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Symposium on 'Metabolic aspects of human nutrition at rest and during physical stress: recent methodological and technical developments'
Session 4: Tracers in metabolic and nutrition research: 1

An introduction to the use of tracers in nutrition and metabolism

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The present article is a review written at a level suitable for students and new workers to the field of techniques in common current use for the measurement of static and dynamic features of metabolism, especially nutritional metabolism. It covers the nature of radioactive and stable-isotope tracers, the means of measuring them, and the advantages and disadvantages of their use. The greater part of the review deals with methods for the measurement of pool sizes and metabolic processes, with the emphasis being on protein metabolism, a field the author knows best. The examples given are from a variety of sources, including the work of the author, but the principles underlying the techniques are universally applicable to all metabolic investigations using tracers.

Isotopic-labelling techniques: Protein metabolism: Radioactive tracers: Stable-isotope tracers

The word metabolism comes from Greek roots meaning dynamic change, and it makes sense therefore that if we want to look at the effects of altered diet or physical activity, we should use methods which enable us to describe the characteristics of the changes, and in particular their rates. Isotopic-labelling techniques are often ideal for this task. In the present review I will discuss the use of isotopic techniques linked with detection by mass spectrometry and liquid-scintillation counting. There is of course a large literature on other techniques, e.g. positron-emission tomography, magnetic-resonance spectroscopy and near-infra-red spectroscopy, all of which have useful applications in biomedical research, but the topic is simply too big to discuss, and other contributors will describe some of these techniques at this meeting.

Nature of isotopes: stable *v.* radio, and methods of measurement

What are isotopes, and which are useful for biomedical research?

Elements which share the same place in the periodic table are termed isotopes (Table 1). Thus, they have the same chemistry, i.e. the same number of protons, but they are different in their atomic mass and usually in other physical properties also that can be used to distinguish between them.

Common stable isotopes such as ^{1}H, ^{12}C, ^{14}N and ^{16}O make up most of our biological environment and their much-less-common stable companions (^{2}H, ^{13}C, ^{15}N, ^{18}O) are by definition rare (0·02–1·1 %). However, the radio-active analogues (^{3}H, ^{14}C, ^{13}N, ^{11}O) are even more

Abbreviation: IRMS, isotope-ratio mass spectrometry.
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Table 1. Isotopes commonly used in biological research

Common stable	Rare stable	Radioactive
^{1}H	^{2}H (0·02 %)	^{3}H
^{12}C	^{13}C (1·1 %)	^{14}C
^{14}N	^{15}N (0·37 %)	^{13}N*
^{16}O	^{18}O (0·04 %)	^{11}O*

* No long-lived radioisotopes of these elements.

uncommon than the rare stable isotopes, and although they also differ in atomic mass, these masses are not constant, but change during radioactive disintegration with the emission of various sub-atomic particles. Thus, isotopes have the same chemistry, but their different physical properties (e.g. mass or radioactivity) provide the means of measurement separately from the common stable isotopes. Modern organic and bio-organic chemistry has given us the ability to label almost any kind of molecule with rare, stable or radioactive isotopes, the limitations in most cases being time and hence cost. However, in some cases such as, for example, hydroxyproline which itself exists in four different isomeric forms, the difficulties of synthesis are almost insuperable by conventional means.

What are the pros and cons of the use of stable and radioisotopes?

The major advantage of the use of stable isotopes is that they are not a source of ionizing radiation, which means that they are effectively safe and non-toxic, allowing studies in children, including infants and perinates and even babies *in utero* (Koletzko *et al*. 1997; Chien *et al*. 1993). Repeat studies and long-term studies in adults are also perfectly safe and practical, which might not be the case if radioisotopes were used. In addition, the existence of stable isotopes of O and N, which are of obvious biological relevance, and the lack of any analogous long-lived radioisotope for these elements has meant that the use of stable isotopes has grown explosively over the past few years.

Nevertheless, there are some disadvantages. In fact the relatively high natural background for most stable isotopes (1 % for C and 0·3 % for N) means that at low levels of enrichment the range over which measurements of stable isotopes can be made is less than that for radioisotopes. Also, most methods for detection and quantification of stable isotopes are less sensitive than those used for radioisotopes. Furthermore, the cost of some of the isotopes (e.g. ^{18}O) and thus of some tracer molecules made incorporating them, even if they are available, can make some studies not feasible. In addition, simply because of market size, the range of molecules labelled with stable isotopes is smaller than that of radioisotopes. The cost of synthesis of molecules labelled with stable isotopes is always going to be higher per unit than that for radioactive probes if the market size is less, which is patently true for many substances.

Last, the costs of measurement instrumentation are substantial. Not only are capital costs high, but these instruments are complicated and difficult to use and maintain; in addition, their use usually means that specialized staff have to be employed to do these tasks. These factors mean that entering into the stable-isotope metabolic club is difficult and expensive, and requires strength in depth, or a factor which is sadly less prevalent than it should be, i.e. collaboration.

Before we completely leave the consideration of radioactive isotopes, we should note that in many cases, in mature adults, the doses of radioactive tracer necessary are so low that the safety aspects are negligible. We do ourselves a disservice if we ignore the safe use of radioactive tracers in situations where they are more convenient, cheaper, and more appropriately labelled.

How do we measure isotope-labelled molecules and implications of the methods?

