes.

The deuterium to hydrogen ratio of the cholesterol or plasma water sample was determined by loading the reduction gas directly into a differential isotope ratio mass spectrometer (Nuclide 3-60 H/D; MAAS, Bellefonte, Pa.). Ratios were determined by comparison of the sample with two gas standards with electrical H_3^+ compensation, and the values expressed relative to the enrichment of standard mean ocean water (SMOW) (Appendices G, H). Samples were re-analyzed if the standard error of the measurement was greater than 1.0 part per mil (°/oo). The coefficient of variation of the analyses performed on the triplicate samples was 3.8%. Triplicate analyses were averaged to obtain the enrichment value for each time point. A small number of individual values were eliminated due to errors in sample preparation or analysis.

III.9. Calculations. Deuterium enrichment of plasma free cholesterol was chosen as an indicator of the rate of cholesterol synthesis occurring in the rapidly exchangeable pool (46, 61). The daily fractional synthetic rate (FSR) was calculated for each of the 12-hour intervals using the model which predicts that the incorporation rate of deuterium into newly synthesized cholesterol is potentially 22 deuterium atoms per cholesterol molecule (57). FSR is a measure of the fraction of the total pool of cholesterol that is synthesized per day. The equation used was:

$$\text{FSR (per day)} = \frac{\delta\ ^2H/^1H \text{ cholesterol } (^o/_{oo}) \times 2}{\delta\ ^2H/^1H \text{ plasma } (^o/_{oo}) \times (22D/27C \times 27C/46H)}$$

where $\delta\ ^2H/^1H$ cholesterol is the difference in cholesterol enrichment over the 12-hour interval and $\delta\ ^2H/^1H$ plasma is the mean plasma enrichment over the entire 48 hour period corrected for the baseline deuterium enrichment. Values were expressed as parts per mil ($^o/_{oo}$) relative to SMOW (see sample calculation in Appendix G). The molecular composition of cholesterol (27 C/46 H) was used to correct for the cholesterol carbon content (61).

III.10. Statistical Analyses. A two-way analysis of variance (ANOVA) was used to analyze differences in the lipid levels of the screening population (78). The differences between clinical characteristics and the dietary intakes of the experimental subject groups were analyzed using independent Student's t-tests (78). Differences in cholesterol FSR were analyzed using an ANOVA of repeated measures (79). Three factors with two levels each were considered in the ANOVA for their effects: apo E phenotype (E2 vs E4), feeding state (fed vs fasted) and time interval (day vs night). The ANOVA of repeated measures was performed using the UBC Statistical Package for the Social Sciences (SPSS-X) program. Subject characteristics other than apo E phenotype were evaluated for their contribution to plasma cholesterol and cholesterol FSR

variations among subjects using simple linear regression or the Pearson product moment correlation with a two-tailed test (78). In all analyses, a value of $p < 0.05$ was accepted as the level of significance.

IV. RESULTS

IV.1. Screening Population Characteristics. Eighteen of the 131 volunteers screened were not considered in the screening group because of either uncertain phenotype identification due to insufficiently resolved protein bands (n=6), plasma cholesterol levels outside the normal range (n=3) or because they were not Caucasian (n=9). The final sample of volunteers who were screened for the study, referred to as the screening population, consisted of 113 normolipidemic Caucasian males (mean (± SE) age = 26.4 ± 0.1) (Appendix A). Apo E phenotype distribution and plasma lipid levels of volunteers screened for the study are presented in Table V. Apo ε-3 allele frequency was greatest (0.752) followed by ε-4 (0.137) and ε-2 (0.111). For analysis of plasma lipid level differences, homozygous E2/2 and E4/4 individuals were grouped with heterozygous E3/2 and E4/3, respectively, to eliminate the problem of performing an ANOVA on groups with an n = 1. Individuals with the ε-2 allele exhibited lower (154.0 ± 1.2), and those with the ε-4 allele higher (164.6 ± 0.9), total plasma cholesterol concentrations (mean ± SEM in mg/dl) than those with the E3/3 phenotype (155.9 ± 0.4). However, these differences were not significant. There were no significant differences in plasma triglyceride levels between any of the phenotype groups. Mean (± SEM) total cholesterol and triglyceride concentrations (in mg/dl) were 157.5 ± 0.2 and 70.0 ± 0.2, respectively, for the total population.

IV.2. Subject Characteristics. The clinical and laboratory data for the 19 subjects are presented in Table VI. Apo E isoform band ratio was used as a criterion for subject selection. The E3/E2 band ratio for the subject group was lower than the mean E3/E2 ratio for the eligible screening population (E3/E2 = 1.00 ± 0.01) indicating the presence of a greater proportion of E2 protein in the subject samples. Subjects in the E4 group had a higher mean E4/E3 ratio, and therefore more E4 protein, compared to the screening group (E4/E3 = 1.07 ± 0.02). At the time of screening,

Table V. Distribution of apo E phenotypes and plasma lipid levels in the screening population.

Apo E Phenotype	n	Frequency (%)	Plasma Lipid Levels* Cholesterol (mg/dl)	Triglyceride
E 2/2	1	0.9	145	95
E 3/2	21	18.6	154.5 (1.3)	70.2 (1.2)
E 3/3	61	54.0	155.9 (0.4)	67.7 (0.4)
E 4/3	27	23.9	163.2 (0.9)	73.6(0.9)
E 4/4	1	0.9	202	55
E 4/2	2	1.7	146.5 (3.9)	82 (15.6)
			ns‡	ns

* values are mean (SEM). ‡ ns = no significant differences between any of the phenotype groups when analyzed as (E2/2+E3/2); E3/3; (E4/4+E4/3); and E4/2.

Table VI. Anthropometric data and plasma lipid levels of the 19 subjects chosen for study.

Subject Code	Apo E Phenotype	Isoform Ratio	Age (yrs)	Height (cm)	Weight (kg)	Body Mass Index (kg/m^2)	Screening Plasma Lipids Cholesterol (mg/dl)	Triglyceride
E2 Group:		(E3:E2)						
CD	E2/2	*	34.7	176.4	82.2	26.4	145	95
KE	E3/2	0.82	20.8	182.1	62.5	18.8	105	69
GK	E3/2	0.97	24.2	177.5	75.7	24.0	182	62
LW	E3/2	0.74	32.9	186.5	82.9	23.8	148	40
RPi	E3/2	0.97	27.5	181.9	84.2	25.4	166	117
DF	E3/2	0.94	22.6	168.7	73.0	25.7	179	117
LVP	E3/2	0.71	26.8	173.5	73.9	24.5	134	57
PG	E3/2	0.98	27.3	173.6	68.1	22.6	150	44
PO	E3/2	0.89	30.2	171.5	64.3	21.9	154	60
Mean		0.88	27.4	176.9	74.1	23.7	151.4‡	73.4
(SEM)		0.01	0.5	0.6	0.9	0.3	2.6	3.3
E4 Group:		(E4:E3)						
MF	E4/4	2.78	28.6	169.3	69.6	24.3	202	55
MB	E4/3	1.57	29.7	187.6	86.9	24.7	147	61
RPe	E4/3	1.15	21.5	175.6	70.8	23.0	128	71
TC	E4/3	1.22	20.3	181.1	75.7	23.1	182	61
TG	E4/3	1.33	23.1	179.6	87.6	27.2	176	53
PH	E4/3	1.18	29.4	186.0	75.3	21.8	184	105
CC	E4/3	1.41	21.5	182.0	79.5	24.0	198	124
DD	E4/3	1.15	23.9	189.1	87.5	24.5	187	82
RH	E4/3	1.77	25.6	172.8	75.4	25.3	174	79
Mean		1.51	24.8	180.3	78.7	24.2	175.3	76.8
(SEM)		0.06	0.4	0.8	0.8	0.2	2.6	2.7
E4/2 Group:								
MG	E4/2	§	30.7	180.9	81.5	24.9	152	104

* this subject had no E3 band. ‡ significantly different from E4 group ($p < 0.05$). § E4:E3 ratio =2.96, E3:E2 ratio = 0.68.

mean plasma cholesterol concentrations in the E2 group (E2/2 + E3/2) were significantly lower than those in the E4 group (E4/4 + E4/3) ($p < 0.05$). Age and body mass index (BMI) were similar in the two groups as were the screening triglyceride levels. The majority of subjects were within the acceptable BMI range of 20 - 25 which is representative of a healthy range of weight. One subject (TG) had a BMI equal to the 27.2 index above which male subjects are considered obese (80). One other subject (KE) had a much lower BMI (18.8) compared with the remaining study group.

IV.3. Evaluation of Previous Dietary Intake. Analysis of three day food records kept by subjects revealed no significant differences between the groups with respect to the proportion of their reported caloric intake resulting from protein, carbohydrate, and alcohol (Table VII). However, subjects in the E2 group derived a significantly smaller proportion of their total calories from fat, compared to members of the E4 group ($p < 0.05$). The fat-derived calories in the E2 group were replaced primarily by carbohydrates (2.9% higher in the E2 group compared to the E4 group), and in five of the nine E2 subjects, alcohol related calories contributed to over 2% of the total. In the E4 group, alcohol contributions of over 2% were found in only three of the nine subjects. The E4/2 subject consumed the greatest proportion of calories as alcohol, accounting for his lower fat and carbohydrate intake. There were no significant differences in the consumption of dietary cholesterol between the subject groups. The mean absolute amount of cholesterol consumed differed by approximately 35 mg.

The nutrient intakes of the subjects reported on three day food records differed from the experimental diet, considered to be representative of the "typical" North American diet (71). The experimental diet provided approximately the same amount of protein, 5.7% more calories as fat, 3.5% fewer calories as carbohydrate, no alcohol, and a dietary cholesterol intake of 66.5 mg/1000 kcal higher than the mean intake reported by all subjects.

Table VII. Major nutrient and energy content of subjects' usual diet*, and caloric content of experimental diet based on estimated energy requirements for each subject.

Subject Code	3 DAY DIET HISTORY						Experimental Energy Intake (kcal/day)
	Calories Derived from:				Cholesterol Intake	Energy Intake	
	Protein	Fat	Carbohydrate	Alcohol			
	(%)				(mg/1000 kcal)	(kcal/day)	
E2 Group:							
CD	15.7	43.0	41.3	0.0	122.2	2750	3160
KE	9.8	30.5	59.7	0.0	138.9	2570	3080
GK	16.5	28.6	48.4	6.5	80.4	2450	3490
LW	14.5	34.7	48.7	2.1	249.6	2550	3350
RPi	16.2	31.2	51.9	0.7	100.0	3080	3380
DF	11.7	32.7	46.1	9.5	185.0	2270	3060
LVP	16.3	26.5	50.3	6.9	114.6	3700	3650
PG	11.2	34.4	54.4	0.0	95.9	3610	3370
PO	11.8	29.3	51.1	7.8	85.0	2260	2820
Mean	13.7	32.3‡	50.2	3.7	130.2	2800	3260
(SEM)	0.3	0.5	0.6	0.4	6.1	60	30
E4 Group:							
MF	13.2	43.7	43.2	0.0	253.1	3180	2940
MB	14.9	41.4	42.3	1.4	180.1	3370	3480
RPe	13.5	36.1	50.4	0.0	71.9	2100	3160
TC	13.8	36.0	49.3	0.9	110.0	2700	3380
TG	11.4	35.5	48.5	4.5	166.9	2810	3460
PH	17.0	34.7	44.7	3.6	88.8	3040	3530
CC	14.0	29.7	56.3	0.0	62.3	3580	3610
DD	13.8	39.0	40.6	6.6	150.0	2920	3850
RH	14.3	35.4	50.3	0.0	155.0	1840	3280
Mean	14.0	36.8	47.3	1.9	137.6	2840	3410
(SEM)	0.2	0.5	0.6	0.3	6.8	60	30
E4/2 Group:							
MG	15.3	29.5	43.2	12.0	125.4	3340	3620
ALL Subjects:							
Mean	13.9	34.3	48.5	3.3	133.4	2850	3350
(SEM)	0.1	0.3	0.3	0.2	2.9	30	10

*determined from three day food records. ‡significantly different from E4 group ($p < 0.05$).

All but three subjects (LVP, PG, MF) reported lower caloric intakes compared to their estimated energy requirements. The energy content of the experimental diet fed to each subject averaged 19 - 24% above the reported intakes for the E2 and E4 groups, respectively, however, no significant weight gains were observed during the feeding days in either group (Table VIII). The mean (± SEM) weight change over the course of the feeding days was a loss of 0.48 ± 0.07 kg in E2 subjects, while E4 subjects had a gain of 0.09 ± 0.07 kg. Daily fluctuations in body weight during the feeding period were only 0.53 and 0.58 % of body weight in the E2 and E4 groups, respectively. All subjects lost weight on the fasting day. Subjects' mean (± SEM) weight after fasting was 1.3 ± 0.02 kg lower in the E2 group and 1.4 ± 0.03 kg lower in the E4 group, compared to the previous day's weight.

IV.4. Plasma Cholesterol Levels During the Experimental Period. Plasma cholesterol levels were measured using fasting plasma obtained one week prior to the experimental period (baseline) and on Days 3, 6, and 7 during the study (Table IX). Cholesterol concentrations from the study days did not vary from baseline in a consistent manner between subjects, so the baseline value was considered as part of the experimental values. During the experimental period, mean cholesterol concentrations for all but two subjects (KE, RPi) were lower than at screening (Table VI). The mean cholesterol level of the E2 group during the experimental period was 9.9% lower than at screening, and the mean E4 cholesterol level was decreased by 12.7%. The differences between the mean plasma cholesterol levels of the two groups at screening and during the experimental period were similar in magnitude, however, unlike the screening difference, the difference between mean experimental cholesterol levels of the E2 and E4 groups was not significant.

IV.5. Effect of Apo E Phenotype on Cholesterol Synthesis Rate. Cholesterol FSR data for each phenotype group are presented in Table X and Figure 1. The results of the repeated measures ANOVA on cholesterol FSR data are compiled in Table XI. A significant overall effect of apo E phenotype on cholesterol FSR was found between subject groups ($p < 0.05$). Individuals carrying

Table VIII. Weight change data for subjects during the experimental period.

Subject Code	Study Weights (kg)							Feeding (Days 1-6) Weight:	
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Change (D6-D1)	Variation (%)
E2 Group:									
CD	82.5	81.9	82.4	82.1	82.3	82.1	81.1	- 0.4	0.27
KE	62.0	62.3	62.1	61.5	62.1	62.1	60.7	+ 0.1	0.44
GK	75.2	76.5	74.7	74.4	74.7	74.6	73.8	- 0.6	1.03
LW	82.4	82.9	82.9	82.9	83.1	82.7	81.8	+ 0.3	0.29
RPi	82.9	83.4	83.3	83.5	83.7	83.0	81.9	+ 0.1	0.36
DF	72.8	72.8	72.9	72.6	72.8	72.2	71.4	- 0.6	0.35
LVP	74.0	73.5	72.6	73.9	73.3	73.1	72.1	- 0.9	0.71
PG	69.5	68.8	68.2	67.9	67.7	67.7	66.7	- 1.8	1.05
PO	64.5	64.9	64.5	64.0	63.6	64.0	63.3	- 0.5	0.73
Mean	74.0	74.1	73.7	73.6	73.7	73.5	72.5	- 0.48	0.58
(SEM)	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.07	0.03
E4 Group:									
MF	69.0	69.1	69.5	69.4	68.8	68.9	68.1	- 0.1	0.40
MB	86.9	87.4	87.4	86.6	86.4	86.9	85.2	0.0	0.47
RPe	70.1	71.8	71.2	70.7	71.1	70.7	69.5	+ 0.6	0.81
TC	75.7	76.4	76.9	76.6	75.7	76.4	74.9	+ 0.7	0.64
TG	88.2	87.6	86.7	86.5	86.6	86.9	85.2	- 1.3	0.77
PH	76.1	75.0	75.5	75.3	75.5	75.6	74.1	- 0.5	0.48
CC	80.8	80.1	80.1	81.1	80.9	81.0	79.2	+ 0.2	0.56
DD	88.0	88.5	89.0	88.3	88.5	88.7	87.2	+ 0.7	0.38
RH	75.8	75.7	76.0	75.9	76.2	76.3	74.9	+ 0.5	0.30
Mean	78.9	79.1	79.1	78.9	78.8	79.0	77.6	+ 0.09	0.53
(SEM)	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.07	0.02
E4/2 Group:									
MG	81.7	81.7	81.9	81.9	82.4	81.9	80.9	+ 0.2	0.13

Table IX. Plasma cholesterol levels of subjects during the experimental period.

