Forage Genetics and Production

Yield and Nitrogen Fixation of Annual Medics

Y. Zhu, C. Sheaffer, M. Russelle and C. Vance

Introduction

Annual medics are winter annual legumes used in the Australian ley farming system, which integrates leguminous pastures into cereal production systems. The ley system produces improved soil fertility and higher subsequent cereal yields. Annual medics also contribute some symbiotically fixed nitrogen (N) to intercropped grasses. In the North Central USA, annual medics have been evaluated for potential use as summer annual forage sources, as smother plants for weed control in corn, and as intercrops with small grains. Information on N contribution of annual medics in these and other cropping systems, however, is needed to determine optimum N management.

Our objectives were to: 1) describe the aboveground biomass accumulation pattern of spring seeded annual medics in Minnesota, and 2) estimate the proportion and amount of N derived from the atmosphere by spring seeded annual medics using the isotope dilution and difference methods.

Methods

Field experiments to determine dry matter accumulation patterns were conducted at Becker, Minnesota, on a Hubbard loamy sand in 1993 in 1995. Four annual medic species, *Medicago truncatula* vs. Mogul, *M. polymorpha* cv. Santiago, *M. scutellata* cv. Sava, and *M. rugosa* cv. Sapo, were planted in the spring in rows spaced 15 cm apart within 3- by 6-m plots at the rate of 484 live seeds m^{-2}. All medics were inoculated with commercial inoculant (a mixture of 5 rhizobial strains) specially selected for annual medics. Plants were sampled beginning 14 days after planting until maximum dry matter was accumulated, which was in early to mid-August. Harvest intervals averaged 12 days in 1993 and 18 days in 1995. Herbage was removed from a 0.2-m^2 area and samples were dried at 60 °C and then weighed. The experimental design was a randomized complete block with treatments in a split plot arrangement. Medic species were main plots and sampling times were subplots. There were six replicates.

Field experiments to estimate symbiotic N_2 fixation were conducted at Becker (1993 and 1994) and at Rosemount (1993), Minnesota. The soil at Rosemount was a Tallula silt loam. Noninoculated *M. rugosa* was used as a reference crop because it is poorly nodulated by indigenous rhizobia. 'Surrey' annual ryegrass (*Lolium multiflorum*) also was grown as a reference crop. Ryegrass was seeded in 15-cm rows at a rate of 1120 live seeds m^{-2}. Ammonium sulfate enriched to 99.4 atom % ^{15}N was applied at a rate of 1.2 kg N ha^{-1} as an aqueous solution on a 2-m^2 subplot within each plot 10 days after seeding. The central 1 m^2 of each plot was harvested in August, when maximum herbage dry matter was reached. Samples were dried, ground, and then analyzed for total N and ^{15}N on an integrated combustion analyzer-mass spectrometer. Standard isotope dilution and difference equations were used to calculate N derived from the atmosphere (Ndfa).

Results and Discussion

Herbage dry matter increased for most medics until 72 days after planting, but *M. polymorpha* did not achieve maximum dry matter yield until 84 days after planting. Dry matter yields averaged about 9100 kg ha^{-1} in 1993 and 5000 kg ha^{-1} in 1995. Yields of the inoculated *M. rugosa* were 8000 kg ha^{-1} in 1993 and 4700 kg ha^{-1} in 1995, whereas noninoculated *M. rugosa* yields were 3500 kg ha^{-1} in 1993 and 870 kg ha^{-1} in 1995, respectively, indicating lower N supply via symbiotic fixation. Nodules were present on the roots of all inoculated medics at the first sampling, 14 days after planting.

Based on the isotope dilution technique, herbage of annual medics at maximum dry matter accumulation contained an average of 86% Ndfa, using ryegrass as the reference crop, or 79% Ndfa, using noninoculated *M. rugosa* as the reference crop. Higher %Ndfa with ryegrass was likely due to its shallower, more fibrous root system and the possibility of small rates of N_2 fixation by the noninoculated *M. rugosa*. We found very few nodules on roots of the latter crop, and conclude that results based on *M. rugosa* probably are more representative than those with ryegrass. At Becker, the %Ndfa was similar for all annual medic species, but at Rosemount, %Ndfa was higher for *M. polymorpha* (81%) than for *M. scutellata* and *M. rugosa* (72%). The correlation between %Ndfa based on the isotope dilution technique and based on the difference technique was 0.88 ($P = 0.01$, n = 12) when noninoculated *M. rugosa* was the control crop.

Based on the isotope dilution technique, the amount of Ndfa in annual medic herbage ranged from 101 to 205 kg N ha^{-1}. At both locations, *M. polymorpha* had the highest Ndfa, whereas *M. rugosa* had the lowest. The correlation coefficient for estimates of Ndfa by the isotope dilution technique and the difference technique was 0.998. This suggests that the simpler, less expensive difference technique could be used to evaluate symbiotic N fixation in annual medics. Nevertheless, we recommend that the isotope dilution technique be used on soils that have abundant inorganic N supplies, because our other research has indicated that substantial differences in soil N uptake can occur between N_2 fixing and nonfixing species.

Our results indicate that annual medics may provide a good source of symbiotically fixed N in cropping systems in northern climates. Their growth habit supports relatively high N fixation rates, and maximum dry matter and N accumulation can be achieved within 70 to 85 days after spring planting.

Fertilizer N and Inoculation Effects on Annual Medics

Y. Zhu, C.C. Sheaffer, C.P. Vance, P.H. Graham, M.P. Russelle and C. Montealegre

Introduction

Annual medics are important winter annual pasture legumes in southern Australia, where they provide forage for livestock, improve soil fertility, and enhance subsequent crop yield. Medics have been evaluated recently in the North Central USA as summer annual forage crops and as intercrops in small grain and corn.

The symbiotic N_2 fixation capacity of annual medics varies with medic species, *Sinorhizobium* strains, and environmental conditions, especially soil inorganic N concentration and pH. Rates of season-long symbiotic N_2 fixation by annual medics in Minnesota range from about 100 to 200 kg N ha^{-1}. There have been no detailed studies of the effect of soil inorganic N supply and the competitiveness of various *Sinorhizobium* strains included in currently available commercial inoculant. The objective of this research was to address both of these questions.

Methods

Effect of fertilizer N and inoculation. Field experiments were conducted at Becker, MN, on a Hubbard loamy sand with no recorded history of annual medic cultivation. Whole plot treatments were factorial combinations of inoculation (+I and –I) and N fertilizer (+N and –N). Seed in the +I treatment received commercial inoculum comprised of 5 *Sinorhizobium* strains before planting. The +N treatment consisted of 100 kg N ha^{-1} broadcast as ammonium nitrate at planting. Subplot treatments were 8 *Medicago* species (*M. littoralis* cv Harbinger, *M. lupulina* cv George, *M. polymorpha* cv Santiago, *M. rugosa* cv Sapo, *M. scutellata* cv Sava, *M. truncatula* cv Mogul and Sephi, and *M. sativa* cv Nitro) seeded in spring (late April to early May) and summer (late July to early August) of 1991 and 1992.

Herbage and roots were harvested in late June to early July for spring seedings and in mid-September for summer seedings. A subsample of 20 root systems was analyzed for nodule mass score, percentage of plants nodulated, and number of roots with nodules. Herbage and root dry mass was determined and herbage was analyzed for total N using near infrared spectroscopy. Apparent symbiotic N_2 fixation was estimated using the difference technique [N derived from the atmosphere = $(\%N_{fixing} \times yield_{fixing}) - (\%N_{nonfixing} \times yield_{nonfixing})$, where the nonfixing plant was noninoculated *M. rugosa*]. All data from three field replicates were subjected to analysis of variance, and means were separated using Fisher's protected LSD ($p < 0.05$).

Nodule occupancy. Inoculated and noninoculated annual medics (*M. polymorpha*, *M. rugosa*, *M. scutellata*, and *M. truncatula* cv Mogul) were seeded in early May 1993 in single rows spaced 15-cm apart at Becker and St. Paul, MN. The soil at St. Paul was a Hayden fine sandy loam. Peat-based inoculum containing five *Sinorhizobium meliloti* strains (102G3, 102A13, 102Z5, 102H2, and 102B11) was applied to the seeds before planting. Nodules were collected from tap and lateral roots from five randomly selected 50-day-old plants in every plot. Separate antisera were developed against each strain and against 102F51, the primary strain in commercial alfalfa inoculum. Cross reactivity was selectively removed by massive adsorption of the antisera with steamed, washed *Sinorhizobium* cells. The indirect ELISA procedure was used to establish nodule strain occupancy on five replicates, and differences were determined using analysis of proportion.

Results

Effect of N fertilizer and inoculation. Although legumes had not been grown at the site for several years, indigenous *Sinorhizobium* capable of nodulating annual medics were present, and at least 75% of plants in all species were nodulated, except *M. rugosa*, which was poorly nodulated. In –N treatments, inoculation improved nodulation only of *M. rugosa*, but with fertilizer N, inoculation improved nodulation of *M.*

rugosa, *M. polymorpha*, and *M. truncatula* cv Sephi compared with –I. Fertilizer N reduced nodulation of most species that received commercial inoculum, but only some species when inoculation was withheld.

Herbage dry matter yield ranged from 1700 to 3100 kg ha^{-1} and was not affected by inoculation or N fertilization of summer seedings. This may have been due to presence of sufficient soil inorganic N for the 43-day-long growth period in this treatment. In contrast, inoculation improved herbage yield of spring seedings. Nitrogen addition improved yield of four entries in the –I treatments, but only of *M. scutellata* in the +I treatment, implying that more effective inoculum is needed for this species. Neither N fertilizer nor inoculation affected herbage yield of *M. lupulina*, *M. littoralis*, or *M. sativa*, indicating that indigenous *Sinorhizobium* were as effective as commercial inoculum for these legumes. Root dry matter yields ranged from 70 to 200 kg ha^{-1} and were not affected by inoculation or N fertilization.

For spring-seeded plots, estimated N_2 fixation ranged from 40 to 86 kg N ha^{-1}, with *M. polymorpha*, both *M. truncatula* entries, and *M. scutellata* having the greatest fixation. For summer-seeded plots, N_2 fixation ranged from 20 to 50 kg N ha^{-1}, with *M. scutellata* and *M. truncatula* cv Mogul having highest values. These are lower values than obtained in other research, but only 60 and 43 days were allowed for growth of spring and summer seedings, respectively.

Nodule occupancy. Strain occupancy of nodules did not differ between lateral and tap roots, and a high proportion of nodules was occupied by two or more strains, as is typical of multilobed nodules. *Sinorhizobium* strains serologically related to 102F51, the most common strain nodulating alfalfa in the Midwestern USA, were the most prevalent in medic nodules in the –I treatment. At least 68% of nodules apparently contained this strain, except in *M. rugosa*, which showed only 5% of plants nodulated in the –I treatment. Among the *Sinorhizobium* strains in the inoculum, 102G3 and 102A13 were consistently among the most prevalent strains in nodules of +I and –I plants of all annual medics, whereas 102H2 was not identified in any nodule. Presence of all other strains in at least some nodules of the –I treatment indicates that these strains occur in soils at both locations in Minnesota.

Results of this research indicate that some strains of *Sinorhizobium* capable of nodulating annual medics can be found in Midwestern USA soils. Inoculation may not be warranted when annual medics are to be grown for very short periods. The existence of differences in nodule occupancy among *Sinorhizobium* strains implies that selection for more competitive strains may be possible. Apparent symbiotic N_2 fixation rates of 0.5 to 1.4 kg N ha^{-1} d^{-1} were found in this research, indicating that annual medics have the potential to add significant amounts of N to cropping systems in relatively short time periods.

^{15}N Labeling of Dairy Feces and Urine for Nutrient Cycling Studies

J.M. Powell and Z. Wu

Introduction

The economic value of animal manure depends on its ability to provide nutrients to crops. The timely delivery and application of known amounts of manure to specific fields is the primary basis for proper manure management. Estimates of manure nutrient availability to crops, otherwise known as "nutrient credits", are currently single nitrogen (N), phosphorus (P) and potassium (K) values given for the type of manure applied (solid or liquid) and method of application (incorporated or not). Nutrient credits are adjusted to account for multiple years of manure application, residual nutrient availability, etc.

Although it has been shown that proper manure management can be profitable through reduced fertilizer costs, many farmers do not credit the nutrients contained in manure. For example, in areas where manure has been land-spread, many farmers continue to apply fertilizers in sufficient quantities for attaining desired crop yield. The lack of manure nutrient crediting by farmers may be due to many factors that make manure an undependable source of plant nutrients, including differences in soil fertility levels where manure application experiments were conducted, inherent shortcomings in the "fertilizer equivalent" approach for estimating nutrient availability, etc.

The stable isotope ^{15}N has been used extensively to evaluate the availability of fertilizer-N to crops. The use of ^{15}N in nutrient cycling studies involving animal manure has been limited. This has been due partially to the high cost of ^{15}N and the large quantities needed to enrich a sufficient amount of forage for feeding and labeling feces and urine. Also, the homogeneous ^{15}N labeling of manure-N components must be assured. A disproportionate labeling of the microbial- and endogenous-N components in feces, or the undigested feed-N excreted in feces may lead to great error when determining the rate and extent of manure-^{15}N mineralization in soils. The objective of the following experiment was to study the ^{15}N enrichment pattern of dairy feces and urine that can be used in various short- (e.g. manure/soil incubations, greenhouse trials) and long-term (field trials) nutrient cycling studies. Relative ^{15}N enrichment of fecal-N fractions was also studied.

Materials and Methods

Corn (NK N1500) and alfalfa (Cenex Trailblazer) plants were enriched in ^{15}N at the University of Wisconsin Hancock Research Station (44^{o} 7' N, 89^{o} 32' W) on a Plainfield sand (mixed, mesic Typic Udipsamment) during the 1997 cropping season. Ammonium sulfate, containing 12.3 atom % ^{15}N, was applied at an equivalent rate of 75 kg N ha^{-1}. The corn plants were harvested at one-third milkline (70% moisture), chopped to three-quarter inch lengths and ensiled in PVC silos. A 20 m^2 area of a second-year alfalfa stand was fertilized with 10 atom % ^{15}N at an equivalent rate of 100 kg N ha^{-1} in each of two applications. Alfalfa was harvested three times during the season and conserved as hay.

Two ruminally-fistulated non-lactating dairy cows weighing approximately 420 kg were utilized in the feeding trial. The animals were adapted to a diet consisting of 55% alfalfa hay and 45% corn silage on a dry matter (DM) basis (atom % ^{15}N at natural abundance) for 7 d. On the last day of the adaptation period, indwelling catheters were inserted into the bladders for urine collection. For 36-h thereafter, ^{15}N-enriched alfalfa hay and corn silage were fed at the 55% and 45% ratio used during the adaptation period. Cows were kept in two adjoining stanchions and bedded with rubber mats. Total feces and urine were collected at 4, 8 or 12-h intervals after initial offer of ^{15}N-enriched forage up to a total of 192-h. Feces and urine from each collection were frozen immediately. Total-N and ^{15}N concentrations in feeds, feces and urine were determined using a Carlo Erba elemental analyzer coupled with a Europa 20/20 tracermass. Cell wall components (NDF) of feeds and feces were determined using the detergent system as neutral detergent fiber. Total-N and ^{15}N contained in cell walls (NDIN) of feeds and feces were determined as neutral detergent insoluble N. The NDF-soluble-N (NDSN) fraction in feed (cell wall contents) and feces

(microbial-N and endogenous-N that was secreted into the digestive tract and excreted) was obtained from the difference between total-N and NDIN.

Results and Discussion

At the end of the growing season, approximately 36% of the applied fertilizer-^{15}N was accounted for in the three alfalfa harvests and 73% in corn silage. The ^{15}N-enriched diet consisted of approximately 55% alfalfa and 45% corn silage DM and had a total-N content of 19.42 g kg^{-1} of which 4.026 atom % was ^{15}N. The pattern of ^{15}N excretion in urine and feces was similar for both cows (Fig. 1). ^{15}N began to appear in urine between 4- to 8-h and in feces between 16- to 24-h after the initial offer of ^{15}N-enriched feed. Peak ^{15}N concentrations were attained by 30-h in urine (1.642 % ^{15}N) and by 54-h in feces (2.341 % ^{15}N). A more rapid ^{15}N excretion in urine than feces reflected rapid absorption of labeled $^{15}NH_3$ from the rumen and its conversion into urea in the liver. ^{15}N enrichment approached basal levels 132-h after feeding for both urine and feces. Peak ^{15}N concentrations attained 41% in urine and 58% in feces of the ^{15}N concentration in feed. Of the total ^{15}N fed, 97% was recovered by 192-h, 53% in urine and 44% in feces. Most (68%) of the ^{15}N fed was recovered within 96-h.

Between 60 to 70% of the total-N excreted in feces was NDSN and 30 to 40% as NDIN (Fig. 2). A comparison of the ^{15}N concentrations in total-N and NDIN (Fig. 3) indicates that ^{15}N labeling of NDIN and NDSN fecal components was uniform.

The various ^{15}N enrichment levels of urine and feces offer possibilities for differential ^{15}N use in short- and long-term nutrient cycling studies involving animal excreta. For example, highly enriched material, such as urine captured between 24- and 72-h and feces captured between 32- and 84-h after feeding (Fig. 1) could be used for long-term field trials aimed at determining crop uptake of manure-N in the first, second and third year after initial manure application. Manure of lower ^{15}N enrichment could be used for shorter term studies, such as manure/soil incubations and greenhouse trials. The minimum atom % ^{15}N abundance of urine or feces required for a particular nutrient cycling study would depend on the expected ^{15}N content of soil extracts, crops, etc. after manure application and the detection limit of the mass spectrophotometer.

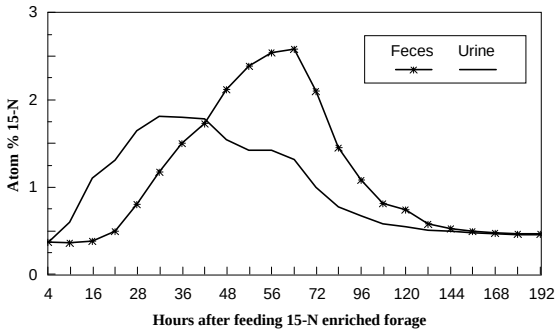

Figure 1. Pattern of ^{15}N excretion in dairy feces and urine.

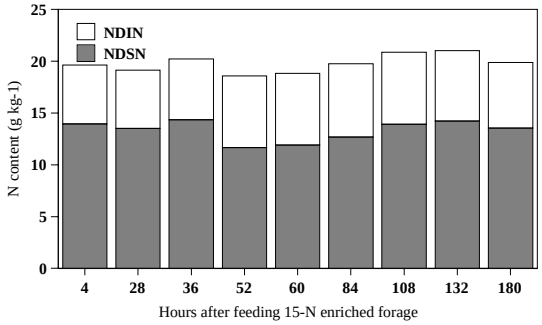

Figure 2. Neutral detergent soluble (NDSN) and insoluble (NDIN) nitrogen in dairy feces

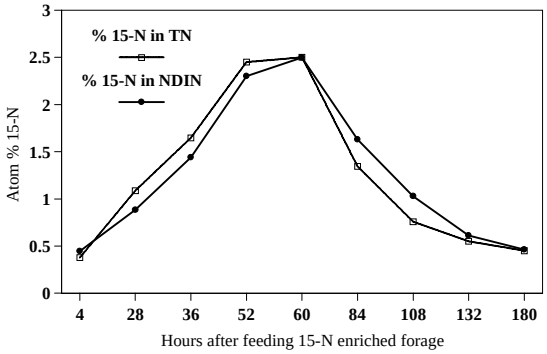

Figure 3. Atom % ^{15}N in total nitrogen (TN) and neutral detergent insoluble nitrogen (NDIN) of dairy feces.

Conclusion

The use of the stable isotope ^{15}N allows for direct measurement of nutrient flow in various aspects of the feed/animal/manure/soil-crop/environment continuum. This study determined the relative efficiency with which fertilizer-N was used to produce forages (alfalfa hay and corn silage), the conversion of forage-N into urine- and fecal-N, and the relative uniformity with which forage-N was incorporated into fecal-N components. The described enrichment pattern of feces and urine and the distribution of fecal ^{15}N between NDSN and NDIN relative to feeding time is useful in determining which components of dairy manure would be most appropriate for short- or long-term nutrient cycling studies in the manure-soil/crop-environment continuum. Uniform labeling of the rapidly decomposable fecal-N pool (microbial- and endogenous-N) and the less decomposable pool (undigested feed-N) must be obtained in order to accurately determine the rate and extent of fecal-N mineralization in soils. The relative effectiveness of using ^{15}N-labeled urine and feces in nutrient cycling studies will depend on its ability to more accurately measure N mineralization in soils than the classical, indirect measurements (e.g. fertilizer equivalent) currently in use.

Reaction of Red Clover and Birdsfoot Trefoil Cultivars and Germplasm to *Mycoleptodiscus terrestris*

R.R. Smith and C.R. Grau

Introduction

Soilborne plant pathogens are regarded as important causes of failures of newly established and mature stands of forage legumes in the North Central Region of the U.S. The fungus *Mycoleptodiscus terrestris* was recovered from decaying roots and stems of birdsfoot trefoil (*Lotus corniculatus* L.) and red clover (*Trifolium pratense* L.) plants sampled from two-year-old plants in 1994. Although recognized in states south of Wisconsin, *Mycoleptodiscus terrestris* has not been implicated in poor health of forage legumes in Wisconsin. *M. terrestris* has been previously reported to be pathogenic on alfalfa, red clover and birdsfoot trefoil in Illinois. The fungus has been reported to be pathogenic on birdsfoot trefoil in Missouri and eastern U.S. Only the trefoil cultivar Dawn and the germplasm CAD have been reported to have some degree of resistance (tolerance) to *M. terrestris.* However, no resistance has been identified in red clover or birdsfoot trefoil germplasm adapted to the northern area of the midwest. Forage legume germplasm has not been characterized extensively for reaction to *M. terrestris.* The objective of this research was to evaluate a select set of cultivars and germplasm populations of red clover and birdsfoot trefoil for their reaction to *M. terrestris*.

Materials and Methods

Three-week-old seedlings of selected cultivars and germplasm of red clover and birdsfoot trefoil were inoculated with a mycelium/sclerotium suspension (one 100 mm standard plate/ l water) as a drench at a rate of 20 ml per 10 seedlings and incubated for three weeks at 25°C. Six-week old seedlings were evaluated for reaction to the respective isolates on a scale of 1 to 5; 1 = no symptoms and 5 = a dead plant.

Results

Significant differences were observed among the 18 birdsfoot trefoil cultivars and germplasm populations evaluated for their response to *M. terrestris* (Table 1). The broad-based cultivar, Norcen, was one of the least susceptible cultivars and AUDewey, selected in Alabama, was the most susceptible. Both the cultivar Dawn and the germplasm CAD were among the more resistant populations. The range between the percent plants in the different DSI classes would suggest that genetic variability does exist for the development of highly resistant germplasm. All red clover cultivars and germplasm populations were quite susceptible to *M. terrestris* (Table 2). On the average, only 15% of the plants were scored in the DSI class of 1 or 2, but this should be sufficient to provide a source for developing resistant germplasm.

Table 1. Response of birdsfoot trefoil cultivars and germplasm to *Mycoleptodiscus terrestris*.

Entry name	Origin	Percent plants with DSI* of 1 & 2's	3's	4 & 5's	Mean DSI**
NORCEN	Nor. Cent.	49	22	29	2.83 a
EMPIRE	New York	43	24	33	2.85 ab
DAWN	Missouri	41	24	35	2.96 abc
MACKINAW	Michigan	40	24	36	3.02 abcd
FERGUS	Kentucky	43	21	36	3.03 abcd
CAD	Missouri	41	20	39	3.07 abcd
VIKING	New York	38	26	36	3.10 abcd
WITT	Wisconsin Gp	37	24	39	3.16 bcd
ARS2620	Missouri	33	30	37	3.12 cd
BONNIE	France	35	22	43	3.23 cd
CARROLL	Iowa	35	23	42	3.24 cde
LEO	Canada	39	18	43	3.26 cde
MU81	Missouri	31	21	48	3.36 cde
MAITLAND	Canada	27	30	43	3.38 cde
TREVIG	Wisconsin GP	30	21	49	3.43 de
BULL	Canada	30	21	49	3.48 de
GEORG 1	Georgia	25	25	50	3.52 de
AUDEWEY	Alabama	21	14	65	3.88 e
All Entries		34	23	33	3.22

*DSI = Disease Severity Index: 1 = healthy plant, 5 = dead plant.

**Average of 275 plants per entry were challenged and entries followed by the same letter are not significantly different at the 5% level.

Note: Coefficient of Variation was 9.5%.

Table 2. Response of red clover cultivars and germplasm to *Mycoleptodiscus terrestris*.

Entry name	Origin	Percent plants with DSI* of 1 & 2's	3's	4 & 5's	Mean DSI**
CONCORD	ABI	22	19	59	3.55
LAKELAND	ARS/Wisconsin	19	26	56	3.58
FGRK01	Forage Gen.	21	18	61	3.64
NY9311	New York	14	28	58	3.67
SCARLETT	Dairyland	15	26	59	3.68
C182	ARS/Wis Exp.	20	21	60	3.69
KENSTAR	Kentucky	16	29	55	3.72
FGR03	Forage Gen.	13	25	62	3.74
WI-1	ARS/Wis GP	19	20	60	3.74
CINNAMON	Farmers For. Res	16	26	58	3.76
ACCLAIM	Allied	14	21	65	3.76
W87A	Northrup-King	17	20	63	3.76
RANDOLPH	Allied	15	22	64	3.82
C328	ARS/Wis Exp.	15	19	65	3.89
ARLINGTON	ARS/Wisconsin	9	21	69	3.93
COMMON	ARS/Wisconsin	16	23	61	4.00
WI-2	ARS/Wis Gp	11	20	69	4.04
MARATHON	ARS/Wisconsin	8	10	82	4.27
All Entries		15	22	63	3.79

*DSI = Disease Severity Index: 1 = healthy plant, 5 = dead plant.

**Average of 120 plants per entry were challenged.

Note: Coefficient of Variation was 10.4% and differences between entries was not significant.

Selection for Resistance to *Mycoleptodiscus* in Red Clover and Trefoil

R.R. Smith and C.R. Grau

Introduction

Soilborne plant pathogens are regarded as important causes of failures of newly established and mature stands of forage legumes in the North Central Region of the U.S. The fungus *Mycoleptodiscus terrestris* was recovered from decaying roots and stems of birdsfoot trefoil (*Lotus corniculatus* L.) plants sampled from two-year-old plants in 1994. Although recognized in states south of Wisconsin, *M. terrestris* has not been implicated in poor health of forage legumes in Wisconsin. *M. terrestris* has been previously reported to be pathogenic on alfalfa (*Medicago sativa* L.), red clover (*Trifolium pratense* L.), and birdsfoot trefoil in Illinois. The fungus has been reported to be pathogenic on birdsfoot trefoil in Missouri and eastern U.S. Only the trefoil cultivar Dawn and the germplasm CAD have been reported to have some degree of resistance (tolerance) to *M. terrestris*. However, no resistance has been identified in trefoil or red clover germplasm adapted to the northern area of the midwest. Forage legume germplasm has not been characterized extensively for reaction to *M. terrestris*. Our goal was to attempt to select birdsfoot trefoil and red clover plants with the resistant reaction to *M. terrestris* using recurrent phenotypic selection.

Materials and Methods

Selection was initiated simultaneously in four populations of birdsfoot trefoil. These populations were derived from Wisconsin germplasm, Minnesota germplasm (provided by Dr. Nancy Ehlke), New York germplasm (provided by Dr. Don Viands), and Nova Scotia germplasm (provided by Dr. Yousef Papadopoulos). The initial red clover germplasm was a composite of the cultivars Arlington and Marathon and selected breeding lines from the USDA/ARS program in Wisconsin. Ten-day-old seedlings established in vermiculite in plastic pans were inoculated with a *M. terrestris* mycelium drench using isolates derived from Wisconsin soils or plants grown in Wisconsin. Inoculated plants were maintained at 25°C for 14 days before removing from the vermiculite and evaluated for disease reaction. Plants were evaluated on a scale of 1 = no necrosis, symptomless to 5 = severely necrotic or dead. Plants with a Disease Severity Index (DSI) of 1 were intercrossed to provide progeny for the next cycle of selection. Two cycles were completed. Seed was harvested by plant in the first cycle of selection in the red clover population and half-sib progeny evaluated to provide an estimate of broad sense heritability. An equal number of first cycle plants representing each of the four trefoil populations were intercrossed to provide half-sib progeny to estimate broad sense heritability among the trefoil populations.

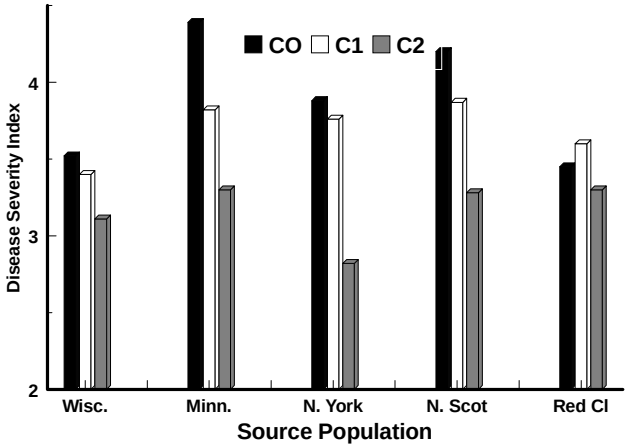

Figure 1. Response to two cycles of selection in trefoil and red clover for resistance to Mycoleptodiscus terrestris.

Results

Slight but significant progress from selection has been realized in each of the trefoil populations (Fig. 1). In red clover, however, the first cycle of selection was not effective, but a response was realized in the subsequent cycle . After two cycles of selection 42, 39, 52, and 38% of the trefoil plants express the resistant reaction in the Wisconsin, Minnesota, New York and Nova Scotia populations, respectively (Table 1). Thirty-one percent of the red clover plants express resistance. Broad sense heritability estimates were 24 and 21% for birdsfoot trefoil and red clover, respectively.

Table 1. Distribution of trefoil and red clover plants by Disease Severity Index (DSI).

Population	Cycle	Percent plants with DSI of 1 and 2	3	4 and 5	Mean	Probable Difference
Trefoil						
Wisconsin	C0	20	12	68	3.52	
	C2	42	18	40	3.11	> 0.10
Minnesota	C0	12	10	77	4.39	
	C2	39	12	48	3.30	> 0.01
New York	C0	18	18	64	3.88	
	C2	52	17	34	2.82	> 0.07
Nova Scotia	C0	15	13	72	4.20	
	C2	38	18	44	3.28	> 0.04
Red Clover						
Composite	C0	16	33	51	3.45	
	C2	31	29	40	3.13	> 0.10

DSI scale: 1 = resistant, 5 = susceptible/dead

Conclusions

1. Two cycles of recurrent phenotypic selection for resistance to *M. terrestris* in birdsfoot trefoil and red clover has been effective.
2. Selection is continuing in all populations to increase the level of resistance. Resistance to this pathogen will improve overall plant health and should aid seedling establishment especially when soil moisture and temperatures are high.

Selection for Resistance to the Northern Root-Knot Nematode in Red Clover

R.R. Smith, C.R.Grau and D.K. Sharpee

Introduction

Little attention has been given to the development of resistance to nematodes in red clover (*Trifolium pratense* L.) even though considerable damage can be caused by nematode diseases in specific sites in the world. In some areas damage may be severe, but generally the effect is more subtle. In this latter case red clover plants tolerate the pest, but yield and stand longevity are reduced. Recently scientists in southern U.S. (Florida) have developed and released red clover germplasm with resistance to several species of the root-knot nematode complex [peanut root-knot nematode: *Meloidogyne arenaria* (Neal) Chitwood: southern root-knot nematode: *M. incognita* (Kofoid and White) Chitwood; and javanese root-knot nematode: *M. javanica* (Treub.) Chitwood]. This resistance greatly enhances the production and longevity of red clover in nematode infested sites.

In northern U. S., the northern root-knot nematode [*M. hapla* (Chitwood)] is the most widely distributed nematode species. *M. hapla* occurs primarily in sandy to sandy-loam soils in Wisconsin. An immediate concern is that *M. hapla* occurs very extensively in our extremely sandy soils in the center of the state. The major crop of the area is the potato (*Solanum tuberosum* L.) which is also extremely susceptible to *M. hapla*. Red clover is used in short rotations with potato to increase the organic matter of the soils, to provide a nitrogen source, and to improve general soil tilth. Resistant red clover germplasm would assist in reducing the population of *M. hapla* in the rotational sequence. Therefore, we initiated a recurrent phenotypic selection program to develop red clover germplasm with resistance to *M. hapla*.

Materials and Methods

The first cycle of selection was developed from intercrosses between selected plants of the cultivars Arlington, Kenstar, and Marathon and the Wisconsin germplasm, C11. Selection was based on both field and greenhouse response to the nematode. All subsequent cycles were conducted in the greenhouse. In cycles 2 through 4 plants were grown and inoculated (15 - 20 juvenile nematodes per plant) in plastic flats containing a mixture of sterilized sand and soil (1:1 v/v). Beginning in cycle 5 individual plants were grown and inoculated in Cone-tainers™ using the same planting media. Nematodes used for inoculation purposes were maintained on tomato (*Lycopersicon lycopersicum* L.). Selection intensity ranged from 3% in cycle 1 to 20% in cycle 6. Selection was based on visual number of galls based on a 1 to 5 scale, where a score of 1 was essentially no galls to 5 which was severely invested with galls.

Results and Conclusions

Progress from selection was variable, but effective, between cycles 1 to 4 with only 15% of the plants resistant (disease score of 1 or 2) after the fourth cycle (Fig.1). The effectiveness of selection increased drastically between the fourth and fifth cycle (37% of plants resistant in the cycle 5). Excellent progress was achieved in the next two cycles of selection such that after seven cycles 75% of the plants express the resistant reaction (Table 1). It is worthy to note that with no selection for resistance to *M. hapla*, Florida germplasm (FLMR7, selected for resistance to *M. arenaria*, *M. incognita*, and *M. javanica)* appears to have some resistance to *M. hapla*.

Cycle 7 germplasm is being prepared for release. Besides resistance to *M. hapla*, this germplasm has good persistence and moderate resistance to northern anthracnose caused by *Auridiobasidium caulivora* (Kirch.) Karak. In addition, it has moderate resistance to *M. arenaria*.

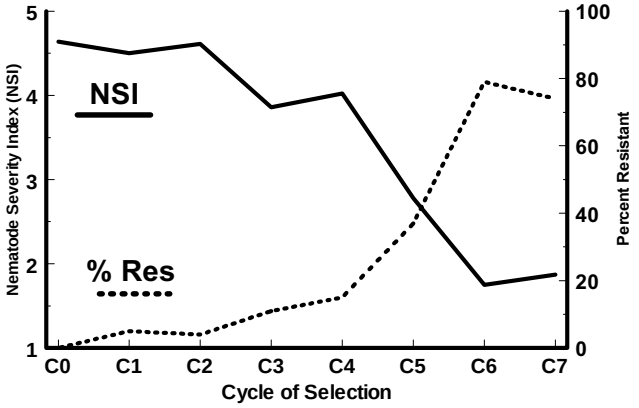

Figure 1. Response to selection for resistance to root-knot nematode in red clover.

Table 1. Response of red clover to seven cycles of selection for root knot nematode resistance.

Cycle/	Percent plants with NSI of							
Material	1's	2's	3's	4's	5's	#Plt	Mean	St Dev
C0	0	0	4	31	65	26	4.62	0.571
C1	0	5	26	21	48	42	4.50	1.221
C2	0	4	4	21	71	28	4.61	0.737
C3	4	7	21	36	32	28	3.86	1.079
C4	0	15	15	23	46	26	4.00	1.131
C5	22	15	37	15	11	27	2.78	1.281
C6	54	25	14	7	0	28	1.75	0.967
C7	59	15	11	7	7	27	1.89	1.311
C7 SYN2	80	20	0	0	0	25	1.20	0.408
Marathon	0	0	4	23	73	26	4.69	0.549
Cherokee*	4	15	22	15	44	27	3.81	1.272
FLMR7**	27	27	19	27	0	26	2.46	1.174
LSD (5%)							0.66	

*Cherokee released in 1993 with resistance to *M. arenaria, M. incognita, and M. javanica.*
**FLMR7, advanced Florida germplasm selected for resistance to *M. arenaria, M. incognita,* and *M. javancia*.

Selection for Divergent Ferulate Cross-Linking and Klason Lignin Concentrations in Smooth Bromegrass

M.D. Casler and H.G. Jung

Introduction

Research by the USDFRC Cell Wall Group has demonstrated that cell walls of grasses have extensive cross-linking of arabinoxylans to lignin by ferulic acid bridges. Using primary cell walls from corn tissue cultures that have been induced to lignify, the degree of ferulate cross-linking is strongly correlated with cell-wall degradability. However, we have had less success showing similar correlations of cross-linking with degradability for intact plants. There is generally a positive correlation between Klason lignin concentration and ferulate cross-linking in grasses. This raises the question of whether cross-linking has a direct effect on cell-wall degradability or if it is merely a reflection of the correlation between cross-linking and lignin. We undertook a selection study in smooth bromegrass to identify genotypes that exhibit repeatable differences in ferulate cross-linking and Klason lignin concentration to more directly determine the independent effects of these two components of grass cell-wall structure on cell-wall degradability.

Materials and Methods

Leaf tissue at the vegetative stage of maturity was collected in 1992 from 300 individual plants taken from each of four smooth bromegrass populations: the cultivars Alpha and Lincoln, and the two synthetic populations WB19e and WB88S derived from elite breeding lines and wild germplasm collected in Russia, respectively. All samples were oven dried, ground, and scanned by near-infrared spectroscopy (NIRS). Eighty samples were analyzed by wet-chemistry for neutral detergent fiber (NDF), ferulate ethers (the only form of cross-linking that can be quantified), and Klason lignin by standard methods. These data were used to generate NIRS calibration equations and then predict composition of all 1200 leaf samples. Ferulate ethers and Klason lignin concentrations were examined on a NDF basis. Twenty plants (five per population) were selected for each of four selection groups; low ferulate ethers: low lignin, high ferulate ethers: low lignin, low ferulate ethers: high lignin, and high ferulate ethers: high lignin. In 1993 the 80 selected plants were re-sampled as done in 1992. All samples were analyzed for ferulate ethers and Klason lignin by wet-chemistry. Based on the means over the two sampling years, 32 plants (two plants per population per selection group) were selected for further evaluation. These 32 plants were clonally propagated and transplanted into a replicated randomized complete block design field trial at Arlington, WI in 1994. Leaf samples were collected from two harvests at the vegetative maturity stage in both 1995 and 1996. All samples were analyzed by wet-chemistry for NDF, etherified ferulic acid, Klason lignin, and 24- and 96-h in vitro NDF degradability.

Results and Discussion

Both ferulate ethers and Klason lignin concentration showed considerable phenotypic variation in all four populations. Based on the two phases of selection (1992 and 1993), it was apparent that both traits were subject to substantial genotype x environment interactions. The results for the 32 selected clones evaluated in 1995 and 1996 are shown in Fig. 1. Selection was successful for ferulate ethers in all populations except WB88S, creating repeatable divergence of 11.2 to 12.5%. Selection was unsuccessful for Klason lignin concentration, most likely due to the genotype x environment interactions. While we were not successful in creating the four targeted divergent phenotypes, the resulting clones showed significant variation for both traits and these two traits were independent ($r = 0.08$; $P > 0.05$) among the selected clones. Both lignin concentration and cross-linking had significant negative effects on 96-h in vitro NDF degradability across three different statistical estimation methods, but little effect on 24-h NDF degradability was observed. For the most divergent and independent clonal comparisons of cross-linking and lignin impact on NDF degradability, 75% of the contrasts showed a significant negative effect of high cross-linking and lignin concentration on degradability (Table 1).

Conclusions

Selection for divergent ferulate cross-linking in smooth bromegrass was successful. Both Klason lignin and ferulate ethers had negative impacts on in vitro NDF degradability, and these effects were of similar magnitude and independent. Our results indicate that recurrent selection for reduced ferulate cross-linking in smooth bromegrass should be a successful strategy for improving forage quality; however, the large genotype x environment interaction will make progress slow. The most divergent clones identified to date provide a valuable resource for conducting animal feeding trials to evaluate the impact of ferulate cross-linking and lignin concentration on animal performance. These genetic materials offer the opportunity to test the impact of these traits in vivo, which has not been possible previously.

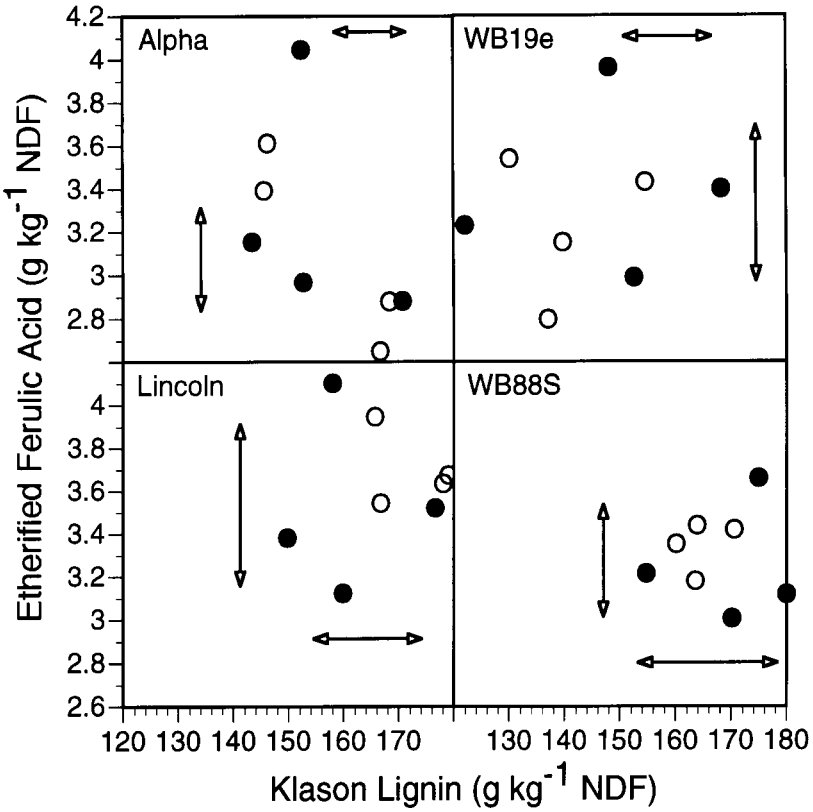

Figure 1. Scatter plots of etherified ferulic acid vs. Klason lignin concentration for clone means of four smooth bromegrass populations. Double-headed arrows represent LSD values for clone means within populations (P < 0.01). Closed symbols represent the most desirable clones within each population for testing the independent effects of lignin concentration and cross-linking on cell-wall degradability.