^{3}H and ^{14}C emit β rays, i.e. electrons, which cause flashes of light in a liquid scintillant which can then be detected in a photomultiplier tube (Fig. 1). The energy spectra of ^{3}H and ^{14}C are different, with ^{14}C radiation having a much higher average energy and thus it is possible to gate the detector to set up energy windows through which pass isotopes of predominantly one kind or another. This process means that we can use dual-labelling techniques with ^{3}H and ^{14}C. However, there is one major disadvantage; although the quantum efficiency of modern photomultipliers is very high and so sensitivity is good, nevertheless the results which are obtained after correction for quenching are measurements of radioactivity only and not of specific radioactivity, i.e. radioactivity per chemical mass. In order to obtain this value we need to measure the chemical mass separately, thus there is an inherent additional error in the calculation of the specific labelling. Stable isotopes, and the tracer probes containing them, differ only in their atomic or molecular mass (i.e. the number of neutrons they contain); they can be separated on this basis, either as native gases or as molecules which are capable of being volatilized. Their isotope ratio gives a direct measure of labelling or enrichment (Fig. 2).

In cases in which the labelled material is a gas such as $^{13}CO_2$, $C^{18}O^{16}O$, $^{1}H^{2}H$, or ^{13}CO, either produced in a metabolic reaction or after reduction by combustion or pyrolysis, the isotopes in the gases can be separated after their conversion to positively-charged molecular ions. This conversion is usually achieved by bombardment with an electron beam which displaces further electrons from the sample gases, producing positively-charged molecular ions (Fig. 2). These molecular ions can then be separated on the

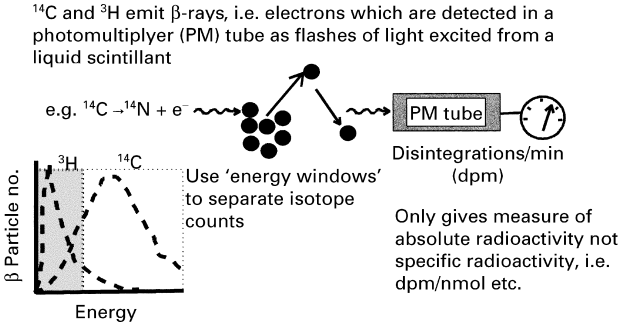

Fig. 1. The principle of measurement of radioactivity of radiolabelled biomolecules.

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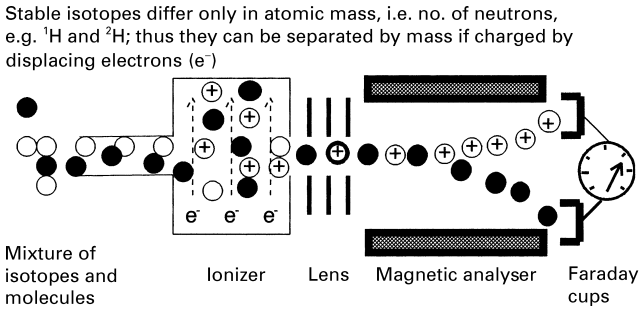

Fig. 2. The principle of measurement of isotope ratios for stable-isotope-labelled molecules or atoms.

basis of their mass : charge ratio, either in a fixed magnetic field produced by a permanent magnet, as in most conventional isotope-ratio mass spectrometers (IRMS), or occasionally by the use of a tuneable (i.e. variable) quadrupole mass spectrometer such as in those used for respiratory gas analysis.

Other kinds of 'front ends' may be used to produce appropriate molecular gases, including elemental (Dumas) analysers or mass spectrometers, or liquid chromatographs coupled to a combustion or pyrolysis module. The full range of so-called hyphenated techniques in mass spectrometry gives us such modes as GC–combustion–IRMS, GC–pyrolysis–IRMS, liquid chromatography–combustion–IRMS etc.

In classical GC mass spectrometry molecules of a relatively large size are first derivatized to make them volatile, using a variety of techniques that add large organic groups to the analyte (Meier-Augenstein, 1997). When these techniques are allied with combustion mass spectrometry (GC–combustion–IRMS), the production of large amounts of unlabelled CO_2 or CO from the adduct produces a substantial dilution of C labelling (Yarasheski *et al*. 1992; Rennie *et al*. 1996). The dilution factor is less of a problem with ^{15}N because most derivatizing agents do not add N to the adduct formed, but there is the major difficulty of making sure that atomospheric N is excluded from the system.

Neither ^{2}H nor ^{18}O can be used in conventional combustion systems because these produce water and CO_2 as products; although CO_2 can be analysed when it contains ^{13}C, it is difficult to analyse CO_2 containing ^{18}O because naturally-occurring CO_2 of mass 44 cannot be used as an internal standard. Pyrolysis (i.e. the partial thermal degradation, rather than complete combustion) of organic compounds and water containing ^{2}H and ^{18}O is a feasible solution to this problem.

Continuous-flow pyrolysis IRMS offers the possibility of measuring the labelling of H and O in microlitre samples of water, and indeed this procedure has been used to measure the metabolic rate of free-living bumble bees (at between 500 and 2500 ml O_2/kg per min!). It furthermore offers the possibility of detection of labelling in organic molecules at very low levels of abundance (e.g. collagen labelled with ^{18}O-containing amino acids and sampled over months to years thereafter).

Generally, for the mass spectrometric methods utilizing magnetic sector instruments on the one hand, or quadrupole analysers on the other, there is a trade-off between sample size and precision of detection limits. GC–mass spectrometry is very sensitive in terms of sample size (femto- to nanomoles) but requires relatively high levels of enrichment (0·5 atom % excess) which can be measured with a precision of approximately 0·5 atom % excess, whereas GC–combustion–IRMS would require 1000-fold more sample, but would allow the limit of detection to be driven down to 0·001 atom % excess with a precision of 0·0002 atom % excess (Table 2).