Subject Code	Plasma Cholesterol Level (mg/dl) Baseline	Day 3	Day 6	Day 7	Mean Level (mg/dl)	Variation (%)
E2 Group:						
CD	129.3	127.3	150.7	126.2	133.4	8.7
KE	114.6	116.6	110.8	110.8	113.2	2.6
GK	141.8	141.8	133.1	126.3	135.8	5.5
LW	128.3	138.5	144.6	132.4	136.0	5.2
RPi	175.1	171.0	159.8	164.9	167.7	4.0
DF	143.6	160.9	152.7	147.6	151.2	4.9
LVP	123.1	120.0	127.1	141.4	127.9	7.4
PG	139.1	129.0	137.1	143.1	137.1	4.3
PO	124.0	116.1	128.1	132.1	125.1	5.5
Mean	135.4	135.7	138.2	136.1	136.4*	5.3
(SEM)	2.0	2.2	1.7	1.7	1.7	0.2
E4 Group:						
MF	175.1	205.7	177.1	177.1	183.8	8.0
MB	153.5	151.6	134.1	139.9	144.8	6.4
RPe		108.8	121.4	126.3	118.8	7.6
TC	147.7	137.0	141.8	130.2	139.2	5.3
TG	176.9	150.5	153.6	168.8	162.5	7.7
PH	139.3	145.4	146.4	151.5	145.7	3.4
CC	150.5	167.8	159.7	172.9	162.7	6.0
DD	186.1	172.1	170.1	171.1	174.9	4.3
RH	142.1	136.1	150.1	153.1	145.4	5.3
Mean	158.9	152.8	150.5	154.5	153.1	6.0
(SEM)	2.2	3.0	1.9	2.1	2.2	0.2
E4/2 Group:						
MG	115.9	118.0	118.9	130.2	120.8	5.3

* no significant difference between E2 and E4 groups.

Table X. Cholesterol fractional synthetic rate (FSR) in experimental subjects.*

Subject Code	FEEDING Day 0-12	FEEDING Night 12-24	FEEDING Total 0-24	FASTING Day 24-36	FASTING Night 36-48	FASTING Total 24-48	Grand Total 0-48 hours
				(FSR per day)			
E2 Group:							
CD	0.039	0.109	0.074	-0.028	0.027	-0.001	0.037
KE	0.053	0.072	0.063	-0.021	0.001	-0.010	0.026
GK	0.032	0.062	0.047	-0.017	0.023	0.003	0.025
LW	0.121	0.077	0.099	-0.001	0.019	0.009	0.054
RPi	0.032	0.051	0.042	-0.008	0.008	-0.0004	0.021
DF	0.058	0.137	0.098	-0.042	0.048	0.003	0.050
LVP	0.032	0.056	0.044	-0.020	0.006	-0.007	0.019
PG	0.101	0.066	0.083	-0.016	-0.003	-0.010	0.037
PO	0.071	0.088	0.079	-0.018	0.002	-0.008	0.036
Mean	0.060	0.080	0.070	-0.019	0.015	-0.002	0.034
(SEM)	0.004	0.003	0.002	0.001	0.002	0.001	0.001
E4 Group:							
MF	0.120	0.136	0.128	-0.008	0.043	0.018	0.073
MB	0.129	0.085	0.107	-0.010	0.032	0.011	0.059
RPe	0.066	0.115	0.091	-0.039	0.007	-0.016	0.038
TC	0.156	0.079	0.117	-0.004	0.048	0.022	0.070
TG	0.087	0.111	0.099	-0.020	0.004	-0.008	0.045
PH	0.076	0.120	0.098	-0.015	0.003	-0.006	0.046
CC	0.104	0.131	0.117	-0.025	0.041	0.008	0.063
DD	0.040	0.055	0.047	-0.013	0.003	-0.005	0.021
RH	0.052	0.078	0.065	-0.017	0.018	0.0004	0.033
Mean	0.092	0.101	0.097	-0.017	0.022	0.003	0.050
(SEM)	0.004	0.003	0.003	0.001	0.002	0.001	0.002
E4/2 Group:							
MG	0.075	0.092	0.084	-0.007	-0.006	-0.006	0.039
ALL Subjects (n=19):							
Mean	0.076	0.091	0.083	-0.017	0.017	-0.0002	0.042
(SEM)	0.002	0.002	0.002	0.001	0.001	0.001	0.001

*see Table XI. for results of ANOVA to identify significantly different values.

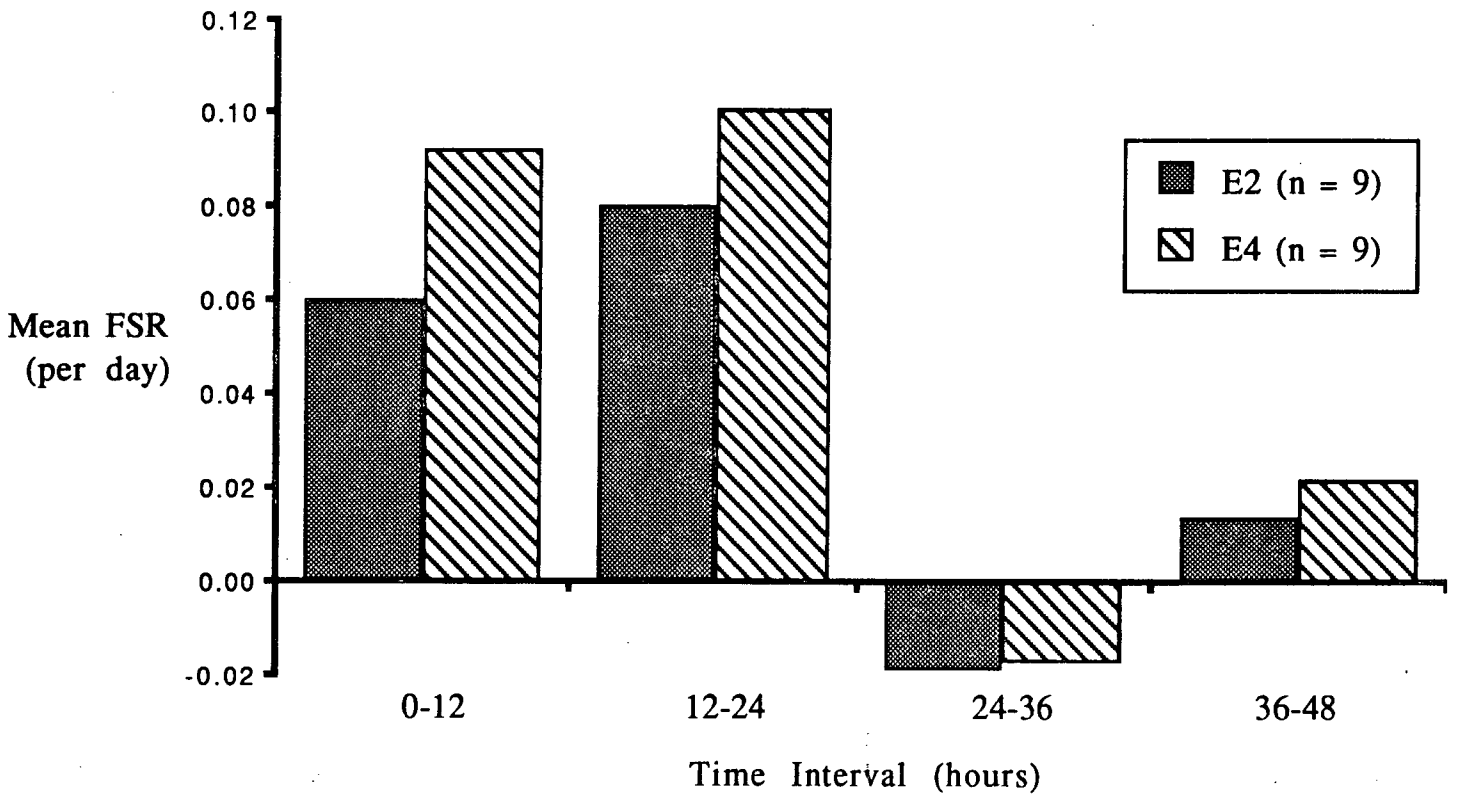

Figure 1. Cholesterol fractional synthetic rates (FSR) in men with different apo E phenotypes. Between subject (E2 vs. E4) comparison: $p < 0.05$)

Table XI. Results of repeated measures ANOVA on cholesterol fractional synthetic rate data in men with different apo E phenotypes.

Comparison	Sum of Squares	Degrees of Freedom	Mean Sum of Squares	F	p
Between Subjects:					
Group*	45.28	1	45.28	4.88	0.042
Subjects within groups	148.53	16	9.28		
Within Subjects:					
F/F‡	1240.85	1	1240.85	135.10	< 0.001
D/N§	115.27	1	115.27	16.66	0.010
F/F by D/N	21.45	1	21.45	5.57	0.031
Group by F/F	21.67	1	21.67	4.81	0.043
Group by D/N	0.36	1	0.36	0.05	0.822
Group by F/F by D/N	3.00	1	3.00	0.78	0.390

* refers to apo E phenotype group (E2 or E4), ‡ refers to feeding condition (fed or fasted), § refers to time interval [day (0730-1930 hrs) or night (1930-0730 hrs)].

the ε-2 allele synthesized cholesterol at a lower rate compared with individuals having the ε-4 allele. Within subject comparisons revealed an interactive effect between apo E phenotype and feeding condition on cholesterol FSR ($p < 0.05$). In particular, FSR differences between groups were significant during the feeding period (0 - 24 hr) with the E2 group FSR being only 72.2% that of the E4 group. The greatest difference in mean cholesterol FSR during the feeding period between the subject groups occurred during the daytime (0 - 12 hr). During this interval a cholesterol FSR difference of 0.032/day was obtained compared with a difference of 0.021/day during the nighttime feeding period. The feeding cholesterol FSR of the E4/2 subject was nearly mid-way between the FSR of the 2 subject groups.

The effect of phenotype group on cholesterol FSR was not as great during the fasting period as that observed during the feeding period. Results of the ANOVA indicated that cholesterol FSR differences between phenotype groups were significant only during feeding. Individuals with different phenotypes responded similarly to the absence of food with a significant drop in cholesterol FSR occurring in both groups during the fasting day (24 - 36 hours). Cholesterol FSR was increased in E2 and E4 groups during the nocturnal periods following both the feeding and fasting days (12 - 24 and 36 - 48 hr) although no significant interaction was found between phenotype group and time interval.

IV.6. Effect of Feeding Condition and Time Interval on Cholesterol Synthesis. The repeated measures ANOVA was used to identify whether an individual's feeding condition affected cholesterol FSR independent of phenotype group, and to determine whether a diurnal variation in cholesterol synthesis rate existed (Figure 2). Feeding condition had the strongest overall effect on cholesterol synthesis compared to phenotype group or time interval. Cholesterol FSR for all subjects was significantly lower during both day- and nighttime fasting periods compared to the day- and nighttime feeding rates ($p < 0.001$) (Figure 3). Negative synthesis rate values were obtained for all subjects during the 24 - 36 hour time interval.

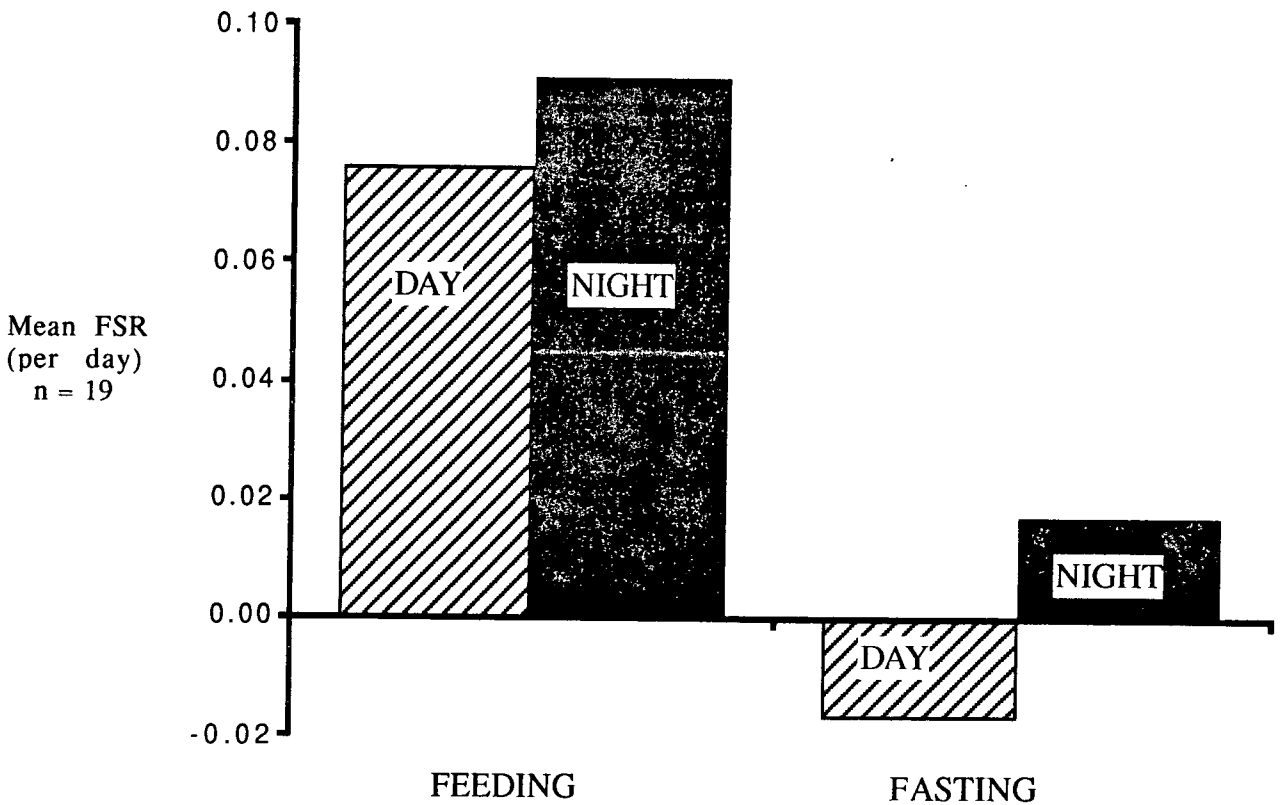

Figure 2. Effect of feeding condition and time interval on cholesterol fractional synthetic rates (FSR) in all subjects.

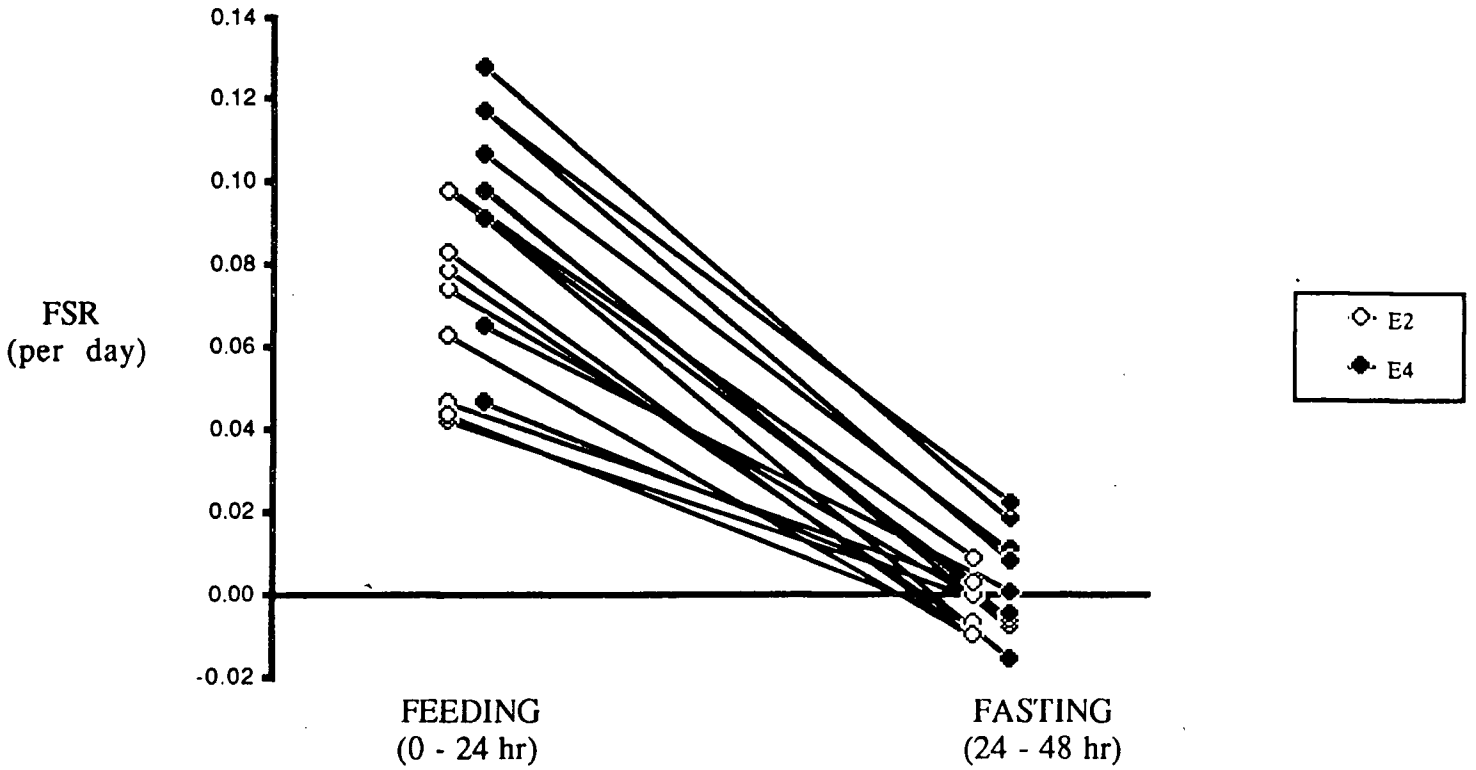

Figure 3. Comparison of cholesterol fractional synthetic rates (FSR) during different feeding conditions in apo E phenotypes. Overall effect of feeding condition on FSR: $p < 0.001$.

