Markers for Quantifying Microbial Protein Synthesis in the Rumen[1]

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ABSTRACT

Measurement of ruminal microbial protein is necessary to quantify ruminal escape of dietary protein and microbial yields. Microbial markers used most widely have been the internal markers, diaminopimelic acid and nucleic acids (RNA, DNA, individual purines and pyrimidines, or total purines), and the external isotopic markers (e.g., ^{15}N and ^{35}S). Combined with digesta flow markers in ruminally and abomasally or intestinally cannulated ruminants, microbial yields can be estimated. An ideal marker system must account for both the bacterial and protozoal pools associated with both the fluid and particulate phases of digesta. No marker has proven completely satisfactory; hence, yield estimates are relative rather than absolute. Total purines represent robust microbial markers that should be adaptable by most investigators. Principal concerns about total purines relate to unequal purine:N ratios in protozoal and bacterial pools and to the need to assume that dietary purines are completely degraded in the rumen. A theoretically sounder, but more costly, method is continuous intraruminal infusion of ^{15}N ammonium salts. However, ^{15}N enrichments of bacterial and protozoal pools are not equal, so the basis for calculating microbial yield in faunated ruminants is uncertain. Urinary purine excretion may prove to be a noninvasive method for estimating microbial protein yields in intact dairy cows.

(**Key words**: microbial markers, microbial protein synthesis, nucleic acids, purines)

Abbreviation key: AEP = 2-aminoethylphosphonic acid, **BCP** = bacterial plus protozoal CP, **DAP** = 2,6-diaminopimelic acid, **DIP** = degraded intake protein, **FAB** = fluid-associated bacteria, **IRMS** = isotope ratio mass spectrometry, **PAB** = particle-associated bacteria, **UIP** = undegraded intake protein.

INTRODUCTION

Quantifying microbial protein synthesis in the rumen is important for ruminant nutrition for a number of reasons. Determination of the contribution of microbial protein to the host's protein and AA requirements has become more important since the development of the new protein systems for ration formulation (2, 55). The NRC protein system (55, 56) assumes that, for lactating cows and cattle fed diets over 40% forage, microbial protein yield (**BCP** = bacterial plus protozoal CP) is a constant function of dietary energy content, as given by the equation:

$$\text{BCP, g/d} = 6.25 \times (-30.93 + 11.45 \times NE_L)$$

where NE_L = daily NE_L intake in megacalories. Because certain grains with essentially equal NE_L, e.g., corn and barley, differ substantially in the extent of ruminal starch fermentation (82), they likely will give different BCP yields. The NRC (56) further assumes

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[1]Mention of commercial products in this paper is for purposes of identification only and does not constitute endorsement by the USDA or the Agricultural Research Service.

that .2 of BCP is nucleic acid N and .8 is true protein N with a true digestibility of .8 (i.e., absorbed protein = .64 × BCP). Although it has been argued that, under conditions of high intakes in dairy cows fed typical diets, BCP will be a relatively constant function of NE_L (36), recent evidence indicates that several factors influence net BCP yield.

The NRC (55, 56) assumed an equivalency among ruminally degraded intake protein (**DIP**) sources for microbial utilization, i.e., NH_3 N was as useful for microbial growth as α-amino N from peptides and AA. Maeng et al. (48) reported that replacing 25% of the NH_3 N in the medium with a mixture of AA significantly increased BCP yield in vitro. Argyle and Baldwin (6) observed that yield increased substantially in batch cultures of mixed ruminal bacteria when 1, 10, and 100 mg of N/L as AA and peptide mixtures were added to media containing only NH_3 N (Table 1). The increased intercept at 100 mg/L, but not at 1 and 10 mg/L, indicated that peptides and AA served as sources of energy only at the highest concentration. In vivo ruminal concentrations of peptides and total AA averaged, respectively, 43 and 72 mg of α-amino N/L in lactating cows fed a diet with 58% alfalfa silage and 42% corn-based concentrate (10). Argyle and Baldwin (6) also found that cell yields increased only with addition of complete AA and peptide mixtures; partial AA mixtures had little effect. Stokes et al. (75) observed that BCP yields increased linearly over a wide range of dietary nonstructural carbohydrates when DIP (peanut meal) supply was increased in continuous culture fermenters charged with mixed ruminal organisms (Figure 1). Although Figure 1 represents a much wider range in DIP concentrations than would likely be encountered in vivo, BCP yield was still increasing at 19% DIP, which suggests that ruminal deficiencies of degraded protein may occur on practical diets formulated from NRC (56) tables.

Additional factors, such as ruminal pH (76), ruminal dilution rate (62), and feeding frequency on high concentrate diets (14), may alter BCP yield. Synchrony of ruminal digestion of energy and DIP may prevent uncoupling of microbial fermentation and growth (42, 59). More slowly degraded proteins may stimulate BCP yield by improving DIP availability over time (8, 25). Simple, reliable, and robust techniques for quantifying ruminal BCP yields under practical feeding regimens with lactating dairy cows are needed to test the reliability of the assumption that BCP supply is a constant function of NE_L. If it is not a constant, factors influencing BCP synthesis in vivo need to be identified. It is important also to quantify microbial biomass production to estimate BCP yields based on truly fermented substrate and to determine the contribution of other nutrients, such as carbohydrates and lipids, from ruminal microbial growth.

The need to determine undegraded intake protein (**UIP**) for feedstuffs to use in the new NRC protein system (55, 56) has increased interest in standard in vivo methods to quantify BCP flow to the abomasum. Although several procedures for UIP estimation have been deve-

TABLE 1. Effect of additions of mixtures of AA and peptides on yield of mixed ruminal bacteria in batch culture (6).

