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COMPARISON OF THREE APPROACHES OF ESTIMATING PROTEIN B2 AND B3 DEGRADATION RATES IN THE RUMEN OF SHEEP

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ABSTRACT: *A method that involved the gravimetric measurement of the amounts of feed protein B2 (feed protein that is insoluble in borate phosphate buffer but soluble in neutral detergent solution) and protein B3 (feed protein that is insoluble in neutral detergent solution but soluble in acid detergent solution) that remain after each in situ incubation period, was used to obtain the degradation rates of these protein pools in six different feeds. These degradation rates were then compared with degradation rates provided by the Cornell Net Carbohydrate and Protein System for nominally similar feeds in order to establish the extent of agreement between these sets of data. Curve peeling technique was also used on the in situ results of this experiment to generate degradation rates for comparison with the gravimetric and the Cornell values. The study showed that the gravimetric, the curve peeling and the Cornell values were not statistically different for the degradation rates of protein B2 even though the gravimetric estimates were the highest followed by curve peeling and then the Cornell values. For protein B3, the degradation rate estimated with the gravimetric method was highest followed by the curve peeling method and then the Cornell values (P<0.01). The degradation rates assigned to protein B3 in the Cornell databank needs re-examination. There is a need for further application of the gravimetric technique to establish if it gives higher estimates of the degradation rates of proteins B2 and B3 in a range of feedstuffs.*

ORIGINAL ARTICLE

Keywords: *Gravimetric method, Cornell, In situ, Degradation rate, Curve peeling*

INTRODUCTION

Many systems, including the UK system of protein evaluation depend on the in situ method to estimate the rumen degradable protein (RDP) (Van Duinkerken et al., 2010). An alternative method, the Cornell model, adopted in the USA has a sub-model that proposes the subdivision of the soluble protein into A and B1 fractions, and the insoluble protein into B2, B3 and C fractions (Lanzas et al., 2008).

For a variety of ruminant feedstuffs, statistically significant correlations between the Cornell and the in situ techniques for estimating RDP have been observed (Avornyo, 1999; Shannak et al., 2000). However, RDP estimated by the Cornell method tended to be lower than the analogous in situ value for ordinary feeds and vice versa for protected feeds. The lack of consistency with Cornell and in situ RDPs for protected and unprotected feeds may be partly due to the cumbrousness of the in situ procedure (Mathis et al., 2001) but may be also due to the degradation rates adopted by the Cornell databank, which can be verified (Lanzas et al., 2008).

The Cornell group utilized in situ and enzymatic data followed by a curve-peeling technique to estimate the degradation rates of protein pools (Sniffen et al., 1992). The use of curve peeling on in situ data introduces an element of subjectivity in the measurement of the rates of degradation, and may affect the accuracy of the results. A way to overcome these problems is to measure the amount of each protein fraction that remains in the in situ residue at each incubation period. A plot of the fraction disappearance against time will generate a curve whose rate will be that of the fraction. This method can however only be applied to protein fractions that are insoluble but degradable over time. These are proteins B2 and B3 since proteins A and B1 are assumed to disappear almost instantly. Protein C on the other hand cannot be degraded and hence has zero degradation rate (Gosselink et al., 2004).

The study therefore aimed to estimate the in situ rates of degradation of protein B2 and B3 fractions gravimetrically and by curve-peeling using six fibrous feeds whose degradability values had been corrected for microbial contamination by the ^{35}S technique.

MATERIALS AND METHODS

Samples

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The samples analyzed in this experiment were the original samples and the in situ residues of dried lucerne (DL; code D 1576, commercial product), maize silage (MS; code D 1578, variety Hudson from a private farm, not wilted, clamp; additive: Bibby maize sil.), peahaulm silage (PHS; code D 1582, from IGER, Aberystwyth, UK), fermented whole crop wheat (FWCW; code D 1583, variety Riband, clamp with additives: salts and inoculants, from Cambridge University Dairy Unit, UK) and two grass silages (Cambridge University Dairy Unit grass silage (DUGS; code D 1579, perennial rye grass wilted, clamp) and a private farm grass silage (PFGS; code D 1580, mainly perennial rye grass, wilted, tower silage, no additive)).