Time interval had a significant overall effect on cholesterol FSR ($p < 0.01$). On both the feeding and fasting days, mean FSR was increased during the nocturnal period. Also, an interactive effect was observed between time interval and feeding condition ($p < 0.05$). Greater FSR variation was observed during fasting when mean nocturnal cholesterol FSR increased by a larger degree compared with the FSR increase during the nocturnal period on the feeding day. Four subjects (2 - E2, 2 - E4), had lower synthetic rates during the nocturnal period following feeding, as compared to daytime FSR. On the fasting day, all subjects had increased FSR during the nocturnal period (Figure 4).

IV.7. Relationship Between Cholesterol Synthesis and Other Subject Characteristics. Subject characteristics other than apo E phenotype were examined in order to identify possible correlations with cholesterol FSR. The 0 - 24 hour FSR was chosen for the correlation analyses because synthesis during this time interval was most affected by phenotype group. Two types of data were analyzed: i) dietary factors, and ii) individual characteristics. A summary of the results of the correlation analyses are presented in Table XII.

i) Dietary factors. The relationships between subjects' usual intake of dietary fat and cholesterol and cholesterol FSR were determined. Subjects' previous dietary fat intake was positively correlated with FSR ($r = 0.42$) (Figure 5). This correlation was strongest for those in the E2 group ($r = 0.47$), however, none of the results were significant using a two tailed test. Dietary cholesterol intake was positively correlated with cholesterol FSR for all subjects ($r = 0.33$) (Figure 6), and the correlation was nearly significant for the E2 group ($r = 0.661$, $p = 0.051$). The correlation between dietary cholesterol intake and cholesterol FSR in the E4 group was not significant.

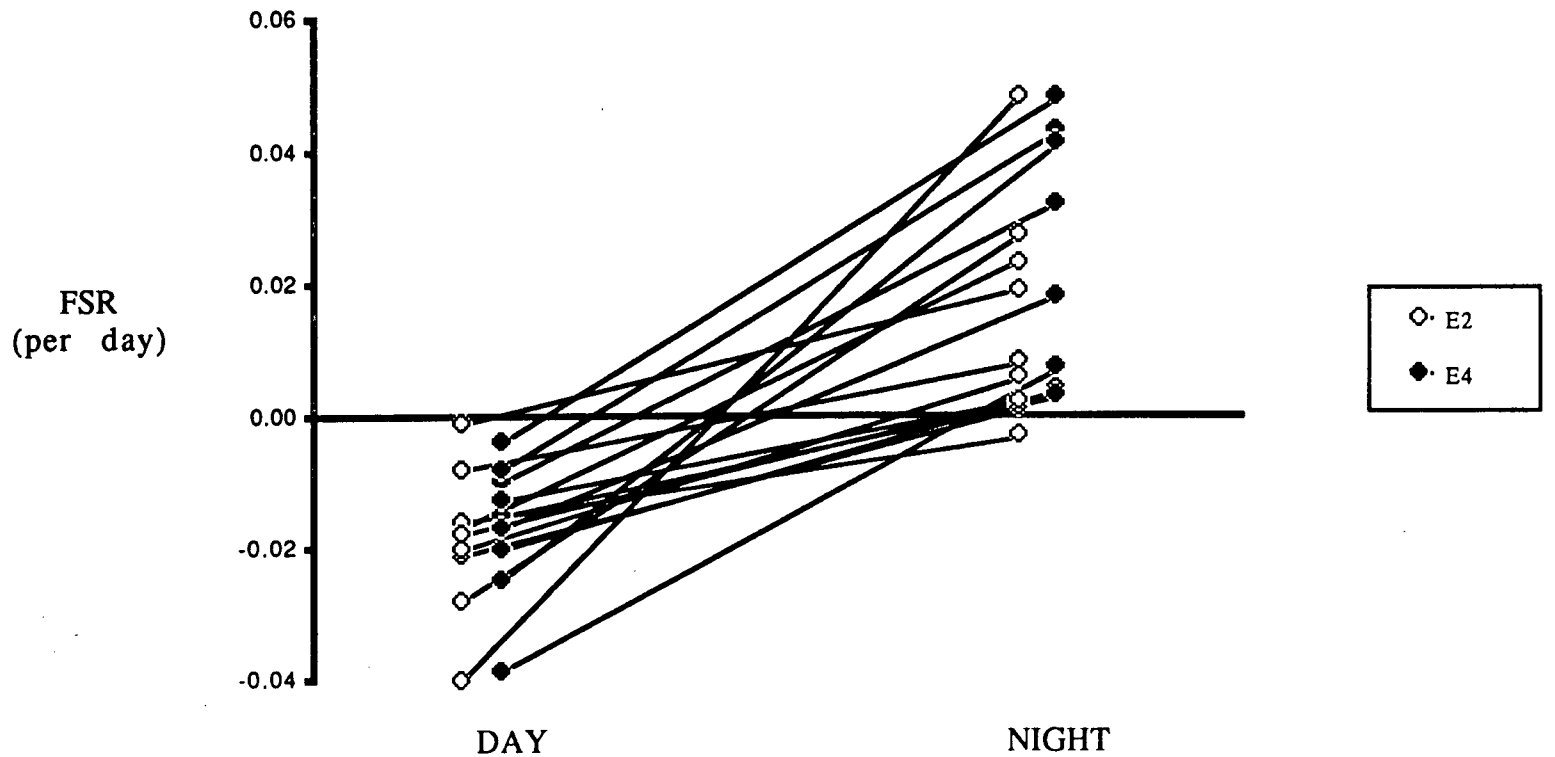

Figure 4. Comparison of day- and nighttime cholesterol fractional synthetic rates (FSR) during the fasting period (24 - 48 hr) in apo E phenotypes. Overall effect of time interval on FSR: $p < 0.01$.

Table XII. Summary of the results of regression and Pearson product moment correlation coefficient analyses.

Variables	All Subjects (n = 19) r	p	E2 Group (n = 9) r	p	E4 Group (n = 9) r	p
1) CHOLESTEROL FSR (0 - 24 hr) and:						
Dietary Fat Intake	0.418	0.072	0.472	0.198	0.043	0.878
Dietary Cholesterol Intake	0.326	0.170	0.661	0.051	0.093	0.798
Plasma Cholesterol Level (during experiment)	0.211	0.390	-0.065	0.842	0.038	0.886
Age	-0.023	0.887	0.259	0.506	0.068	0.839
Body Weight	-0.028	0.874	-0.100	0.785	-0.350	0.358
BMI	0.006	0.929	-0.004	0.939	-0.178	0.649
Band Density Ratios (E2 = E3:E2; E4 = E4:E3)	(NA)		-0.042	0.884	0.398	0.289
2) PLASMA CHOLESTEROL LEVEL and:						
Age	-0.079	0.743	0.064	0.845	0.250	0.522
Body Weight	0.441	0.057	0.647	0.058	0.266	0.494
Dietary Fat Intake	0.418	0.072	0.112	0.766	0.313	0.416
Dietary Cholesterol Intake	0.358	0.129	0.042	0.879	0.609	0.080

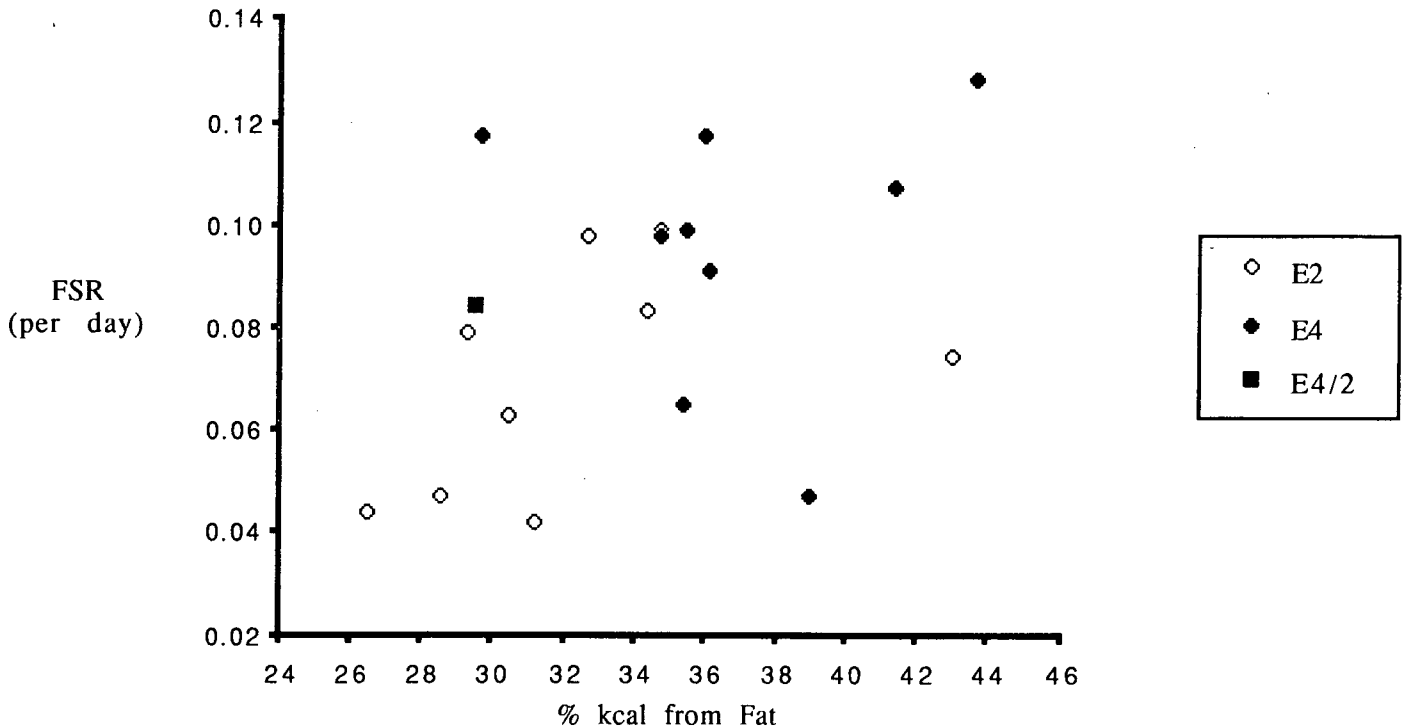

Figure 5. Relationship between usual dietary fat intake and cholesterol fractional synthetic rate (FSR) (0-24 hr). All subjects: r = 0.42, E2: r = 0.47, E4: r = 0.04.

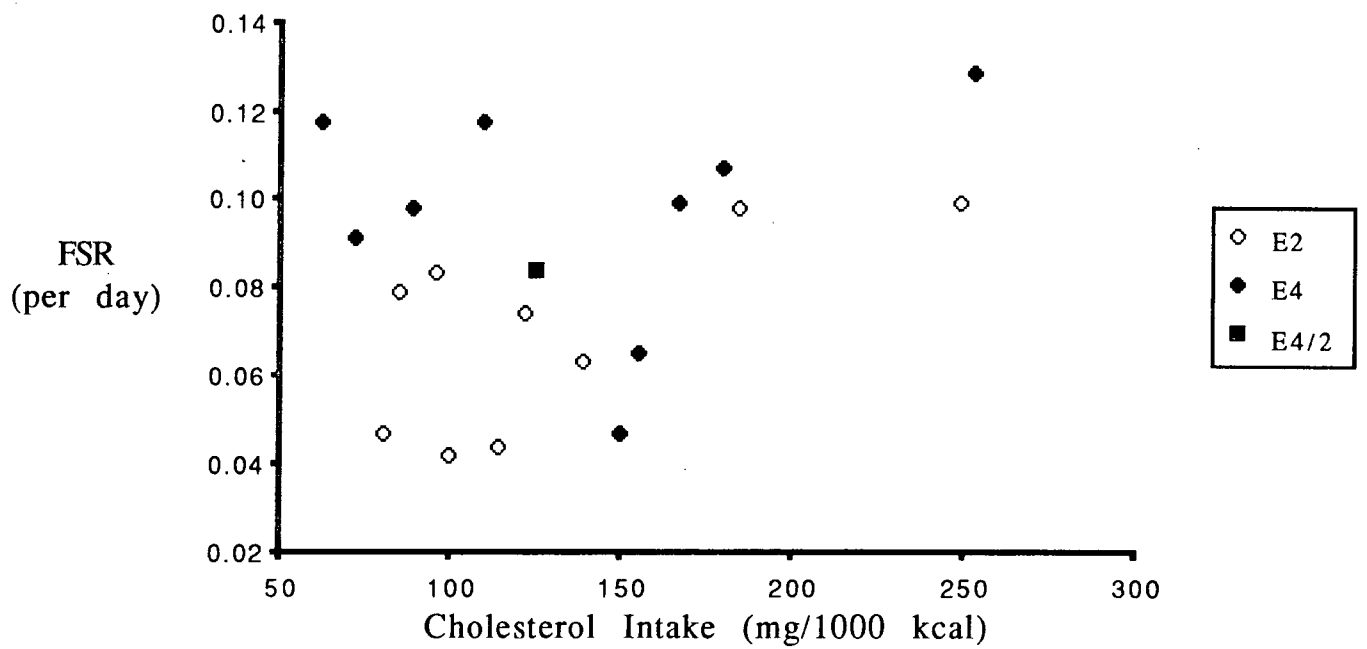

Figure 6. Relationship between usual dietary cholesterol intake and cholesterol fractional synthetic rate (FSR) (0-24 hr). All subjects: r = 0.33, E2: r = 0.66, E4: r = 0.09.

ii) Individual Characteristics. There were no significant correlations between subject groups' mean plasma cholesterol levels during the study period and cholesterol FSR for either phenotype group or for all subjects (Figure 7). Subject's age, BMI, and weight were also considered, but found to have no relationship to FSR. Finally, isoform band densities for subjects in each phenotype group were examined to determine if the proportion of protein coded for by the rare alleles was related to cholesterol synthesis. No significant findings were obtained.

Certain characteristics were also examined for their contribution to the variation in plasma cholesterol levels among the subjects. There was no significant relationship between age and plasma cholesterol level. Body weight, dietary cholesterol and dietary fat intake were positively correlated with plasma cholesterol levels, although none of the correlations were significant (Figures 8 - 10). Body weight was found to be most highly correlated with plasma cholesterol level ($r = 0.441$), particularly in the E2 group ($r = 0.647$).

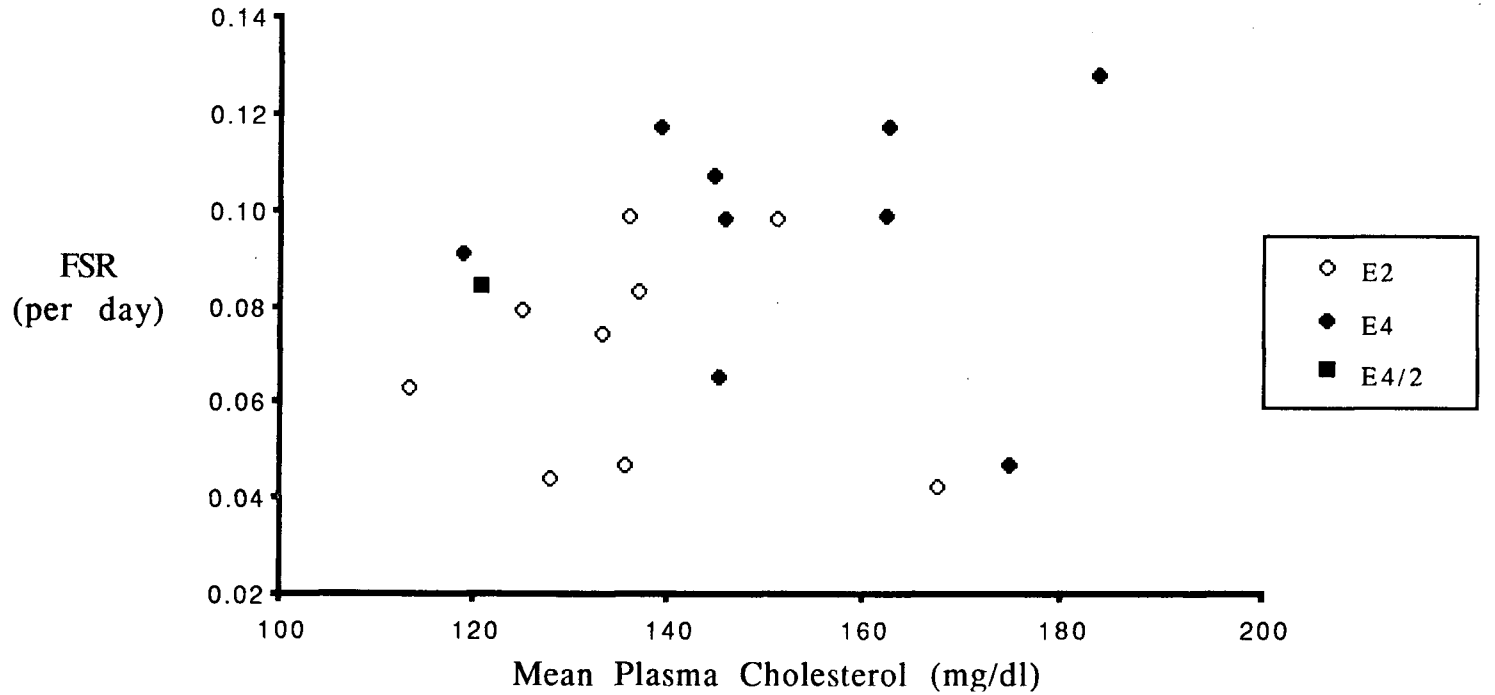

Figure 7. Relationship between mean plasma cholesterol level during the experimental period, and cholesterol fractional synthetic rate (FSR) (0-24 hr). All subjects: r = 0.21, E2: r = -0.07, E4: r = 0.04.

