Metabolism of Peptides and Amino Acids During In Vitro Protein Degradation by Mixed Rumen Organisms[1]

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ABSTRACT

In vitro inoculum enriched with particle-associated organisms was prepared using rumen contents from a cow fed a 60% forage, 40% concentrate diet. Treatment of in vitro inoculum with cetyltrimethylammonium bromide was used to release intracellular free amino acids from mixed rumen organisms. Addition of 10 m*M* tosylarginine methyl ester, a competitive inhibitor of trypsin, decreased degradation rate and intracellular free amino acids in incubations containing either casein or serum albumin. Extracellular peptides increased rapidly to a maximum at 60 min in casein incubations but were not different from zero in albumin incubations. Accumulation of intracellular free amino acids was maximal at 60 min in casein and albumin incubations; the concentration observed with albumin was about one-fourth that with casein. Ammonia production from intact casein was slightly greater than that from acid and enzymatically hydrolyzed casein and about 80% greater than that from albumin. Ammonia production and appearance of extracellular free amino acids lagged behind accumulation of intracellular free amino acids. Results suggest that formation and metabolism of extracellular peptides are important in controlling the rate of protein degradation by mixed rumen organisms.

INTRODUCTION

Degradation of dietary protein in the rumen can reduce protein utilization because degradation often exceeds resynthesis by ruminal microbes. The process of microbial protein degradation in the rumen is not completely understood. It is known that proteolytic enzymes are closely associated with the ruminal microbes (18), free amino acid concentrations are generally low in cell-free rumen fluid (1, 7), and extracellular peptides can accumulate (28). Hence, peptides rather than free amino acids may be the protein fragments principally metabolized during ruminal protein catabolism.

The purpose of these experiments was to study the time course of appearance and metabolism of protein degradation products, particularly extracellular peptides and intracellular free amino acids, during in vitro incubations of soluble N sources with mixed rumen microbes.

MATERIALS AND METHODS

Incubation Conditions

Rumen contents were obtained from a ruminally cannulated lactating Holstein cow, weighing 635 kg, fed a diet of (DM basis) 35% alfalfa hay, 25% corn silage, 28.8% corn grain, 10% soybean meal, .7% dicalcium phosphate, and .5% trace-mineralized salt, plus 1600, 300, and .3 IU/kg DM, respectively, of vitamins A, D, and E. Equal portions of diet were fed 4 times per d at 6-h intervals; intake averaged 18 kg DM/d. Whole rumen contents were obtained 4 h after feeding and used to prepare in vitro inoculum as described by Craig et al. (12). The

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inoculum consisted of equal volumes of strained rumen fluid and McDougall's buffer (21), which was used to extract organisms associated with the solids. Microbial DM and N contents of each inoculum were determined in quadruplicate by analysis of pellets from high speed centrifugation (31,000 × *g*, 15 min, 2°C). Mean (± SE) microbial DM and N (2) of final incubation media were 8.79 (± .44) and .596 (± .009) mg/ml, respectively. The medium also contained 2 m*M* mercaptoethanol, and O_2-free CO_2 (17) was used in buffer preparation and all other applications. No inhibitors or energy sources were added to the inoculum. Resazurin (.1 mg/ml) was maintained in the reduced state.

Incubations were conducted in duplicate in spinner flasks (No. 1969-00250, Bellco, Vineland, NJ) equipped with bunsen valves and were maintained at 39°C in a warm room. Warmed inoculum (200 ml) was dispensed to each vessel. Incubations were begun by adding 100 ml (final volume 300 ml) of warmed McDougall's buffer alone (blank) or with N source dissolved in the buffer; vessels were gassed with CO_2. The N sources (Sigma, St. Louis, MO) studied were casein (No. C5890), bovine serum albumin (BSA; No. A9647), enzymatically hydrolyzed casein, type II (CHE; No. C4523), and acid-hydrolyzed casein (CHA; No. C9386). The N sources were prepared as .50% wt/vol solutions; final concentrations during incubations were .167% wt/vol.

