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IN VIVO MEASUREMENT OF FLUXES THROUGH METABOLIC PATHWAYS: The Missing Link in Functional Genomics and Pharmaceutical Research

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■ **Abstract** In the postgenomic era of biology, much attention has been given to functional genomics, or the relation between genes and higher levels of organization in the cell. The latter are typically represented as mRNA, protein, or organic metabolite complements. The theme of this review is that the operational unit of function in complex biological systems is more properly seen as the fully assembled metabolic pathway in the whole organism. Due to the connectivity, interactions, and complexity of metabolic pathways, the measurement of components is an inadequate method for predicting phenotype. Measurement of the outputs of pathways (molecular fluxes) involves different tools than static measures of components, however. Here, we review recently developed stable isotope-mass spectrometric tools for measuring fluxes through metabolic pathways in vivo, focusing on the response to dietary macronutrients (carbohydrates and fats). Methods discussed include measurement of lipid dynamics, DNA replication, hepatic assembly of lipoproteins, and long-lived protein synthesis. Measuring fluxes through multiple pathways concurrently allows regulatory themes to emerge. Use of ^2H_2O-labeling is emerging as a particularly powerful approach for multiple concurrent biosynthetic flux measurements. Several examples demonstrate that pathway flux results are often unexpected and not predicted by classic biochemistry or the expression of genes and proteins.

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INTRODUCTION

A central paradox of contemporary biology and medicine is the dissonance between our understanding of the parts and the whole in complex biological systems. The radically uneven development in knowledge about the components of biochemical networks (reductionist understanding) compared to the intact assemblages (integrated understanding) tends to be taken for granted. The thesis of this review is that this state of affairs has profound implications for several of the signature areas of modern biological research. The severe limitations of even a perfect understanding within a purely reductionist paradigm of molecular components are not widely appreciated, and will be reviewed here. These limitations force recognition of the importance of higher levels of biochemical organization. Within this context, a definition of the true unit of function in biochemical control (the integrated metabolic pathway) is presented and emerging techniques are described for characterizing multiple metabolic pathway fluxes concurrently in living organisms.

FUNCTIONAL GENOMICS

Stimulated by the Human Genome Project and recent technologic advances, a new vocabulary has become familiar to biologists: genomics, gene expression profiling (or transcriptomics), proteomics, organeomics (or metabolomics), and informatics. This general approach—systematic characterization of every element within a class of molecules in a cell as an approach to the complexity of living systems—has received considerable publicity in the context of genetics, disease

research, and drug discovery. The ascendance of these approaches is based on the general belief that the parameters measured reflect and therefore may reveal fundamental loci of control in living systems.

Fundamental Unpredictability and Emergent Property of Cellular Metabolism

It is not widely appreciated, however, that none of these levels of information reveal in a predictable or reliable manner the activity of integrated metabolic pathways. The catalytic networks that characterize biochemical systems are extraordinarily complex, with interactions that comprise a higher level of control. The "architecture" of this control, which is defended by complementary and multi-tiered feedback and feedforward loops, arises from the connectivity and interactions among the components (4, 10, 45, 46). The notion that rules of behavior (organizational principles of a system) are different from rules of construction (components of a system) is hardly new in engineering or other fields. In molecular biology and biochemistry, however, this principle remains insufficiently appreciated. Indeed, the "central dogma" of modern biology (49)—that DNA codes for life—may be true from the point of view of information transmission, but it is fair to say that this view is misleading for understanding of control in higher level systems, based on several lines of evidence:

METABOLIC ENGINEERING OF BACTERIA Nowhere is this limitation better appreciated than in the field of metabolic engineering (4, 45, 46). Here, the goal is to induce bacterial cells to produce certain biochemical products (amino acids, proteins, antibodies, lipids, etc.). This field has had to face the remarkable capacity of evolved cellular pathways to resist flux alterations, despite imposed over- or under-expression of various enzymes. The surprising fact is that genetic engineering of cells has generally failed to direct metabolic pathways to the efficient production of desired metabolites (45, 46). The interactions among individual enzyme reactions that generate the pattern of fluxes ("flux distributions") characteristic of a particular cell or organism resist simplistic attempts at redirection. Based on these observations, concepts and terms such as "network rigidity," "flux mapping," and "control architecture" have arisen in this field to describe the integrated behaviors of metabolic systems (46). The resistance of metabolic systems to external modulation is frustrating to biochemical engineers but is of fundamental importance to our concepts of metabolic control and the nature of biochemical disturbances in disease. Metabolic engineers have learned that metabolic phenotypes even in microorganisms are "emergent" properties—that is, they are not predictable in a simple way from their physical components but operate by their own rules and organizational principles (4).

UNPREDICTABLE DISTAL PHENOTYPIC CONSEQUENCES OF INBORN ERRORS OF METABOLISM IN HUMANS The phenotypic consequences of single-gene defects have also proven to be complex and unpredictable (31). An excellent example

comes from glycogen storage disease (GSD). GSD-type 1, the most common variety, is due to a deficiency in the enzyme glucose-6-phosphatase in liver. This enzyme is required for the transport and release of glucose from the liver into the bloodstream. Infants often present with hypoglycemia but hypoglycemia is not in fact the ultimate phenotype that emerges in GSD-1 (33). The clinical complications of GSD-1 include hyperlipidemia, fatty liver, liver tumors, gout and, growth retardation—rather than symptoms of hypoglycemia. Though explicable post hoc on the basis of spillover of metabolites into secondary pathways (33), these phenotypic consequences would not have been predicted by classic metabolic maps. Indeed, if glucose-6-phosphatase had been discovered genetically without knowing its catalytic action, it might be categorized as tumor suppressor gene in the liver!

PHENOTYPES OF TRANSGENIC AND KNOCK-OUT MICE The phenotypes of transgenic and knock-out mice are often unexpected or even frankly contradictory to molecular biologic teaching and expectations. This topic has been discussed in detail elsewhere (25, 27).

GENETIC ANALYSIS OF COMPLEX DISEASES OR TRAITS Attempts to understand disease-susceptibility genes for complex disorders have been largely disappointing. Candidate gene searches and other strategies have generally not been fruitful for common diseases such as type 2 diabetes or obesity (12), other than for rare Mendelian subtypes, usually with a striking or characteristic clinical phenotype.

Is There a Solution to this Tension Between the Whole and the Parts?

The thesis of this review is that there is, in principle, a solution available to experimental biologists for investigating metabolic control. It is not necessary to treat the cell as a black box, or somehow rely on a nonmolecular approach, simply because genes and proteins do not by themselves fully explain biological function. Principles from systems theory suggest an alternative approach, subject to experimental inquiry: identification of the operational units of function, or intermediate levels of control, and improvement of their "observability."

DEFINING THE OPERATIONAL UNIT OF FUNCTION: MOLECULAR FLUXES THROUGH PATHWAYS A solution to this problem that combines analytic (reductionist) and integrated (holistic) approaches is to identify units that are emergent (i.e., reflect connectivity and interaction terms and exhibit the unpredictability and context dependence of assembled systems) but are also objectively measurable by experimental techniques and are of functional significance (i.e., are quantifiable and have intrinsic phenotypic consequences). Fluxes of molecules through metabolic pathways, if properly defined, exhibit these properties.