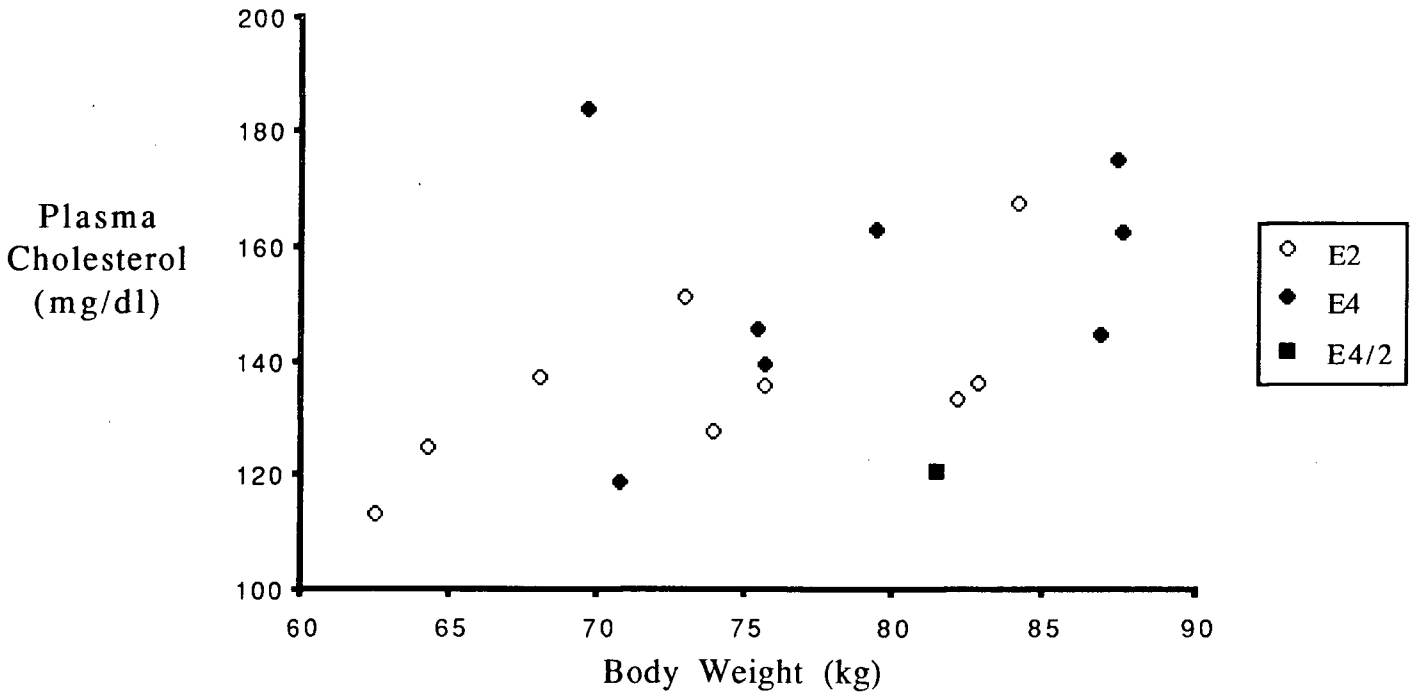

Figure 8. Relationship between body weight and mean plasma cholesterol level during the experimental period. All subjects: r = 0.44, E2: r = 0.65, E4: r = 0.27.

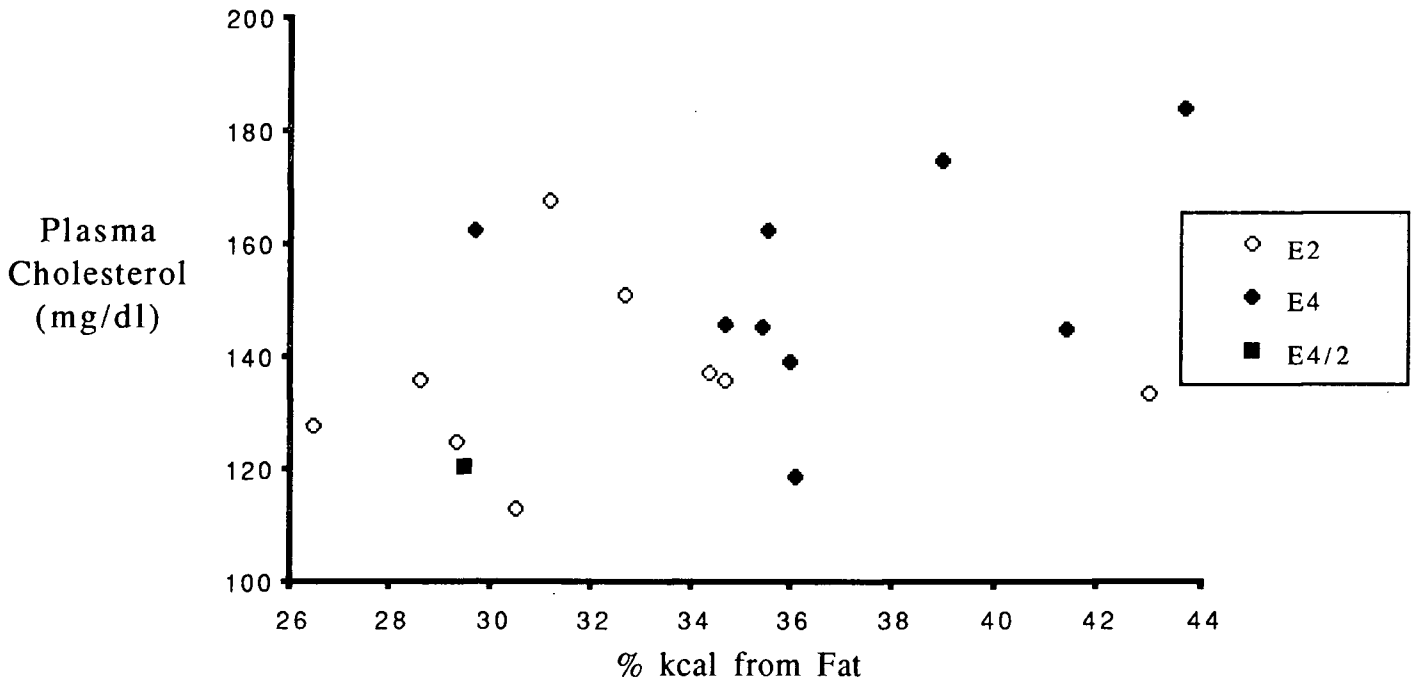

Figure 9. Relationship between usual dietary fat intake and mean plasma cholesterol level during the experimental period. All subjects: r = 0.42, E2: r = 0.11, E4: r = 0.31.

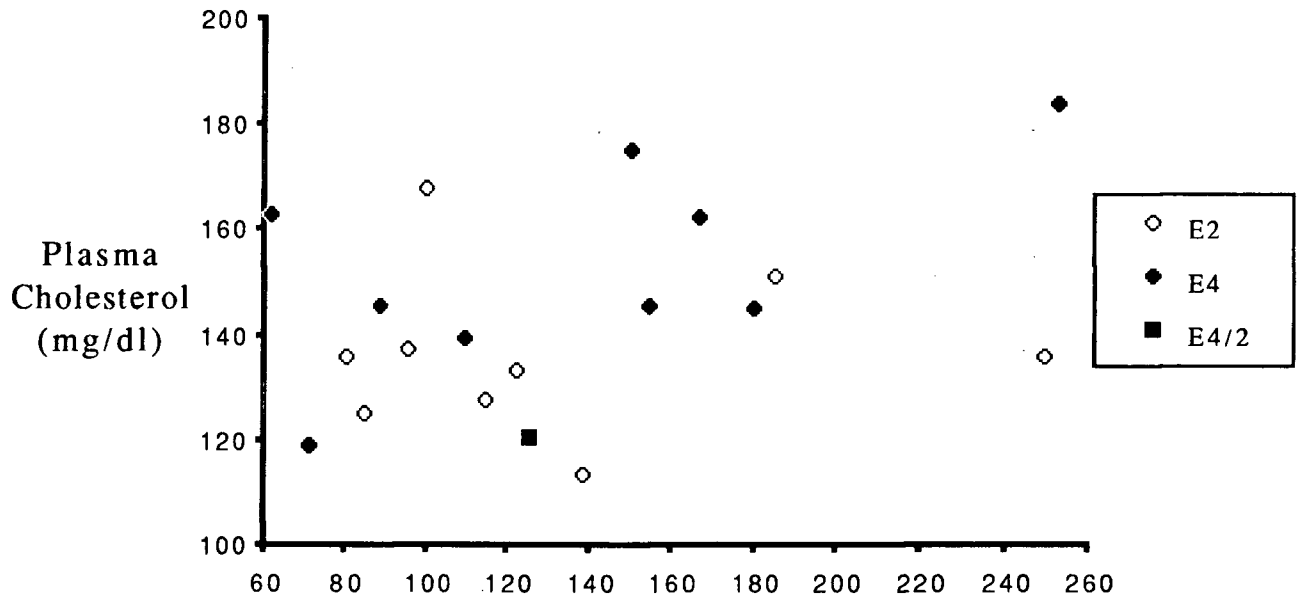

Figure 10. Relationship between usual dietary cholesterol intake and mean plasma cholesterol level during the experimental period. All subjects: r = 0.36, E2: r = 0.04, E4: r = 0.61.

V. DISCUSSION

The primary goal of the present study was to examine the relationship between apo E phenotype and the rate of endogenous cholesterol synthesis. Cholesterol synthesis measurements were carried out over four consecutive 12-hour time intervals in two groups of individuals with different apo E phenotypes. Subjects were studied under conditions of feeding and fasting to evaluate if the cholesterol synthesis differences between groups were altered in response to the absence of influence from dietary components.

V.1. Apo E Allele and Phenotype Distribution in the Screening Population. The apo E allele distribution pattern in the Vancouver screening population was identical to that predicted by all other studies (8-11, 17, 22, 29-33). The apo ε-3 allele frequency was greatest followed by ε-4 and ε-2 frequencies. The size of the allele frequencies varied from those found in other populations. Vancouver's sample population had an ε-2 allele frequency that was slightly higher (0.111), and an ε-4 frequency that was slightly lower (0.137), than the mean allele frequencies calculated for a sub-sample of other Caucasian populations (mean ε-2 = 0.088; mean ε-4 = 0.162) (Table XIII). These differences arose from the larger number of E3/2 individuals and smaller number of E4/4 individuals in the Vancouver group. For example, E3/2 frequency was 8.8% higher and E4/4 frequency was 3.0% lower in the Vancouver population compared with the phenotype frequencies in the normolipidemic population sample from Ottawa, Ontario (8).

The slight differences between the Vancouver and Ottawa population samples are intriguing due to the presence of different rates of IHD across Canada. The rate of deaths attributed to IHD in males is 18% lower in British Columbia compared to Ontario (81). Therefore, Vancouver, with its lower ε-4 and higher ε-2 allele frequency, may also have a rate of IHD that is lower than that of the

Table XIII. Frequency of apo E alleles and phenotypes in selected Caucasian populations.*

	Canada				Mean
	Vancouver (n = 113)	Ottawa (n = 102)	Nancy, France (n = 223)	Helsinki, Finland (n = 615)	Frequency (from Table II)
Alleles					
ε-2	0.111	0.078	0.130	0.063	0.088
ε-3	0.752	0.770	0.742	0.698	0.750
ε-4	0.137	0.152	0.128	0.239	0.162
Phenotypes (%)					
E2/2	0.9	2.0	0.9	0.5	
E3/2	18.6	9.8	20.6	10.1	
E3/3	54.0	61.7	55.2	47.3	
E4/3	23.9	20.6	17.5	34.8	
E4/4	0.9	3.9	2.2	5.8	
E4/2	1.7	2.0	3.6	1.4	

* from references 8, 9, 29.

Ottawa population. The occurrence of IHD has been shown to be related to apo E phenotype and to the cholesterol altering effects of the apo E alleles (11, 12). However, more information about the significance of the apo E allele frequency distribution differences and the presence of other differences in IHD risk factors between Ottawa and Vancouver is needed before conclusions regarding the contribution of the apo E alleles to the incidence of IHD in these two populations can be reached.

It is also possible that the size of each phenotype group, and the relative allele frequencies in the Vancouver screening population may have been under- or overestimated due to problems inherent in the isoelectric focusing technique used for phenotype identification. Sialylated E3 proteins migrating to the E2 position may have caused E3:E2 band ratios to fall on the borderline between E3/3 and E3/2 phenotype classification. Therefore, some individuals of the E3/3 phenotype may have been incorrectly identified as E3/2s, resulting in an overestimation of the size of the E3/2 group and the ε-2 allele frequency, and an underestimation of the ε-3 allele frequency. The apo E phenotype identification method employed in this study has been widely used in other apo E phenotype surveys (8, 30, 66, 67). However, with the recent development of monoclonal antibodies, a method has been developed that eliminates the problems caused by interference of the sialylated proteins allowing more accuracy and confidence in phenotype identification (82). Nevertheless, the method used in this study enabled accurate identification of potential subjects for the cholesterol synthesis measurement study; those with the densest E2 or E4 bands.

V.2. Effect of Apo E Polymorphism on Plasma Lipids. In the majority of studies reported in the literature, apo E polymorphism significantly affects the plasma lipid level distribution in the population under study (8, 9, 11, 17, 26, 29, 30). Compared with individuals of the E3/3 phenotype, individuals with the ε-2 allele tend to have lower, while those with the ε-4 allele tend to have higher, plasma cholesterol levels. In the Vancouver screening population, a trend towards increasing total plasma cholesterol concentrations from individuals with the ε-2 allele to those with

the ε-4 allele was present, although this trend was not statistically significant (Table V). Furthermore, the effect of the ε-2 allele on plasma cholesterol levels, relative to the mean plasma cholesterol level of the E3/3 group, was smaller than the ε-4 allele effect. The mean plasma cholesterol level of individuals with the ε-2 allele (E2/2 + E3/2) was only 1.8 mg/dl lower than the mean plasma cholesterol level of E3/3 individuals. The E4 group (E4/4 + E4/3) had a mean cholesterol level that was 8.7 mg/dl greater than the E3/3 group and 10.5 mg/dl greater than the E2 group. These findings are in contrast with most other reports on cholesterol level distributions between apo E phenotype groups where the effect of the ε-2 allele is usually two to three times greater than that of the ε-4 allele (10, 83). In a sub-sample of populations, the difference between the cholesterol-altering effects of the ε-2 and ε-4 alleles was much greater than that found in the present study, ranging from 13 - 30 mg/dl (10). It is not clear why the effects of the apo E alleles in the Vancouver screening population would differ from those found in other Caucasian populations. The number of volunteers screened for the study was adequate for detection of appropriate subjects, however, a more accurate representation of plasma cholesterol variations would probably have been obtained had the sample size been increased. In addition, it is likely that other genetic and environmental factors were influencing plasma lipid levels in these individuals. The limited information obtained from the volunteers was not adequate to suggest what those factors may be.

There were no significant differences between the plasma triglyceride levels of the phenotype groups in the screening population. This finding is consistent with other reports on normolipidemic subjects suggesting that the relationship between apo E phenotype and plasma lipid levels is specific to cholesterol.

V.3. Comparison of the Physical Characteristics of the Two Study Groups. Anthropometric and clinical characteristics of the subject groups were compared in order to identify the presence of factors other than apo E phenotype that might affect cholesterol FSR. Age and body weight have

been identified as being predictive of some of the variations in model parameters in the three pool model of human cholesterol turnover developed by Goodman et al (47). These parameters include the size of the rapidly exchangeable pool of cholesterol, and the cholesterol production rate (cholesterol absorption + cholesterol synthesis). Age and relative body weight may also contribute to plasma cholesterol variations in a given population (2, 9). The E2 and E4 study groups were not significantly different with respect to the group mean age, weight, and body mass index (BMI) (Table VI). The study group as a whole had a lower mean age compared with most of the studies reported in the literature, although no age-related effects of apo E phenotype distribution on plasma cholesterol levels have been identified (10). Age, BMI and body weight were found to have no significant correlation with cholesterol FSR or plasma cholesterol level (Table XII, Figure 8). It is therefore unlikely that individual differences in subject characteristics between the two groups contributed significantly to variations in cholesterol FSR.