AA Added[1]	Peptides added[1]	Intercept[2]	Yield[3]	Relative yield
(mg of N/L)		(mg of N per culture)	(mg of N/g of CHO[4])	(%)
0	0	38.3	5.2	100
1	1	39.0	11.9	230
10	10	33.7	15.7	304
100	100	47.1	20.4	396

[1]Medium with no added AA or peptides contained only ammonia N. Amino acids were an equal weight mixture of 20 protein AA. Peptides were supplied as trypticase.

[2]Intercept = Growth per culture without added CHO.

[3]Bacterial yields measured as RNA. Cells contained 10.7% RNA and 8% N.

[4]CHO = Carbohydrate.

loped using in vitro (9, 44, 64, 67, 69) and in situ techniques [e.g., (58)], in vivo estimates are required to verify these methods. In vivo UIP determinations depend upon calculations of difference, i.e., UIP is computed as the difference between total protein flow, usually measured as NAN, and BCP flow. Such difference computations are subject to error because imprecision magnifies the overall error of the method.

Microbial markers may be classified as internal markers [inherently present in microorganisms, e.g., 2,6-diaminopimelic acid (**DAP**)] and external markers (markers added to the rumen to label the microorganisms, e.g., ^{15}N). Characteristics of an ideal microbial marker include (38) being 1) easy to quantify, 2) not present in the feed (i.e., unique to BCP), 3) present at constant ratio under specific experimental conditions, and 4) biologically stable. Internal and external markers commonly used to estimate BCP synthesis in the rumen are listed in Table 2.

Internal and external marker procedures generally have been used to quantify ruminal protein yield by assuming a single ratio of marker:protein that can be determined for the total ruminal BCP. There are at least two reasons why this simple assumption is inaccurate. First, both internal and external markers usually are found at different marker: protein ratios in different populations of ruminal bacteria and protozoa. For example, with pulse dosing or continuous infusion of $^{15}NH_3$ into the rumen, ^{15}N first is incorporated into bacterial protein; protozoal protein is labeled only indirectly through protozoal predation of bacteria. Protozoa also obtain part of their N from unlabeled feed CP. Hence, protozoal protein will be less ^{15}N-enriched than bacterial protein, and enrichment of bacterial protein will not describe accurately the enrichment of BCP as a whole. Second, different growth rates between the protozoa and bacteria and within the fluid-associated bacteria (**FAB**) and particle-associated bacteria (**PAB**) may result in different marker:protein ratios for different microbial pools. Microbial protein flowing from the rumen represents a mixture of these various pools that leave the rumen in different proportions. An internal marker present in both bacteria and protozoa, such as nucleic acids, is found at different marker:protein ratios in each pool (31).

The implication of these concerns is that there is no simple ideal marker system with which to quantify BCP yield in the rumen. However, it may be possible to establish a reliable, interim procedure that could be used to study factors influencing BCP yields and ruminal protein escape. This might be advantageous rather than using many different systems that may not be comparable, each with different inherent errors.

INTERNAL MICROBIAL MARKERS

DAP

Work (86) reported the discovery of a new AA, DAP, in the Gram-negative organism *Corynebacterium diphtheriae*. Subsequently, DAP was found in oligopeptides that crosslink repeating peptidoglycan units in bacterial cell walls (46). Purser and Buechler (65) found that, although DAP concentrations ranged from zero to more than four times the overall mean in 22 strains of ruminal bacteria, DAP:protein ratios of mixed ruminal bacteria were relatively constant (24). Hence, DAP appeared to be a satisfactory marker for ruminal bacterial protein (47). Since it was first suggested as a microbial marker (77), DAP has been the most common internal marker for estimating microbial (bacterial) protein synthesis in the rumen, and it has been used in several hundred reports (46). Presence of DAP only in the bacterial

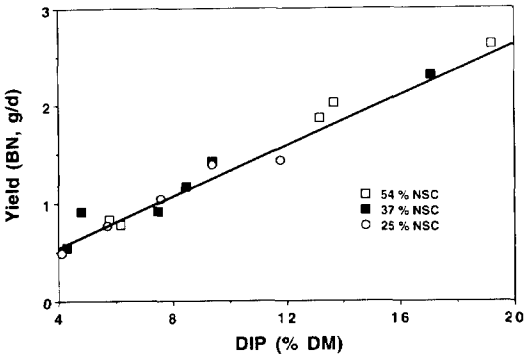

Figure 1. Relationship of yields of mixed ruminal bacteria N (BN) to dietary concentrations of degraded intake protein (DIP) in continuous culture fermenters fed rations containing 25, 37, and 54% nonstructural carbohydrates (NSC) (75).

TABLE 2. Commonly used internal and external markers for quantifying bacterial and protozoal protein (BCP) synthesis in the rumen.

Type of marker[1]	BCP Fraction estimated	Suggested method
Internal		
DAP	Bacteria	Czerkawski (24)
D-Alanine	Bacteria	Garrett et al. (34, 35)
AEP	Protozoa	Ling and Buttery (47)
ATP	Bacteria and protozoa	Not recommended
Nucleic acids	Bacteria and protozoa	
DNA		Not recommended
RNA		Ling and Buttery (47)
Individual purines and pyrimidines		Schelling and Byers (70) and Schelling et al. (71)
Total purines		Zinn and Owens (87, 88) and Ushida et al. (81)
External		
$^{15}NH_3$	Bacteria and protozoa	Siddons et al. (72) and Firkins (31, 32)
$^{35}SO_4$	Bacteria and Protozoa	Beever et al. (7)
^{32}P-Phospholipid	Bacteria and protozoa	Not recommended