Various kinds of devices which allow fine sprays of fluids containing analytes, or which permit what is effectively sublimation of solid samples (as a result of heat or radiation treatment), will enable relatively large molecules to be analysed in the gas phase. Such techniques allow volatilization of peptides and nucleic acid fragments, but these techniques do not normally allow the measurement of the labelling of the individual fragments. For metal ions, thermal-desorption and plasma-ionization techniques allow examination of the isotope ratios of mixes of Zn, Ca, Se and other metal ions of biological interest with great sensitivity, accuracy and precision (Turnlund *et al*. 1982; Turnlund, 1991).

The dilution principle applied to determination of isotopic labelling

The dilution principle stems directly from the Law of Conservation of Mass. Since atoms can be neither created nor destroyed, the total mass of isotopic tracer added to a biological system, including that which is metabolized and exhaled or excreted, must remain constant. Thus, if it is known how much tracer is added to a system, sampling of the various pools into which the tracer mixes and measurement of its dilution in those pools will provide a measure of the size of the pools (Fig. 3). This static dilution technique has been used with great success in the measurement of body composition. Models of body composition may be constructed at atomic, molecular, cellular and tissue levels, and at each of these levels it is possible to use isotopes to define the size of one of the compartments. Thus, exchangeable H, O, Na, Cl and K (e.g. ^{18}O, ^{2}H, ^{3}H, ^{35}Cl, ^{42}K etc.) can be used to measure extracellular and intracellular water etc. These isotopes can be analysed by conventional spectrometric or radiometric methods, as appropriate. The natural radioactivity of the

Table 2. Sample size, accuracy and precision of mass spectrometers (MS): characteristics of MS methods for measurement of enrichment of samples labelled with a stable-isotope tracer

Mode	GC–MS	Off-line IRMS	GC–C–IRMS
Minimum sample size	1 pmol	1 μmol	1 nmol
Detection limit of enrichment (atom % excess)	0·5	0·001	0·0005
Precision (atom % excess)	0·5	0·0005	0·0001

IRMS, isotope-ratio MS; C, combustion.

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body due to the ^{40}K left over from the 'big bang' produces a very sensitive means of measuring the body cell mass (Forbes, 1987). When bodily elements are made radioactive as a result of small doses of neutron irradiation, a large number of elements can be detected as a result of their own short-term nuclear decay, including N, K, Na, C, P, Cl etc., and this process enables almost every compartment of the body to be analysed with great accuracy and precision (Forbes, 1987).

Knowing the mass of different compartments of the body is obviously important, but in metabolic terms it is often less interesting than knowing the rates of exchange between the compartments or knowing the rates of metabolism of the components within those compartments. For these purposes, dynamic tracer dilution is required (Fig. 4).

In the simplest version of this technique, tracer is added to a particular body pool (whose metabolism is to be examined) at a constant rate and samples are taken at defined intervals. The tracer will eventually equilibrate throughout the pool, enabling the rate of flux of the tracer and its tracee to be calculated rather simply. The movement of metabolite through the compartment (in jargon terms the 'flux') is simply infusion rate : extent of labelling in a steady-state. Of course, there are a variety of ways of applying the tracer and a number of choices in determining the pools to be sampled, and also many different ways in which the resulting information can be analysed mathematically and statistically (Waterlow, 1984; Cobelli *et al*. 1987; Wolfe, 1992).

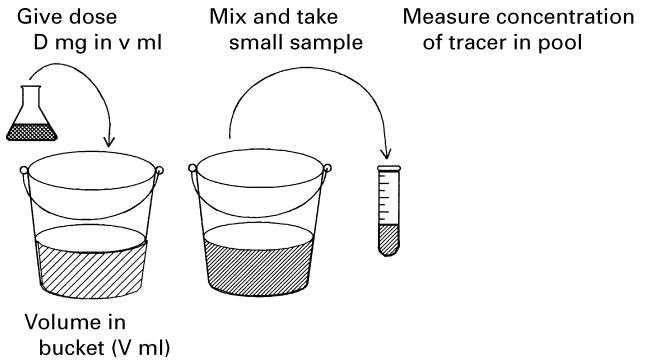

Fig. 3. The dilution principle applied to a static pool. Concentration (c) in the sample (mg/ml) = D/(V + v), where D is the dose (mg) in volume v (ml) and V is the volume of the pool (ml). Thus, V = (D/C) – v.

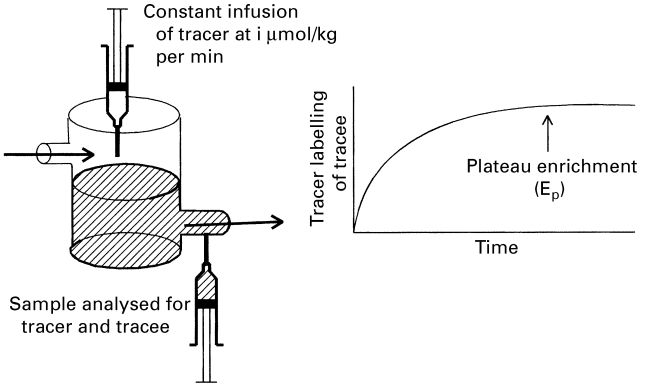

Fig. 4. The dilution principle applied to a dynamic pool. Flux through the pool (μmol/kg per min) = i/Ep, where i is infusion rate of the tracer (μmol/kg per min) and Ep is the plateau enrichment.

