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REGULATION OF HEPATIC DE NOVO LIPOGENESIS IN HUMANS

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ABSTRACT

The enzymatic pathway for synthesis of fatty acids from acetyl-coenzyme A, or de novo lipogenesis (DNL), is present in human liver and, to a lesser extent, in adipose tissue. Although the molecular and enzymatic regulation of the components for DNL are well characterized, the quantitative importance of the assembled pathway and its physiologic functions have remained uncertain. We review methods that have been used for measuring DNL in vivo, their limitations and the conclusions based on them. Two new methods for direct measurement of DNL in humans are discussed—mass isotopomer distribution analysis (MIDA), a mass spectrometric technique based on combinatorial probabilities, and ^2H_2O incorporation. Recent findings with these methods in a variety of dietary and hormonal settings are reviewed. In particular, we focus on the question of whether or not surplus carbohydrate energy is converted to fat by the liver in humans. A somewhat surprising model of the response to carbohydrate overfeeding emerges from these studies, with a number of implications for metabolic regulation in health and disease. We close by speculating on potential functions of DNL in physiology and pathophysiology if storage of surplus carbohydrate energy is not an important function of DNL. The availability of techniques for quantifying DNL in vivo should make it possible to resolve these uncertainties regarding its functions and regulation in humans.

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INTRODUCTION

Although carbohydrate (CHO), fat, or protein may be present in excess in the diet, adipose fat is the only substantial storage form for surplus energy in animals. In contrast to triglycerides, the capacity for storage of glycogen is limited (15), and no protein has been identified in humans whose sole function is to serve as an amino acid reservoir (unlike beans, for example, which have storage proteins). Thus, no mechanism exists for direct storage of a long-term surplus of CHO or protein in the diet. These simple considerations lead to the inference that the body must be capable of transforming surplus nonfat energy into fat. The discovery of an enzymatic pathway for de novo lipogenesis (DNL) provided biochemical confirmation of this inference. Accordingly, DNL has been widely assumed to represent the final common fate for surplus nonfat energy; conversely, the main function of DNL has been assumed to be in the macronutrient energy economy.

Experimental findings in humans have not supported so simple a model of the energy economy or of the functions of DNL, however. In this review, we discuss the history of ideas about DNL, the experimental methods available

for measuring DNL, and the evidence both indirect and direct concerning the regulation and quantitative importance of DNL in the energy economy. We conclude with speculations on other potential functions of DNL in cellular and organismal homeostasis. The methods by which DNL is measured are discussed in detail, with emphasis on recent methodologic advances that allow accurate measurements in humans. Our hope is to convince the reader that for DNL, as is so often the case in experimental biology, expectations concerning biological functions have not been borne out when tested directly, and that the result is a more interesting, though incompletely understood, regulatory model.

HISTORICAL REVIEW

In the early 1840s, the German chemist Liebig suggested that starch and sugar may be converted to fat (81). He observed that farmers fattened domestic animals by feeding them grains and hypothesized that the chemical reaction to make fat from sugar would simply require the removal of oxygen from sugar molecules (56). This hypothesis contradicted the general laws proposed by French chemists Dumas and Boussingault. They believed that plants were capable of reduction reactions, i.e. those adding hydrogen or yielding free oxygen, but that animals were capable only of oxidation, which used oxygen and resulted in the breaking down of organic compounds (18). According to these rules, animals could make fat only from plant fat. Thus, Dumas and Boussingault immediately challenged Liebig's hypothesis of DNL. French scientists tried to prove that the quantity of fat in plants was sufficient to account for all the fats produced by animals. They based their denial of synthesis of fats in animals on the conviction that food provided quantitatively all the fat in the body. But in 1845, after conducting their own rigorous balance trial experiments with geese (97) and pigs (14), they finally had to admit that in the fattening of these animals, much more fat was assimilated than could be found in their rations. In 1866, Lawes & Gilbert (73) published decisive data showing that fats could arise from dietary protein in the pig. Balance experiments of a similar nature confirmed the formation of fats from CHO or protein in different species such as geese, dogs, and cats (36).

ELUCIDATION OF THE BIOCHEMICAL PATHWAYS

The biochemistry of DNL was elucidated during the first two thirds of the twentieth century. Raper speculated about the two-carbon building blocks for DNL in 1907 (99): "The formation of fatty acids in animals from carbohydrates, and the occurrence in natural fats, such as butter, of all the fatty acids containing an even number of carbon atoms, from two to twenty, suggest that their fatty acids are produced by the condensation of some highly reactive

substance containing two carbon atoms and formed in the decomposition of sugar." Forty-five years later, in the 1950s, the actual structure of the two-carbon building blocks, acetyl-coenzyme A (CoA), was confirmed (40). At that time, the question arose as to whether or not biosynthesis of fatty acids was the reverse of the recently discovered ß-oxidation process. The speculation was brought to an end in the late 1950s when the synthesis of long-chain fatty acids from acetyl-CoA was demonstrated in vitro in the absence of any of the enzymes of the ß-oxidation sequence (40). The enzymes of the fatty acid-synthesizing system were then isolated and characterized by the Vagelos and Wakil groups from extracts of *Escherichia coli* (40). The energetic cost of DNL was recognized early (34).

SUMMARY OF BIOCHEMICAL PATHWAYS

The biosynthesis of fatty acids is a complex cytosolic polymerization with acetyl-CoA as a primer to initiate the process and palmitate one end product (Figure 1). Acetyl-CoA is the principal building block of fatty acids for DNL,

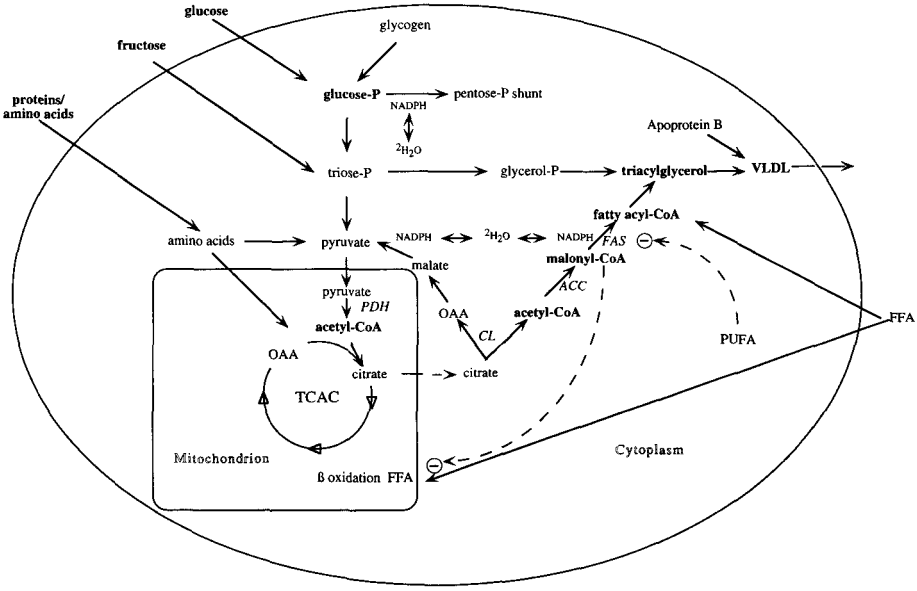


Figure 1 Metabolic pathways related to DNL. A liver cell is shown, with very-low-density lipoprotein (VLDL) secretory pathway and fructose uptake; otherwise, the pathways also apply in adipocytes. Abbreviations: P, phosphate; PDH, pyruvate dehydrogenase; TCAC, tricarboxylic acid cycle; OAA, oxaloacetate; FFA, free fatty acids; CPT-1, carnitine palmitoyl transferase I; CL, ATP:citrate lyase; NADPH, production or utilization of nicotinamide adenine dinucleotide phosphate (reduced form); ACC, acetyl-coenzyme A (CoA) carboxylase; FAS, fatty acid synthetase; PUFA, polyunsaturated fatty acids. (*circled minus*) Inhibitory action.

after activation to malonyl-CoA by the multifunctional polypeptide acetyl-CoA carboxylase (ACC). This reaction is the controlling step in fatty acid synthesis and takes place from acetyl-CoA, bicarbonate, and ATP in the cell cytosol. The overall synthesis of fatty acids is catalyzed by the fatty acid synthase complex, a single polypeptide containing seven distinct enzymatic activities. Condensation reactions accompanied by decarboxylation and two reductions with NADPH as a hydrogen donor are successively repeated until formation of a palmitate molecule is achieved.

Phyllogenic Survey

It is clear that many species are capable of converting CHO to fat in substantial quantities, including pigs (73, 86), rats (113), mice (5), ruminants (5), and dogs, cats, and birds (36). In rodents, lipogenesis is reported to be adaptive in response to energy surplus (113). Dietary polyunsaturated fatty acids suppress lipogenic enzymes in rodents (see below). The adaptive changes in response to energy surplus exhibit both immediate and delayed components (5).

THE REGULATION OF DNL IN HUMANS: EX VIVO EVIDENCE

Evidence concerning the activity and functions of DNL in humans has taken several experimental forms. We first review ex vivo evidence.