Feed Analyses

The Cornell method: Samples of DL, MS, PHS, FWCW, DUGS and PFGS were taken. The wet samples among them were first oven-dried at 55^0C to steady state. Then all the samples were milled through a 1 mm sieve and analyzed in triplicate. The Cornell fractions were determined with the method proposed by Licitra et al. (1996).

The in situ method: A corresponding in situ experiment was carried out on DL, MS, PHS, FWCW, DUGS and PFGS to estimate the degradation rates of proteins B2 and B3. The method provided by the AFRC Technical Committee (1992) for estimating protein loss from in situ bags was followed. Monofilament polyester bag (20 x 9 cm) with a pore size of 41 μ was used. Each bag contained approximately 5 g dry matter of sample.

Ruminal infusion of ^{35}S: A stock solution with a concentration of 20 μCi/ml (^{35}S in the form of $Na_2{}^{35}SO_4$) containing 100 μg/ml anhydrous Na_2SO_4 was made. Some of the stock solution was further diluted with water in a large bottle to supply 190 μCi/sheep/day. Dosing of sheep with the 190 μCi of ^{35}S was continuous at 480 ml of water per sheep per day through tubes with the help of a peristaltic pump. Microbial protein formed in the rumen of sheep was marked with ^{35}S by the continuous intra-ruminal infusion of $Na_2{}^{35}SO_4$ for a total of two periods of 14 days each. The method used to obtain solid-associated microbes was originally reported by Whitehouse et al., (1994).

The incubation procedure: Two bags were staggered on each semi-rigid stalk and wetted before placing in the rumen of sheep. Bags were inserted simultaneously into all three sheep. Incubation times were 0, 2, 5, 8, 16, 24, 48 and 72 h. Zero hour disappearance values were obtained by subjecting duplicate bags containing sample material to 24 min of machine washing with the continuous flow of cold water. There were six replicates (3 sheep x 2 trials) of each feed for each incubation time. When removed, the bags were machine-washed and rinsed with cold water in a wash cycle similar to the zero hour determinations. Bags containing the feed residues were then stored frozen at -20^0C followed by freeze drying. All residues, in bags, prior to analysis were vacuum-dried at 70^0C for 1h. On drying, the bags were placed in a dessicator and weighed. After weighing, the samples were allowed to air-equilibrate before grinding to 1 mm to ensure homogeneity, and analyzed for dry matter by the Association of Official Analytical Chemists (AOAC) method, crude protein (Kjeldahl) and microbial contamination.

^{35}S:NAN ratio: Estimation of the extent of microbial contamination of in situ bag residues used a method similar to that described by Mathers and Aitchison (1981). The ^{35}S:N (disintegrations/min per mg N) was computed for both microbe and residue. The proportion of microbial N in residual N was calculated as: ^{35}S:N (residue)/^{35}S:N (microbial).

The Gravimetric Method

Protein B2 pool by the gravimetric method: Estimation of the protein B2 pool by the gravimetric method was done on zero h in situ residues. About 0.3 g of zero h residues were boiled in neutral detergent (ND) solution following the recommended method of Licitra et al. (1996) for the estimation of the neutral detergent insoluble protein (NDIP) content of a feed. The pool size of protein B2 was estimated using the following equation:

$$PB2_{0\ h\ residue}\ (g) = CP_{0\ h\ residue}\ (g) - NDIP_{0\ h\ residue}\ (g), \quad (1)$$

where:

$PB2_{0\ h\ residue}$ was the estimated protein B2 content of the feed, by the gravimetric method.

$CP_{0\ h\ residue}$ was the crude protein content of the zero h residue, and

$NDIP_{0\ h\ residue}$ was the neutral detergent insoluble protein content of the zero h residue.

Degradation rate of protein B2: About 0.3 g samples of in situ residues, of known CP concentrations, obtained at the various incubation times were boiled separately in neutral detergent solution to dissolve any remaining protein B2. In terms of equation, the amount of protein B2 remaining in the in situ residue at any incubation time was given by:

$$PB2_{residue}\ (g) = CP_{residue}\ (g) - NDIP_{residue}\ (g) - MN_{residueNDS}\ (g), \quad (2)$$

where:

$PB2_{residue}$ was the protein B2 remaining in the residue,

$CP_{residue}$ was the protein content of the residue,

$NDIP_{residue}$ was the neutral detergent insoluble protein content of the residue, and

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$MN_{residueNDS}$ was the microbial protein solubilized by the ND method, which was found to be 0.975 of the microbial protein associated with the residue (Avornyo, 1999).