In contrast to the larger screening population, of the subjects selected for study, subjects in the E4 group had significantly higher plasma cholesterol levels than those in the E2 group at the time of screening. The heterozygous subjects selected were those who had the greatest proportion of E4 or E2 protein relative to E3. The more pronounced cholesterol differences in the heterozygotes of the study group suggested that the significance of the effect of the rare alleles might depend on the quantity of the particular apoprotein present on the cholesterol-carrying lipoproteins. However, no significant correlations were found between the amount of rare protein coded for by the apo E allele and cholesterol FSR (Table XII). This indicated that the greater amounts of E2 or E4 protein in the study subjects had not caused them to synthesize cholesterol at a different rate from others in the screening population of the same phenotype.

Although the difference between the screening plasma cholesterol levels of the subject groups was significant, during the experimental period the mean plasma cholesterol differences were not significant (Table IX). Plasma cholesterol determinations are known to vary widely due to

variations within an individual, and to difficulties encountered with the cholesterol assay which cause poor reproducibility of results (4, 84). A consistent decrease in plasma cholesterol concentration occurred in both groups from the time of screening to the time of the experiment. It is likely that this was a result of a systematic difference in the cholesterol assay and not a physiological difference. The experimental plasma cholesterol level was derived from the mean of values from four time points throughout the study period and samples were assayed using the same cholesterol reagent. It is therefore likely that the experimental values were more representative of the subjects' actual plasma cholesterol concentration.

V.4. Dietary Intakes of the Subject Groups. The objective of the dietary assessment was to determine if the two subject groups had similar "usual" nutrient intakes. Major nutrient and energy content of subjects' diets were compared, as were certain dietary factors such as fat and cholesterol intake, and P:S ratio, that may influence individual plasma cholesterol levels (6, 7). Subjects were placed on the four day stabilization diet in order to standardize the nutrient and energy intake of all subjects and to minimize the effects of any pre-experimental dietary differences.

All subjects completed food records for the three days preceding their scheduled experimental period. The three days consisted of two week days (Thursday and Friday) and one weekend day (Saturday) to improve the accuracy of determining subjects' usual daily intake (85). A number of observations emerged regarding the recording abilities, dietary habits and food intake patterns of the entire group (Table VII). The majority of foods consumed by subjects were prepared and consumed at home. Three subjects, however, consumed nearly all meals in the school cafeteria or at restaurants. As a group, subjects appeared capable of recording major food items, although side items, snacks, or "extras" were often omitted and only discovered during follow up questioning. In many cases it appeared that subjects failed to estimate portion sizes carefully, for example, reporting consumption of "one cup" of milk when the actual intake was not exactly 250 ml.

All but three subjects reported consuming a level of energy that was below their requirement as estimated from subjects' height, weight, age and activity schedule. E2 subjects consumed 85.5% and E4 subjects consumed 83.3% of their estimated requirement. These findings are not consistent with reports indicating that a subject's true energy intake can be determined from three days of record keeping (76). The discrepancy between reported and estimated intake is likely due to difficulties encountered with both determinations. Subjects may have under-reported their actual food intake. In addition, their requirements may have been overestimated by the use of the 1.7 activity factor. Daily body weight measurements were taken to evaluate whether subjects were remaining in a state of energy balance. During the five day feeding period of the experiment no significant weight gains were observed in any subject (Table VIII), even for those with the largest variations in reported versus estimated intakes (RH, RPe, GK, and DF), suggesting a variable degree of under-reporting by all subjects. On the contrary, measurement of body weight may not have been adequate to detect weight gains resulting from a mean excess of 460 kcal/day (E2) or 570 kcal/day (E4) for 5 days (E2 = 2300 total kcal; E4 = 2850 total kcal). These weight fluctuations may also have resulted from differences in the hydration state of the subjects. Subjects were instructed to consume six - 250 ml portions of fluid per day, although most did not appear to make a conscious effort to monitor fluid intake, or to replace fluid lost during exercise.

In general, subjects' mean protein intake was near the level considered typical of the North American intake (71) and was adequate for meeting the Canadian recommended intake of dietary protein for individuals of their age and sex category (86). Subjects' mean carbohydrate intake was higher than the typical North American diet (71), although it was closer to what is presently considered a desirable intake (5). The mean intake of alcohol-related calories in both groups could be attributed to intake by only a few subjects who consumed 3 - 8% of their calories as alcohol. Alcohol-related calories may have been overestimated because subjects recorded their intakes on Friday and Saturday, times at which the majority of alcohol consumption occurs for some

individuals. These weekend alcohol-related calories would probably have accounted for a smaller percentage of total intake if seven day records had been collected.

As a group, subjects' mean fat intake was lower than that considered representative of the typical North American diet (71). A significant difference between the fat intakes of the E2 (32.3% of total calories) and E4 (36.8%) groups was observed. All subjects may have under-reported their actual fat intake judging by answers to follow-up questions on cooking methods and the use of extra foods. Underestimation of fat consumption would also account for the lower-than-estimated energy intakes but it is likely that this under-reporting would occur to the same extent within each subject group and would not alter the differences between groups. Unfortunately, it was not possible to accurately determine a P:S ratio due to widespread omission of brand names, and uncertain sources of fat contained in cafeteria or restaurant meals reported on the food records.

Cholesterol intakes were similar between subject groups when expressed per unit of energy intake. The higher energy intake in the E4 group resulted in a net cholesterol intake that was approximately 35 mg greater in the E4 group than in the E2 group. Both groups' mean cholesterol intake was lower than the usual North American intake of 214 mg/1000 kcal (71). Determination of group cholesterol intake was probably the least accurate of all dietary determinations because estimates of the recording time required for accurate assessment of cholesterol intake vary from 9 to 13 days (76, 87).

Statistical analyses required in evaluating the dietary intake and physical characteristics of the subject groups may have produced erroneous findings. The probability of committing a type I error and rejecting the null hypothesis at the $p = 0.05$ level when indeed the null hypothesis was true is approximately 50% with the performance of seven Students' t-tests (78). It is therefore possible that the finding of a significant difference between the dietary fat intakes of the two subject

groups was in error. Regardless, the presence of this difference was considered for its possible influence on cholesterol FSR.

V.5. Evaluation of Subject Compliance with Experimental Protocol. Few problems were encountered with subject participation in the experiment. With a few exceptions, all subjects were on time for all meals and blood sampling appointments. Subjects consumed their entire meal on each occasion. Initially, subjects complained about the size of the breakfast meal, however, by Day 3 most of the subjects seemed to have adjusted to the isocaloric meals. One of the most difficult situations arose with one subject who usually consumed 6 - 8 cups of coffee or tea per day. This subject complained of headaches and drowsiness which he attributed to the absence of caffeine-containing beverages from the experimental diet.

It is believed that subjects complied with the experimental protocol. Subjects reported consuming no food other than that which was provided. However, no sensitive method was available for determining if extra food had been consumed. Body weight measurements indicated that most subjects remained in a state of energy balance. On the fasting day, most subjects remained in the UBC testing facility during the intervals when not in class or at work. Plasma was observed for the presence of chylomicrons which may have indicated that a subject had not complied with the fasting protocol. No chylomicrons were detected visually in the fasting plasma.

Finally, it is believed that subjects also complied with the deuterium labelling procedure. The priming dose of D_2O was consumed under supervision. Body water deuterium enrichment rose to the expected range in all subjects, and varied by a mean value of 2.9% in the E2 group and 4.7% in the E4 group (Appendix H). These values suggest that appropriate amounts of the deuterium labelled water had been consumed during the experimental period.

V.6. Major Findings from the Calculation of Cholesterol FSR in the Two Subject Groups. Three major findings were obtained by the determination of cholesterol FSR in the two groups of subjects with different apo E phenotypes. These major findings include:

1. cholesterol FSR was related to apo E phenotype
2. cholesterol FSR was related to an individual's feeding condition, and
3. cholesterol FSR was related to the time interval during which the measurements were made.

These major findings are qualified by two significant interactive effects on cholesterol FSR occurring between the factors under study. Namely:

1. an interaction between an individual's phenotype group and their feeding condition influenced cholesterol FSR, and
2. cholesterol FSR was affected by an interaction between feeding condition and time interval.

Each of these major findings will be discussed as follows:

V.6a. Relationship Between Apo E Phenotype and Cholesterol FSR. The present results indicate that the fractional rate of endogenous cholesterol synthesis was influenced by apo E phenotype. Significantly lower cholesterol FSR was observed in the E2 group compared with the E4 group, particularly during the feeding period (0 - 24 hr) (Figure 1). Cholesterol FSR during this period may therefore be a contributor to the different concentrations of plasma cholesterol usually observed in these groups of individuals.

It was interesting to note that the cholesterol FSR of the subject with both an ε-2 and an ε-4 allele (E4/2 phenotype) synthesized cholesterol at a level that was mid-way between those in the E2 and E4 groups. This finding indicates that the effects of the two alleles on cholesterol FSR may be additive. However, it is impossible to determine this conclusively using data obtained from a single subject.

Determination of cholesterol FSR during the fasting period provided an opportunity to evaluate whether apo E-related differences could be demonstrated at near-baseline rates of cholesterol synthesis in the two groups. During fasting, feedback regulation of cholesterol synthesis was expected to be reduced due to the absence of dietary cholesterol. However, there was no significant effect of apo E phenotype group on cholesterol synthetic rate during the fasting period. Findings of the study indicated there were no differences in the ways that individuals of different apo E phenotypes responded to the removal of dietary influences on cholesterol metabolism. It is possible that the magnitude of the effect of feeding condition on cholesterol FSR obscured any effect of apo E phenotype group that may have been present.

The rates of cholesterol synthesis calculated during the feeding period in this study are inconsistent with the predictions made by the current hypothesis on the mechanisms by which apo E affects plasma cholesterol levels. Utermann and others focus on the regulation of cholesterol homeostasis by the production of LDL receptors (10, 17, 37, 38, 83). They suggest that defective E2 binding may cause up-regulation of the LDL receptor system resulting in increased receptor production and enhanced removal of LDL from plasma. Also, the increased catabolic rate of E4-containing lipoproteins may cause down-regulation of the system due to an increase in cellular cholesterol concentration. This would result in a reduced rate of synthesis of LDL receptors and an accumulation of LDL in plasma. Regulation of the LDL receptor system by these mechanisms would predict that endogenous cholesterol production would be higher in individuals carrying the ε-2 allele and lower in individuals carrying the ε-4 allele. Over the long term, however, if these hypothetical production rates were to continue, higher cholesterol levels in E2 individuals and lower plasma cholesterol levels in E4 individuals would be predicted. This study's finding of endogenous cholesterol synthesis rates opposite to those predicted by Utermann's theory suggests that regulation of the LDL receptor system primarily by synthesis of LDL receptors may contribute only partially to the cholesterol-altering potential of the apo E alleles.

Only one other study of cholesterol synthesis in men with different apo E phenotypes has been reported. Kesaniemi and co-workers found increased rates of synthesis in E2 subjects compared with E4 subjects using measurements of serum lathosterol as an indicator of cholesterol synthesis (17). The findings of these researchers are consistent with the direction of cholesterol synthesis predicted by Utermann's hypothesis. However, serum sterol measurements, while useful for determining relative rates of synthesis under certain metabolic conditions, must be interpreted with caution due to the possibility of daily fluctuations in sterol precursor concentrations (51). Furthermore, serum lathosterol results were expressed relative to the cholesterol concentration of the sample because expression of absolute concentrations of lathosterol may not correlate well with HMG Co A reductase activity (88). This presentation may artificially enhance lathosterol differences because plasma cholesterol levels between the groups were significantly different. Cholesterol synthesis, when measured using sterol balance methods, was not different in the phenotype groups in the same study.

The method employed in the present study measured cholesterol FSR throughout an entire 24-hour period, rather than at a fixed time point. Although FSR data are not direct measurements of the amount of cholesterol synthesized, they can be related to the estimated total pool size to yield absolute production values. Since there were no significant differences in the mean BMI or body weights of the two subject groups, and since mean plasma cholesterol levels of the two groups were only slightly different, it is not unreasonable to assume that differences in the total body cholesterol pool sizes between groups were small. The absolute amount of cholesterol synthesized would therefore be greater in the E4 group as compared to the E2 group.

A possible mechanism for increased cholesterol synthesis in individuals carrying the ε-4 allele compared to those with the ε-2 allele may involve fluctuations in the daily pattern of cholesterol synthesis. Following a normal meal, rapid clearance of exogenous cholesterol-containing

remnants by E4 individuals may indeed down-regulate the LDL receptor system and cause an accumulation of LDL in plasma (37). Over the course of the day, however, in the absence of short -term influences of dietary cholesterol, up-regulation of the receptor system may occur and the synthetic rate of cholesterol may rise. In E2 individuals, delayed clearance of chylomicron remnant particles may cause initial up-regulation of the LDL receptor system and removal of LDL from plasma (37). A period of down-regulation and reduced synthesis could then occur as exogenous and endogenous cholesterol eventually become incorporated into hepatic cells leading to a longer-term inhibition of cholesterol synthesis. It is not clear how much time would be required for these long-term changes in the regulation of cholesterol synthesis to occur. Additional information about the daily pattern of cholesterol synthesis regulation by the LDL receptor system would be helpful in explaining these findings, however a review of the literature failed to locate studies that had been conducted to investigate this important area of research. What is clear from the findings of this study is that the total daily production rate of cholesterol is greater in individuals of the E4/4 or E4/3 phenotypes, and lower in individuals of the E2/2 or E3/2 phenotypes. The overall effects of these cholesterol synthetic rate differences would be the possibility of E4 individuals developing higher plasma cholesterol levels compared to E2 individuals.

The finding of higher rates of cholesterol synthesis in subjects in the E4 group compares favorably with the higher levels of plasma cholesterol in the same group. However, while the mean plasma total cholesterol concentration in the E4 group was higher than that of the E2 group, the difference was not statistically significant. In the majority of studies on plasma cholesterol variations in apo E phenotypes, a significant difference has been observed between the cholesterol levels of E4 and E2 individuals. A few recent studies, however, have not found this relationship to be significant even though the trend of increasing plasma total cholesterol from E2/2 to E4/4 individuals was present (37, 89). However, plasma LDL cholesterol concentrations have been found to be significantly higher in individuals with the E4/4 and E4/3 phenotype compared to E3/2 individuals, in the absence of differences in plasma total cholesterol (37). This suggests a need to better characterize

the cholesterol distribution among the various lipoprotein fractions, particularly cholesterol carried within the LDL since cholesterol variations between phenotype groups are mainly attributed to differences in LDL cholesterol concentration (38). Unfortunately, LDL cholesterol concentrations were not determined in the present experiment.

One assumption of the deuterium incorporation model is that subjects in both groups were receiving the same amount of absorbed dietary cholesterol in order to impose identical degrees of feedback regulation on the LDL receptor system. In this study it was not possible to ensure that the absolute amount of cholesterol being absorbed was identical in all subjects. For practical reasons, subjects consumed a level of dietary cholesterol that was proportional to their caloric intake. The mean cholesterol intake of the groups differed by approximately 35 mg. Kesaniemi and co-workers have recently suggested that E4 individuals have an increased efficiency of cholesterol absorption compared to E2 individuals (17). However, this difference in dietary cholesterol input may only affect the short-term regulation of synthesis rates. Differing amounts of incoming dietary cholesterol may also alter the size of the free cholesterol pool under study and affect the accuracy of FSR determinations. This could result in underestimation of FSR during the feeding day. If E4 individuals were indeed absorbing more cholesterol than E2s, greater underestimation of cholesterol FSR would result, further increasing the differences in calculated daily synthetic rates.

One final factor that may have contributed to the variations in cholesterol FSR between subjects in the apo E phenotype groups was their dietary intake prior to the experimental period. Subjects' usual dietary fat intake was positively correlated with cholesterol FSR during the feeding period, although this correlation was not significant (Figure 5). Further, subjects in the E2 group showed a nearly significant positive correlation between usual dietary cholesterol intake and cholesterol FSR (Figure 6). Metabolic studies have demonstrated that subjects respond with increases in their plasma cholesterol concentrations when placed on diets high in fat, and in particular saturated fat

(6). The opposite response occurs when subjects are placed on a low fat diet (90). The plasma cholesterol response to dietary cholesterol is usually more limited and varied in outpatient populations (91). It appears that individuals differ in their ability to regulate plasma cholesterol concentrations in response to dietary influences because consistent responses are not seen in all subjects (6, 7, 91).

The mechanisms by which dietary fat and cholesterol influence plasma cholesterol concentrations are likely related to regulation of all stages of cholesterol metabolism including cholesterol absorption (92), synthesis of cholesterol and LDL receptors (7, 91), and cholesterol excretion (93). Although the subjects in the two study groups usually consumed different amounts of fat, all were placed on the experimental diet for four days before cholesterol synthesis rate measurements were carried out. This stabilization period should have been long enough to reduce or eliminate influences the pre-experimental diets may have had on cholesterol metabolism within each subject.