Enzyme Regulation

Classic enzyme regulation in the DNL pathway is well characterized (13, 72, 118). The most important regulatory steps in the pathway are generally agreed to be ACC and perhaps fatty acid synthetase, in addition to the rate of delivery of acetyl-CoA subunits to the cytosolic site of biosynthesis (13, 72, 82, 118). Citrate plays a central role in the integration of substrate availability and enzyme activity. Because acetyl-CoA from oxidation of most nutrients (CHO, fatty acids, amino acids) is generated in the mitochondria whereas DNL occurs in the cell cytosol, acetyl-CoA must be transported from the interior of the mitochondria to the cytosol for DNL. The primary mechanism for transporting mitochondrial acetyl-CoA to the cytosol (82) involves transport of citrate out of the mitochondria, followed by its cleavage to acetyl-CoA and oxaloacetate by the cytosolic enzyme ATP:citrate lyase (Figure 1). In addition to its role in acetyl-CoA transport, citrate activates ACC (72, 118), the enzyme that catalyzes the committed first step in DNL and produces malonyl-CoA.

The hypothesis that mitochondrial citrate serves as an integrating signal in the regulation of DNL fits nicely with the teleologic notion that the primary function of DNL is to provide a disposal route for excess CHO energy.

Citrate concentration may represent a marker of the integrated state of CHO energy available to a cell, because the rates of both production and removal of citrate are influenced by the availability of energy in the form of CHO. Synthesis of citrate is catalyzed by citrate synthase. The reaction rate is sensitive to the concentration of its substrate oxaloacetate (OAA), because the intramitochrondrial OAA concentration is below the K_M of the enzyme (70). OAA synthesis, in turn, is affected by pyruvate availability and the activity of pyruvate carboxylase, which in turn is activated by acetyl-CoA, whereas pyruvate availability is affected by glycolytic flux (107, 120). Availability of mitochondrial pyruvate reflects the availability of glucose and insulin to the cell and, thereby, controls the production of citrate. Metabolism of mitochondrial citrate is regulated by the activity of the tricarboxylic acid (TCA) cycle, the rate of which controls the energetic requirements of the cell—TCA cycle flux being controlled primarily by NAD/NADH, ADP/ATP, and CoA/acetyl-CoA ratios (70). To summarize these regulatory interactions, mitochondrial citrate concentrations are high when glycolysis is active relative to oxidation of pyruvate via pyruvate dehydrogenase, thereby allowing synthesis of OAA and citrate, and when ATP, NADH, and acetyl-CoA are abundant, thereby slowing TCA cycle flux. Citrate concentrations are therefore well positioned to reflect the availability of surplus CHO energy in a lipogenic cell.

Support for the citrate-regulatory model of DNL has been documented in a variety of physiologic conditions. Lane and colleagues (87, 120) have provided evidence, for example, that glucagon inhibits DNL in cultured hepatocytes by inhibiting glycolysis, thereby reducing production of pyruvate, OAA, and citrate. Pyruvate added to in the medium completely reversed the inhibition of DNL by glucagon and caused increases in OAA and citrate concentrations, providing further evidence that substrate availability mediates the antilipogenic actions of this hormone. The action of other activators or inhibitors of DNL may be explained by this substrate regulatory model (24, 84).

McGarry & Foster (84, 85) extended this model to include integration with fatty acid oxidation. Their work demonstrated that the first committed metabolite in the DNL pathway (malonyl-CoA, figure 1) is also a potent inhibitor of mitochondrial fatty acid oxidation by virtue of its inhibition of carnitine palmitoyl transferase I (CPTI). By inhibiting transport of fatty acyl-CoAs into the mitochondria for ß-oxidation, malonyl-CoA serves a dual function as substrate for synthesis of fat and regulator of oxidation of fat. The citrate/malonyl-CoA/CPTI model thereby provides a simple and elegant biochemical explanation for the mutual exclusivity of ß-oxidation and DNL in tissues. These regulatory findings are consistent with a model of DNL as an overflow pathway for surplus CHO energy in the macronutrient energy economy.

Activity of DNL Enzymes in Different Tissues

In several nonhuman species (see above), the levels of the enzymes of DNL respond to dietary and hormonal perturbations (5, 20, 36, 38, 86, 113, 115). On the other hand, the total capacity of human liver or adipose tissue for DNL, when calculated from in vitro enzyme activities (106, 109, 121), is quite low (Table 1). An important quantitative role for DNL is not possible if these ex vivo estimates are valid. Tissue content of enzymes cannot, of course, provide definitive evidence concerning actual flux through an assembled system in vivo, because local conditions, availability of substrates and cofactors, existence of metabolite channeling, and other determinants of integrated enzyme activities in an assembled pathway cannot be reproduced with fidelity ex vivo (50, 64, 92).

Gene Regulation

The expression of genes that encode the enzymes involved in DNL is regulated by hormones and nutrients (20, 38, 39, 54, 110, 115). The promoters of several of the genes for lipogenic enzymes, including those for fatty acid synthetase and ACC, are regulated by dietary state and hormones. Insulin or CHO feeding, for example, increase transcription of these genes, whereas fatty acids reduce gene expression (20, 38, 39, 54, 110, 115)

Summary of Biochemical Evidence

Although important for providing the anatomic map, the regulatory themes, and the general constraints of the DNL pathway, biochemical observations of the type just summarized cannot provide a definitive answer to questions regarding quantitative fluxes through the intact lipogenic system in vivo. For quantitative and functional questions like these, measurements of the process itself in situ are required in each organism and condition of interest.

IN VIVO EVIDENCE IN HUMANS: INDIRECT MEASUREMENTS

Until recently, direct measurement of DNL in humans has been problematic, so that most published studies have been based on indirect methods of measurement. These indirect approaches are reviewed first.

Indirect Calorimetry

The technique of indirect calorimetry is based on the measurement of gas exchange and urinary nitrogen. Intracellular oxidation of carbohydrates, lipids, and proteins provides the only source of energy useful to the human

Table 1 Tissue enzyme activities in humans and rat[a]

Enzyme	Liver		Adipose						
	Human	Rat	Human			Rat			
			pr	sc	om	pr	sc	om	ep
PDH	1.5	16.4		2.0					23.6
ATP citrate lyase	1.0	0.8	0.9	ND	1.5	73.1	33.5	36.8	
FA synthase	0.8	6.4	1.0	0.9	1.0	52.2			54.3

[a]Measurements shown in milliunits per milligram of protein. Abbreviations: pr, perirenal; sc, subcutaneous; om, omental; ep, epididymal; ND, not detectable. Adapted from References 106, 121. Data on human tissues were from subjects on typical western diets (fat content up to 40%). No differences were observed after a high-carbohydrate diet for three days in these studies.

organism and follows a specific stoichiometry for each nutrient. Because approximately 1 g of urinary nitrogen arises from the oxidation of 6.25 g of protein, protein oxidation is easily estimated. Thus, for each 1 g of protein oxidized, a known amount of oxygen is consumed and a known amount of carbon dioxide is produced. By difference, it is possible to determine non-protein carbon dioxide production due to carbohydrate and fat oxidation. Several more-comprehensive reviews of the topic have been published (32, 59, 112). The stoichiometry of the oxidation of glucose (*a*) and palmitate (*b*) is as follows:

$$(a)\ C_6H_{12}O_6 + 6\ O_2 \rightarrow 6\ H_2O + 6\ CO_2 \qquad (RQ = 1.0);$$
$$(b)\ C_{16}H_{32}O_2 + 23\ O_2 \rightarrow 16\ H_2O + 16\ CO_2 \qquad (RQ = 0.7).$$

The nonprotein (NP) respiratory quotient (*RQ*) is the quotient of carbon dioxide production ($NPVCO_2$) to oxygen consumption ($NPVO_2$) and is an indicator of the relative proportion of carbohydrate and fat oxidation. The *RQ* is 1.0 when glucose is oxidized and 0.7 when fat is oxidized. The conversion of glucose into fat (DNL) increases the *RQ*. One possible stoichiometric equation for DNL is:

$$4.5\ C_6H_{12}O_6 + 4\ O_2 \rightarrow C_{16}H_{32}O_2 + 11\ H_2O + 11\ CO_2 \qquad (RQ = 2.75).$$

Different stoichiometries have been proposed for DNL, and this has resulted in some doubts about the validity of the method when the NP *RQ* is greater than 1. The validity of indirect calorimetry has been addressed elsewhere by an algebraic approach with minimal assumptions about stoichiometries (32). Yet, it is crucial to realize that during metabolic processes such as gluconeogenesis or DNL, indirect calorimetry does not give the actual oxidation rate,

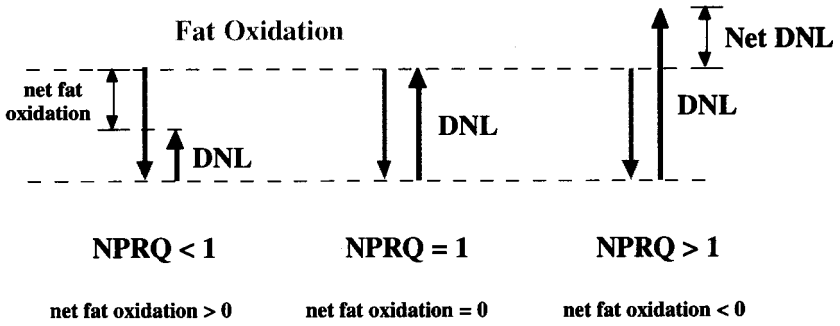


Figure 2 Net versus absolute DNL by indirect calorimetry. Indirect calorimetry measures the difference between fat oxidation and de novo fat synthesis. Only when synthesis of fat is in excess of oxidation of fat is net DNL observed.

it gives the net oxidation rate of the substrate considered. For example, glucose could be either directly oxidized to $CO_2 + H_2O$ or first converted to fatty or amino acids and then oxidized to $CO_2 + H_2O$; there would be no difference by indirect calorimetry. Indirect calorimetry accounts for the complete oxidation of each substrate to $CO_2 + H_2O$, independent of the metabolic pathways followed (112). If, for example, fat is being produced from glucose in the liver and fat is concurrently oxidized in muscle, the indirect whole-body calorimetry will measure these combined processes as net glucose oxidation. In normally fed subjects less fat is generally made de novo than is being oxidized. Consequently, the NP *RQ* is less than 1, and indirect calorimetry does not reveal the presence of DNL (Figure 2). Only when the amount of fatty acids made de novo exceeds the amount concomitantly oxidized is the NP *RQ* greater than 1. Under these conditions, indirect calorimetry measures net DNL by giving a net negative rate of fat oxidation; the difference corresponds to the net quantity of fat made de novo (Figure 2).

