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[13] Quantitating Isotopic Molecular Labels with Accelerator Mass Spectrometry

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Abstract

Accelerator mass spectrometry (AMS) traces isotopically labeled biochemicals and provides significant new directions for understanding molecular kinetics and dynamics in biological systems. AMS traces low-abundance radioisotopes for high specificity but detects them with MS for high sensitivity. AMS reduces radiation exposure doses to levels safe for use in human volunteers of all ages. Total radiation exposures are equivalent to those obtained in very short airplane flights, a commonly accepted radiation risk. Waste products seldom reach the Nuclear Regulatory Commission (NRC) definition of radioactive waste material for ^{14}C and ^{3}H. Attomoles of labeled compounds are quantified in milligram-sized samples, such as 20 μl of blood. AMS is available from several facilities that offer services and new spectrometers that are affordable. Detailed examples of designing AMS studies are provided, and the methods of analyzing AMS data are outlined.

METHODS IN ENZYMOLOGY, VOL. 402 0076-6879/05 $35.00
DOI: 10.1016/S0076-6879(05)02013-6

Introduction

Isotopic labels remain the standard for tracing chemically exact analogues of biochemicals, whether they are drugs, toxins, nutrients, or macromolecules, through biological systems. Isotopes are one or more equivalent atomic forms of a chemical element that contain that element's signature number of nuclear protons and atomic electrons, but that may have varying numbers of nuclear neutrons that give each isotope of an element a distinctive mass. The number of neutrons in an isotope nucleus may render it unstable against several forms of radioactive decay. These radioisotopes may have lifetimes of billions of years or fractions of a second. Some elements form stable nuclei from only one configuration of protons and neutrons and have a single isotopic mass, such as aluminum (atomic weight = 27 g/mol) or indium (113 g/mol). The next element beyond indium, tin, has 10 stable isotopic forms from mass 112 g/mol with a natural terrestrial abundance of 0.97% to mass 124 g/mol at a natural abundance of 5.79%. Most elements have a dominant atomic mass, such as carbon-12 (^{12}C) (98.89% abundance), with one or more low abundance stable isotopes like carbon-13 (^{13}C) (1.11%). Hydrogen is 99.985% mass 1 and 0.015% mass 2 (deuterium). Both radioisotopes and stable isotopes are used as labels that give a molecule a distinctive molecular signature in an isolated biological component. The altered molecular mass generally has no effect on the molecule's chemical interactions, although an entire field of enzyme kinetic research does depend on effects resulting from the careful placement of an isotope in a particularly reactive molecular location (Cleland, 2003, and references therein).

The ultimate sensitivity for quantifying an isotopic label depends on three factors: the rarity of the isotope, the precision and sensitivity of the isotopic detection technique, and the number of isotope labels in the traced compound. The number of isotope labels in a molecule has linear significance at most; the number may not exceed the natural amount of the element in the compound without changing its chemical properties. Isotope abundance and detection provide a more complex space from which to choose an experimental design. The more rare an isotope, the more distinctive it will be as a molecular label, assuming equivalent efficiency and precision of detection. The rarest stable isotope, ^{3}He, has a natural occurrence of 1.4 ppm but is useless as a label because of its noble chemistry. The decay of radioisotopes ensures that they have very low natural abundance, unless there are continuous production mechanisms, such as the creation of ^{14}C in the upper atmosphere. The natural concentration of ^{14}C in the living biosphere remains approximately a part per trillion (ppt) ($1:10^{12}$), compared to the part-per-100 abundance of ^{13}C. Doubling the concentration of

both isotopes in 1 mg of carbon requires 900 nmol of ^{13}C label or 98 amol of ^{14}C. Equivalent detection of the two isotopes gives a 10 billion increase in sensitivity for compounds labeled with ^{14}C rather than ^{13}C. Routine laboratory measurements of these isotopes are not equivalent, however, and fail to exploit the inherent sensitivity of low abundance radioisotope labels. Isotope quantitation falls into two broad technologies: detection of decay products from radioactive isotopes or isotope ratio MS (IRMS) for stable isotopes. AMS, a type of high-energy IRMS, now quantifies certain long-lived radioisotopes for biochemical research at efficiencies similar to those for stable isotopes but with much higher sensitivity.

AMS arose in the late 1970s from two distinct research threads with a common goal: an improvement in radiocarbon dating that would make efficient use of datable material and that would extend the routine and maximum reach of radiocarbon dating. Attempts to use IRMS for ^{14}C were hampered by difficult isobar backgrounds (Aitken, 1978), but nuclear physicists were able to use negatively charged ions and high-energy collisions to remove these interferences (Bennett *et al.*, 1977; Nelson *et al.*, 1977). AMS is routinely used in geochronology and archaeology, but biological applications began appearing in 1990 (Turteltaub *et al.*, 1990) and were available only from a few laboratories. AMS is now more accessible for biochemical quantitation from several industrial concerns, at a National Institutes of Health (NIH) Research Resource at Lawrence Livermore National Laboratory, or through the development of smaller affordable spectrometers. This chapter provides a tutorial for biochemical tracing of ^{14}C- and ^{3}H-labeled compounds using AMS.

