CHAPTER 1

BASIC CHARACTERISTICS OF ISOTOPIC TRACERS

1.1 WHAT IS A TRACER?

A tracer is a compound that is chemically and functionally identical to the naturally occurring compound of interest (the *tracee*) but is distinct in some way that enables detection. By following the fate of a tracer in the body, information can be obtained regarding the metabolism of the tracee.

There are three general ways in which tracers are used in metabolic research. In one case, the tracer is bound to a compound that is injected into the body, and the fate of the compound, and possibly its metabolites, is followed by virtue of the convenience of the distinct property of the tracer. Injection of a pharmacological compound labeled with a tracer is a common application of this approach. For example, if a pharmaceutical compound is labeled with radioactive iodine, the kinetics of the compound can be determined by simply counting the radioactivity, rather than performing the potentially tedious task of measuring the concentration of the compound. The second use of a tracer is to measure its rate of incorporation into another compound. This can be used to calculate the rate of synthesis of a product (e.g., protein), or the rate of oxidation of a compound (by measuring the rate of incorporation of labeled carbon into CO_2). Also certain aspects of metabolism can be measured using the incorporation principle, such as measuring the rate of gluconeogenesis from a particular precursor or quantifying specific pathways of degradation. The third general use of a tracer is called the "tracer dilution" technique. The most common use of this method is the measurement of the rate of appearance of a substrate into the plasma.

An ideal tracer can be detected with sufficient precision so that it can be given in such a small dose that the metabolism of the tracee produced in the body (the endogenous substrate) is not affected. Alternatively, a tracer can be used in a larger dose, provided that account can be taken of the effect of the tracer on the endogenous kinetics of the tracee. Also the metabolism of tracer must reflect the metabolism of the tracee. In other words, the labeling of the tracer cannot make it so distinct from the tracee that the two compounds are metabolized at different rates.

1.2 TYPES OF TRACERS

A tracer is made by labeling a molecule otherwise identical to the tracee with one or more atoms that are distinct from the most abundant form of that atom. Tracers are conventionally labeled with either *radioactive* or *stable* isotopes of one or more atoms in a molecule. Atoms are composed of a dense core of positively charged protons and

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uncharged neutrons. In the lighter elements, such as carbon, hydrogen, oxygen, and nitrogen, there are approximately equal numbers of neutrons and protons. The number of protons plus neutrons is called the mass number. The mass number is traditionally denoted by the symbol A. The number of protons is the atomic number (symbol Z). Given that the mass units of protons and neutrons are each equal to one, early students of the atom questioned why atomic weights were not whole numbers. In 1911 Soddy used equipment developed the previous year by Thompson that accurately determined relative nuclear charges and masses, and he demonstrated experimentally that certain elements were composed of atoms that were chemically identical but that differed slightly in weight. He proposed the term *isotopes* for such atoms. Mass differences of isotopes are due to different numbers of nuclear neutrons. The number of neutrons in the atom does not affect the chemical properties of the atom, which are determined by the electronic configuration. Thus a commonly used radioactive isotope of carbon (^{14}C) is the same mass as the most abundant isotope of nitrogen (^{14}N), yet these two atoms are chemically distinct as carbon and nitrogen.