A large literature exists in which indirect calorimetry has been used to estimate DNL in humans.

NORMAL DIET Under conditions of a normal western diet (i.e. high fat, eucaloric), NP *RQ* is less than 1.0 throughout the day (1–3, 8, 21, 57, 96, 103), and net DNL is not observed either in the fasted or in the fed state. A single large meal containing up to 500 g of CHO does not cause NP *RQ* to exceed 1.0 (2).

OVERFEEDING DNL is clearly not responsive to acute modulations in CHO energy availability over the course of a single day in humans, at least by this

assessment technique. Surplus CHO in a meal is stored as glycogen or is oxidized, not converted to fat (1, 2, 21, 57, 103). Overfeeding with CHO for 3–5 days also does not result in a significant stimulation of net DNL (44–48). The finding that surplus dietary CHO fails to induce net DNL is an important conclusion based on indirect calorimetry.

After periods of CHO overfeeding longer than three to five days, however, NP *RQ* values greater than 1.0 have been observed. Acheson et al (1) observed a NP *RQ* of 1.17, with a calculated net DNL of 150 g/day, after 7–10 days of massive CHO overfeeding (surplus 4500 kcal/day, on average 2000 g of CHO/day). Net DNL was not observed the first day but only after two days of overfeeding, or after a positive whole-body CHO balance of 800 g had occurred. Their interpretation (1) was that body glycogen stores must be filled to maximum capacity before substantial rates of DNL are induced, and that glycogen storage capacity of normal-weight humans may be greater than was previously estimated. Interestingly, the net DNL was associated with a sixfold elevation of plasma triglyceride concentrations after seven days of overfeeding (8.6 versus 1.3 mM).

OTHER CIRCUMSTANCES Other instances of a NP *RQ* greater than 1.0 have been documented in humans. Administration of intravenous nutrients at high rates, particularly in sick patients, has been associated with induction of net DNL (7, 42). An interesting example of this is the sick, premature infant placed on intravenous nutrition, in whom NP *RQ* values of 1.15–1.20 are not unusual (W Chwals, personal communication).

SPECIFIC NUTRIENTS Certain nutrients such as ethanol, fructose, and sucrose are suspected to stimulate DNL on the basis of the pathways by which they are assimilated in the liver (Figure 1), and based on animal studies (80, 83, 105). Ingestion of ethanol increases NP *RQ* but typically not to greater than 1.0 (111). Suter et al (111) concluded, based on indirect calorimetry, that ethanol reduces oxidation of fat but does not stimulate net DNL in humans. It is worth recalling that if hepatic DNL were induced by ethanol while fat oxidation still occurred in the body, the apparent effect by indirect calorimetry would be a reduction in the oxidation of fat, not occurrence of the lipogenesis/fat oxidation cycle. The inability to exclude concurrent fat oxidation and DNL represents an important limitation of indirect calorimetry.

In summary, indirect calorimetry has indicated a limited function for net DNL under all but the most extreme dietary conditions, but this technique is not able to measure true, unidirectional rates of DNL.

Adipose Tissue Fatty Acid Composition

Hirsch proposed an original and elegant technique for estimating DNL over 30 years ago (55). He compared the fatty acid compositions of adipose tissue

and diet in normal-weight humans. If substantial DNL were occurring, one would expect an increase in the proportion of nonessential fatty acids (e.g. palmitate, stearate) relative to essential fatty acids (e.g. linoleate). Adipose fatty acid composition was found to match closely the composition of the diet. After imposition of a controlled diet with a different fatty acid composition for six months under metabolic ward conditions, the adipose fat composition varied in parallel with the diet. He concluded that "we are what we eat" in terms of fatty acids—that is, DNL is not important under normal dietary conditions in humans (55). This approach, however, cannot exclude preferential oxidation of newly synthesized fatty acids. Nor is it practical to investigate the effect of various dietary perturbations (overfeeding, high-carbohydrate diet, ethanol, etc) by this method.

Whole-Body Macronutrient Balances

On rare occasions, simple stoichiometry may be sufficient to allow inference of DNL in the organism. If accumulation of body fat exceeds total intake of dietary fat, for example, then nonfat energy must have been converted to fat. This is commonly observed in livestock animals—e.g. pigs, chickens (5, 73, 86, 97, 113). There is also one notable example in the modern scientific literature in humans—the Guru Walla model (95). This is a traditional fattening ritual that takes place in Cameroon, wherein adolescent boys are intentionally and massively overfed for two months as part of a rite of passage to manhood. The diets are high in carbohydrates (ca. 70%). These boys were documented to eat close to 7000 kcal per day and to gain 12 kg of fat over 10 weeks while eating only 4 kg of fat. These calculations of net fat accumulation therefore require that dietary CHO have been converted to fat under these highly unusual dietary conditions. DNL was not directly measured, however. Actual DNL flux (as compared to net accumulation of fat), the timing of induction for DNL in relation to whole-body CHO balances, the circadian pattern of DNL, and the tissue or tissues responsible for DNL were not determined. It must be emphasized that the dietary conditions imposed in the Guru Walla (95) are highly unusual and do not apply to normal physiology or to the kind of energy storage that may be observed under ad libitum overfeeding conditions in humans. This observational study strongly suggests, however, that quantitatively important rates of DNL can occur in humans.

IN VIVO EVIDENCE ABOUT THE REGULATION OF DNL IN HUMANS: DIRECT MEASUREMENTS

As for any biosynthetic process, direct measurement of DNL requires the use of isotopic labels (49, 101, 122, 123). All measurements of biosynthesis attempt

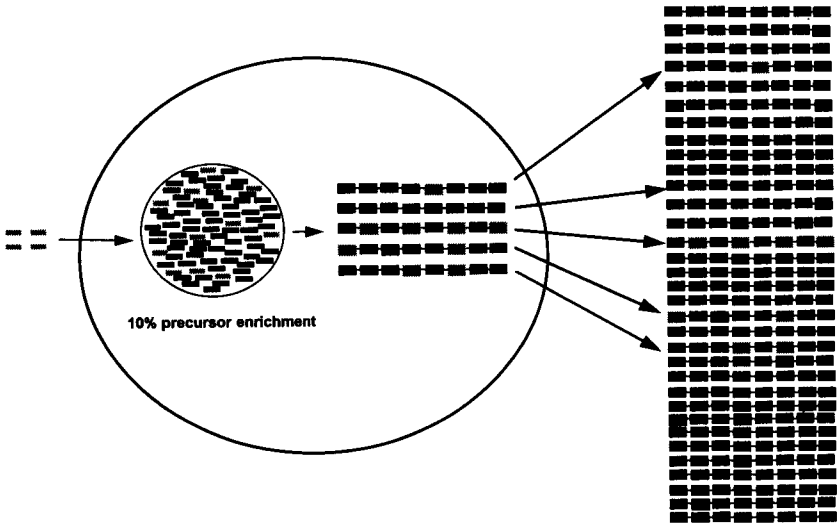


Figure 3 Schematic representation of biosynthesis and its measurement with isotopes. A labeled precursor such as [^{13}C]acetate (*diagonal lines*) is introduced into an unlabeled precursor pool, in this case cytosolic acetyl-CoA. The precursor pool also contains monoisotopic (*solid*) and natural abundance isotopic-containing (*stippled*) subunits. The labeled subunits achieve a 10% proportion in the pool, from which a polymer containing eight subunits (i.e. palmitate) is synthesized. The newly synthesized polymer molecules in the cell exhibit a characteristic distribution of labeled subunits. These newly synthesized polymers then mix with preexisting polymers that contain the natural abundance distribution of precursor subunits. The objective of a biosynthesis measurement is to quantify the fraction of newly synthesized polymers in the final mixture. This is achieved by identifying the isotope content of the newly synthesized polymers and calculating their dilution in the mixture. The isotope content of the newly synthesized polymers can be calculated from the proportion of labeled subunits in the precursor pool.

to answer the following question with respect to a population of biochemical molecules (Figure 3): Which product molecules were newly synthesized over a period of time and which ones were preexisting? Thus, the essential task in measuring biosynthetic kinetics is to identify and quantitate in an in vivo mixture of product molecules those that were newly synthesized. One can then calculate the rate of metabolite flow through the biosynthetic pathway. Biosynthesis typically involves polymerization, in which monomeric subunits combine to form a polymeric end product (Figure 3). This chemistry has led to an investigative strategy: namely, to introduce a labeled metabolite that enters the pool of monomeric subunits and to quantify the passage of these labeled subunits through the biosynthetic pathway and into the polymeric end product. Isotopes are necessary for this purpose because they are identifiable—one can distinguish between labeled and unlabeled molecules and thereby can

differentiate newly synthesized from preexisting polymers by use of a suitable instrument (e.g. liquid scintillation counter or mass spectrometer).