[1]DAP = 2,6-Diaminopimelic acid; AEP = 2-aminoethylphosphonic acid.

cell wall implies that its concentration in total ruminal bacterial protein would vary with growth conditions that alter mean cell size. Conditions favoring larger cells would lower ratios of cell wall (proportional to surface area) to protoplasm (proportional to volume) and decrease DAP:protein ratios. For a spherical coccus, doubling the cell diameter decreases the surface area:volume ratio by half; however, doubling the length of a 1- by 2-μm rod to 1 by 4 μm decreases the surface area:volume ratio by only 6%. Shifts in conditions of the rumen resulting in increased or decreased numbers of low DAP or DAP-free strains and species obviously will alter the overall DAP: protein ratio. Despite these concerns, the DAP: protein ratio was assumed to be a constant, and some workers reported BCP yields and values for dietary protein escape based on literature estimates of the DAP:protein ratio without accounting for possible experimental or methodological effects. Research has shown that DAP:protein ratios vary substantially within the same animals on the same diets with time after feeding (47) and between FAB and PAB (53). Nevertheless, many workers (24, 47, 68, 78) have used DAP successfully by determining the DAP:protein ratios under their specific conditions.

A more alarming problem with the DAP marker approach is the presence of substantial amounts of DAP in feed sources (68, 78). Rahnema and Theurer (68) reported that the DAP:N ratios in common feedstuffs were 18 to 40% of those found in isolated ruminal bacteria: they suggested that abomasal DAP flows should be corrected for dietary DAP intake. Feed DAP concentrations reported by Rahnema and Theurer (68) were too high to be attributed solely to bacterial contamination. Analytical problems, possibly interference from alloisoleucine (68), may have contributed to some of this difficulty. Ion-exchange chromatography in conjunction with the colorimetric ninhydrin assay at pH 5.5 was used to quantify DAP (68). However, DAP can be difficult to resolve from other ninhydrin-positive compounds in a typical AA chromatogram (40); it is better assayed using acid-ninhydrin reagent (pH 2.0 or less), which excludes most interfering AA except proline and cystine (40). Unless care is taken to exclude O_2, cystine is largely lost during acid hydrolysis of proteins. Czerkawski (24) used acid-ninhydrin reagent, in conjunction with small ion-exchange columns to separate DAP and proline, as the basis for a routine DAP assay. Some DAP also was detected in feedstuffs using an acid-ninhydrin method, but concentrations were only about one-third to one-half of those reported by Rahnema and Theurer (68) using conventional ninhydrin analysis of simi-

lar feeds (Table 3). Interestingly, C. G. Schwab (1991, personal communication) found substantially lower DAP in feeds despite using the method of Rahnema and Theurer (68). Applying the DAP:N ratio from isolated ruminal bacteria to feed samples indicated that microbial N accounted for 18 to 22, 16 to 32, 8 to 10, and 5 to 8% of the total N in corn silage, haycrop silage, low protein grains, and high protein grains, respectively. Nevertheless, any BCP determinations based on DAP should employ an acid-ninhydrin assay in conjunction with chromatographic separation of DAP from proline and residual cystine.

Ruminal protozoal preparations contain some DAP (24, 68), partly because of bacterial contamination of the protozoa during isolation by differential centrifugation, but mainly because of presence within protozoa of undigested bacterial matter engulfed during bacterial predation (21). Hence, DAP flow at the abomasum or intestine partly reflects protozoal protein. However, protozoa have a lower DAP: protein ratio than do bacteria (Table 3).

Variation in concentrations among microbial pools and contamination of feed are not problems unique to DAP; these concerns about DAP have been addressed (47). However, a potentially more serious problem with DAP as a microbial marker has been raised. Denholm and Ling (26) observed that ^{3}H-labeled DAP in intact Gram-positive and Gram-negative bacteria was catabolized at substantially different rates by ruminal protozoa and bacteria in vitro. In vivo data from Masson et al. (49), plotted in Figure 2, show that the mean proportion of total DAP in duodenal digesta that was cell-associated varied from 69% 1 h before feeding to as little as 29% 5 h after feeding. The pattern in ruminal digesta was similar (49). Thus, variable proportions of the DAP leaving the rumen are contributed from intact bacterial cells, and often more than half of the DAP may be in either free or peptidyl form that is noncell-bound. Intraruminal degradation of bacterial protoplasmic proteins appears to be more rapid and extensive than cell-wall residues and their constituent DAP (46). Bacterial "ghosts" may remain stably attached to particles despite catabolism of their protoplasm. Thus, total DAP flowing out of the rumen may be disproportionately high, and use of DAP:protein ratios determined with isolated bacteria may lead to substantial overestimates of bacterial protein yields. Ling (46) explains the report of Nikolic and Jovanovic (57) of greater than 100% bacterial NAN passing the abomasum on this basis and suggests that other workers may have been reluctant to publish similarly aberrant results. Therefore, despite numerous reports of bacterial protein and BCP yields based on DAP, this marker appears to be seriously and perhaps fatally flawed.

D-Alanine

Garrett et al. (34) suggested a novel marker, D-alanine, to estimate postruminal flow of bacterial protein. The oligopeptides crosslinking bacterial cell-wall peptidoglycan contain D-alanine as well as DAP (46). D-Alanine content of the peptidoglycan generally is greater than DAP; D-alanine is more widely distributed than DAP among ruminal bacterial species (34) and was not detected in feedstuffs (35). D-Alanine was used successfully by those workers to estimate bacterial protein flow for in vivo measurement of BCP synthesis and dietary protein escape (35). However, Quigley and Schwab (66) occasionally found that BCP flow exceeded total protein flow at

TABLE 3. 2,6-Diaminopimelic acid (DAP) concentrations reported in ruminal microbial preparations and common feedstuffs.

