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Partial Characterization of a Protein–Carbohydrate Complex from the Rumen of Steers Fed High-Quality Forages

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Four ruminally and abomasally cannulated steers fed coastal Bermuda grass and alfalfa hay were used to investigate and characterize cell-free rumen fluid. The cell-free rumen fluid contained carbohydrates and uronic acids bound to an unidentified component that interacted with Sephadex and prevented the determination of molecular weight. Because Klason lignin or phenolic acids were not found, the strong 280-nm ultraviolet absorption and the interaction with the Sephadex indicated that proteins were involved. Amino acid analysis verified the presence of proteins; solid-state ^{13}C NMR indicated that proteins were present in the complex, but further indicated very low levels of phenolic components. The data support the conclusion that the material isolated from the rumen was a protein–carbohydrate complex and not a lignin–carbohydrate complex (LCC). This finding suggests that cattle consuming these high-quality forages produce little or no soluble LCC in the rumen.

Rumen contents are composed of stratified layers of particulate matter and solutes suspended in a buffered liquid phase. Particulate matter is composed of feed particles at various stages of digestion and the microflora and microfauna that inhabit the rumen. Solutes originate from ingested feed or liquids, saliva, secretions into the rumen, or metabolites of microorganisms. Solutes of particular nutritional importance are the nitrogenous components, the volatile fatty acids as well as other organic acids, minerals, vitamins, and buffers.

During the investigation of microbiol fermentation of carbohydrate components of feeds, Gaillard and Van't Kooster (1973) reported that polysaccharides and glycoproteins were added to the rumen liquor. In further studies on the digestion of glycoproteins, Gaillard and Richards (1975), using centrifugation, separated the digesta into discrete fractions and discovered that the cell-free rumen fluid (CFRF) contained sizable quantities of carbohydrate- and lignin-derived components. According to these authors, the CFRF contained 20% carbohydrate and 50% Klason lignin and gave UV and IR spectra characteristic of the aromatic components of lignin. In addition, polymers in the CFRF did not pass a dialysis membrane. When purified by gel filtration, an interaction between the polymers and Sephadex occurred, preventing the determination of molecular weight. From these results, Gaillard and Richards (1975) concluded that the CFRF was a lignin–carbohydrate complex.

This investigation was conducted to characterize the CFRF from steers fed high-quality alfalfa and coastal Bermuda grass hay.

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MATERIALS AND METHODS

Feeding Trial. Four Holstein steers (averaging 160 kg) fitted with permanent ruminal and abomasal cannulae were used to obtain rumen fluid. The rumen fluid was obtained in conjunction with a 4 × 4 Latin square arrangement of treatments to evaluate hemicellulose digestibility by steers fed alfalfa and coastal Bermuda grass as hay or drum dehydrated (Windham et al., 1987). However, only the rumen fluid from steers consuming coastal Bermuda grass hay (CBG-H) and alfalfa hay (Alf-H) were used in this study due to the complex and time-consuming laboratory analyses.

Isolation of Cell-Free Rumen Fluid. Ruminal fluid (4 L) for isolation of CFRF was collected prior to the 10:00 a.m. feeding on day 5 of each collection period, which followed a 10-day diet adjustment period. The rumen fluid was filtered through four layers of cheesecloth and transported to the laboratory. The CFRF was collected by centrifuging the fluid (100 mL/min, 27000*g*; Gaillard and Richards, 1975) in a Sorvall Model SS-3 centrifuge equipped with a Szent-Gyorgyi-Blum continous-flow system. The CFRF from each steer was dialyzed (10000 molecular weight cutoff) against running water for 2 days and then freeze-dried (Gaillard and Richards, 1975).

Analyses. Portions of the CFRF were analyzed for moisture by drying at 105 °C for 24 h and for crude protein (AOAC, 1976). Amino acid hydrolysates of the CFRF were prepared by refluxing aliquots of the CFRF in 6 N HCl under N_2 for 24 h (Wilkinson et al., 1968); amino acids were analyzed as described previously (Amos et al., 1976). Neutral carbohydrates were quantitated by hydrolyzing aliquots in 2 N trifluoroacetic acid for 30 min at 121 °C and determined by high-pressure liquid chromatography (HPLC) (Windham et al., 1987). Total carbohydrate was determined on the dialyzed CFRF by the phenol–sulfuric acid method (Dubois et al., 1956) and expressed as a percentage of CFRF. The percentage of carbohydrates obtained from acid hydrolysis and HPLC analysis was used to construct a standard curve for the phenol–sulfuric acid method. For evaluation of phenolic acids, CFRF was hydrolyzed with 50 mL of 1 N NaOH for 2.5 h and analyzed by capillary gas chromatography as described by Akin et al. (1987). Uronic acids were determined as outlined by Blumenkrantz and Asboe-Hansen (1973). Gel permeation chromatography was carried out on a 94 × 2.5 cm column

