



# Isotope Fractionation: Why Aren't We What We Eat?

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The isotopic composition of an element records information about its history. Given a fossil, it is possible to analyse the isotopic composition of the elements in the fossil and to use this to reconstruct the diet that the animal consumed. The process of dietary reconstruction, however, is far from simple. Biological systems are quite complex and can themselves introduce isotopic fractionations that may distort the dietary information. The aim of this paper is to review the concepts of isotope fractionation under steady-state conditions to provide a framework for discussion of dietary reconstruction. Among the elements of interest for dietary reconstruction, nitrogen bears a distinct role. This is because nitrogen is almost unique to protein. A secondary aim of this paper is then to review nitrogen metabolism. The final aim is to combine these in postulating a simple isotopic model of nitrogen metabolism. © 1999 Academic Press

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## Introduction

The light elements of major biological importance including hydrogen, carbon, nitrogen, and oxygen all possess naturally occurring stable isotopes. For three of the elements, the minor isotopes are one mass unit heavier than the major or most abundant isotope. The fourth, oxygen, has two minor isotopes that are one and two mass units heavier than the major isotope. Although the isotopes have exceedingly similar chemical properties, they are not identical. The difference in mass results in slight differences in reaction kinetics and bond energies. Thus, as elements participate in chemical reactions, the various isotopes may react at slightly different rates, or if equilibrium is established, partition themselves differently between products and reactant. Not surprisingly as the elements wind their way through the biological and geochemical cycles, an isotopic fine structure develops in which the relative isotope abundances of these elements differ between the various reservoirs of the element in any given environment. This isotopic fine structure can become a source of information about reaction mechanisms, the reaction environment, and/or the source or fate of a compound. The latter of course is of particular interest in a dietary context, as the isotopic information can be used to trace and quantitate food webs in human and animal diets (DeNiro & Epstein, 1981; Koch, Fogel & Tuross, 1994). Unusual metabolic pathways in a plant species can provide an isotopic label that can be used to identify the contribution of that plant to the food web (van der Merwe & Vogel, 1978); geographic variations in isotopic abundances can provide a signal that can help identify the source of a food item in a community (Nakamura *et al.*, 1982); or metabolic influences can alter isotopic abundances of element as they pass through the food web to provide infor-

mation regarding trophic level (Minagawa & Wada, 1984).

Understanding and interpreting these isotopic signals, unfortunately, is not always easy. The aim of this paper is to provide background information about isotope biogeochemistry and nitrogen metabolism that will aid in the interpretation of these isotopic signals.

## Isotope Fractionation

Although numerous reviews of isotope fractionation have been written (Dansgaard, 1964; Vogel, 1980; O'Leary, 1981), most assume that the reader has a significant understanding of isotope biogeochemistry. Two reviews that are more useful for the general reader are those of Hayes (1982, 1993).

Isotopes differ with regard to the number of neutrons and hence their atomic weight. The isotopes that have the greater atomic mass due to the presence of an extra neutron(s) are termed heavy, while those with the lower atomic mass are referred to as the light isotope. Among the elements of biological interest, the light isotopes are far more abundant than the heavy isotopes (Table 1). It should also be noted that while the abovementioned differences in the natural abundances of the heavy isotopes are easily measured using modern instrumentation, the isotopic differences are actually quite small, typically being seen in the third or fourth significant digit. Because of this, data are rarely presented as a fractional abundance. Instead, they are presented as a relative change in the ratio of the heavy to light isotope using the delta per mil notation (McKinney *et al.*, 1950).

$$\delta = (R_{(\text{sample})} - R_{(\text{standard})}) * 1000 / R_{(\text{standard})}$$

where  $R$  is the ratio of the heavy to light isotope. While appearing cumbersome, the delta per mil is simply 10