Methods

Decay Counting Efficiency

The contrast between radioisotope quantitation by decay counting and by MS is indicated by the definition of "activity," A, in which the decays per time (e.g., dpm) are related to the total number of that isotope present through the mean isotopic life (τ = half-life divided by the natural log of 2) in Eq. (1). The mean life of ^{14}C is 8270 yr (4.35×10^9 min), and that of tritium is 17.8 yr (9.36×10^6min).

$$A = \frac{\partial N}{\partial t} = \frac{N}{t_{1/2}/\ln(2)} = \frac{N}{\tau} \quad (1)$$

The maximum efficiency (ε) of detecting a fraction of the ^{14}C depends directly on the length of time used to detect the decays, no matter which

decay counting method is used:

$$\partial t = \frac{\partial N}{N} \times \tau = \epsilon \times \tau \tag{2}$$

Thus, 1% of the ^{14}C in a sample can be counted in, at best, 83 yr. A sample decaying at 1 dpm (0.45 picocurie [pCi], 17 millibecquerel [mBq]) requires a week of counting to provide 10,000 counts for 1% statistical precision, momentarily ignoring the usual instrumental background that prevents quantitating 1 dpm with most decay counters. The sample contains 7.2 fmol of ^{14}C:

$$N = \frac{\partial N}{\partial t} \times \tau = \frac{1}{\text{min}} \times 4.35 \times 10^{9}\ \text{min} \times \frac{\text{mol}}{6.02 \times 10^{23}} = 7.22\ \text{fmol} \tag{3}$$

AMS has isotope-counting efficiencies greater than 1% in short measurements of a few minutes (Vogel *et al.*, 1989). New spectrometers count natural ^{14}C (ppt) at 300 counts/s or more (Brown *et al.*, 2000). The above 7.2 fmol ^{14}C in 1 mg of carbon is 87 ppt, which provides 10,000 ^{14}C counts in less than 1s by AMS counting. Sensitivity is only slightly less striking for shorter lived isotopes, such as tritium. One dpm of tritium arises from 15.5 amol (10^{-18} mol) of the isotope. In 1 μl of water, this amount creates an isotope ratio of 0.14 ppt ($^{3}H/H$), from which AMS counts 10,000 counts in 1000s (17 min).

Replacing decay counting by MS yields a sensitivity gain of 1000 to 1 billion for radioisotope quantitation. This gain can be used to reduce ^{14}C and ^{3}H use below operational definitions of radioactive, isolate much smaller and more specific biochemical fractions for measurement, trace compounds at physiological rather than pharmacological levels in host animals or in all human populations (including children), obtain higher sample throughput for studies requiring many samples, or some combination of all of these goals.

Accelerator Mass Spectrometry

AMS produces high detection efficiency using techniques outlined in Fig. 1, which is drawn to diagram ^{14}C analysis but applies to a number of isotopes without fundamental changes. Atoms from about 1 mg of carbon are ionized with an added electron and accelerated to a moderate energy before a mass analysis at 14 atomic mass units (amu). About 1% of the ion beam from the source passes this analysis, although the true ^{14}C represents only 1 ppt, emphasizing the early difficulty in detecting ^{14}C with simple mass spectrometers. The vast majority of these ions are molecular hydrides of the lighter carbon isotopes: $^{12}CH_2$, ^{12}CD, and ^{13}CH. Lithium enters the

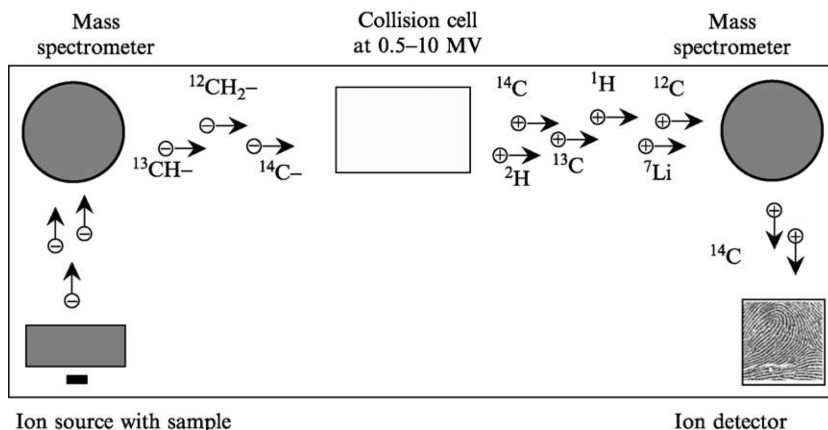

FIG. 1. Schematic of the basic ideas behind accelerator mass spectrometry (AMS): Tandem MS (MS/MS) with a charge-changing collision cell at million-volt potentials between the spectrometer elements. Each ion is "fingerprinted" by how it loses energy in the final detector.

prepared samples from the glass reaction vessels used in reducing all carbon samples to a graphitic form (Loyd *et al.*, 1991) and forms a mass 14 molecule, $^{7}Li_2$. Nitrogen, which is 99.63% the mass 14 isotope, does not form a negative ion and does not appear in the initial ion beam. This discovery was one of the fundamental steps in making AMS possible for ^{14}C detection (Middleton, 1974). As in tandem collision MS, molecules are broken by collisions in a thin foil or gas cell. Macromolecules dissociate at kiloelectron-volt (keV) energies, but an acceleration through several million volts is necessary to destroy hydrides in a single collision. Multiple collisions at sub-megavolt potentials have been used to destroy these molecules, ushering in a series of smaller and more affordable spectrometers (Hughey *et al.*, 1997; Suter *et al.*, 1997). Molecular dissociation produces positive ions that accelerate away from the high positive potential back to ground potential. Multiple magnetic and electrostatic elements follow the accelerations to select the desired high-energy ^{14}C ions from the molecular debris composed of H, D, ^{7}Li, ^{12}C, and ^{13}C. This selection is effective to parts per billion or better and reduces the filtered ion stream to hundreds of particles per second. Scattered background ions are rare in the high-energy spectrometer because ion energies are too high for atomic scattering and the nuclear scattering probabilities are very low. Transport through the high-energy spectrometer ends at a detector that uniquely identifies the ion by quantifying how it loses energy.

The specificity of AMS lies in the two processes that require high energies provided by inclusion of an accelerator in the tandem mass spectrometer: destruction of molecular isobars in energetic collisions and identification of each high-energy ion through quantifying the rate of energy loss. These two processes are present in AMS detection of all isotopes and are straightforward for ^{3}H and ^{14}C, because their nuclear isobars (isotopes of other elements having the same mass as the counted ion) do not form negative ions. Other elements often require ''tricks'' of chemistry or physics to reduce the counting rate of the detector to acceptable levels from an interfering isobar. For example, ^{41}Ca is initially accelerated as $^{41}CaF_3$ because $^{41}KF_3$ is not stable, and a thick foil is placed before the final detector in quantifying ^{10}Be because the higher atomic number of ^{10}B forces it to lose energy faster than ^{10}Be, which passes through.