Table 1. Mean differences from high vs. low clonal comparisons for etherified ferulic acid, Klason lignin, and 24- and 96-h in vitro NDF degradability in smooth bromegrass.

		Impact of Divergence			
		Concentration		In Vitro NDF Degradability	
Population	Clonal Comparison	Ferulate Ether	Klason Lignin	24-h	96-h
		- - - - - g kg^{-1} NDF - - - - - - -		- - - - - - - g kg^{-1} - - - - - -	
Ferulate Contrast					
Alpha	5 - 4	1.07**	0.3	-33	-51**
WB19e	1 - 4	0.97**	-4.5	-50*	-22*
Lincoln	3 - 4	0.98**	-1.9	-23	-11
WB88S	8 - 2	0.65**	4.8	-10	-30*
Klason Lignin Contrast					
Alpha	6 - 1	-0.28	27.3**	-8	6
WB19e	3 - 7	0.17	46.3**	-26	-28**
Lincoln	1 - 6	0.29	29.3**	-8	-25*
WB88S	4 - 7	-0.10	25.5**	-19	-30*

*,**Effect is significantly different from zero (P < 0.05 and 0.01, respectively).

Quantitative Trait Loci for Cell-Wall Traits in Corn

W. Ni, H.G. Jung and R.L. Phillips

Introduction

Improving forage quality by increasing cell-wall digestibility is a major goal of our research program. In this study we attempted to map and characterize quantitative trait loci (QTLs) associated with the polysaccharide and hydroxycinnamate components in corn cell walls. Advances in molecular marker technology have enabled the development of dense genetic linkage maps for major crops such as corn. These maps have been used to associate DNA markers with important physiological and morphological traits. Complex traits can be dissected into discrete factors, also referred to as QTLs. Identification of QTLs affecting traits of interest can be the first step in the map-based cloning of genes related to quantitative traits. Markers linked to QTLs can also be used in modern breeding programs to increase genetic progress. Objectives of our research were to (*i*) determine phenotypic correlations among corn cell-wall traits and (*ii*) ascertain the number and the chromosomal locations of QTLs and estimate the genetic effects of these QTLs involved in cell-wall trait expression.

Materials and Methods

A population of 48 recombinant inbred corn lines (RIL) was developed by Burr and co-workers by crossing the Cm37 and T232 inbreds. Plants were grown in two randomized blocks in St. Paul and Rosemount, MN in 1995. Mature leaves from the sixth and seventh internode of three or four plants per field replicate were collected at the pollen shedding stage of development, and oven dried at 50°C. The following cell-wall components were measured using standard procedures: arabinose, galactose, glucose, mannose, xylose, uronic acids, *p*-coumarate and ferulate esters and ethers, and Klason lignin and syringyl-to-guaiacyl (S/G) ratio of lignin composition. The linkage map was obtained from Brook Haven National Laboratory and a subset of 315 markers was selected to provide relatively even spacing (6-7 centimorgans) for QTL analysis. The composite interval mapping method available in the Plabqtl program were used for estimating QTL positions and effects. The probability of the presence of a QTL at a particular location was expressed as a LOD score ($\log_{10}$ of the likelihood odds ratio). The LOD threshold was estimated for each data set using the QTLcartographer program with permutations set at 1000. Because of the small population size of RILs used in this study, we adopted a stringent ($P < 0.01$) LOD score requirement to declare a QTL significant.

Results and Discussion

Significant phenotypic correlations were found among most cell-wall lignification traits in this RIL population. *p*-Coumaric (PCA) and ferulic (FA) acid esters were highly correlated to their corresponding ethers ($r = 0.90$ and 0.74, respectively). In contrast, only half of cell-wall polysaccharide components had significant correlations with each other. Arabinose was highly correlated with galactose ($r = 0.82$) and glucose was moderately correlated ($r = 0.59$) with xylose. All hydroxycinnamate esters and ethers were positively associated with arabinose, galactose, and xylose, possibly indicating the coordinated deposition pattern of these components during cell-wall development. However, lignin lacked association with these three sugars. High correlations of S/G ratio with arabinose and galactose were also observed, but the physiological significance of these correlations is debatable. Genotypic variances among RILs were highly significant ($P < 0.01$) for all traits except Klason lignin. Location also exerted significant effects on concentrations of most sugars, FA ether, lignin and S/G ratio. Genotype x environment interactions were detected for glucose, xylose, and FA esters and ethers. A total of 44 QTLs were detected for five cell-wall polysaccharide components, ranging from five to 14 QTLs for each trait. These QTLs explained 59 to 91% of the phenotypic variance. Very few QTLs displayed QTL x environment interactions except for uronic acid where seven of nine QTL exhibited such interactions. Forty QTLs were detected for five lignification traits, accounting for 52 to 85% of the

phenotypic variance. Two markers associated with FA ether QTLs were located in the vicinity of two known brown midrib loci (bm3 and bm4). Some of the cell-wall polysaccharide and lignification traits shared the same molecular markers, an indication that genes associated with these overlapping QTLs may be responsible for the coordinated deposition of polysaccharide and lignin components in the cell wall.

Conclusions

Little previous work has been done on QTL analysis for forage quality traits in corn. Previous research has used acid detergent fiber (ADF, a gravimetric measurement after extraction) as the cell-wall trait of interest. LOD scores and variance explained by the QTLs for ADF were relatively low. In contrast, our study of the molecular components of the cell wall found high LOD scores and coefficients of determination. However, population size may have contributed to the difference between the studies. The data suggest that these markers could be successfully used for breeding or map-based cloning of cell-wall traits.

Table 1. Quantitative trait loci (QTLs) associated with concentrations of cell-wall polysaccharide and lignification components of 48 recombinant inbred corn lines grown in two environments.

Trait	Number of QTLs	Chromosomes	Range of LOD Scores for QTLs	Variance Explained (r^2)
Polysaccharide Components				
Arabinose	5	5, 9, 10	5.83 - 16.41	0.61
Galactose	7	1, 4, 5, 6, 9, 10	6.03 - 17.97	0.79
Glucose	9	1, 3, 5, 7, 8, 9, 10	5.86 - 16.76	0.80
Xylose	14	1, 2, 4, 5, 6, 7, 9, 10	6.24 - 21.42	0.91
Uronic Acids	9	1, 2, 3, 4, 5, 9	8.10 - 13.67	0.59
Lignification Components				
Ferulate Esters	10	1, 3, 5, 6, 7, 8	8.31 - 18.15	0.85
p-Coumarate Esters	7	1, 2, 6, 7, 8, 9, 10	6.33 - 13.37	0.69
Ferulate Ethers	11	1, 2, 3, 4, 7, 9, 10	7.03 - 14.46	0.80
p-Coumarate Ethers	7	1, 2, 6, 7	7.02 - 16.47	0.74
S/G Ratio	5	2, 3, 4, 7	4.94 - 8.19	0.52

Phytase Feed Supplements from Transgenic Alfalfa

R.G. Koegel, S. Austin-Phillips and R.J. Straub

Introduction

Buildup of phosphorus in the environment and the resulting degradation of water resources is of mounting concern. Much of this buildup is traceable to human activities. Important among these is livestock production. Monogastric animals, such as poultry and swine which can solubilize only a small fraction of the phosphorus in their grain-based rations, while excreting the remainder, have come under increased scrutiny. Supplementation of inorganic phosphorus into rations to meet animal nutritional requirements exacerbates the problem.

Much of the phosphorus in grain is in the form of insoluble phytates. Researchers have shown that supplementing poultry and swine rations with the enzyme phytase can lead to solubilization of the phosphorus, thus reducing the need for phosphorus supplementation and concurrently reducing the level of phosphorus in the excrement to approximately one-half of that normally experienced.

The enzyme phytase derived from *Aspergillus niger* has, to date, generally been produced in fermentation vats using genetically engineered microorganisms. Currently the cost of phytase supplementation is slightly higher then the cost of conventional supplementation with dicalcium phosphate.

As an approach to reducing the cost of phytase production, a multi-disciplinary ARS-UW team at Madison, Wisconsin has produced transgenic alfalfa with the capability of expressing phytase. This phytase can be recovered from juice extracted from the herbage. Other constituents of the juice including xanthophyll (used to pigment egg yolks and broiler skin), high levels of dietary protein, and various vitamins and minerals add to its value in rations. Leaf meal is another feedstuff which could be used to add phytase to monogastric rations. The use of whole alfalfa herbage, however, would not be desirable due to its high fiber content. Since phytase would potentially be needed in great quantities, but not in very pure or concentrated form, it is believed that the economic advantage of production in "plant bioreactors" such as alfalfa could be great. The advantage in capital costs is particularly great. Ideally, the cost of phytase supplementation should be competitive with the traditional dicalcium phosphate supplement, with the environmental benefits as an added incentive.

Methods

Sixteen phytase-producing alfalfa transformants were originally created. Early bioassays indicated that these transformants produced phytase at a range of levels. These transformants were vegetatively propagated in the greenhouse during the winter and spring of 1997 and approximately 7500 plants were set out into the field in mid-May in both replicated research plots as well as larger "production plots." The plant densities in the two plot types were 9680 and 139396 per acre, respectively. Both sets of plots were harvested in late May and early June, with herbage from the research plots being dried and separated for production of leaf meal while the herbage of the highest phytase producers in the "production plots" was macerated and the juice expressed. Some of the juice was sprayed on ground corn at a 1:1 ratio (wet basis). Half of the total juice was sprayed on at a time with the resulting mix being sun-dried after each juice addition. The dried mix could then be stored. The remainder of the juice was stored in a freezer.

The leaves from approximately 1/3 of the research plots were detached by passing the herbage through an impacting rotor running at approximately 3450 RPM while the remaining herbage was passed

between rubber rolls with differential surface speeds to strip the leaves. Phytase content, crude protein content, and fiber content of selected leaf meal samples were determined.

Results

Leaf Separation. The leaf to herbage ratios (d.b.) for three groups of herbage is shown in Table 1. The high ratio for the first group was due to the inclusion of many small stem fragments caused by the impact rotor and the inability to separate these from the true leaves. The ratio of .44 for the second group appears to be realistic based on previous work. Since this was first cutting herbage with a lower leaf:stem ratio than for subsequent cuttings, it would not be unrealistic to expect a ratio of 0.5 or greater for the season. The ratio for group 3 which was harvested one week later than group 2 was considerably lower. While the herbage mass (presumably non-leaves) increased by approximately 20% during the additional week, an absolute loss of leaves would have been necessary to explain the low ratio. If an average value for phytase activity of 180 units/g leaf meal is used along with a per acre herbage dry matter yield of 10,000 lb and a leaf yield of 0.5, the result is 400 x 10^6 phytase units/acre. At the rate of 400 phytase units/lb ration, 500 tons of ration could be dosed. If a cost of $3.00/ton for inorganic phosphorus is eliminated, the value of the leaf meal, for phytase content only, would be about $1500/acre. The protein and pigmenting (xanthophyll) value of the leaf meal would be in addition.

Wet Fractionation. Wet fractionation of five production plots containing the highest phytase producers resulted in average juice yields of 46.0% w.b. (s = 3.8) and 21.5% d.b. (s = 1.7). The phytase content of the juice averaged approximately 50 units/ml or 22,680 units/lb juice. At a herbage fresh weight of 50,000 lb/acre-yr, a juice yield of 0.46, a dosage of 400 units/lb feed, and potency of 22,000 units/lb juice, 630 tons of feed could be treated. If $3.00/ton for inorganic phosphorus supplementation is eliminated, the value of the juice would be $1890/acre-year plus the value of protein and pigmenting substances.

When juice was added to ground corn at a 1:1 ratio w.b. the activity calculated should have been 45 units/g mix, while the assayed value was around 33 units/g. It is not yet known whether this apparent activity loss of around 27% is real or a result of the assay method.

Conclusions

Economically significant phytase yields were achieved from transgenic alfalfa in its second year in field plots. In research reported elsewhere, the efficacy of this phytase was demonstrated in poultry and swine rations.

Table 1. Yields of leaf meal relative to herbage.

Group	Plants	Harv. Date	Rows*	Wt/plant (lb w.b.)	Separation Method	"Leaf" wt/ Herbage wt (d.b.)
1	Reps 1 & 2 (partial)	5/26/98	18	2.2	Impact Rotor, Screening	.65, s = .08
2	Reps 1 & 2 (partial)	5/26/98	19	2.2	Rubber Rolls, Screening	.44, s = .06
3	Rep 3	6/2/98	19	2.6	Rubber Rolls, Screening	.25, s = .04

*10 plants/row

Feeding Trials Involving Transgenic Alfalfa Phytase

R.G. Koegel, M.E. Cook, T.D. Crenshaw, S. Austin-Phillips and R.J. Straub

Introduction

Phosphorus is abundant in the grain-based rations typically fed to poultry and swine. Because much of this phosphorus is contained in insoluble phytates, however, monogastric animals normally can utilize only about one-third of it. As a consequence, rations must be supplemented with inorganic phosphorus for optimum animal performance. This practice involves cost for the supplement and results in excessive phosphorus, contained in the feces, being added to the environment.

Researchers have shown that the addition of the enzyme phytase (from *Aspergillus niger*) to monogastric rations can approximately double the amount of phosphorus derived from the grain by the animals. This can reduce or eliminate the need for inorganic phosphorus supplementation and reduce the phosphorus content of the feces to as low as 50% of typical levels.

Phytase has been produced, at economically significant levels in alfalfa, by researchers in Madison, Wisconsin. Research was needed, however, to verify that the efficacy of alfalfa-produced phytase is equivalent to phytase produced by *Aspergillus niger* in fermentation vats.

Groups of chicks and weanling pigs were fed at different levels of inorganic phosphorus supplementation (0-max.). Gains of these animals were compared with those of animals with no phosphorus supplementation and phytase from either transgenic alfalfa (juice or leaf meal) or from a commercial source.

Results

Figure 1 shows 3-week gains of chicks at different levels of inorganic phosphorus supplementation. Phytase was fed in the form of alfalfa juice dried on ground corn (1:1 w.b.) at a rate of 800 phytase units per kilogram of ration. The gain from the alfalfa-produced phytase was as great or greater than that of the optimum level of inorganic phosphorus supplementation. While it appeared to be greater than that produced by the commercial phytase product, no valid comparison could be made due to discrepancies in assays for phytase activities and losses in phytase activity during storage.

In the case of weanling pigs (Fig. 2), phytase was added to rations in the form of (1) alfalfa juice, (2) alfalfa leaf meal, and (3) a commercial phytase product. While the gains from the "optimum" level of inorganic P appear slightly higher than those from the various forms of phytase, there was no statistical difference. Additional feeding trials with larger numbers of animals will be carried out to enable valid statistical comparisons to be made.

Conclusions

Research, to date, indicates that phytase from transgenic alfalfa, in various forms, can totally replace inorganic phosphorus supplementation in poultry and swine while maintaining growth rates.

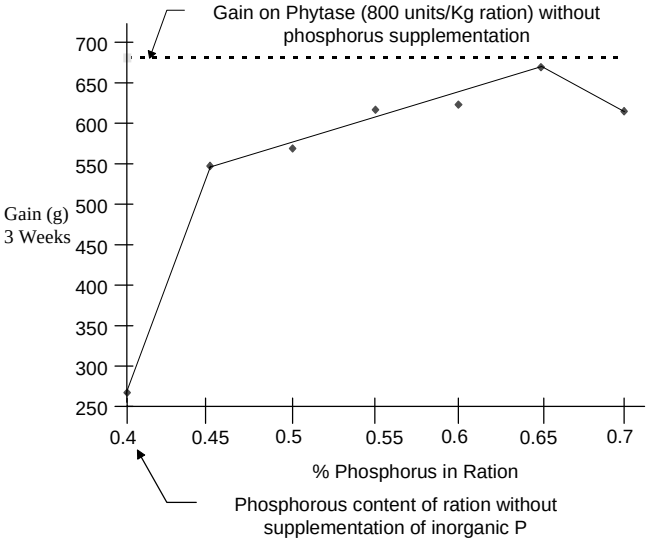

Figure. 1. 3 week gain of chicks vs. phosphorus content of ration compared to phytase with no phosphorus supplementation.

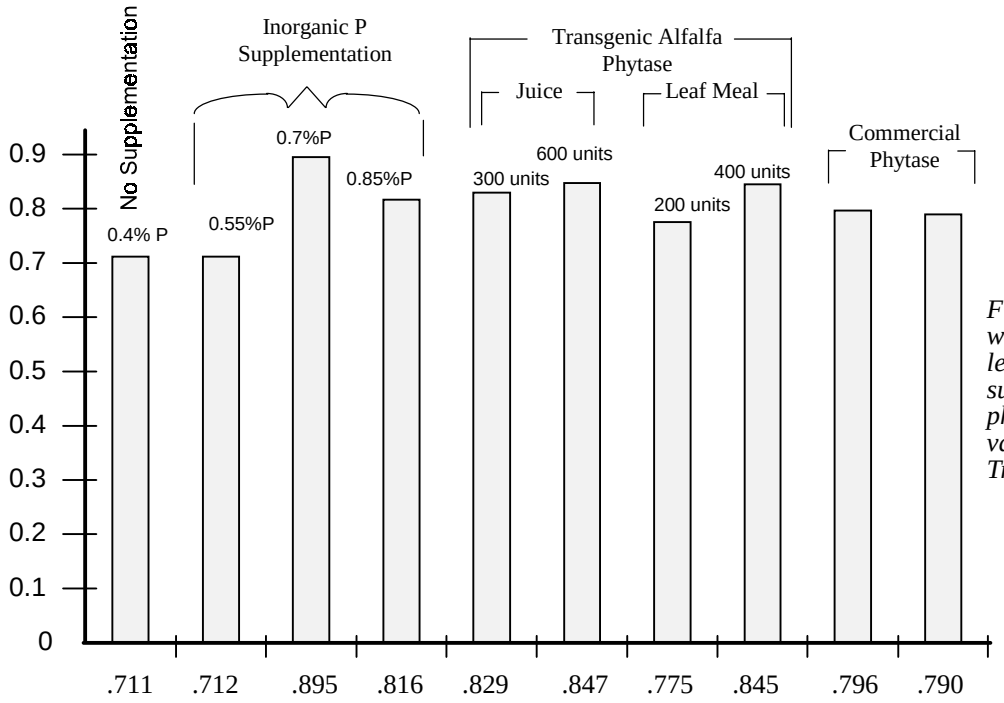

Figure 2. Daily gains of weanling pigs fed at various levels of inorganic phosphorus supplementation or with phytase supplementation from various sources.
Trial Durations: 18-21 days

Separation and Concentration of Alfalfa Juice Components by Dynamic Membrane Filtration

C. O'Donnell, R.G. Koegel and R.J. Straub

Introduction

Value-added products, including industrially valuable enzymes are currently being produced in transgenic alfalfa by researchers at Madison, Wisconsin. One method of recovering the desired substances is from juice expressed from fresh herbage (wet fractionation). The juice contains both soluble and particulate components, largely broken chloroplasts.

Concentration of the protein in the whole juice by ultrafiltration has not been practical due to fouling and resultant flux reduction caused by the particulates. If the particulates are removed by some other means, such as centrifugation, ultrafiltration of the resulting clarified juice appears to work relatively well to concentrate the desired soluble proteins.

In the past, a technique for reducing filter blinding and fouling has been high tangential flow velocity of the feedstock in an attempt to scour the filter surface clean. This requires recirculating the feedstock many times at high velocity. The result is high power requirement, temperature rise of the feedstock, and possible degradation due to mechanical shear of the protein during many passes through the pump. An alternative is to have a filter surface moving at a high velocity with either rotational or oscillatory motion. This produces the desired relative motion between the filter surface and the feedstock in a more efficient way which should alleviate the problems of the conventional tangential flow filter.

The objective of this research is to assess whether dynamic membrane filtration could play a practical role in the concentration and/or fractionation of plant juice. In particular, could microfiltration remove particulates from the juice followed by ultrafiltration to concentrate the soluble protein in the resulting clarified juice?

Methods

Filtration was carried out in a pilot apparatus (V-SEP) rented from New Logic International, Emeryville, CA. Eleven-inch diameter filter membranes with nominal pore sizes ranging from a minimum of 10 kD (kilo Daltons » 10,000 molecular weight cutoff) to a maximum of 3.0 mm (microns) were used. The membrane oscillated about its axis at a frequency of 60 Hz and an amplitude of 25 mm at the periphery. The flow rate or flux ($lm^{-2}h^{-1}$) and the fouling index (FI = water flow rate/feedstock flow rate after two hours) were measured or calculated.

Results

The results for a given set of filter operating conditions varied widely. It is recognized that the juice varies from day to day and that the physical properties of a given batch of juice can change with time, temperature, microflora, and mechanical shear. Figure 1 shows three different runs using identical membranes where the flux of the "best" run is approximately twice that of the "worst" run.

Table 1 shows that while the initial (water) flux (F.I. x J) of the larger pore sizes is great, the fouling index is also great, leading to greatly diminished flux at the end of two hours. Surprisingly, at the end of two hours the smallest pore size (10 kD) with the F.I. of 2 has the greatest two-hour flux (with one exception). It could be conjectured that the particles are too big to enter the very small pores and can thus be swept from the membrane surface by its motion. Figure 2 shows another example of a 10 kD membrane exhibiting a flux almost double that of a 1.0 mm membrane over an eight hour period.

Figures 3 and 4 show increase in phytase activity as juice dry matter is concentrated with time. Based on phytase molecule size, it might be expected that it would be retained by the 10 kD membrane, but passed into the permeate through the 0.1 mm membrane. It appears, however, to be retained by both membranes. This was subsequently confirmed by the low level of phytase activity found in both permeates. This phenomenon is believed to be due to a retained layer of material on the membrane surface

with an effective pore size smaller than that of the membrane. This could increase the difficulty of separating particulate from soluble protein.

Conclusion

At the maximum flux of 100 $lm^{-2}h^{-1}$ shown in Table 1, processing of 1 t juice per hour would require a filter area of 10 m^2. One dynamic filter on the market has a maximum membrane area of approximately 30 m^2 and could thus be expected to process about 3 t/h of juice. It appears likely under present conditions that most of the soluble protein will be in the retentate regardless of membrane pore size.

In the future pretreatments of the juice, including thermal and pH adjustment will be evaluated in an attempt to aggregate the particulates into a coarser floc. This might allow use of a greater membrane pore size which might, in turn, lead to improvements in flux, lower fouling index, or permit separation of soluble protein from particulates.

Since membranes are expensive, an estimate of their total useful life needs to be made. This includes determining to what extent the original flux rate can be restored by cleaning techniques.

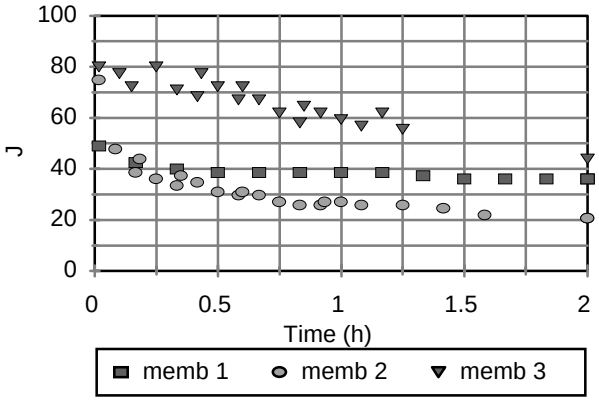

Figure 1. Flux (l/m²h) through 0.2mm membrane with permeate recycled.

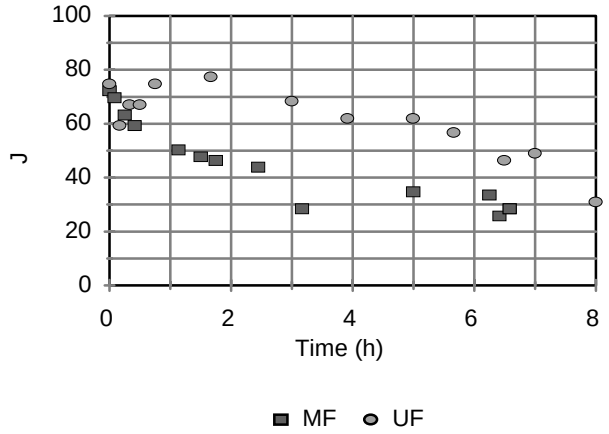

Figure 2. Flux (l/m²h) through 1.0 mm MF and 10 kD UF membranes for concentration of alfalfa juice expressing phytase.

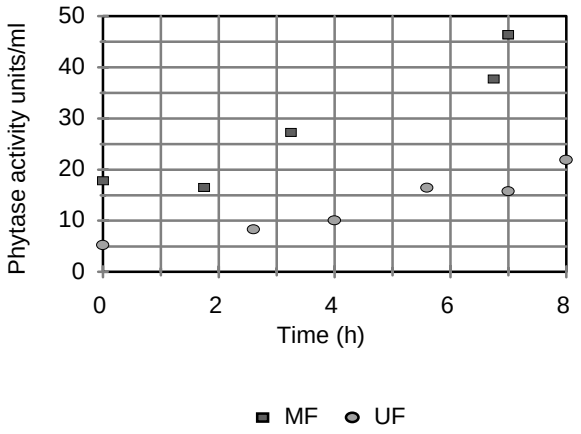

Figure 3. Phytase activity (U/ml) in the concentrated juice processed through 0.1 mm MF and 10 kD UF membranes.

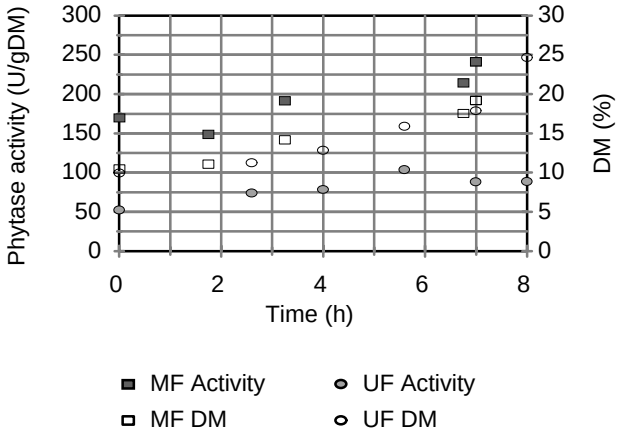

Figure 4. Phytase activity (U/gDM) and DM (%) in the concentrated juice processed through 0.1 mm MF and 10 kD UF membranes.

Table 1. Flux and fouling index of alfalfa juice.

Membrane		Flux, J, ($lm^{-2}h^{-1}$)			Fouling Index		
Pore Size	h	max	min	mean	max	min	mean
3.0 mm	2	85.2	74.8	80.0	50	43	46.5
1.0 mm	4	139.4	32.3	69.7	83	40	58.8
0.5 mm	3	126.5	12.3	74.7	212	40	99.0
0.2 mm	3	43.9	20.6	33.5	116	54.5	78.8
0.1 mm	2	64.5	19.4	42.0	189	57	123
100 KD	2	55.4	51.6	53.5	10	9	9.5
10 KD	1	100.6	100.6	100.6	2	2	2

Production of Lactic Acid From Alfalfa Fibrous Fraction

R.G. Koegel, H.K. Sreenath and R.J. Straub

Introduction

Fractionation of alfalfa herbage is carried out to yield a number of value-added products including enzymes (from transgenic plants), carotenoids, and soluble and particulate protein concentrates. The value of these products can be in the range of $1000 - $2000 per acre-year. The fibrous component from fractionation is about 75% of the initial herbage dry matter. While it makes a good feed for ruminants, other economic uses are being evaluated. Since a large part of the fiber is made up of polymerized sugars, it is possible to hydrolyze the fiber into sugars, by means of selected enzymes, and then to ferment it into target chemicals using appropriate microorganisms.

Both ethanol and lactic acid have been produced through fermentation of saccharified alfalfa. Lactic acid appeared more promising because of its higher yield and higher unit price. Cargill has developed a process for making a biodegradable plastic ("ECO-PLA") from lactic acid. This could create a large market for lactic acid.

Methods

Alfalfa fibrous fraction, resulting from wet fractionation was fermented with or without a liquid hot water (LHW) pretreatment (220°C for 2 min.). The pretreatment partially hydrolyzes the material resulting in a liquid and solid fraction referred to as "extract" and "residue".

The untreated fiber and the extract and residue from the pretreatment respectively were dosed with commercially available enzymes found to be effective for saccharification in previous work and were inoculated with one of four species of *Lactobacillus*. The four species of *Lactobacillus* were: *delbrueckii*, *plantarum*, *pentoaceticus,* and *xylosus*. Fermentations were carried out at near-optimum conditions for the respective species. Levels of lactic acid, acetic acid, and unfermented sugars were monitored periodically.

Results

The enzymatic hydrolysis of plant fiber to sugars results in both six-carbon sugars (hexoses) and five-carbon sugars (pentoses). Most organisms found in nature can ferment one or the other but not both. *L. plantarum* is an exception with the ability to ferment both hexoses and pentoses. Since the extract resulting from the LHW pretreatment is rich in pentoses and the residue is rich in hexoses, the ability of *L. plantarum* to ferment pentoses is shown by comparing yields for the four organisms from the extract (Fig. 1). Figure 2 shows roughly equal production for the four species from the hexose-rich residue. Figure 3 shows total yields from the extract plus the residue. Here the yields from *L. plantarum* after LHW pretreatment are roughly 44% greater than for *L. delbrueckii* because of the ability of the former to ferment pentoses. Figure 4 shows relative lactic acid yields by *L. plantarum*

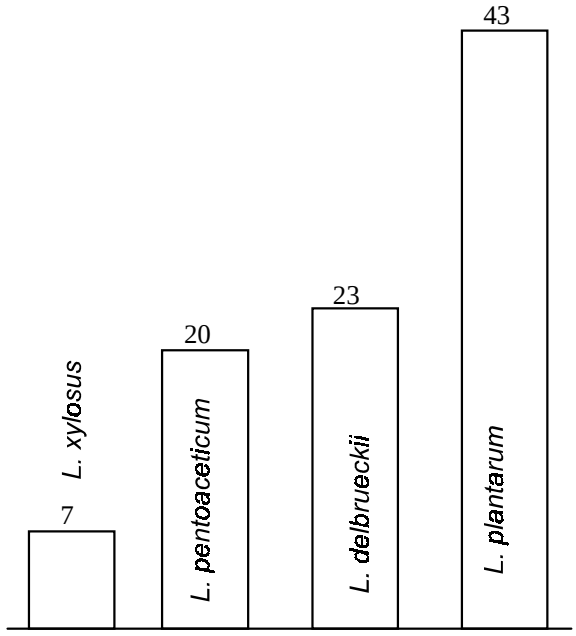

Figure 1. Lactic acid yield from LHW extract resulting from 100g alfalfa fiber.

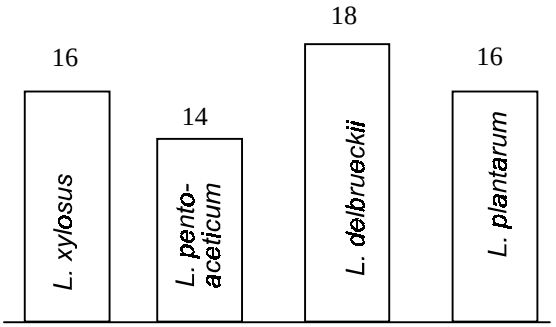

Figure 2. Lactic acid yield from LHW residue resulting from 100g alfalfa fiber.

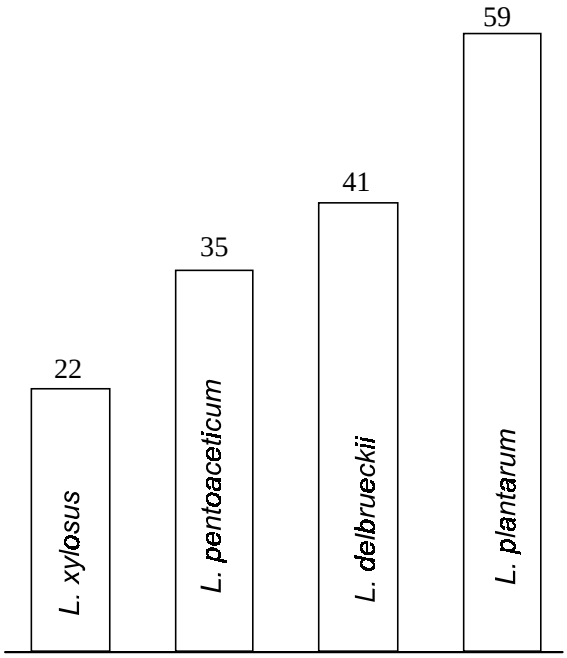

Figure 3. Total lactic acid yield from 100g alfalfa fiber subjected to LHW pretreatment.

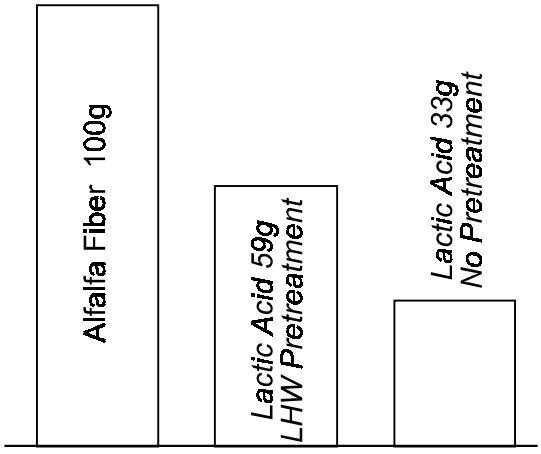

Figure 4. Lactic acid yields from 100g alfalfa fiber using L. plantarum with and without LHW pretreatment.

with and without the LHW pretreatment. While the LHW pretreatment lead to an almost 80% yield increase in this case, the economic advantage of the pretreatment is still unclear because of its considerable capital and operating cost.

The 33g of lactic acid production for the untreated fiber shown in Fig. 4 occurred at 24 h of fermentation. At this time there was 15g of unfermented sugars and 5g of acetic acid for a total of 53g of hydrolysis products. It appears therefore, that, with improved management, there may be the potential for increasing the yield of lactic acid from untreated fiber.

Conclusions

Yields as high as 59g of lactic acid were obtained from 100g of pretreated alfalfa fiber using *L. plantarum*. While yields from untreated fiber were only 56% of this, there appears to be potential for increasing this yield.

Factors Affecting Bunker Silo Densities

R.E. Muck and B.J. Holmes

Introduction

Attaining a high density in a silo is important for two primary reasons. Firstly and most importantly, density and dry matter content determine the porosity of the silage. Porosity, in turn, sets the rate at which air can move into the silo and subsequently the amount of spoilage that can occur during storage and feedout. Secondly, the higher the density the greater the capacity of the silo. Thus, higher densities generally reduce the annual cost of storage per ton of crop by both increasing the amount of crop entering the silo and reducing crop losses during storage.

The factors affecting density in bunker and pile silos are not well understood. General recommendations have been to spread the crop in 15 cm layers and pack continuously with heavy, single-wheeled tractors. In a survey of alfalfa silage in 25 bunker silos, Ruppel et al. (1995) found that tractor weight and packing time (min/t as fed or min/m^2) were the most important factors affecting density. However, both factors only explained a small fraction of the variation observed, and layer thickness was not measured. The objectives in our study were to measure density in a wider range of bunker silos and correlate those densities with filling practices.

Methods

Twenty collaborating county extension agents in Wisconsin measured densities in over 160 bunker silos containing either haycrop or corn silage. Density was measured with a 5-cm diameter corer, taking cores at approximately chest height at four locations across the silage face. Core depth, distance from the top and distance from the floor were recorded. Cores and a grab sample were express mailed to the Center for determination of weight, dry matter content and particle size distribution.

A survey was filled out for each silo sampled. Information requested from farmers included: number of packing tractors, tractor weight, number of tires per tractor, tire pressure, tire condition, number of drive wheels, silage delivery rate, packing time per day, harvest time per day, filling time, filling technique, initial layer thickness, silo dimensions, maximum silage height, crop, crop maturity, and theoretical length of cut. These factors were then correlated with measured dry matter densities.

Results

The range of densities and dry matter contents observed in haycrop and corn silages are shown in Table 1. Ranges of dry matter densities were similar for both haycrop and corn silages. Densities on the low end suggested little packing whereas the highest densities were in the range observed in tower silos. Average dry matter densities were slightly higher than a recommended minimum density of 225 kg/m^3.

Preliminary analyses indicate that dry matter densities were most closely correlated with total tractor weight (Fig. 1) and initial packing layer thickness (Fig. 2). Use of rear duals or all duals on packing tractors did not have a large effect on density. Packing time per ton appeared to be less important than total tractor weight or layer thickness; however, this may have been due to an inverse relationship between silage height and packing time. Taller silos tended to receive less packing time per ton, but density increased with silage height, indicating more self-compaction in deeper bunker silos.

Another issue raised in the preliminary analyses was packing time relative to crop delivery rate to the silo. Packing time per ton was highest (1 to 4 min/t as fed) under low delivery rates (<30 t as fed/h) and generally declined with increasing delivery rate. Packing times were consistently less than 1 min/t as fed at delivery rates above 60 t/h in our survey. These results suggest that farmers using contractors for harvesting their silage crops probably will need to pay particular attention to spreading the crop in a thin layer and would benefit from using several packing tractors simultaneously.

Conclusions

Densities in bunker silos across Wisconsin were highly variable. Preliminary analyses of the results indicate that weight of the packing tractor, initial crop layer thickness and packing time per ton are the most important factors. Finally, the results suggest that continuous packing with a single tractor may not be sufficient to obtain a high density in silos receiving high delivery rates such as with custom harvesting.

Reference

Ruppel, K.A., Pitt, R.E., Chase, L.E. and Galton, D.M. 1995. Bunker silo management and its relationship to forage preservation on dairy farms. J. Dairy Sci. 78:141-153.

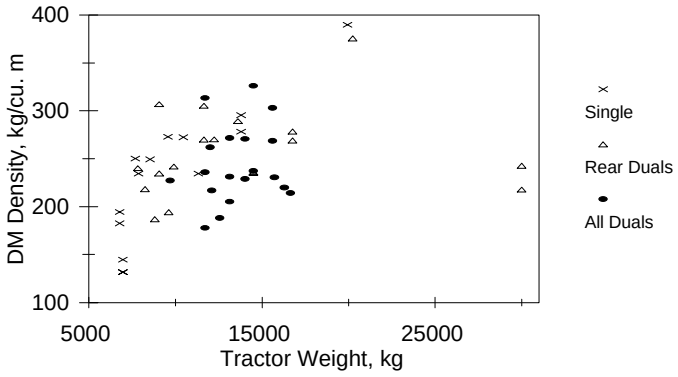

Figure 1. Silage dry matter density as related to total weight of packing tractors for silos with a 3.6 m wall height.

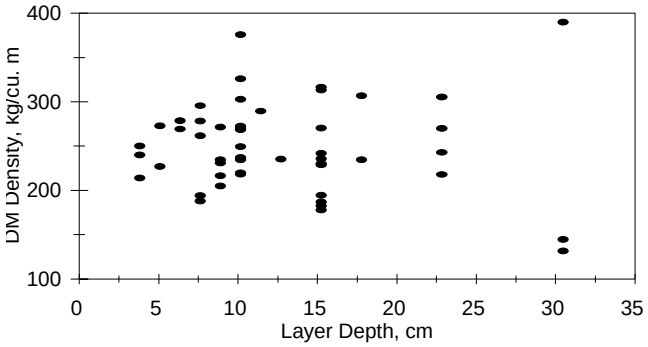

Figure 2. Silage dry matter density as related to crop layer thickness after initial spreading for silos with a 3.6 m wall height.

Table 1. Summary of core samples collected from 168 bunker silos.

	Haycrop Silage (87 silos)			Corn Silage (81 silos)		
Characteristic	Average	Range	Std. Dev.	Average	Range	Std. Dev.
Dry Matter, %	42	24-67	9.50	34	25-46	4.80
Wet Density, kg/m^3	590	210-980	175	690	370-960	133
Dry Density, kg/m^3	237	106-434	61	232	125-378	46
Avg. Particle Size, mm	11.7	6.9-31.2	3.8	10.9	7.1-17.3	2.0

Modified Atmosphere to Reduce Proteolysis in Alfalfa Silage

C. O'Donnell and R.E. Muck

Introduction

Alfalfa is now commonly conserved as silage. While ensiling has many advantages, one particular problem is the breakdown of true protein to soluble nonprotein nitrogen (NPN). In alfalfa silage, soluble NPN typically accounts for 50 to 70% of total nitrogen. This loss of true protein makes it more difficult to develop a ration where the nitrogen will be utilized efficiently by cattle, and leads to excess excretion of nitrogen in manure.

Currently, there are no cost-effective methods of preserving true protein in alfalfa during ensiling. Modified atmospheres, containing nitrogen, carbon dioxide and low levels of oxygen, have been used to maintain fruit and vegetable quality by minimizing respiration but keeping enough oxygen present to keep cells intact. Such success with preserving fruits and vegetables suggested that a modified atmosphere (MA) might preserve protein in silage, and this was shown in earlier research at the Center. The studies reported here were performed to further understand and potentially enhance the benefits of modified atmosphere on protein preservation in alfalfa.

Methods

Three experiments were performed. Treatments in the first two experiments were similar, but one was conducted on direct-cut alfalfa and the other on wilted alfalfa. The alfalfa was ensiled in 3 l PVC silos. Half the silos were ensiled normally under anaerobic conditions and the other half were maintained under MA (3% O_2, 12% CO_2, 85% N_2). For both atmospheric conditions, there were four treatments: control, sugar addition at 5% DM, ammonium hydroxide addition at 0.8% DM, and reduced temperature. All silos were emptied and analyzed after 7 d.

