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COMPARING THE METABOLIC FATES OF CHYLOMICRON FATTY ACIDS:
EVIDENCE FOR HIGHER FRACTIONAL OXIDATION OF OLEATE THAN PALMITATE

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate School
of The Ohio State University

By

Dorothy Elaine Schmidt, B.S.

The Ohio State University
1998

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Approved by
Ohio State University Nutrition Program
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Dorothy Elaine Schmidt

1998
ABSTRACT

Public health recommendations regarding dietary fat focus exclusively on limiting intake, due to evidence linking high fat intake and degenerative diseases. However, diets rich in oleate may benefit plasma lipids and glycemic control. Differences in acute oxidation of dietary fatty acids could contribute to their differing health effects. Tracer oxidation studies provide evidence of such differences, particularly that unsaturated fatty acids are more readily oxidized than saturated ones. However, other inconsistent results may be due to confounding variables such as grossly unequal amounts of tracee fatty acid being ingested or differences in true precursor enrichment.

The primary aim of this research was to compare acute oxidation of dietary oleate and palmitate. Two secondary aims were to compare their clearance from circulation as non-esterified fatty acids (NEFA) and their appearance in very-low-density lipoproteins (VLDL). Ten healthy, young, non-obese adults were fed a eucaloric formula diet into which [1-\(^{13}\)C]-labeled oleate or palmitate was emulsified. Total fat was 40% of energy. Oleate and palmitate each contributed 16% of energy. Small frequent meals were ingested for seven hours to produce steady-state excretion of \(^{13}\)C label in breath CO\(_2\). Subjects were studied twice with identical protocols except for fatty acid tracer given. Enrichment of breath CO\(_2\) was analyzed by gas isotope ratio mass spectrometry.
Enrichment of plasma fatty acids was analyzed by gas chromatography/mass spectrometry. Plasma fatty acids were quantified by gas chromatography with flame ionization detector.

Absolute rates of fatty acid oxidation were defined as rate of excretion of label corrected for precursor enrichment and isotope exchange. Relative oxidation (Rel$_{ox}$) was defined as the intra-subject ratio between fractional oxidation of oleate and palmitate. Rel$_{ox} = 1.21$ (P<0.05), indicating that oleate oxidation was 21% greater than that of palmitate. Relative clearance of oleate versus palmitate was defined as the amount of oleate versus palmitate in NEFA relative to chylomicrons. On this basis, oleate was more efficiently cleared from peripheral circulation than palmitate by 45% (P<0.05). Relative incorporation into VLDL was defined as the ratio of amounts of the two tracers in VLDL relative to chylomicrons. There was no significant difference in relative VLDL incorporation.
Dedicated to my maternal grandmother

Mrs. Dorothy Belle Poffenberger
ACKNOWLEDGMENTS

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<td>2,5-anhydro-D-mannitol</td>
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<tr>
<td>a-v</td>
<td>arterio-venous</td>
</tr>
<tr>
<td>apoB 48</td>
<td>apolipoprotein B 48</td>
</tr>
<tr>
<td>apoB 100</td>
<td>apolipoprotein B 100</td>
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<tr>
<td>apoC-II</td>
<td>apolipoprotein C-II</td>
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<tr>
<td>apoE</td>
<td>apolipoprotein E</td>
</tr>
<tr>
<td>AT-LPL</td>
<td>adipose tissue lipoprotein lipase</td>
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<td>APE</td>
<td>atoms percent enrichment</td>
</tr>
<tr>
<td>BMI</td>
<td>body mass index; weight/height$^2$</td>
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<tr>
<td>$^{13}$C</td>
<td>carbon-13; stable isotope of carbon</td>
</tr>
<tr>
<td>$^{14}$C</td>
<td>carbon-14; radioisotope of carbon</td>
</tr>
<tr>
<td>CE</td>
<td>cholesterol ester</td>
</tr>
<tr>
<td>CHO</td>
<td>carbohydrate</td>
</tr>
<tr>
<td>CM</td>
<td>chylomicron</td>
</tr>
<tr>
<td>CPT-I</td>
<td>carnitine palmitoyl transferase I</td>
</tr>
<tr>
<td>CVD</td>
<td>cardio-vascular disease</td>
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<tr>
<td>DHA</td>
<td>docosahexaenoate</td>
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<td>Abbreviation</td>
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<td>DIO</td>
<td>diet-induced obesity</td>
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<tr>
<td>EPA</td>
<td>eicosapentaenoate</td>
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<tr>
<td>FABP</td>
<td>fatty acid binding protein</td>
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<td>FAME</td>
<td>fatty acid methyl ester</td>
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<tr>
<td>GC-FID</td>
<td>gas chromatography-flame ionization detection</td>
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<td>GCMS</td>
<td>gas chromatography/mass spectrometry</td>
</tr>
<tr>
<td>GCRC</td>
<td>General Clinical Research Center</td>
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<tr>
<td>GLA-oil</td>
<td>oil blend high in γ-linolenate</td>
</tr>
<tr>
<td>^{3}H</td>
<td>hydrogen-3: radioisotope of hydrogen</td>
</tr>
<tr>
<td>HDL</td>
<td>high-density lipoprotein</td>
</tr>
<tr>
<td>K_d</td>
<td>dissociation constant</td>
</tr>
<tr>
<td>K_M</td>
<td>Michaelis-Menten equilibrium constant</td>
</tr>
<tr>
<td>LCAD</td>
<td>long-chain acyl dehydrogenase</td>
</tr>
<tr>
<td>LDL</td>
<td>low-density lipoprotein</td>
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<tr>
<td>LPL</td>
<td>lipoprotein lipase</td>
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<tr>
<td>M-LPL</td>
<td>muscle lipoprotein lipase</td>
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<tr>
<td>MCAD</td>
<td>medium-chain acyl dehydrogenase</td>
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<td>MIDA</td>
<td>mass-isotopomer distribution analysis</td>
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<td>MPE</td>
<td>moles percent enrichment</td>
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<td>MUFA</td>
<td>monounsaturated fatty acid</td>
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<td>NEFA</td>
<td>non-esterified fatty acid</td>
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<td>NHANES</td>
<td>National Health and Nutrition Examination Survey</td>
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<td>Abbreviation</td>
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<tr>
<td>NIDDM</td>
<td>non-insulin-dependent diabetes mellitus</td>
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<td>PC</td>
<td>phosphatidyl choline</td>
</tr>
<tr>
<td>PL</td>
<td>phospholipid</td>
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<tr>
<td>PUFA</td>
<td>polyunsaturated fatty acid</td>
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<tr>
<td>REE</td>
<td>resting energy expenditure</td>
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<tr>
<td>SCAD</td>
<td>short-chain acyl dehydrogenase</td>
</tr>
<tr>
<td>$S_f$</td>
<td>Svedberg flotation rate</td>
</tr>
<tr>
<td>TAG</td>
<td>triacylglycerol</td>
</tr>
<tr>
<td>VCO$_2$</td>
<td>rate of production of CO$_2$</td>
</tr>
<tr>
<td>VLCAD</td>
<td>very-long-chain acyl dehydrogenase</td>
</tr>
<tr>
<td>VLDL</td>
<td>very-low-density lipoprotein</td>
</tr>
<tr>
<td>$V_{max}$</td>
<td>maximal reaction rate</td>
</tr>
<tr>
<td>VO$_2$</td>
<td>rate of consumption of O$_2$</td>
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CHAPTER 1

INTRODUCTION

Comparative fatty acid metabolism is currently a matter of intensive interest both in nutrition research and among the public at large. This is owing in part to the evidence linking dietary fat with non-insulin dependent diabetes mellitus (NIDDM) and cardiovascular disease (CVD), both directly via effects on insulin sensitivity and lipoprotein metabolism, and indirectly through promotion of obesity. In addition, the most recent US demographic data show increasing prevalence of obesity across all age groups and socioeconomic strata of our society (Kuczmarski et al. 1994).

Recent dietary recommendations have emphasized the negative consequences of eating a high-fat diet, with no balancing message to the public regarding the essential role of dietary fat. For example, the USDA’s Food Guide Pyramid (USDA 1992) categorizes fat with sweets, as something to only “use sparingly.” Similarly, the Dietary Guidelines for Americans (USDA & USDHHS 1995), recommend that one “choose a diet low in fat” without any indication that there is a lower limit. The Guidelines further set upper limits on specific types of fat, including 10% of energy as polyunsaturates. This category includes essential linoleate (C18:2 n-6), for which the Recommended Daily Allowance is that it should make up at least one to two percent of dietary energy. As a consequence of the unrelenting message to consumers that fat should be avoided, fat-free substitutes for high-fat foods such as ice cream and salad dressing have become readily available. This has made it possible for people to nearly eliminate fat from their diets, the public health consequences of which remain to be seen.
Apart from the potential danger of essential fatty acid deficiency, there is good evidence that, as dietary energy derived from fat decreases, fat synthesis increases (Hudgins et al. 1996). Unfortunately, the fatty acid primarily synthesized by humans is saturated palmitate (C16:0) (Stryer 1988 p 485), one of the most atherogenic of all fatty acids (Kris-Etherton & Yu 1997). In addition, persons with NIDDM or hypercholesterolemia who follow the current dietary guidelines for fat intake as a therapeutic measure often develop fasting hypertriglyceridemia (Grundy 1986, Mensink & Katan 1987). This may be caused by increased synthesis of fat by the liver.

Oleate (C18:1), which makes up one-third of all dietary fat as well as most of the monounsaturated fat that we eat (Gurr 1992), was initially regarded by researchers as "neutral" in terms of its effects on CVD (Mattson & Grundy 1985). It was consequently treated as irrelevant. However, interest in oleate increased following reports in the 1980's of low rates of CVD among Mediterranean populations, in spite of a moderately high fat intake (Mattson & Grundy 1985). However, the principal dietary fat source in that region is olive oil, which is composed primarily of oleate. It has since been demonstrated that a diet high in oleate improves plasma cholesterol profiles and insulin sensitivity when compared to a diet high in saturated fat (Garg et al. 1994). These benefits are comparable to those seen with the currently recommended high-carbohydrate (CHO) diet, but oleate does not elevate plasma triacylglycerols (TAG).

Although high-oleate diets containing up to 50% of energy as fat have been shown to be at least as beneficial as those based on the currently recommended fat intake of less than 30% (Garg et al. 1988), their use remains controversial (Franz 1989, Sheard 1995). This may be partly because information about the mechanisms for the metabolic differences between oleate and saturated fatty acids has been lacking. One potential factor is differences in acute oxidation of dietary fatty acids. Among the numerous complex interactions between various dietary fatty acids and metabolic outcomes that have been documented, however, differences in oxidation may be one of the least-understood. Although the preponderance of evidence suggests that unsaturated fatty acids in a meal are more readily oxidized than long-chain saturated fatty acids (Cenedella &
there have also been several conflicting results (Toorop et al. 1979, Coots 1964, Jones 1994). Interpretation of these discrepancies is difficult due to the wide range of methodologies employed. Whether differences in oxidation of various fatty acids really exist, and under what circumstances they might be physiologically relevant, remains to be determined.

**Statement of the problem**

Oxidation of fatty acids is assessed by administering tracers, i.e., fatty acids labeled with the isotopes of carbon, $^{13}$C or $^{14}$C, and measuring the appearance of labeled carbon in breath CO$_2$. In previous comparisons of dietary fatty acid oxidation, relative oxidation has been determined by giving a single dose of labeled fatty acid tracer, then measuring CO$_2$ isotope enrichment over an arbitrary time period. Discrepancies in outcomes mentioned above may be due to numerous methodological differences, such as experimental subjects used, habitual dietary fat intake, CO$_2$ collection times, and method of tracer administration. They may also be affected by confounding variables such as grossly unequal amounts in the diet of the fatty acids being studied in some studies and possible differences in true precursor enrichment, which have not been measured.

In order to simplify interpretation of the results, the present research was designed to minimize sources of variability unrelated to the metabolism of the fatty acids themselves. One source of variability in ultimate oxidation of any nutrient is its digestion and absorption by the gut. When fatty acid oxidation has been assessed in rats, the amount remaining in the intestine has been directly measured following sacrifice. In humans, total absorption can be assessed from fecal collection, but the time course of absorption cannot. However, following absorption, long-chain fatty acids are delivered to arterial circulation via the lymphatic system in the form of chylomicrons (CM), so that their availability to body tissues (other than the gut itself) can be assessed by sampling
arterial blood. That is, CM can be used as the precursor pool for oxidation of long chain dietary fatty acids.

The fraction of tracer label which appears in breath cannot be calculated without knowing the rate of delivery of the fatty acids in CM. This can be estimated by assuming that all of the dietary fat reaches circulation, endogenous contributions to CM are negligible, and that arterial concentration is a direct function of delivery, with clearance remaining constant. However, these assumptions are obviated if a steady state in CM appearance is produced, which was the approach used in the present research. That is, the relative oxidation of two or more fatty acids can be assessed if the tracers are fed in separate protocols, with identical diets, such that delivery of CM also remains constant between protocols. Further, secondary effects such as oxidation following reappearance in very-low-density lipoproteins (VLDL) or as non-esterified fatty acids (NEFA) from adipose tissue, which could be significant in the latter part of the collection intervals for single-dose studies, can be minimized by the use of small, frequent meals to obtain constant levels of hormones, such as insulin, which regulate fat and glucose metabolism. If a steady state in CO$_2$ isotope enrichment is achieved, it provides good evidence that fat delivery and oxidation are indeed being held constant.

In addition to absorption issues, overall metabolism of all fatty acids may be affected by the presence of other fatty acid species. For example, as will be seen in Chapter 2, CM catabolism and VLDL secretion are influenced by fat composition, and various enzymes are unequally affected by different fatty acids. To determine whether observed differences in metabolic fates involve the fatty acid per se, or some secondary effect of fat composition on fat metabolism, it is necessary to know how fatty acids compare when fed in equal amounts, such that they are able to compete on an equal basis for various metabolic processes involved.

Although many fatty acids are of potential interest, oleate and palmitate were chosen for this research as being the most relevant to the question of immediate oxidation. This is not only because of their apparently very different health effects, but also because they are the two most common fatty acids in the diet (Gurr 1992).
Aims of the research

The current research sought to compare the metabolic fate of dietary oleate and palmitate immediately following their delivery to the bloodstream. At the same time, it also served to demonstrate whether the novel experimental approach taken would yield interpretable data for both of the fatty acids studied. Although the main emphasis was on comparing oxidation of the two fatty acids in CM, the composition of two additional plasma lipid fractions was analyzed for comparison, to see if they might shed light on a mechanistic interpretation of the oxidation data. First, NEFA served as a measure of relative clearance of the fatty acids derived from CM during their catabolism, as well as an index of dilution from endogenous sources. Second, VLDL were used to assess liver uptake of the dietary fatty acids and subsequent re-assembly and export.

The primary aim of this research was to conduct a randomized crossover trial comparing postprandial metabolism of oleate and palmitate in non-obese, healthy, young adults. Three sub-aims were to analyze data collected during these studies to test the following main hypotheses:

1) The appearance of labeled CO2 derived from oleate was predicted to be greater than from palmitate, relative to the abundance of each in circulating CM.

2) The fractional amount of each fatty acid in the plasma NEFA relative to its fractional amount in CM was predicted to be inversely related to oxidation, with palmitate greater than oleate.

3) Appearance of diet-derived fatty acids in VLDL was predicted to be inversely related to oxidation, with palmitate greater than oleate.
CHAPTER 2

REVIEW OF LITERATURE

The first section of this review provides a context for investigating differential fatty acid oxidation. This is achieved by demonstrating the key role of dietary fat in human health. To that end, the ways in which dietary fat influences health are discussed, including the role of fat in obesity and diseases such as NIDDM and CVD. In this context, current recommendations for dietary fat as well as relevant aspects of actual US fat intake will also be discussed.

The middle section focuses on dietary fat metabolism with particular attention to those aspects that influence fatty acid oxidation. These can be divided into two categories: processes that mediate fatty acid delivery to cells, and cellular metabolism. The first category includes digestion and absorption, CM formation and catabolism, the sources and fates of NEFA in a fed state, and VLDL metabolism. The second includes cellular uptake, intracellular transport, and the actions of various enzymes that regulate fat oxidation.

The third section examines experimental evidence of differences in metabolism among fatty acids. This includes previous comparisons of fatty acid tracer oxidation, effects of dietary fat type on CM and VLDL metabolism, and fatty acid specificities of enzymes and transporters. These findings provide the rationale for expecting to find differences in metabolism of oleate and palmitate, as well as potential explanations for the experimental results.
Human health and dietary fat

Dietary fat intake in the US

The second *National Health and Nutrition Examination Survey*, NHANES II, was carried out in 1978 to 1987. Data from the survey indicate that the average intake of dietary energy as fat ranged from 35 to 38% across sex and age categories (Block & Subar 1992), which is higher than the national policy goal of 30% or less specified in *Healthy People 2000* (USDHHS 1990). Furthermore, a breakdown of the distribution of intakes shows that the upper quartile for the various categories ranges from 39 to 43%. Since publication of NHANES II, NHANES III phase 1 has been completed, and limited results published. These data indicate that in the period of 1988-91 overall average US fat intake had decreased from 36% to about 34% (anonymous 1994). For the purposes of this discussion however, the NHANES II data will be used as representative of the composition of US dietary fat.

NHANES II breaks down the composition of dietary fat into total saturated fat, oleate, and linoleate categories (Block & Subar 1992). Mean intakes of these reported in the survey were 16 to 40 grams of saturated fat, 17 to 40 grams oleate and 7 to 18 grams linoleate. For each of these categories, intakes for women are lower than for men because women’s energy intakes are less. From these data it can be inferred that oleate is the most common fatty acid in the diet, since amounts of oleate and total saturated fat are about equal, whereas the category of saturated fat includes several common fatty acids, the most common being palmitate (C16:0), stearate (C18:0), and myristate (C14:0) (Gurr 1992). For convenience, nomenclature of all fatty acids discussed herein is summarized in Appendix A.

Interpretation of the literature regarding health effects of dietary fat is enhanced by considering the composition of dietary fat sources. Of the saturated fatty acids, the largest fraction by weight in most commonly consumed fat sources is palmitate (Gurr
Exceptions to this rule include the highly saturated tropical oils, coconut and palm kernel, which contain more myristate than palmitate, and sometimes beef fat, which may contain as much stearate as palmitate. The high fraction of dietary fat represented by oleate in the NHANES data (Block & Subar 1992) is corroborated by data regarding the composition of various food fats. In animal sources, oleate is generally the most common fatty acid, although this can be greatly influenced by the animal's diet (Gurr 1992). In milk fat, oleate and palmitate content are similar, although there is generally more oleate.

In highly-unsaturated vegetable oils such as corn, sunflower and soy, linoleate typically makes up roughly half of the total fat, oleate is between one quarter and one third, and palmitate is less than one sixth. Hence, wide variations in fatty acid composition are achievable by shifting the source of dietary fat among naturally occurring foods.

It is also important to realize that, although single fat sources are often referred to as simply "saturated," "monounsaturated" or "polyunsaturated," all naturally occurring fats are a combination of all three categories, in varying proportions. In most fats it is rare for a single fatty acid species to make up more than 60% of the total. Olive oil, which is about three-quarters oleate, is an exception to this rule and is therefore sometimes the fat source used in high-oleate diet interventions (Mensink & Katan 1987, Baggio et al. 1988), although high-oleate varieties of safflower (Grundy 1986, Mattson & Grundy 1985, Grundy et al. 1988) or sunflower (Temme et al. 1996) oils or even fresh, whole avocado (Colquhoun et al. 1992, Lerman-Garber et al. 1994) have also been used. Palm oil is also a unique fat source in that oleate and palmitate are about equal and together make up about 86% of the total. In most other fat sources having equal oleate and palmitate, such as (common variety) safflower oil, they are both minor components (Gurr 1992).

In addition to fatty acid composition, the positional structure of fats has been suggested to influence health effects of fats, because the 2-position is largely conserved during digestion (Mattson & Volpenhein 1964, Kayden et al. 1967). In general, unsaturated fatty acids tend to be preferentially found in the sn-2 position of TAG, and saturated fatty acids tend to be on the sn-1 or -3 positions (Gunstone 1986).
of saturated fatty acids may be enhanced by randomizing their position on the TAG in some circumstances (Lien et al. 1993, Tomarelli et al. 1969). However, the reported effects on plasma lipids of manipulating positional structure of diet fats have thus far been minimal. In humans, no significant effects were seen in hormonal and plasma lipid responses to feeding (Zampelas et al. 1994). Slight but significant effects were seen in fasting lipoprotein concentrations of men but not women (Zock et al. 1995). Slight differences were also found in structural composition of circulating TAG in VLDL (Zock et al. 1996). In piglets, altered plasma cholesterol was found (Innis et al. 1993).

Dietary fat and degenerative disease

Current public recommendations for dietary fat are based on several decades of research into the role of diet in the etiology and treatment of CVD and NIDDM. Both the American Diabetes Association (ADA 1987) and American Heart Association (AHA 1988) have adopted the recommendations for fat intake of the Dietary Guidelines for Americans (USDA & USDHHS 1995). These state that total fat should provide not more than 30% of dietary energy, that saturated fat should be less than 10%, and polyunsaturated fat (PUFA), which is primarily composed of linoleate, may be up to 10%. Although no specific mention of oleate is made, if the guidelines were met, mono-unsaturated fat (MUFA), which is primarily oleate (Gurr 1992), would contribute about 10 to 20% of total energy. From the NHANES II data it can be estimated that, between 1978 and 1987, Americans were consuming an average 15% energy as saturated fat, 15% oleate and 7% linoleate. Thus, fat consumption in excess of recommended levels was primarily in the form of saturated fatty acids.

The primary aim of dietary fat recommendations is to alter concentrations of various plasma lipid fractions which have been identified as risk factors for CVD (AHA 1988). In particular, the goal is to reduce fasting plasma TAG and low-density lipoprotein (LDL) cholesterol, and increase or maintain high-density lipoprotein (HDL) cholesterol. In the case of NIDDM, an additional goal is to enhance blood glucose
control and insulin sensitivity. It should be noted that NIDDM and CVD are often concurrent. In fact, the designation "syndrome X" has been coined to describe a set of symptoms that include deranged plasma lipids, hypertension, and insulin resistance (Reaven 1988), with the suggestion that a common underlying pathology may be present.

The breakdown in recommendations by type of fat reflects the differences among individual fatty acids in their apparent atherogenicity. This has been the subject of much research in itself, and was the focus of a recent supplemental issue of the American Journal of Clinical Nutrition (Vol. 65, No. 5S). A comprehensive review of human studies in that issue (Kris-Etherton & Yu 1997) concluded that stearate, oleate and linoleate all reduce LDL as compared with any of the C12:0 to C16:0 saturated fatty acids. Linoleate also reduces HDL however, whereas the results for oleate in this regard have been less consistent. This discrepancy may involve the presence of varying amounts of antioxidant nutrients in the fats being consumed, rather than, or in addition to, the fatty acids themselves. For example, in one study no benefit to plasma lipids of olive oil over the more saturated palmolein was found (Choudhury et al. 1995). This was attributed to the high vitamin E content of the palmolein. Since linoleate is more susceptible to oxidative damage than oleate or saturated fatty acids, oxidative damage to plasma lipids may be a factor in the reduction of HDL seen in diets high in linoleate.

It should be noted that, although eating "too much" fat is obviously a health risk, there is also substantial risk in giving one-sided advice against eating fat. Linoleic acid, for example, is a dietary essential (NRC 1989), and other fatty acids can have therapeutic value in preventing heart attacks (Hunt & Groff 1990). Nevertheless, the Guidelines (Kennedy et al. 1996) urge selecting a diet "low in fat" but "moderate in sugars," as well as use of alcohol "in moderation." Alcohol and sugar would appear, by implication, to be better than fat, although neither is essential to health. In addition, dietary oleate may have a unique role among fatty acids in promoting health, the evidence for which will be considered next.
High-oleate diet interventions

The potential benefit of dietary oleate was first suggested by the relatively high olive oil consumption and low CVD rates found among populations in the Mediterranean geographic region in the 1960's (Helsing 1995). Although numerous aspects of the Mediterranean type of diet have been found to be beneficial to health (Kushi et al. 1995), consumption of oleate per se remains an active area of research. A "Mediterranean diet pyramid" has recently been formulated in which olive oil has its own category, just above the fruit & vegetable tier, but below (i.e., to be consumed more frequently than) the dairy and meat tiers (Willett et al. 1995). Even so, the traditional Mediterranean diet is estimated to have only 25 to 35% of energy as fat (Willett et al. 1995). The apparent discrepancy lies in the fact that dietary recommendations such as the Pyramid rely on the concept of "servings" (USDA 1992). Thus, whereas a typical serving of milk is 250 ml (about 8 ounces) or more, a serving of fat is about 5 ml.

Unfortunately, dietary oleate per se was at first ignored by researchers because it was perceived to be "neutral" (Mattson & Grundy 1985). However, while diets low in fat and high in complex CHO were successful in reducing LDL, they sometimes raised fasting TAG concentrations. This, and the HDL-lowering effects of diets high in PUFA, prompted an investigation of the effects of substituting oleate for other fats in relatively high-fat diets. In the first of these (Mattson & Grundy 1985), patients with high plasma cholesterol were fed diets containing 40% of energy as fat for four weeks in a crossover comparison of three types of dietary fat substituted at about 10% of energy. The high-oleate and high-PUFA diets reduced plasma LDL similarly, compared to a high-saturated fat diet, but effects on plasma TAG and HDL were inconclusive. Next, a similar study compared saturated fat, oleate, and complex CHO (Grundy 1986). Again, 40% of the energy in the two high-fat diets was from fat, and the substitutions equaled 20% of energy. Compared to saturated fat, oleate and CHO reduced LDL similarly, but the high-CHO diet also raised TAG and lowered HDL, unlike the high-oleate diet. Similar results
were found in healthy persons fed high-oleate or high-CHO diets for 36 days (Mensink & Katan 1987). The two high-fat diets in that study contained about 40% of energy as fat, and the experimental diets replaced about 14% of saturated fat energy with MUFA or CHO. Another study in healthy men, with similar diet interventions but of six weeks' duration, also showed benefits to cholesterol with MUFA over saturated fat or CHO (Grundy et al. 1988).

Because patients with NIDDM also suffer disturbances in plasma lipids and lipoproteins, assessing the effects of high-MUFA diets in this population was a logical next step. Several studies have compared the effects of high-MUFA and high-CHO diets in patients with NIDDM. In the first (Garg et al. 1988), patients were fed each of the two diets for 28 days. The high-MUFA diet was 50% total fat, 33% MUFA and 35% CHO. In the high-CHO diet 25% of fat energy was replaced with complex CHO. The high-MUFA diet resulted in lower plasma TAG and VLDL cholesterol, higher HDL cholesterol, and similar LDL cholesterol, in agreement with previous studies. In addition, mean plasma glucose levels and insulin requirements were reduced by the high-MUFA diet. Similar results were obtained in a second study (Parillo et al. 1992), in which each diet was fed for 15 days, and the high-MUFA diet was 40% total fat, 29% MUFA, and 40% CHO, and the high-CHO diet substituted 20% of energy. In that study a euglycemic hyperinsulinemic clamp was used to show that insulin-mediated glucose disposal was improved by the high-MUFA diet.

To determine whether the apparent benefits of replacing some complex CHO with oleate would persist beyond the few weeks that had previously been used, a 14-week study was undertaken (Garg et al. 1994). NIDDM patients were fed either a high-CHO diet with 30% total fat, or one with 15% of CHO energy substituted with oleate so that total fat was 45%. Elevated TAG and VLDL persisted throughout the 14 weeks of high-CHO feeding, as did elevated plasma glucose and insulin concentrations, as compared with the high-MUFA diet. Finally, a high-MUFA diet was also shown to increase insulin sensitivity in healthy young women, relative to a high-CHO diet (Uusitupa et al. 1994). That the various benefits of high-MUFA diets are found in healthy persons suggests a
fundamental difference in metabolism between oleate and the more atherogenic saturated fatty acids.

In the above discussion, it should be noted that the category of MUFA also includes small amounts of fatty acids other than oleate. Although researchers have typically reported only total MUFA content, little is known about how different MUFA's like different saturated fatty acids, differ in their health effects. However, two studies have looked at this. In one study, various fatty acids were added to the diets of weanling pigs at 10g/100g of feed weight (Smith et al. 1996). Stearate, linoleate, and palmitate did not differ significantly in their effects on plasma lipoproteins, but a mixture of myristoleate (C14:1) plus palmitoleate (C16:1) produced the highest LDL. A second study compared palmitate, oleate or palmitoleate in hypercholesterolemic men (Nestel et al. 1994). In this three-week crossover trial, 40% of energy was fat, and substituting just 4% of energy as palmitate or palmitoleate resulted in small but significant and similar increases in plasma LDL relative to oleate. Until more data on MUFA other than oleate are available, it would seem prudent to assume that only oleate is responsible for any benefits observed in high-MUFA diet interventions.

It can be seen from the preceding discussion that diets relatively high in MUFA consistently result in beneficial effects on risk factors of heart disease and diabetes. Yet controversy remains as to optimal recommendations for dietary fat. Part of the source of this controversy involves the definition of how high is high and how low is low, so to speak. Thus, it is useful to know whether there are limits to fat oxidation, and where those limits might be found.

Metabolic limits of fat oxidation

Mechanistic explanations for the effects of dietary fat on lipoproteins and insulin sensitivity have been notably lacking in the literature. However, such information would be valuable in formulating future diet recommendations, particularly if they could be individualized, as has been suggested for persons with NIDDM (Sheard 1995). In terms
of total quantity of fat in the diet, both the upper and lower extremes involve specific mechanistic limitations that should be considered. At very low fat intakes, fat synthesis is stimulated. The evidence for this in humans is limited, but consistent. When healthy men were overfed a diet with 83% CHO and 2% fat energy in one study, their rate of net fat synthesis, assessed by indirect calorimetry, precisely matched the excess energy intake (Aarsland et al. 1997).

The above result, that excess energy intake as CHO is converted to fat for storage, is not surprising. However, there is also evidence for fat synthesis on eucaloric diets. This is more difficult to assess, because simultaneous fat synthesis and oxidation cannot be measured by indirect calorimetry. Two novel methods have recently been used to address this question (Hudgins et al. 1996). Mass isotopomer distribution analysis (MIDA) uses incorporation of $^{13}$C-labeled acetate into palmitate and stearate of VLDL TAG. Linoleate dilution uses changes in linoleate content of VLDL relative to that of adipose stores to infer synthesis of other fatty acids. When a eucaloric diet with 10% of energy as fat and 75% CHO was fed to healthy men for 25 days, 30 to 57% of VLDL fatty acids were calculated to have been newly synthesized (Hudgins et al. 1996). In the same study, even when a 40% fat diet was consumed, some de novo fatty acid synthesis was detectable by MIDA. However, the 45% of energy in the diet that was CHO was in the form of glucose polymers, which the authors noted may be more likely to stimulate fat synthesis than complex CHO.

In fact, palmitoleic acid has been shown to be a biomarker for adherence to a low-fat diet (Phinney et al. 1996). Even slight increases in plasma palmitoleate are easily detectable because of its scarcity in dietary and adipose fat. One reason fat synthesis may not be desirable is that humans synthesize palmitate for the most part (Stryer 1988 p 485). Although a small fraction of the palmitate synthesized is then desaturated to form palmitoleate (Rhee et al. 1997, Emken et al. 1993), this is probably similar to palmitate in its effect on plasma lipoproteins (Nestel et al. 1994).

At the opposite extreme, diets very high in fat have been shown to cause insulin resistance (Storlien et al. 1986, Kraegen et al. 1991). In one study, a diet having 59% of
energy as safflower oil was fed to rats for 24 days and insulin resistance was observed in skeletal muscle, liver, and brown adipose tissue (Storlien et al. 1986). In a further study using a similar protocol, the time course for developing impairments in insulin function was investigated (Kraegen et al. 1991). After only three days, adipose tissue uptake of fat and hepatic glucose output showed impaired responses to insulin. Overall peripheral glucose disposal was not significantly impaired at three days, but was by three weeks, at which time muscle TAG storage was double that of high-starch-fed controls. Recall that human diet intervention studies have demonstrated benefits of oleate at total fat intakes of up to 50%, at least for short periods of time, so the precise point at which fat intake causes insulin resistance, and how fat type might affect this point, is not known.

The glucose fatty acid cycle

Insulin resistance is, by definition, reduced sensitivity of metabolic processes to regulation by insulin. Although insulin resistance per se is not a disease state, persons with insulin resistance frequently progress to NIDDM (Defronzo et al. 1992), so it poses a serious health threat. The net effect of insulin resistance is to shift substrate oxidation away from glucose and towards fat if insulin concentration is held constant. Thus, it is not surprising that it should occur when fat intake exceeds the body’s capacity to up-regulate fat oxidation. A reciprocal relationship between regulation of glucose and fat metabolism was first proposed by Randle, et al. in 1963, who termed the (then putative) processes involved “the glucose fatty acid cycle.” In that paper Randle proposed that, while the mutual inhibition of each-other’s metabolism by glucose and fatty acids may be a primitive mechanism for maintaining plasma glucose concentrations, it also represents a method by which fat can induce insulin resistance (Randle et al. 1963).