Reference	Source	DAP N
		(mg/g of total N)
Czerkawski (24)[1]	Small bacteria	7.3
	Large bacteria	4.7
	Protozoa	.9
	Hay	1.2
	Dried grass	.3
	Barley	.8
Rahnema and Theurer (68)[2]	Bacteria	7.1
	Protozoa	2.4
	Alfalfa hay	3.5
	Sorghum grain	2.5
	Barley	2.0
	Corn	1.3
	Casein	3.3
	Soybean meal	1.9

[1]Acid-ninhydrin system (pH < 2.0) for DAP determination.

[2]Conventional ninhydrin system (pH 5.5) for DAP determination.

the abomasum when D-alanine was used as a microbial marker. This finding and a paucity of other reports on D-alanine as a microbial marker have diminished its earlier theoretical promise. Concerns discussed earlier regarding disproportionate catabolism of bacterial protoplasm and cell-wall components such as DAP (46) probably apply to D-alanine also.

Aminoethyl-Phosphonic Acid

Horiguchi and Kandatsu (39) isolated an AA, 2-aminoethylphosphonic acid (**AEP**), that was present in ciliate protozoa but appeared to be absent from common ruminant feedstuffs. Although AEP is most abundant, two other aminophosphonic acids have been found in rumen protozoa (38). Analogous to DAP, AEP concentration varies substantially among genera of ruminal ciliate protozoa (84). Use of AEP as a marker for protozoal protein has been reviewed by Horigane and Horiguchi (38). Unique chemicai properties of its phosphorus to carbon bond allow AEP analysis in the presence of a plethora of phosphate compounds (1, 38). Horigane and Horiguchi (38) concluded that the use of AEP as a specific protozoal marker is compromised by widespread distribution of AEP among ruminal bacteria and presence of AEP in feedstuffs. They argue that such occurrence of AEP is not merely an artifact. These authors state that AEP and other aminophosphonic acids are incorporated by ruminal bacteria, that plants can take up AEP and other aminophosphonic acids originating from soil microorganisms, and that fish meal may contain AEP originating from phytoplankton. Ankrah et al. (4) found that AEP concentrations in mixed ruminal bacteria from defaunated sheep were one-third of mixed ruminal protozoa, and AEP concentrations in ruminal digesta from defaunated sheep were 55% of that in faunated sheep.

Nucleic Acids

The high concentrations of DNA and especially RNA in unicellular organisms led to recognition of their potential as markers for ruminal BCP. Ellis and Pfander (28) assessed microbial flow from the rumen based on total polynucleotide flow. Topps and Elliott (80) reported on use of urinary nucleic acid excretion to estimate microbial supply. Subse-

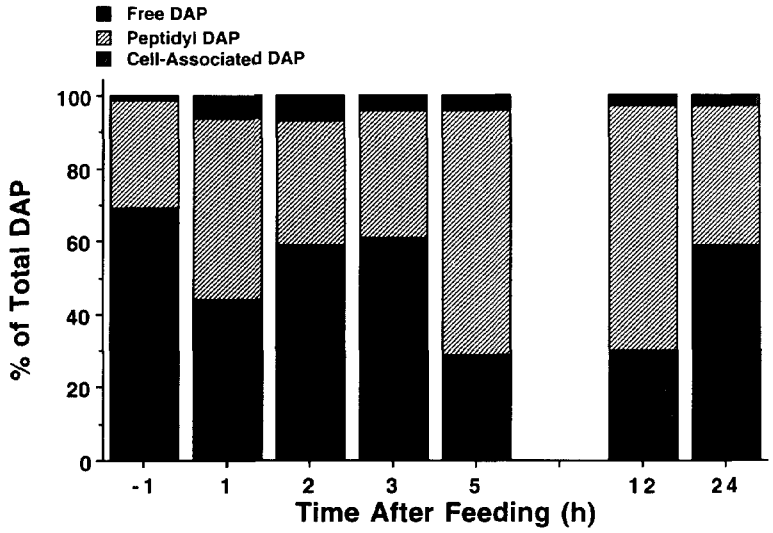

Figure 2. Proportions of total 2,6-diaminopimelic acid (DAP) present at various times after feeding as free, peptidyl-bound, and cell-associated DAP in duodenal digesta from sheep (27).

quently, microbial protein yields were estimated using RNA (47, 73), total nucleic acids (20, 81, 87, 88), or individual bases (70, 71). Use of nucleic acids as BCP markers is well established. Schelling et al. (71) reviewed the use of nucleic acids as microbial markers.

Theoretical and practical concerns regarding the use of nucleic acids as markers for BCP synthesis include 1) presence of nucleic acids in dietary ingredients, 2) differential nucleic acid:protein ratios in bacteria and protozoa and among the FAB and PAB pools within the rumen, and 3) possible disproportionate metabolism of microbial nucleic acids following intraruminal turnover of microbial cells. The third concern has been addressed by McAllan and Smith (51, 52), who reported that DNA and RNA from isolated unicellular organisms, when added to the rumen, were rapidly and completely catabolized. Although many common feedstuffs are much lower in purines than ruminal microbes, others, such as fish meal, have total purine:N ratios that approximate those of ruminal bacteria and protozoa (Table 4). However, McAllan and Smith (51, 52) used their data on degradation of exogenous purines to conclude that the first concern, abomasal and intestinal passage of dietary nucleic acids, was insignificant. Schelling and Byers (70) reported that some dietary adenine, but little cytosine, escaped the rumen. Koenig [cited in (71)] estimated that, except for shortly after feeding, ruminal escape of dietary nucleic acids from alfalfa hay, which is rich in nucleic acids, was relatively minor. Nevertheless, further research is necessary to establish nucleic acids as completely reliable microbial markers.