The constant-infusion method (Fig. 5(a)) is the simplest conceptually, and as long as sufficient samples are taken it provides information on the degree to which equilibrium (or at least a metabolic steady-state) has been achieved, signalled as the relative constancy of labelling of the metabolic pool in question. An alternative approach is to deliver the tracer as a bolus to one of a number of accessible metabolic compartments (Fig. 5(b)). This approach obviously provides dynamic information on those pools that can be sampled directly or, in the case of breath and urine, of the whole body treated as a single pool. However, by using a spike input of tracer into one metabolic pool together with sampling from another interconnected pool, it is possible to gain information about the movement of substances and interconversion in metabolic pools that are otherwise inaccessible. Thus, injection of tracer into plasma and sampling from plasma nevertheless may provide information about other pools such as extracellular and intracellular compartments. In order to obtain reliable information the input function must be well defined and should, if possible, be instantaneous (or at least occurring over a known time period), mixing should be rapid (or at least over a defined period), a steady-state must be assumed, and rapid sampling must be possible in at least one of the pools. Some extremely sophisticated methods of analysis have been applied, together with imaginative sampling protocols, to enable us to gain large amounts of information about the metabolic interchange turnover and compartmental size for a variety of metabolic processes. Among the major difficulties of compartmental analysis, however, are that often the pools are only notional, being the conglomerate of groups of pools which exist in a variety of different tissues (e.g. the extracellular space); furthermore, it is often very difficult to obtain dynamic information for more than four interconnected pools, especially as the labelling of the primary pool from which the sample is taken starts to approach zero, and often large numbers of laborious experiments have to be conducted to examine the behaviour of secondary metabolites. This situation occurs particularly when we are examining changes in patterns of nutrient intake or composition, physical activity and disease state.

If compartmental modelling is avoided, then a single bolus input combined with measurement of the tracer in a metabolic pool or total production of some metabolite of the tracer can give large amounts of useful information. The simplest example of this procedure is the use of the doubly-labelled water method for measuring energy expenditure, in which both H and O in water are labelled. The dose divided by the tracer labelling at time zero (extrapolated from the results of analyses of later samples) provides a measure of the total body water and thus of lean body mass (Wolfe, 1992). As long as it is assumed that the rate of loss of ^{2}H and ^{18}O labelling from the body water pool each follow a single exponential decay (of the form $E_t = E_0\, e^{-kt}$), where E_t and E_0 are the enrichments at zero time and time t and k is a constant, then the difference in the rates of loss of ^{2}H and ^{18}O will provide a measure of CO_2 production; given an assumed RQ of 0·85, this measurement provides a measure

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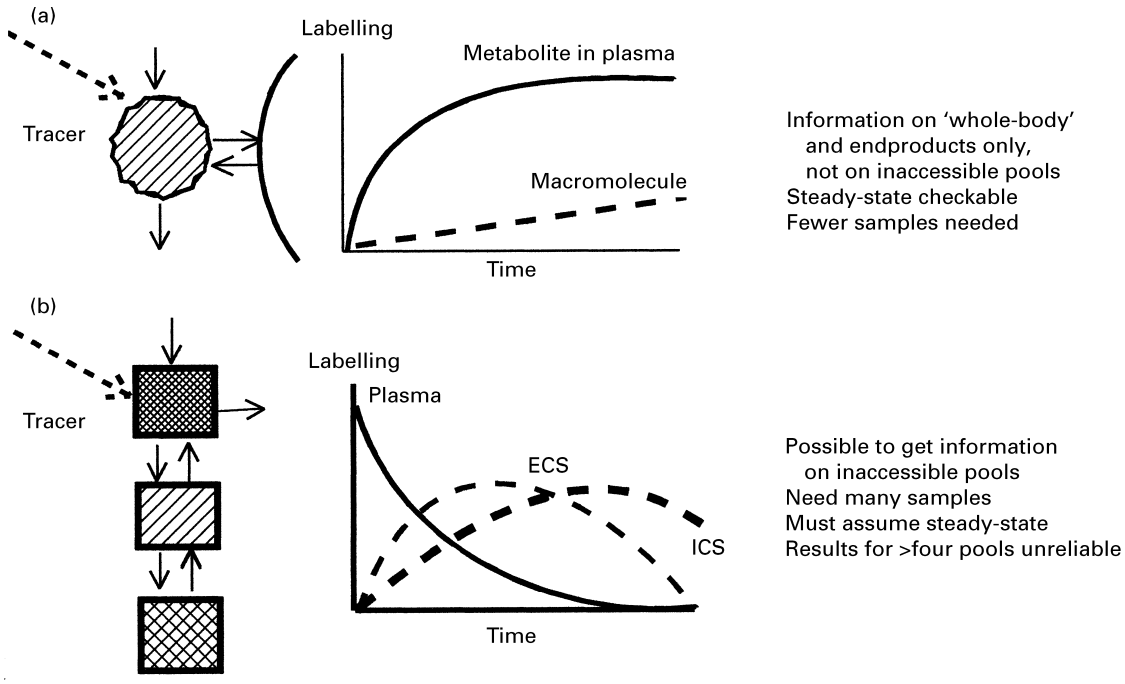

Fig. 5. (a) The constant-infusion method and (b) the bolus-injection method, with compartmental (pool) analysis, for measuring isotopic labelling of a metabolic pool. (▩), Plasma; (▨), (▭), extracellular (ECS) and intracellular (ICS) space.

of O_2 consumption and thus of energy expenditure. This topic is explored in more detail by Klaas Westerterp (1999). If a metabolic end product is measured (e.g. CO_2 in the breath or urinary urea and/or NH_3 after administration of, for example, [^{13}C]leucine or [^{15}N]glycine), then the flux through the pool is equal to the dose divided by the area under the endproduct curve, assuming that the tracer has as much chance of getting into the metabolic endproduct as does the tracee. This factor is not usually a problem in the case of relatively simple metabolic processes such as the production of CO_2 from glucose, but when a particular amino acid (e.g. [^{15}N]glycine) is used as being representative of all other amino acids, then unless its metabolism truly is representative (which in the case of glycine it almost certainly is not; Matthews *et al*. 1981) difficulties of interpretation may arise.

