Chapter Eight
Radio and Stable Isotopes in Lipid Nutrition Research

E. A. Emken
Northern Regional Research Center
Agricultural Research Service
U.S. Department of Agriculture
1815 N. University St.
Peoria, IL 61604

Radio and stable isotopes have had an enormous impact on the physical and biological sciences. The basic methodology and applications of radioisotopes are familiar to most lipid chemists, and have become standard biochemical and analytical tools. In contrast, the stable isotopes have not been widely utilized in lipid research. The radio and stable isotopes that have been most extensively utilized in lipid research are summarized in Table 1. Radioisotopes of iodine are included by virtue of the ease with which iodine can be used to tag lipid enzymes and unsaturated fats. However, problems with the stability of iodine-labeled compounds limit their applications, and the effect of iodine on the metabolism of labeled fatty acids needs to be considered.

There are hundreds of publications that review the synthesis, analysis

<table>
<thead>
<tr>
<th>Isotope</th>
<th>Radio</th>
<th>Stable</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbon</td>
<td>$^{14}\text{C}$</td>
<td>$^{13}\text{C}$</td>
</tr>
<tr>
<td>Hydrogen</td>
<td>$^{3}\text{H}$</td>
<td>$^{2}\text{H}$</td>
</tr>
<tr>
<td>Oxygen</td>
<td>$-\text{X}$</td>
<td>$^{17}\text{O}^{\text{L}},^{18}\text{O}$</td>
</tr>
<tr>
<td>Phosphorous</td>
<td>$^{32}\text{P}$</td>
<td>$-\text{X}$</td>
</tr>
<tr>
<td>Iodine</td>
<td>$^{125}\text{I},^{131}\text{I}$</td>
<td>$-\text{X}$</td>
</tr>
</tbody>
</table>

TABLE 8-1.
Common Isotopes Used in Lipid Research
and applications of isotope-labeled compounds. The topics covered in these reviews range from general (textbook) to very specific (chapters and journal articles), and until the last few years have generally been limited to radioisotopes. A few of the many books and chapters that cover synthesis, analysis and biological applications of isotope-labeled compounds are listed in Table II (1–24), providing an introduction to the enormous volume of literature available. The last five citations (19–24) in Table II are more specifically concerned with the synthesis and analysis of isotope-labeled fatty acids and their applications in lipid research. The only relatively comprehensive coverage of biological studies with isotope-labeled lipids is Mead's review (23), Radioisotope Studies of Fatty Acid Metabolism, published in 1960.

Application of Isotopes

Historically, isotope-labeled lipids have been primarily used in short-term experiments designed to identify metabolic products, follow the transport and distribution of fatty acids, measure turnover rates, investigate enzyme kinetics and specificities, or quantitate lipids isolated from tissue. Labeled fatty acids can also provide valuable complementary data to long-term lipid nutrition and physiology studies which measure the dietary effects on parameters such as organ size, body weight, arterial lesion development and change in serum lipids.

Single and dual isotope methods

The relative simplicity of the experiments and the definitive results obtained are responsible for the widespread use of tracer techniques. Tracer studies basically involve administering one or more isotope-labeled compounds and then following the distribution of the labeled compound(s) or formation of metabolic product(s). Applications that use a single-isotope tracer are the most common; however in many cases, dual- and triple-labeled experiments would have provided additional useful data without much extra effort.

A variety of enzyme reactions with labeled isomeric fatty acids has been reported and a few examples that utilized single isotope tracer studies are cited (25–29). These in vitro studies normally involve incubating a radioisotope-labeled fatty acid isomer with an enzyme preparation, and then following the enzymatic reaction product as a function of time. Many similar in vivo experiments also have been reported, and some representative
## TABLE 8-II.