The protein B2 disappearing at any incubation period was given by:

$$PB2_{lost}\,(g) = PB2_{feed}\,(g) - PB2_{residue}\,(g), \quad (3)$$

where:

$PB2_{lost}$ was the protein B2 lost during the incubation,

$PB2_{feed}$ was the protein B2 content of the original feed before incubation, determined by the Cornell chemical analysis, and

$PB2_{residue}$ was the protein B2 remaining in the residue.

Protein B3 pool by the gravimetric method: Estimation of the size of protein B3 by the gravimetric method was done on the zero h in situ residues. About 1 g samples of the zero h residues were boiled in acid detergent (AD) solution using the procedure described by Licitra et al. (1996) for the estimation of acid detergent insoluble protein (ADIP). The amount of protein B3 was given by:

$$PB3_{0\ h\ residue}\,(g) = NDIP_{0\ h\ residue}\,(g) - ADIP_{0\ h\ residue}\,(g), \quad (4)$$

where:

$PB3_{0\ h\ residue}$ was the estimated protein B3 content of the feed, by the gravimetric method.

$NDIP_{0\ h\ residue}$ was the neutral detergent insoluble protein content of the zero h residue.

$ADIP_{0\ h\ residue}$ was the acid detergent insoluble protein content of the zero h residue.

Degradation rate of protein B3: About 1 g residue samples obtained at all the incubation time points were boiled individually in acid detergent solution and the indigestible protein C content estimated. In terms of equation, the amount of protein B3 remaining in the in situ residue at any incubation time was given by:

$$PB3_{residue}\,(g) = NDIP_{residue}\,(g) - ADIP_{residue}\,(g) - MN_{residueNDI}\,(g), \quad (5)$$

where:

$PB3_{residue}$ was the protein B3 remaining in the residue,

$NDIP_{residue}$ was the neutral detergent insoluble protein content of the residue,

$ADIP_{residue}$ was the acid detergent insoluble protein content of the residue, and

$MN_{residueNDI}$ was the microbial protein insoluble in the ND method, that is, 0.025 of the microbial protein associated with the residue.

The protein B3 disappearing at any incubation period was given by:

$$PB3_{lost}\,(g) = PB3_{feed}\,(g) - PB3_{residue}\,(g), \quad (6)$$

where:

$PB3_{lost}$ was the protein B3 lost during incubation,

$PB3_{feed}$ was the protein B3 content of the original feed before incubation, determined by the Cornell chemical analysis, and

$PB3_{residue}$ was the protein B3 remaining in the residue.

Curve peeling: The curve peeling technique described by Nocek and English (1986) was employed. The equation for describing the constants of each linear component of the curve was:

$$R = -kt + R_0 \quad (7)$$

where:

R was the natural logarithm of percent available protein of the feed protein that is present in the residue at time t,

k was the degradation rate constant of the protein fraction,

t was the incubation time,

R_0 was the natural logarithm of percent available protein of the feed protein that is present at incubation time zero.

Calculations and statistical analyses

The cumulative disappearance of each feed protein B2 and B3 fractions were regressed against time.

Two-way Analysis of Variance (ANOVA) was used to remove the effect due to feed (block) and compare the means due to the method (treatment) of estimating the feed protein fraction. The GLM procedure of SAS (1990) was used to compare the means from the degradation rate data.

RESULTS

The disappearance curves

Feed protein fraction disappearance after correcting for microbial contamination by ^{35}S is shown in Figures 1 and 2. Information is given on those fractions in which a regression of their disappearance on time was statistically significant. Protein B2 loss at zero hour incubation ranged from 0.17 in FWCW to 0.69 of total protein B2 in MS (Figure 1b). As incubation progressed, the cumulative fractional protein B2 degradation tended to exceed unity. A slow protein B2 disappearance was observed for PHS.

