

## PROCEEDINGS OF THE NUTRITION SOCIETY

*The Two Hundred and Sixtieth Scientific Meeting (One Hundred and Third Scottish Meeting) was held at the Animal Breeding Research Organization, King's Buildings, West Mains Road, Edinburgh, on 12 October 1973.*

### SYMPOSIUM ON 'TRACER TECHNIQUES IN NUTRITION'

#### Isotope techniques for studying the dynamics of nitrogen metabolism in ruminants

By J. V. NOLAN and R. A. LENG, *Department of Biochemistry and Nutrition, University of New England, Armidale, New South Wales 2351, Australia*

##### *Introduction*

Isotope tracer methods offer the only means of making quantitative estimates of nitrogen metabolism in the body of normal, feeding animals. Since the radioactive N nuclides have short half-lives (i.e. less than 11 min), the stable isotope  $^{15}\text{N}$  is probably the only one suitable for long-term studies of N metabolism. The absence of a suitable radioactive isotope and the unique technical problems associated with measurement of  $^{15}\text{N}$  content in biological materials are probably important reasons why relatively few metabolic studies using  $^{15}\text{N}$  have been made since the early experiments of Schoenheimer & Rittenberg (1939).

Further difficulties occur in interpretation of  $^{15}\text{N}$  isotope kinetics. A common approach to the problem of analysing kinetic data has been to use compartmental analysis as a means of obtaining explicit solutions for relatively simple, assumed models (see, for example, Baker, Shipley, Clark & Incefy, 1959). However, models that describe the multiplicity of N exchanges occurring in the animal are such that it is not practicable to obtain sufficient information in a single experiment to allow explicit solutions of such a complex system. Isotope studies of N metabolism in the whole animal are therefore likely to require another approach.

In this paper, the experience gained in our laboratory using  $^{15}\text{N}$  to study the dynamics of N movement in the body of feeding sheep is discussed together with consideration of the assumptions that are necessarily made in interpretation of kinetic data, and of potential errors in  $^{15}\text{N}$  analysis.

##### *A. Theoretical considerations*

*Models of N metabolism for the whole animal.* The general approach in our laboratory has been to study normal, feeding animals. The experimental design is influenced by the concept of an abstract, whole-animal, steady-state model (see Nolan & Leng, 1972). The model represents a hypothesis which, as such, is always subject to change and refinement. Therefore to represent the animal system in

terms of an invariable model is clearly unsatisfactory. It seems important, therefore, that the methods used for analysis of isotope dilution results should not require presuppositions about the nature of the model. The analysis must be valid for a system with an unknown number of pools in which there may also be lag components. In practice, it is impracticable to sample all accessible pools in one experiment and so we have adopted a policy of making several experiments on the same, or similar animals, under defined conditions.

Quantitative information about the system has been obtained largely by using isotope dilution techniques with [ $^{14}\text{C}$ ]- and [ $^{15}\text{N}$ ]urea and [ $^{15}\text{N}$ ]ammonium sulphate.

In our laboratory, injections have been made into three sites in sheep, i.e. into the ruminal fluid ammonia pool, caecal fluid ammonia pool and blood urea pool. These pools, into which isotopes are injected, have been termed primary pools. Pools in the system which are linked directly or indirectly to the primary pool are termed secondary pools. Thus when an isotope is injected into the blood urea pool, the ruminal fluid ammonia pool becomes a secondary pool.

*Single injection and continuous infusion methods.* Most of our metabolic studies made with  $^{15}\text{N}$  have used single injection rather than continuous infusion methods. Theoretically, provided that the early part of the enrichment-with-time curve is accurately defined, the same information can be obtained from either method. In practice, pool size, total flux rate and recycling rate (see below) tend to be estimated with less precision from data from continuous infusion results, since the equation to the curve is less accurately estimated. This is because errors associated with  $^{15}\text{N}$  analysis of samples of low enrichment, such as occur in the early stages of continuous infusion experiments, are greater than those occurring in analysis of samples taken during the same period in single injection experiments made under comparable conditions.