IMPROVING "OBSERVABILITY" OF THE FULLY ASSEMBLED SYSTEMS In order to direct any complex process toward a desired end, it is essential to see where you are going and what you have just done. This notion is central to the term "cybernetics" (coined by Norbert Wiener in the 1940s, from the Greek kybernos, the helmsman). Exerting feedback control is ultimately trivial when the process being affected is "observable," even when the physical basis of the system is not understood. A driver of a car, for example, does not need to understand the internal combustion engine, alloys of rubber in tires, etc., to steer the vehicle. The paradox of contemporary research is that discovery in science has been from the bottom up, which has resulted in a kind of ideology of the pieces (molecules), but control is from the top down. The missing link in contemporary functional genomics, which includes the areas of disease pathogenesis and pharmaceutical research, is therefore the capacity to observe and thereby control the outputs of the true units of function (biochemical pathway fluxes) in living systems.

The remainder of this review will focus on tools for observing (measuring) the flow of molecules through integrated metabolic pathways in living organisms.

TOOLS FOR MEASURING FLUXES DIFFER FROM TOOLS FOR MEASURING STATIC CONCENTRATIONS

Fluxes differ from static measurements in the same way that motion pictures differ from snapshots: The dimension of time is included. The tools for measuring biochemical dynamics are therefore fundamentally different from the tools for measuring static concentrations (like movie cameras or videos differ from a still camera). Observability of biochemical networks must take the form of molecular motion detectors.

Fluxes are usually measured using isotopes, because isotope-labeling studies generate asymmetry in the dimension of time—the isotope was not present, then it was. This feature allows the dimension of time to be introduced and thereby allows kinetic processes to be measured.

Recent Developments in Stable Isotope/Mass Spectrometric Methods: Application to the Metabolic Effects of Dietary Carbohydrates and Fats

Quantitative mass spectrometry is an analytic tool for metabolic flux measurements that has many of the attractive features of gene expression chips or proteomics techniques: high throughput, capacity for automation, multiplicity of concurrent measurements, accuracy, precision, and the possibility of informatics. A number of methodological advances have occurred over the past several years that involve the use of stable isotope labeling with mass spectrometric analysis and allow measurement of fluxes through metabolic pathways in vivo. These methods have been applied in many areas inside and outside the field of nutrition. I focus here

on the consequences of carbohydrate and fat intake on metabolic pathways. The repertoire of classic isotopic approaches and principles available to metabolic investigators has been reviewed in detail by others (23, 54, 55).

MEASUREMENT OF LIPID PATHWAY DYNAMICS IN RESPONSE TO MACRONUTRIENT INTAKE

De Novo Lipogenesis (DNL)

We have reviewed the regulation and physiologic functions of the de novo lipogenesis (DNL) pathway previously in the *Annual Review of Nutrition* (19). A key methodologic advance that allowed DNL to be measured was the development of the MIDA technique, or combinatorial analysis (16–19). MIDA is based on a model of combinatorial probabilities. Polymerization biosynthesis can be conceptualized as a combinatorial process, with monomeric subunits from a precursor pool combining into a polymeric collection or assemblage. If the monomeric subunits are of more than one distinctive type, i.e., labeled and unlabeled, then the population of assembled polymers will not be of uniform isotopic composition. The polymers will exist as distinguishable species containing varying numbers of the different types of subunits. Some species will contain no labeled subunits, some will contain one labeled subunit, some will contain two, and so on. The relative proportion of each species of polymer is uniquely determined by the proportion of labeled subunits present in the monomeric precursor pool and can be calculated from the binomial (or multinomial) expansion. This isotope pattern in the polymeric product can be measured by mass spectrometry. Conversely, the proportion of labeled monomeric precursors can then be inferred from the isotope pattern in the polymer. This approach allows precursor-product strategies for measuring fluxes through pathways involving polymerization biosynthesis, such as DNL, to be easily applied in living organisms, including humans. A key operational point is that only the polymer itself needs to be analyzed to establish the isotopic content of both the precursor and the product.

Measurements of DNL have resulted in a number of unexpected findings.

How Are Plasma Lipid Levels Maintained in Chylomicron-deficient Mice?

One setting where DNL might be expected to be elevated is when dietary fat absorption is impaired, resulting de facto in a high-carbohydrate (CHO) diet. A mouse model of chylomicron deficiency was developed by Young et al. (56). These mice express a human apolipoprotein (apo) B transgene in the liver but do not synthesize any apoB in the intestine and do not absorb fat by the classic chylomicron pathway. Despite severe intestinal fat malabsorption, normal concentrations of plasma lipids and apoB 100–containing lipoproteins are maintained (56). We hypothesized (22) that plasma lipids are maintained by an increased rate of DNL and

cholesterogenesis (i.e., endogenous fatty acid and cholesterol synthesis). Hepatic and plasma triglyceride (TG) concentrations and plasma FFA fluxes were not different between chylomicron-deficient mice and normal controls, despite reduced body fat stores in the former group (22). The contribution from DNL to hepatic TG was only modestly higher in chylomicron-deficient mice ($12 \pm 5\%$ versus $4 \pm 3\%$; Figure 1), whereas cholesterogenesis was markedly elevated. The contribution from plasma FFA to hepatic TG was greatly elevated in the chylomicron-deficient animals (65% versus 23%). Accordingly, 73% of hepatic TG was neither from DNL nor plasma FFA in controls, presumably reflecting TG stores largely derived from chylomicron remnants, compared to only 23% in the chylomicron-deficient group. Body fat accumulation was lower in chylomicron-deficient animals, reflecting their fat malabsorption, and the rate of whole-body absolute DNL was significantly lower, not higher, than in controls (54 ± 7 mg palmitate synthesized over 30 days versus 149 ± 4 mg in controls, $p < 0.01$). We concluded (22) that plasma and hepatic TG pools and hepatic secretion of apoB-containing particles are maintained at normal levels in chylomicron-deficient mice not by de novo fatty acid synthesis, as had been anticipated (56), but by avid re-esterification of plasma FFA, replacing the normally predominant contribution from chylomicrons; that normal adipocyte lipolytic rates are maintained in chylomicron-deficient mice despite markedly reduced body adipose tissue mass; and that some dietary fat can be absorbed by apoB-independent mechanisms (Figure 1). Thus, the presence of a high proportion of CHO and a low proportion of fat in absorbed nutrients does not result in functionally significant increases in DNL in mice, but other mechanisms are brought into play to maintain the production of hepatic lipoproteins. Attempts at therapeutic targeting of intestinal fat absorption to reduce hyperlipidemia must account for these metabolic adjustments. In particular, the combination of reducing fat absorption while inhibiting endogenous fat synthesis (e.g., with DNL inhibitors) is unlikely to be effective, contrary to intuition and to the experience with hypocholesterolemia (combining bile acid–binding resins and statins to target absorption and endogenous synthesis).

