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Estimating Forage Protein Degradation in the Rumen

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ABSTRACT: Forage proteins are degraded rapidly by rumen microorganisms and therefore supply relatively small quantities of undegraded intake protein (UIP). Growing cattle with high metabolizable protein requirements and lactating beef and dairy cows respond to UIP supplementation when fed high-forage diets, even though degradable intake protein (DIP) is adequate. This observation suggests that an accurate estimate of forage UIP is needed to establish optimal supplementation conditions. Microbial protein must be quantitated in duodenal or in situ residue samples to accurately measure forage UIP. Purines commonly are used as a microbial protein marker. Recent reports suggested that the original purine procedure generates interfering compounds that reduce estimates of microbial protein. Reanalysis of samples with a modified purine procedure yielded three to four times more purines in both duodenal samples and NDF residue incubated in situ. An alternative in situ procedure removes the microorganisms by refluxing with neutral detergent after ruminal incubation. This alternative correlates highly to the purine-corrected in situ procedure, and it is less variable and simpler to perform. Differential centrifugation may be inappropriate for obtaining clean samples of rumen microbes for determination of purine-to-N ratio because it does not represent particle-associated microorganisms. We suggest an alternative method of measuring that ratio in which NDF is incubated in situ and the particle-associated microorganisms can be measured. Rate of passage is used along with in situ rate of degradation to calculate UIP. We propose that a lag time associated with passage should be added to that calculation. Degradation with no passage (applying a lag) reduced UIP values of forage with high potentially available UIP pools. Enzyme analysis and near infrared reflectance spectroscopy show promise for measuring UIP when fistulated cattle are unavailable. Both are useful predictors of UIP and need more validation research to firmly establish their efficacy. Methods suggested in this paper may improve the accuracy and precision of UIP estimation. The in situ NDIN procedure appears to be a simple and acceptable method of UIP determination. When measurement of purines is needed, the modified assay described in this paper should be considered.

Key Words: Detergents, Forage, Protein Degradation, Purines

Introduction

A metabolizable protein (**MP**) system more accurately describes cattle requirements and types of proteins in feedstuffs (NRC, 1985, 1996) than does a CP system. Degradable intake protein (**DIP**) must be supplied to meet the needs of rumen bacteria. Ruminants with high MP requirements for growth or lactation cannot usually meet their MP needs without supplemental undegraded intake protein (**UIP**) being supplied.

Forage proteins are rapidly degraded by rumen microorganisms and, therefore, tend to be good sources of DIP and relatively poor sources of UIP. Since 1988, we have conducted eight experiments with yearling beef cattle grazing cool- and warm-season grasses during the summer. Although DIP seemed to be adequate, cattle gains were improved by UIP supplementation in all of the studies (Klopfenstein, 1996; Lardy et al., 1999; and Wilson et al., 2000). Broderick (1995) demonstrated that the degradability of alfalfa protein was high and that lactating dairy cows needed supplemental UIP. Patterson et al. (2000) have demonstrated a positive response in reproduction to UIP supplementation of gestating beef heifers. Ferguson et al. (1988, 1993) and McCormick et al. (1999) demonstrated that excessive DIP reduced fertility in dairy cows.

Accurate measures of forage protein degradability are necessary to predict animal performance or to appropriately supplement ruminants to meet MP requirements when high amounts of forages are included in the diets. The methodology for determining forage protein

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degradation is evolving. Thus, our discussion targets some important developments that may enhance our ability to measure forage protein degradability with speed, accuracy, and precision. The issues to be discussed are purine analysis; microbial isolation from rumen contents; use of neutral detergent to remove attached microorganisms; incorporation of lag time in rate of passage estimates; enzyme assays; and near infrared reflectance spectroscopy (**NIRS**).

Discussion

Purine Analysis

The importance of correcting for microbial attachment to feedstuffs has been documented by Nocek (1988). The Zinn and Owens (1986) procedure has been used widely to determine microbial protein concentrations in ruminal, duodenal, and in situ bag contents. The procedure assumes a constant ratio of purines to N in microbial cells within a given animal and(or) feeding situation.

In the past year, research was reported that challenged the accurate analysis of purines and their use as a marker (Makkar and Becker, 1999; Obispo and Dehority, 1999). Makkar and Becker (1999) reported essentially 100% recoveries of purines using the Zinn and Owens (1986) procedure if the source of nucleic acids was pure RNA or microbial cells. However, when the hydrolysis with $HClO_4$ was conducted in the presence of NDF, starch, cellulose, or undigested feed particles, recovery was poor (about 50%). They concluded that the hydrolysis produced interfering substances from the carbohydrates. They further demonstrated that hydrolysis with 0.6 M $HClO_4$ or 2 M $HClO_4$ did not produce interfering substances, but did completely hydrolyze the RNA. Interfering substances were produced with 12 M $HClO_4$ as used in the Zinn and Owens (1986) procedure.

