Application of New Methods and Analytical Approaches to Research on Polyunsaturated Fatty Acid Homeostasis

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ABSTRACT: New methods and analytical approaches are important to challenge and/or validate established beliefs in any field including the metabolism of polyunsaturated fatty acids (PUFA; polyunsaturates). Four methods that have recently been applied toward obtaining a better understanding of the homeostasis of PUFA include the following: whole-body fatty acid balance analysis, magnetic resonance imaging (MRI), ^{13}C nuclear magnetic resonance (NMR) spectroscopy, and gas chromatography–combustion–isotope ratio mass spectrometry (GC–C–IRMS). Whole-body balance studies permit the measurement of both the percentage of oxidation of linoleate and α-linolenate and their conversion to long-chain PUFA. This method has shown that β-oxidation to CO_2 is normally the predominant metabolic fate of linoleate and α-linolenate. Furthermore, models of experimental undernutrition in both humans and animals show that β-oxidation of linoleate and α-linolenate markedly exceeds their intake, despite theoretically sufficient intake of linoleate or α-linolenate. Preliminary results suggest that by using MRI to measure body fat content, indirect whole-body linoleate balance can be done in living humans. ^{13}C NMR spectroscopy provided unexpected evidence that linoleate and α-linolenate were metabolized into lipids synthesized *de novo*, an observation later quantified by tracer mass balance done using GC–C–IRMS. This latter method showed that within 48 h of dosing with ^{13}C-α-linolenate, >80% underwent β-oxidation to CO_2 by suckling rats, whereas 8–9% was converted to newly synthesized lipids and <1% to docosahexaenoate. Further application of these recently developed methods in different models should clarify the emerging importance of β-oxidation and carbon recycling in PUFA homeostasis in mammals including humans.

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Progress in scientific research depends on a mixture of new ideas and new methods. Sometimes new ideas can be tested using existing, and even old, methods. Often, however, a new method may be required to undertake the appropriate experiment. Applying new methods to an old problem can sometimes reveal the information that was hoped for. Unanticipated results that perhaps contradict prevailing concepts may also occur in the process. It takes originality, patience, and luck to optimize experimental and analytical methods to research objectives.

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Abbreviations: GC, gas chromatography; GC–C–IRMS, GC–combustion–IRMS; IRMS, isotope ratio mass spectrometry; LC, long chain; MRI, magnetic resonance imaging; NMR, nuclear magnetic resonance spectroscopy; PUFA, polyunsaturated fatty acids.

Research on the metabolism of polyunsaturated fatty acids (PUFA; polyunsaturates) has benefited from new methods almost since the inception of this field in the original studies of Burr and Burr in 1929–1930. Of any single analytical method, gas chromatography (GC) has arguably made the greatest contribution to the fields of fatty acid and sterol analysis. The application of deuterium isotope ratio mass spectrometry (IRMS) to problems in lipid metabolism is older than GC, but refinements in the 1990s have pushed this method into the forefront of PUFA research. Nuclear magnetic resonance (NMR) spectrometry was considered to be a tool of chemists until people began to use it to measure tracer metabolism in isolated tissues and live animals in the 1980s. These methods are by no means the only analytical developments that have contributed to an improved understanding of PUFA homeostasis.

WHOLE-BODY LINOLEATE AND α-LINOLENATE BALANCE

In a nutritional context, balance methodology refers to comparing "what goes in to what comes out." It has been applied extensively to the metabolism of nutrients that cannot be synthesized, i.e., minerals and indispensable amino acids. Because there is no endogenous synthesis of "essential" nutrients in mammals, the difference between what goes in (dietary intake) and what comes out (excretion and β-oxidation) represents the amount accumulating in some form or other in the organism. If animals are used, what accumulates can be measured directly and compared with intake or excretion under the experimental conditions in question.