**Reviews on Synthesis, Analysis, and Applications of Isotopes**

<table>
<thead>
<tr>
<th>Topic covered</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Stable isotopes: General</strong></td>
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<tr>
<td>Biological references to deuterium for 1971–1976</td>
<td>Klein (1978a)</td>
</tr>
<tr>
<td>Biological reference to carbon-13</td>
<td>Klein (1978b)</td>
</tr>
<tr>
<td>Biological reference to nitrogen-15</td>
<td>Klein (1978c)</td>
</tr>
<tr>
<td>Biological reference to $^{17}$O/$^{18}$O/$^{34}$S</td>
<td>Klein (1978d)</td>
</tr>
<tr>
<td>Biological application of stable isotopes</td>
<td>Baillie (1978)</td>
</tr>
<tr>
<td>Biological effects of deuterium</td>
<td>Thomson (1963)</td>
</tr>
<tr>
<td>Deuterium labeling in organic chemistry</td>
<td>Thomas (1971)</td>
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<td><strong>Radioisotopes: General</strong></td>
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<tr>
<td>Tritium and its compounds</td>
<td>Evans (1966)</td>
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<tr>
<td>Synthesis with tritium</td>
<td>Murry (1958a)</td>
</tr>
<tr>
<td>Synthesis with carbon-14</td>
<td>Murry (1958b)</td>
</tr>
<tr>
<td>General overview of carbon-14</td>
<td>Calvin (1949)</td>
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<tr>
<td>General overview of carbon-14</td>
<td>Raan (1968)</td>
</tr>
<tr>
<td>Chemistry and applications of isotopes</td>
<td>Elvidge (1979)</td>
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<tr>
<td>Isotope effects on enzyme reactions</td>
<td>Cleland (1977)</td>
</tr>
<tr>
<td>Radiotracer methodology in biological science</td>
<td>Wang (1965)</td>
</tr>
<tr>
<td>Biological applications of radiotracers methods</td>
<td>Kobayashi (1974)</td>
</tr>
<tr>
<td>Radiochemical manual</td>
<td>Wilson (1966)</td>
</tr>
<tr>
<td><strong>Fatty acids and lipids: Stable and radioisotopes</strong></td>
<td></td>
</tr>
<tr>
<td>Synthesis and mass spectral analysis of perdeuterated fatty esters</td>
<td>Nguyen (1978)</td>
</tr>
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<td>Synthesis of deuterium labeled fatty acids</td>
<td>Rakoff (1982)</td>
</tr>
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<td>Synthesis of isotope labeled fatty acids</td>
<td>Emken (1979)</td>
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<tr>
<td>Synthesis and analysis of isotope labeled fatty acids</td>
<td>Emken (1978)</td>
</tr>
<tr>
<td>Radioisotope studies of fatty acid metabolism</td>
<td>Mead (1960)</td>
</tr>
<tr>
<td>Labelling and radio purity of lipids</td>
<td>Snyder (1966)</td>
</tr>
</tbody>
</table>
studies are cited (30–32). The main difference between in vitro and in vivo experimental designs is that, for in vivo studies, the isotope-labeled fatty acid is fed orally or injected, and its distribution, interconversion or catabolism is followed by analysis of blood, urine or organ samples. The specific design of single isotope experiments and the information that can be obtained appear to be limited only by the ingenuity of the lipid chemist. Most readers already have a working knowledge of single radioisotope techniques, and the specific details pertaining to experimental design strategies and calculations. Since these points have been well reviewed, they will not be discussed.

Dual-labeled experiments, involving the administration of two differently labeled lipids at the same time, have been used in both in vitro and in vivo studies (33–39). This approach allows experimental data for a control and an experimental lipid to be directly compared under identical conditions. The technique is particularly useful in in vivo experiments where variation in the data occurs because of uncontrollable experimental parameters, such as biological variation and fluctuation in diets. The technique of reverse-labeling is recommended when the dual-labeled technique is used. Reverse-labeling consists of reversing the isotope label on the experimental and control compounds. For example, a typical set of radio-isotope experiments may consist of feeding a combination of $9t$-18:1-$^3$H and $9c$-18:1-$^{14}$C and repeating the experiments by feeding $9t$-18:1-$^{14}$C and $9c$-18:1-$^3$H. Any variation in the isotope ratios obtained from the experimental data is an indication of problems that may be due to loss of label, to isotope effects or to unintentional fractionation of the labeled isomers.

A problem can occur in in vivo dual-labeled experiments if the enzyme reaction order for the labeled substrates changes as a function of substrate concentration. This problem arises because radioisotope experiments use small amounts of labeled fatty acids; therefore, a large dilution by unlabeled endogenous fatty acid occurs when labeled fatty acids such as stearic, oleic or linoleic are fed. In contrast, little dilution of labeled fatty acid isomers occurs because these fatty acids are not biosynthesized in mammals and normally are not present in large amounts in endogenous lipids. This difference in isotope dilution should not affect $^3$H/$^{14}$C ratios since the difference in specific activities resulting from dilution is self-compensating for the difference in fatty acid concentration if first order enzyme kinetics are assumed. For example, after dilution by endogenous fatty acids, an enzyme involved in product formation would be presented with a large pool containing a labeled fatty acid at a low specific activity and a small pool containing a labeled fatty acid at high specific activity. Experimentally we find $^3$H/$^{14}$C ratios are not influenced by dilution of the specific activities.
with endogenous fatty acids, but this potential problem should not be ignored when designing new experimental approaches.

An alternative approach to using two or more differently labeled lipids is to label different portions of the same lipid molecule with two or three different isotope labels. Labeling a single molecule in two places with the same isotope also has been used as an effective strategy. These various approaches are referred variously as "dual-," or "double-" and "triple-labeled" experiments and have resulted in some confusion in nomenclature. Examples of "double labeling" are experiments with triglycerides containing a fatty acid-\(^{14}\)C and glycerol-\(^{3}\)H or phosphatidylcholine containing both \(^{14}\)C- and \(^{3}\)H-labeled fatty acids (see Fig. 1) (40–41). Use of lipids labeled in this manner provides direct evidence as to whether the lipid undergoes a transformation as an intact molecule or if it is first hydrolyzed and then reassembled. Advantages of multiple-isotope techniques and points to consider in designing experiments are discussed in more detail in the section on multiple deuterium isotope methodology.