In the third experiment, the length of MA exposure was investigated. Direct-cut alfalfa was ensiled in pint glass jars. Six treatments were imposed: normally ensiled or MA for 1, 2, 3, 5 or 7 d. For the various MA exposure times, two normally ensiled and two MA silos were opened and analyzed for quality and cell viability. The remaining contents of the MA silos were reensiled and allowed to go anaerobic. An additional two MA silos were opened after 3 and 7 d, inoculated with lactic acid bacteria at 10^5 cfu/g crop, reensiled and allowed to go anaerobic. After a total storage period of 28 d, silos of all treatments were opened for sampling and analysis.

Results

Modified atmosphere reduced soluble NPN (Tables 1 and 2) and improved protein preservation in all three experiments. However, in experiments 1 and 2, there was extensive mold growth and high pHs in the MA alfalfa after seven days storage. In the third experiment, no mold growth was observed in the MA alfalfa. In that experiment, pH and DM contents were generally similar to initial conditions for the first 3 days. Thereafter, DM content decreased and pH increased in the MA alfalfa indicating increasing respiration losses. Such changes coincided with the loss of cell viability in the MA treatments between days 2 and 3 (Fig. 1).

In experiments 1 and 2, the sugar and ammonia treatments did not significantly reduce soluble NPN (Table 1) or improve protein preservation in either the normally ensiled or MA alfalfa. Low temperature reduced soluble NPN in the direct-cut alfalfa, particularly in the MA alfalfa. Similar trends were observed in the wilted alfalfa, but they were not statistically significant.

When the MA treatments in experiment 3 were reensiled, little or no fermentation was evident after 28 d of storage. The only reductions in pH occurred in the 1-day MA treatment. Inoculation of the 3-day and 7-day treatments with lactic acid bacteria produced no difference in pH relative to the corresponding uninoculated MA treatments. These results suggest that the MA treatment resulted in the production of compounds that inhibited lactic acid bacterial growth.

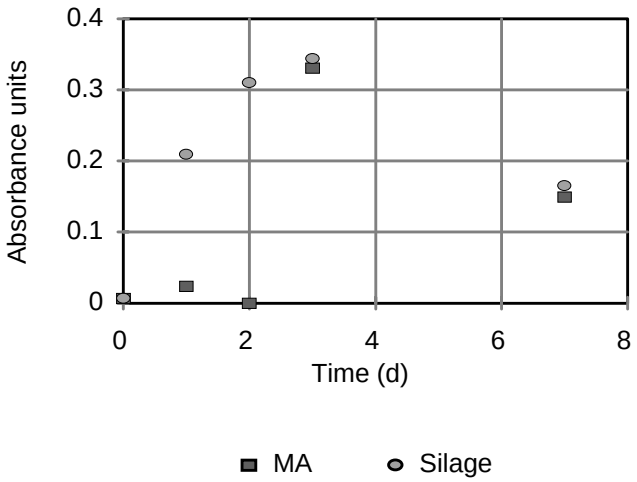

Figure 1. Absorbance of Evans Blue (an indicator of cell viability) by alfalfa cells from MA and silage treatments in Experiment 3.

Conclusions

Modified atmosphere was able to enhance protein preservation in all three experiments. However, our results suggest that MA treatment needs to be limited to early in ensiling. MA was capable of extending cell viability in the silo for two to three days and in this period little DM loss occurred. There was no benefit to extending the MA beyond this period as longer treatment times increased DM losses substantially and increased the probability for the development of spoilage microorganisms such as molds. More research is needed to develop optimum schemes that promote both protein preservation and a normal fermentation.

Table 1. Soluble NPN (% total N) in alfalfa silages in Experiments 1 and 2.

						Significance	
Atmosphere	Control	Sugar	Ammonia	Low Temp	SEM	MA	Treatment
Experiment 1 (alfalfa at 17% DM)							
Silage	50.2	56.0	58.9	46.9	5.5	***	**
MA	36.7	39.6	49.3	19.0			
Experiment 2 (alfalfa at 64% DM)							
Silage	22.4	18.7	24.2	20.7	2.5	*	NS
MA	17.3	16.9	22.9	13.3			

*** = P<0.001, ** = P<0.01, * = P<0.05, NS = not significant

Table 2. Soluble NPN (% total N) in alfalfa silages (20% DM) in Experiment 3.

							Significance	
Atmosphere	Day 1	Day 2	Day 3	Day 5	Day 7	SEM	MA	Day
Silage	35.6	40.1	52.8	49.9	55.0	3.2	***	***
MA	16.0	22.2	26.9	26.1	37.8			

Red Clover PPO: PPO Activity in Different Tissues

R.D Hatfield, C. Neumier[1] and K. Rueden[1]
[1]Wisconsin High School Biotech Program

Introduction

Red clover contains significant amounts of polyphenol oxidase (PPO), an enzyme that appears to have a role in decreasing proteolytic losses during the ensiling of red clover. Not a great deal of information is known about the red clover PPO enzyme so we have undertaken several small studies to determine some of its basic physiological characteristics. Here we report on the PPO activity within different tissues of red clover.

Materials and Methods

Red clover plants were grown in the greenhouse under mercury lamps with a 14:10 hour day:night cycle. All plants were asexually propagated and maintained in four inch clay pots containing growth media (soil:sand:vermiculite 1:1:1) with fertilization (20:20:20, N:P:K) every other week. Plant tissues (ca 3 g) were harvested, quick frozen in liquid nitrogen, and ground to a fine powder in a cold mortar and pestle. Ground samples were quantitatively transferred to a 50 mL beaker with the aid of 10 mL of 100 mM Tris buffer (pH 7.0, 2mM DTT, 5mM ascorbic acid). Samples were gently stirred on ice for 15 min before filtering through 2 layers of Miracloth. Extracts were centrifuged at 20,000 xg to pellet remaining insoluble material. Polyphenol oxidase activity was measured in extracts using caffeic acid (10 mM) as the primary substrate and reduced 5,5-dithiobis(2-nitrobenzoic acid) (TNB) as quinone trap. Activity of red clover PPO was measured as the loss of absorbance at 412 nm due to the reaction of quinones with TNB over a five minute time interval.

Results and Discussion

Since there seems to be a strong connection between loss of proteolytic activity in red clover silages and PPO activity it is important to know the distribution of PPO within red clover tissues. We have compared the PPO activity in the four major tissues of red clover. All tissues were harvested from the same plant and the experiment was replicated three times.

All tissues tested positive for PPO activity. This is not surprising as at least certain forms of PPO are ubiquitously found in plastids of plants. A comparison of red clover tissues indicates that leaves contain a much higher level of PPO activity on a per gram fresh weight basis (Table 1). The total PPO activity mirrors total protein concentrations within red clover plants. Leaves contain 25 to 33 times as much PPO activity as petioles and stems respectively. The high level of PPO in red clover leaves along with abundant levels of soluble phenolics probably accounts for the rapid discoloration of red clover under normal field drying conditions.

For a comparison we evaluated the PPO activity in leaves of alfalfa and tomato. Tomatoes, as well as other members of the Solanaceae family, have been reported to have high levels of PPO. It is clear from the data in Figure 1 that red clover leaves have markedly higher levels of PPO activity on a gram fresh weight basis. Alfalfa leaves contain PPO activity but at levels 2500 times less than in red clover. We do not know at this time if this difference in activity is due to increased expression of the PPO gene(s) or a more active form of the enzyme. Current work is pursuing the answers to these questions.

Table 1. PPO activity in different tissues of red clover plants.

Plant Tissue	A/min/g fresh weight
Flower	0.005
Stem	0.066
Petiole	0.086
Leaves	2.280

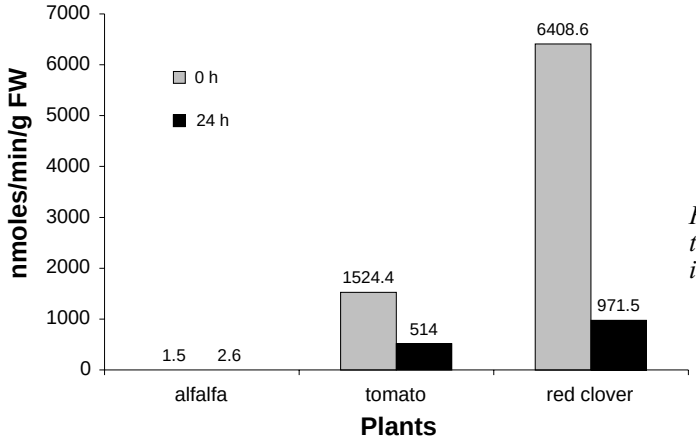

Figure 1. Comparison of PPO activities in leaves of alfalfa, tomato and red clover. Activities were measured immediately after extraction and after 24 hours at 4 ℃.

Variations in Lignin: What do Recent Studies on Lignin-Biosynthetic-Pathway Mutants and Transgenics Reveal about Lignification?

J. Ralph, R.D. Hatfield, R.R. Sederoff and J.J. MacKay

Introduction

Lignification produces the complex lignin polymer in the plant cell wall that is vital for structural integrity of land-based plants, for defense against pathogens, and to facilitate various functions such as water transport. Lignin is unusual compared to other abundant natural polymers due to the low degree of order and the high degree of heterogeneity in its structure. We have recently been interested in understanding how plants respond to deficiencies in their ability to produce the normal precursors for lignification. Some such deficient plants occur as natural mutants; others can be developed using genetic engineering approaches. The potential to improve plant utilization by ruminants and in various other natural and industrial processes by engineering the amount, composition, and structure of lignin is currently attracting considerable interest.

Unexpected variation in lignin subunit composition has been found recently, particularly in an unusual mutation affecting the wood of loblolly pine (Ralph et al. 1997, MacKay et al. 1997, U.S. Dairy Forage Research Center 1997) and also in mutants and genetically engineered variants of higher plants. These results have significant implications regarding the traditional definition of lignin, and highlight the need for a better understanding of the lignin precursor biosynthetic pathway. We believe that the observed variation in composition and structure of lignin is still best explained by variation of the monolignol precursors and their abundance in the lignifying zone [see p.33]. The plasticity in lignin composition reveals new potential that extends beyond the traditional monolignol pathway for modification of the polymer by genetic engineering.

Our arguments for a greater level of plasticity in lignin, through variation in precursor composition, have recently been challenged (Gang et al. 1998, Lewis and Davin 1998). Here, we review and extend our results to support our structural findings and present our conclusions that these structures represent normal lignification with unusual precursors, consistent with a traditional paradigm for lignification. Our view is in sharp contrast with a recent model of lignin biosynthesis requiring template dependent stereospecific control of lignin polymerization (Lewis and Davin 1998, Davin et al. 1997) [see p. 33]. Structural information has long been used to guide the search for underlying mechanisms for important biological processes, and the biosynthesis of lignin is no exception. The combination of current methods of structural chemistry, biochemistry, cell biology and genetics should continue to elucidate the nature and origin of the lignin polymer.

Lignin is conventionally defined as a complex hydrophobic network of phenylpropanoid units derived from the oxidative polymerization of one or more of three types of hydroxycinnamyl alcohol precursors. These alcohols give rise to *p*-hydroxyphenyl, guaiacyl and syringyl subunits in lignin (Fig. 1). The precursors are themselves derived from phenylalanine by deamination, followed by hydroxylation of the aromatic ring, methylation, and the reduction of the terminal acidic group to an alcohol. These alcohols have long been thought to be the direct precursors for lignin (monolignols). The lignin precursors can radically couple at several sites with each other, or, more frequently, with the growing lignin oligomer, to produce a complex polymer with a variety of intermolecular linkages. Here, we focus on a mutation in the last step of the precursor pathway: the formation of the monolignol coniferyl alcohol from coniferaldehyde. This step is catalyzed by the enzyme cinnamyl alcohol dehydrogenase (CAD; EC 1.1.1.195) encoded by a single gene in loblolly pine.

Discovery of a CAD-deficient pine mutant. The discovery of a recessive mutant allele of the *cad* gene in loblolly pine, *cad-n1*, has permitted the study of pines with severe deficiencies of CAD enzyme (MacKay et al. 1997, Ralph et al. 1997). The secondary xylem (wood) in *cad-n1* homozygous seedlings acquires a brown color, distinct from the nearly white color of wild-type pine wood. CAD deficiency causes dramatic changes in the

accumulation and nature of soluble phenolics; it also alters the structure of the lignin polymer that is deposited in the cell wall. The color and many of the changes in wood chemistry are similar to those observed in transgenic plants and in brown midrib mutants with suppressed CAD activity. However, the incorporation of one compound, dihydroconiferyl alcohol (DHCA), a major lignin precursor in pines with the *cad-n1* mutation, has not yet been noted in other plants with decreased CAD activity. These plants need to be re-examined using newer analytical methods. Detailed biochemical and molecular characterization of the CAD enzyme in loblolly pine and genetic analysis of the *cad-n1* allele have allowed us to establish a strong link between severe CAD deficiency and the incorporation of DHCA. Characterization of the gene and the CAD-deficient plants has been described in detail elsewhere (O'Malley et al. 1992, MacKay et al. 1995, MacKay et al. 1997).

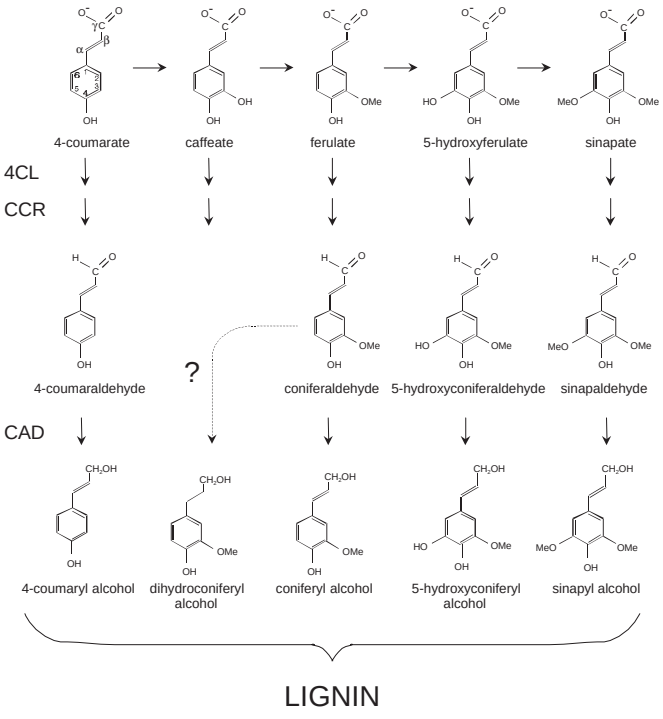

Figure 1. Lignin precursor biosynthesis. The general monolignol precursor biosynthetic pathway is outlined from 4-coumarate to five lignin precursors. Only three enzymes are shown: 4CL (hydroxycinnamate:CoA ligase), CCR (hydroxycinnamoylCoA:NADPH oxidoreductase) and CAD (cinnamyl alcohol dehydrogenase).

Accumulation of the substrate of CAD and the mutant. Chemical analyses of the wood from *cad-n1* homozygotes all indicate a dramatic increase in the levels of coniferaldehyde, the predominant substrate of CAD enzyme in pine, and in structures derived from coniferaldehyde. Simple extraction of ground wood with alcohol or other solvents revealed a several fold increase in soluble phenolics and a large increase in coniferaldehyde and vanillin. The resultant lignin polymer contains coniferaldehyde and vanillin at levels significantly higher than the wild type. This inference is based on results from isolated lignin samples analyzed by NMR, FTIR and UV spectroscopy, and from extractive free wood samples by microphoto-spectrometry, FTIR, PyrMS and thioacidolysis. The development of a brown or red-brown color in lignified tissues of plants that have suppressed CAD activity is well documented in both transgenics and mutants. In addition, *in vitro* synthesis of lignin oligomers (DHPs) starting with a mixture of coniferaldehyde and coniferyl alcohol generates dark red product, as opposed to a nearly colorless product when the aldehyde is absent or at low levels.

Discovery of dihydroconiferyl alcohol (DHCA) subunits in *cad-n1* homozygotes. The lignin from *cad-n1* homozygotes shows a large increase of unexpected subunits derived from DHCA. The identity and abundance of these subunits were first determined in an isolated lignin preparation by diagnostic NMR experiments. These findings were confirmed by direct chemical analysis of the isolated lignin using the DFRC method and analysis of extractive free wood samples by DFRC, Pyrolysis-MS, and thioacidolysis. Greatly elevated levels of DHCA have been observed in wood of several *cad-n1* homozygous seedlings and trees obtained from several crosses as outlined above; DHCA accumulation was not detected in any of the wild-type seedlings. The association between this phenotype and the *cad-n1* homozygous genotype is strong and was not dependent on environmental or biotic stress. DHCA is a *known* minor component of lignins. It is therefore not a new product in the mutant, but one found at highly elevated levels.

Dihydroconiferyl alcohol-derived subunits are components of lignin. Combined chemical degradation and NMR provide unambiguous evidence

that DHCA is a *bona fide* and abundant component of the lignin polymer. The non-extractable lignin fraction can be dissolved in propionyl bromide, where NMR confirms the presence of DHCA. Similar results in support of this claim are obtained whether the analyzed material is milled wood lignin, total wood, or fully solubilized residual lignin from the mutant pine. Comparison with synthetic lignins and model compounds indicates that approximately 50% of the subunits are in 5–5-coupled structures (Fig. 2). The presence of monomeric DHCA in solvent extracts and its predominance in 5–5-linked structures argues strongly against the suggestion that the DHCA components are the result of a modification of lignin *following coupling of coniferyl alcohol*. Detailed examination of dimers from degradation of normal pine lignin by the DFRC method provides evidence that the coniferyl alcohol monomer is essentially not involved in 5–5-coupling reactions. It has also been suggested that DHCA may be a dioxane:water extractable oligomerized lignan artifact (lignans are nonstructural dimeric phenolic metabolites). However, DHCA is present in all lignin fractions; the "careful recalculations" done by Lewis et al. are therefore flawed by their invalid assumption that DHCA is only an extractable artifact. In addition, the MWL of a CAD deficient tree had a weight-average molecular weight of ~17,000 and did not contain a low molecular weight fraction. Therefore, the abundance of DHCA is unlikely to be attributable to contaminating lignans. In fact, since lignans in *Pinus taeda* appear to be optically active, the inability to detect optical activity in the mutant lignin [see p. 35] may be a sufficient counter-argument.

The origin of DHCA in the mutant pine and in other normal softwoods is unknown. An NADPH-dependent enzyme activity reported to reduce the cinnamyl alcohol double bond in b-5 dilignol dehydrodiconiferyl alcohol to produce dihydrodehydrodiconiferyl alcohol will also convert coniferyl alcohol to dihydroconiferyl alcohol. This activity is an interesting candidate for involvement in the conversion of coniferaldehyde to dihydroconiferyl alcohol and should be carefully tested.

Composition and content of lignin in other mutants and transgenic plants. Mutant or transgenic plants with genetic deficiencies affecting enzyme activity in the lignin biosynthetic pathway often have novel lignin structures or modified lignin composition, suggesting a high level of metabolic plasticity in the formulation of lignin precursors. A mutation in the enzyme ferulate-5-hydroxylase results in lignin without a syringyl component, whereas an overexpressing transgenic variant produces a lignin that is almost entirely composed of syringyl units. Minor components of normal lignins can become more significant when other key enzymes are depleted. Naturally occurring mutants (*e.g.*, the brown-midrib (bm3) mutants of maize and sorghum) and transgenic plants deficient in *O*-methyl transferase (OMT) contain significant amounts of units derived from 5-hydroxyconiferyl alcohol. Tobacco downregulated in CCR shows a striking increase in tyramine ferulate, a logical sink for the anticipated build-up of feruloyl-CoA. Coniferaldehyde becomes more significant in the CAD deficient pine mutant, radically coupling with aldehydes or with lignin monomers/oligomers. Similarly, sinapaldehyde becomes a major component of antisense-CAD tobacco transgenics.

Variability of lignin composition indicates a high level of metabolic plasticity based upon precursor supply. The ability of plants to adapt to diverse and

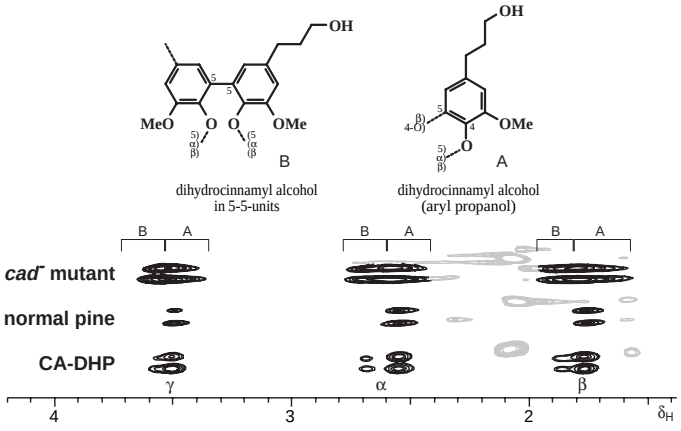

Figure 2. Comparison of DHCA units in the CAD deficient mutant, normal pine, and a coniferyl alcohol synthetic lignin (DHP). The normal pine, with only low levels of DHCA has units which couple only with traditional monolignols. In the CAD deficient mutant, the level is so high that DHCA units are coupling with each other, resulting in ~50% of the units being 5-linked. In the DHP, the level of DHCA is about 3% of the coniferyl alcohol; a small amount of homocoupling occurs. The spectra are partial 2D HMQC-TOCSY NMR plots through a and b on DHCA units showing corrections to the a, b, and g sidechain protons.

large changes in the precursor supply indicates that there is considerable metabolic plasticity in the assembly of the lignin polymer. Lignin must encompass a wider array of phenolic structures, with its composition and structure primarily guided by the precursor supply in the lignifying zone within plant tissues. The precursor supply varies among plant taxa; it also varies among cell types and within the cell wall, thus resulting in macro and micro heterogeneity of lignin itself. The increase in *p*-hydroxyphenyl subunits in lignin from compression wood is well-established. The precursor supply is also affected by genetic lesions or variants that create additional variation in lignin structure as discussed here.

Other nontraditional subunits are found in lignins. It is important to recognize that lignins, even in 'normal' plants, are extremely variable in their composition. The definition of lignin as a polymer resulting from the three monolignols has long been recognized as too narrow. Many plants have lignins containing significant levels of other unusual components (Fig. 3), and it is likely that no plant contains lignins that are solely derived from the three "primary" precursors. For example, all lignins contain aldehyde groups – it is this feature that provides the diagnostic lignin staining reaction with acid phloroglucinol. Evidence from mutants and genetic variants where aldehydes accumulate strongly supports the view that aldehydes are incorporated as precursors, because, in these variants, more aldehydes are found in the lignin.

Many lignins are biosynthesized by incorporating esterified monolignols into the lignification scheme. Thus, grasses utilize *p*-coumarates, hardwoods and some dicots such as kenaf utilize acetates, and some plants, notably bamboo, aspen and willow, use *p*-hydroxybenzoates as 'monomers' for lignification. Ferulates and diferulates are found intimately incorporated into all grass and some dicot lignins, where they are equal partners in the free-radical polymerization process and may even be nucleation sites for lignification. Amides may also be incorporated; although it is a wounding response product, tyramine ferulate is found in various lignins, *e.g.*, in tobacco. A general definition of lignin must include more than the traditional three hydroxycinnamyl alcohols, or the phenolic polymers in many plants serving the structure and function of lignin, *e.g.*, in grasses, might not be considered lignin. Lignin components do appear to be derived from phenylpropanoids as a general class; this classification has been used frequently.

Conclusions

Recent genetic studies have shown that manipulating specific lignin-biosynthetic-pathway genes produces profound alterations in the phenolic components of

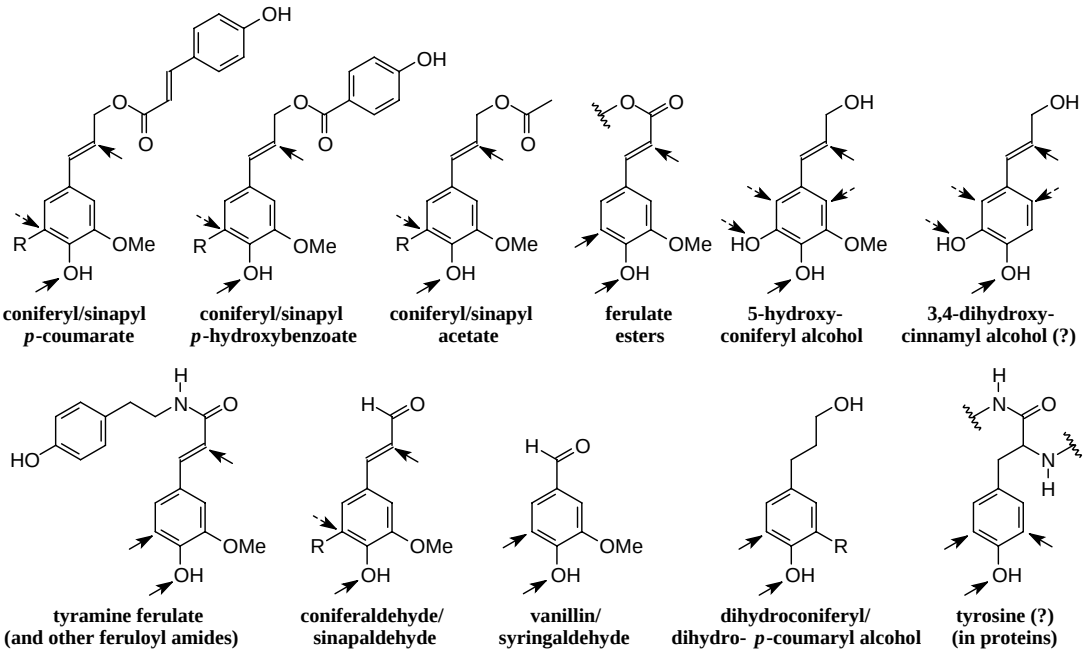

Figure 3. Non-monolignol "monomers" that are known or thought to be intimately incorporated into lignins. Sites for radical coupling are indicated with an arrow; a dashed arrow indicates other possible sites (coupling behavior not known).

plants. Whether the polyphenolic components produced by radical coupling reactions should be called lignin is little more than semantics. Although the 'lignins' in mutant and transgenic plants may appear to be strikingly different from 'normal lignins,' findings indicate that they represent merely broad compositional shifts. All of the novel units that have been found to date appear to be minor units in normal lignins. The recognition that such minor units can incorporate into lignin provides significantly expanded opportunities for engineering the composition and consequent properties of lignin.

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Order and Randomness in Lignin and Lignification: Is a New Paradigm for Lignification Required?

J. Ralph, R.D. Hatfield, R.R. Sederoff and J.J. MacKay

Lignification and the Definition of Lignin

Lignification is a process essential to the nature and evolution of vascular plants that is still poorly understood, even though it has been studied for more than a century. Lignin is unusual compared to other abundant natural polymers due to the apparent low degree of order and the high degree of heterogeneity in its structure. It is also unusual as a plant polymer in that there are no plant enzymes for its degradation.

Definitions of lignin that are both specific enough and general enough are difficult to find. For example, a recent book on "Lignin and Lignans" does not provide such a definition even in the first chapter, entitled "Lignin and Lignan Biosynthesis: Distinctions and Reconciliations" (Lewis et al. 1998). Attempts to define lignin in terms of its function within the plant are also not clear-cut. Lignin is often defined for specific applications, *e.g.*, it is often regarded as little more than the (non-extractable) phenolic component that must be fragmented or degraded to produce pulp and paper. We prefer to consider lignin as an operational term for a diverse class of naturally occurring phenolic polymers that need to be more precisely defined in each case, based upon the source and the method of purification or analysis. It is often difficult to distinguish cell wall lignin from other polymerized infusions, *e.g.*, normal lignification *vs*. wound-response lignin. Until there is better agreement on how lignin should be defined, it will not be surprising if there is continued controversy about its origin, composition, properties and biosynthesis.

Order in Lignin

The issue of randomness or the degree of order in lignin formation remains controversial. Researchers occasionally speculate about the possibility of crystallinity. Studies on simple lignin trimers led one of us to describe lignin as a stereochemical nightmare with crystallinity in the traditional sense being astronomically improbable (Ralph 1993). That is not to say that other aspects of order in the polymer, such as the alignment of aromatic rings indicated by Raman spectroscopy (Atalla and Agarwal 1985), are impossible. Randomness has been used to imply that the process appeared to have no direct enzymatic control. Obviously, the polymerization of lignin is not completely random, and only a subset of the possible linkages are found. In fact, prior descriptions as 'random' presumably never implied a totally random distribution of coupling products; clearly, the coupling of two monomers must be weighted by the propensity for each type of coupling. However, lignin formation has little to do with monomer coupling — lignification, unlike lignan formation, almost entirely involves coupling of a single monomer to a growing oligomer. The idea that the coupling would depend on the types of units involved, their concentrations, the matrix and so on are all issues that can determine what type of coupling arises at any step.

Order in the lignin polymer could result from processes of self-assembly, or alternatively, specific finely orchestrated steps might be involved in the assembly. Lewis has argued that, "It is inconceivable that lignin formation would be left to the vagaries of such a wide range of enzymes or be realized in a haphazard manner" (Davin et al. 1997). A more general opposing view is succinctly stated by Denton (Denton 1998), who argued that "Biological defense is well served by loosely ordered chemistry. Where many randomly linked products are needed, sloppy pathways are economical."

A New Paradigm for Lignification?

Lewis *et al*. claimed that we have argued (Gang et al. 1998, Lewis et al. 1998) for a new and unsupported paradigm for lignin polymerization from our findings relating to lignin-biosynthetic-pathway mutants (Ralph et al. 1997) and transgenics (Ralph et al. 1998). This is clearly not the case. Rather, we believe that the properties of abnormal lignins are simply due to changes in the relative abundance of precursors likely

to be found in normal lignins. For example, the lignin in *Arabidopsis* can be converted from a guaiacyl-syringyl lignin to a predominantly guaiacyl lignin or to an almost exclusively syringyl lignin by modifying the level of expression of the gene for ferulate-5-hydroxylase (Meyer et al. 1998). Much of the variation in lignin, whether due to genetic or environmental causes, can be readily explained by a change in the relative abundance of the precursors delivered to the lignifying zone. All that is required beyond delivery of precursor is that the mechanism for polymerization be a general one.

Our results do challenge the need for the new paradigm proposed by Lewis and Davin (Lewis and Davin 1998). Recent isolation of a "dirigent" protein from *Forsythia* which facilitates coniferyl alcohol radial coupling to produce the lignan pinoresinol in a regio- and stereoselective manner has led to a proposed new mechanism for lignin biosynthesis because of the similarity of the phenolic precursors. According to their model, lignins form from template arrays of dirigent proteins and are synthesized with absolute structural control. With the possibility that the synthesized lignin chain then structurally dictates the next chain by a template-polymerization process (Sarkanen 1998), the model resembles the mechanism of biosynthesis of more highly ordered biological polymers. This idea is intriguing, but highly speculative and currently devoid of evidence.

Exquisitely synthesized polymers should produce an array of discrete products following degradation by such procedures as ether cleaving reactions (acidolysis, thioacidolysis, the DFRC method, or high temperature base). Instead, such degradative methods produce a continuous array of oligomers with no members of the series obviously missing. We have not been able to detect any hint of optical activity in various isolated lignins nor in degradation products which retain the optical centers produced in the coupling step [see p. 35]. The new paradigm proposal cites two possible explanations for the "perceived lack of optical activity of lignins" (Lewis and Davin 1998). One explanation is that "two distinct types of proteins each encoding formation of complementary chains that effectively cancel out any measurable optical activity." This idea requires that the plant would go to the energetically extreme measure of creating an optically active lignin polymer only to carefully negate that structural feature via a complementary set of proteins for which additional (complimentary) biochemical pathways must also be supported. This effort is to produce two complementary lignin polymers when each has *identical physical properties*, identical to those of the racemic mixture — some reason would have to be envisaged, although producing a variety of structures/stereochemistries is an asset in defense. The template argument (that "complementary mirror images form via template replication") has less serious detractions, although no evidence of any structural replication ability has yet appeared. Since lignins are found intimately associated with hemicelluloses, it is not clear how discontinuities in the alignment of one lignin chain might contribute to excess optical activity (of the incompletely replicated section). We look forward to cogent arguments, rationales and diagnostic experimental evidence for the complex issues involved in the proposed new lignification paradigm. Until experimental evidence is provided, the extension of the lignan dirigent protein observations to lignin, as a paradigm for lignification, is without substance.

Control of Monomer Supply

We have not seen any experimental data that require a precisely controlled synthesis of structurally-defined lignin, *i.e.*, data that cannot be supported by simply recognizing that the plant does exquisitely, temporally and spatially, control the supply of monolignols, oxidizing enzymes and oxidative species. The matrix environment of the polymerization may alter the interunit linkage composition, as has been well demonstrated in synthetic lignification experiments. The regulated differences in lignification in various cells and various regions of the cell, in wounding or in stress, demand little more than changes in monomer supply.

Conclusions

Lignification is a complex process that currently defies exact definition. Evidence to date points to lignin as being a product of chemically (rather than

biochemically) controlled radical coupling reactions. A recently proposed new paradigm which aims to bring lignin into the realm of other exquisitely synthesized biological polymers is currently devoid of evidence. In fact, all of the variation in lignin composition and structure, including the dramatic changes that occur in lignin-biosynthetic-pathway mutants and transgenics, appears to be the result merely of controlled precursor supply (including oxidases, and co-factors, *e.g.*, peroxide) to the lignifying zone. How the plant controls the temporal and spatial aspects of precursor supply will presumably be subjected to more extensive study in the future. Although interesting, there are overwhelming problems with the proposed new paradigm for lignification resulting from extrapolation of lignan observations. No evidence currently available challenges the established views on lignification.

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Are Lignins Optically Active?

J. Ralph, J. Peng, F. Lu and R.D. Hatfield.

Introduction

Lignin is a complex polymer produced in the plant cell wall by radical coupling reactions. It provides no nutritive value to ruminants and further ties up potentially digestible polysaccharides. Other plant polymers such as polysaccharides and proteins are synthesized by the plant with careful attention to structure. As such, their monomeric components and the polymers themselves, are optically active — that is, they have chirality, or "handedness" (as our right and left hands differ by being mirror images). It has long been assumed that lignins are not optically active since they derive from monomers with no chirality and appear to be the result of chemical reactions that are independent of enzymatic control. To date, however, there does not appear to be any rigorous proof of this assertion. Recently, the accepted derivation of lignins from non-(enzymatically)-controlled reactions has been challenged, and it is relevant to ascertain unequivocally whether lignin is, or is not, optically active.

Lignans, dimers of monolignols, are also produced by many plants, and are often in optically active forms. For example, the lignan pinoresinol, and its derived matairesinol, were found only as their *(-)*-isomers in suspension-cultured *Pinus taeda* (Eberhardt et al. 1993). A recent isolation of a "dirigent" protein from Forsythia which facilitates coniferyl alcohol radial coupling to pinoresinol in a regio- and stereospecific manner has lead the discoverers to extrapolate their lignan observations to lignins (Davin et al. 1997), as is discussed in an accompanying article [see p. 33]. Although their new paradigm may not require the resultant lignin to be optically active, the finding of chiral lignin would significantly strengthen the idea.

If lignification were to be controlled in a structural and stereochemical sense by dirigent proteins, it would be possible to alter the properties of lignin, potentially to our advantage, by targeting these proteins using biogenetic engineering methods. The question of lignin optical activity therefore needs to be addressed. This report describes a two-pronged approach to resolve the issue as unequivocally as possible.

Materials and Methods

Isolated lignins were dissolved in 10:3 acetonitrile:water, a particularly good solvent for UV studies. DFRC conditions were those described previously (Lu and Ralph 1997b; Lu and Ralph 1997a). Circular dichroism (CD) spectra were run on an AVIV model 62A-DS circular dichroism spectrometer (Lakewood, NJ) driven by an Apple Macintosh computer running IGOR-Pro 3 software (Wavemetrics, Lake Oswego, Oregon). The lamp current was set at 26 amps; temperature was 25 °C; all conditions were standard. For lignin dimers, the solvent was acetonitrile (HPLC grade, Baker); for isolated lignins, the solvent was 10:3 acetonitrile:water. Sample concentrations were adjusted so that optical densities measured by the dynode were just below the maximum of 600 V at maximal absorptions in the spectral range. Spectra were obtained using 10-s averaging in 1 nm steps. Spectra were baseline subtracted (using scans of the blank solvents). Raw non-smoothed spectra are shown in the figures.

Results and Discussion

Of the various methods for determining optical activity, circular dichroism (CD) was chosen for its sensitivity and because the association of CD phenomena only with UV-active absorptions allows optical activity in lignins to be determined even in the presence of contaminating (optically active) polysaccharides.

Lignin dimeric degradation products. Dimeric lignin fragments were isolated from pine wood following DFRC (**d**erivatization **f**ollowed by **r**eductive **c**leavage). As seen in Fig. 1, the bonds originally formed in the radical coupling step remain unaltered from their native states following this degradation. Determination of chirality in these compounds therefore reflects chirality in the original lignin. This is particularly clear-cut with b–5 and b–b-compounds (where the crucial bonds do not cleave), but less so

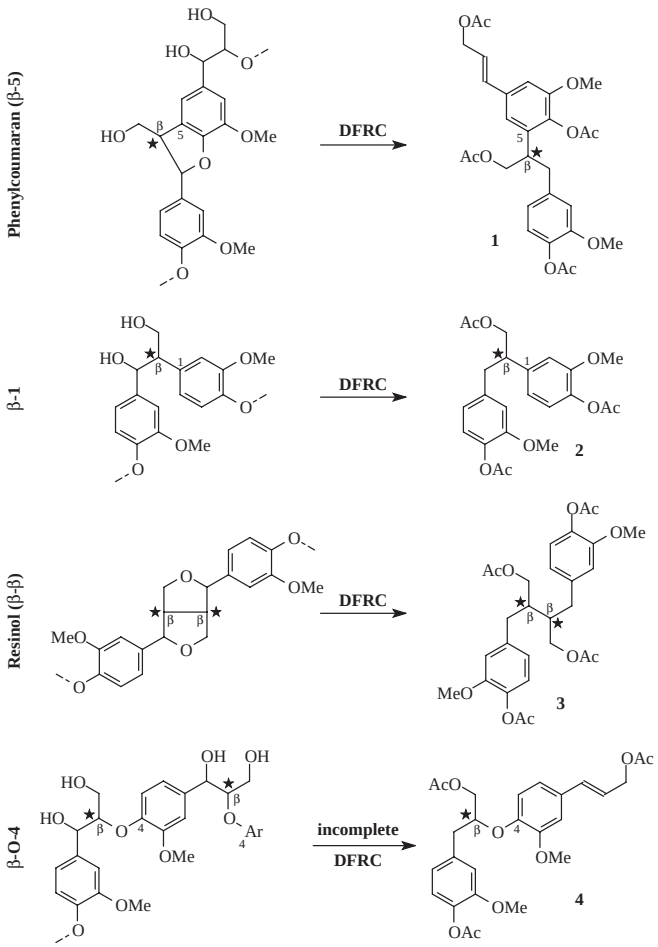

*Figure 1. Dimeric DFRC products from various structural units in softwood lignins. Optical centers at b-carbons formed by radical coupling reactions during lignification are denoted with a ★. Note: the b–O–4-dimer **4** is only produced in small amounts from incomplete ether cleavage.*

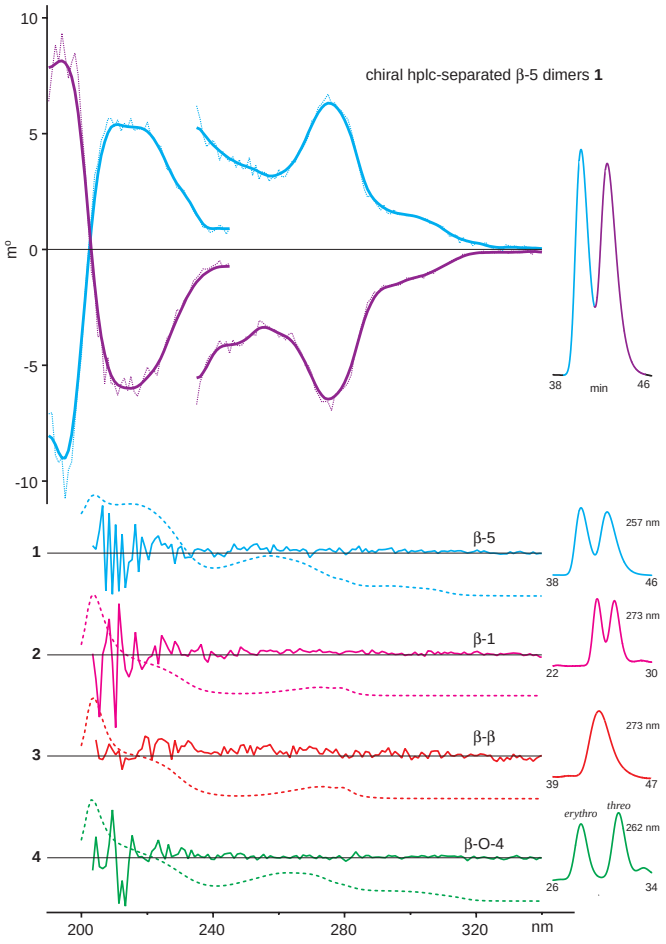

*Figure 2. CD spectra of isolated DFRC dimers **1-4** along with the chiral-HPLC-separated b–5-dimer **1**. UV spectra are shown with dashed lines; the CD spectra are more noisy at high UV absorptions, but the CD rotations are also strongest in the region of strong UV absorption. Chiral-column HPLC traces are shown to the right of the figure; the b–b-enantiomers **3** were not resolved, nor were the b–O–4-enantiomers **4**, although the threo- and erythro- (syn- and anti-) diastereomers were. The resolved b–5-enantiomers **1** showed strong CD spectra; the region from 190-245 nm was run using one quarter the concentration used for the 240-350 nm region.*

for b–1's (where there are still some uncertainties regarding how they arise) and, perhaps, b–O–4's (where it is less clear that the b–O–4 bond has not been broken and reformed in the production of the fragment dimers).

Figure 2 shows CD spectra of dimers **1-4** isolated from pine lignin following DFRC, along with spectra from both enantiomers of the b–5-dimers **1** (which were separated by chiral HPLC). The spectra are a little noisy, particularly near 200 nm, where the absorption is high and the total optical density of the sample limits the sample concentration that can be used for CD. However, high CD rotations are also expected at high absorbtion wavelengths, so high sensitivity is expected in this area. This may be seen from the 190-245 scans of the b–5-enantiomers **1** where the concentrations were reduced five-fold and still produce pronounced CD rotations. At 280 nm, a maximum in lignin UV spectra, the noise level is sufficiently low to allow relatively sensitive determination of optical activity. No hint of such activity was found.