Table I. Composition of Dialyzed Cell-Free Rumen Fluid Obtained from Steers Fed Sun-Cured Alfalfa and Coastal Bermuda Grass Forages

		component,[d] %							
		carbohydrate							
forage	dry matter[a]	b	c	Rha	Xyl	Ara	Glu	Gal	uronic acid
alfalfa	251.50	11.05**[f]	11.04**	2.45+	1.58*	0.99**	3.08	2.94**	3.91**
GBG	241.82	15.16	7.20	2.08	2.09	0.00	3.03	0.00	5.46
SEM[e]	16.64	0.90	0.34	0.11	0.08	0.06	0.18	0.27	0.20

[a] Values correspond to milligrams of cell-free rumen fluid/100 mL of rumen fluid. [b] Carbohydrate determined by phenol–sulfuric acid method. [c] Total recovered carbohydrate and monomers determined by high-pressure liquid chromatography. [d] Values correspond to percent of dry matter. [e] Standard error of the mean. [f] Key: +, $P < 0.10$; *, $P < 0.05$; **, $P < 0.01$.

of Sephadex G-100. Aliquots of the CFRF were dissolved in deionized water (9.4 mg/mL), and 3 mL was applied to the column with 0.15 M sodium chloride as the eluent. Fractions collected were monitored for total carbohydrate (phenol–sulfuric acid) and uronic acid and for absorbance at 280 nm. Data were treated by analysis of variance for a replicated 2 × 2 reversal with the statistical analysis system described by SAS (1982).

Solid-State ^{13}C NMR Spectroscopy. The techniques of cross-polarization and magic-angle spinning (Schaefer and Stejskal, 1979) in conjunction with a JEOL FX-270 NMR spectrometer operating at 67 MHz for carbon-13 were used to obtain a solid-state NMR spectrum of the CFRF. The system was equipped with high-power amplifiers and a narrow-bore magic-angle spinning probe supplied by Chemagnetics. The freeze-dried, dialyzed CFRF was packed in a bullet-type Kel-F rotor having a sample volume of 0.4 cm^3 and was spun at 2.8 kHz. The data collected represented 30 000 pulses with a pulse delay of 2 s and a 1-ms contact time. The spectrum was externally computer-referenced to hexamethylbenzene at 132.3 ppm (frequency 67 MHz).

RESULTS AND DISCUSSION

The yield of dialyzed CFRF differed ($P < 0.05$) from steer to steer for each forage. The concentrations ranged from 151 to 332 and 208 to 328 mg/100 mL of rumen fluid for CBG-H and Alf-H, respectively. This variation is in agreement with Neilson and Richards (1978) who reported that the concentration of CFRF from four different steers on the same spear grass hay diet varied from 104 to 227 mg/100 mL of rumen fluid. However, the composition of the CFRF did not differ ($P < 0.05$) due to animal. The carbohydrate concentration from CFRF by the phenol–sulfuric acid method was greater ($P < 0.01$) from the steers consuming CBG-H (Table I). These percentages are comparable to the carbohydrate content of CFRF obtained from steers grazing urochloa and buffelgrass in early regrowth but considerably lower than that obtained from steers consuming spear grass hay (Neilson and Richards, 1978) and a Holstein cow consuming a semipurified diet containing 45% ground corncobs or 38% ground sawdust (Williams et al., 1979). The concentration of carbohydrate based on recovery of component monosaccharides from HPLC analysis was equal to the phenol–sulfuric method for Alf-H CFRF but 52.5% less for CBG-H CFRF. These data suggest that some other component of CBG-H CFRF was reacting with the phenol–sulfuric reagent. The carbohydrate components of the CFRF are in general agreement with Neilson and Richards (1982) but differ substantially from work on the monosaccharide composition of CBG-H and Alf-H hemicellulose (HC) (Windham et al., 1987). Lower proportions of xylose and arabinose were present in the CFRF compared to the predominance of these monosaccharides in HC from CBG-H and Alf-H. In addition, arabinose and galactose composed 4.2 and 1.7%, respectively, of CBG-H HC. Although these sugars were present in the HC of CBG-H, they were absent in the CFRF of CBG. The relative proportions of rhamnose and uronic acids were greater in the CFRF than in Alf-H and CBG-H HC, indicating their resistance to degradation. Neilson and Richards (1982) also reported an increase in rhamnose in the CFRF compared to its concentration in spear grass hemicellulose and deduced that rhamnose was resistant to degradation and was, therefore, associated with lignin in the cell wall.