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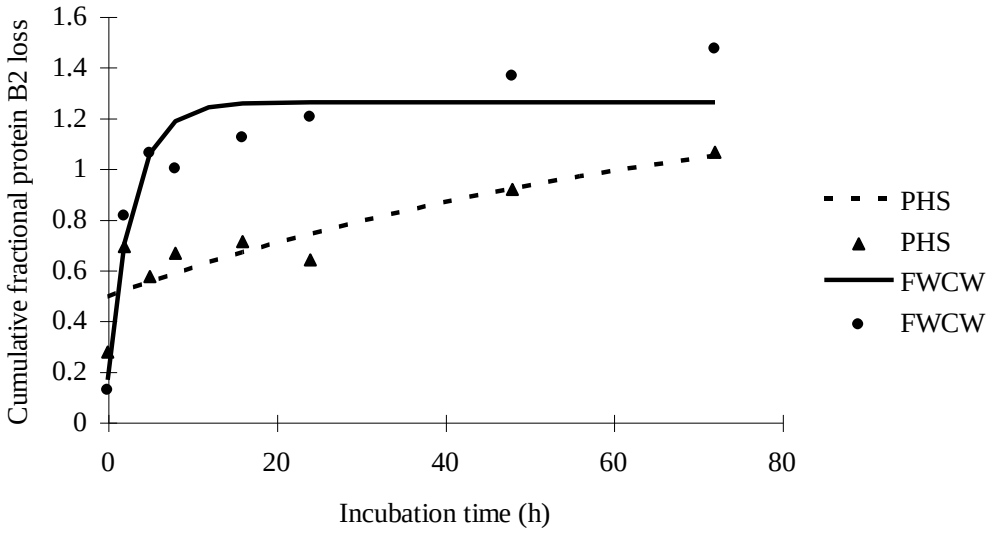

Figure 1a - Protein B2 disappearance in peahaulm silage (PHS) and fermented whole crop wheat (FWCW) after correction for microbial contamination of in situ residues by the ^{35}S technique

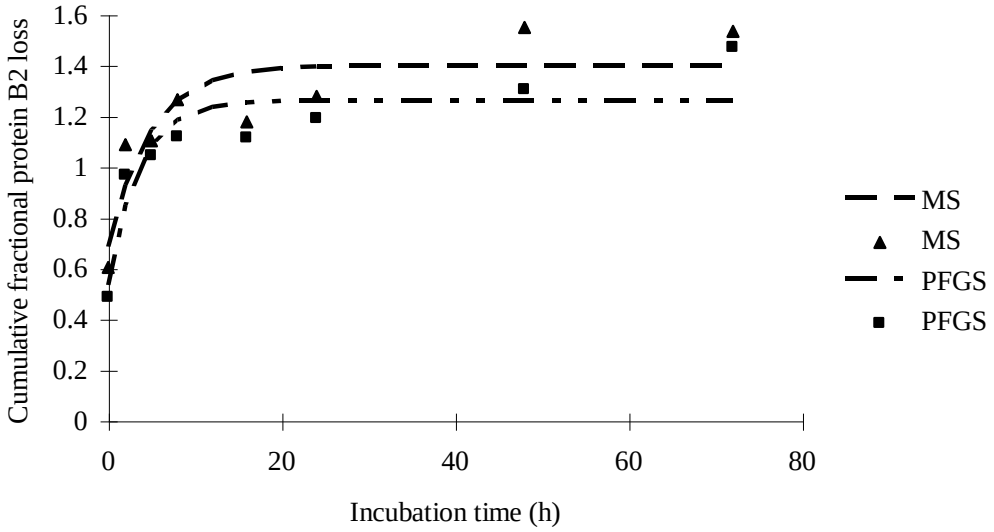

Figure 1b - Protein B2 disappearance in maize silage (MS) and private farm grass silage (PFGS) after correction for microbial contamination of in situ residues by the ^{35}S technique

