Evaluation of a mathematical model of rumen digestion and an in vitro simulation of rumen proteolysis to estimate the rumen-undegraded nitrogen content of feedstuffs

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1. Twelve grain mixtures, one lucerne (*Medicago sativa*) hay and one maize silage which had been used in mixed diets for which dietary nitrogen undegraded in the rumen (UDN) had been estimated with duodenally-cannulated cows, were studied. Total N in the feeds was fractionated into pool A (N soluble in borate–phosphate buffer), pool B (total N−(pool A+pool C)) and pool C (acid-detergent-insoluble N or residual N after 24 h incubation in protease solution).

2. N solubilization in protease solution containing 6·6 units/ml (substrate-saturating enzyme concentration) indicated the presence of subfractions in pool B, with different rates of solubilization. Such subfractions were not detectable from *in situ*, Dacron bag, estimates of N solubilization.

3. UDN was estimated using a dynamic mathematical model and rate-constants obtained from N solubilization in protease solution or *in situ*. For three grain mixtures tested using the protease technique the model predicted UDN values of 7, 10 and 12% compared with values of 47, 66 and 59% estimated in vivo. The full range of experimental feeds was tested using the *in situ* technique and UDN values predicted by the model were used to derive UDN values for twelve mixed diets. The latter values were significantly but not closely correlated with those determined in vivo (r^2 0·41, $P < 0·05$).

4. An attempt was made to simulate rumen proteolysis in vitro by choosing a protease enzyme concentration (0·066 units/ml) providing a proteolytic activity similar to that of whole rumen fluid. The experimental samples of feed were subjected to simulated rumen proteolysis for 18 or 48 h to resemble the mean retention times in the rumen for grain mixtures and roughages respectively. The residual N at the end of incubation was considered as an estimate of UDN. The UDN values estimated from simulated rumen proteolysis and those determined in vivo for twelve mixed diets were in close agreement (r^2 0·61, $P < 0·01$).

5. Simulated rumen proteolysis can serve as a simple, rapid and sensitive method to estimate UDN in a variety of feedstuffs.

The nutritive value of feed proteins for the ruminant varies inversely with the extent of degradation of the proteins in the rumen (Chalmers & Synge, 1954). The estimation of rumen-degraded nitrogen (RDN) and undegraded dietary N (UDN) is the central theme of improved systems for protein rationing (see Waldo & Glenn, 1982) but, as yet, there are no simple techniques to estimate these fractions. Techniques available to estimate rumen protein degradation include the use of abomasally- or duodenally-cannulated animals (Faichney, 1975; MacRae, 1975) and in vitro measurements using either rumen inoculum (Mahadevan *et al.* 1979) or commercially-available proteases of microbial origin (Pichard, 1977; Chamberlain & Thomas, 1979; Poos *et al.* 1980). Because these techniques do not recognize the importance of rumen dynamics, attempts have been made to predict dietary N degradation by means of mathematical models (Ørskov & McDonald, 1979; Broderick & Craig, 1980; Van Soest *et al.* 1982). Such models recognize the need to allow for factors such as rate of passage, rate of degradation and the lag time before degradation begins. The various models proposed are similar in their assumptions of heterogeneity of feed N

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and first-order kinetics for degradation and passage of proteins from the rumen, but they differ in the techniques used to estimate the rates of degradation. These differences are important because the accuracy with which rumen degraded N is determined by a mathematical model depends not only on the ability of the model to describe biological function, but also on the accuracy of the laboratory estimation of the model's criteria.

This study was conducted with the objective of evaluating *in situ* (Dacron bag) and in vitro, protease techniques to estimate the rates of solubilization of feed N in the rumen and the applicability of these rate constants in a dynamic mathematical model to estimate the UDN content of a range of mixed diets. An in vitro technique designed to simulate rumen proteolysis was also investigated as an alternative approach to the use of a mathematical model.

MATERIALS AND METHODS

Feed samples

Twelve samples of grain mixtures, one sample of maize silage and one sample of lucerne (*Medicago sativa*) hay were obtained from the University of Wisconsin, Madison. *In situ* estimates of N solubilization, made using the Dacron bag technique, and in vivo estimates of dietary N undegraded in the rumen, made using duodenally-cannulated cows, had previously been undertaken on the samples at Madison. The compositions of the grain mixtures are presented in Table 1. Total N, buffer-insoluble N (Krishnamoorthy *et al.* 1982) and acid-detergent-insoluble N (ADIN; Goering & Van Soest, 1970) in these samples and in samples of maize and soya-bean meal were estimated by macroKjeldahl (Pierce & Haenisch, 1940).