Of the linkage types that might have shown optical activity, the b–b may be the most likely. The lignans from *Pinus taeda* are reported to be optically active. There is the possibility that even carefully pre-extracted wood meal contains lignans or polymerized lignans. The lack of any optical activity in the b–b-dimer, which was obtained from whole wood, not

isolated lignin, appears to provide evidence in addition to that already at hand that the dimers arise from true racemic lignin, rather than some poly-lignan (that would likely be optically active). Similar conclusions arise from examining CD-spectra of isolated lignins (see below).

Isolated lignins. A valuable and exploitable feature of CD spectra is that CD is associated only with UV bands. Unlike other optical measurements (direct optical rotation, or optical rotatory dispersion), CD spectra can therefore be run on impure samples containing chiral impurities providing the UV-spectra of the component of interest has diagnostic absorptions well separated from those of the chiral contaminants. Isolated lignins always contain contaminating carbohydrates, which are optically active. However, the UV spectra of carbohydrates do not extend much above 200 nm. A major lignin peak of interest is at 280 nm, with shoulders out at around 300+ nm. The range 250-375 nm was sufficiently diagnostic for lignins while devoid of carbohydrate interference. CD spectra of maize, kenaf, pine, and CAD-deficient mutant pine lignins all showed no detectable optical activity, Fig. 3. Improved signal-to-noise was obtained by longer averaging in noisy sub-regions (not shown).

An indication of the level of a chiral component required to observe optical activity can be gained from spiking studies. Fig. 4 shows the CD spectra from pine lignin with ~5% and ~10% levels of the isolated

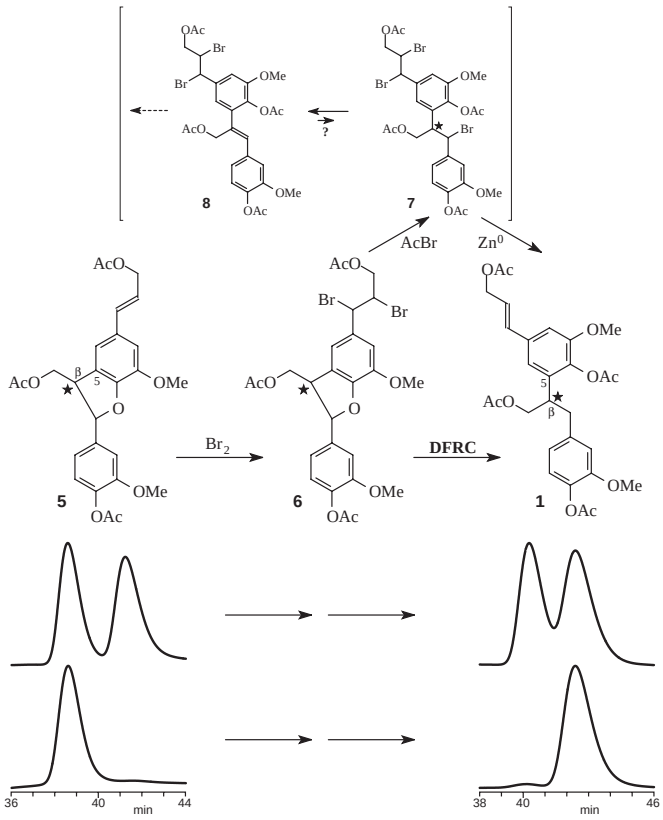

*Figure 3. Proof that the DFRC method does not scramble the crucial b-carbon stereochemistry in b–5-units. Dimeric model **5** was first brominated so that the unsaturated sidechain would be generated giving the same b–5-product **1** as isolated from DFRC of lignin. The potential for scrambling the center by HBr elimination from the bromo-intermediate **7** is shown, but as noted in the text, is not anticipated. Chiral HPLC (bottom traces) indicate that a single enantiomer of **5** produces a single enantiomer of **1** following DFRC, proving that the optical center is not scrambled.*

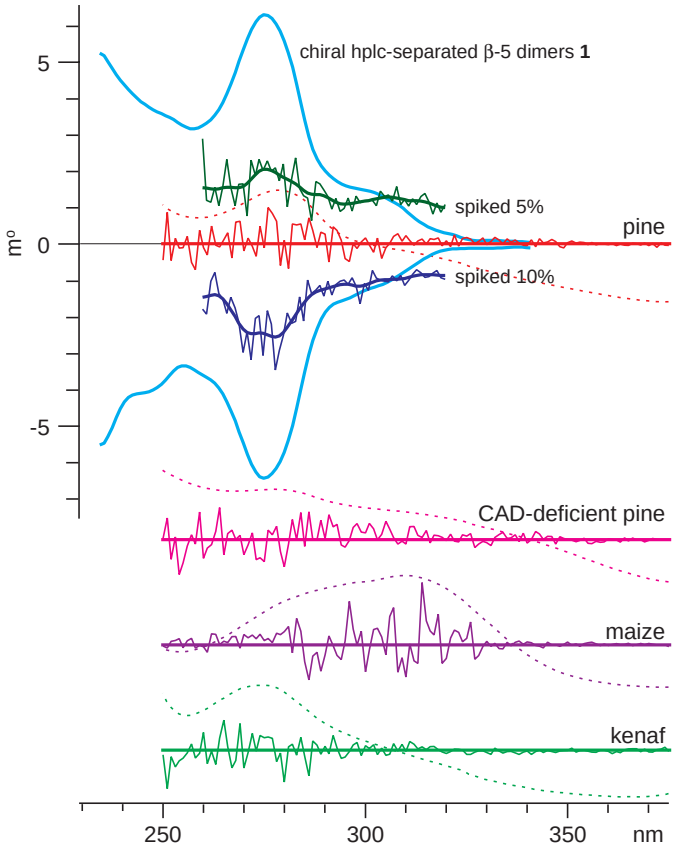

*Figure 4. CD spectra of various lignin isolates, illustrating no detectable optical activity. CD spectra of the separated b–5-enantiomers **1** are shown again for comparison. To test the sensitivity of detection, a resolved enantiomer of **1** was spiked into the pine lignin sample at a 5% and a 10% level; the raw data and the curve smoothed by the CD software indicate that a 5% component is reasonably easily detected (although the required level may be higher in the complex lignin sample where various units might have opposing contributions).*

chiral b-5 dimer **1** added. The optical activity can be readily seen at the 5% level. Admittedly, with several potential chiral centers in lignin, and the possibility of non-reinforcing optical CD spectra, the ability to determine chirality at levels as low as 5% may still be difficult under our experimental conditions. Unlimited access to long-term-averaging would improve signal-to-noise and should allow an improvement of these determinations.

The maize lignin has high UV absorption centered at ~310 nm, resulting in considerable noise in the CD spectrum in that region. The other lignins look rather similar. The CAD-deficient pine mutant was of particular interest because it has been suggested that this isolated lignin is merely a polymerized lignan artifact, an issue that we address more fully elsewhere [see p. 28]. If this were so, the mutant's lignans must be (unexpectedly?) optically inactive since the isolated lignin contains no hint of optical activity.

Conclusions

Our inability to detect optical activity in lignins seems to strengthen the view that lignin is indeed synthesized by the plant without direct (regio- or) stereo-control over the exact course of radical coupling events. An ability to produce a cell wall polymer with appropriate properties for water transport, defense, and other tasks, with a compositional (and structural) flexibility that is determined by monomer supply is presumably well suited to surviving environmental, gravitropic, and biological stresses. Indeed, the recently noted abilities of plants to remain viable by circumventing biogenetic obstacles placed upon their lignin biosynthetic pathways, in a single generation, without the benefit of evolution, appears to be an endorsement of their flexible strategy. A more extensive discussion of the implications for lignification is in an accompanying article.

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Isochroman Structures in Lignin: a New b-1 Pathway

J. Ralph, J. Peng and F. Lu

Introduction

Lignins are produced principally from a dehydrogenative polymerization of coniferyl and sinapyl alcohols. With four available coupling sites (4-O-, 1-, 5-, and b-) on the coniferyl alcohol monomer radical and at least three (4-O-, 1- and 5-) on its subsequent dimer and higher oligomer radicals, the resulting polymer is structurally and stereochemically complex. Structural details are still emerging from model studies coupled with NMR, and from closer examination of the products of various degradation methods. Only recently were dibenzodioxocins discovered in softwood lignins. They have now been found as important branch-point structures in hardwoods, grasses and legumes.

In our efforts to understand the roles and mechanisms by which lignins in forages inhibit forage digestibility by ruminants, we recently developed a new analytical method for lignin analysis, the "DFRC" method (U.S. Dairy Forage Research Center 1996). Applications of this method are beginning to reveal new details about lignin, as illustrated here. The use of pine in this study is not a move away from forages; it represents merely the study of a simplified system (pine lignin contains no sinapyl alcohol) which allows us to make observations about the general lignin pathways.

Results and Discussion

New aryl isochroman products **5** were discovered in the trimer fraction of pine wood degraded by the DFRC procedure. These structures implicate a new pathway following b-1 coupling between a coniferyl alcohol radical **1** and a lignin oligomer radical **2**. The plausible mechanism for biosynthesis of this structural unit, Scheme 1, suggested that it might be found in native lignins. Examining *in situ* lignins with the resolution required to identify such a structure is not possible, but we report here its firm identification in isolated pine milled wood lignins.

HMQC or HSQC spectra of various pine milled wood lignin isolates showed a small but diagnostic correlation between δ_C 40.3 and δ_H 3.59 ppm (not shown). Regrettably, the other carbon/proton correlations are in congested regions of the spectra but peaks are present at the correct locations. Evidence for the entire proton coupling network was further confirmed by TOCSY experiments, Fig. 2, where correlations corresponding to all four protons on the aryl isochroman ring are clear. The correlations represent 5 simultaneous NMR chemical shifts (1 carbon, 4 proton) that correspond exactly with the shifts in the isolated trimer (as shown on Fig. 2), constituting significant proof.

Although the identification of the aryl isochroman structure in an isolated milled wood lignin can be made firmly, the apparently low amount visible in spectra does not account for the significant amounts of derived b-1 products that arise from DFRC-degradation, or various other acidolytic methods. The possibility remains that it is a product of isolation and that its precursor **4**, for example, may be the *in situ* product,

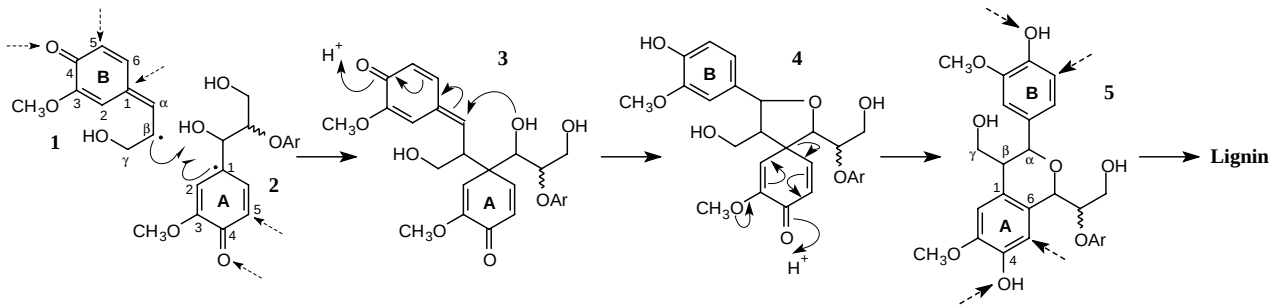

*Figure 1. Proposed lignification mechanism in which b-1 coupling of a coniferyl alcohol radical **1** and a preformed lignin oligomer radical **2** produce a quinone methide intermediate **3** which is internally trapped to produce the spiro-compound **4** (rather than fragmenting via the 'normal' b-1 pathway (Lundquist 1965). Sidechain migration produces the aryl isochroman **5**. Dashed arrows indicate potential sites of further radical coupling during lignification. Numbering follows normal lignin conventions.*

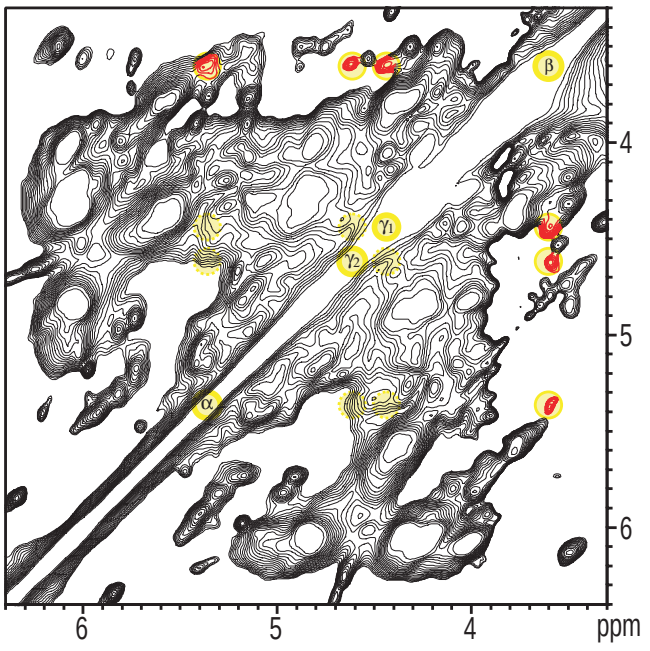

*Figure 2. Sidechain region of a TOCSY (spin lock time, 100 ms) spectrum of Pinus taeda acetylated milled wood lignin, clearly showing the H-a/H-b/H-g/H-g' coupling network. Correlations from the isolated compound **5** [Ar = (OMe)Ph-CH=CH-CH$_2$OAc], are at the center of the overlying circles. Other correlations present but not fully resolved are shown with dotted circles.*

as has been proposed by Brunow. Either way, however, structure **5** provides compelling evidence of the internal cyclization pathway from b-1 intermediate **3**, where no evidence has previously been presented.

Conclusions

The isolation of compound **5** and various related dimers following DFRC-degradation of pine wood adds further support for the occurrence of b–1-coupling during lignification. The structures suggest the existence of a plausible new pathway following the radical coupling step, a pathway which involves intramolecular trapping of the b–1 quinone methide followed by sidechain migration. Compounds assigned as having b–6-linkages have resulted from other degradative procedures; the aromatic-ring substitution patterns vary from those identified here and should be carefully re-examined in light of the more reasonable mechanism shown in Fig. 1. Products with a–6 linkages can result under acidic conditions, and it is possible that the dienone-phenol rearrangement (**4** ® **5**, Fig. 1) is mediated by the acidic conditions of the DFRC procedure. The mechanistic plausibility of the reaction pathway, the isolation of expected degradation dimers, and the identification of the aryl isochroman structure in pine milled wood lignins by NMR, all suggest that such structures, or their precursors, are present in native lignins.

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The DFRC Method for Lignin Analysis: Detection and Determination of Acetylated Units in Lignins

J. Ralph and F. Lu

Introduction

Some lignins have long been known to be naturally acylated by various acids, although the biochemistry and even the full role of such acylation remains unresolved. All grasses (both C_3- and C_4-grasses) are *p*-coumaroylated; some hardwood lignins, notably willow and aspen, and some grass lignins, notably bamboo, are *p*-hydroxybenzoylated; and acetates have been implicated in many hardwood and some dicot lignins. In the cases of forage grasses and legumes, the implications of these units on digestibility of the plant cell wall, by ruminants, has not yet been fully resolved.

The recently developed DFRC (Derivatization Followed by Reductive Cleavage) method is a degradation procedure that produces analyzable monomers **5** and dimers by cleaving a- and b-ethers in lignins **3** (U.S. Dairy Forage Research Center 1997, 1996). One feature of the method is that g-ester groups on lignins **4** remain intact. Without modification, however, any information about acetate groups is lost since the DFRC method utilizes various acetate-containing reagents in its protocol. Information on natural lignin acetates has also been scarce from NMR studies, where lignins are frequently acetylated for improved NMR properties, or are purified using acetic acid where acetylation artifacts might arise.

The need for an independent method to determine acetates on native lignin follows from our discovery of high levels of acetate on kenaf bast fiber lignins, and a general disbelief of the result by other researchers. Acetylation in lignins is important as it represents another form of lignin acylation and presumably invokes another transferase enzyme that could be targeted for biogenetic modification to possibly improve fiber degradability.

Methods

Cell wall and lignin isolations. Cell walls were isolated simply by solvent-extracting ground (2 mm, Wiley mill) lyophilized plant-stem material. The kenaf was from the bast fiber fraction only because its core contains lignin of a different composition. Solvent extraction was sequentially with water, methanol, acetone, and chloroform. Lignin isolations were from ball-milled material following standard procedures. No purification steps involving acetic acid were used, and no steps in the extractions or isolations involved any acetyl-containing reagents.

The modified DFRC (DFRC') procedure. Lignin (5 mg) was stirred overnight at room temperature with propionyl bromide in propionic acid (1:2, 3 ml). Alternatively, the solvent-extracted cell wall sample (30 mg) was stirred for 16 h. at 50 °C with 5 ml of the same reagent. The solvents and excess bromide were removed at 50 °C under a stream of nitrogen, and then at high vacuum (~50 mtorr). The product was then dissolved in 8 ml dioxane:propionic acid:water (1:1:0.1), and 100 mg powdered Zn added. After stirring for 30 min, the product was worked up as usual. Propionylation with propionic anhydride in pyridine completed the procedure. Analysis was by GC and/or GC-MS as described previously. Absolute yields were not calculated since response factors for the variously acetylated and propionylated monomers were not determined. Monomer yields from normal DFRC on these materials were approximately 1260 mmoles/g for kenaf lignin, and 900 mmoles/g for aspen lignin.

Results and Discussion

The modification to the basic DFRC method simply involves replacing all acetate-based reagents with their propionate analogues. It is then a simple matter to differentiate fully propionylated monomers **7** (which come from normal unacetylated lignin units) from any g-acetylated monomers **6** which must come from g-acetylated units in the native lignin, Fig. 1. Propionyl bromide in propionic acid readily derivatized and dissolved lignin samples, but was a little less efficient than the normal acetyl bromide system for whole-cell-

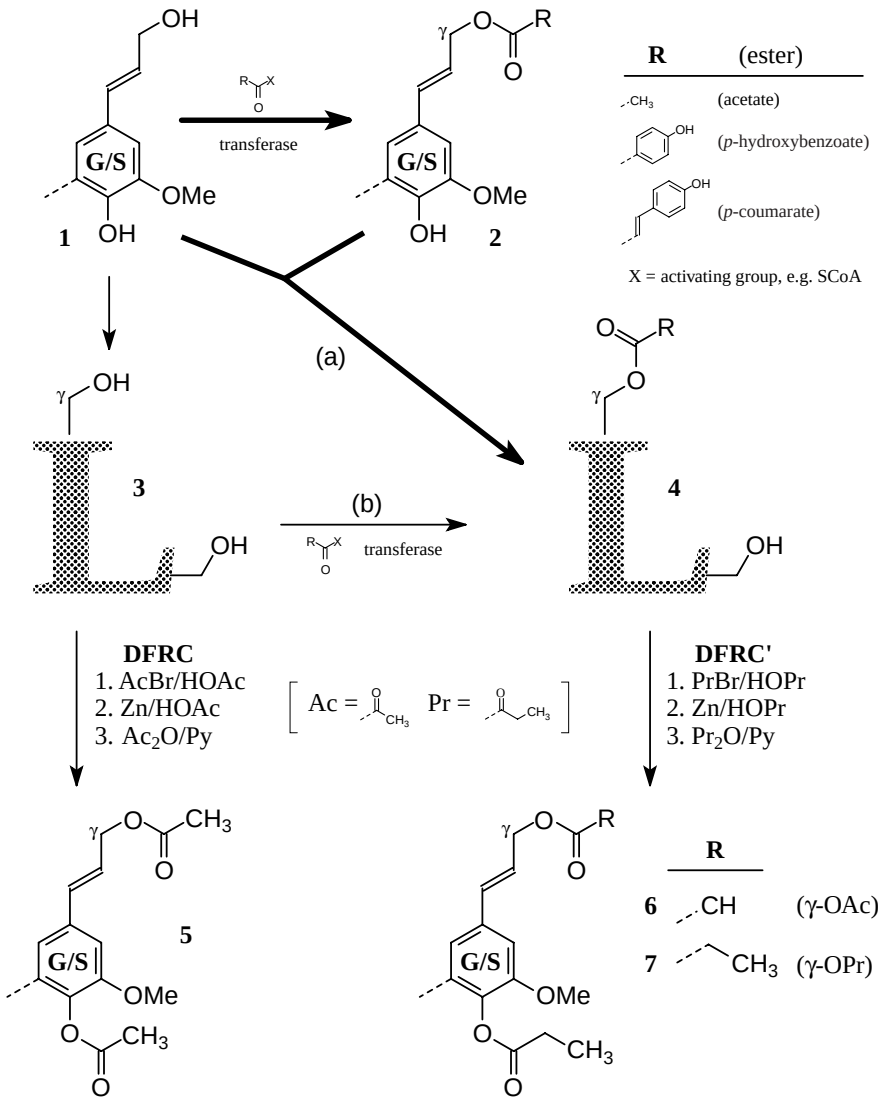

*Figure 1. Formation and degradation pathways for acylated lignins **4**. Monolignols **1** can be acylated by suitable activated acids (acetic, p-hydroxybenzoic, p-coumaric, and others are known in nature) via transferase enzymes to monolignol esters **2**, presumably before diffusing into the cell wall. Esters **2**, along with monolignols **1**, can then be incorporated via radical coupling reactions into lignins **4** with partial γ-acylation (pathway a). Alternatively, normal lignins **3** can be subsequently acylated (pathway b). The normal DFRC protocol cleaves α- and β-ethers (in unacylated lignins **3**) releasing, following acetylation, quantifiable monomers **5**. Lignin γ-esters (such as in **4**) are not cleaved during the normal DFRC protocol and the method can be used for identifying p-coumarate, p-hydroxybenzoate (and presumably other) esters (not specifically shown in this figure). A modification to the DFRC protocol, substituting all acetate-based reagents with their propionate analogues (DFRC' protocol), allows determination of acetates that are naturally on lignin γ-positions. Thus, normal lignin β-ether units can release the 4,γ-dipropoxy monomers **7**, whereas units originally bearing γ-acetates will release 4-propoxy-γ-acetoxy monomers **6**. Releasable units originally bearing acetates can then be readily distinguished from normal (unacetylated) units by GC.*

wall samples; some finely divided insoluble material remained. Use of fresh propionyl bromide was crucial: reagent from an old bottle of unknown purity and composition did not dissolve even isolated lignin samples.

The GC spectra are easy to interpret, particularly when aided by MS. Fig. 2a shows the monomeric products **6**, **7** that resulted from the modified DFRC procedure applied to isolated lignin from kenaf bast fiber; this lignin was never subjected to acetyl reagents in any form. Compounds **7** arise from normal (γ-OH) units in lignins whereas compounds **6** (γ-OAc) arise from originally γ-acetylated units in lignins **4** (R = CH_3). The predominant 4-propoxy-sinapyl-γ-acetate peak **S-6** proves that syringyl units were highly acetylated in the isolated lignin. The same procedure applied to whole cell walls from kenaf bast fiber produced the chromatogram in Fig. 1b. This sample had been extracted only with toluene:ethanol to remove extractives. Obviously, the unisolated cell wall lignin also had syringyl units which were heavily acetylated. In both cases, only minor amounts of acetate were on guaiacyl units, **G-6**. Although the guaiacyl component of kenaf lignins is strikingly low, the preference for acetylation of syringyl components suggests a specific enzymatic process (although chemical acetylation may also be selective). Presumably this is the acetylation of sinapyl alcohol **S-1** via a transferase and activated (e.g. S–CoA) acetic acid.

The modified DFRC procedure also allowed detection of minor acetate components in hardwood lignins.

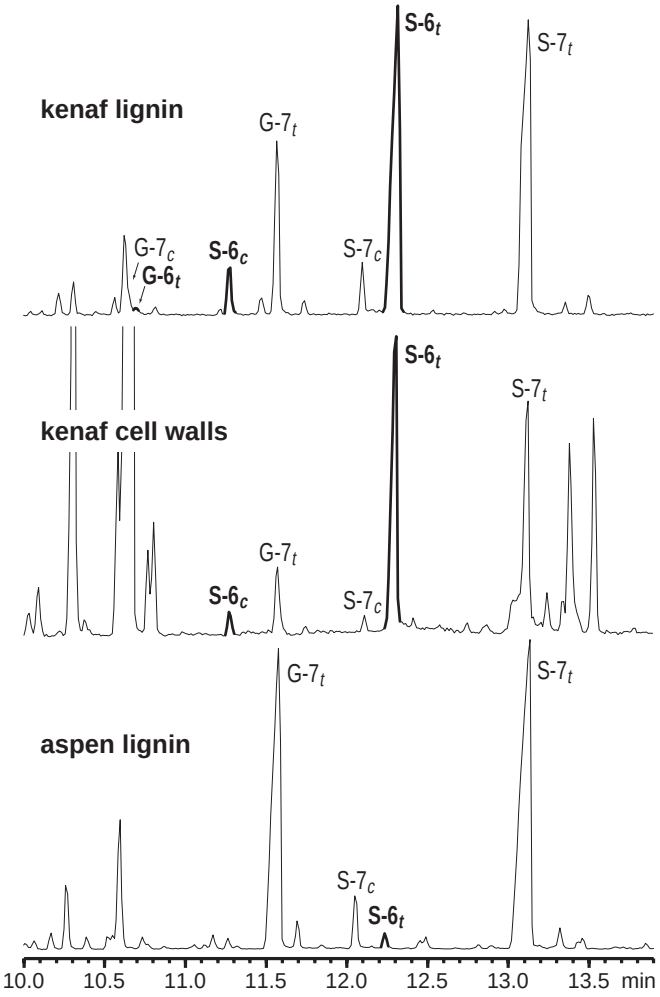

*Figure 2. GC-MS total-ion chromatograms of monomers from the modified DFRC procedure applied to a) isolated Tainung kenaf lignin, b) Tainung kenaf whole-cell walls, c) isolated aspen lignin. Components **S-6** and **G-6** (bold peaks) were from acetylated units in the original lignin; **S-7** and **G-7** were from normal (unacetylated) units. c = cis, t = trans. The peak labeled **S-6**$_t$ in c) contains a significant co-eluting component that was not identified. GC-MS is therefore crucial for compound authentication.*

Thus, for example, the chromatogram from isolated aspen lignin also showed a small 4-propoxy-sinapyl-g-acetate **S-6** peak; the labeled peak actually contains two components so the compound really is quite minor but definitely present as confirmed by MS. The analogous guaiacyl **G-6** peak could not be detected. The methods applied here do not exclude acetylation at other positions in hardwoods, but apparently, as in kenaf, a similarly selective transferase enzyme exists in aspen for acetylating sinapyl alcohol prior to its export to the wall for lignification.

Conclusions

Modification of the DFRC protocol by use of propionate analogs of normal reagents and solvents allows acetates in lignins to be unambiguously detected and confirms their presence at the g-positions of lignin side-chains. Kenaf bast fiber lignin is naturally highly acetylated as originally reported, overwhelmingly on syringyl units; releasable sinapyl monomers were over 50% acetylated in Tainung kenaf (reported here) and in 3 other varieties. Supporting literature observations, selected hardwood lignins were only slightly g-acetylated, again predominantly on syringyl units. The results suggest relatively specific acetylation of sinapyl alcohol prior to lignification. The modified DFRC protocol is a powerful new method for analyzing naturally acetylated lignins and pinpointing acetylation sites. It will allow researchers to screen hardwoods and other plant materials for the presence of acetates on lignins, although it is recommended that GC-MS (or LC-MS) be used for product authentication. The propionyl modification described provides a useful complement to the acetyl DFRC method and other lignin analytical methods.

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The DFRC Method for Lignin Analysis: The Behavior of Cinnamyl End-groups

F. Lu and J. Ralph

Introduction

Lignins are complex natural polymers arising from an enzyme-mediated dehydrogenative polymerization of phenylpropanoid precursors, primarily coniferyl and sinapyl alcohols. Softwood guaiacyl lignins are derived primarily from coniferyl alcohol whereas hardwood and non-woody plant (e.g. forage grasses and legumes) guaiacyl-syringyl lignins come from a mixture of coniferyl and sinapyl alcohols. Lignification involves free-radical coupling reactions, sometimes combined with nucleophilic additions to quinone methide intermediates, to form three-dimensional polymers. Sarkanen and Ludwig's seminal Lignin book reviewed the two types of polymerization processes for synthetic lignin (DHP) formation *in vitro* originated by Bernd Lehman and John M. Harkin (Harkin, personal communication). The 'Zutropf' DHPs, formed by adding lignin precursors slowly and continuously, were called 'end-wise' polymers and structurally resembled isolated lignins more closely than 'Zulauf' DHPs or 'bulk' polymers, formed by adding the precursors in a single batch.

One characteristic difference between end-wise and bulk synthetic lignin polymers is that there are fewer cinnamyl end-groups in the former than in the latter, because bulk lignification involves substantial immediate dimerization. End-wise polymerization more frequently involves addition of a monomer to a growing lignin oligomer. Since the number of cinnamyl end-groups in lignin is relatively low, lignification in the plant cell wall is believed to be an end-wise polymerization, although there is considerable evidence for cytochemical heterogeneity in lignins. Recently we found that a milled tobacco lignin, like DHPs, has a high content of cinnamyl end-groups, b–5 and b–b linkages. The content of end-groups in lignins is therefore an important characteristic of lignin structure. It would be helpful to be able to quantify lignins' end-groups for a better understanding of lignin biosynthesis.

The DFRC (**d**erivatization **f**ollowed by **r**eductive **c**leavage) method is a recently developed analytical tool for lignin characterization (U.S. Dairy Forage Research Center 1996). Through DFRC, b-aryl ether linkages in lignin are cleaved releasing monomers which are quantified by GC. Most monomeric and dimeric DFRC products have been identified. In this study several lignin models with cinnamyl end-groups were subjected to DFRC degradation and major monomers isolated and identified. Mechanisms leading to the formation of these diagnostic monomers are addressed.

Methods

For GC-mass spectrometric analysis, 5-10 mg substrates were used for DFRC. For preparative scale DFRC, 100-150 mg starting materials were used. AcBr treatment conditions used were standard. AcBr treatment products were separated on normal-phase preparative (2-mm thickness) TLC plates (Alltech, Deerfield, IL) using $CHCl_3$/EtOAc (20:1) as solvent. The major DFRC final products **3**, **4**, and **7** were isolated from C_{18} reverse-phase 1-mm TLC plates (Alltech) using MeOH/water, 6:4, following normal-phase TLC ($CHCl_3$/EtOAc, 20:1) from preparative DFRC of 4-hydroxycinnamyl alcohols **1** and 4-hydroxycinnamaldehydes **5**.

Results and Discussion

Coniferyl alcohol **1a**, sinapyl alcohol **1b**, coniferaldehyde **5a**, and sinapaldehyde **5b** were subjected to the DFRC procedure, Fig. 1. Although such units in lignins are completely etherified, reactions on these phenolic models helped elucidate some DFRC pathways; similar reactions with appropriately etherified models, not described here, produced analogous results. Compounds were identified by their mass spectral data, and their structures were authenticated by NMR following isolation.

The major DFRC products from coniferyl alcohol **1a** were 4-acetoxy-guaiacylcyclopropane **3a** and the guaiacylpropyl bromide **2a**. The major monomers from sinapyl alcohol **1b** were the analogous compounds **3b** and **2b**. The unusual cyclopropyl

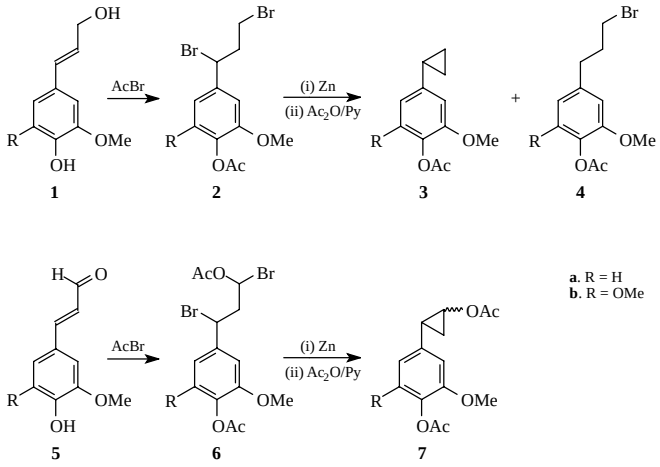

*Figure 1. The formation of arylcyclopropane compounds **3** and **7**.*

compounds **3** were isolated from preparative DFRC of cinnamyl alcohols **1**. Identification was made by the usual series of NMR experiments (^{1}H, ^{13}C, DEPT, 2D COSY, 2D gradient-enhanced HMQC and HMBC). In the proton NMR spectra of **3**, two multiplet peaks at δ_H 0.7 and 0.9, integrating for two protons each, indicated the presence of 2 pairs of cyclopropane protons. Those protons correlated with only one carbon peak in HMQC experiments suggesting a symmetry. Carbon–1 of the aromatic ring correlated with all side-chain protons in HMBC experiments, an occurrence not encountered in typical aryl-*n*-propyl side-chains. Thus compounds **3** were identified as arylcyclopropanes.

The major DFRC monomers from coniferaldehyde **5a** and sinapaldehyde **5b** were diagnostic *cis*- and *trans*-arylcyclopropyl acetates **7**, Fig. 1. Two low-field multiplet signals around δ_H 1.2-1.3 in the ^{1}H-NMR spectra, and the corresponding δ_C 11.4-11.5 methylene signals in the ^{13}C-NMR spectra, indicated the presence of cyclopropane protons in compounds **7**. Singlets integrating for 3 protons at δ_H 1.8-2.0 indicated a side-chain (aliphatic) acetate attached to tertiary carbons. In HMBC experiments, C–1 on the aromatic ring again correlated with all protons on the sidechain, confirming its cyclic nature.

To understand the formation of DFRC monomeric products from cinnamyl alcohols **1** and cinnamaldehydes **5**, the intermediates produced during acetyl bromide (AcBr) treatment were also isolated by preparative TLC and identified by NMR. 4-Hydroxycinnamyl alcohols **1** resulted primarily in aryl-1,3-dibromopropanes **2**, presumably formed via allylic bromination to the cinnamyl bromide, then HBr addition across the double bond, followed by acetylation. Coniferaldehyde **5a** and sinapaldehyde **5b** reacted with AcBr in acetic acid in a similar way to the alcohols resulting in compounds **6** as major products.

The final compounds are then logical Zn-reductive products of the corresponding intermediate bromides **2** and **6**. Phenylcyclopropane has been obtained by treatment of 1-phenyl-1,3-bromopropane with a Zn–Cu couple in dimethylformamide. Similar ring closure was also observed when comparable reducing conditions were applied to 1,3-dihilades. Hence compounds **3** and **7** likely resulted from the 1,3-

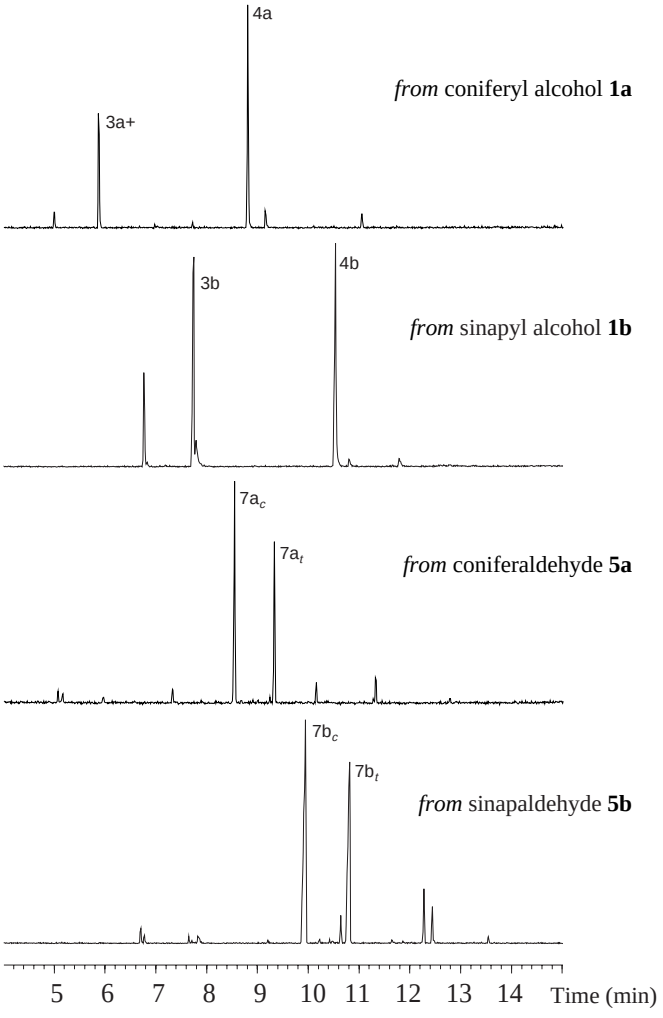

*Figure 2. Total-ion chromatograms of DFRC products from coniferyl and sinapyl alcohols **1a** and **1b**, and coniferaldehyde **5a** and sinapaldehyde **5b**.*

dibromides **2** and **6** formed in the AcBr treatment step. When isolated **2a** was treated with Zn dust in dioxane/acetic acid/water mixed solvent, as in the reductive step of the DFRC procedure, compounds **2a** and **3a** were indeed produced. Compounds **7** were produced analogously when isolated compounds **6** were treated with Zn under DFRC conditions.

Conclusions

Diagnostic products were formed from cinnamyl alcohol and cinnamaldehyde end-groups in lignins following DFRC treatment. The reactions are not as clean as the ether-cleaving reactions that form the basis of the DFRC method, but nevertheless provide valuable markers for studying end-groups in lignins. Cinnamaldehyde end-groups produce characteristic arylcyclopropyl acetates, so the DFRC method could find value in understanding compositional changes in mutant and transgenic plants where aldehyde build-up is suspected. Cinnamyl alcohol groups produce 1-aryl-3-bromopropanes, along with more diagnostic arylcyclopropanes and other more minor products. Although the product mixtures are more complex, the production of relatively diagnostic "fingerprint" products from cinnamyl alcohol endgroups also allows the DFRC method to provide useful data on these features of lignins.

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3D NMR Experiments for Use in Cell Wall Research

J. Ralph

Introduction

NMR (nuclear magnetic resonance spectroscopy) is a powerful tool that has made dramatic impacts into the understanding of cell wall chemistry. Despite the complexity of the wall in general and of the lignin component in particular, intimate structural details are revealed by diagnostic NMR experiments. And now that we understand and routinely apply 1D and 2D NMR (!!??), it seems appropriate to add to the complexity by throwing in a third dimension.

NMR is not limited to 1- and 2-dimensions. Three-dimensional experiments are now commonplace, and 4D-, 5D-, and higher-D-experiments have been applied to labeled proteins. Much of the value of these experiments comes from the further dispersion realized by correlating over the additional dimensions. This is particularly valuable in proteins where ^{13}C, ^{1}H, and ^{15}N dimensions are available. For uniformly ^{13}C-enriched lignins, the 3D HMQC-TOCSY experiment (with one ^{13}C and two ^{1}H axes) has been applied with some success. The increased complexity and data size, and the reduced resolution provide less than compelling advantages over 2D experiments, although their value is becoming appreciated. The expectation that 3D experiments necessarily are more time demanding overlooks the fact that signal-to-noise is gained on the *total number of scans in the entire experiment*. Although labeled materials facilitate 3D (as well as 1D and 2D) experiments, valuable spectra from unlabeled materials are readily obtained.

Results and Discussion

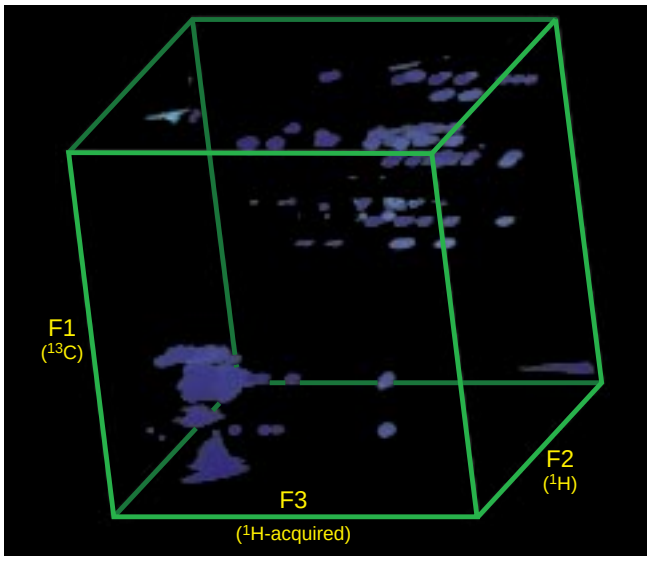
Figure 1a shows a 3D spectrum of the "methoxy-less" lignin, with natural ^{13}C-abundance. In a weekend, this 3D experiment provides ample sensitivity for a synthetic lignin. The spectrum is obtained without applying any of the sensitivity- or resolution-enhancement treatments such as linear prediction in the 3D processing. More informative 2D sub-spectra for the prominent structures in lignins are shown in Figs. 1b–d. Note how "clean" most of these sub-spectra are — a result of dispersion into a third dimension. The particular pulse program used for Figures 1a-e (Bruker's "invbml3d") is not a gradient experiment and has two features requiring comment. Firstly, the sequence, like its 2D-HMQC-TOCSY counterpart ("invbmltp") uses a pre-excitation BIRD sequence to reduce the unwanted intensity from protons attached to NMR-inactive ^{12}C. The BIRD sequence takes about one third of the experimental time; no real data are collected during this time. The other 'feature' is that the directly bonded C–H pair that is excited in the HMQC portion of the experiment retains its $^{1}J_{C-H}$ coupling (~140 Hz). This is illustrated with the slices that are plotted in Figs. 1c-e.

