The use of tritiated water in livestock research in Africa: Theory, application, methodology and potential errors of the technique

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Summary

TRITIUM, *the radioactive isotope of hydrogen has a number of applications in livestock research. Combined with the water molecule, it forms tritiated water (HTO) which can be used to estimate total body water, body composition, water turn-over, milk intake and feed intake. Since the application of HTO in livestock research in Africa is likely to become more widespread with the increasing availability of scintillation counters, a review is made of the theoretical aspects of the technique and its potential errors. A comprehensive methodology is also given. The latter is of particular importance given the lack of suitable guidelines for research workers in Africa.*

Introduction

Tritiated water has been used for animal research for many years but its application in Africa in livestock research has been limited, not least by the scarcity of the sophisticated and expensive equipment needed to measure it. Moreover, much of the data in the literature lack credence because the values are often unvalidated, and insufficient or even no information is given on counting procedure, which can be a major source of error in the method.

Since validation usually requires slaughter, total dissection and a laborious dessication procedure, it is rarely undertaken, so that detailed description of sample counting becomes essential if other workers are to be able to repeat the results. The absence of such information has led to questionable results, even to the extent that tritiated water space is often reported as total body water space, the two being regarded as synonymous. This in part is caused by a reluctance of biologists to study the radiation biology and applied physics which are the basis of the technique, and partly because such information is often to be found only in highly specialised literature.

For those who may wish to consider tritiated water as a tool for livestock research in Africa, a better understanding of the theory behind the technique may lead to greater accuracy and more convincing results. This paper presents a theoretical outline of the technique, highlights its potential errors, and describes a methodology for the use of tritiated water under field conditions.

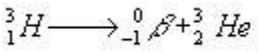
What is tritiated water?

Tritiated water is water which has been labelled with the radioactive isotope of hydrogen, tritium (^{3}H). This is distinct from water which is labelled with deuterium (^{2}H), the stable isotope of hydrogen. Tritiated water is variously known as tritium oxide, HTO, TOH or $^{3}H_2O$, while

deuterated water is known as heavy water, deuterium oxide, D_20, ^2HHO, $^2H_20$, HDO or DOH. For the purposes of this article, the abbreviation HTO will be used for tritiated water.

Tritiated water is used in animals because it is cheap and relatively safe. For ethical reasons, deuterium oxide is usually used for humans since deuterium is a stable isotope and exposure to radiation is obviated. Measurement of deuterium is achieved by mass spectrometry.

Being an unstable isotope, tritium decays with the emission of a neutron to a rare isotope of helium (He) as shown in the following equation:


(1)

The emitted *ß* particle has a maximum energy of 18.6 keV and a maximum range in air of 6 mm. The time taken for a given quantity of decaying atoms to reach half their initial activity is known as the radio-chemical half-life, and for tritium this is 12.26 to 12.43 years.

The activity of radioactive material is measured in S.I. (Système international) units of the becquerel (Bq) which replaces the curie (Ci). One Bq is equal to one disintegration per second ($d.\ sec^{-1}$) and one Ci is equal to $3.7 \times 10^{10} d.sec^{-1}$. The Bq is the reciprocal of the Ci and is equivalent to $2.703 \times 10^{-11} sec.d^{-1}$. Since the radioactive material is normally supplied with a 'carrier' of the stable isotope of the same element, the amount of isotope is expressed per unit mass. This is the `specific activity' which is measured in Bq, Ci or disintegrations per minute per mole (or per gram) of mixture.

In biological experiments, activities used are usually measured in millicuries (mCi), microcuries (µCi) and nanocuries (nCi). However, since such activities are increasingly described in units of gigabecquerels (GBq), megabecquerels (MBq) and kilobecquerels (kBq), both sets of units will be used in this paper.

HTO as a marker

Animals are made up of between 50 and 80% water, and 99% of the molecules in an organism are water molecules (Macfarlane and Howard, 1972). The administration of a known quantity of HTO will allow an estimate of body water volume (HTO space) to be made *in vivo* using the dilution principle. HTO (the tracer) is assumed to mix completely with body water (the tracee), and a sample of body fluid after equilibration of the tracer will allow body water to be estimated following the preparation of standard solutions.

Measurements of HTO

Liquid scintillation counting

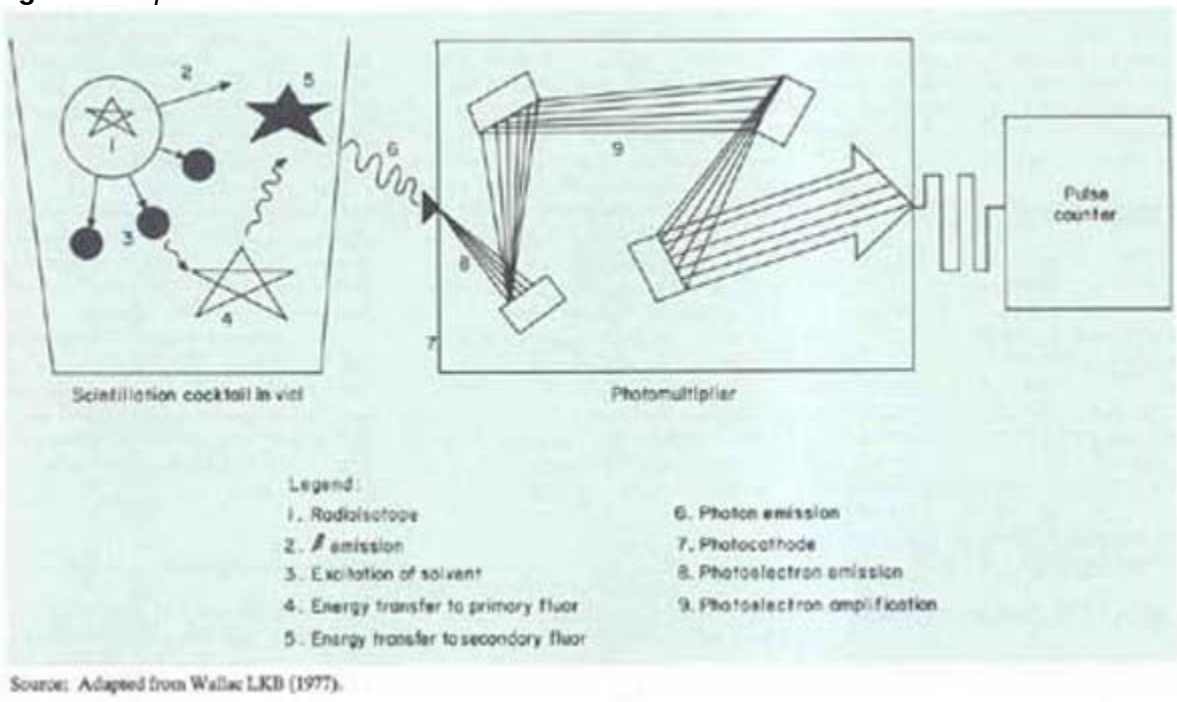
Radiation can be detected and quantified by two methods: gas ionisation and excitation. Since HTO is a soft *ß* emitter, its measurement using gas ionisation techniques such as the gas-flow counting Geiger—Muller tube is still only about 5% efficient. Instead, HTO can be measured indirectly as a result of the ability of certain compounds to fluoresce in the presence of *ß* particles. This method is known as liquid or internal scintillation counting.