This method can be applied to two important questions in PUFA metabolism, i.e., (i) How much dietary linoleate (18:2n-6) or α-linolenate (18:3n-3) is converted to the respective long-chain (LC) PUFA? (ii) How much linoleate or α-linolenate undergoes β-oxidation, i.e., what is their true bioavailability under normal conditions? The only apparent condition on such fatty acid balance studies is that, because linoleate and α-linolenate can both be chain-elongated from their respective 16-carbon precursors, which are present in edible green vegetables, and because each of these precursors can be converted to several LC-PUFA, the dietary PUFA in a whole-body fatty acid balance study should be only linoleate and α-linolenate. This is not usually a difficult condition to meet as long as semipurified diet ingredients are used. PUFA accumulation would then have two components, (i) accumulation of linoleate or α-linolenate and (ii) accumulation of all

n-6 or n-3 LC-PUFA, namely, the percentage of conversion from linoleate or α-linolenate.

If the analysis is done correctly, the difference between intake minus the sum of both excretion and the combined accumulation of linoleate or α-linolenate plus their respective LC-PUFA represents the disappearance of linoleate or α-linolenate from the whole-body pool of n-6 or n-3 PUFA, i.e., their β-oxidation. In growing animals, a balance period of only a few days is sufficient, but it can be several weeks if it is desirable. The advantages of this analytical approach to studying both net desaturation-chain elongation and β-oxidation of PUFA are as follows: (i) No tracers are involved; thus the data are not dependent on isotope equilibration with various pools. (ii) β-Oxidation exceeding intake can be measured, which is a surprisingly common phenomenon (see below). (iii) Whole-body rather than single compartment synthesis of LC-PUFA can be measured. (iv) The analytical method (quantitative fatty acid extraction and analysis by capillary GC) is relatively simple and well established. The single main disadvantage is that LC-PUFA also undergo β-oxidation, although to a lesser extent than linoleate or α-linolenate. This component of the equation can be estimated from published values (1) but cannot be measured directly; thus, there is an inherent although modest error in its estimation.

The whole-body fatty acid balance method provided the first clear indication of the overall capacity to synthesize LC-PUFA in rats. Subcellular preparations, tracer conversion, or changes in precursor to LC-PUFA ratios simply cannot provide this information because they are isolated from the integrated influences on PUFA homeostasis in the whole body. Predictable changes such as increased LC-PUFA synthesis during pregnancy or impaired LC-PUFA synthesis in nutritional deprivation such as moderate zinc deficiency (Table 1) confirm the plausibility of the results obtained using whole-body fatty acid balance methodology. Perhaps most surprising are the relatively low levels of conversion [3–4% to arachidonate (20:4n-6) and 1–2% to docosahexaenoate (22:6n-3)], especially relative to β-oxidation of the parent PUFA.

TABLE 1
Various Nutritional Manipulations Probing Key Aspects of Whole-Body Homeostasis of Linoleate and α-Linolenate, i.e., Conversion to Long-Chain Polyunsaturated Fatty Acids (LC-PUFA) or β-Oxidation

	Linoleate	α-Linolenate	Reference
	(% of intake)		
Conversion to LC-PUFA[a]			
Normal, young rats	3–4	1–2	2
Pregnant rats	8	45	3
Pregnant + mild zinc deficiency	4	24	3
β-Oxidation			
Normal, young rats	70	80	2
Pregnant rats	62	35	3
Fasted, refed pregnant rats	106	142	4
Weight-cycled male rats	81	89	5
Linoleate-deficient male rats	485	ND[b]	6

[a]18- to 22-carbon LC-PUFA in each family.
[b]ND, not determined.

This method also shows that whole-body β-oxidation of linoleate and α-linolenate is generally in the range of 70–80% (Table 1), values that are in broad agreement with whole-body tracer data (1). Predictably, β-oxidation of PUFA is reduced by pregnancy and increased by a variety of conditions involving nutritional deprivation (Table 1). One important advantage of this method is that because whole-body analysis is done at two time points to determine accumulation, a net loss over the balance period (negative accumulation) can be measured. Hence, unlike with tracer methods, depletion of PUFA from body stores is easily quantified. A significant degree of whole-body PUFA depletion occurs with weight loss, fasting-refeeding, and dietary linoleate deficiency (Table 1).