EFFECTS OF CHO SURPLUS ON DNL AND GLUCOSE METABOLISM If a high percent CHO in the diet does not induce DNL, what are the effects of increased absolute CHO intake (CHO overfeeding) on DNL? CHO excess is known to induce the expression of genes and proteins related to DNL (20, 47). When normal-weight men were placed on five-day controlled diets containing surplus carbohydrate or fat energy, however, the absolute rate of DNL remained quantitatively insignificant (42). Only about 3 g of fat were synthesized by the liver per day, even on a diet with 1500 surplus kcal/day of added CHO (Table 1). Although not converted to fat, surplus CHO caused striking alterations in whole body fuel selection. The fasting NP RQ increased; CHO was able to replace fat almost completely in the fuel mix, even in the postabsorptive state. Fasting hepatic glucose production was more than 40% higher on surplus CHO compared to deficient CHO diets. This elevation occurred despite significantly higher serum insulin concentrations, thereby representing a

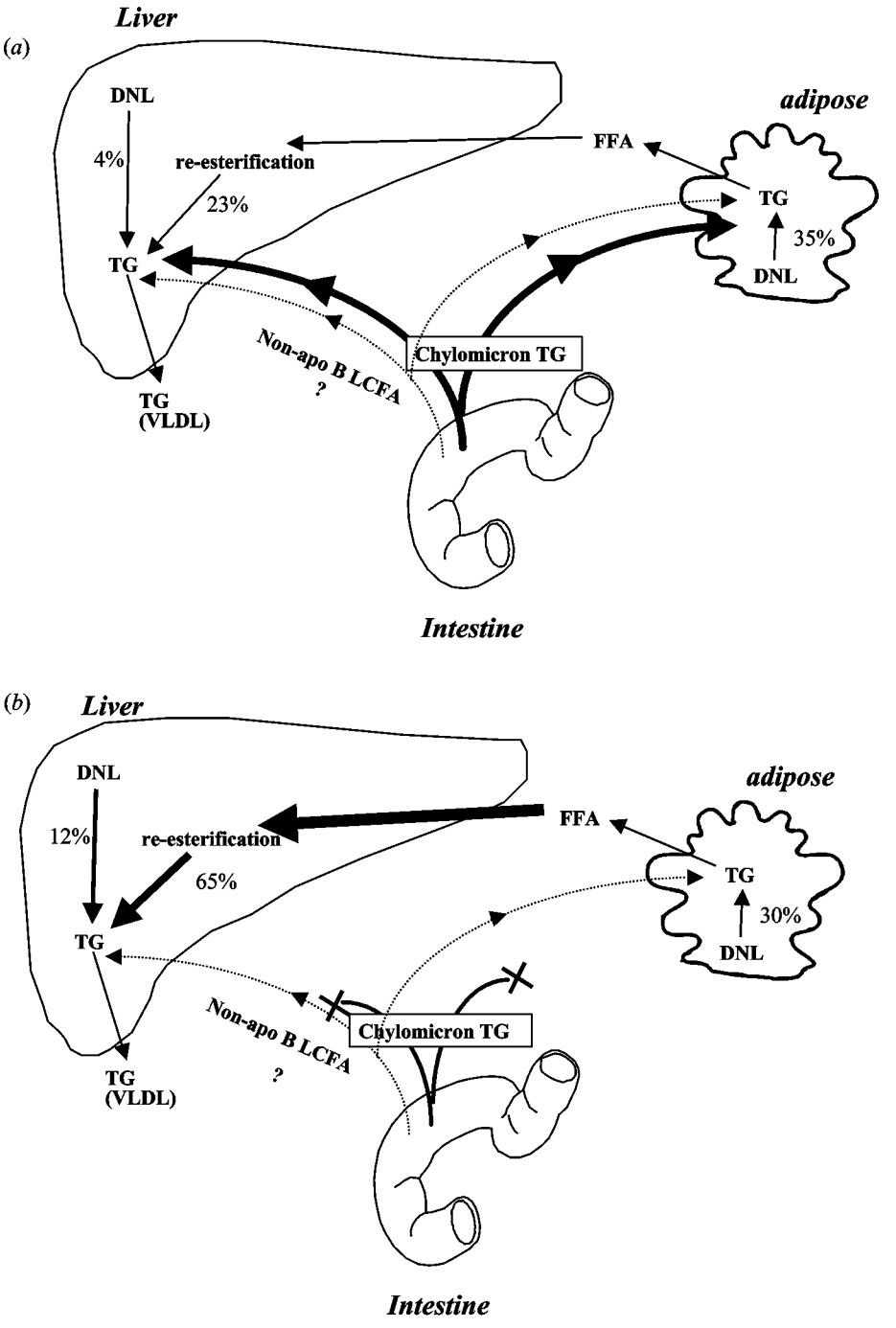

TABLE 1 Absolute DNL in normal human subjects on surplus (+50% energy) CHO diet for five days[a]

Fractional DNL (%)	K_s (Hr^{-1})	$T_{1/2}$ (Hr)	[TG] (mg/dl)	TG prod. (g/d)	Abs DNL (g/d)	Glc to fat (g/d)
25.4 ± 3.1	0.370 ± 0.059	2.21 ± 0.44	104 ± 7	28.0 ± 5.0	3.3 ± 0.8	9.3 ± 2.3

[a]Normal human subjects (n = 6) were placed on 50% surplus CHO energy diets for five days under metabolic ward conditions (42). DNL and TG kinetics were measured by MIDA from plasma VLDL-triglyceride, using [2-^{13}C]-acetate. K_s, fractional synthesis rate of VLDL-triglyceride; $T_{1/2}$, half-life; TG prod., absolute triglyceride production rate; Abs DNL, absolute rate of DNL; Glc to fat, conversion rate of glucose to fat via DNL.

form of hepatic insulin resistance. Lipolysis was also markedly reduced. No effects on metabolism or fuel selection were observed on diets containing surplus dietary fat (42).

Thus, adding CHO energy to a mixed diet will make a person fatter, but not by CHO being converted to fat—instead, CHO spares the oxidation of dietary or adipose fat but does not itself traverse the DNL pathway (19, 42). The first and quantitatively most important response to increased CHO intake is to increase whole body CHO oxidation, not to increase CHO conversion to fat (Figure 2). This quiescence of the DNL pathway in the face of marked CHO surplus was not predicted by enzymologists (20, 24, 53) nor is it apparent from measurements of gene or protein expression (20, 47). The failure to use DNL as a pathway for disposal for excess CHO energy has important implications for regulation of macronutrient balances in people, however, as Flatt (11) has pointed out. The absence of a "safety valve" allowing surplus CHO to be converted to fat combined with the limited capacity for whole body glycogen storage means that all dietary CHO must be oxidized over the course of a few days. The macronutrient regulatory system is therefore organized around matching fuel selection to recent CHO intake (11). Not only is CHO oxidation reduced in times of energy starvation to preserve lean body mass by reducing gluconeogenic needs from amino acids (5, 37), but CHO oxidation is increased in times of CHO surplus. Moreover, both adaptations involve the liver and modulation of endogenous glucose production.

The simple metabolic observation that CHO and fat are not functionally connected through DNL but have independent economies has led to important metabolic predictions. One is that the liver is more likely to contribute to obesity by overproducing glucose and reducing peripheral tissue fat oxidation than by

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Figure 1 Schematic model of hepatic and adipose lipid sources in (*a*) normal control mice and (*b*) chylomicron-deficient mice. FFA, free fatty acids; LCFA, long-chain fatty acids. The percentages shown reflect the contribution from the pathway. Thickness of arrows reflects quantitative flux through pathway (from Reference 22).