Extensive experimentation in the University of Illinois laboratory has addressed the second of the cited concerns. Using the total purine analysis method of Zinn and Owens (88), as modified by Ushida et al. (81), Cecava et al. (15) assessed the relative purine:N ratios of FAB, PAB, and mixed ruminal bacteria. Those workers observed different ratios among these three sources of bacteria, particularly between FAB and PAB (Table 5). However, mixed ruminal bacteria had purine:N ratios that were similar to those of PAB [Table 5 (15)]. Craig et al. (23) observed significant changes in total purine:N ratios of both FAB and PAB over the feeding cycle; the lowest ratios occurred immediately after feeding (Figure 3). Earlier findings revealed that typically 20% (22, 33) and, depending on time after feeding, as little as 10% (54) of total ruminal microbial biomass was associated with the FAB fraction. Thus, Cecava et al. (15) concluded that PAB or mixed ruminal bacteria, isolated from digesta obtained over the entire feeding cycle, were the most appropriate for determining the purine:N ratio of "typical" bacteria leaving the rumen. Because FAB are more easily isolated and are not extensively contaminated with feed residues, earlier workers often used FAB for marker:N ratios. Using the data of Cecava et al. (15), estimated net ruminal BCP yield would be 21% lower, and estimated dietary UIP would be 20% greater, if the FAB ratio rather than the PAB ratio were used to compute bacterial protein passing the abomasum in steers fed alfalfa-corn diets. However, the purine:N ratio for mixed ruminal bacteria was not intermediate between FAB and PAB in our study.

Employing only the bacterial purine:N ratios may underestimate BCP yields. Protozoa passing to the abomasum or duodenum contribute to total purine flow, but protozoal purine:N ratios typically are about half those of ruminal bacteria (Table 4; 31). Thus, the contribution of protozoal protein would be underestimated if total BCP flow were computed

TABLE 4. Concentration of total purines in preparations of isolated ruminal bacteria and protozoa and in common feedstuffs.

Source	Reference: Titgemeyer et al. (79)	Reference: Firkins et al. (31)
	(purines/g of N[1])	
FAB[2]	. . .	.80
PAB	. . .	.78
Mixed ruminal bacteria	.90	. . .
Mixed ruminal protozoa	. . .	.44
Soybean meal	.17	. . .
Corn gluten meal	.17	. . .
Blood meal	.03	. . .
Fish meal	.67	. . .

[1]Purine units are grams of yeast RNA equivalents per gram of N.

[2]FAB = Fluid-associated bacteria; PAB = particle-associated bacteria.

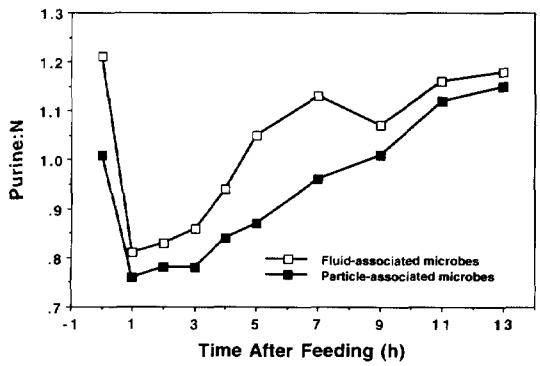

Figure 3. Changes in total purine:N ratios of fluid-associated and particle-associated microbes isolated from the rumens of dairy cows at various times after feeding (23).

based on the bacterial purine:N ratio. Quantifying protozoal protein also is problematic because of possible selective retention of protozoa within the rumen (37). Thus, ruminal pool sizes of bacteria and protozoa may not be indicative of the true proportion of protozoal protein in total BCP flowing from the rumen.

Average UIP values for the alfalfa-corn diets fed by Cecava et al. (14, 15) were computed to be 55 and 67% of total dietary N based on the reported purine:N ratios of mixed ruminal bacteria and FAB, respectively. The UIP of these diets computed from NRC (56) data were 35%. It is possible that true BCP flow was underestimated using purine markers, suggesting that underestimation of protozoal protein may be more important than ruminal escape of dietary nucleic acids. However, errors that are due to flow markers, nonuniform sampling, and variation in UIP among feeds also may explain the differences among estimates of protein escape. Moreover, Cecava et al. (13) found that use of total purines or ^{15}N to estimate ruminal protein escape from diets containing one-third of dietary CP as either soybean meal or a mixture of corn gluten meal and blood meal yielded estimates, respectively, of 36 and 56% UIP (total purines) and 33 and 52% UIP (^{15}N). The relative UIP values computed from NRC (56) data were 35% for soybean meal and 68% for the mixture of corn gluten meal plus blood meal. Ling and Buttery (47) compared internal and external microbial markers and concluded the nucleic acids (measured as RNA) were as reliable as ^{35}S.

Several alternative nucleic acid assays have been used in the last two decades, including determination of individual purine or pyrimidine bases. Schelling and Byers (70) and Schelling et al. (71) used HPLC determination of adenine or cytosine to quantify BCP flow. Koenig [cited in (71)] reported that ruminal catabolism of adenine and especially cytosine was extensive; this may obviate interference from escape of dietary nucleotides. Although HPLC equipment is sophisticated and expensive, such instruments are becoming more common, so availability may not preclude use of individual nucleic acid bases as "simple" microbial markers.