Analytical Methods

Ammonia and total amino acids (TAA) were determined (6) in incubation samples, CHE and CHA solutions, and acid hydrolyzates (20 h, 6 N HCl, 105°C) of casein, BSA and CHE. Peptide-bound amino acids (PBAA) were determined by the difference method of Gardner (15), except samples were deproteinized with 5% wt/vol TCA, rather than sulfosalicyclic acid. High speed TCA supernatants were analyzed by ninhydrin-color reaction for TAA before and after hydrolysis under N_2 with 6 N HCl (20 h, 105°C); differences in TAA were considered to be PBAA. Determinations of PBAA also were made using a ninhydrin-CO_2 assay adapted to an autoanalyzer (19). This method is based on formation of CO_2 from reaction of amino acids with ninhydrin at pH 2.5; free amino acids (FAA), but not ammonia or peptides, give rise to CO_2 in the assay (16). Relative responses of individual FAA and 32 specific di-, tri-, and oligopeptides were determined using both ninhydrin assays. Concentrations of PBAA also were determined on samples obtained via rumen cannulae from cows fed alfalfa silage, high moisture corn plus protein supplements (8). Samples were treated with TCA (5% wt/vol) for ninhydrin-color assay or with H_2SO_4 (1% vol/vol) for the ninhydrin-CO_2 assay because TCA decomposes to CO_2 upon heating (19). In all determinations of ammonia, TAA, and PBAA, concentrations were corrected for recoveries of added ammonia and leucine (the standard amino acid). Casein, BSA, CHE, and CHA were analyzed for total N by micro-Kjeldahl (2). Extent of degradation of each N source was computed as described by Broderick (5).

Intracellular Amino Acids

The treatment used to release intracellular FAA was a modification of the procedure of Annison (1). In this method, 1-ml aliquots of inoculum were added to 1 ml of McDougall's buffer (blank) or 2% wt/vol cetyltrimethylammonium bromide (CTAB; Sigma, St. Louis, MO). After vortex mixing, samples were held on ice for 45 min and then centrifuged (15,300 × *g*, 30 min, 2°C). The CTAB procedure was compared to sonication and French press treatment with or without acidification to .2 N H_2SO_4 (13) prior to processing. Sonication (Model 185 Sonifier; Branson Sonic Power Co., Danbury, CT) at 90 W for 1 min was done using a micro-tip probe in 12 × 75-mm disposable centrifuge tubes held on ice. Treatment with the French press (American Instruments Co., Silver Spring, MD) was conducted at 137,000 KPa and 4°C. Sonicated and French press treated samples were held on ice for 30 min, then centrifuged (15,300 × *g*, 30 min, 2°C). Treatments were applied to duplicate samples of rumen inoculum obtained on 3 separate d.

Effectiveness of CTAB for releasing anticellular FAA was compared with TCA extraction in studies in which rumen organisms were preincubated with radioactive α-aminoisobutyric acid (AIB), a nonmetabolizable amino acid (31). Rumen inoculum was prepared as described. Aliquots (50 ml) of inoculum were

dispensed to two flasks containing 25 ml .50 m*M* unlabeled AIB in McDougall's buffer (background), and two flasks containing 25 ml .50 m*M* unlabeled AIB plus 5 μCi of [1-^{14}C]AIB (specific activity 52.6 μCi/nmol; New England Nuclear, Boston, MA). Flasks were gassed with CO_2, stoppered, then incubated at 39°C with mixing. After 120 min, flasks were placed in an ice bath and quadruplicate aliquots were mixed with equal volumes of McDougall's buffer, 2% wt/vol CTAB, or 10% wt/vol TCA. Samples were processed as described, and duplicate .5-ml aliquots of each supernatant were transferred to scintillation vials containing 15 ml of tritosol (14). Radioactivity was counted using a liquid scintillation spectrometer (Tricarb 3330; Packard Instruments, Downers Grove, IL) with correlation for counting efficiency using [^{14}C]toluene (New England Nuclear, Boston, MA) internal standard. Three separate experiments were conducted.

Trypsin Inhibition

The effect of tosylarginine methyl ester (TAME; Sigma, NO. T-4626), a trypsin substrate that also acts as a competitive inhibitor of trypsin (3), was studied by adding 10 m*M* TAME, directly to the inoculum. Incubations were conducted as described using casein and BSA. Duplicate samples were taken at 0, .5, 1.0, 2.0, and 3.0 h. Sample processing and analysis were as described. Incubations were repeated three times.