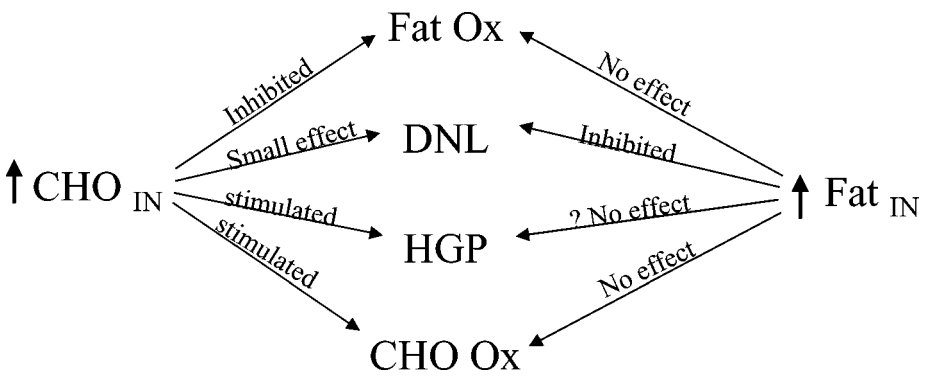

Figure 2 Consequences of surplus CHO or fat intake on macronutrient metabolic pathways in humans (after 42). Fat Ox, whole-body oxidation of fat; HGP, hepatic glucose production; CHO Ox, whole-body oxidation of CHO. Note that overfeeding of CHO does not substantially increase conversion to fat (DNL) but alters fat oxidation and CHO metabolism, while short-term overfeeding of fat has relatively modest effects on macronutrient metabolism.

converting glucose to fat (42). Indeed, Pagliassotti et al. (38) have shown that nonsuppressibility of hepatic glucose production by insulin is the best predictor of obesity in response to high-fat diets in rats, and Valera et al. (52) reported that overexpression of the gluconeogenic enzyme PEP-CK in the liver results in obesity. Another prediction is that short-term changes in dietary CHO intake should have a large effect on hepatic insulin resistance, since the liver must sensitively disburse dietary glucose to peripheral tissues for oxidation in response to variations in CHO intake. This prediction has been borne out therapeutically in the setting of type 2 diabetes mellitus (6). Finally, the absence of a safety valve for CHO and the primacy of CHO over fat in the fuel selection hierarchy (11, 42) means that the glucose-fatty acid cycle of Randle (41) stops being a cycle, but is unidirectional, when both fat and CHO are present in excess. Sidossis et al. have termed this "the Randle cycle reversed" (43), wherein surplus CHO unidirectionally suppresses fat oxidation, rather than the opposite. Therapies to improve glucose utilization by inhibiting fatty acid oxidation (41) are therefore unlikely to be effective in settings where surplus CHO is available and already being oxidized.

METABOLIC FATE OF ETHANOL Another setting where high flux rates through the hepatic DNL pathway might be expected is after ethanol (EtOH) intake. Significant changes in whole body lipid balances are induced by EtOH (48), but the precise metabolic pathways traversed had been difficult to establish with certainty. In particular, the question as to whether EtOH is directly converted to fat in the liver via DNL or instead is oxidized in peripheral tissues, thereby sparing fat from the fuel mixture, is of fundamental importance. Because the site of initial EtOH metabolism to acetate is the liver, and hepatic acetate can be directly converted to

acetyl-CoA, the general conception has been that EtOH is converted to fat in the liver in significant amounts (26).

We (44) studied this question directly in human subjects given 24 g of ethanol. Measurements included DNL (by MIDA), lipolysis (by dilution of [1,2,3,4-$^{13}C_4$] palmitate and [$^{2}H_5$]glycerol), conversion of alcohol to plasma acetate (by incorporation from [1-$^{13}C_1$]ethanol), and plasma acetate flux (by dilution of [1-$^{13}C_1$]acetate). The fractional contribution from DNL to very-low-density lipoprotein-triglyceride-palmitate rose following alcohol from 2 $\pm$ 1% to 30 $\pm$ 8%; nevertheless, the absolute rate of DNL (0.8 g/6 hours) represented <5% of the ingested alcohol dose (Figure 3). 77 $\pm$ 13% of the alcohol cleared from plasma could be accounted for as acetate flux entering plasma; acetate flux increased 2.5-fold following EtOH intake. Adipose release of free fatty acids into plasma decreased 53%, serum FFA concentrations fell by 47%, and whole body lipid oxidation decreased by 73%.

These observations form the basis of a somewhat surprising model of the integrated lipid response to EtOH consumption (Figure 3). EtOH is converted in the liver to acetate, but only a small portion is then converted to fatty acids. Most of the acetate is released into the circulation (28), where it affects peripheral tissue metabolism; adipocyte release of FFA is decreased (1) and acetate replaces lipids in the fuel mixture. Despite the availability of surplus 2-carbon units in the liver, a lipogenic organ, DNL is not used as a quantitatively significant metabolic pathway.

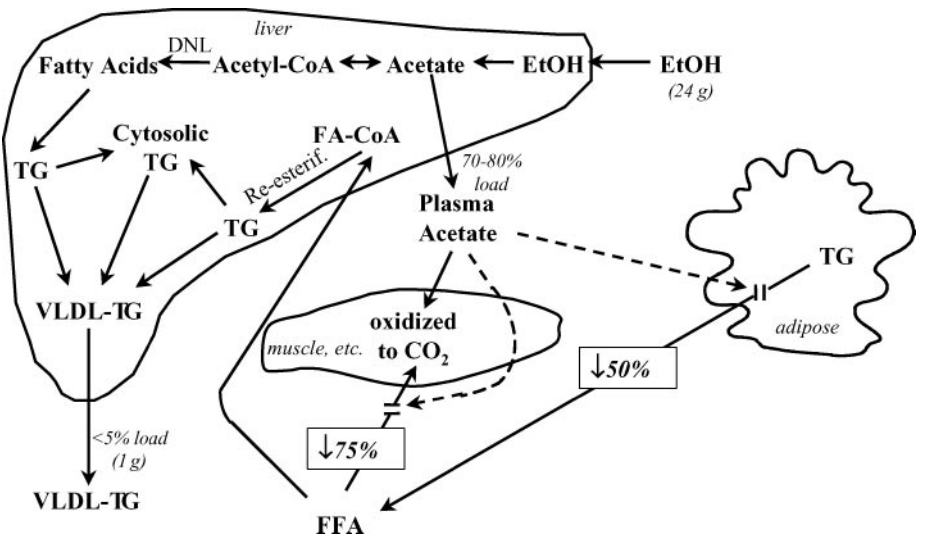

Figure 3 Metabolic model of EtOH ingestion (24 g over one hour). FA-CoA, fatty-acyl-CoA; re-esterification, re-esterification of FA-CoA to TG; FFA, free fatty acids.

Comment

The response of the liver to the availability of surplus 2-carbon units after EtOH ingestion is in many ways analogous to the hepatic response to carbohydrate overfeeding. In both instances, the liver alters whole-body lipid balances not by synthesizing lipid but by changing fuel selection of peripheral tissues (i.e., by releasing an oxidizable fuel in the form of glucose or acetate). In both cases, the inability of hepatic DNL to provide a quantitative disposal route for surplus substrates plays an essential permissive role in the system, allowing the liver to export the surplus to peripheral tissues for oxidation.

Although the metabolic responses to surplus 2-carbon or 6-carbon substrate availability are in many ways contrary to expectation, it is apparent that a similar pattern emerges for the two fuels. The fact that pathway fluxes are not predictable from regulatory models derived from ex vivo studies should therefore not prevent us from seeking and recognizing higher-level principles of organization and control in the whole organism. It should also be evident from these observations that pathway fluxes must be measured directly, and that tools for measuring multiple pathway fluxes concurrently are much more informative than measurement of single pathways in isolation.