Use of isotope methods in mechanism and kinetic studies

As illustrated by the enzyme-isomeric fatty acid experiments cited earlier, kinetic and mechanistic information can be obtained by incubating the enzyme preparation with a labeled fatty acid and determining reaction rates by sampling the reaction mixture at appropriate time intervals. Turnover, half-life and enzyme binding constants can then be calculated from reaction rates based on product formation and/or substrate disappearance using typical Michaelis-Menten equations and Lineweaver-Burk plots.

Strategic placement of the isotope label at various positions of the lipid molecule has been useful to gain insight into reaction intermediates and mechanistic pathways. This approach involves following the loss of label, appearance of labeled fragments in reaction products and determination of isotope effects. The stereochemistry of lipid reactions, for example, often is elucidated by stereospecifically labeled substrates.

For in vivo studies, half-lifes, turnovers and reaction rates can be calculated from pulse and constant infusion techniques (42–45). The concepts, equations and calculations have been described by Segal (46) and others (15–17). The constant infusion technique is more widely used because of the higher reliability of the data. The approach typically involves infusing a labeled fatty acid of known specific activity until a steady-state concentration of the labeled fatty acid is reached in the circulatory system. The infusion and removal rates are then assumed to be equal, and the turnover
Incorporation of Double-Labeled (³H and ¹⁴C) Phosphatidylcholine and Monopalmitin

\[
\begin{align*}
H_2C-O-C-R-3H &= (67 \times 10^6 \text{ dpm}) \\
HC-O-^{14}C-R &= (1.7 \times 10^6 \text{ dpm}) \\
H_2C-O-P-O-(CH_2)_2N(CH_3)_3OH &= \text{in vivo} \xrightarrow{\text{Isolated PC from liver}} \text{Rat} \\
³H/¹⁴C &= 35 \\
³H/¹⁴C \text{ Ratio} &= 39.4
\end{align*}
\]

Hoppe-Seyler's Z. Physiol. Chem. 353:949 (1972)

\[
\begin{align*}
H_2C-O-^{14}C-R &= (9,978 \text{ dpm} \ ¹⁴C) \\
³H-OH &= (18,714 \text{ dpm} \ ³H) \\
H_2C-OH &= \text{Intestinal mucosal cells} \\
\text{Monoglyceride} \ ³H/¹⁴C &= 1.90 \\
\text{Diglyceride} \ ³H/¹⁴C &= 1.89 \\
\text{Triglyceride} \ ³H/¹⁴C &= 1.82
\end{align*}
\]


Fig. 8-1. Incorporation of double-labeled (³H and ¹⁴C) phosphatidylcholine and monopalmitin by rat liver and intestinal mucosal cells (40, 41).

of the plasma fatty acid is proportional to the decrease in specific activity of the labeled fatty acid and its rate of infusion. As an alternative to measuring the isotope-labeled fatty acid in the plasma, expired CO₂ can be collected and used as an indication of fatty acid turnover. Unfortunately, the CO₂ breath-test has some fundamental experimental problems, but its noninvasive feature is an attractive advantage.

A recent report of a new “double-label” radioisotope technique to measure bile acid turnover rates and pool size should be useful for lipids in general, if modified somewhat (47). The technique involves infusing cholic acid-¹⁴C intravenously on day 1 and then injecting cholic acid-³H exactly 24 hr later. On day 3, a sample of bile is collected via a duodenum tube. The radioactivity and total bile acid content in the sample are determined and used to calculate the cholic acid turnover rate and pool size. Results agreed very well with data obtained from the classical single isotope dilution technique described by Lindstedt (48). The technique requires only one sample to be taken and analyzed. Calculations are based on the ratio of the administered cholic-¹⁴C and cholic-³H compared to the ¹⁴C/³H ratio in the
sample. First order kinetics and constant pool sizes are assumed. The decay constant (k) is expressed by equation 1:

\[
(1) \quad \frac{S_{a_t} - ^{14}\text{C}}{S_{a_t} - ^{3}\text{H}} = \frac{S_{a_0} - ^{14}\text{C}}{S_{a_0} - ^{3}\text{H}} e^{-k}
\]

where: 
- \( S_{a_t} \) = specific activity of the isolated sample
- \( S_{a_0} \) = specific activity of the infused sample

Rearranging equation 1 gives:

\[
(2) \quad k = \ln \left( \frac{S_{a_0} - ^{14}\text{C}}{S_{a_0} - ^{3}\text{H}} + \frac{S_{a_t} - ^{14}\text{C}}{S_{a_t} - ^{3}\text{H}} \right)
\]

Since equation 2 requires only that the ratios for the specific activities be known, the \(^{14}\text{C}/^{3}\text{H}\) ratio in disintegrations per minute (dpm) can be substituted for the specific activities to give equation 3:

\[
(3) \quad k = \ln \left( \frac{dpm - ^{14}\text{C}}{dpm - ^{3}\text{H}} + \frac{dpm - ^{14}\text{C}}{dpm - ^{3}\text{H}} \right)
\]