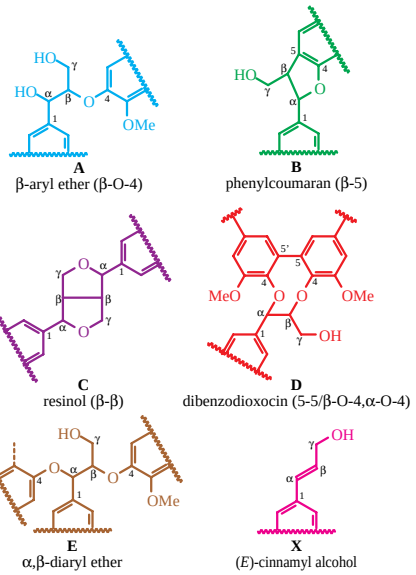
More modern versions using gradient selection decrease the time requirement. A more efficient TOCSY-HSQC experiment has been implemented by Bruker ("mleviietf3gs3d" which can be modified to a two-channel version, "mleviietgs3d") and provides beautiful HSQC sub-spectra in the F_2–F_3 plane, Figs. 1f-k, and TOCSY sub-spectra in F_1–F_3 (not shown). Figures 1h-j show how, if a proton chemical shift is unique, it is possible to observe HSQC spectral planes that are purely from a single substructure in lignin (as seen for structures **A**, **B**, and **C**). Where protons are not unique, HSQC spectra of several units are obtained, such as is the case at 4.45 ppm, where g-protons of substructures **A**, **B**, **C**, **D**, **E** and **X** all resonate, Fig. 1k.

Understanding 3D spectra

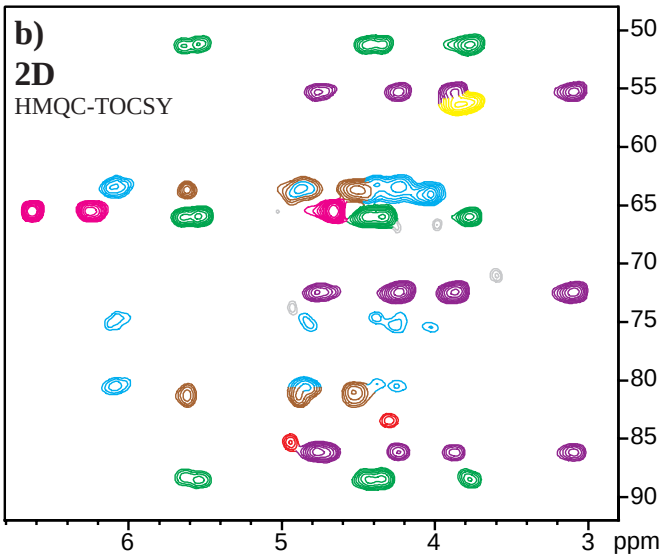
The following brief introduction is provided for those new to the 3D experience. The 3D HMQC-TOCSY experiment is acquired with three orthogonal dimensions, labeled F_1, F_2 and F_3. The acquired dimension is F_3 and, for sensitivity reasons, is proton. F_2 is also proton, and F_1 is carbon. Since this is an HMQC-TOCSY experiment, a 2D F_1F_3 plane is basically a 2D ^{13}C–^{1}H HMQC-TOCSY spectrum at a given proton chemical shift (defined by the distance along the proton F_2 axis). Thus, e.g. Fig. 1d(vii), at the plane through the H–a proton frequency of

a)
DHP
3D HMQC-TOCSY

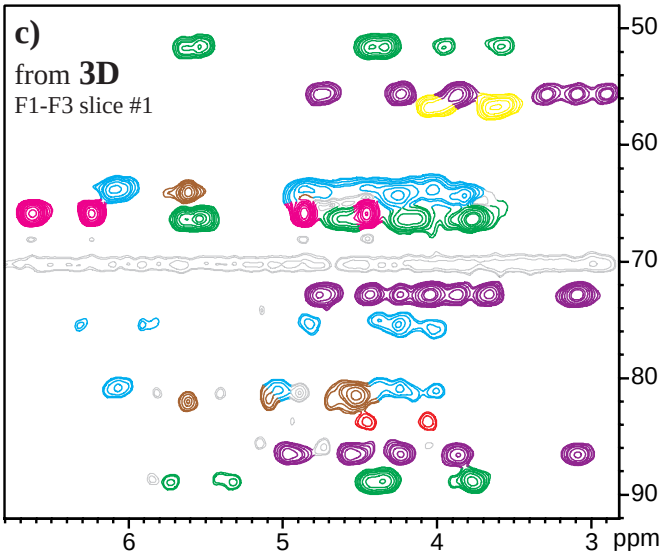




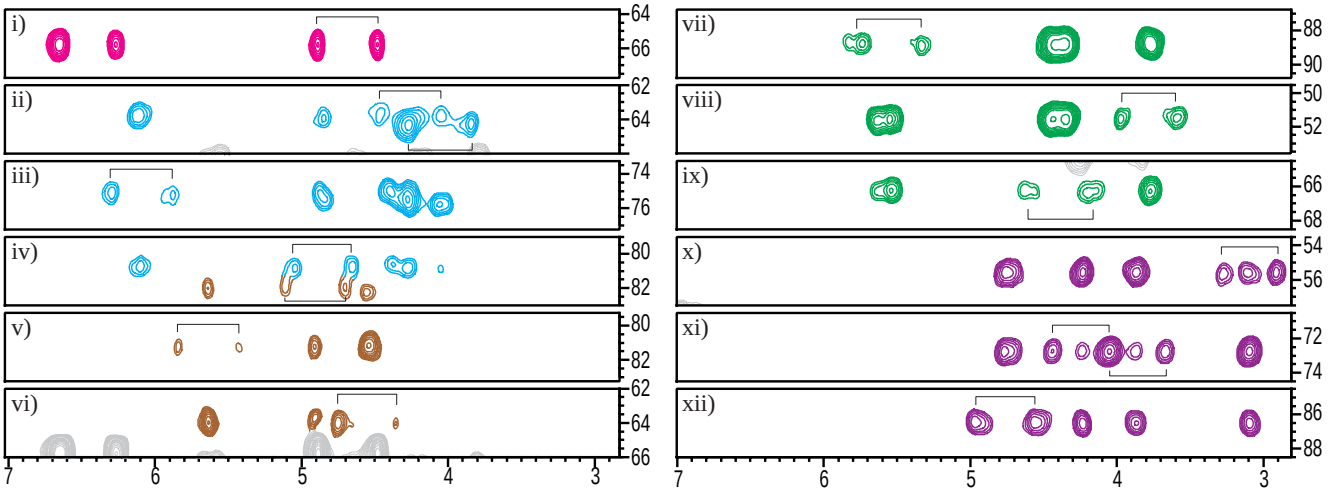
b)
2D
HMQC-TOCSY



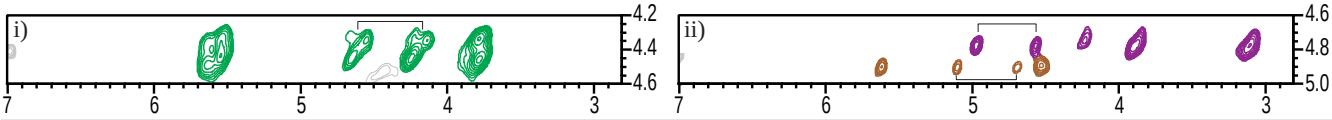
c)
from **3D**
F1-F3 slice #1



d) F1-F3 Slices



e) F2-F3 Slices



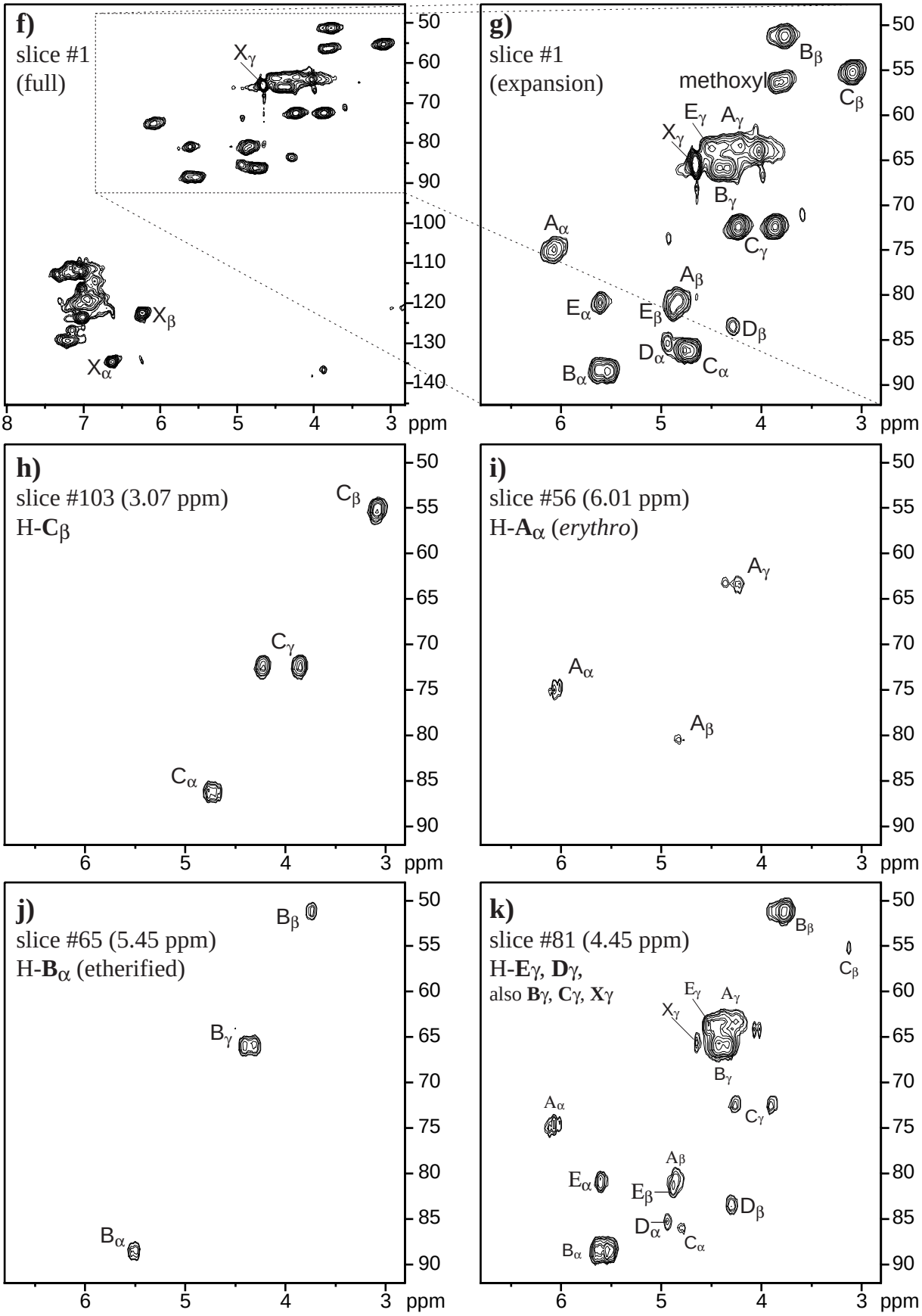

f)
slice #1
(full)
X_γ
X_β
X_α
g)
slice #1
(expansion)
B_β
methoxyl
C_β
E_γ A_γ
X_γ
B_γ
A_α
C_γ
A_β
E_α
E_β
D_β
D_α
C_α
B_α
h)
slice #103 (3.07 ppm)
H-$\mathbf{C}\beta$
C_β
C_γ
C_α
i)
slice #56 (6.01 ppm)
H-$\mathbf{A}\alpha$ (*erythro*)
A_γ
A_α
A_β
j)
slice #65 (5.45 ppm)
H-$\mathbf{B}_\alpha$ (etherified)
B_β
B_γ
B_α
k)
slice #81 (4.45 ppm)
H-**E**γ, **D**γ,
also **B**γ, **C**γ, **X**γ
B_β
C_β
E_γ A_γ
X_γ
B_γ
A_α
C_γ
A_β
E_α
E_β
D_β
D_α
C_α
B_α
ppm

*Figure. 1. a-e: 3D-HMQC-TOCSY experiment (100 ms TOCSY) on a synthetic DHP, at natural abundance. a) presentation of the 3D data-set, rather pretty but spectacularly useless until 2D projections or slices are made for viewing and plotting; b) a 2D HMQC-TOCSY (as seen previously in Fig. 8a) for comparison with the following; c) the first 2D slice in the F_1-F_3 plane is essentially the same as the 2D experiment in b) — differences with this particular experiment are the residual 1-bond ^{13}C–^1H coupling between the excited proton-carbon pair (see text for details); d) F_1-F_3 slices at various proton frequencies (in F_2) showing the beautiful resolution of the major units in HMQC-TOCSY-type sub-spectra; the selected proton corresponding to the slice (e.g. **X**g) is noted; e) two F_2-F_3 slices to show the TOCSY-type data available at selected carbon frequencies (in F_1); the selected carbon corresponding to the slice (e.g. **B**g) is noted. f–g: Gradient-edited 3D-TOCSY-HSQC experiment (100 ms TOCSY) on the same sample, F_3–F_2 slices, 3D plot not shown. f) the first 2D slice in the F_2-F_3 plane is essentially the same as a 2D HSQC experiment; g) expansion of the major sidechain region of slice 1; h) slice through δ_H = 3.07 (H_b of etherified **C** units) showing a pure HSQC spectrum of the sidechain of etherified structures **C**; i) slice through δ_H = 6.01 (H_a of erythro-**A** units) showing a pure HSQC spectrum of the sidechain of erythro-structures **A**; j) slice through δ_H = 5.45 (H_a of etherified **B** units) showing a pure HSQC spectrum of the sidechain of etherified structures **B**; k) slice through δ_H = 4.45 (H_g of structures **A**, **B**, **C**, **D**, **E**, and **X** all overlap) showing composite HSQC spectra of the sidechains of all of these structures.*

phenylcoumaran **B** structures, we see C–a (~88.5 ppm) correlating (in TOCSY fashion) with the H–a (~5.6 ppm, split by $^1J_{Ca–Ha}$), H–b (3.76 ppm), and H–gs (~4.4 ppm). Similarly, a 2D F_2F_3 plane is basically a 2D ^1H–^1H TOCSY spectrum at a given carbon chemical shift (defined by the distance along the carbon F_1 axis). Thus, e.g. Fig. 1e(i), at the plane through the C–gcarbon frequency of phenylcoumaran **B** structures, we see H–a (~5.6 ppm) correlating (in TOCSY fashion) with the H–b (3.76 ppm), and H–gs (~4.4 ppm, split by $^1J_{Cg-Hg}$). Although the profile looks similar, note that the vertical axis of the 2D sub-plot is ^{13}C for Figs. 1d and ^1H for Figs. 1e. The first plane in either dimension is, like in a 2D experiment, very much like a projection of all resonances onto that plane (but with lower signal-to-noise since it represents only a single plane). Therefore, Fig. 1c, the 2D F_1F_3 first plane, is similar to the 2D HSQC-TOCSY spectrum, Fig. 1b (seen previously in Fig. 8). [Unfortunately, we acquired one nasty artifact at about 70 ppm by having the acquired dimension end right on a signal – we were lucky that no other peaks of interest came at a carbon chemical shift of 70 ppm!] See the Web-version at http://www.dfrc.ars.usda.gov for a color version that is easier to interpret!

The 3D gradient-selected TOCSY-HSQC spectrum (3D plot not shown) is understood similarly. In this case, the acquired dimension is again F_3 (proton), but this time F_2 is carbon and F_1 is proton. F_2–F_3 planes are basically 2D ^{13}C–^1H HSQC spectra. The proton shift (on F_1) of the F_2–F_3 plane can be considered as the initially excited proton, which then transfers magnetization to all of the protons in its coupling network. For example, Fig. 1i, an F_1 proton shift of 6.01 corresponds to the a-sidechain proton of b-aryl ether units **A** (in fact just the *erythro*-isomers); that proton is in the same coupling network as the b- and the two g-protons on the same sidechain. During the subsequent HSQC step, each of those sidechain protons correlates with their respective attached carbons. The result in the ideal case is a clean HSQC spectrum of one single unit type, e.g. Figs 1h–j for structures **C**, **A**, and **B** respectively. Such clean spectra derived from individual units in a complex polymer like lignin are an obvious asset for identifying structures —the early lignin structure pioneers could certainly have benefited from such a powerful diagnostic tool. In a similar fashion to F_2–F_3 slices in the non-gradient HMQC-TOCSY experiment described above (Fig. 1e), The F_1–F_3 planes show TOCSY correlations for the proton attached to the carbon at the F_2–frequency of the plane with all of the other protons in the coupling network of that attached proton (not shown).

Conclusions

In the future we will see more and more applications where 3D experiments provide diagnostic information on new structures in lignins. The ability to trace out individual units in the complex polymer is a particularly attractive feature.

Inoculum Preparation Affects In Vitro Gas Production During Early Fermentation

D.R. Mertens

Introduction

Gas production during microbial fermentation may be a useful marker for estimating the rate and extent of digestion of feeds. Measuring gas production instead of the disappearance of substrates such as fiber or dry matter has several advantages. It measures the sum total of all fermentation and estimates the total energy contribution of the rumen to the cow from fiber, soluble carbohydrates and proteins. It is not contaminated by microbial residues like residual dry matter, which complicates the use of dry matter residues to estimated digestion kinetics. Gas production is much easier to measure than fiber disappearance because serial samples do not have to be analyzed individually.

Recent work indicated that the source of inoculum had a significant impact on in vitro results. Preliminary experiments suggested that the method for preparing the inoculum also affected results. Initally, inoculum was prepared by blending ruminal solids with chilled in vitro media to detach microbes that cling to particles. Strained ruminal fluid was combined with the strained blended media to produce the inoculum. However, this inoculum yielded relatively high gas production for blanks (in which no sample material is included), and cooling the inoculum probably slowed initial fermentation rates. The objectives of these studies were to determine if removing solids by centrifugation would reduce blank gas production and affect gas production kinetics, to determine if blending of ruminal solids (which increases blank gas production) improves the rate of gas production kinetics, and to determine if preparing inoculum with warm buffer affects early fermentation.

Materials and Methods

Transistorized pressure transducers were used with a computer data acquisition system to continuously monitor pressure accumulation in sealed serum bottles with a head space for gas of about 48 ml after media is added. Approximately .1100 gm of material was fermented. Bottles containing the sample, a small Teflon-coated stir bar, 8 ml of media (6 ml when blended ruminal inoculum was used) were warmed in a water bath at 39 °C. Each was individually purged with CO_2 before .3 ml of reducing solution was added and bottles were lightly stoppered. At the time of inoculation, bottles were moved from the water bath, injected with about 2 ml of ruminal fluid (or 4.1 ml of blended ruminal inoculum), and the inoculum weight was recorded. Each bottle was stoppered, sealed with an aluminum crimp, immediately connected to a transducer using a 20-gauge needle. Gas pressure was recorded every 0.1 hr for at least 3 hr. Sample materials included glucose, purified starch and citrus pectin, an alfalfa hay standard, and 20 alfalfa genotypes.

Rumen contents (both liquid and solids) were collected in the morning (AM) and afternoon (PM) on three days from two lactating cows that were fed a mixed ration containing alfalfa silage, corn silage, high moisture corn, soybean meal and minerals. Cows were fed ad libitum with feed offered once in the morning of each day. Within 10 min after collection, the top 3-5 cm of solids were discarded and the remaining contents were strained through 2 layers of cheesecloth. In experiment 1, 50 gm of strained solids and 100 ml of strained ruminal fluid from each cow were combined and blended for 60 sec. The ruminal fluid and blended material were each strained through four layers of cheesecloth to obtain strained ruminal fluid (SRF) and blended ruminal fluid (BRF) that was used as inocula. Both SRF and BRF were also centrifuged at 500g for 5 min to produce centrifuged SRF (CSRF) and BRF (CBRF). In experiment 2, 50 ml of strained ruminal solids from each cow were combined with 220 ml of previously warmed and reduced in vitro media and blended for 60 sec.

Blended material and 100 ml of strained ruminal fluid from each cow were strained through four layers of cheesecloth to obtain blended ruminal media (BRM).

Results and Discussion

In experiment 1, SRF obtained a larger volume of fermentative gas production on samples than BRF (Table 1). Although centrifuging reduced the amount of gas produced by the blanks for both CSRF and CBRF, it also resulted in lower gas production by samples. The lower gas production by the BRF compared to SRF was surprising because in previous studies BRF had provided greater extents of fiber digestion when measured at longer fermentation. The dramatic depression in gas production when ruminal fluids were centrifuged was also unexpected. The low centrifugal force that was used could not have sedimented bacteria unless they were attached to particles. It is suspected that the reduced activity of centrifuged inocula may be due to temperature decreases during centrifugation. Temperature of these inocula were about 33 °C at the time of inoculation. Temperatures of BRF were also less than 39 °C at the time of inoculation due to cooling of the ruminal fluid during blending.

In experiment 2, ruminal solids were blended with warmed in vitro media that had been purged with CO_2 and reduced. It was hypothesized that blending of solids with warmed media would detach additional ruminal microorganisms and improve initial rates of gas production. As shown in Table 1, BRM resulted in slightly higher blank gas production, but also significantly increased gas production of samples. In both experiments, inocula collected in the afternoon had higher blank gas production, but the gas production of samples was higher in the PM in experiment 1 and lower in the PM in experiment 2. There were differences among days, but the day effect could be removed by using a standard hay or purified pectin as a covariate. In experiment 2 we observed an effect due to location in the incubator that is unexplained, but could be accounted for statistically.

Summary and Conclusion

It appears that both the source and method of preparing inoculum can affect in vitro gas production during early fermentation. To maximize the rate of gas production and ensure that the method used in the in vitro system is not limiting digestion kinetics, ruminal solids should be blended with warmed and reduced in vitro media to extract additional microorganisms. It is recommended that ruminal contents be collected from at least 2 and preferably 4 cows fed different diets and that strained ruminal fluid from these cows be mixed with warmed, reduced media that has been blended with ruminal solids and strained.

Table 1. Volume of fermentative gasses produced by blanks and samples when the ruminal fluid is strained (SRF) or blended with solids (BRF) and then centrifuged (CSRF or CBRF, respectively) in experiment 1 or when using SRF or strained ruminal fluid plus ruminal solids blended with in vitro media (BRM) in experiment 2.

Fermentation time	ml of CO_2 equivalent produced							
	Blanks				Sample average			
Experiment 1	SRF	CSRF	BRF	CBRF	SRF	CSRF	BRF	CBRF
1 hr	4.04	2.14	4.40	3.28	1.76	0.95	1.23	0.84
2 hr	5.26	2.28	5.06	3.52	4.38	2.76	3.60	2.52
3 hr	6.08	2.38	5.82	3.68	7.46	5.30	6.29	5.02
Experiment 2	SRF		BRM		SRF		BRM	
1 hr	3.32		3.52		1.60		2.68	
2 hr	4.06		4.72		3.91		5.47	
3 hr	4.60		5.52		6.53		8.27	

Interactions Among Ruminal Cellulolytic Bacteria in Defined Cocultures Under Cellobiose Limitation

J. Chen and P.J. Weimer

Introduction

Cellulose is the major component of plant material and thus a major energy source for forage-fed ruminants. In the rumen, cellulose hydrolysis produces cellodextrins and cellobiose, which can be utilized by both cellulolytic and noncellulolytic ruminal bacteria. Individual species of cellulolytic bacteria differ in their fermentation endproducts and in their interaction with noncellulolytic species, suggesting that the cellulolytics have an effect on both the overall microbial ecology of the rumen, and on the endproducts of the ruminal fermentation. Previously, we have shown that the three predominant species of ruminal cellulolytic bacteria can establish stable tricultures when grown under limiting amounts of cellulose. The success of each species in the competition depends on several factors, including its ability to attach to cellulose; to effectively utilize low concentrations of hydrolytic products; and to produce products that inhibit the growth of competitors. During growth on soluble products of cellulose hydrolysis, the outcome of the competition may be expected to differ, because attachment to cellulose is no longer a factor in the competition. The purpose of this study was to examine the outcome of competition among the three predominant ruminal cellulolytic species in defined coculture with the soluble substrate cellobiose as growth substrate, and to determine how the competition is affected by the presence of noncellulolytic cellobiose-utilizing competitors.

Materials and Methods

Fibrobacter succinogenes S85, *Ruminococcus flavefaciens* FD-1 and *Ruminococcus albus* 7 were simultaneously inoculated in similar (~ 3 mL) quantities into a 139 mL reactor fed a modified Dehority medium that contained ~ 4 g of cellobiose/L. The reactor was continuously sparged with CO_2, and was fed at dilution rates in the range of 0.016 to 0.046 h^{-1}. In some of the experiments, the noncellulolytic bacteria *Selenomonas ruminantium* (either with strain D, or a mixture of strains D, GA192, HD4, and H18) or *Streptococcus bovis* JB-1 were included in the inoculum. After achievement of steady state (3-5 dilutions), samples (5 mL) of culture were aseptically removed and analyzed for pH, residual soluble sugars (by a phenol-sulfuric acid method), and fermentation endproducts (by HPLC). After concentrating the cells by centrifugation, RNA was extracted from the cells by a chloroform-phenol method, and the recovered RNA hybridized on nylon membranes to species-specific oligonucleotide probes to 16S ribosomal RNA. Hybridized RNA was quantitated by chemluminescence and densitometry. Because the cells in the chemostats at steady state had equivalent growth rates, it was assumed that cell mass corresponding to each species was proportional to the relative abundance of species-specific RNA in the sample. Owing to the unavailability of effective probes for *S. ruminantium* and *S. bovis*, estimates of relative population densities were determined only for the three cellulolytic species, and were expressed as a percentage of the total cellulolytic population. However, the production of lactate by *S. bovis*, or of propionate by *S. ruminantium*, were taken as indicators of their presence in the coculture, as none of the three cellulolytic strains used here produce these compounds in significant amounts.

Results and Discussion

The data on microbial populations are summarized in Table 1. For all of the defined mixed cultures, steady states were achieved in which the species distributions reached constant values. In the three tricultures of the cellulolytic species, *R. albus* was the dominant member of the culture, while *R. flavefaciens* FD-1 and *F. succinogenes* S85 were below the detection limit of the oligonucleotide probe method. Based on previous triculture studies in batch mode on cellobiose (Odenyo et al. 1994) and in cellulose-fed chemostats (Weimer and Chen 1998), it appears that *R. albus* 7 produced a compound that inhibits the growth of the normally more competitive *R. flavefaciens*. This was

confirmed by the demonstration that culture supernatants of *R. albus* 7 reduced the growth rate of *R. flavefaciens* FD-1 in liquid culture.

Inclusion of the succinate-decarboxylating, sugar-fermenting, noncellulolytic *Selenomonas ruminantium* resulted in a dramatic shift of the cellulolytic population away from *R. albus* and toward *R. flavefaciens*. These cultures demonstrated a nearly complete conversion of succinate to propionate, and microscopy revealed the abundance of *S. ruminantium*. Because succinate conversion by *S. ruminantium* does not yield energy necessary to support growth, this species grew in the coculture via effective competition for cellobiose. This reduced the density of *R. albus*, and the resulting decrease in the level of inhibitor enhanced the prominence of *R. flavefaciens*.

Inclusion of sugar-fermenting *Streptococcus bovis* in the cellulolytic triculture resulted in only a slight change in the cellulolytic population at D = 0.021 h^{-1} (Table 1). However, at D = 0.045h^{-1}, the cellulolytic population was shifted in a manner similar to that observed in the *Selenomonas*-amended cultures.

Conclusions

R. albus can effectively outcompete *R. flavefaciens* and *F. succinogenes* under conditions of cellobiose limitation, but this effect is attenuated by the presence of noncellulolytic cellobiose-utilizing bacteria. In these defined cocultures, *Selenomonas ruminantium* (a major ruminal propionate producer) enhanced *R. flavefaciens* (a major ruminal producer of the propionate precursor, succinate) at the expense of *R. albus*. This effect may counterbalance the suppression of *F. succinogenes* (which also produces succinate) observed previously in cellulose-limited cocultures (Weimer and Chen 1998).

References

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Weimer, P.J., and J. Chen. 1998. Interactions among ruminal cellulolytic bacteria in defined cocultures under cellulose limitation. U.S. Dairy Forage Res. Ctr. 1997. Res. Sum., USDA-Agricultural Research Service, Washington, DC., p.53-54.

Table 1. Quantitative distribution of cellulolytic species in cellobiose-limited tricultures of *Fibrobacter succinogenes* S85, *Ruminococcus albus* 7, and *R. flavefaciens* FD-1under steady state conditions in the presence or absence of the noncellulolytic bacteria *Selenomonas ruminantium* or *Streptococcus bovis*.

Culture	D (h^{-1})[a]	pH	Percentage of the cellulolytic population[b]		
			F.succinogenes	*R.albus*	*R.flavefaciens*
F.s. + *R.a.* + *R.f.*	0.016	6.36	< 2.1 AB	> 97.1 G	< 0.8 A
	0.026	6.67	< 2.1 AB	> 97.1 G	< 0.8 A
	0.046	6.63	< 2.1 AB	> 97.1 G	< 0.8 A
F.s. + *R.a.* + *R.f.* + *S. ruminantium* D	0.021	6.63	< 2.1 AB	3.3 BC	94.6 F
F.s. + *R.a.* + *R.f.* + four strains of *S. ruminantium*[c]	0.034	6.68	< 2.1 AB	5.8 D	92.2 E
F.s. + *R.a.* + *R.f.* + *S. bovis* JB1	0.021	6.49	< 1.9 AB	3.4 BC	94.7 F
	0.045	6.71	< 1.9 AB	93.6 EF	4.5 CD

[a]Dilution rate in reciprocal hours
[b]Values with different capital letters in column or row differ ($p < 0.05$)
[c]*Selenomonas ruminantium* strains D, GA192, HD4, and H18

Comparative Growth of the Ruminal Bacteria *Fibrobacter succinogenes* and *Ruminococcus flavefaciens* on Cellulose and Cellobiose: Analyzing the Cost and Benefit of a Cellulolytic Mode of Growth

P.J. Weimer

Introduction

Cellulose is the primary component of plant cell walls and thus serves as a major feed component in forage-based dairy rations. In the rumen, a specialized group of bacteria converts cellulose to acetic acid and succinic acid (a precursor to propionic acid) used by ruminants for energy and milk production. Growth of the bacteria during the fermentation also results in conversion of ammonia to microbial cell protein, which serves as a protein source for the ruminant. While quantitative information of growth of ruminal cellulolytic bacteria is important for modeling the ruminal fermentation, there is little information available on how microbial growth yield may differ when these bacteria are growing on cellulose versus on soluble sugars that can also serve as growth substrates. Growth on cellulose may require additional expenditures of cellular energy and materials to synthesize cellulolytic enzymes and a glycocalyx that facilitates attachment of cells to cellulose. On the other hand, growth on cellulose can provide energetic advantage through more efficient activation of hydrolytic products via the intracellular enzyme cellodextrin phosphorylase (van Walsum and Lynd 1997). The purpose of this study was to compare the growth yields of two prominent ruminal cellulolytic bacteria on cellulose and cellobiose. By comparing the true catabolic growth yields and maintenance coefficients obtained during growth in media differing only in energy source (cellulose vs. cellobiose), the relative cost or benefit of a cellulolytic mode of growth can be determined.

Materials and Methods

Pure cultures of *Fibrobacter succinogenes* S85 and *Ruminococcus flavefaciens* FD-1 were grown in cellobiose-limited continuous culture under a CO_2 gas phase. The bioreactors (working volume 139 mL or 150 mL) contained a modified Dehority medium supplemented with 25 mL of clarified ruminal fluid and 4 g of cellobiose per liter. Experiments were conducted at seven different dilution rates (range 0.016 to 0.101 h^{-1}). Cultures were sampled 4 to 7 times over a 3 d period after reaching steady state ($\geq$ 4 dilutions). Culture samples (5 mL) were analyzed for residual sugars via a phenol-sulfuric acid assay, for soluble fermentation products (acetate, succinate and formate) by HPLC, and for total particulate N (a measure of cells and cell-associated enzymes and glycocalyx) by combustion analysis. The data obtained from these cellobiose-limited cultures was compared to data obtained for growth on the same medium with cellulose instead of cellobiose (Shi and Weimer 1992, Weimer 1993). Comparisons were made on a N basis to avoid potential confounding by differences in the amounts of intracellular storage polysaccharides.

Results

Growth on cellobiose of both *F. succinogenes* S85 and *R. flavefaciens* FD-1 displayed linear Pirt plots (inverse of observed yield vs. inverse of dilution rate). Comparison of the growth data on cellobiose versus that obtained on cellulose (Table 1) revealed that, for both cultures, the true catabolic yield (Y_G) and the maintenance coefficient (m) were higher on cellobiose than on cellulose.

The parameters Y_G and m were used to solve the Pirt equation at different bacterial growth rates

$$1/Y = 1/Y_G + m/\mu,$$

where Y = observed growth yield and μ = growth rate (equivalent to dilution rate in chemostat culture). The data reveal that these strains display a higher growth yield on cellulose than on cellobiose only at very low growth rates (0.017 h^{-1} for *F. succinogenes* S85, and 0.040 h^{-1} for *R. flavefaciens* FD-1).

Cultures of both strains contained only low levels of extracellular protein, and low activities of extracellular Avicelase and CMCase enzymes. Analysis of fermentation products revealed that the molar yield of

acetate and succinate (the two carbohydrate-derived organic fermentation products of these strains) was considerably lower for cellobiose-grown cultures (Table 1). This value is expected to be 2.0 in the absence of cell growth and anabolism, and in the range of 1.6-1.7 at the growth yields observed in these experiments. It is likely that synthesis and degradation of storage polysaccharides, known to occur extensively in both strains, proceeds to a greater extent during growth on cellobiose, even low at growth rates equivalent to those on cellulose. Calculations based on recovery of carbon in cells, acetate, and succinate reveal that the amount of substrate partitioned into the synthesis and degradation of storage polysaccharides during growth on cellobiose and on cellulose, respectively, were 23 and 13 per cent for *F. succinogenes*, and 27 and 16 per cent for *R. flavefaciens*. Unproductive cycling of substrate through storage polysaccharides may account for the larger maintenance coefficients for these strains on cellobiose.

Conclusions

Under most growth conditions, the cellulolytic mode of growth generally reduces the growth yield of two prominent species of ruminal bacteria. Utilization of cellulose, while less efficient for microbial growth, apparently represents an adaptation to a specialized lifestyle in response to the availability of cellulose, an abundant growth substrate utilizable by few other bacterial species.

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Table 1. Growth parameters for *Fibrobacter succinogenes* S85 and *Ruminococcus flavefaciens* FD-1, determined in continuous cultures limited by cellobiose or cellulose.

Strain	Substrate	Y_G (g N/ g AHG)	m (g AHG/ g N/ h)	Mean molar Yield A+S
F. succinogenes S85	Cellobiose	0.041	0.73	1.19
	Cellulose	0.026	0.46	1.35
R. flavefaciens FD-1	Cellobiose	0.085	2.10	1.14
	Cellulose	0.029	1.04	1.45

AHG = anhydroglucose, A = acetate, S = succinate

Grain-Feeding and the Dissemination of Acid-Resistant *Escherichia coli* From Cattle

F. Diez-Gonzalez, T.R. Callaway, M.G. Kizoulis and J.B. Russell

Introduction

Foods can be cooked or irradiated to kill bacteria, but there are approximately 30 million food-borne illnesses each year in the United States. *Escherichia coli* is a normal inhabitant of the gastrointestinal tract, some strains (e.g. O157:H7) produce toxins and are pathogenic. Hamburger has been frequently contaminated with pathogenic *E. coli*, and cattle are a natural reservoir.

The ability of bacteria to act as a food-borne pathogen depends on their capacity to survive the low pH of the gastric stomach and colonize the intestinal tract of man. Pathogenic and non-pathogenic *E. coli* cultures only develop extreme acid resistance when they are grown at mildly acidic pH. If *E. coli* is grown at neutral pH, it is acid-sensitive and killed by the low pH of gastric juice.

Since World War II, fattening beef cattle in the United States have been fed large amounts of grain (starch) and very little hay, but the impact of grain feeding on acid-resistant *E. coli* had not been examined. Many forms of starch pass through the pregastric stomach (rumen) to the intestines, and cattle are deficient in the starch-degrading enzyme, amylase. Starch can be fermented in the colon, and starch fermentation in the colon produces volatile fatty acids that decrease pH .

Materials and Methods

Colonic digesta from cattle fed hay, grass and varying amounts of rolled corn were diluted 10-fold with sterile anaerobic water, and the pH was measured. Coliforms were enumerated by serial dilution in lauryl sulfate. *E. coli* was determined by lactose fermentation, gas production, indole production, the methyl red reaction, Voges-Proskauer test and citrate fermentation. Acid shock was performed by diluting digesta samples 100-fold into Luria broth that had been adjusted to pH 2.0. After 1.0 h at pH 2.0, viable cell numbers were determined by serially diluting into lauryl sulfate broth.

Cattle were fed medium quality timothy hay (14% crude protein, 40% neutral detergent fiber) and a grain mixture (89% rolled (cracked) corn and 11% soybean meal) every 2 h (10 kg dry matter/d). The diets were 0, 45 and 90% grain with the remainder being hay. Samples obtained from the rumen as well as the colon were centrifuged, and fermentation acids were analyzed by high-pressure liquid chromatography. Total count of anaerobic bacteria was determined by serially diluting in a nonselective medium. *E. coli* strains were obtained from sorbitol MacConkey's plates.

Results and Discussion

A survey of 61 cattle indicated that grain could increase total and acid-resistant *E. coli* numbers. Cattle fed either hay or pasture had a colonic pH > than 7.0, the total *E. coli* count was only 20,000 cells per g, and virtually all of these bacteria were killed by acid shock. Moderate amounts of grain (60%) did not cause a decrease in pH , but the total *E. coli* were 6,300,000 per g. Some of the *E. coli* were killed by acid shock, but acid-resistant were > 25,000 per g. When animals were fed > 80% grain, the pH was lower, and acid-resistant *E. coli* were 250,000 per g.

When cattle were fed increasing amounts of grain, the volatile fatty acids in the rumen did not increase significantly, but the concentration in the colon increased approximately 4-fold. Under these conditions, ruminal pH remained essentially constant, but the pH of the colon decreased when the volatile fatty acids accumulated. Grain had little impact on the numbers of anaerobic bacteria in the rumen, but the in colonic count increased 1000-fold. Cattle fed hay had less than 10^5 colonic coliforms, but those fed 90% grain had approximately 10^8 coliforms per g digesta. Only a small fraction of the ruminal coliforms were *E. coli*, but virtually all of the colonic coliforms were identified as *E. coli*. Cattle fed hay had a low concentration of volatile fatty acids in their colons, and acid shock killed more than 99.99% of the *E. coli*. When diets were supplemented with either 45 or 90% grain, acids accumulated, colonic pH declined, and a much larger percentage of the *E. coli* survived acid shock.

The idea that grain, by promoting acid production in the colon, was regulating acid resistance in vivo was corroborated by in vitro experiments. When *E. coli* strains isolated from cattle were grown in the laboratory with a high concentration of glucose, acetic acid accumulated in the medium, pH declined, and the cell survival after acid shock was high. If the glucose concentration of the medium was low, little acid was produced, and cell survival was extremely low. Cattle adapted to a 90% grain diet had an acid resistant *E. coli* count $>10^6$ viable cells per g digesta. Upon change to a hay diet, the viable cell number immediately declined, and after 5 days acid resistant *E. coli* were nearly 10^6 lower.

Strains isolated from cattle fed forage or grain, and *E. coli* O157:H7 behaved similarly, and this result indicated that grain-feeding was inducing acid resistance rather than selecting a different population of *E. coli*. None of our *E. coli* isolates (n = 155) tested positive for O157:H7 antigens. The absence of *E. coli* O157:H7 in our cattle is not surprising. The percentage of O157:H7 positive animals in herds directly linked to outbreaks was less than 2%.

Conclusions

The finding that grain-feeding increased the number and acid resistance of *E. coli* in cattle could have implications for food safety. Not all *E. coli* are pathogenic, but at least some cattle will harbor pathogenic strains. Because acid resistance is a factor in the dissemination of *E. coli* from cattle to humans, it is reasonable to suggest that the induction of acid resistance could increase the risk of foodborne illness. Our studies indicated that the time needed to decrease *E. coli* numbers was relatively short. Grain-feeding is a practice that promotes cattle production, and it is unlikely that cattle will ever be fed diets consisting only of hay. However, our studies indicate that cattle could be given hay for a brief period immediately before slaughter to reduce the risk of food borne *E. coli* infection.

The Importance of pH in the Regulation of Ruminal Acetate to Propionate Ratio and Methane Production In Vitro

J.B. Russell

Introduction

Ruminal fermentation end products are dependent on diet, and acetate to propionate ratio is generally lower for cereal grains than for forages. When the acetate to propionate ratio decreases, there is a decline in CH_4 production, and energy retention by the cattle increases. Blaxter noted that the fraction of dietary energy that is converted to CH_4 declined significantly when the feed intake of the cattle increased, but the cause of this effect was not explained.

Cereal grain fermentation often causes a decrease in ruminal pH, and this effect is most dramatic when feed intake is high. Recent work has indicated that ruminal methanogens are sensitive to even modest decreases in pH. The question then arises, are decreases in acetate to propionate ratio that are dependent on cereal grain a product of low ruminal pH and the inability of methanogens to use reducing equivalents?

Materials and Methods

Ruminally fistulated cows were fed diets containing either 100% forage or 90% cereal grain and 10% forage (10 kg DM per day). Ruminal contents (1 L total) were squeezed through cheesecloth, and the pH was determined immediately. Concentrated ruminal bacteria were suspended in a medium containing salts, tricarballylate, branched chain volatile fatty acids, cysteine and transferred to tubes that contained 100 mg of hay or 50 mg of cracked corn and incubated for 48 h at 39°C. CH_4 and H_2 were measured with a gas chromatograph. Fermentation acids were analyzed with a high pressure liquid chromatograph.