Although pyrimidines are largely catabolized after absorption by the ruminant, absorbed purines originating from ruminal microbes are either reutilized or excreted as urinary allantoin and other metabolites by cattle and sheep (50). This has led to the suggestion that urinary purine derivatives are potential markers for quantifying BCP contribution to the intestine (80). Earlier workers considered the nature of urinary purine excretion to

TABLE 5. Effect of bacterial source on the total purine:N ratio (grams of yeast RNA equivalents per gram of N) (15).

	NE_L of		Feeding frequency	
Bacterial source[1]	1.36 Mcal/kg of DM	1.73 Mcal/kg of DM	2/d	12/d
Mixed bacteria	1.30[c]	1.30[b]	1.32[b]	1.28[b]
FAB	1.56[a]	1.56[a]	1.64[a]	1.52[a]
PAB	1.39[b]	1.33[b]	1.39[b]	1.35[b]

[a,b,c]Means in same columns with different superscripts differ ($P < .05$).

[1]FAB = Fluid-associated bacteria; PAB = particle-associated bacteria.

be potentially complex (W. C. Ellis, 1980, personal communication). However, Chen et al. (19) reported that excretion patterns in cattle were relatively simple; essentially all of the urinary purine derivatives were present in allantoin and uric acid. When using urinary purines to quantify BCP contribution, it is necessary to assess that portion originating from endogenous metabolism. Chen et al. (17) found that, as the exogenous supply of purines was increased by abomasal infusion in sheep maintained by intragastric feeding, endogenous metabolism contributed a progressively lower proportion of urinary purine derivatives. Exogenous supply replaced all endogenous synthesis when daily total purine excretion in sheep exceeded .6 mmol/kg of $BW^{.75}$; beyond that point (about 10 mmol/d of abomasal purine), the regression of urinary purine output on exogenous supply was linear with slope = .84 [Figure 4 (17)]. Thus, microbial purine flow to the small intestine might be estimated by dividing total urinary purine excretion by .84. Perkins (63) reported that daily allantoin excretion was equivalent to 2.2 mmol/kg of $BW^{.75}$ in dairy cows fed diets with a wide range of protein concentrations. It appeared that 16% of purine excretion occurred via other routes (17); about 10% of total purine excretion was recycled to the rumen as salivary allantoin and uric acid and completely degraded there (16). The preliminary results of Antoniewicz et al. (5) estimating ruminal microbial N flow from urinary allantoin were promising. However, further research is necessary to confirm that reliable estimates of postruminal purine flow and, hence, BCP flow may be made from urinary purine excretion in dairy cattle. This approach suggests itself as a potentially noninvasive, albeit approximate, technique for use with large numbers of lactating cows, particularly if urine grab samples and total urine outputs, estimated from creatinine concentration (63), obviated the need for total urine collection.

ATP

Forsberg and Lam (33) used ATP determination by bioluminescence in a classic study to quantify the proportions of FAB and PAB. Others (29, 85) also have used ATP to estimate the microbial biomass in the rumen. However, Wallace and West (83) found that ATP content of abomasal digesta was substantially lower than that of ruminal digesta. Also, the ATP content of ruminal digesta from faunated sheep was more than four times that of digesta from defaunated sheep (83), suggesting that protozoal biomass is substantially higher in ATP. Wallace and West (83) concluded that, although ATP may be an approximate indicator of microbial biomass in active fermentations, rapid ATP hydrolysis coupled with little or no ATP formation in inactive or dead cells precluded its use for estimating flow of BCP at the abomasum or small intestine.

EXTERNAL MARKERS

^{15}N

Inorganic ^{15}N has been used extensively as a tracer for labeling BCP in vivo. Several advantages accrue to ^{15}N as an external marker for ruminal BCP. 1) Because ^{15}N is a stable isotope, it is not an environmental hazard. 2) Generally, ^{15}N is not found in the diet above natural enrichment; hence, intraruminal dosing with ^{15}N ammonium salts in short-term studies will only label microbial N. Feed N, of course, will be unlabeled, and body tissue proteins will be essentially unlabeled until incorporation of microbial ^{15}N labeled AA has occurred. 3) The ^{15}N-labeled ammonium salt will label all microbial N pools (although enrichments will be different). Bacterial N is labeled by direct incorporation of $^{15}NH_3$; protozoal N is labeled indirectly through predation of bacteria. 4) Inorganic ^{15}N is relatively inexpensive. One source (EG&G Mound Applied Technologies, Miamisburg, OH) sells $(^{15}NH_4)_2SO_4$ for $109/g of ^{15}N with enrichments of 60 to 85% (plus a processing charge of $76 per order). Using isotope ratio mass spectrometry (**IRMS**) for ^{15}N determination, about 13 g of ^{15}N (total cost $1500) was sufficient isotope for a continous infusion study with six adult dairy cows for a total of 36 cow-infusion days (6 cows × 6 infusion days; Hristov and Broderick, 1991, unpublished). Often, ^{15}N of lower enrichment is available at somewhat lower prices; $(^{15}NH_4)_2SO_4$ with 10% ^{15}N, which would be adequate to conduct the continuous infusion study just described, is available from another source (Isotech, Miamisburg, OH) at $99/g of

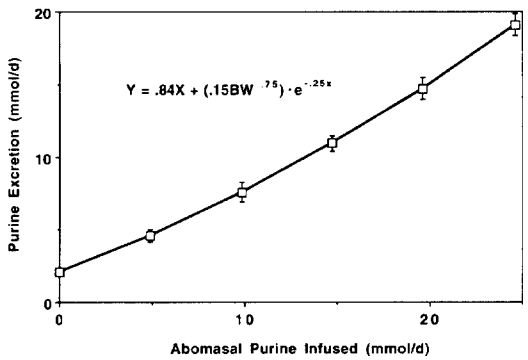

Figure 4. Relationship of total purine (allantoin, uric acid, and xanthine plus hypoxanthine) excretion in the urine of sheep receiving abomasal infusions of a microbial nucleic acid concentrate (17).