1.3 NUMBERING OF MOLECULES

The exact position in which a tracer molecule is labeled and the specific isotope label are denoted by the numbering of the molecule. Molecules are numbered according to a system approved by the International Union of Pure and Applied Chemistry (IUPAC). In general, the longest continuous chain of C atoms is considered to be the "parent" hydrocarbon, and any branching alkyls (carbons and hydrogens) or functional groups (e.g., carboxyl, amino groups) attached to it are considered to be substituents of that parent. The parent chain is numbered so as to give the lowest possible set of numbers to the carbons bearing the alkyl or functional groups. For substrates involved in metabolic studies, the functional group is often a carboxyl group (COOH), and consequently the carboxyl carbon is generally the only carbon of the molecule. The hydrogens and oxygen atoms are not specifically numbered but are referred to in relation to the number of the carbon atom to which they are attached. The specifically enriched atom is referred to by identification of the weight of the atom by a superscript prior to the letter. Thus ^{12}C, ^{13}C, and ^{14}C refer to carbon atoms of atomic masses 12, 13, and 14, respectively. ^{12}C is the most abundant mass, approximately 99%. ^{13}C is the naturally occurring stable isotope, and ^{14}C is the radioactive isotope. The position within a molecule of an enriched carbon is referred to by the appropriate carbon number, preceding the abbreviation of the description of the isotope used as a tracer. Thus 1-$^{13}C_1$-glucose refers to a molecule of glucose in which the 1 position is labeled with carbons enriched with the stable isotope of mass 13. The subscript following the C refers to the number of specifically enriched atoms of carbon (in this example, one) in the molecule. If the 1 and 2 positions were both specifically enriched with ^{13}C, then the compound would be called 1,2-$^{13}C_2$-glucose. If all carbons are labeled, it is considered to be uniformly labeled, which is abbreviated by U. Thus a glucose molecule with all positions containing ^{13}C-enriched atoms would be U-$^{13}C_6$-glucose, since there are 6 carbons in glucose. If hydrogen is used as a tracer, it is denoted as ^{2}H for deuterium, the stable isotope of hydrogen of mass 2, and ^{3}H for tritium, the radioactive isotope of hydrogen of mass 3. The number of carbon to which the hydrogen is attached is denoted by the first number. Thus 2-^{3}H-glucose refers to glucose labeled with one hydrogen atom, of mass 3, which is the radioactive isotope (also called *tritium*), attached to the 2 carbon of the molecule. In the case of hydrogen it is possible for more than one

labeled atom to be attached to the same carbon. The commonly used tracer [6,6,-$^2H_2$]-glucose is an example, in which 2 hydrogens attached to the 6 carbons are specifically enriched with atoms of mass 2 (deuterium). The abbreviation d is sometimes used for deuterium, so that 6,6,-d_2-glucose might also be used to describe the same tracer.

1.4 RADIONUCLIDES

Radionuclides have been the most commonly used tracers for metabolic studies. A radioactive nuclide spontaneously disintegrates to form an atom of another element, with radiation being emitted in the process.

There are three distinct types of radiation: alpha, beta, and gamma rays. ^{3}H and ^{14}C are the most commonly used radionuclides for metabolic studies. Both ^{3}H and ^{14}C emit beta rays. Beta rays are actually comprised of electrons with a mass of about 1/1800 that of the hydrogen atom and travel at about the speed of light. In spontaneous decay, one of the neutrons in the nucleus becomes a proton and an electron, the latter of which is emitted from the nucleus. Energy accompanies the stream of emitted electrons from an atom undergoing decay. For example, $^{14}_{6}$C has 8 neutrons and 6 protons. The superscript 14 refers to the sum of the neutrons and protons, and the 6 refers to the number of protons. When undergoing decay, a neutron becomes a proton. In the case of $^{14}_{6}$C, a neutron becoming a proton when an electron is released resulting in the formation of nitrogen, which has 7 neutrons and 7 protons (denoted). Thus

$$^{14}_{6}\mathrm{C} \rightarrow {}^{14}_{7}\mathrm{N} + {}^{0}_{-1}\mathrm{e}$$

The atomic mass units (amu) of reactants and products are equal to the masses of neutral atoms of C and N:

Reactant. Mass of $^{14}_{6}$C atom: 14.003242 amu

Product. Mass of $^{14}_{7}$N atom: 14.003074 amu

Loss of mass in reaction. 0.000168 amu

The energy (E) equivalent to this loss of mass is

$$E = 0.000\,16\,8 \text{ amu} \cdot \bullet\ 931 \text{ MeV amu}^{-1} = 0.156 \text{ MeV},$$

where MeV is millielectron volts. Thus the emitted electrons have an energy of 0.156 MeV.