In studies using continuous infusions, estimation of irreversible loss rate of tracee from the sampled compartment requires estimates of the rate of infusion of tracer and the plateau enrichment (i.e. at time infinity). Thus, experimentally, estimates of irreversible loss rate of tracee can be made from plateau enrichment obtained from a single estimate of enrichment of N in the sampled pool after a sufficiently long period of infusion. This simplifies the experimental procedure, but the difficulty in practice is not knowing how long is 'sufficiently long' unless the model is already known.

*Analysis of isotope ratio-with-time curves in primary compartments.* Assuming the animal is in steady state, which means that the pool sizes remain constant and the rate of inflow of material is equal to the rate of outflow, the change in isotope ratio (E) in a primary pool with time after a single injection of isotope tracer is given by a multi-exponential curve of the form:

$$E = \sum_{i=1}^n A_i e^{-m_i t} \quad (1)$$

where  $t$ =time,  $A$ =zero-time intercept of each exponential component,  $m$ =rate

constant of each component,  $n$ =number of components and  $i$ =component number.

Such multi-exponential curves are usually fitted by conventional curve 'peeling' processes or by minimization of variance, often by using a digital computer. From this equation, the following can be calculated:

1. Size ( $Q$ ) of the primary compartment (pool);

$$Q = \frac{P}{\sum_{i=1}^n A_i} \quad (2)$$

where  $P$ =number of  $^{15}\text{N}$  atoms injected.

2. Total flux rate ( $F$ ), which is the rate (mass  $\div$  unit time) at which all tracer enters and leaves a primary compartment which is in steady state. It is given by:

$$F = Q \left\{ \sum_{i=1}^n A'_i m_i \right\} \quad (3)$$

where  $A'_i$  are fractional zero-time intercepts, e.g.

$$A'_2 = \frac{A_2}{\sum_{i=1}^n A_i}$$

and therefore,

$$\sum_{i=1}^n A'_i = 1.$$

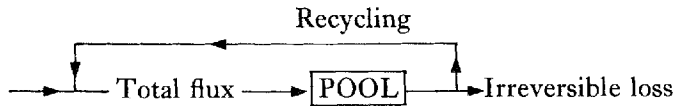
3. Irreversible loss rate ( $L$ ), which is that fraction of the total flux rate (mass  $\div$  unit time) through the primary compartment that leaves but does not return during the experimental period. It is given by:

$$L = \frac{P}{\sum_{i=1}^n \frac{A_i}{m_i}} \quad (4)$$

which is:  $\frac{P}{\text{area under the curve}}$ .

4. Recycling rate is defined as that part of the total flux rate (mass  $\div$  unit time) which leaves the compartment and returns to it during the experimental period. It is given by the difference between the total flux and irreversible loss rates.

The three parameters can be described diagrammatically:



*Application of results to the primary pool.* An important feature of equations 1, 2, 3 and 4 is that they can be applied to a primary compartment in any model, regardless of the number of other interconnected pools and their configurations and routes of inflow and outflow (see Shipley & Clark, 1972).

Equation 4 may be particularly valuable in tracer studies, since instantaneous administration of tracer is not a necessary assumption for analysis of isotope dilution curves provided the area under the curve can be accurately estimated. A corollary of this theory is that steady-state conditions are not a necessary assumption for estimation of the mean irreversible loss rate over the experimental period, provided the curve can be adequately defined and the area calculated.

Mathematically, the area under the curve is calculated by integrating the equation to the isotope ratio-with-time curve to time infinity. In practice, little error is introduced by having a relatively early cut-off time. Thus planimetry or numerical quadrature may be used to estimate area and these methods are applicable even when the complex nature of these curves precludes the use of the usual multi-exponential curve fitting methods. Procedures using the digital computer, where applicable, may have the theoretical advantage of reducing possible operator bias. However, in our hands computer methods have given no particular advantages and in practice, absence of computer facilities should not be a handicap to making these estimates.