Thus, the information about biosynthesis that can in principle be learned uniquely from isotopic incorporation studies is the fraction of molecules in the product pool that were newly synthesized over a given period of time. How does an investigator calculate the fraction of newly synthesized molecules in a mixture? The answer is by knowing how much isotope label was incorporated in the newly synthesized molecules. If one knows the isotope content of newly synthesized molecules, a simple ratio between label in the mixture relative to label in the newly synthesized molecules reveals the biosynthetic fraction (Figure 3). Unfortunately, within a population of biomolecules there is no physical method for distinguishing between newly synthesized and preexisting molecules, so the isotope content of the new molecules cannot be determined in any simple manner from measurements on the mixture. It is here that the underlying chemistry of polymerization from monomeric subunits can be exploited. Instead of the newly synthesized molecules, one can measure how much label is in the subunit precursor pool from which new molecules were synthesized (Figure 3). The only requirement, then, is to isolate from the cell the true precursor subunits and measure their isotopic content.

Although this requirement to measure isotopic enrichment of the precursor pool may seem straightforward, it has represented a challenge to researchers for over 40 years. Problems arise from the complexity of subcellular organization. Cell biology is characterized by inhomogeneity and complexity: membrane-associated microenvironments, metabolite channeling through multienzyme complexes, functional compartmentalization of metabolite pools, regulation of the channeling mechanisms (64, 92), etc. Every biosynthetic system that has been carefully evaluated has revealed complexity in the metabolic and isotopic behavior of its precursor pool. In the case of lipogenesis and cholesterogenesis, for example, the acetyl-CoA used in the liver for synthesis of ketones and for citrate oxidized in the TCA cycle differs from the pool used for DNL and cholesterol synthesis(25–27). As a consequence, calculation of precursor-specific activity based on indirect measures of acetyl-CoA labeling—such as labeling of secreted ketones or administered acetate—have resulted in "serious errors in the estimation of true precursor labeling and biosynthetic rates" (26). In the case of protein synthesis, as well, the amino acid pool used for synthesis of tRNA-amino acids is variable, regulated by ambient nutrient and hormonal conditions and different from tissue to tissue (4, 11, 68, 119). Therefore, differences in isotope incorporation into a protein product even when corrected for specific radioactivity of the extracellular amino acid may reflect differences of label entry into the tRNA-amino acid pool rather than true differences in protein synthesis (119). Analogous problems have been observed for synthesis of carbohydrates (65, 70), nucleic acids (53), and other polymers.

Moreover, this is not just a case-by-case problem; it is a fundamental one for classic biochemistry. In principle it is not sufficient to isolate the putative precursor molecule from the cell and measure its isotopic content because one can never be certain whether or not a putative precursor molecule isolated from a cell (acetyl-CoA from liver, tRNA-leucine from muscle, etc) represents the correct pool, i.e. the one used for polymerization to a particular polymer. A point that is obvious but that has been convenient to ignore is that any subunit that a biochemist can isolate from the cell by definition did not get selected to enter the polymer. How well it represents the true biosynthetic pool can therefore can be called into question, i.e. the pools isolated biochemically from cells may be precisely those molecules that are not in the functional precursor pool desired.

There are two potential theoretical strategies to resolve this problem of identifying the isotopic content of the true biosynthetic precursor pool amidst the complexity of subcellular biological systems. One would be to identify definitively the particular subunits that actually entered the polymer and to measure their isotope content. The second solution would be to use a precursor molecule were used that does not exhibit subcellular compartmentalization or isotopic inhomogeneity. Then, any metabolite pool sampled would represent the true biosynthetic precursor pool. These two strategies may not sound feasible, but in fact they form the basis of recently developed stable isotope-mass spectrometric techniques that resolve this fundamental problem of biosynthetic measurements. The first technique, called mass isotopomer distribution analysis (MIDA), is based on the mathematics of combinatorial probabilities. Analysis of the pattern of labeling in a polymer itself reveals the isotopic content of the precursor subunits from which new molecules were synthesized (33, 44, 47, 49, 51). The second technique involves incorporation of ^2H_2O (62, 63, 77) because membranes are freely permeable to water, and it should not exhibit compartmentalization. These two techniques are described in detail.

MIDA

MIDA is based on the mathematics of combinatorial probabilities and on the principle that the subunits present in a polymer itself represent unequivocally the precursor pool used for its own synthesis. The key principles of MIDA can be summarized as follows: (*a*) Polymers are composed of repeating monomeric subunits; (*b*) the isotope distribution or relative proportions of polymers containing different numbers of labeled subunits—unlabeled, single-labeled, double-labeled, etc, species—is determined by the proportion of labeled monomers in the precursor pool, in accordance with combinatorial probabilities calculable from the binomial or multinomial expansion (Figure 4); (*c*) the

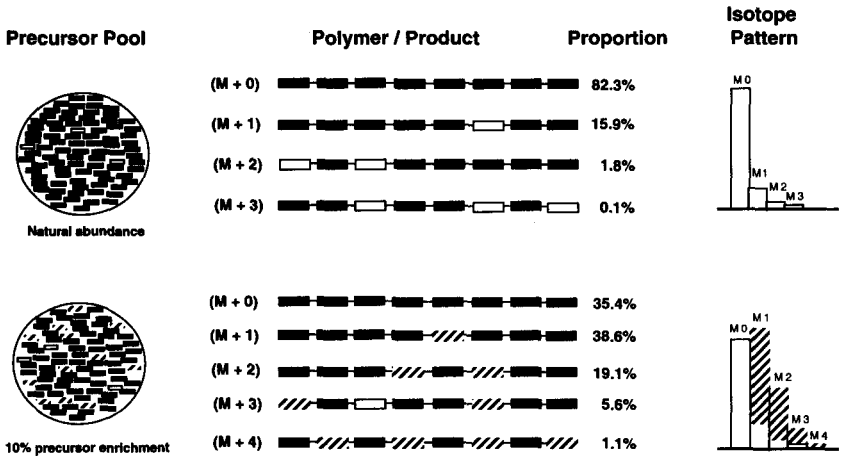
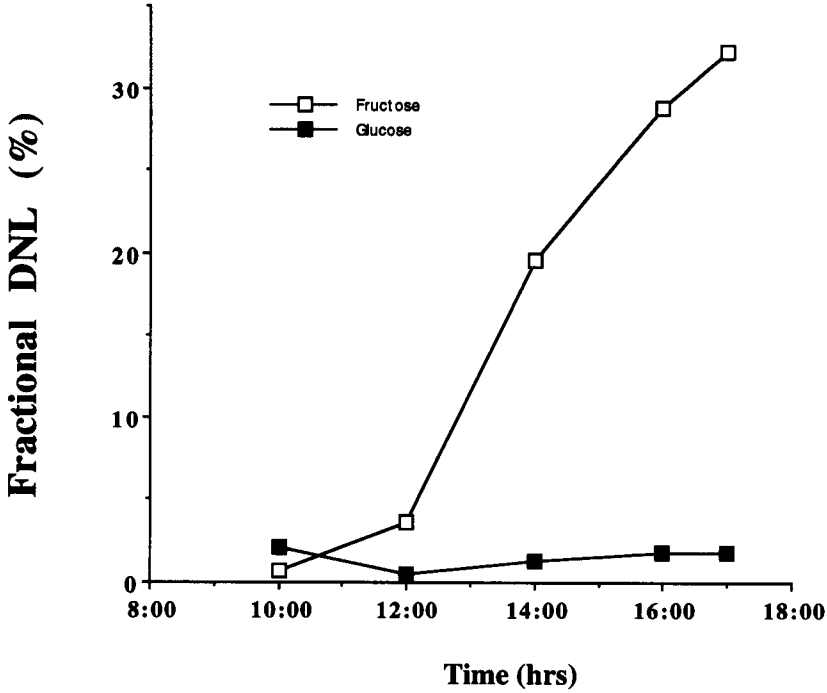
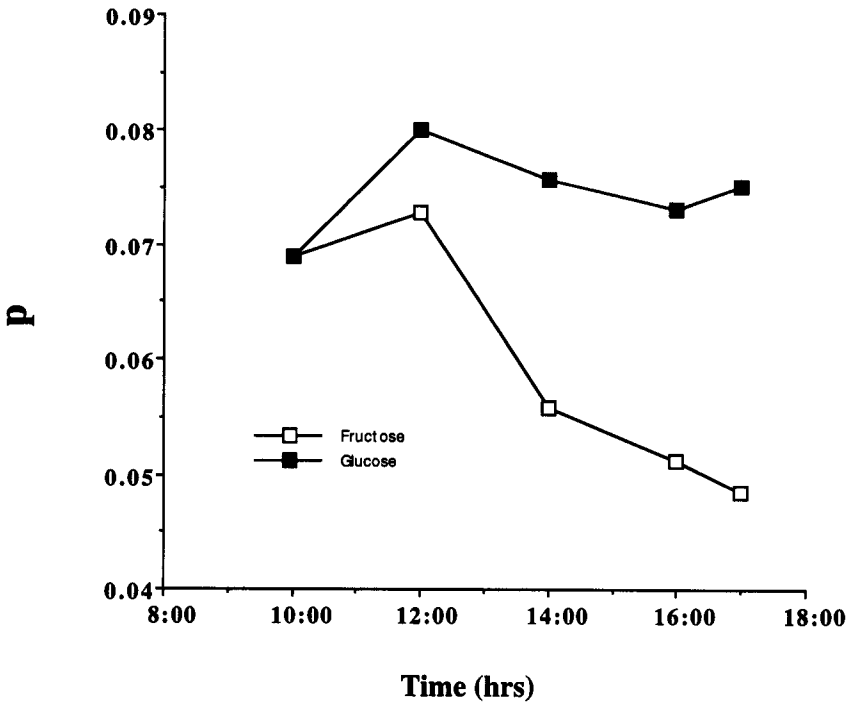