Results

Cows that were fed 90% concentrate had lower ruminal pH values (6.22 vs. 6.86), higher VFA concentrations (85 vs. 68 mM), and lower acetate to propionate ratios (2.24 vs. 4.12) than did cows that were fed forage only. When mixed ruminal bacteria from cows that were fed 90% concentrate or 100% forage were incubated (48 h) with hay (10 g/L) or cracked corn (5 g/L) in a medium containing bicarbonate (38 mM) and tricarballylate (50 mM), final pH values were less than 0.3 units lower than the initial pH. At final pH values less than 5.7, hay fermentation was inhibited, the acetate to propionate ratio and CH_4 production declined more than two-fold, and the inoculum source was without effect. Small amounts of H_2 were detected at pH values less than 5.5. Total VFA production from cracked corn decreased when pH declined, but only if the inoculum was obtained from cows that were fed 90% concentrate. The acetate to propionate ratio of cracked corn incubations declined from 1.2 to 0.6 when final pH was decreased from 6.5 to 5.3, and CH_4, as a percentage of total VFA production, also decreased. At pH values less than 5.3, the acetate to propionate ratio of cracked corn increased more than four-fold, and large amounts of H_2 could be detected. Over the final pH range of 6.5 to 5.3, CH_4 production was highly correlated with acetate to propionate ratio, which was dependent on pH and substrate (CH_4 = 0.02 + 0.05 pH, r^2 = 0.80). Calculations based on the differences between pH 6.5 and 5.8 indicated that as much as 25% of the decrease in acetate to propionate ratio could be explained by the effect of pH alone.

Discussion

Inoculum source had no effect on total VFA from hay, and this result supports the assumption that ruminal cellulolytic bacteria cannot easily adapt to low pH. Our cracked corn fermentations were less sensitive to low pH than were hay fermentations, and the inocula differed in their pH sensitivity. Bacteria from cows that were fed 90% concentrate produced nearly as much VFA from corn at a final pH 5.2 as they did a pH of 6.5, but the bacteria from cows that were fed forage were strongly inhibited at pH values less than 5.5. This result supports the idea that some, but not all, starch-fermenting bacteria can adapt to low pH.

Low pH caused a marked decrease in the acetate to propionate ratio, and this decline was mirrored by a

reduction in CH_4 production. This result is consistent with the idea that propionate production and methanogenesis are competing and alternative mechanisms of reducing equivalent disposal, but this result alone provides little insight on the regulation. Was the increase in propionate driving the decrease in CH_4 or vice versa?

The pH had only a small impact on the acetate to propionate ratio and CH_4 production of cracked corn fermentations until the final pH was less than 5.5, and H_2 was not detected until the final pH was less than 5.5. Based on these results, it appears that many starch-fermenting bacteria prefer to produce propionate, and H_2 would not be available for methanogenesis.

Changes in the acetate to propionate ratio and CH_4 production were dependent on substrate and pH, but this simple correlation did not indicate which variable was more important (substrate or pH). By calculating the production of acetate and propionate from each feedstuff at pH 6.5 and 5.7, it was possible to develop a model to compare the effects of pH and substrate. When hay was replaced in stepwise fashion with corn, the acetate to propionate ratio always increased even if pH did not change, and this result indicated that substrate had a major impact on the fermentation end products .

Conclusions

The effect of pH was more subtle than substrate, but as much as 25% of the change in the acetate to propionate ratio could be explained by the change in pH. The relative effect of pH declined at high ratios of hay to corn, but cows that were fed hay do not usually have a low ruminal pH.

The Relationship Between Intracellular Phosphate, Protonmotive Force, and the Energy Spilling Rate of *Streptococcus bovis* JB1

D.R. Bond and J.B. Russell

Introduction

The growth efficiency (yield) of bacteria is generally much lower than the amount that would be predicted from growth and maintenance, and previous work indicated that the ruminal bacterium, *Streptococcus bovis*, was able to spill large amounts of energy via a futile cycle of protons through the cell membrane. The energy spilling of ruminal *S. bovis* was greatest when amino nitrogen was not available, but the regulation was not understood. Because mixed ruminal bacteria behave in a similar fashion, we decided to study the energy spilling of *S. bovis* in greater detail.

Materials and Methods

S. bovis JB1 was grown anaerobially on glucose in batch and continuous cultures in a medium that had ammonia or ammonia plus Trypticase as a nitrogen source. Cell suspensions were centrifuged through silicone oil, treated with perchloric acid, and assayed spectrophotometrically for fructose-1,6-diphosphate (FDP). Intracellular phosphate was assayed via an assay employing ammonium heptamolybdate, malachite green, and Sterox color reagent. The pH gradient across the cell membrane (ΔpH) and the electrical potential ($\Delta \Psi$) were determined by methods employing silicon oil centrifugation, the distribution of 3H-tetraphenylphosphonium bromide (3H-TPP^+) and ^{14}C-benzoate across the cell membrane, and the Nernst equation ($-2.3\ RT/\mathcal{F} \times \log$ [concentration in]/[concentration out]). ATP was assayed with a luminometer using luciferin-luciferase. Fermentation acids were analyzed by high-pressure liquid chromatography. Glucose was determined via hexokinase and glucose-6-phosphate dehydrogenase. Protein was determined by the Lowry method.

Results

When the rate of glucose addition to non-growing *S. bovis* cell suspensions was increased, the fermentation was homolactic, fructose-1,6-diphosphate (FDP) increased, intracellular phosphate (P_i) declined and the energy spilling rate increased. ATP and ADP were not significantly affected by glucose consumption rate, but the decrease in P_i was sufficient to cause an increase in the free energy of ATP hydrolysis ($\Delta G'p$). The increase in $\Delta G'p$ was correlated with an increase in protonmotive force (Δp). *S. bovis* continuous cultures (dilution rate of 0.65 h^{-1}) that were provided with ammonia as the sole nitrogen source also had high rates of lactate production and energy spilling. When Trypticase was added as a source of amino acids, lactate production decreased, a greater fraction of the glucose was converted to acetate, formate and ethanol, and the energy spilling rate decreased. Trypticase also caused a decrease in FDP, an increase in P_i and a decrease in Δp. The change in Δp could be explained by P_i-dependent changes in the free energy of ATP hydrolysis ($\Delta G'p$). When P_i declined, $\Delta G'p$ and Δp increased. The ratio of $\Delta G'p$ to Δp (mV/mV) was always greater (> 4) at low rates of energy spilling, but declined when the energy spilling rate increased. Based on these results, it appears that Δp and the energy spilling rate are responsive to fluctuations in the intracellular phosphate concentration.

Discussion

Previous work indicated that amino acid limitation (due to growth on ammonia nitrogen) increased the energy spilling rate of *S. bovis* energy-excess batch cultures, and the present experiments indicated that amino nitrogen was also able to regulate the energy spilling rate of energy-limited continuous cultures. Other workers reported that bacteria growing in rich media had lower Δp values than bacteria growing in minimal media, but a relationship between Δp and energetic efficiency was not considered. When *S. bovis* continuous cultures were supplemented with a source of amino acids (Trypticase), Δp and energy spilling both declined.

The energy spilling rates of growing and non-growing *S. bovis* cells could be correlated with a decline in FDP and increase in intracellular P_i. When P_i increased, both the $\Delta G'$ of ATP hydrolysis and the Δp declined. Creation of the Δp is driven by the $\Delta G'$ of ATP hydrolysis, and some researchers have assumed that Δp is in equilibrium with $\Delta G'p$. However, the cell

membrane is not a perfect insulator. If proton flux into the cell is rapid (e.g. high rates of energy spilling), Dp should be less than the amount predicted by DG'p. When *S. bovis* was spilling energy at a slow rate, the ratio of DG'p to Dp was greater than 4, but this ratio declined to 3.3 when the energy spilling rate was high. Other workers have noted a similar variation. The DG'p to Dp of *Lactococcus lactis* ranged from 3 to 4.3, and the DG'p to Dp of *Lactococcus cremoris* ranged from 4.5 to 2.

Previous work indicated that the energy spilling reaction of *S. bovis* required a decrease in membrane resistance and an increase in proton conductance. Because the non-growth energy dissipation rate was as high as 70 mmol ATP/g protein/h and the H+/ATP stoichiometry of the F_1F_0 ATPase can be high as 4, the proton permeability of *S. bovis* could be as high as 280 mmol H^+/g protein/h. The passive proton permeability of *L. lactis* is approximately 1.6 µS/cm^2 (approximately 1.5 mmol H^+/g protein/h at a Dp of -120 mV). Mammalian mitochondria have ion channels that can increase non-growth energy dissipation, but flux through these channels decreases Dp. *S. bovis* cells had higher (not lower) Dp when rates of energy spilling were high. The bacterial protein, colicin E1, is a Dp-dependent (voltage-gated) ion channel, with a threshold of approximately 80 mV. Further work is needed to see if *S. bovis* uses a similar mechanism to regulate membrane resistance and energy spilling rate.

Conclusions

The energy spilling rate of *S. bovis* is regulated by an elegant cascade of metabolic changes that ultimately change the resistance of the membrane to protons. This regulation can be triggered by a deficiency of amino nitrogen.

Effect of Method Variation on the Determination of aNDF Using the ANKOM Filter Bag System

D.R. Mertens

Introduction

The ANKOM system is a semiautomatic method for measuring fiber that allows 24 samples to be analyzed simultaneously. Samples are sealed in filter bags that are extracted in a pressurized chamber with vertical agitation. Pressure is achieved by heating reagent solutions in the extraction chamber after it is sealed. This pressure prevents the filter bags from ballooning which helps to ensure that reagent solutions pass through the filter bags during extraction. The ANKOM system has the advantage that filtering difficulties associated with the use of crucibles are eliminated, the number of samples that can be analyzed daily is increased, and imprecision due to variation among technicians in analytical technique may be reduced because the system is semiautomatic. However, the effects of variation in the procedure used with the ANKOM system have not been evaluated or compared with the traditional crucible reflux method. The objective of this research was to determine which steps in the ANKOM procedure have a significant impact on the determination of amylase-treated neutral detergent fiber (aNDF).

Materials and Methods

Three experiments were conducted to identify critical steps and evaluate the effects of small differences in the recommended procedure (ruggedness) on the analysis of aNDF using the ANKOM filter bag system. Each experiment was an incomplete factorial design. In experiment 1, alfalfa silage, corn silage, red clover hay, barley hay, corn stover, alfalfa pellets, citrus pulp, wheat midds, corn grain, roasted soybeans, and expeller soybean meal were analyzed in duplicate within batches. Duplicate samples were separated to be on either the top and bottom trays in each batch and a specific set of seven differences in method were evaluated in one of 8 batches:

(**A**) mixing sodium sulfite and amylase in neutral detergent (ND) solution before adding it to the extraction chamber vs. (**a**) adding ND solution, sulfite and amylase to the chamber sequentially without mixing,

(**B**) using 1800 ml of ND vs. (**b**) using 2100 ml of ND,

(**C**) start with extraction chamber at room temperature or cooled with cold tap water vs. (**c**) start with extractor warm after a previous extraction,

(**D**) mixing amylase with hot wash water before adding to the chamber vs. (**d**) adding hot water and amylase sequentially,

(**E**) using boiling water and heating chamber with lid sealed vs. (**e**) using hot water (80-90 ºC) with no heating,

(**F**) soaking with water for 5 min each time (first 2 with amylase followed by 2 without) vs. (**f**) soaking for 3 min (first 2 with amylase followed by 1 without), and

(**G**) after ND extraction, soaking with 240 mls of acetone for 5 min with swirling at 0, 2, and 4 min. vs. (**g**) soak in minimum amount of acetone (about 200 mls) for 3 min. without swirling.

In experiment 2, the effects of mixing sodium sulfite and amylase (treatments A vs. a and D vs. d) were investigated using a washing method that maximized pressure in the chamber during the water soakings:

(**H**) using 2100 mls of hot water (80-90 ºC) and heating the chamber with the lid sealed vs. (**h**) using boiling water with no heat.

In this experiment, 2000 mls of ND was used and other factors were held constant using treatments C, F, and G. In addition to the materials used in experiment 1 (except citrus pulp, roasted soybeans, and expeller soybean meal), single samples of brewer's grains, grass silage, hominy feed, distiller's grains, wheat

straw, meat meal, oat grain, SoyPlus, bermudagrass hay, birdsfoot trefoil hay, corn gluten feed, sunflower meal, and high moisture ear corn were evaluated in each batch and the tray location of samples within the extractor was varied among batches. Materials were selected to have a greater proportion of feeds that were heated or contained starch which should be most sensitive to sulfite and amylase mixing.

In experiment 3, factors A, C, D, F, and G were held constant and deviations in soaking (treatments H vs. h) were evaluated with variations in pre-extraction methods:

- (**I**) pre-extract all samples twice in 240 mls of acetone that was shaken 10 times then soaked for 10 min vs. (**i**) pre-extract all samples once in 240 mls of acetone for 10 min without shaking or swirling, and
- (**J**) increase last soaking of treatments I and i to 6 hr vs. (**j**) no 6 hr soaking of treatments I and i.

Materials were the same as in experiment 2 except that alfalfa pellets, wheat midds, hominy feed, meat meal, and high moisture corn were replaced with raw soybeans, a feed mixture containing fat, roasted soybeans, Puma cottonseed, and rice mill feed (containing fat) to increase the number of materials that contained fats.

In all three experiments, statistical analysis was done on results expressed as deviations from the traditional crucible reflux method for each material. Least square means for each sample-treatment combination were tested with all other treatments adjusted to their mean response.

Results and Discussion

In experiment 1, the effect of post-extraction with acetone (G vs. g) was highly significant overall primarily due to its effect on roasted soybean material (G = +2.10 vs. g = +13.29). The high deviation and large variation in aNDF caused by this treatment on roasted soybeans that were not pre-extracted, tended to mask all other treatments; therefore, the roasted soybean material was removed from the data set for the remaining statistical analyses. Deviations in aNDF from the crucible method was different for soaking time (F = +0.58 vs. f = +0.85) and location in the extraction chamber (bottom half = +0.62 vs. top half = +0.82). The ANKOM system contains 8 vertical trays that each hold 3 bags. In experiment 1, there was a linear effect due to tray with a change in aNDF of -0.063 per tray from top to bottom. The effect of soaking time (F vs. f) was due primarily to significant differences for alfalfa pellets, alfalfa silage, and red clover hay (average F = +0.25 vs. f = +0.98 for these legume materials). Although mixing amylase and sulfite with ND (A vs. a) was not significant over all materials, this treatment was significant for two starch containing feeds, corn silage and wheat midds (average A = -0.01 vs. a = +0. 70). When both crucible and ANKOM aNDF were adjusted for blanks, mixing amylase in the soaking water before adding it to the chamber (D vs. d) was significant (D = +0.08 vs. d = +0.32).

In experiment 2, the overall deviations in aNDF were different when water was heated during the soaking procedure (H = +0.64 vs. h = +0.98) and there was a significant linear effect due to tray location from top to bottom (-.090/tray). Heating affected deviations in aNDF for corn stover and sunflower meal (average H = -.06 vs. h = +1.45). Although aNDF measured by the ANKOM method had large deviations from the crucible method for distiller's grains and meat meal, heating the water during soaking reduced the magnitude of the discrepancy (average H = +3.48 vs. h = +4.48). The lack of an overall effect of mixing sulfite and amylase with ND may have been related to an unexplained significant inverse effect for distiller's grain, meat meal, and sunflower meal (average A = +3.57 vs. a = +2.27) compared to all other materials. Mixing of amylase with soaking water was not significant overall or for any single material.

In experiment 3, shaking of materials in acetone during pre-extraction significantly lowered the deviations from aNDF measured by the crucible reflux method (I = -0.41 vs. i = +0.27) apparently due to the loss of particles from many materials, especially those that were ground finely. Of the materials containing fat, shaking improved the measurement of aNDF only for Puma cottonseed (I = +1.32 vs. i = +2.86). Soaking materials in acetone for 6 hr did not improve the

measurement of aNDF in Puma cottonseed (J = +2.78 vs. j = +1.27), but it lowered the deviation in aNDF from the crucible method for brewer's grains, one of the roasted soybeans and wheat straw (J = -0.64 vs. j = +0.76). Heating the water during soaking significantly lowered the deviations in aNDF when corrected for blanks (H = -0.46 vs. h = -.14) for all samples, and also for one of the roasted soybeans and sunflower meal (average H = -1.23 vs. h = +.06). The negative overall average deviation for treatment H was due to losses of fiber on many samples during pre-extraction. There was linear effect due to tray location (-.085/tray) from top to bottom.

Summary and Conclusion

It appears that small differences in volume of ND (B vs. b) or starting temperature of the extraction chamber (C vs. c) had little effect on aNDF analyses using the ANKOM system. Treatment E may have been ineffective because using boiling water and heating it in a sealed chamber did not create enough pressure to collapse the filter bags and aid the washing of fiber residues. The lack of significant difference for most treatments and the small magnitude of the difference (about 0.3%-unit of aNDF) when they were statistically significant indicates that the ANKOM system is relatively rugged for the average material. To be valid, however, an analytical method must obtain accurate results for each type or sample of material. Averaging results across all samples can mask the need for a particular procedural treatment for a specific sample or type of material. Significant differences for procedural treatments for individual samples or materials were often greater than 0.7%-units. The procedural treatments (and the material affected by it) that are recommended for the ANKOM system include: A (starchy materials), D (all materials), F (all materials, especially legumes), G (fatty materials that are not pre-extracted), and H (all materials, especially corn stover, sunflower meal, roasted soybeans, distiller's grains, and meat meal). Treatment I improved aNDF analysis for only one of the fat-containing materials, but resulted in a significant loss of fiber when used for all materials and cannot be recommended. Treatment J did not improve aNDF analysis for fatty materials and caused several feeds to have deviations from the crucible reflux method that went from significantly positive to significantly negative and cannot be recommended.

Dietary Characteristics Affecting Ruminal Acidosis

D. Sauvant* and D.R. Mertens
*Departement des Sciences Animales, Institut National Agronomique Paris-Grignon and Institut National de la Recherche Agronomique

Introduction

Ruminal acidosis is a concern in modern nutrition of ruminants because increased production is typically associated with increased proportions of concentrates in diets. The decreased chewing activity and salivary buffer secretion when ground, low fiber concentrates are consumed, coupled with the rapid digestion rate of concentrate carbohydrates can result in dramatically decreased ruminal pH. Ruminal acidosis can lead to several production disorders such as negative digestive interactions, reduced milk fat content, and digestive and metabolic pathologies. Mild acidosis occurs when ruminal pH is between 5.5 and 6.25 and severe acidosis occurs when pH is less than 5.5. When ruminal pH is below 6.0, fiber digestion is decreased, the ratio of acetate to propionate is shifted to values below 2.0; and milk fat production is often depressed. Moderate to severe acidosis is often associated with foot problems and metabolic disorders that affect the long term health of lactating females.

Our objective was to systematically study published data on ruminal pH with the purpose of developing quantitative relationships between diet characteristics and ruminal pH. Both acid-producing as well as the buffering or neutralizing effects of the diet were investigated to identify ways of estimating the risks associated with ruminal acidosis which could be used to create new or improved systems for formulating dairy rations.

Materials and Methods

A database of digestion information was compiled from 223 publications that reported results for 945 combinations of animal and treatment. Because of differences among publications in methods and experimental approaches, unique codes were assigned each experiment data set and used as a classification variable in statistical analyses. Two statistical approaches were used for meta analyses. First, an overall or global relationship (**g**) was determined without removing the variation among experiments. Second, the global relationship was adjusted for among-experiment differences by including the experiment class variable in the regression model to obtain a pooled within-experiment relationship (**p**). The weighted average intercept is reported for pooled regression models. In each statistical analysis, the number of combinations of animals and treatments (n), number of experimental classes (nexp), correlation coefficient (r), and standard error of regression (SE) were reported.

Results and Discussion

For most published research, the criteria for measuring ruminal acidosis was mean ruminal pH that was averaged over 24 h or less. Because fiber digestion is inhibited during the time when ruminal pH is below 6.0 (**pH < 6**), some research also reported the duration that pH was at or below this value. These two criteria of ruminal acidosis were highly correlated:

pH < 6 (min/d) = 1224 - 1316 (pH -5.5);
n = 80, nexp = 35, r(p) = -0.94, SE(p) = 112.

For pH to remain above 6.0 throughout the day, average pH must be 6.4 or higher. An average threshold pH of 6.25 would result in pH being less than 6 for about 4 h/d. Several ruminal and dietary characteristics were related to this index of rumen irregularity (min/d that pH < 6) including ruminal ammonia [r(p) = -0.91], ruminal acetate to propionate ratio [r(p) = -0.91], dietary crude protein [r(p) = -0.94], and dietary starch [r(p) = +0.91].

The primary factor acidifying the rumen is concentration (mM) of volatile fatty acids (**VFA**). The global relationship in this data set was:

pH = 7.20 - .0091 (VFA);
n = 406, r(g) = -0.75, SE(g) = 0.25.

The relatively large SE(g) of this equation suggests that at a given VFA concentration, pH can vary substantially. Although dietary starch was not related to ruminal pH [r(g) = +0.17], it was related to the residual variation between pH and VFA. A significant portion of this residual variation (**RV**) can be explained by percentage of starch in ration dry matter (**Starch**) and mg/l of ruminal ammonia ($\mathbf{NH_3}$):

$RV = 0.15 - .011 (Starch) + .0014 (NH_3)$;
n = 120, nexp = 49, $R^2(p) = 0.98$, SE(p) = 0.19.

Numerous factors affect ruminal pH including fermentable organic matter, ruminally digestible starch, amount and composition of fiber and nonfibrous carbohydrate, and particle size and buffering capacity in the diet, intake of silage and total dry matter, rate of feed intake, chewing activity, capture of hydrogen ions by products of digestion, changes in the stoichiometry of fermentation, and absorption or passage of VFA. Although each of these factors may play a role in the phenomenon of ruminal pH, we propose a simple two-stage approach to estimating pH based on easily measured or estimated variables.

Stage 1. Establish a baseline ruminal pH that is estimated from the intake of concentrates and forages which every nutritionist should be able to measure or estimate. From the database we derived an equation based on the intake of forage (**%FI**) and concentrate (**%CI**) expressed as a %BW/d:

pH = 6.54 - 0.054 (%FI) - 0.18 (%CI);
n = 527, r(g) = 0.42, SE = 0.28.

Using the relatively simple variables of %FI and %CI, this equation takes into account the effects of intake and differences in the fermentability and particle size between forages and concentrates.

Stage 2. Adjust the baseline ruminal pH for differences in percentage NDF, particle density (**PD**) which is the inverse of mean particle size (mm), and percentage of ruminally degradable starch (**RDS**) in the ration:

$DpH = 0.60 [(\log_{10}NDF) - 1.57]$;
n = 484, SE = 0.13,
DpH = -0.06 (DP - 0.42);
n = 71, SE = 0.15, and
DpH = -0.012 (RDS - 17.2);
n = 117, SE = 0.10.

Summary and Conclusion

Equations developed from a database of ruminal pH data compiled from the literature can be used to estimate the risk of mild or severe acidosis from readily available information. Assuming a minimum threshold for ruminal pH of 6.25 for animals of moderate production levels, it is possible to define corresponding recommendations for various dietary characteristics. Thus, it is recommended that the diet dry matter (DM) would have to contain a minimum of 35% of total NDF, 25% of forage NDF, and 40% of particles that are larger than 2 mm or have a minimum mean particle size of 2.5 mm. Conversely, the percentage of concentrate in the diet dry matter must remain below 45%, and starch and ruminally degradable starch must be less than 25% and 20% of diet DM, respectively.

The large nutrient demands of high producing animals requires that the proportion of concentrate is increased and fiber is decreased in the diet. These animals typically consume 4% of BW/d of a diet that contains 55% concentrate and 28% NDF with a mean particle size of 2 mm. This situation increases the risk of acidosis and the system of equations that are proposed estimates that average ruminal pH for these animals will be 5.9-6.0. Therefore, it is recommended that feeding management for these animals be consistent from day to day and that supplemental buffers may be beneficial.

Effect of Replacing Alfalfa Silage With High Moisture Corn on Nutrient Utilization and Milk Production

S.C. Valadares Filho, G.A. Broderick, R.F.D. Valadares and M.K. Clayton

Introduction

Alfalfa silage (**AS**) is one of the most important forages fed to dairy cows in the U.S. However, during ensiling, more than half of the CP in AS is degraded to NPN. Utilization of the NPN in AS is stimulated by high moisture ear corn (**HMC**) and other high energy feeds because its extensive fermentation increases microbial protein formation in the rumen. However, feeding rates for high energy feeds must be limited because lactating cows require adequate amounts of dietary fiber to maintain rumen health. General guidelines for providing adequate effective fiber in the diet and maintaining optimal DM intake include feeding between 25 to 35% total NDF, maintaining a minimum of 18% forage NDF, and feeding between 33 to 40% non-fiber carbohydrates. The objective of this experiment was to determine how much HMC-based concentrate can be safely fed in AS based diets to maximize utilization of the NPN in AS.

Materials and Methods

Twenty-four multiparous Holstein cows, averaging 610 kg BW and 40 kg/d milk yield, were assigned to six 4 X 4 Latin squares with 3-wk periods (total 12 wk). Data from ruminal sampling is reported in the companion Research Summary. Four diets (Table 1), fed as TMR, contained (DM basis) 80, 65, 50 or 35% AS as the sole forage plus 20, 35, 50 or 65% concentrate. The HMC was ground through a 1-cm screen using a hammer mill just prior to feeding. Diets were held isonitrogenous by adding soybean meal and urea as AS was decreased; urea was added to maintain NPN at 43% of total N. All cows were injected with bST. Milk samples were collected on d-12 and d-19 of each period and analyzed for fat, protein, SNF and milk urea N (**MUN**). Fecal grab samples also were collected from each cow to estimate apparent nutrient digestibility using indigestible ADF as an internal marker. Wk-1 of each period served as adaptation time, and milk yield and DM intake data were collected during wk-2 and wk-3 of each period. The general linear models procedure of SAS was used for all statistical analyses. Dietary concentrate level (% of DM) at the maximum response was determined for significant quadratic equations.

Results and Discussion

Alfalfa silage fed in this trial averaged 37% DM, 20.7% CP and 47.6% NDF (DM basis), and 55% NPN (% of total N). The NPN and CP content was similar to that of AS found in commercial tower silos; however, NDF content was higher and more typical of AS from bunker silos. Thus, all diets, including that with 65% concentrate, contained greater than the recommended minimum of 25% total NDF and 18% NDF from forage (Table 1). Each replacement of 15 percentage units of AS with concentrate decreased total NDF by about 5% and increased NE_L (computed from NRC tables) by an average 0.09 Mcal/kg DM (Table 1). There was a linear increase in apparent OM digestibility with decreasing AS in the diet (Table 2); this may be attributed to reduced fiber and increased content of more digestible nonstructural carbohydrates in the diet. The linear decline in NDF and ADF digestibility with increasing dietary concentrate (Table 2) was in agreement with many reports; increased intake of nonstructural carbohydrates likely would reduce ruminal pH and, thus, depress fiber digestibility. Digestibility of ADF had a significant quadratic response to increasing concentrate with a maximum predicted at 32% dietary concentrate (Table 3).

We anticipated quadratic responses in this trial, including a decline in feed intake, with decreasing AS, due to adverse ruminal effects of high concentrate feeding. Significant linear and quadratic effects of replacing AS with concentrate were observed on intakes of DM, NDF and digestible OM (**DOM**), and on BW change in this study (Table 2). Intake of DM ranged from 4.0 to 4.2% of BW for cows fed 35 to 65% concentrate, similar to the 4% of BW cited by the NRC for 600 kg cows producing 40 kg/d of FCM. There was a quadratic response in DM intake (Fig. 1), with a maximum at 51% dietary concentrate (Table 3). As expected, maximal NDF intake occurred at high AS—27% dietary concentrate (Table 3). Intake of NDF was unusually high and, except at 65% concentrate, exceeded the 1.2% of BW suggested as maximal by Mertens (Proc. 46th Ga. Nutr. Conf., Univ. Georgia, 1985), reaching 1.5% of BW on the two highest AS diets (Table 2). Feed intake may have been limited at higher AS levels in this trial by rumen fill of undigested feed residues. Maximum DOM intake, a measure of TDN intake, occurred at 71% concentrate, which was greater than the highest concentrate level actually fed in this trial. Gain of BW was greatest on 35 and 50% concentrate, intermediate on 65% concentrate, while cows fed 20% concentrate lost BW. The maximum for BW gain was at 44% dietary concentrate (Table 3).

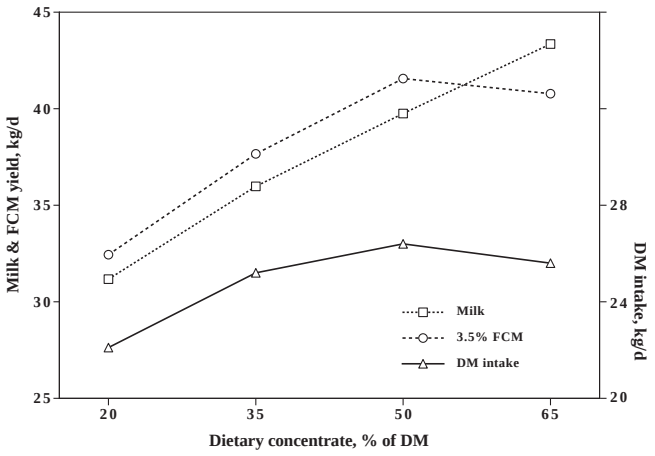

Figure 1. Mean daily yield of milk and 3.5% FCM and DM intake over the last two wk of the 3-wk Latin square for cows fed diets containing (% of DM) 20, 35, 50 and 65% concentrate.

Data on milk yield and composition are in Table 2. Milk yield increased linearly with the increased concentrate in the diet (Fig. 1). The linear increase of milk yield may be attributed to increased DOM intake with decreased dietary AS. Production efficiency (milk/DM intake) followed a response that had different shape from the other quadratic curves: a minimum for milk/DM intake was found at 27% concentrate in the diet (Table 3). However, yield of 3.5% FCM followed a quadratic response (Fig. 1); maximum FCM was predicted at 57% dietary concentrate (Table 3). Milk fat content was unchanged from 20 to 50% concentrate, but declined 0.6 percentage unit at 65% dietary concentrate. This classic pattern of depressed milk fat content with elevated intake of nonstructural carbohydrates resulted in a quadratic response in milk fat yield (Table 3), despite milk secretion being greatest at 65% concentrate. Maximum fat yield occurred at 43% concentrate and 57% AS in the diet. The changes in the pattern of ruminal fermentation, including reduced acetate, increased propionate, and reduced acetate: propionate ratio (described in the companion Research Summary), reflected the lower fiber digestion and intake (Table 2) at the highest levels of concentrate.

Effectiveness of high concentrate feeding to stimulate utilization of AS NPN should be indicated by production responses. Milk content and yield of protein and SNF increased linearly as greater amounts of dietary concentrate replaced AS (Table 2). Elevated microbial protein synthesis with greater energy intake would be expected to increase amino acid supply for milk protein synthesis. Diets containing 35 to 65% concentrate also were supplemented with increasing amounts of soybean meal. Soybean meal CP likely contributes more escape protein than does AS CP. Increased ruminal propionate formation with increased dietary concentrate would be expected to spare amino acids from catabolism for lactose synthesis. Lowest MUN concentration, reflecting improved ruminal utilization of degraded protein and recycled urea, was obtained in cows fed 65% concentrate (Table 2). Maximal MUN was estimated to occur at 31% dietary concentrate (Table 3).

Summary and Conclusion

As dietary concentrate was increased, OM digestibility increased linearly but NDF digestibility was linearly depressed. A number of maxima were identified in this trial when HMC-based concentrate replaced AS in lactating cows fed AS as the only forage. Maximum NDF intake occurred at 27% concentrate while maximal intake of DM and DOM occurred at, respectively, 51 and 71% concentrate. Maximal fat yield occurred at 43% dietary concentrate, while maximal yield of FCM occurred at 57% dietary concentrate. However, yields of milk, protein and SNF did not give quadratic responses and were continuing to increase at 65% dietary concentrate.

Table 1. Composition of diets.

	Dietary concentrate (% of DM)			
Item	20	35	50	65
	- - - - - - - - - - - - (% of DM) - - - - - - - - - - - -			
Alfalfa silage	79.60	65.26	50.30	35.30
High moisture ear corn[1]	18.80	30.87	43.63	56.44
Solvent soybean meal	. . .	1.87	3.54	5.22
Urea	. . .	0.41	0.84	1.26
Dicalcium phosphate	0.60	0.60	0.61	0.61
Sodium bicarbonate	0.50	0.50	0.50	0.50
Potassium magnesium sulfate[2]	. . .	0.09	0.18	0.27
Salt	0.30	0.30	0.30	0.30
Mineral and vitamin premix[3]	0.10	0.10	0.10	0.10
Chemical composition				
CP	19.5	20.1	19.9	19.7
NE_L,[4] Mcal/kg	1.40	1.48	1.57	1.66
NDF	42.9	38.2	32.6	27.7
ADF	33.5	29.5	23.9	18.9
Indigestible ADF	19.9	16.8	13.1	9.7
OM	88.5	88.8	90.8	92.3
K	2.12	1.83	1.52	1.23

[1]High moisture ear corn was ground with a hammer mill through a 1.0 cm screen.
[2]Contained (per kilogram) 111 g Mg, 184 g K, and 222 g S.
[3]Provided (per kilogram of DM) 27 mg of Mn, 27 mg of Zn, 17 mg of Fe, 7 mg of Cu, 0.40 mg of I, 0.30 mg of Se, 0.10 mg of Co, 3880 IU of vitamin A, 730 IU of vitamin D, and 0.73 IU of vitamin E.
[4]Computed from estimated NE_L content of alfalfa and from NRC tables.

Table 2. Effect of replacing dietary alfalfa silage with concentrate on nutrient digestibility and intake, and on yield of milk and milk components.[1]

	Dietary concentrate (% of DM)						
Item	20	35	50	65	SEM	L	Q
OM digestibility, %	55.1[d]	59.5[c]	62.6[b]	67.6[a]	0.5	< 0.001	0.603
NDF digestibility, %	37.4	36.7	36.3	35.0	0.7	0.016	0.693
ADF digestibility, %	38.1[a]	38.4[a]	37.6[a]	35.0[b]	0.7	0.104	0.032
DM intake, kg/d	22.1[b]	25.2[a]	26.4[a]	25.6[a]	0.5	< 0.001	< 0.001
NDF intake, % of BW	1.51[a]	1.53[a]	1.37[b]	1.13[c]	0.03	0.002	< 0.001
DOM intake, kg/d	10.8[c]	13.5[b]	15.0[a]	15.9[a]	0.3	< 0.001	0.004
BW Change, kg/d	-0.17[b]	0.63[a]	0.58[a]	0.11[ab]	0.14	< 0.001	< 0.001
Milk, kg/d	31.2[d]	36.0[c]	39.8[b]	43.4[a]	0.6	0.002	0.338
Milk/DM intake	1.41[b]	1.42[b]	1.50[b]	1.71[a]	0.03	0.069	0.005
3.5% FCM, kg/d	32.4[c]	37.7[b]	41.6[a]	40.8[a]	0.8	< 0.001	< 0.001
Fat, %	3.77[a]	3.83[a]	3.77[a]	3.16[b]	0.09	< 0.001	< 0.001
Fat, kg/d	1.32[b]	1.43[ab]	1.49[a]	1.32[b]	0.04	0.001	0.001
Protein, %	2.85[c]	2.94[bc]	3.01[ab]	3.06[a]	0.03	< 0.001	0.516
Protein, kg/d	1.01[c]	1.09[bc]	1.19[ab]	1.28[a]	0.03	< 0.001	0.982
SNF, %	8.37[c]	8.51[bc]	8.64[ab]	8.68[a]	0.04	< 0.001	0.216
SNF, kg/d	2.96[c]	3.19[bc]	3.41[ab]	3.64[a]	0.07	< 0.001	0.998
MUN, mg/dl	25.0[ab]	25.7[a]	24.0[b]	20.6[c]	0.35	< 0.001	< 0.001

[a,b,c,d]Means in rows with different superscripts differ ($P < 0.05$).
[1]DOM = digestible OM, L = probability of linear effect, MUN = milk urea N, Q = probability of quadratic effect.

Table 3. Significant linear and quadratic regressions on dietary concentrate level.[1]

Variable (Y)	Type	Equation	$(R^2)^2$	Maximum[3]
Apparent digestibility				
OMD, %	Linear	Y = 49.7 + 0.271 C	0.871	. . .
NDFD, %	Linear	Y = 38.5 - 0.0520 C	0.488	. . .
ADFD, %	Quadratic	Y = 35.1 + 0.210 C - 0.00325 C^2	0.529	32.3%
Intake and BW change				
DM intake, kg/d	Quadratic	Y = 14.9 + 0.448 C - 0.00437 C^2	0.738	51.3%
NDF intake, % of BW	Quadratic	Y = 1.32 + 0.0153 C - 0.000280 C^2	0.821	27.3%
DOM intake, kg/d	Quadratic	Y = 5.97 + 0.281 C - 0.00198 C^2	0.808	71.0%
BW change, kg/d	Quadratic	Y = - 2.09 + 0.125 C - 0.00141 C^2	0.464	44.3%
Milk yield and MUN				
Milk yield, kg/d	Linear	Y = 26.1 + 0.269 C	0.913	. . .
Milk yield/DM intake	Quadratic	Y = 1.39 - 0.0116 C + 0.000214 C^2	0.762	(27.1%)[4]
3.5% FCM yield, kg/d	Quadratic	Y = 19.7 + 0.760 C - 0.00667 C^2	0.861	57.0%
Fat, %	Linear	Y = 4.17 - 0.0126 C	0.613	. . .
Fat yield, kg/d	Quadratic	Y = 0.901 + 0.0264 C - 0.000304 C^2	0.750	43.4%
Protein, %	Linear	Y = 2.77 + 0.00462 C	0.613	. . .
Protein yield, kg/d	Linear	Y = 0.885 + 0.00602 C	0.770	. . .
SNF, %	Linear	Y = 8.24 + 0.00726 C	0.746	. . .
SNF yield, kg/d	Linear	Y = 2.66 + 0.0151 C	0.827	. . .
MUN, mg/dl	Quadratic	Y = 21.2 + 0.282 C - 0.00448 C^2	0.891	31.4%

[1]C = Dietary concentrate (% of DM), ADFD = ADF digestibility, DOM = digestible organic matter, MUN = milk urea N, NDFD = NDF digestibility, and OMD = organic matter digestibility.
[2]Coefficient of determination.
[3]Dietary concentrate content (% of DM) at maximum determined by taking first-derivative of quadratic equations, where significant.
[4]The quadratic equation for milk yield/DM intake has the opposite shape and taking the first-derivative identifies the dietary concentrate content (27.1% of DM) at the minimum.

Effect of Replacing Alfalfa Silage With High Moisture Corn on Ruminal Protein Synthesis Estimated From Urinary Excretion of Purine Derivatives

R.F.D. Valadares, G.A. Broderick, S.C. Valadares Filho and M.K. Clayton

Introduction

More than half of the protein in alfalfa silage (**AS**), one of the most important forages fed to dairy cows in the U.S., gets broken down to NPN in the silo. Utilization of AS NPN is stimulated by high energy feeds, such as high moisture ear corn (**HMC**), by increasing microbial protein formation in the rumen. However, over-feeding of high energy feeds can result in adverse effects in the cow's rumen. Thus, it is important to determine the amount of HMC that can be fed to obtain the maximum utilization of AS NPN without impairing animal performance. Urinary excretion of purine derivatives (**PD**) has been used to measure microbial protein synthesis in the rumen. This trial quantified ruminal microbial protein from urinary PD excretion in lactating cows fed alfalfa silage that was supplemented with varying levels of HMC-based concentrate.

Materials and Methods

Twenty-four multiparous Holstein cows, including 8 with ruminal cannulae, were assigned to six 4 X 4 Latin squares with 3-wk periods (total 12 wk). Four diets (see Table 1 of the companion Research Summary), fed as TMR, contained (DM basis): 80, 65, 50 or 35% AS as the sole forage plus 20, 35, 50 or 65% concentrate. The concentrate consisted mainly of ground HMC; soybean meal and urea were added to make diets equal in CP and NPN (43% of total N). Allantoin was determined in milk samples taken on d-12 and d-19 of each period. Ruminal contents were taken at 0, 1, 2, 3, 4 and 6 h postfeeding on d-15 of each period and analyzed for pH, NH_3, total free AA and VFA. Total, 24-h urine collections were made using indwelling bladder catheters on d-19 of each period; daily urinary excretion of allantoin, uric acid, creatinine and urea was determined. Total PD excretion was the sum of urinary allantoin and uric acid excretion plus milk allantoin excretion. Ruminal microbial protein synthesis was computed using these equations (Chen and Gomes 1992): Total absorption of microbial purines (mmol/d) = (total PD excretion - 0.385 $BW^{0.75}$)/0.85, where 0.385/$BW^{0.75}$ is endogenous PD excretion (mmol/d) and 0.85 is purine absorptive efficiency; and 2) net synthesis of microbial N (g/d) = (purine absorption x 70) / (0.116 x 0.83 x 1000), where 70 is the N content of purines (mg N/mmol), 0.116 is the ratio of purine-N: total-N in mixed rumen microbes and 0.83 is the assumed digestibility of microbial purines. Other trial details are described in the companion Research Summary.

Results and Discussion

Daily urine volumes were 48.3, 48.4, 41.8, and 31.5 kg/d for diets containing, respectively, 20, 35, 50, and 65% concentrate. Urine output decreased linearly with increasing concentrate (Table 3) probably due to the decrease in dietary K, lowest to highest concentrate level, from 2.12 to 1.23% K (DM basis). Replacing AS, the major dietary source of K, with HMC and soybean meal accounted for this decrease. Daily metabolite excretions are summarized in Table 1. As expected, urinary creatinine excretion was a constant function of BW. Urinary excretion of allantoin and uric acid, and total PD excretion, were higher in cows fed 50 than 20% concentrate. All three traits were influenced quadratically by concentrate level, with maxima averaging about 48% dietary concentrate (Table 3). Daily urinary excretion of allantoin varied from 369 to 535 mmol/d. The proportion of urinary allantoin in total PD excretion was constant, ranging from 90.2 to 90.7%. Milk allantoin secretion increased linearly with concentrate ranging from 18.7 to 28.6 mmol/d and accounted for 4.2 to 5.7% of the total PD excretion. Urea N excretion declined from a maximum of 342 to 239 g/d with increasing concentrate, indicating better NPN utilization with greater energy intake. Maximum urea N excretion was predicted to occur at 36% concentrate (Table 3). Total PD excretion ranged from 423 to 613 mmol/d and was highest for cows fed 50% concentrate (Table 1). Ruminal microbial N yields, computed from PD

excretion using the equations of Chen and Gomes (1992), ranged from 278 to 419 g/d (Table 1). Of course, maximum microbial yield also was predicted to occur at 48% concentrate (Table 3). This was consistent with the maximum DM intake estimated at 51% dietary concentrate (companion Research Summary). Microbial N yields in the rumen also were computed using dietary NE_L, calculated from apparent OM digestibilities, and DM intakes using the NRC equation: Microbial N = - 30.93 + 11.45 x NE_L (Mcal/d). Estimates of 280, 356, 395 and 420 g/d were obtained for, respectively, diets containing 20, 35, 50 and 65% concentrate. Subtracting these from estimates computed with PD excretion yielded differences of -2, -23, +24 and -85 g/d, respectively. The large difference only at the highest level of concentrate is interesting and may be attributed to reduced pH and other changes in the environment of the rumen that may depress net yield of microbial protein. Mean ruminal pH over the 6 h after feeding declined from 6.5 (20% concentrate) to 6.1 on both 50 and 65% concentrate (Table 2). That microbial N yield was 20% lower (335 versus 419 g/d) on 65 than on 50% dietary concentrate suggested that factors in addition to low ruminal pH contributed to depressed microbial protein formation on the highest concentrate diet. Overall, microbial N yields estimated from total PD excretion were consistent with observed yield of milk and milk components on the same diets.