Half-life \((T_{1/2})\) is obtained from equation 4:

\[
(4) \quad T_{1/2} \text{ (days)} = \frac{0.693}{k}
\]

\( S_{a_0} - ^{3}\text{H} \) and \( S_{a_0} - ^{14}\text{C} \) can be calculated from general equation 5:

\[
(5) \quad S_{a_t} = S_{a_0} e^{-kt}
\]

where \( S_{a_t} \) is the experimentally determined specific activity in dpm/g at time \( t \)

Pool size is calculated by equation 6:

\[
(6) \quad \text{Pool size (g)} = \frac{\text{dpm of cholic-}^{14}\text{C injected}}{S_{a_0} - ^{14}\text{C in dpm/g}}
\]

Use of isotopes in analytical measurements

The simple technique of isotope dilution is useful for the determination of sample loss or recovery during extraction, purification and derivatization
of lipids. The procedure involves addition of a known quantity of the isotope-labeled lipid to the original sample and determination of the amount of recovered labeled lipid in the purified sample. The ratio of the recovered isotope to added isotope is proportional to the amount of lipid in the original sample. Radioisotopes are usually used rather than stable isotopes because of the ease of determination of radioactivity by scintillation counting.

An example of the isotope dilution technique is as follows: 10,000 dpm of phosphatidylcholine-$^{14}$C (PC-$^{14}$C) is added to a lipid extract; after separation by preparative TLC, 4 mg of PC is isolated that contains 7,000 dpm of $^{14}$C. Ratio of added to recovered $^{14}$C = $\frac{10,000}{7,000}$ or 1.43, and total PC in original lipid extract = $1.43 \times 4$ mg or 5.7 mg.

Stable isotope dilution techniques combined with gas chromatography-mass spectrometry (GC-MS) analysis have been used to quantitate prostaglandins (49-52) and steroids (53,54). In the procedure for prostaglandins (PG), a deuterated prostaglandin serves both as a carrier for the undeuterated prostaglandin and for quantitation based on the deuterated to undeuterated PG ratio. The inverse isotope dilution procedure described by Green uses tetradeuterated prostaglandins, and the basic technique has been extended to the analysis of a number of prostaglandins and prostaglandin metabolites (49-52). A standard curve is plotted for the PG-d$_0$/PG-d$_4$ ratios vs. mg PG-d$_0$/µg PG-d$_4$ ratios in a set of standard mixtures. The curve is used to correct for any difference between the measured $^1$H/$^2$H ratio and actual PG-d$_0$/PG-d$_4$ ratios and to establish the accuracy and limits of detection. In Green's procedure (49), 8-10 µg of PGE$_2$-d$_4$ or PGF$_{2\alpha}$-d$_4$ is added to the 20-ml blood samples before isolation and derivatization of PGE$_2$ or PGF$_{2\alpha}$. GC-MS analysis is used to determine the $^1$H/$^2$H ratio, which is in the range of 1:1000 to 16:1000. The calculations are similar to those used for radioisotopes, except that atom percent excess (APE) is used in place of specific activities. APE represents the percent of stable isotope over and above that naturally present and is calculated from the $^1$H/$^2$H ratio. The lower limits for detection in experimental samples were about 200 pg/ml for PGF$_{2\alpha}$ and 100 pg/ml for PGE$_2$.

Other examples of analytical procedures based on radioisotope dilution include radio-enzyme assay, radioimmunoassay, double-isotope dilution and assorted clinical tests. The double-isotope dilution procedure has been largely replaced by radioimmunoassay methods, but it deserves mention because it does not require an analytical method for determining the weight of the isolated product. The technique has been used mainly for the analysis of steroids but requires careful laboratory technique to give reliable data. These various methods have not been generally applied to analysis of fatty acids and will not be discussed further.
Comparison of Stable and Radioisotope Methods

Choice of isotope method

Whether to use radio or stable isotopes depends on the experimental situation and the information desired. Both types of isotopes have advantages, which are summarized in Table III.

TABLE 8-III.
Comparison of Radio and Stable Isotope Methods

<table>
<thead>
<tr>
<th>Advantages of radioisotopes:</th>
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<tbody>
<tr>
<td>Higher sensitivity</td>
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<tr>
<td>Rapid and simple detection</td>
</tr>
<tr>
<td>Ease of sample preparation for analysis</td>
</tr>
<tr>
<td>Small sample size administered</td>
</tr>
<tr>
<td>Greater variety of commercially labeled lipids available</td>
</tr>
<tr>
<td>Lower overall cost and effort</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Advantages of stable isotopes:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human studies permitted</td>
</tr>
<tr>
<td>No licensing requirements</td>
</tr>
<tr>
<td>Safety factors</td>
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<tr>
<td>MS analysis provides structural information</td>
</tr>
<tr>
<td>Useful for NMR studies</td>
</tr>
<tr>
<td>Multiple label approach feasible</td>
</tr>
<tr>
<td>Ease of labeled compound synthesis</td>
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</tbody>
</table>