It is not clear why the dietary cholesterol intake of subjects in the E2 group would be more highly correlated with cholesterol FSR than that of the E4 subjects. Both groups consumed similar amounts of dietary cholesterol, yet the E2 subjects had a significantly lower rate of cholesterol synthesis than the E4 subjects. The mean plasma cholesterol concentration of subjects in the E2 group was 16.7 mg/dl lower than the mean concentration for subjects in the E4 group. It is possible that a relationship between usual dietary cholesterol intake and cholesterol synthesis exists only in individuals who have plasma cholesterol concentrations below a certain threshold level. A significant relationship between dietary cholesterol intake and total plasma cholesterol concentration has been found in the Tarahumara Indians of Mexico whose mean cholesterol intake is 71 mg/day and mean plasma cholesterol concentration is 134 mg/dl for adult males (94). Seven of the subjects in the E2 group, compared to only two in the E4 group, had plasma cholesterol concentrations near this mean value for the Tarahumaras. It is interesting that the correlation between cholesterol intake and cholesterol FSR was found only in the E2 subject group. Absence

of a correlation between plasma cholesterol level and dietary cholesterol intake, as was found in the Tarahumaras, is not surprising considering the usual intake of cholesterol by the subjects, and the cholesterol content of the experimental diet, was well above that consumed by the Tarahumaras. The correlation between cholesterol intake and cholesterol FSR in the E2 and not the E4 group may simply have been a factor of the inability to accurately determine cholesterol intake from three day records (76, 87). In addition, the stabilization diet was designed to reduce any influence of previous dietary intake on cholesterol metabolism during the experimental period. Nevertheless, it would be interesting to look further into the differences in regulation of cholesterol synthesis occurring in individuals of various plasma cholesterol concentrations.

V.6b. Relationship Between Feeding Condition and Cholesterol FSR. The data demonstrate a significant relationship between cholesterol FSR and an individual's feeding condition. Feeding condition had the strongest overall effect on cholesterol synthesis (Figure 3). During the fasting period, physiological changes occurring in all subjects resulted in a decrease in daytime cholesterol FSR from the previous feeding period. A small but significant recovery in cholesterol FSR occurred during the night (36 - 48 hr).

Fasting-induced reductions in cholesterol synthesis rates have been clearly documented in experimental animals (95, 96). The exact mechanisms which regulate cholesterol synthesis during fasting remain unclear although cellular cholesterol balance, hormones, and the body's demand for energy are probably involved. The absence of dietary cholesterol would cause a reduction in hepatic cellular cholesterol concentration. This condition would cause up-regulation of cholesterol synthesis and LDL receptor production (35). Findings of the present study contradict those predicted by the regulation of the LDL receptor system suggesting the presence of other factors during fasting that are responsible for regulation of cholesterol metabolism.

Hormonal changes during the fasting period could affect regulation of cholesterol synthesis by altering HMG Co A reductase metabolism. In the feeding state, insulin stimulates not only the amount of enzyme present, but the proportion of enzyme present in the active state (97). On the contrary, catecholamines and glucagon have been found to suppress HMG Co A reductase activity in the liver and intestine of laboratory animals (96). Thus, the marked decrease in FSR in humans may be related to decreased insulin and increased glucagon levels that occur as a result of fasting.

Further inhibition of cholesterol synthesis could arise due to the reduced availability of substrates required for energy production during the short-term fast. Cholesterol synthesis is dependent upon the concentration of and competition for the precursor acetyl Co A (98). In the fed state, acetyl Co A is produced by the metabolism of carbohydrate and the production of pyruvate. High concentrations of acetyl Co A allow use of this substrate for cholesterol and fatty acid synthesis as well as for energy production by way of the tricarboxylic acid cycle. During a fast, acetyl Co A production from glucose is reduced due to the shift in cellular metabolism towards gluconeogenic processes. However, the cell's need for acetyl Co A for energy production continues.

In this study, during the 24 - 36 hour period on the fasting day, subjects' energy requirements were likely being met by the oxidation of free fatty acids. Some of the acetyl Co A produced during this process would usually be made available for cholesterol biosynthesis. However, energy required by the fasting subjects could also have been derived from a shift in the use of acetyl Co A units from cholesterol synthesis towards pathways of energy production (61). The net result would be a reduction in cholesterol synthesis, as was demonstrated in this study. During the nocturnal period following fasting, increased cholesterol FSR may have resulted due to less of a demand for energy, allowing acetyl Co A to once again be used as a substrate for cholesterogenesis even though changes in hormone status remained constant.

Reduced rates of cholesterol synthesis in humans during fasting has been previously observed. Using the deuterium incorporation technique, Jones and co-workers demonstrated a significantly reduced rate of cholesterol synthesis during the nocturnal period following fasting (0.004 ± 0.006/day) as compared to following feeding (0.052 ± 0.005/day) in five healthy males (61). The rates during both periods were lower than the findings obtained in the present study. The order of the measurement periods differed from that of the present study by having the fasting period before the feeding period. Perhaps the cholesterol FSR following feeding was underestimated due to a lag effect of fasting on feeding day cholesterol FSR. HMG Co A reductase activity is thought to be extremely sensitive to nutritional state in animals, requiring more time to recover from a fasting period than to disappear upon the initiation of fasting (95). Whether human HMG Co A reductase is sensitive to nutritional state is not known, but if so, it may have contributed to the delayed rise in cholesterol FSR on the feeding day. Changes in cholesterol synthesis during fasting have been measured most often using serum sterols as indirect indicators of cholesterol synthesis (99). Reduced cholesterol synthesis in humans following 12 days of fasting has been documented using serial measurements of serum mevalonic acid (51).

V.6c. Relationship Between Time Interval and Cholesterol FSR. Findings of this study revealed the presence of daily fluctuations in cholesterol synthesis. Knowledge of cholesterol FSR during the period following feeding was essential for evaluation of the net rate of synthesis during the 24-hour feeding period. The nocturnal period may also provide an important baseline measurement. Upon entering the body cholesterol pool, dietary cholesterol may impose feedback regulation of cholesterol production, and/or increase free cholesterol pool size. These effects would be minimized during the latter portion of the nocturnal period after the absorption of dietary components had been completed.

Time interval had a significant overall effect on cholesterol FSR. Mean nocturnal FSR was greatest in both phenotype groups compared to daytime FSR indicating the presence of diurnal

variations in cholesterol synthesis. Individual responses were more variable, as four out of 19 subjects showed decreases in cholesterol FSR during the nocturnal period following feeding. Differences between mean day- and nighttime FSR were greatest on the fasting day (Figure 4).

Animals exhibit significant diurnal variations in cholesterol synthesis (100, 101). Cholesterol synthesis peaks during the dark cycle, the time during which most laboratory animals are active, possibly as a response to feeding (102). However, in animals fed ad libitum diets, cholesterol synthesis rates are highest six hours after the onset of the dark cycle suggesting that a circadian rhythm exists that is not entirely dependent upon food intake (95). Both the activity and the cellular content of HMG Co A reductase are increased during an animal's dark cycle resulting in a high active enzyme/total enzyme ratio and higher rates of cholesterol synthesis (98). Other researchers have found that during the light cycle the rate of cholesterol synthesis, rather than the amount of enzyme present, is reduced (95), suggesting that regulation occurs primarily through regulation of HMG Co A reductase activity.

In humans, diurnal variation in cholesterol synthesis probably exists as a result of fluctuations in the activity and amount of HMG Co A reductase, similar to findings in animals (103). However, cholesterol synthesis measurements have not been routinely made over short time intervals so little is known about these daily fluctuations in cholesterol FSR. Serial measurements of certain cholesterol precursors were found to vary by several-fold during the day- and nighttime periods (103). These measurements suggested that nocturnal rates of cholesterol synthesis were greater than those in the daytime.

The findings of the present study differ from those reported by Jones et al (61). Using deuterium incorporation methodology, these investigators reported that nocturnal FSR following fasting was lower than the daytime fasting FSR. In addition, cholesterol FSR was increased following the feeding period to a greater extent than that found in the present study. It is not clear why these

different results were obtained. The difference may be due to the reversed order of the feeding and fasting days, and the carry over effects of fasting into the feeding period.

V.7. Evaluation of the Deuterium Incorporation Methodology. The method employed for this study to determine cholesterol FSR was based on a number of assumptions. The finding of negative synthetic rates in all subjects during the fasting day indicates problems with certain aspects of the method. Negative fractional synthetic rates clearly suggest that actual FSR has been underestimated for all time periods evaluated during the study. It is likely that this underestimation is a result of the entrance of unlabelled free cholesterol into the plasma compartment. Since the negative values were determined on the fasting day, dietary cholesterol would not be considered a significant cause of the dilution of the free plasma cholesterol pool. The most likely source of free cholesterol would be the result of de-esterification of cholesterol esters within the central pool (104). Entrance of free cholesterol from either of the two side pools could also contribute to the findings of negative cholesterol FSR since a small fraction of these pools is turning over each day (46). If the free cholesterol had been newly synthesized in the side pools, the molecules would contain a similar proportion of deuterium atoms since D_2O is able to penetrate all cell compartments. However, the rate of cholesterol synthesis in these other tissues may not be identical to that occurring in the central pool leading to additional errors in cholesterol FSR calculations.

It is also possible that some newly synthesized cholesterol within the central pool was being removed soon after synthesis, preventing exchange of the labelled cholesterol with the unlabelled cholesterol carried on lipoproteins. Deuterium-enriched cholesterol may have been used for bile acid production soon after synthesis. Newly synthesized hepatic free cholesterol makes up 25% of the cholesterol substrate available for bile acid synthesis (105). Also, esterification of newly synthesized cholesterol immediately following synthesis would cause enrichment of the cholesterol ester pool rather than the free cholesterol pool. Since this method assumes that newly synthesized

cholesterol remains unesterified during the measurement interval, greater underestimation of cholesterol FSR would occur. Analysis of cholesterol ester deuterium enrichment would provide valuable information on the behavior of the newly synthesized cholesterol during the 48 hour measurement period.

A baseline level of cholesterol synthesis most likely is always present in all cells in order to meet their need for cholesterol. It is not known to what level the negative enrichment values would fall were the period of fasting increased, or other means of suppressing cholesterol synthesis employed. It would be interesting to determine the minimum rate of cholesterol synthesis occurring in the central pool. Perhaps a study could be designed using cholesterol-free diets where cholesterol synthetic rates could be determined in the absence of the influence of dietary cholesterol, but under otherwise normal physiological conditions.

Nevertheless, the deuterium incorporation methodology allowed determination of differences in the cholesterol FSR occurring between two groups of individuals with different genetic backgrounds, and clearly identified an influence of feeding condition, apo E phenotype, and time interval on regulation of cholesterol synthesis. Further refinement of the FSR model may be necessary to improve the accuracy of this calculation.

VI. SUMMARY AND CONCLUSIONS

In summary, cholesterol synthetic rates were determined in two groups of subjects having different genetic backgrounds. This feature of cholesterol metabolism was examined in order to identify differences in regulation of endogenous cholesterol production that may contribute to variations in plasma cholesterol concentrations usually observed in these two groups of subjects. Current research into the mechanisms by which the genetic characteristic, apo E phenotype, affects plasma cholesterol levels has focused on regulation of cholesterol homeostasis by LDL receptor production. Knowledge of the relationship between apo E phenotype and endogenous cholesterol production is important since cholesterol homeostasis is also maintained through regulation of the activity of HMG Co A reductase, the rate-limiting enzyme in cholesterol production.

Subjects in the two study groups were chosen from a group of 113 Caucasian volunteers having normal plasma lipid concentrations. Subjects participated in an experiment consisting of a four day stabilization period and a two day measurement period. Cholesterol FSR was determined during the two measurement days by administration of deuterium oxide and the subsequent analysis of the incorporation of deuterium from body water into newly synthesized free cholesterol. Subjects were studied under conditions of feeding and fasting to identify differences in the response of cholesterol metabolism to dietary influences.

A significant effect of apo E phenotype, feeding condition, and time interval on cholesterol FSR was identified. Subjects of the apo E2/2 or E3/2 phenotypes synthesized cholesterol at a lower rate than those having the E4/4 or E4/3 phenotypes. This finding may suggest a reason for the lower levels of plasma cholesterol seen in individuals carrying the ε-2 allele compared to those with the ε-4 allele. Fasting caused a marked reduction of cholesterol synthesis in all subjects and may have masked any differences in the way cholesterol synthesis is regulated in the two groups of subjects

in the absence of food. A diurnal variation in cholesterol synthesis was discovered, primarily during the fasting period when cholesterol synthesis was increased during the nocturnal period from that in the daytime.

These results have provided valuable information not only about fluctuations in cholesterol synthesis in response to nutritional state or time interval, but about the contribution of genetics to the regulation of cholesterol synthesis in humans. Genetic influences on cholesterol metabolism and plasma cholesterol levels are now beginning to be recognized as being more significant in the evaluation of how a patient will respond to dietary treatment for various lipid disorders. Identification of a patient's apo E phenotype may prove to be a useful screening tool when used in combination with the determination of plasma lipid levels in the prediction of an individual's risk of developing ischemic heart disease.

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Appendix A. Plasma lipid levels and apo E phenotype identification of the screening population.

Phenotype Group/ Subject Code	Age (yr)	Plasma Cholesterol (mg/dl)	Plasma Triglyceride (mg/dl)	Isoform Band Ratio
E2/2				E3:E2
CD	34	145	95	(no E3 band present)
E3/2				
CChe*	26	192	64	1.13
AC	22	144	42	1.12
JCr	26	152	53	1.05
CE	39	198	121	0.78
KE	20	105	69	0.82
DF	22	179	117	0.94
IF	30	207	118	1.16
RGe	32	135	70	1.06
PGu	27	150	44	0.98
EH	22	134	66	1.10
AHr	24	129	54	1.16
PJ	30	134	61	1.07
GK	24	182	62	0.97
DK	18	153	83	1.14
POr	29	154	60	0.89
RPie	27	166	117	0.97
RPit	34	203	71	1.05
JP	21	163	55	1.12
BQ	23	142	55	1.11
GRe*	23	202	89	0.86
MT	27	132	59	-
LVP	26	134	57	0.71
LW	32	148	40	0.74
E3/3				
JA	31	162	66	1.94
TA	35	180	78	1.48
PB	29	138	72	1.31
LB	22	134	51	1.82
RBa	23	154	52	1.29
RBu	30	172	115	1.55
FC*	22	149	65	1.65
JCho*	33	195	67	1.34
JChr	26	128	53	1.31
GC	22	171	108	1.29
MC	21	132	77	1.61
FD	18	152	52	3.04
PDun	40	161	54	2.52
PDur	25	167	98	1.65

Appendix A. (continued)

Phenotype Group/ Subject Code	Age (yr)	Plasma Cholesterol (mg/dl)	Plasma Triglyceride (mg/dl)	Isoform Band Ratio
(E3/3 - continued)				(E3:E2)
SF**	20	87	60	1.25
GF	29	138	76	1.37
NF	22	159	45	1.26
PGo	37	193	81	1.36
JG	20	155	75	1.47
RGr	21	150	95	1.58
GHa	24	116	41	1.95
AHa	32	154	23	1.22
NH	28	147	49	1.59
MH	23	132	51	1.35
CH*	22	126	86	1.49
GHo	25	154	55	1.86
GHu	25	159	77	1.23
KJ	19	134	36	1.39
TJ	28	176	58	1.29
AJ	26	165	147	1.72
JK	30	168	80	1.25
PKl	20	100	67	1.36
SK	21	134	39	1.34
PKr	38	157	80	1.28
JL*	29	129	73	2.82
UM	29	165	90	1.80
JM	29	147	66	1.40
DMc	29	167	112	1.47
DMe	22	112	44	2.20?
PM	34	179	55	1.37
KM	25	182	81	1.40
BN	37	205	53	1.44
SN	34	172	74	1.33
DN	26	162	59	1.30
MO'C	30	164	68	1.39
MO'G	28	167	99	1.32
MP	20	110	41	1.52
FP	21	159	57	1.37
JRa	23	176	73	1.67
GRa	-	146	108	1.53
CSc	28	177	38	1.68
SS**	24	76	10	1.82
MS	19	203	70	1.92
ES	21	127	70	1.38
CStan	30	154	41	1.41
CSto	26	155	41	1.31
JSt	20	147	71	1.38
JSu*	22	176	91	1.51

Appendix A. (continued)

Phenotype Group/ Subject Code	Age (yr)	Plasma Cholesterol (mg/dl)	Plasma Triglyceride (mg/dl)	Isoform Band Ratio
(E3/3 - continued)				(E3:E2)
CTa	31	197	58	1.44
GT	27	153	84	1.37
TTo*	20	137	29	1.80
TTu	28	162	52	1.50
MV	19	164	69	1.36
CV	29	139	66	1.44
JW	30	154	34	1.23
SW	20	149	75	1.93
EW	30	142	37	1.31
KW	22	167	73	1.58
DW	30	166	120	1.72
E4/3				E4:E3
AB	30	150	42	0.98
MBo	33	179	125	1.57
MBu	29	147	61	1.15
JB	31	191	81	0.87
CCha	23	166	83	0.75
TC	20	182	61	1.15
CCu	21	198	124	1.41
DD	23	187	82	1.15
MD	24	174	47	0.91
GG	25	149	62	0.96
TG	22	176	53	1.33
PH	29	184	105	1.18
AHi	30	148	56	0.96
RHs*	21	173	31	0.95
RHu	25	174	79	1.77
RK	22	159	59	0.80
BK	29	179	65	0.72
CL	28	168	73	0.87
DRL	22	159	48	0.92
IM**	22	224	53	0.80
SM	22	131	53	0.76
GM	21	130	59	0.77
HM	32	163	75	0.93
RPe	21	128	71	1.15
JRo	18	123	86	0.69
AS	32	208	98	-
CTh	34	148	111	-
ST	26	123	36	0.70
CW	22	182	99	0.81

Appendix A. (continued)

Phenotype Group/ Subject Code	Age (yr)	Plasma Cholesterol (mg/dl)	Plasma Triglyceride (mg/dl)	Isoform Band Ratio	
E4/4				E4:E3	
MF	28	202	55	2.78	
E4/2				E3:E2	E4:E3
MGa	24	141	60	0.54	2.00
MGr	30	152	104	0.68	2.96
Uncertain					
RD		208	108		
ML		183	162		
DMo		128	49		
CStanz		172	164		
AT		217	94		
POu		140	47		

* subjects were not considered for participation in the cholesterol synthesis measurement study because they were not Caucasian. ** subjects were not considered for participation in the cholesterol synthesis measurement study because of plasma cholesterol levels outside the normal range (100 - 220 mg/dl).