MULTIPLE PHENOTYPIC MEASUREMENTS PERFORMED CONCURRENTLY WITH ^2H_2O

A powerful new approach has emerged for systematically measuring multiple metabolic pathway fluxes concurrently, using ^2H_2O as a near-universal label for the biosynthesis of macromolecules. A single experimental procedure can be used in an animal or person (administration of ^2H_2O by mouth, as drinking water). In the context of macronutrient metabolism, the capacity to measure adipose tissue acylglyceride synthesis, adipocyte proliferation, mitochondrial biogenesis, and muscle protein synthesis represent useful advances and will be described next.

Measurement of All-Source Lipogenesis and Lipolysis (Adipose TG Turnover)

In addition to the measurement of DNL, it would be useful to have tools for measuring all source TG synthesis and breakdown. The central component of acylglycerides is the glycerol moiety; TG-glycerol kinetics therefore best represents kinetics of the intact TG molecule. Labeled glycerol is not an efficient precursor for adipose lipids, however, because adipose tissue does not contain glycerol kinase (55). A potential labeling strategy for all-source acylglyceride synthesis became apparent from the observation that $^2H_5$-glycerol is an inefficient labeling precursor compared to ^{13}C-glycerol, for TG made in the liver (40, 42), a tissue containing glycerol kinase. The nearly complete loss of $^2H_5$-glycerol label to body water prior to conversion to the glycerol moiety of acylglycerides in liver suggested that the

opposite process must also occur—namely, exchange of ^2H_2O from body water into C–H bonds of glycerol in acylglycerides. ^2H_2O labeling has been used (50) for measurement of acylglyceride kinetics.

ADIPOSE TG TURNOVER: DIFFERENCES AMONG DEPOTS The kinetics of adipose TG turnover were determined by ^2H_2O administration in rats and mice (50). Ad-libitum chow-fed rats were sacrificed sequentially after 0 to 12 weeks of ^2H_2O intake. The time course of ^2H-glycerol labeling in adipose TG from various fat depots is shown (Figure 4). The replacement rate constants (k_s) for adult rats were 0.04–0.06 d^{-1} for epididymal fat and 0.21 d^{-1} for mesenteric fat, representing half-lives of 12–15 and 3–4 days, respectively. Remarkably, 60% of mesenteric TG was newly synthesized by day 7 (Figure 4). Accordingly, the ^2H_2O labeling method supported the previously difficult-to-prove hypothesis that visceral adipose TG is metabolically more active than subcutaneous TG.

Measurement of Cell Proliferation (DNA Synthesis)

Although cell division is usually framed in terms of cell biology and molecular biology, it can also be framed as a biosynthetic question: What is the rate of DNA synthesis in a population of cells? We (13, 15, 29, 30, 36) recently developed a stable isotope-mass spectrometric technique for measuring DNA synthesis. The deoxyribose (dR) moiety of dNTPs in replicating DNA can be labeled endogenously, through the de novo nucleotide synthesis pathway, using stable isotope-labeled glucose or deuterated water (^2H_2O). The isotopic enrichments of purine deoxyribonucleosides are then determined by gas chromatographic/mass spectrometric (GC/MS) analysis, after isolation and hydrolysis of genomic DNA. Because no radioactivity or genotoxic agents are involved, this technique is safe for use in humans. Use of this approach has allowed a number of questions traditionally considered nutritional to be addressed for their impact on cell proliferation.

ADIPOGENESIS IN OB/OB MICE The ob/ob mouse has a mutation in the leptin gene that results in obesity, insulin resistance, hyperphagia, sterility, and other metabolic and hormonal disturbances (21). There have been reports that leptin inhibits differentiation of preadipocytes (3), and reverses differentiation of mature adipocytes (57), but these effects had not been established in vivo.

We (36, 51) measured the proliferation rates of a mature adipocyte-enriched (stromal-vascular cell-depleted) fraction isolated from adipose tissue depots of mice, using the ^2H_2O labeling method (Figure 5). The proliferation rate was low (~1%–1.5% new cells produced per day) in control mice. Absolute rates of cell proliferation were markedly elevated in ob/ob mice (Figure 4). Thus, leptin deficiency results in hyperproliferation of adipocytes in addition to effects on energy and fat balance.

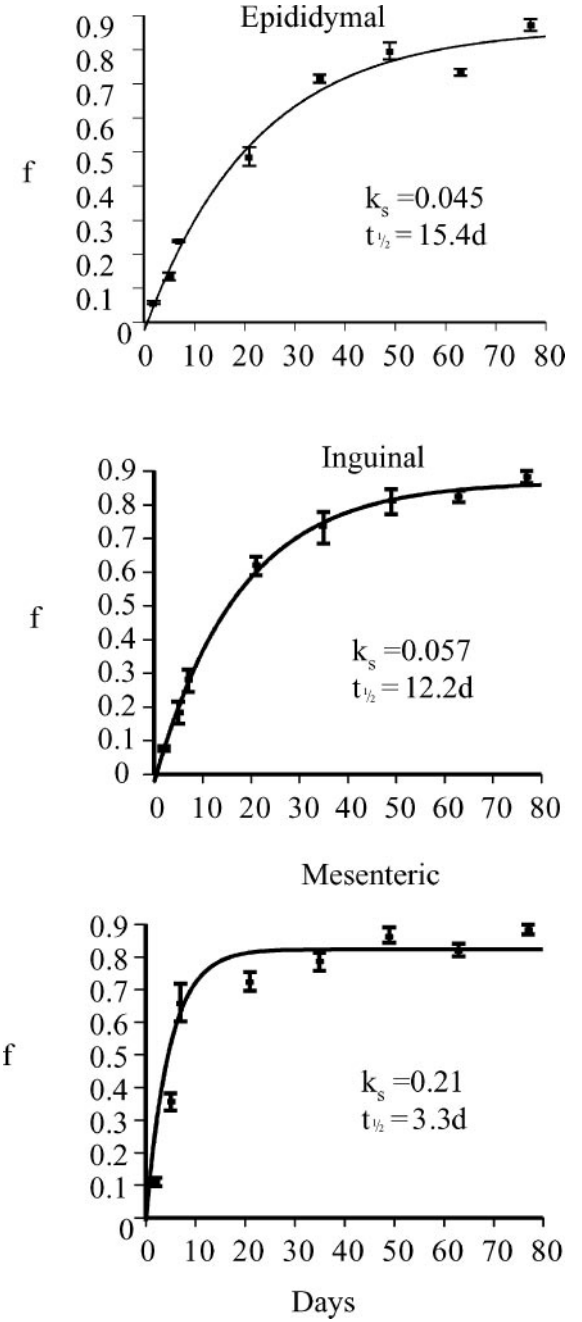

Figure 4 Time course of label incorporation into glycerol moiety of adipose tissue acyl-glycerides during ^2H_2O administration, in adult rats, for different depots (n = 4 per time point). Insets show the calculated replacement kinetics (from Reference 50).