^{15}N.

Analysis of ^{15}N for use as a BCP tracer has been reviewed (41) previously. Determination of ^{15}N enrichment of microbial N is tedious because the N fraction under study must be isolated prior to analysis by IRMS. However, accuracy and precision of IRMS are excellent: as little as .001% enrichment (above the natural ^{15}N abundance of .368%) can be detected, and .010 to .05% enrichment is adequate for typical enrichments in most studies (12). Analysis by IRMS requires about .5 to 1.0 mg of total N as N_2 (which is produced at the IRMS by chemical reduction of ammonium salts) for precise determination of ^{15}N enrichment. These instruments are very expensive, costing about $150,000 to $200,000. However, newer instruments, consisting of benchtop IRMS hooked in series to Dumas-style N analyzers, are only slightly less precise. These machines obviate the need for Kjeldahl digestion of samples, require as little as 10 μg of total N per analysis, and sell for about $75,000 above the cost of the N analyzer (e.g., VG Isomass, Isotope Services, Summit, NJ). Commercial analyses with these newer IRMS instruments are available from cooperating universities and ARS facilities for about $5 to $12 per ^{15}N determination, depending on the amount of processing prior to submission of samples (M. R. Russelle, 1991, personal communication). The continuous infusion experiment with six cows described above required approximately 500 determinations of ^{15}N enrichment and would have had a total cost for isotope plus analyses of about $4500 to $9000.

Radioactive Isotopes

In a manner analogous to use of ^{15}N, inorganic ^{35}S can be infused into the rumen and will be incorporated into BCP. Typical methods [e.g., (7, 43)] involve dosing $Na_2{}^{35}SO_4$ into the rumen, where sulfate-^{35}S, after reduction to sulfide-^{35}S, is incorporated into bacterial protein via de novo synthesis of cystine and methionine. The ^{35}S also will be incorporated into other sulfur compounds, such as coenzyme A. Indirectly, ^{35}S eventually will label protozoal proteins. A "typical" sample of ruminal microbes must then be isolated to provide the standard tracer:BCP ratio with which to compute BCP flow from the rumen. Potential health hazards of the weak ß-emissions from ^{35}S probably are low, and its relatively short half-life (87 d) would rapidly dissipate radioactivity from the environment. However, radioactivity accumulates in tissues and milk, thus precluding their use for food. Thus, routine use of ^{35}S as a microbial tracer in lactating dairy cows would be inconvenient because of waste disposal problems.

Bucholtz and Bergen (11) used ^{32}P as a tracer for ruminal microbes. Inorganic ^{32}P was incorporated into microbial phospholipids and showed considerable promise as a general tracer for both bacterial and protozoal biomass. However, ^{32}P has the same limitations as ^{35}S, except that its radioactivity represents a greater environmental hazard. Thus, ^{32}P cannot be recommended for widespread application without the substantial precautions that attend radioactive tracers.

RECOMMENDED MARKER SYSTEMS

We recommend use of two microbial marker systems: 1) the total purine method to meet the immediate need for a simple procedure with which to quantify BCP yields under practical feeding conditions in lactating dairy cows and 2) the $^{15}NH_3$ method, used with either continuous intraruminal infusion or discontinuous dosing via feed. Whenever possible, investigators should use both techniques to strengthen the data base with which other in-

formation may be compared (32). Work should be conducted to study use of 3) urinary excretion of purine metabolites on a wide variety of diets to test the general applicability of this procedure and 4) sampling of rumen contents only, relative to conventional sampling of abomasal or intestinal contents, in ^{15}N studies to test the potential of this less invasive technique for estimating postruminal BCP flows.

Total Purine Technique

An appropriate total purine assay for BCP yield in the urine would include the total purine isolation-quantitation procedure described by Zinn and Owens (87, 88), as modified by Ushida et al. (81). Aharoni and Tagari (3) have suggested additional modifications of the reagents used in this assay. We recommend that the purine:N ratio for BCP be determined using mixed bacteria isolated from ruminal contents (prepared from whole digesta pooled over time after feeding), as described by Cecava et al. (15), except that the ruminal contents from which the microbes are isolated should be treated with 1% (wt/vol) formaldehyde (22). Formaldehyde must be added to digesta, which are stored frozen prior to preparation of bacterial isolates; this is not necessary if microbes are isolated from fresh digesta. Experimental ruminants should be fitted with cannulas in the rumen and the abomasum or, preferably, the proximal duodenum; dairy cows at medium to high production would be most appropriate because of their high DMI. Standard marker methods for quantifying flows of digesta, total purines, and total NAN should be employed (60, 61).