Since then, various proposed metabolic mechanisms of the glucose fatty acid cycle have been demonstrated experimentally. A powerful tool in this field has been the metabolic clamp technique, in which plasma concentrations of insulin and other hormones, and sometimes glucose, are held constant by means of infusions. In addition
to the well-known role of insulin in stimulating glucose uptake and oxidation, it has been shown to exert dose-dependent suppression of fat oxidation and of lipolysis of adipose TAG stores (Boden et al. 1993, Campbell et al. 1992, Bonadonna et al. 1990b). Similar inhibition of fat oxidation is obtained with drugs that inhibit lipolysis directly (Li et al. 1993), indicating that this inhibition is the direct cause of the shift in substrate oxidation, rather than some other effect of insulin. Adipose lipolysis is more sensitive than fat oxidation to insulin, presumably due to oxidation of intracellular lipid stores in metabolically active tissues. Plasma glucose can also inhibit lipolysis, independent of the effects of insulin (Carlson et al. 1991). On the other hand, when fatty acid delivery to cells is enhanced by infusion of fat emulsions and heparin, the effects of insulin on glucose metabolism are impaired. This is true for glucose oxidation (Yki-Jarvinen et al. 1991, Laville et al. 1995, Boden et al. 1991), whole-body glucose disposal (Yki-Jarvinen et al. 1991, Saloranta et al. 1993), and hepatic glucose production (Laville et al. 1995). Taken together, these results demonstrate that fat and CHO metabolism are closely linked, and any investigation of one implicitly involves the other.

The importance of the glucose fatty acid cycle lies in its role in disease states. Uncomplicated obesity results in hyperinsulinemia (Bonadonna et al. 1990a), particularly in persons with excess visceral fat (Mauriege et al. 1990), and is a risk factor for developing NIDDM (DeFronzo et al. 1992). Clamp studies have demonstrated that the effects of insulin on various aspects of fat metabolism are blunted in obese subjects. These include whole body absolute rate of lipolysis (Campbell et al. 1994), although not when expressed per fat mass (Groop et al. 1992), and production of VLDL apoB-100 protein by the liver (Lewis et al. 1993). In obese subjects with glucose intolerance, fat metabolism following a mixed meal was also found to be insulin resistant (Coppack et al. 1992). Diabetes may also involve impaired insulin sensitivity of fat metabolism independent of obesity. Lean patients with NIDDM also show impaired suppression of lipolysis during clamp studies compared with control subjects (Groop et al. 1989). A four-way comparison of lean versus obese and healthy versus NIDDM subjects found that, although lipolysis was insulin-resistant in both the lean NIDDM and healthy obese
subjects, it was most resistant in the obese NIDDM patients (Groop et al. 1991). Of course, one outcome of elevated lipolysis is elevated reesterification within the liver, and therefore elevated VLDL production (Cummings et al. 1995). This in turn elevates circulating TAG, which may be a risk factor for CVD. Also, there is consequently an increased rate of production of the end product of VLDL catabolism, LDL cholesterol.

**Dietary fat and obesity**

To say that weight gain is caused by overeating is a tautology. It is more instructive to examine the causes of eating in excess of one’s energy expenditure, which are many. Almost certainly, one of these is a failure to maintain fat balance (Flatt 1987). That is, when proportional fat intake exceeds the body’s ability to match oxidation to intake, progressive fat accretion and thus obesity will result. Thus, excess fat intake may be caused by the need to maintain CHO intake at a certain level when the ratio of fat to CHO in the diet exceeds some threshold amount. This threshold is presumably influenced by genetic and behavioral factors, and possibly by the composition of dietary fat.

Animal models of diet-induced obesity (DIO) support this idea. When mice were fed ad libitum diets containing amounts of fat from 1 to 64% of energy, their fat mass and incidence of obesity increased with dietary fat (Salmon & Flatt 1985). Also, individual variability increased greatly among mice fed 32 to 64% fat, compared with those fed 1 or 13%. This is a common feature of DIO studies, i.e., that individual animals in a healthy population vary greatly in susceptibility to DIO. One approach frequently used in this field is to feed high-fat diets to animals, and define those in the upper quartile of weight gain as “DIO”, and the lower quartile as “resistant.” In one such study, rats were fed diets with either 20 or 60% fat energy, and the average weight of the low-fat-fed animals did not differ from that of the resistant group eating the high-fat diet (Chang et al. 1990). The resistant animals also had a higher 24-hour fat oxidation than the DIO group, as would be expected. When insulin sensitivity was assessed by euglycemic insulin clamp, that of the
resistant animals was also higher than in DIO. In a prospective study with rats fed 60% fat diets for four weeks, the DIO animals had a lower percentage of red muscle fibers than the resistant animals, both before and after fat feeding (Mrad et al. 1992). Red muscle fibers are the type which primarily oxidize fat. Of course, rodents possess a metabolic mechanism for increasing fat oxidation, futile cycling in brown adipose tissue, which is probably not significant in adult humans except as a thermoregulatory mechanism under circumstances of cold exposure (Macdonald 1992).

In humans, there are several lines of evidence to support a role for impaired fat balance in the development of obesity. One of these is correlational. In two studies, three-day food records were used to calculate percent fat intake in 244 men (Tremblay et al. 1989) or in 344 men plus 355 women (George et al. 1990). When upper and lower quartiles of fat intake were compared, greater adiposity was associated with higher fat intake in both studies. Similarly, in 205 women, fat intake was positively correlated with body fatness after controlling for various factors such as age and activity level (Tucker & Kano 1992). A similar result was obtained in a study with 48 children aged 9 to 11 years (Gazzaniga & Burns 1993).

To address whether expanded fat mass leads to increased fat oxidation, as is suggested by the fat balance model (Flatt 1987), the relationship between body fatness and fat oxidation has also been examined. In 106 weight-stable obese women, fasting fat oxidation was positively correlated with body fat (Schutz et al. 1992). In a similar study with 427 men and 293 women however, no such relationship was observed (Nagy et al. 1996). In that study, subjects varied in body fatness from 4% to 55%, suggesting that the relationship may be specific to obesity (Nagy et al. 1996). On the other hand, when 24-hour fat oxidation was measured in 38 obese women and 35 non-obese controls, a positive correlation with body fat was observed for all subjects combined (Astrup et al. 1994b). If fat oxidation is a factor in maintaining fat balance, it must occur over the course of the entire day, not just at the end of an overnight fast. In the above study, the authors estimated that an increase in dietary fat energy of just 1.5 ± 1.1% as a proportion
of intake would require a 10 kg increase in fat mass to restore fat balance. If so, it would be interesting to determine whether a threshold exists for this effect.

The cross-sectional studies discussed above are complemented by others which looked at spontaneous intake of diets with differing fat contents but similar appearance, density and palatability. In one crossover study, 24 women with a wide range of body fatness consumed diets with three fat levels for two weeks each (Lissner et al. 1987). Compared with a diet having 30 to 35% fat energy, spontaneous consumption of a 15 to 20% fat diet was reduced 11%, and consumption of a 45 to 50% fat diet was increased 15%. Similarly, both lean and obese subjects consuming diets having either 26 or 52% fat also over-consumed the high-fat diet (Thomas et al. 1992). However, in lean subjects 24-hour fat oxidation was highly correlated to fat intake, but in obese subjects no relationship was found. That is, lean subjects were apparently better able to match fat oxidation to intake than were obese. Furthermore, formerly-obese women were found to have impaired ability to match fat oxidation to intake compared to never-obese controls in spite of controlled caloric intake (Astrup et al. 1994a). After three days of eating a 50% fat diet, the 24-hour fat balance of control subjects was -9 grams, whereas that of the post-obese was +11 grams. In a year’s time, 11 grams of fat per day would add up to 4.0 kg. Recently, Saltzman et al. reported no significant difference in intake of a 40% fat versus a 20% fat diet in seven male monozygotic twin pairs fed each diet for nine days (Saltzman et al. 1997). However, the high-fat diet in this study was not as high in fat as any of the previously mentioned results. Also, individual twin pairs varied in their response to the diets, supporting the idea that genetic susceptibility is a significant determinant of this effect.

It is generally true that healthy people quickly match their proportional (net) oxidation of fat to CHO, under conditions of moderate fat intake, if the energy intake is experimentally held constant. In a recent study where healthy adults stayed in a respiration chamber, after switching from 30 to 60% of energy intake as fat with constant total energy intake, fat balance was reestablished by day seven (Schrauwen et al. 1997). This is in agreement with the results of a crossover study with weight-stable obese
women (Roust et al. 1994). Following isocaloric intake of diets having 42% versus 27% fat for four weeks each, no changes in body weight or composition were observed.

The evidence considered thus far suggests that dietary fat may play a crucial role in obesity and related health problems. It also indicates substantial genetic variation in susceptibility to negative consequences of high fat intake. This, in turn, results from variation in ability to regulate fat oxidation to match intake. Obesity, in this context, may be a very-long-term method for reestablishing fat balance when short-term regulatory capacity is exceeded. However, in interpreting these effects, it would be useful to understand the proximate cause of spontaneous overeating in response to a high-fat diet. This will be considered next.

Regulation of food intake

Spontaneous alterations in food intake among healthy persons suggests an effect on metabolic hunger and satiety signals. Therefore, it will be useful to consider the origins and nature of such signals, especially with regard to how they are influenced by macronutrient intake. In general, there are two categories of regulatory signals for eating behavior. One is peripheral in origin, and appears to be a function of energy charge in the liver, and to involve signaling between liver and pancreas. The other is central, and appears to integrate hypothalamic processing of nutrients and neural signals from other areas of the brain as well as from the periphery (Carlson 1981). For the purposes of this review, only the peripheral signals will be discussed in depth, because these involve processes related to whole-body fat and glucose oxidation.

In an elegant series of studies, Friedman and colleagues have presented compelling evidence that integrated fuel oxidation in the liver is a key element in the peripheral hunger signaling system (Tordoff et al. 1988, Park et al. 1996, Tordoff et al. 1991, Rawson et al. 1994, Park et al. 1995). In these studies the fructose analogue, 2,5-anhydro-D-mannitol (2.5-AM), was used to induce a metabolic state in the liver similar to that which initiates feeding. The mechanism for the effects of 2.5-AM is phosphate
trapping and consequent reduction of ATP. Like fructose, it is taken up only by the liver and phosphorylated, but unlike fructose it cannot be further metabolized (Rawson et al. 1994). The evidence for this has been summarized elsewhere (Park et al. 1995).

In the first of these studies, 2.5-AM induced feeding in rats when administered peripherally, and caused shifts in plasma nutrients similar to those seen with mild food deprivation (Tordoff et al. 1988). Its effects on insulin and epinephrine also paralleled those of fasting (Park et al. 1996). Also, hepatic branch vagotomy blocks the feeding response to low doses of the fructose analogue (Tordoff et al. 1991), suggesting strongly that its effect is mediated by the liver. Another effect of 2.5-AM in rats that is similar to fasting is a shift in nutrient oxidation towards fat (Park et al. 1995). Interestingly, in a species of migratory bird kept on a short light cycle, a low dose of the drug had no effect on feeding, and a higher dose actually suppressed feeding (Boswell et al. 1995). The higher dose also increased plasma NEFA, suggesting that, in these birds adapted to relying almost entirely on stored body fat for fuel, elevated NEFA may be instrumental in satiety signaling.

Eating regulation for rats is likely more representative of what is found in humans than that of migratory birds. In the studies so far discussed, rats were fed a standard low-fat chow diet. However, when rats were fed a 60% fat diet for two weeks they became insensitive to the effects of 2.5-AM, despite experiencing similar changes in plasma nutrients and insulin (Rawson et al. 1996). Recall that this level of fat intake was sufficient to induce insulin resistance in liver of rats (Storlien et al. 1986, Kraegen et al. 1991). This was reflected in the depletion of liver glycogen found in the high-fat-fed animals (Rawson et al. 1996). On the other hand, rats adapted to a 40% fat diet remained responsive to 2.5-AM although the feeding response in these animals appeared to be more variable than that of rats eating 13% fat (Rawson et al. 1996). Inhibition of fat oxidation potentiated the effect of 2.5-AM on food intake, but did not alter plasma fuel or insulin responses. The authors concluded from this that the feeding signal produced by the liver is not solely dependent on CHO oxidation, but integrates information about both CHO and fat oxidation within this organ.
The findings discussed above may have clinical relevance for reduced-obese persons, who have been found to have impaired ability to oxidize dietary fat both as compared with their own pre-obese status (Ballor et al. 1996) and to never-obese controls (Raben et al. 1994, Larson et al. 1995a). This may be due to up-regulation of mechanisms favoring storage of dietary fat (Yost & Eckel 1988, Kern et al. 1990).

Authors of a recent cross-sectional study relating maintenance of weight loss to physical activity concluded that at least 90 minutes per day of moderate-intensity exercise is required for long-term maintenance of weight loss (Klem et al. 1997). Exercise promotes fat oxidation in at least three ways. First, during moderate exercise, fat becomes the primary fuel source (Romijn et al. 1993). Second, exercise lasting an hour or more increases insulin sensitivity for up to 24 hours, such that daily exercise results in de facto long-term improvement in insulin sensitivity (Rogers et al. 1990). It has been suggested that insulin resistance is a mechanistic adaptation by which the body restores fat balance (Eckel 1992). This amount of exercise may also be sufficient to increase resting fat oxidation, which has been demonstrated to occur in elite endurance athletes (Sjodin et al. 1996). Increasing fat oxidation arguably has the potential to restore fat balance for post-obese individuals without restoration of body fat stores.

**Metabolism of dietary fat**

To correctly interpret the results of experimental manipulations of dietary fat, it is necessary to understand the various metabolic processes that act on ingested fat to determine its short-term fate. Although this review will not attempt to cover these comprehensively, the aspects relevant to interpreting the research presented herein will be discussed. These include digestion and absorption of dietary fat; formation, composition and catabolism of CM and VLDL; and the sources and uptake of plasma NEFA in a fed state. They are diagrammed in Figure 1. Transporters and regulatory enzymes of fat oxidation will also be described, since specificities in these may contribute to differential
fates of dietary fatty acids. Finally, human genetic alleles of a few of these proteins known to affect fat metabolism will be described.

Digestion & absorption of dietary fat

The first step in metabolism of ingested fat is digestion and absorption. The processes involved in this are hydrolysis in the intestinal lumen of TAG to NEFA and monoacylglycerol (MAG) or glycerol, uptake by enterocytes, resynthesis to TAG, assembly into CM, and delivery to peripheral circulation via the thoracic duct of the lymphatic system. Phospholipids, which are present in smaller quantities than TAG, are handled similarly. This is entirely distinct from the absorption of CHO and protein, which are delivered to the liver via the portal vein.

Some methodological variations that should be noted in the following discussion of fat absorption include: how much fat is given; whether fat is given alone or with other nutrients; if it is given alone, is it emulsified; and if it is intubated without being emulsified, is it a stomach or duodenal tube. When fatty acid tracers are employed, there is the additional issue of whether the tracers are given as NEFA or TAG, and whether it is mixed with unlabeled TAG or given alone.

These considerations are important because there is evidence that under certain conditions substantial fat may be transported directly from the duodenum to portal circulation, bypassing CM altogether (Mansbach et al. 1991). In fasted rats given constant duodenal infusions of 135 μmol/hr ^3H-labeled triolein emulsified in water with bile salts, the radioactivity recovered from portal circulation by six hours was 39% of the infusion rate. Lymph was collected throughout to prevent the label from reaching circulation via that route. However, a later study from the same laboratory found that adding 9 μmol/hr phosphatidyl choline (PC) to the infusate reduced portal recovery to 1.4%, and that reducing the triolein infusion to 27 μmol/hr further reduced portal recovery to 0.5% of the infusion rate (Mansbach & Dowell 1993). PC has been suggested to be a rate-limiting factor in CM formation (Mansbach et al. 1985, Tso et al. 1978). In
one study a bile fistula was shown to impair CM formation (Mansbach et al. 1985) and in another replacing the PC restored CM secretion, but replacing bile salts alone did not (Tso et al. 1978).

Although the role of PC in dietary fat digestion is not fully known, it is the principal phospholipid (PL) in bile and in plasma lipoproteins (Skipski 1972), and has emulsifying properties due to its chemical structure of hydrophobic diacylglycerol tail plus hydrophilic phosphate-choline head. It may have a role in emulsifying dietary lipids in the lumen during digestion (Hernell et al. 1990). Emulsification of dietary TAG is generally assumed to occur primarily in the gut via the actions of bile salts and peristalsis (Linder 1988). However, significant emulsification of ingested dietary fat in the stomach has been demonstrated in adult humans (Armand et al. 1994). A coarsely emulsified test meal containing 70 grams each olive oil and sugar plus 93 grams of egg (which contributed 5.8 mM PL) was fed, and gastric aspirates collected hourly. The size distribution of fat particles was considerably reduced with time, with most of the emulsifying effect occurring in the first hour. This raises the possibility that duodenal infusions of TAG do not necessarily reflect normal fat digestion. This is not supported by a similar study in which a coarsely emulsified test meal was intubated to the stomach or duodenum and samples aspirated after one hour (Armand et al. 1996). A comparison of resulting lipid particle sizes revealed no significant difference in emulsification by stomach versus duodenum. One should still interpret studies using unemulsified fats with caution, since emulsification of lipids is necessary for lipolysis to occur (Linder 1988).

Another common assumption regarding fat digestion is that it occurs entirely within the duodenum and jejunum (Linder 1988). This assumption was recently tested in dogs given meals in which either 15 or 60 grams corn oil were emulsified into a protein solution (Lin et al. 1996). Patterns of recovery of fat from chyme via duodenal and midgut fistulas suggested involvement of the entire small intestine in fat absorption, and further, that this pattern is influenced by dose of ingested fat. That is, digestion of larger amounts of fat involved the latter segments of the intestine to a greater degree. Some differences in metabolism of absorbed fatty acids by proximal versus distal small
intestine have also been observed (Wu et al. 1975. Wu 1980). This could create experimental confounding factors related to the amount of fat used. It also has implications for observations involving the time course of delivery of digested fat to the periphery.

An additional methodological consideration involves potential malabsorption of NEFA as compared with TAG. For example, in rats given NEFA tracers in a fat-free diet, absorption of palmitate was only 30% after 7 hours and 40% after 24 hours (Toorop et al. 1979). Absorption of linoleate in that study was 59% and 92%, respectively. When tracers were dissolved in olive oil, absorption was 60% for both palmitate and linoleate after 6 hours in one study (Cenedella & Allen 1969), and 97-98% after 24 hours in another (Leyton et al. 1987). In those studies, all tracers were in the form of NEFA but by dissolving them in TAG they were presumably digested similarly to the TAG. For example, they might be protected from formation of calcium soaps. Fat absorption in humans can only be assessed by fecal collection, and so no inference of time course can be made in human studies. However, total absorption of stearate tracer given to humans in capsule form in one study was 78% whereas dietary stearate absorption was 92% (Jones et al. 1985b). In this same study, capsule-fed oleate tracer was 97% absorbed and linoleate tracer was 99% absorbed. In another study, \([1-^{13}C]\)-palmitate was mixed with butter and fed to healthy women with bread and juice, and fecal excretion of tracer was 14 ± 10% in the first trial and 32 ± 25% in a second trial (Murphy et al. 1995). Tracer excretion was also unrelated to total lipid excretion in that study. It should also be noted that fecal collection does not rule out colonic fermentation of fatty acids, which has been demonstrated in rats (Segal et al. 1990).

Chylomicron composition

Most fatty acids in the diet and in CM are in the form of TAG, but some are also esterified to phospholipids and, to a lesser extent, cholesterol. In the gut, additional PL are introduced via bile, which helps in the digestive process by promoting emulsification
of hydrophobic TAG. Like cholesterol, PL, especially PC, are also components of cell membranes. Their presence in the diet is therefore variable but quantitatively minor by comparison with TAG. In both rats (Mattson & Volpenhein 1964) and humans (Kayden et al. 1967) only about 25% of TAG are hydrolyzed completely to NEFA and glycerol. The majority are absorbed as 2-MAG plus 2 fatty acids, which are reesterified to form TAG within the enterocyte. In both of those studies, radio-labeled tracer fatty acids esterified to specific positions on TAG were emulsified and introduced directly either to the duodenum or stomach in a fasting state, and lymph was collected via thoracic duct cannula. Phospholipids have two fatty acids esterified in the 1- and 2- positions, and only the 2-position is cleaved in the gut by phospholipase A2 (Linder 1988). The resulting lyso-PL is also reesterified within the enterocyte, so there is the potential for some exchange of fatty acids between TAG and PL in the enterocyte.

The lipid content of CM is about 89 to 95% TAG, PL contributes about 3 to 6%, and cholesterol ester (CE) is about 2 to 5% (Skipski 1972). Although one might expect most of the TAG in CM to come from dietary sources, there is evidence that a large portion of the fatty acids in CM TAG are of endogenous origin. First, there are substantial differences between fatty acid composition of diet and CM (Lambert et al. 1996). In rats tube-fed 0.5 ml of various fats (Lambert et al. 1996), significant alterations in fatty acid composition of CM relative to dietary fat were observed. The fats were chosen to represent extremes of composition, and included palm oil, butter, fish oil, olive oil, and corn oil. In all cases the CM compositions were more homogeneous than those of the parent fats. For example, linoleate content of diet fats ranged from 1% in fish oil to 58% in corn oil, but in CM the range was 13% to 50%. Also, eicosapentaenoate (EPA, C20:5 n-3) and docosahexaenoate (DHA, C22:6 n-3) were not detectable in any of the diet fats except fish oil, but were consistently present in CM of non-fish oil fed rats, at 0.5 to 0.9% for EPA and 1.3 to 2.1% for DHA.

Results from two tracer feeding studies are consistent with the idea that CM lipids are substantially formed from endogenous sources (Karmen et al. 1963, Emken et al. 1987). In the first of these, radiolabeled fatty acids were given to rats by stomach
intubation with various amounts and compositions of unlabeled fat. and chyle was recovered from the thoracic duct for 24 hours after tracer administration (Karmen et al. 1963). Total recoveries of label in CM ranged from 39 to 67%, and were up to 79% in total lymph. Dilution of CM lipid by endogenous sources was calculated to be 40-45%, depending on the fatty acid and experimental protocol. In two human subjects, 38 or 27 grams of deuterium-labeled fatty acids in TAG form were emulsified into a liquid meal with protein and sugars and fed in place of breakfast (Emken et al. 1987). At the peak label recovery time, dilution of label in CM was calculated to be 39 or 62% for the two subjects. However, since the second subject also consumed a lunch meal with unreported fat content two hours before the maximal sample was collected, it was probably also diluted by the new, unlabeled dietary fat.

A third tracer study in humans produced contradictory results to those cited above (Nguyen et al. 1996). Triolein labeled with $^3$H was incorporated into a liquid diet with 32% fat energy, and fed in hourly doses for six hours, with total energy intake equaling one-third of total daily energy expenditure. CM TAG enrichments equal to those of the diet fat were reported, although the time course for this was not given, and a plateau of CM concentration was not achieved until about the last 40 minutes of the protocol.

Potential sources of endogenous CM lipids include bile, fat remaining in the enterocyte from previous meals, or NEFA taken up from plasma. NEFA may originate from mesenteric fat stores or peripheral hydrolysis of TAG from circulating lipoproteins. There is evidence to support all three of these options. First, in one study most of the lipid in lymph of fasting rats appeared to come from bile PL (Shrivastava et al. 1967). Not only did the lymph lipid closely resemble bile PL in fatty acid composition, during bile collection the lymph lipid flow reductions (of about 80%) were closely matched to bile output within animals, and lymph lipid output was not restored by infusions of bile salts to the gut, suggesting that emulsification was not the critical factor.

Second, when humans were fed two sequential meals with substantially different fatty acid compositions. CM composition during peak plasma CM concentration following the second meal substantially reflected the fatty acids from the first meal.
(Fielding et al. 1996). This was interpreted by the authors to reflect fat that had been retained in the enterocyte between the two meals.

Finally, tracer fatty acids injected peripherally have been recovered from intestinal cells of rats (Gangl & Ockner 1975, Hultin et al. 1996) and humans (Gangl & Renner 1978), albeit in relatively small quantities. However, some researchers have suggested that these NEFA are incorporated into a separate lipid pool in the enterocyte from the one which subserves CM formation (Gangl & Ockner 1975, Mansbach & Dowell 1992).

**Kinetics of chylomicron delivery**

Finally, the time course of CM formation and delivery will be considered, although exiting data are limited. One kinetic tracer study evaluated the fractional turnover rate of the CM precursor pool in rats, using tracer washout data with a constant duodenal lipid infusion (Mansbach & Arnold 1986). When 135 μmol/hr of labeled triolein was infused, fractional turnover was 0.61 to 0.74 pools per hour, versus 1.03 pools per hour when 27 μmol triolein was infused. Thus, fractional turnover increased as infusion rate decreased, consistent with a saturable process. Another study in rats found that, when emulsions of labeled tristearin were infused with triolein or tripalmitin, a steady state labeling of lymph TAG was observed by about the 4th hour (Bergstedt et al. 1991).

When constant enteric infusions of fat have been given to humans, a steady-state CM concentration has been achieved by five or six hours (Nguyen et al. 1996, Grundy & Mok 1976). Also, appearance of CM in plasma following fat ingestion has been reported to occur within as little as 30 minutes (Hultin et al. 1996).

If one assumes a 30 minute delay and fractional turnover of 0.6 pools per hour, CM production and composition should be within 6% of its theoretical asymptotic (i.e., steady-state) value by three hours. Alternatively, with an hour's delay and turnover of 1.0 pool per hour, it would take five hours to reach the same point. Thus, in the studies above, the time course for achieving constant plasma CM concentration is consistent with
the kinetics of fatty acid turnover by enterocytes as assessed by tracer infusion. It might also, however, be influenced by alterations in the clearance of the CM being delivered, mediated by hormonal changes that occur during the transition from fasting to satiation.

Chylomicron catabolism

The disposition of fatty acids following their delivery to arterial blood in CM is central to the present research, since CM-derived fatty acids were used as the precursor pool for metabolic processing of diet-derived fatty acids. Three aspects of CM catabolism will be discussed next. These are: mechanisms for catabolism and removal from circulation, kinetics of the removal process, and tissue-specific distribution of uptake.

First, particles in the CM size category are not known to be taken up into any tissue directly. Both CM and VLDL in the periphery are partially hydrolyzed by lipoprotein lipase (LPL) in capillary beds before their remnant particles are cleared from circulation (Linder 1988). The interaction of CM with LPL is facilitated by the CM surface protein cofactor ApoC-II (Hussain et al. 1996). A majority of core TAG is hydrolyzed by LPL before the remnant is removed from circulation, but cholesterol esters and retinol esters are retained in the remnant rather than being taken up peripherally.

LPL is synthesized within tissues, then is transported to the lumenal surface of endothelial cells. Although the gene encoding LPL in all tissues has a single isoform, it is regulated differently in adipose (AT-LPL) and muscle (M-LPL) tissues (Farese & Eckel 1991). In the fed state, insulin up-regulates the AT-LPL and down-regulates M-LPL in healthy persons (Farese & Eckel 1991). This regulation of LPL is one of the metabolic processes that becomes resistant to the effects of insulin in obesity. Obese women in one study experienced a 45% rise in M-LPL activity during a 6-hour glucose/insulin infusion, as compared with the 18% drop found in lean controls (Yost et al. 1995). In another study, obese women had fasting AT-LPL activities greater than those of lean women, although activities following a six-hour glucose infusion were similar for both groups
(Yost & Eckel 1992). That is, during fasting, circulating lipids were more readily diverted back to storage in the obese. However, fat oxidation tends to be maintained in obesity by elevated circulating TAG and consequent elevated whole-body delivery of fatty acids to the periphery. Also, in reduced-obese persons, AT-LPL activities are consistently elevated both in the fasted (Yost & Eckel 1988, Kern et al. 1990, Schwartz & Brunzell 1981) and fed (Yost & Eckel 1988) states. This would favor redeposition of the fat that had previously been lost.

Diet composition may also play a role in LPL regulation by insulin. In healthy humans infused for six hours with insulin and glucose, ingestion of 67 grams of emulsified corn oil virtually eliminated the AT-LPL response at three and six hours, compared with that of subjects who ingested no fat (Sadur et al. 1984). This represents a mechanism for diverting dietary fat away from storage and towards oxidation and VLDL formation when intake is high. In reduced-obese women, however, this response was not seen (Yost & Eckel 1988). This is in agreement with the previously mentioned failure of reduced-obese women to adjust fat oxidation to a high-fat diet (Astrup et al. 1994a).

CM have a remarkably short half-life in circulation of less than 10 minutes for mammalian species (Hussain et al. 1996). However, it has been suggested that clearance is slowed in persons with hypertriglyceridemia, because their CM concentrations are elevated relative to those of control subjects following fat ingestion (Grundy & Mok 1976). Genetic alleles of apoE also influence CM clearance (Weintraub et al. 1987a). The kinetics of CM catabolism have been assessed by injection of a bolus tracer dose of labeled CM and use of compartmental modeling to compute label kinetics. Labeled CM are obtained by feeding a large bolus of fat with various tracers to a “CM donor,” followed by collection of lymph from donor animals, or of plasma from human subjects. The CM fraction is separated by centrifugation, then injected into another animal, or into the same human subject following a washout period. In general, there is an initial first-order rapid disappearance of label from plasma which is thought to represent conversion of CM to remnants (Hultin et al. 1996, Hultin et al. 1995). Separate labels are sometimes used to assess clearance of CM remnants, or “core lipids” from plasma, including retinol.
palmitate and CE. These species are generally assumed to stay with remnants and to not be hydrolyzed by LPL, and this does appear to be the case in vitro at physiologic concentrations of LPL (Hultin et al. 1996).

Another question addressed in those studies is the fate of newly hydrolyzed fatty acids. Although it would be reasonable to suppose that NEFA hydrolyzed by LPL in capillaries would be taken up immediately and not returned to venous blood, this is not always the case. Although plasma NEFA may be undetectable following ingestion of a low-fat meal, suggesting that immediate peripheral uptake is virtually complete, following a high-fat meal NEFA concentrations have been shown to equal fasting levels (Griffiths et al. 1994). In that study, dietary origin of plasma NEFA was inferred both from the rise in NEFA concentration and from fatty acid composition of NEFA, which was similar to that of the diet. There is also direct evidence from tracer studies that CM-derived NEFA circulate (Nguyen et al. 1996, Hultin et al. 1996). Again, the “spill-over” of NEFA into venous circulation when the rate of hydrolysis is high represents a mechanism for increasing oxidation of fat from a high-fat meal by delivering the excess fatty acids to metabolically active tissues such as heart muscle, even if their local LPL is down-regulated by insulin.

Fatty acid tracers have also been used to measure regional uptake of CM-derived fatty acids. Tissue-specific uptake has been assessed in animals by measuring label recovered from various tissues. Results generally demonstrate that uptake of CM fatty acids occurs in all types of tissues studied, although more is recovered from the liver of sacrificed animals than from any other tissue category (Hultin et al. 1996, Levy et al. 1991). This technique does not distinguish between fatty acids hydrolyzed before uptake and those derived from remnants, however. Another technique sometimes used is arteriovenous (a-v) balance. Regional flow, regional venous label concentration (usually as specific activity of radioisotope tracers), and arterial label concentration are measured, and uptake is calculated from the label a-v difference and flow rate. This technique requires a steady-state turnover of CM in plasma. In a study with humans, this was achieved by feeding small, frequent meals containing triolein tracer for six hours (Nguyen
et al. 1996). A-v balance data were interpreted to show that 71% of CM were catabolized in the splanchnic bed in men, versus 20% in women. Catabolism by the legs of men and women were similar at about 10%. In a similar study using dogs or sheep, when dogs were given constant infusions of labeled CM, 22% of CM TAG was cleared by the liver, and in sheep this was 10% (Bergman et al. 1971). Note that splanchnic circulation includes all of the visceral organs and their surrounding adipose stores.

The circulation time of CM remnants is somewhat longer than that of CM, but is still fairly short. In one study it was calculated to be 7.5 minutes, from disappearance of core label (Hultin et al. 1995). However, remnant clearance appears to be slowed in persons with elevated fasting TAG (Lewis et al. 1991, O'Meara et al. 1992, Weintraub et al. 1987b). Remnant clearance may also be saturable. In one study, when healthy adults ingested 70 grams of fat in the form of heavy cream, retinol palmitate concentrations in plasma exceeded the apparent saturation level for seven hours (Berr 1992).

There is also evidence for a system of feedback regulation of LPL at very high rates of fat entry to circulation (Karpe et al. 1992, Peterson et al. 1990, Saxena et al. 1989). This involves LPL release from the capillary endothelium in proportion to NEFA concentration. Evidence includes positive correlations between NEFA and LPL concentrations in plasma of humans given TAG emulsions either orally (Karpe et al. 1992) or by infusion (Peterson et al. 1990), and cell culture release of labeled LPL with increasing NEFA-to-albumin ratios (Saxena et al. 1989). Thus, as the ability of albumin to bind NEFA is exceeded, LPL is released into plasma. Further, LPL was shown to enhance remnant binding to hepatic receptors (Beisiegel et al. 1991), and so may facilitate clearance of remnants from plasma as well. It is clear from the above discussion that the process of dietary fat clearance from circulation is complex, and may vary greatly depending upon the feeding protocol used.