Table 1. Isotopic abundances of the light isotopes

Element	Isotope	Fractional abundance	‰ change	International standard
Hydrogen	$^1\text{H}$	0.999844		Standard Mean Ocean Water (SMOW)
	$^2\text{H}$	0.000156	0.00000156	
Carbon	$^{12}\text{C}$	0.98889		Peedee Belemnite Limestone (PDB)
	$^{13}\text{C}$	0.01111	0.00001123	
Nitrogen	$^{14}\text{N}$	0.99634		Air
	$^{15}\text{N}$	0.00366	0.00000367	
Oxygen	$^{16}\text{O}$	0.99755		Standard Mean Ocean Water (SMOW)
	$^{17}\text{O}$	0.00039	0.00000039	
	$^{18}\text{O}$	0.00206	0.00000207	

times the percent difference in the isotope ratio relative to a standard. Moreover, the  $\delta$  values are far simpler to tabulate, compare and remember than the multidigit fractions listed in Table 1.

The differences in the relative isotopic abundances of the elements that arise in two different molecular species or two different pools of the same molecular species that are observed in the biogeosphere are termed isotope fractionation. As indicated above, isotope fractionation results from differences in reaction rates associated with the two isotopes. While isotope fractionation can only result as a consequence of isotope discrimination and while virtually every reaction is associated with some isotopic discrimination not all isotope discrimination is expressed as isotope fractionation. The expression of isotope discrimination will depend on whether the discrimination is large enough to introduce a measurable isotope difference and whether the reaction yield is less than 100%. If the reaction proceeds to completion, the isotopic abundance of the product will be equal to that in the reactant because all of the atoms originally present in the reactant are converted to product and thus any isotope discrimination cannot be expressed as isotope fractionation. If, however, the reaction does not proceed to completion, the discrimination will be expressed as fractionation and the fractionation will be a function of the yield (Figure 1).

If the reaction is not chemically reversible or if the products and reactant are separated in a manner that prevents the reverse reaction, then the discrimination is said to be under kinetic control and the relationship between yield and fractionation will be linear as indicated in Figure 1(a). If the reaction is reversible and the reactant and products reach equilibrium, the discrimination will be under equilibrium control and the relationship will be parabolic as indicated in (b).

While this knowledge can be exploited to create a model for isotope fractionation in a biological system, the number of reactions in any metabolic route is too great and data on isotope discrimination for each reaction too limited to permit a complete model to be developed. Fortunately, the task of modelling isotope fractionation can be simplified. Sequential, non-

branching reactions that occur after commitment to a metabolic route will not express isotope fractionation, because each molecule that enters the sequence will exit the sequence at the other end. Thus, from an isotopic standpoint, the reaction sequence can be treated as a single isotopic entity, and considerable progress can be made toward modelling of isotopic fractionation *in vivo* based on the identification of major metabolic pools and their branch points (Hayes, 1993).

## Isotope Fractionation Models

Isotopic fractionation will be reflected in a shift in the  $\delta$  value. If the discrimination is against the heavy isotope the  $\delta$  value will be less (or more negative) and