Table I lists long-lived isotopes of elements that are routinely measured at the LLNL Center for AMS. Most AMS facilities have ^{14}C capabilities, and many quantify other isotopes. Chemical isolation of the chosen element is often a limiting technology in applying AMS to a given isotope. Suppression of nuclear isobars in the chemical preparation is fundamental to the use of AMS for many elements, because high count rates in the ion detector degrade its ability to identify individual ions. Methods for detection of the isotopes in Table I are published in the AMS literature with the last AMS conference proceedings as a suitable resource (Nakamura *et al.*, 2004).

TABLE I
LONG-LIVED RADIOISOTOPES MEASURED AT CAMS/LLNL

Element	Isotope	Half-life (yr)	Mean life (yr)	Mean life (min)
Hydrogen	^{3}H	12.33	17.79	9.36e6
Beryllium	^{7}Be	0.15	0.22	1.14e5
	^{10}Be	1.51 *M*	2.18 *M*	1.15e12
Carbon	^{14}C	5730	8270	4.35e9
Aluminum	^{26}Al	740 k	1.07 *M*	5.61e11
Chlorine	^{36}Cl	301 k	434 k	2.28e11
Calcium	^{41}Ca	103 k	149 k	7.82e10
Nickel	^{59}Ni	108 k	156 k	8.20e10
	^{63}Ni	100.1	144	7.60e7
Technetium	^{99}Tc	210 k	303 k	1.59e11
Iodine	^{129}I	15.7 *M*	22.6 *M*	1.19e13
Uranium	Various	Various	Various	Various
Plutonium	Various	Various	Various	Various

Units

Radiocarbon occupies a special place among isotopes, because it is produced in the atmosphere and is incorporated in all living systems, whether plant, animal, microbe, etc. The "distance" of a living creature along the food chain from the atmospheric source is reflected in the concentration of ^{14}C that it contains. Fresh deepsea fish or soil microbes, for example, have lower ^{14}C levels than a new blade of grass. The popular application of ^{14}C is for radiocarbon dating that depends on the steady radioactive decay of the isotope within the carbon of a previously living creature or plant. Atmospheric ^{14}C is variable over many centuries, because of production influences of the solar cycle and the earth's magnetic field. Testing nuclear weapons in the 1950s and 1960s almost doubled the amount of ^{14}C in the atmosphere, but that excess has been absorbed into the oceans with an atmospheric mean lifetime of 15 years. The atmosphere is now only a few percentages above its "natural" level. The ^{14}C dating community and many AMS facilities report ^{14}C concentration in a unit that reflects this expected natural concentration of atmospheric ^{14}C: Modern. The unit is convenient in biochemical AMS to give an immediate feel for the level of labeled compound in a living host. It is also a convenient way to report ^{14}C concentrations that can be robustly converted to one of several other units desired for analysis. "Modern" represents a ^{14}C concentration, an amount of ^{14}C within a specific amount of carbon. Contrast this with a measure of decay products (e.g., dpm, microcurie) that represents an amount of ^{14}C that gives rise to the radioactivity of a sample independently of the sample size, as defined in Eq. (1). Table II lists several equivalent numerical values of Modern, with 13.56 dpm/g carbon being the fundamental definition (Stuiver and Polach, 1977). AMS samples often contain about 1 mg of total carbon, so some units are expressed per-milligram of carbon.

TABLE II
EQUIVALENT CONCENTRATIONS OF ^{14}C AND ^{3}H IN VARIOUS UNITS

^{14}C Value	Unit	^{3}H Value	Unit
1.0	Modern (Mod)	1.0	Tritium unit (TU)
1.176×10^{-12}	atom/atom C	1.0×10^{-18}	atom/atom H
1.176	pmol/mol C	1	amol/mol H
13.56	dpm/g C	64.38	μ dpm/mg H
226.0	μ Bq/mg C	1.073	μ Bq/mg H
6.108	fCi/mg C	29.00	aCi/mg H
97.89	amol/mg C	0.992	zmol/mg H

Tritium naturally arises from radioactive decay products in the earth and from atmospheric sources similar to ^{14}C, but at much lower concentrations. The unit of natural ^{3}H is the ''tritium unit'' (TU), which represents one ^{3}H atom in 10^{18} hydrogen atoms, or approximately 3.2 pCi/L of water. Various sources produce a background in our present environment of about 10 TU.

Range, Resolution, and Sample Size

AMS was developed for radiocarbon dating, which quantified the loss of ^{14}C in a sample compared to the natural level of 98 amol/mg carbon. AMS quantifies ^{14}C concentrations in materials as old as 50,000 yr (0.2% Mod) with precisions as high as 0.1%. The amount of ^{14}C in 1mg of 50,000-year-old material is 0.23 amol. A 0.1% precision on measurement of 1 mg, 1 Mod sample also quantifies $\pm$0.1 amol. For several decades, earth scientists have routinely quantified atto- and even zepto- (10^{-21}) moles of ^{14}C in milligram-sized samples. The chemical methods for handling large numbers of biological samples are less rigorous than those for dating applications, raising the useful sensitivity limit of biological AMS to 1% Mod, or about 1 amol of isotope label in a milligram-sized sample. Samples can be as small as 100 μg, however, leading to quantitations in zmol of label. Sensitivity for tritium extends to similar concentrations at less than 100 TU in 200-μg samples for tracing limits less than 1 amol.