The sample to be counted by liquid scintillation is dissolved in a solvent (usually toluene, xylene, anthracene or cumene) which fluoresces. The light emitted is of such a short wavelength that it is immeasurable, and this necessitates the presence of another compound, the primary fluor, which absorbs the light energy and converts it to light of a longer wavelength. A common primary fluor is PPO (2,5-diphenyloxazole). Fluors are heteroaromatic compounds with several double bonds, one part of which is formed by sigma-electrons constituting a rigid bond while the other part is formed by delocalised pi-electrons which absorb the energy from a *ß* particle and jump to a higher energetic, or excited, state when they release extra energy in the form of photons (Wallac LKB,1977).

Depending on the photochemistry of the primary fluor, a secondary fluor may be used to reconvert the light into a detectable wavelength. Some primary fluors such as Butyl-BPD (2–(4'-t-butylphenyl)–5–(4"-bi-phenyl)–1,3,4-oxydiazole) emit light at a detectable wavelength and thus do not need secondary fluors. The commonest secondary fluors are bis-MSB [p-bis-(o-methylstyryl) benzene] and POPOP [1,4-di-(2–(5-phenyloxazolyl)) benzene].

The combination of solvent, primary and secondary fluors is known as the scintillation cocktail. The light emitted is converted into an electric pulse via a photomultipper tube; the pulse is directly proportional to the energy of the initial radioactive event. The whole process is shown diagrammatically in Figure 1. The efficiency of detection of ^{3}H by liquid scintillation is frequently up to 50% of total disintegrations.

Figure 1. *Liquid scintillation and tritium detection*.



Source : Adapted from Wallac LKB (1977)

Quenching

The errors that can arise from liquid scintillation counting may be due to the limitations of the scintillation counter or to sample preparation. However, as the manufacturers of modern

scintillation counters have eliminated such electronic errors as photomultiplier noise, the major error source is likely to be in the sample preparation and the use of, or failure to use, counting standards.

Interference with the passage of light from the sample to the photomultiplier is known as quenching. Quenching reduces the number of single photon events that are recorded, and it can take three forms:

- Optical quenching, which occurs when the counting vials vary in thickness or are dirty;
- Colour quenching, which occurs when colour in the sample absorbs light prior to its leaving the vial; and
- Chemical quenching, which occurs when some chemical in the sample interferes with the various energy transfer processes in the scintillation cocktail.

Perhaps the major source of error in liquid scintillation counting off biological materials is the assumption that all samples are chemically identical and that it is sufficiently accurate to use relative counting (counts per minute, c.p.m. or $ct.min^{-1}$). However, as biological materials vary in quench characteristics between animals, between samples (milk, plasma, urine), and at different times, every sample should be converted to absolute counts (disintegrations per minute, d.p.m. or $d.min^{-1}$) unless the efficiency of counting is known to be identical in all samples. Even if pure water is extracted from the biological material, counting efficiency may vary since water itself is a quenching agent.

When a standard solution is used in body-composition or water-turnover studies, the degree of quenching in the standard must be similar to that of the sample being counted in order to avoid conversion to $d.min^{-1}$. However, background samples are frequently taken from well-fed animals while labelled samples may come from fasted animals, and this has been the cause of variations in quench in both milk and plasma owing to changes in blood and milk chemistry (Nicholson, unpublished results).

Efficiency determination

The determination of quenching is synonymous with the determination of sample counting efficiency. Reliance on $ct.min^{-1}$ assumes that the efficiency of counting is constant, which in practice it will not be since chemical and optical quenching can vary unpredictably. When biological materials are counted directly there is the added problem of colour quenching.

The three commonest methods of quenching correction are a) the internal standard method, b) the external standard ratio method, and c) the sample channel's ratio method (Kobayashi and Maudsley, 1974; Wallac LKB, 1977).

Internal standard (IS) method. With this method the sample is counted, a known and constant amount of non-quenching radioactive standard is added, and the sample is recounted. The standard will have been counted previously in the absence of quenching agents. The counting efficiency coefficient is then calculated as:

$$[(Z–X)/Y] \times 100 \quad (2)$$

where:

X = quenched sample count ($ct.min^{-1}$),

Y = unquenched standard count ($ct.min^{-1}$), and

Z = quenched sample plus standard count ($ct.min^{-1}$).

The isotope in the standard must be the same as the one in the sample and it should not itself be quenched, so that non-quenching agents such as ^{3}H-toluene, ^{3}H-n-hexadecane and ^{3}H-cholesterol should be used. Water and benzoic acid can also be used, but as they are both quenching agents very small volumes should be added. The internal standard count in the sample should be at least 10 times as high as the sample count alone (Whisman et al, 1960).

The method suffers from a number of drawbacks:

- Highly accurate pipetting of the standard is essential and minor errors are inevitable.
- The sample cannot be reused or recounted as it will be contaminated by the standard.
- Opening the vial to add the standard will result in evaporation, oxygen quenching and water absorption, all of which are likely to cause variations in the second count.
- Reorientation of the vial causes slight alterations in the geometry of the vial relative to the photomultiplier tube.
- The small change in volume (100 µl of standard is frequently added) may change the degree of quenching.
- The sample and the standard must be thermally equilibrated to prevent moisture from condensing in the vial.
- Additional time is required to count.

As a result of these disadvantages, the linked errors in the estimates of the sample and of the internal standard reinforce each other. Nevertheless, reasonable care will ensure that the method is the most precise estimate of quenching available (Rogers and Moran, 1966).

External standard ratio (ESR) method. Following initial counting the vial and sample are irradiated by an external source of gamma radiation (^{226}Ra or ^{137}Cs) which is housed in a 40-mm lead shield in the instrument. The principle behind this method is that gamma radiation can dissipate the energy of a photon via the Compton effect. The incident gamma radiation photon reacts with a planetary electron causing the ejection of a recoil, or Compton, electron which behaves like a *ß* particle. Any quenching of the sample causes proportionate quenching of the Compton electrons, which are counted by an additional pulse-height analyser set to detect very high energy particles coming exclusively from the Compton electrons. A calibration curve must be prepared and this is then used to calculate the efficiency of counting in the unknown samples.

