Estimation of the proportion of non-ammonia-nitrogen reaching the lower gut of the ruminant derived from bacterial and protozoal nitrogen

BY W. D. STEINHOUR, M. R. STOKES, J. H. CLARK, J. A. ROGERS AND C. L. DAVIS

Department of Dairy Science, University of Illinois

AND D. R. NELSON

College of Veterinary Medicine, University of Illinois, Urbana, Illinois 61801, USA

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1. A method for estimating the proportions of bacterial- and protozoal-N in the total non-ammonia-N reaching the lower gut of the ruminant under steady-state conditions was evaluated. Three trials using two different diets were conducted with a Holstein steer equipped with a rumen cannula and duodenal re-entrant cannulas.

2. An intraruminal primed infusion of $(^{15}NH_4)_2SO_4$ was administered for 68 h during each trial. Bacteria and protozoa samples were isolated from rumen fluid at approximately 6 h intervals during each infusion period. Total non-ammonia-N was isolated from duodenal digesta samples taken at approximately the same times. All of these samples were analysed for ^{15}N enrichment. A computer program was used to fit equations to the ^{15}N-enrichment curves of bacterial- and protozoal-N. Models of both bacterial- and protozoal-N kinetics consisted of a small pool which equilibrated rapidly with rumen NH_3 and a large pool with a fractional turnover rate of 0·045–0·070/h for bacterial-N and 0·056–0·069/h for protozoal-N.

3. Abomasal fluid turnover was estimated by a single injection of polyethylene glycol (molecular weight 4000) into the rumen followed by sampling of rumen fluid and duodenal digesta.

4. Estimates of abomasal fluid turnover, bacterial-N turnover, and protozoal-N turnover were entered into an equation which was adjusted by computer iteration to fit the ^{15}N-enrichment curve of duodenal digesta non-NH_3-N generated from each $(^{15}NH_4)_2SO_4$ infusion period. The computer fit of this equation to the observed results gave estimates of 0·39–0·45 and 0·22–0·41 for the proportion of duodenal non-NH_3-N derived from bacterial-N and protozoal-N respectively.

5. This method is potentially useful in estimating microbial protein passage to the lower gut in ruminants. Sampling digesta from the omasum rather than the duodenum would simplify the method and possibly increase the reliability of the estimates.

The study of microbial protein synthesis in the rumen has been severely limited by the lack of a reliable technique to measure microbial protein passage to the lower gut of the ruminant. Proposed markers of microbial protein in vivo include diaminopimelic acid (DAP) (Hutton *et al.* 1971), aminoethylphosphonic acid (AEP) (Abou Akkada *et al.* 1968), RNA (Smith & McAllan, 1970), ^{35}S (Beever *et al.* 1974) and ^{15}N (Mathison & Milligan, 1971). AEP, the only protozoal marker of those listed, has been found in foodstuffs and bacteria (Ling & Buttery, 1978), and therefore is of questionable value for such measurements. The other markers listed have previously been used in ways that either underestimate the contribution of protozoa to microbial protein passage or measure only bacterial protein. Because bacteria and protozoa are present in the rumen in approximately equal quantities on a mass basis under many dietary conditions (Hungate, 1966), an adequate technique for estimating microbial protein flow to the lower gut should estimate both bacterial and protozoal protein. This report describes an initial evalution of a technique currently being developed in this laboratory which is designed to estimate the proportions of bacterial- and protozoal-N present in duodenal digesta of the ruminant.

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MATERIALS AND METHODS

Animal surgery and post-operative treatment

The 550 kg Holstein steer used in this experiment was fitted with a rumen cannula. Re-entrant cannulas were placed in the duodenum between the pyloric valve and entrance of the bile duct, and positioned to allow gravitational flow of digesta, using a procedure similar to that of Otchere *et al.* (1974). The steer had access to a trace-mineral-containing salt block at all times after surgery and was given lucerne (*Medicago sativa*)–brome grass (*Bromus inermis*) hay *ad lib.* for at least 2 weeks before adaptation to the experimental diet.

Construction of duodenal re-entrant cannulas

Duodenal re-entrant cannulas were constructed from 150 mm × 200 mm × 1 mm reinforced Silastic Medical Sheeting (Dow Corning Corporation, Midland, Michigan) glued together with Silastic Medical Adhesive (Silicone Type A; Dow Corning Corporation). Nalgene strips (cut from reagent bottles) were glued between the layers of Silastic sheeting along the neck and base of the cannulas to add rigidity.

Experimental diets

This study was conducted in three separate periods. Two diets (Table 1) were formulated to be similar in all respects except the supplemental N source. Both diets contained 18·4 g N/kg dry matter (DM). Diet U, containing urea, was fed during periods 1 and 3 (Table 2). Diet S, containing soya-bean meal, was fed during period 2. Each diet was offered hourly by the use of an automatic feeder (Stokes *et al.* 1979) to provide approximately 8·1 kg DM and 150 g N/d. This level of food intake was sufficient to maintain the steer and ensure rapid consumption of food at each hour. The steer was adapted to a respective diet at least 3 weeks before sampling in each experimental period.