The upper limit of isotope concentration in AMS comes from the counting ability of the identifying detector. There are several kinds of detectors, and the electronic systems connected to them can correct for missed counts (''live-time'' corrections) to some degree, but rates greater than 100,000 cps lead to larger uncertainties in quantitation (Vogel *et al.*, 2004). Typical counting rates for Modern material are 100 cps or more, and samples up to 1000 Mod are easily measured. Experiments should be designed to reliably provide samples below 100 Mod for ^{14}C, or 100 mega-TU for tritium. The ion source can become contaminated at concentrations a factor of 100 above these, and a time-consuming cleaning process is required to recover sensitivity for lower levels after such a sample.

The range of readily measured samples, thus, spans 5 orders of magnitude, with the highest precision available in the middle 2 orders, with reproducibility of 1–3% for ^{14}C and 2–5% for ^{3}H, as measured by repeated isotope ratio measurements each having less than 1% counting statistics. Uncertainties in quantitation expand to 5–10% for the high decade ($\geq$100 Mod) and for the lowest decade (1–10% Mod). Natural biological hosts do contain Modern carbon, and the ability to quantify below this level may appear spurious. The extreme sensitivity of AMS makes extensive sample definition through highly selective fractionation possible. Chemical

processing for AMS measurement needs more material than found in the fractions, and a well-defined mass of "carrier" compound is added to these isolated fractions that may contain only 1 ng or μg of natural carbon after solvents are removed. This carrier is chosen to have a low, but non-zero, ^{14}C or ^{3}H concentration. HPLC identification of metabolites has been done this way (Buchholz *et al*., 1999). Many separation media, such as acrylamide gel, are made from ^{14}C- and ^{3}H-free petroleum stock, in which isolated proteins that bind labeled compounds are easily distinguished at submicrogram amounts using the separation media directly as the carrier material (Vogel *et al*., 2001).

Measurement precision is highest at Modern ^{14}C concentrations, and tissue samples only a few percentages above natural levels can be confidently quantified using self-controls that are collected from a biological host just before dosing with the labeled compound. Resolution can be as little as 5% above the presumably Modern control, providing quantitation as low as 1 fmol/g of tissue (tissue ≈20% carbon):

$$(105 - 100)\% \times \text{Modern} \Rightarrow 0.05 \times 98\frac{\text{amol}}{\text{mgC}} \times 0.2\frac{\text{mgC}}{\text{mg}} \approx 1\frac{\text{amol}}{\text{mg}} = 1\frac{\text{fmol}}{\text{g}} \tag{4}$$

Even restricting intake to keep samples at less than 10 Mod results in a 200:1 dynamic range above minimal resolution:

$$10\ \text{Modern} \Rightarrow 10 \times 98\frac{\text{amol}}{\text{mgC}} \times 0.2\frac{\text{mgC}}{\text{mg}} \approx 200\frac{\text{amol}}{\text{mg}} = 200\frac{\text{fmol}}{\text{g}} \tag{5}$$

Tissue concentration of the label is directly derived from an isotope ratio knowing only the carbon content of the measured tissue. The size of the individual sample does not enter the calculation, reducing the number of manipulations and increasing the precision of the determination.

Conversion of carbon to the solid fullerene (Vogel, 1992) or hydrogen to the titanium hydride (Chiarappa-Zucca *et al*., 2002) forms preferred in AMS quantitation work best if approximately 1 mg of the element is present. The carbon and hydrogen contents of some biological samples are listed in Table III, along with the amount of that sample that is useful for AMS sampling. Capillary collection using finger pricks for humans or tail punctures for rodents is possible for the occasional blood sample. These small blood volumes are also readily obtained during postmortem animal sampling without the use of skilled cardiac punctures. Indwelling catheters are useful when a kinetic study requires many closely spaced collections from humans or large animals (Dueker *et al*., 2000). The small AMS samples are a benefit to invasive collections such as blood or cells but

TABLE III
CARBON AND HYDROGEN CONTENTS OF VARIOUS BIOLOGICAL MATERIALS AND THE APPROXIMATE AMOUNT OF EACH REQUIRED IN MAKING AN AMS SAMPLE

	%C	Sample	%H (wet)	Sample	%H (dry)	Sample	Collection
Plasma	4	25 μl	11	3 μl	5	3 mg	Capillary, syringe, catheter
RBC	17	5 μl	11	4 μl	8	3 mg	Capillary, syringe, catheter
Blood	10	10 μl	11	3 μl	6	3 mg	Capillary, syringe, catheter
Urine	0.3–1.5	200 μl	11	3 μl	2	6 mg	Cup
Feces	10	10 mg	7	4 mg	5	6 mg	Bag
Cells	22	5 mg	10	3 mg	6	5 mg	Various, e.g., buccal scrape
Organ	15	7 mg	10	3 mg	7	4 mg	Biopsy, dissection
Fat	64	1.5 mg	12	3 mg	12	3 mg	Biopsy, dissection
Protein	28	4 mg			5	6 mg	Biopsy, purification
DNA	29	4 mg			4	6 mg	Biopsy, purification
Nerve	13	8 mg	11	3 mg	9	3 mg	Biopsy, dissection
Muscle	11	8 mg	10	3 mg	6	5 mg	Biopsy, dissection
Breath	1	80 ml	1	60 ml			Tube, absorbent

complicate the use of the copious excreta samples that must be homogeneously sampled. Careful homogenization of urine and feces maintains relevance in the isotope ratio. Postmortem tissue or needle biopsy samples are 5–10 mg, because most tissue is 10–20% carbon. A few test samples can be weighed to provide a "feel" for the proper sample size, but a known sample size is not needed for quantitation. The elimination of sample weighing reduces the incidence of accidental contamination. Contamination control is of utmost importance when the instrument sensitivity reaches attomoles (Buchholz *et al.*, 2000).