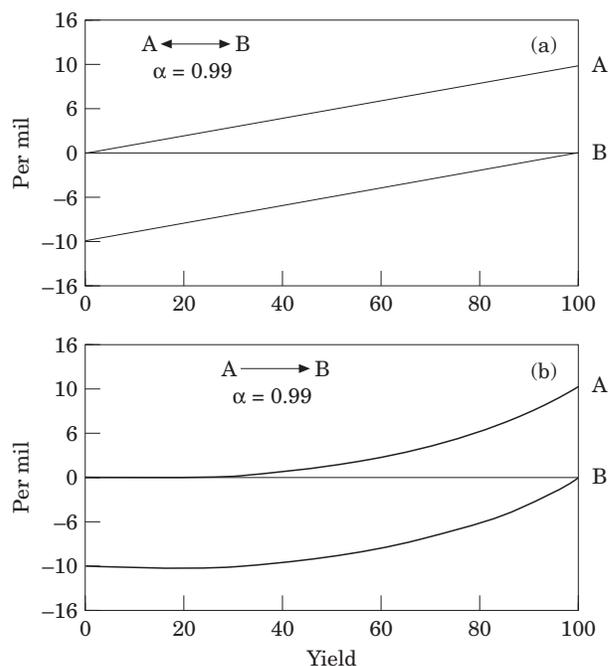


Figure 1. Effect of isotope discrimination on the isotopic abundances of the reactant (A) and product (B) as a function of yield in a closed system under conditions of (a) equilibrium control and (b) kinetic control.

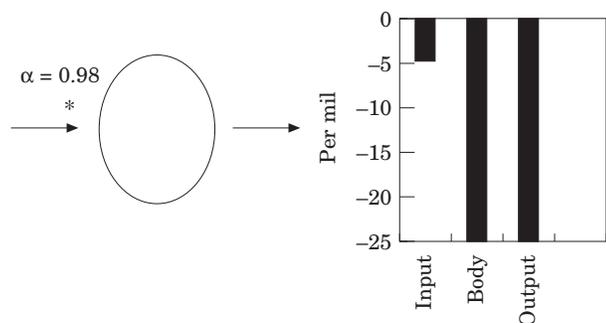


Figure 2. Effect of isotope discrimination on the isotopic abundances of an open system with a single compartment and one input and one output. Isotope discrimination (\*) occurs at the input from the compartment.

the product is said to be lighter. The  $\delta$  for the remaining reactant will therefore become more positive and this residual will be described as heavier. Discrimination factors are expressed as either  $\alpha$  or  $\epsilon$ , where  $\alpha = R_{\text{product}}/R_{\text{reactant}}$ . Because  $\alpha$  typically has a value quite close to 1, it is often expressed as  $\epsilon$  where  $\epsilon = \alpha - 1$ .

Modelling of biological systems requires consideration of two additional issues. Unlike the closed test tube systems illustrated in Figure 1, a biological system is generally an open system. It is open in that it has an input and output connecting it to a much larger biosphere. Furthermore, the models require an assumption of steady-state, i.e., the absence of measurable change. In reality, a perfect steady state in which the pool sizes remain constant is never achieved, but a practical steady state in which the changes are too small to introduce a measurable change is relatively easy to achieve. In general, steady state is assumed in the absence of an acute change in pool size exceeding 5% or a systematic change exceeding a couple of percent for a period of one biological half-life. Thus, on a day-to-day basis, humans are probably in steady state except during acute illness or rapid growth (the first 6 months of life, the adolescent growth spurt, and pregnancy).

At the lowest level of complexity, an organism can be modelled as a single compartment with a single input and single output (Figure 2). If there is isotopic discrimination at entry with  $\alpha = 0.98$  (discrimination against the light isotope), then the pool of that element in the body will be depleted relative to the material entering the body. The isotopic abundance of the material exiting the body, which in this model is not subjected to discrimination, will exit the body with the same isotopic abundance as the material in the body and thus be fractionated relative to the input.

Although this might appear to be an exception to the rule that fractionation does not occur except at branch points were there is an incomplete yield, it should be realized that the input into the body is a branch point in which material can either enter into the body or

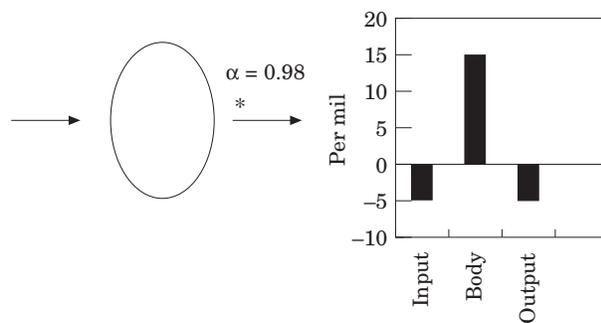


Figure 3. Effect of isotope discrimination on the isotopic abundances of an open, steady-state system with a single compartment and one input and one output. Isotope discrimination (\*) occurs at the output from the compartment.