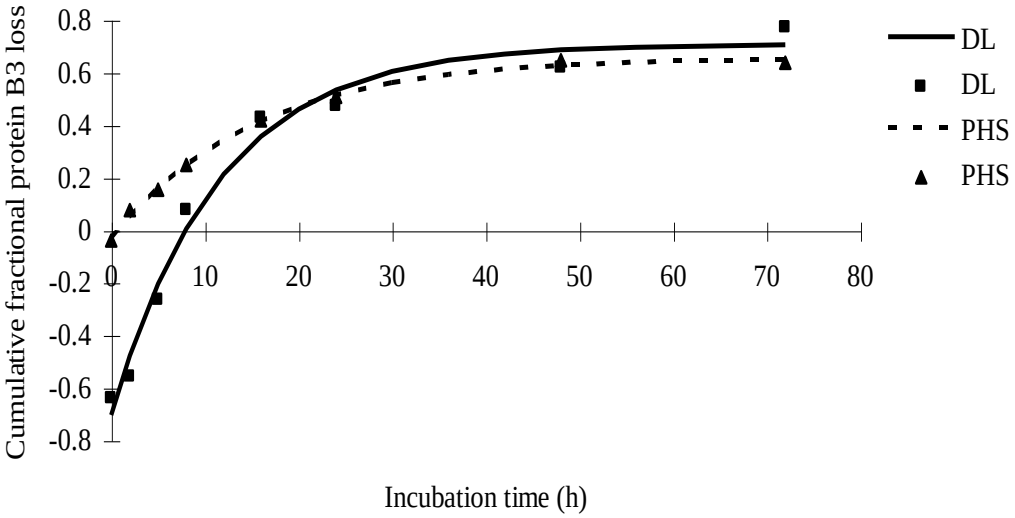

Figure 2a. Protein B3 disappearance in dried lucerne (DL) and peahaulm silage (PHS) after correction for microbial contamination of in situ residues by the ^{35}S technique

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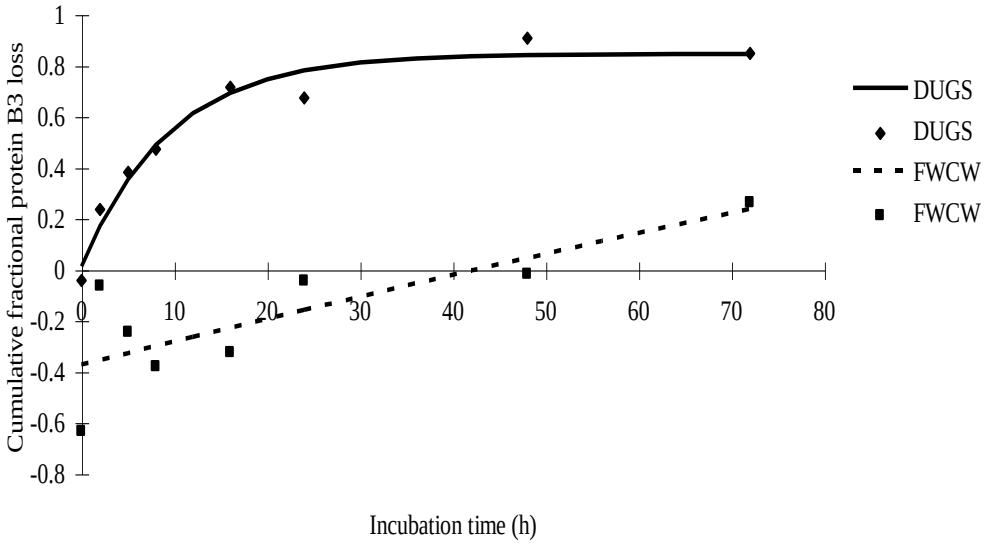

Figure 2b - Protein B3 disappearance in dairy unit grass silage (DUGS) and fermented whole crop wheat (FWCW) after correction for microbial contamination of in situ residues by the ^{35}S technique

The washing loss of protein B3 of DL and FWCW were negative values (Figures 2a and 2b) and the asymptotes of the curves were below one.

Feed fractions

Soluble crude protein determined with borate phosphate buffer was compared with that estimated as the quickly degradable protein "a" in the in situ procedure (Table 1). While the values by the two methods correlated (r^2 0.80; P<0.05), protein solubility in borate buffer was considerably lower than that estimated by the in situ method (P<0.01).

Table 1 - Protein soluble in borate phosphate buffer (Cornell) and the in situ soluble crude protein ("a") obtained by fitting ^{35}S corrected nitrogen disappearance data to the exponential equation of Mehrez and Orskov (1977) for the test feeds[a], fraction of total protein

	Feed							
Method	**DL**	**MS**	**PHS**	**DUGS**	**PFGS**	**FWCW**	**Mean**	**P**
Cornell	0.35	0.56	0.41	0.61	0.70	0.74	0.56	
In situ	0.57	0.73	0.58	0.74	0.86	0.74	0.70	0.022; P<0.01

[a]Feed: DL, dried Lucerne; MS, maize silage; PHS, peahaulm silage; DUGS, dairy unit grass silage; PFGS, private farm grass silage; FWCW, fermented whole crop wheat

The protein B2 pools by the gravimetric method were compared with those by the Cornell chemical method and by the curve peeling method (Table 2). There was largely no correlation between the values produced by the methods. The results of the Cornell chemical method were higher than the equivalent gravimetric and curve peeling methods (P<0.01). The data sets produced by the gravimetric and curve peeling did not differ significantly.