In vivo estimates of UDN passing to the duodenum were made for twelve mixed diets (consisting of a grain mixture, maize silage and lucerne hay) in three different experiments at Madison (Stern & Satter, 1982; Stern *et al.* 1983). Lactating Holstein cows surgically-prepared with rumen cannulas and T-type cannulas at the proximal duodenum were used as the experimental animals in the three experiments each of a 4×4 Latin-square design. Cows were given four meals/d and dry matter intake ranged from 14 to 18 kg daily. Each experimental period was 14 d in duration with the first 10 d serving as an adjustment period, followed by a 4 d period for collection of digesta. Twelve samples of duodenal digesta were collected every 8 h with one 6 h interval to allow a shift in sampling times. Lanthanum was used as an indigestible marker to measure digesta flow (Stern *et al.* 1983). N in pooled digesta was fractionated into ammonia-N, non-ammonia-N and bacterial N. The latter was determined, using diaminopimelic acid (DAPA) as a marker, from the ratio N:DAPA in isolated rumen bacteria and duodenal digesta (Hutton *et al.* 1971). Dietary N undegraded in the rumen was calculated as the difference between the total N and the sum of ammonia-N and bacterial N reaching the duodenum.

Rates of N solubilization

In vitro. Protease enzyme from *Streptomyces griseus*, type XIV (5·4 units/mg protein, Sigma Chemical Co., St Louis, Missouri; 1 unit represents the quantity of enzyme that will hydrolyse casein to produce colour with Folin–Ciocalteu reagent equivalent to 1 μmol tyrosine/min at pH 7·5 and 37°) was used because of the similarity in activity of this enzyme (Matsubara & Feder, 1970; Trop & Birk, 1970) and that of *Bacteroides amylophilus* strain H 18 (Blackburn, 1968). Enzyme solution was prepared fresh in borate–phosphate buffer (BP; NaH_2PO_4 H_2O 7·6 g/l, $Na_2B_4O_7$. $10H_2O$ 13·17 g/l), filtered through Whatman no. 54 filter paper and the filtrate used for incubation studies. Optimum pH and enzyme activity (V_{max}) were measured with azocasein substrate (Charney & Tomarelli, 1947). The enzyme had an optimum pH of 8·0 and V_{max} (mg azocasein hydrolysed/min per unit

Table 1. *Composition of grain mixtures (g/kg)*

Grain mixtures*... Ingredient	1	2	3	4	5	6	7	8	9	10	11	12
Ground maize	480	480	480	480	540	540	400	410	710	630	550	460
Ground oat straw	100	60	80	80	110	170	—	50	180	120	50	—
Dried molasses	30	30	30	30	40	50	20	30	40	60	80	100
Maize starch	40	—	—	—	—	—	—	—	—	10	20	30
Mineral vitamin mix	30	30	30	30	20	20	20	20	20	20	20	20
Urea	—	—	—	—	—	—	10	10	10	10	10	10
Soya-bean meal	320	—	—	—	290	—	—	—	—	—	—	—
Whole soya-bean	—	400	—	—	—	—	—	—	—	—	—	—
Whole soya-bean extruded at: 132°	—	—	380	—	—	—	—	—	—	—	—	—
extruded at: 149°	—	—	—	380	—	—	—	—	—	—	—	—
Maize-gluten meal	—	—	—	—	—	220	—	—	40	150	270	380
Brewers' grain	—	—	—	—	—	—	550	—	—	—	—	
Distillers' dried grain	—	—	—	—	—	—	—	480	—	—	—	—

* From Stern & Satter (1982) and Stern *et al.* (1983).

enzyme) of 1·36. To stop enzyme activity in the incubation studies with feed samples, *t*-butyl alcohol, 600 ml trichloroacetic acid (TCA)/l and immersion of the incubation mixture in boiling water for 1 min were tested. *t*-Butyl alcohol was found to be ineffective. TCA and immersion in boiling water were found to be effective inhibitors, but could not be used because of the difficulty of separating precipitated soluble proteins from insoluble N. Therefore, immediately after their removal from the water-bath the incubated feed samples were filtered through a Whatman no. 54 filter paper on a suction manifold and then washed with distilled water to stop enzyme activity on insoluble feed N.

The enzyme concentration necessary to saturate protein substrates was estimated by incubating 800 mg soya-bean meal with graded amounts (0·5–8·8 units/ml incubation medium) of enzyme at 39° for 1 h. This concentration was 6·6 units/ml in the final incubation medium (see Van Soest *et al.* 1982).

An air-dry sample (0·5 g) ground through a 1 mm screen was weighed into a series of 125 ml Erlenmeyer flasks. BP buffer (40 ml), pH 8·0, was added and the sample soaked at 39° for 1 h. Protease solution (33 units/ml; 10 ml) was added to all flasks except two which served as the control or the zero incubation. Two flasks were removed at 0·25, 0·50, 1, 2, 4, 8, 12, 18, 24, 32, 40 and 48 h and filtered immediately through Whatman no. 54 filter paper on a suction manifold. The residue was washed eight to ten times with portions of 400 ml distilled water. Enzyme and buffer solution without feed sample subjected to a similar filtration and washing procedure was used as a blank. The residual N was estimated by a macroKjeldahl procedure.