Principle of method

The method by which microbial protein passage to the small intestine was estimated in this experiment is based on the differing rates of incorporation of ^{15}N into bacterial- and protozoal-N observed during intra-ruminal infusion of $(^{15}NH_4)_2SO_4$ (Pilgrim *et al.* 1970; Mathison & Milligan, 1971). A priming dose of $(^{15}NH_4)_2SO_4$ was administered into the rumen followed by a 3-d continuous infusion of the isotope. During the infusion, ^{15}N enrichment was determined in samples of bacteria and protozoa isolated from the rumen and in duodenal digesta non-NH_3-N collected from the re-entrant cannula. Equations were derived containing parameters that were adjusted by a computer program to fit the bacterial and protozoal ^{15}N-enrichment curves resulting from the infusion. Since the ^{15}N-enrichment of non-NH_3-N reaching the small intestine is essentially a weighted sum of the ^{15}N enrichment of bacterial- and protozoal-N, the contribution of these fractions to the total can be estimated by fitting an appropriate equation to the observed ^{15}N enrichment at the lower gut. In simplified terms, this equation can be expressed as:

$$E_D = Xf_B(t) + Yf_P(t) \quad (1)$$

where E_D is the atoms excess ^{15}N in non-NH_3-N of duodenal digesta at time t, $f_B(t)$ is the atoms excess ^{15}N in bacterial-N at time t, $f_P(t)$ is the atoms excess ^{15}N in protozoal N at time t, X is the proportion of duodenal non-NH_3-N contributed by bacteria and Y is the proportion of duodenal non-NH_3-N contributed by protozoa.

The actual equation used to estimate X and Y was somewhat more complex because the fractional turnover rate of digesta in the abomasum had to be taken into account. (See eqn (10), for greater detail.) Since the animal was fed hourly, steady-state conditions were

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Table 1. *Composition of diet (g/kg dry matter)*

	Diets	
	Urea (U)	Soya-bean meal (S)
Maize silage	540	550
Ground maize	438	394
Urea (480 g N/kg)	7·8	—
Soya-bean meal (94 g N/kg)	—	43·4
Trace mineral-containing salt*	6·4	5·9
Dicalcium phosphate	6·4	5·9
Vitamins A† and D‡	0·3	0·2
Sodium sulphate	0·4	0·4

* Contains (g/kg) iodine 0·005, iron 1·25, copper 0·25, cobalt 0·005, manganese 2·0, zinc 2·5, sodium chloride 985·0.

† Contains (μg/g) retinylacetate 3440.

‡ Contains (μg/g) ergocalciferol 31·25.

Table 2. *Design of experiment**

Period†	Diet‡	$(^{15}NH_4)_2SO_4$ (single injection)	$(^{15}NH_4)_2SO_4$ (primed infusion)	Polyethylene glycol‖ (single injection)
1	Urea		×	
2	Soya-bean meal	× ×	×	
3	Urea	×	×	× ×

* The techniques were performed once or twice (×, × ×) respectively in each period.

† For details, see pp. 419 and 420.

‡ For details, see Table 1.

‖ Used to measure abomasal-fluid turnover.

assumed to apply to digesta turnover rates in the rumen and abomasum and for bacterial- and protozoal-N uptake.

Infusion and sampling procedures

Period 1. A priming dose of 80 mg $(^{15}NH_4)_2SO_4$ (99% ^{15}N; Isotope Labeling Corporation, Whippany, New Jersey) was administered intra-ruminally and mixed into the rumen contents by hand. The priming dose was followed immediately by a constant infusion of approximately 25 mg $(^{15}NH_4)_2SO_4$/h. The pH of the infusate was approximately 4. The infusate contained 110 mg $(^{15}NH_4)_2SO_4$/l and was delivered by a continuous, automatic infusion-withdrawal pump (Harvard Apparatus Company, Inc., Dover, Massachusetts) for 68 h.

Samples of digesta were collected from the proximal duodenal re-entrant cannula at 5–7 h intervals beginning 10 h before the start of the $(^{15}NH_4)_2SO_4$ infusion. This was continued until the end of the 68 h infusion. Approximately 500 ml samples of digesta were collected and immediately placed in a dry ice-ethanol bath. Samples were subsequently stored at −20°.

At 6 h intervals, beginning 10 h before the $(^{15}NH_4)_2SO_4$ infusion, samples of rumen fluid were withdrawn from four areas of the rumen and pooled. These composite samples were acidified to pH 2 with sulphuric acid (500 ml/l) and stored at −20°.

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At the same time as the rumen fluid samples were collected, two samples of whole rumen contents (for isolation of bacteria and protozoa) were collected near the reticulo-omasal orifice into 250 ml wide-mouth centrifuge tubes by removing the stopper after location of the site. The tubes were immediately stoppered and placed in water at 35–40°.

To reduce cell lysis during the isolation of bacteria and protozoa the samples of rumen contents were gassed with carbon dioxide that was freed of oxygen by passing over a heated copper column. Samples of rumen contents used for bacteria isolation were blended for 1 min at a low speed in a Waring blender. The resulting slurry was strained through four layers of cheesecloth. Solids were discarded. The liquid was centrifuged at 1000 *g* for 5 min at 15–20° to remove food particles and protozoa. The supernatant fraction was pipetted into a centrifuge tube and spun at 16000 *g* for 25 min. The pellet was washed twice in anaerobic buffer (final concentration (g/l): K_2HPO_4, 4·5; KH_2PO_4, 4·5; NaCl, 9·0; $(NH_4)_2SO_4$, 4·5; $MgSO_4.7H_2O$, 1·8; $CaCl_2.2H_2O$, 1·2; Na_2CO_3, 40·0; cysteine hydrochloride, 5·0; resazurin, 0·02). The final pellet was resuspended in distilled water, frozen, and lyophyllized. The lyophyllized samples were ground to a fine powder with a mortar and pestle. This procedure yielded preparations of bacteria that were relatively free of food contamination as judged by microscopic examination of the final pellet.