Figure 4 Principle of MIDA technique for measuring biosynthesis of polymers, such as fatty acids. Precursor pools at natural abundance or containing 10% of an isotopically labeled subunit (e.g. [^{13}C]acetate) are shown. The population of polymers containing eight 2-C units (palmitate) synthesized from each of these two pools will contain characteristic statistical distribution of M + 0 (monoisotopic), M + 1, M + 2, etc, molecular species. Palmitate molecules formed from a pool with 10% of acetyl-CoA subunits labeled, for example, will have much higher fractional abundances of M + 1 and M + 2 species than those synthesized from a population of precursors with natural abundance (38.6 and 19.1% M + 2 in palmitate from 10% labeled acetyl-CoA compared to 15.9 and 1.8%, respectively, in palmitate from natural-abundance acetyl-CoA). These proportions in the polymer can be represented as a frequency histogram of the isotope pattern (*right*), measured by mass spectrometry. The isotope pattern in the polymer, after correction for natural abundance, is characteristic of products synthesized from precursors at a particular level of enrichment. Accordingly, the degree of enrichment of the precursor pool can be calculated by comparing measured isotope patterns in the polymer to theoretical ones.

isotope distribution in newly synthesized polymers is not altered by mixture with unlabeled polymers but remains unique and characteristic of the newly synthesized polymers through their biological lifetime; (*d*) the isotope pattern of a polymer can be measured by mass spectrometry and altered by introduction of a stable isotope-labeled subunit into the biosynthetic precursor pool; and (*e*) the proportion of labeled monomers in the biosynthetic precursor pool can therefore be calculated from the isotope pattern in the polymer mixture by comparing the isotope pattern in the polymers to expected statistical distributions.

These principles of combinatorial probabilities as applied to polymerization biosynthesis are shown pictorially in Figures 3 and 4. A labeled precursor enters the intracellular pool of biosynthetic precursors. Newly synthesized polymers are formed from this pool and reflect an isotope distribution characteristic of the proportion of labeled precursors present when these





newly synthesized polymers were formed. These label-enriched polymers mix with preexisting, unlabeled polymers whose isotope distribution reflects natural isotope abundances. The difference in isotope pattern between the natural abundance (baseline) and an isotopically enriched mixture of polymers reveals the degree of enrichment of the precursor pool. This allows determination of the isotope content of newly synthesized polymers, which in turn allows determination (by dilution) of the fraction of newly synthesized polymers present.

In practice, the polymer is isolated before adding lebeled precursor (baseline) and after incorporation of a stable-isotope labeled precursor and isotopomer abundances are quantified by mass spectrometry. The isotope distribution in the enriched mixture is corrected for the natural abundance isotope distribution, and biosynthetic parameters are calculated (precursor pool enrichment, fractional synthesis, replacement rate of the polymer, and absolute synthesis rate). MIDA has been applied to the measurement of fatty acids (12, 33, 44, 47, 48, 51, 89, 94, 103), cholesterol in plasma and bile (9, 33, 88, 100), glucose (43, 90, 91), glycogen (90), glucuronate (79, 90), and proteins (16, 93).

An example of the biosynthetic parameters for DNL that are measurable by MIDA is shown (Figure 5). In this study (102), fructose (10 mg/ kg of lean body mass/min) was administered orally to a normal-weight man after an overnight fast. Sodium [1-^{13}C]acetate was infused prior to and during fructose administration. Plasma very-low-density lipoprotein (VLDL) was isolated by ultracentrifugation and transmethylated to generate methyl-esters of palmitate and stearate, which were analyzed by gas chromatography/mass spectrometry. The fractional abundances of the parent molecular species (M0), the species containing a single [^{13}C]acetate subunit (M1), and those containing two [^{13}C]-acetate subunits (M2) were determined for the enriched and unenriched states. The ratio of excess M2/excess M1 molecules is characteristic of the true enrichment (p) of precursors for newly synthesized palmitate molecules, i.e. the isotopic enrichment of hepatic acetyl-CoA. In Figure 5, p is 0.08 prior to fructose and 0.05 after fructose, indicating an influx of unlabeled acetate units derived from fructose. Fractional DNL (f) rose markedly from fasted values of 1% to fructose-stimulated values of 32%. The kinetics of the increase in DNL was then modeled (103) to calculate calculate turnover of VLDL-triglyceride (TG) and absolute rate of DNL (see below).

Since our original description of MIDA (33, 44, 47, 51), other algorithms have been independently developed for this combinatorial probability-based

Figure 5 Effects of equicaloric fructose versus glucose ingestion (10 mg/kg of lean body mass/min) on hepatic fractional DNL (*top*) and on the enrichment (p) of the precursor pool (hepatic acetyl-CoA) (*bottom*).

approach. Lee et al (74–76) have used a matrix-correction algorithm to correct for natural isotope abundances, whereas Kelleher and colleagues (66, 67, 69) independently described a nonlinear least-squares, best-fit approach for solving for p and f simultaneously. These approaches differ only in the mathematical strategy used to solve for p and f; they are based on the same principles of combinatorial probabilities and molecular mixing and give identical results when compared directly (MK Hellerstein, RA Neese, unpublished observations; T Masterson, personal communication).

Incorporation of Labeled Water (^2H_2O)

Water diffuses freely across membranes and within cells, so it is reasonable to assume that functional pools with different isotope enrichments do not exist. Cellular water is a relevant precursor to DNL because some of the hydrogens of H_2O are incorporated into fatty acids (FA) during biosynthesis (Figure 1). They are derived from cellular H_2O directly or via NADPH (Figure 1). The ^3H_2O incorporation technique was developed almost 30 years ago (63) for measurement of lipid synthesis. Cellular H_2O is taken to represent the true precursor for hydrogen in newly synthesized lipid molecules, by way of H_2O or NADPH; H_2O specific activity is determined by sampling any body fluid. The precursor-product relationship is then applied (Figure 3).

The central problem with this method is that the proportion of NADPH hydrogens derived from water is not known a priori and depends on the metabolic pathway by which NADPH is generated in the cell (28). NADPH can come from the pentose phosphate pathway, malic enzyme, etc (Figure 1), and these pathways result in different degrees of exchange with hydrogen of H_2O. The investigator must choose a hydrogen:carbon (H:C) ratio—e.g. for cholesterol, an H:C value of 22 H/mol of cholesterol is usually selected (6, 28), and for palmitate, H:C is estimated to be 14 per mol (63). Unfortunately, the techniques used for estimating the H:C ratio in newly synthesized lipids have been both flawed and inconsistent. A range of values for cholesterol, for example, from 21 to 27 per mol has been reported (28). Recently, Lee et al used an elegant variation on the MIDA technique to calculate the H:C ratio in cholesterol (74). Instead of solving for p (the isotopic enrichment of the hydrogen atoms in H_2O) at a known n (number of hydrogen atoms in cholesterol that derived from H_2O), they solved for n at a known p (determined from cellular ^2H_2O enrichment). By this method, n ranged from 20–30, depending on the tissue, metabolic conditions, etc (74). Selmer & Grunnet (104) had previously shown that ethanol affects the H:C ratio. Calculation of H:C by MIDA (74) is not currently feasible in humans, unfortunately, because of constraints on ^2H_2O dosage. This uncertainty regarding H:C ratio represents the major unsolved problem of the method.

An important recent advance has been the adaptation of the ^3H_2O incorporation method to stable isotopes (^2H_2O) by use of isotope ratio/mass spectrometry by Jones et al (61, 62, 77, 78). ^3H_2O doses required for this procedure are far too high for safe use in humans and had previously prevented use of labeled H_2O incorporation methods in humans. The development of ^2H_2O technique (61, 62, 77, 78) represents an important advance for human studies. Again, however, a constant H:C value is assumed (62, 77).

REGULATION OF HEPATIC DNL IN HUMANS

With the development of MIDA and ^2H_2O incorporation techniques, evidence has begun to emerge concerning the regulation of hepatic DNL in humans. We discuss these results in the context of several basic questions regarding hepatic DNL.