Following CM conversion to remnants and some apoprotein remodeling, especially acquisition of apoE from HDL, remnants are taken up primarily by the liver, but also by bone marrow or spleen depending on the species (Hussain et al. 1996). Significant amounts of core label have also been recovered from other tissues in rats.
(Hultin et al. 1996), including 14% in muscle and 6% in adipose tissue. As these tissues are not recognized to take up remnants, the reason for this finding is not clear. It is liver uptake that is of primary interest in this review however, as that is the route by which dietary fat from remnants is incorporated into VLDL. In addition, the liver expends a considerable fraction of metabolic energy at rest, and more so during digestion and absorption than when fasting, so the liver may represent a major site of fat oxidation in a fed state.

**VLDL metabolism**

Delivery of fat from a meal can occur over many hours, and NEFA taken up by the liver can be secreted in VLDL in as little as 10 minutes (Hultin et al. 1996). Presumably, TAG from remnants can also be processed by liver in this way. In one study, 14% of TAG label from injected CM was recovered from liver of rats after 20 minutes (Hultin et al. 1995). In another study, chow-fed rats were injected with lipoproteins labeled with $^{14}$C-oleoyl cholesterol. After 65 minutes, more than half of the injected dose had been taken up by liver, half of that had been synthesized into TAG, and nearly half of the TAG had been secreted into plasma (Moir & Zammit 1992). In fasting humans, injected $^3$H-labeled palmitate appeared in plasma TAG within 15 minutes, and peaked after one hour (Eaton et al. 1969). In another, production of apoB 100 in VLDL was increased in feeding versus fasting in healthy men given hourly meals containing 40% fat energy (Cohn et al. 1990). This is significant because each secreted VLDL particle contains a single apoB 100 protein (Davis & Boogaerts 1982). Also, insulin coordinately regulates fatty acid metabolism in the liver by inhibiting oxidation and simultaneously stimulating PL synthesis (Zammit 1996). Taken together, these findings suggest that a substantial fraction of dietary fatty acids may recirculate in VLDL.

The contribution of VLDL to fat oxidation in a fed state is unknown, but it is likely much less than that of CM. VLDL have a much longer half-life in circulation than do CM: on the order of one to three hours in healthy persons (Linder 1988). In addition,
CM appear to be the preferred substrate for LPL versus VLDL in a fed state (Potts et al. 1991, Schneeman et al. 1993). This has the potential to further slow VLDL catabolism following a high-fat meal. In one a-v balance study, extraction of VLDL TAG by forearm adipose tissue decreased following a meal whereas clearance of CM TAG rose (Potts et al. 1991). In another study, 80% of the increase in the number of plasma TAG-rich particles following a meal was in lipoproteins of hepatic origin, and only 20% was of enteric origin (Schneeman et al. 1993), suggesting reduced VLDL clearance. However, VLDL are typically two to three orders of magnitude smaller than CM (Linder 1988). Therefore, the rise in TAG from hepatic particles in this instance would have been small relative to the rise in CM TAG. Similarly, in healthy humans given a high-fat meal the postprandial peak increase in particles of enteric origin was over five-fold, whereas the increase in the number of hepatic particles was less than double the fasting concentration (Cohn et al. 1988).

The two studies just cited used the apoprotein B subtype distribution in plasma lipoprotein fractions to determine particle origin. This difference is an important tool in fat metabolism research. Each CM has a single apoB48 and each VLDL has an apoB100 protein incorporated into its structure during its formation. Unlike other apoproteins, which can be transferred between lipoprotein species in the plasma, apoB proteins form an integral part of CM and VLDL, with several hydrophilic regions that protrude at various locations on the surface of the lipoprotein, connected by interior hydrophobic regions (Chatterton et al. 1991). The fact that the two isoforms of apoB are different in liver versus intestine in humans (Linton et al. 1991) permits identification of the origins of circulating particles in the size ranges that have been established to represent the two categories, with the boundary between them being somewhat arbitrary (Lindgren & Jensen 1972). During fat digestion the two sources, liver versus intestinal, have size distributions that overlap slightly, so the exact size cutoff at which separation is maximal may vary, but has most often been considered to be at Svedberg flotation rate, $S_f = 400$, or the equivalent of 757 Angstroms (Å) (Lindgren & Jensen 1972, Dole & Hamlin 1962). Lymph of rats contains substantial VLDL-sized particles during fasting (Ockner et al.
1969a), but not during fat feeding (Windmueller et al. 1970, Ockner et al. 1969b). In this review, "VLDL" refers to lipoproteins of hepatic origin, and "CM" refers to those of enteric origin, unless otherwise noted.

**Cellular fatty acid metabolism**

The first step in cellular metabolism of fatty acids is their initial uptake. Whether the mechanism by which NEFA cross cell membranes involves a protein transporter (permease) or the lipid bilayer itself is not known. However, the binding of NEFA to membranes has been shown to be saturable and non-competitive for palmitate and oleate binding to "ghost" membranes from human erythrocytes (Bojesen & Bojesen 1996a). This indicates that it is not a matter of simple passive diffusion, and that individual fatty acids do not rely on a common transporter. It was recently suggested that phospholipid microdomains of the lipid bilayer, mediated by the presence of membrane proteins, act as the transporters for NEFA (Bojesen & Bojesen 1996a).

Following the uptake of fatty acids by cells, they must be transported to appropriate intracellular sites for further metabolism. A family of intracellular proteins known as fatty acid binding proteins (FABP) are thought to transport fatty acids and thereby facilitate their metabolism. There are several lines of evidence for this. Competitive inhibition for uptake of oleate and palmitate has been demonstrated in perfused rat liver (Sorrentino et al. 1996) and human intestinal cell culture (Trotter et al. 1996). This suggests that facilitated transport occurs within the cell. Rat liver exhibits sex differences in clearance of plasma NEFA which may be related to FABP. Female liver clears NEFA more efficiently than male liver, and has a higher cytosolic FABP content (Sorrentino et al. 1992). Cytoplasmic fatty acid diffusion rate in hepatocytes from female rats was also found to be higher than in males (Luxon & Weisiger 1993). FABP and fat oxidation are also linked. They are positively correlated in heart and skeletal muscle of rats (Veerkamp & van Moerkerk 1993). Regulation of FABP also responds to manipulations of fat oxidation. Increasing dietary fat intake from 10% to 40% of energy...
Increased FABP content of liver and adipose tissue of rats (Veerkamp & van Moerkerk 1993). Insulin was shown to up-regulate adipose (Melki & Abumrad 1993) and heart (Glatz et al. 1994) FABP in rats. Fasting doubled FABP in rat skeletal muscle (Carey et al. 1994). Thus, FABP appears to have an important role in uptake and oxidation of fatty acids.

Regardless of their fate, fatty acids are first activated by esterification to CoA esters, which is mediated by fatty acyl-CoA synthetase. Further metabolism of fatty acyl-CoA’s is regulated by various enzymes, in a tissue-specific fashion. Although small amounts of fat are used in synthetic processes, especially cell membrane PL synthesis, the majority of dietary fat is either stored as TAG or oxidized.

The rate-limiting step in long chain fatty acid β-oxidation is mediated by carnitine palmitoyl transferase I (CPT-I) (Stryer 1988 p 473). This enzyme transfers long-chain fatty acids from CoA to carnitine so that they can be transported into the mitochondrial matrix. Following reconversion of fatty acids to CoA esters, β-oxidation proceeds by sequential removal of 2-carbon units in the form of acetyl-CoA, which involves four enzymes. The first committed step is mediated either by long-chain acyl-CoA dehydrogenase (LCAD), which is active towards fatty acids in the range of 6 to 20 carbons, or very-long chain acyl-CoA dehydrogenase (VLCAD), which is active towards fatty acids with 12 to 24 carbons (Izai et al. 1992). There are also medium- and short-chain dehydrogenases (MCAD and SCAD) with activities toward 4 to 12 carbon and 4 to 6 carbon fatty acids, respectively. CPT-I is inhibited by malonyl-CoA, an intermediate of fat synthesis, such that simultaneous synthesis and oxidation of fat is minimized.

Following delivery of fatty acids to the mitochondrial matrix, the rate of β-oxidation is regulated by product inhibition at each step. The acyl-CoA dehydrogenases are inhibited by their 2-enoyl fatty acyl-CoA products. 2-enoyl hydratase is inhibited by β-hydroxy fatty acyl-CoA’s, β-hydroxyacyl-CoA dehydrogenases are inhibited by β-ketoacyl-CoA’s and NADH, and β-keto thiolases are inhibited by acetyl-CoA. Note that NADH and acetyl-CoA levels are also related to the overall energy charge in the cell.
Although acetyl-CoA is the end product of β-oxidation, complete oxidation of a fatty acid to CO₂ also involves delivery of this substrate to the tricarboxylic acid cycle.

Some β-oxidation of fatty acids also occurs in peroxisomes, which have a separate set of enzymes from the mitochondrial ones, and which have the capacity to shorten fatty acids without completely catabolizing them to acetyl-CoA. Peroxisomal β-oxidation is variable, but in healthy, fed humans it is thought to be minor (Osmundsen et al. 1991). Also, the primary function of peroxisomal β-oxidation is thought to be catabolism of substrates that do not undergo mitochondrial oxidation, such as certain very-long-chain fatty acids. The primary role of mitochondrial β-oxidation, on the other hand, is energy production.

Knowing where and when fat is oxidized is also relevant to this discussion. During exercise, skeletal muscle consumes a large proportion of the total energy being used, but at rest, liver and other organs account for the majority of the body’s energy consumption (Elia 1992b). In the “reference human” liver and brain each are estimated to account for about 21% of total daily energy expenditure, and heart and kidney about 8 to 9% each, although these proportions are higher at rest than during exercise, especially in a fed state. Although brain oxidizes glucose almost exclusively except during long fasts, the rest of the organs oxidize both fat and glucose, with the selection of fuel regulated by various hormonal and enzymatic processes.

Along with oxidation, storage is the principal fate of dietary fat. Although small quantities of fat are stored in muscle and other metabolically active cells, most stored fat is in adipocytes, where NEFA taken up are reesterified to TAG. The enzyme responsible for hydrolysis of stored TAG, hormone-sensitive lipase, is inhibited by insulin in a dose-dependent fashion, so NEFA release in a fed state is suppressed. However, some NEFA release does occur under physiologic feeding conditions (Roust & Jensen 1993). This is particularly true of abdominal as opposed to peripheral fat (Mauriege et al. 1990, Roust & Jensen 1993, Marin et al. 1992). There is also evidence that the abdominal hormone-sensitive lipase of men is more resistant to insulin than that of women (Jensen 1995).
Common alleles of several proteins that affect fat metabolism have been reported in humans, including apoE (Weintraub et al. 1987a, Bergeron & Havel 1996, Martin et al. 1993), LPL (Wang et al. 1996, Pimstone et al. 1996), and human intestinal FABP (Baier et al. 1995). Three alleles of apoE, designated E2, E3 and E4, exist with relative frequencies of about 15, 75, and 10%, respectively. This results in a total of six possible phenotypes, three homozygous and three heterozygous. Because apoE is involved in recognition of remnants by receptors responsible for remnant uptake (Hussain et al. 1996), it has a central role in determining lipemia in the fed state. Remnant clearance has been found to be slowed in persons with an E3/2 phenotype, relative to the E3/3 phenotype (Weintraub et al. 1987a). On the other hand, results for the E4 allele are mixed. For example, E4/3 accelerated clearance relative to E3/3 in a high-fat challenge protocol (Weintraub et al. 1987a), but the opposite result was observed following ingestion of a meal containing one third of daily calories and with 39% of that energy as fat (Bergeron & Havel 1996). Both of these studies used healthy volunteers with normal fasting lipid profiles, so it appears that apoE phenotypes do not necessarily influence fasting lipoprotein metabolism. However, significant effects of apoE phenotype on the response of HDL to dietary cholesterol have been observed, with E4/3 being beneficial and E3/2 slightly detrimental relative to E3/3 (Martin et al. 1993).

Numerous mutations of the LPL gene have been discovered, having various effects on the function of LPL. A comprehensive review of these has been recently published (Wang et al. 1996). Given the central role of this enzyme in the clearance of plasma lipids, it is not surprising that the more severe defects lead to a serious condition known as chylomicronemia syndrome. However, more subtle variations also exist. One of these was shown to result in impaired CM clearance in normolipemic volunteers (Pimstone et al. 1996). Another study found a significant correlation between a common
LPL mutation and the presence of diabetes, as well as severity of coronary artery disease, independent of levels of circulating lipoproteins (Wang et al. 1996).

Finally, a recently identified polymorphism of human intestinal FABP was discovered in the Pima Indian population (Baier et al. 1995). Pima Indians have extremely high rates of obesity and the highest reported prevalence of NIDDM of any population in the world (Lillioja et al. 1993), and so have been the subject of a great deal of research into these health problems. The two phenotypes differ by a single amino acid substitution, with relative frequencies of 70% and 30%. The less common allele was associated with insulin resistance and higher fat oxidation, and was further shown to have enhanced binding affinity for long-chain fatty acids. Whether this affinity difference is related to the observed metabolic derangement is not immediately apparent.

Differences in metabolism among fatty acids

Since the primary focus of the present research is comparative fatty acid oxidation, this discussion will first examine evidence for the existence of differences in acute oxidation of various fatty acids. This includes both direct comparisons and indirect evidence in the form of fat accretion and altered insulin sensitivity. It will also consider other metabolic factors that might contribute to such differences. These include effects of fatty acid composition on CM and VLDL formation and clearance, substrate specificities of the enzymes and transporters involved in fatty acid metabolism, and regulation by specific fatty acids of the enzymes involved in fat oxidation.

Tracer comparisons of acute fatty acid oxidation

Numerous comparisons have been made of acute oxidation of various ingested fatty acids, the results of which are summarized in Figure 2. All have used a single dose of fatty acids labeled with carbon isotopes in the carboxyl- or 1-position, with oxidation assessed by appearance of labeled carbon in breath CO$_2$ over a somewhat arbitrary period
of time. Animal studies have used radioactive $^{14}\text{C}$ (Jones 1994, Leyton et al. 1987, Toorop et al. 1979, Cenedella & Allen 1969, Coots 1964, Mead et al. 1956), whereas human studies have used the stable isotope $^{13}\text{C}$ (Jones et al. 1985b, Watkins et al. 1982). In the later animal studies oxidation was normalized for absorption by measuring radioactivity remaining in the intestinal lumen at the end of the experiment (Jones 1994, Leyton et al. 1987, Toorop et al. 1979, Cenedella & Allen 1969). In earlier animal studies, results are reported on the basis of percent of tracer fed (Coots 1964, Mead et al. 1956). In one human study total absorption was assessed by fecal analysis (Jones et al. 1985b), although this technique is limited in that time course of absorption is unknown and colonic fermentation would increase apparent absorption.

On balance, the results of those studies indicate that unsaturated fatty acids may be more readily oxidized following a meal than are saturated fatty acids (Leyton et al. 1987, Jones et al. 1985b, Watkins et al. 1982, Cenedella & Allen 1969, Mead et al. 1956). However, some of the results are inconsistent with this conclusion (Toorop et al. 1979, Coots 1964, Jones 1994). This may be due to differences in methodology, some of which are outlined in Figure 2, including collection time, method of tracer administration, and usual diet. The implications of these were discussed under "Digestion & chylomicron formation."

Oleate and palmitate have been compared in three studies, with mixed results. First, when tracers were transesterified onto soy oil TAG, emulsified in a liquid diet and given to rats by stomach tube, recovery of label from oleate and palmitate was similar throughout a 51-hour collection period, at the end of which 66 and 70% had been recovered, respectively (Coots 1964). Total fat given was about 1.6 ml. By contrast, when tracers were dissolved in 0.2 ml olive oil (Leyton et al. 1987), 24-hour recovery of oleate label was nearly twice that of palmitate, at 57% versus 32% respectively. Similarly, in healthy human children given $^{13}\text{C}$-labeled tracers in a lipid emulsion, 12% versus 7% of oleate and palmitate tracer labels were recovered in 6 hours (Watkins et al. 1982).
Palmitate and linoleate have also been compared in three studies, also with mixed results. In one, rats were given tracers dissolved in one ml olive oil by stomach tube, then allowed ad libitum access to chow (Cenedella & Allen 1969). Between five and six hours, 11% of absorbed palmitate label was recovered in breath, versus 21% of linoleate label. When rats in another study were fed fatty acid tracers as methyl esters with 10 grams amount of a fat-free diet, 7% of palmitate label and 4% of linoleate were recovered in the first 8 hours, but by 24 hours, total recovery for each was 26% (Toorop et al. 1979). When the same tracers were fed with cellulose, 35% of each label was recovered in 8 hours. Interestingly, similar amounts of the two tracers were recovered from body fat at 8 hours, but by 24 hours more linoleate than palmitate was found in fat pads. In a third study, tracers were dissolved in 0.2 ml olive oil and given by stomach tube, and 32% of palmitate versus 48% of linoleate label was recovered in 24 hours (Leyton et al. 1987).

One study already mentioned also compared oleate and linoleate, with appearance oleate label being higher, at 57% versus 48% (Leyton et al. 1987). Three additional studies also compared these two unsaturated fatty acids. In one, tracers were given to mice with an unspecified amount of corn oil, and 45% of oleate versus 27% of linoleate label was recovered in eight hours (Mead et al. 1956). In a human study, men were given tracers in capsules with a mixed meal, and 12% versus 8% of label was recovered over nine hours (Jones et al. 1985b). Finally, when rats were fed diets with equal oleate, linoleate, and α-linolenate (C18:3 n-3) for ten weeks with total fat providing 58% of energy, recoveries of all three labels in 12 hours were 26-27% (Jones 1994). This result for α-linolenate also disagrees with previous results in which 64% of α-linolenate label was recovered in 24 hours versus 48% of linoleate (Leyton et al. 1987).

Stearate is the only fatty acid for which relative oxidation results are consistent. That is, it is poorly oxidized as compared with all of the fatty acids previously discussed (Leyton et al. 1987, Jones et al. 1985b, Mead et al. 1956, Coots 1964). Only arachidonate (C20:4 n-6) oxidation has been found to be less than that of stearate (Leyton et al. 1987). Some stearate is desaturated in the liver and in enterocytes to form oleate (Rhee et al. 1997). This may be a factor in its surprising lack of atherogenicity relative to all other...
saturated fatty acids (Kris-Etherton & Yu 1997), although less than 15% of stearate is desaturated acutely (Rhee et al. 1997, Emken et al. 1993).

Taken together, the above results demonstrate the difficulty in interpreting results of fatty acid oxidation comparison studies. In particular, variability among protocols makes any mechanistic interpretation of conflicting results difficult.

Fat accretion with dietary fat interventions

Indirect evidence for differential fatty acid oxidation is found in studies involving fat accretion and insulin sensitivity with variations in diet fat composition. In one such study, month-old rats were fed diets with 18% of energy as fat, with either all fat being soy oil or half of the fat as a high-γ-linolenate (C18:3 n-6) oil blend (GLA oil), such that γ-linolenate provided 13% of fat energy or 2.3% of total energy (Takada et al. 1994). After four weeks, the γ-linolenate group had 15% less body fat than controls, adjusted for reduced absorption of the GLA oil. Body fat differences were also found in a study which compared beef tallow and safflower oil fed at 45% of energy to month-old rats (Shimomura et al. 1990). Safflower oil is about 80% linoleate while tallow is distributed mainly between oleate, palmitate, and stearate. The two groups were fed equal metabolizable energy, but after four months the safflower group had 30% less carcass fat than tallow-fed, reduced plasma insulin and TAG (fed and fasting), reduced AT-LPL response to feeding, and increased M-LPL activity in a fed state.

Partially contradictory results to the preceding study were obtained in older, three- to four-month-old, rats fed different diets for six months (Hill et al. 1993). In that study, corn oil versus lard fed at 45% of energy resulted in equal fat accretion and fasting insulin. Although fasting plasma TAG was higher in lard-fed rats at three months, by six months it was equal in the two groups. However, those researchers also compared fish oil with other fats, and did find reduced body fat per food intake, reduced fasting TAG and cholesterol, and increased insulin sensitivity in rats fed fish oil versus other fats. Although specific composition of fats used in that study was not reported, corn oil is
primarily linoleate. Lard is similar to tallow but usually has less stearate, and fish oil has substantial EPA and DHA. It must be noted that differences in amount of fat fed, time course of studies, developmental stage of experimental animals, and genetic differences among strains must all be considered potential confounding factors in comparing the studies above.

Finally, in a strain of mice which are susceptible to developing obesity and NIDDM when fed a high-fat diet, different dietary fats produced different health effects (Ikemoto et al. 1996). After 19 weeks of eating diets with 60% fat energy, soy oil-fed mice had the highest body fat, fish oil had the lowest, and palm oil, lard, and safflower oil were in between. Fasting insulin however was highest in palm oil, less for soy, safflower and lard, and least for fish oil, so the relationship between insulin and body fatness was not consistent.

The foregoing results may be explained in part by the finding that certain dietary fatty acids stimulate activities of enzymes involved in β-oxidation in rat liver. Those fatty acids are EPA (Aarsland et al. 1990, Willumsen et al. 1996), γ-linolenate (Takada et al. 1994), and α-linolenate (Ide et al. 1996, Kabir & Ide 1996). They were compared with equal amounts of more common dietary fatty acids (Aarsland et al. 1990, Takada et al. 1994, Ide et al. 1996, Kabir & Ide 1996), or in one case with DHA (Willumsen et al. 1996). The affected enzymes include CPT-I (Aarsland et al. 1990, Takada et al. 1994, Ide et al. 1996, Kabir & Ide 1996), which regulates mitochondrial β-oxidation; various enzymes of mitochondrial β-oxidation, including acyl-CoA dehydrogenase (Ide et al. 1996, Kabir & Ide 1996); and acyl-CoA oxidase (Ide et al. 1996, Takada et al. 1994), which catalyzes the initial, rate-limiting reaction in peroxisomal β-oxidation. None of the diets used provided more than 30% of energy as fat. Concomitant increases in β-oxidation of palmitate were also observed in three of those studies (Ide et al. 1996, Kabir & Ide 1996, Willumsen et al. 1996). Thus, certain fatty acids directly stimulate fat oxidation in rats via long-term regulation of enzymes.
It has already been noted that feeding rats diets with more than 50\% of energy as fat can cause insulin resistance within a few days (Kraegen et al. 1991). However, not all fatty acids are equal in this regard. In rats maintained on isoenergetic diets in which 59\% of energy was from fat, but with varying fatty acid composition, not all fats induced insulin resistance (Storlien et al. 1991). Specifically, safflower oil, olive oil, and a mixture of tallow and safflower (40:60) all resulted in severe insulin resistance after 30 days. When a blend of fish oil and safflower oil (30:70) was fed, such that 11\% of fatty acids were EPA or DHA, insulin action assessed by euglycemic clamp was equal to that of chow-fed animals. A blend of linseed oil and tallow (21:79), also normalized insulin sensitivity, although linseed and safflower oils in the same proportions did not. Linseed oil is high in α-linolenate, which made up 11\% of the fatty acids in these diets, and which can be converted in vivo to EPA and DHA. These results are consistent with those discussed above regarding stimulation of β-oxidation by α-linolenate and EPA.

An additional candidate mechanism for the effects of chronic fat intake on insulin sensitivity is altered membrane PL composition. Although membrane composition is more tightly regulated than is adipose storage (Field et al. 1985), dietary fatty acid composition does influence membrane fatty acid distribution and therefore degree of saturation (Van Amelsvoort et al. 1986, Field et al. 1989). Indices of saturation were correlated with insulin receptor function in rat adipose cells (Van Amelsvoort et al. 1986, Field et al. 1989) and insulin sensitivity in humans (Borkman et al. 1993). It is likely than this effect is mediated by the influence of fatty acid composition on membrane fluidity since unsaturated fatty acids increase fluidity over saturated ones (Amatruda & Finch 1979).
Fatty acid effects on chylomicron kinetics

The physical characteristics of CM, especially size and kinetic characteristics such as half-life of CM TAG and remnants, are affected acutely by the type of fat being fed. In general, as diet fat becomes more unsaturated, CM size increases (Feldman et al. 1983, Ockner et al. 1969b, Sakr et al. 1997) and half-life decreases (Levy et al. 1991). In one study, CM were harvested from rats chronically fed diets containing different types of fat, then injected into chow-fed rats (Levy et al. 1991). Safflower-oil-derived CM had a half-life of only 1.8 minutes versus 3.1 minutes for coconut oil. The average diameters of CM from the two oils were 1850 Å and 1400 Å, respectively. Similarly, in rats consuming diets in which 75% of fat was one of four fatty acids, chyle lipoproteins were smaller when palmitate or stearate were fed than with oleate or linoleate (Feldman et al. 1983). In another study rats were given three different fatty acids mixed with monoolein and the bile salt taurocholine, and the amount of lymph TAG in VLDL-sized particles versus CM-sized was determined (Ockner et al. 1969b). In fasting rats, nearly half the TAG was in the smaller particles. When the rats were fed palmitate, this was reduced to 31%, and in oleate- or linoleate-fed, only 17% of TAG was in the VLDL-sized fraction. Qualitatively similar CM size differences were obtained in plasma of healthy women who ingested test meals containing 60 grams of various fats in a milk-shake type emulsion (Sakr et al. 1997).

"CaCo2" cell cultures, which are derived from human colon cells, have also been employed to examine CM formation, and have shown substantial differences in effects of various fatty acids (Field et al. 1988, van Greevenbroek et al. 1996, van Greevenbroek et al. 1995). Uptake of NEFA and subsequent secretion of lipoproteins into culture medium by these cells is most strongly stimulated by oleate of all fatty acids studied thus far (Field et al. 1988, van Greevenbroek et al. 1996). Linoleate is the next most potent (Field et al. 1988), followed by palmitate (Field et al. 1988, van Greevenbroek et al. 1995), which is (surprisingly) similar to stearate (van Greevenbroek et al. 1996) and laurate (C12:0)
(Field et al. 1988). The higher rates of secretion were characterized by a higher ratio of lipid to apoB, indicating larger particle size. It is not certain how similar these cells are to mature cells of the small intestine, since they originate from colon cells and some of their features are characteristic of fetal rather than mature enterocytes. However, the differences observed are in keeping with in vivo results discussed above.

Two studies have compared acute versus chronic effects of dietary fat composition on CM clearance; one in humans (Weintraub et al. 1988) and one in rats (Green et al. 1984). Human subjects were fed for 25 days diets high in saturated fat, linoleate, or fish oil, with total fat being 42% of energy. They were then given challenge meals containing 50g/m² of one of the three fat types with retinol added as a marker for plasma CM, from which clearance was inferred. Fish oil facilitated CM clearance relative to vegetable oil, whereas it was slowed by saturated fat. Both acute and chronic diets influenced CM clearance, although the chronic effect was more pronounced. The authors suggested that this was due to competition from VLDL, which were elevated by saturated fat. However, since retinol was used as a marker, these results reflect remnant clearance to an unknown extent, and the large dose of fat used may have saturated remnant clearance (Berr 1992). However, similar results were seen in rats maintained on diets with 47% of energy from palm oil or a corn oil and triolein mixture, then injected with ¹H-oleate-labeled CM from rats acutely fed one of the two diets (Green et al. 1984). That is, basal concentration of TAG had a more pronounced effect on clearance than did fat type fed to donor animals, although both acute and chronic suppression of CM clearance was seen with saturated fat. The clinical relevance of these findings stems from the concern that elevated postprandial TAG may be a risk factor for atherosclerosis, independent of plasma cholesterol or fasting TAG levels, and longer half-lives of CM result in higher concentrations of TAG.

Decreasing half-life with increasing size may appear contradictory. However, it is consistent with a model in which individual CM are catabolized in a single place, i.e., at a single LPL site, rather than gradually. Thus, differences in efficiency of CM clearance could result from differences in how readily they interact with LPL during catabolism.
based on physical characteristics such as surface curvature or fluidity of the surface phospholipid monolayer.

In addition to CM differences per se, recall that when plasma concentrations of TAG are high LPL is displaced from capillary endothelium (Karpe et al. 1992, Saxena et al. 1989). This occurs when large amounts of fat are fed, and there is evidence for fatty acid specificity of this effect. In one cell culture study which used porcine aorta, release of LPL from the cell surface by oleate was higher than any other fatty acid studied at 78% (Saxena & Goldberg 1990). Release by arachidonate and linoleate was somewhat less than oleate, and palmitate was relatively ineffective, releasing only 28% of LPL.

The synthesis and secretion of LPL is also affected by fatty acid composition. In embryonic adipocytes cultured with various fatty acids, linoleate and EPA suppressed LPL expression far more effectively than oleate (Montalto & Bensadoun 1993). Of course, such findings cannot be generalized to the in vivo situation, but they do raise the possibility that changing dietary fat composition can change the fate of all of the CM fatty acids via LPL. That is, overall clearance of CM TAG from a high-fat meal could be reduced when the fatty acid composition favors LPL displacement. Alternatively, the balance of clearance could be shifted away from the periphery towards the liver.

Fatty acid effects on VLDL secretion

A significant amount of CM-derived fatty acids are taken up by liver, with the potential to reenter the bloodstream as VLDL. Oxidation and VLDL TAG synthesis therefore represent major competing pathways for the short-term fate of dietary fatty acids within the liver. Hence, specificity in how fatty acids affect VLDL synthesis is relevant to the fate of dietary fats. Such differences do appear to exist, both at the level of whole-body incorporation of fatty acids into VLDL and at the level of cellular uptake and synthesis.

In one study, fasting VLDL secretion in rats was altered by chronic fat intake (Lai et al. 1991). Rats maintained on a diet with 30% of energy as beef tallow secreted 60%
more VLDL in a fasting state than rats eating a 30% corn-oil diet, and olive oil produced intermediate results. One early study compared incorporation of palmitate and linoleate into VLDL in healthy men (Nestel & Barter 1971). During constant infusions of [9,10-3H]-palmitate and [1-14C]-linoleate, fractional incorporation of plasma palmitate into VLDL was consistently higher than that of linoleate despite a higher fractional turnover of plasma linoleate. This was true whether the men were fasting or ingesting sucrose. This study could not distinguish uptake by liver from that of other tissues, however. That is, it is not clear whether liver was resecreting palmitate at a higher rate, or just taking up less of the total linoleate.

Several studies have also compared metabolism of different fatty acids by isolated hepatocytes in cell cultures (Davis & Boogaerts 1982, Pai & Yeh 1996, Bruce & Salter 1996, Kvilekval et al. 1994). In these, hepatocytes were incubated with various fatty acids and albumin, at concentrations designed to be within a physiological range, and the outcomes were assessed in various ways. One used 3H-labeled glycerol to measure stimulation of total VLDL TAG secretion by rat hepatocytes during a two hour period (Davis & Boogaerts 1982). Oleate-stimulated secretion was highest, at about seven times the control rate; palmitate-stimulated secretion was least, at twice the control rate; and myristate, linoleate and linolenate results were intermediate.

Another study with rat hepatocytes used fatty acid tracers to compare total incorporation of different fatty acids into glycerolipids (TAG and PL), (Pai & Yeh 1996). After a four-hour incubation, similar amounts of oleate, palmitate, and myristate were incorporated into TAG; that of linoleate was somewhat less; and stearate incorporation was only about one tenth of other fatty acids. However, more palmitate than oleate was incorporated into PL. In a similar protocol using hamster hepatocytes, palmitate and oleate were incorporated into VLDL TAG equally, but stearate much less so (Bruce & Salter 1996). Stimulation of total TAG synthesis and secretion was similar for the three fatty acids, however. Interestingly, in each of these two studies, oxidation of oleate was higher than that of palmitate (Pai & Yeh 1996, Bruce & Salter 1996).
Finally, fatty acid tracers were used to compare uptake, incorporation, and kinetics of TAG formation in rat hepatocytes (Kvilekval et al. 1994). This study differs from the others in that concentrations of fatty acid added to the medium were adjusted to produce equal unbound fatty acid concentrations, using published values for albumin binding (Spector et al. 1969). The time course of total uptake was similar for all fatty acids. However, the maximal reaction velocity at saturated substrate concentration, $V_{\text{max}}$, was highest for oleate, somewhat less for linoleate and palmitate, and very low for stearate, on the order of 10% that of the other fatty acids. The equilibrium constants, $K_M$, for incorporation of oleate and linoleate into TAG were about twice that of palmitate, and four times that of stearate. Thus, affinity of TAG synthesis for stearate was higher than for other fatty acids when the effect of albumin affinity is controlled, assuming the albumin binding values used are accurate. Its saturation concentration was quite low, however, which was reflected in the finding that 60 to 80% of the stearate taken up was still in nonesterified form when only 1% of oleate remained in nonesterified form.

As was the case for whole-body relative oxidation, the results for stearate are most consistent, but there is also evidence that unsaturated fatty acids are more readily incorporated into VLDL than palmitate. Thus, whether dietary fatty acids appear in VLDL involves partitioning initially in the periphery and later within hepatocytes.