The relative ease with which radioisotopes can be detected by liquid scintillation counting is the major reason for their widespread use compared to stable isotopes. This advantage requires that there be a specific overriding reason before stable isotopes are used in place of radioisotopes. A common reason for choosing stable isotopes is the necessity to obtain experimental data from human subjects. Present regulations in the U.S. do not permit radioisotope studies with children or pregnant women and restrict the use of radioisotopes with other human subjects to $10 \mu\text{Ci}$ of $^{14}\text{C}$ and $50 \mu\text{Ci}$ of $^{3}\text{H}$ except for therapeutic reasons. These levels are too low to provide the accuracy and sensitivity needed in many in vivo lipid experiments, especially when much of the labeled fatty acid is rapidly lost via $\beta$-oxidation. Also, ethical considerations cause difficulty in obtaining protocol approval from human research committees for administration of even these low levels of $^{14}\text{C}$ and $^{3}\text{H}$ to healthy subjects.
Another application where carbon-13 and deuterium but not radioisotopes are useful is in membrane studies where nuclear magnetic resonance (NMR) is used to detect lipid acyl chain interactions. In addition to deuterated prostaglandins, deuterated steroids are also used in isotope dilution mass spectrometry for steroid analyses. For prostaglandin analysis, radioimmunoassay methods (55) have been developed and should theoretically be reliable. However, comparison of radioimmunoassay and mass spectrometry methods indicates the radioimmunoassay method overestimates the levels of prostaglandins by several fold, possibly due to cross-specificity of the antigen preparations.

Detection methods for isotopes

Various analytical techniques for radio and stable isotopes are listed in Table IV; the choice of detection methods depends on the available sample size, sensitivity requirements, purification or separation procedures available and information required.

**TABLE 8-IV.**
Detection and Analytical Methods

<table>
<thead>
<tr>
<th>For tritium and carbon-14 labeled lipids:</th>
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</thead>
<tbody>
<tr>
<td>Liquid scintillation counting</td>
</tr>
<tr>
<td>Radio-TLC scanning</td>
</tr>
<tr>
<td>GC-liquid scintillation counting</td>
</tr>
<tr>
<td>Radio-HPLC flowthrough detector</td>
</tr>
<tr>
<td>X-ray film</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>For deuterium and carbon-13 labeled lipids:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mass spectrometer</td>
</tr>
<tr>
<td>Infrared spectrometer</td>
</tr>
<tr>
<td>NMR spectrometer</td>
</tr>
<tr>
<td>Neutron activation analysis</td>
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<tr>
<td>Laser-Raman spectroscopy</td>
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</tbody>
</table>

The radioisotopes of carbon and hydrogen are normally quantitated by scintillation counting, which is the most sensitive and accurate method available. Scintillation counters, which have been commercially available for almost 30 years, allow most laboratories to easily use radioisotopes. The use of modern three-channel scintillation counters allows carbon-14, tritium and phosphorous-32 to be detected simultaneously and greatly facili-
Radio and Stable Isotopes

states the use of dual and triple radioisotope experiments. Combining
gas chromatography (GC), thin-layer chromatography (TLC), or high-
performance liquid chromatography (HPLC) with radioisotope detection
(54,56,57) provides very useful techniques for lipid analysis. Examples of
radioisotope data obtained by GC/liquid scintillation counting and radio-
TLC for $^3$H and $^{14}$C labeled methyl esters are shown in Figures 2 and 3.

![Graph showing radio-gas chromatography of egg phospholipid methyl esters containing $^3$H and $^{14}$C labeled fatty acids. The curve was reconstructed from sequentially collected effluent GC fractions after radioactivity was measured by liquid scintillation counting (22).]

Fig. 8-2. Radio-gas chromatography of egg phospholipid methyl esters containing $^3$H and $^{14}$C labeled fatty acids. The curve was reconstructed from sequentially collected effluent GC fractions after radioactivity was measured by liquid scintillation counting (22).

By far the most common method for analysis of stable isotope-labeled
lipids is mass spectroscopy. Figure 4 shows the analysis of a mixture containing
soybean oil methyl esters plus five deuterium-labeled fatty methyl esters by
chemical ionization-gas chromatography-mass spectrometry (CI–GC–MS).
The major advantage of the CI–GC–MS method compared to scintillation
counting is that data on the molecular structure are obtained. GC–MS data
drastically reduce the possibility that artifacts or impurities are contribut-
ing to the analysis. By comparison, a common problem with scintillation
counting is the need to eliminate or correct radioactive counts due to
radioactive impurities, background, fluorescence or quenching. The major
disadvantages of mass spectroscopic methods are the time, equipment costs, training and experience required to obtain reliable data.