Table 2 - The protein B2 and B3 (fraction of total protein) pools, of the analyzed feeds[a], estimated by the Cornell chemical method, the gravimetric method and the curve peeling method

	Protein B2			Protein B3		
Feed	**Gravimetric**	**Curve peeling**	**Cornell**	**Gravimetric**	**Curve peeling**	**Cornell**
DL	0.12	0.21	0.42	0.26	0.14	0.16
MS	0.13	0.05	0.33	0.13	0.14	0.05
PHS[b]	0.26	0.04	0.37	0.17	0.41	0.16
DUGS	0.10	0.05	0.16	0.19	0.16	0.18
PFGS	0.10	0.10	0.19	0.01	0.05	0.07
FWCW	0.14	0.14	0.16	0.07	0.02	0.04
Mean	0.14	0.10	0.27	0.14	0.15	0.11
s.e.m.; P[c]		0.032; P<0.01			0.029; P=0.58	
[d]r^2		0.12	0.21		0.23	0.59
P[e]		*P*=0.51	*P*=0.36		*P*=0.34	*P*=0.07

Feed: DL, dried lucerne; MS, maize silage; PHS, peahaulm silage; DUGS, dairy unit grass silage; PFGS, private farm grass silage; FWCW fermented whole crop wheat. [b]peas in the sample were not crushed before the in situ analysis. [c]P was the level of significance of the difference between the means. [d]r^2 was obtained by relating the gravimetric values to the other values. [e]*P* was the corresponding significance of the r^2 values.

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Protein B3 sizes by the three methods did not reveal significant difference although curve peeling was the highest followed by the gravimetric and then the Cornell estimates.

Degradation rates

The rates of degradation of the protein B2 pools determined gravimetrically, with the exception of PHS and MS, tended to be higher than those given in the Cornell data bank, and determined by curve peeling (Table 3). The protein B2 rates of degradation were not significantly correlated between the methods. However, when the three methods were compared again with regard to their estimation of the degradation rate of protein B3, a real difference (P<0.01) was revealed with the gravimetric values being the highest followed by curve peeling before the Cornell data bank values.

Table 3 - The degradation rates (per hour) of the feed[a] proteins B2 and B3 fractions estimated by the Cornell[b] chemical method, the gravimetric method and the curve peeling method

Feed	Protein B2			Protein B3		
	Gravimetric	Curve peeling	Cornell	Gravimetric	Curve peeling	Cornell
DL	NE	0.20	0.09	0.09	0.04	0.01
MS	0.20	0.26	0.12	NE	0.06	0.002
PHS[c]	0.01	0.21	0.14	0.07	0.02	0.02
DUGS	NE	0.11	0.15	0.10	0.04	0.01
PFGS	0.30	0.08	0.15	NE	0.03	0.01
FWCW	0.30	0.05	0.10	0.002	0.01	0.002
Mean	0.20	0.15	0.13	0.07	0.03	0.01
(±)s.e.m.	0.051	0.042	0.042	0.010	0.009	0.009
P[d]		P=0.53			P<0.01	
[e]r^2		0.47	0.10		0.85	0.32
P[f]		*P*=0.31	*P*=0.68		*P*=0.09	*P*=0.43

Feed: DL, dried lucerne; MS, maize silage; PHS, peahulm silage; DUGS, dairy unit grass silage; PFGS, private farm grass silage; FWCW fermented whole crop wheat. [b]The Cornell values were taken from their data bank that has been installed on a computer in the Nutrition Laboratory, 307 Huntingdon Road, Cambridge, UK. [c]peas in the sample were not crushed before the in situ analysis. NE, not estimated. [d]P was the level of significance of the difference between the means. [e]r^2 was obtained by relating the gravimetric values to the other values. [f]*P* was the corresponding significance of the r^2 value.