Gaillard and Richards (1975) accounted for 34% of the total CFRF dry matter (300 mg/100 mL of rumen fluid) as carbohydrate, protein, and ash. Sephadex gel filtration of the CFRF resulted in an interaction between CFRF and the gel that prevented the determination of molecular weight. In addition, there was a coincidence between absorbance at 280 nm and carbohydrate. They concluded that the absorbance at 280 nm was due to the aromatic molecules derived from lignin and that the carbohydrates were bound to lignin that interacted with the gel, indicating that the material was a lignin–carbohydrate complex (LCC). Gaillard and Richards (1975) thus accredited the remaining 198 mg (i.e., 66% of CFRF) to the aromatic molecules derived from lignin. The Klason lignin concentration of the LCC was 52%.

The difference in the expected lignin content and that from the 72% sulfuric acid method was probably due to loss of lignin during the analysis (Hartley, 1973; Neilson and Richards, 1978). However, it is unclear as to the nature of this apparent "Klason lignin". Unless protein is totally removed prior to Klason lignin determination, the insoluble residue cannot be considered to be a quantitative indication of lignin (Sarkanen and Ludwig, 1971). On the basis of these data and the assumption that the volume of fluid leaving the rumen and entering the abomasum was approximately 150 L/day, Gaillard and Richards (1975) stated that the presence of 198 mg of lignin in the LCC/100 mL of rumen fluid corresponds to approximately 43% of the total lignin intake. Under the conditions of our experiment, deducting carbohydrate by HPLC, uronic acids and total amino acid nitrogen (N) (Table II) left 133.7 and 127.4 mg of dry matter/100 mL to be accounted for in the CFRF of CBG-H and Alf-H, respectively. The steers consumed 3341 and 3273 g of dry matter of CBG-H and Alf-H containing 3.7 and 8.7% lignin, respectively (Amos et al., 1984), with a volume of 57.1 L/day leaving the rumen. These calculations were based on liquid digesta flow to the abomasum as determined by poly(ethylene glycol) (Amos et al., 1984). Thus, the material unaccounted for would correspond to 62.0 and 24.8% of the total lignin intake for CBG-H and Alf-H, respectively. However, when CBG-H and Alf-H CFRF were analyzed for Klason lignin, 100% of the material was solubilized, indicating that no lignin was present. In addition, no saponifiable phenolic acids were detected. These data suggest that cattle consuming these high-quality forages produce little or no soluble LCC in the rumen. The data of Neilson and Richards (1978) support this suggestion. They found that cattle grazing fresh young high-quality forages produced a significantly lower level of

Table II. Amino Acid Composition (%) of Dialyzed Cell-Free Rumen Fluid Obtained from Steers Fed Sun-Cured Alfalfa and Coastal Bermuda Grass Grass Forages

amino acid	coastal Bermuda grass[b]	alfalfa[b]	SEM[a]
Lys	2.71[c]	2.81[c]	0.08
His	0.52[c]	0.56[c]	0.02
Arg	1.02[c]	1.16[c]	0.06
Asp	4.41[c]	4.62[c]	0.07
Thr	2.00[c]	2.15[c]	0.05
Ser	1.40[c]	1.49[d]	0.02
Glu	4.08[c]	4.41[d]	0.02
Pro	1.21[c]	1.22[c]	0.03
Gly	1.71[c]	1.77[c]	0.03
Ala	2.33[c]	2.56[c]	0.06
$^1/_2$Cys	0.20[c]	0.26[c]	0.03
Val	1.91[c]	2.10[d]	0.02
Met	0.85[c]	0.86[c]	0.02
Iso	1.69[c]	1.80[d]	0.01
Leu	2.19[c]	2.36[d]	0.02
Tyr	1.85[c]	2.03[d]	0.03
Phe	1.84[c]	2.07[c]	0.01
Dap	0.14[c]	0.15[c]	1.23
total	32.07	34.38	
recovered N as AA	82.54[c]	82.92[c]	1.02

[a] Standard error of the mean. [b] Values correspond to percent dry matter (Table I). [c,d] Row means with unlike superscripts differ ($P < 0.05$).

soluble LCC with a low carbohydrate percentage.