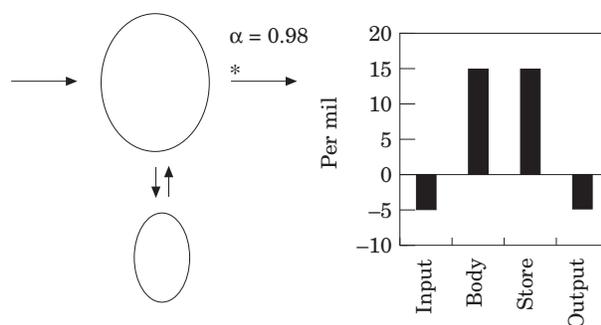


Figure 4. Effect of isotope discrimination on the isotopic abundances of an open, steady-state system with two compartments in equilibrium and one input and one output. Isotope discrimination (\*) occurs at the output from the compartment.

remain in the large reservoir of material outside of the organism. Fractionation of this type does not occur frequently in regard to solid or liquid materials, but is common for gaseous materials such as molecular oxygen in mammals (Epstein & Zeire, 1988) or carbon dioxide in plants (O'Leary, 1981).

If the discrimination occurs at the output from the organism, then a different pattern of isotopic fractionation emerges (Figure 3). Under this condition, the body is isotopically heavy relative to the input, but the output is isotopically identical to the input. This exemplifies the principle of lack of fractionation with regard to a committed step (entry into the organism) in which all the material entering exits via the same route (Hayes, 1993). The intermediate pool, which in this example is the whole organism, will be heavy relative to input under steady-state conditions.

It is instructive to consider the next level of complexity to modelling, which is the presence of two metabolic pools. A common organismal example would be one with a common metabolic pool and a storage pool. This first example simply adds a second pool in equilibrium to the central pool that was depicted in the previous example (Figure 4). Again, because the discrimination occurs only at the output step, the output is isotopically identical to the input at steady state, but

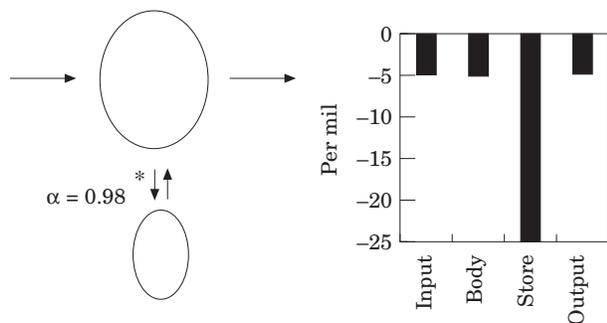


Figure 5. Effect of isotope discrimination on the isotopic abundances of an open, steady-state system with two compartments in equilibrium and one input and one output. Isotope discrimination (\*) occurs between the compartments.

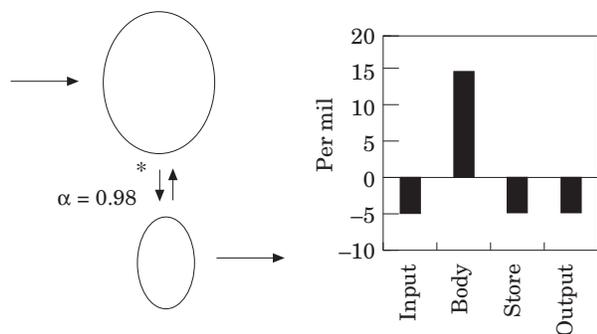


Figure 6. Effect of isotope discrimination on the isotopic abundances of an open, steady-state system with two compartments in equilibrium and one input and one output, but with the input and output in different compartments. Isotope discrimination (\*) occurs between the compartments.

the body becomes isotopically heavy. Because there is no discrimination between the two pools in the body, there is also no isotopic difference between them and both pools are equally heavy relative to the input.