Samples of rumen contents for protozoa isolation were strained through four layers of cheesecloth and incubated in 250 ml separatory funnels at 37° for 30–45 min. The lower portion of liquid in the separatory funnel was transferred to a centrifuge tube, adjusted to 150 ml by adding anaerobic buffer, and centrifuged at 160 *g* for 1 min. The pellet was washed three times with anaerobic buffer. The final pellet was resuspended in distilled water, frozen, lyophyllized, and finely ground. This procedure yielded relatively pure preparations of protozoa containing very low levels of food and bacterial contamination as judged by microscopic examination of the final pellet.

Periods 2 and 3. A single injection technique was employed to estimate rumen-NH_3 kinetics in periods 2 and 3 because it was found in period 1 that rumen-NH_3 ^{15}N-enrichment measurements made during a continuous infusion of $(^{15}NH_4)_2SO_4$ were subject to large mixing or sampling errors or both. A combined dose of 100–150 mg $(^{15}NH_4)_2SO_4$ and 40–50 g polyethylene glycol (PEG) was injected into the rumen and mixed by hand. Samples of rumen fluid, for NH_3 ^{15}N-enrichment determination, were taken every 30 min for 5 h and hourly for the next 19 h. Samples were acidified to pH 2 with H_2SO_4 (500 ml/l) and frozen. Rumen fluid for PEG analysis to determine rumen fluid volume was obtained at hourly intervals for eight consecutive hours and frozen. This experiment was conducted 1 week before and 1 week after the $(^{15}NH_4)_2SO_4$ infusion for period 2, and 1 week after the infusion for period 3. A 3-d primed continuous infusion of $(^{15}NH_4)_2SO_4$ was conducted during periods 2 and 3 as was described for period 1.

The passage rate of liquid from the rumen to the abomasum was estimated from two single injection experiments using PEG as a liquid marker. For both experiments a dose of 150 g PEG was given intra-ruminally and mixed into the rumen contents by hand. Samples of rumen fluid and duodenal digesta were collected hourly for 24 h and frozen. A summary of the techniques used in each period is shown in Table 2.

Preparation of samples for ^{15}N analysis

Rumen-NH_3. Samples were centrifuged at 27000 *g* and 0–5° for 15 min. A portion (20 ml) of the supernatant fraction was steam-distilled over magnesium oxide and trapped in boric acid (20 g/l; AOAC, 1970). Approximately 5 ml ethanol was distilled between samples to displace traces of NH_3 adhering to the glassware (Bremner, 1965). The boric acid was acidified with excess 0·05 M-H_2SO_4 and evaporated to dryness at 55°. The dried residue was resuspended in 3–5 ml 0·05 M-H_2SO_4 and stored at 3° until analysed for ^{15}N enrichment.

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Bacteria and protozoa. Isolated samples of bacteria and protozoa containing 1–5 mg N were analysed for total N by the micro-Kjeldahl technique. Concentrated H_2SO_4 (3 ml) containing selenium oxychloride (12 g/l) was used to digest the samples overnight. Sodium hydroxide (300 g/l; 15 ml) was added to the flasks after digestion, and the ammonia was steam-distilled into boric acid (20 g/l). Ethanol was distilled between samples, and the boric-acid solutions containing NH_4^+ were concentrated as described for rumen-NH_3 samples.

Duodenal digesta. Samples of duodenal digesta were thawed and blended in a Waring blender at low speed for 1 min to mix the sample and to reduce particle size. The digesta was then rapidly stirred on a magnetic stirrer, and 2–4 ml subsamples were removed with a large-bore pipette. The pH of the subsamples was adjusted to pH 10 with 5 M-NaOH and incubated in a waterbath at 60–70° for 30 min while N_2 was bubbled through the samples to remove NH_3. The remaining non-NH_3-N was prepared for ^{15}N analysis as described for bacterial- and protozoal-N samples.

^{15}N analysis

^{15}N enrichment in rumen-NH_3, bacterial-N, protozoal-N, and duodenal non-NH_3-N was measured by mass spectrometry. A portion (3–5 ml) of sample containing 1–5 mg N was mixed with 3 ml sodium hypobromite solution (prepared by adding 6 ml bromine drop-wise while stirring, to 200 ml of an ice-cold solution of sodium hydroxide (100 g/l) and potassium iodide (1 g/l)) in an evacuated Rittenburg tube. The resulting N_2 was analysed directly on a mass spectrometer (Nuclide Corporation, State College, Pennsylvania). Results were corrected for background enrichment and expressed as atoms excess ^{15}N. Atoms excess ^{15}N was calculated as $^{15}N/(^{14}N+{}^{15}N)$.

Determination of NH_3 and PEG

Rumen fluid and duodenal digesta samples were centrifuged at 27000 *g* for 15 min. NH_3 was assayed in the supernatant fraction after micro-diffusion as described by Umbreit *et al.* (1964). PEG in the supernatant fraction was quantified by the turbidimetric procedure of Hyden (1955) as modified by Smith (1959).

Food samples

N was assayed by the Kjeldahl technique (AOAC, 1970). Dry matter was determined by heating at 55° for at least 18 h.

Mathematical analysis of results

Rumen-NH_3 kinetics. Rumen-NH_3 kinetics were estimated from the $(^{15}NH_4)_2SO_4$ and PEG single-injection experiments of periods 2 and 3. Observed NH_3 ^{15}N-enrichment values were fitted to the equations used by Nolan & Leng (1972):

$$E_{NH_3} = \sum_{i=1}^{n} A_i \, e^{-m_i t} \tag{2}$$

where E_{NH_3} is the atoms excess ^{15}N in rumen NH_3, A_i is the zero-time intercept of the ith component, m_i is the fractional turnover rate (/h) of the ith component, i is the identification number of the component and n is the total number of components.