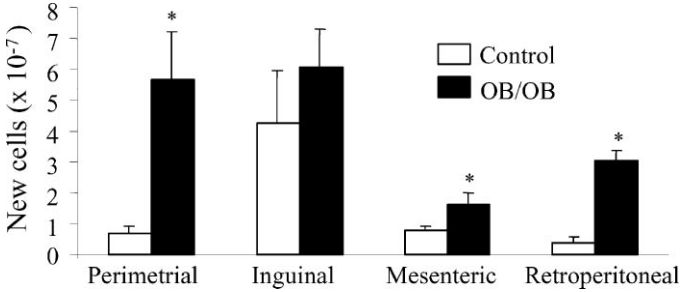

Figure 5 Cell proliferation rates in the mature adipocyte-enriched fraction isolated from adipose depots of control (n = 7) and ob/ob (n = 4) mice. Animals received 4% ^2H_2O in drinking water for 21 days. Controls weighed 18.5 +/− 0.2 g (mean ± S.E.) at the start of ^2H_2O administration and 20.8+ /− 0.4 g at the end. Ob/ob weighed 26.3 +/− 0.6 g and 35.7 +/− 0.9 g, respectively (36).

ADIPOSE LIPID AND CELL DYNAMICS IN OB/OB MICE: EFFECTS OF LEPTIN VERSUS FOOD RESTRICTION By combining the measurement of DNA replication, TG synthesis, and DNL from ^2H_2O, it was possible to compare the integrated effects of leptin versus energy restriction on adipose tissue dynamics in ob/ob mice (51). Four groups of C57/BL6J female mice were studied: ad libitum fed normal control mice (controls); ad libitum fed ob/ob mice (*ob/ob*); food restricted ob/ob mice (*ob-r*); and ob/ob mice treated with a constant subcutaneous infusion of leptin (2 μg/day) (*ob-lep*). ^2H_2O (4%) was administered for 21 days to measure TG synthesis, palmitic acid synthesis (DNL), and adipose tissue DNA replication. Compared to controls, the absolute rates of TG synthesis ($\sim$1.0 versus 0.2 g/21 days) and DNL (0.2 versus 0.05 g/21 days) were up to 4–5 times elevated in *ob/ob* (Figure 6). Food restriction in the ob/ob mouse resulted in only a modest reduction in the rates of DNL, TG synthesis, or adipogenesis. In contrast, leptin administration markedly reduced all three synthetic measurements. We concluded that leptin deficiency per se results in abnormal elevations in DNL, TG synthesis, and adipose cell proliferation and that these abnormalities are not driven primarily by hyperphagia. Moreover, these alterations are regulated in a coordinated manner by leptin availability.

It is of interest to compare these flux measurements to gene expression patterns in the adipose tissue of ob/ob mice (34, 35). Oligonucleotide microarrays from adipocytes of of ob/ob mice revealed significantly decreased expression of genes associated with normal adipocyte differentiation, including SREBP1, ATP-citrate lyase, PPAR-gamma, and glycerol 3-phosphate dehydrogenase (34, 35). The authors concluded that adipocytes from obese mice have "dramatically decreased lipogenic capacity, similar to preadipocytes" and that, although lipid engorged, adipocytes from obese mice had significantly reduced capacity to synthesize fatty acids. Our direct measurements of fatty acid synthesis and total lipogenesis (Figures 5 and 6) demonstrate that these inferences from gene expression (mRNA)

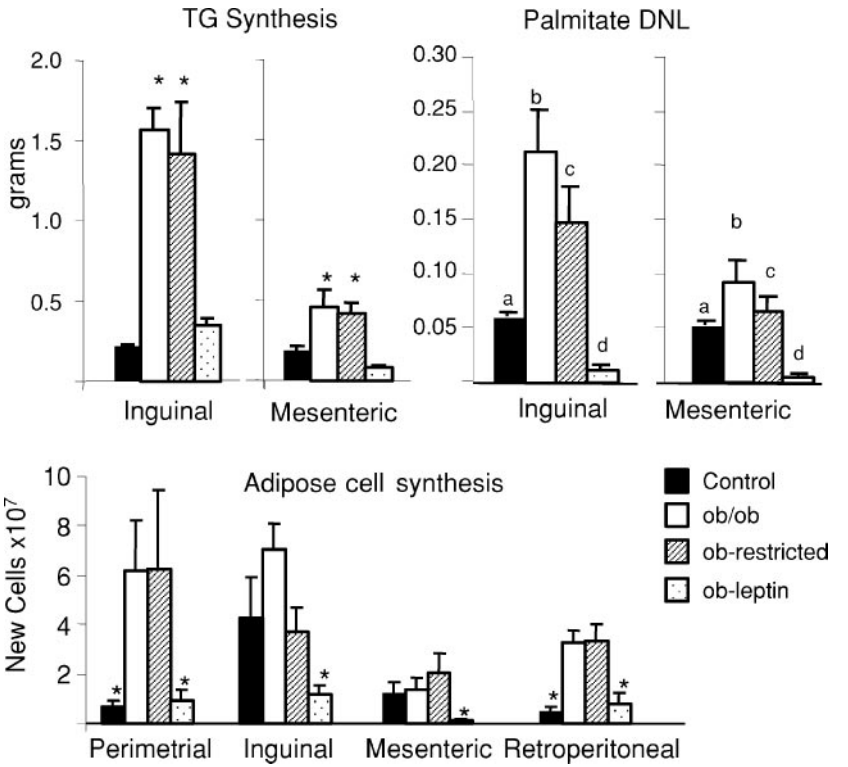

Figure 6 Dynamics of adipose lipid components in ob/ob mice and controls. Effects of food-restriction (pair-feeding) and leptin administration are compared.

data were incorrect—adipocytes from ob/ob mice exhibit markedly *increased* new fat synthesis and total lipogenesis, not decreased.

A number of explanations can be put forward for misleading gene expression results, as was the case here, e.g., post-transcriptional regulation of protein synthesis, disinhibition of enzyme activities by metabolites or hormones, alterations in competing pathways, increased input of substrates into the pathway, etc. The key point, however, is that the only way to know for sure what the flux is through a complex pathway is to measure it directly.

Dynamics of Mitochondrial Components Measured In Vivo Using ^2H_2O Labeling

Another area of classic energy metabolism research that can be posed as a question of integrated lipid synthesis and DNA replication is the area of mitochondrial biogenesis. The number or mass of mitochondria per cell varies depending upon physiologic factors, even in cells that are terminally differentiated (7, 9).

Aerobic exercise also has well-established effects on muscle mitochondrial mass and whole-body energetics (9). The ^2H_2O labeling method was applied to the measurement of mtDNA and phospholipid synthesis (8). Mitochondria were isolated from muscle and mtDNA was isolated. PCR confirmed absence of nuclear DNA contamination. Synthesis of muscle mtDNA was observed in nongrowing adult female rats (Figure 7*a*). Average half-lives were approximately 150 days in cardiac muscle and 350 days in hind-limb muscle. Higher synthesis rates of mtDNA compared to genomic DNA were observed in both cardiac and hind-limb muscle tissues (Figure 7*a*), consistent with mitochondrial turnover independent of somatic growth.