Assuming that residual N beyond 24 h incubation represented ultimately unavailable N, potentially available residual N (PARN) was calculated from the difference between residual N at different times of incubation before 24 h and the average value for the residual N at 24, 32, 40 and 48 h. If the plot of the natural log PARN *v.* time was non-linear, the solubilization rate constants of the individual PARN fractions were estimated by a curve-peeling procedure analogous to that used for the graphic analysis of the composite decay curve of a mixture of two or three radioisotopes (Wang *et al.* 1975).

In situ. *In situ* N solubilization for grain mixtures, lucerne hay and maize silage were carried out at Madison (Stern & Satter, 1982; Stern *et al.* 1983). Dacron bags (pore size 25–75 μm) containing 0·5 g test sample were suspended in the rumen of a cow given a high

forage diet (g/kg: 600 lucerne hay, 290 maize meal, 60 oats and 40 soya-bean meal) twice daily. Bags were removed at 1, 4, 8, 12, 17 and 24 h, washed with tap-water and residual N determined by Kjeldahl analysis. An empty bag suspended in the rumen served as a blank.

ADIN was used as the estimate of the ultimately unavailable N fraction (Pichard & Van Soest, 1977). PARN remaining in Dacron bags at 1, 4, 8, 12, 17 and 24 h was estimated by subtracting ADIN from Dacron bag residual N. From the plot of ln of PARN *v.* time, as no non-linearity in the shape of any curve was visible, the rate of solubilization was estimated by linear regression of ln PARN *v.* time.

Evaluation of the model for prediction of UDN

Fractionation of feed N. Total N was fractionated into pool A (buffer soluble N, pH 6·7 for Dacron bag values and pH 7·8 for in vitro protease values), pool C (ADIN for Dacron bag values, 24–48 h residual N in vitro protease values) and pool B or PARN [total N−(pool A+pool C)]. Pool B was further divided into subfractions B_1, B_2 and B_3 by means of curve peeling of ln PARN *v.* time.

Prediction of UDN. The rate-constants and pool size of N fractions obtained from the in vitro protease technique and those calculated from Dacron bag studies were entered in the UDN prediction model (Van Soest *et al.* 1982),

$$\text{UDN} = \sum_{i=1}^{n} B_i\left[\left(\frac{k_{pi}}{k_{si}+k_{pi}}\right)(e^{-k_{pi}l_i})+(1-e^{-k_{pi}l_i})\right]$$

where UDN is the percentage dietary N passing undegraded from the rumen, B_i is the pool size (% of total N in feed) of N fractions (i.e. A, B_1, B_2, B_3 and C), k_{pi} is the fractional turnover rate due to passage, k_{si} is the fractional turnover rate due to degradation, l_i is the lag time. Rates of passage (0·04/h for grain mixtures and 0·034/h for forages) reported by Hartnell & Satter (1979) and a lag time of 0·5 h (Van Soest *et al.* 1982) were used in the calculations. UDN values predicted from *in situ* and in vitro rate-constants were compared with those estimated in experiments in vivo.

The UDN for total mixed rations (grain mixture, lucerne hay and maize silage) was calculated from the equation:

$$\text{UDN} = Ax+By+Cz \qquad (1)$$

where UDN is the estimated percentage UDN for total mixed ration, x is the predicted UDN (%) for grain mixture, y is the predicted UDN (%) for lucerne hay, z is the predicted UDN (%) for maize silage and A, B, C are the proportions of N in mixed ration contributed by grain mixture, lucerne hay and maize silage respectively (Table 2).

Simulation of rumen proteolysis

Rumen fluid was obtained from a cow given a complete mixed diet (g/kg: 700 grass legume hay, 220 cracked maize, 70 soya-bean meal, 10 salt and mineral mixture) once daily at a maintenance level of feeding. The fluid was collected and prepared according to Goering & Van Soest (1970) at 3 h before feeding and 1, 5, 9, 13 and 17 h after feeding. Rumen fluid proteolytic activity (V_{max} and K_m) on azocasein substrate (Mahadevan *et al.* 1979) was measured using Michaelis–Menten kinetics (Segel, 1976). Based on these values, simulation of rumen proteolysis in vitro was attempted using the protease from *Streptomyces griseus* previously used in in vitro N solubilization studies. Samples of grain mixtures, lucerne hay and maize silage on which UDN for the total mixed diet had been estimated in vivo, were subjected to simulated rumen proteolysis in vitro. The incubation procedure was similar to that described earlier for the studies of rate of solubilization except that a lower enzyme concentration was used. To 40 ml BP buffer (pH 8·0) containing 0·5 g air-dry