In modern counters, the external standard technique is fully automated, with the gamma source placed adjacent to the vial while counting is continued for a further minute. The three advantages of the method are that efficiency can be calculated in any sample irrespective of its radioactivity, it is rapid and it requires no further handling of the sample. However, the disadvantages are fivefold:

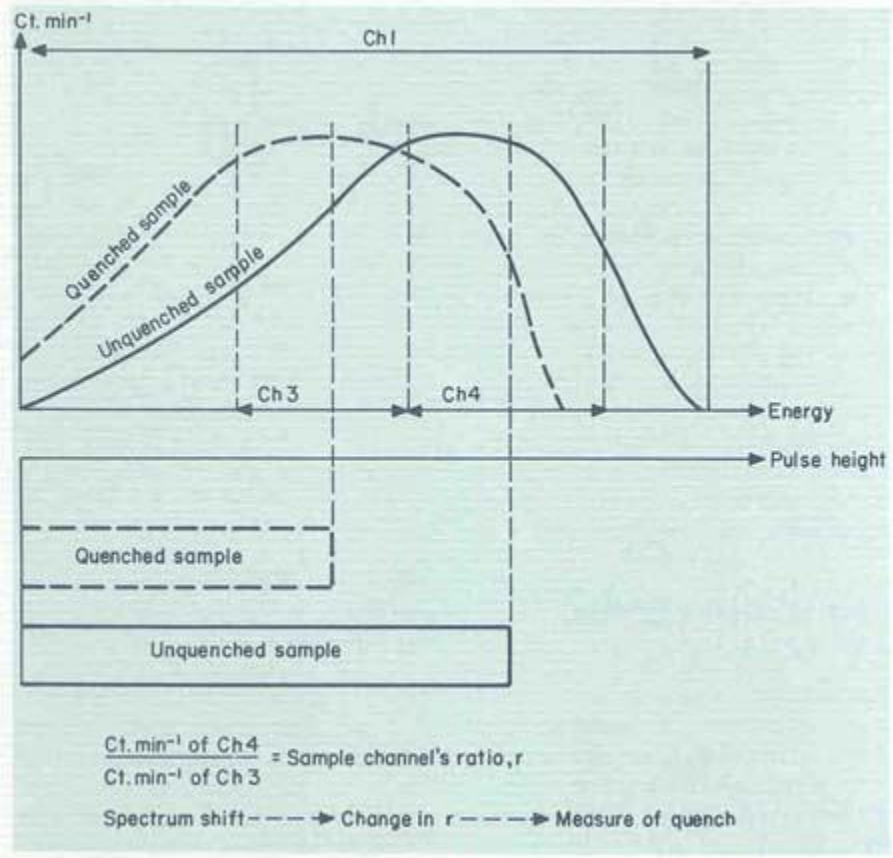
- The efficiency with which gamma radiation enters the vial can be variable.

- Irreproducibility occurs since it is impossible to ensure constant geometry between the gamma source and the vial, due both to instrument limitations and to variations in glass thickness.
- It is highly sensitive to electronic fluctuations e.g. in voltage.
- In practice, Compton electrons do not behave exactly like *ß* particles.
- The process giving rise to Compton electrons is mass dependent and hence volume dependent.

Owing to these disadvantages, the method tends to be the least effective of the three methods of quench correction (Rogers and Moran, 1966).

Sample channel's ratio (SCR) method. Quenching involves a shift in the overall spectrum of pulse heights (Figure 2). Liquid scintillation counters have different channels to count at different energy spectra, normally a narrow-window and a wide-window efficiency. The channel's ratio is the narrow-window efficiency divided by the wide-window efficiency. The ratio is constant for an unquenched isotope; it is therefore necessary to establish a quench calibration or channel's ratio curve in a series of standards of identical activity but different degrees of quenching (Figure 2). The ratio decreases as the quenching increases and the relationship is usually linear or curvilinear.

Figure 2. *Channel's ratio method: The effect of quenching on energy and pulse height spectrum.*



Source: Adapted from Wallac LKB (1977).

A major advantage of this method is that the sample to be counted does not need to be handled and can be reused as its composition is unaltered. For efficiencies down to 12%, the accuracy of the channel's ratio method is very similar to that obtained by internal standardisation (Rogers and Moran, 1966) but for highly quenched samples, its accuracy falls off sharply. The method is unsuitable where small amounts of radioactivity are present, as a prohibitively long time will be required to accumulate a statistically acceptable number of counts. In modern counters the SCR technique is frequently combined with the automatic ESR technique in a method known as the external standard channel's ratio (ESCR) method in which the ESR technique is used to check how good the scintillation cocktail is.

Biological sources of error in the use of HTO

Tritiated water is used in two different ways. First, it allows an estimate to be made of body water and, indirectly, of body composition, following injection and equilibration based on the dilution principle. Second, by following the decline in activity over time, it is possible to calculate water flux, and this can be extended to measurements of milk or feed intake.

Nagy and Costa (1980) have examined the six assumptions that were made by Lifson and McClintock (1966) as fundamental to the use of HTO. These assumptions are particularly relevant to HTO work when fluxes are being measured; if they are invalid, significant errors in calculated water fluxes are likely. Nagy and Costa's examination of the six assumptions is regarded as of sufficient importance to be discussed in detail.

Body water volume

When calculating water flux, it is convenient to assume that body water volume is constant over a given period of time. This means that the animal is neither gaining nor losing weight, nor altering its body composition. Under field conditions this is seldom the case, particularly with growing animals, and the calculations must take into account such changes.

Constant water flux rates

The equations used to determine flux rates assume that the rate of flux and hence body water volume are constant. In fact they are not, since almost all influx and efflux events (drinking, eating, defaecation) are both independent of each other and sporadic. Major changes can occur especially under intermittent watering when cattle have been recorded as drinking up to 104 litres of water in 4 minutes (Nicholson, 1985). Such changes can greatly upset flux calculations, unless sampling times are adjusted to watering times (see page 15). Errors as a result of injudicious sampling times can vary from –2 to + 14.4% of true flux rates (Nagy and Costa, 1980).

Isotope labelling

Water molecules freely dissociate and their hydrogen (H) atoms exchange with the H atoms of organic molecules, or become incorporated with newly synthesised molecules (Culebras and Moore, 1979). Incorporation of the isotope into non-aqueous compounds will cause an overestimate of body water volume if exchange occurs prior to equilibration, and a higher calculated flux rate if exchange is relatively slower. Lewis and Philips (1972) found that it took 5

days for non-aqueous and aqueous 3H to equilibrate in neonatal calves. They also reported that bound ^{3}H was sufficient to label 3.5% of body weight. The overestimate of body water volume by isotopic exchange alone can be as high as 13% since appreciable amounts of ^{3}H have been found in desiccated tissue (Nagy and Costa, 1980), although Richmond et al (1962) reported that exchange was usually no more than 2%.

Loss of ^{3}H to non-aqueous compounds is regarded as a source of significant error in growing animals and in animals with low water flux rate. When water flux alone is being calculated it is advisable to wait several days until ^{3}H exchange is complete prior to taking the first sample. However, when specific activity becomes very low in the animal's body water, as may occur at the end of a water flux trial, the process is likely to reverse and specific activities rise again as ^{3}H reenters the aqueous phase. To avoid this the specific activity of the end sample should not approach 1% of the activity of the first sample (Nagy and Costa, 1980).

A further error can be caused by the loss of HTO prior to equilibration, and although it is possible to extrapolate back to the time of injection using subsequent turnover rate, this is not necessarily valid since pre-equilibration turnover rate will be slow (due to fasting) and because the behaviour of HTO on injection is unclear. Failure to allow complete equilibration will give rise to errors (see page 15). In dehydrated camels for example, 18 hours may be required for equilibration (Seibert and Macfarlane, 1971). Nagy and Costa (1980) therefore recommend that workers report equilibration time in their studies.

Loss of non-aqueous bound tritium

The flux rate assumes that ^{3}H is lost only as HTO. It has been found that while the loss of bound ^{3}H in the dry matter of faeces and urine amounted to only 1 % in kangaroo rats (Siri and Evers, 1962), such losses could be higher (although probably still quite small) in ruminants due to comparatively low digestibilities and the fact that each unit of cellulose has three exchangeable atoms (Nagy and Costa, 1980).