Obispo and Dehority (1999) obtained poor recoveries of purines using the Zinn and Owens (1986) procedure, and the problem was traced to solubility of the silver salt of adenine in the acid-wash solution. When the precipitating solution was used as the wash, recovery of the purines was over 97%. Creighton et al. (2000) investigated modifications of the Zinn and Owens (1986) procedure as suggested by the previous reports. Ten brome and alfalfa omasal samples were used to test the stringency of hydrolysis acid on purine recovery. Hydrolysis was conducted with either 12 or 2 M $HClO_4$ at 95°C for 1 h. Purine recovery with 2 M acid was higher (14.86 vs 4.46 mg/g) and more precise (3.14 vs 14.87% CV) than with 12 M acid. In a second experiment, after hydrolysis with 2 M acid, the precipitate was washed with 0.005 N H_2SO_4 plus 0.005 M Ag NO_3 or with the initial precipitation solution (buffer + $HClO_4$ + $AgNO_3$). The precipitation solution gave higher mean purine values (4.32 vs 3.40 mg/g) and higher precision (2.84% vs 9.85% CV).

The data suggest that analyses for purines from digesta or in situ residues using the Zinn and Owens (1986) procedure should be interpreted with great caution. However, the procedure seems to work well with modifications suggested by Makkar and Becker (1999).

Microbial Isolation

In order to use purines to determine bacterial crude protein (**BCP**) content of digesta or in situ residues, it is necessary to measure a ratio of purines to BCP in the microbial population being studied. Typically, this is done by collecting rumen contents and by separating the bacterial cells by differential centrifugation (Broderick and Merchen, 1992). Clark et al. (1992) reported wide variation (30% CV) for both purine content and purine to N in mixed bacteria prepared by differential centrifugation. As discussed previously, this variation could be due in part to inaccurate purine analysis. In addition, variation could be caused by biological variation in the bacteria, such as growth stage, or by contamination of the bacterial pellet with very small feed particles (Obispo and Dehority, 1999). Obispo and Dehority (1999) presented good evidence for contamination of the bacterial pellet with NDF, but their purine analysis was with 12 M $HClO_4$. Although this would not have caused interference with the pure cultures, there may have been some interference with the mixed cultures because of contamination with plant carbohydrates.

A further complication of using differential centrifugation is the concern that fluid-associated bacteria are harvested and they may have a purine to N ratio different than particulate-associated bacteria (Broderick and Merchen, 1992). Craig et al. (1987a) stated that only 32 to 52% of the particle-associated bacteria were removed; therefore, we must assume that those 32 to 52% represent all particle-associated bacteria. Further, Craig et al. (1987b) reported that over 80% of the microbial mass was associated with the particles. This observation is especially important for our discussion of forage proteins as we would expect the majority of the bacteria to be attached to the forage particles.

Mass et al. (1999) and Creighton et al. (2000) proposed another method of determining the purine to N ratio of attached bacteria. They incubated NDF in situ and measured N and purines on the residue that remained undigested after 12 h. The NDF residue was extracted with neutral detergent, and then N was determined (**NDIN**). Mass et al. (1999) determined that neutral detergent removed microbial cells (purines) from forage fiber. Total N and purine also were determined on the unincubated NDF residue. Total N minus NDIN was considered to be bacterial N for calculating the purine to N ratios.

The purine to N ratio (adenine and quanine standards) obtained by Mass et al. (1999) using the original Zinn and Owens (1986) procedure was 0.14 for high-quality, smooth bromegrass hay. Creighton et al. (2000), using the modified Zinn and Owens (1986) pro-

cedure, obtained values ranging from 0.28 for higher quality forages (alfalfa, brome, and high-quality meadow hays) to 0.36 and 0.41 for low-quality meadow hay and switchgrass hay, respectively. These values fall between the ratios for mixed organisms by differential centrifugation (0.20) and pure cultures (0.59) reported by Obispo and Dehority (1999).

We believe this technique has potential to determine purine to N ratios in bacteria from forage-fed ruminants. The ratio should be appropriate for in situ studies because it should measure the same organisms as those flowing to the omasum. Because the majority of bacteria are attached to fiber particles in forage-fed ruminants, the ratio determined on the in situ NDF residue should represent the ratio in duodenal contents at least as well as bacteria separated by differential separation.