A computer program using the method of Provencher (1976) was employed to estimate values for n, A_i and m_i. Rumen-fluid volume was estimated from the zero-time PEG concentration as determined by linear regression of the natural log of PEG concentration *v.* time (Bauman *et al.* 1971). Rumen-fluid volume was multiplied by mean rumen-NH_3

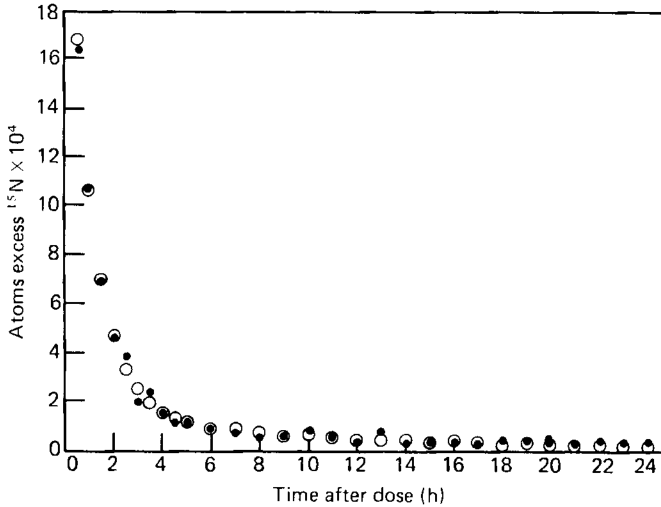

Fig. 1. A typical isotope decay curve of rumen-NH_3 ^{15}N enrichment resulting from an intra-ruminal dose of approximately 100 mg $(^{15}NH_4)_2SO_4$. ●, Observed data points; ○, computer fit of eqn (2).

concentration to calculate NH_3 pool size. This was used to correct the A_i values estimated from ^{15}N-enrichment values.

Rumen-NH_3 kinetics were found to fit a two-pool model in all instances. An example of the data is given in Fig. 1. For period 2, the mean values of A_1, A_2, m_1 and m_2 were calculated from the two single-injection experiments. NH_3 kinetics in period 1 were assumed to be quantitatively similar to that in period 3. Rumen-NH_3 ^{15}N enrichment during intra-ruminal infusion of $(^{15}NH_4)_2SO_4$ was assumed to be described by the equation of Steele *et al.* (1956):

$$E_{NH_3} = \frac{1}{Q}\left[\left(P_2 - \frac{F}{m_1}\right) \quad (A_1' e^{-m_1 t}) + \left(P_2 - \frac{F}{m_2}\right) \quad (A_2' e^{-m_2 t})\right] + \frac{F}{Q}\left(\frac{A_1'}{m_1} + \frac{A_2'}{m_2}\right) \qquad (3)$$

where Q is the rumen-NH_3 pool size (mmol), P_2 is the priming dose of $(^{15}NH_4)_2SO_4$ (mmol N), F is the infusion rate of $(^{15}NH_4)_2SO_4$ (mmol N/h), A_1', A_2' are fractional pool components ($A_1' + A_2' = 1$) and m_1, m_2 are fractional turnover rates of the respective components (/h). A_1', A_2', m_1, m_2 were taken from the computer fit of eqn (2) to $(^{15}NH_4)_2SO_4$ single-injection values.

Bacterial-N kinetics. Rumen-NH_3 was assumed to be the direct source of all ^{15}N taken up by bacteria during the constant infusion of $(^{15}NH_4)_2SO_4$. A small proportion of the bacterial-N was assumed to equilibrate rapidly with rumen-NH_3 and thus would have the same level of ^{15}N enrichment as rumen-NH_3. The remaining bacterial-N constituted a pool with a slower turnover rate. The instantaneous rate of change in ^{15}N enrichment of this major bacterial-N pool during the $(^{15}NH_4)_2SO_4$ infusion was described by the equation:

$$\frac{dE_B}{dt} = K_1(P_1 E_{NH_3} - E_B) \qquad (4)$$

where E_B is the atoms excess ^{15}N in this major bacterial-N pool at time t, K_1 is the fractional turnover rate of this bacterial-N pool (/h) and P_1 is the proportion of this bacterial-N pool that was derived from rumen-NH_3.

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This equation was solved for E_B algebraically by integration with respect to time assuming $E_B = 0$ at $t = 0$. ^{15}N-enrichment in total bacterial-N ($f_B(t)$) is represented as:

$$f_B(t) = (1-Z_1)E_B + Z_1 E_{NH_3} \quad (5)$$

where Z_1 is the proportion of rumen bacterial-N in rapid equilibrium with rumen-NH_3. Z_1, P_1 and K_1 were adjusted to make eqn (5) fit the observed values by iteration with a computer program (Berman & Weiss, 1974). This is a non-linear least squares method. Data were equally weighed.

Protozoal-N kinetics. Protozoa also were assumed to have a small nitrogen pool which equilibrated rapidly with rumen-NH_3, having ^{15}N enrichment which equalled rumen-NH_3 enrichment (eqn (3)). The remaining protozoal-N was assumed to acquire ^{15}N only from the uptake of bacterial-N. The instantaneous rate of change of ^{15}N enrichment of this major protozoal-N pool was described by the equation:

$$\frac{dE_P}{dt} = K_6(P_3 f_B(t) - E_P) \quad (6)$$

where K_6 is the fractional turnover rate of this protozoal-N pool (/h) and P_3 is the proportion of this protozoal-N pool that was derived from bacterial-N.