Gel permeation chromatography and solid-state ^{13}C NMR spectroscopy were used to evaluate the type of material that had been isolated. The highest yield of CFRF (331.7 mg/100 mL) was obtained from the steer consuming CBG-H in period 2 of the Latin square, and therefore, subsequent work was confined to this material because of its abundance and availability. The chromatogram using G-100 is shown in Figure 1 and is similar to that reported by Gaillard and Richards (1975). Peak 1 shows coincidence of carbohydrate and uronic acid and of 280-nm absorbance, and because the peak eluted at V_o, this "complex" or mixture of compounds must have a molecular weight greater than 150 000. Peak 2 also shows coincidence between the components of the material and eluted well beyond the volume where solutes of low molecular weight (less than 4000) emerge from the column. This figure is evidence that the material isolated was a high molecular weight complex and that the carbohydrate constituents are bound to some other type of material retarded by the gel. Such interactions were also found by Gaillard and Richards (1975) and can be accredited to aromatic molecules derived from lignin because the nitrogen content of the LCC was too low to account for the strong absorbance at 280 nm. However, the strong ultraviolet absorption in our material is quite likely associated with protein.

The amino acid (AA) compositions of CBG and Alf CFRF are shown in Table II. Although there were some differences ($P < 0.05$) in the amounts of AA between CBG and Alf, the overall AA profiles were similar. This is reflected in the total AA recovered in CBG and Alf CFRF. In addition, approximately 83% of the N present in the CFRF was AA N as compared to the Kjeldahl N. The remaining 17% of the N not accounted for by amino acids is probably associated with the carbohydrate moiety of the complex. These data clearly indicate that approximately 33% of the CFRF DM was composed of amino acids and thus could account for the strong absorbance at 280 nm. Of particular interest was the finding that the protein of the CFRF also contained diaminopimelic acid (DAP). Because DAP is indicative of bacterial protein and not

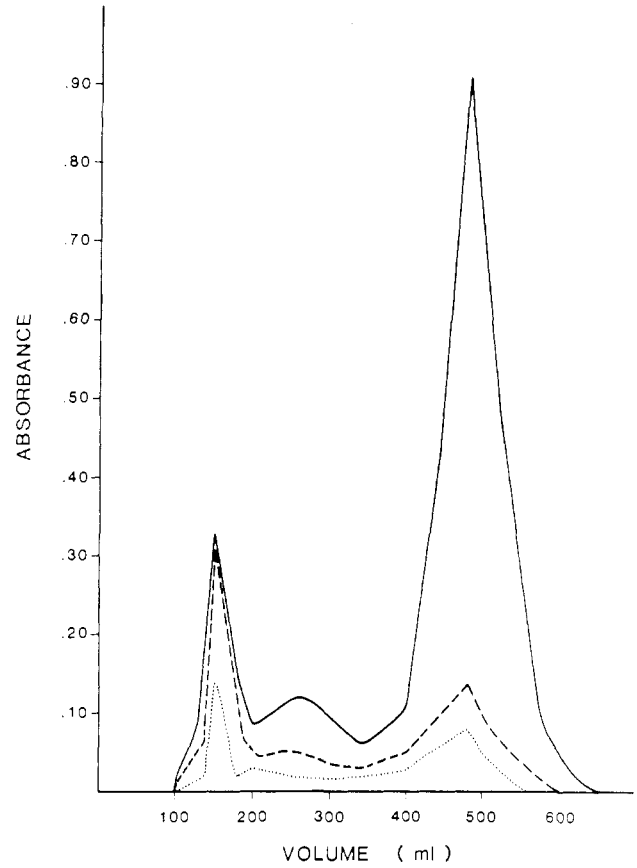

Figure 1. Fractionation of dialyzed cell-free rumen fluid on Sephadex G-100: —, A_{280}; - - -, carbohydrate; ···, uronic acid.

commonly found in forage protein, we suggest that this protein–carbohydrate complex is, in fact, of bacterial origin and a product of anabolism rather than catabolism. In addition, DAP comprised approximately 0.43% of the total recovered amino acids (proteins), which was consistent with the values published by Amos et al. (1984) for partially purified bacterial protein. The presence of this bacterial protein–carbohydrate complex in the CFRF could result either from the lysis (due to death, damage, etc.) of intact bacterial cells with the subsequent release of bacterial glycoproteins or from the secretion of an extracellular protein–carbohydrate complex by the viable rumen bacterial cells and/or the release of ionically bound glycoprotein from the bacterial cell wall fragments or "ghosts" due to the ionic conditions of the rumen fluid. These possibilities and the effect of DAP in this fraction on the estimate of bacterial protein synthesis are currently being investigated.