Tissue samples can be smaller than those outlined here, perhaps down to 10% of that listed, but special handling is required in preparing them. Even smaller samples are augmented with carrier compounds to obtain the desired process size. Two cases are most easily analyzed: either the carbon in the sample must arise from the natural tissue, or the added carrier carbon should dominate (>95%) the sample. Samples containing 10–50 μg of carbon or hydrogen fall between these limits and are problematic for solid sample AMS. Ion sources that accept combustion products directly are used for this mass range and allow more direct coupling of separation instruments (Liberman *et al.*, 2004). A new technique of

measuring sample masses between 0.1 and 100 μg permits quantitative isotope dilution by addition of a known amount of carrier carbon to the measured mass of the sample before combustion of the sample for AMS measurement (Grant *et al.*, 2003).

Dose Estimation

AMS quantitates most accurately for isotope ratios near 10^{-12}. Experiments designed for AMS quantitation need to keep samples within the range of high precision while introducing sufficient isotope label to have the needed dynamic range for the biological process being studied. In this section, we provide examples of dose estimations based on the data and constraints in the previous section. The calculations will use the data from the ''reference human'' listed in Table IV to allow for normalizations to other cases.

Decay counting experiments are designed to avoid a loss of signal at the low end. If there is an excess of isotope label, the high-end samples are just counted faster. AMS turns this around, because it has high precision for distinguishing signals near the natural background concentrations, but the instrument can be harmed by carelessly high isotope concentrations. Maximum concentration arises in plasma shortly after the absorption of a bioavailable or injected dose of a hydrophilic compound that remains isolated in the plasma. Such isolation in plasma is unlikely, and corrections are considered below. A labeled dose adding 10 Mod to the naturally Modern plasma leaves room for error and individual variation without approaching the upper limit of 100 Mod. The total dose for a 70-kg person is found from the 1mg of carbon available from 25 μl of the 3 liter plasma,

TABLE IV
VALUES FOR PHYSICAL PROPERTIES OF THE REFERENCE HUMAN USED IN CALCULATING DOSES

Weight	70 kg
Body water	42 L
Intracellular water	24 L
Blood	5 L
Plasma	3 L
Soft tissue	45 kg
Fats	12 kg
Muscle	28 kg
Daily urine	2 L
Daily feces	1 kg

and Modern is expressed in any of the desired units (curie, becquerel, mole):

$$10 \text{ Mod} \Rightarrow 10 \times 6.1 \frac{\text{fCi}}{\text{mgC}} \times \frac{1 \text{ mgC}}{25\,\mu\text{l}} \times 31 = 7.3 \text{ nCi} \tag{6}$$

A total ^{14}C dose of about 10nCi is optimal if a chemical enters the plasma through complete gastrointestinal tract absorption or through intravenous injection and is expected to clear from plasma without extensive redistribution. Many compounds are labeled with ^{14}C at 10 Ci/mol (16.0% of the molecules containing a ^{14}C; pure ^{14}C = 62.4 Ci/mol, 2.3 teraBq/mol), yielding this ^{14}C dose from only 45 nmol, or 9 μg of a 200g/mol compound in the 70-kg human. Even physiological concentrations are greater than this 130 ng/kg, leading to the possibility of diluting labeled compounds to parts per hundred or more with unlabeled equivalents to make up the desired chemical dose at this radioactive content.

A similar calculation for tritium in plasma is given in Eq. (7), where the tritium concentration is found for a sample that yields 1 cps by AMS:

$$0.01 \text{ megaTU} \Rightarrow 10^4 \times \frac{0.99 \text{ zmol}}{\text{mgH}} \times \frac{1 \text{ mgH}}{9.5\,\mu\text{l}} \times 31 = 3 \text{ nmol} \tag{7}$$

This represents 90 μCi of ^{3}H (pure ^{3}H = 29.1 kCi/mol, 1077 teraBq/mol), 3 μmol of a compound that is labeled at 1 in 1000, or 60 μg of a 200 g/mol compound for the 70-kg subject. A 0.01megaTU sample decays at 0.6 dpm, but AMS provides a dynamic range 3 orders of magnitude down from this concentration, and a 3% counting precision is available in 20 min rather than 30 h/sample.

The retention of a hydrophilic compound fully in the 3 liter of plasma is unrealistic, because such a compound is at least likely to expand into the interstitial water for a total distribution of 18 liters, increasing the estimated dose of ^{14}C to about 45 nCi and of ^{3}H to 560 μCi. The maximum distribution is limited to the full 42 liters of all body water, requiring a factor of 14 more isotope to obtain the same plasma concentrations as found in Eqs. (6) and (7). The human ^{14}C dose would then become 102 nCi, a dose that has been used in successfully tracing the hydrophilic folate vitamin within humans (Clifford *et al.*, 1998; Lin *et al.*, 2004). It is instructive to calculate the natural ^{14}C in our 70-kg reference human assuming a total body carbon abundance of 23%:

$$1 \text{ Mod} \Rightarrow 1 \times 6.1 \frac{\text{fCi}}{\text{mgC}} \times \frac{23 \text{ mgC}}{100 \text{ mg}} \times 70 \text{ kg} = 98.2 \text{ nCi} \tag{8}$$

The ^{14}C dose for tracing the hypothetical hydrophile only doubles the ^{14}C in humans over the period that the compound requires for metabolism and elimination. These dose estimates contain sufficient leeway for variations in bioavailability and volume of distribution over factors of 5 or more. If bioavailability is expected to be less than 20%, the initial test dose should be increased. Label doses also increase for hydrophobic compounds that partition into the lipids and are less available in easily collected fractions. Beta-carotene was labeled at 200 nCi of ^{14}C per subject for studies that traced it and its derivative vitamin A for more than 6 months from a single dose (Dueker *et al.*, 2000; Hickenbottom *et al.*, 2002; Lemke *et al.*, 2003).