Fatty acid specificities of enzymes and transporters

One logical source of differences in metabolism of various fatty acids lies in the affinities of proteins with which they interact. Recall that these include the transporters, albumin and FABP, synthetic enzymes that esterify fatty acids to CoA and carnitine, and the complex of enzymes responsible for oxidation. Data in this area are somewhat limited, and not always in agreement with the whole-animal findings discussed above. In addition, there are always potential confounding factors involved with in vitro assays as compared with in vivo function. Still, it is useful to examine what has been found and explore its potential significance for differential fatty acid oxidation.
It has already been noted that a small fraction of the dietary fatty acids in CM are incorporated into PC or CE (Skipski 1972). Specificity in this process might contribute to the conflicting results that have been found in comparisons of oxidation. That is, if a tracee fatty acid is present in very small quantities, and there is a preference for its incorporation into non-TAG lipids, acute oxidation of the tracer might be less than if large amounts of tracee were present. Specificity among fatty acids in PL and CE esterification do exist. For example, in rats fed 14C-labeled fatty acid tracers, PC incorporation showed a strong preference for stearate and to a lesser degree linoleate, as compared with palmitate or α-linolenate, and least of all for oleate (Whyte et al. 1963). CE synthesis showed specificity for oleate versus palmitate, stearate or linoleate (Karmen et al. 1963). It should be noted that 94 to 98% of recovered tracer appeared in the TAG fraction for all but stearate, for which 90% was in TAG. In a similar study with humans, deuterated tracers were fed to four subjects and recoveries compared in various CM lipid components (Emken et al. 1990). In that study, CE and PL synthesis both showed specificity for linoleate, and the 1-position of PL also demonstrated specificity for stearate and palmitate. Again, the vast majority of label was recovered from TAG even when tracee amounts were quite small, indicating that the contribution of such differences to metabolic fate may be minor.

Transport proteins also have the potential to influence fatty acid disposition. In plasma, NEFA are primarily transported bound to albumin, which has multiple fatty acid binding sites. The role of albumin in NEFA uptake is demonstrated by the fact that the rate of NEFA uptake by isolated hepatocytes is a function of the fatty acid-to-albumin ratio rather than the absolute concentration of fatty acids in the medium (Sorrentino et al. 1992, Kvilekval et al. 1994).

A recent study found differences in binding affinities of serum bovine albumin for palmitate and oleate (Rose et al. 1994), with a lower affinity for oleate than palmitate at their respective primary and tertiary sites, but a higher affinity for oleate than palmitate at the secondary sites. Affinity for laurate was much lower than the other fatty acids at all sites. As affinity decreases, the unbound concentration of fatty acid increases. This raises
the possibility that the relative kinetics of non-esterified oleate and palmitate may depend on total fatty acid concentration of plasma. An earlier study found a high affinity for stearate versus other long-chain fatty acids in human albumin (Goodman 1958). Finally, the binding capacity of albumin for oleate, but not stearate or palmitate, was recently reported to depend on the molar ratio of fatty acid to albumin (Pai & Yeh 1996). At a 2:1 ratio, oleate binding was 99.94%, stearate binding was 99.84%, and palmitate was 99.45%. At a ratio of 5.6:1, oleate binding decreased to 99.53%, and at 10:1 it was 96.54%. Clearly, albumin binding of NEFA in plasma has the potential to effect differential cellular uptake of fatty acids.

The next step in uptake of NEFA is that of traversing the cell membrane. Limited evidence using erythrocyte ghosts suggests that binding of palmitate and oleate to cell membranes is saturable and not competitive (Bojesen & Bojesen 1996a). This is consistent with dependence on a transport mechanism rather than passive diffusion. In that study, the membrane total binding capacity for oleate was slightly higher than for palmitate while that of arachidonate was much lower. However, in vivo arachidonate concentration would also tend to be much lower. Also, ratios of inner-membrane:outer-membrane binding were quite different for oleate versus palmitate. In any case, it remains to be seen if there is tissue specificity in membrane binding and whether that affects fatty acid uptake.

FABP’s have a similar capacity to that of albumin to influence fatty acid metabolism. In a recent comparison of FABP affinities from various tissue types and animal species, striking differences between tissues were noted, although species differences were slight (Richieri et al. 1994). Tissue sources included intestine, heart, liver, and adipose. The authors concluded that the first three tissues would be capable of buffering plasma fatty acid uptake. The lower affinity of adipocyte FABP, however, would tend to favor secretion of fatty acids under fasting conditions, as would be expected. Substrate specificities in the dissociation constant, \( K_D \), were found in all FABP’s tested in that study. In rat liver, affinities of oleate and stearate were higher than of palmitate or linoleate. In rat or bovine heart and rat intestine, affinity was highest for
stearate, less for palmitate or oleate and lowest for linoleate. In addition, $K_d$ values for the linolenate and arachidonate were consistently higher than any other fatty acid tested. A more recent study using rat intestine FABP, however, reported much higher affinity for oleate than palmitate or arachidonate (Kurian et al 1996). Human liver and skeletal muscle FABP have been shown to bind oleate and palmitate with similar affinity (Maatman et al. 1994). These results are generally consistent with an overall faster turnover of long-chain PUFA's and slower turnover of stearate than of oleate or palmitate.

One extreme difference has been reported in FABP binding of oleate versus palmitate (Xu et al. 1996). In that study, murine brain FABP was found to bind oleate readily but palmitate not at all, at physiological concentrations. Of course, since the brain oxidizes almost no fat, this would not be important in terms of comparative fat oxidation, but it does demonstrate that FABP’s can exhibit extreme differences in specificity. Also, it may be related to the essential role of oleate in brain tissue as a precursor to myelin synthesis.

Net rates of uptake of various fatty acids are ultimately determined by the overall kinetics for the various intra- and extra-cellular transporters. Some limited selectivity for net uptake has been observed in humans subjects. Whole-body fractional turnover of plasma nonesterified linoleate was about twice that of palmitate in healthy men (Nestel & Barter 1971). Two a-v balance studies found that fractional hepatic uptake in fasting subjects increased with increasing number of unsaturated bonds and decreasing chain lengths of fatty acids (Hagenfeldt et al. 1972, Hagenfeldt 1975). Specifically, the pattern of uptake was: laurate, myristate, palmitoleate, and linolenate > linoleate, oleate, and palmitate > stearate and arachidonate. However, there is a lack of specificity for extraction by human muscle tissues, including forearm (Hagenfeldt et al. 1972), leg (Hagenfeldt 1975), and heart (Wisneski et al. 1987). It may be that in some tissues, but not others, albumin binding specificity is offset by intracellular specificities of FABP or synthetic enzymes.
Mobilization of stored TAG from adipose cells occurs primarily in fasting conditions. However, it is worth considering in any discussion of the fates of different fatty acids because no fed-state difference in oxidation can be significant if it is counteracted in the fasting state that follows. Marked differences in mobilization of individual fatty acids from cultured rat adipocytes have been demonstrated (Raclot & Groscolas 1993). In particular, mobilization increased exponentially with increasing desaturation and decreasing chain length. Altogether, 27 fatty acids were examined, with chain lengths from 14 to 22 carbons and having up to 6 double bonds. Relative mobilization was defined to be presence in incubation medium of the fatty acid relative to its presence in adipocyte TAG. For palmitate this was 1.07, and for oleate it was 0.90, so neither was very far from unity. Nor were linoleate at 0.91, and α-linolenate at 1.08, although the number for γ-linolenate was 1.42. Overall, the highest relative mobilization, 2.76, was seen in EPA, and the lowest, 0.38, was for behenate (C22:0).

Of course, if there is substantial net imbalance in retention of different fatty acids, it would be reflected in the composition of adipose stores. The composition of depot fat is typically quite similar to that of the diet (Field et al. 1985). On the other hand, the ratio of MUFA to saturated fatty acids in adipose tissue of healthy humans is consistently higher than that computed from diet records, while the proportion of PUFA closely reflects that of the diet (Beynen et al. 1980, Garland et al. 1998). Also, depletion of α-linolenate and linoleate occurs with weight cycling in young rats (Chen et al. 1996, Chen et al. 1997), so under certain circumstances the observed differences in adipose mobilization do appear to be significant.

It may be that during normal 24-hour cycling between fed and fasting states the differences which occur in one state tend to be balanced by differences in the other state, thereby maintaining body fat composition similar to that of the diet. That is, a more efficient uptake and storage in the fed state might be balanced by greater release and oxidation in the fasting state. The relatively greater amounts of MUFA found in adipose tissue versus diet at the expense of saturated fatty acids may also reflect desaturation of
stearate and palmitate to oleate and palmitoleate, respectively (Emken et al. 1993, Rhee et al. 1997).

Finally, several studies have assessed substrate specificities in intracellular enzymes having a direct role in fat oxidation. The first of these to interact with fatty acid substrates is acyl-CoA synthetase. Two studies have reported specificities for acyl-CoA synthetase, although not in humans (Black et al. 1997, Weis & Bercute 1997). In one, enzyme from *E coli* had a higher maximal activity towards myristate than oleate (Black et al. 1997). In the other, rabbit heart and liver enzymes had the pattern of maximal activity: palmitate > oleate > arachidonate, but greater affinity (lower $K_M$) for oleate than palmitate (Weis & Bercute 1997).

The next synthetic enzyme in the pathway of delivering fatty acids to be oxidized is CPT-I, the substrates of which are the CoA esters of fatty acids. Two studies have reported specificity of CPT-I in rat liver (Ide et al. 1995, Gavino & Gavino 1991). In one, $V_{max}$ was highest for $\gamma$-linolenoyl-CoA, intermediate for palmitoyl- and linoleoyl-CoA, and least for $\alpha$-linolenoyl-CoA (Ide et al. 1995). In addition, $K_M$ for $\gamma$-linolenoyl-CoA was less than half that the other substrates, indicating higher affinity. Similarly, the second study (Gavino & Gavino 1991) found $V_{max}$ values in the order: $\gamma$-linolenoyl-CoA > oleoyl-, palmitoyl-, eicosapentaenoyl- and linoleoyl-CoA > stearoyl-, arachidonoyl- and docosahexaenoyl-CoA. Those researchers also found $K_M$ values in the order: stearoyl-CoA > $\gamma$-linolenoyl-, oeloyl- and linoleoyl-CoA > palmitoyl- and arachidonoyl-CoA. These results are in agreement with whole-body oxidation results for stearate versus other fatty acids (Jones et al. 1985b, Coots 1964, Mead et al. 1956). In liver CPT-I from a species of Antarctic fish, on the other hand, $V_{max}$ was highest for palmitoyl-CoA, similar for oleoyl-, palmitoyl- linoleoyl-, docosahexaenoyl-, and eicosapentaenoyl-CoA, and much less for linolenoyl-and arachidonoyl-CoA (Crockett & Sidell 1993). This demonstrates that species differences exist for CPT-I, so that results in rats cannot necessarily be generalized to humans.
Summary

In this review, we have seen that recommendations for dietary fat may play an important role in the future of public health in the US. Manipulations of dietary fat can interact with a genetic predisposition to obesity, CVD and NIDDM and thus profoundly affect long-term health. At the same time, current dietary recommendations may be too simplistic to allow individuals and health professionals to optimize fat intake, given the rather one-sided advice cautioning against eating “too much” fat. Dietary fat is essential to health.

There is also compelling evidence that different types of fat have very different effects on disease risk factors such as plasma lipids in fasting and fed states, and functional indicators of metabolism such as insulin sensitivity and acute fat balance. There is also plentiful evidence that various enzymes and transporters involved in fatty acid metabolism have the capacity to mediate differences in their respective metabolic fates.

Despite this evidence, mechanistic explanations of the observed differences in health effects are lacking for the most part. In this context it would be useful to complement these findings with experiments designed to isolate and systematically manipulate individual mechanisms which may be involved. The research described herein represents a first step in the investigation of one such mechanism: comparative acute oxidation of dietary oleate and palmitate. It also seeks to test whether differential fatty acid oxidation is linked to plasma fatty acid turnover and VLDL incorporation.
Figure 1. Experimental model of dietary fatty acid metabolism. CM, chylomicron; TAG, triacylglycerol; MAG, monoacylglycerol; VLDL, very low density lipoprotein; LDL, low density lipoprotein; LPL, lipoprotein lipase.
<table>
<thead>
<tr>
<th>Reference</th>
<th>fatty acid tracers</th>
<th>tracer label recovered as CO₂ (mean±SEM)</th>
<th>time after tracer feeding (hours)</th>
<th>absorbed tracer (%) b</th>
<th>tracer feeding method c</th>
<th>usual diet fat: % of energy, tracee amount, duration</th>
<th>subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jones, 1994</td>
<td>[1-14C]-oleate, linoleate, alpha-linolenate</td>
<td>% of fed 26±1, 27±1, 27±1</td>
<td>12</td>
<td>not reported</td>
<td>intragastric, dissolved in oil (0.2 ml) with equal amounts of tracees</td>
<td>58% of energy, 23%</td>
<td>male Sprague-Dawley rats, n=10</td>
</tr>
<tr>
<td>Leyton et al., 1987</td>
<td>[1-14C]-oleate, linoleate, alpha-linolenate, arachidonate, laurate, myristate, palmitate, stearate</td>
<td>% of fed 57±2, 48±3, 64±3, 14±2, 63±8, 40±7, 32±3, 25±3</td>
<td>24</td>
<td>not reported</td>
<td>intragastric, dissolved in olive oil (0.2 ml)</td>
<td>not reported</td>
<td>female Sprague-Dawley rats, n=7-10</td>
</tr>
<tr>
<td>Jones et al., 1985b</td>
<td>[1-13C]-oleate, linoleate, stearate</td>
<td>% of absorbed 12±5, 8±3, 2±2</td>
<td>3 days d</td>
<td>97, 100, 78</td>
<td>encapsulated, fed with mixed meal having 1/3 of daily energy intake</td>
<td>40% of energy, 25%</td>
<td>healthy adult men, n=6 crossover design (continued)</td>
</tr>
</tbody>
</table>

a. Times are for cumulative label recovery following tracer administration unless otherwise noted.
b. Absorption assessed by measuring tracer in feces and gastrointestinal tract unless otherwise noted.
c. Tracers were administered to fasting subjects in all cases.
d. Absorption was inferred from complete fecal collections performed for 3 days following tracer administration.

Figure 2. Previous tracer studies comparing in vivo whole-body oxidation of various dietary fatty acids. Tracers were labeled with carbon isotopes in the 1- position as indicated, and oxidation was assessed by recovery of label in breath CO₂. "Tracees" are unlabeled fatty acids corresponding to tracers.
Figure 2 (continued).

<table>
<thead>
<tr>
<th>Reference</th>
<th>Fatty acid tracers</th>
<th>Tracer label recovered as CO₂ (mean±SEM)</th>
<th>Time after tracer feeding (hours)</th>
<th>Absorbed tracer (%)</th>
<th>Tracer feeding method</th>
<th>Usual diet fat: % of energy, tracee amount, duration</th>
<th>Subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Watkins et al., 1982 (control group)</td>
<td>[1-13C]-trioctanoin triolein palmitate</td>
<td>% of fed 28±10 11±7 7±4</td>
<td>6</td>
<td>not reported</td>
<td>fed, dissolved in 0.7 g/kg fat emulsion: 8% palmitate, 30% oleate, 56% linoleate</td>
<td>not reported</td>
<td>healthy children, n=10 crossover design</td>
</tr>
<tr>
<td>Watkins et al., 1982 (fat malabsorption group)</td>
<td>[1-13C]-trioctanoin triolein palmitate</td>
<td>% of fed 7±8 1±2</td>
<td>6</td>
<td>not reported</td>
<td>fed, dissolved in 0.7 g/kg fat emulsion: 8% palmitate, 30% oleate, 56% linoleate</td>
<td>not reported</td>
<td>children with pancreatic insufficiency, n=5-6 crossover design</td>
</tr>
<tr>
<td>Toorop et al., 1979 (Experiment II)</td>
<td>methyl [1-14C]-linoleate palmitate</td>
<td>% of absorbed 4±1</td>
<td>8</td>
<td>8</td>
<td>fed, mixed with 300 mg methyl linoleate or 700 mg methyl palmitate &amp; equal cellulose</td>
<td>fat-free, 15 g/d fed for 6 days</td>
<td>male Sprague-Dawley rats n=6</td>
</tr>
<tr>
<td>Toorop et al., 1979 (Experiment III)</td>
<td>methyl [1-14C]-linoleate palmitate</td>
<td>% of absorbed 35±3</td>
<td>8</td>
<td>71</td>
<td>fed, mixed with 300 mg methyl linoleate or 700 mg methyl palmitate &amp; equal cellulose</td>
<td>fat-free, 15 g/d fed for 6 days</td>
<td>male Sprague-Dawley rats n=8</td>
</tr>
<tr>
<td>Toorop et al., 1979 (Experiment IV)</td>
<td>methyl [1-14C]-linoleate palmitate</td>
<td>% of absorbed 25±1</td>
<td>24</td>
<td>93</td>
<td>fed, mixed with 300 mg methyl linoleate or 700 mg methyl palmitate &amp; 10 g of non-fat diet</td>
<td>15 g/d of fat-free with 450 mg linoleate or 1050 mg palmitate, fed for 6 days</td>
<td>male Sprague-Dawley rats n=10</td>
</tr>
</tbody>
</table>

(continued)
Figure 2 (continued),

<table>
<thead>
<tr>
<th>Reference</th>
<th>Fatty acid tracers</th>
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<th>Time after tracer feeding (hours)</th>
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<th>Tracer feeding method</th>
<th>Usual diet fat (% of energy, tracee amount, duration)</th>
<th>Subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cenedella and Allen, 1969</td>
<td>[1-14C]-linoleate palmitate</td>
<td>% of absorbed 21±7 11±3</td>
<td>the one-hour interval between 5 &amp; 6</td>
<td>60</td>
<td>Intragastric, dissolved in olive oil (1 ml), followed by ad libitum Chow</td>
<td>Purina Rat Chow</td>
<td>Male Wistar rats, n=4</td>
</tr>
<tr>
<td>Coots, 1964</td>
<td>[1-14C]-oleate, elaidate, palmitate, stearate (all esterified to triacylglycerols)</td>
<td>% of fed 66±4 66±2 70±2 57±1</td>
<td>51</td>
<td>96</td>
<td>Intragastric, transesterified to soy oil, in 5.5 g of liquid diet having 27% fat by weight</td>
<td>Not reported</td>
<td>Male Holtzman rats, n=3-7</td>
</tr>
<tr>
<td>Mead et al., 1956</td>
<td>Methyl [1-14C]-oleate, linoleate, stearate (control group, graphical data)</td>
<td>% of fed 45 27 15</td>
<td>8</td>
<td>Not reported</td>
<td>Intragastric, as methyl esters dissolved in corn oil</td>
<td>Not reported</td>
<td>Male mice, n=3-7</td>
</tr>
<tr>
<td>Mead et al., 1956</td>
<td>Methyl [1-14C]-oleate, linoleate, stearate (control group, tabular data)</td>
<td>% of fed 58 38 29</td>
<td>24</td>
<td>Not reported</td>
<td>Intragastric, as methyl esters dissolved in corn oil</td>
<td>Not reported</td>
<td>Male mice, n=3-7</td>
</tr>
<tr>
<td>Mead et al., 1956</td>
<td>Methyl [1-14C]-oleate, linoleate, stearate (fat-deficient group, tabular data)</td>
<td>% of fed 49 58 51</td>
<td>24</td>
<td>Not reported</td>
<td>Intragastric, as methyl esters dissolved in corn oil</td>
<td>Fat-free, fed for 3 months</td>
<td>Male mice, n=3-4</td>
</tr>
</tbody>
</table>
CHAPTER 3

METHODS

Subjects

Ten healthy adult volunteers were studied. Exclusion criteria included: obesity, defined as body mass index (BMI) greater than the 85th percentile for height, age, sex, and race (Must et al. 1991a, Must et al. 1991b); fasting blood lipid abnormalities, including TAG or low density lipoproteins above the 85th percentile or high density lipoproteins less than the 15th percentile for age and sex (Kwiterovich 1989 pp 291-295); and chronic health problems or use of drugs that affect lipid metabolism, including oral contraceptives and nicotine.

Fasting plasma lipids were analyzed at The Ohio State University Medical Center laboratory. Each subject was given a complete physical examination before admission, and verified to be afebrile on the morning of each study. Female subjects were studied during the first ten days of the menstrual cycle (i.e., early- to mid-follicular phase) to minimize variation in estrogen levels. Onset of menstruation was determined by self-report.

There were six women and four men, seven white and three black subjects, ranging in age from 20 to 34 years, and with body mass index (BMI) ranging from 19.2 to 27.3 kg/m². Characteristics of all subjects are given individually and summarized in Figure 3.
Experimental protocol

The experimental protocol was approved by the Biomedical Sciences Institutional Review Board for Research Involving Human Subjects, The Ohio State University. Subjects were recruited by fliers posted in the General Clinical Research Center (GCRC) and at the College of Human Ecology of The Ohio State University. Informed written consent was obtained from each subject prior to participation.

Each subject was studied on two occasions separated by at least three days and not more than 30 days. This resulted in a washout period of at least 36 hours, to prevent carry-over in breath enrichment and progressive adaptation to the diet. The same formula diet was fed during each study, but on one occasion [1-¹³C]-palmitate was added to the diet, and on the other [1-¹³C]-oleate was added. The order in which the two tracers were given was alternated within each sex category. On the first day of each study, the subject reported to the General Clinical Research Center following an overnight fast. The entire day and following night were then spent in the unit. During that time, subjects consumed only the liquid diet formulated for this experiment, which is described below. Following an overnight fast, on the second day of each study 30 to 60 ml of the liquid diet was administered each 20 minutes for a total of nine hours: during the first two hours without tracer, and during the last seven hours with tracer added. During each study, baseline breath and blood samples were collected two hours after ingestion of the first non-tracer meal, or nominal time = 0:00, and at 4:00, 5:00, 6:00, 6:20, 6:40, and 7:00 hours after the baseline sample. Additional breath samples were collected at 6:10, 6:30, and 6:50, resulting in breath collection each 10 minutes during the final hour. In a subset of subjects, breath samples were also obtained at 3:00 hours. The protocol timeline is diagrammed in Figure 4.

In a preliminary study to demonstrate the time course for achieving a plateau in breath enrichment, the protocol used was similar, except for the following: The subject did not stay overnight in the hospital, and the study was carried out in the Children's
Hospital Clinical Study Center. Feeding of the diet with tracer lasted 8:00 hours and breath collection lasted 8:40 hours. Only one study was done, using palmitate tracer. A priming dose of 6.0 μmol/kg $^{13}$C-bicarbonate was ingested after one hour of palmitate tracer feeding. The purpose of a priming dose is to shorten the time required to achieve a plateau, or steady state, in label appearance in breath CO$_2$ during constant-infusion tracer protocols, and the primer is typically given at the outset of tracer infusion. However, in the present experiment, tracer was ingested rather than infused. Administration of the primer dose was delayed because there is a delay between ingestion of fat and its appearance in plasma CM (Wu et al. 1975). Thus, the primer dose was timed to coincide approximately with the onset of tracer appearance in CM.

**Diet and tracers**

**Diet composition**

The test diet used was a liquid formula diet formulated specifically for this experiment by Ross Products Division of Abbott Laboratories. Its formulation was based on that of the commercial product Pediasure®, except for the fat source. The TAG in the test diet was 93% palm oil and 7% high-oleic acid safflower oil. The composition of the diet was analytically verified by Abbott Laboratories, and the macronutrient content is shown in detail in Figure 5. It provided 1.06 kcal/ml, with 40% of energy as fat, 46% as CHO, and 14% as protein. Calcium was 0.5% of dry weight and the Ca:P ratio was 1.2. By design, palmitate and oleate each contributed about 16% of total energy. Because the energy derived from oxidation per gram of oleate and palmitate is approximately equal (Livesey & Elia 1988), the test diet also contained similar weights of the two fatty acids. Oleate was 41.0% of the diet fat by weight and palmitate was 39.5%. Linoleate contributed 4% of diet energy and 10.1% of fat by weight, stearate, 2% of diet energy and 4.9% of fat, and other fatty acids made up less than 2% of diet energy, or 4.5% of fat.
**Fatty acid tracers**

The $[^{13}C]$-oleate and $[^{13}C]$-palmitate tracers were purchased from Isotech, Inc. (Cincinnati, Ohio). The tracers were added to the diet in amounts equal to 5% of their respective total dietary fatty acids. Since the dietary intake of each subject was calculated on the basis of 150% of resting energy expenditure (REE) (see next section), the tracer dose was also based on REE. Thus, total tracer dose was computed as follows:

$\frac{\text{tracer dose (g)}}{\text{total oleate or palmitate (g)}} = 0.05$; therefore

$\frac{\text{tracer dose (g)}}{\text{tracer dose (g) + diet oleate or palmitate (g)}} = 0.05$.

Rearranged: $\text{tracer dose (g)} = 0.05 \times \frac{1}{(1.0 - 0.05)} \times \text{diet oleate or palmitate}$.

Where

$\text{diet oleate or palmitate (g)} = 1.5 \times \text{REE (diet kcal/hr)} \times 7 \, \text{hr} \times 0.40 \times 0.42 \frac{\text{kcal oleate or palmitate}}{\text{fat kcal}} \times \frac{1}{9} \frac{\text{kcal/kg body weight}}{\text{oleate or palmitate}}$.

The resulting tracer dose was $(\text{mean ± SEM}) 784 ± 42 \text{ mg total, } 112 ± 6 \text{ mg per hour, or } 1.79 ± 0.05 \text{ mg per kg body weight per hour.}$ When converted to a molar basis, the doses for the two tracers are no longer equal, being $6.98 ± 0.21 \mu\text{mol per kg body weight per hour for palmitate and } 6.29 ± 0.19 \mu\text{mol per kg body weight per hour of oleate}$.

Tracer fatty acids were emulsified into the diet on the day preceding each protocol. Fifteen grams of granular PC (commercially available purified egg yolk PC) was dissolved in one cup of water at room temperature using a blender. Oleate tracer, which is liquid at room temperature, was added directly to this mixture and emulsified in a blender on low power. Palmitate is a waxy solid at room temperature, and has a melting point of about 58°C. Therefore, in order to emulsify this tracer, the PC mixture was gradually heated to about 60°C, and the metal blender jar was also heated in an oven to about 100°C before adding the lecithin solution and palmitate, in order to ensure the tracer would not re-solidify before it could be emulsified. This mixture was then stirred into the formula diet, divided into 21 equal parts, sealed in plastic cups, and refrigerated.
Because oleate is highly susceptible to oxidation, oleate tracer was stored at -80°C. Palmitate tracer was stored at 4°C.

**Rationale for rate of diet intake**

The rate of dietary energy intake was set equal to 150% of the individual REE of each subject, which had been measured on a previous occasion following an overnight fast. The rationale for feeding 150% of REE was that this would be equivalent to the fed state that results when food is consumed in several meals during the course of a day. There were two considerations involved in arriving at the specific rate of food intake used in this experiment. First, the rate of energy expenditure during the feeding protocol was expected to be higher than REE, and second, the digestion of food typically occurs during a period that is less than 24 hours, so that in a fed state, energy delivery exceeds expenditure. A discussion of these two factors follows.

First, on average, total energy expenditure in sedentary persons is 30 to 50% higher than fasting REE (Ravussin & Rising 1992). There are two sources of energy expenditure which contribute to an average daily total metabolic rate greater than REE. These are the thermic effect of food, which refers to the energy expended in consuming and assimilating food (James 1992), and physical activity. In the present protocol physical activity was minimal, since subjects were confined to bed except when using the bathroom. However, the magnitude of this energy expense can be estimated from the results of a study in which sedentary subjects (n=118) were confined to a respiratory chamber with floor dimensions of 3.33 m × 2.65 m (Ravussin et al. 1986). In that study, the energy cost of “spontaneous activity” averaged 15% of total 24-hour energy expenditure. In subjects confined to bed, the energy cost of activity would be somewhat less than in a chamber where subjects could move around at will.

The other source of energy expenditure above basal is the thermic effect of food. This is a function of numerous factors, including meal composition and the nutritional status of the subject (James 1992), but is about 7 to 13% of the energy consumed (Elia
In the study previously mentioned (Ravussin et al. 1986), the average thermic effect of food was 7% of total energy expenditure, making the sum of non-basal energy expenditure 22% of the total daily energy expenditure in that study. Therefore, basal metabolic rate accounted for 78% of the total, and non-basal expenditure was equal to 28% of basal expenditure (i.e., \( \frac{0.22}{0.78} \times 100 = 28\% \)). On the basis of available published data, therefore, it was predicted that the rate of energy expenditure in the present protocol would not exceed 130% of measured REE, and probably would be less than that.

In addition to meeting the energy expenditure of subjects in a fed state, the aim of the present feeding protocol was to provide additional food intake at a rate that would constitute what may be termed an “average fed state.” That is, suppose that a person consumes three equal meals at six-hour intervals, e.g., at 7:00 a.m., 1:00 p.m., and 7:00 p.m. Then assume that one-third of that person’s food energy for the day is digested and absorbed during each of the six-hour intervals following the three meals. Altogether, digestion and absorption would therefore occur during a total of 18 hours. Now suppose that the total energy intake for the day is 2400 kcal, or 100 kcal per hour. The average rate of delivery of food energy during the 18-hour “fed state” would then be equal to 2400 kcal / 18 hr or 133 kcal/hr. That is, the average rate at which food energy is assimilated in this example is 33% greater than the average metabolic rate for the entire day, assuming weight maintenance. If one assumes instead that each meal is assimilated in five hours rather than six, this difference becomes 60% since \( \frac{2400}{15} = 160 \) kcal/hr.

The margin between the rates of intake and expenditure is overestimated somewhat in the example above, since energy expenditure in the awake, active, fed state is higher than when sleeping. In addition to activity and thermic effect of food, simply being awake increases energy expenditure slightly over the sleeping state (Ravussin et al. 1986). To illustrate, if the person in the example above expends 80 kcal/hr while sleeping for 8 hours, and 110 kcal/hr on average while awake, then the average rate of energy assimilation during waking hours is only 21% greater than energy expenditure in
the first case (i.e., meals at six-hour intervals), and 45% in the second case (five-hour intervals). That is, \((133-110) \text{ kcal/hr} / 110 \text{ kcal/hr} \times 100 = 21\%\) and \((160-110) \text{ kcal/hr} / 110 \text{ kcal/hr} \times 100 = 45\%\). In the metabolic chamber study discussed above (Ravussin et al. 1986), energy intake averaged over 15 hours was 42% higher than average daytime energy expenditure.

The fact that energy intake typically exceeds expenditure in the fed state is an important consideration. This is because there are hormonal changes that occur in response to feeding, most notably in insulin, that are influenced by rate of nutrient absorption and delivery to circulation (Hunt & Groff 1990), and the aim of the experiment was to compare metabolism of fatty acids in a fed state. On the other hand, it was important to avoid overfeeding, that is, an intake rate that would suppress fat oxidation entirely (Bonadonna et al. 1990b, Carlson et al. 1991) or stimulate fat synthesis (Aarsland et al. 1997). In the present protocol, it was anticipated that actual energy expenditure would not exceed 130% of REE, and might in fact be quite close to REE. Hence, the feeding rate of 150% of REE was chosen to ensure that intake would exceed energy expenditure without being extreme.

One final point that should be addressed is the distinction between basal metabolic rate and resting energy expenditure, or REE. Basal metabolic rate is measured in subjects who have awakened from sleeping for a night before they get out of bed or perform any other physical activity (Elia 1992a). In the present protocol, subjects’ energy expenditure was measured in a resting state, but they had to come to the GCRC from their homes that morning, so their metabolic rate was probably slightly above the basal level. It was also the subjects’ first exposure to the metabolic cart and hood, and the unfamiliarity of the situation may have also increased the metabolic rate of some individuals (Elia 1992a). However, to the extent that the REE measurement in the present protocol exceeded true BMR, the result was an increased margin between energy expenditure and intake.
Sample analysis

Fatty acid analysis

Blood samples were drawn from a small forearm vein via an indwelling catheter and arterialized using a flexible heating pad on the high setting, applied for at least 10 minutes before each sample was drawn. Blood was collected in heparinized tubes and immediately placed in an ice bath. Samples were centrifuged within one hour at 4°C for 10 minutes at 3,000 rpm to separate the plasma fraction. Plasma was then transferred to microcentrifuge tubes (3 or 4 per sample) and stored at -20°C until analysis. Plasma samples were thawed immediately before analysis.

The CM (S_r > 400) and VLDL (S_f; 20-400) fractions were isolated by sequential ultracentrifugation (Hellerstein et al. 1991) in a Beckman Model L8-55 ultracentrifuge with SW50.1 swinging bucket rotor (Beckman Instruments, Schaumburg, IL). Approximately 3.7 ml saline (d=1.0063) with EDTA (0.1 mg/ml) was layered over 0.8 ml of plasma. Samples were centrifuged at 33,500 rpm for 30 minutes, after which the surface layer of solution was pipetted off with an extra-fine tipped pipet (about 0.5 ml), and the total volume restored by adding fresh saline. CM were visible as a milky film floating on the surface. This procedure was then repeated once. VLDL were then separated from the same sample by centrifugation at 40,000 rpm for 17 hours. VLDL were also usually visible on the surface of the solution. CM and VLDL samples were transferred to microcentrifuge tubes (three or four per sample) and stored at -20°C. Fatty acids were isolated from CM and VLDL preparations and derivatized to methyl esters (FAME's) using 3-Normal methanolic HCl (Hellerstein et al. 1991). The detailed procedure is given in Appendix B. In a subset of samples, separation of CM from VLDL was tested qualitatively by separation of the "large CM" fraction (S_r > 1000), as distinct from remnants (Lindgren & Jensen 1972), which may contain some liver-derived
particles. These samples were centrifuged at 33,500 rpm for 12 minutes and their fatty acid enrichments compared with that of their corresponding Sf > 400 fraction.

NEFA were prepared according to the method of Wolfe (Wolfe 1992) using 0.6 ml of plasma per sample. After lipid extraction, NEFA were isolated by thin layer chromatography and the NEFA band was identified using an external standard and rhodamine B dye. Fatty acids were then derivatized to FAME's using 14% BF₃-CH₃OH. The detailed procedure is given in Appendix C.