Indirect approaches using whole-body fatty acid balance methodology can also be used to estimate requirements for certain PUFA such as docosahexaenoate (7). Docosahexaenoate accumulation at 10 mg/d in the human neonate was estimated from a collation of tissue data. Taking into consideration an estimate that 50% of dietary docosahexaenoate undergoes β-oxidation in neonates (8) left an apparent requirement for docosahexaenoate intake and/or synthesis of 20 mg/d. Although breast milk almost invariably provides at least 60 mg/d docosahexaenoate, no data obtained by balance or tracer methods in an animal or human model suggest that α-linolenate can be converted to docosahexaenoate at the necessary rate of 20 mg/d, i.e., a conversion rate of 5% (7). These results, although preliminary and potentially in need of revision, demonstrate the applicability of whole-body fatty acid balance to important questions in PUFA research in both infants and animal models.

MAGNETIC RESONANCE IMAGING (MRI) AND *IN VIVO* WHOLE-BODY FATTY ACID BALANCE

One significant limitation to applying whole-body fatty acid balance to understanding PUFA metabolism in humans is the need for an accurate measure of the whole-body content of the fatty acids in question, i.e., the need for tissue analysis. Under some circumstances, MRI may be able to provide the necessary information to estimate the whole-body pool of PUFA such as linoleate in living humans (9). The key requirements of this method can be met without direct lean tissue PUFA analysis, at least under the following experimental conditions: (i) Whole body MRI scans provide accurate lean and fat tissue volumes for the whole body. (ii) Lean and fat tissue densities and water content are known; thus, the actual mass of individual fatty acids in these compartments can be determined. (iii) Linoleate is predominantly in body fat and because fat is the only tissue (besides plasma) that can be routinely biopsied; fatter individuals reduce the error of having only a sample of subcutaneous fat with which to analyze linoleate levels. (iv) Linoleate levels seem to be the same at all fat locations (10), allowing one to generalize that the data obtained from one biopsy site is valid across all fat depots. (v) Studying linoleate β-oxidation during energy deficit and moderate weight loss essentially eliminates the need to

measure linoleate conversion to n-6 LC-PUFA because energy deficit impairs desaturation (11). (vi) Moderate energy deficit leading to <15% weight loss does not change lean tissue linoleate levels in animals (12); thus, only the size of the lean tissue compartment but not a change in its PUFA content must be known.

In our initial experiments with this *in vivo* version of the whole-body fatty acid balance method, it became clear that β-oxidation of linoleate during weight loss of 13 kg in obese men can markedly exceed its intake, despite a nominally adequate linoleate intake (9). This was not evident in the PUFA content of plasma fatty acid profiles. Because of their already large linoleate stores and ongoing "normal" linoleate intake, this whole-body loss exceeding intake by two- to threefold was unlikely to be a significant risk, at least immediately. However, the point is that indirect whole-body linoleate balance can be done in living humans under minimally invasive and ethically acceptable circumstances. This method appears to provide plausible estimates of linoleate β-oxidation and indicates for the first time that under conditions of apparent linoleate adequacy, linoleate β-oxidation can considerably exceed its intake. This may lead to assessment of conditions in which linoleate sufficiency is less certain, i.e., during pregnancy, lactation, early infancy, or chronic risk of undernutrition.

In one such future application, we are beginning an evaluation of fetal MRI to assess the feasibility of determining fat accumulation during the third trimester *in utero*. Fetal adipose tissue accumulates uniquely during the third trimester. Growth retardation *in utero* or premature birth are both associated with a higher risk of compromised neurological development postnatally and with lower fetal fat accumulation. At birth, adipose tissue contains more docosahexaenoate than α-linolenate (7); thus, this depot is potentially important in both normal postnatal development and early postnatal PUFA homeostasis. The PUFA balance methodology is analogous to that used in living adults (9) in which the changes in total fetal fat volumes will be combined with PUFA profiles obtained from separate autopsy samples. In principle, this should allow the estimation of PUFA accumulation in fetal adipose tissue.