Mean ruminal pH of cows fed 20% concentrate was higher than at 50 and 65% dietary concentrate (Table 2) and remained higher than on the other three diets over the 6 h after feeding (Fig. 1). Ruminal pH on 35% dietary concentrate was intermediate (Table 2) and did not change due to time after feeding (Fig. 1). Reductions of ruminal pH of short duration likely cause only moderate, transient depression in fiber digestion; however, pH reductions for longer periods may cause washout of the ruminal organisms associated with fiber digestion and severely reduce fiber and OM digestion and microbial protein yield. Ruminal pH after feeding fell below 6 at only one time (6-h after feeding) on 50% concentrate but was below pH 6 at three time-points on 65% concentrate (Fig. 1). Ruminal NH_3 was very high at all times after feeding (Fig. 2) and there were no differences among diets in mean concentration (Table 2). High ruminal NH_3 is not surprising at dietary CP levels of 19.5 to 20.1%, with 43% of the CP equivalent coming from NPN. There were differences in total AA concentrations after feeding (Fig. 3); overall, total AA tended to be higher on the 65% concentrate diet (Table 2), the diet that contained the most soybean meal. Peak ruminal NH_3 (Fig. 2) and total free AA concentrations (Fig. 3) for all diets occurred 1-h postfeeding. Ruminal total VFA were unaffected by diet (Table 2). Molar proportions of acetate decreased linearly and propionate increased linearly

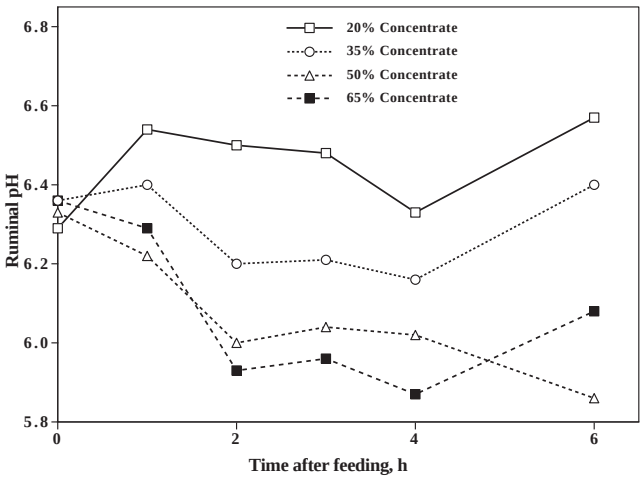

Figure 1. Mean hourly pH in the rumens of cows fed diets with all forage from alfalfa silage and 20, 35, 50 or 65% of dietary DM as a concentrate mix based on ground high moisture ear corn.

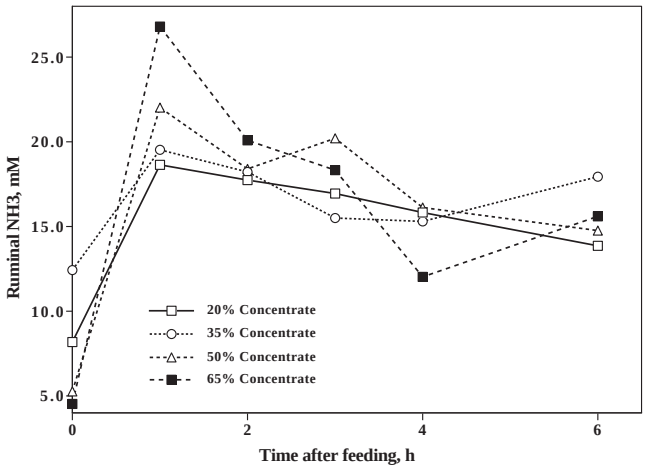

Figure 2. Mean hourly NH_3 concentration in the rumens of cows fed diets with all forage from alfalfa silage and 20, 35, 50 or 65% of dietary DM as a concentrate mix based on ground high moisture ear corn.

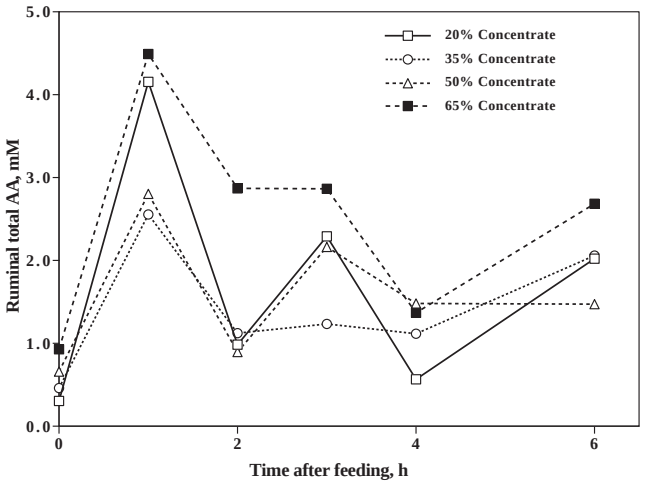

Figure 3. Mean hourly total AA concentration in the rumens of cows fed diets with all forage from alfalfa silage and 20, 35, 50 or 65% of dietary DM as a concentrate mix based on ground high moisture ear corn.

with decreased AS and increased concentrate in the diet; acetate: propionate ratio also declined linearly, from 3.58 to 2.00, with increased dietary concentrate (Table 2). These changes are typical when more nonstructural carbohydrate and less fiber is fermented in the rumen. Molar proportions of isobutyrate and isovalerate plus 2-methylbutyrate declined linearly with decreasing AS in the diet (Table 2). Branched-chain VFA are formed from microbial catabolism of branched-chain AA and their concentrations would be directly related to the intake of free AA and peptides in AS NPN.

Summary and Conclusion

Purine derivative excretions, measured in 24-h total urine collections, gave satisfactory estimates of microbial N yields in lactating cows fed varying levels of dietary concentrate to replace AS. Maximum yield of microbial N was observed at 48% dietary concentrate, suggesting that this was the optimum feeding level for HMC-based concentrate to maximize utilization of the NPN in AS.

Reference

Chen, B. and M.J. Gomes. 1992. Int. Feed Res. Unit, Occasional Publ., Rowett Res. Inst., Aberdeen, U.K. pp. 75-82.

Table 1. Effect of replacing dietary alfalfa silage with concentrate on daily excretion of milk allantoin and urinary creatinine, and of allantoin, uric acid and urea N measured in total urine collections and on microbial N synthesized in the rumen as estimated from total excretion of purine derivatives.[1]

	Dietary concentrate (% of DM)						
Item	20	35	50	65	SEM	L	Q
Milk allantoin, mmol/d	18.7^{d}	22.7^{c}	25.9^{b}	28.6^{a}	0.5	0.001	0.195
Total urine collection							
Creatinine, mg/kg BW/d	28.6	28.4	30.0	28.9	0.5	0.209	0.281
Allantoin, mmol/d	369.1^{b}	435.2^{ab}	534.8^{a}	428.9^{ab}	31.1	0.005	0.011
Uric acid, mmol/d	35.5^{b}	43.4^{ab}	52.4^{a}	42.3^{ab}	3.7	0.009	0.017
PD[2], mmol/d	423.3^{b}	501.5^{ab}	613.2^{a}	499.8^{ab}	34.1	0.004	0.009
Allantoin, % of PD	90.7	90.2	90.5	90.6	0.5	0.454	0.462
Microbial N, g/d	278.4^{b}	336.0^{ab}	419.3^{a}	335.1^{ab}	25.3	0.004	0.009
Urea N, g/d	303.7^{b}	342.0^{a}	307.9^{b}	238.7^{c}	7.5	< 0.001	< 0.001

^{a,b,c}Means in rows with different superscripts differ ($P < 0.05$).

[1]L = Probability of linear effect, PD = total purine derivatives (allantoin + uric acid), Q = probability of quadratic effect.

[2]Including milk allantoin excretion.

Table 2. Effect of replacing dietary alfalfa silage with concentrate on ruminal pH, ruminal concentration of NH_3, total AA and total VFA, and on molar proportions of ruminal VFA.[1]

	Dietary concentrate (% of DM)						
Item	20	35	50	65	SEM	L	Q
pH	6.51[a]	6.29[ab]	6.08[b]	6.08[b]	0.11	0.001	0.195
NH_3, m*M*	15.20	16.49	16.13	16.23	2.32	0.005	0.011
Total AA, m*M*	1.72[ab]	1.42[b]	1.58[ab]	2.53[a]	0.61	0.009	0.017
Total VFA, m*M*	114.1	129.5	133.0	130.0	5.0	0.089	0.074
Molar proportion, mol/100 mol of total VFA							
Acetate (A)	64.4[a]	62.7[ab]	58.5[b]	52.7[c]	1.2	< 0.001	0.190
Propionate (P)	18.2[b]	19.1[b]	22.5[b]	28.6[a]	1.5	0.001	0.093
A: P ratio	3.58[a]	3.30[ab]	2.79[b]	2.00[c]	0.18	< 0.001	0.146
Butyrate	11.8	12.5	14.0	14.0	0.5	0.092	0.190
Isobutyrate	1.42[a]	1.38[a]	1.15[b]	0.97[c]	0.04	< 0.001	0.482
Isovalerate + 2-methylbutyrate	1.93[a]	2.04[a]	1.65[a]	1.20[b]	0.10	0.003	0.996
Valerate	2.20	2.28	2.23	2.47	0.14	0.674	0.072

[a,b]Means in rows with different superscripts differ ($P < 0.05$).

[1]L = Probability of linear effect, Q = probability of quadratic effect.

Table 3. Significant linear and quadratic regressions on dietary concentrate level.[1]

Variable (Y)	Type	Equation	$(R^2)^2$	Maximum[3]
Total urinary excretion				
Urine volume (kg/d)	Linear	Y = 58.6 – 0.373 C	0.795	. . .
Allantoin (mmol/d)	Quadratic	Y = 66.3 + 18.3 C – 0.192 C^2	0.402	47.6%
Uric acid (mmol/d)	Quadratic	Y = 3.80 + 1.99 C – 0.021 C^2	0.396	47.5%
PD (mmol/d)	Quadratic	Y = 82.8 + 20.6 C – 0.215 C^2	0.411	48.0%
Urea-N (g/d)	Quadratic	Y = 182 + 8.74 C – 0.120 C^2	0.816	36.4%
Microbial N (g/d)	Quadratic	Y = 26.0 + 15.3C – 0.159 C^2	0.413	48.1%
Ruminal VFA proportion				
Acetate (A)	Linear	Y = 70.7 – 0.262 C	0.842	. . .
Propionate (P)	Linear	Y = 12.3 + 0.231 C	0.782	. . .
A: P	Linear	Y = 4.41 – 0.035 C	0.820	. . .
Isobutyrate	Linear	Y = 1.68 – 0.010 C	0.879	. . .
Isovalerate + 2-methylbutyrate	Linear	Y = 2.44 – 0.017 C	0.758	. . .

[1]C = Dietary concentrate (% of DM), and PD = total purine derivatives (allantoin + uric acid).

[2]Coefficient of determination.

[3]Dietary concentrate content (% of DM) at maximum determined by taking first-derivative of quadratic equations, where significant.

Potential of Biochlor and Fermenten for Improving Nitrogen Utilization in Lactating Dairy Cows

G.A. Broderick and N. De Leon Gatti

Introduction

A number of fermentation byproducts are reputed to improve microbial protein synthesis in the rumen. One that has received considerable attention in this regard is Biochlor, a byproduct of microbial fermentations producing MSG. The objective of this research was to determine whether the commercial fermentation byproducts Biochlor and Fermenten (a product similar to BioChlor but with perhaps a "better" ion balance) can serve as effective N sources, relative to standard supplements for lactating dairy cows. Urea served as the standard NPN source and solvent soybean meal (**SBM**), known to be an effective N source for microbial protein formation that also provides about 35% ruminal escape protein, was used as the standard protein N source. A third N source, a mixture of three fermentation products from Ajinomoto Co. (**AP1**), also was fed.

Materials and Methods

Twenty-five Holstein cows, 20 multiparous (five of which were fitted with ruminal cannulae) and five primiparous cows, with mean (± SD) 627 (± 76) kg BW, parity 2.7 (± 1.4) and 39 (± 5) kg/d of milk yield were blocked by days-in-milk and randomly assigned to five 5 x 5 Latin squares with 2-wk periods (total 10 wk). Five diets that differed only in source of supplemental N but with approximately equal energy were fed as TMR (Table 1). All cows were injected with bST. Milk yield from wk-1 was discarded and data analyzed from wk-2 of each period. Milk samples were collected on d-11 of each period and analyzed for fat, protein, lactose, SNF and milk urea N (**MUN**). On d-13 of each period, ruminal samples were taken at 0, 1, 2, 3, 4 and 6 h after feeding from cannulated cows for determination of pH, NH_3, total AA and VFA. Spot urine samples also were taken on d-13 and analyzed for creatinine, allantoin and uric acid to estimate microbial N from output of purine derivatives assuming a daily creatinine excretion of 0.256 mmol/kg BW (R.F.D. Valadares, unpublished). Results were analyzed as a replicated 5 x 5 Latin square using the general linear models procedure of SAS. When dietary treatment effects were significant ($P \leq 0.05$), mean separation was by LSD at the 5% level of probability.

Results and Discussion

Alfalfa silage fed in this trial averaged 22.1% CP and 45.4% NDF (DM basis), pH 5.2 and 49% NPN (% of total N); corn silage and high moisture corn contained, respectively, 7.3 and 8.0% CP and 39.6 and 16.4% NDF (DM basis). Thus, the composition of major ingredients in these diets was typical for dairy cattle rations in the U.S. Diets were about isonitrogenous and supplements supplied nearly equal proportions of the total dietary CP: 28, 26, 27, 27 and 26% of CP in, respectively, diets A, B, C, D and E (Table 1). Intake of DM, BW gain, DM and N efficiencies, and milk yield and composition data are in Table 2. Intake of DM and BW gain were greatest on the diet containing SBM, with generally lower intakes and weight gains on urea, Biochlor and AP1 (a mixture of Protoferm, CMS and wheat middlings). Intake was lower and BW loss occurred on the Fermenten diet. Yield of milk, FCM, fat, protein, lactose and SNF all were highest on the SBM containing diet. As for intake and BW gain, yields of milk and milk components were intermediate on urea, Biochlor and AP1, and lowest on Fermenten. Efficiency of capture of dietary N as milk N also was greatest with cows fed the diet with SBM; however, this observation was confounded by the fact that diet B had slightly lower CP content (Table 1).

Generally, milk protein yield in lactating cows is a function of the absorbable protein supplied by microbial protein synthesized in the rumen plus feed protein that escapes the rumen. The SBM diet provided the most absorbable protein, possibly because about 35% of the protein in SBM can escape the rumen (NRC, 1989). That protein yields on Biochlor and AP1 were similar to urea suggested that

neither of these two fermentation products gave rise to greater ruminal microbial protein and neither was superior to urea as an NPN source. The very low milk protein yield on Fermenten indicated that it was an inferior source of NPN for the dairy cow. Several interesting effects were noted on concentrations of milk components and MUN. Milk fat content was highest on Fermenten; this may have been related to mobilization of body fat because this was the only diet on which there was BW loss (Table 2). It was surprising that MUN content was lowest, and not different from SBM, on Biochlor; MUN was intermediate on AP1 and Fermenten and highest on urea (Table 2). Levels of MUN possibly were influenced by urine volume, which was estimated to be numerically greatest on the Biochlor diet (Table 4). Milk protein, despite supplemental N coming from NPN sources on four out five diets, was relatively high, ranging from 3.19 to 3.29%. Average milk protein concentration found in 25 trials at the Dairy Forage Center in cows fed 80 different diets was 2.99%. Increased protein content of milk is now more valuable in the U.S. with the advent of component pricing to determine the farm price of bulk milk. Rather than being constant, lactose concentration in milk from cows fed urea and SBM both were higher in milk from cows fed the other NPN sources (Table 2).

Ruminal pH was lower on Biochlor, Fermenten and AP1 than on SBM; this may reflect the acid load coming from these three fermentation byproducts (Table 3). Total VFA concentration was greater on the urea diet than on other NPN containing diets; this may explain why ruminal pH on urea was intermediate between SBM and the other three diets. As expected, ruminal NH_3 was greater on the four NPN sources than on SBM. Ruminal NH_3 concentrations are the resultant of production and absorption; absorption is reduced at lower ruminal pH. It is interesting that feeding AP1 gave rise to higher ruminal NH_3 than feeding comparable levels of NPN as urea, Biochlor and Fermenten at similar ruminal pH (Table 3). Ruminal concentrations of isobutyrate were greater on the diet containing SBM than on the NPN supplemented diets; this likely was due to catabolism of valine released from degradation of SBM true protein. Urinary excretion of the purine derivatives allantoin and uric acid by dairy cows arises largely from body catabolism of absorbed purines originating from microbial growth in the rumen. Concentrations of allantoin and purine derivatives (allantoin plus uric acid) were greater in spot urine samples taken from cows fed urea, SBM and Fermenten than in urine from cows fed Biochlor and AP1 (Table 4). However, creatinine concentrations also were greater in these

Table 1. Diet composition.

Ingredient	A Urea	B SBM	C Biochlor	D Fermenten	E AP1
			(% of DM)		
Alfalfa silage	28.2	28.2	28.4	28.4	28.2
Corn silage	30.5	30.5	30.7	30.7	30.5
High moisture corn	32.2	32.2	32.4	32.4	32.2
Wheat middlings	7.15	0	0	0	4.71
Urea	0.94	0	0	0	0
Solvent SBM	0	7.95	0	0	0
Biochlor	0	0	7.45	0	0
Fermenten	0	0	0	7.48	0
Protoferm	0	0	0	0	1.85
CMS	0	0	0	0	1.30
DiCal	0.30	0.30	0.30	0.30	0.30
Bicarb	0.39	0.39	0.40	0.40	0.39
TMS	0.30	0.30	0.30	0.30	0.30
Vitamins ADE conc.	0.10	0.10	0.10	0.10	0.10
Composition (DM basis)					
CP, %	15.3	14.8	15.2	15.2	15.0
NE_L, Mcal/kg	1.66	1.68	1.66	1.66	1.67
NDF, %	33	31	32	32	33

The corn silage was rolled before ensiling and the high moisture ear corn was rolled just before feeding.

same urine samples, indicating urine from cows fed the other diets was more diluted. Because urinary creatinine excretion per unit BW is constant, volume of urine output was estimated to be greater in cows fed the Biochlor and AP1 diets (Table 4). Although there were no significant differences in estimated purine derivative excretion and microbial protein synthesis, both were numerically lower on Biochlor and Fermenten. This also suggested that, in this trial, ruminal microbial growth was not greater on these two commercial fermentation byproducts than on urea.

Summary and Conclusion

Feeding the fermentation NPN sources Biochlor and Fermenten did not result in better utilization of their N, relative to urea and a third fermentation product (AP1), in lactating dairy cows. The true protein N source SBM was superior to all NPN sources. Similar milk protein yields on Biochlor and AP1 as on urea suggested that neither gave rise to greater microbial protein production in the rumen than urea. The same yields of ruminal microbial protein were computed from estimated urinary excretion of purine derivatives for cows fed Biochlor and Fermenten. In these studies, Fermenten was the least effective NPN source for lactating dairy cows.

References

Vagnoni, D.B., G.A. Broderick, M.K. Clayton, and R.D. Hatfield. 1997. Excretion of purine derivatives by Holstein cows abomassally infused with incremental amounts of purines. J. Dairy Sci. 80:1695-1702.

Vagnoni, D.B. and G.A. Broderick. 1997. Effects of supplementation of energy or ruminally undegraded protein to lactating cows fed alfalfa hay or silage. J. Dairy Sci. 80:1703-1712.

Table 2. Effect of feeding supplemental CP as urea, solvent soybean meal (SBM), Biochlor, Fermenten, or a mixture of Ajinomoto fermentation products (AP1) on DM intake, BW change, DM and N efficiencies, and yield of milk and milk components.

Item	Diet					SEM[1]	$P > F^2$
	A Urea	B SBM	C Biochlor	D Fermenten	E AP1		
DM intake, kg/d	23.7^{b}	24.9^{a}	23.0^{bc}	22.9^{c}	23.7^{b}	0.3	< 0.001
BW gain, kg/d	0.65^{ab}	1.13^{a}	0.27^{b}	-0.48^{c}	0.52^{ab}	0.25	< 0.001
Milk yield, kg/d	32.3^{b}	34.4^{a}	31.1^{cd}	30.3^{d}	32.0^{bc}	0.4	< 0.001
DM efficiency[3]	1.38	1.39	1.36	1.34	1.36	0.02	0.450
N efficiency[4]	27.9^{b}	30.2^{a}	28.4^{b}	27.9^{b}	28.7^{b}	0.5	0.012
3.5% FCM, kg/d	33.6^{b}	35.3^{a}	32.0^{b}	32.0^{b}	32.3^{b}	0.6	< 0.001
Fat, %	3.75^{ab}	3.67^{b}	3.65^{b}	3.89^{a}	3.54^{b}	0.07	0.025
Fat, kg/d	1.21^{ab}	1.25^{a}	1.14^{b}	1.17^{b}	1.13^{b}	0.03	0.012
Protein, %	3.19^{b}	3.29^{a}	3.24^{ab}	3.25^{ab}	3.23^{ab}	0.02	0.029
Protein, kg/d	1.03^{b}	1.13^{a}	1.01^{bc}	0.98^{c}	1.04^{b}	0.02	< 0.001
Lactose, %	4.83^{a}	4.78^{ab}	4.73^{b}	4.70^{b}	4.72^{b}	0.03	0.029
Lactose, kg/d	1.56^{b}	1.65^{a}	1.48^{cd}	1.42^{d}	1.52^{bc}	0.02	< 0.001
SNF, %	8.77	8.84	8.72	8.70	8.70	0.04	0.056
SNF, kg/d	2.84^{b}	3.05^{a}	2.72^{bc}	2.63^{c}	2.80^{b}	0.04	< 0.001
MUN, mg/dl	14.53^{a}	11.31^{c}	10.82^{c}	12.74^{b}	12.68^{b}	0.28	< 0.001

^{a,b,c,d}Means in rows with no common superscripts are different ($P < 0.05$).

[1]SEM = Standard error of the mean.

[2]Probability of a significant effect of diet.

[3]Milk yield : DMI.

[4]Milk N yield : N intake.

Table 3. Effect of feeding supplemental CP as urea, solvent soybean meal (SBM), Biochlor, Fermenten, or a mixture of Ajinomoto fermentation products (AP1) on ruminal pH, NH_3, total AA and VFA patterns.

Item	Diet					SEM[1]	$P > F^2$
	A Urea	B SBM	C Biochlor	D Fermenten	E AP1		
pH	6.07[ab]	6.16[a]	5.97[b]	6.00[b]	5.98[b]	0.05	0.059
NH_3, mM	13.53[b]	8.84[c]	13.88[ab]	14.52[ab]	17.35[a]	1.18	0.004
Total AA, mM	0.99	1.56	1.48	1.53	1.80	0.28	0.383
Total VFA, mM	140.5[a]	131.9[ab]	130.0[b]	128.3[b]	127.5[b]	3.0	0.065
Molar proportion, mol/100 mol of total VFA							
Acetate (A)	60.7	62.1	60.4	61.4	59.6	0.9	0.338
Propionate (P)	21.7	19.8	21.2	20.4	22.4	1.0	0.437
A: P ratio	2.86	3.14	3.03	3.03	2.71	0.15	0.357
Butyrate	12.8	13.3	14.1	13.8	13.7	0.6	0.622
Isobutyrate	1.04[bc]	1.10[a]	0.98[c]	1.07[ab]	1.01[bc]	0.02	0.013
IV+ 2MB[3]	1.82	1.77	1.50	1.51	1.48	0.11	0.122
Valerate	1.95	1.89	1.82	1.84	1.86	0.05	0.433

[a,b]Means in rows with no common superscripts are different ($P < 0.05$).
[1]SEM = Standard error of the mean.
[2]Probability of a significant effect of diet.
[3]Isovalerate plus 2-Methylbutyrate.

Table 4. Effect of source of supplemental CP on urinary excretion of purine derivatives (PD) and creatinine, and estimated urine volume, PD excretion and microbial protein formation.

Item	Diet					SEM[1]	$P > F^2$
	A Urea	B SBM	C Biochlor	D Fermenten	E AP1		
Allantoin, m*M*	30.2[a]	31.7[a]	22.8[b]	30.6[a]	23.7[b]	1.2	< 0.01
Uric acid, m*M*	3.36[b]	3.87[a]	2.73[c]	3.38[ab]	2.92[bc]	0.18	< 0.01
PD,[3] mM	33.5[a]	35.5[a]	25.5[b]	34.0[a]	26.6[b]	1.3	< 0.01
Allantoin, % of PD	89.8	88.8	89.0	89.8	88.9	0.5	0.21
Creatinine, m*M*	10.6[a]	10.78[a]	8.07[b]	10.83[a]	8.15[b]	0.38	< 0.01
Urine volume,[4] L/d	16.2[b]	16.3[b]	22.2[a]	16.2[b]	22.8[a]	1.1	< 0.01
PD excretion,[5] mmol/d	529	559	517	517	537	11	0.12
Microbial CP,[6] g/d	1984	2125	1928	1930	2025	50	0.12

[a,b,c]Means in rows with no common superscripts are different ($P < 0.05$).
[1]SEM = Standard error of the mean.
[2]Probability of a significant effect of diet.
[3]Allantoin plus uric acid.
[4]Urine volume estimated from creatinine concentration, assuming daily creatinine excretion equal to 0.256 mmol/kg BW (R.F.D. Valadares, unpublished, 1998).
[5]Urinary PD excretion estimated using the equation of Vagnoni et al. 1997.
[6]Microbial CP computed from estimated PD excretion assuming CP: purine ratio in ruminal microbes equal to 3.99 g/mmol (Vagnoni and Broderick 1997).

Summary of the Effects of Feeding Macerated Alfalfa Silage to Lactating Dairy Cows

G.A. Broderick, R.G. Koegel and T.J. Kraus

Introduction

Improving the energy content of alfalfa forage would increase its value to dairy cows because less concentrate would need to be fed to maintain performance. A number of small trials conducted at the Dairy Forage Center showed that macerating alfalfa concurrent with mowing improved ruminal fiber digestion. Work has continued on the maceration-mat machine; four large scale feeding studies and one smaller digestibility trial were conducted during the past two years to assess the possible benefits of maceration on the utilization of alfalfa silage. This report summarizes the findings from these recent studies.

Materials and Methods

Alfalfa was harvested using either a conventional mower-conditioner (Control) or the prototype maceration-mat machine (Macerated), field wilted to 40 to 50% DM and ensiled in upright concrete stave silos for three cuttings during 1996 and for two cuttings during 1997. One digestibility trial (third cutting) and two lactation trials (first and second cutting) were conducted with alfalfa harvested in 1996; two lactation trials (first and second cutting) were conducted with alfalfa harvested in 1997. A total of 141 cows were used in the four lactation trials. Apparent digestibility of DM, OM, NDF, ADF and CP was determined using both external (Yb) and internal (indigestible ADF) markers in the digestibility trial; apparent digestibility was determined using the internal marker only in all other trials. Three diets were fed in lactation trials: Negative Control (control alfalfa) and Macerated (macerated alfalfa) diets were formulated with about 60% DM from alfalfa, and Positive Control (control alfalfa) with about 50% DM from alfalfa (Table 2). Cows were fed their diets for 10-wk without switching in lactation trials conducted using 1996 forage; cows were fed diets in 3X3 Latin square arrangements of treatments (4-wk periods, 12-wk total) in lactation trials conducted using 1997 forage. Cows were injected biweekly with rBST; intake, milk yield, and BW changes were measured in all lactation trials. The general linear models procedure of SAS was used in the overall statistical analysis by weighting performance responses by the number of cows in each trial.

Results and Discussion

Overall, CP content and pH content of Macerated alfalfa silage were similar to Control; however, Macerated alfalfa contained greater amounts of ash, NDF and ADF (Table 1). This suggested that more soil contamination and possibly greater leaf loss occurred when Macerated alfalfa was harvested for our studies. The 8% reduction in NPN in Macerated alfalfa suggested that its fermentation was more rapid; this would be beneficial to CP utilization by the cow. The mean composition of the diets fed in the four lactation studies (Table 2) indicated that the Negative Control and Macerated diets were nearly identical, except for the source of alfalfa silage. Also, it should be noted that the Positive Control diets contained about 7 percentage units more high moisture corn, 3.6 percentage units more soybean meal, plus sodium bicarbonate to buffer the rumen. The same apparent digestibility was obtained in the digestibility study using the internal (indigestible ADF) and external (Yb) markers, indicating that the internal marker technique could be applied reliably in the lactation trials. Apparent digestibilities determined in the four lactation trials for the 60% forage diets only are in Table 2. Overall, maceration improved apparent digestibility of DM but especially of OM; trends of about a one percentage unit of improved digestibility of fiber and CP were not significant.

Although maceration significantly increased ($P < 0.05$) DM intake in two out of four lactation trials, this trend was not significant overall (Table 4). There was no effect of diet on BW gain. However, the most milk

Table 1. Mean composition of macerated and non-macerated (control) alfalfa silage.[1]

Component	Control	Macerated	SEM[2]	$P > F$[3]
CP, %	20.9	20.6	0.3	0.35
pH	5.00	4.88	0.07	0.27
Ash, %	10.2	11.2	0.2	0.03
NDF, %	43.3	44.6	0.5	0.05
ADF, %	34.5	35.6	0.4	0.06
NPN, %	44.0	40.3	1.4	0.06

[1]Alfalfa harvested at one maturity during each of three cuttings in 1996, and harvested at two maturities during first cutting and one maturity during second cutting in 1997.
[2]SEM = Standard error of the mean.
[3]Probability of a significant effect of maceration.

was produced on the Positive Control diet and milk yield also was greater on Macerated than Control alfalfa silage. Despite these highly significant effects on milk yield, there were no differences in FCM because of the decline in milk fat content with increased dietary concentrate (Table 4). That milk fat content on Macerated diet was intermediate between the Negative and Positive Controls suggested that some alteration in ruminal VFA patterns may have occurred with the feeding of Macerated alfalfa. However, ruminal sampling done during the trials conducted using alfalfa harvested in 1996 showed no differences in ruminal VFA between the two diets with 60% forage (data not shown). There were no differences due to diet in milk content of protein, lactose and SNF. However, the overall pattern of significance among diets in yields of protein, lactose and SNF was the same as for milk: Greatest on Positive Control, intermediate on Macerated and lowest on Negative Control (Table 4). Maceration increased yields of milk, protein, lactose and SNF all by about 4% over the Negative Control. Over all four trials, there was a clear advantage to feeding Macerated alfalfa versus Control alfalfa silage.

Overall weighted means (Table 4) from the four lactation trials were used to estimate how much maceration increased NEL content of alfalfa silage. The NEL requirements for maintenance (using mean BW) and BW gain were computed based on NRC (1989) values; NEL requirements for milk production were computed from both composition and yield (Table 5). After deducting the NEL estimated to come from the concentrate portion of each diet, dividing the remaining NEL by OM intakes from alfalfa silage yielded estimates of the NEL contents of Control and Macerated forages. Based on these computations, Macerated alfalfa silage fed in these trials had 4.5% greater NEL per unit OM than did Control alfalfa silage.

Summary and Conclusion

Compared to Control, macerating alfalfa immediately after cutting using a maceration-mat machine depressed NPN content when ensiled; however, Macerated alfalfa harvested using our methods had elevated ash and fiber content versus Control. Apparent digestibility of DM and OM was increased by maceration; trends for increased fiber digestibility were not significant. Over the course of four lactation trials, yield of milk, protein, lactose and SNF each was increased by about 4% on Macerated alfalfa silage over that produced feeding equal amounts of Control alfalfa. There was a reduction in milk fat content with Maceration of alfalfa; milk fat content was intermediate on Macerated alfalfa between the Negative and Positive Controls. The NEL content of the OM in Macerated alfalfa silage was increased by about 5% over Control. Maceration improves the nutritive value of alfalfa silage for lactating dairy cows.

Table 2. Mean composition of diets fed during lactation trials.[1]

Ingredient	Negative Control	Macerated	Positive Control
		(% of DM)	
Control alfalfa silage	60.6	0	49.7
Macerated alfalfa silage	0	60.5	0
Processed high moisture corn	34.5	34.6	41.5
Solvent soybean meal	1.0	1.0	4.6
Roasted soybeans	0.8	0.8	0.8
Low-solubles fish meal	2.4	2.4	2.4
Sodium bicarbonate	0	0	0.40
Dicalcium phosphate	0.28	0.28	0.22
Trace mineral salt (+ Se)[2]	0.30	0.30	0.30
Potassium magnesium sulfate[3]	0.04	0.04	0.04
Vitamin ADE concentrate[4]	0.10	0.10	0.10
Composition (DM basis)			
CP, %	18.0	17.8	18.1
NDF, %	30.8	31.6	27.4

[1]Mean compositions of rations fed in five lactation studies using Control and Macerated alfalfa silage harvested during two cuttings each in 1996 and 1997.
[2]Provided (/kg of DM): Mn, 27 mg; Zn, 27 mg; Fe, 17 mg; Cu, 7 mg; I, 0.40 mg; Se, 0.30 mg; and Co, 0.10 mg.
[3]Provided (/kg of DM): Mg, 110 mg; K, 180 mg; S, 220 mg.
[4]Provided (/kg of DM): vitamin A, 3880 IU; vitamin D, 730 IU; and vitamin E, 0.73 IU.

Table 3. Effect of macerating alfalfa silage on apparent digestibility of nutrients in diets containing about 60% forage.[1]

Nutrient	Control (60.6%)[2]	Macerated (60.5%)[2]	SEM[3]	$P > F$[4]
	- - - - - - - - - - - - - % - - - - - - - - - - - -			
DM	60.0	61.1	0.4	0.08
OM	61.9	63.8	0.4	< 0.01
NDF	44.2	45.0	0.6	0.17
ADF	37.9	38.8	0.5	0.32
CP	53.0	54.4	0.7	0.31

[1]Apparent digestibility was estimated using indigestible ADF as an internal marker.
[2]Mean proportion DM from either Control or Macerated alfalfa silage during the four trials.
[3]SEM = Standard error of the mean.
[4]Probability of a significant effect of maceration.

Table 4. Mean performance data from feeding Control or Macerated alfalfa silage to lactating cows.[1]

	Diet[2]				
Item	Negative Control	Macerated	Positive Control	SEM[3]	$P > F^4$
DM intake, kg/d	25.8	26.3	26.3	0.8	0.82
BW change, kg/d	0.38	0.50	0.35	0.20	0.38
Milk, kg/d	36.8[c]	38.2[b]	40.4[a]	0.9	< 0.01
3.5% FCM, kg/d	36.4	37.1	38.4	1.1	0.79
Fat, %	3.50[a]	3.41[b]	3.30[c]	0.12	< 0.01
Fat, kg/d	1.27[b]	1.28[ab]	1.30[a]	0.05	0.04
Protein, %	3.18	3.19	3.32	0.05	0.24
Protein, kg/d	1.16[c]	1.20[b]	1.32[a]	0.03	< 0.01
Lactose, %	4.77	4.80	4.82	0.04	0.59
Lactose, kg/d	1.75[c]	1.83[b]	1.93[a]	0.05	< 0.01
SNF, %	8.66	8.71	8.85	0.09	0.47
SNF, kg/d	3.17[c]	3.30[b]	3.53[a]	0.09	< 0.01
Milk yield : DMI	1.45[b]	1.46[b]	1.55[a]	0.06	0.02

[a,b,c]Means in rows with different superscripts differ ($P < 0.05$).

[1]Mean performance data from five lactation trials weighted for the number of cows in each trial.

[2]Diets contained on average: Negative Control (60.6% Control alfalfa silage); Macerated 60.5% Macerated alfalfa silage); and Positive Control (49.7% Control alfalfa silage).

[3]SEM = Standard error of the mean.

[4]Probability of a significant effect of diet.

Table 5. Effect of maceration on NEL contents of alfalfa silage (AS) estimated from intake and performance data.[1]

	Diet[2]		
Component	Negative Control	Macerated	Positive Control
Maintenance (638 kg), Mcal/d	10.2	10.2	10.2
BW gain, Mcal/d	1.9	2.5	1.8
Milk (composition), Mcal/d	25.8	26.6	28.0
NEL Requirement, Mcal/d	37.9	39.3	39.9
Total DM intake, kg/d	25.7	26.2	26.2
Concentrate DM intake, kg/d	10.2	10.4	13.2
Concentrate NEL,[3] Mcal/d	18.9	19.3	24.5
NEL from AS, Mcal/d	19.0	20.0	15.3
AS DM intake, kg/d	15.5	15.8	13.0
AS NEL, Mcal/kg DM	1.22	1.26	1.18
AS OM intake, kg/d	14.0	14.1	11.7
AS NEL, Mcal/kg OM	1.36	1.42	1.31
Macerated/Control, %	104.5		108.2

[1]Mean performance data from the four lactation trials was weighted for the number of cows in each trial.

[2]Diets contained on average: Negative Control (60.6% Control alfalfa silage); Macerated 60.5% Macerated alfalfa silage); and Positive Control (49.7% Control alfalfa silage).

[3]Mean NEL content of the concentrate portion of the three diets was computed to be 1.86 Mcal/kg DM from NRC (1989) tables.

Milk Production During the Complete Lactation of Dairy Cows Fed Diets Containing Different Amounts of Protein

Z.Wu and L.D. Satter

Introduction

The increase in milk production following incremental additions of protein to the dairy cow diet is a diminishing response, and the point of maximum profitability is likely to be at a dietary protein level that is slightly below that needed for maximum milk production. The objective of this study was to determine milk production response in high producing cows to dietary supplementation of different amounts of protein having low rumen degradability.

Materials and Methods

Fifty-eight multiparous Holstein cows were used in a 44 wk lactation trial. Diets (Table 1) containing 15.4, 17.4 or 19.3% CP were fed during weeks 1 through 16 of lactation to groups of 15, 29 or 14 cows, respectively. Beginning at week 17 of lactation, cows were changed to diets that were fed for the remainder of lactation. Cows that were on the diet with 15.4% CP during the first 16 weeks were kept on a low protein diet (16.0% CP) for the rest of the lactation. Fourteen of the 29 cows fed the 17.4% diet in early lactation were kept at a similar level of protein (17.9% CP) for the rest of lactation, while the remaining 15 cows in that group were fed the 16.0% CP diet. Cows receiving the 19.3% CP diet in early lactation were switched to the 17.9% CP diet at week 17. Thus, the following four treatments were formed over the entire lactation with respect to dietary CP percentages: 15.4 ® 16.0, 17.4 ® 16.0, 17.4 ® 17.9, and 19.3 ® 17.9, with the change occurring at the beginning of week 17. Higher than expected protein content of alfalfa silage fed in the last 28 weeks of lactation resulted in small increases in total dietary protein for this phase of lactation.

Results and Discussion

Milk yield for the 44 week lactation for the 17.4 - 16.0% CP treatment (10,832kg) was 776 kg higher ($P < 0.12$) than that for the 15.4 - 16.0% CP treatment (10,056 kg), and similar to the yields of 11,095 kg and 11,132 kg for the 17.4 - 17.9 and 19.3 - 17.9% CP treatments (Table 2 and Fig. 1). The amount of excreted N, estimated from intake N minus milk N (assuming no net change in tissue N), largely reflected N intake. The efficiencies for converting feed N to milk N, ranging from the lowest to the highest dietary protein treatments, were 28.8, 25.9, 24.1 and 24.7%. Means for dry matter intake did not differ ($P < 0.05$) among dietary protein levels for weeks 1-16 of lactation, but may have been slightly higher for the two high treatments for weeks 17-44 of lactation. Dry matter intake for the lowest protein group was often less than for the other treatments for the first 31 weeks of lactation.

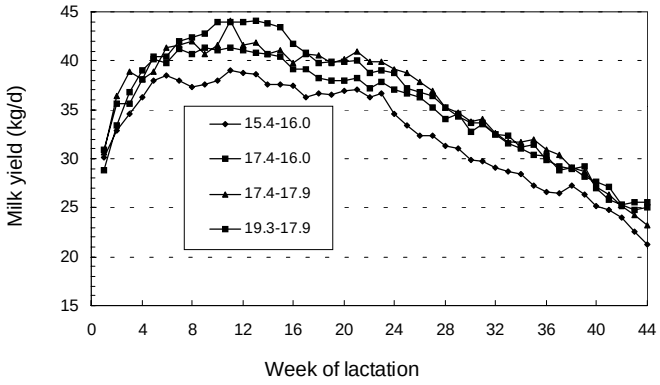

Figure 1. Lactation curves of cows fed diets containing different levels of CP: 15.4% during lactation wk 1 to 16 and 16.0% during wk 17 to 44 (15.4 - 16.0, □), 17.4% during wk 1 to 16 and 16.0% during wk 17 to 44 (17.4 - 16.0, ■), 17.4% during wk 1 to 16 and 17.9% during wk 17 to 44 (17.4 - 17.9, ○), and 19.3% during wk 1 to 16 and 17.9% during wk 17 to 44 (19.3 - 17.9, ●).

Conclusions

Issues related to nutrient management as well as costs associated with excessive protein supplementation point to the need for closer matching of animal requirements with dietary allowance of protein. It is suggested that early lactation diets contain approximately17.5% CP, 35-37% of which is rumen undegradable. Reduction of dietary protein in later

lactation as milk production declines needs to be done cautiously, and for cows receiving BST and producing approximately 11,000 kg/lactation or more, this reduction should not occur before midlactation, and then not be reduced to below approximately 16% CP. This recommendation assumes that the supplemental protein offered throughout lactation will have approximately 50% rumen undegraded protein.