Table 4 - A comparison of the amounts of protein B2 and B3 measured in the original feed sample and in its zero h in situ (gravimetric determination) residue. Values are fraction of total feed protein

Feed[b]	Protein fractions[a]					
	PB2 in original feed	PB2 in zero h residue	PB3 in original feed	PB3 in zero h residue	B2+B3 in original feed	B2+B3 in zero h residue
DL	0.42	0.11	0.16	0.26	0.58	0.37
MS	0.33	0.13	0.05	0.14	0.38	0.27
PHS	0.37	0.26	0.16	0.16	0.53	0.42
DUGS	0.16	0.09	0.18	0.19	0.34	0.28
PFGS	0.19	0.10	0.07	0.01	0.26	0.11
FWCW	0.16	0.14	0.04	0.06	0.20	0.20
Mean	0.27	0.14	0.11	0.14	0.38	0.28
s.e.m; P	0.030; P<0.05		0.017; P=0.28		0.021; P<0.05	

Feed: DL, dried lucerne; MS, maize silage; PHS, peahulm silage; DUGS, dairy unit grass silage; PFGS, private farm grass silage; FWCW fermented whole crop wheat. [a]Protein fractions: PB2, protein B2; PB3, protein B3.

DISCUSSION

The gravimetric method quantitatively measured feed protein fraction disappearance over time. In the process, many variables such as dry matter (DM), crude protein (CP), NDIP, ADIP and microbial contamination were estimated on the in situ residue, each with its associated error. Therefore feeds with a low amount of proteins B2 and B3, for example, PFGS with only 0.07 and MS with only 0.05, of CP as protein B3, did not produce meaningful curves for these fractions.

The in situ prediction of the immediately soluble and completely degradable protein "a" was higher when compared to the protein soluble in borate buffer. An examination of the measured zero h residue indicated that apart from a suggestion of fine particle loss, there was probably a redistribution of the protein B2 fraction. Some amount of protein B2 probably became soluble and was lost during the machine washing, which explains the bigger in situ "a" than the Cornell soluble crude protein (Table 1). Table 4 also shows a lower value for protein B2+B3 in the zero h residue compared to that in the original feed. This was in spite of the apparently bigger values for protein B3 in the zero h residues compared to that in the original feed. Hence there was a bigger loss of protein B2 in the zero h residue than could be offset by the increase in protein B3 in the zero h residue. Pichard and Van Soest (1977) have noticed the presence of a very rapidly degradable insoluble protein fraction with a half-life of about 10 min. After only machine washing of the feeds to determine the zero h incubation, the protein B2 of the resulting residue was found to be lower (P<0.05) than that measured in the feed while the protein B3 appeared to be higher than that determined in the feed. It is likely that the in situ process had affected protein B2 to render a portion

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resistant to solubility in neutral detergent solution. Lanzas et al. (2008) arrived at a conclusion that in order to improve upon the Cornell protein model, protein fractions B2 and B3 should be merged because they were convinced that B3 was degraded at a faster rate than given in the Cornell model. The new rates they assumed were still found to be too slow for prediction of undegraded B3 flow at the omasum. Their new B3 degradation rates still seem to be lower than the rates determined by the gravimetric method.

Summation of the B2 and B3s of the feeds studied was 0.10 less in the zero h residue than the original feed (Table 4). This means varying amounts of protein B2 were also probably lost during machine washing.

The separate comparisons of the protein B2 and B3 sizes obtained by curve peeling in this study, and those reported in Mansbridge (1996) also indicated a lower protein B2 and a higher protein B3 than estimated by the Cornell chemical method. The method of curve peeling may be less accurate because at shorter incubation time points, substantial amounts of protein B2 would have disappeared from in situ bags. The rate of degradation of protein B2 is therefore likely to be underestimated by the curve peeling method. Suggestively higher degradation rates were estimated by the gravimetric method that calls for an evaluation of the Cornell data bank values. The lack of a good correlation between the rates estimated by the gravimetric method and by curve peeling or from the Cornell data bank may be because the Cornell degradation rates were those of feeds which were only nominally similar to the ones studied, and would have different physical and chemical characteristics.

CONCLUSION

The gravimetric method gave higher rates of degradation of protein B2 and B3 ($P<0.01$) of the feeds, when compared to those listed in the Cornell data bank and those determined by curve peeling. Further testing of the gravimetric technique may be needed to ascertain its usefulness.

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