If the discrimination against the heavy isotope occurs during the equilibration step between the central pool and the storage pool, the storage pool will be isotopically light relative to the central pool (Figure 5). The central pool will be unfractionated relative to the input because there is no discrimination at either the input or output.

If the output occurs not from the central pool, but instead from the pool in equilibration with the central pool, the pattern will be slightly different (Figure 6). Again, because the discrimination occurs after the material passes the committed step, i.e., entry into the body, there is no fractionation between the input and output. The central body store, however, is isotopically heavy relative to the input, because of the discrimination against the heavy isotope during the equilibration between the pools.

If a second storage pool is added to the model in Figure 5 and discrimination occurs at both equilibration steps, then a more complex isotopic distribution is established in the body (Figure 7). The pattern,

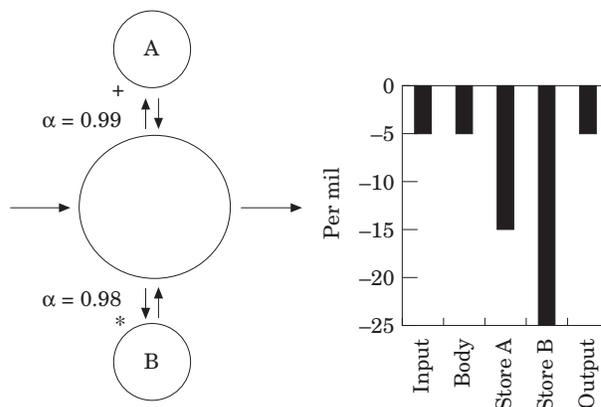


Figure 7. Effect of isotope discrimination on the isotopic abundances of an open, steady-state system with three compartments in equilibrium with discrimination between the central compartment and the two side compartments.

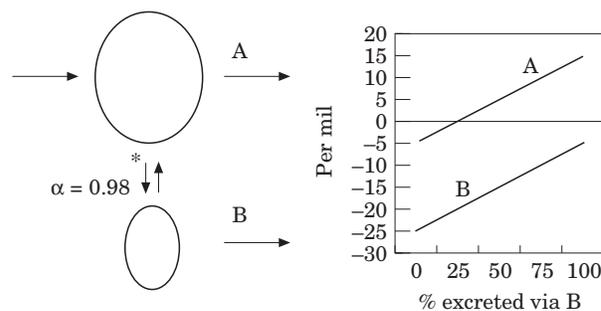


Figure 8. Effect of isotope discrimination on the isotopic abundances of an open, steady-state system with two compartments in equilibrium and one input and two outputs, one from each compartment. Isotope discrimination (\*) occurs at the input from the compartment.

however, is a straightforward extension of that in Figure 5. Because there is no discrimination at either the input or output step, the central pool and the output are not fractionated relative to the input. Both side pools or storage pools, however, will be isotopically heavier than the central pool as both discriminate against the heavy isotope at the equilibration step. Because the discrimination factors have not been set to the same value, however, the two storage pools will be isotopically distinct.

A final example, which is quite common in organism models, is a model similar to that in Figure 6, except that material exits the body through two routes, one from each pool (Figure 8). Unlike the other models, this model will result in variable isotopic fraction, with the fractionation being a function of the partitioning of material between the two metabolic routes. Because the discrimination occurs at a step after the commitment step, the output must be isotopically equal to the input. However, because there are two outputs, the two outputs may be fractionated relative to the input as long as the weighted sum of the two outputs equals that of the input (Hayes, 1993). In this example, the

isotopic abundances of the material in the body pools are equal to those of the output because no discrimination occurs at the output step. The two limiting conditions are those with all the output occurring through only a single output route and in each case the output is isotopically equal to the input as it was in previous examples with fractionation occurring after the commitment step. As the fraction of material exiting the body changes and output of material occurs via both routes, the isotopic abundances shift linearly in relation to the partitioning between the two routes.