Minimum dose calculations ensure quantitation for extended kinetic studies. For example, minimum ^{14}C for detection at least 10% above natural levels in urine is calculated in Eq. (9) for 1 wk after a dermal exposure to a compound with 5% absorption whose biological mean life is 1 day. The urine is assumed to sample the 24 liters of interstitial body water, and the ^{14}C is found in approximately 1.5 mg carbon obtained in 200 μl:

$$(1.1 - 1)\ \text{Mod} \Rightarrow 0.1 \times 6.1\frac{\text{fCi}}{\text{mgC}} \times \frac{1.5\ \text{mgC}}{200\ \mu\text{l}} \times 24\,\text{l} = 110\ \text{pCi} \qquad (9)$$

Because the urine is collected 7 mean lives after the exposure, the body water needs to be a factor of 1097 (=e^7) higher on the exposure day, giving a total interstitial water load of 120 nCi shortly after exposure. This absorbed amount arises from 2.4 μCi of ^{14}C applied dermally with a 5% chemical absorption (=120 nCi/0.05). This compares well with the actual human doses of [^{14}C]-atrazine whose metabolites were identifiable by HPLC-AMS 1 wk after dermal exposures (Buchholz *et al.*, 1999). Tritium does not have to overcome a large natural background, but a minimum concentration of about 100 TU will provide a readily quantifiable count rate in AMS:

$$100\ \text{TU} \Rightarrow 100 \times 29\frac{\text{aCi}}{\text{mgH}} \times \frac{1\ \text{mgH}}{10\ \mu\text{l}} \times 24\ \text{l} = 7\ \text{nCi} \qquad (10)$$

The same factors apply for a 1-wk loss through excretion (7.7 μCi) and a 5% bioavailability to yield a minimum ^{3}H dermal dose of 150 μCi. Extrapolation of these simple examples of kinetics calculations to animals from human examples are usually done with a simple assumption of body mass scaling.

Biopsies or dissections provide tissues for quantitating molecular interactions with macromolecules such as DNA or proteins (Kautiainen *et al.*, 1997; Vogel *et al.*, 2002). Adjustments to the simple dose calculations must be made to allow for chemical partitioning into the cells or for binding

probabilities. It is difficult to predict cellular uptake and interaction equilibria for chemicals at low doses within animals, and ranging experiments beginning with low doses are the best way to determine a final dose for an AMS experiment. Mauthe *et al.* (1999) compared tissue-available doses and DNA adduction of a labeled compound in humans, rats, and mice. All received approximately 60-pCi/g doses: 4.2 μCi total ^{14}C for the humans, 15 nCi for rats, and 1.5 nCi for mice. Colon tissue was sampled 4 h later by surgery of the human volunteers and through dissection of the rodents. The tissue was 8–30 Mod above natural ^{14}C, and the extracted purified DNA was 0.4–4.0 Mod above natural levels. DNA samples were small and augmented by carrier carbon before measurement. ^{3}H- and ^{14}C-labeled compounds were quantified in rat liver after doses containing 1.4 μCi ^{3}H and 50 pCi of ^{14}C, but quantitation of the same compounds bound to liver proteins required doses that were 10 times higher (Dingley *et al.*, 1998b). Binding of a labeled compound to proteins available from simple blood samples, albumin and hemoglobin, was compared in humans and rats given 4.2 μCi and 0.10–120.00 μCi, respectively. ^{14}C concentrations in the isolated hemoglobin doubled at low doses and ranged to 20 Mod at high dose. Albumin, being more accessible to circulating chemicals, was measured at 1–300 Mod above natural ^{14}C (Dingley *et al.*, 1998a). A starting dose for quantifying macromolecular interactions within living mammals is 50 pCi/g for ^{14}C and 5 nCi/g for ^{3}H. Similar uptake calculations and assumptions are needed in estimating exposures for cell cultures.

Data Interpretation

AMS measures an isotope ratio, expressed as ^{14}C per mass of carbon or ^{3}H per H. No other information is available. The isotope ratio is interpreted by knowing the sources of carbon or hydrogen isolated in the quantified sample. This is expressed mathematically in Eq. (11):

$$R_{meas} = \left({}^{14}C/C\right)_{meas} = \frac{{}^{14}C_{trace} + {}^{14}C_{natural} + {}^{14}C_{carrier} + {}^{14}C_{other}}{C_{trace} + C_{natural} + C_{carrier} + C_{other}} \quad (11)$$

Experimental design provides the data to unfold this equation into the desired quantitation. The $^{14}C_{other}$ represents unexpected isotope contamination that is assumed negligible until proven otherwise. C_{other} is also expected to be zero. C_{trace} is the mass of carbon due to the labeled compound within the isolated sample and is usually negligible, because 1 pmol or less of the compound is quantified in milligram-sized samples. These assumptions leave us with two easily calculated cases. The first comes from isolated samples that contain enough carbon for immediate chemical processing without addition of any carrier material:

$$R_{meas} = \frac{^{14}C_{trace} + {}^{14}C_{natural}}{C_{natural}} = \frac{^{14}C_{trace}}{C_{natural}} + \frac{^{14}C_{natural}}{C_{natural}} = \frac{^{14}C_{trace}}{C_{natural}} + R_{natural} \quad (12)$$

The tissue concentration of the labeled compound (grams of compound per gram of tissue) is quantified from the tissue sample, a control sample of similar tissue ($R_{natural}$), the carbon content of the tissue (C_t), the compound's label concentration (L), and its molecular weight (W):

$$Conc(g/g) = \frac{^{14}C_{trace}}{C_{natural}} \times C_t \times W/L = (R_{meas} - R_{natural}) \times C_t \times W/L \quad (13)$$

For example, the tissue dose of a 200-g compound, labeled at 10 Ci/mol, in liver ($C_t = 14.4\%$), measured as 1.6 Mod is

$$Conc = (1.6 - 1) \times 6.1 \frac{fCi}{mgC} \times \frac{\frac{14.4\ mgC}{100\ mg} \times 200 \frac{fg}{fmol}}{10 \frac{fCi}{fmol}} = 10.5 \frac{fg}{mg} = 10.5 \frac{pg}{g} \quad (14)$$

The mass of the measured sample is not required to calculate this tissue concentration, but an elemental analyzer is useful in checking the carbon and hydrogen content of specific animal tissues, because Table III is only adequate for dose estimations.