Question 1: What Is the Extent of Hepatic DNL on Standard Western (High-Fat) Diets?

The results with MIDA and ^2H_2O confirm the conclusion based on previous indirect methods—that DNL is quantitatively quite low under normal conditions on typical high-fat diets. We (33, 47, 48, 89) have studied normal, weight-stable men on standard western diets (30–40% fat calories). DNL accounted for less than 3% of VLDL-palmitate or stearate in the postabsorptive state and less than 5–7% in the fed state. Absolute DNL was less than 1 g synthesized per day—a very small number compared with dietary intake of fat, which is typically 100–200 g/day. Jones et al (76) reported almost identical values of DNL using ^2H_2O incorporation. These direct isotopic measurements of DNL therefore exclude the fatty acid synthesis/fat oxidation cycle and confirm that the indirect calorimetric estimates are essentially correct in this setting.

Question 2: Is Hepatic DNL Regulated (Qualitatively or Quantitatively) by Nutrients or Hormones in Conditions Other Than CHO Overfeeding?

The effects of a number of dietary or endocrine conditions on hepatic DNL have been studied in humans by use of MIDA or ^2H_2O incorporation techniques.

FRUCTOSE VERSUS GLUCOSE INTAKE We (102) administered equicaloric amounts of glucose or fructose (7–10 mg/kg of lean body mass/min for 6 h orally) to healthy normal-weight men. Fructose stimulated fractional DNL to greater than 32% (Figure 5), whereas glucose had essentially no effect (DNL

remaining less than 3%). The absolute rate of conversion of fructose to fat still represented less than 10% of the fructose load ingested, however, and the absolute rate of hepatic DNL remained small (less than 1 g/palmitate synthesis/h). Long-term fructose in the diet (15% of calories for three weeks in a eucaloric diet) did not alter fractional or absolute DNL in the fasted state or in response to acute fructose ingestion. Thus, fructose has a striking qualitative effect on DNL but not an important quantitative one. Whether fructose intake causes hypertriglyceridemia in normal or diabetic humans is still controversial (10), but these results suggest some mechanisms by which hepatic production of TG-containing lipoprotein particles might be affected. First, the activation of lipogenesis presumably reflects increased malonyl-CoA concentrations and an antiketogenic milieu in the liver (see above and Figure 1), which would favor reesterification of plasma free fatty acids (FFA) (84, 85). Second, recent studies in isolated hepatocytes (30, 37) indicate that hepatic FA made by DNL are more effective than hepatic FA taken up from the extracellular medium at stimulating entry of TG into the secretory (VLDL) pathway relative to the cytosolic storage pathway. FA from DNL appear to be especially efficient at mobilizing apolipoprotein B and inducing VLDL particle assembly, which may, in turn, increase hepatic cholesterol synthesis as well (37). Dietary fructose may thereby alter hepatic lipoprotein production out of proportion to the grams of fat produced. Dietary sucrose has not yet been studied in this manner.

VERY-LOW-FAT, HIGH-CHO EUCALORIC DIETS Very-low-fat (e.g. 10–20%), high-CHO (e.g. 65–75%) diets in the absence of surplus energy have important effects on serum lipoproteins and body fat stores (23, 29). The role of DNL in these processes has recently been addressed (58) by combining the MIDA technique with a modification of the essential FA dilution technique of Hirsch (55; see above). Normal-weight subjects were placed on weight-maintaining formula diets containing either a typical fat/CHO content or very-low-fat/high-CHO content (10% fat/70% CHO) for three weeks, under in-patient metabolic ward conditions. The FA composition of the formula diet was matched to the FA composition of each subject's adipose tissue. At the end of the very-low-fat high-CHO diet period only, fractional hepatic DNL (as measured by MIDA) was increased to greater than 20% (fasting) and about 30% (fed state). A diurnal pattern was observed with maximal DNL in the late evening. Changes in fractional DNL based on dilution of dietary/adipose linoleic acid (18:2) in VLDL-TG paralleled changes observed with the MIDA measurement. Interestingly, the estimate of DNL from 18:2 dilution was systematically higher than by the MIDA measurement, consistent with some selectivity of FA metabolism (differential tissue uptake of essential from nonessential FA) under these low-fat dietary conditions. Also of interest, marked hypertriglyceridemia was observed in some subjects, as has frequently been

reported on low-fat diets (23, 29). The same mechanistic considerations discussed for dietary fructose (see above) are relevant to low-fat diets with regard to hypertriglyceridemia.

In quantitative terms, however, absolute DNL was still a small number: fewer than 10 g of fat synthesized per day by MIDA, and NP *RQ* remained less than 1.0 in both fasted and fed states. From an energy balance viewpoint, there was no stochiometric requirement for conversion of CHO to fat in these studies because these were eucaloric diets rather than overfeeding experiments. The question of whether or not humans can perform large-quantity DNL was therefore not addressed. Moreover, since these were controlled diets, the impact of short-term fluctuations in CHO energy intake under conditions of chronic low-fat high-CHO diets was not addressed. If dietary fat suppresses DNL (20, 115), it is possible that day-to-day alterations in energy intake on low-fat diets might induce quantitatively important flux through DNL. No direct evidence has yet been presented on this hypothesis.

MEAL FREQUENCY The issue of nibbling versus gorging was addressed by Jones et al (61), who used ^2H_2O incorporation to measure hepatic DNL. Although moderate increases in DNL were observed on the larger, less-frequent meals regimen, the effects were minor.

ETHANOL INTAKE Ethanol (EtOH) is known to have substantial but incompletely understood effects on whole-body fat metabolism in humans (80, 111). Suter et al (111) reported that acute intake of EtOH suppressed oxidation of fat, based on the indirect calorimetric observation of increased NP *RQ* and the assumption that all EtOH was oxidized. An alternative explanation can be proposed that is consistent with their results—that hepatic DNL was stimulated by EtOH (Figure 1), whereas FA oxidation continued, with the net effect appearing to be reduced oxidation of fat (see above). These questions are relevant not only to body weight and macronutrient balances (111), but to the etiology of fatty liver and hypertriglyceridemia, both of which can be brought on by chronic EtOH intake in susceptible humans (80). The role of DNL versus reesterification of exogenous FFA in hepatic TG synthesis induced by EtOH has long been studied in experimental animals (80) but never previously in humans.

We (108) have performed preliminary studies of DNL and FFA metabolism after EtOH intake in normal-weight human subjects. Ingestion of 30 g of EtOH (the equivalent of two mixed drinks) increased fractional DNL from less than 3% to greater than 30% for VLDL-palmitate and from less than 2% to 18% for stearate. VLDL-TG production, determined from the kinetics of rise to plateau for fractional DNL, was 1.08 g/h with a half-life of 1.1 h. Absolute hepatic DNL was calculated to be less than 0.8 g out of the 30 g of EtOH

ingested. It is possible that some newly synthesized FA were not secreted from the liver over the 4– to 6–h period monitored following EtOH intake. The preferential secretion into the VLDL pathway of newly synthesized FA (30) argues against this possibility. We also observed a marked inhibition of lipolysis and FFA release into the circulation, concurrent with increased NP *RQ*. These combined results suggest that acute EtOH ingestion indeed reduces fat mobilization and oxidation, as concluded by Suter et al (111), and does not induce a quantitatively major amount of DNL. The effects of chronic EtOH intake have not yet been investigated.

CYTOKINE MEDIATORS OF INFECTION Hypertriglyceridemia and abnormalities of nutrient partitioning favoring fat over lean tissue are commonly observed in states of infection or inflammation (41). Cytokines such as tumor necrosis factor, interleukin-1, interleukin-6, etc, increase VLDL-TG production and DNL in a variety of animal models (12, 41). Grunfeld et al (41) have proposed that the hypertriglyceridemia of infection serves a function in host defense, because circulating VLDL binds lipophilic materials such as bacterial cell walls (endotoxin) and certain viruses and thereby modulates the acute-phase response. We (80) observed threefold elevated DNL in patients with weight loss due to acquired immunodeficiency syndrome (AIDS). This finding is paradoxical according to classic starvation theory (15, 38): The last thing a person who has lost weight should do is convert lean tissue to fat; rather, fat is supposed to be oxidized to preserve lean tissue, in adapted starvation. Our finding of increased DNL in patients with AIDS-wasting provided early evidence that an abnormal metabolic milieu exists in at least a subset of AIDS patients, presumably mediated by increased cytokine production (45).

Interestingly, asymptomatic HIV-positive patients also exhibited somewhat elevated DNL (48), as well as triglyceride concentrations (41). These metabolic results provided the first evidence that early HIV infection is not a state of true viral latency. We have also asked whether or not measurement of DNL could be useful as a clinical management test, to select appropriate anabolic therapies in these patients (see below).

GONADAL HORMONES AND MENSTRUAL CYCLE Women have proportionately more fat than men. We asked whether or not hepatic DNL differs between the genders and if that could explain body composition differences. Young women were studied during the follicular and luteal phases of their menstrual cycles (33). DNL in the luteal phase was not increased; but it was increased two- to threefold in the follicular phase compared to that in men. Absolute DNL was still very low, however (less than 3 g/day), and would impose an estimated yearly fat burden of less than 1 kg (trivial compared to yearly intake of fat of 35–70 kg). Serum estradiol concentrations only weakly correlated with DNL.