This equation was solved for E_P algebraically by integration with respect to time, assuming $E_P = 0$ at $t = 0$. ^{15}N enrichment of the total protozoal-N ($f_P(t)$) was represented as:

$$f_P(t) = (1-Z_2)E_P + Z_2 E_{NH_3} \quad (7)$$

where Z_2 is the proportion of rumen protozoal-N in rapid equilibrium with rumen-NH_3. Z_2, P_3 and K_6 were adjusted to fit observed values by iteration with a computer program (Berman & Weiss, 1974). Data were equally weighted.

Liquid flow from rumen to duodenum. The fractional turnover rate of rumen fluid was estimated from the PEG single-injection experiments. Concentration of PEG in rumen fluid following an intra-ruminal single injection was described by the equation:

$$C_R = A_R e^{-K_R t} \quad (8)$$

where C_R is mg PEG/l rumen fluid at time t, A_R is mg PEG/l rumen fluid at $t = 0$, K_R is the fractional turnover rate of rumen fluid (/h) and t is time after dosing (h). Eqn (8) was converted to the form $\ln C_R = \ln A_R - K_R t$. Values for A_R and K_R were estimated by linear regression of observed $\ln C_R$ *v.* t.

The concentration of PEG in duodenal digesta was assumed to equal that of abomasal fluid. Instantaneous rate of change in the concentration of PEG in abomasal fluid was assumed to be described by the equation:

$$\frac{dC_A}{dt} = K_A\,(P_4 A_R e^{-K_R t} - C_A) \quad (9)$$

where C_A is mg PEG/l abomasal fluid at time t, K_A is the fractional turnover rate of abomasal fluid (/h) and P_4 is liquid outflow from the rumen:liquid outflow from the abomasum.

C_A was solved algebraically by integration with respect to time assuming $C_A = 0$ at $t = 0$. A_R and K_R had already been estimated from eqn (8). P_4 and K_A were estimated by computer iteration as described previously. These parameters were assumed to be the same for all three experimental periods.

Bacterial and protozoal flow to the duodenum. The instantaneous rate of change in

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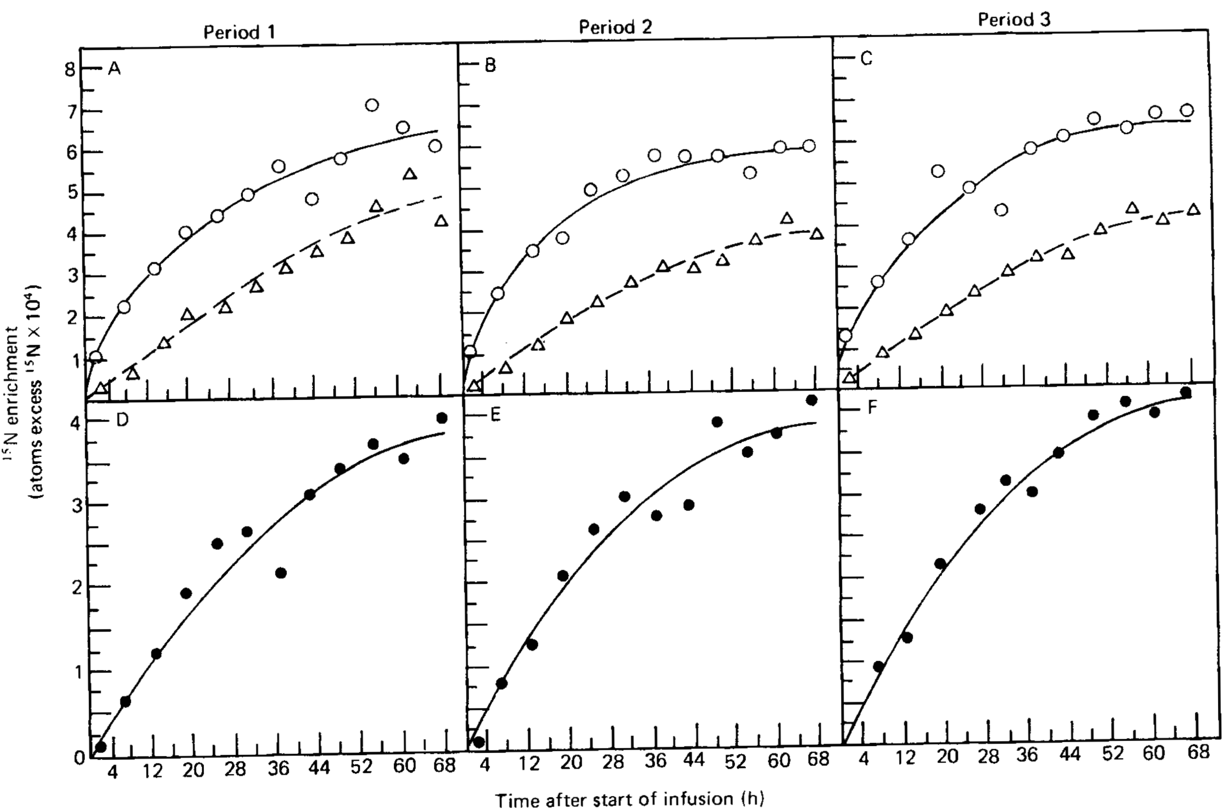

Fig. 2. Isotopic labelling of bacterial-N (A, B, C; —○—), protozoal-N (A, B, C; - - - △ - - -), and duodenal non-ammonia-N (D, E, F; —●—) during intra-ruminal primed infusion of $(^{15}NH_4)_2SO_4$. Priming dose was 80 mg $(^{15}NH_4)_2SO_4$. Infusion rate was 25 mg $(^{15}NH_4)_2SO_4$/h. —○—, - - -△- - -, —●—, Computer fit of eqns (5), (7) and (10) respectively.