Table 2. *Contribution of nitrogen from different sources in the mixed diets*

	% total N		
Diet*	Grain mixture†‡	Lucerne (*Medicago sativa*) hay‡	Maize silage‡
1	70	14	16
2	69	15	16
3	69	14	17
4	70	14	16
5	73	12	15
6	75	11	14
7	70	15	15
8	73	12	15
9	58	16	26
10	67	13	20
11	73	10	17
12	77	9	14

* From Stern & Satter (1982) and Stern *et al.* (1983).
† For composition, see Table 1.
‡ For N content, see Table 5.

sample incubated at 39° for 1 h, 10 ml protease solution of 330×10^{-3} units/ml was added. The incubation was stopped at 18 h for grain mixtures and 48 h for forages.

RESULTS

Rates of N solubilization

In vitro. Residual N at different times of incubation and ADIN in ground maize, soya-bean meal and three grain mixtures are shown in Table 3. N solubilization was very rapid reaching an asymptote beyond 24 h. ADIN was comparable to 24–48 h residual N in maize and grain mixture 4. Pool sizes and rate constants for different fractions of N are presented in Table 4. B_2 and B_3 fractions of PARN (intercepts derived from regression analysis of the curves obtained by curve peeling procedure) did not add up to 100%. Therefore, the difference between PARN at 0 h (100%) and the sum of B_2 and B_3 was considered as the instantly solubilizable pool (B_1), perhaps representing N solubilized in less than 15 to 30 min in the presence of protease enzyme. Hence, the rate-constant of ∞ was assigned to this fraction. Ground maize had more of the slowly-solubilizable fraction (B_3) than soya-bean meal. The rate constants for B_2 and B_3 fractions differed between feedstuffs.

In situ. The ADIN (pool C), buffer-soluble N (pool A) and PARN or pool B [total N − (pool A + pool C)] in Dacron bags at different incubation times are presented in Table 5. Since there was no non-linearity in the semilogarithmic plot of PARN in Dacron bags *v.* time, the rate-constant (k_s) for PARN was estimated by treating this fraction as a single pool. The k_s for grain mixtures varied from 0·02 to 0·10 h.

Evaluation of model for prediction of UDN

UDN predicted from the mathematical model (Van Soest *et al.* 1982) using in vitro rate constants were 7, 10 and 12% for grain mixtures 4, 6 and 7 respectively. However, the measured in vivo UDN (solving for A in eqn (1) by substituting the in vivo UDN values for lucerne hay (B) and maize silage (C) reported by Stern & Satter (1982) for sheep) for the three grain mixtures were 47, 66 and 59% respectively. Predicted UDN for twelve mixed diets using *in situ* rate-constants for PARN (Table 5) are plotted *v.* the in vivo UDN

Table 3. *Residual nitrogen at different times of incubation with protease solution (6·6 units/ml) at 39° and acid-detergent-insoluble N (ADIN) in ground maize, soya-bean meal and grain mixtures*

Period of incubation (h)	Residual N (% total N)				
	Ground maize	Soya-bean meal	Grain mixtures*		
			4	6	7
0	71·3	88·1	91·4	90·7	82·2
0·25	50·2	49·2	—	—	—
0·50	42·7	35·1	29·8	62·3	42·2
1	35·2	18·7	19·5	42·1	32·4
2	26·3	9·1	15·1	28·0	24·8
4	18·2	5·2	10·3	14·3	17·5
8	9·6	2·6	6·2	6·1	11·6
12	7·3	1·9	4·6	3·9	8·5
17	6·0	1·7	—	—	—
24	5·4	1·1	3·2	3·1	7·1
32	5·5	1·2	—	—	—
40	5·1	1·2	—	—	—
48	4·1	0·9	3·2	2·8	6·5
Unavailable N†	5·0	1·1	3·2	2·9	6·8
ADIN	4·6	2·7	3·5	6·5	10·8

* For composition, see Table 1.
† Mean of 24, 32, 40 and 48 h residual N.

in Fig. 1. The correlation between the two estimates (r^2 0·41; $P < 0·05$) was poor even though statistically significant.

Simulation of rumen proteolysis

V_{max} of whole rumen fluid (mg azocasein hydrolysed/h per ml) at 3 h before feeding and 1, 5, 9, 13 and 17 h after feeding were 2·4, 1·3, 1·3, 1·9, 3·5 and 3·4 respectively. The V_{max} of protease (mg azocasein hydrolysed/min per unit) was 1·36. Therefore, an enzyme concentration ranging from 0·02 to 0·05 units/ml incubation medium was considered to simulate the observed lower and upper V_{max} of rumen proteolysis. The pattern of soya-bean meal N solubilization at these two concentrations of enzyme is presented in Fig. 2.