Microbial Correction with Neutral Detergent

Sniffen et al. (1992) hypothesized that NDIN is the primary UIP fraction in feedstuffs. This assertion is likely true of forages because they do not contain proteins such as zein that are neutral detergent soluble but not readily degraded in the rumen. Although Sniffen et al. (1992) did not specifically state that NDIN is the primary UIP fraction, it is implied because rates of digestion of the neutral detergent soluble proteins are 10 times or more rapid than the NDIN fraction. Because neutral detergent removes purines and presumably bacteria from fiber (Mass et al., 1999), we theorized that NDIN of incubated in situ residues would be a direct measure of UIP. Eight forages ranging from 5.6 to 30% CP (DM basis) and 49.4 to 73.9% IVDMD were incubated in situ for time periods between 4 and 24 h. Residues were analyzed for N, NDIN, and purines. The UIP values were determined using N (uncorrected for bacterial N), purine-corrected N, and NDIN. The UIP values were calculated from fractional rates of digestion (k_d) and passage (k_p).

The UIP estimates made with total N, uncorrected for microbial attachment, were higher than the two corrected estimates (Figure 1). Overall means for UIP using either purine-correction (Zinn and Owens, 1986, without modification) or NDIN were not different (2.75 and 2.70% of DM, respectively). The relationship of the two measures of UIP was good ($r^2 = 0.96$); however, the slope of the regression was 0.74. It is not clear whether the difference was associated with the purine correction or with the assumptions inherent in the NDIN procedure. These assumptions are that protein insoluble in ND escapes rumen digestion, protein soluble in ND is ruminally degradable, and ND removes microbes.

Coblentz et al. (1999a) also compared the NDIN correction procedure to the more traditional in situ procedure. However, they did not correct for microbial attachment. They stated that "purine concentrations were found to be negligible" in the in situ residues. This result might be due to interference in the purine proce-
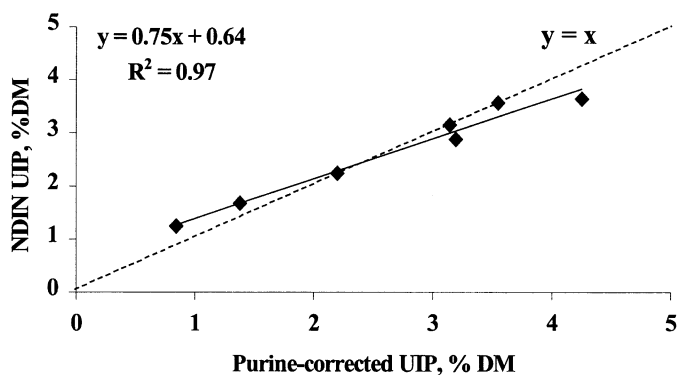

Figure 1. Comparison of undegraded intake protein (UIP) concentrations for eight forages calculated using either purine-corrected UIP (Zinn and Owens, 1986, without modification) or neutral detergent insoluble N (NDIN) UIP (Mass et al., 1999).

dure (Zinn and Owens, 1986; Makkar and Becker, 1999). Coblentz et al. (1999a) found an excellent relationship between the NDIN procedure and the traditional, uncorrected total N procedure ($r^2 = 0.93$). However, the slope of the regression line was only 0.53.

Following the report by Makkar and Becker (1999) and the revision of the purine procedure (2 M HClO$_4$ hydrolysis; Creighton et al., 2000), we made another comparison of the NDIN procedure to the purine-corrected procedure for estimating UIP. Twenty-four forages were included in the experiment. Eight forages were low quality (<5.5% CP), seven forages were medium quality (5.5 to 10% CP), and nine forages were high quality (>10% CP). Ruminal incubations were for 2, 12, and 96 h. Rate of digestion was calculated using the 2- and 12-h values corrected for the 96-h extent of digestion. The values at 2 and 12 h were regressed to 0 h to obtain the potentially degradable UIP pool. Rates of passage (estimated) and purine to N ratio (adenine and quanine standards) for the low-, medium-, and high-quality forages were: 2%/h, 0.417; 3%/h, 0.360 and; 4%/h, 0.286, respectively.

The UIP values ranged from about 1 to 7% of DM (Figure 2). The relationship of NDIN to purine-corrected UIP values was good ($r^2 = 0.97$; SE = 0.03; N = 0.01). In this case, the slope of the regression was 0.98. We concluded that the NDIN procedure gives results equivalent to purine correction. Previous results showing a slope different from one may have been due to inaccurate or imprecise purine analysis.