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duodenal non-NH_3-N ^{15}N enrichment during $(^{15}NH_4)_2SO_4$ infusion can be described by the equation:

$$\frac{dE_D}{dt} = f_B(t)P_5K_A + f_P(t)P_6K_A - E_DK_A \qquad (10)$$

where P_5 is the proportion of duodenal non-NH_3-N derived from bacteria and P_6 is the proportion of duodenal non-NH_3-N derived from protozoa.

K_A had been estimated from eqn (9). The functions $f_B(t)$ and $f_P(t)$ had been mathematically described by eqns (5) and (7). The equation was solved for E_D algebraically by integration with respect to time assuming $E_D = 0$ at $t = 0$. P_5 and P_6 were estimated by fitting eqn (10) to the observed values using a computer program (Berman & Weiss, 1974). Data were equally weighted.

RESULTS AND DISCUSSION

^{15}N enrichment of bacterial-N

^{15}N-enrichment curves for bacterial-N resulting from intra-ruminal primed infusions of $(^{15}NH_4)_2SO_4$ in periods 1, 2, and 3 are shown in Fig. 2. Eqn (4) was inadequate for describing bacterial-N-labelling because it underestimated the ^{15}N enrichment of the initial bacterial-N samples in all three periods. Eqn (5) more closely fits the observed values. The latter equation is based on the assumption that a small proportion of the bacterial-N is in rapid equilibrium with rumen-NH_3. As shown in Table 3, this pool represented 1·9–7·1% of the total bacterial-N. It probably corresponds to the rapidly labelling amide-N pool in rumen bacteria reported by Salter *et al.* (1979). The other bacterial-N pool incorporates ^{15}N from rumen-NH_3 at a slower rate. This pool is made up largely of proteins and nucleic acids. Its fractional turnover rate (Table 3) is thus indicative of the total growth rate of rumen bacteria under the conditions of this experiment. The estimate of the fractional turnover rate of this major bacterial-N pool for period 1 differs markedly from the estimates for periods 2 and 3. This is probably due to error in estimation of rumen-NH_3 kinetics for period 1. Since a $(^{15}NH_4)_2SO_4$ single-injection experiment was not performed for period 1, rumen-NH_3 kinetics were assumed to be similar to NH_3 kinetics in period 3. The animal was given diet U (see Table 1) in both of these periods. Any error in this assumption would affect estimates of the fractional turnover rate of the major bacterial-N pool in period 1. The estimated fractional turnover rates of the major bacterial-N pool in periods 2 and 3 were similar to the fractional turnover rates of rumen fluid in these experiments as measured by PEG dilution (Table 4). This indicates that either bacterial-N flows out of the rumen with the liquid phase, and that very little bacterial lysis occurs in the rumen under these conditions, or that bacterial-N flows out of the rumen at a slower rate than rumen fluid, and the bacterial growth rate includes replacement of lysed cells. The latter explanation seems more likely in view of the significant intra-ruminal recycling of N reported by Nolan & Leng (1972) and the ability of rumen bacteria to adhere to plant particles within the rumen (Cheng *et al.* 1977). In the three infusion periods, 37–48% of the total bacterial-N was derived from sources other than rumen-NH_3 (Table 3). This agrees well with observations made by other workers (Pilgrim *et al.* 1970; Mathison & Milligan, 1971; Kennedy & Milligan, 1978) and indicates the importance of peptides and amino acids as N sources for rumen bacteria.

^{15}N enrichment of protozoal-N

Eqn (5), which was based on the assumption of direct microbial uptake of rumen-NH_3, did not accurately describe the ^{15}N-enrichment pattern of protozoal-N during the $(^{15}NH_4)_2SO_4$ infusions. This is in agreement with previous observations that protozoa do not incorporate

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Table 3. *Turnover rates of bacterial N and source of N for bacterial protein synthesis*

Period*	Fractional turnover rate† (/h)	Estimated SD‡(/h)	Proportion of pool derived from rumen-NH_3†	Estimated SD‡	Proportion of bacterial-N in equilibrium with rumen-NH_3	Estimated SD‡	Proportion of total bacterial-N not derived from rumen-NH_3
1	0·045	0·013	0·498	0·036	0·041	0·048	0·48
2	0·070	0·010	0·609	0·018	0·019	0·042	0·38
3	0·057	0·017	0·606	0·036	0·071	0·069	0·37

* For details, see Table 2.
† Pool of bacterial-N assumed not to be in rapid equilibrium with rumen-NH_3.
‡ Standard deviation for each parameter was estimated based on variation about the fitted lines by the computer program (SAAM 25) used for curve fitting, 9 error degrees of freedom (Berman & Weiss, 1974).

Table 5. *Turnover rates of protozoal-N and sources of N for protozoal protein synthesis*

Period*	Fractional turnover rate† (/h)	Estimated SD‡ (/h)	Proportion of pool derived from bacterial-N†	Estimated SD‡	Proportion of protozoal-N in equilibrium with rumen-NH_3	Estimated SD‡	Proportion of total protozoal-N not derived from rumen-NH_3‖
1	0·056	0·023	0·822	0·081	0·024	0·025	0·56
2	0·065	0·015	0·675	0·036	0·031	0·020	0·56
3	0·069	0·013	0·683	0·024	0·023	0·016	0·56

* For details, see Table 2.
† Pool of protozoal-N assumed not to be in rapid equilibrium with rumen-NH_3.
‡ Standard deviation for each parameter was estimated based on variation about the fitted lines by the computer program (SAAM 25) used for curve fitting, 9 error degrees of freedom (Berman & Weiss, 1974).
‖ Includes both dietary N and bacterial-N that did *not* pass through the rumen-NH_3 pool.