Isotopic and biological fractionation

The HTO method assumes that the concentration of ^{3}H in the water lost is equal to its concentration in the body. Studying dehydration in zebu cattle, Nicholson (unpublished results) counted faecal water in a cow 6 hours after she had drunk 80 litres of water and found that specific activity in the faeces was lower than in the blood. A similar finding was reported by McClintock and Lifson (1958). The workers in both studies concluded that since much of the water drunk continues down the alimentary tract, and since rates of faecal water loss rose rapidly within a few hours of drinking, the HTO in the body water did not have sufficient time to equilibrate fully with water in the alimentary tract prior to defaecation. Although Lifson and McClintock (1966) provide an adjustment equation for this unequal loss, under field conditions it is not possible to correct for this error unless total faecal and urine collections are made as part of the experiment.

Since ^{3}H atoms are much heavier than H atoms, the rate of loss of ^{3}H atoms (or HTO) by evaporation may be less than that of H atoms from normal water molecules. In an ingenious experiment using toads, Nagy and Costa (1980) found that this isotopic fractionation yields a 407% underestimate of the rate of water efflux by evaporation and a 9% underestimate when evaporation was from a ,glass beaker, the difference being attributable to ‘effective’

fractionation. The likely error varies according to animal species and the site and process of evaporation. For example, Rubsamen et al (1979) found that HTO levels in the pulmonocutaneous vapour of rabbits were between 5 and 50% lower than in the blood, but Nagy and Costa (1980) concluded that it is difficult to keep pulmonary vapour free from unlabelled contaminated water vapour.

Cutaneous and pulmonary water influx

Another assumption in the calculation of water flux is that unlabelled water from outside is not exchanging with labelled water. However, Nagy and Costa (1980) found that influx of water via the skin and lungs overestimated actual water influx rates, due to a constant exchange of unlabelled and labelled water. Nevertheless, this appears to be of importance only in insects and very small animals, particularly burrowing animals, in humid environments. Although probably not important in ruminants, Nagy and Costa (1980) regard this as the largest unavoidable source of error (up to ± 50%) in HTO measurement of water flux.

Applications of HTO in livestock research

In livestock research HTO is used to estimate body water, body composition, water turnover, milk intake and feed intake.

Body water estimation

Although most animals have a fairly narrow range of body water space (% of body mass that is water) there are occasions when measurements of body water volume *per se* are required, as for example in dehydration studies. It is important to make it clear that using HTO gives estimates of body water space, and that HTO usually overestimates water space (Little, 1983; King and Finch 1982; King et al, 1978) and hence water flux rates.

Body composition

An extension of body water estimation is the fact that the more fat an animal carries the less its total body water space is, so that comparisons can be made based on a single injection of HTO and sampling at equilibration between individuals or groups of animals. In both body water and body composition estimation HTO space is being measured, and for reasons stated earlier this is not synonymous with total body water space, although HTO space is likely to be ± 20%, but usually ± 10%, of the true value.

In hydrated animals there is an inverse relationship between total water space and total body fat. Pace and Rathbun (1945) suggested a simplified formula for calculating body-fat content, based on the assumption that body-water content is constant at 73.2% of fat-free tissue. However, since gut water can be a substantial source of body water, this equation does not hold (Widdowson, 1968). Using the results of various workers, Little (1983) cited the empirical relationships between fat and body water, and reference should be made both to this author and to Panaretto (1963). Caution should be exercised when predicting body-fat content from body-water content, not least because HTO is not an absolute measurement of total body water as validated by dissection.

Water turnover

Different animal species or animals in different environments have different rates of water turnover. Tritiated water allows measurement of this flux through the fractional rate constant, which is described by the equation:

$$k = \frac{In(H_1^{\bullet} / H_2^{\bullet})}{t} \qquad (3)$$

where:

$H_1^{\bullet}, H_2^{\bullet}$ = initial and final specific activities of the isotope in the animal, and

t = time interval (days).

In units of ml $H_2O.kg^{-1}.day^{-1}$ this is transformed to:

ml H_20 flux.$kg^{-1}.day^{-1}$ =

$$\frac{1000 Q In(H_1^{\bullet} / H_2^{\bullet})}{W} \qquad (4)$$

where:

Q = the HTO space, and

W = the animal's weight (kg).

This equation assumes a steady-state model where Q is constant. In such a case:

$H_2^{\bullet}$ = half the activity $H_1^{\bullet}$,

In $H_2^{\bullet} / H_1^{\bullet}$ = 0.693, and

$T_{\frac{1}{2}}$ = biological half-life calculated as:

$$T_{\frac{1}{2}} = \frac{0.693}{k} \qquad (5)$$

When Q is not constant, equations 17 and 18 apply.

Milk intake

The water intake of suckling livestock will be directly proportional to the milk intake when they are neither drinking water nor eating any food apart from milk. In addition to the water turnover, a qualitative analysis of the milk is required, both in terms of total solids and proportions of lactose, fat and protein, to allow calculations of metabolic water to be made (Coward et al, 1982a). Milk intake has been successfully estimated by the water turnover method using HTO in growing calves over prolonged periods of time, and this allows simultaneous measurement of body composition (Yates et al, 1971; Macfarlane et al, 1969).

Milk intake by older livestock that are still suckling can be estimated through the dam efflux method (Coward et al, 1982b). The mother is injected and the efflux of HTO is measured by sampling dam and offspring over a period of time. A two compartment (mother and offspring) steady-state model describes water fluxes (Figure 3), and the fractional rate constants (Shipley and Clark, 1972) in dam and calf are given as:

$$\frac{H_m^*(t)}{H_m^*(o)} = e^{-k_{mm}t} \qquad (6)$$

and

$$\frac{H_c^*(t)}{H_m^*(o)} = \frac{F_{cm}(e^{-k_{mm}t} - e^{-k_{cc}t})}{Q_c(k_{cc} - k_{mm})} \qquad (7)$$

where:

H_m^*, H_c^* = specific activities in the mother's milk and the calf plasma, corrected for back ground activity,