Appendix B. Sample sheet containing information and instructions for experimental subjects.

THE EFFECT OF HUMAN APOLIPOPROTEIN E PHENOTYPE ON CHOLESTEROL SYNTHESIS RATES AS MEASURED BY DEUTERIUM INCORPORATION

Investigators: Dr. Peter J. Jones 228 - 6253
Rebecca P. Roe 228 - 2502

Division of Human Nutrition, School of Family and Nutritional Sciences
University of British Columbia, Vancouver, B. C.

INFORMATION FOR PARTICIPANTS

Thank you in advance for agreeing to participate in the second phase of our research project. At the conclusion of the study we expect to be able to answer an important question regarding the contribution of a person's genetic background to their plasma cholesterol level, and ultimately to their risk of developing coronary heart disease.

This letter contains important information regarding your participation. As you can see, you have been assigned to a 1-week experimental period between January and April, 1988. Enclosed is a detailed description of events that will take place during the week prior to your scheduled experimental period, and during the experimental period itself. We have also included a copy of the consent form which you will be asked to sign one week before participating in the study.

Please do not hesitate to call us if you have questions regarding the experimental schedule or procedures.

EXPERIMENTAL SCHEDULE

Sun.	Jan. 24	- Sat.	Jan. 30:	MB, KE, RPe
Sun.	Feb. 7	- Sat.	Feb.13:	TC, GK, LW
Sun.	Feb. 14	- Sat.	Feb. 20:	CD, MF, RPi
Sun.	Feb. 28	- Sat.	Mar. 5:	DF, MGr, LVP
Sun.	Mar. 6	- Sat.	Mar. 12:	(MGa - withdrew from study), TG, PH
Sun.	Mar. 20	- Sat.	Mar. 26:	CC, DD, RH
Sun.	Mar. 27	- Sat.	Apr. 2:	PG, PO

THE STUDY WILL BE CONDUCTED IN ROOM 140 OF THE FAMILY AND NUTRITIONAL SCIENCES BUILDING. YOU SHOULD REPORT TO THIS ROOM FOR ALL MEALS AND BLOOD SAMPLING APPOINTMENTS.

DURING THE WEEK PRIOR TO YOUR EXPERIMENTAL WEEK (on Tues, _____, 8 - 8:30 am) YOU WILL BE ASKED TO REPORT TO ROOM 140 FOR THE FOLLOWING:

- determination of height and weight so that we may estimate your energy requirements.
- initial blood sampling (sample #1) to determine baseline cholesterol level.
- instructions on keeping a 3-day food record.
- signing of consent form.

YOU SHOULD HAVE NOTHING TO EAT OR DRINK, EXCEPT WATER, FOR 12 - 14 HOURS PRIOR TO REPORTING FOR THIS PRELIMINARY MEETING.

Appendix B. (continued)

MEAL TIMES WILL BE AS FOLLOWS

Breakfast:	7:30 am
Lunch:	12:30 pm
Supper:	5:30 pm

EXPERIMENTAL DIET

The meals that will be served to you will consist of solid foods such as omelettes, macaroni and cheese, and baked chicken. The 3-meal menu plan will be repeated on each of the 5 feeding days. Each meal will contain the same number of calories and the same proportions of fat, carbohydrate and protein. You may find that breakfast is larger, and supper smaller, than you are used to. If you are unable to finish any of the meals the food will either be given to you to eat at a later time, or it will be saved for determination of its nutrient content. We will indicate to you which food items are most important for you to eat.

In addition to the meals served, you should try to consume 6 cups (250 ml each) of fluid per day (as water or as diet soda provided by the investigators). Diluted deuterium-labelled drinking water will be given to you on Thursday and Friday.

WE WILL ASK THAT YOU CONSUME ONLY THE FOOD SERVED TO YOU DURING THE EXPERIMENTAL PERIOD. YOU MUST REFRAIN FROM CONSUMING CAFFEINATED COFFEE AND TEA, SNACKS AND ALCOHOL. NO FOOD IS TO BE CONSUMED ON DAY 6 WITH THE EXCEPTION OF BEVERAGES PROVIDED BY THE INVESTIGATORS.

SCHEDULE OF MEALS AND BLOOD SAMPLING TIMES DURING THE EXPERIMENT

DAY 1 (SUN):	Normal Meals	
DAY 2 (MON):	Normal Meals	Blood Sample #2 (7:30 am)
DAY 3 (TUES):	Normal Meals	
DAY 4 (WED):	Normal Meals	
DAY 5 (THURS):	Normal Meals* *Report at 7:00 am	Blood Sample #3 (7:30 am) Blood Sample #4 (7:30 pm)
DAY 6 (FRI):	FAST DAY	Blood Sample #5 (7:30 am) Blood Sample #6 (7:30 am)
DAY 7 (SAT):	Optional Breakfast	Blood Sample #7 (7:30 am)

CONSUMPTION OF DEUTERIUM-LABELLED WATER

Before breakfast on Day 5 you will be asked to drink a specific quantity of deuterium-labelled water. This quantity (approximately 20 - 30 ml) will be determined based on the measurement of your body weight. You will then be requested to drink diluted deuterium-labelled water in place of tap water for the remainder of Day 5 and throughout Day 6.

ACTIVITY

Your activity level should remain constant throughout the feeding days of the experimental period. The energy content of the food served to you will be determined based on your reported "usual" level of activity, and it will be the same on each of the 5 feeding days. The goal is that your body weight remain constant during the study.

On the fasting day we will ask that you restrict your activity. We will also request that you remain in the testing facility as much as possible during that day.

REMUNERATION

You will be given a Department of Finance voucher for $125 upon completion of the study. If you withdraw from the study prior to its completion you will be given a pro-rated fraction of this amount.

Appendix C. Sample Consent Form.

CONSENT FORM PATIENT NAME_____

THE EFFECT OF HUMAN APOLIPOPROTEIN E PHENOTYPE ON CHOLESTEROL SYNTHESIS RATES AS MEASURED BY DEUTERIUM INCORPORATION

Investigators: Dr. Peter J. Jones and Rebecca P. Roe

Division of Human Nutrition, School of Family and Nutritional Sciences
University of British Columbia, Vancouver, B. C.

I. PURPOSE OF THE STUDY AND DESCRIPTION OF THE PROCEDURE:

The study you are volunteering for is designed to determine whether individuals having different phenotypes for apolipoprotein E (apo E) synthesize cholesterol at different rates.

In the body, fat is transported along with proteins in molecules called lipoproteins. Apo E is a protein found on these lipoproteins. The structure of apo E is genetically determined. Individuals have apo E that is one of three forms: apo E2, E3 or E4. Apo E3 is the most common form. Individuals having genes for apo E2 or apo E4 are less common.

We are interested in studying cholesterol synthesis in individuals with the genes for apo E2 and/or apo E4.

The study will be conducted in 2 phases. Phase I, which you have already participated in, was the screening phase during which we determined your apo E phenotype and your plasma cholesterol and triglyceride levels. You have been asked to participate in phase II, the experimental phase, because you have the appropriate apo E phenotype and you have normal lipid levels.

Phase II will take place over a 7-day period. During the first 4 days you will be given prepared meals that will stabilize your cholesterol level and maintain you body weight. On the morning of Day 5 you will be given deuterium-labelled water to drink, and you will consume the same meals as were served to you on Days 1 - 4. Five blood samples (30 ml each) will be taken at 12-hour intervals for the next 48 hours. You will be asked to fast following supper on Day 5 until breakfast on Day 7. You will be requested to remain in the testing facility from 7:30 am to 7:30 pm on Day 6. Deuterium-labelled drinking water will also be given to you on Day 6. The study will conclude on Day 7 following the morning blood sample.

II. POTENTIAL RISKS AND/OR BENEFITS

The drinking water you will be given on Days 5 and 6 will contain deuterium, a stable isotope of hydrogen that occurs naturally in the environment. The amount given to you will increase your body deuterium level to 3-times its usual amount. There are no known hazards of side effects associated with drinking water containing this level of deuterium. The deuterium in your body should return to its usual level within 2 to 3 months following the experimental trial.

Blood sampling will be performed by a Registered Laboratory Technician in the testing facility of the Department of Family and Nutritional Sciences. There are no known risks from this procedure other than those normally associated with blood taking.

Appendix C. (continued)

Your plasma cholesterol and triglyceride levels and your apo E phenotype identification will be made available to you at the conclusion of the study.

III. CONSENT

The nature of the project and procedures associated with it have been fully explained to me. I have had the opportunity to ask questions concerning the project and procedures involved. I understand that I may continue to ask questions once the study has begun.

Confidentiality of records concerning my participation in the project will be maintained by the investigators.

I understand that I will receive $125.00 upon completion of the study. If I decide to withdraw before the study, I will receive a pro-rated fraction of this amount. I understand that even though I participated in the screening phase, I am in no manner obligated to participate in the experimental study.

I am aware that I may withdraw my consent at any time.

I, _____, agree to participate in this research project and acknowledge receiving a copy of this consent form.

Signature of Participant: _____

Signature of Witness: _____

Signature of Investigator: _____

Date: _____ Time: _____am/pm

Appendix D. Sample instruction sheet provided to subjects for keeping a three day food record.

EFFECT OF HUMAN APOLIPOPROTEIN E PHENOTYPE ON CHOLESTEROL SYNTHESIS RATES AS MEASURED BY DEUTERIUM INCORPORATION

INSTRUCTIONS ON KEEPING A 3-DAY FOOD RECORD

Please keep a record of everything you eat and drink on the attached forms for the following 3 days:

_____ _____ _____

1. Write down EVERYTHING you eat and drink. Be sure to include all SNACKS and ALCOHOL. Record immediately after each meal and smack to ensure accuracy.

2. Write down HOW MUCH you eat and drink:

   A) Use VOLUME measures (cups, Tbsp, tsp, or mls) for cereals, cooked rice and pasta, vegetables, canned fruit, peanut butter, mayonnaise, salad dressings, butter, margarine, sauces, gravies, soups, sugar, jam, beverages, etc.

   B) Use COOKED WEIGHTS (ounces or grams) for meat, fish, poultry and cheese. Note: the weight of meat, poultry and fish decreases by about 25% during cooking. Examples:
   4 oz. raw beef shrinks to 3 oz cooked beef.
   6 oz. raw cod shrinks to 4.5 oz. cooked cod.

   C) Use SIZE for raw fruits, muffins, crackers, cakes, pies, cookies, desserts, etc. Give DIMENSIONS (eg 1 oatmeal cookie, 1" in diameter), or specify small, medium, or large serving based on the attached diagrams.

   D) Be specific about the TYPE OF FOOD, BRAND NAME IF APPLICABLE, HOW THE FOOD WAS PREPARED, AND CONTENT OF MIXED DISHES.

   E) For combination items, list each item separately, eg a cheeseburger would be described as: bun, cooked ground beef, processed cheese, butter (for cooking), relish, etc.

   F) IF THE FOOD IS PREPARED BY SOMEONE OTHER THAN YOURSELF: please try to estimate the portion size and describe the contents of the dish that is served to you.

   G) Don't forget the EXTRAS! eg sugar on cereal or in coffee, dressing on salad, candy, soft drinks, alcohol.

EXAMPLE:

Time	Food Item Description	Amount	Location
Breakfast	2% milk	1/2 cup	UBC
	whole wheat bread	2 slices	Hospital
	margarine	2 tsp	Cafeteria
	strawberry jam	1 tsp	
	omelette: eggs	2 large	
	cheddar cheese	1/2 oz.	
	orange juice	6 oz	

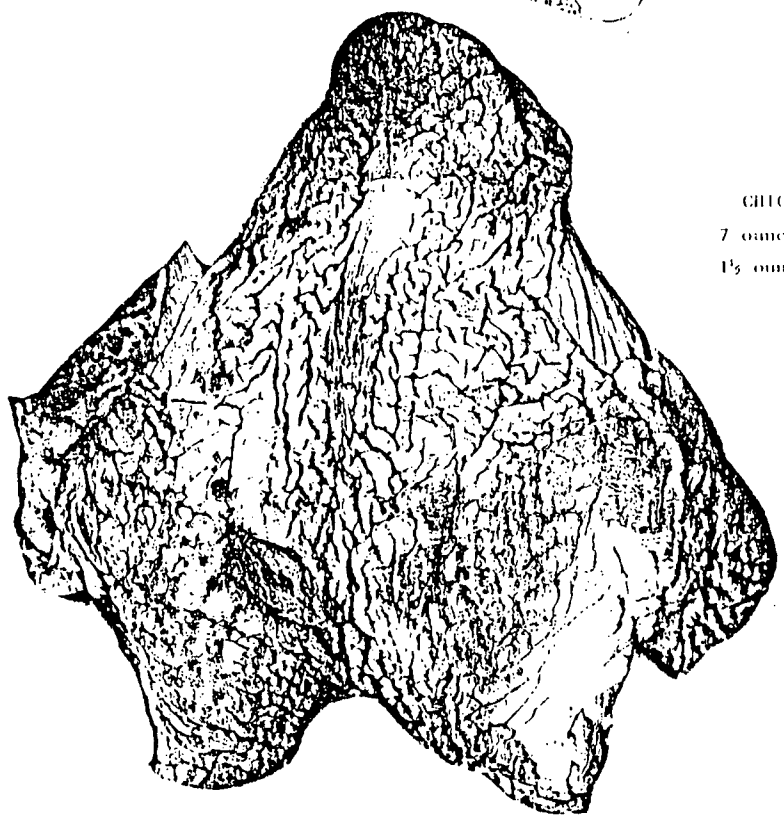
Diagrams of Meat, Poultry, Fish, and Cheese to Help You Estimate Cooked Weights

CHICKEN LEG (cooked)

3 ounces lean flesh

(thigh equals 2 ounces)
(drumstick equals 1 ounce)

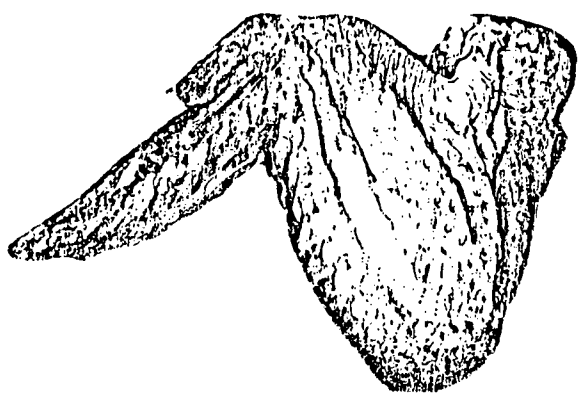
½ ounce skin plus fat



CHICKEN BREAST (cooked)

7 ounces lean flesh

1½ ounces skin plus fat

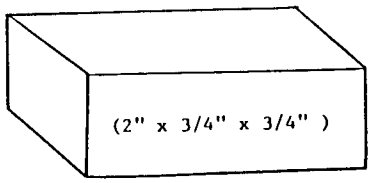


CHICKEN WING (cooked)

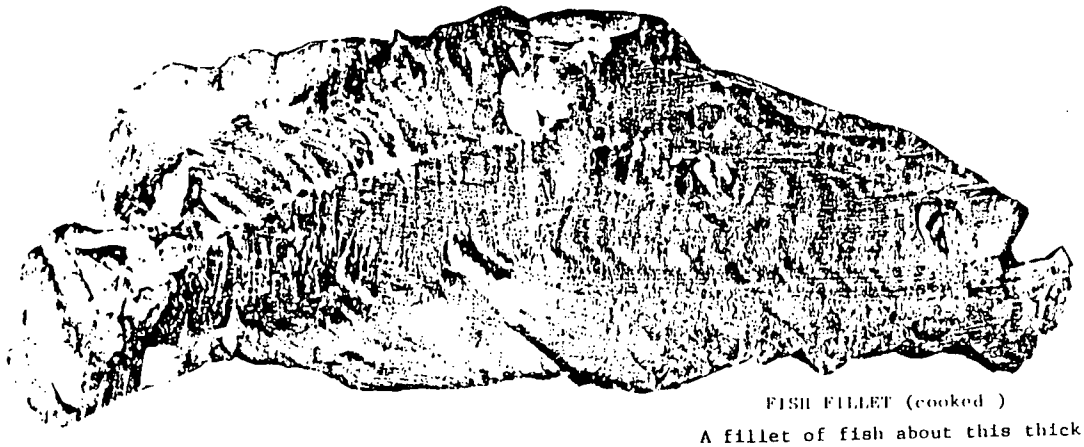
½ ounce lean flesh

½ ounce skin plus fat

(reduced by 30%)

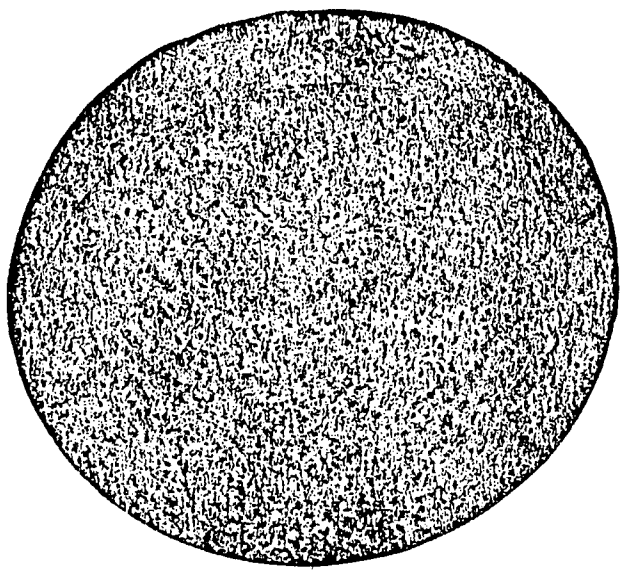


A piece of cheese this size is equal to 1 ounce.