The rate at which the atoms undergo transformation is directly proportional to the number of radioactive atoms present. As the number of radioactive atoms decreases due to the radioactive transformations, the rate of emission of beta particles decreases. The half–life of a radionuclide is the time required for half of the atoms to be transformed through radioactive decay. The half–life of ^{14}C is 5730 years, and therefore the decay of stored ^{14}C, for example, need not be considered as an important factor in terms of loss of tracer. It also means that any ^{14}C infused into a subject as part of a substrate will be lost entirely as a function of the metabolic turnover of the infused tracer, as opposed to spontaneous decay. The half–life of ^{3}H is 12.3 years, which is still lengthy in relation to physiological turnover of molecules, including water, in the body. However, the half-life could be pertinent if a tritiated nuclide is stored for a few years and then used, since, in that example, 10% of the radioactivity would be lost after 2 years as a result of spontaneous decay. Such spontaneous decay can easily be

accounted for by determining the decays per minute per milliliter of a stock solution of the tracer before mixing a solution for infusion.

1.5 STABLE ISOTOPES

In contrast to radioactive isotopes, there is no spontaneous decay of stable isotopes (hence the name *stable* isotope). Stable isotopes of an atom contain variable numbers of neutrons in the nucleus. This alters the mass of the atom but not its chemical nature. The most commonly occurring isotope has the lowest mass in the case of carbon, hydrogen, oxygen, and nitrogen. However, this is not true for all atoms. Table 1.1 shows a partial list of stable isotopes. In the case of selenium, for example, there are six naturally occurring stable isotopes, with none comprising more than 50% of the total. Further the lowest atomic weight (74) is the least common isotope, and the most common atomic weight (80) is almost the heaviest isotope. In the discussion of calculation of isotopoic enrichment by mass spectrometry, it is therefore important to note that the assumption that the lowest atomic weight of the tracer atom is its most abundant isotope is implicit in the calculation of enrichment by the technique described most extensively in this book. It is also assumed that the natural abundance of other isotopes is infrequent.

The use of stable isotopes as metabolic tracers in vivo actually predates the use of radioactive isotopes by almost 20 years. In early studies at Columbia University, Schoenheimer and Rittenberg [1, 2] used the stable isotope of hydrogen (^{2}H, deuterium) to study fat metabolism in mice. They soon extended their isotopic studies by using ^{15}N-labeled glycine to demonstrate the dynamic nature of the protein pool of the body [3].

TABLE 1.1 Partial List of Stable Isotopes

Element	Stable Isotope	% Natural Abundance	Element	Stable Isotope	% Natural Abundance
H	1	99.985	Fe	54	5.82
	2	0.015		56	91.66
				57	2.19
C	12	98.89		58	0.33
	13	1.11			
			Zn	64	48.89
N	14	99.63		66	27.81
	15	0.37		67	4.11
				68	18.57
0	16	99.76		70	0.62
	17	0.037			
	18	0.204	Se	74	0.87
				76	9.02
S	32	95.00		77	7.58
	33	0.76		78	23.52
	34	4.22		80	49.82
				82	9.19
Si	28	92.21			
	29	4.9			
	30	3.09			

Experiments were also performed as early as the 1930s with ^{13}C [4, 5] and ^{18}O [6]. With the advent of scintillation counting and the availability of a wide variety of radioactive tracers, most studies in which metabolic rates were determined (i.e., kinetic studies) in the 1950s and 1960s used radioactive tracers. In the 1970s a resurgence of the use of stable, nonradioactive isotopes began. The most important reasons for this were probably availability of tracers labeled with stable isotopes and the improved ease of analysis due to the availability of the quadruple mass spectrometer interfaced with the gas chromatograph (GCMS), which made possible the convenient use of selective ion monitoring for the quantitation of isotopic enrichment. An increased awareness of the health hazards of radioactivity for human investigations also stimulated the use of stable isotopes.