Fatty acids from each lipid fraction were quantified by gas chromatography with a flame-ionization detector (GC-FID) (Varian Star 3600 GC, System ID 3600-01313, Varian Analytical Instruments, Sugarland, TX). For each study, samples analyzed were those from time = 0:00, 4:00, and one from the final hour (t = 6:00 through 7:00). Therefore, six samples per subject were analyzed. In addition to oleate and palmitate, stearate (C₁₈:0) and linoleate (C₁₈:2) peaks were quantified. These four fatty acids comprised about 97% of the diet fat, so together they provide a qualitative index of how closely the CM composition reflected that of the diet. Identities of these peaks were verified using external standards. Each sample was analyzed in duplicate with acceptable repeatability defined to be less than 10% in the ratio of oleate to palmitate peak areas. Relative quantities of the oleate and palmitate peaks for a given fraction and subject were calculated by averaging the ratios from the six samples. Sample GC-FID plots for the three fatty acid fractions are shown in Appendix D.

Isotope enrichment of fatty acids was determined by electron impact ionization gas chromatography with mass spectrometer (GCMS) (Model 5971A, Hewlett Packard Co., Palo Alto, CA). A Supelco Omegawax 250 column, 0.25 mm ID x 30 m, was used for GC separation of FAME's. For each FAME, the M+0 and M+1 peaks were assessed: ions 270 and 271 for palmitate, 296 and 297 for oleate. The (M+1) / (M+0) isotope ratio was calculated, and enrichment defined as the difference between ratios of enriched and unenriched samples x 100, or moles percent excess (MPE) (Wolfe 1992), corrected against a standard curve. Each enriched sample was analyzed in duplicate and unenriched samples were analyzed in quadruplicate. Acceptable repeatability was defined to be less than
than 0.5%, which resulted in a difference in enrichment of less than 0.15 MPE. In selected samples, the stearate (C18:0) (M+1) / (M+0) ratio (ions 298 and 299) was calculated to assess whether measurable elongation of palmitate was occurring. To avoid confounding of enrichment data by concentration dependence of ion ratios for fatty acids (Patterson & Wolfe 1993), concentrations of samples being compared (i.e., enriched versus unenriched) were within a range of 15% of each other, and the MS was tuned such that voltage was maximized. Samples being compared were also analyzed within a period of not more than 24 hours to minimize variation over time (Wolfe et al. 1980).

Relative to palmitate enrichment, assessment of oleate enrichment is complicated by two factors. They are: (1) the greater fragmentation of oleate in the source, and (2) the proximity of the stearate and linoleate peaks to the oleate peak. The methodological considerations involved in analyzing oleate enrichment are discussed in Appendix E.

In order to assess the extent to which peripheral NEFA originated from CM catabolism versus release of endogenous fatty acids from adipose stores or catabolism of VLDL, enrichment of NEFA was determined in the t = 4:00 and t = 7:00 hour samples of each palmitate study, and in a smaller subset of oleate studies. These values were then compared with CM and VLDL enrichments from the same samples.

**ApoE phenotyping**

ApoE phenotyping was by isoelectric focusing followed by immunoblotting, using whole plasma, according to the method of Kamboh (Kamboh et al. 1988), as modified by Kataoka (Kataoka et al. 1994).

**Breath $^{13}$CO$_2$ isotope enrichment**

Expired air was collected in Quintron collection bags and stored in non-siliconed sterile Venoject tubes (four 15 ml tubes per sample) for not more than six months. The CO$_2$ was cryogenically purified and the atoms percent enrichment (APE)
was measured by gas isotope ratio mass spectrometry (IRMS) as previously described (Kien et al. 1992) (Model Delta E, Finnigan MAT, San Jose, CA). Reproducibility is 0.24% for replicate (n=5) samples prepared and analyzed in the natural abundance range of $^{13}$C. The detailed procedure for CO$_2$ purification is given in Appendix F.

**Indirect calorimetry**

Oxygen consumption (VO$_2$), and CO$_2$ production (VCO$_2$), were measured via indirect calorimetry, using a metabolic cart (Deltatrac, Sensor Medics Corporation, Yorba Linda, CA). A one-hour measurement of fasting REE was used in calculating the amount of diet to be fed to each subject. Subjects reported to the GCRC and were asked to rest in bed for at least 30 minutes before this fasting measurement. In addition, two 20-minute measurements were performed during each feeding protocol, and the final 15 minutes of each were analyzed. One of these measurements was made during the fifth hour of the feeding protocol (between t = 2:00 and 3:00), and the other was made in the ninth hour (between t = 6:00 and 7:00). These were used to determine whether intra-subject VCO$_2$ and VO$_2$ were consistent between and during the two studies.

Total energy expenditure is calculated from VO$_2$ and VCO$_2$ by the Deltatrac computer once each minute. Percent of energy derived from fat oxidation was estimated from energy expenditure and RQ (Livesey & Elia 1988). Average RQ was defined as the ratio of the averages of VCO$_2$ to VO$_2$, and protein oxidation was assumed to equal intake with a respiratory quotient of 0.835, as recommended by Livesey and Elia (Livesey & Elia 1988). Absolute rate of fat oxidation during the protocol was then computed from total energy expenditure and percent of energy derived from fat oxidation.

Absolute rate of appearance, Ra, of $^{13}$C derived from tracers was computed from the average VCO$_2$ during each protocol and the plateau value of APE as follows:

$$\text{Ra} \ ^{13}\text{C label (\mu mol/hr)} = \frac{\text{VCO}_2\ (ml/min) / 22.4 \text{ ml/mmol} \times 10^3 \mu \text{mol/mmol} \times 60 \text{ min/hr} \times \text{APE} / 100.}$$
The fraction of $^{13}$C label which appeared in breath CO$_2$ during each study was then computed for each subject by dividing the rate of label appearance by the rate of feeding of [1-$^{13}$C]-fatty acids. This was computed for comparison with results of studies shown in Figure 2, in which this was the primary or only outcome measure.

**Experimental model**

The present model is designed to expand upon previous comparisons of oxidation of different dietary fatty acids. Several features of the experimental design reflect that goal. In general, these features are designed to minimize, if not eliminate, metabolic sources of difference in oxidation. That is, the experiment was designed to maximize the probability that oxidation of the two fatty acids would be equal. The possible sources of metabolic variability are discussed in detail in Chapter 5. One of these, however, would be low-capacity, high-affinity enzymes or transport proteins, such as those which are involved in metabolism of branched-chain amino acids (Bender 1985 p 180). If such enzymes exist for fatty acids, they could greatly affect the fate of a given substrate depending on whether it were present in relative scarcity versus excess. To control for this possibility, equal amounts of the tracee fatty acids were fed. Another possible source of difference in oxidation was the processes involved in digestion, absorption, and incorporation into CM of both tracers and tracees. To control for these, CM were taken as the precursor pool to oxidation of diet-derived fat, and $^{13}$C enrichments and relative quantities of fatty acids in CM were measured. The outcome measures were then controlled for differences in digestive processes, including the presence of endogenous sources of substrate (Karmen et al. 1963, Emken et al. 1987, Nguyen et al. 1996), by computing relative oxidation on the basis of fractional oxidation of the two fatty acids in CM. This is discussed in the next section.

An additional goal was to preclude temporal differences in processes involved in the metabolic cycle of meal feeding, which could complicate interpretation of results. For example, one study found differences among fatty acid tracers in the time course of
digestion in rats (Leyton et al. 1987). (See Figure 2.) Such differences cannot be directly measured in humans, since fecal collection only measures what is neither absorbed nor fermented. To control for such potential sources of variability, tracers were fed with small, frequent meals to achieve steady-state delivery and metabolism of dietary fat, and steady-state appearance of label in breath CO₂. On the other hand, the protocol duration was limited to preclude colonic fermentation of undigested tracers. The use of a steady-state protocol also permits use of steady-state equations in analyzing the results.

In addition to minimizing sources of metabolic differences in fat oxidation, the model also is designed to incorporate certain aspects of physiological feeding conditions. First, the fat content of the test diet, 40% of energy, is approximately equal to the upper quartile of the range of usual intakes in the American diet reported in NHANES II (Block & Subar 1992). Second, although there is no single “typical” eating pattern for humans, the maintenance of a eucaloric feeding condition was intended to represent the fed state during the course of a day, as described above. The specific intent was to avoid extreme overfeeding, with consequent fat synthesis (Hudgins et al. 1996, Aarsland et al. 1997) and/or saturation of fat digestion (Wu et al. 1975) or CM remnant clearance (Berr 1992). On the other hand, the continuous delivery of CHO and protein throughout the study was expected to maintain a consistent level of insulin secretion. Insulin suppresses release of fatty acids from adipose stores (Boden et al. 1993, Campbell et al. 1992, Bonadonna et al. 1990b) and activates adipose tissue lipoprotein lipase which in turn stimulates triacylglycerol hydrolysis and clearance in peripheral tissues (Linder 1988).

**Derivation of equations**

**Absolute oxidation of chylomicron-derived fatty acids**

The principal aim of the present experiment was to compare oxidation of different fatty acids, rather than to assess their absolute oxidation. However, the expression of absolute substrate oxidation is the most commonly used equation in tracer oxidation
studies. Therefore, as an introduction to the rationale for the expression of relative oxidation which was used, this equation will first be derived and discussed. In a steady-state protocol, the absolute rate of oxidation of a given substrate (\( \text{SUB}_\text{ox} \)) is equal to the rate of substrate delivery to, or appearance in, the precursor pool for oxidation (\( \text{Ra}_\text{SUB} \)) multiplied by the fraction of substrate that is oxidized (\( \text{SUB}_\text{fractox} \)) (Wolfe 1992). By design, the precursor pool is also the pool being sampled. This relationship can be expressed:

\[
\text{SUB}_\text{ox} (\mu\text{mol/min}) = \text{Ra}_\text{SUB} (\mu\text{mol/min}) \times \text{SUB}_\text{fractox}. \tag{1}
\]

To assess oxidation of a given substrate, tracers labeled with carbon isotopes, \(^{13}\text{C}\) or \(^{14}\text{C}\), are used. These can be generically designated \(*\text{C}\). When the tracer is oxidized, the \(*\text{C}\) label appears in the breath as \(*\text{CO}_2\). When tracers are infused at a constant rate, designated \(F\), this rate can be used to determine \(\text{Ra}_\text{SUB}\), because isotope enrichment of the substrate precursor pool, \(\text{IE*SUB}\), is equal to the fraction of the precursor pool that is derived from tracer. This fraction is in turn equal to the ratio between appearance of labeled and unlabeled substrate. This assumes that the tracer is "massless." That is, the rate of tracer infusion is negligible compared to rate of appearance of endogenous substrate. Therefore:

\[
\text{IE*SUB} = \frac{F (\mu\text{mol/min})}{\text{Ra}_\text{SUB} (\mu\text{mol/min})}, \tag{2a}
\]

which can be rearranged to become:

\[
\text{Ra}_\text{SUB} (\mu\text{mol/min}) = \frac{F (\mu\text{mol/min})}{\text{IE*SUB}}. \tag{2b}
\]

Fractional substrate oxidation, \(\text{SUB}_\text{fractox}\), can also be computed from the rate of label excretion, \(*\text{C}_\text{EX}\), relative to the infusion rate, \(F\). This is based on the assumption that the fractional oxidation of the tracer, \(\text{TR}_\text{fractox}\), is the same as that of the unlabeled substrate. Thus.

\[
\text{SUB}_\text{fractox} = \frac{\text{TR}_\text{fractox} = *\text{C}_\text{EX} (\mu\text{mol/min})}{[F (\mu\text{mol/min}) \times c]}. \tag{3}
\]

The isotope exchange correction factor, \(c\), is discussed in detail in Chapter 5 under "Isotope exchange and label recovery." Briefly, it accounts for the temporary loss of a portion of labeled carbon due to exchange with endogenous pools of unlabeled \(\text{CO}_2\) and
tricarboxylic acid cycle intermediates (Wolfe 1992, Sidossis et al. 1995b). By rearranging the equation above, rate of label excretion in breath CO₂ can be expressed:

\[ *C_{EX} (\mu mol/min) = \text{SUB}_{\text{fract OX}} \times F (\mu mol/min) \times c. \]

However, the rate of label excretion is also equal to the total rate of excretion of CO₂ in breath multiplied by enrichment of breath \(^{13}\text{CO}_2\):

\[ *C_{EX} (\mu mol/min) = *\text{CO}_2_{EX} (\mu mol/min) = \text{VCO}_2 (\mu mol/min) \times \text{IE} \cdot \text{CO}_2. \]

Therefore, the two expressions above can be set equal and rearranged to become:

\[ \text{SUB}_{\text{fract OX}} = \text{VCO}_2 (\mu mol/min) \times \text{IE} \cdot \text{CO}_2 / [ F (\mu mol/min) \times c ]. \] (3)

Substituting the expressions for \( \text{R}_{\text{SUB}} \) and \( \text{SUB}_{\text{fract OX}} \) from equations 2b and 3 into equation 1 yields:

\[ \text{SUB}_{\text{OX}} (\mu mol/min) = F (\mu mol/min) / \text{IE} \cdot \text{SUB} \times \text{VCO}_2 (\mu mol/min) \times \text{IE} \cdot \text{CO}_2 / [ F (\mu mol/min) \times c ], \]

which, simplified, becomes:

\[ \text{SUB}_{\text{OX}} (\mu mol/min) = \text{VCO}_2 (\mu mol/min) \times \text{IE} \cdot \text{CO}_2 / ( \text{IE} \cdot \text{SUB} \times c ). \] (4)

In the present experiment, the substrates for oxidation being studied are oleate (OA) and palmitate (PA). Therefore, the expression for absolute oxidation of oleate is:

\[ \text{OA}_{\text{OX}} (\mu mol/min) = \text{VCO}_2 (\mu mol/min) \times \text{IE} \cdot \text{CO}_2 / ( \text{IE} \cdot \text{OA} \times c ). \] (5a)

Similarly, the expression for absolute oxidation of palmitate is:

\[ \text{PA}_{\text{OX}} (\mu mol/min) = \text{VCO}_2 (\mu mol/min) \times \text{IE} \cdot \text{CO}_2 / ( \text{IE} \cdot \text{PA} \times c ). \] (5b)

Because the aim herein was to compare the oxidation of these two substrates, some additional annotation of terms is required. In formulating a single expression for relative oxidation, it is necessary to denote which tracer was being fed in each of the terms. Also, fatty acids from three plasma lipid fractions were analyzed. Therefore, the specific fatty acid fraction under consideration will also be denoted henceforward. In the case of oxidation of dietary fat, the precursor pool is the chylomicron fraction, which will be indicated by a "CM" subscript. The expression for oleate oxidation thus becomes:

\[ \text{OA}_{\text{OX}} (\mu mol/min) = \text{VCO}_2 \text{OA} (\mu mol/min) \times \text{IE} \cdot \text{CO}_2 \text{OA} / ( \text{IE} \cdot \text{OA}_{\text{CM}} \times c_{\text{OA}} ). \] (6a)

Where
$VCO_{2\text{OA}}$ is the rate of excretion of $CO_2$ during oleate tracer ingestion.

$IE\cdot CO_{2\text{OA}}$ is the $^{13}C$ enrichment of $CO_2$ during ingestion of $[1-^{13}C]$-labeled oleate (APE/100; dimensionless), and

$IE\cdot OA_{\text{CM}}$ is the $^{13}C$ enrichment of the fatty acid derived from CM (MPE/100; dimensionless).

Similarly, for palmitate oxidation:

$PA_{\text{OX}} (\mumol/min) = VCO_{2\text{PA}} (\mumol/min) \times IE\cdot CO_{2\text{PA}} / ( IE\cdot PA_{\text{CM}} \times c_{\text{PA}} ). \quad (6b)$

**Relative fatty acid oxidation**

The present experiment differs from one using a constant infusion of a single tracer in certain fundamental respects, which must now be addressed. Tracer infusion studies typically assess how substrate oxidation varies with varying metabolic state, and therefore absolute oxidation rate, e.g., $\mu$mol oxidized per minute, is the appropriate measure for comparison. Since the present research sought to compare oxidation of two substrates on the basis of equal availability, the logical basis for comparison is the fractional oxidation, rather than absolute rate. This compares oxidation on the basis of amount oxidized versus amount delivered. Note that the experiment was designed to equalize availability of dietary fat, and if the calculation were performed on the basis of what was fed, relative oxidation computed on an absolute or fractional basis would give the same answer. However, since relative oxidation is being computed using CM-derived fatty acids rather than dietary fat, using fractional oxidation provides a means for correcting for variations in availability between the two fatty acids in CM. In order to assess relative oxidation, then, the expressions for fractional oxidation of the two substrates were used. Revising the annotation for equation 3, the expression for fractional oxidation, to reflect the oxidation of oleate results in the following expression:

$OA_{\text{frac OX}} = VCO_{2\text{OA}} (\mumol/min) \times IE\cdot CO_{2\text{OA}} / [ F_{\text{OA}} (\mumol/min) \times c_{\text{OA}} ] . \quad (7a)$

Similarly, for palmitate oxidation:

$PA_{\text{frac OX}} = VCO_{2\text{PA}} (\mumol/min) \times IE\cdot CO_{2\text{PA}} / [ F_{\text{PA}} (\mumol/min) \times c_{\text{PA}} ] . \quad (7b)$
Relative oxidation of the two fatty acids, \( \text{Rel}_{\text{OX}} \), was defined as the ratio of the two fractional oxidation rates, or \( \text{OA}_{\text{frac oxy}} / \text{PA}_{\text{frac oxy}} \). In this ratio, if one assumes that metabolic conditions remain constant between the two tracer-feeding protocols, two of the terms cancel. These are \( c \) and \( VCO_2 \). That is, \( c_{\text{OA}} \) is assumed to be equal to \( c_{\text{PA}} \) and \( VCO_2_{\text{OA}} \) is assumed to be equal to \( VCO_2_{\text{PA}} \). The simplified expression then becomes:

\[
\text{Rel}_{\text{OX}} = (\frac{\text{IE} \cdot \text{CO}_2_{\text{OA}} / F_{\text{OA}}}{\text{IE} \cdot \text{CO}_2_{\text{PA}} / F_{\text{PA}}}).
\]  

(8)

When a tracer is infused directly to the circulation, its rate of entry, \( F \), is known precisely. In the present protocol, however, there is uncertainty in this value due to the possibility of incomplete incorporation of tracer into CM. Therefore, an expression of \( F \) is needed that enables the computation of \( \text{Rel}_{\text{OX}} \) from the available data. Rearranging equation 2b and modifying the notation to reflect an oleate tracer study yields:

\[
F_{\text{OA}} (\mu\text{mol/min}) = \frac{\text{Ra}_{\text{OA-CM}} (\mu\text{mol/min}) \times \text{IE} \cdot \text{OA}_{\text{CM}}}{\text{IE} \cdot \text{CO}_2_{\text{OA}} / (\text{Ra}_{\text{OA-CM}} (\mu\text{mol/min}) \times \text{IE} \cdot \text{OA}_{\text{CM}})}.
\]  

(9a)

Similar ly, for a palmitate tracer study:

\[
F_{\text{PA}} (\mu\text{mol/min}) = \frac{\text{Ra}_{\text{PA-CM}} (\mu\text{mol/min}) \times \text{IE} \cdot \text{PA}_{\text{CM}}}{\text{IE} \cdot \text{CO}_2_{\text{PA}} / (\text{Ra}_{\text{PA-CM}} (\mu\text{mol/min}) \times \text{IE} \cdot \text{PA}_{\text{CM}})}.
\]  

(9b)

Substituting equations 9a and 9b into equation 8:

\[
\text{Rel}_{\text{OX}} = \left(\frac{\text{IE} \cdot \text{CO}_2_{\text{OA}} / (\text{Ra}_{\text{OA-CM}} (\mu\text{mol/min}) \times \text{IE} \cdot \text{OA}_{\text{CM}})}{\text{IE} \cdot \text{CO}_2_{\text{PA}} / (\text{Ra}_{\text{PA-CM}} (\mu\text{mol/min}) \times \text{IE} \cdot \text{PA}_{\text{CM}})}\right).
\]  

(10)

Like \( F \), the rates at which oleate and palmitate in CM enter the circulation are not known. However, in a steady-state protocol the ratio between these two rates is equal to the ratio of their concentrations, \( C \), in CM. This is analogous to the reasoning used in formulating the equation for substrate enrichment, 2a. When the two substrates are measured within the same sample, the volume terms cancel. Thus:

\[
\text{Ra}_{\text{OA-CM}} (\mu\text{mol/min}) / \text{Ra}_{\text{PA-CM}} (\mu\text{mol/min}) = C_{\text{OA-CM}} (\mu\text{mol}) / C_{\text{PA-CM}} (\mu\text{mol}).
\]  

(11)

The relative concentrations of oleate and palmitate are given on a carbon mass basis by their peak areas from the GC-FID data. That is because the peak areas are directly proportional to the carbon mass. This relationship can be expressed:

\[
A = k \times M,
\]
where \( A \) = the peak area, \( k \) is a constant response factor relating number of carbon atoms to area counts, and \( M \) is the mass of carbon atoms present in a given peak. Within a given GC-FID sample analysis, since \( k \) remains constant, it cancels when the ratio of two peak areas is taken. Hence the ratio of oleate versus palmitate present in a CM sample can be expressed:

\[
\frac{A_{OA-CM}}{A_{PA-CM}} = \frac{M_{OA} (\mu g \text{ oleate carbon})}{M_{PA} (\mu g \text{ palmitate carbon})}. \tag{12}
\]

Where

\( A_{OA-CM} \) and \( A_{PA-CM} \) are the GC-FID-derived peak areas of oleate and palmitate, respectively, in CM-derived samples, and \( M_{OA} \) and \( M_{PA} \) represent the mass of carbons per oleate or palmitate peak.

It remains to convert the above ratio to the basis of \( \mu \text{mol} \) rather than grams. This can be accomplished by dividing the peak area terms by the number of acetyl-CoA, \( \text{acCoA} \), molecules derived per molecule of substrate in \( \beta \)-oxidation of the fatty acids, which will be designated "\( n_{SUB} \)." That is,

\[
C (\mu \text{mol}) = \frac{M (\mu g)}{n_{SUB} \times 2 \times (\text{carbons/ acCoA}) \times \text{carbon AMU} \ (\text{g/mol})}. \]

When the ratio of \( M_{OA} / M_{PA} \) is taken, two of the terms, \( 2 \times \text{carbons/ acCoA} \) and \( \text{carbon AMU} \), cancel, and the resulting expression is:

\[
R_a \frac{OA-CM (\mu \text{mol/min})}{PA-CM (\mu \text{mol/min})} = \frac{C_{OA-CM} (\mu \text{mol})}{C_{PA-CM} (\mu \text{mol})} = \left( \frac{A_{OA-CM}}{n_{OA}} \right) / \left( \frac{A_{PA-CM}}{n_{PA}} \right). \tag{13}
\]

For the substrates of this analysis, \( n_{OA} = 9 \), and \( n_{PA} = 8 \), since there is one \([1-^{13}\text{C}]\)-acetyl-CoA per 9 acetyl-CoA's formed in the oxidation of \([1-^{13}\text{C}]-\text{oleate} \), and one \([1-^{13}\text{C}]\)-acetyl-CoA per 8 acetyl-CoA's formed in the oxidation of \([1-^{13}\text{C}]\)-palmitate. Note that there is no difference in the final answer between using number of carbons (18/16) versus number of acetyl-CoA's (9/8). However, there is a logical reason for using units of acetyl-CoA rather than carbon atoms per molecule of substrate. The first and second carbon molecules in each acetyl-CoA do not equally label the \( \text{CO}_2 \) pool (Wolfe & Jahoor 1990). Although this is not an issue in the present experiment, it is an important distinction that should be recognized.
This conversion factor can be viewed more intuitively as follows: In the present experiment, equal amounts of the two fatty acids of interest were fed on the basis of energy derived, which meant that equal masses (grams) of each were fed. Also, the two fatty acids were enriched equally in the diet, at 5% MPE each, such that equal gram amounts of the two tracers were also fed. Each labeled CO₂ is produced from the oxidation of the first acetyl-CoA to be formed during β-oxidation of a [1-¹³C]-fatty acid. If 100% of both tracers were oxidized, the enrichment of ¹³CO₂ during the oleate protocol would be lower than during the palmitate protocol, because each gram of palmitate contains more moles of the fatty acid, and, hence, produces more labeled acetyl-CoA, than a gram of oleate, even though the total moles of acetyl-CoA generated in oxidation are about equal.

Substituting equation 13 into equation 10:

\[
\text{Rel}_{\text{OX}} = \left( \frac{\text{IE} \cdot \text{CO}_2 \text{OA}}{\text{AO} \cdot \text{CM} / \text{n OA} \times \text{IE} \cdot \text{OA}_\text{CM}} \right) / \left( \frac{\text{IE} \cdot \text{CO}_2 \text{PA}}{\text{PA} \cdot \text{CM} / \text{n PA} \times \text{IE} \cdot \text{PA}_\text{CM}} \right)\]

rearranging gives the final expression of relative oxidation:

\[
\text{Rel}_{\text{OX}} = \left( \frac{\text{IE} \cdot \text{CO}_2 \text{OA} \times \text{n OA} \times \text{A} \cdot \text{PA} \cdot \text{CM} \times \text{IE} \cdot \text{PA}_\text{CM}}{\text{IE} \cdot \text{CO}_2 \text{PA} \times \text{n PA} \times \text{A} \cdot \text{OA} \cdot \text{CM} \times \text{IE} \cdot \text{OA}_\text{CM}} \right).
\]

Relative oxidation, as defined by this equation, can then be conceptualized as the "efficiency" with which dietary oleate is oxidized relative to palmitate.

Finally, in the present research, the two fatty acids were fed on the basis of equal energy of oxidation. For this reason, a comment should be added regarding the relationship between comparing oxidation on the basis of fraction of moles or mass versus energy of oxidation. In the case of the two fatty acids being considered herein, the two approaches are nearly identical. That is because the ratio between numbers of acetyl-CoA’s is approximately equal to the ratio of energy derived from oxidation. That is, when palmitate is oxidized, there is a net yield of 129 ATP (Stryer 1988 p 477), which equals 16.125 ATP per acetyl-CoA. When oleate is oxidized, net ATP yield is 144, or 16.0 ATP per acetyl-CoA, so the difference between the two fatty acids in ATP produced per acetyl-CoA is less than 1%.

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Relative appearance of dietary fatty acids in VLDL

The appearance of dietary oleate in VLDL was compared with that of palmitate in the following way. The situation is partially analogous to that of relative oxidation, although simpler because no substrate conversion to other forms (i.e., CO₂) takes place. First, the fractional rates at which labeled and unlabeled substrate are incorporated are assumed to be the same. Also, as with relative oxidation, the comparison between the two substrates is on the basis of their fractional, not absolute, rate of incorporation. That is, the comparison is made on the basis of availability of the precursor fatty acids derived from CM. Modifying equation 7 to reflect incorporation into VLDL of CM-derived fatty acids, rather than their oxidation, yields:

\[ \text{OA}_\text{fract VI} = \frac{\text{Ra}_{\text{OA-VLDL}} \times \text{IE•OA}_{\text{VLDL}}}{\text{F}_{\text{OA}}} \]  \hspace{1cm} (15)

Where \( \text{OA}_\text{fract VI} \) is the fractional incorporation of oleate into VLDL by the liver; \( \text{Ra}_{\text{OA-VLDL}} \) is the total rate of incorporation of oleate into plasma VLDL; and \( \text{IE•OA}_{\text{VLDL}} \) is enrichment of VLDL oleate. Note that \( c \) does not appear in equation 15 since it is a consequence of processes which occur during oxidation of fatty acids.

As discussed above, the rate of delivery of labeled fatty acids from CM, \( F \), is not known precisely, but data from the CM fraction can be used to derive an expression for relative incorporation. Thus, substituting equation 9a, into equation 15 for oleate:

\[ \text{OA}_\text{fract VI} = \frac{\text{Ra}_{\text{OA-VLDL}} \times \text{IE•OA}_{\text{VLDL}}}{(\text{Ra}_{\text{OA-CM}} \times \text{IE•OA}_{\text{CM}})} \] \hspace{1cm} (16a)

Similarly, for palmitate,

\[ \text{PA}_\text{fract VI} = \frac{\text{Ra}_{\text{PA-VLDL}} \times \text{IE•PA}_{\text{VLDL}}}{(\text{Ra}_{\text{PA-CM}} \times \text{IE•PA}_{\text{CM}})} \] \hspace{1cm} (16b)

Relative incorporation is defined to be the ratio of the two expressions for fractional incorporation:

\[ \text{Rel}_\text{VI} = \frac{\text{OA}_\text{fract VI}}{\text{PA}_\text{fract VI}} \]
Substituting area ratios from GC-FID data for Ra ratios as in equation 14 and simplifying yields:

\[
\text{Rel}_V = \frac{\text{IE} \cdot V_L \cdot \text{A}_{\text{oa-osl}} \times \text{IE} \cdot \text{P} \cdot \text{A}_{\text{cm}} \times \text{A}_{\text{pa-cm}}}{\left( \text{IE} \cdot \text{P} \cdot \text{A}_{\text{osl}} \times \text{A}_{\text{pa-cm}} \times \text{IE} \cdot \text{O} \cdot \text{A}_{\text{cm}} \times \text{A}_{\text{oa-cm}} \right)}
\]

Note that, because there is no conversion of substrate to CO\(_2\), no acetyl-CoA conversion factor is needed in this expression, as opposed to equation 14. If they were included, they would simply cancel.

\textit{NEFA clearance}

Relative clearance of oleate versus palmitate from the plasma NEFA fraction was defined to be the proportion of plasma NEFA made up of oleate divided by the proportion of CM made up of oleate, compared to the same relationship for palmitate, and will be denoted R_{\text{NEFA}}. The expression for this is derived as follows. First, the proportion, P, of NEFA that is made up of oleate in a given fraction (NEFA or CM) on the basis of mass can be written:

\[
P_{\text{oa-nefa}} = \frac{M_{\text{oa-nefa}}}{\Sigma M_{\text{nefa}}}
\]

\[
P_{\text{oa-cm}} = \frac{M_{\text{oa-cm}}}{\Sigma M_{\text{cm}}}
\]

Where \(\Sigma M\) is the sum of all fatty acid masses in the respective plasma fractions.

The next step rests on the assumption that fatty acids newly hydrolyzed from CM are the main source plasma NEFA. The proportion of oleate in NEFA relative to the proportion of oleate in CM, R_{\text{p oa}}, is then a measure of how its peripheral clearance from plasma compares with the average for all fatty acids present in CM. This can be expressed:

\[
R_{\text{p oa}} = \frac{P_{\text{oa-nefa}}}{P_{\text{oa-cm}}}
\]

\[
= \left( \frac{M_{\text{oa-nefa}}}{\Sigma M_{\text{nefa}}} \right) \left/ \left( \frac{M_{\text{oa-cm}}}{\Sigma M_{\text{cm}}} \right) \right.
\]

(18)
As previously discussed with regard to equation 12, the individual fatty acid masses are proportional to the peak areas from the GC-FID analysis, so the peak areas can be substituted in equation 18:

\[ \text{RP}_{\text{OA}} = \frac{A_{\text{OA-NEFA}} / \Sigma A_{\text{NEFA}}}{A_{\text{OA-CM}} / \Sigma A_{\text{CM}}} \]  

Similarly, for palmitate:

\[ \text{RP}_{\text{PA}} = \frac{A_{\text{PA-NEFA}} / \Sigma A_{\text{NEFA}}}{A_{\text{PA-CM}} / \Sigma A_{\text{CM}}} \]  

The expressions above relate clearance of each fatty acid to that of the total pool of CM-derived fatty acids. However, in this analysis, the aim was to compare oleate to palmitate, which can be accomplished by taking the ratio of equations 19 and 20. The \( \Sigma A \) terms cancel in this expression, which then becomes:

\[ \text{RC}_{\text{NEFA}} = \frac{\text{RP}_{\text{OA}}}{\text{RP}_{\text{PA}}} = \frac{A_{\text{OA-NEFA}} / A_{\text{OA-CM}}}{A_{\text{PA-NEFA}} / A_{\text{PA-CM}}} \]  

Note that, in this expression, all four terms have the same units, so there is no difference in computing this number on the basis of moles versus mass.