Complexity may be added, with fractionation occurring at one or both outputs, or for that matter at the input. The effect of the added discrimination would be additive. Thus, if discrimination with  $\alpha=0.98$  occurred at the point of entry, then the isotopic abundances of A and B would be decreased 20‰ relative to the values shown in Figure 8. If the discrimination were added at one of the output steps, the principle of no net isotopic fractionation would still hold, and the weighted sum of outputs A and B would equal  $-5$ . In fact the output function would be unchanged. The isotopic abundances of the pools within the body would be displaced in the same manner as they were in previous examples.

## Nitrogen Metabolism

Nitrogen abundance analysis has been applied to the analysis of trophic level in ecosystems based on the observation that nitrogen abundances increase by about 2‰ with each step up the food chain (Minagawa & Wada, 1984). The discrimination processes that lead to this phenomenon, however, are not well understood. A review of nitrogen metabolism and some of the isotopic data is thus a valuable first step in understanding the above observation.

Nitrogen in the body and in the diet is present almost exclusively as protein and the amino acids from which protein is synthesized. It is also present in nucleic acids, urea and ammonia, but about 98% of nitrogen is found in protein and amino acids. Human breast milk constitutes the major exception to this as about 20% of the nitrogen is present as something other than protein. Over half of this urea and the remainder is distributed in a large number of nutrients (Donovan *et al.*, 1991). The isotopic consequences of this are not known.

Proteins are synthesized from 20 common amino acids and several minor amino acids. The 20 common amino acids are divided into two subsets, essential and non-essential amino acids (Young & El-Khoury, 1995). An essential amino acid is one in which the organism is not capable of synthesizing the carbon skeleton of the essential amino acids. It must be derived from the diet, hence the term essential. In contrast, the carbon skeleton of non-essential amino acids can be synthesized by the organism. The carbon skeleton is distinguished

from the complete amino acid because nitrogen can be moved from one amino acid to another via transamination. Transamination, however, does not occur to the same extent in all amino acids, but varies from none to complete equilibration. It should also be noted that the essentiality of any given amino acid depends on the species and it must be not be assumed that all species are the same (Anon, 1994; Anon, 1995). Even within a species, essentiality can change with age and illness (Young & El-Khoury, 1995).

As indicated earlier, nitrogen intake is almost exclusively in the form of protein. The generally accepted nutritional requirement for adults is 0.75 g of dietary protein per kg body weight (RDA), but intakes may vary even between individuals meeting their needs for energy. While a typical modern western diet contains about 100 g protein for an average 70 kg subject, an individual subsisting on a diet made up mostly of meat would consume over 200 g of protein, while a vegan would consume about 90 g of protein each day. The ingested protein is denatured in the stomach and hydrolysed to amino acids and short polypeptides. In the process, dietary protein is mixed with an additional 70 g of protein in the form of digestive enzymes and intestinal cell sloughage. The amino acids and short polypeptides are absorbed and transported to the liver via the portal vein. The liver and other splenic tissue takes up 75% of the amino acids. Those in excess of the requirement are quickly catabolized producing ammonia, of which most is converted to urea. Both are water soluble and distribute throughout the pool of body water. Most is cleared during passage through the kidney, such that about 88% of total nitrogen excretion is found in urine. Most of the nitrogen is present as urea, but about 7% is present as ammonia and 10% as creatinine and other molecules (Linder, 1985). Not all urea, however, is cleared by the kidney. A portion passes into the intestine where it is subjected to bacterial modification, producing ammonia and bacterial proteins. The latter are eliminated from the body in faeces. An additional major route of nitrogen output would be milk production in a lactating female and minor losses due to sweat, sloughage of skin and growth of hair and nails.