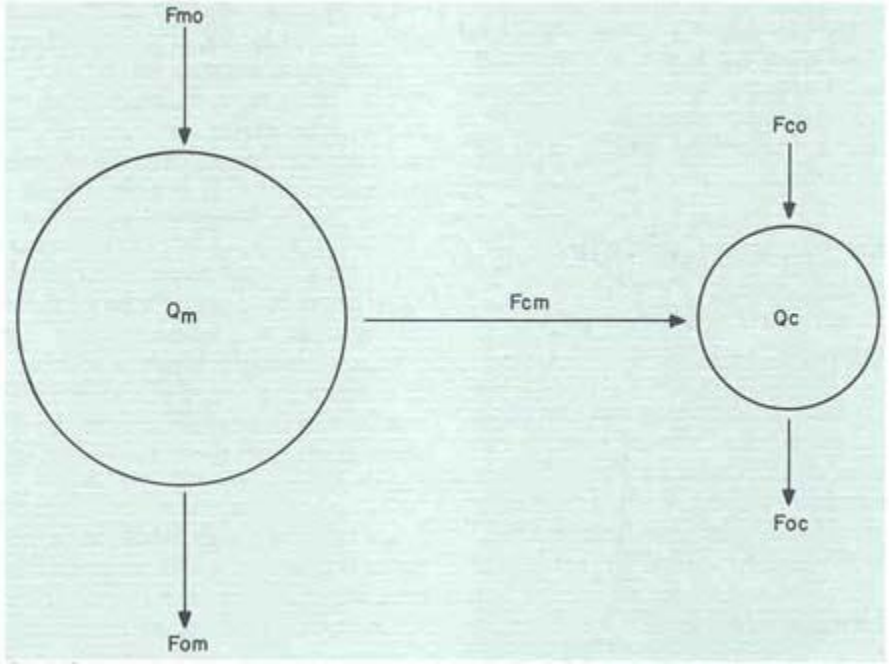
Q_C = the HTO space of the calf,

F_{cm} = water flux from the dam to the calf,

k_{mm}, k_{cc} = fractional rate constants ($k_{mm} = F_{om} + F_{cm}/Q_m$ and $K_{cc}=F_{oc}/Q_c$), and

$k_{mm}, H_m^*(o)$ = slope and intercept of the mono-exponential curve for the disappearance of ^3H from the dam's milk.

Figure 3. *Steady-state model of water fluxes in mother (m) and offspring (c).*



Legend:
Q_m,Q_c = estimated body water volume (HTO space) of dam and calf respectively;
F_{mo},F_{co},F_{om},F_{oc} = directional water fluxes with second subscript indicating where is water coming from and first referring to where it is going.
Source: Adapted from Coward et al (1982a).

Measured specific activities must be analysed by a computer program which fits numerical data directly to the compartmental model (Feldman, 1977). Since a steady-state does not exist in growing animals due to the changes in total body water and flow rates, the analysis initially requires that the estimates for unknown parameters be primed and subsequently refines them to converge on best-fit values.

Feed intake

Dry matter (DM) intake can be determined quite accurately from the water influx rate provided that the diet consists of <70% DM (Nagy, pers. comet.) and preformed water in the diet is the basis of the calculations. The calculations are extremely sensitive to small errors in DM estimates: in dry diets water influx from other sources (metabolic water, pasture or cutaneous exchange) is likely to have a major effect on estimated DM intake.

Metabolic water in non-drinking animals (where metabolic water is a constant fraction of total water flux) can be estimated from total water flux described by Nagy's (1975) equation:

$$\text{ml } H_2O_m^{-1} = \frac{ml H_2O_{m/g\,food}}{ml\ H_2O_{m/g\,food} + ml\ H_2O_{p/g\ food}} \text{ ml } H_2O_T \cdot day^{-1} \quad (8)$$

where subscripts M, P and T denote metabolic, preformed and total water.

Metabolic water per gram of food can be measured or calculated from oxidation yields of basic food components: 1 g each of protein, fat and carbohydrate yields 0.41, 1.07 and 0.55 ml water respectively on complete oxidation (Bergmann et al, 1974).

Benjamin et al (1975) used HTO dilution and standing biomass estimations to measure the intake of young barley by sheep. He found that the measurements from the two methods differed by less than 18% and concluded that the HTO method was a useful field technique. Dry matter intake (DMI) was estimated as:

$$DMI = \frac{a}{b} \times c \qquad (9)$$

where:

a = water turnover (litres.sheep^{-1}.day^{-1}),

b = water content (%) of food, and

c = DM content (%) of pasture.

The experiment could be refined to avoid errors due to dew, pasture growth and the varying DM content of the grass, but where this is not possible food intake can be satisfactorily determined by the HTO method.

Methodology of HTO use

HTO availability

Tritiated water is available in a variety of specific activities from Amersham International PLC, White Lion Road, Buckinghamshire, England HP7 9LL. A practical amount is 0.2 ml with an activity equivalent to 185 GBq.ml^{-1}(5 Ci.ml^{-1}).

Dose and toxicity

Opinion varies as to optimum dose to be used but generally, the smaller the dose the better. The most important criterion is that the sample taken at the end of the experiment should have a level of activity measurably higher than the background radiation. The author of this paper considers equilibration levels of 3.7 kBq.ml^{-1}(100 nCi.ml^{-1}) of body water a convenient rule of thumb. In some small animals a larger dose is required due to the small blood sample (Nagy and Costa, 1980), and 37 kBq.ml^{-1} (1 µCi.ml^{-1}) would be most appropriate. For body water and body composition studies, 370 Bq.g^{-1} body mass (10 nCi.g^{-1} body mass) or less are adequate in large animals since only one sample is needed. For body water turnover and milk intake studies, a high level of HTO concentration is required, especially in the dam efflux method because after 20 days the HTO in the cow's milk is further diluted in the calf's plasma.

Both HTO and HDO are toxic in high concentrations due to the ‘heaviness’ of water and radiation. The ionising radiation is a function of dose and its biological (as opposed to chemical) half-life. Radiation dose is given as:

$Gy = 1.0973 \times 10^{-4} C^*T_{1/2}$ (10)

where:

Gy = radiation dose in grays (Gy),

C* = initial specific activity in $kBq.g^{-1}$ body mass, and

$T^{1/2}$ = biological half-life.

For a 300-kg cow injected with 740 MBq (20 mCi) FITO with a biological half-life of 4 days the dose per gram would be 1.1×10^{-3}Gy. This is 0.07% of a dose needed to induce acute effects and 0.7% of the threshold for chronic effects (impaired fertility, cancer etc) (Pizzarello and Witcofski, 1967). Seven-hundred-and-forty MBq of HTO injected into a 400-kg cow is about 0.05% of the maximum suggested non-injurious dose of 3.7 $MBq.g^{-1}$ body mass (Feinendegen,1967).

To avoid errors due to isotope decay, all the animals in a trial should be injected with the same batch of HTO. Similarly, all samples must be counted together. In practice, owing; to the relatively long half-life of ^3H, several weeks must elapse before lower counts due: to decay significantly affect the result. If necessary, decay correction can be calculated for samples not analysed simultaneously using the equation:

$$\text{In } H_{2c}^{\bullet} = In(H_1^{\bullet} + k_d t_a)\, In(H_1^{\bullet} / H_2^{\bullet}) \quad (11)$$

where:

$H_{2c}^{\bullet}$ = the correct $H_1^{\bullet}$,

k_d = the rate of tritium decay (1.53×10^{-4} disintegrations. day^{-1}), and

t_a = the time between the analyses of $H_1^{\bullet}$ and $H_2^{\bullet}$ (not the time between taking the samples).

Alternatively, a standard can be counted on each occasion a sample is analysed.

Stock solution

In a fume cupboard, the contents of a 0.2 ml vial with a concentration of 185 $GBq.ml^{-1}$ (5 $Ci.ml^{-1}$) are made up to 200 ml with physiological saline solution (0.09% NaCl) to give an activity of 185 $MBq.ml^{-1}$ (5 mCi. ml^{-1}). For small animals, a stock solution can be made up to 1 litre, giving an activity of 37 $MBq.ml^{-1}$(1 $mCi.ml^{-1}$). To store the solution, add a drop of toluene and keep in coloured glass bottles at 4°C or in smaller plastic bottles in the refrigerator. Label each bottle with date of manufacture so that decay can be calculated. Make up enough of both stock and standard solutions for all trials in a study, because small differences in dilution between standards could be a further source of error. An aliquot of stock solution is injected into the animal and the sample taken after equilibration is compared with the standard.