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Table 4. *Rumen and abomasal fluid turnover rates*

Diet*	Fractional turnover rate of rumen fluid (/h)	Fractional turnover rate of abomasal fluid (/h)	Estimated SD† (/h)	Rumen-fluid flow:abomasum-fluid flow	Estimated SD†
Urea	0·072	0·339	0·027	0·687	0·015
Urea	0·072	0·278	0·021	0·733	0·015

* For details, see Table 1.

† Standard deviation for each parameter was estimated based on variation about the fitted lines by the computer program (SAAM 25) used for curve fitting, 20 error degrees of freedom (Berman & Weiss, 1974).

N directly from rumen-NH_3 (Coleman, 1975). Eqn (6), which is based on the assumption that all protozoal-N consists of a single pool obtaining ^{15}N indirectly through the engulfment of bacteria, did not fit the observed protozoal-N ^{15}N enrichment because it underestimated the ^{15}N enrichment of the initial samples. Eqn (7) fits the observed protozoal-N ^{15}N enrichment values. This equation was based on the assumption that a small proportion (Table 5) of the protozoal-N equilibrates rapidly with rumen-NH_3. This assumption was used because it represented the least complicated model that could be adjusted to fit the values. This small protozoal-N pool could consist of free intracellular NH_3 or an intracellular amino acid pool that turns over much more rapidly than protozoal proteins and nucleic acids. Although protozoa incorporated ^{15}N at a lower rate than bacteria during the intra-ruminal $(^{15}NH_4)_2SO_4$ infusions (Fig. 2), the fractional turnover rate of the major N pool of protozoa (Table 5) is similar to that estimated for bacterial-N (Table 3). The fractional turnover rates estimated for protozoal-N correspond to a generation time of 11–13 h. While this is shorter than the 16–18 h protozoal generation time observed by Singh *et al.* (1974) in buffalo calves, Warner (1962) has reported a generation time as short as 5·5 h for *Entodinia* in the rumen of sheep. Approximately 56% of the N in protozoa did not pass through the rumen-NH_3 pool in these experiments (Table 5). This estimate is within the range of 36–69% reported by Pilgrim *et al.* (1970) and Mathison & Milligan (1971).

Turnover rate of abomasal fluid

A single dose of PEG into the rumen of the experimental animal was used to generate the PEG concentration curves in rumen fluid and duodenal digesta shown in Fig. 3. As can be seen from the plots of the computer fit of eqn (9) to the observed duodenal digesta PEG concentrations, this model did not precisely fit the experimental values. Modifying the model to allow for mixing in an omasal fluid pool or a simple time delay in the omasum did not improve the fit. Manual mixing of the PEG dose in the rumen appears to have temporarily stimulated abomasal fluid outflow as evidenced by the rapid peak in duodenal PEG concentration that could not be simulated by eqn (9). This problem has not been encountered in sheep, where manual mixing is precluded by the small rumen fistula size (Faichney & Griffiths, 1978). The mean of the fractional turnover rates for abomasal fluid reported in Table 4 was used for K_A in eqn (10) for fitting this equation to the observed duodenal non-NH_3-N ^{15}N enrichment during the $(^{15}NH_4)_2SO_4$ infusions.

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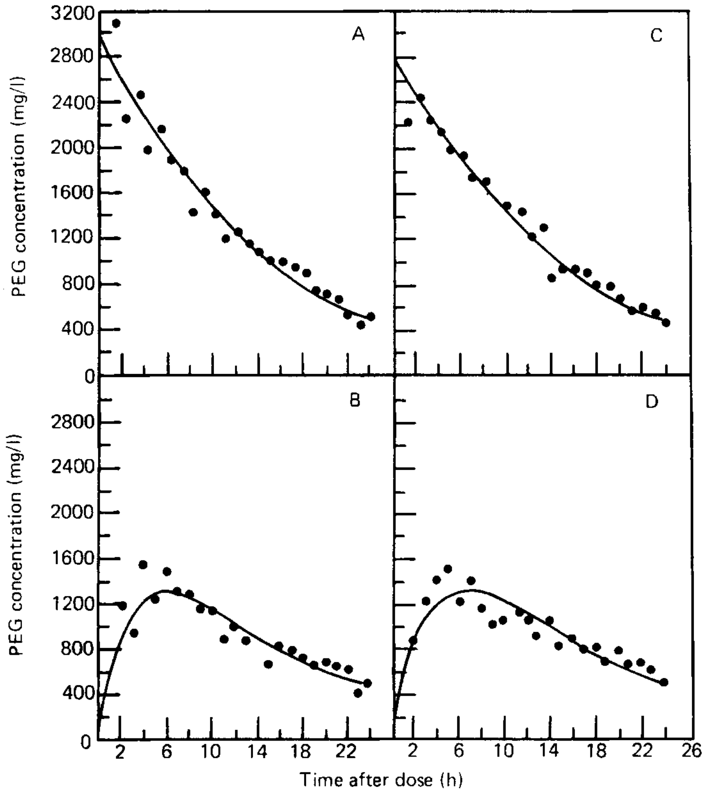

Fig.3. Polyethylene glycol (PEG) concentrations in rumen fluid (A, C) and duodenal digesta (B, D) in two separate experiments in which 150 g PEG was administered intra-ruminally. ——, Computer fit of eqns (8) and (9).