Reproducibility. Residual N in soya-bean meal at different times of incubation with protease (22×10^{-3} units/ml) is presented in Table 6. A low coefficient of variation (less than 7%) indicates good reproducibility of the technique.

Comparison of in vitro and in vivo estimates of UDN. In vitro estimates of UDN for twelve complete mixed diets estimated according to eqn (1) are shown in Table 7 together with corresponding in vivo estimates. The correlation between the two estimates was statistically significant ($P < 0·01$), the r^2 value being 0·61.

DISCUSSION

Kinetics of digestion

In kinetic studies of fibre digestion in the rumen the need to determine the indigestible fractions in order to increase the accuracy of estimation of the rate-constants has been emphasized (Blaxter *et al.* 1956; Waldo & Smith, 1972; Mertens, 1973). It has also been pointed out that the potentially-digestible cell wall can have more than one rate-constant characteristic of the different cell wall components such as cellulose and hemicellulose (Smith

Table 4. *Pool size and the rate of degradation constants* (k_s) *for nitrogen fractions in ground maize, soya-bean meal and grain mixtures using protease solution (6·6 units/ml)*

Feedstuff	N fractions	Pool size: % PARN	Pool size: % total N	k_s (/h)	r^2
Ground maize	A	—	28·7	∞	—
	B_1	20·0	13·3	∞	—
	B_2	31·5	20·8	−1·56	0·99
	B_3	48·5	32·2	−0·22	0·99
	C	—	5·0	0	—
Soya-bean meal	A	—	11·9	∞	—
	B_1	13·6	11·8	∞	—
	B_2	70·9	61·7	−2·11	0·99
	B_3	15·5	13·5	−0·29	0·99
	C	—	1·1	0	—
Grain mixture					
4*	A	—	8·6	∞	—
	B_1	60·2	53·1	∞	—
	B_2	21·8	19·2	−1·38	0·92
	B_3	18·0	15·9	−0·21	0·99
	C	—	3·2	0	—
6*	A	—	9·3	∞	—
	B_1	8·3	7·3	∞	—
	B_2	47·9	42·0	−1·17	0·97
	B_3	43·8	38·5	−0·31	0·99
	C	—	2·9	0	—
7*	A	—	17·8	∞	—
	B_1	6·1	4·6	∞	
	B_2	62·1	46·8	−3·33	0·99
	B_3	31·8	24·0	−0·25	0·99
	C	—	6·8	0	—

PARN, potentially available residual N = total N − (pH 7·8 buffer-soluble N + unavailable N) (see Table 3). Table 3).

* For composition, see Table 1.

et al. 1971). Similar considerations apply to rumen protein digestion because of the heterogeneous nature of feed N (Pichard & Van Soest, 1977; Krishnamoorthy *et al.* 1982). Variable amounts of feed N can occur in the form of lignin, tannin–protein complexes and heat-damaged Maillard products that are highly resistant to mammalian and microbial enzymes. It has been suggested that ADIN can be used as an estimate of this fraction (Goering & Van Soest, 1970; Pichard & Van Soest, 1977). The potentially-digestible fraction of feed N can have different components varying in susceptibility to enzymic hydrolysis due to differences in physicochemical properties (Mahadevan *et al.* 1980). The heterogeneity of feed N fractions with respect to their susceptibility to enzymic hydrolysis can be detected under conditions where the rate of hydrolysis is not limited by the enzyme concentration (i.e. the reaction is first-order). The principle behind the technique employed here was similar to that used in the detection of individual radioisotopes in a mixture of two or three isotopes emitting similar radiation with varying half-lives (Wang *et al.* 1975). However, it is crucially important that in in vitro techniques to estimate solubilization rate-constants the enzyme concentration used is sufficient to saturate the substrate. Under conditions where the enzyme concentration is limiting, the end-products accumulated during the period of incubation can lead to progressive inhibition of enzyme activity causing a deflection in the

Table 5. *Buffer-(pH 6·7) soluble nitrogen, acid-detergent soluble N (ADIN), PARN in Dacron bag and* in situ *rate of disappearance constants* (k_s) *for PARN in grain mixtures*, lucerne* (Medicago sativa) *hay and maize silage*