^{13}C NMR SPECTROSCOPY: APPLICATION TO A NEW PATHWAY IN PUFA METABOLISM

NMR spectroscopy has several applications in PUFA metabolism, particularly in determining the physiological properties of fatty acids such as docosahexaenoate (13). Our particular interest in this method was in the potential application of ^{13}C NMR to the noninvasive measurement of LC-PUFA synthesis in organs such as brains of living animals and humans. As detailed elsewhere (14), technical limitations still prevent useful information on this potentially valuable application of NMR to studying PUFA metabolism. However, in the process of attempting to measure LC-PUFA synthesis noninvasively from uniformly ^{13}C-labeled linoleate and α-linolenate in live neonatal rat pups, we made the serendipitous observation that the tracer was appearing in substantial amounts in brain lipids containing saturated carbons, i.e., cholesterol, saturated, and monounsaturated fatty acids. This was clearly evident in the *in vivo* ^{13}C NMR spectra because peaks for saturated carbons of all lipids cluster in one region of the spectrum, whereas the peaks containing unsaturated carbons cluster in a separate region distinct from the saturated carbon peaks.

At the time, we were unaware of Andrew Sinclair's seminal work with ^{14}C-α-linolenate showing essentially the same thing in suckling rats (15); skeptical then of our *in vivo* NMR data, we proceeded with ^{13}C NMR analysis of brain total lipid extracts (16). Spectra of these extracts unequivocally confirmed the ^{13}C enrichment in saturated fatty acids and cholesterol that appeared in the poorly resolved *in vivo* ^{13}C NMR spectra (16). More sophisticated NMR methods, including generating double quantum, two-dimensional spectra, demonstrated the carbon pairing of telltale satellite peaks that indicated exactly which cholesterol carbons came into the molecule as intact ^{13}C-enriched carbon pairs directly from the dosed ^{13}C-PUFA (17). Further analysis by continuous flow IRMS confirmed the occurrence of substantial "carbon recycling" from 18-carbon PUFA, probably through ketones, into brain lipids synthesized *de novo* (16).

The point of this example is that our goal, i.e., to demonstrate synthesis of LC-PUFA in an *in vivo* model, had been quite different from the main results it produced. Had we used routine methods to examine LC-PUFA synthesis, the cholesterol fraction would not have been retained and we would not have learned about "carbon recycling" from PUFA into *de novo* lipid synthesis. This is also one of the drawbacks to GC/MS with selected ion monitoring, i.e., the investigator selects the ions, and therefore the molecules of interest, and discards or ignores the others regardless of their information content. In light of the data to be presented in the following section, unfortunately, this is a lost opportunity to evaluate a pathway of PUFA metabolism that appears to be, quantitatively, more important than LC-PUFA synthesis.

Carbon recycling from PUFA into *de novo* lipid synthesis appears to occur *via* β-oxidation of the PUFA, incorporation of the PUFA carbons into ketones, and use of the labeled ketones for *de novo* lipid synthesis in the brain (18). This pathway has not been investigated but would be an ideal subject for ^{13}C NMR analysis because this method is well suited to identifying the water-soluble products (ketones, but probably other metabolites as well) through which the ^{13}C enrichment is transferred.

GAS CHROMATOGRAPHY–COMBUSTION–ISOTOPE RATIO MASS SPECTROMETRY (GC–C–IRMS): APPLICATION TO CARBON RECYCLING OF PUFA

IRMS is the most sensitive and precise way to measure the increase in ^{13}C (or other tracer) above background during a tracer experiment. Because this method requires that the isotope in question be gaseous, it is limited to low-molecular-weight isotopes, mostly of hydrogen, oxygen, nitrogen, and

carbon. Gaseous samples such as breath require a purification step but can otherwise be sent straight to the mass spectrometer for analysis. Nongaseous samples must be combusted, a process that used to be done manually, with the gaseous sample then being purified and fed through a vacuum system to the mass spectrometer. In the past two decades, "continuous flow" systems have combined automated combustion and purification of the samples with the subsequent MS, thereby greatly reducing the need for manual input. In the past decade, the further refinement of adding a gas chromatograph ahead of the combustion system has been commercialized.