*Errors due to non-instantaneous mixing of isotope in primary pools.* A basic assumption for any analysis of isotope dilution results is that the pool sampled can be validly regarded as a kinetic entity (i.e. a 'compartment') and hence that tracer introduced is rapidly mixed through the pool. This assumption is largely valid when the pool sampled is in blood, but presents difficulties in tracer studies of the rumen or caecum, where the assumption of instantaneous mixing is always, to a greater or lesser degree, a source of error. It is important to have some indication of the extent to which incomplete mixing causes errors.

Our approach has been to inject a non-absorbable water marker ( $^{51}\text{Cr}$  EDTA; Downes & McDonald, 1964) in solutions containing [ $^{15}\text{N}$ ]ammonia when injections are made into the rumen or caecum. This has several purposes:

(a) if samples are taken frequently after administration of isotopes, the kinetics of the injection solution in the organ, as indicated by  $^{51}\text{Cr}$  EDTA, are defined (and the rate of dispersal of the injection solution is also indicated);

(b) for any experiment, the kinetics of the soluble, non-absorbable marker will indicate whether first-order kinetics for water turnover occurred throughout the experimental period and hence whether the isotope dilution curves are influenced by changes in water kinetics;

(c) if mixing of the tracer is relatively slow, so that the early part of the decay

curve is not a true indication of tracee kinetics in the organ, a correction can be made to the tracer decay curve to force it through a zero-time intercept compatible with the fluid volume (which is estimated from the volume of distribution of  $^{51}\text{Cr}$  EDTA); it is assumed that  $^{15}\text{N}$  and tracee N are distributed in the same volume of  $^{51}\text{Cr}$  EDTA; equations 1, 2, 3 and 4 can then be applied to the corrected curve;

(d) estimates of the time of transit of digesta through segments of the gut can be made from the time of appearance of  $^{51}\text{Cr}$  EDTA; these are useful in defining the lag components in the model and explaining the complex nature of the curves describing isotope appearance in some secondary pools.

*Effects of quantity of  $^{15}\text{N}$  in an injection.* The injection of tracer ideally should not perturb the system. The amount of  $^{15}\text{N}$  injected should be as small as possible consistent with there being sufficient isotope in the system to permit the terminal part of the isotope ratio-with-time curves to be satisfactorily defined. In practice, when injections of  $^{15}\text{N}$  are made into relatively small organs such as the caecum, it may be difficult to avoid perturbation of the primary pool if sufficient N is injected to adequately label the secondary pools of interest.

*Application of results to the model of the whole system.* The irreversible loss rate is applicable to the system (i.e. the whole organism) only when all defined material passes through the sampled compartment. For example, urea synthesis rate in the body is given by the irreversible loss rate estimated by injecting isotope into, and taking samples from, the blood urea pool, because all the urea synthesized in the body passes through that pool before disposal. On the other hand, there is an assumption to be made if ammonia synthesis rates in the rumen or caecum are to be estimated by analysing samples of ammonia from ruminal fluid or caecal fluid. The necessary qualification is that all ammonia produced in these organs entered and mixed throughout the fluid pool from which the ammonia was isolated; it is conceivable that ammonia may be produced and utilized by micro-organisms without entering the fluid pool, with the result that total production of ammonia in the organ would be underestimated.

*The use of two tracers simultaneously.* The quantity of urea synthesized in the body, degraded to ammonia in the digestive tract and subsequently resynthesized into urea in the body can be estimated from the difference between the rates of irreversible loss of urea-C and urea-N from plasma, as estimated by using simultaneous injections of [ $^{14}\text{C}$ ]- and [ $^{15}\text{N}$ ]urea. A necessary assumption is that the quantity of  $^{14}\text{C}$  recycled to [ $^{14}\text{C}$ ]urea following urea degradation is negligible, and thus the irreversible loss of urea-C as estimated with  $^{14}\text{C}$  is also an estimate of total flux of urea out of the body urea pool, and the difference between estimates made with  $^{14}\text{C}$  and  $^{15}\text{N}$  represents the recycling rate of urea-N. The precision of the estimate depends on the accuracy of analytical procedures necessary for estimation of results of both tracers.