A role of ad libitum dietary changes could not be excluded as an explanation of the higher-follicular-phase DNL in these free-living subjects. Although it will be worth pursing the hormonal or dietary factors modulating DNL in young women, hepatic synthesis of fat does not appear to explain gender differences in body composition in humans.

BODY WEIGHT CHANGES AFTER CESSATION OF CIGARETTE SMOKING As a group, smokers weigh less than nonsmokers, and weight gain after cessation of cigarette smoking (CS) is extremely common (17, 46). Fear of gaining weight is also one of the most common reasons given for not quitting, especially in young women. Some authors have suggested that weight gain after smoking cessation has a metabolic basis—e.g. hungry fat cells (17). We (46, 89) asked whether hepatic synthesis of fat was increased or whether whole-body oxidation of fat was decreased after cessation of CS. Whether subjects were maintained on eucaloric diets (46) or allowed ad libitum food intake (89), no differences in DNL were present during CS or non-CS phases. Basal mobilization of FFA and oxidation of fat were also not different after cessation of CS (although CS acutely increases lipolysis). We concluded that gain of weight and fat after cessation of CS can not be attributed to a primary metabolic abnormality independent of standard energy balance factors (thermogenic and appetite-modulating effects of CS).

OBESITY AND INSULIN RESISTANCE One might postulate that a defect in the suppressability of DNL by dietary fat could contribute to obesity, much as the failure to suppress cholesterogenesis by dietary cholesterol promotes hypercholesterolemia. We measured hepatic DNL in obese men with fasting hyperinsulinemia or normal serum insulin concentrations (102). Fasting DNL was moder- ately elevated (fractional DNL = 5–7%) in the hyperinsulinemic obese men only. Again, the rate of DNL could not account for even a minor part of the excess body fat stores present in obesity. It is interesting to speculate on the cause of a qualitatively elevated DNL in obese, hyperinsulinemic men. Insulin stimulates hepatic DNL (114), and we have observed a large increase in hepatic DNL in subjects with poorly controlled diabetes after an injection of insulin (MK Hellerstein, RA Neese, unpublished observations). For insulin to be responsible for elevated DNL in these insulin-resistant subjects implies differential insulin sensitivity among tissues or even among metabolic pathways within the liver (e.g. resistance to suppression of hepatic glucose production but sensitivity to stimulation of hepatic DNL). Alternatively, increased DNL in these subjects may reflect recent positive energy balance (see below), which would suggest a different model of obesity-related metabolic disturbances.

MISCELLANEOUS CONDITIONS Some other conditions have not yet been studied but are likely to exhibit very increased DNL. One of these conditions is

glycogen storage disease type 1 (GSD-1), or glucose-6-phosphatase deficiency. Individuals affected by this inborn error of metabolism exhibit glycogen and fat-laden livers, the tendency toward hypoglycemia, extreme hypertriglyceridemia, lactic acidemia, and antiketogenesis (116). These manifestations are seemingly all explicable by their inability to release hepatic hexose-phosphates into the circulation, with secondary accumulation of glycogen and glycolytic metabolites. Substrate-mediated activation of hepatic pyruvate dehydrogenase and DNL becomes one of the routes for disposal available for these metabolites. Plasma FFA flux is also increased in GSD-1 patients because of peripheral hypoinsulinemia. The role of hepatic DNL versus reesterification of FFA in the often massive hypertriglyceridemia exhibited by these individuals remains uncertain. Regardless, persons with GSD-1 seem to be an excellent population in which to look for quantitatively significant hepatic DNL.

A dietary factor that may induce substantial DNL is medium chain triglycerides (MCT). These are used as a fat source in patients with gastrointestinal disorders in whom absorption of long-chain FA is impaired. Unique features about the metabolism of MCT are that the CPTI/malonyl-CoA transport system (see above) is not used for entry into the mitochondrial ß-oxidation sequence. In addition, reesterification of MCT apparently does not occur to a significant extent. MCT are therefore believed to be converted quantitatively to mitochondrial acetyl-CoA. Indirect evidence for stimulation of DNL by MCT feeding has been presented by Van Lith et al (117). Direct measurement of DNL in MCT-fed humans, has not yet been reported, however.

CONCLUSIONS There are clearly a number of factors that modulate hepatic DNL qualitatively in humans—nutrients, hormones, cytokines, inherited enzyme alterations. In none of the circumstances so far discussed has DNL been documented to play a major quantitative role in the energy economy, however.

Question 3: Is DNL Stimulated to a Quantitatively Important Level by Surplus CHO Energy in the Diet?

Studies of CHO overfeeding for fewer than five days have uniformly revealed suppression of whole body fat oxidation but NP $RQ \leq 1.0$, without significant net DNL (see above). Many questions remained unanswered, however.

We have addressed the effects of surplus dietary CHO energy on DNL. First, DNL was measured by the MIDA technique in eight subjects allowed to eat ad libitum under metabolic ward conditions (89). For comparison, six subjects were maintained on a constant eucaloric diet (103). Both groups were followed for 14 days. Several of the subjects on ad libitum diets were found to ingest 5500–6000 kcal/day, including 750–1000 g of CHO/day (89). Fractional DNL increased to 15.2% (fasted) in comparison to the eucaloric diet

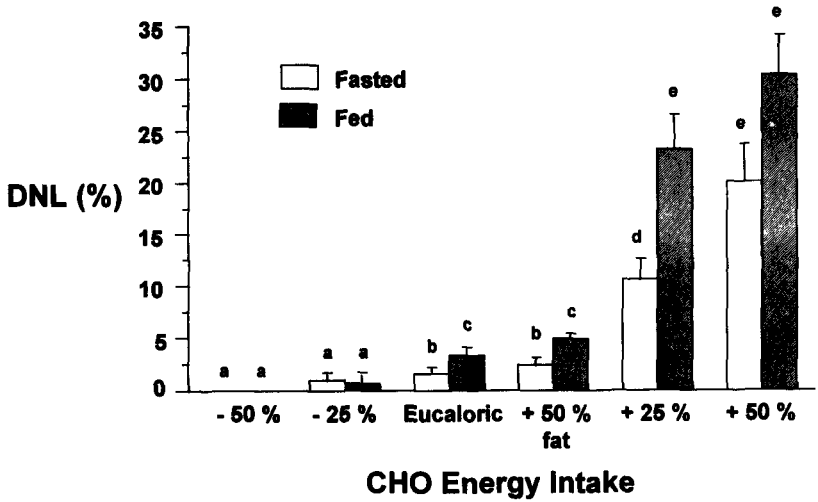


Figure 6 Effect of varying energy content and source of fractional DNL in normal-weight human subjects (from Reference 103, with permission). Percentages at bottom (–50% to +50%) refer to deficient or surplus CHO energy added to diet for five-day periods preceding measurement. %, Percent of palmitate in VLDL that was synthesized de novo from acetyl-CoA.

group's values of 2.5%. We also observed a highly significant direct correlation between excess energy intake and fractional DNL. Nevertheless, absolute DNL only accounted for 5 g of FA synthesized per day in the ad libitum group. The daily intake of dietary fat is 150–200 g and of dietary CHO is 750-1000 g; thus, DNL represents a minor pathway.

We repeated the study under controlled dietary conditions (103). Normal-weight men were placed on sequential five-day dietary periods containing varying CHO and fat energy, in surplus or deficit. Dietary CHO was either added or subtracted from a mixed diet, to provide 50% surplus energy, 25% surplus energy, 25% deficient energy, or 50% deficient energy. Periods with these diets were compared to those with eucaloric diets and diets containing 50% surplus fat energy. A strong direct relationship between dietary CHO energy content and fractional DNL was again observed (Figure 6), whereas surplus fat energy had no effect on DNL. In terms of the whole-body energy economy, however, the absolute rate of DNL was once again relatively insignificant, representing only 3.3 ± 0.8 g of fat synthesized per day, or 9.3 ± 2.3 g of CHO converted to fat, even on the diet with 50% surplus CHO.

We concluded that the addition of surplus CHO on the order of 1500 kcal/day to a mixed diet did not induce substantial flux through hepatic DNL,

at least when given for five days. What then were the metabolic fates of the surplus CHO? Although not converted to fat, surplus CHO caused striking alterations in on whole body fuel selection and intermediary metabolism. The fasting NP *RQ* changed from 0.77 ± 0.02 to 0.95 ± 0.01, on 50% deficient versus 50% excess CHO diets. Thus, extra CHO was able to replace fat almost completely in the fuel mix, even in the postabsorptive state. The metabolic mechanisms by which this occurred are of interest. Hepatic glucose production (HGP) was more than 40% higher on surplus CHO compared to deficient CHO diets, which is roughly equal to the difference in HGP between diabetics and nondiabetics. The elevated HGP occurred despite significantly higher serum insulin concentrations, thereby representing a form of hepatic insulin resistance that evolved over only five days. Lipolysis was also markedly reduced and the efficiency of oxidation of fuels entering the bloodstream varied in parallel with dietary CHO. No effects on metabolism or fuel selection were observed on diets containing surplus dietary fat. Clore et al (21) recently reported remarkably similar effects on HGP and whole-body fuel selection in response to CHO overfeeding.