Time Course Studies

Two sets of rumen in vitro incubations were conducted to follow the time course of peptide and amino acid metabolism. In the first, blank, casein, BSA, CHE, and CHA incubations were conducted on 4 different d as described earlier, except aliquots were removed from each spinner flask at 0, 10, 20, 30, 45, 60, 90, and 120 min. Duplicate 1-ml samples from each incubation were mixed with equal volumes of McDougall's buffer, 2% wt/vol CTAB, or 10% wt/vol TCA. Samples were held on ice for 45 min, then centrifuged (15,300 × *g*, 15 min, 2°C). The supernatants were analyzed for ammonia and TAA (6) and PBAA.

The second series of incubations involved only blank and plus-casein vessels, and also was replicated on 4 different d. Samples were removed at 0, 2.5, 5.0, 7.5, 10, 15, 20, and 30 min; 1-ml duplicates were mixed with equal volumes of McDougall's buffer or 2% wt/vol CTAB. Further processing and analysis were as described.

Statistical Analyses

One-way ANOVA was used to analyze data (29). Separation of means where significant treatment effects were observed was by least significant difference. Computation of intraclass correlations, procedures for least squares regression, and statistical tests for differences between linear regression coefficients were those of Steel and Torrie (29).

RESULTS AND DISCUSSION

Intracellular Amino Acids

Treatment with the cationic detergent CTAB yielded intracellular FAA that were equal to or greater than several other chemical and physical treatments. Annison (1) theorized that CTAB released amino acids associated with the cell wall. It also seems possible that CTAB disrupts the permeability barrier of the cytoplasmic membrane, allowing small molecular weight compounds such as FAA to diffuse out.

The highest apparent intracellular FAA concentration was used as the criterion of greatest cell disruption. However, treatments that result in extensive hydrolysis of protein or peptides would also appear to elevate intracellular FAA. There were increases in apparent intracellular FAA in samples treated by sonication and French pressing when reanalyzed after storage for 3 d at 4°C. The effect of adding .2 N H_2SO_4 (found previously to stop microbial casein proteolysis) was tested in conjunction with CTAB, sonication, and French-press treatments. Estimated intracellular FAA were reduced slightly with H_2SO_4 addition to CTAB samples; however, H_2SO_4 greatly decreased FAA in both sonicated and French-press-treated samples (Table 1). Forsberg and Lam (13) used extraction with dilute H_2SO_4 to determine intracellular ATP; however, their use of cationic exchange resin would be expected to reduce amino acid recoveries. Results obtained with H_2SO_4 in the present studies suggest that much

TABLE 1. Effect of H_2SO_4 addition on intracellular free amino acids (FAA) in mixed rumen organisms treated with cetyltrimethylammonium bromide (CTAB), sonication, or French press.

Treatment[1]	Intracellular FAA, (μmol/g DM)[2]	
	$\bar{X}$	SE
CTAB	52.0[a]	.1
H_2SO_4/CTAB[1]	45.0[a]	3.0
Sonication	48.4[a]	8.7
H_2SO_4/Sonication[1]	6.2[c]	2.6
French press	26.2[b]	2.8
H_2SO_4/French press[1]	2.8[c]	.8

[a,b,c]Means without common superscript differ ($P<.05$).

[1]Inoculum acidified by addition of .05 ml 4 N H_2SO_4/ml prior to dilution with CTAB or McDougall's buffer (sonication and French press treatments).

[2]Concentrations of FAA (μmol/g microbial DM) in inoculum.

of the apparent intracellular pool of FAA with physically disrupted cells may have been artifacts resulting from solubilized proteolytic activity (18). Subsequent work (5) indicated that addition of 1% wt/vol CTAB followed by chilling was as effective as TCA in arresting proteolysis by mixed rumen organisms.

The model amino acid AIB is transported but not metabolized by bacteria (31); hence, preincubation of mixed rumen microbes with [1-^{14}C]AIB should serve to label the intracellular FAA pool. Recovery of radioactivity using direct dilution of medium with equal volumes of 2% wt/vol CTAB or 10% wt/vol TCA was used to test the effectiveness of both treatments for releasing intracellular FAA. Direct treatment with CTAB gave essentially complete recovery of all [^{14}C]AIB present in the intracellular pool (Table 2). Direct treatment with TCA was nearly as effective as CTAB (91 vs. 99% recovered; Table 2), indicating TCA released relatively more of the model amino acid AIB than total FAA in preliminary studies (28.8 vs. 54.9 μmol/g DM). This difference may be related to molecular weight or amino acid charge. It would be of interest to see if basic or acidic amino acids are released to the same extent as neutral amino acids with TCA treatment. Meznarich (22) reported that the highest estimates of intracellular FAA and ammonia pools were obtained with CTAB followed, in order, by boiling plus CTAB, boiling alone, CTAB plus TCA, and TCA alone.