An alternative means of making the same estimate is offered by a novel technique which depends on a finding (Walser, George & Bodenlos, 1954) that when urea is oxidized with alkaline hypobromite solution, each diatomic molecule of  $\text{N}_2$  evolved is derived from a single molecule of urea. In [ $^{15}\text{N}$ ]urea of high enrichment (99

atoms % excess) most of the urea molecules are doubly labelled with  $^{15}\text{N}$  (i.e.  $^{15}\text{NH}_2\text{-CO-}^{15}\text{NH}_2$ ). In the body, during resynthesis of urea from ammonia that arises from degradation of urea in the digestive tract, mostly singly labelled urea molecules are formed (i.e.  $^{14}\text{N},^{15}\text{N}$ ), at a rate which is dependant on the extent of urea-N recycling in the body.

Since there is negligible recycling of  $[^{15}\text{N},^{15}\text{N}]$ urea, in this instance equation 4 gives an estimate of total flux rate. The difference between total flux rate, as estimated from turnover of  $[^{15}\text{N},^{15}\text{N}]$ urea, and irreversible loss rate, as estimated from the turnover of  $[^{15}\text{N}]$ urea (which includes all  $^{15}\text{N}$  in the three species of urea molecule), gives an estimate of recycling of urea-N.

*Analysis of isotope ratio-with-time curves in secondary pools.* Information can be obtained from the tracer build-up or dilution curve in any secondary pool, irrespective of model.

It can be shown that the ratio of the area under the secondary curve (*b*) to that under the primary curve (*a*) gives the proportion of tracer (and tracee) irreversibly disposed of from the secondary pool that was derived from the primary pool. Thus (*F*), the rate of flow of N to pool *b* from pool *a*, is given by:

$$F = \frac{\text{area } b}{\text{area } a} \times \text{irreversible loss from pool } b.$$

The rate of irreversible loss of substrate from pool *b* may be obtained in the same experiment by isotope dilution techniques using another tracer or in a separate experiment under the same conditions using  $^{15}\text{N}$  (see Nolan & Leng, 1972).

### *B. Analytical considerations*

A discussion of  $^{15}\text{N}$  tracer studies would be incomplete without some consideration of the essential technology and, in particular, of sample preparation procedures and analysis of samples for  $^{15}\text{N}$  content on the mass spectrometer. In the following discussion some recommendations are made concerning routine technology.

It is essential that commercial sources of  $^{15}\text{N}$ -labelled compounds used in tracer studies be checked for chemical purity and  $^{15}\text{N}$  content. A sample of the injection solution should always be analysed with unknown samples from the same experiment.

Following injection of isotope, samples should be taken often enough to accurately define the isotope dilution curve; more frequent sampling should be used for the parts of the curve with the greatest rate of change. Ideally, sampling should continue for several days so that the slower exponential components of the curves are well defined.

It is important to ensure that the sample N is not contaminated by extraneous N during laboratory procedures and that metabolism in the sample is stopped immediately, so that the enrichment of the isolated N accurately reflects that in the pool when the sample was taken. This is achieved by rapid sample preparation techniques and storage of samples at low temperatures ( $-20^\circ$ ). For example, when ruminal ammonia samples are taken, microbial fermentation should be stopped immediately,

but preferably without causing lysis of micro-organisms or leakage of extracellular contents.

A routine procedure for isolation of N and estimation of  $^{15}\text{N}$  enrichment should be adopted, and maintained. In general, the steps in analysis are (a) conversion of the N in the sample by specific methods into ammonium sulphate, (b) purification by steam distillation to remove interfering substances, (c) conversion of the purified ammonium sulphate to gaseous  $\text{N}_2$  by oxidation with alkaline hypobromite solution and (d) measurement of  $^{15}\text{N}$  content using a mass spectrometer.