In summary, isotope studies have forced a reconsideration of the simple conception of how hepatic DNL fits into the energy economy and where surplus CHO calories go. Adding CHO energy to a mixed diet will make a person fatter, but not by CHO being converted to fat—-that is, CHO spares the oxidation of dietary or adipose fat but does not itself traverse the DNL pathway. The metabolic implications of this revised model are potentially far-reaching. First, it is not valid to invoke the high thermogenic cost of DNL as a factor that might mitigate energy storage from dietary CHO, because DNL is not a highly used pathway. Second, the necessity to mobilize and oxidize glycogen rather than fat may explain fluctuations in the sensitivity of HGP to suppression by insulin as an unavoidable physiologic feature of this system. Such fluctuations in hepatic insulin sensitivity in response to dietary CHO balance may be important in the management of diabetic patients. Third, a pharmacologic strategy of reducing body fat by administering inhibitors of DNL can not be effective if this is the only mechanism of action involved. Nor does it make sense that polymorphisms in the genes for lipogenic enzymes be sought as candidate genes in the etiology of obesity.

Question 4: What Happens to Surplus CHO on a Low-Fat Diet?

The evidence just summarized refers only to surplus CHO added to a mixed (fat-containing) diet. But what happens if surplus CHO energy is ingested as part of a very-low-fat diet? There are no controlled dietary studies addressing this point. The best surrogate is the Guru Walla study of Pasquet et al (95)

discussed above, but the details have not been explored. The apparent absence of human subjects who spontaneously eat extremely high-calorie, low-fat diets or exhibit massive DNL suggests that a physiologic control system exists that prevents this (e.g. a feedback on appetite if whole-body CHO stores rise high enough to induce DNL).

IMPLICATIONS AND SPECULATIONS

Relevance to Macronutrient Balance Theory

One of the more interesting and systematic theories of obesity focuses on macronutrient balances rather than on energy balance per se. According to this conception, formulated by Flatt (35), there is no active mechanism for achieving energy balance; instead, there are independent mechanisms for the major energy-yielding macronutrients, CHO and fat. The theory holds, in brief, that intake of CHO controls its own utilization and intake, whereas intake of fat controls neither utilization nor intake in the short-term and requires alteration in body-fat stores before fat balance is attained. One of the key assumptions of this model is that CHO and fat are not interconvertible in the body, otherwise their signals and stores would not be independent. The conversion of fats to CHO is accepted to be impossible in animals, but DNL provides a potential channel from CHO to fat. The results discussed above support the macronutrient balance approach, since a functional block does appear to exist between CHO and fat, even if the enzymatic pathway is present.

What Are the Functions of DNL in Humans?

The low flux through DNL under most conditions in humans is different from other species. Thus, it is appropriate to speculate on the functions of DNL in humans. A number of possibilities deserve consideration.

1. There are no functions of DNL (it is a vestigial pathway in humans). In view of the complexity and regulation of the pathway, and the apparent absence of humans or animals lacking DNL enzymes, this seems unlikely.
2. DNL is an embryonic pathway only (it is a vestigial pathway in postnatal life). The developing human fetus has enormous needs for lipids, particularly for the growing central nervous system and subcutaneous adipose tissue in the third trimester. Placental lipid transfer capacity is low, which suggests an important role for DNL. After delivery, exogenous fat may suppress DNL. The extremely high net DNL rates observed in certain stressed premature infants (see above) may therefore represent a persistence of the fetal pattern.
3. DNL is suppressed by modern high-fat diets (it is a vestigial pathway

culturally). In the context of human dietary evolution, one of the most dramatic differences between the diet of the hunter-gatherer and the present western diet is the enormous increase in fat, from 20–25% fat calories in the late Paleolithic diet to 35–40% in our current diet (31). The current state of suppressed DNL may simply reflect the shift from a diet consisting of 75–80% CHO and proteins to the modern diet, which contains 60–65% CHO and proteins. This hypothesis suggests that the large differences among species are really dietary differences. Humans, when faced with a transient surplus of CHO or protein in the setting of a chronic low-fat diet, might increase flux quantitatively through DNL—but this has not yet been confirmed.

4. DNL requires longer periods for induction. If whole-body glycogen reserves have to be filled to capacity before DNL is induced, the typical 3– to 5–day overfeeding study (1–3, 8, 57, 96, 103) may be of insufficient duration. Acheson et al (1) reported a substantial increase in net DNL between days 4 and 7 of a CHO overfeeding experiment by indirect calorimetry. Their dose-response curve indicated a net positive CHO balance of 8000 kcal before DNL was fully induced, after which point an estimated net of 150 g of fat was synthesized per day. This hypothesis remains to be tested directly.
5. DNL occurs in an extrahepatic site. If DNL occurs in adipose tissue rather than in liver, isotope studies of VLDL will fail to quantify it properly. Although extrahepatic DNL is contrary to prevalent notions (106, 109), it needs to be excluded by direct measurement under conditions of CHO surplus. Alternatively, DNL may be reserved for specialized tissues such as lactating mammary gland.
6. DNL even at low values has regulatory effects. Hepatic gluconeogenic substrates intersect with DNL at a potential branch point [pyruvate dehydrongenase (Figure 1)]. Diversion of even 5 g of fat produced per day could reduce gluconeogenic flux by 10–20% and serve a regulatory function over HGP.
7. DNL serves as a signal (regulatory function). DNL is known to serve certain regulatory functions in the cell, independent of any role in storage of overflow CHO energy. Malonyl-CoA, the first and committed metabolite in the DNL pathway (Figure 1), plays an important role in fuel selection (24, 84, 85, 87, 120), and newly synthesized FA in the liver protect apolipoprotein B from degradation and induce VLDL assembly and secretion more efficiently than exogenous FA does (30, 37). Palmitoyl-CoA may serve as a signal for insulin secretion in the pancreatic ß-cell (22, 98). Myristate has an important function in the posttranslational modification of proteins (60), but the source of myristate is not known: Is it an essential dietary FA or is it synthesized de novo? If the latter holds, then DNL may be essential by providing special FAs for cellular regulation.

Is There a Role for DNL in Human Diseases?

Even within the constraints of relatively low rates of DNL, some diseases could be affected by lipogenesis.

HYPERTRIGLYCERIDEMIA In vitro, hepatic FA synthesized de novo may contribute disproportionately to VLDL assembly, apoB secretion, and triglyceride overproduction (30, 37). In humans, conditions associated with relatively increased DNL—such as low-fat, eucaloric diets (23, 29, 58), inflammatory diseases (41), EtOH intake (108), CHO overfeeding (1)—are also often associated with hypertriglyceridemia.

ALCOHOLIC LIVER DISEASE Alcoholic liver disease typically progresses from fatty liver to fibrosis and parenchymal destruction. Although a pathogenic link is not certain, factors contributing to fatty infiltration of the liver might play a causal role in the progression of disease. DNL induced by EtOH (53, 109) may contribute to hepatic fat disproportionately to its quantitative significance in the whole organism.

ACUTE-PHASE RESPONSE If the increase in circulating lipids observed in infections (41, 45, 52) serves a homeostatic function, DNL may be useful under these circumstances.

Does Measurement of DNL Have Any Uses in Patient Diagnosis or Management?

Values of fractional DNL (the contribution to VLDL-palmitate from the endogenous pathway) change by an order of magnitude or more in response to certain stimuli (see above). The absolute values remain low only because baseline values are so low. But the large proportional changes raise the possibility that this measurement could be useful as an indicator of various underlying processes in clinical or research settings.

One potential use of DNL is as a biomarker of recent CHO energy balance, because either CHO overfeeding or underfeeding has a dose-related effect on fractional DNL (103) (Figure 6). An objective biomarker of recent diet could be useful in a number of circumstances—to validate diet compliance in energy-restriction studies, for example, or to investigate the pathophysiologic role of overfeeding in insulin resistance, or to categorize non-insulin dependent diabetes mellitus patients according to their sensitivity to energy restriction (19). Another potential use of DNL is in wasting conditions, such as AIDS. Different patient subgroups exist in these conditions, differentiated by the underlying pathogenic process responsible for weight loss—e.g. simple starvation or abnormalities of metabolism (45, 48). Some AIDS patients with

wasting exhibit high values of fractional DNL, presumably mediated by cytokines (12, 41), whereas other have normal values of DNL (45). The former group tends to do poorly over the short-term (52) and to be resistant to lean tissue accrual when treated with dietary supplements. Therapeutic stratification into nutrient-responsive versus unresponsive patients may therefore be possible by using DNL as an index of the underlying cytokine milieu. Although mass spectrometric techniques have not traditionally been seen as clinical tests, there is no reason that dynamic measurements such as DNL could not someday be used in the patient-care setting.

SUMMARY AND CONCLUSIONS

The fact that DNL exists as a pathway cannot be disputed. It is also probable that humans can synthesize fat in large amounts, under extreme dietary conditions. What is surprising is the reluctance of humans to use the DNL pathway even when presented with a large surplus of CHO energy; perhaps DNL serves more subtle functions than that of an energy overflow pathway in humans. The large qualitative variations in DNL in response to diet and hormones suggest that some regulatory function is being served. Clearly, much remains to be learned about DNL. The availability of accurate methods to measure this biosynthetic process in vivo should make it possible to address these fundamental questions in the coming years.

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