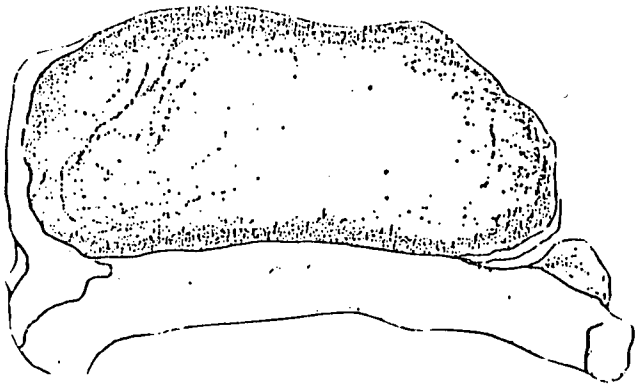


FISH FILLET (cooked)

A fillet of fish about this thick is equal to 4 ounces.



A slice of bologna or other luncheon meat this thick is equal to 1 ounce.



PORK CHOP (cooked)

A pork chop this thick is equal to 3 ounces.

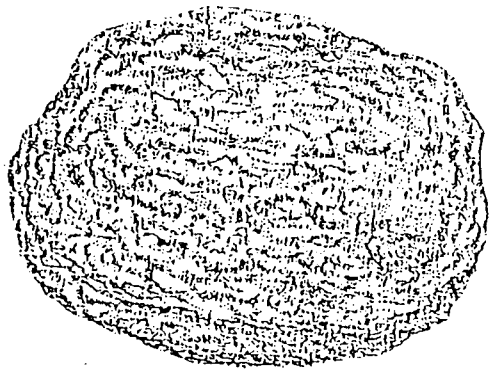
A pork chop this thick is equal to 2 ounces.

(reduced by 30%)

MEAT (cooked)

One slice of meat this thick is equal to 3 ounces.

One slice of meat this thick is equal to 1½ ounces.



HAMBURGER PATTY (cooked)

One hamburger patty this thick is equal to 3 ounces.

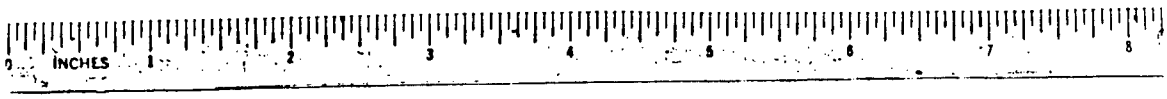
One hamburger patty this thick is equal to 2 ounces.

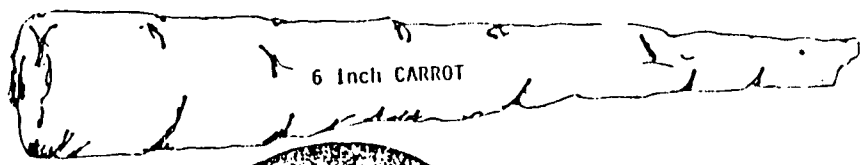
STEWING MEAT (cooked)

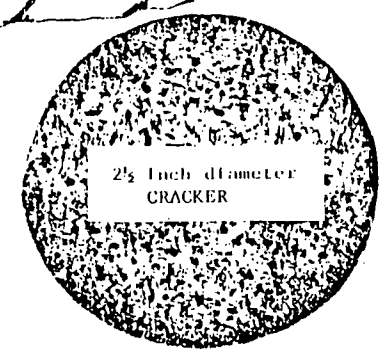
One piece of cooked stewing meat this size is equal to ½ ounce.

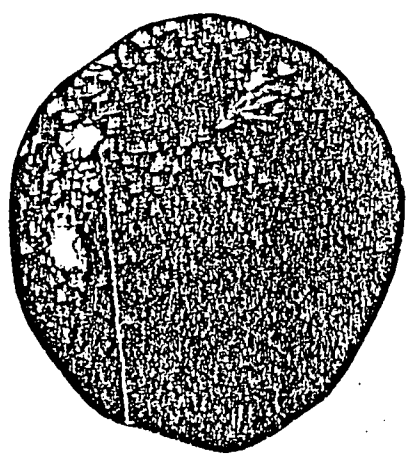
(reduced by 30%)

Scale and Diagrams to Help You Determine Sizes of Raw Fruits, Crackers, Cake etc









MEDIUM APPLE

* Use this diagram to describe peaches, oranges, potatoes, tomatoes, etc

MEDIUM MUFFIN

(100 grams)

(reduced by 30%)

Appendix E. Calculation of subjects' energy requirements for the experimental feeding period.

Subject Code	Surface Area* (m^2)	kcal/m^2/hr‡	Additional Energy for Activity§ (kcal)	Total Energy Required** (kcal/day)
E2 Group:				
CD	2.00	38.68		3156
KE	1.82	41.43		3076
GK	1.96	40.24	270	3488
LW	2.09	39.34		3355
RPi	2.06	40.24		3382
DF	1.84	40.82		3064
LVP	1.89	40.24	548	3651
PG	1.82	40.24	384	3372
PO	1.76	39.34		2825
E4 Group				
MF	1.81	39.81		2940
MB	2.14	39.81		3476
RPe	1.87	41.43		3161
TC	2.00	41.43		3381
TG	2.08	40.82		3464
PH	2.01	39.81	262	3527
CC	2.02	41.43	197	3611
DD	2.16	40.82	250	3847
RH	1.90	40.24	166	3285
E4/2 Group:				
MG	2.04	39.34	343	3617

* determined using the Mayo Clinic Nomogram (reference 72) from subjects' height and weight.
‡based on subjects' age - using the Mayo Clinic Nomogram. § see calculations in Appendix F.
** Total = [(cal/m^2/hr) x 24 hr x 1.7] + additional energy.

Appendix F. Calculation of additional energy required for subjects reporting a high level of activity.

Subject Code	Usual Weekly Activity	Time/Activity	Approximate energy cost*	Total kcal	Additional Energy/Day
GK	swim	2 days x 20 min	10 kcal/min	1350/	
	run	2 days x 35 min	10 kcal/min	5 days	270
	run	1 day x 25 min	10 kcal/min		
LVP	weight training (estimate 50 min activity/day)	4 days x 2 hr	0.185 kcal/min/kg	2740/5 days	548
PG	cycling	6 days x 20 min	8 kcal/min	1920 /	
	soccer (estimate 120 min activity for soccer)	1 day x 3 hr	8 kcal/min	5 days	384
PH	run	3 days x 25 min	10 kcal/min	1310/	
	weight training (estimate 40 min activity total for weight training)	2 day x 30 min	0.185 kcal/min/kg	5 days	262
CC	weight training (estimate 67 min activity total for weight training)	2 days x 50 min	0.185 kcal.min/kg	985/ 5 days	197
DD	rugby	1 day x 125 min	10 kcal/min	1250/5 days	250
RH	weight training (estimate 60 min activity total)	2 days x 45 min	0.185 kcal/min/kg	830/5 days	166
MG	hockey (estimate 45 min activity for hockey)	1 day x 60 min	8 kcal/min		
	weight training (estimate 90 min activity total for weight training)	2 day x 60 min	0.185 kcal/kg/min	1717/ 5 days	343

* determined from references 74, 75.

Appendix G. Deuterium enrichment of cholesterol samples extracted from plasma collected at various time points throughout the deuterium labelling experiment.* (Sample cholesterol FSR calculation included.)

Subject Code	$^2H/^1H$ of cholesterol baseline	12 hr	24 hr	36 hr	48 hr
	(parts per thousand (°/oo) relative to SMOW‡)				
E2 Group:					
CD	-308.1 (5.6)	-264.0 (3.5)	-140.4 (4.0)	-172.5 (5.3)	-142.4 (1.4)
KE	-299.9 (3.6)	-249.6 (1.2)	-180.9 (3.0)	-200.9 (7.0)	-199.5 (3.0)
GK	-290.4 (2.8)	-261.8 (2.4)	-207.0 (1.1)	-222.4 (0.6)	-201.8 (2.2)
LW	-310.7 (1.7)	-193.2 (3.6)	-118.8 (3.3)	-120.1 (1.3)	-101.8 (3.9)
RPi	-309.0 (2.7)	-275.2 (0.7)	-222.0 (5.2)	-230.7 (2.2)	-222.8 (1.1)
DF	-321.5 (1.5)	-259.7 (5.4)	-112.8 (1.8)	-157.8 (3.6)	-106.3 (0.6)
LVP	-297.8 §	-266.8 (3.1)	-212.7	-231.7 (3.7)	-226.4 (3.0)
PG	-309.9 (5.4)	-200.8 (4.1)	-129.7 (2.2)	-147.2 (0.4)	-150.3 (5.5)
PO	-297.7 (2.2)	-224.4 (1.9)	-133.3 (6.0)	-151.7 (4.9)	-149.9 (4.2)
E4 Group:					
MF	-325.5 (5.6)	-189.1 (2.8)	-35.2 (2.2)	-43.9	5.3 (0.6)
MB	-307.0 (3.3)	-181.7 (4.1)	-98.6 (7.1)	-108.7 (2.6)	-77.2 (9.3)
RPe	-297.0 (2.8)	-230.8 (1.6)	-116.1 (6.5)	-154.5 (6.5)	-147.5 (3.9)
TC	-297.1(12.1)	-124.9 (1.0)	-37.7 (3.3)	-41.6 (6.3)	11.0 (3.8)
TG	-312.7 (5.8)	-215.9 (2.2)	-92.3 (4.0)	-114.1 (0.5)	-110.1 (1.5)
PH	-307.0 (2.0)	-233.0 (1.6)	-115.4 (2.8)	-129.6 (5.0)	-126.3 (4.9)
CC	-311.6 (0.3)	-203.2 (0.6)	-67.3 (0.2)	-93.5 (8.8)	-50.6 (2.7)
DD	-316.9 (0.5)	-278.3 (1.3)	-224.9 (5.5)	-238.0 (3.2)	-235.4 (1.9)
RH	-300.0 (4.0)	-244.6 (5.4)	-161.3 (4.8)	-179.9 (4.0)	-160.5 (7.9)
E4/2 Group:					
MG	-311.0 (9.4)	-235.6 (2.2)	-142.9 (4.1)	-149.8 (3.3)	-155.6 (4.8)

*values are mean (± SD) of 3 samples. ‡Standard Mean Ocean Water. § if no SD listed, value determined from only 1 sample.

Sample Cholesterol FSR Calculation: (see equation in Methods section)

Subject CD:
Cholesterol FSR for 12 - 24 hr interval
12 hr cholesterol enrichment = -264.0 °/oo
24 hr cholesterol enrichment = -140.4 °/oo
mean plasma enrichment over 48 hrs (see Appendix H) = 4714.7 °/oo

$$\text{FSR (per day)} = \frac{[-140.4\ °/oo - (-264.0\ °/oo)\ \text{per 12 hr}] \times 2}{4714.7\ °/oo \times (22D/27C \times 27C/46H)} = 0.109\ \text{per day}$$

Appendix H. Deuterium enrichment of plasma water samples derived from plasma collected at various time points throughout the deuterium labelling experiment.*

Subject Code	$^2H/^1H$ of plasma baseline	12 hr	24 hr	48 hr	mean enrichment 0-48 hr	corrected‡	vari-ation
	(parts per thousand (°/oo) relative to SMOW§)						(%)
E2 Group:							
CD	-88.9 (2.0)	631.1 (7.4)	699.4 (7.8)	698.4 (5.6)	676.3	4714.7	5.8
KE	-85.6 (1.3)	562.2 (6.9)	535.4 (4.3)	551.2 (1.6)	549.6	3951.2	2.5
GK	-57.8 (4.0)	520.3 (5.8)	492.9 (1.4)	512.5 (8.6)	508.6	3677.2	2.8
LW	-90.2 (0.4)	560.3 (2.1)	560.3 (6.6)	568.6 (5.7)	563.1	4036.6	0.9
RPi	-80.6 (7.7)	616.0 (6.8)	631.5 (4.1)	607.2 (6.6)	618.2	4358.0	2.0
DF	-54.6 (3.1)	666.2 (3.3)	642.2 (7.0)	607.1 (1.5)	638.5	4453.6	4.7
LVP	-81.2 (2.5)	552.6 (4.2)	567.2(13.2)	561.4	560.4	4011.6	1.3
PG	-74.5 (0.8)	680.4 (8.3)	636.6 (4.8)	612.6 (1.4)	643.2	4501.7	5.3
PO	-67.4 (3.2)	617.9 (2.9)	617.4 (6.9)	603.8 (4.2)	613.0	4313.6	0.5
E4 Group:							
MF	-84.3(10.2)	671.2 (6.6)	696.3 (5.2)	671.4(19.7)	679.6	4730.1	2.1
MB	-68.9 (4.7)	528.9 (6.1)	610.4 (0.7)	569.9 (2.1)	569.7	4055.3	7.2
RPe	-83.8 (2.7)	578.4 (6.1)	585.6 (4.2)	584.5 (2.1)	582.8	4148.8	6.7
TC	-86.7 (2.1)	650.1 (5.2)	707.1 (7.0)	618.7 (0.4)	658.6	4606.5	6.8
TG	-81.3 (8.0)	723.0 (5.2)	657.4 (2.6)	619.4 (0.8)	666.6	4648.9	7.9
PH	-74.4 (1.1)	546.1 (9.3)	612.4 (7.2)	558.0 (1.6)	572.2	4075.4	6.2
CC	-80.9 (1.9)	632.0 (4.7)	613.7 (7.4)	598.3 (1.6)	614.7	4336.9	2.7
DD	-80.4(15.8)	564.4 (7.8)	567.5 (0.0)	574.1 (0.2)	568.7	4060.4	0.9
RH	-85.6 (3.4)	625.7 (5.9)	641.0 (2.4)	626.6 (6.1)	631.1	4440.2	1.4
E4/2 Group:							
MG	-87.3 (1.1)	615.2 (2.9)	572.5 (2.7)	574.0(2.6)	587.2	4178.7	4.1

*values are mean (±SD) of 2 samples. ‡corrected for dilution with, and deuterium enrichment of 5% bovine serum albumin (BSA) solution (see sample calculation). §Standard Mean Ocean Water.

Sample Calculation for Corrected Mean Plasma Enrichment:

Subject CD:
dilution of plasma: 5 parts BSA:1 part plasma (except baseline plasma) = 6 fold dilution
mean plasma enrichment = 676.3 °/oo
baseline plasma enrichment = -88.9 °/oo
enrichment of BSA = -113.6 °/oo

Corrected Mean Plasma Enrichment :
= (°/oo undiluted plasma) - (°/oo BSA solution) - (°/oo baseline plasma)
= (676.3 °/oo x 6) - (-113.6 °/oo x 5) - (-88.9 °/oo)
= 4714.7