**Use of steady-state equations**

In the derivations above, steady-state equations are used throughout. This is based upon the assumption of a constant metabolic state. Although the protocol was also designed to achieve a plateau in \(^{13}\text{C} \text{O}_2\) enrichment, it is crucial to distinguish between the metabolic steady state to which the equations used apply, and the steady-state appearance of labeled fatty acids or \(^{13}\text{C} \text{O}_2\) that is demonstrated by achieving a plateau in their enrichments. In the case of the former, it is the metabolism of unlabeled substrate that is assumed to remain constant; e.g., the rates of appearance and disappearance from the pool being sampled, and hence the size of that pool. In the case of stable isotopes, the source(s) of unlabeled substrate are also assumed to remain constant, since otherwise the background \(^{13}\text{C}\) content may change (Wolfe et al. 1984, Barstow et al. 1990, Jones et al. 1985a).
A plateau in product enrichment, on the other hand, is required to compute either fractional or absolute rates of conversion of substrate to product, e.g., oxidation to CO$_2$ or VLDL synthesis. That is, to the extent that enrichments are below their true plateau values, rates of conversion are underestimated. In the expressions for relative oxidation and VLDL incorporation derived herein, however, their validity does not depend upon plateau enrichments, if the two enrichments being compared are subject to the same kinetic parameters. However, the closer to a plateau the enrichment curve comes, the better the accuracy of the result. Mathematically, this can be shown by considering a monoexponential function describing the time course for enrichment of a single, well-mixed pool following the onset of constant infusion of a tracer:

$$\text{IE}(a)_t = \text{IE}(a)_\infty \times (1 - e^{-\lambda t}) = \text{IE}(a)_\infty \times (1 - 2^{-\frac{t}{T}}).$$  \hspace{1cm} (22a)

Where $\text{IE}(a)_t$ is the enrichment at time = t; $\text{IE}(a)_\infty$ is the theoretical enrichment at time = infinity, or a perfect plateau value for a given rate of tracer infusion; $F(a)$, $\lambda$ is the fractional turnover rate of the substrate pool; and $T$ is the pool half-life. Similarly, for a second tracer infusion rate, $F(b)$:

$$\text{IE}(b)_t = \text{IE}(b)_\infty \times (1 - e^{-\lambda t}) = \text{IE}(b)_\infty \times (1 - 2^{-\frac{t}{T}}).$$  \hspace{1cm} (22b)

If equation 22a is divided by equation 22b it can be seen that, at any given time, $t$, the resulting expression simplifies to:

$$\frac{\text{IE}(a)_t}{\text{IE}(b)_t} = \frac{\text{IE}(a)_\infty}{\text{IE}(b)_\infty}.$$  \hspace{1cm} (23)

Hence, even if a perfect steady state in enrichment is not achieved, the ratio of the enrichments is equal to what it would be at steady state. On the other hand, as a plateau in enrichment is approached, the absolute values of the two enrichments increase, and their rates of change decrease, thereby increasing the accuracy of their experimental determination. In addition to this consideration, the present protocol was designed to achieve a functional plateau in order to compare the absolute oxidation of the dietary fatty acids with that of previous comparisons of fatty acid oxidation. On the other hand, the protocol was limited to seven hours of tracer intake to preclude significant "recycling" of label from endogenous pools, which eventually become measurably labeled as a
consequence of isotope exchange. For practical purposes, a functional plateau may be thought of as that point at which additional data generated by extending the protocol would be of negligible value.

Statistical analysis

CM-derived oleate and palmitate were defined to be the precursors in computing three outcome measures: fatty acid oxidation, NEFA clearance, and VLDL incorporation. CM fatty acid $^{13}\text{C}$ enrichment was defined a priori as the average MPE during the final hour of each study (four data points). To determine whether there was evidence of unequal absorption of the two tracers, the amounts of labeled oleate and palmitate in CM during the final hour were compared by paired t-test. For the purposes of this computation, the amount of labeled fatty acid was defined as $\text{IE}_{\text{CM}} \times A_{\text{CM}}$. (See equations 12 and 13.) Because the half-life of CM is typically less than ten minutes (Hussain et al. 1996), it was anticipated that the enrichment of CM fatty acids would be constant during the final three hours of tracer ingestion ($t = 4:00$ through $7:00$), when enriched samples were collected. To assess whether this was the case, a multiple regression analysis was performed upon CM enrichment with tracer, time, and subject as the independent variables. Similarly, to assess whether a plateau was achieved in VLDL enrichment, a multiple regression analysis with tracer, time, and subject as the independent variables was performed. In addition, the kinetic parameters for enrichment of CM and VLDL were estimated by curve-fitting the averaged data for each tracer to a simple monoexponential function. This necessitated estimation of three parameters for each curve: half-life of the fatty acids in CM or VLDL, theoretical steady-state enrichment, and time at which the $^{13}\text{C}$ label first appeared in circulation after beginning of tracer ingestion.

Enrichment of NEFA was compared with that of CM and VLDL as a means of inferring the origins of circulating plasma NEFA. A multiple regression was performed for NEFA enrichment with the independent variables being time and the enrichments of
CM and VLDL. If enrichment of CM was a significant predictor of NEFA enrichment, that would support the conclusion that circulating CM were a significant source of plasma NEFA. Similarly for VLDL, a predictive relationship in enrichment would support the conclusion that some of the plasma NEFA originated from VLDL catabolism.

A plateau in breath $^{13}$CO$_2$ enrichment was defined as a slope of not more than 10% of average APE during the final hour (seven time points). Each study's APE data were analyzed individually. In the event that a visual inspection revealed suspected outliers, a test for exclusion of outliers was performed (Tietjen 1986). No data were excluded on this basis. Variability in APE values during the final hour limited the statistical power of slope calculations for individual subjects. Therefore, slopes were also computed for the averages of all subjects for each tracer, for the $t = 4:00$ through 6:00 period, during the final hour ($t = 6:00$ through 7:00), and during the final half-hour ($t = 6:30$ through 7:00). In order to estimate the theoretical steady-state $^{13}$CO$_2$ enrichments, the averaged data were fitted to a simple monoexponential function, as was done for CM and VLDL enrichment.

Results of the 4 measurement periods of indirect calorimetry were compared by 2-way ANOVA (day x time) with repeated measures. Time, in this case, refers to morning versus afternoon measurements. Since no such differences were found, all data were pooled for each subject (60 time points). Comparing the two times provided a measure of how consistent substrate oxidation was throughout the protocol.

Statistical significance was defined to be $P<0.05$. The EXCEL software package was used in computing all statistical tests.
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<th>age (years)</th>
<th>Height (m)</th>
<th>Weight (kg)</th>
<th>BMI (kg/m²)</th>
<th>fasting TAG (mg/dL)</th>
<th>fasting LDL (mg/dL)</th>
<th>fasting HDL (mg/dL)</th>
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Figure 3. Anthropometric characteristics of participants. BMI is Body Mass Index; TAG is triacylglycerol; LDL is low-density lipoprotein cholesterol; HDL is high-density lipoprotein cholesterol. Apolipoprotein E (ApoE) determination was by isoelectric focusing (Kamboh 1988, Kataoka 1994).
Figure 4. Experimental protocol timeline. Liquid formula diet was fed each 20 minutes beginning two hours before baseline samples were taken, at time = 0. Diet with [1-13C]-labeled palmitate or oleate tracer was fed each twenty minutes for seven hours beginning at time = 0. Indirect calorimetry was performed for 20 minutes during each of the two sampling periods.
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<th>fat energy (%)</th>
<th>nutrient source and distribution (%)</th>
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Figure 5. Macronutrient composition of test diet. The liquid formula diet used was formulated specifically for this experiment by Ross Products Division of Abbott Laboratories (Product EN9543).
CHAPTER 4

RESULTS

Indirect calorimetry

Indirect calorimetry data for individual subjects are shown in Figure 6. No significant intra-subject differences between the two study days were found in VCO$_2$ or VO$_2$, so data from both studies were pooled. The average energy derived from fat oxidation was 42 ± 4%, which is not significantly different from the fraction of energy provided by the dietary fat, which was 40%. Thus, on average, there was no evidence for a tendency to oxidize dietary CHO in preference to fat during the feeding protocol. Interindividual variability in percent fat oxidation was high, however, ranging from 15% to 61%. Consequently, calculated net fat oxidation rates ranged from 22 to 81% of the rate at which fat was ingested.

Estimated thermic effect of food, shown in Figure 6, ranged from 0 to 18%. This was only an estimated value, since fasting REE was measured on a separate day from the feeding protocols, and subjects did not stay overnight in the GCRC before this REE measurement. The significance of this, however, is that subjects were ingesting between 28 and 50% in excess of their individual rates of energy expenditure during the feeding protocol. That is, the purpose of the REE measurement was to calculate the amount of diet that would be fed to subjects, which was set at 150% of REE. Thus, this result is consistent with the experimental goal of food energy intake within a physiologic range, as was discussed in the section of Chapter 3 entitled “Rationale for rate of diet intake.”
Plasm a fatty acid composition

Proportional contributions of the four major dietary fatty acids to the three plasma lipid fractions, CM, NEFA and VLDL, are presented in Figures 7 through 9, respectively. Because it was the purpose of the present research to investigate diet-derived fatty acids, the fractions are computed on the basis of fraction of the sum of the peak areas of the four principal dietary fatty acids, which comprised 95.5% of the dietary fat. However, in all three plasma lipid fractions, numerous additional peaks were also present, as illustrated in Appendix D. In all cases, palmitate and oleate were the two largest peaks. Although these two fatty acids combined made up 80.5% of the dietary fat, they made up only about half of the plasma fatty acids by weight. Likewise, the four principal dietary fatty acids combined made up only about two-thirds of the total plasma fatty acids in all three fractions. Of the additional peaks, the areas of most were less than 10% of the area of the largest peak (palmitate or oleate). There were two peaks consistently present having areas which were sometimes greater than 10% of the largest peak, and one peak was consistently present with an area equal to about half that of the largest peak. The significance of these additional peaks is discussed in Chapter 5.

The average fatty acid composition of each lipid fraction for all subjects is depicted in Figure 10, along with the diet composition for comparison. As in Figures 7 through 9, all values are expressed as a fraction of the sum of the four major dietary fatty acids (e.g., oleate area / Σ 4 areas). As was discussed in the derivations of fractional oxidation in Chapter 3, this is equivalent to the fraction of the sum of carbon mass and will be referred to as fractional amounts, or relative amounts in the case of direct comparisons between oleate and palmitate. Note that relative amounts are not affected by the presence of fatty acids other than oleate and palmitate, since the denominators for fractional amounts are equal within a given sample. That is, \( \frac{A_{OA}}{\Sigma 4 \text{ areas}} / \frac{A_{PA}}{\Sigma 4 \text{ areas}} = \frac{A_{OA}}{A_{PA}} \).
The most notable difference between the diet and CM composition is the amount of linoleate, which comprised 22 ± 2% of CM, versus only 10% of dietary fat. (See Tables 3 and 5.) There was also a larger proportion of stearate in CM than diet: 8 ± 1% versus 5%. Thus, for all subjects, CM contained relatively greater proportions of linoleate and stearate as compared with the diet, and the proportions of oleate and palmitate were therefore reduced.

By comparison with CM-derived fatty acids, NEFA, shown in Figure 8, contained somewhat less of the two unsaturated fatty acids relative to the two saturated fatty acids. Individual subjects varied more in their fractional amounts of various NEFA than CM-derived fatty acids, particularly linoleate, which varied from a few percent to over one quarter of the NEFA. The composition of VLDL was closer to that of CM than was the case for NEFA, although it contained slightly higher fractional amounts of the two unsaturated fatty acids on average than did CM. (See Figure 9.) On average, the sum of the two unsaturated fatty acid fractions equaled 58% in CM, 60% in VLDL, and 40% in NEFA.

**Enrichment of plasma oleate versus palmitate**

Enrichments of CM- and VLDL-derived fatty acids during the final hour of the protocol for individual subjects are shown in Figure 11. In CM, oleate enrichment was higher than that of palmitate in all but subject 3024, with average oleate enrichment being 0.71 MPE (26%) greater than palmitate during the last hour. This is identical to the results of the multiple regression shown in Figure 12 (oleate β=0.71, P<0.001), which includes the four- and five-hour time points. Average enrichment of VLDL during the last hour, from Figure 11, was 0.32 MPE higher for oleate than palmitate. However, from the multiple regression of Figure 13, oleate enrichment was 0.26 MPE greater than palmitate enrichment (P<0.001). This reflects the fact that VLDL enrichments were diverging over time, because they had not yet approached a plateau by four hours.
Plasma fatty acid enrichment kinetics

Average enrichments versus time for all subjects' CM during oleate and palmitate tracer feeding are depicted in Figure 14. Results of the multiple regression analysis of CM enrichment, shown in Figure 12, demonstrate that enrichments were significantly lower at four and five hours after beginning tracer administration than at seven hours (P<0.001), but the six and seven hour enrichments were not significantly different (P=0.3). Quantitatively, enrichment at four hours was on average 0.84 MPE less than at seven hours, and 0.54 MPE less at five hours than seven. Fitting the data for each tracer to a simple monoexponential function yielded the following equations:

\[
\begin{align*}
IE_{OA}^t &= IE_{OA}^x \times (1 - 2^{-(t - t_i)/\tau}) = 3.91 \times (1 - 2^{-(t - 65 \text{ min})/113 \text{ min}}) \\
IE_{PA}^t &= IE_{PA}^x \times (1 - 2^{-(t - t_i)/\tau}) = 3.06 \times (1 - 2^{-(t - 69 \text{ min})/114 \text{ min}}).
\end{align*}
\]

Note that, in contrast to equation 22 from Chapter 3, time is computed as time after beginning tracer ingestion, \(t\), minus time before tracer is estimated to have first appeared in plasma, \(t_i\). This is equivalent to the time at which tracer administration is begun in an infusion protocol. Thus, the kinetics of appearance of the two tracers in CM appear to be similar. Consistent with this, the average enrichment of oleate during the final hour, from Figure 11, is equal to 87% of the calculated theoretical steady-state enrichment, \(IE_{OA}^x\), shown above, and average palmitate enrichment in the last hour was 88% of \(IE_{PA}^x\). On the other hand, the fact that the theoretical steady state values are higher than the actual values suggests that the enrichments were, in fact, still rising during the last hour.

Although the multiple regression of CM enrichment (Figure 12) shows no significant differences in enrichment among the time points during the last hour (6:00, 6:20 6:40 and 7:00), the average slopes during this time were 5.7% per hour for oleate and 5.4% per hour for palmitate. Thus, although the enrichment was apparently still increasing during the final hour of the protocol, the rate of change was sufficiently low to conclude that a functional steady state was achieved in the final hour.
Average enrichments versus time for VLDL are shown in Figure 15. The multiple regression analysis of VLDL enrichment (Figure 13) shows that the average enrichment increased monotonically with time, and the differences among hourly time points were significant in all cases. Fitting the data for each tracer to a simple monoexponential function yielded the following equations:

\[ E_{OA_t} = E_{OA_0} \times (1 - 2^{-\left(\frac{t - t_i}{T}\right)}) = 3.30 \times (1 - 2^{-\left(\frac{t - 141}{194}\right)}) \quad (25a) \]

\[ E_{PA_t} = E_{PA_0} \times (1 - 2^{-\left(\frac{t - t_i}{T}\right)}) = 2.65 \times (1 - 2^{-\left(\frac{t - 135}{189}\right)}) \quad (25b) \]

Like CM, the kinetic parameters for VLDL enrichment of oleate versus palmitate are similar, supporting the conclusion that the underlying processes of incorporation to and catabolism from VLDL are the same for oleate and palmitate. Unlike CM, however, the data indicate that a functional steady state was not achieved for VLDL, given that the average slopes during the last hour were 13.4% per hour for oleate and 13.7% per hour for palmitate, and the enrichments in the final hour (Figure 11) are only 63% and 64% of the theoretical steady-state values for oleate and palmitate, respectively. Equations 24 and 25 are plotted in Figure 16.

**NEFA enrichment**

NEFA enrichment in a subset of samples averaged 93% ± 10% that of CM. There was no difference the ratio of NEFA-to-CM enrichments of oleate (8 samples) versus palmitate (22 samples) (P=0.9). Thus, there was no measurable difference in endogenous dilution between the two fatty acids. In a multiple regression analysis of combined oleate and palmitate samples, CM enrichment was a highly significant predictor of NEFA enrichment (P<0.001), whereas VLDL enrichment and sample time were not significant. The multiple regression results are shown in Figure 17.
Elongation of palmitate

Because the enrichment of palmitate was so much lower than that of oleate, there was concern as to whether a significant fraction of the tracer was being converted to stearate by elongation. If so, measurable $^{13}$C enrichment of stearate during palmitate tracer administration would be expected. In two subjects (3029 and 3031), CM and VLDL samples at $t = 6:00$ were analyzed for evidence of elongation of palmitate by determining whether there was measurable enrichment of the stearate peaks during palmitate tracer feeding. There was no difference in the stearate M+1/M+0 ratios of plasma samples between oleate versus palmitate studies. That is, the M+1/M+0 ratios for stearate of each $t = 6:00$ palmitate sample was within 1%, or ± 0.10 MPE of the corresponding $t = 6:00$ oleate sample.

Breath $^{13}$CO$_2$ enrichment kinetics

Individual plots of $^{13}$CO$_2$ enrichment (APE) are shown in Figure 18. The average values of APE for all subjects at each time point are plotted in Figure 19. Few of the individual slopes of APE versus time were statistically significant during the last hour of the protocol, which limited the assessment of whether a functional steady-state was achieved (slope < 10% of average APE per hour). This was of concern because the raw computed slopes ranged from -4% per hour to +34% per hour. However, computing the average value for all subjects at each time point provided a means of examining the average kinetics of $^{13}$CO$_2$ enrichment. Therefore, slope calculations were performed on these averages for the $t = 4:00$ through $6:00$ times (3 points), the final hour (7 points), and for the final half-hour (4 points). The slope from $t = 4:00$ through $t = 6:00$ was 26% and 23% of the final APE per hour for oleate and palmitate, respectively. During the last (7th) hour, the slopes were 17% and 16% per hour, and during the final 30 minutes, the slopes were 2% and 7% per hour, indicating that the definition of plateau was met by the
final half-hour, on average. Fitting the data for each tracer to a simple monoexponential function yielded the following equations:

\[ I_{E':CO_{21}} = I_{E':CO_{2x}} \times (1 - 2^{-\left(t - t_{0}\right)/T}) \]

\[ = 0.0176 \times (1 - 2^{-\left(t - 111 \text{ min}\right)/137 \text{ min}}) \] (26a)

\[ = 0.0129 \times (1 - 2^{-\left(t - 111 \text{ min}\right)/137 \text{ min}}) \] (26b)

for oleate and palmitate, respectively. Unlike the procedure employed for fatty acid \(^{13}\text{C}\) enrichments, the curves above were computed simultaneously, with the \(IE_x\) terms being forced into a ratio equal to their ratio during the final hour of the protocol. This was done because of the absence of evidence for unequal kinetic parameters of the CM-derived fatty acid precursors to oxidation of \(^{13}\text{C}\) label. The average values attained in the last hour of the experiment, shown in Figure 20, are equal to 76% of the resulting estimated values for steady-state enrichment.

The results above are in contrast to the results of a preliminary study, which are plotted in Figure 21. In that study, palmitate tracer was fed for eight hours, and a slope in \(^{13}\text{CO}_{2}\) enrichment of 8% was achieved in the sixth hour, which decreased to 3% in the seventh hour, and to -1% in the eighth hour. However, the primer dose of \(^{13}\text{C}\)-bicarbonate given one hour after beginning tracer ingestion in that study probably accelerated the process of achieving a plateau.

**Label excretion in \(\text{CO}_2\)**

Tracer dose and label excretion data for individual subjects are shown in Figure 20. It should be noted that the molar dose of palmitate is 12.5% greater than oleate because equal weights were fed in order to produce equal molar enrichment of the dietary fatty acids. The average fraction of label recovered in breath \(\text{CO}_2\) was 50% higher for oleate than for palmitate. The excreted fractions of the two labels during the final hour of the protocol were 17.9% of oleate and 11.9% of palmitate. (See Figure 20.) However,
using the theoretical steady-state enrichments computed by curve-fitting the data. the
excretion values increase to 23.5% of oleate and 15.7% of palmitate.

**Relative oxidation of fatty acids**

The major hypothesis of this experiment, that oxidation of CM-derived oleate
would be greater than that of palmitate, is accepted. That is, the relative oxidation of
dietary oleate versus palmitate, \( \text{Rel}_{\text{ox}} \), was found to be 1.21 ± 0.05 (x ± SEM) with a 95%
confidence interval of 1.10 to 2.31. Thus, on average, 21% more of the oleate in CM
was oxidized than palmitate. Figure 22 shows the individual values of relative label
recovery and CM enrichment and composition. These are the three factors used in
computing relative oxidation. When individual ratios of fatty acid enrichment are taken
(second column), oleate enrichment averaged 69 ± 20% higher than that of palmitate.
The high variability in this parameter is largely due to the maximum value (248%
difference, subject 3029). Without this subject, average oleate label recovery was 46%
more than palmitate. This subject was included in the analysis, however, because after
correcting for precursor pool data, the \( \text{Rel}_{\text{ox}} \) value was no longer the maximal value. By
contrast to enrichment, the contribution of fatty acid composition of CM to calculated
\( \text{Rel}_{\text{ox}} \) was minor. That is, the average ratio of oleate peak area to palmitate area was only
1.04, indicating that there was only a 4% difference in oleate carbon mass and palmitate
carbon mass in CM, on average.

**NEFA clearance**

Of the two secondary hypotheses, the first, that relative clearance of oleate from
plasma NEFA is more efficient than palmitate, is accepted. Relative clearance of plasma
NEFA, or \( \text{RC}_{\text{NEFA}} \), averaged 0.69 overall, with a 95% confidence interval of 0.43 to
0.95, as shown in Figure 23.
Incorporation into VLDL

The hypothesis that relative incorporation of CM-derived palmitate into VLDL is greater than that of oleate is rejected. Relative appearance of the two dietary fatty acids in VLDL as compared with their presence in CM, $\text{Rel}_{\text{vl}}$, averaged $1.11 \pm 0.08$, as shown in Figure 23. That is, during the seventh hour of tracer ingestion, 11% more of the oleate than palmitate that originally appeared in CM was present in circulating VLDL, which is in the opposite direction from the hypothesized outcome. However, this number was highly variable, ranging from 0.75 to 1.50, and the mean is not statistically different from unity.
<table>
<thead>
<tr>
<th>Subject number</th>
<th>VCO₂ (ml/min)</th>
<th>Fed EE (kcal/24 hr)</th>
<th>fat oxidation (% of energy)</th>
<th>fat oxidation (g/hr)</th>
<th>fat oxidation (% of fed)</th>
<th>thermic effect of food (%)</th>
</tr>
</thead>
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<tr>
<td>3020</td>
<td>232</td>
<td>1793</td>
<td>36</td>
<td>3.0</td>
<td>70</td>
<td>16</td>
</tr>
<tr>
<td>3022</td>
<td>247</td>
<td>1674</td>
<td>58</td>
<td>4.5</td>
<td>81</td>
<td>8</td>
</tr>
<tr>
<td>3024</td>
<td>198</td>
<td>1536</td>
<td>32</td>
<td>2.3</td>
<td>48</td>
<td>16</td>
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<tr>
<td>3025</td>
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<td>1223</td>
<td>49</td>
<td>2.8</td>
<td>71</td>
<td>12</td>
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<tr>
<td>3026</td>
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<td>2180</td>
<td>41</td>
<td>4.1</td>
<td>62</td>
<td>17</td>
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<td>74</td>
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<td>1410</td>
<td>38</td>
<td>2.5</td>
<td>53</td>
<td>7</td>
</tr>
<tr>
<td>3029</td>
<td>273</td>
<td>2030</td>
<td>15</td>
<td>1.4</td>
<td>22</td>
<td>13</td>
</tr>
<tr>
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<td>147</td>
<td>1245</td>
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<td>3.5</td>
<td>79</td>
<td>0</td>
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<tr>
<td>3031</td>
<td>173</td>
<td>1372</td>
<td>43</td>
<td>2.7</td>
<td>66</td>
<td>18</td>
</tr>
<tr>
<td>Mean</td>
<td>201</td>
<td>1585</td>
<td>42</td>
<td>3.0</td>
<td>63</td>
<td>12</td>
</tr>
<tr>
<td>± SEM</td>
<td>14</td>
<td>104</td>
<td>4</td>
<td>0.3</td>
<td>6</td>
<td>2</td>
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</table>

Figure 6. Metabolic data during feeding protocol assessed by indirect calorimetry. All values are calculated on the basis of the average values of CO₂ production and O₂ consumption during four 15-minute measurements per subject. Thermic effect of food was estimated from fed energy expenditure relative to fasting resting but non-basal energy expenditure measurement on a separate day.
<table>
<thead>
<tr>
<th>Subject number</th>
<th>oleate C18:1 (g/Σg)</th>
<th>palmitate C16:0 (g/Σg)</th>
<th>linoleate C18:2 (g/Σg)</th>
<th>stearate C18:0 (g/Σg)</th>
</tr>
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<td>3020</td>
<td>0.374</td>
<td>0.352</td>
<td>0.208</td>
<td>0.067</td>
</tr>
<tr>
<td>3022</td>
<td>0.383</td>
<td>0.364</td>
<td>0.196</td>
<td>0.057</td>
</tr>
<tr>
<td>3024</td>
<td>0.379</td>
<td>0.343</td>
<td>0.215</td>
<td>0.063</td>
</tr>
<tr>
<td>3025</td>
<td>0.297</td>
<td>0.333</td>
<td>0.263</td>
<td>0.107</td>
</tr>
<tr>
<td>3026</td>
<td>0.345</td>
<td>0.351</td>
<td>0.188</td>
<td>0.117</td>
</tr>
<tr>
<td>3027</td>
<td>0.335</td>
<td>0.299</td>
<td>0.270</td>
<td>0.096</td>
</tr>
<tr>
<td>3028</td>
<td>0.297</td>
<td>0.300</td>
<td>0.310</td>
<td>0.111</td>
</tr>
<tr>
<td>3029</td>
<td>0.401</td>
<td>0.387</td>
<td>0.160</td>
<td>0.060</td>
</tr>
<tr>
<td>3030</td>
<td>0.404</td>
<td>0.357</td>
<td>0.169</td>
<td>0.089</td>
</tr>
<tr>
<td>3031</td>
<td>0.356</td>
<td>0.356</td>
<td>0.214</td>
<td>0.074</td>
</tr>
</tbody>
</table>

Mean ± SEM  
0.357 ± 0.012  
0.344 ± 0.009  
0.219 ± 0.015  
0.084 ± 0.007

Figure 7. Fatty acid composition of chylomicrons. Values shown are proportional amounts on a weight basis of the four principal fatty acids in the diet as analyzed by GC-FID (i.e., grams of carbon in each peak/grams of carbon in total of all four peaks). Subjects were fed a liquid formula diet with 40% of energy as fat in small frequent meals for nine hours. Samples were collected between two and nine hours after the start of feeding. Values for individual subjects are based on the mean of six samples analyzed in duplicate.
<table>
<thead>
<tr>
<th>Subject number</th>
<th>oleate C18:1 (g/Σg)</th>
<th>palmitate C16:0 (g/Σg)</th>
<th>linoleate C18:2 (g/Σg)</th>
<th>stearate C18:0 (g/Σg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3020</td>
<td>0.169</td>
<td>0.557</td>
<td>0.000</td>
<td>0.067</td>
</tr>
<tr>
<td>3022</td>
<td>0.144</td>
<td>0.585</td>
<td>0.010</td>
<td>0.057</td>
</tr>
<tr>
<td>3024</td>
<td>0.145</td>
<td>0.577</td>
<td>0.023</td>
<td>0.052</td>
</tr>
<tr>
<td>3025</td>
<td>0.346</td>
<td>0.366</td>
<td>0.191</td>
<td>0.097</td>
</tr>
<tr>
<td>3026</td>
<td>0.411</td>
<td>0.338</td>
<td>0.148</td>
<td>0.103</td>
</tr>
<tr>
<td>3027</td>
<td>0.418</td>
<td>0.331</td>
<td>0.164</td>
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<tr>
<td>3028</td>
<td>0.317</td>
<td>0.439</td>
<td>0.277</td>
<td>0.131</td>
</tr>
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<td>3029</td>
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<td>0.153</td>
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<td>0.442</td>
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<td>Mean</td>
<td>0.288</td>
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<td>0.112</td>
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<tr>
<td>± SEM</td>
<td>0.032</td>
<td>0.030</td>
<td>0.028</td>
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</table>

Figure 8. Fatty acid composition of plasma non-esterified fatty acids. Values shown are proportional amounts on a weight basis of the four principal fatty acids in the diet as analyzed by GC-FID (i.e., grams of carbon in each peak/grams of carbon in total of all four peaks). Subjects were fed a liquid formula diet with 40% of energy as fat in small frequent meals for nine hours. Samples were collected between two and nine hours after the start of feeding. Values for individual subjects are based on the mean of six samples analyzed in duplicate.
<table>
<thead>
<tr>
<th>Subject number</th>
<th>oleate C18:1 (g/Σg)</th>
<th>palmitate C16:0 (g/Σg)</th>
<th>linoleate C18:2 (g/Σg)</th>
<th>stearate C18:0 (g/Σg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3020</td>
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<td>0.225</td>
<td>0.063</td>
</tr>
<tr>
<td>3022</td>
<td>0.390</td>
<td>0.341</td>
<td>0.198</td>
<td>0.070</td>
</tr>
<tr>
<td>3024</td>
<td>0.402</td>
<td>0.313</td>
<td>0.234</td>
<td>0.052</td>
</tr>
<tr>
<td>3025</td>
<td>0.347</td>
<td>0.321</td>
<td>0.273</td>
<td>0.058</td>
</tr>
<tr>
<td>3026</td>
<td>0.411</td>
<td>0.303</td>
<td>0.220</td>
<td>0.067</td>
</tr>
<tr>
<td>3027</td>
<td>0.334</td>
<td>0.332</td>
<td>0.237</td>
<td>0.097</td>
</tr>
<tr>
<td>3028</td>
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<td>0.304</td>
<td>0.277</td>
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<td>0.325</td>
<td>0.241</td>
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<tr>
<td>Mean</td>
<td>0.370</td>
<td>0.327</td>
<td>0.227</td>
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</tr>
<tr>
<td>± SEM</td>
<td>0.009</td>
<td>0.008</td>
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<td>0.006</td>
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Figure 9. Fatty acid composition of very-low-density lipoproteins. Values shown are proportional amounts on a weight basis of the four principal fatty acids in the diet as analyzed by GC-FID (i.e., grams of carbon in each peak/grams of carbon in total of all four peaks). Subjects were fed a liquid formula diet with 40% of energy as fat in small frequent meals for nine hours. Samples were collected between two and nine hours after the start of feeding. Values for individual subjects are based on the mean of six samples analyzed in duplicate.
Figure 10. Relative quantities of the four principal fatty acids in the diet and three plasma fatty acid fractions. Values shown are averages for all subjects. Six samples per subject were analyzed. CM, chylomicrons; NEFA, non-esterified fatty acids; VLDL, very-low-density lipoproteins. A liquid formula diet with 40% of energy as fat was fed each 20 minutes for nine hours.
<table>
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<th>Subject number</th>
<th>oleate tracer study</th>
<th>palmitate tracer study</th>
</tr>
</thead>
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<td></td>
<td>CM enrichment (MPE)</td>
<td>VLDL enrichment (MPE)</td>
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<tr>
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<td>2.02</td>
</tr>
<tr>
<td>3022</td>
<td>3.10</td>
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</tr>
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<td>3024</td>
<td>2.32</td>
<td>1.78</td>
</tr>
<tr>
<td>3025</td>
<td>3.21</td>
<td>1.71</td>
</tr>
<tr>
<td>3026</td>
<td>3.62</td>
<td>2.02</td>
</tr>
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<td>3027</td>
<td>3.29</td>
<td>2.36</td>
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<td>2.21</td>
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<td>3029</td>
<td>3.26</td>
<td>1.62</td>
</tr>
<tr>
<td>3030</td>
<td>4.12</td>
<td>2.10</td>
</tr>
<tr>
<td>3031</td>
<td>4.45</td>
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<td>Mean</td>
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<td>2.08</td>
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<tr>
<td>± SEM</td>
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</table>

Figure 11. Fatty acid $^{13}$C enrichment during the last hour of each protocol. Samples were collected from six to seven hours after beginning fatty acid tracer feeding. MPE is moles % enrichment. CM is chylomicron-derived fatty acids. VLDL is very-low-density lipoprotein-derived fatty acids. CM and VLDL fractions were separated from plasma samples by ultracentrifugation. Subjects were fed a liquid formula diet with [1-$^{13}$C]-labeled fatty acid tracers in small frequent meals for seven hours. Dietary fatty acid enrichments were 5.0 MPE for each tracer.
Regression Statistics

<p>| | |</p>
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<td>Multiple R</td>
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</tr>
<tr>
<td>R Square</td>
<td>0.788</td>
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<tr>
<td>Adjusted R Square</td>
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<td>Standard Error</td>
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<td>Observations</td>
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Analysis of Variance

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<th>Sum of Squares</th>
<th>Mean Square</th>
<th>F</th>
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<td>60.188</td>
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<td>2.32E-28</td>
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<td>Residual</td>
<td>104</td>
<td>16.171</td>
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<td></td>
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<td>Total</td>
<td>119</td>
<td>76.360</td>
<td></td>
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<table>
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<th>P-value</th>
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<td>Intercept</td>
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<td>18.844</td>
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<tr>
<td>tracer = oleate</td>
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<tr>
<td>time = 4:00</td>
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<td>-6.733</td>
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<td>-1.087</td>
<td>-0.592</td>
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<tr>
<td>5:00 hours</td>
<td>-0.54</td>
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</tr>
<tr>
<td>6:00 hours</td>
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<tr>
<td>6:20 hours</td>
<td>-0.10</td>
<td>-0.760</td>
<td>0.449</td>
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<tr>
<td>6:40 hours</td>
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<tr>
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</tr>
<tr>
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<td>-2.306</td>
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<td>-0.691</td>
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Figure 12. Multiple regression analysis of chylomicron-derived fatty acid $^{13}$C enrichment. Units are moles % enrichment. Independent variables are tracer, time after beginning tracer administration, and individual subjects. All of the independent variables were treated as categorical variables. Reference value is palmitate enrichment for subject 3020 at time = 7:00 hours. Subjects were fed a liquid formula diet with [1-$^{13}$C]-labeled fatty acid tracers in small frequent meals for seven hours.
### Regression Statistics

- Multiple R: 0.857
- R Square: 0.735
- Adjusted R Square: 0.683
- Standard Error: 0.354
- Observations: 80

### Analysis of Variance

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<td>6:00 hours</td>
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<td>0.177</td>
<td>4.454</td>
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Figure 13. Multiple regression analysis of very-low-density lipoprotein-derived fatty acid $^{13}\text{C}$ enrichment. Units are moles % enrichment. Independent variables are tracer, time after beginning tracer administration, and individual subjects. All of the independent variables were treated as categorical variables. Reference value is palmitate enrichment for subject 3020 at t = 7:00 hours. Subjects were fed a liquid formula diet with $[1-^{13}\text{C}]$-labeled fatty acid tracers in small frequent meals for seven hours.
Figure 14. Average fatty acid $^{13}$C enrichment of chylomicrons for all subjects. Tracer $[1^{13}$C$]-$labeled fatty acids were fed each 20 minutes for seven hours in a liquid formula diet. Diet fatty acid enrichment was 5.0 MPE for both tracers. Oleate enrichment was higher than palmitate enrichment at each time point ($P<0.05$). Enrichment of each fatty acid was greater at six to seven hours than at four or five hours ($P<0.05$). MPE, moles % enrichment.
Figure 15. Average $^{13}$C enrichment of very-low-density lipoprotein-derived fatty acids for all subjects. Tracer [$\text{L}^{13}$C]-labeled fatty acids were fed each 20 minutes for seven hours in a liquid formula diet. Diet fatty acid enrichment was 5.0 MPE for both tracers. Oleate enrichment was higher than palmitate enrichment at seven hours ($P<0.05$). Enrichment of each fatty acid was greater at six to seven hours than at four or five hours ($P<0.05$). MPE, moles % enrichment.
Figure 16. Theoretical $^{13}$C enrichment of chylomicron- and very-low-density lipoprotein-derived fatty acids. Curves represent best fit of average data for ten subjects to a monoexponential function during ingestion of $[1^{-13}\text{C}]$-oleate and -palmitate tracers. Tracers were fed each 20 minutes for seven hours in a liquid formula diet. Diet fatty acid $^{13}$C enrichment was 5.0 moles percent enrichment (MPE) for both tracers.
Regression Statistics

Multiple R  0.927
R Square  0.860
Adjusted R Square  0.844
Standard Error  0.283
Observations  30

Analysis of Variance

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<th>P-value</th>
<th>Lower 95%</th>
<th>Upper 95%</th>
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<td>CM enrichment (MPE)</td>
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<td>0.147</td>
<td>0.07</td>
<td>0.943</td>
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</table>

Figure 17. Multiple regression analysis of non-esterified fatty acid $^{13}$C enrichment. Independent variables are: time after beginning tracer administration, chylomicron (CM) enrichment in the same sample, and very-low-density lipoprotein (VLDL) enrichment in the same sample. MPE is moles % enrichment. Subjects were fed a liquid formula diet with [1-$^{13}$C]-labeled fatty acid tracers in small frequent meals for seven hours.
Figure 18. Breath $^{13}$CO$_2$ enrichment for individual subjects. Tracer [1-$^{13}$C]-labeled fatty acids were fed each 20 minutes for seven hours in a liquid formula diet. APE, atoms % enrichment. Open circles, o, oleate tracer; closed circles, •, palmitate tracer.
Figure 18 (continued).

\[ \delta^{13}C_{\text{APE}} \]

\[ 0.018 \]

\[ 0.015 \]

\[ 0.012 \]

\[ 0.009 \]

\[ 0.006 \]

\[ 0.003 \]

\[ 0.000 \]

\[ 0 \]

3024

\[ 0 \]

2 4 6

time after beginning tracer ingestion (hours)

3025

(continued)
Figure 18 (continued).