Multiple Deuterium Labeled Methodology

Single vs. multiple tracer experiments

The advantage of simultaneously administering two or more differently labeled fatty acids has been exploited in many dual-labeled experiments (33–41,45,58) However, this methodology has been applied only to a limited extent with deuterated fatty acids in spite of the advantages of multiple
Fig. 8-4. Analysis of multiple deuterium-labeled standard mixture by chemical ionization GC-MS using methane as the ionization reagent. Sample contained soybean methyl esters plus 16:0-d_{2}, 18:0-d_{6}, 9c-18:1-d_{0}, 9t-18:1-d_{4}, and 9c,12c-18:2-d_{4}.

label methodology compared to single label experiments, as summarized in Table V.

**TABLE 8-V.**

**Advantages of Multiple Label Compared to Single Label Experiments**

<table>
<thead>
<tr>
<th>Advantage</th>
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</thead>
<tbody>
<tr>
<td>Internal control reduces problem of biological variation</td>
</tr>
<tr>
<td>Analytical errors are self compensating</td>
</tr>
<tr>
<td>Easier interpretation of data</td>
</tr>
<tr>
<td>More data per subject or experiment</td>
</tr>
<tr>
<td>Reduced effort and cost</td>
</tr>
</tbody>
</table>

Multiple-labeled experiments using only deuterated fatty acids are feasible if mixtures of fatty acids containing 2, 4, 6 or more deuterium atoms are administered. For mass spectrometry analysis, varying the number of deuterium atoms per fatty acids is in effect the same as using both deuterium and carbon-13 isotopes, because the mass spectrometric analysis depends only on differences in molecular weight of the labeled fatty acids. Both deute-
rium and carbon-13 labeled fatty acids are nonradioactive and nontoxic and can be used in human experiments. The advantage of using deuterated fatty acids is that the available synthetic procedures for deuterium labeling are easier and more versatile than for carbon-13 labeling. It is also much simpler to place the deuterium label at various positions on the fatty acid acyl chain and to incorporate different numbers of deuterium atoms per molecule. Although both isotopes increase the molecular weight of the labeled fats by one mass unit per isotope, the cost of deuterium is much less than for carbon-13. For mass spectrometric analysis, it is desirable to increase the molecular weight of the labeled fatty acid at least two mass units above the endogenous unlabeled fatty acid to minimize the correction for the $M+1$ peak due to the 1.11% natural abundance of $^{13}$C, which results in a probability that approximately 21% of the $C_{18}$ fatty methyl ester molecules will contain at least one $^{13}$C atom.

**Results of multiple deuterium experiments**

The data in Figures 5–8, from human studies with isomeric fatty acids, illustrate the results of triple-labeled experiments with deuterated fatty acids (59–63). The basic protocol used was to have the subject drink a mixture containing triglycerides composed of three different deuterated fatty acids blended with water, caseinate, sucrose and glucose. Blood samples were drawn, and the various plasma lipid fractions were analyzed as their methyl esters by selective ion monitoring (SIM) GC–MS (64). In each experiment, deuterated cis-9-octadecenoic acid (9c-18:1) was included as one of the fatty acids in the mixture and served as an internal control. Plasma phosphatidylcholine data for deuterated 9c-18:1 from eight subjects are plotted in Figure 5 to illustrate the variability for incorporation of 9c-18:1. This large variability represents the results that would have been obtained from single tracer studies. It is obvious that even after statistical treatment of the data, it would be difficult to compare and interpret the results in Figure 5 for the control (9c-18:1) fatty acid with data for the experimental fatty acid isomers plotted in Figure 6. In fact, the attempt to interpret such data has probably been responsible for some of the controversy involving the results of human experiments with cholesterol and fatty acid isomers.

In contrast, the data in Figure 6 for incorporation of the control (9c-18:1) and fatty acid isomers into Plasma-PC can be readily compared and interpreted, without the benefaction of statistical treatment since the fatty acids were fed at the same time. The underlying reason is that each fatty acid has experienced the same biological effects, isolation and purification
Fig. 8-5. Distribution of deuterium labeled 9c-18:1 in human plasma phosphatidylcholine samples from eight subjects (60–63).

Experimental design

The design of multiple-labeled experiments is not without difficulties. Some of the many considerations applicable to both stable and radio multiple labeled experiments are listed in Table VI. Of particular importance is the need to be aware of the limitations of the SIM–GC–MS detection method and the potential problem of interconversion of the deuterated fatty acids. For example, if the reported (65,66) conversion of 16:0 and 18:0 to 18:1 occurs to a significant extent in an experiment utilizing 16:0-d$_2$ and 18:0-d$_2$, the 11c-18:1-d$_2$ and 9c-18:1-d$_2$ formed would interfere with interpretation of 9c-18:1-d$_2$ data if 18:1-d$_2$ were also included in the fed mixture. Nondeuterated and natural $^{13}$C abundance in unlabeled fats also cannot be ignored. The molecular weight of 18:0-d$_0$ (298) is essentially the same as for 9c-18:1-d$_2$; because it is present in much higher concentration than 9c-18:1-d$_2$, 18:0-d$_0$ can introduce serious errors in the GC–MS data if the GC separation of 18:0 and 18:1 is not complete. A correction for the contribution of the M + 2 peak due to the natural abundance of $^{13}$C in 9c-18:1-d$_0$ must also be
Fig. 8-6. Distribution of deuterated fatty acids in human plasma phosphatidylcholine samples from three subjects fed mixtures of triglycerides containing specifically deuterated fatty acid isomers and deuterated 9c-18:1 as an internal standard (60–63).
made because its molecular weight is the same as 9c-18:1-d_2. A secondary problem occurs because the synthesis of deuterated 9c-18:1-d_2 does not yield 100% 9c-18:1-d_2 but will contain varying amounts of 9c-18:1-d_0, -d_1, -d_2, -d_3, -d_4, etc., depending on the method of preparation. This distribution pattern must be determined and appropriate correction of the mass spectral data made.