A second reduction of the isotope ratio is calculated for samples that contain very little "natural" carbon to which carrier compound has been added, such as HPLC eluent fractions. Equation (11) then reduces to Eq. (15):

$$R_{meas} = \frac{^{14}C_{trace} + {}^{14}C_{carrier}}{C_{carrier}} = \frac{^{14}C_{trace}}{C_{carrier}} + \frac{^{14}C_{carrier}}{C_{carrier}} = \frac{^{14}C_{trace}}{C_{carrier}} + R_{carrier} \quad (15)$$

The amount of labeled compound in the isolated fraction is found from the known amount of carrier added, the measured isotope ratio for control carrier aliquots, and the compound's label concentration:

$$Comp_{fraction} = {}^{14}C_{trace}/L = C_{carrier} \times (R_{meas} - R_{carrier})/L \quad (16)$$

Figure 2 shows HPLC aliquots around an amino acid peak from an AMS-Edman sequencing of a ^{14}C-labeled protein (Miyashita *et al.*, 2001). Carrier carbon was added to each aliquot as 50 μl of a methanol solution at 40 mg/ml. Evaporation of the methanol left 1.19 mg of carbon from the 59.5% carbon material, tributyrin. Multiple aliquots of the carrier averaged to 8.3 ± 0.4% Mod, whereas HPLC eluents on either side of the glycine peak were 7.2% Mod. Loss of ^{14}C is unexpected, but the result could

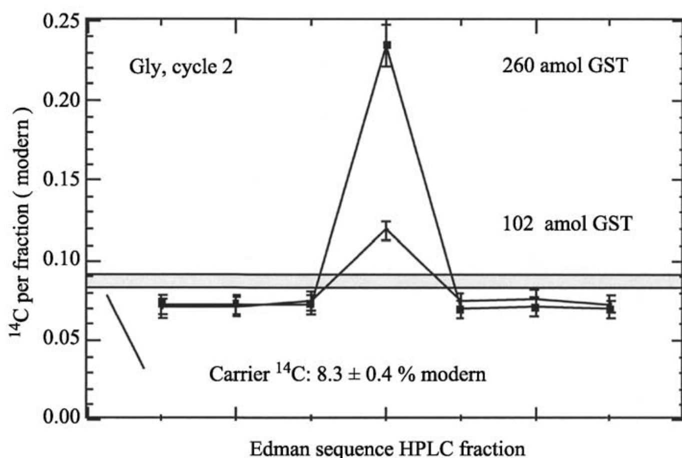

Fig. 2. ^{14}C content in high-performance liquid chromatography (HPLC) eluent fractions around a single amino acid peak. Three aliquots of carrier compound added to the fractions averaged 8.3 Mod, but the signal-free fractions averaged 7.2 Mod. Unexpected carbon from acetate buffer lowered the isotope ratio.

arise from about 100 μg of unaccounted ^{14}C-free carbon (“C_{other}”) in each fraction. A review of the protocol found nonvolatile acetate in the HPLC solvent. Correcting for this oversight, the second Edman cycle of glutathione sulfur transferase produced 5.9 ± 1.1 amol of ^{14}C-glycine from 102 amol of protein and 20.4 ± 1.5 amol from 260 amol of protein with 1.29 mg of the 7.2% Mod effective carrier.

Radiation Exposure and Wastes

The radiation dose produced by an ingested radioisotope depends on the type and energy of the emitted decay product, electrons in the case of ^{3}H and ^{14}C, at average energies of 6.2 k and 52 k eV, respectively. The standard international unit of radiation dose equivalent is the sievert (Sv), which equals a joule of energy deposited in a kilogram of material by energetic electrons. Many people are still familiar with the radiation equivalent unit, the rem, which is 0.01 sieverts. An electronvolt is an amount of energy equal to the charge of the electron (1.6×10^{-19} coulomb [C]) brought across a 1 V (joule/coulomb) potential, so that the average decay energy deposited by ^{3}H is 992 aJoule and by ^{14}C is 8.32 fJoule. Because we all contain ^{14}C at about 1 Mod, we are constantly exposed to radiation energy from ^{14}C decays, d, within us:

$$1\ \text{Mod} \times 13.6\frac{\text{dpm}}{\text{grC}} \times \frac{23\ \text{grC}}{100\ \text{gr}} \times 70\ \text{kg} \times 8.3\frac{\text{fJoule}}{\text{d}} \times 60\frac{\text{min}}{\text{hr}} = 110\frac{\text{nJoule}}{\text{hr}} \quad (17)$$

This natural ^{14}C is spread throughout our bodies, so the energy deposition is converted to radiation dose equivalent by dividing by the body mass, 70 kg, to get 1.6 nSv/h or 160 nrem/h. We also contain other natural radioisotopes that produce greater radiation exposures than these from ^{14}C.

Isotope-labeled compounds do not provide a constant radiation exposure, because they are metabolized and excreted. Exact exposure calculation requires a full kinetic profile, but estimations are made using a model that incorporates instantaneous absorption followed by an exponential elimination characterized by a single time constant, the biological mean life, equal to biological half-life divided by ln(2). This exponential loss must be integrated over the expected life of the subject after dosing, perhaps 50 yr. However, the biological mean lives of drugs (<24 h) and nutrients (<60 days) are small compared to 50 yr, which is an effectively infinite time in comparison. Integrating the exposure to infinity allows a simple closed formula that overestimates the exposure by a small amount:

$$\text{Exposure} = \frac{E_d}{M_d} \times \text{Dose} \times \int_0^\infty e^{-t/\tau_{bio}}\,dt = \frac{E_d}{M_d} \times \tau_{bio} \times \text{Dose} \quad (18)$$