Fewer difficulties arise if the probe used is metabolized rapidly to an endproduct via a metabolic process which may often be rate limiting. This was the case in a study we conducted of a child with Crohn's disease investigated before and after treatment with steroids, when the difference in the appearance of $^{13}CO_2$ from orally-administered [^{13}C]leucine was very apparent (Fig. 6; Thomas *et al*. 1992). In this case the process of interest was absorption via the gut. In general such breath tests can give a substantial amount of information concerning gastric emptying, oro-caecal transit time, the presence or not of *Helicobacter pylori* infection, gut bacterial overgrowth, the extent of liver function, fat digestion, and absorption, protein digestion, and energy expenditure. All these processes use ^{13}C exhalation in the breath CO_2 as a probe for the physiological function of interest.

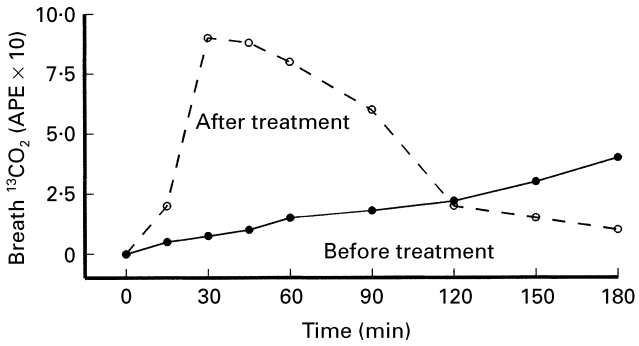

Fig. 6. The effect of treatment on labelled carbon dioxide in expired breath after ingestion of [1-^{13}C]leucine in a child with Crohn's disease. The difference presumably represents more efficient small intestinal absorption. APE, atom % excess. Flux = dose/$\int E_{^{13}CO_2}$, where $E_{^{13}CO_2}$ is ^{13}C enrichment of CO_2. Mean residence time = pool size/flux. It is difficult here to estimate pool size as dose/E_0, where E_0 is the enrichment at zero time. (From Thomas *et al.* 1992.)

Intermediary metabolism and protein turnover

When more extensive information is required about interrelated metabolic processes (e.g. in the case of leucine which may be incorporated into body protein, or appear from body protein in the processes of protein turnover, or which may be transaminated to its α-keto acid, which is thereupon oxidized), a much greater range of analytes will need to be sampled in order to provide such information. In my opinion such experiments are easiest to carry out under conditions in which primed constant infusions of tracer have been applied, in which case it is possible to make

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measurements in the steady-state of a number of integrated processes. A good example is the measurement of the whole-body leucine rate of appearance and oxidation which, when subtracted, provides information on whole-body leucine disappearance into protein, i.e. protein synthesis. (Matthews *et al*. 1981; Fig. 5(a)). Individual tissue protein synthesis can be calculated using the same tracer administration protocol, while in addition measuring the rate of increase in enrichment of the protein sampled by biopsy (Fig. 7; Smith & Rennie, 1990).

Methods for measurement of macromolecular turnover

This topic has been touched on earlier, where it was shown that tissue protein synthetic rate can be determined by means of the change in enrichment with time divided by the average extent of labelling in the precursor pool. (Fig. 7). An alternative method is to measure the rate of dilution of the tracer across an organ (ideally assuming that the tracee is

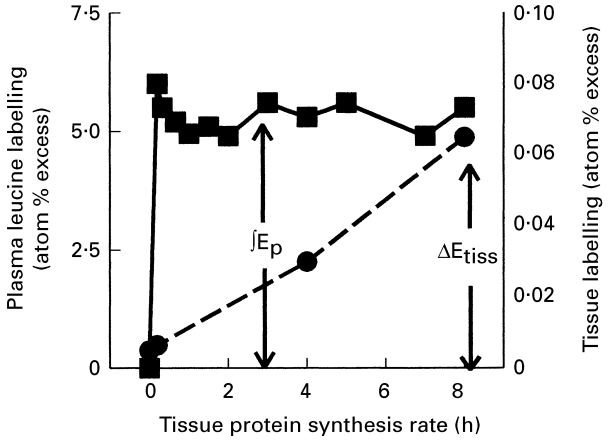

Fig. 7. Typical pattern of labelling of plasma free leucine and tissue protein leucine during a primed constant infusion of [1-^{13}C]leucine. Tissue protein synthesis rate (%/h) = $\Delta E_{tiss}/\int E_p \times 1/t$, where E_p is plateau enrichment, ΔE_{tiss} is change in tissue enrichment and t is time.