During these procedures it is important to avoid contamination of the sample with non-specific N compounds or with  $\text{N}_2$  from the air. For example, blood urea-N must be converted to ammonia and then gaseous  $\text{N}_2$  without additions of other N during the conversion steps involved. The likelihood of serious errors due to addition of extraneous N becomes greater when the quantities of N isolated are small (i.e. less than 100  $\mu\text{g}$  N).

N contamination commonly occurs as a result of incomplete isolation of sample N, use of impure chemical reagents, absorption of ammonia from laboratory air, and additions of ammonia and  $\text{N}_2$  dissolved in the solution used to oxidize the ammonium sulphate samples.

Cross-contamination between consecutive samples of high and low enrichment in an analytical series is a further source of error. These 'memory' effects are often due to adsorption of N onto the surfaces of laboratory glassware, and onto ice at temperatures such as those of liquid- $\text{N}_2$  traps. 'Memory' effects can also occur if the gas handling system and analyser tube of the mass spectrometer are not adequately evacuated between samples.

Mass spectrometer variation is also a source of error, but with sensible procedures and experienced operators, these errors are likely to be less serious than those occurring in sample preparation.

The sources of likely errors in  $^{15}\text{N}$  analysis have been reviewed in detail by Bremner, Cheng & Edwards (1964), Martin & Ross (1968) and Nolan (1972).

A large source of error in enrichment estimates occurs where the background abundance of  $^{15}\text{N}$  is not accurately known. In our experience, the estimated background abundance varies from the natural abundance of  $^{15}\text{N}$  in air. Whether these deviations from natural abundance of  $^{15}\text{N}$  in body N result from isotopic discrimination which occurs in biological systems (Wellman, Cook & Krouse, 1968; Focht, 1973) or from artefacts of the  $^{15}\text{N}$  analysis is not known. The important practical consideration is that background abundances of  $^{15}\text{N}$  should be estimated for the various body N pools before injection of  $^{15}\text{N}$ , to allow enrichment values to be accurately estimated.

Another practical consideration is that abundance and enrichment are expressed as 'atoms %' and 'atoms % excess'. These are units of 'atomic weight' or 'equivalents', not units of mass, since the atomic weight of N changes from 14 to 15 as the  $^{15}\text{N}$  content varies. The total content of N in samples should, therefore, be estimated by titration rather than by weighing methods. Failure to recognize this may lead to errors

in isotope dilution studies, particularly where the injected tracer solution contains N of high enrichment.

## REFERENCES

- Baker, N., Shipley, R. A., Clark, R. E. & Incefy, G. E. (1959). *Am. J. Physiol.* **196**, 245.
- Bremner, J. M., Cheng, H. H. & Edwards, A. P. (1964). In *The use of Isotopes in Soil Organic Matter Studies* p. 429. Vienna: International Atomic Energy Agency.
- Downes, A. M. & McDonald, I. W. (1964). *Br. J. Nutr.* **18**, 153.
- Focht, D. D. (1973). *J. environ. Qual.* **2**, 247.
- Martin, A. E. & Ross, P. J. (1968). *9th International Congress of Soil Science Transactions, Adelaide* Vol. 3, p. 521 [J. W. Holmes, editor]. New York: Elsevier.
- Nolan, J. V. (1972). Dynamics of protein metabolism in sheep. PhD Thesis, University of New England, Australia.
- Nolan, J. V. & Leng, R. A. (1972). *Br. J. Nutr.* **27**, 177.
- Schoenheimer, R. & Rittenberg, D. (1939). *J. biol. Chem.* **127**, 285.
- Shipley, R. A. & Clark, R. E. (1972). In *Tracer methods for in vivo kinetics. Theory and Applications*. New York and London: Academic Press.
- Walser, M., George, J. & Bodenlos, L. J. (1954). *J. chem. Phys.* **22**, 1146.
- Wellman, R. P., Cook, F. D. & Krouse, H. R. (1968). *Science, N.Y.* **161**, 269.