E_d is the energy deposited per decay in joules; M_d is the affected mass in kilograms; dose is the amount of radioactivity in dpm or becquerels (dps); and τ_{bio} is the biological mean life of the labeled compound in minutes or seconds. The absorbed dose decreases with radioactive decay and with chemical elimination, but the correction is negligible as long as the radioactive life of the isotope is much longer than the biological life of the compound, as is true in these cases. For cases of similar biological and radioactive mean lives, τ_{bio} in Eq. (18) is replaced by $\tau_{bio}\tau_{rad}/(\tau_{bio}+\tau_{rad})$, where τ_{rad} is the radioactive mean life of the isotope. The examples of Eqs. (9) and (10) assume a 1-day (1440 min) mean life of the compound with a total absorbed radioactive dose of 210 nCi of ^{14}C and 7.7 μCi of ^{3}H that would produce whole-body exposures of

$$\text{Exposure}(^{14}\text{C}) = \frac{8.3\ \text{fJ}}{70\ \text{kg}} \times 1440\ \text{min} \times 210\ \text{nCi} \times 2200\frac{\text{dpm}}{\text{nCi}} = 79\frac{\text{nJ}}{\text{kg}} = 79\ \text{nSv} \quad (19)$$

$$\text{Exposure}(^3\text{H}) = \frac{0.99\ \text{fJ}}{70\ \text{kg}} \times 1440\ \text{min} \times 7700\ \text{nCi} \times 2200\frac{\text{dpm}}{\text{nCi}} = 345\frac{\text{nJ}}{\text{kg}} = 345\ \text{nSv} \tag{20}$$

These exposures assume that the compounds spread evenly throughout the body. If these compounds concentrated in the 1.8-kg liver, the exposures are 3.1 and 13 μSv to that organ, replacing the 70-kg affected mass with the liver mass.

Human experimental subjects are not asked to accept risks to their health that are significantly greater than commonly accepted risks in everyday life, unless their health benefits. The lifetime exposures calculated in Eqs. 17 and 18 are equivalent to 2 and 9 days of the natural ^{14}C radiation and are acceptable risks to many. A common exposure to radiation occurs in aircraft at usual cruising altitudes, where the radiation dose is 5 μSv/hr (Friedberg *et al.*, 2000). This radiation is primarily long-range energetic protons and muons that produce the same exposure to all tissues, compared to the electrons emitted from ^{3}H and ^{14}C, which stop within a few millimeters of their emittance. The liver and brain receive 5 μSv/hr in a plane, as does the leg or arm. Thus, even the exposures calculated for our example compound concentrated in the liver are lower than those obtained in 1–4 h of flying, a commonly accepted radiation risk, even for young children and pregnant women. Figure 3 shows the whole-body radiation dose from 100 nCi of ^{14}C and 1 μCi of ^{3}H in a 70-kg person as a function of biological mean life of the compounds incorporating the isotopes as labels. The left axis provides the exposure in nanosieverts, and the right axis converts the exposure to equivalent time in a cruising aircraft. Most drugs and toxins have short biological lives of less than a day, but nutrients can be recycled for many months.

Absorbed isotope doses to humans in AMS experiments range from 0.0014 nCi/g (100 nCi/70 kg) of ^{14}C to 0.14 nCi/g (10 μCi/70 kg) of tritium, with animal doses scaling proportionately to mass. Nuclear regulations allow disposal of wastes from biological experiments if the ^{14}C or ^{3}H content is less than 50 nCi/g, up to a total of 1 μCi/yr (U.S. Code of Federal Regulations, Title 10, Section 20.2005, 1991). Many experimenters can dispose of all their wastes from AMS experiments as nonradioactive. Others will reduce their radioactive waste streams by several orders of magnitude using AMS quantitation, for a significant savings in operation costs.

Conclusion

AMS increases the applicability of radioisotope labels for definitive molecular tracing in all biological systems, including humans. Chemicals are labeled with low specific activity, reducing synthesis costs and wastes.

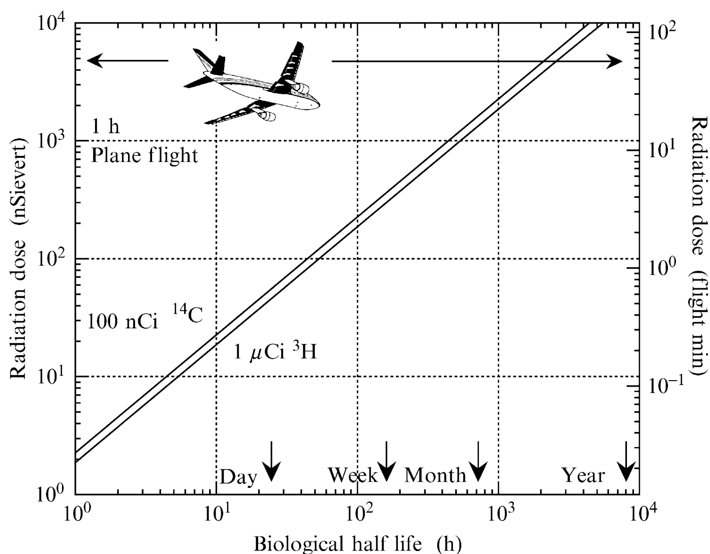

FIG. 3. Absorbed radiation-dose equivalents in a 70-kg human dosed with 100 nCi ^{14}C and 1 μCi ^{3}H are given as functions of the biological half-life of the compound containing the isotopic label. The radiation is expressed as nanosieverts and as equivalent minutes in an aircraft at typical flying altitude.

Laboratory safety is enhanced through low radioactivity usage. AMS provides high precision and throughput despite the greatly decreased isotope concentrations. Radiation exposures are below commonly accepted risks, even for children for whom many drug and nutrient studies can now be designed. AMS is available through an NIH Research Resource and from established academic and industrial facilities worldwide. A new class of smaller spectrometers is now available for institutions that can benefit from dedicated instruments.

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