Feedstuff	Total N × 6·25 (g/kg dry matter)	Buffer-soluble N (pool A)	PARN (pool B)	ADIN (pool C)	Percentage PARN in Dacron bag Period of incubation (h) 1	4	8	12	17	24	k_s for PARN (/h)	r^2
Grain mixture												
1	214	19·1	76·0	4·9	104·9	95·3	67·0	50·8	37·6	15·8	0·081	0·96
2	207	28·6	66·0	5·4	92·5	74·7	45·2	28·0	19·6	8·1	0·104	1·00
3	207	5·8	88·7	5·5	88·0	85·3	75·0	68·5	61·3	45·9	0·029	0·98
4	212	6·6	89·9	3·5	88·3	86·8	78·6	68·7	65·1	53·3	0·024	0·97
5	210	18·5	77·1	4·4	91·1	84·1	57·0	44·0	21·8	15·4	0·082	0·98
6	218	5·8	87·7	6·5	92·0	88·7	84·4	77·8	70·1	63·3	0·018	0·98
7	195	15·6	73·6	10·8	76·2	70·2	44·3	34·8	6·9	14·1	0·098	0·80
8	199	24·9	59·4	15·7	80·3	74·6	70·5	56·5	38·9	35·8	0·042	0·95
9	145	31·2	62·1	6·7	74·8	68·9	66·4	61·6	57·2	44·9	0·026	0·86
10	212	14·5	80·0	5·5	86·6	84·7	78·7	74·3	61·1	52·4	0·024	0·96
11	281	1·8	93·4	4·8	78·3	75·2	71·8	70·6	63·3	51·6	0·021	0·86
12	346	8·1	87·0	4·9	86·4	84·4	81·6	78·4	73·0	64·3	0·015	0·91
Lucerne hay	149	26·2	63·0	10·8	85·8	71·9	58·0	49·6	39·6	27·8	0·051	0·98
Maize silage	73	49·8	44·3	5·9	113·0	92·9	79·3	75·9	55·3	46·0	0·036	0·96

PARN, potentially avaiable residual N = total N − (buffer-soluble N + ADIN).
* For composition, see Table 1.

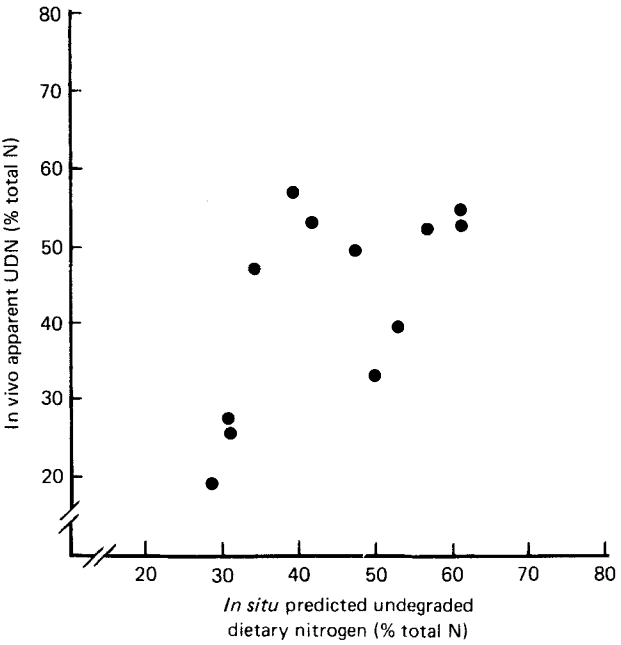

Fig. 1. Relationship between *in situ* predicted undegraded dietary nitrogen (UDN) (% total N) and in vivo apparent UDN (% total N) for twelve mixed diets (r^2 0·41, $P \leqslant 0{\cdot}05$).

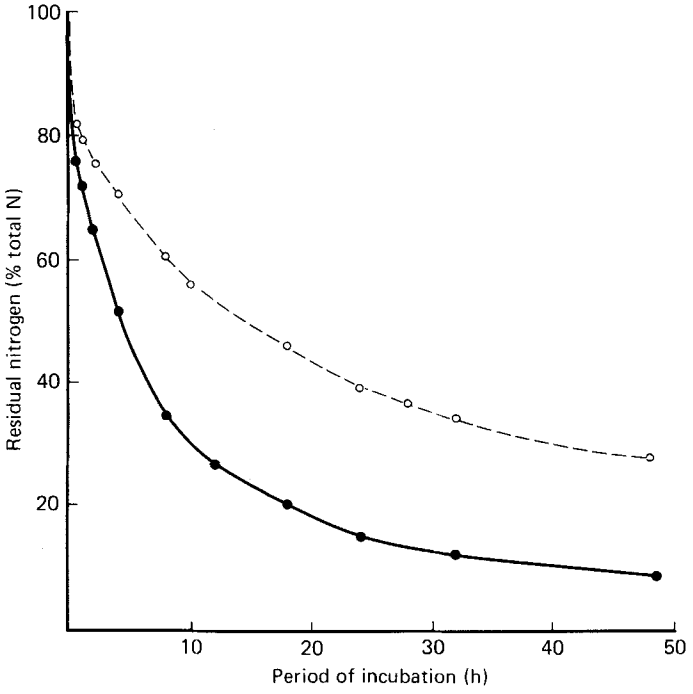

Fig. 2. Pattern of soya-bean meal nitrogen solubilization in protease solution of two concentrations: (●), 66×10^{-3} units/ml; (○), 22×10^{-3} units/ml.