^{15}N Technique

The method that we recommend involves continuous introduction of ^{15}N ammonium salts with enrichments of 10 to 85% (whichever is most economical) for about 48 h (31, 72); this is accomplished most satisfactorily by continuous infusion via the ruminal cannula. Including ^{15}N ammonium salts in feed may be a convenient alternative means of introducing the tracer into the rumen in a semicontinuous manner over longer periods (H. Tagari, 1991, personal communication). Firkins et al. (31) reported that continuous infusions of 7 and 8 d were required to approach a plateau for ^{15}N enrichments of duodenal and ileal NAN. However, there may be no need to reach constant specific enrichment of ruminal or postruminal N pools; what is sought is uniform distribution of ^{15}N tracer throughout the microbial N pools. After 2 d of continuous infusion, there will be little difference in ^{15}N enrichment in ruminal and postruminal microbial pools sampled at the same time. Mean turnover rate for ruminal NAN-^{15}N reported by Firkins et al. (31) was .027/h, which was equivalent to a retention time of 37 h. The regression equation (31) describing ^{15}N enrichment indicated that duodenal NAN reached .400 and .407 atom % excess after, respectively, 48 and 85 h of continuous infusion—suggesting that BCP flows estimated with a 48-h infusion would have an error of less than 2%. Except for continuous infusion for substantial periods, the FAB, PAB, and protozoal N pools will not equilibrate to nearly equal ^{15}N enrichment (31). The "representative" ^{15}N: microbial N rato for ruminal BCP should be determined using mixed bacteria isolated from formalin-treated, pooled ruminal contents as described (15, 22).

Simultaneous determinations of BCP yields made using both total purine and ^{15}N methodology could be used to address questions related to marker use. Firkins et al. (31) observed similar purine:N ratios (Table 4) and ^{15}N enrichments with intraruminal $^{15}NH_3$ infusion (Table 6) in FAB and PAB isolated from steers fed a 50% forage diet. However, mean protozoa:bacteria ratios were different for the two markers: protozoa:bacteria ratios were .69 (Table 6) for ^{15}N and .56 (Table 4) for total

TABLE 6. Differential ^{15}N enrichment of microbial fractions obtained with intraruminal infusion of $^{15}NH_3$ in steers fed a 50% forage diet (31).

Item	Daily DMI 2.1% of BW	Daily DMI 1.4% of BW
Atom percentage excess of ^{15}N		
Particle-associated bacteria	.039[a]	.038[a]
Fluid-associated bacteria	.038[a]	.038[a]
Protozoa	.027[b]	.026[b]
Protozoa:bacteria ratio	.70	.68

[a,b]Enrichments within columns with different superscripts differ ($P < .05$).

purines. Subsequently, those workers (32) reported on use of both purines and $^{15}NH_3$ infusion to quantify protein escape and BCP yield in steers fed the 50% forage diet at 1.4 and 2.1% of BW; UIP and BCP yield estimated by either method were not different at the lower feed intake, but ^{15}N predicted lower UIP and greater BCP yields at higher intake. Mean UIP predicted by both methods was 54% (32); UIP computed for this diet from NRC (56) tables was 44%. Further comparisons of ^{15}N to purine might test whether dietary purines were extensively degraded in the rumen: in defaunated ruminants, ^{15}N enrichment of purines passing the abomasum or duodenum are diluted, relative to the ^{15}N enrichment of purines isolated from mixed ruminal bacteria, by escape of dietary purines. Comparisons among diets also might help to assess the magnitude of dietary purine passage, e.g., high purine diets based on alfalfa versus low purine diets based on corn (71).

Differences between bacteria and protozoa in purine:N ratio (Table 4) and ^{15}N enrichment (Table 6) probably partly account for errors in BCP flows estimated by either technique. When purine-free semipurified diets are fed (88), the proportion of total abomasal or duodenal purine flow of protozoal origin can be computed from differences in ^{15}N enrichment of purine N in digesta, protozoa, and mixed bacteria. That there is apparently less than proportionate passage of protozoal mass out of the rumen is well established (37). Steinhour et al. (74) used a novel kinetic approach with ^{15}N to estimate proportions of bacterial and protozoal N in duodenal NAN. Faunated, unfaunated, defaunated, and refaunated experimental ruminants also might be used to study this problem.

Adjunct Methods

The procedures of Chen et al. (17, 19) are the basis for a recommended noninvasive procedure to assess influence of dietary and other factors on ruminal BCP yields using urinary excretion of purine metabolites. Hristov and Broderick (1992, unpublished data) are assessing the relationships between microbial yields, estimated from the total purine and ^{15}N procedures described, and total urinary excretion of allantoin (18, 45) and uric acid (18). Although total urine output was measured using catheterized cows, less invasive methods are available for total urine collection (30). However, total urine collection is inconvenient for large numbers of cows. We suggest that total excretion of urinary purines be estimated in lactating cows from partial collection of daily urine output (63) or from urine grab samples using purine:creatinine ratios and assuming constant excretion of creatinine in proportion to muscle mass (27). A substantial data base on factors influencing microbial yields of the rumen might be developed using such an approach.

Estimates by Ruminal Sampling

Ruminally cannulated ruminants can be prepared and maintained for years without apparent adverse effects on the cow or sheep and with little inconvenience to the investigator. However, in this era of increased awareness of animal welfare issues, preparation and maintenance of dairy cows fitted with cannulas in the abomasum or proximal duodenum, and possibly also the terminal ileum, are increasingly problematic. Public relations concerns may restrict the numbers of such animals housed in facilities that are accessible to the general public. Common sense dictates that scientists use the least invasive methods possible.

We suggest that approaches using ruminal cannulas should be tested for quantifying BCP yields. A brief outline of a research protocol might be 1) infusing $^{15}NH_3$ into the rumen to label microbial pools (as described), 2) isolating representative samples of microbial pools to determine the microbial ^{15}N:protein ratios, and 3) sampling digesta from the reticulum or preferably through the reticulo-omasal orifice. This approach was used by Nagel and Broderick (54) to quantify the effects of formic acid or formaldehyde treatment of alfalfa silage on BCP yield and ruminal protein escape. Reliability of sampling from the reticulum or omasal orifice as representative of digestive leaving the rumen should be addressed in multiply cannulated dairy cows at ad libitum feed intakes. However, such methods might prove to be useful for estimation of microbial yields under practical feeding regimens.

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