The accuracy of the GC–MS data depends primarily on the percentage of deuterated fatty methyl ester in the sample (see Table VII). Reported standard deviations for 0.5 μg triple-labeled samples containing 2–3% of each deuterated fatty acid are approximately 0.2 to 0.4, which translates to a 5% to 20% error (64). However, GC–MS accuracy and sensitivity are expected to improve considerably as the methodology is refined.

Applications of Carbon-13 Labeled Fats

Carbon-13 labeled compounds are used as an alternative to carbon-14 labeled compounds in CO_2 breath tests where regulations do not permit use of radioisotopes. The ^13CO_2 breath test has been used in a number of human studies with carbon-13 enriched glucose, amino acids and drugs (67–69). More recently trioctanoin-1-^13C and natural ^13C enriched maize oils have
Fig. 8-8. Distribution of deuterated fatty acids in human plasma cholesteryl ester samples from three subjects fed mixtures of triglycerides containing specifically deuterated fatty acid isomers and deuterated 9c-18:1 as an internal standard (60-63).
TABLE 8-VI.
Protocol Considerations in Multiple Labeled Experiments

<table>
<thead>
<tr>
<th>Placement of label</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interconversion of fatty acids</td>
</tr>
<tr>
<td>Sample size</td>
</tr>
<tr>
<td>Isotope effects and loss of label</td>
</tr>
<tr>
<td>Analytical interferences</td>
</tr>
<tr>
<td>Reverse labeling</td>
</tr>
</tbody>
</table>

been used to evaluate the use of $^{13}$C in place of $^{14}$C for detection of fat malabsorption via the CO$_2$ breath test (68,70). The $^{13}$CO$_2$ breath test is an isotope dilution method that is based on determination of changes in expired $^{13}$CO$_2$/$^{12}$CO$_2$ ratios by MS. Compared to measuring deuterated fatty acids in blood plasma, the CO$_2$ breath test has the advantage of being a noninvasive method.

Other features of the $^{13}$CO$_2$ breath test include the small sample requirements if highly enriched $^{13}$C compounds are fed (1 mg/kg body wt), availability of automated MS instrumentation, and ease of sample preparation. Infrared spectrometry methods are also available for $^{13}$CO$_2$ measurements (71,72). The carbon-13 breath test is considered to be as accurate as the carbon-14 breath test, but both methods suffer from problems caused by variability in (a) rates of gastric emptying, (b) size of triglyceride pool, (c) $\beta$-oxidation rates, and (d) fecal transit times (73,74).

The relative insensitivity of mass spectral methods compared to radioisotope methods for detection of intact lipids has been a limiting factor in the use of stable isotope labeled fatty acids for metabolic experiments. Improved mass spectral methods are now available that are reported to allow the turnover rate and half-life of 16:0-1-$^{13}$C (90% $^{13}$C enriched) to be measured in dogs infused with 0.042 $\mu$ mole/kg body wt/min or about 20 mg 16:0-1-$^{13}$C for a 2-hr study (75). The procedure is reported to have a sensitivity close to the $^{13}$CO$_2$/$^{12}$CO$_2$ ratio method but still retains the advantage of monitoring the molecular ion of methyl palmitate. Another recent report has compared turnover and oxidation data from dogs infused with 0.035 $\mu$ mole/kg/min of 16:0-$^{13}$C to data obtained with 16:0-$^{14}$C (76). Both investigations reported the use of 16:0-1-$^{13}$C in combination with MS detection to be an accurate and effective technique. This basic procedure has been applied to determine the epinephrine plasma threshold levels needed to induce lipolysis in normal human subjects (77). The procedure consisted of infusing about 77 mg of 16:0-1-$^{13}$C (85% enrichment) at a rate of 0.033 $\mu$ mole/kg/min for 2 hr. The percent 16:0-1-$^{13}$C enrichment in 1-ml blood samples drawn at
<table>
<thead>
<tr>
<th>Method and sample size</th>
<th>Total label (%)</th>
<th>( \bar{x} )</th>
<th>s</th>
<th>% RSD</th>
<th>( \bar{x} )</th>
<th>s</th>
<th>% RSD</th>
<th>( \bar{x} )</th>
<th>s</th>
<th>% RSD</th>
<th>( \bar{x} )</th>
<th>s</th>
<th>% RSD</th>
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</thead>
<tbody>
<tr>
<td>EI, 50</td>
<td>56.5 1.23</td>
<td>2.2</td>
<td>13.6 0.33</td>
<td>2.4</td>
<td>15.4 0.15</td>
<td>1.0</td>
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<tr>
<td>0.5 µg 2</td>
<td>92.8 0.73</td>
<td>0.8</td>
<td>1.88 0.35</td>
<td>18.6</td>
<td>2.59 0.17</td>
<td>6.6</td>
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<tr>
<td>CI, 50</td>
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<td>0.6</td>
<td>0.50 0.36</td>
<td>72.0</td>
<td>0.70 0.09</td>
<td>12.8</td>
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<tr>
<td>0.5 µg 2</td>
<td>57.2 0.46</td>
<td>0.8</td>
<td>13.3 0.22</td>
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<td>0.3</td>
<td>1.90 0.27</td>
<td>14.5</td>
<td>2.64 0.12</td>
<td>4.5</td>
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<tr>
<td>0.05 µg 2</td>
<td>98.5 0.22</td>
<td>0.2</td>
<td>0.13 0.19</td>
<td>146</td>
<td>0.69 0.13</td>
<td>18.8</td>
<td>0.72 0.15</td>
<td>20.8</td>
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<tr>
<td>CI, 50</td>
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<td>14.2 0.41</td>
<td>2.9</td>
<td>14.9 0.44</td>
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<td>14.6 0.37</td>
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<tr>
<td>0.05 µg 2</td>
<td>91.4 1.86</td>
<td>2.0</td>
<td>2.87 1.04</td>
<td>36.1</td>
<td>3.01 0.51</td>
<td>16.9</td>
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<td>23.1</td>
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<tr>
<td>2</td>
<td>97.2 1.01</td>
<td>1.0</td>
<td>1.02 0.76</td>
<td>74.5</td>
<td>0.89 0.12</td>
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</tbody>
</table>