1.6 ADVANTAGES OF STABLE ISOTOPES

1.6.1 Biological Effects

The most obvious advantage of stable isotopes is that they are nonradioactive and present little or no risk to human subjects. Carbon 13 is a naturally occurring isotope present to the extent of approximately 1.1% of the major isotopic species, carbon 12 (Table 1.1). Since carbon 13 naturally contributes 1.1% of the carbon pool, and since it has not been possible to demonstrate more than trivial in vitro isotopic effects on chemical reactions with carbon 13–labeled substrates [7, 8], significant side effects in vivo are not expected from administration of "tracer" doses of carbon 13. In fact Gregg et al. [9] raised mice on ^{13}C enriched algae, thereby raising the carbon 13 content of the total body pool from 60% to 70% of total carbon without discernible effect on the animals. Replacement of H_2O with D_2O, however, can affect the growth of microorganisms. To see an effect, however, the deuterated water must be in excess of 20% of the total water [10]. Similarly $H_2^{18}O$ [10], carbon 13, and ^{15}N can be shown to affect certain parameters of cell function at extremely high levels of enrichment that would never be attained in an in vivo study in humans [11]. There is no evidence that stable isotope tracers present an identifiable risk to human subjects at the highest levels of enrichment that might reasonably be achieved, with the possible exception of ^2H_2O given at a dose sufficient to raise the enrichment of the total body pool by about 5%.

1.6.2 Enzymatic Effects

The use of a labeled tracer requires the assumption that the labeled molecule will not be discriminated from the unlabeled molecule and that the labeled molecule will trace the movement of the unlabeled molecules. However, certain enzymatic effects of stable isotopes have been reported, such that the isotope will be selectively fractioned from its more abundantly occurring counterpart [12–15]. Potential isotope effects when carbon, nitrogen, and oxygen are used as tracers are rarely of concern. However, it is possible that sufficient isotope effects might occur with ^3H or ^2H to be of physiological significance. For example, an isotope effect was claimed in the clearance of 3-^{3}H-glucose from blood [15], as well as for 6-^{3}H-glucose and 6,6-$^2H_2$-glucose [16]. However, it seems likely that these possible isotope effects can largely, or entirely, be explained by tracer contamination [17] or problems in modeling. Thus, although concern for isotope effects is appropriate, in vivo studies provide little evidence that such errors are of concern when the tracer atom is not directly involved in a metabolic

reaction. On the other hand, isotope effects are of more potential concern when the tracer in question is directly involved in a metabolic reaction (e.g., loss of ^{13}C from a molecule of $^{13}CO_2$). In any case, with the stable isotope tracers of carbon (^{13}C) and hydrogen (^{2}H), the mass displacement from the most abundant isotope is less than with the corresponding radioactive isotopes ^{14}C and ^{3}H), so less pronounced isotope effects will occur with the stable isotopes. In reality, in most cases, variability is generally greater than isotope effects in human tracer studies. An exception is the doubly labeled water technique, discussed in Chapter 8. In that case the entire technique is predicated on the different handling by the body of $H_2{}^{18}O$ and $^{2}H_2O$.

In addition to the safety of stable isotopes for human use and the fact that isotopic effects should be minimized, there are other advantages of stable isotopic tracers [18]. Obviously there are no practical radioisotopes of nitrogen (N) or oxygen (O), whereas there are stable isotopes of each element (^{15}N and ^{18}O). Also, when analysis by GCMS is used, it is often possible to determine enrichment on small samples of blood. Further, simultaneous and repeated use of several tracers is possible in the same subject, since the process of mass spectrometry isolates individual compounds before analysis. For example, several amino acids enriched with ^{13}C can be given simultaneously, and the enrichment of each tracer measured independently of the other labeled compounds. On the negative side, because of analytical insensitivity it is often necessary to give so much "tracer" that the endogenous kinetics of the tracee are affected. This problem will be discussed in detail in subsequent chapters.