It was difficult to assess effectiveness of our treatments because of a lack of literature data on intracellular FAA. Mandelstam (20) reported total intracellular FAA ranged from 3 to 8 mg/g DM in cell extracts from *Escherichia coli*. Assuming an average molecular weight of 100, this corresponds to 30 to 80 μmol/g DM. Tempest and Meers (30) reported highly variable intracellular FAA in extracts of three gram-negative organisms, ranging from 32 to more than 200 μmol/g DM under different culture conditions. Our estimates of intracellular FAA in mixed rumen microbes fall within these ranges.

The method used to release the intracellular FAA pool in further studies was: mixing of equal volumes of rumen in vitro medium with 2% wt/vol CTAB (final concentration 1% wt/vol), followed by chilling for 45 min and centrifugation (15,300 × *g*, 15 min, 2°C).

TABLE 2. Release of radioactivity from mixed rumen organisms preincubated with [1-^{14}C]α-aminoisobutyric acid ([^{14}C]AIB).

Treatment	Recovered [^{14}C]AIB (nmol/ml)[1]	Estimated intracellular pool[2]	
		Net (nmol/ml)	(%)
Buffer (extracellular)	944	. . .	. . .
CTAB[3]	1231	287	99
TCA	1209	265	91

[1]Recovered [^{14}C]AIB computed from observed dpm/ml medium and specific radioactivity of added [^{14}C]AIB (52.6 μCi/nmol).

[2]Theoretical intracellular pool of [^{14}C]AIB computed from difference between total [^{14}C]AIB added (1235 nmol/ml) and extracellular [^{14}C]AIB (944 nmol/ml, estimated from samples diluted with buffer alone) was 291 nmol/ml.

[3]CTAB = Cetyltrimethyl ammonium bromide.

Trypsin Inhibition

Addition of the artificial trypsin substrate, TAME, which acts as a competitive inhibitor of trypsin (3), reduced ($P<.05$) the degradation rates of casein from .459 to .162/h and BSA from .145 to .035/h, decreases of 65 and 76%, respectively. That similar degrees of inhibition were obtained with both proteins implies the importance of trypsin-like activity in ruminal microbial protein degradation (3, 4). The trypsin inhibitor also suppressed intracellular FAA accumulation during casein and particularly BSA degradation (Figure 1). Concomitant reduction in degradation rate and the intracellular FAA pool suggested the relationship of the latter to ruminal protein degradation.

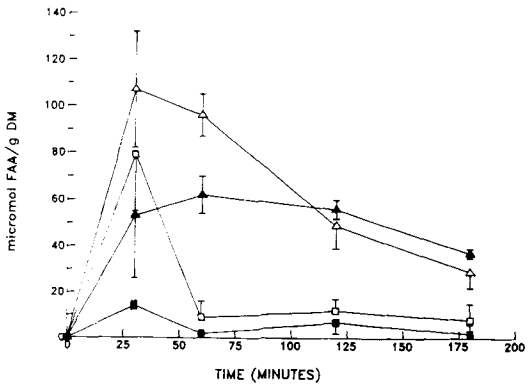

Figure 1. Effect of tosylarginine ethyl ester (TAME) on net (blank-corrected) concentrations of total intracellular free amino acids (FAA) during rumen in vitro incubations of casein with 0 TAME (▲) and 10 m*M* TAME (Δ) and bovine serum albumin with 0 TAME (■) and 10 m*M* TAME (□). Vertical lines represent ± 1 SE.