The evidence of a protein–carbohydrate complex was further verified by solid-state ^{13}C NMR spectroscopy (Figure 2). This spectrum showed that the material contained a considerable amount of protein as indicated by signals at 21 and 23 ppm (attributable to methyl groups in Ala, Leu, or Val), 32 ppm (attributable to the CH_2 in Val), 131 ppm (which can arise from protonated aromatic rings), and 173 ppm (primarily due to the amide carbonyls but also to carbonyls of acidic amino acids). Carbohydrates are indicated by the large signal at 72 ppm (for hydroxymethylenes) and the signals at 94, 97, and 102 ppm (for anomeric carbons). Many aromatic amino acid signals appear in the region from 115 to 140 ppm, which is somewhat obscured by a carbonyl signal sideband (SB). There is an indication of phenolic aromatic components in the region from 140 to 160 ppm, but these signals are mainly due to the ϵ-carbons of Arg and Tyr or the phenolic

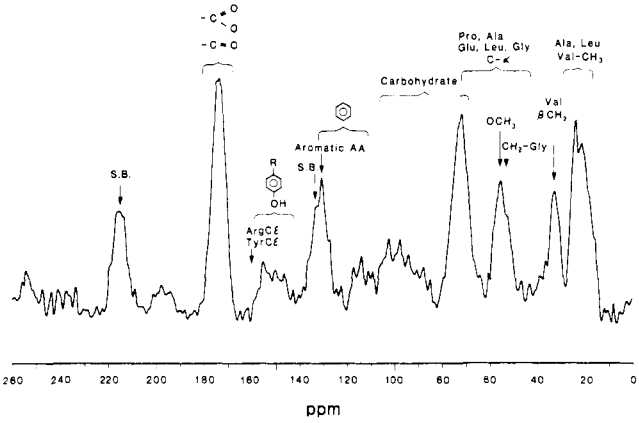

Figure 2. Solid-state CP/MAS ^{13}C NMR spectrum at 67 MHz of cell-free rumen fluid obtained from a steer consuming coastal Bermuda grass hay (SB = sideband, AA = amino acid).

carbon in Tyr. There is also the possibility that some low molecular weight, lignin-type materials are giving rise to signals in this region of the spectrum.

However, these spectral results and the data of Tables I and II support the general conclusion that the material isolated was essentially a protein–carbohydrate complex and not a lignin–carbohydrate complex. Approximately 50% of this complex was composed of protein and carbohydrate. The composition of the remaining 50% is not known at this time. Gaillard and Richards (1975) found 2.0% protein N within the LCC obtained from steers fed spear grass hay. Williams et al. (1979) reported protein N contents of cell-free rumen fluid of 3.9 and 3.2% from a Holstein cow fed a semipurified diet plus ground corncobs and sawdust, respectively. These later authors suggested that N present was from glycoproteins added to the digesta from saliva. However, McDougall (1948) reported that protein N content of sheep's saliva was variable but usually on the order of 0.1–0.2%.

The detection of no soluble LCC in the rumen has significant implications in interpretation of the ruminant digestion process. Apparent lignin digestibility or incomplete recovery of lignin has been reported (Fahey et al., 1979; Gaillard and Richards, 1975; Muntifering, 1982) to be possibly due to apparent digestion obtained by formation of soluble LCC that passes from the rumen as a polymer in the liquid phase of the rumen digesta. The other probable reasons for incomplete lignin recoveries as summarized by Muntifering (1982) are partial destruction of fecal lignin fractions by reagents used in the analytical methods (Fahey et al., 1979) and physical and/or chemical differences between feed and feces in the nature of material empirically defined as lignin. In order to credit apparent lignin digestibilities to the formation of a soluble LCC, it is obviously relevant to know whether production of a soluble LCC was sizable in light of the findings reported here.

Registry No. Raffinose, 512-69-6; D-xylose, 58-86-6; D-arabinose, 10323-20-3; D-glucose, 50-99-7; D-galactose, 59-23-4; Lys, 56-87-1; His, 71-00-1; Arg, 74-79-3; Asp, 56-84-8; Thr, 72-19-5; Ser, 56-45-1; Glu, 56-86-0; Pro, 147-85-3; Gly, 56-40-6; Ala, 56-41-7; Cys, 56-89-3; Val, 72-18-4; Met, 63-68-3; Iso, 73-32-5; Leu, 61-90-5; Tyr, 60-18-4; Phe, 63-91-2.

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