![Graph](image)

$^{14}CO_2$ enrichment (APE)

_time after beginning tracer ingestion (hours)_

(continued)
Figure 18 (continued).

![Graph showing the enrichment of $^{13}$CO$_2$ over time.](image)

(continued)

112
Figure 18 (continued).

\[ ^{13}\text{CO}_2 \text{ enrichment (APE)} \]

0.018 -

0.015 -

0.012 -

0.009 -

0.006 -

0.003 -

0.000 -

0 2 4 6 8

Time after beginning tracer ingestion (hours)

\[ ^{13}\text{CO}_2 \text{ enrichment (APE)} \]

0.018 -

0.015 -

0.012 -

0.009 -

0.006 -

0.003 -

0.000 -

0 2 4 6 8

Time after beginning tracer ingestion (hours)
Figure 19. Average breath $^{13}$CO$_2$ enrichment for all subjects. Tracer [1-$^{13}$C]-labeled fatty acids were fed each 20 minutes for seven hours in a liquid formula diet. APE, atoms % enrichment.
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<td>$^{13}$CO$_2$ enrichment (APE)* (n=7)</td>
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<tr>
<td>3022</td>
<td>433</td>
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<tr>
<td>3024</td>
<td>364</td>
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<td>303</td>
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<tr>
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<td>521</td>
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<tr>
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<tr>
<td>± SEM</td>
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</table>

* Average of seven data points during final hour of study.

Figure 20. Tracer dose and $^{13}$C label excretion in ten healthy adults. Subjects were fed a liquid formula diet with fatty acid tracers in small frequent meals for seven hours. Samples were collected from six to seven hours after beginning [1-$^{13}$C]-fatty acid tracer feeding. APE is atoms % enrichment. Percent of ingested tracer dose is calculated to be rate of tracer excretion in breath during final hour of protocol relative to rate of feeding.
Figure 21. Breath $^{13}$CO$_2$ enrichment in a preliminary study with primer dose of [1-$^{13}$C]-labeled acetate administered at one hour after beginning tracer fatty acid feeding. Tracer [1-$^{13}$C]-labeled palmitate was fed each 20 minutes for eight hours in a liquid formula diet. APE, atoms % enrichment.
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<th>chylomicron enrichment (OA/PA)</th>
<th>fractional chylomicron composition (OA/PA)</th>
<th>relative oxidation, $\text{Rel}_{\text{ox}}$</th>
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<td>0.02</td>
<td>0.05</td>
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<td>0.98 - 1.09</td>
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* Corrects for molar difference in production of labeled acetyl CoA from palmitate (n=8) versus oleate (n=9).

Figure 22. Relative oxidation of oleate (OA) versus palmitate (PA) and the contributing components in ten healthy adults. Subjects were fed a liquid formula diet with [1-$^{13}\text{C}$]-labeled fatty acid tracers in small frequent meals for seven hours. Values computed on the basis of enrichments of plasma and breath samples collected from six to seven hours after beginning fatty acid tracer feeding.
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<th>fractional VLDL composition (OA/PA)</th>
<th>VLDL incorporation v. CM, Rel V&lt;sub&gt;I&lt;/sub&gt;</th>
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<th>NEFA clearance v. CM, RC&lt;sub&gt;NEFA&lt;/sub&gt;</th>
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<td>0.04</td>
<td>0.08</td>
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</table>

95% C.I. 0.96 - 1.79 1.04 - 1.23 0.94 - 1.28 0.44 - 0.96 0.43 - 0.95

a. Calculated from enrichment × fractional ratios ÷ analogous ratios for chylomicrons.
b. Calculated from fractional ratio of NEFA ÷ fractional ratio for CM.

Figure 23. Oleate (OA) versus palmitate (PA) relative clearance of non-esterified free fatty acids (NEFA) and incorporation into very-low-density lipoproteins (VLDL) in ten healthy adults. Analogous chylomicron (CM) values for fractional composition and relative enrichment shown in Figure 11. Relative clearance is computed from plasma concentration of NEFA versus CM. Incorporation into VLDL is computed from plasma concentration and enrichment of VLDL versus CM. Subjects were fed a liquid formula diet with fatty acid tracers in small frequent meals for seven hours.
CHAPTER 5

DISCUSSION

The results presented herein demonstrate that, under certain dietary conditions, a larger fraction of the oleate derived from CM is oxidized than palmitate. This is the first study to make a direct comparison of the metabolic fates of two fatty acids on a fractional basis, with correction for their delivery as chylomicrons. As such, it represents a new approach for studying comparative fatty acid metabolism. Previous comparisons of acute oxidation of fatty acids, summarized in Figure 2, have produced results that have sometimes been contradictory. Thus, these have left many questions unanswered, especially with regard to mechanisms for observed differences. By design, this research sought to minimize mechanisms which might cause oxidation differences, including those of digestion and absorption, endogenous dilution of chylomicrons, and grossly unequal availability of tracee fatty acids. The present findings may shed light on previous discrepant results, at least in the case of oleate versus palmitate, by demonstrating that significant differences in acute oxidation persist in spite of equalizing their dietary availability and correcting for rate of delivery to arterial circulation.

Mechanisms for differential fatty acid oxidation

There are numerous opportunities for specificity of fatty acid metabolism, as was seen in Chapter 2. Further, there are numerous examples of differences in metabolism among various fatty acids. Yet, there is no readily apparent, simple explanation for differential oxidation of oleate and palmitate. Differences are known to exist at several
steps in the process of converting fatty acids from TAG in CM to CO₂. However, the complex nature of interactions among the various steps in fatty acid metabolism precludes any definite conclusion on the basis of an isolated finding of, for example, a single difference in enzyme affinity. Nevertheless, it would be useful to review the existing body of data with particular focus on processes with potential to contribute to more efficient oxidation of oleate versus palmitate in the present protocol.

The first such process is LPL hydrolysis of fatty acids from the TAG core of CM. There is no evidence for fatty acid specificity at this step, but the rationale for its potential role is as follows. First, various fatty acids occur in different positions on TAG with unequal frequencies (Gunstone 1986). Second, LPL only hydrolyzes the sn-1 and -3 positions of TAG (Morley & Kuksis 1972, Paltauf et al. 1974). If 2-monoacylglycerol (2-MAG) is taken up by cells it might be more prone to resynthesis into TAG than NEFA, as is the case in enterocytes (Mattson & Volpenhein 1964, Kayden et al. 1967). However, there is negligible production of 2-MAG when TAG are incubated with LPL in plasma (Higgins & Fielding 1975, Chajek et al. 1978), so plasma 2-MAG hydrolase probably efficiently hydrolyzes the remaining fatty acid (Fielding 1981). Therefore, this an unlikely mechanism for differential oxidation.

It is also conceivable that LPL might prefer one type of TAG over another, such that, for example, triolein is more likely to be hydrolyzed while tripalmitin is more likely to remain in the remnant core and be taken up by the liver. However, the naturally occurring distribution of individual fatty acids on TAG precludes segregation of this type (Gunstone 1986). Another argument against this possibility is the similarity in composition between CM and their remnants (Lambert et al. 1996). A fraction of CM TAG is, however, taken up by the liver in the form of remnants. Within the hepatocyte, TAG may not be completely hydrolyzed, suggested by the finding of slight conservation of the sn-2 position of dietary TAG in VLDL (Zock et al. 1996). This is quantitatively minor, however, especially in the context of overall dietary fatty acid metabolism.

Another opportunity for differential handling of fatty acids involves albumin binding, for which specificity in binding site affinities has been demonstrated (Rose et al.
1994). Rose et al. found that the primary and tertiary binding sites of palmitate have a higher affinity than the corresponding sites for oleate, whereas the secondary sites have the opposite preference. This could cause more palmitate to circulate bound to albumin while more oleate was taken up at the site of hydrolysis, but this would depend on the concentrations of the two fatty acids. If so, however, one might expect palmitate oxidation to be greater, because more hydrolysis of CM fatty acids occurs in adipose tissue than muscle or organs in a fed state.

The next potential site for differential handling of fatty acids occurs at the cell membrane. Although the evidence suggests that oleate and palmitate do not compete for this process (Bojesen & Bojesen 1996a), if membrane binding is saturable at physiologic NEFA concentrations, it could contribute to fatty acid differences in uptake. In that study, the membrane total binding capacity for oleate was slightly but not significantly higher than for palmitate (34 versus 29 nmol/g). However, the distribution of binding between inner and outer membrane was remarkably different, with oleate having an inner/outer ratio of 0.45 while the ratio for palmitate was 4 to 5. Intuitively, this would seem to support greater palmitate uptake than oleate. Also, efflux of intracellular albumin-bound oleate to the medium was slower than that of palmitate.

Within the cell, there are several processes by which differences in metabolism of fatty acids might occur. A role in the specificity of intracellular fatty acid transport by the family of FABP’s is suggested not only by the existence of fatty acid specificity of binding affinity, as discussed in Chapter 2 (Kurian et al. 1996, Maatman et al. 1994, Richieri et al. 1994), but also by the fact that FABP expression and properties are markedly tissue-specific (Gossett et al. 1996, Xu et al. 1996). For the most part, these specificities do not help to explain the present findings. In most FABP’s oleate and palmitate binding was similar, including human liver, muscle (Maatman et al. 1994), and adipose (Richieri et al. 1994), although rat liver FABP had a higher affinity for oleate (Richieri et al. 1994). However, this is a relatively new area of research and this family of proteins certainly has the potential to mediate differences in the fates of fatty acids within the cell.
Net uptake of plasma fatty acids is a function of interactions between plasma albumin, cell membrane transport, and intracellular processes, including FABP binding. Thus, no single isolated binding specificity has meaning outside of the context of other, in a sense competing, processes. A few early studies which measured fractional uptake of fatty acids by human heart (Wisneski et al. 1987), liver (Hagenfeldt et al. 1972, Hagenfeldt 1975) or forearm (Hagenfeldt et al. 1972) or perfused rat liver (Soler-Argilaga et al. 1973) failed to demonstrate any difference between palmitate and oleate. Since these all represent fasting metabolic conditions, however, they cannot be generalized to the fed state of this experiment.

In addition to FABP transporters, several intracellular enzymes regulate fatty acid metabolism. First, acyl-CoA synthetases from rabbit heart and liver (Weis & Bercute 1997) and from E. coli (Black et al. 1997) have marked substrate specificities, although human data are lacking. Of these, only the rabbit study compared oleate and palmitate, and reported a higher affinity for oleate in rabbit heart, but a higher maximal activity for palmitate versus oleate (Weis & Bercute 1997).

CPT-I is the first enzyme for which activity is specifically related to fatty acid oxidation. It converts the CoA esters of fatty acids to carnitine esters, permitting their entry to mitochondria. One study which compared substrate specificities in rat liver CPT-I found a lower affinity for oleoyl-CoA than palmitoyl-CoA, although the \( V_{max} \) values were similar (Gavino & Gavino 1991). In another study, activity of this enzyme towards oleoyl-CoA was more sensitive to inhibition by malonyl-CoA than was activity towards palmitoyl-CoA (Ide et al. 1995). Both of these findings favor oxidation of palmitate versus oleate, although they cannot be generalized to humans.

Finally, there are numerous enzymes involved in mitochondrial β-oxidation. Oxidation of both oleate and palmitate is initially mediated either by LCAD or VLCAD (Izai et al. 1992). Oleate also requires the \( \Delta3,\Delta2 \)-enoyl CoA isomerase, due to the location of its double bond. The regulation and organization of these and the other enzymes of β-oxidation have recently been reviewed elsewhere (Eaton et al. 1996). However, all of the evidence indicates that once a fatty acid is delivered to the
mitochondrial matrix it will be completely oxidized to acetyl-CoA. Thus, the β-oxidation enzymes are not generally believed to regulate fatty acid oxidation, but are probably “demand-led.”

Of course, there are many additional ways in which metabolism of fatty acids may vary, such as synthetic enzymes for TAG, PL, and other products, but an exhaustive treatment of these is beyond the scope of this discussion. Also, whole-body oxidation is the sum of numerous parallel processes occurring in the various tissues and organs of the body, and differential fatty acid oxidation is probably not determined by any single metabolic pathway.

Health implications of findings

The present method has potential applications in illuminating long-term health effects of dietary fat manipulation. The most obvious of these is regulation of body composition via maintaining fat balance. There is intuitive appeal in the notion that greater oxidation of fat during its initial delivery to the body should result in reduced fat accretion in the long term. This premise has been tested in various ways, with somewhat mixed results, as was discussed in Chapter 2.

In this context, it is interesting to extrapolate the present results arithmetically to speculate on the long-term consequences of differential fatty acid oxidation. For example, suppose that a person ingests 2400 kcal per day, and that one-fourth of this intake is stored. Further assume that, as in this protocol, 40% of the energy is in the form of fat, and 40% of that is palmitate. If there is no difference among macronutrients in partitioning between oxidation versus storage, then palmitate stored per day during the fed state would be: 600 kcal × 0.40 × 0.40 = 96 kcal, and palmitate oxidized would be: 1800 kcal × 0.40 × 0.40 = 288 kcal. If half of the palmitate was replaced with oleate, and if this resulted in a 21% increase in oxidation of that portion of fat during digestion and absorption, then an additional 30 kcal or 3.4 grams of palmitate would be burned in the fed state. If this difference in fed-state oxidation was not reversed by reciprocal
differences in the fasting state, over the course of a year the high-palmitate diet would result in accretion of about 1.2 kg of fat. This example assumes that either spontaneous intake is increased, or energy expenditure is decreased, as a result of the extra stored fat.

Of course, the preceding discussion is purely speculative and highly simplistic. It assumes, for example, that any CHO not burned in the fed state is stored rather than being converted to fat. Even when energy balance is maintained, fat synthesis may be substantial on a high-CHO diet (Hudgins et al. 1996). Similarly, it assumes that a shift in fed-state fat oxidation will not be reversed via a reciprocal shift during the overnight fast. It also ignores possible long-term adaptations to diet manipulation. Certainly, there is significant capacity among healthy humans to adjust net oxidation of CHO to match intake under conditions of weight maintenance (Roust et al. 1994, Schrauwen et al. 1997, Astrup et al. 1994a, Snitker et al. 1997, Saltzman et al. 1997).

On the other hand, there is a tendency to overeat a high-fat diet versus one lower in fat (Lissner et al. 1987, Thomas et al. 1992, Tremblay et al. 1989, Stubbs et al. 1995). This may be particularly so for persons genetically prone to obesity, such as Pima Indians (Larson et al. 1995b, Larson et al. 1995c). It has been argued that this is because CHO oxidation is tightly regulated in response to intake such that glycogen stores are maintained (Stubbs et al. 1993, Snitker et al. 1997, Flatt 1995). Fat oxidation, moreover, does not respond directly to intake (Schutz et al. 1989, Bennett et al. 1992) but is instead reciprocally regulated by CHO oxidation (Flatt 1987, Jebb et al. 1996) via operation of the glucose fatty acid cycle (Randle et al. 1963, Carlson et al. 1991).

Thus, immediate oxidation of ingested fat is probably not regulatory in a direct sense, but may have an indirect effect on long-term body composition via its influence on food intake. That is, if a high-oleate diet shifts fuel oxidation in a fed state towards fat and away from CHO, in the absence of fat synthesis, greater glycogen storage would result. This could prolong availability of glucose in the post-absorptive (fasting) state and delay the onset of hunger. The net effect on fat balance would be to increase the percent of energy that could be consumed as fat without promoting adipose accumulation. Stated another way, the obligatory glucose oxidation required to support oxidation of dietary fat
would be reduced if the ability of fat to compete with glucose for oxidation was enhanced. Recall that the peripheral “feeding signal” appears to integrate fat and CHO oxidation information within the liver (Rawson et al. 1996).

The speculation above is consistent with findings regarding long-term effects of dietary fat manipulation on the risk factors for NIDDM. Diets relatively high in oleate improve glycemic control in patients with NIDDM relative to saturated fat, without the detrimental effects on HDL and TAG seen with high-CHO diets (Garg et al. 1994). More efficient oxidation of oleate than of palmitate during digestion of a mixed meal could contribute to this effect.

The fact that there was considerable variation herein among subjects’ relative fatty acid oxidation may also be related to the above discussion in that people vary greatly in their susceptibility to obesity and NIDDM. Two of the ten had virtually equal oxidation of the two fatty acids, half of the subjects’ oleate oxidation was between 11 and 21% higher than palmitate, and the remaining three subjects’ oleate oxidation was 36 to 55% higher than palmitate (Figure 22). One potential source of explanations for this variability was anthropometric characteristics of individual subjects. Although all subjects were healthy, young, non-obese adults, they did represent a diverse group in several respects, most notably race and sex. Because previous fatty acid oxidation comparisons used either male rats or mice, or humans of one sex and race (Figure 2), there was no rationale for selecting a single sex or racial group. Therefore, subjects were included in this study without regard to these factors. Consequently, there were four men, one of whom was black, and six women, two of whom were black (Figure 3). There was no pattern in the data to suggest that race or sex were factors in the outcome, however. For example, of the three subjects with the greatest difference in oxidation, there was one white male, one white female, and one black female (Tables 2 and 13). Similarly, none of the other known characteristics of the subjects appeared to have any relationship to relative oxidation, including percent of energy from fat oxidation (Figure 6), apoE allele, and fasting lipid profiles (Figure 3).
Origins and interactions of plasma fatty acids

Chylomicrons

The fatty acid composition of CM was critical to the present research, since this fraction was defined as the precursor pool for dietary fat metabolism following its digestion, absorption, and processing by the enterocyte. Although the processes of fat digestion contribute substantially to its eventual oxidation, these were not the focus herein. The CM quantitative fatty acid composition and enrichment was used, therefore, to control for potential differences in absorption of tracers and/or tracee (unlabeled) fatty acids. This was a matter of concern since the tracers were given as free fatty acids, and dietary fat is primarily TAG. This approach also controls for potential endogenous dilution during the process of CM formation, which is important because CM composition does not perfectly reflect dietary composition (Karmen et al. 1963, Emken et al. 1987). Also, inducing a steady state in label excretion obviates assumptions concerning how fat absorption rates affect appearance of label, since these may not be equal for all fatty acids (Toorop et al. 1979, Field et al. 1988, van Greevenbroek et al. 1996) and cannot be directly assessed in humans.

The experimental diet was designed such that oleate and palmitate were equal on a weight basis, and the two together comprised 80.5% of total fatty acids. Further, equal fatty acid enrichments (5.0 MPE) were fed by design. Therefore, it was anticipated that the amounts and enrichments of oleate and palmitate would be similar, and any corrections made on the basis of actual CM composition would be quantitatively minor. In the case of relative amounts this proved to be true, although there was on average 4% more oleate than palmitate in CM. In the case of three subjects, however, there was a greater than 10% difference between the two fatty acids (Figure 22). For two of these, there was more oleate, and for the third, more palmitate. Since the 95% C.I. for the true...
mean includes 1.0, these data do not support the conclusion that TAG-derived oleate was better absorbed from the diet than palmitate, on average.

In contrast to the similarity between the quantities of oleate and palmitate in chylomicrons, average enrichment of CM oleate was 34% greater than enrichment of palmitate (Figure 22). In fact, the enrichment difference constituted more than 90% of the overall difference between the two fatty acids in CM data (i.e., enrichment x quantity). This could have been caused by at least four factors:

1) Impaired absorption of dietary oleate relative to palmitate. This is highly unlikely, considering the fractional composition data discussed above. In addition, oleate absorption from dietary TAG is >97% in healthy rats (Leyton et al. 1987) and humans (Jones et al. 1985b), and slightly more oleate than palmitate was recovered from CM. The feeding protocol employed, i.e., small frequent meals in a low-fiber liquid formula, is optimal for nutrient absorption from the gut (Zeman 1991). Also, if there is a difference between monoacylglycerol and NEFA uptake and incorporation into CM due to formation of calcium soaps, oleate should be favored over palmitate since the majority of oleate is on the sn-2 position of palm oil TAG (Gunstone 1986).

2) More palmitate than oleate from endogenous sources. Since the formula diet was fed for 24 hours before the experimental protocol, such dilution could not have been due to a previous meal's fatty acids remaining within the enterocyte. Alternatively, enterocytes are capable of extracting fatty acids from plasma in a fasted state (Gangl & Ockner 1975, Hultin et al. 1996, Gangl & Renner 1978). However, such fatty acids have been shown not to be used as precursors for CM TAG formation (Gangl & Ockner 1975, Mansbach & Dowell 1992). Also, this should if anything cause greater oleate dilution than the reverse, since there is generally about twice as much oleate as palmitate present in human adipose stores (Field et al. 1985). This might seem to contradict the present report of less oleate in NEFA than palmitate but recall that this was taken as a sign of more efficient clearance by cells at the site of hydrolysis rather than poor availability to cells. And, as with (1), the nearly equal amounts of oleate and palmitate in CM argue against this possibility.
3) Retention of palmitate label in enterocytes relative to unlabeled. Since the majority of palmitate in palm oil is found in the sn-1,3 positions of TAG (Gunstone 1986), most palmitate is cleaved before absorption into the enterocyte, so there should be no distinction between tracer and tracee once they are absorbed.

4) Impaired absorption of palmitate tracer. This is the most likely explanation, although emulsifying and mixing tracers into the diet was expected to result in virtually complete tracer absorption. In one previous human study, stearate tracer given in a capsule was poorly absorbed as compared with TAG-derived stearate, or with oleate and linoleate tracers (78 versus 92, 97, and 99%) (Jones et al. 1985b). (See Figure 2.) Similarly, methyl-palmitate tracer absorption by rats was impaired relative to methyl-linoleate when each was mixed with unlabeled methyl esters (Toorop et al. 1979). However, in another study, palmitate, oleate, and linoleate tracers were all ≥ 97% absorbed by rats when dissolved in olive oil (Leyton et al. 1987). In the present study, differences in tracer and TAG-derived fatty acid absorption due to formation of calcium soaps prior to TAG hydrolysis in the gut cannot be ruled out, since calcium made up 0.5% of the dry weight of the diet.

It should be noted that endogenous dilution of both fatty acids is not excluded by these findings. That is, even oleate enrichment in CM was only 68 ± 4% of the enrichment in the diet (Figure 11). On the other hand, palmitate enrichment in CM was lower still, being only 75 ± 14% that of oleate. Also, the overall composition of CM suggests that a substantial proportion of fatty acids were of endogenous origin, so a combination of endogenous dilution plus differences in tracer absorption is plausible. Soap formation may be higher for palmitate than oleate due to its higher melting temperature.

A further exploration of the evidence for and nature of the putative endogenous CM fatty acids is warranted. First, of the four principal fatty acids in the diet, linoleate contributed 10%, and stearate 5%. In CM, however, linoleate contributed between 16 and 31% of the total of these four fatty acids, and stearate made up 6 to 12%. (See Figure 7.) The CM composition reported here is comparable to that found in chyle harvested from
rats tube-fed palm oil (Karmen et al. 1963). In that study, the oil contained 10% linoleate and the CM contained 19%, when expressed as a percent of the four major fatty acids.

In addition to the principal dietary fatty acids, recall that numerous additional peaks were present in the GC-FID data (Appendix D), some of which were consistently present in all subjects and fractions. Although the identities of those additional peaks is not directly related to the experimental outcome, it might shed light on the nature of the endogenous dilution of dietary fatty acids in CM. First, an attempt was made to identify the peaks using fatty acid standards. Although none of the larger peaks was identified by this procedure, some possibilities were eliminated. These included linolenate, arachidate (C20:0), behenate, arachidonate, EPA, and DHA. This was surprising, given the biological importance of some of these fatty acid, although some were identifiable as smaller peaks in some samples. Also, in the study already mentioned (Karmen et al. 1963) small but significant amounts of EPA and DHA were consistently present, although they were undetectable in the palm oil used. Other investigators have reported arachidonate in CM at more than 1% of the total TAG following feeding of fats devoid of this fatty acid (Karmen et al. 1963, Bergstedt et al. 1991, Griffiths et al. 1994).

Failing a positive identification of the larger peaks, some generalizations can be made. They eluted much later than any of the major dietary fatty acids, suggesting that they were longer and/or more unsaturated than linoleate since the column used separates fatty acids primarily by length, and secondarily by polarity. Polarity increases with increasing number of unsaturated bonds. In fact, the largest of the unidentified peaks eluted later than DHA, which was the latest of the identifiable fatty acids tested. Thus, the identities of these peaks remains unknown.

Another unanticipated finding was the delayed time course for plateau enrichment of CM fatty acids (Figure 14). CM have a plasma half-life on the order of 2 to 10 minutes (Hussain et al. 1996), and dietary fat appears in plasma CM within one hour after feeding (Hultin et al. 1996). Thus, assuming a one-hour delay and 10-minute half-life, it was estimated that CM enrichment would be within 2% of steady-state by the end of two hours. That is, after two hours, 60/10 half-lives would have elapsed, and $0.5^{60/10} = 0.02$. 129
However, CM enrichment increased significantly between five and six hours after beginning tracer administration (Figure 12). Two possibilities could account for this.

One is contamination of the CM fraction with large particles of liver origin, which would have a lower enrichment, and more delayed turnover time, as can be seen in Figure 15. This would also account for part of the decrement seen between diet and CM enrichment. However, to account for this decrement, the quantitative contribution of liver-derived particles would have to be about 40% of the total, as estimated algebraically from the enrichments at four hours versus the last hour. This is highly implausible, considering typical separation reported in the literature. For example, in one study, separation of apoB 48 and apoB 100 particles from centrifugally separated CM and VLDL in fed humans was virtually complete, although in the same subjects, fasting CM-sized fractions contained traces of apoB 100 (Potts et al. 1991). Furthermore, when only very large CM ($S_f < 1.000$) were separated from selected samples in the present study, their enrichments were only slightly higher than those of the corresponding entire CM fraction. In fact, the apparent difference (~0.10 MPE) was smaller than could be reliably detected by GCMS.

Alternatively, there could be a pool of fatty acids within the enterocyte with a longer half-life than that of CM in plasma. This is suggested by some earlier findings. In one study substantial amounts of fat from the first meal of the day appeared in the post-meal TAG peak, as inferred from the fatty acid compositions of the two meals versus that of CM (Fielding et al. 1996). Since the liquid formula diet in this study was fed without tracer for two hours prior to beginning tracer ingestion, and on the previous day, it is plausible that increasing CM enrichment is due to turnover of an intracellular fatty acid pool. In rats, a fractional turnover rate of the precursor TAG pool for CM formation of $\lambda = 0.61$ to 1.03 pool-equivalents per hour has been reported, with the lower turnover at higher rates of fat infusion (Mansbach & Arnold 1986). Even a fractional turnover of 0.61 is faster than the rate of $\lambda = 0.37$ per hour of this experiment, as computed from equation 24. This may be partially due to the fact that the turnover rate herein also
includes plasma CM turnover, although it may reflect a species difference. Whether the delay in CM enrichment is due to intracellular turnover could be tested by feeding a priming dose of fatty acid tracer at the outset of the protocol.

Finally, it is notable that the basic conclusion, that oleate was oxidized more efficiently than palmitate, is not changed by controlling for the size and enrichment of the precursor pool, but the magnitude of the effect was reduced by two-thirds. That is, about two-thirds of the difference in appearance of label was explained by differences in CM proportions and enrichments, in spite of controlled feeding conditions designed to minimize such differences. This illustrates the utility of the present approach in comparing oxidation of different fatty acids.

NEFA

The half-life of plasma NEFA is only a few minutes (Wolfe 1992, Sidossis et al. 1995b). In the fasting state, NEFA originate primarily from adipose stores, and must circulate to reach the organs and muscles where they are taken up to be oxidized. In a fed state, muscle LPL is partially down-regulated by insulin, while adipose LPL is up-regulated, so if CM fatty acids are oxidized, they still must circulate to some extent. Some are presumably taken up immediately into adipose tissue, but the NEFA enrichment of this experiment demonstrates that circulation of CM-derived NEFA can occur, even at moderate rates of fat intake. Some oxidation of CM fatty acids may also occur in the liver as a result of remnant uptake and catabolism, so the $^{13}$CO$_2$ enrichment herein cannot be attributed entirely to oxidation of plasma NEFA.

Since the processes involved in NEFA production and clearance are so rapid, the composition of arterial NEFA provides a kind of snapshot of whole-body lipolysis and uptake of fatty acids. Hence, the composition of plasma NEFA was used in this study in two ways. First, enrichment of NEFA was compared with that of CM in order to quantify the contribution to peripheral NEFA by sources other than CM fatty acids. On average, NEFA enrichment was about 93% of CM enrichment. This suggests that CM did indeed
comprise the major contributor to fatty acids delivered to peripheral tissues during the feeding protocol, but there may have been minor contributions from other sources. These will be considered next.

Non-CM sources of arterial NEFA include those hydrolyzed from VLDL concurrently with CM and those released from adipose storage. In the fed state, insulin (Boden et al. 1993, Campbell et al. 1992, Bonadonna et al. 1990b) and glucose (Carlson et al. 1991) both suppress adipose tissue lipolysis. The conditions of this experiment were designed to produce steady delivery of CHO and proteins to the bloodstream, and hence, continuous insulin production. However, lipolysis may not have been completely suppressed, given the moderate rate of feeding and the dose-dependent relationship between insulin and inhibition of lipolysis. This represented an experimental trade-off, in that factors which inhibit adipose lipolysis also inhibit fat oxidation. Reduced fat oxidation would make differences in breath enrichment more difficult to detect, both by reducing the signal-to-noise ratio, and by narrowing the magnitude of such differences.