Time Course of Degradation

The difference method for determination of PBAA has been criticized because peptides as well as FAA yield ninhydrin color. If significant, this problem would result in underestimation of PBAA because of overestimation of FAA prior to HCl-hydrolysis. Therefore, we determined the reactivity of 12 dipeptides, 20 tripeptides and oligopeptides, and 20 individual FAA using the ninhydrin-color and ninhydrin-CO_2 assays. Responses of individual FAA in the ninhydrin-color system were similar to those reported earlier (6). Responses of the 32 peptides ranged from 1.6 to 79.3% of the molar response of free leucine. Responses of most peptides were less than 30% of leucine, and 10 peptides were less than 10%. However, responses (leucine = 100%) were high for peptides with N-terminal glycine (53 to 79%), tryptophan (42 to 46%), threonine (45%), serine (45%), or glutamate (50%). Reactivity of all peptides was very low in the ninhydrin-CO_2 system: 15 peptides gave no response and 16 gave responses which were .5 to 2.4 % of free leucine. Only aspartyl-alanine had substantial reactivity (12.0% of leucine). Setting leucine = 100%, mean molar responses of 19 FAA was 101.2 (SE = 3.5)%; aspartic acid gave a relative response of 190.6%. Because of its β-carboxyl group, free aspartate potentially can yield 2 CO_2/molecule (16).

The problem of peptide interference in the determination of PBAA based on the ninhydrin-color assay was studied further using in vivo rumen samples from cows fed alfalfa silage based diets (8). Overall mean PBAA concentrations were 3.09 and 2.95 m*M*, and intraclass correlations (29) from repeated PBAA analyses were .92 and .83 by the ninhydrin-CO_2 and ninhydrin-color assays, respectively. That PBAA values determined by ninhydrin-color were comparable to those obtained with the theoretically sounder ninhydrin-CO_2 assay may be due partly to use of dialysis in the autoanalyzer sample stream. Dialysis in our system reduced mean relative response (leucine = 100) of all 32 peptides to 62.5% (SE = 2.2) of that without dialysis. Mean relative response of the 20 individual FAA with dialysis was 103.4% (SE = 4.5) of that without dialysis. These results indicated that the ninhydrin-color assay in conjunction with the difference method would yield satisfactory estimates of PBAA concentrations. Chen et al. (11) approached the problem of peptide interference in the ninhydrin-color assay by assuming that FAA were negligible and by measuring PBAA as total ninhydrin reactivity following HCl-hydrolysis. However, FAA concentrations have been reported to be of similar magnitude to peptides in the rumen (9).

The pattern of metabolism of extracellular PBAA is in Figure 2A. There was rapid release of PBAA from casein to a maximum of 803 μmol/g DM at 60 mn before declining to 551