Although the vast majority of nitrogen in the body is present in amino acids, it should not be assumed that all nitrogen is isotopically identical. In fact, significant inter-amino acid isotopic variations have been demonstrated for nitrogen. Hare & Estep (1983) isolated individual amino acids from modern, bovine tendon collagen. The range of  $^{15}\text{N}$  abundances was 19‰, with threonine nitrogen being the lightest and leucine nitrogen being the heaviest. This range may at first be surprising, but it should be remembered that the amino acids are far from metabolically identical. As indicated above, some of the amino acids are essential while others are not. Furthermore, nine amino acids contain nitrogen in a form other than an amine linked to the carboxyl carbon (Linder, 1985). This nitrogen has little

or no opportunity to mix with other sources of nitrogen and thus is likely to be isotopically different from amino nitrogen. In addition, it has been shown that isotopic discrimination differs between transamination reactions and that this might also be an explanation of the inter-amino acid isotopic differences (Macko *et al.*, 1986). For it to be a complete explanation of the isotopic differences, however, would require that the body amino nitrogen pool reach equilibrium and this is unlikely because of intra-cellular compartmentalization (Meijer, 1995). Finally, amino acids serve important metabolic functions beyond those of being precursors for protein synthesis. For example, alanine participates in the Cori cycle as a nitrogen carrier from peripheral tissues to the liver and glycine is a vital energy substrate for the intestine. Although the cause(s) of inter-amino acid nitrogen isotopic variation has not been identified, the existence of these differences does help explain isotopic variations between proteins as amino acid composition differs between proteins, and the composite nitrogen abundance of a protein will reflect the different contributions of each amino acid.

When nitrogen isotope abundances are measured in the same protein or in tissue samples that contain a large number of proteins, the inter-amino acid differences in nitrogen abundances become less of an issue. In the case of the former, the reason is obvious—the amino acid composition is identical. In the latter case, the large number of proteins results in an averaging of amino acid composition and thus a smaller likelihood that amino acid compositions will vary between samples. As indicated in the introductory statements, when comparisons of this type have been made between species, it has been noted that the  $^{15}\text{N}$  abundance increases by about 2‰ with each step up the food chain (Minagawa & Wada, 1984). The isotopic discrimination(s) that produces this fractionation, however, has not been identified.

### Speculations on a Nitrogen Isotope Model

The simplest isotopic model would be a two-compartment model with one output from each compartment similar to that in Figure 8. The two compartments would represent protein and non-protein nitrogen, while the outputs would be faeces and urine. An example of the use of this model is the work of Steele & Daniel (1978) who measured nitrogen isotope balance in cows and steers. In steers, it was reported that the body pool as sampled from blood was 4.2‰ heavier than the diet, while urinary nitrogen was about 2‰ lighter than the diet and faecal nitrogen 2‰ heavier. The relationship between diet and faecal nitrogen might suggest that there is fractionation during digestion and absorption with preferential uptake of the lighter  $^{14}\text{N}$ . This, however, would suggest that body nitrogen should be isotopically light, when in fact

it is heavy. Another, more plausible explanation is that dietary nitrogen will mix with a roughly similar mass of endogenous nitrogen in the intestine. Because endogenous nitrogen is 4‰ heavier than dietary nitrogen, an equal mixture would be 2‰ heavier than the diet. Inclusion of this mixing requires the introduction of an additional compartment to represent the gastrointestinal track.