Table 6. *Reproducibility of in vitro protease technique, as indicated by incubations of soya-bean meal in protease solution*

Period of incubation (h)	No. of observations	Residual nitrogen (% total N)		Coefficient of variation (%)
		Mean	SE	
0·5	9	81·3	0·35	1·3
1	10	79·6	0·27	1·1
2	10	75·5	0·30	1·2
4	10	70·4	0·36	1·6
6	9	65·5	0·32	1·5
8	10	60·9	0·61	3·2
10	10	56·1	0·93	5·2
18	9	46·0	0·96	6·2
24	10	39·3	0·83	6·7
28	10	36·8	0·44	3·8
32	10	33·7	0·35	3·3
48	10	27·5	0·21	2·5

Table 7. *Comparison of in vitro and in vivo estimates of undegraded dietary nitrogen (UDN) for mixed rations*

Diet*	Estimates of UDN (% total N)	
	in vitro†	in vivo‡
1	33	27
2	37	20
3	32	34
4	39	40
5	28	29
6	63	55
7	47	48
8	48	54
9	42	56
10	55	49
11	63	52
12	65	54

* For composition, see Table 1.
† Calculated from 18 and 48 h protease residual N, see p. 558 and eqn (1).
‡ Stern & Satter (1982) and Stern *et al.* (1983).

solubilization curve which might be attributed to the presence of a different fraction of N. With *in situ* techniques, this problem is considered not to arise because of the small quantity of test substrate relative to rumen volume, the continuous removal of fermentation end-products either through passage or absorption and the continuous production of enzymes by the microbes. Perhaps for these reasons, nutrient digestion in the rumen is generally considered to follow first-order kinetics despite the lack of unequivocal evidence to support that view.

Rates of N solubilization

In vitro. The rapid decline in residual N in all the feedstuffs, irrespective of protein content, followed by the asymptote beyond 24 h indicated a residual N fraction resistant to enzymic

degradation. Residual N between 24 and 48 h was lower than the ADIN for soya-bean meal and grain mixtures nos. 6 and 7. This contrasts with the findings of Pichard & Van Soest (1977) who reported that the ADIN was lower than 48 h protease residual N but the reason for this discrepancy may be the higher concentration of enzyme used in the incubations in this study. A higher value of ADIN compared with protease residual N indicates the presence of small quantities of proteins that have been precipitated by acid-detergent solution (see Krishnamoorthy *et al.* 1982). Therefore in the present experiments, instead of ADIN, the 24 h or 48 h residual N or both was considered as the ultimately unavailable N to derive PARN. The presence of fractions in PARN (Table 4) with varying degradation rate-constants confirms the heterogeneity of feed N and illustrates that such fractions could be identified by the technique used here.

In situ. The single rate-constant (accompanied by high r^2 value) for PARN of grain mixtures (Table 5) reflects linearity in the ln plot of N disappearance from the bag *v.* time and is consistent with homogeneity of the N source. However, this conclusion is difficult to reconcile with the fact that the grain mixtures contained various N sources (Table 1), differing in susceptibility to enzymic hydrolysis (see Table 4 for ground maize and soya-bean meal). Failure to identify more than one fraction from the kinetic analysis of Dacron bag values could be due to the lack of sensitivity of the technique.

Even though the in vitro protease procedure was more sensitive than the *in situ* technique for the study of N solubilization kinetics, solubilization in protease solution was rapid compared with that in *in situ* (Tables 3–5). This may be due to the substrate-saturating enzyme concentration used in the incubation medium to create substrate-limiting conditions. However, if protein substrates in the rumen are limiting and microbial enzyme activity is in excess (Pichard, 1977; Broderick, 1978) the rate-constants for solubilization obtained from *in situ* and in vitro should have been in reasonable agreement.

The lower sensitivity of the Dacron bag technique to changes in the kinetics of digestion and the high degradation rates obtained from the kinetically sensitive protease technique question the suitability of the rate-constants from either technique to predict UDN through mathematical models based on first-order kinetics.

Evaluation of the model for prediction of UDN

Mathematical models proposed so far (Ørskov & McDonald, 1979; Broderick & Craig, 1980; Van Soest *et al.* 1982) are similar in their assumption of heterogeneity of feed N and first-order kinetics for the rates of passage and degradation. However, the one proposed by Van Soest *et al.* (1982) offers potential advantages because it incorporates a lag factor. This factor allows for the fact that the process of digestion of insoluble nutrients can begin only after hydration and microbial contact with feed particles has occurred. Since passage of UDN from the rumen is a function of the interaction between rates of passage and digestion, a delay in the onset of digestion can increase the UDN value to a considerable extent.