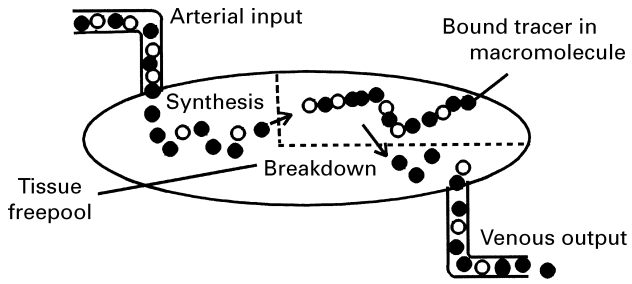

Fig. 8. Arterio–venous tracer exchange model which can give values for transport of amino acids into and out of muscle or other tissues, as well as values of disappearance into protein (synthesis) and appearance from protein (breakdown). (○), Tracer; (●), tracee. Balance = $C_a - C_v$, where C_a and C_v are the arterial and venous concentrations respectively. Breakdown = $1 - E_a/E_{tiss} \times \text{flow} \times C_a$, where E_a and E_{tiss} are the arterial and tissue enrichments respectively. Synthesis = balance + breakdown. Using this method it is also possible to measure the tracee, and tracee transport in and out of the tissue.

not produced metabolically (as distinct from via turnover) by that organ, which is often the case) and then taking the measurement of the tracer dilution as a measure of the breakdown of a macromolecule to its monomer. This method would work very well for, for example, a measurement of protein breakdown as dilution of tracer phenylalanine via release of unlabelled phenylalanine. The analysis in the case of a study of muscle is facilitated by the fact that phenylalanine is an essential amino acid (i.e. is not synthesized *de novo* in mammals) and shows no intermediary metabolism. Thus, the rate of dynamic dilution is equivalent to the rate of protein breakdown, and the rate of protein synthesis is therefore simply the input minus output of phenylalanine, i.e. the net phenylalanine balance across the tissue or organ under consideration, plus protein breakdown (Bennet *et al*. 1990; Rennie *et al*. 1990). A major and very useful refinement of the method has been to model not only rates of protein metabolism but also transport into and out of tissues (Biolo *et al*. 1992; Fig. 8).

The caveats which Ian Macdonald (1999) discussed for the normal measurement of arterio–venous flux in organs are doubly important when it comes to the measurement of tracer exchange, i.e. there must be a steady-state of concentrations (particularly of tracee and its tracer) and the flow and transit time of the measured substances across the organ must be less than the intervals of sampling. However, in the case of tracers another condition needs to be fulfilled, and that is that the pool in which the greatest extent of dilution is taking place must be sampled in order for the model to be applied accurately. In many cases the venous output has been used, but actually this, because of shunting between the arterial and venous blood, provides an underestimate of the extent of dilution of label. Ideally it should be sampled from the extracellular space (e.g. by means of the dialysis technique; see Arner, 1999) or even in the intracellular free amino acid pool, if this were truly possible. Usually workers in the field make do with either the venous plasma labelling or that of the tissue free amino acid, which is mainly intracellular.

An alternate way to measure protein breakdown, applicable to any tissue for which the tissue free amino acid pool can be sampled, is the fractional breakdown rate method (Fig. 9; Zhang *et al*. 1996). The method depends on measuring the dilution in the venous blood draining a tissue, or in the tissue free pool, of the tracer after its delivery has been stopped. This dilution is a measure of the appearance of unlabelled amino acid from tissue protein, i.e. protein breakdown.

The precursor–postcursor problem

The problem of adequately sampling a particular labelled pool the results of which will be used to calculate a particular metabolic function, is endemic in the study of metabolic processes using tracers. In the study of the synthesis of macromolecules it is known as the precursor problem, but in fact it could well be called the postcursor problem (if that is not too ugly a neologism) when applied to the measurement of the extent of dilution of a tracer in the free pool as the result of breakdown of the tracer. If a tracer amino acid is added in such a way (e.g. by constant infusion)

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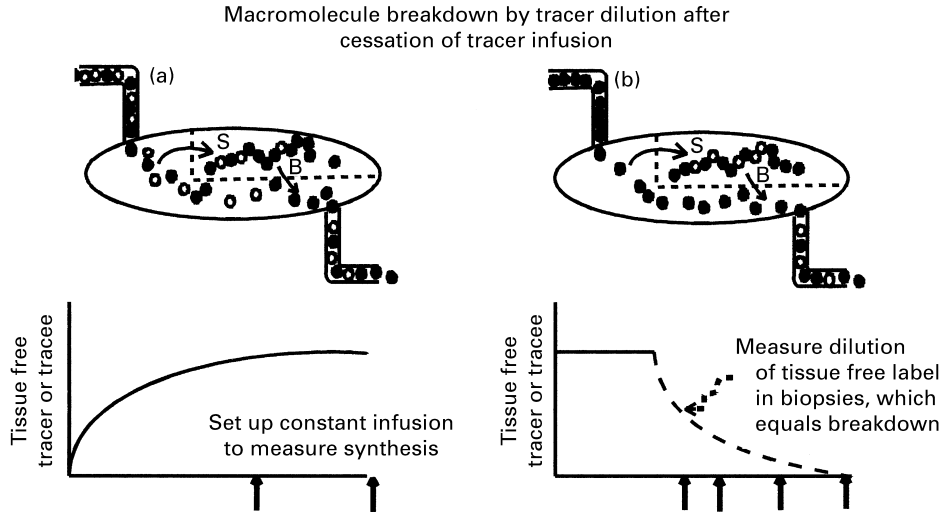

Fig. 9. The fractional breakdown rate method. (a) Tracer is infused at a constant rate in order to measure synthesis rate and (b) dilution is measured after infusion is stopped as a measure of breakdown. (●), Tracee; (○), tracer; S, synthesis; B, breakdown; ↑, sampling times. (Zhang *et al.* 1996.)