The enrichment of body nitrogen relative to the diet could be explained by either isotope fractionation favouring heavy nitrogen during protein synthesis from amino acids, because the vast majority of body nitrogen is in the form of protein, even in blood; or isotope fractionation favouring light nitrogen in the process of nitrogen elimination. Fractionation during protein synthesis is highly unlikely, however, because the committed step in protein synthesis occurs in the binding of amino acids to tRNA and this binding does not involve the amino nitrogen and thus should not discriminate between the nitrogen isotopes. Thus, the more likely site of fractionation would be nitrogen elimination. Fractionation in this process would also result in the body nitrogen pool becoming isotopically heavy as illustrated in Figure 2. This fractionation could occur in the transamination processes that are involved in the movement of amino nitrogen from the periphery of the body to the liver for urea synthesis, deamination, or urea synthesis. Isotopic data comparing the nitrogen in urea and ammonia fractions of urine would be valuable to any further discussion. For any further discussion, however, it should be noted that there is a differential contribution of amino acids to urea and ammonia (Linder, 1985) and that the inter-amino acid nitrogen isotope abundances are not equal (Hare & Estep, 1983). It is also interesting to note that birds and fish exhibit a tropic nitrogen isotope effect similar to mammals (Minagawa & Wada, 1984). Birds and fish, however, excrete nitrogen as uric acid or ammonia, respectively, instead of urea (Campbell, 1970) suggesting that either the isotope fractionation is similar in the three processes or that fractionation occurs prior to these steps. Data regarding the isotopic abundance of nitrogen in each of the separate chemical forms and waste products would be very helpful in furthering the understanding of nitrogen isotope biochemistry.

A final addition to the simple model, i.e., a model that does not include all of the inter-amino acid isotopic differences, would be addition of the path for urea re-utilization in the intestine (Figure 9). This re-utilization involves the action of bacteria, which use urea as a nitrogen source for protein synthesis. The isotope fractionation associated with this process has been investigated, but only *in vitro* using ammonia as the nitrogen source rather than urea (Wattiaux & Reed, 1995). That work demonstrated that the bacteria discriminated against heavy nitrogen producing isotopically light protein. This could explain the enrichment of body nitrogen, but only if the light protein is

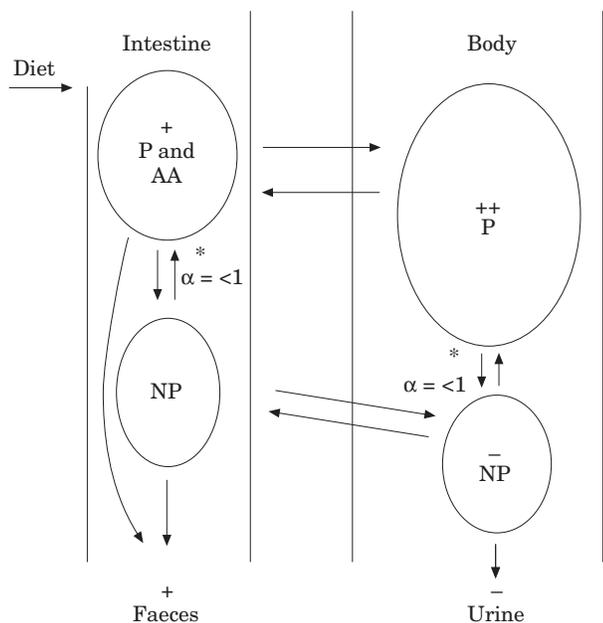


Figure 9. Hypothesized steady-state model of mammalian nitrogen isotope metabolism. The model is simplified in that only two anatomical compartments are considered (gut and body), and only two chemical entities are considered. These are protein (P) and non-protein nitrogen (NP). Pools heavier than diet nitrogen are indicated as (+) and those lighter than diet nitrogen are indicated as (-).

eliminated in faeces, which is not consistent with the report of isotopically heavy faecal nitrogen (Steele & Daniel, 1978). Thus, this process, while probably important to the model, is most likely a minor pathway that by itself does not explain the nitrogen isotope trophic effect. Urea recycling, however, may be an important process in understanding the variability in the trophic effect.

In summary, a simplified, organismal isotopic model permits speculation that can explain the trophic effect observed for nitrogen isotopes. The speculation is that the primary isotope discrimination occurs somewhere between amino acid catabolism and urinary nitrogen excretion. Data, however, are lacking and further research is required to test this hypothesis.

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