Standard solution

To prepare standard solution the stock solution should be diluted by a similar order of magnitude as it will be diluted when injected into the animal.

Example: A cow of 286 kg will be approximately 70% water. So if 5 ml of stock HTO (185 $MBq.ml^{-1}$) is injected into that animal, the dilution will be:

DILUTION

5 ml into 286 x 0.70 x 1000 ml = 1:40 040

The preparation of the standard is as follows:

a) 0.2 ml stock HTO (185 $MBq.ml^{-1}$) is made up to 0.5 litre with physiological saline (0.09 NaCl) into a standard solution of 74 kBq. ml^{-1}. 1:2500

An ultra-micropipette previously calibrated by weighing or by a known weight of stock should be used to weigh the components to the nearest 0.1 mg in order to obtain maximum precision in the preparation of the standard.

DILUTION

b) 0.2 ml standard solution (74 $kBq.ml^{-1}$) + 3.0 ml background plasma or fat-free milk. 1:40 000

The dilution factors must be altered according to circumstances. For example, for a 40-kg goat injected with 1 ml of stock HTO with a concentration of 37 $MBq.ml^{-1}$, the dilution will be:

1 ml into 40 x 0.7 x 1000 ml = 1:28 000

The preparation of this standard will be:

c) 0.2 ml stock HTO (37 MBq. ml^{-1}) made up to 1 litre gives a standard of 7.4 $kBq.ml^{-1}$ 1:5000

d) 0.2 ml standard solution (7.4 $kBq.ml^{-1}$) + 0.8 ml background plasma 1:25 000

The concentration of the standard should be ± 20% of the expected dilution in the animal.

Injection of the animal with HTO

Unless the experiment is designed to investigate aspects of dehydration, the animals should normally be hydrated, although it is usual to withhold food and water for 8 to 12 hours before injection in order to avoid losses of HTO in the urine and faeces prior to equilibration and to prevent labelling of water which is not strictly body water in the food. Preferably, the animals should be injected at 1800 h so that equilibration can take place overnight without the risk of excessive water (and hence HTO) loss through evaporation.

Before injecting the animal, a background blood sample should be taken using an evacuated tube to which an anticoagulant has been added (lithium or sodium heparin are suitable). The injection syringe with needle and its cover should be weighed to the nearest 0.1 mg before and as soon as feasible after injection to prevent evaporation of residual HTO. When administered intramuscularly care should be taken to avoid blood vessels so as to prevent bleeding with possible loss of HTO on withdrawal of the needle as well as contamination of the needle with blood. The difference between the full and empty syringe weights is the volume injected.

Route of administration and equilibration time

In theory, HTO injected into the body of an animal will equilibrate after a certain number of hours. The author prefers the intramuscular route of injection for two reasons. First, HTO injected intravenously causes an immediate rise in specific activity in the blood, and second, since samples are drawn from the bloodstream, an `equilibration' sample taken prematurely could have abnormally high levels of specific activity. The opposite, of course, could be said of the intramuscular route. However, intravenous administration is more likely to cause losses of specific activity in the urine as some diuresis will be occurring in the early stages of water deprivation. Since urine and faeces are likely to be voided prior to taking the equilibration sample, the smaller the loss of unequilibrated HTO via these routes the better.

Equilibration is a function of body weight and may vary in time from 1 hour in lambs to between 10 and 18 hours in camels. Once equilibration has been reached, and provided no food or water is offered, HTO levels will remain constant as it is assumed that ^{3}H leaves the body at the same rate as unlabelled water. Although this is a questionable assumption, for practical purposes 12 to 14 hours post-injection is a satisfactory interval for taking an equilibration sample from cattle. The animal should be weighed at equilibration and can then resume normal eating and drinking.

If there is any doubt about equilibration time, it is better to take two samples an hour or two apart. The sample with the highest activity will then be the equilibration count.

End sample

At the end of the trial, a blood sample is taken in order to calculate water turnover, and the animal is reweighed. For cattle, 10 to 20 days after the trial is a suitable period for calculating; water turnover. An animal with a slower water turnover would still have a countable activity up to 30 days after the trial, so there is no reason why the period should not be flexible to suit the convenience of the operator.

When working with infrequently watered stock, care should be taken to adjust the timing of the end sample to watering time. For example, measuring water turn-over in 3-day watered cattle involves injecting them on the night before they are normally watered and taking an equilibration sample an hour or so before watering. This gives the HTO space of a dehydrated animal. Water consumption is recorded and the hydrated HTO space (expressed in %) is calculated using the equation:

$$\text{Hydrated HTO space} = \frac{\text{HTO space} + I}{W_2} \times 100 \qquad (12)$$

where:

HTO space = tritiated water space (litres) of a dehydrated animal,

I = water consumption (litres), and

W_2 = rehydrated weight (kg).

The end sample is taken 9, 12, 15 or 18 days later prior to watering. If the animal is watered first, not only will its water turnover rate be affected, but HTO will be unequilibrated unless several hours are allowed for the equilibration of residual activity with ruminal water.

However, since HTO space overestimates total body water (TBW) space in large ruminants, relying on a validated regression equation may be more useful in determining true TBW change between dehydration and rehydration. In validation studies in cattle, Little (1983) found the relationship between HTO space (litres) and TBW space (litres) to be:

TBW = 0.78 HTO + 7.96 (13)

($r = 0.98$, $SE_b = 0.058$)

Having converted HTO space to TBW space for a dehydrated animal, we can now calculate the TBW space of a rehydrated animal as follows:

$$TBW_r = \frac{TBW_d + I}{W_2} \quad (14)$$

where:

TBW_r = rehydrated TBW space (litres),

TBW_d = dehydrated TBW space (litres), and

I = water consumption (litres).

Thus if a 300-kg cow with a TBW_d of 198 litres (66% of fasted liveweight) drinks 67 litres of water, her TBW_r becomes 265 litres, which is 72% of fasted liveweight.

Since body composition or body water volume may change over the trial period, HTO space can be recalculated at the end of the trial using another dose of HTO followed by sampling. This dose can be very small since only an equilibration sample will be required.

Sample preparation

Blood should be centrifuged immediately after sampling and plasma transferred either into sealed bottles or directly into counting vials in order to avoid spontaneous haemolysis and further colour quenching.

Plasma should be refrigerated or frozen. Freezing can cause precipitation of some proteins, so an accurate determination of the plasma solid concentration of the supernatant is necessary. In any event, plasma solids must be determined gravimetrically at 60°C.

The commonest methods available for sample preparation and subsequent counting are vacuum distillation, acid precipitation, ethanol precipitation and direct sample counting.

Vacuum distillation

In the early days of HTO work, vacuum distillation was the customary method of HTO counting (Till and Downes, 1962). It involves distillation of water from the plasma using Thunberg tubes, dry ice, ethanol and a vacuum apparatus. If the equipment is available, vacuum distillation has the great advantage of separating water from protein avoiding both dilution or quenching agents. The method is time-consuming but accurate even though it can cause isotopic fractionation (Riley and Brooks, 1964). Although vacuum distillation has largely been replaced by other methods (Springell, 1969), it is still occasionally and successfully used (Little, 1983).