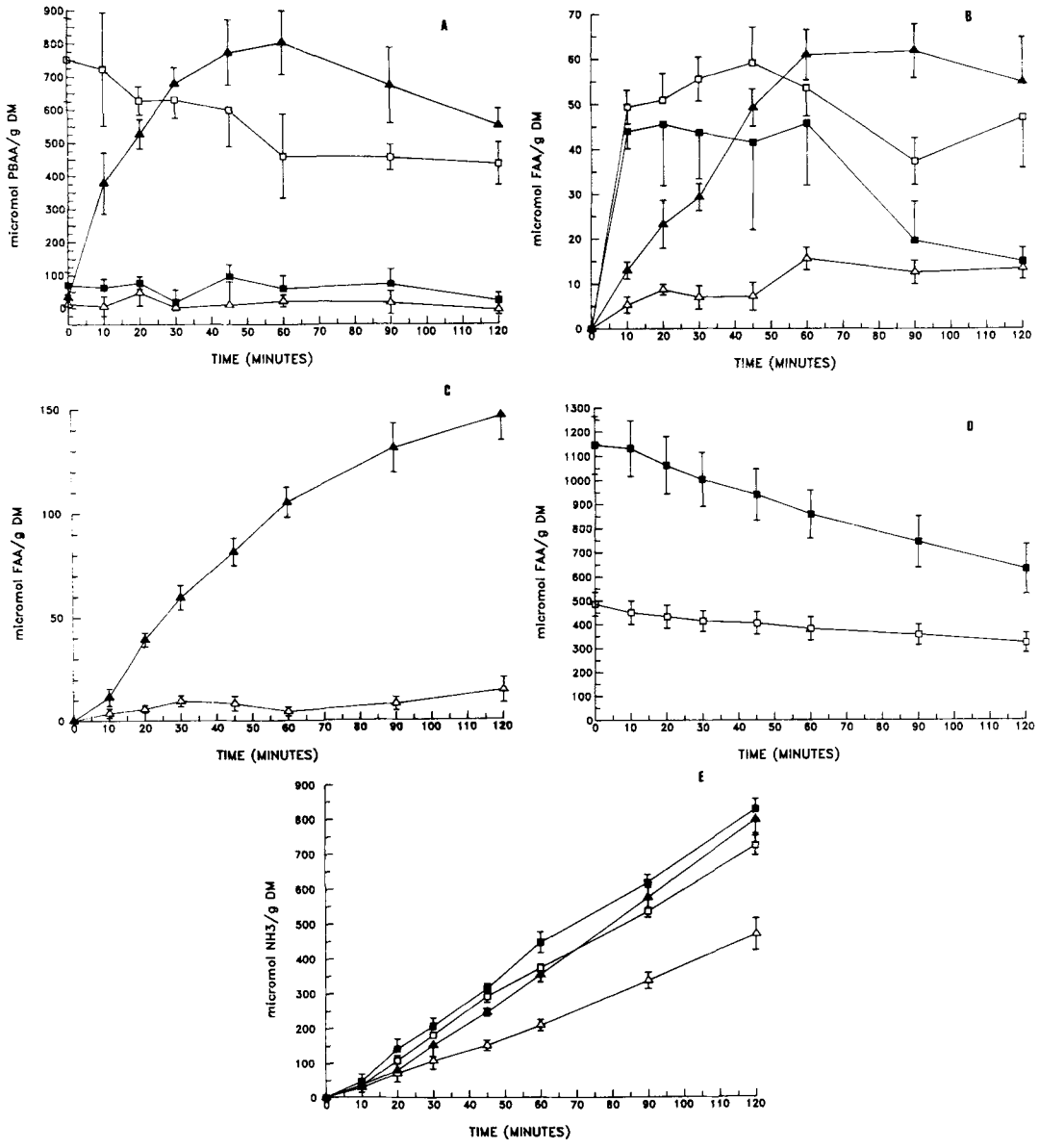

Figure 2. Net (blank-corrected) concentrations of protein degradation products during rumen in vitro incubations with casein (▲), bovine serum albumin (BSA, Δ), acid-hydrolyzed casein (CHA; ■) and enzymatically hydrolyzed casein (CHE; □). A) Extracellular peptide-bound amino acids (PBAA). B) Total intracellular free amino acids (FAA). C) Total extracellular FAA (casein and BSA). D) Total extracellular FAA (CHA and CHE). E) Total (intracellular plus extracellular) ammonia. Vertical lines represent ± 1 SE.

μmol/g DM at 120 min. Degradation of BSA did not yield PBAA accumulations at any time which were different from zero ($P>.10$). A similar relative pattern of ruminal peptide accumulation was observed in vivo in sheep fed a diet with 5% CP equivalent from casein; however, peptides did not accumulate in the rumen when comparable amounts of ovalbumin were fed (9). Mean PBAA at 0 h in CHE incubations was 752 μmol/g DM, decreasing exponentially

to 436 μmol/g DM at 120 min. The larger SE and the fact that mean PBAA in incubations to which CHA (which contained no peptides) was added were slightly greater than zero reflect the PBAA variability. Wallace (32) reported .3 μg/ml undigested casein was solubilized with TCA treatment of rumen in vitro incubations. This corresponds to an error of 35 μmol PBAA/g DM, much less than the SE of all our PBAA measurements (Figure 2A).

The relatively slow rate of clearance of peptides from the casein and CHE incubations was similar to the in vitro observations of Russell et al. (28), who reported that peptides from casein degradation by mixed rumen organisms remained elevated after 7 h. Accumulation of extracellular peptides over an extended time suggests their metabolism may limit ruminal casein degradation. The relatively slow clearance of casein peptides may be related to digestion of larger peptides to sizes small enough to diffuse through the cell envelope (to the region of cell membrane permeases) or to transport into the cell (24). Work by Payne and Gilvarg (25) with *Escherichia coli* suggested that the upper size limit for transport corresponded to peptides of 5-amino acid residues. However, Pittman et al. (26) reported that this limit was about 20 amino acid residues in the rumen anaerobe *Bacteroides ruminicola*. Nugent and Mangan (23) could not detect significant quantities of peptides during ruminal degradation of both casein and fraction I leaf protein. Chen et al. (10) reported peptides persisted in the rumen of cows fed conventional diets.

Intracellular FAA (determined by CTAB treatment) rose rapidly in both the CHE and CHA incubations and remained elevated over the first 60 min (Figure 2B). Concentrations in CHA incubations then declined to about one-third of maximum by 120 min, whereas CHE decreased to a lesser extent. The intracellular FAA in casein incubations rose steadily to a maximum of 61 μmol/g DM at 60 min and remained at about this concentration over the last 60 min of the incubation. The pattern of intracellular FAA with BSA was similar to but only about one fourth the concentrations observed with casein.