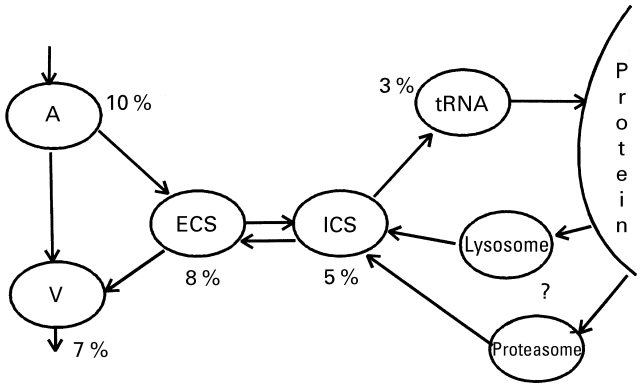

Fig. 10. Notional values for tracer : tracee (% enrichment) for a non-metabolizable tracer amino acid in various free pools in a tissue such as muscle or liver. Where there is *de novo* synthesis and metabolic chanelling the tRNA pool may be substantially less than that of the intracellular space (ICS; e.g. as for proline in the skin). A, arterial blood; V, venous blood; ECS, extracellular space.

that it achieves a particular labelling in the arterial blood, dilution of the tracer results in a much lower enrichment of other pools (Fig. 10).

The tracer may be diluted to an unknown extent in some pools, such as the lysosome and proteosome. The difficulty is that in order to measure the true rate of protein synthesis or the true rate of protein breakdown, it is imperative to characterize the labelling in the appropriate pool. The situation is no different when it comes to measurement of synthesis or breakdown of, for example, glycogen (in which the appropriate precursor pool would be that of glucose-6-phosphate) or triacylglycerol (in which case it would be useful to know the extent of the labelling of free fatty acids and newly-synthesized glycerol-3-phosphate), or of RNA (in which case it would be important to know the amount of labelling of, for example, ribose, or of a free purine or pyrimidine, or of the nucleotide phosphate).

The importance of getting the value right is demonstrated by results of an experiment my colleagues and I carried out in which we presented leucine or valine labelled with ^{13}C either intragastrically or intravenously, and observed that when we measured the labelling of the free tracee in the human duodenal mucosa mixed-tissue water there was a twofold difference in the tracer : tracee value (Nakshabendi *et al.* 1995; Fig. 11). However, there was also a twofold difference in the labelling of duodenal mucosa protein, so that when protein synthesis was calculated on the basis of the labelling in the free pool of the tissue in each case (irrespective of whether the tracer had been administered intragastrically or intravenously, or whether it was leucine or valine) the rate of protein synthesis was found to be approximately 2·5 %/h. In this case we did not measure the labelling of intracellular tRNA, but where we have done so (e.g. in the pig stomach) we have shown that leucyl tRNA reaches to a value which is approximately that of the gastric mucosal intracellular pre-water labelling and also of the plasma α-ketoisocaproate labelling (Watt *et al.* 1992). In these circumstances it is therefore possible to use the plasma α-ketoisocaproate labelling to provide an indication of the intracellular labelling, as we have done most successfully for muscle in which the intracellular free pool, the leucyl tRNA and the plasma α-ketoisocaproate labelling give similar results (Watt *et al.* 1991). In the case illustrated in Fig. 11, the protein synthetic rates estimated from the protein labelling and the intracellular pools were identical for both tracers used and both routes of administration.

It is also possible to measure the rate of synthesis of secreted proteins, e.g. pepsin as the rate of incorporation of ^{13}C into pepsin protein isolated from gastric juices (Corbett *et al.* 1995). Other workers have used the same techniques to measure pancreatic protein synthesis or the synthesis of export proteins from liver such as albumin, apolipoprotein B100 etc. (Collins *et al.* 1992; Bennet *et al.* 1993). However, in some circumstances, for example if we wanted to measure the rate of labelling of a macromolecule synthesized in a relatively inaccessible pool, it would be very difficult to use this kind of methodology. For example, in bone and skin, collagen is made by cells which are

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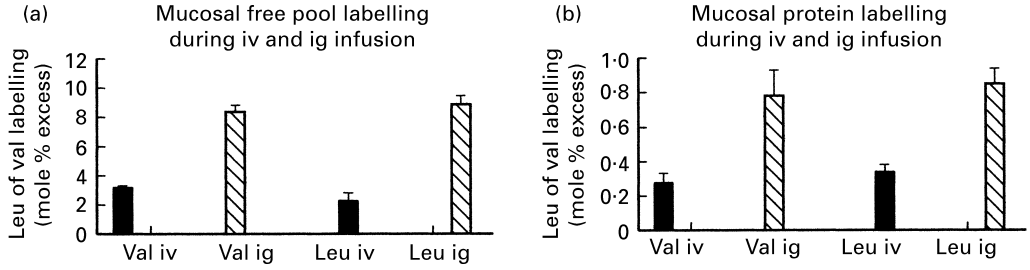

Fig. 11. Differences in ^{13}C labelling in (a) the free and (b) the protein-bound amino acid pools when leucine (leu) or valine (val) labelled with ^{13}C were administered either intravenously (iv; ▬) or intragastrically (ig; ▨). Values are means with their standard errors represented by vertical bars for *n* 6-8 determinations. (From Nakshabendi *et al.* 1995.)

embedded in an extracellular matrix which almost certainly does not, for example, participate in the transamination of leucine to α-ketoisocaproate at rates sufficiently high to give us confidence that plasma α-ketoisocaproate provides a good measure of intracellular leucine labelling, and in these circumstances application of the so-called flooding-dose technique might be particularly useful (Scrimgeour *et al*. 1993; Rennie *et al*. 1996) The idea here is to equilibrate the free pools by providing so much (in a chemical sense) of the labelled amino acid that an equilibrium is forced.