Protein precipitation by acid

Using 10 to 20% TCA (trichloroacetic acid) to coagulate plasma proteins, and centrifugation to separate the coagulated proteins from the plasma, this method gives a clear supernatant for counting. One of its disadvantages is that although colour quenching is reduced, chemical quenching may be increased by the acid. In addition to the error inherent in any dilution technique, the rapid coagulation of the proteins can cause; plasma trapping, which means that some; activity fails to reach the acid-water supernatant. This will give rise to a lower count and an overestimate of body water. However, accurate pipetting and crushing of the coagulated protein pellets to release trapped HTO will overcome the problem. Nicholson (unpublished results) used 2 ml of sample to 1 ml of TCA and found that when the coagulant was crushed, the method proved to be the most suitable for preparing a sample for counting.

Protein precipitation by ethanol or acetone

Plasma proteins can also be precipitated by ethanol or acetone. Plasma is mixed with 96% ethanol in Nunc tubes and spun at 10 000 rpm at $^{-}$20°C. The method suffers from the same disadvantages as the acid precipitation technique, but its precipitate is easier to resuspend in order to count residual activity. Acetone is not recommended as it is a strong quenching agent. Both the ethanol and acetone methods and the acid technique indicated some residual activity in the precipitate after washing and resuspending (Nicholson, unpublished results).

Direct sample counting

Water-based biological material can be counted directly if solubilising agents, detergents or more sophisticated solvents are added to the sample. Provided that a suitable quench correction procedure is adhered to, this method overcomes the problem of dilution errors. However, a protein precipitate occurs in the counting vial which, if shaken or disturbed, greatly increases quenching.

Counting

Before counting begins, 4.5 ml of scintillation cocktail is added to 0.5 ml of sample (not the other way round). This sample-to-cocktail ratio (or 'counting geometry') is critical, and advice should be sought from the manufacturers of the scintillation counter on the correct ratio. If the quantity of radioactive sample is very small, absorption of the sample onto the glass walls of the counting vial can produce a change from 4 to 2 pi geometry and this may result in a highly significant change in counting efficiency (Davidson and Oliveiro, 1965). Duplicates are necessary and new pipette tips must be used for each pair of samples.

For plasma pipetting, the 'reverse' technique of micropipetting is recommended since this reduces volume errors due to plasma viscosity. The technique involves complete expulsion of the air to the second stop, pipetting the sample and expelling to the first stop.

Having mixed the sample with the cocktail, the mixture will be scintillating. At this stage, photons may be produced as a result of the chemical reaction releasing extra energy in the form of single photon events (Wallac LKB, 1977). This chemiluminescence can be avoided by one of three options:

- Cooling the vials to 4°C for a period of 1 hour to several days until cloudiness has disappeared;
- Adding 0.5 to 1.0 ml of 0.5 M HCI to acidify the sample;
- Heating the vials to 35°C for 2 to 3 hours to speed up the chemical reaction, cooling and subsequent counting.

Quench correction

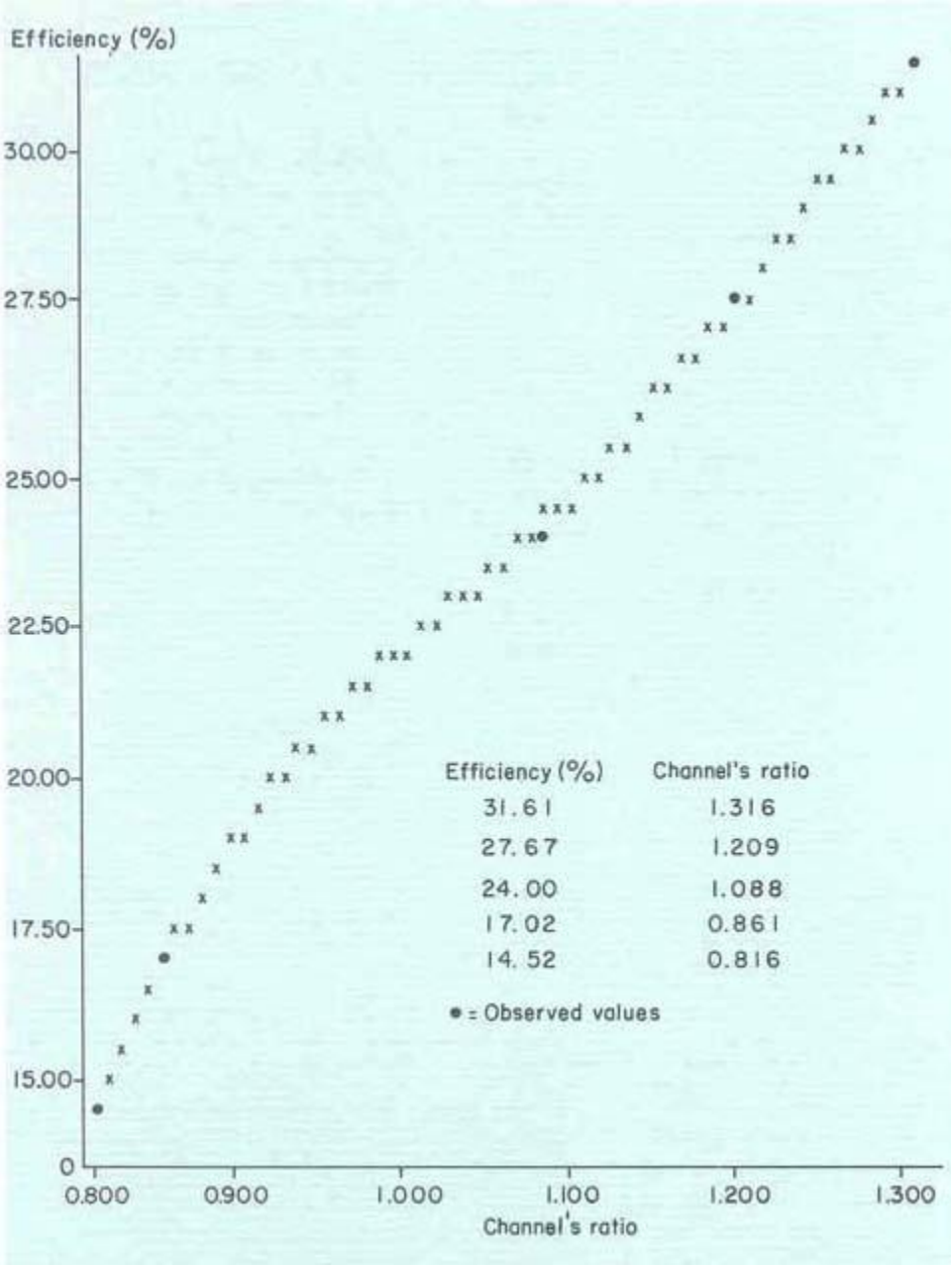
Use either the internal standard or the external standard channel's ratio method to correct for quenching. For the internal standard method, count 100 ml of high-activity HTO or ^{3}H toluene in sextuplicate. The main source of error will be the volume added to the cocktail. The mean count of the six vials should be used for efficiency determination (equation 2).