However, UDN predicted from this model using in vitro rate constants resulted in a gross underestimate when compared with in vivo estimates. This is consistent with the view that the first-order in vitro rate-constants for N solubilization do not reflect the rate of degradation in the rumen. An overestimated rate of digestion from the in vitro protease technique could possibly be rectified by reducing enzyme concentration in the incubation medium to simulate rumen proteolysis, under which circumstances the reaction is likely to deviate from first-order due to limiting enzyme activity (Sniffen *et al.* 1979; Van Soest *et al.* 1982). This poses an interesting problem as to whether protein solubilization in the rumen (*in situ*) can be treated as a first-order reaction. While the experiments by Broderick (1978) support first-order kinetics, those of Nugent & Mangan (1981) demonstrate that it could

be zero as well as first-order. As the proteolytic activity in the rumen fluctuates to a considerable extent (Krishnamoorthy, 1982) and the concentration of protein substrates in the rumen can vary in relation to amount and frequency of feeding, it is possible that the proteolysis in the rumen can follow zero-, second- and first-order kinetics. Fluctuation of proteolytic activity may be one of the reasons for the insensitivity of the Dacron bag technique to identify subfractions of PARN, though the lack of sensitivity could also reflect influx and efflux of feed particles from the bag and microbial contamination of feed N (Mathers & Aitchison, 1981). A better understanding of the kinetics of N digestion in the rumen is essential to develop improved prediction models and techniques to estimate the criteria required in such models.

Simulation of rumen proteolysis in vitro

The major difficulty in simulating rumen proteolysis is the fluctuation in proteolytic activity of rumen fluid and uncertainty about substrate-limiting conditions in the rumen. An earlier study (Sniffen *et al.* 1979) showed that under enzyme-limiting conditions the quantity of protein solubilized in a given time varies with sample size and enzyme concentration. Therefore, other factors associated with rumen dynamics were taken into account in deciding the conditions for the simulation. The sample size in the incubation medium was decided on the basis that the dry matter content of rumen digesta was normally 50–200 g/kg. The buffer volume was decided on the basis of estimates of rumen liquid dilution rate (for example, a 10 ml incubation medium containing 50 g dry matter/kg, at a dilution rate of 0·08 h for 48 h would require an additional 40 ml buffer). The choice of an enzyme concentration (66×10^{-3} units/ml incubation medium, equivalent to 20% greater than the maximum V_{max} observed in vivo) was designed to bring about the maximum extent of degradation. The low dry matter, high dilution rate and marginally higher concentration of enzyme chosen were beneficial from the standpoint of minimizing end-product inhibition. The pH of the incubation medium was kept at the optimum for the enzyme.

The significance of enzyme concentration on N solubilization is shown in Fig. 2. Another factor of note in this figure is the importance of the duration of incubation on N solubilization. If the incubation time is equated with the mean retention time of feed in the rumen, and N solubilized with RDN, the residual N at different times of incubation should reflect variations in UDN resulting from varied retention times. However, from a practical point of view, it is desirable to have values for UDN which apply under typical feeding conditions. Thus, a sample size of 0·5 g incubated for 18 h in the case of concentrates and 48 h for forages may be considered adequate unless true retention times have been previously determined.

Comparison of in vitro and in vivo estimates of UDN. The in vivo and in vitro estimates of UDN were in good agreement (r^2 0·61; $P < 0·01$). However, it is important to recognize some problems associated with these estimates. Because of the failure to correct duodenal non-ammonia, non-bacterial N for protozoal and endogenous N the in vivo values for UDN may be overestimates. The N:DAPA value found in isolated rumen bacteria was used to estimate bacterial N in duodenal digesta and contamination of the isolated rumen bacteria with feed proteins (Nugent & Mangan, 1981) could lead to an overestimate of duodenal bacterial N passsage and thus to an underestimation of UDN. In vitro estimates are similarly subject to errors due to the assumption that all solubilized N is completely degraded in the rumen. However, this assumption is acceptable for feedstuffs wherein solubilization of insoluble proteins is the step limiting the pool size of soluble proteins in the rumen and where only a small amount of soluble true protein escapes degradation.

Because in vivo estimates of true UDN reported in the literature are scarce, and due to the continuing problem in their estimation, the validity of this in vitro simulated rumen

proteolysis procedure to estimate true UDN cannot be fully assessed. However, the technique can be recommended to evaluate feedstuffs on a relative basis and appears to provide results in reasonable accord with those obtained in vivo. It also offers the potential to allow assessment of the influence of rumen dynamic criteria such as proteolytic activity and retention of feed, as well as level of feeding, on the predicted UDN values by manipulation of enzyme concentration, incubation time and sample size in the incubation medium.

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