Constant infusion or flooding dose?

The so-called flooding dose approach was first used by scientists interested in the measurement of protein turnover in tissue culture; it was first applied to living mammals by McNurlan & Garlick (1980), who used it to study protein turnover in tissues of the rat. When it was applied to adult human subjects the tracer used was leucine labelled with ^{13}C; later, ^{2}H-labelled tracer was used because of the ability of GC–mass spectrometry to measure [^{2}H]phenylalanine in hydrolysates of protein into which the tracer had been incorporated (McNurlan *et al*. 1991; Calder *et al*. 1993). One of the difficulties with this method is that, although it appears to be perfectly acceptable for measuring decreases in the rate of protein synthesis, it seems to have very little dynamic range in detecting increases above the normal physiological value, at least in muscle. In our group we were particularly worried, when looking at values first obtained, that the method appeared not to be able to detect the effects of feeding on protein synthesis, and therefore we hypothesized that the flooding technique itself apparently stimulated protein synthesis. In a long series of experiments we have now shown that when muscle protein synthesis is measured in human subjects using a flooding dose of an essential amino acid there is an apparent stimulation (which may, in fact, be a true stimulation of muscle protein synthesis), but when nonessential amino acids such as glycine, proline, serine or arginine are used there is no such apparent stimulation (Smith *et al*. 1992*a*,*b*,*c*, 1998; Fig. 12).

This fascinating result will not be discussed further here, but I have to say that it strongly suggests that there is some control by amino acid availability and type on the rate of lean mass accretion in the body. In addition, it may turn out to be very useful that proline does not invoke this effect, since it has been possible to measure the rate of bone and skin collagen synthesis using the flooding-dose protocol (Scrimgeour *et al*. 1993).

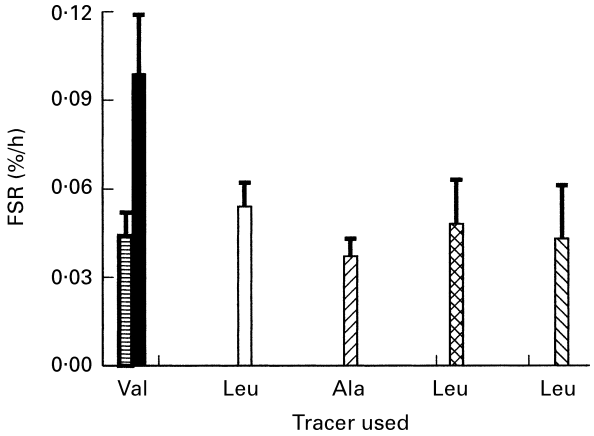

Fig. 12. Effect of flooding dose of various amino acids, both essential and nonessential, on muscle protein synthesis. (▤), Tracer alone; (▬), leucine (leu) flood; (▭), glycine flood; (▨), proline flood; (▩), serine flood; (▧), arginine flood. Val, valine; ala, alanine; FSR, fractional synthetic rate. Values are means with their standard errors represented by vertical bars for *n* 6-8 determinations.

Measurement of nucleic acid turnover using stable-isotope techniques

The measurement of the turnover of RNA and DNA is of great interest to those of us concerned with regulation of body metabolism. There have been a number of attempts to measure the breakdown of RNA and DNA using specific non-reutilizable metabolites such as dimethylguanosine and pseudouracil (Marway *et al*. 1996), but the measurement of nucleic acid synthesis *in vivo* has been much more difficult. There are a large number of labelling choices for the study of the synthesis of RNA and DNA, since molecules which could be incorporated include phosphate, ribose, glycine, glutamine and, of course, the bases themselves (Berthold *et al*. 1995; Boza *et al*. 1996; Perez & Reeds, 1998). In addition, some of the constituents are methylated from methionine, which increases the number of possibilities of labelling. Unfortunately, the situation is markedly complicated by the existence of salvage pathways which recycle the bases into nucleic acids. Furthermore, the labelling of

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the bases with adenosine and guanosine is difficult because of the substantial pool sizes.

In another approach, Macallan and co-workers (Hellerstein *et al*. 1999) used glucose as a precursor of ribose to measure leucocyte labelling *in vivo*. Unfortunately, because of the relatively small production of ribose from glucose they had to use glucose at 20 atom % in order to achieve sufficient labelling. This approach is probably inordinately expensive as a means of routinely measuring nucleic acid turnover. Obviously, if these techniques can be made to work with mRNA it will be possible, at long last, to make molecular biology quantitative!

Summary

The techniques outlined here depend, to a large extent, on the availability of instrumentation which requires substantial capital expenditure, and on the availability of trained scientists and technicians who have particular expertise in maintaining and operating modern mass spectrometers. Although the user-friendliness of the technology is improving year-by-year, and increasing numbers of stable-isotope-labelled compounds are becoming become available, so that almost any metabolic problem could theoretically be tackled with the equipment currently on offer, for most people starting in the field there is a very substantial barrier. In my view the best way we can overcome this problem is by collaborating between groups so that individuals with particular expertise in, for example vitamin metabolism, may co-operate with others with mass spectrometry expertise in order to make kinetic measurements of vitamin turnover. To date, the nutritional and metabolic community has been very poor at this kind of collaboration, but without it, it will be much more difficult to tackle the many interesting and important questions which currently confront us.

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