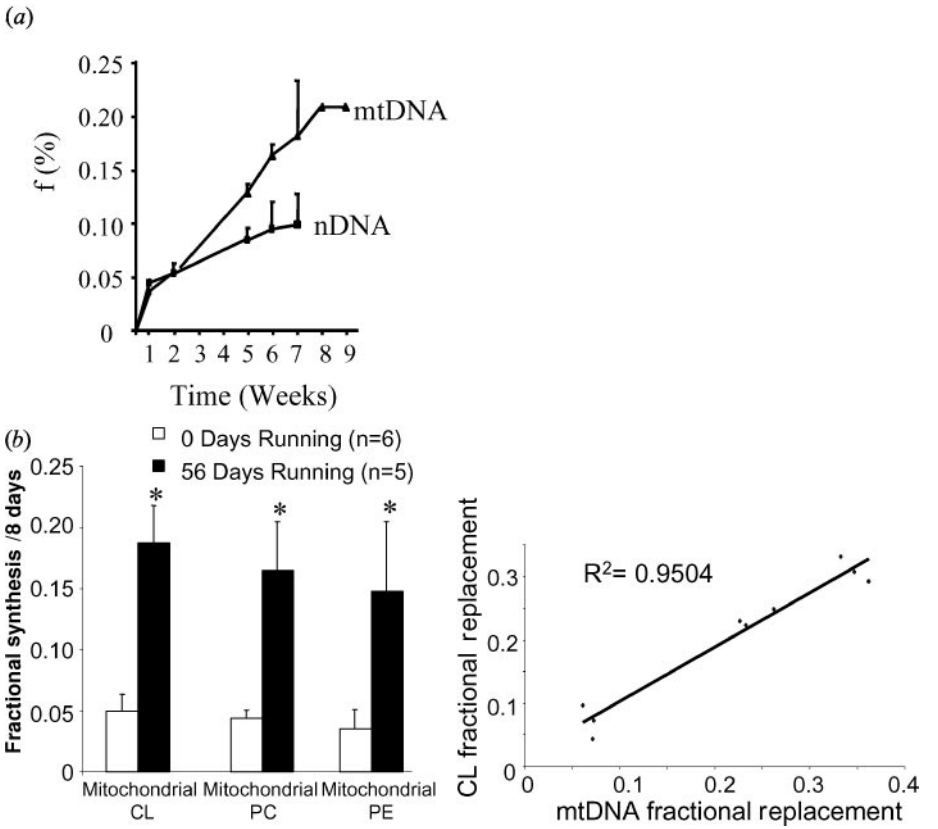

Figure 7 (*a*) Comparison of mtDNA to nuclear DNA synthesis in hind-limb muscle of weight-stable female rats (mean +/− S.D.); f, fractional replacement (8). (*b*) *Left*, synthesis of mt phospholipids in hind-limb muscle of rats, and effect of exercise training (voluntary wheel running). Animals received ^2H_2O for eight days. CL, cardiolipin; PC, phosphatidylcholine; PE, phosphatidyl-ethanolamine; *, $P < 0.05$ versus control rats. *Right*, correlation between mtDNA and mtCL synthesis.

Mitochondrial biogenesis also includes the accrual of mitochondrial phospholipids, such as cardiolipin (CL). CL is an interesting lipid for the ^2H_2O labeling technique because each CL molecule contains three glycerol moieties. The synthesis of mtDNA and CL can be measured at the same time, by administration of ^2H_2O. Voluntary wheel running in female rats resulted in marked increases in mtCL synthesis (Figure 7*b*) and mtDNA synthesis in skeletal muscle. The mtDNA and CL synthesis rates were highly correlated (Figure 7*b*). These results are consistent with the model that mitochondria are assembled as units. Thus, the ^2H_2O-labeling approach represents a simple and powerful strategy for studying the regulation and integration of mitochondrial biogenesis pathways. The relationships among mitochondrial biogenesis, metabolic fitness, and whole-body energy expenditure are of great interest but remain largely unexplored because of previous limitations in measuring the pathways involved.

Comment

Cell division and mitochondrial biogenesis can be seen as complex metabolic pathways culminating in, and best reflected by, replication of DNA. The measurement of DNA synthesis and breakdown, in this context, clearly has a wide variety of applications to classic questions in metabolism and nutrition, including obesity, energy balance, exercise, and fitness.

MEASUREMENT OF HEPATIC VLDL ASSEMBLY

Another example where macronutrient intake has an impact on both lipid and protein metabolism is in the assembly of VLDL particles in the liver. VLDL assembly is complex and fundamental to the pathogenesis of dyslipidemias. Several intersecting pathways meet in the assembled VLDL particle, including TG synthesis, cholesterogenesis, phospholipid and cholesterol-ester synthesis, DNL and apolipoprotein B synthesis. Approaches using multiple tracers can be used in animals and humans, to dissect out the sources of hepatic VLDL-TG and the integration of VLDL-TG assembly. One example with potential public health implications is CHO-induced hypertriglyceridemia (HTG). The potential atherogenicity of CHO-induced elevations in TG has been the subject of debate (2, 39). Differences in lipoprotein dynamics between the two forms of HTG (Western diet–associated and high CHO–induced) might indicate differential risk for cardiovascular disease.

We (40) studied subjects with low TG [normolipidemia (NL), TG $= 61 \pm 7$ mg/dL] and those with moderately elevated TG [hypertriglyceridemia (HTG), TG $= 149 \pm 14$ mg/dL]. Each group was studied on both a control (35% fat) and a low-fat, high-carbohydrate (15% fat) diet. The group with baseline HTG exhibited a striking overproduction state for VLDL-TG and VLDL-ApoB with no significant

TABLE 2 Kinetics of VLDL-ApoB and TG in human subjects with normolipidemia (NL) and hypertriglyceridemia (HTG)

	NL subjects		HTG subjects		Comparison of change scores *p*	Overall treatment effect *p*
	Control diet	LF/HC diet	Control diet	LF/HC diet		
VLDL ApoB						
Concentration (μg/ml)	3.4 ± 1.1	6.6 ± 1.6	12.8 ± 3.8	19.3 ± 5.2	0.86	0.03
Transport rate (μmol·kg TBW^{-1} h^{-1})	0.11 ± 0.05	0.13 ± 0.04	0.24 ± 0.05	0.32 ± 0.08	0.03	—
K_s (h^{-1})[2]	0.36 ± 0.05	0.25 ± 0.04	0.25 ± 0.05	0.22 ± 0.06	0.74	0.09
Clearance rate (ml/min)	17.5 ± 4.4	12.4 ± 2.2	16.7 ± 4.4	15.1 ± 5.3	0.64	0.32
VLDL-TG						
Concentration (mg/dL)	5.0 ± 1.6	8.1 ± 1.9	10.2 ± 2.0	17.8 ± 5.9	0.29	0.04
Transport rate (μmol·kg FFM^{-1} h^{-1})	10.3 ± 1.5	12.5 ± 1.5	27.9 ± 10.8	18.9 ± 4.2	0.26	0.58
K_s (h^{-1})	0.44 ± 0.04	0.32 ± 0.06	0.37 ± 0.08	0.19 ± 0.05	0.41	0.03
Clearance rate (ml/min)	23 ± 3	19 ± 3	29 ± 10	12 ± 4	0.78	0.05

Data are mean ± SEM. LF/HC, low fat, high carbohydrate; Ks, fractional replacement rate; VLDL, very low density lipoprotein; TBW, total body water; FFM, fat-free mass.

Adapted from Reference (40).

differences in clearance (Table 2), consistent with previous studies (reviewed in 39), in comparison to NL on baseline diet. The low-fat, high-CHO diet resulted in 60–80% elevations in TG concentrations in both NL and HTG groups, but no significant change in VLDL-ApoB or VLDL-TG secretion rates (Table 2). Instead, a 37% reduction in VLDL-TG clearance and an 18% reduction in whole-body fat oxidation were observed. Significant elevations in fasting apoB48 concentrations (representing chylomicra) were also observed on the low-fat, high-CHO diet in HTG subjects. In both groups, fasting DNL was low (<5% of VLDL TG-fatty acids), regardless of diet (39).