\( \bar{x} \) = mean percent deviation  
\( s \) = standard deviation (N = 6)  
% RSD = percent relative standard deviation  
GC–MS (EI) = M-31 and M-32 ions monitored  
GC–MS (CI) = M + 1 ions monitored

**TABLE 8-VII.**

Accuracy of SIM–GC–MS Electron Impact Ionization (EI) and Chemical Ionization (CI) Methods for Triple Labeled Standard Mixtures (64)
Radio and Stable Isotopes

10-min intervals was measured by multiple ion detection mass spectrometry. Turnover rates were calculated from the 16:0-13C infusion rate and the 16:0-13C/16:0-12C ratio after 16:0-13C plasma levels reached a steady state equilibrium. To calculate turnover rate, the percent 16:0-13C in the 0 time and steady state samples were determined from the 13C/12C ratio. The values were subtracted and the difference or increase in percent 16:0-13C is termed atom percent enrichment (APE) or mole percent excess enrichment. Examples of the equations used to calculate turnover and oxidation rates are given in Figures 9 and 10.

**Plasma FFA Turnover Rate by Steady State Infusion of 16:0-1-13C**

Assume: Rate of 16:0-13C infusion equals rate of 16:0-13C disappearance

Data: Infusion Rate (IF) = 0.06 μmole/kg/min 16:0-13C (85% enriched)

16:0-13C/16:0-12C ratio expressed as 13C Isotope Enrichment (IE)

\[ IE_N = 19.79\% \quad IE_E = 22.69\% \]

Atom Percent Excess (APE) = \( %IE_E - %IE_N = 2.9\% \)

\[ \text{Turnover of 16:0} = \frac{\text{APE of infused 16:0-13C}}{\text{APE of plasma 16:0-13C}} \times IF \]

\[ = \left( \frac{0.85}{0.029} - 1 \right) \left( 0.06 \right) = 1.7 \mu\text{mole/kg/min} \]

Turnover of Total FFA = 16:0 turnover ÷ 16:0% in plasma

\[ = 1.7 \div .30 = 5.67 \mu\text{mole/kg/min} \]


Fig. 8-9. Calculation of plasma free fatty acid turnover in dogs based on steady state infusion of 16:0-1-13C (76).
Plasma FFA Oxidation Rate by Steady State Infusion of 16:0·1-13C

Data: Rate of CO2 expired = 210 μmole/kg/min
APE of 13CO2 0.013% APE of plasma 16:0·13C = 2.9%

Percent CO2 from 16:0·13C = \frac{\text{APE 13CO2}}{\text{APE 16:0·13C} \times 0.92} \times 100
\quad = \frac{0.013}{2.9 \times 0.92} \times 100 = 0.48%

Where 0.92 is correction factor for 13CO2 −2 ion in plasma

16:0 Oxidation rate = 0.0048 \times 210 = 1.0 \mu \text{mole/kg/min}

Percent 16:0 oxidized = \frac{\text{Oxidation rate}}{\text{Turnover rate}} \times 100
\quad = \frac{1.0}{1.7} \times 100 = 58.8%


Fig. 8-10. Calculation of plasma free fatty acid oxidation in dogs based on steady state infusion of 16:0-1-13C (76).

References