The NDIN procedure is much simpler than purine correction and probably more precise (Mass et al., 1999). The NDIN procedure consists of refluxing the residue in neutral detergent solution, rinsing, drying, and analyzing for N. In fact, the neutral detergent analysis can be completed in the same in situ bag used for the rumen incubation. No transfer is required, and therefore there is no chance for transfer error. The purine correction method requires several steps, each re-
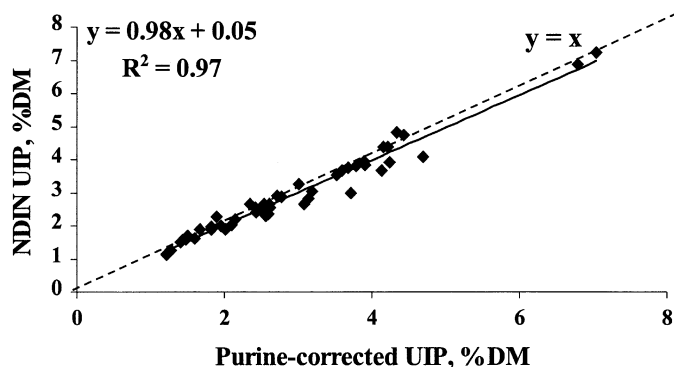
Figure 2. Comparison of undegraded intake protein (UIP) concentrations for 24 forages (in duplicate) calculated using either purine-corrected UIP or neutral detergent insoluble N (NDIN) UIP.
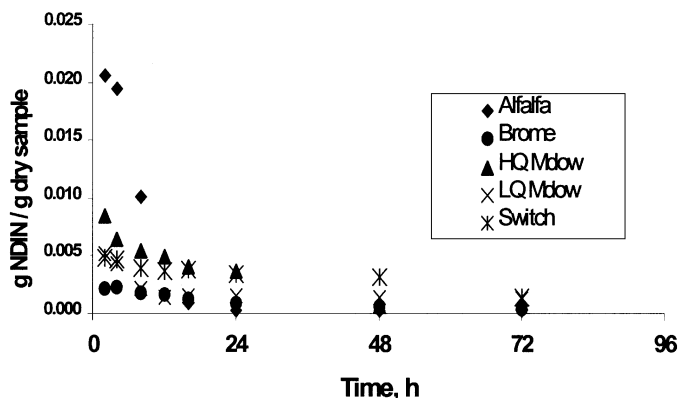

Figure 3. In situ disappearance of neutral detergent insoluble N (NDIN) in the rumen. All points corrected for 96-h extent of degradation (Creighton et al., 2000).

quiring time and adding potential for additive "errors." Further, the purine correction procedure requires a purine-to-N ratio that can be difficult to determine and may vary with physical or physiological phase of the microorganisms. The NDIN procedure does not require measurement of a purine to N ratio.

Lag in Rate of Passage

Based on the original work of Orskov and MacDonald (1979), Broderick (1994) described the use of k_d and k_p in calculating UIP values of forages with the equation:

$$UIP = B \times [k_p/(k_p + k_d)] + C$$

where B is the UIP potentially degradable (insoluble) pool and C is the undegradable pool. However, this equation assumes that ingested forage particles are ready for passage from the rumen instantaneously. This may be true for concentrates but it is not the case for forages. Forage particles are too large or buoyant to immediately pass from the rumen, or they may be trapped in the digesta mat layer (Ellis et al., 1994). The very existence of the mat layer is evidence of particles too large and buoyant to pass. These observations suggest that we must include a lag time in calculating UIP values of forages and that values reported in the literature may overestimate UIP content. This includes our UIP values discussed previously in this paper.

Ellis et al. (1994) addressed this problem with an excellent discussion of models for calculating passage pools and rates. They made measurements on a steer with ruminal and duodenal cannulae. By sampling rumen contents, duodenum contents, and feces, they were able to partition retention times into three major pools. The pools were: a lag-rumination pool; a mass action turnover pool, and an intestinal pool. They further demonstrated that all three pools could be estimated from marker appearance in the feces. For purposes of protein degradation in the rumen, the intestinal pool is not

important. As indicated, the Broderick (1994) model includes the mass action turnover pool but not the lag-rumination pool.

The forage fed by Ellis et al. (1994) was a low-quality bermudagrass hay (about 50% digestible DM). Rate of passage (based on the mass action turnover pool) was 2.61%/h, which is consistent with other literature reports. The rate of movement from the lag-rumination pool to the mass action turnover pool was 10.6%/h. It may be easier to conceptualize this model in terms of compartment mean retention times (**MRT**). The MRT for the mass action turnover pool was 38 h, whereas the MRT for the lag-rumination pool was 9.4 h. This total of 48 h is much longer than the 12 to 24 h used for in situ UIP determinations discussed previously (Mass et al., 1999). Total MRT of 48 h does not seem excessive for a low-quality hay, especially if we are considering fiber digestion. We routinely use 48 h for IVDMD determinations. The UIP in forages is related physically to the fiber; therefore, it is logical that similar retention times and fermentation times should be considered for fiber and protein. In fact, Burns et al. (1997) suggested that 48-h IVDMD is not long enough for low-quality forages.