These results were in many ways contrary to expectation. The most likely mechanism of high CHO-induced HTG has been assumed to be stimulation of hepatic DNL and VLDL-TG secretion, but these were not observed. Rather, whole-food, low-fat, high-CHO diets reduced VLDL-TG clearance and did not increase VLDL-TG secretion or DNL. Chylomicron remnants were present in the fasting state, despite much lower daily fat intake, implying delayed clearance of constitutively released intestinal lipoproteins (possibly competing with VLDL-TG for clearance). Finally, the assembly, production, and clearance of elevated plasma VLDL-TG in response to low fat/high CHO differed dramatically from that for elevated TG on higher-fat diets, suggesting that not all HTG should be considered identical in terms of atherogenic potential. A similar divergence between lipid and apoB kinetics in VLDL, in response to weight loss, was recently reported by Mittendorfer et al. (32). These findings suggest that characterization of metabolic pathway fluxes may stratify different forms of hyperlipidemia by mechanism and potential atherogenic risk.

MEASUREMENT OF SYNTHESIS OF SLOW TURNOVER PROTEINS USING ^2H_2O LABELING

In addition to the dynamics of lipids and DNA, the regulation of protein turnover is an area of fundamental interest in metabolic regulation and physiology. Techniques for measuring protein turnover have long been problematic, however. The complex subcellular organization of amino acid (AA) pools accounts for some of the methodologic difficulties (23, 54). Another problem derives from the very slow turnover rate of some physiologically important proteins (e.g., bone collagen, muscle myosin, erythrocyte hemoglobin). Recently, a continuous label administration, rise-to-plateau approach using the precursor-product method for measuring protein synthesis has been developed, based on the incorporation of ^2H_2O into nonessential amino acids (NEAA) and the use of MIDA (14). H-atoms from H_2O are incorporated into C–H bonds of AA only during specific enzyme catalyzed reactions on free AA. Once bound in peptides or proteins, C–H bonds in AA are not labile in physiologic solution. Accordingly, the incorporation of ^2H from ^2H_2O into the C–H bonds on protein-bound AA represents newly synthesized proteins (i.e., assembled from free AA during the period of ^2H_2O exposure).

This ^2H_2O technique has been used to measure the synthesis of muscle and bone proteins (14). Rats were administered 4% ^2H_2O as drinking water for up to 12 weeks. The combinatorial pattern (ratio of double- to single-labeled NEAA species) revealed the number of metabolically exchanging C–H atoms (n) present in each NEAA by use of MIDA (14). The value of n was 4 for alanine and 2 for glycine, reflecting essentially complete exchange. Knowledge of the number of active hydrogen atoms allowed use of the precursor-product method for measuring protein synthesis. In skeletal muscle, fractional replacement rate (k) of mixed proteins was ca. 0.22 wk^{-1} ($t_{1/2} = 3$ weeks); in cardiac muscle, k was 0.31 wk^{-1} (Figure 8*a*).

The ^2H_2O label incorporation approach was also applied to bone collagen synthesis (Figure 8*b*). In growing mice, bone collagen fractional synthesis rate (k) was 0.035 d^{-1}. In weight-stable adult female rats, k was 0.17 wk^{-1} (Figure 8*b*). Administration of estrogen pellets (200 mcg) to ovariectomized, adult female rats reduced k of collagen by 35%. Thus, dynamics of the major components of lean tissue (e.g., proteins in muscle, bone, and other organs) can be measured concurrently with dynamics of body lipid components (see above), in studies of body composition, by use of a single experimental technique (^2H_2O labeling).

SUMMARY AND CONCLUSIONS

Several messages should emerge from these experimental measurements of fluxes through complex metabolic pathways in living organisms. First, and most striking, is that fluxes through pathways are often surprising. Neither classical metabolic teaching nor measurement of gene and protein expression reliably predicts what

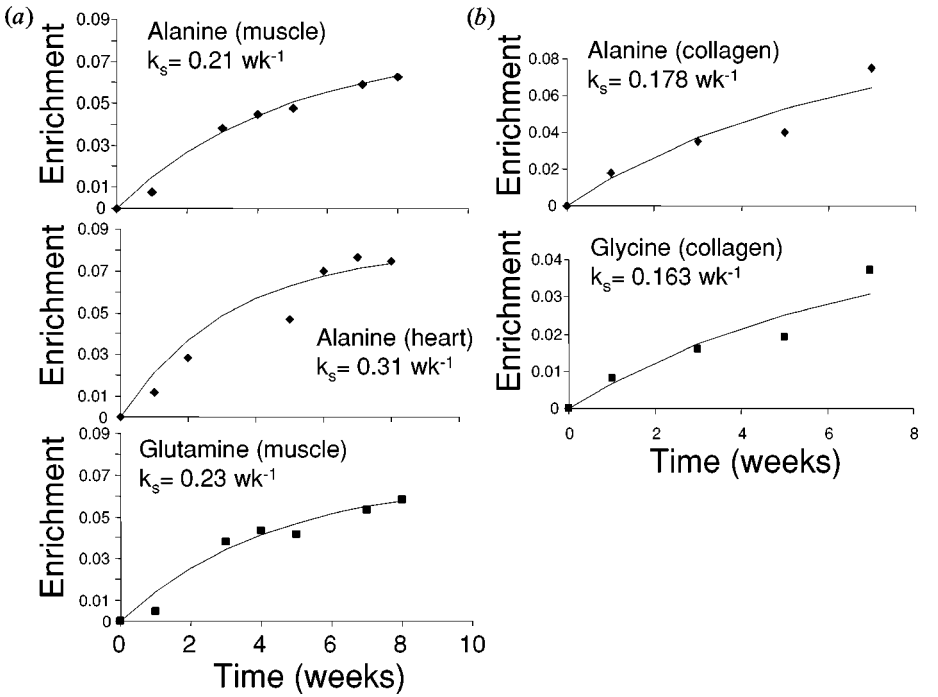

Figure 8 (*a*) Synthesis of skeletal muscle and cardiac muscle protein from ^2H_2O in adult female rats. Data shown as molar excesses. Calculated rate constants are also shown. (*b*) Synthesis of bone collagen in adult female rats. Data shown as molar excesses. Rate constants shown.

actually happens in vivo. Pathway fluxes are not predictable when measured in mammalian organisms, just as they are not predictable or controllable in the simple case of microorganisms (4, 10, 45, 46), but need to be measured experimentally. Moreover, the interconnected nature of metabolic pathways makes it most informative to measure multiple pathway fluxes at the same time, as I have emphasized in this review. Availability of tools that allow multiple pathway fluxes to be monitored concurrently also allows regulatory themes and patterns to become apparent. Fortunately, there is a growing repertoire of tools that allow multiple pathway fluxes to be measured in an increasingly simple and high-throughput manner, as discussed here. Use of ^2H_2O has emerged as a particularly powerful approach toward this end.

This review has also emphasized the importance of improved tools for measuring pathway fluxes in fields not generally considered part of metabolic regulation or tracer techniques, such as DNA synthesis. Hopefully, continued advances in metabolic methodologies will continue to drive advances in these signature areas of modern biology.

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