Since CM is the preferred substrate for LPL activity relative to VLDL (Potts et al. 1991, Schneeman et al. 1993), and VLDL turnover is much slower than that of CM (Linder 1988), the contribution of VLDL to circulating NEFA was probably small. It is not possible to distinguish between contributions to NEFA from adipose stores versus VLDL by comparing enrichments of NEFA and CM, but some inferences can be drawn. For example, if NEFA enrichment was consistently below that of VLDL, it would be evidence for substantial adipose lipolysis, but this was not the case for any of the samples analyzed. On the other hand, if NEFA enrichment increased with time proportionally to the increasing enrichment of VLDL, that would be evidence that VLDL was a significant source of NEFA. Multiple regression analysis of NEFA enrichment (Figure 17) did not reveal a significant effect for either time or VLDL enrichment, however, so contributions to NEFA from VLDL could not be detected by the present method.

The fractional, i.e., quantitative NEFA data were used as an index of peripheral uptake of fatty acids, after normalizing for CM composition (Figure 23). It was hypothesized that more efficient oxidation might be accompanied by more efficient
uptake of oleate. This possibility was suggested by a previous study in which plasma NEFA composition only partially reflected CM composition following a high-fat meal (Griffiths et al. 1994). That is, although oleate comprised about one-fourth of the CM fatty acids, its proportion of NEFA dropped as a percent of the fasting value, a finding the authors were unable to explain. In general, the present results support the notion of more efficient uptake of oleate and linoleate than of palmitate and stearate in the periphery, since the ratio of unsaturated-to-saturated fatty acids was lower in NEFA than in CM. In three subjects, the ratio of NEFA oleate to palmitate normalized for that in CM was less than one-third. In four it was between 0.61 and 0.73, and in the remaining three the ratio was greater than unity. Unfortunately, these categories are entirely unrelated to the CM composition categories previously discussed, so no mechanistic connection can be inferred.

**VLDL**

This aspect of the research was somewhat exploratory in nature. If oleate is more readily taken up and oxidized in the periphery than palmitate, it would be logical to expect more of the dietary palmitate to appear in VLDL. This could contribute to long-term effects of these fatty acids on LDL concentrations. However, although there was a trend in the opposite direction to what was hypothesized, no significant difference was found. (See Figure 23.)

Quantitatively, the fractional composition of VLDL was similar to that of CM. (See Figures 7 and 9.) There were also similar amounts of the previously discussed unidentified peaks, presumed to be fatty acids, present in VLDL as were found in CM. This supports the conclusion that the CM peaks came from endogenous sources rather than from non-fatty acid components of the diet such as fat-soluble vitamins. It is also consistent with substantial endogenous dilution of tracee fatty acids in CM. This raises the question of the source of these endogenous contributions. One possibility could be uptake of NEFA derived from VLDL. Alternatively, the liver may draw on the same fatty
acid pool in synthesizing bile components as it does for VLDL synthesis, so the non-diet CM fatty acids may come from bile.

**Breath $^{13}$CO$_2$ enrichment**

*Enrichment kinetics*

Neglecting isotope exchange, which will be considered in the next section, during a constant tracer infusion of labeled CO$_2$, the enrichment of the rapid-turnover pool from which breath CO$_2$ is excreted will approach the net enrichment of its input sources approximately as a monoexponential function. Thus, at time $= t_{1/2}$ the output enrichment is 50% that of the input enrichment, at time $= 2 \times t_{1/2}$ it is 75% of the input, at time $= 3 \times t_{1/2}$ it is 87.5% of the input, and so on. For example, in one study, healthy adults were given a constant infusion of CO$_2$ tracer in a basal state, and the $t_{1/2}$ of CO$_2$ was computed from the time course of enrichment to be about 37 minutes (Heiling et al. 1991).

In addition to CO$_2$ kinetics, the overall half-life for CO$_2$ isotope enrichment derived from fatty acid tracer oxidation is also a function of acetyl-CoA kinetics. It has been argued that fatty acids entering cells may first mix with an intracellular TAG pool before being oxidized (Jensen et al. 1990), which would introduce still another delay in CO$_2$ enrichment. This suggestion was based on the longer half-life for labeled acetate as compared with CO$_2$. However, in a direct comparison of the kinetics of $^{13}$CO$_2$ derived from [1-$^{14}$C]-acetate versus [1-$^{13}$C]-palmitate, researchers did not find a significant difference (Sidossis et al. 1995b), indicating that the delay is attributable to acetate kinetics. In that study, the half-life of palmitate's labeling of CO$_2$ averaged 107 minutes versus 97 minutes for acetate. If there is a slight delay between the two, it is probably due to slower uptake of plasma NEFA versus acetate.

In the present research, CO$_2$ kinetics are also a function of enrichment of fatty acids in CM and, perhaps, VLDL. From equation 24, the average calculated $t_{1/2}$ for CM
fatty acids was 113 minutes, with an initial appearance, $t_0$, of about 67 minutes. Extrapolating theoretical enrichment to eight hours yields a predicted rise in enrichments of only about 4% had the protocol been extended another hour. Parameters for VLDL from equation 25, were $t_{1/2} = 138$ minutes and $t_0 = 191$ minutes. The theoretical curves for CM and VLDL enrichment are depicted in Figure 16. Despite the later appearance and longer half-life of VLDL, extrapolating its enrichment predicts only a 7% increase in enrichment between seven and eight hours. Thus, it would appear that little would have been gained in plasma fatty acid enrichments by a longer protocol than was used, since the closer one approaches a steady state, the less return there is in terms of approaching the asymptote, or true input enrichment.

On the other hand, the average slope for $^{13}$CO$_2$ enrichment during the last hour of the protocol was 16%, which does not satisfy the a priori definition of a functional plateau established in this research. However, during the final 40 minutes of the protocol, a slope of < 10% was achieved. More importantly, there was no evidence that the two tracer fatty acids were kinetically different from each other, so the final outcome measures for relative oxidation would have been identical had the protocol been extended. The only difference would have been the percent of label recovered. The theoretical asymptotes for $^{13}$CO$_2$ enrichment can be estimated, from equation 26, at 24% higher than what was found.

Isotope exchange & label recovery

The steady-state recovery of a carbon label as CO$_2$ derived from substrate oxidation is governed by the existence of large pools of various metabolites with relatively slow turnover rates. (For more in-depth discussion of this, see: Wolfe 1992, Sidossis et al. 1995b, Irving et al. 1983, Kien 1989). As is the case for kinetic parameters, when fatty acids are the substrates for oxidation, these pools include acetyl-CoA and CO$_2$. Also, when labeled CO$_2$ is infused, about 2% of label is lost in urine (Elia
et al. 1992), and some is presumably temporarily fixed in forms which turn over so slowly as to make recycling undetectable, such as calcium carbonate in bone. However, there are pools with turnover rates short enough to permit achieving an apparent plateau in breath enrichment within a few hours, but from which significant recycling of label can eventually occur. The exact nature of these pools is a matter for conjecture (Armon et al. 1990, Barstow et al. 1990), and is not relevant to the present discussion. It is important, however, to recognize their existence and implications when interpreting label recovery data. (See Figure 20.)

When labeled CO₂ is infused in healthy adults, under basal (i.e. resting, fasting) conditions, plateau label recovery is about 80% (c = 0.8) (Wolfe 1992). However, when labeled acetate is infused, label recovery is reduced, and a recovery factor of c = 0.56 has recently been proposed for computing absolute oxidation of fatty acids labeled at the 1-carbon (Sidossis et al. 1995a). This difference is due to the greater opportunity for temporary fixation of carbon. During β-oxidation, fatty acids are shortened by sequential removal of 2-carbon units which are esterified to form acetyl-CoA. This can enter the TCA cycle, or be used in synthetic processes such as acetylation reactions, ketone formation, and cholesterol synthesis. Acetyl-CoA entering the TCA cycle is first incorporated into citrate, and neither carbon is lost in the first spin of the TCA cycle. Labeled carbon can therefore also be incorporated into the products synthesized from TCA cycle intermediates, as well as products of the gluconeogenic pathway. This has been demonstrated by the appearance of labeled glutamate and lactate in plasma during labeled acetate infusion in fasting subjects (Sidossis et al. 1995b). Also, because only the 1-carbon is oxidized to CO₂ on the second spin, and only half of the 2-carbon is lost on each subsequent spin, more of the label in the 2-position than in the 1-position ofacetate is exchanged (Wolfe & Jahanor 1990). For the purposes of this research, however, it is the 1-carbon that is of interest, since it is labeled by β-oxidation of [1-¹³C]-labeled fatty acids.
Validity of the model assumptions

The validity of any experimental model rests on the validity of the assumptions on which it is based. The key assumptions of the present model are discussed below:

1) *Dietary fat is exclusively incorporated into CM and delivered to arterial circulation via the lymphatic system.* Under certain conditions, a large fraction of fat in the duodenum may pass directly into the portal vein rather than being incorporated into CM (Mansbach et al. 1991). However, this can be reduced to as little as 0.5% when PC is present, when other macronutrients are present, and with reduced rates of fat infusion (Mansbach & Dowell 1993). Under the circumstances of the present study, therefore, delivery of dietary fatty acids to portal circulation is extremely unlikely.

2) *The kinetics of fatty acid oxidation to CO\textsubscript{2} are approximately first-order.* The data shown in Figures 16 and 18 generally support the validity of this assumption. That is, there does not appear to be any “recycling” of label during the course of the protocol, which would manifest itself as an acceleration in the rate of increase of $^{13}$CO\textsubscript{2} enrichment following the initial leveling-off. However, there may be a small contribution to $^{13}$CO\textsubscript{2} enrichment from oxidation of VLDL, undetectable in the present data. From equations 24 and 25, enriched VLDL began to appear in plasma at $t = 2:18$, versus $t = 1:07$ for CM. This could have resulted in a slightly “sway-backed” $^{13}$CO\textsubscript{2} enrichment curve, given a sufficiently high contribution of VLDL to fatty acid oxidation. That is, between $t = 5:00$ and 7:00 hours, $^{13}$CO\textsubscript{2} enrichment was leveling off (Figure 19) while VLDL enrichment was still rising rapidly (Figure 15). The lack of significant effect of time or VLDL enrichment on NEFA enrichment (Figure 17) also supports the idea that the contribution of VLDL fatty acids to fat oxidized by peripheral tissues was much less than that of CM.

3) *Sufficient dietary fat is oxidized in the fed state to produce measurable enrichment of $^{13}$CO\textsubscript{2}.* The present protocol produced measurable enrichments in all cases.
even in subject 3029, whose palmitate enrichment was only 1.62 MPE, and whose RQ data indicated he only derived 15% of energy from fat oxidation.

4) **CM composition represents an unbiased measure of delivery of tracee fatty acid to cells.** The unequal distribution of oleate and palmitate on the sn-2 versus sn-1 and -3 positions of the glycerol backbone of TAG in palm oil (Gunstone 1986) introduces several potential confounding factors which must be considered. The first is unequal absorption of tracee fatty acids, which has been discussed above. Second, to the extent that oleate tracer was over-represented on the sn-1 and -3 positions relative to its tracee, there was the opportunity for differential fates of tracer and tracee oleate in the periphery. This is because LPL only hydrolyzes the 1- and 3-positions of TAG (Zock et al. 1996), as discussed above in "Mechanisms for differential fatty acid oxidation." In humans, the effects of position investigated thus far have been negligible (Zampelas et al. 1994, Zock et al. 1995). Also, it is likely that hydrolysis of fatty acids from TAG in plasma is complete, so that no 2-MAG is taken up (Fielding 1981).

Similarly, incorporation of the two tracers into species other than TAG such as CE, PL, and retinol esters may not be identical, given that definite substrate preferences that exist for the synthetic enzymes which catalyze these reactions. To the extent that this is the case for tracees liberated from TAG, it can be argued that it reflects the usual physiological fate of dietary fatty acids. Therefore, if the preference is for palmitate, as is the case for retinol esters formed within the enterocyte (Hultin et al. 1996), it is not a confounding factor because most tracee would also be hydrolyzed before enteral uptake. Oleate tracer, on the other hand, might be protected from oxidation relative to its tracee by differential incorporation to species that are not hydrolyzed in the periphery but remain in CM remnants until they are taken up. This would not explain the finding that more oleate tracer was oxidized, but might contribute to greater incorporation to VLDL.

The effects of position could be tested by a protocol in which both tracee and tracer fatty acids are randomized in TAG form before feeding. If there were a difference, however, the effect on tracer oxidation in the present study should be minimal, since most of both tracers were presumably esterified to the 1- and 3-positions.
5) **There is no carry-over of $^{13}$CO$_2$ enrichment within subjects.** It can be estimated from the $t_{1/2}$ of 137 minutes shown in Equation 26 that $^{13}$CO$_2$ enrichment should have returned to within 1% of baseline 16 hours after the tracer was discontinued. That is less than half of the minimum 36-hour washout period used. In the preliminary study (Figure 21), $^{13}$CO$_2$ enrichment fell by 20% between 40 and 60 minutes after ingestion of the last tracer dose. This is an even faster drop than would be predicted from the CM enrichment kinetics. VLDL enrichment, similarly, would be predicted to be 1% of baseline within 24 hours.

6) **Analytical measurements and data interpretation are unbiased.** All diet preparation and administration, as well as preparation of plasma samples, was performed by one person. Therefore, it was not possible to carry out the study in a completely double-blinded fashion. However, the subjects and the technician who analyzed the breath samples were unaware of the expected outcome of the experiment.

7) **The within-subject isotope exchange rate is similar.** To the extent that this assumption was not met it represents a source of variability which could not be controlled. However, the isotope exchange factor, c, is highly dependent on metabolic states such as rest, exercise, and eating (Wolfe et al. 1984), as are VO$_2$ and VCO$_2$. The observed replication of VO$_2$ and VCO$_2$ rates between study days does indicate that similar metabolic states were achieved.

8) **Composition of the CM fraction represents composition of CM produced by enterocytes.** The use of the heated hand technique to arterialize blood samples was intended to maximize the CM content of samples, since CM are delivered to arterial circulation, and to minimize that of CM remnants. Concentration of remnants should be higher in venous blood, following passage through peripheral capillary beds. The significance of this is that a larger fraction of CM lipid is in the form of TAG, so that remnant composition of total fatty acids may be different from that of the precursor CM. Evidence from the literature supporting the validity of this assumption was discussed in Chapter 2 under "VLDL metabolism."
9) **VLDL fraction is not significantly contaminated by CM remnants.** There is evidence that uptake of CM remnants is saturable (Berr 1992), but at a rate of fat digestion more than twice that of the present intake, so a progressive accumulation of CM remnants in the present protocol is unlikely. The half-life of remnants has been measured in the range of 7 to 30 minutes (Hultin et al. 1995, Berr 1992), much less than that of VLDL, which also argues against progressive dilution of VLDL with remnants. In fact, the upper end of this range was obtained using a dose of fat sufficient to saturate the CM remnant clearance process. Further, the time course observed herein for VLDL fatty acid enrichment is similar to that seen in apoB 100 enrichment during a constant infusion of labeled leucine (Cohn et al. 1990). In that study, synthesis of apoB 100 was assessed in humans fed hourly with a liquid diet very similar in macronutrient composition to the one used in this experiment. This is consistent with VLDL enrichment's coming from newly synthesized particles of hepatic origin, rather than accumulated CM remnants.

10) **Feeding small frequent meals is a physiologically relevant approach.** There is no single eating pattern for humans. Eating patterns range from a single large meal per day to “grazing” throughout the day. The appearance of label reported herein tends to be somewhat higher than that reported for an experimentally imposed single meal for a similar time interval (Cenedella & Allen 1969, Watkins et al. 1982), although they are closer to recoveries reported after 24 hours (Leyton et al. 1987, Toorop et al. 1979, Jones 1994). This may indicate that a smaller fraction of what was fed in the present research was stored during digestion in this protocol than in single-meal comparisons.

11) **A functional steady state is achieved.** As was discussed in Chapter 3, it is not necessary to obtain a perfect steady state in $^{13}$CO$_2$ enrichment if the underlying kinetic parameters are equal. There are, in addition, disadvantages in extending a feeding protocol for many more hours than the seven used herein. For example, fatty acid tracer that is not absorbed in the small intestine may undergo bacterial fermentation (Segal et al. 1990), thereby increasing the apparent oxidation of tracee fatty acid within the body. Since there appeared to be significant malabsorption of palmitate tracer in the present experiment, this would be a serious source of error. There is also increasing probability
for measurable isotope recycling from the slow-turnover pools previously mentioned. However, the data must be sufficient to compute the kinetic parameters and predicted steady-state values are needed to calculate absolute oxidation. The slope of less than 10% in $^{13}$CO$_2$ enrichment achieved in the last 30 minutes of the protocol indicates this criterion was met.

12) A metabolic steady state was achieved. Although the use of steady-state equations relies on the assumption that a physiological steady state is achieved, in practice there is no perfect steady state in biological systems. Two major sources of temporal variation are sleep-wake and fed-fasted cycles, which lead to continually changing hormonal states. However, the lack herein of significant differences in substrate oxidation and energy expenditure between morning and afternoon supports the assumption that substrate oxidation remained constant during the course of the protocol.

13) $\beta$-oxidation is complete. There are no published data to suggest that either palmitate or oleate is merely chain-shortened and not completely oxidized in healthy adults. Since there are distinct families of mitochondrial dehydrogenases which mediate the $\beta$-oxidation of short-, medium- and long-chain fatty acids (Eaton et al. 1996) incomplete oxidation is conceivable. If this were to happen, however, if both were shortened to the same length, then the proportional difference in oxidation would be even greater than what has been reported. (Recall that fatty acid tracers labeled at the 1-carbon lose their label on the first round of $\beta$-oxidation.) If, on the other hand, oleate were shortened to palmitoleate (C16:1) and then transported back out of the mitochondria, the energy derived from oleate oxidation would be overestimated. However, this is unlikely, given the paucity of palmitoleate in human adipose tissues (Phinney et al. 1996). Also, when medium- and short-chain fatty acids are fed, they are oxidized more rapidly than long-chain fatty acids. Nor are fatty acids with fewer than 14 carbons found in stored or structural human lipids (Field 1985, Phinney 1994). Even myristate is only about 2 to 3% of total fatty acids in adipose tissue.

14) There is no preferential hydrolysis of CM-derived oleate versus palmitate. As was previously discussed, available evidence argues against partial hydrolysis of TAG
in the periphery (Fielding 1981). There is some evidence for conservation of the sn-2 position of dietary TAG in liver-derived VLDL, however, suggesting that positional isomerism may have some influence on the ultimate fate of fatty acids. The arguments in (4) above, however, also apply in this case.

15) The presence of other fatty acids in CM does not differentially affect fractional oleate versus palmitate metabolism. If, for example, linoleate competed with oleate but not palmitate for a rate-limiting step in their metabolism, it could reduce the fractional oxidation of oleate. Recall that arachidonate appears to compete with palmitate but not oleate for binding to erythrocyte ghost membranes (Bojesen & Bojesen 1996a). The magnitude of such an effect would be minimized in the present protocol by the fact that oleate and palmitate comprised 80% of dietary fat. Further, most of the balance of the diet fat was linoleate and stearate, which are commonly present both in food fats, and in endogenous lipids. Thus, if such competition exists, it can be argued that it represents the physiological norm.

Limitations

Interpretation of the present results is necessarily limited by the experimental conditions used. For example, obese persons or those with disorders of lipid metabolism may respond differently than the lean, healthy volunteers of this experiment. Also, the use of a liquid formula, low-residue diet may not produce the same result as would more usual, solid foods. It would also be useful to know if the fed pattern observed herein would persist into a fasted state, or perhaps be reversed. Since human TAG stores have been consistently shown to contain more oleate and less saturated fat than the usual dietary intake (Beynen et al. 1980, Garland et al. 1998), it is not plausible to conclude from these data that palmitate accumulates progressively in body stores.

There are several specific limitations related to dietary fat, per se. First, only acute effects were examined. Numerous aspects of fat metabolism are influenced by the composition of dietary fat that is chronically consumed, including CM clearance
(Weintraub et al. 1988), acute fat oxidation (Takada et al. 1994), insulin sensitivity (Storlien et al. 1991), VLDL secretion (Lai et al. 1991), and body fat accretion (Shimomura et al. 1990, Hill et al. 1993). Second, equal amounts of the two fatty acids of interest were fed, in order to induce equivalent metabolic states during the two protocols. However, dietary fat composition acutely affects CM size (Levy et al. 1991, Feldman et al. 1983, Ockner et al. 1969b, Sakr et al. 1997) and turnover (Levy et al. 1991), CM remnant uptake (Lambert et al. 1994), bile acid synthesis (Bravo et al. 1996), and VLDL synthesis by cultured hepatocytes (Kvilekval et al. 1994, Pai & Yeh 1996, Bruce & Salter 1996). Therefore, in order to determine if any of these phenomena might change the observations made herein regarding relative metabolic fates, they would have to be investigated in separate studies.

Finally, although oleate and palmitate are the two most common fatty acids in the US food supply (Gurr 1992), several other fatty acids are certainly significant. Linoleate and α-linolenate are essential nutrients, and the minimum requirement for linoleate has been set at 1 to 2% of energy (NRC 1989). EPA, α-linolenate and γ-linolenate stimulate fat oxidation in rat hepatocytes (Ide et al. 1996, Kabir & Ide 1996, Willumsen et al. 1996). Fish oils high in EPA and DHA have been promoted in the diet for their anti-thrombotic properties and appear to reduce fat accretion and insulin resistance in rats fed high-fat diets (Hill et al. 1993, Ikemoto et al. 1996, Storlien et al. 1991).

Of course, each of the limitations discussed also represents an opportunity for further exploration of the mechanisms underlying differential fates of dietary fatty acids.

Summary

The present research compared the metabolic fates of what are arguably the two most important fuel fatty acids in the diet, i.e., oleate and palmitate, as a means of investigating three potential mechanisms for their differing health effects. These were: first and most importantly, oxidation; second, incorporation of the two fatty acids into
VLDL; and third, appearance in, and clearance from, plasma NEFA of CM-derived fatty acids.

The well-established link between dietary fat and degenerative disease is the basis for public policy recommendations to limit fat intake. However, fatty acids vary considerably in their effects on human health, so it is important to establish balanced guidelines that recognize the essential role of dietary fat. The present research has investigated relative oxidation of two fatty acids as one potential mechanism by which they may differ in metabolic fate, and hence, in their health effects. It also sought to determine whether differences in peripheral clearance of fatty acids might help to explain differences in oxidation, and whether such differences would be reflected in incorporation of dietary fat into VLDL by the liver. The main result, that dietary oleate was oxidized at a higher fractional rate than palmitate, supports the idea that such differences in immediate metabolic fate may contribute to the benefits of high-oleate diets.

The substantial effect of the present experimental control measures on the final outcome also demonstrates the usefulness of such controls in interpreting comparative oxidation of fatty acids in the fed state. Further experiments are needed to delineate how such results will be affected by dietary manipulations, other lifestyle factors such as exercise, and by genetic variation in proteins which mediate fat metabolism.


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APPENDIX A

FATTY ACID NOMENCLATURE

Saturated fatty acids

Laurate (C12:0)
Myristate (C14:0)
Palmitate (C16:0)
Stearate (C18:0)
Arachidate (C20:0)
Behenate (C22:0)

Monounsaturated fatty acids

Myristoleate (C14:1)
Palmitoleate (C16:1)
Oleate (C18:1)
Polyunsaturated fatty acids

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Chemical Formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linoleate *</td>
<td>(C18:2 n-6)</td>
</tr>
<tr>
<td>α-linolenate *</td>
<td>(C18:3 n-3)</td>
</tr>
<tr>
<td>γ-linolenate</td>
<td>(C18:3 n-6)</td>
</tr>
<tr>
<td>Arachadonate</td>
<td>(C20:4 n-6)</td>
</tr>
<tr>
<td>Eicosapentaenoate</td>
<td>(C20:5 n-3)</td>
</tr>
<tr>
<td>Docosahexaenoate</td>
<td>(C22:6 n-3)</td>
</tr>
</tbody>
</table>

* Essential fatty acids.
APPENDIX B

PREPARATION OF TRIACYLGLYCEROL-DERIVED
FATTY ACID METHYL ESTERS

A. Ultracentrifugation
1. To each wettable centrifugation tube, add 0.75 ml plasma. Without overfilling, layer about 4 ml mock solution (physiological saline) on top of plasma.
2. Weigh tubes with lids to within 0.01 g of each other.
3. Ultracentrifuge for 30 minutes at 33.5K & 4°C in a Beckman 50Ti rotor. no brake. slow acceleration.
4. Use plastic Pasteur pipet to skim off not more than top 0.7 ml of chylomicon-containing solution, drawing air with water. Chylomicrons appear as a milky residue floating on the surface.
5. Add enough mock solution to each tube to replace what was withdrawn.
6. Repeat steps 2 through 5.
7. Divide CM-containing solution into 2 equal parts by weight.
8. Ultracentrifuge for 17 hours at 40K. slow acceleration. no brake.
9. Skim off top 1 ml of VLDL-containing solution as in (4).

B. Separation/Extraction
1. To one 16 x 100 mm plain tube per sample, add 3 ml methanol:chloroform. If layers do not separate, add 1 ml water. Vortex.
2. Pipet each lower phase into a thick-walled 16 x 125 mm screw-top tube.
3. Add 2.5 ml each chloroform and water to each tube. Vortex.
4. Centrifuge for 10 minutes at 2,000 rpm.
5. Pipet off top (aqueous) layer and discard.
6. Add Na-sulfate to tubes to precipitate water.
7. Decant to 16 x 125 mm screw-top tubes.
8. Dry under N2 at 40°C.

C. Derivatization/Transmethylation
1. Add 1 ml each chloroform and 3N-methanolic HCl.
2. Add 2 ml 5% NaCl to each.
3. Add 3 ml hexane and vortex.
4. Decant to 16 x 100 mm tubes.
5. Pipet top (hexane) layer into a 13 x 100 ml tube.
6. Repeat steps 4 and 5.
7. Add Na-sulfate to precipitate water.
8. Decant to 16 x 100 mm tubes.
9. Dry under N2 (about 20-30 minutes).
APPENDIX C

PREPARATION OF NON-ESTERIFIED FATTY ACID-DERIVED

FATTY ACID METHYL ESTERS

A. Extraction.
1. Thaw and vortex plasma samples. Centrifuge samples for 10 minutes.
2. Pipet 0.6 ml to screw-top tubes.
3. Add internal standard (C15:0) to each sample.
4. Vortex.
5. Add 5 ml extraction solution to each tube.
6. Cap tubes & shake for 30 minutes.
7. Add 2 ml water to each tube.
8. Add 3 ml heptane to each tube.
9. Vortex each tube 2 minutes.
10. Shake tubes 15 minutes.
11. Pipet upper phase into small (12 x 75) tubes.
12. Dry under N2 at 40°C. (Wipe catheters w/alcohol before and after.)

B. Thin Layer Chromatography Separation.
1. Bake chromatography plates for 60 minutes at 90-100°C to activate. Cool at least 5 minutes at room temp.
2. Add 50 microliters of (2:1) chloroform:methanol to each tube and one external standard tube.
3. Mark starting line one inch from bottom of chromatography plate. Mark lanes.

4. Spot extract from each tube in a band across one lane.

5. Mix TLC solvent: 80 ml heptane, 2 ml glacial acetic acid, 20 ml ethyl ether (add last).

6. Place filter paper upright in tank. When paper is saturated, place plate in tank and cover.

7. When solvent is within 1.5 inches of top (about 25-30 min), remove plate from tank and dry at 30°C for 10 minutes.

8. Spray with rhodamine dye until saturated. Air dry overnight. If spots are not sufficiently distinct, use UV light or let the color fade for a day.

9. Scrape spots into heavy-walled screw-top tubes.

C. Derivatization/Esterification.

1. Add 50 microliters hexane to each tube.

2. Add 1 ml 14% BF3-CH3OH to each tube.

3. Seal with teflon-lined caps (unchipped).

4. Vortex to break up silica gel.

5. Heat for 4 minutes at 100°C.

6. Pipet 1 ml H2O and 2 ml hexane to each tube.

7. Shake on shaker for 15 minutes.

8. Centrifuge 10 minutes at 2000 rpm.

9. Pipet upper phase into small (12 x 75) tubes.

10. Dry under N2 at 40°C.
Figure 24. Sample gas chromatography-flame ionization detector results for chylomicron-derived fatty acid methyl esters. A, solvent peak; B, standard (C15:0); C, palmitate; D, stearate; E, oleate; F, linoleate.
Figure 25. Sample gas chromatography-flame ionization detector results for very low density lipoprotein-derived fatty acid methyl esters. A, solvent peak; B, standard (C15:0); C, palmitate; D, stearate; E, oleate; F, linoleate.

Figure 26. Sample gas chromatography-flame ionization detector results for non-esterified fatty acid-derived fatty acid methyl esters. A, solvent peak; B, standard (C15:0); C, palmitate; D, stearate; E, oleate; F, linoleate.
APPENDIX E

GAS CHROMATOGRAPHY/MASS SPECTROMETER
ANALYSIS OF OLEATE $^{13}$C ENRICHMENT

The analysis of oleate enrichment by GC/MS is more complex than that of palmitate for several reasons. These arise primarily from two sources: increased degradation of oleate in the MS source, and the presence of other fatty acids of significant amounts of fatty acids differing from oleate by only 2 amu in the sample. These factors require a somewhat more rigorous analysis of oleate peaks than is the case for palmitate.

The increased degradation of oleate in the source is due to the presence of a double bond, and results in peak areas of about 15% as large as are obtained for the same concentration by weight of palmitate. This increases the signal-to-noise ratio (s:n) for oleate peaks by a factor of 6 or 7, since the peak height is reduced, while the peak width remains the same. Figure 14 depicts this phenomenon for a standard sample containing equal weights of various FAMES. In addition, the later elution time of oleate also increases the ratio peak width to peak height relative to palmitate, further increasing s:n.

The plasma fatty acids analyzed herein contained significant amounts of stearate and linoleate, meaning that the column separated them from oleate only on the basis of polarity, which is a function of the number of double bonds. Note that, although the absolute quantity of stearate was small, it is less degraded than the unsaturated fatty acids, and so the peak areas for stearate were similar to those of oleate. Figure 15 illustrates the potential error introduced by incomplete separation of these three peaks. The stearate
peak contains an M-2 component which has the same amu as the M+0 component of oleate (ion 296). Ideally, if these two peaks are not completely separated, they are integrated such that a vertical drop to baseline is performed at the nadir between them. However, if the peak is integrated such that these two peak areas are added together, the calculated ratio of M+1/M+0 is reduced. If, on the other hand, the baseline of the oleate M+0 peak begins at the nadir, the ratio is increased. Similarly, the linoleate peak contains a small M-1 peak with the same amu as oleate M+1 (297), with the same potential to introduce error.

Intuitively, it would seem that these errors should not result in significant analytical error. However, because the ratios of enriched samples are subtracted from that of unenriched samples, a small error in peak area can propagate to a large error in MPE. For example, if the enriched ion ratio = 0.250, and unenriched ion ratio = 0.200. MPE = 5.0. If the enriched M+1 area is decreased by just 4%, then the sample ratio becomes 0.240, and calculated MPE = 4.0, or 25% less than the actual enrichment. Note that, in each calculation, 4 separate peak areas are involved, such that multiple errors in baseline computation can produce additive results much larger than in this example.

Reproducibility of ratios for repeated samples provides one means of screening for the occurrence of the errors described above. However, since such errors are reproducible to the extent that the automatic integration algorithm uses the same strategy on repeated injections of the same sample. Therefore, any results that appear anomalous relative to other samples in the same series must be examined individually to determine how the integration was performed.
Figure 27. Results of gas chromatography/mass spectrometer analysis of a standard. Standard contained equal weights of methyl esters of palmitate, stearate, oleate, linoleate, and α-linolenate, in order of elution.
Figure 28. Variability in integration of oleate peaks. Upper panel shows oleate M+1 peak baseline drawn from the minimum height between its leading edge and the trailing edge of the stearate M-1 peak area. Lower panel shows a drop-to-baseline at that point.
APPENDIX F

BREATH CO₂ PURIFICATION PROCEDURE

Numbers refer to apparatus labels as follows:
1 = valve from syringe to water trap
2 = valve from water trap to cold finger
3 = cold finger valve
4 = valve to pumps
5 = Roughing Pump
7 = Diffusion Pump

Two days before beginning, turn on the roughing pump

Set-up:
1. Turn on water to diffusion pump.
2. Turn on diffusion pump.
3. Set up dry ice methanol trap.
4. When the diffusion pump gage stabilizes, fill the liquid nitrogen trap.
5. After turning off the diffusion pump, wait at least an hour before turning off water.
Sample purification:

1. Draw sample into syringe, holding positive pressure for at least 30 seconds on each vial.
2. Install syringe. Open 2. stopcock to apparatus.
3. When RP stabilizes, close 5. open 7 (DP).
4. When DP stabilizes, close 2. open stopcock to syringe.
5. Close stopcock to apparatus. and 1.
6. Wait 3 minutes to freeze water. (Remove syringe.)
7. Put cold finger in N₂ trap.
8. Close 4, or you will lose your sample.
9. Open 2. close 7, open 5.
10. Wait 2.5 minutes to freeze CO₂.
11. Close 2. open 4 (RP) to vacuum N₂ gas off of sample.
12. When RP stabilizes. close 5. open 7 (DP), adding N₂ to trap as needed.
13. When DP stabilizes. close 3. remove N₂ trap from cold finger.
16. Open stopcock, close 2. and close stopcock.
17. Remove cold finger and replace with a new cold finger. Open 4 to establish a vacuum.
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