In more recent reports and reviews (Ellis et al., 1999; Wylie et al., 2000), Ellis and colleagues have concluded that lag-rumination time is 10 ± 1 h with no clear relationship to fiber content or DM digestibility. Based on their conclusion and the underlying biology of rumination and buoyancy, we have chosen to make calculations based on a constant lag time in passage for all forages.

We had protein degradation curves of five forages being used for in vivo protein escape studies (Creighton et al., 2000; Figure 3). Rates of degradation were calculated for 2 to 12 h for k_{d_1} and from 12 to 24 h for k_{d_2}. Then, the UIP values (% DM) were calculated using the 10-h lag with degradation at rate k_{d_1} and then further degradation using:
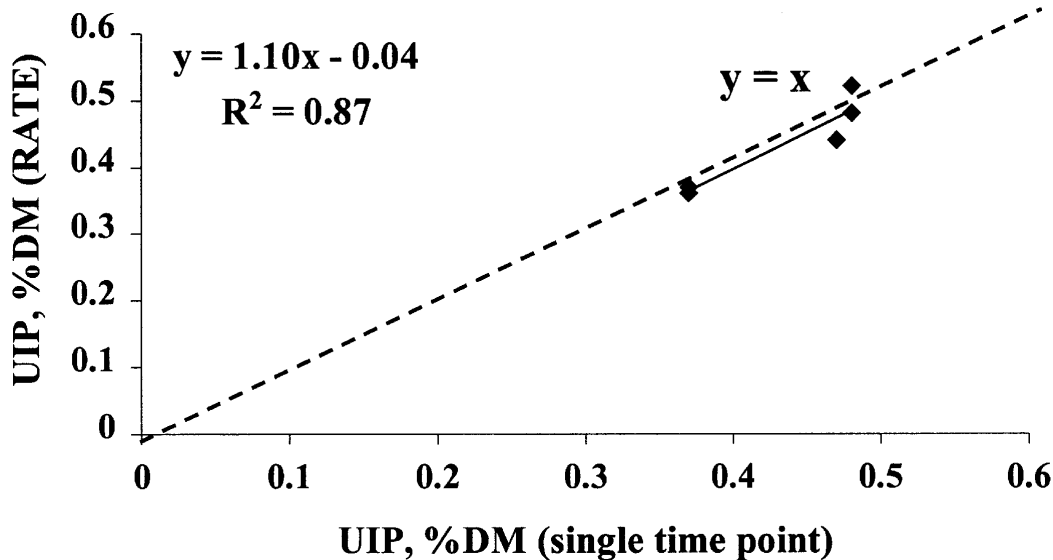
Figure 4. Relationship between undegraded intake protein (UIP) concentrations calculated either by a single in situ incubation time point or two time points used to calculate a degradation rate (RATE). The five forages shown are the same as those in Figure 3.

$$\frac{k_p}{k_p + k_{d_2}}$$

The UIP values also were calculated by interpolating between the 24- and 48-h values. Our objective was to determine if the values determined using lag, rates of digestion, and passage gave similar results to measurements made at one discrete time (mean retention time plus lag). The means were similar (0.42 and 0.44), and the values were correlated ($r^2 = 0.88$; Figure 4). Rates of digestion were more than two times greater from 2 to 12 h than 12 to 24 h. Further, UIP values decreased very little from 24 to 48 h (less than 5.4% for four of the five forages). On average, 77% of the UIP (range of 57 to 96%) was the C fraction (NDIN remaining at 96 h). This indicates that with reasonably long rumen retention times, probably 25 to 45 h, most of the potentially degraded protein has been degraded and only the very resistant (96 h) protein is remaining as UIP.

A pertinent question is whether digestion lag should be included in the in situ UIP calculation. It is certainly not clear from the data in Figure 3 or from the literature if a digestion lag exists for protein degradation. A digestion lag for fiber digestion is logical because of the need for microbial attachment prior to digestion of fiber. It is not clear that a similar mechanism is necessary for protein degradation. Coblentz et al. (1999a) determined protein digestion lags of 2.1 to 8.3 h for whole plant alfalfa and eastern gama grass. This calculation depends upon assumptions made in defining pools A, B, and C. Until the biology of a protein digestion lag is clearly elucidated, we have chosen not to include it in our calculations.