For the ESCR method it is necessary to obtain the background count (ct.min^{-1}) from six replicates of the material to be counted. The procedure is then as follows:

- Add one ^{3}H capsule of known activity (about 2 x l0^{5}d.min $^{-1}$) to 100 1 of unlabelled plasma and 4.7 ml of scintillation cocktail. This is the *standard* sample.
- Program the scintillation counter computer as per manufacturer's instructions with counting time, mode, capsule activity, decay correction coefficient, operating channels, mean background count etc.
- Count the standard sample, pipette 5 µl of CCl_4 into it and recount.
- Add 10, 20 and 30 µl of CCl_4 to the standard sample and recount after each addition. There are now five standard points and the computer will automatically calculate the quench curve and print it out (Figure 4).
- Start counting the samples. The computer will automatically calculate d.min^{-1}, which must be used in all calculations, and the efficiency ratio.

The standards are made by adding standard solution to a known volume of unlabelled material. Since unlabelled material will contain a significant amount of background radiation, this must be measured and deducted from the gross activity of the labelled material.

Figure 4. *Quench calibration curve for acidified fat-free milk in pseudocumene prepared using manual serial addition of CC14; observed values joined using a spline function*.



Source: Nicholson (unpublished results)

Body water volume (Q, in litres) is estimated from the following equation:

$$Q = \frac{H_S^{\bullet} \times \text{Vol injected} \times \text{Dilution factor}}{H_1^{\bullet x} 1000} \qquad (15)$$

where:

$H_s^{\bullet}$ = net count of the standard (d.min^{-1}), and

$H_1^{\bullet}$ = net count of the equilibration sample (d.min^{-1}).

The net count of the standard must be equated with the net count of the equilibration sample by adjusting for water content. Thus, if plasma is 92% H_20, the standard will contain (0.9 ml x 0.92) + 0.1 ml standard solution = 93% H_2O, and the standard count must be adjusted down by multiplying by a coefficient of 0.99 (0.92/0.93). The volume of the standard can be different to the sample provided further adjustment is made for the differences in actual water volume. The HTO space turnover (litres.day^{-1}) is then calculated as follows:

HTO space turnover = HTO space (%) x k (16)

where:

$$\text{HTO space (\%)} = \frac{\text{HTO space (l)} \times 100}{\text{W (kg)}} \qquad \text{and}$$

k = rate constant.

It is important to realise that HTO space is an estimate of body water volume and that if necessary, validation needs to be carried out.

Growing animals

Under steady-state conditions, turnover is equal to water influx which is equal to water efflux. For animals which are growing, or gaining or losing weight, Ttn is not the time required to turn over half the body water since body-water volume (Q) is not constant. Coward et al (1982a) showed that water influx in growing animals could be calculated from the equation:

$$\int_{t_1}^{t_2} F_{in}(t)\,dt = \frac{Q_1 - Q_2}{In\frac{Q_1}{Q_2}} \cdot In\frac{H_1^{\bullet}}{H_2^{\bullet}} \qquad (17)$$

where:

$Q_{1,2}$ = initial and final HTO spaces (litres), and

$H^{*}_{1,2}$ = initial and final specific activities.

Nagy and Costa (1980) transformed Lifson and McClintock's (1966) equation for water influx by using body water (Q) changing linearly with time:

$$F = \frac{2000\,(Q_2 - Q_1)\,In\,(H_1^{*}Q_1 / H_2^{*}Q_2)}{(W_1 + W_2)\,In(Q_2 / Q_1)t} + \frac{2000\,(Q_2 - Q_1)}{t(W_1 + W_2)} \qquad (18$$

where: F = water influx (ml H_2O influx. $Kg^{-1}.day^{-1}$),

Q = body-water volume (litres),

$W_{1,2}$ = animal weights (kg) at two points in time, and

$\frac{2000\,(Q_2 - Q_1)}{t(W_1 + W_2)}$ = water stored.

In practice, equations 17 and 18 give similar estimates of water influx.

Using equation 18 and the equations by Green (1972) and Nagy (1972), Nagy and Costa (1980) calculated the likely error involved in estimating water efflux. They found that the larger the change in body-water volume, the larger the error in efflux-rate estimation, particularly when body-water volume fell.

Worked example

Equations 15 and 17 can be used to calculate the milk intake by a growing calf, provided that the ratio of the variable input [F(1)] and output [F(t)] rates is constant. The following data are given:

W_1 = 66.75 kg

W_2 = 74 kg

H_1^{*} = 4750 $d.min^{-1}$(adjusted)

H_2^{*} = 1938 $d.min^{-1}$(adjusted)

H_s^{*} = 5521 $d.min^{-1}$(adjusted)

Composition of milk: 14.54% solids (including 5.4% fat, 3.3% protein and 4.9% lactose) and 85.46% water.

Using these data:

$$Q_1 = \frac{5521 \text{x } 0.93 \text{x } 1 \text{x } 50000}{4750 \text{x } 1000} = 54.05 \text{ litres}$$

where:

0.93 = correction factor for water content of standard,

1 = 1 ml HTO injected, and

50 000 = dilution factor.

At the end of the trial, body-water volume (Q_2) was found to be 62.9 litres. Thus the total water intake [$F_{in}(t)$] was:

$$\int_{t_2}^{t_1} F_{in}(t)\,dt = \frac{54.05\text{-}62.9}{In\frac{54.05}{62.9}} \cdot In\frac{4750}{1938} = 50.98 \text{ litres}$$

Complete oxidation of the milk will yield 9.83 g H_2O/100 ml milk, so that total water yield will be 85.46 + 9.83 = 95.29 g and the total milk intake by the calf would be 50.98 x 0.953 = 48.59 litres if the calf did not have any other source of water input. Validation done by weighing the calf before and after suckling over a 10-day period showed its milk intake to be 50.5 kg.

Statistical guidelines

Beta emission is a random process, such that an identical sample will not give exactly the same count over repeated counting periods. Similarly, replicates will not give identical counts so that, for samples of low activity in particular, counting time should be extended or a greater number of replicates used in order to get a better estimate of the true mean

Radiotracer experiments use the relative standard deviation (relative σ which is defined as $100\sqrt{x}$ where $\bar{x}$ is the mean count) to estimate the true mean. They are designed to have a relative σ of 1%, which means that it is necessary to have 10 000 counts per sample. Since decay follows a Poisson distribution, one can then state that there is a 68.3% probability that the base count is 10 000 ± 100. It follows that to obtain optimum accuracy it is better to count for a preset number of counts.

Conclusions

This comprehensive review of the use of HTO in animal experiments has been designed for workers in Africa, who have difficulty accessing the literature, especially some of the more specialised but obscure journals on radioisotopes. The HTO method has sometimes been abused because of a failure to grasp, or be aware of, the potential errors in the technique.

These errors can be reduced by accurate experimentation, but only to a point where unavoidable biological and biochemical phenomena interfere with the distribution and exchange of tritium atoms. Nevertheless, the scope for its use in Africa is large over a wide range of applications, and it is hoped that this article will serve as a solid base, both theoretically and practically, from which to start.

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