Contribution of bacterial- and protozoal-N to duodenal non-NH_3-N

Duodenal non-NH_3-N ^{15}N enrichment curves during the three $(^{15}NH_4)_2SO_4$ infusions are presented in Fig. 2. The proportions of duodenal non-NH_3-N contributed by bacteria and protozoa are given in Table 6. The proportion of bacterial-N in duodenal non-NH_3-N was fairly constant among the three periods at 39–45%. These findings are similar to those of other scientists who have used DAP to estimate bacterial protein reaching the lower gut of ruminants given similar diets. Ling & Buttery (1978), using DAP as a marker, estimated that 42–47% of the duodenal-N was of bacterial origin in sheep given high-barley diets. Slightly higher estimates ranging from 48 to 71% have been reported for the contribution of bacterial-N to total digesta-N at the lower gut in cows (Hutton *et al.* 1971) and steers (Smith *et al.* 1978) given hay and concentrate diets.

Protozoal-N was estimated to make up 22–41% of the total non-NH_3-N reaching the duodenum in the three infusion periods. There was no clear dietary effect. Variation in the estimates among periods was probably due to uncertainty in the computer fitting of eqn (10) to the observed values. Assessment of the validity of these estimates is difficult because of considerable disagreement among scientists as to the contribution of protozoal-N to total-N at the lower gut of ruminants. Weller & Pilgrim (1974) estimated that protozoal-N

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Table 6. *Contribution of bacterial-N and protozoal-N to duodenal non-ammonia-nitrogen*

Period*	Bacterial-N: duodenal non-NH_3-N	Estimated SD†	Protozoal-N: duodenal non-NH_3-N	Estimated SD†	Non-microbial N: duodenal non-NH_3-N‡
1	0·449	0·092	0·217	0·153	0·334
2	0·392	0·096	0·408	0·172	0·200
3	0·394	0·059	0·388	0·107	0·218

* For details, see Table 2.
† Standard deviation for each parameter was estimated based on variation about the fitted lines by the computer program (SAAM 25) used for curve fitting, 10 error degrees of freedom (Berman & Weiss, 1974).
‡ Estimated by difference.

Table 7. *Effect of changes in fractional turnover rate of abomasal fluid on estimates of bacterial and protozoal-N in duodenal non-ammonia-nitrogen*

Fractional turnover rate of abomasal fluid (/h)	Bacterial-N: duodenal non-NH_3-N	Estimated SD*	Protozoal-N: duodenal non-NH_3-N	Estimated SD*
0·308†	0·392‡	0·096	0·408‡	0·172
0·208	0·474‖	0·099	0·285‖	0·180
0·408	0·345‖	0·095	0·479‖	0·169

* Standard deviation for each parameter was estimated based on variation about the fitted lines by the computer program (SAAM 25) used curve fitting, 10 error degrees of freedom (Berman & Weiss, 1974).
† Mean determined in period 2, for details see Table 4.
‡ Determined in period 2, for details see Table 6.
‖ Estimated assuming fractional turnover rate (/h) of abomasal fluid was 0·208 or 0·408 respectively.

leaving the rumen represented no more than 2% of the N intake for sheep given a variety of diets. Their method, however, did not include measurement of protozoa leaving the rumen attached to food particles. In contrast to these results, Harrison *et al.* (1979) found that protozoa contribute 23–28% of the total amino acid-N reaching the duodenum of sheep given a semi-purified diet. Other scientists have reported that estimates of microbial flow to the lower gut of ruminants based on DAP as a marker are often lower than estimates based on ^{35}S (Walker & Nader, 1975; Ling & Buttery, 1978) or RNA (Ling & Buttery, 1978; Smith *et al.* 1978). These workers concluded that DAP underestimated total microbial protein flow out of the rumen because it does not include protozoal protein. Other evidence indicating that protozoa contribute significantly to duodenal-N is the lack of effect of defaunation on ruminant performance (Abou Akkada & El-Shazly, 1964; Eadie & Gill, 1971; Williams & Dinusson, 1973). If protozoa were sequestered in the rumen, one would expect that the presence of protozoa in the rumen would lead to decreased efficiency of growth of the ruminant due to wasteful recycling of N within the rumen. Also, because faunated animals have lower rumen bacterial counts than defaunated animals (Eadie & Gill, 1971), less microbial protein would reach the lower gut in faunated than in defaunated ruminants if protozoa did not flow out of the rumen. The fact that defaunated ruminants generally do not perform better than faunated ruminants on most diets indicates that protozoa are probably not sequestered within the rumen.

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The major limitation of the results of this experiment is the assumption that the non-NH_3-N pool in the abomasum is a well-mixed pool that turns over at the same rate as abomasal fluid. Faichney & Griffiths (1978) reported that in sheep given a pelleted high-barley diet, solids in the abomasum turn over at a slower rate than abomasal fluid. The presence of two pools of non-NH_3-N in the abomasum with different fractional turnover rates presents a serious problem in the use of the method proposed in this experiment of estimation of microbial protein reaching the lower gut. This is probably the main reason for the large extent of variation between periods and the high estimated standard deviations associated with the estimates of proportions of bacterial- and protozoal-N in duodenal non-NH_3-N in these experiments (Table 6). We examined the sensitivity of our final estimates of bacterial- and protozoal-N to deviations in estimated turnover rates. The results are given in Table 7. A modification to this method which would circumvent this difficulty would be to collect digesta samples from the sulcus omasi rather than duodenum. In this way, the ^{15}N enrichment of the non-NH_3-N leaving the rumen at various times during the infusion of $(^{15}NH_4)_2SO_4$ could be accurately measured. Estimates of bacterial- and protozoal-N leaving the rumen would not be complicated by mixing in one or more N pools in the abomasum. Although the method as performed in this experiment does have this limitation, it is the only method currently available which allows estimation of both bacterial and protozoal contribution to non-NH_3-N flow out of the rumen. With the previously mentioned refinement, this method could be useful in leading to a greater understanding of the interactions of rumen bacteria and protozoa in N metabolism of the ruminant.

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