After a lag of about 10 min, extracellular FAA with casein rose in a curvilinear manner throughout the incubation (Figure 2C); however, extracellular FAA increased only slightly over the course of the BSA incubations. Clearance of extracellular FAA in the CHA and CHE incubations was linear (Figure 2D) at rates of 4.48 and 1.25 μmol·g DM^{-1}·min^{-1}, respectively. Linear rates of ammonia production were observed for all four N sources (Figure 2E). Rate of total ammonia production from CHA and CHE were 6.97 and 6.14 μmol·g DM^{-1}·min^{-1} and were not different ($P>.10$). Prins et al. (27) reported that in vitro degradation of casein amino acids by mixed rumen organisms was substantially faster when added as peptides rather than FAA. In our work, disappearance of extracellular FAA accounted for 32% of the ammonia production rate from CHE. The proportion of total N added as FAA in CHE incubations was 39%, suggesting similar contributions to ammonia formation from FAA and PBAA.

A lag of about 8 min was observed in ammonia production from casein; however, no lag was observed with BSA. Overall rate of ammonia formation from BSA was 56% of that from casein (3.95 vs. 7.00 μmol·g DM^{-1}·min^{-1}), whereas fractional degradation rate of BSA in these studies was only 33% of casein (.126 vs. .377/h). This reflects the greater proportion of casein degradation products that accumulated as FAA, whereas ammonia accounted for almost all (94% at 120 min) of the N released during BSA degradation. Ammonia production from casein, CHA, and CHE was similar, and, after the lag with casein, linear over the course of the incubation. This suggests that intracellular FAA with these three substrates quickly reached concentrations that were saturating for amino acid deamination.

The early time course of formation of FAA and ammonia from casein also was examined (Figure 3). Although not different ($P>.10$) at 2.5 min, intracellular FAA were higher ($P<.05$) than extracellular FAA at 5, 7.5, and 10 min of incubation. Except for a small spike at 5 min, total ammonia accumulation lagged behind appearance of FAA in the intracellular pool. These data suggest that intracellular FAA may begin to accumulate during protein degradation prior to appearance of extracellular FAA.

The metabolic pattern for degradation products from rapidly degraded casein suggests that rapid accumulation of peptides precedes appearance of intracellular FAA. Proteases associated with the outside of the cell coat (18)

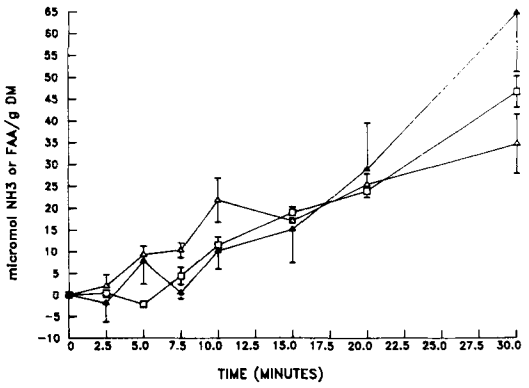

Figure 3. Early time course of appearance of net (blank-corrected) concentrations of total free amino acids (FAA) in the intracellular (Δ) and extracellular (□) pools and total (intracellular plus extracellular) ammonia formation (▲) during rumen in vitro incubations with casein. Vertical lines represent ± 1 SE.

probably are involved in formation of these peptides, and trypsin-like activity may be rate-limiting in the process (3, 4). These peptides may be transported into rumen microbial cells (26) prior to cleavage to FAA rather than extracellular proteolysis completely to FAA. However, recent research indicates metabolism of extracellular peptides is complex and may involve extracellular cleavage by dipeptidyl peptidases (Wallace and McKain, personal communication).

The rate of degradation of soluble BSA was substantially slower than casein and similar to that of largely insoluble feed proteins such as soybean meal (5). Little accumulation of peptides or FAA occurred during BSA degradation, indicating peptide metabolism was more rapid than formation. Intracellular deamination also was sufficient to prevent intracellular or extracellular accumulation of FAA. These results suggest that, under the conditions of our incubations, casein degradation (to ammonia) was limited by clearance of extracellular peptides or intracellular amino acids while BSA degradation was limited by proteolytic steps preceding peptide formation.

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