We propose that in situ UIP values be determined by incubating samples for times equivalent to mean retention times (for example, k_p of 6 = 17 h and k_p of 3 = 33) plus a lag (10 h) for totals of 27 to 43 h. The in situ bags and contents can be extracted intact with neutral detergent followed by N analysis of the residues. The N remaining ($\times 6.25$) divided by the DM into the bag is the UIP value. This is a simple, accurate, precise, and inexpensive method. Validation with in vivo values is needed, however.

There are three primary concerns with this proposed procedure. There is concern that some neutral detergent soluble protein escapes rumen degradation. If that fraction (B_2) is rapidly solublized in the rumen and escapes at the rate of the soluble fraction, some of it could escape rumen degradation and be unaccounted for in the UIP. This fraction would not be measured in either the purine-corrected method or the NDIN method as it is a concern for the basic in situ procedure. It is not clear if the B_2 fraction is rapidly solublized and leaves the fiber fraction, and rate of degradation of the B_2 fraction has not been critically measured.

The second concern is that a 10-h lag (MRT) for all forages seems intuitively too simple. This is based on one reference (Ellis et al., 1999). Certainly further research is needed to determine these very important passage lag times. Mertens and Ely (1979) assumed three rumen pools, large, medium, and small particles. They assumed the particles were reduced in size according to first-order kinetics. However, they did not consider buoyancy. It is not clear how rates of particle size degradation were applied to forages of varying fiber digestion characteristics.

It is not completely clear that particle-size reduction follows first-order kinetics, although that may well be the case. Buoyancy may not follow first-order kinetics. It is not clear if particle size or buoyancy is a more important factor in passage lag.
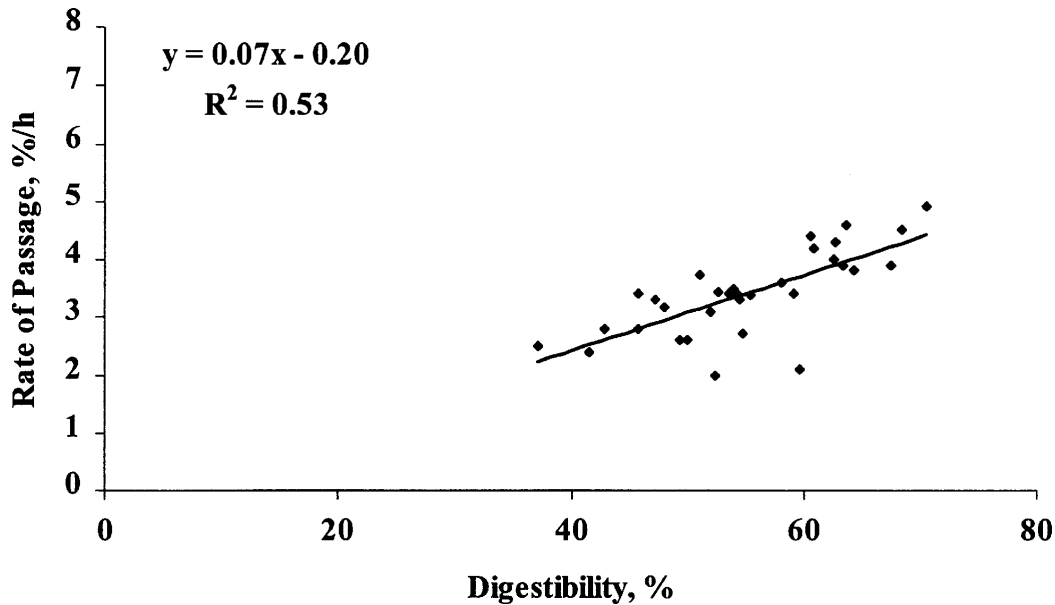
Figure 5. Relationship of rate of passage to digestibility.

The third concern is in the use of MRT rather than kinetic data. Orskov and MacDonald (1970) showed that using MRT overestimates degradation compared to using rates of degradation and passage. Broderick (1994) further emphasized that point. Our goal is to provide a simple, straightforward procedure and the use of two rates of digestion, one rate of passage and one rate of movement of particles from the lag-rumination pool into the mass action turnover pool is not simple!

When in situ data are being used in a model that requires degradation rates, then the use of a specific time of incubation is not appropriate. The procedure proposed here would be applicable when larger numbers of samples are being analyzed.