1.7 ISOTOPOMERS

An *isotopomer* is a molecule with an isotopic tracer incorporated somewhere in the molecule. *Positional isotopomers* are molecules with isotope tracers incorporated into different specific positions in the molecule. For example, 1-^{13}C-leucine and 2-^{13}C-leucine would be positional isotopomers—the mass of the two molecules is the same, but the position of the label is different. *Mass isotopomers* refers to two functionally identical molecules that differ in molecular weight because of the incorporation of a different number of stable isotope tracers. For example, 1-^{13}C-palmitate and 1,2-$^{13}C_2$-palmitate are mass isotopomers.

1.8 PRINCIPLES OF MEASUREMENT: THE UNITS OF ISOTOPIC ENRICHMENT

The measurement of the presence of a radioactive nuclide is conventionally accomplished by detecting the decay rate of the sample by counting. The amount of tracer present is directly proportional to the number of decays/minute. For the commonly used beta emitters ^{3}H and ^{14}C, detection is accomplished by scintillation counting. Briefly, the isotope tracer is dissolved in a fluid ("scintillant") that emits light for each decay that occurs. When placed in a scintillation counter, the photons of light resulting from excitation by beta rays are counted. Therefore it is possible to use a radioactive isotope for tracing the fate of an injected compound that is not naturally occurring in the body, such as a pharmacological product, because detection involves directly determining the total amount of tracer in a sample. However, the most common unit that is used in conventional tracer methodology, whether by the product incorporation approach or by tracer dilution, is the ratio of the labeled tracer/unlabeled tracee. This

is because tracing an endogenous metabolic reaction is the normal goal. In the case of a radioactively labeled tracer, direct determination of counts enables quantification of the amount of tracer. The concentration of the unlabeled tracee must then be quantified separately, and the ratio of the two calculated. The ratio of the amount of radioactive tracee/amount of unlabeled tracee is called the *specific activity*, SA.

The amount of a stable isotope tracer in the blood or tissue cannot be measured by a means analogous to counting the amount of radioactivity. By virtue of the fact that there is no spontaneous decay, the amount of stable isotope cannot be determined by counting. Further the natural occurrence of stable isotopes means that most stable isotope tracers will have some naturally occurring counterparts in the body before the infusion starts. Thus a stable isotope tracer will *enrich* the amount of heavy isotope present. The abundance of a stable isotope is generally determined by the measurement of the molecular weight of the compound of interest. A molecule enriched with 1 or more heavy stable isotopes (e.g., ^{13}C) will be increased in mass correspondingly (hence the term *mass* isotopomer). Mass spectrometry is conventionally used to determine the molecular mass. Mass spectrometry allows determination of the ratio of molecules with heavy stable atoms somewhere in the molecule to molecules of the lowest possible molecular weight. Because of the natural abundance of heavy stable isotopes, there are different ways of expressing the extent of enrichment. The unit "tracer/tracee ratio" (t/T ratio) is analogous to the term SA for radioactive isotopes and will be used most commonly in this book. An alternative unit is atom percent excess (APE), or mole percent excess (MPE). These units express the amount of tracer as a ratio to the sum of the tracer + tracee. Because of the relatively low sensitivity of mass spectrometry analysis, it is often necessary to give enough tracer that the terms APE or MPE are different numerically from t/T ratio where the amount of tracer may be 5% or more of the total substrate in the body. Consideration must therefore be given in modeling to the unit of enrichment. Examples of the differences in the units will be given in Chapter 3.

In contrast to the situation with radioactive tracers, the absolute amount of tracer present is not measured. Rather, the ratio of tracer/tracee, or some function of that ratio (APE, MPE) is measured. Consequently a stable isotope does not help in the tracing of a compound that is not naturally occurring in the body, such as a drug. For this reason, tracer models are based on the ratio of labeled to unlabeled compound present, rather than the absolute amount. To determine the amount of a stable isotope present requires first measuring the tracer/tracee ratio and then multiplying by the independently measured concentration.

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