In principle, any organic compounds that can be separated by GC can have isotopic enrichments analyzed by GC–C–IRMS. For instance, it is now reasonably common to measure, in both animal models and humans, synthesis of ^{13}C-labeled n-3 LC-PUFA after dosing with ^{13}C-α-linolenate (8,19–22). Following on from our preliminary data showing that carbon recycling into *de novo* lipid synthesis appeared to capture a significant amount of the carbon skeleton of a mixture of linoleate and α-linolenate (16), we applied GC–C–IRMS to quantifying the fate of a physiologic oral dose of ^{13}C-α-linolenate in suckling rat pups. Total lipids of brain, liver, gut, lung, and remaining carcass were extracted; the ^{13}C enrichment in fatty acids and sterols was analyzed separately; tissue fatty acid and sterol levels were quantified; and the data were combined so as to determine the distribution of ^{13}C from α-linolenate in body lipids. In addition, the total recovery of ^{13}C in body lipids would indicate by mass balance how much ^{13}C was missing and therefore lost through β-oxidation.

Results for the brain, liver, and gut have already been reported (23), from which it was clear that recycling of α-linolenate carbon into *de novo* lipid synthesis exceeded that going into docosahexaenoate by 5- to 40-fold, depending on the tissue and time of sampling after dosing with the tracer. More recently, we confirmed these data in the lung and are compiling the carcass and whole-body data. A nearly complete whole-body estimate showed that 48 h after dosing, <10% of ^{13}C-α-linolenate remained in total body lipids in any form, i.e., that ~90% underwent complete β-oxidation. Of the remaining 10%, <5% stayed as ^{13}C-α-linolenate, and <5% was converted to n-3 LC-PUFA. Less than 1% of the dosed ^{13}C-α-linolenate appeared to be going into docosahexaenoate in the whole body of the suckling rat. If our remaining analyses and those of others working in this field confirm these preliminary conclusions (Fig. 1), these data will corroborate our whole-body balance data showing that 1–2% of dietary α-linolenate is normally used by the growing rat to make n-3 LC-PUFA (2).

The influence on n-3 LC-PUFA synthesis of preformed docosahexaenoate in the milk consumed by the suckling rats will still have to be assessed; the point remains, however, that IRMS is a reliable and sensitive analytical method for quantitatively assessing the bioavailability and whole-body homeostasis of PUFA.

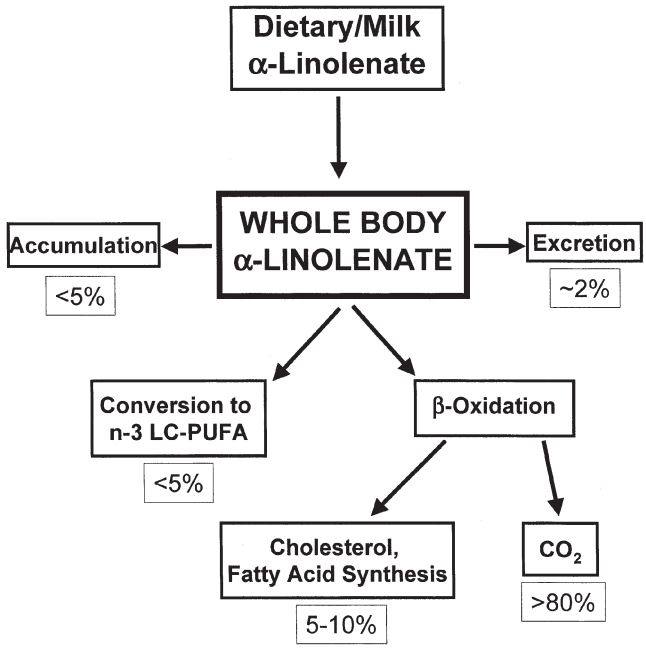

FIG. 1. Scheme outlining α-linolenate homeostasis in suckling rats based on metabolism of an oral dose of ^{13}C-α-linolenate 48 h after dosing in 6-d-old rat pups. The values are derived from published (23) and unpublished data. LC-PUFA, long-chain polyunsaturated fatty acids.

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