We further propose that rates of passage (MRT) can be estimated by determining IVDMD. In order to use forage UIP values in a model such as the NRC (1996), an energy value is needed so IVDMD analysis is likely. Ellis et al. (1999) proposed that indigestible NDF is related to rate of passage. Indigestible NDF is related to digestibility so we extended the concept using other literature values. We used literature data to develop a regression of rate of passage on digestibility (Gates et al., 1987; Goetsch et al., 1987; Pond et al., 1987; Krysl et al., 1989; Prigge et al., 1984, 1990; Allen, 1996; Lamb, 1996; Burns et al., 1997; Villalobos et al., 1997). Because of the lack of precision in measuring rate of passage, the r^2 is not especially high (0.53) but gives a reasonable prediction of passage rate from digestibility (Figure 5). Unless rate of passage (MRT) is known or can be reasonably estimated, the equation in Figure 5 may be used to determine in situ incubation times as suggested previously.

Ellis et al. (1999) used this basic concept and obtained excellent correlation with in vivo values. Both in vivo and in situ values were corrected for microbial attachment using diaminopimelic acid. Lag was included in passage estimates. However, the feedstuffs used were primarily protein concentrates, and only one forage was included (ryegrass).

Enzyme Assay

Nocek (1988) reviewed the development of enzyme assays for determining UIP values of feedstuffs. Emphasis was on concentrate feeds, although some research was reported for forages. Broderick (1994) concluded that free proteolytic enzymes may not be satisfactory for assessing ruminal protein degradability. However, he based this conclusion on work primarily with protein concentrates (Luchini et al., 1996). Alternatively, the French (Aufrére et al., 1991) adopted the enzymatic (*Strep. griseus*: **SGP**) method as an acceptable laboratory method for their metabolizable protein systems (**PDI**).

DeBoever et al. (1997) evaluated the SGP, ficin assay, and solubility. They used 29 concentrate feeds and 12 forages. The SGP worked well with the forages (R^2 = 0.821 to 0.921) and not the concentrates. Licitra et al. (1998, 1999) studied enzyme concentration, pH, and incubation time in the SGP. They offered a procedure by which in vivo and in situ values could be calibrated for enzymatic activity. They worked primarily with concentrate feeds, however.

More recently, researchers at Kansas State University (Abdelgadir et al., 1997) studied the SGP strictly for forages. They found that carbohydrases were not necessary in the incubation. They compared the SGP procedure to the in situ procedure using 20 diverse forages including grasses and legumes of varying maturities (Coblentz et al., 1999b). Single time-point esti-

Table 1. Linear regressions of single end-point estimates of rumen-degraded protein by *Streptomyces griseus* protease at 6.6 activity units/mL on standard rumen-degraded protein estimates derived from in situ analysis and corrected for microbial contamination (Study 2). Data from all forages (n = 20) were used. For all five regressions, slopes and intercepts did not differ ($P > 0.05$) from unity and 0, respectively[a]

Incubation time, h	Regression statistic				
	Slope	Se_{slope}	Intercept	$Se_{intercept}$	r_2
			%		
1	1.00	0.07	−9.4	4.7	0.918
2	0.88	0.06	3.5	4.0	0.925
3	0.88	0.07	3.3	4.7	0.898
4	0.96	0.06	−2.8	4.3	0.926
5	0.91	0.06	2.5	4.3	0.917

[a]Coblentz et al., 1999b.

mates of UIP using SGP were highly correlated to in situ estimates of UIP (Table 1). Slopes and intercepts were not different ($P > 0.05$) from 1 and 0, respectively, when 6.6 activity units/mL were used compared to lower concentrations of the enzyme. Relationship to in situ estimates was good ($r^2 = 0.898$ to 0.926). Degradation was essentially complete by 2 h with SGP, so the authors recommended a time point between 2 and 5 h. This procedure shows promise for forages because of the simplicity of the procedure and because a fistulated animal is not needed.

However, there is need to further validate the SGP procedure. It overestimated UIP values of two forages (alfalfa and prairie hay) which had in vivo estimates. Further, the in situ procedure used did not account for a passage lag (as discussed previously), and the purine procedure used was Zinn and Owens (1986), using $12\,M$ $HClO_4$ (as discussed previously). Coblentz et al. (1999b) also used 3%/h passage rate for all forages that may not be correct. Therefore, the SGP procedure needs to be validated against in situ results obtained using a passage lag, correct passage rates, and with microbial attachment accounted for with a modified purine procedure or NDIN.

Near Infrared Reflectance Spectroscopy

Near infrared reflectance spectroscopy is probably the least expensive and simplest means of estimating nutritional characteristics of feedstuffs, especially forages. With major improvements in NIRS hardware and software, it is used commonly in commercial forage testing (Shenk and Westerhaus, 1994). If it could be truly developed as a means of estimating forage UIP, it would be a great asset for implementation of MP systems.

Recent literature indicates potential for NIRS; however, not all of the data are positive. Probably the biggest limitation is a good wet chemistry procedure to use for calibration and validation. It is doubtful that we will have sufficient in vivo data to develop calibrations. In addition, in vivo data are suspect because of

limitations of flow markers, microbial markers, purine analysis, and estimates of endogenous nitrogen. Calibrations for NIRS will likely need to be developed using in situ UIP data. As discussed, the in situ technique has several limitations. Hopefully, the developments made in the past few years will help us develop precise and accurate in situ UIP values.

Halgerson et al. (1995) used ficin enzyme and in situ incubation, uncorrected for microbial attachment, to develop data for NIRS calibrations. They developed equations for cool-season grasses and alfalfa. Acceptable equations were obtained when wet chemistry values were expressed as DIP/CP. Hoffman et al. (1999b) suggested that because of a high correlation between DIP and CP, the NIRS equations were just measuring CP.

Mass et al. (1998) used a diverse set of forage masticate samples (n = 574) to develop NIRS calibrations. The samples contained cool- and warm-season grasses and legumes that varied in maturity. They were separated into high quality (>10% CP and 60% IVDMD) and low quality. The UIP was determined using in situ NDIN at 2 and 12 h to calculate k_d, and k_p was set at 5%/h for high-quality and 2%/h for low-quality forages. Passage lag was not included in the calculations. The UIP was expressed both as UIP/DM and UIP/CP.

Although NIRS predictions of CP and IVDMD were reasonably well correlated to wet chemistry values ($r^2 = 0.80$ to 0.92), the NIRS predictions and UIP values were not well correlated ($r^2 < 0.50$). The unacceptable NIRS predictions may have been due to the heterogeneity of the forage species in the sample sets. Further, ignoring passage lag in the wet chemistry calculations may have biased the UIP values.

Researchers at Wisconsin have had very good success in predicting UIP with NIRS (Hoffman et al., 1999a,b,c) using grass and legume silages. The UIP (wet chemistry) was determined with a 24-h in situ incubation corrected for microbial attachment using NIRS. The NIRS equation was developed from purine assays (Zinn and Owens, 1986; Brehm et al., 1997). These researchers

developed an excellent protocol for UIP determinations. As discussed previously, a single time point may be appropriate for in situ incubations. They chose 24 h which may be appropriate for these forages. With a 10-h passage lag, that would be 7.1%/h passage rate (1/14). The UIP was expressed as UIP/CP.

The NIRS prediction of in situ UIP was excellent (r^2 = 0.94). However, one concern is that the UIP values are expressed as a percentage of CP. Higher protein forages, such as immature alfalfa, have high CP and high DIP as mentioned previously. When UIP is expressed as a percentage of CP, it is inversely correlated to CP. In fact, Hoffman et al. (1999a) found a high negative relationship between UIP/CP and CP (r^2 = 0.80). Therefore, the NIRS only explained 14 additional units (0.94 to 0.80) above that explained by CP alone.

Near infrared reflectance spectroscopy has great potential for predicting UIP in practical situations, such as commercial laboratories. However, much needs to be done to validate NIRS including the measurement of accurate in vivo values.

Conclusions

We have tried to emphasize the importance of accurate UIP and DIP values in the successful application of metabolizable protein systems. We are proposing an in situ procedure for the laboratory determination of UIP for forages. The procedure is simple, relatively inexpensive, and quite precise. Forage samples are incubated in small, heat-sealed Dacron bags for a time period based on MRT. The MRT can be estimated from IVDMD. After incubation, the bags are placed in neutral detergent solution, extracted, washed, dried, and weighed. The N is determined on the residue. The UIP is the remaining N ($\times 6.25$) as a percentage of the initial DM weighed into the bag.

We believe this procedure is preferable to other in situ procedures. However, it must be validated with accurate in vivo data before the in situ results can be used in metabolizable protein systems. Once this NDIN in situ procedure is validated, it can be used to determine whether the enzyme assay or NIRS procedures can be used for routine, commercial laboratory analyses.

Implications

The widely used purine assay needs to be modified to improve accuracy and precision. The suggested modifications include reducing perchloric acid concentration and use of the precipitating solution for washing. An in situ method, based on neutral detergent extraction, accurately and precisely measures undegraded intake protein in forages. This technique has good potential for research laboratories, and near infrared reflectance spectroscopy and enzyme assays have potential for commercial laboratories.

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