Table 1. Ingredients and chemical analysis of diets varying in crude protein content during lactation weeks 1 to 16 and 17 to 44.

	wk 1 - 16			wk 17 - 44	
Item	15.4% CP	17.4% CP	19.3% CP	16.0% CP	17.9% CP
	- - - - - - - - - - - - - - - - (% of DM) - - - - - - - - - - - - - - - -				
Alfalfa silage	33.0	33.0	33.0	33.0	33.0
Corn silage	22.0	22.0	22.0	22.0	22.0
HMEC[1], finely ground	32.0	27.0	22.0	32.6	27.6
Soybeans, roasted	10.0	10.0	10.0	10.0	10.0
Soybean meal, expeller process[2]	…	5.0	10.0	…	5.0
Mineral and vitamin mix	3.0	3.0	3.0	2.4	2.4
Chemical analyses					
CP	15.4	17.4	19.3	16.0	17.9
RUP, % of CP[3]	33.5	35.8	37.6	33.5	35.8
NDF	29.3	29.6	30.0	29.0	29.4
ADF	20.1	20.3	20.5	20.0	20.2
NE_L, Mcal/kg[3]	1.64	1.64	1.65	1.61	1.61

[1]High moisture ear corn.
[2]Soyplus® (West Central Cooperative, Ralston, IA).
[3]Estimated based on feedstuff values (NRC, 1989).

Table 2. Milk yield, intake N, milk N, and manure N of cows fed diets varying in CP content during 308-d lactation.

	Treatment[1]					P*		
Item	15.4-16.0 (n = 15)	17.4-16.0 (n = 15)	17.4-17.9 (n = 14)	19.3-17.9 (n = 14)	SEM	a > b	b > c	a > c
	- - - - - - - - - - - (kg) - - - - - - - - - - -							
Milk	10,056[c]	10,832[b]	11,095[a]	11,132[a]	349	…	0.12	0.04
3.5% FCM	10,690b[b]	11,628[a]	11,804[a]	11,559[a]	368	…	0.11	0.07
Intake N	177.8[c]	189.1[b]	213.7[a]	214.2[a]	4.0	0.01	0.05	0.01
Milk N	51.2	48.9[b]	51.5	53.0[a]	1.6	0.08	…	…
Manure N[2]	126.6[c]	140.2[b]	162.2[a]	161.2[a]	3.8	0.01	0.01	0.01

*Values without superscripts do not differ ($P > 0.15$) from other values within a row.
[1]Treatments varying in dietary CP content: 15.4% during lactation week 1 to 16 and 16.0% during wk 17 to 44 (15.4-16.0), 17.4% during wk 1 to 16 and 16.0% during wk 17 to 44 (17.4-16.0), 17.4% during wk 1 to 16 and 17.9% during wk 17 to 44 (17.4-17.9), and 19.3% during wk 1 to 16 and 17.9% during wk 17 to 44 (19.3-17.9).
[2]Calculated from intake N - milk N, assuming no deposition or mobilization of tissue N.

Effect of Corn Particle Size, Moisture Level and Frequency of Feeding on Performance and Nutrient Utilization by Lactating Dairy Cows

F. San Emeterio, R.B. Reis, W.E. Campos and L.D. Satter

Introduction

Corn processing can be important for improving ruminal starch utilization as well as overall starch digestion. The objective of this experiment was to evaluate the effect of moisture level and particle size of corn grain on milk production, nutrient utilization and ruminal fermentation. An additional objective was to evaluate once or twice daily feeding of high energy diets to relatively high producing dairy cows.

Materials and Methods

Thirty-six Holstein cows were assigned to six squares according to their lactation number and the presence of a rumen cannula. Eighteen cows were assigned to the six different treatments and were fed once daily. Within this group of 18 cows, six had a rumen fistula and were used to evaluate nutrient digestibilities and ruminal fermentation. The remaining 18 cows were similarly assigned, except that they were fed twice daily. Cows were randomly assigned to each of the treatments in a 6 x 6 Latin square design replicated three times for each frequency of feeding. Periods were 21-d long; the first 14-d were considered as an adaptation period, and the last 7-d constituted the sampling period. Six diets, fed as TMR, were identical except for the corn portion of the diet. Corn treatments tested were dry shelled corn (DSC), high moisture ear corn (HMEC) and high moisture shelled corn (HMSC), either coarsely (CG) or finely (FG) ground. The six dietary treatments were DSC-CG, DSC-FG, HMEC-CG, HMEC-FG, HMSC-CG and HMSC-FG.

Diets were formulated to contain 50% DM, 1.69 Mcal NEL/kg DM, 17.5% CP, 32.8% NDF, 19.7% ADF and 37.2% NSC. Forage to concentrate ratio was 45:55. Dry matter content of the grain was: 88.9%, DSC; 70.0%, HMEC; and 68.1%, HMSC. Mean particle size (mm) for the corn treatments were 3.28, DSC-CG; 1.11, DSC-FG; 4.43, HMEC-CG; 1.32, HMEC-FG; 3.78, HMSC-CG; and 1.02, HMSC-FG.

Ytterbium was used as an external marker to estimate apparent total tract digestibility. Purine derivatives measured in a urinary spot sample were used as an indirect method for estimating rumen microbial protein synthesis.

Results and Discussion

Cows fed once daily. Production of milk was not affected by treatment (Table1). Percentage of milk fat and milk fat yield trended lower (P = .09) for the high moisture corn and for the finely ground corn. Milk protein content and yield were not affected by treatment. Dry matter, OM, NDF and ADF digestibilities were not affected by treatment, but digestibilities of starch and free glucose were highest with the finely ground high moisture corn treatments. The allantoin: creatinine ratio was highest for the HMSC-FG treatment, indicating greater microbial protein production in the rumen.

Cows fed twice daily. Milk yield was significantly higher with the HMSC treatments, and milk fat test was lower (Table 1). Fine grinding of corn tended to reduce fat test across all treatments. Fine grinding increased the digestibility of starch, and tended to increase the allantoin:creatinine ratio. Ruminal pH was lowest for the HMSC treatments.

Effect of feeding frequency. Production of fat corrected milk was increased (P < .07) (Table 2), along with total milk fat production (P < .07) when cows were fed twice rather than once daily.

Conclusions

Feeding HMSC compared to HMEC resulted in higher milk production and slightly lower milk fat

percentage, reflecting the higher energy content of HMSC. The digestibility of starch + free glucose was improved by feeding high moisture corn and by fine grinding. Higher starch digestibility tended to increase microbial growth in the rumen. Feeding twice daily rather than once daily tended to increase milk production, but had little effect on nutrient utilization.

Table 1. Performance of cows.

	DSC CG	DSC FG	HMEC CG	HMEC FG	HMSC CG	HMSC FG	SEM	Trt P
Once daily feeding								
Milk, kg/d	37.7	37.2	34.9	36.3	37.9	36.2	1.57	0.26
Milk fat, %	3.60	3.58	3.49	3.29	3.49	3.25	0.14	0.09
Milk protein, %	3.02	3.05	3.08	2.96	3.03	3.13	0.05	0.12
Intake, kg/d								
Dry matter	25.8	24.7	23.3	22.8	24.6	24.9	1.58	0.25
Starch[1]	9.13	8.73	8.26	8.09	8.73	8.86	0.56	0.25
Digestibility, %								
Dry matter	62.6	69.3	64.9	67.9	67.4	68.5	2.39	0.31
Starch[1]	80.4^{e}	88.1^{bc}	85.5^{cd}	90.2^{ab}	84.1	91.8^{a}	1.37	<.01
Fecal starch[2]	18.8^{a}	13.7^{b}	14.8^{b}	10.7^{c}	15.7^{b}	9.32^{c}	0.79	<.01
Urine constituents								
A:C ratio	2.67^{bc}	2.54^{bc}	2.34^{c}	2.96^{ab}	2.79^{ab}	3.10^{a}	0.20	0.01
Rumen								
pH	6.12	6.09	6.10	5.99	5.96	5.94	0.09	0.09
NH_3, mM	10.2^{a}	9.32^{a}	8.74^{ab}	6.79^{c}	7.43^{bc}	6.98^{c}	0.61	<.01
Tot VFA, mM	121.7	125.0	122.6	127.5	28.2	130.9	3.84	0.30
C_2:C_3 ratio	3.19^{a}	3.05^{a}	3.15^{a}	2.95^{a}	2.84^{ac}	2.47^{bc}	0.24	0.04
Twice daily feeding								
Milk, kg/ d	37.2^{c}	38.9^{abc}	38.0^{bc}	37.8^{c}	39.3^{ab}	40.3^{a}	1.19	<.01
Fat, %	3.82^{a}	3.67^{ab}	3.78^{ab}	3.72^{ab}	3.59^{b}	3.31^{c}	0.12	<.01
Protein, %	3.10	3.08	3.03	3.09	3.11	3.09	0.04	0.46
Intake, kg/ d								
Dry matter	26.3	26.8	25.2	25.2	25.8	25.4	1.87	0.69
Starch[1]	9.31	9.47	8.94	9.53	9.16	9.03	0.76	0.81
Digestibility, %								
Dry matter	65.6	65.9	62.7	66.6	67.1	69.2	2.45	0.55
Starch[1]	84.4^{c}	86.5^{bc}	83.2^{c}	89.7^{ab}	86.7^{bc}	91.2^{a}	1.48	0.01
Fecal starch[2]	18.7^{a}	13.8^{b}	15.9^{b}	10.8^{c}	14.3^{b}	10.1^{c}	1.08	<.01
Urine constituents								
A:C ratio	2.58	2.69	2.93	3.36	2.78	3.04	0.22	0.17
Rumen								
pH	5.99^{a}	5.97^{a}	5.94^{ab}	5.81^{bc}	5.80^{c}	5.80^{c}	0.07	0.02
NH_3, mM	10.9^{a}	10.1^{ab}	8.88^{bc}	7.53^{c}	8.61^{bc}	7.04^{c}	0.67	<.01
Tot VFA, mM	127.8^{c}	132.2^{bc}	131.9^{bc}	135.9^{ab}	137.2^{ab}	140.2^{a}	3.10	0.02
C_2:C_3 ratio	3.18^{a}	3.05^{a}	2.87^{ab}	2.5^{bc}	2.46^{bc}	2.13^{c}	0.24	<.01

[1]Includes free glucose

[2]Includes free glucose, and is expressed as % of fecal DM.

Table 2. Effect of frequency of feeding.

	Fed once daily	Fed twice daily	SEM	Frequency *P*
Milk, kg/d	36.7	38.6	1.07	0.22
4% FCM, kg/d	33.6	36.5	1.11	0.07
Milk fat, %	3.45	3.65	0.09	0.15
Milk protein, %	3.04	3.07	0.04	0.48
Intake, kg/d				
Dry matter	24.3	25.8	1.54	0.51
Digestibility, %				
Dry matter	66.7	66.2	1.33	0.77
Urine constituents				
A:C ratio	2.74	2.90	0.14	0.39
Rumen				
pH	6.04	5.89	0.07	0.10
NH_3, mM	8.24	8.85	0.32	0.17
Tot VFA, mM	126.0	134.2	2.36	0.01
C_2:C_3 ratio	2.94	2.70	0.18	0.35

Increased Conjugated Linoleic Acid (CLA) in Milk Fat of Grazing Cows is not Explained by More CLA Production in the Rumen

Z. Wu, M.N. Lahlou, L.D. Satter, L. Massingill and M.W. Pariza

Introduction

Grazing and feeding high-oil diets increase the concentration in milk of conjugated linoleic acid (CLA), a demonstrated anticarcinogen. The increase has been attributed to more production of CLA as an intermediate in biohydrogenation of linoleic acid (18:2(n-6)) in the rumen. This theory explains the increase in milk CLA during oil feeding but cannot satisfactorily explain the increase during grazing, because formation of CLA (*cis* 9, *trans* 11 18:2) from 18:2(n-6) is known but formation from 18:3(n-3) is unknown. The predominant fatty acid in pasture forage is linolenic acid (18:3(n-3)) (60 to 70%) rather than 18:2(n-6) (5 to 20%). The objective of this study was to determine the relationship between production of CLA in the rumen and its secretion in milk, utilizing cows fed a TMR in confinement or grazing a mixed grass-legume pasture.

Materials and Methods

The experiment was conducted in June to July. Eight ruminally cannulated multiparous Holsteins were paired based on milk yield and each member of the pair was fed a TMR or grazed on a grass-legume (2 : 3) mixed pasture. Grazing cows were supplemented with a grain mix. After 21 days, cows within a pair were changed to the opposite diet and continued with the new diet for another 21 days. The TMR consisted of alfalfa silage, corn silage, and a concentrate portion containing primarily corn and soybean meal. The grain supplement offered to the grazing cows was the same as the concentrate contained in the TMR. The supplement was fed at the rate of 8.4 kg/day (DM), accounting for about 40% of the total feed intake. Rumen contents, omasal contents, blood plasma, and milk were sampled during the last 7 days in each of the 21-day periods and analyzed for CLA concentrations.

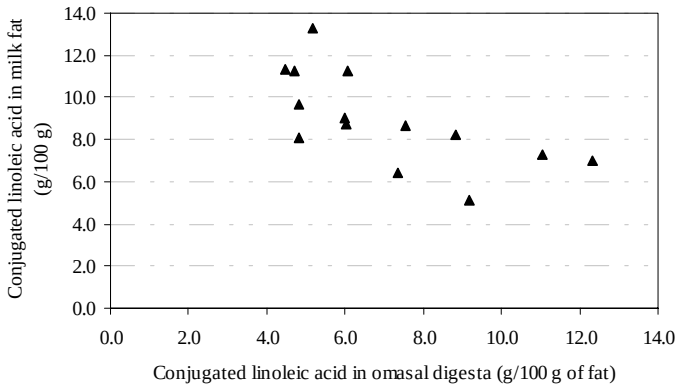

Figure1. Concentration of CLA in milk and omasal digesta fat.

Results and Discussion

Fat content of milk did not differ between treatments, but the concentration of CLA in milk fat was approximately 50% higher ($P < 0.05$) during grazing than during TMR feeding (Table 1). Unlike milk fat, fatty acids contained in ruminal and omasal digesta had lower ($P < 0.05$) concentrations of CLA during grazing than during TMR feeding. Figure 1 illustrates a negative relationship between CLA content in milk fat and CLA content of fat in omasal digesta.

Because the increased concentration of CLA in milk during grazing cannot be explained by production of CLA in the rumen, sources of milk CLA other than from metabolism in the rumen must exist. Possibly, CLA can be formed from *trans* 18:1(n-7) in body tissues by the activity of D-9-desaturase, an enzyme known to convert 18:0 to 18:1(n-9) in mammary microsomes. Conversion of *trans* 18:1(n-7) to *cis*-9, *trans*-11 18:2 (CLA) by this enzyme has not been demonstrated in vivo. However, a recent study (Corl et al. 1998) showed increased concentrations of conjugated *cis*-9, *trans*-11 18:2 and non-conjugated *cis*-9, *trans*-12 18:2 in milk fat when *trans* 18:1 isomers (n-7) and (n-6) were infused into the abomasum, substantiating this possibility. In the present study the concentration of CLA in blood plasma doubled during grazing as compared with TMR feeding, supporting the idea of synthesis of CLA in the body. It also suggests that the synthesis can occur in tissues other than the mammary gland.

If CLA is synthesized in the body, then why does grazing enhance this synthesis? Conceivably, availability of the essential cofactors that are involved in the D-9-desaturase activity could be a factor. However, a hypothesis that is gaining support is related to milk fat liquidity. A liquid form of milk fat is necessary for milk fat droplets to move to the surface of the secretory cell to be pinched off and for fat globules to be suspended in milk (Moore and Christie 1979). Normally, liquidity is assured by acylation of short chain fatty acids (low melting point) to the *sn*-3 position of the glycerol moiety. If short chain fatty acids are less available, 18:1(n-9), also low in melting point (14 °C), could be substituted. In general, short and medium chain fatty acids decrease in milk fat during grazing. Thus, more CLA may be synthesized during grazing in response to reduced supply of short and medium chain fatty acids. Because *trans* 18:1(n-7) (melting point 44 °C) does not exist as a liquid at body temperature and physicochemically resembles 18:0 (melting point 69 °C) rather than 18:1(n-9), its conversion into CLA (*cis*-9, *trans*-11 18:2) would be physiologically sound. Interestingly, desaturation of 18:0 is inhibited by short and medium chain fatty acids. If this was also true for desaturation of *trans* 18:1(n-7), more CLA would be produced when short and medium chain fatty acids are in low supply.

Conclusions

The content of CLA in milk was higher when cows were grazed on pasture than when fed a TMR. The increase in milk CLA could not be explained, however, by more CLA production in the rumen. Rather, higher CLA content of milk fat in grazing cows appeared to be related to desaturase activities in the body. It is possible that increased CLA production in the body is in compensation for a decreased supply of short and medium chain fatty acids required for maintenance of milk fat liquidity.

Reference

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Table 1. Fatty acids in rumen contents, omasal contents, blood plasma, and milk fat of cows fed a total mixed ration or grazed on pasture.

Fatty acid	TMR	Grazing	SEM	*P*
Rumen contents				
Total fatty acids, % of DM	2.99	3.38	0.07	0.01
CLA, g/100 g of total fatty acids	0.43	0.27	0.04	0.02
Omasal contents				
Total fatty acids, % of DM	3.12	3.39	0.13	0.19
CLA, g/100 g total fatty acids	0.88	0.52	0.07	0.02
Blood plasma CLA, mg/l	0.53	1.04	0.16	0.07
Milk fat, %	3.21	3.19	0.09	0.89
CLA, g/100 g of total fatty acids	0.72	1.07	0.07	0.02

Milk Production of Fall Calved Cows Fed Total Mixed Rations in Early and Mid/ Lactation Followed by Grazing Grass or Grass-Clover Pastures in Late Lactation

Z. Wu, L. Massingill, V.R. Kanneganti, L.D. Satter, R.P. Walgenbach and M.C. Wiltbank

Introduction

Intensive rotational grazing can be an economic way of feeding dairy cows. Achieving high milk yield in early lactation, however, is difficult. To better capitalize on the potential economic advantage of grazing, as well as the cow's milking potential in early lactation, a fall calving strategy was employed. Cows were fed a total mixed ration under confinement for the first 200-225 days of lactation to maximize milk production. This was followed by grazing for the latter stage of lactation.

Most unimproved pasture in the northern United States consists of grass species, with a small proportion of legumes. Inclusion of legumes can increase the nutritive value of pasture. In addition to the fall calving strategy, milk production response to frost seeding of red clover in the pasture was measured.

Materials and Methods

The experiment was carried out for two years using pasture located at the U.S. Dairy Forage Research Farm in Prairie du Sac, Wisconsin. In year 1, the pasture had two distinct types of paddocks, one containing mixed grasses only and one containing mixed grasses, white clover, and red clover. Red clover and white clover accounted for 26% of the forage in the mixed paddocks. Kentucky bluegrass, quackgrass, and smooth bromegrass were the principal grasses. In year 2 all paddocks were similar, containing almost all grass, with only negligible clover. For both years, cows calved during September and October. Fall-calving was achieved by synchronizing for breeding using GnRH and PGF_{2a}. After calving, cows were fed a total mixed ration, then grazed from April to August when pasture was available. Grazing lasted 11 weeks in year 1 and 18 weeks in year 2. At the beginning of grazing, cows averaged 220 (SD 15) days in milk in year 1 and 203 (SD 17) days in year 2.

A supplement mix consisting mainly of high moisture ear corn and roasted soybeans was fed during grazing. The amount of supplement dry matter fed was 6.2 kg/d in year 1 and 7.9 kg/d in year 2. This accounted for approximately 35 to 40% of total dry matter intake. In year 2, cows were administered bST every 2 weeks. In each year, 40 cows (20 primiparous in year 1 and 12 primiparous in year 2) were grazed. Of the 40 cows in year 1, 27 (14 primiparous) grazed the grass paddocks and 13 (6 primiparous) grazed the mixed paddocks. In year 2, all 40 cows grazed grass paddocks as one group. Intensive rotational grazing was practiced by using electric fences to allocate grazing areas. Fresh paddocks were provided every 24 hr.

Results and Discussion

Milk yield declined upon turning cows out to pasture (Fig. 1). The decline was less in year 1 with the grass-legume pasture than for the all grass pasture. The decline in milk production upon turning cows out to pasture in year 2 was even more evident (Fig. 2). This may reflect the higher milk production level when cows were placed on pasture, probably due to the use of bST, as well as the absence of legume species in the pasture. Total lactation yields for a 308 day lactation were: (year 1) 19,520 lb for cows on all grass, and 20,120 lb for the mixed grass-legume pasture; (year 2) 22,120 lb for all cows given bST and on all grass pasture.

Conclusions

Total milk production was about 4,000 lb higher in this study for fall calved cows pastured in late lactation compared to spring calved cows pastured in early lactation (Dhiman and Satter 1996). While milk production still declined in late lactation upon turning cows out to pasture, the cumulative loss was much less than the early lactation cows experienced.

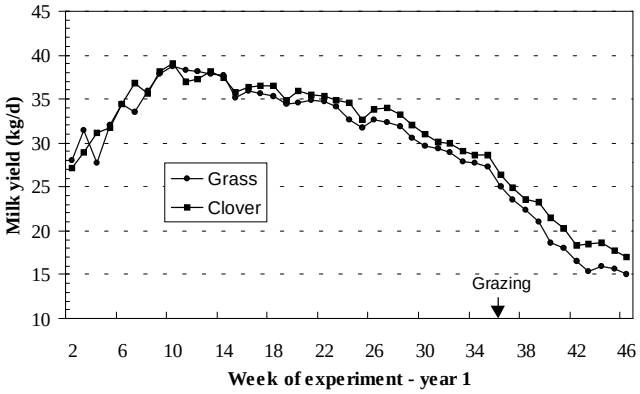

Figure 1. Milk yield of cows grazing grass pasture or grass-clover pasture during wk 36 to 46 of year-one experiment.

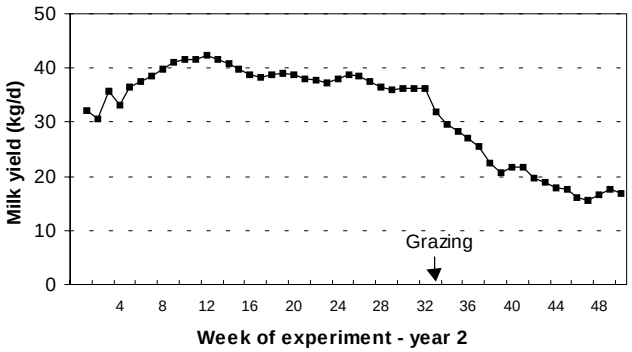

Figure 2. Milk yield of cows grazing grass pasture during wk 33 to 50 of year-two experiment.

Reference

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Milk Production and Reproductive Performance of Dairy Cows Fed Low or Normal Phosphorus Diets: Year Two

Z. Wu and L.D. Satter

Introduction

Last year we reported that cows receiving no supplemental phosphorus (P) produced as much milk during a complete lactation and had similar reproductive performance as cows supplemented with phosphorus. The experiment has been continued for another lactation, with the objective being to determine response of lactating cows to reduced dietary P over a minimum of two lactations.

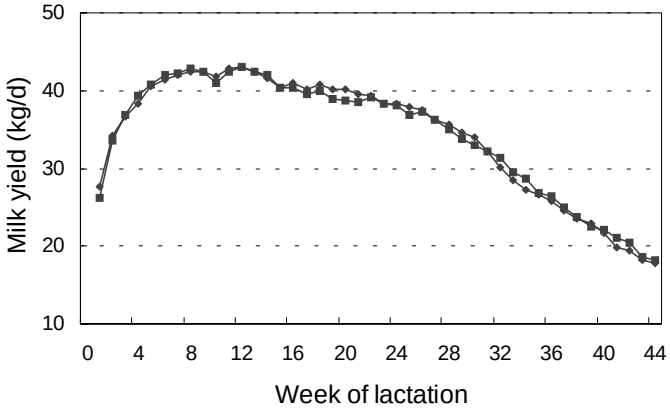

Figure 1. The lactation curve of cows fed diets containing low (0.37%, ◆) or high (0.47% ■,) P.

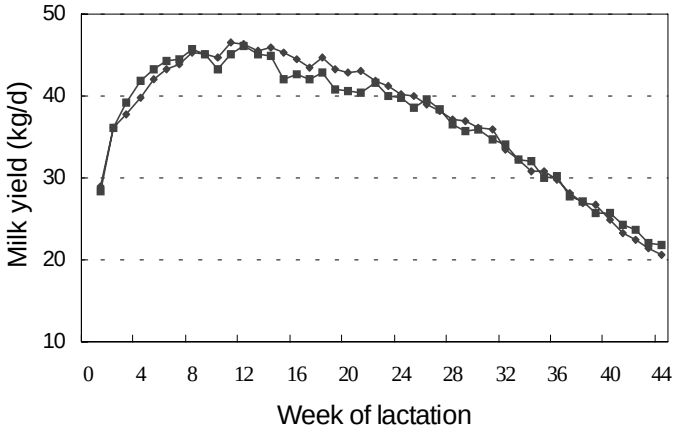

Figure 2. The lactation curve of cows fed diets containing low (0.37%, ◆) or high (0.47% ■,) P for a second year of lactation.

Materials and Methods

Fifty-three cows were used in this second year trial. The lactation period included confinement feeding for approximately the first 2/3 of lactation, and grazing for the last 1/3 of lactation. Thirteen of the 53 cows were in confinement for the entire lactation. A supplemental grain mix was fed during grazing. Twenty-six cows received no supplemental P (dietary P was ~ .37% of dry matter during confinement and ~.31% during grazing) and 27 cows received NaH_2PO_4 and $CaHPO_4$ to increase dietary P to ~ .47% during confinement and ~.44% during grazing. Thirty of the cows were in their second year of experiment.

Results and Discussion

Milk yields for the entire lactation were not different between treatments. This was true when all 53 cows were used to obtain treatment averages, and also when just the 30 cows in their second year of the experiment were evaluated (Table 1). At no time during lactation did milk yield for the two groups appear different (Fig. 1 and 2)

The lack of difference in lactation performance due to dietary P level is consistent with results of several other long (Brintrup et al. 1993; Steevens et al. 1971) or short term (Dhiman et al. 1995) studies. The dietary P concentrations compared in these studies were .33 vs. .37%, .4 vs. .6%, and .39 vs. .65%, respectively. In another study we conducted, milk yield was similar for cows given either .40 or .49% P in their diet dry matter (11,226 vs. 11,134 kg/308 d), but was reduced (10,790 kg/308 d) when only .31% P was fed, resulting from lower production during the late part of lactation. These studies indicate that feeding .32% P may not harm milk production of low producing cows (7,500 kg per lactation), but .37 to .40% P is recommended for high producing cows (> 10,000 kg per lactation).

Feeding low P showed no adverse effect on reproductive performance of cows (Table 2). This is consistent with eight other studies reviewed. Data summarized from these experiments involving 730 cows indicate no influence of dietary P level on reproductive efficiency. Impaired production appears unlikely unless P levels are extremely low (< .25%). This rarely occurs with lactating dairy cows because their diets usually include protein supplements, which are high in P.

Conclusions

Consistent with the results in year one, reducing dietary P from .47 to .37% did not affect milk production or reproductive performance. Cows receiving the low P diet for a second year performed normally. Phosphorus at .37-.40% is adequate for high producing cows.

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Table 1. Lactation performance of cows fed diets containing low or high phosphorus.

	All cows				Second year cows			
Item	Low P (n = 26)[1]	High P (n = 27)[1]	SEM	*P*	Low P (n = 14)	High P (n = 16)	SEM	*P*
Milk, kg/308 d	9,864	9,898	340	0.94	11,457	11,358	404	0.86
Milk fat								
%	3.78	3.65	0.10	0.38	3.66	3.63	1.20	0.85
kg/d	1.23	1.20	0.04	0.68	1.39	1.34	0.04	0.47
Milk protein								
%	3.14	3.14	0.04	0.96	3.06	3.15	0.05	0.23
kg/d	1.01	1.03	0.03	0.73	1.16	1.17	0.03	0.88
Milk lactose, %	4.81	4.87	0.05	0.44	4.79	0.05	0.74	
Milk SNF, %	8.72	8.77	0.07	0.65	8.60	8.71	0.08	0.31
Milk SCC, 10^3/ml	230	407	82	0.14	259	373	102	0.44

[1]Includes 6 primiparous cows in each group.

Table 2. Reproductive measures of lactating cows fed diets containing low or high phosphorus.

	All cows				Second year cows			
Measure	Low P (n = 26)	High P (n = 27)	SEM	*P*	Low P (n = 14)	High P (n = 16)	SEM	*P*
Days to first estrus	47.8	60.7	8.4	0.28	48.7	73.6	10.0	0.09
Days to first AI	65.6	72.2	5.7	0.42	67.2	80.7	7.4	0.21
Days open[1]	103.3	104.7	12.6	0.94	104.8	109.8	17.1	0.83
Conception rate at first AI, %	42.3	28.0	- -	- -	42.9	12.5	- -	- -
Pregnancy rate								
Before 120 DIM, %	61.5	50.0	- -	- -	64.3	43.8	- -	- -
End of lactation, %	96.4	86.4	- -	- -	85.7	56.3	- -	- -
Services/conception1	1.6	2.1	0.2	0.13	1.5	2.0	0.2	0.13

[1]Includes only the cows that ultimately became pregnant.

Effect of Mechanical Processing on the Utilization of Whole-Plant Corn Silage by Lactating Dairy Cows

M. Bal[1], R. Shaver[1] and L.D. Satter
[1]Dairy Science Department, University of Wisconsin

Introduction

Corn silage harvested at a maturity greater than 2/3 milk line (less than 60% whole-plant moisture) results in reduced milk yield mostly due to inefficient starch utilization in the rumen. Breaking of whole corn kernels in corn silage can decrease the passage of undigested starch to the manure. The objectives of this experiment were to examine the effect of rolling corn silage on silage composition, intake, total nutrient digestibilities, milk production, milk composition, and body weight gain of lactating dairy cows.

Materials and Methods

Corn silage was harvested at the 1/2 milk line stage of kernel maturity with or without rolling using a John Deere self propelled chopper. Theoretical length of cut was increased with the rolled silage to give similar mean particle length for the rolled (9.8 mm) and unrolled (9.9 mm) silages. Twenty-nine multiparous (47 days in milk) and thirteen primiparous (52 days in milk) Holstein cows were randomly divided between the two groups. A 2-wk covariant adjustment period preceded a 16-wk experimental period. Diets (Table 1) consisted of 50% forage (2/3 corn silage: 1/3 alfalfa silage) and 50% concentrate (DM basis). Dry matter intake and milk production were recorded daily. Milk composition was determined from biweekly a.m. and p.m. samples. Total tract nutrient digestibility was determined once during the 8th week of the experiment using ytterbium as an external marker.

Results and Conclusions

Overall, results indicate that mechanical rolling of corn silage did not improve performance of lactating dairy cows in this experiment (Table 3). The high proportion of broken kernels in the control (82%) relative to the rolled corn silage (99%) may have been responsible for this. Although starch digestibility was improved with roller milling, the small proportion of unbroken kernels in the control silage may have precluded a better response in this trial. There was no effect of corn silage processing on body condition or body weight change in this experiment.

Table 1. Ingredient and chemical composition of diets.

Ingredient	Control	Rolled
	% DM basis	
Alfalfa silage	16.0	16.0
Control corn silage	34.0	-
Rolled corn silage	-	34.0
High moisture ear corn	27.0	27.0
Roasted soybeans	12.0	12.0
Soybean meal	8.0	8.0
Limestone	1.0	1.0
Dicalcium phosphate	.7	.7
MgO	.2	.2
Trace mineralized salt	.5	.5
Sodium bicarbonate	.6	.6
Chemical composition		
Crude protein	16.2	16.3
ADF	16.7	16.9
NDF	27.8	28.4
Starch	24.7	25.2

Table 2. Chemical composition of control and rolled silages.

	Control	Rolled
Moisture		
Ensiled	62.5	62.7
Fresh	64.8	61.5
	% of DM	
Crude Protein	7.1	7.0
NDF	39.4	38.2
ADF	23.7	23.0
Starch	24.5	23.2

Table 3. Dry matter intake, milk yield, milk composition and yield, and nutrient digestibilities.

	Control	Rolled	P <
Dry matter intake, kg/d			
Primiparous	21.9	21.9	NS
Multiparous	26.6	26.5	NS
Milk yield, kg			
Primiparous	37.5	35.4	.01
Multiparous	44.2	43.6	NS
Milk fat, %			
Primiparous	3.51	4.12	.01
Multiparous	3.66	3.76	NS
Milk fat, kg/d			
Primiparous	1.37	1.45	NS
Multiparous	1.60	1.65	NS
Milk protein, %			
Primiparous	3.22	3.14	NS
Multiparous	2.99	3.06	.09
Milk protein, kg/d			
Primiparous	1.23	1.12	.05
Multiparous	1.32	1.33	NS
Starch Digestibility, %			
Primiparous	85.6	86.6	NS
Multiparous	83.8	87.9	.09

Farm/Herd Report - Wisconsin

U.S. Dairy Forage Research Center - Annual Dairy Operations Report January 1999

L.L. Strozinski - Herd Manager

1998 has been a very good year at the farm. Favorable weather with adequate moisture and moderate temperatures coupled with favorable milk production and prices has made for smooth sailing throughout the year. Herd size has increased slightly from what it was a year ago, however, planned culling will maintain the milking herd in the 350 cow range. Calf mortality remains very low in our herd and even though we have sold a few heifer calves which were very small at birth, herd replacement numbers are up. This should increase our culling abilities or perhaps provide some marketing opportunities in the future. Current average milk production per cow per day is 74.6 pounds. Our DHIA rolling herd averages for milk, fat and protein decreased somewhat during the year but is now on the upswing with numbers similar to those of a year ago. The farm "mailbox" net price received per hundred weight of milk ranged from $12.58 to $17.53 with an average of $16.48 for 1998.

A number of our cows have received special recognition this year by the Holstein Association and DHIA for their performance. In addition, a few daughters of young sires have been receiving a lot of attention by some of the bull studs. In 1998 we contracted the sale of a bull calf from one of our cows to Accelerated Genetics for $3500.

Cull cattle prices have generally remained low during 1998. Bull calf prices on the open market have remained extremely low. In 1997 we received an average of $34.97 per calf for our bull calves. In 1998 we began marketing our calves through a new program initiated by the local Cooperative. They are purchasing all bull calves weighing 80 pounds and over for $63.00 plus a $10.00 coupon which can be used for feed purchases at the Co-op. Calves are taken to contract growers. This program has added significantly to our cattle sales income; however 1998 revenues lag behind last year's total.

Research activities in the dairy operation continued at a high level in 1998 with 408 animals involved in 15 different experiments during the year. A cooperative project was undertaken in 1998 with the USDA Livestock Behavior Research Unit at Purdue University. Approximately 20 calves were used in a tail-docking/fly avoidance experiment at the farm.

In last year's report I announced the initiation of a cooperative program with Trans-Ova Genetics. During the year we have collected and sold colostrum from more than 200 cows to Trans-Ova. In addition, we have sold cull cows to them for use in their program. Revenues from this program totaled more than $10,000. The leukosis free status and the potentially Johnne's free status of our closed herd is of keen interest to transgenic dairy development groups. Future developments could be interesting!

A new cooperative program was started this year with the Wisconsin Department of Agriculture, Trade and Consumer Protection. We assist the Milk Standards Unit by providing milk from select cows in the herd to be used as standards for instrument calibration and daily performance controls for dairy plants throughout the state. It's a small project but it generates good relationships.

Numerous facility improvements were made in 1998. A new tile floor was installed in the milkroom and a new computer controlled clean-in-place wash system was purchased for the milking equipment. Two years ago we installed a sprinkling cooling system for the cows in our free-stall facility. Although it worked well, I felt it could be improved if fans could be added to provide more evaporative cooling. When the UW Graduate School and the All Campus Animal Care and Use Committee undertook an animal facilities improvement initiative, I submitted a proposal to add fans to our system. My request was approved and the

$5300 cost was split by the Graduate School and CALS. New drive-by feed bunks on gravel pads were constructed at two of our pasture lots. These feeding areas greatly reduced feeding time and pasture destruction around the feeding areas.

During the year the Agricultural Research Stations Department received permission to hire Experimental Farm Laborers using a critical recruitment procedure which streamlined the hiring system. The system seems to work well and the dairy crew is currently fully staffed.

Herd Statistics		Change from previous year
Herd Inventory		
Milking cows	314	+34
Dry cows	41	-16
average cow age	46 months	+1
percent first lactation	35%	-6
percent second lactation	29%	+2
percent third lactation	17%	0
percent greater than third	17%	+2
Herd replacements	339	+24
Total	**694**	**+42**
Rumen Fistulated Cows	22	0
Herd Performance		
Cows calved	391	+58
Heifer calves live	181	+21
Heifer calves born dead	25	+12
Bull calves born live	169	-12
Bull calves born dead	39	+25
Heifer calves died < 1 year old	2 (1.1%)	-2
DHIA rolling herd average		
milk	21,889 lbs	-579
protein	692 lbs.	-28
fat	810 lbs.	-66
Milk sold in 1998	6,986,355 lbs.	+48,584
Heifer calves sold	14	-3
Bull calves sold	169	-11
Cows sold	106	-38
cows culled for:		
reproduction problems	25	-18
poor production	9	-6
poor udder	19	-8
poor feet and legs	9	-2
mastitis	29	-2
other	15	-2
Cattle sales revenue	$62,475.52	-10,079.91
Herd Reproduction		
Average days open	120	-5
Average calving interval	12.95 months	-.04
Average services per conception	2.1	-.5
Average age at first calving	24 months	0

U.S. Dairy Forage Research Center - Annual Field Operations Report January 1999

R.P. Walgenbach - Farm Manager

The spring of 1998 began the growing season with above average temperatures. The 1998 spring followed three consecutive springs where temperatures were well below normal. The winter was very mild which left little to no frost in the soil in early spring. A lack of frost contributed to ideal conditions for early planting (Table 1) and a very good start to forage growth in the spring. Accumulated growing degree days were well above average for the growing season. The mild winter did little damage to overwintering alfalfa and other perennial forage crops. The warm spring stimulated rapid alfalfa growth in April and early May. Alfalfa growth and development appeared to be about 2 to 3 weeks ahead of average spring growth which resulted in the cutting of our first alfalfa field on 16 May (Table 2). Typically our first alfalfa cutting will occur around 27 to 30 May. This early start to cutting set the stage for four growing season harvests for several fields and along with timely rainfall produced excellent yields of dry matter (DM). Rainfall recorded at the farm entrance rain gauge in inches were 3.40 in April, 5.62 in May, 7.30 in June, 2.32 in July, 4.64 in August, 3.77 in September, 2.58 in October, 1.91 in November and 0.12 in December. We continue to plant a large number of acres of all crops with little or no tillage, although we did increase the amount of surface tillage this past cropping season. Fields that are being planted to forage crops have been tilled to facilitate traveling over fields in the harvesting process. Also in fields where high rates (9,000 + gallons) of manure have been applied, surface tillage was accomplished with the Aer-way tillage implement. Manure application rates of over 9,000 + gallons per acre combined with soybean and corn residues develop an insulating mat, significantly reducing the rate of drying and warming of the soil surface. Disturbing the crusted residue at the soil surface has increased corn plant stands and their uniformity while still maintaining adequate amounts of surface residue and the improved soil tilth developed during previous years of no-till planting.

The excellent growing season produced very high yields of corn, soybeans, red clover and alfalfa (Table 3). The oat crop appeared to be headed for high yields but this did not happen. Untimely, hot weather during grain fill and unexpected rust development produced poor yields and very low (24 lbs/bu) test weights for oats. Oat grain filling is particularly sensitive to hot temperatures. Soybean plants thrived this past growing season, and even though white mold was present, it had minimum impact on yields.

We continue to be plagued with lightening troubles to both our drive over scale and our electronic gate access to the Badger Army Ammunition Plant (BAAP). An interim system that uses phone lines to transmit video and gate opening and closing signals has been installed. This phone line system is less susceptible to lightening damage; however, anytime a storm passes through this area we disconnect the system to prevent potential damage. We will likely be required to maintain this type of access to BAAP at least through the 1999 growing and harvest season.

Several projects were worked on this past season. We added some permanent laneways to the grazing research area to facilitate cattle and equipment movement during wet weather. The field crew also worked on a large fencing project at the BAAP. This project will facilitate our leasing cropland closer to our building complex. We acquired a new Ag Bag machine (fills 8 ft. diameter bags) which we used very extensively this season. Ten bags were filled for various research projects and eight bags were filled with general herd feed. The farm also acquired a unique soybean roaster that uses heated oil to roast the soybeans. This roaster will allow us to economically roast soybeans grown at the farm and maintain control over the degree of roasting the soybeans receive.

Mike Rogers, one of our farm equipment operators, resigned in February 1998 to pursue a full-time assessing career. We appreciated Mike's many contributions to the Research Center's programs and wish him much success in his new career. Dan Wendt, a former farm equipment operator at the University of

Wisconsin's Arlington Experiment Station was hired in August 1998 to fill the vacant Agriculture Project Supervisor position. We welcome Dan and look forward to working with him.

It now appears that the BAAP is definitely no longer going to be required by the Department of Defense. The General Service Administration (GSA), the broker for all Federal Property, has begun in earnest to develop a reuse plan for the land and assets of the BAAP. This process is governed and regulated by federal laws and procedures established by congressional acts over the years. As a federal agency the USDA has a first opportunity to request property if it has a justifiable use for properties no longer required by other federal agencies. The USDA has submitted a formal application on behalf of the USDFRC to the GSA to acquire approximately 1400 acres of crop and pasture land needed in our research program. The only other federal agency to express an interest in acquiring land from the BAAP has been the Bureau of Indian Affairs on behalf of the Winnebago Ho Chunk tribe. The GSA and former Congressman Scott Klug have held numerous informational meetings concerning the reuse of the BAAP. The land occupied by the BAAP contains about 7,400 acres and it is located at the base of the Baraboo Bluffs near Devils Lake State Park. There is a great deal of history to this area from pre-settlement to the present. This large contiguous property and its unique location have peaked the interest of local and state conservation and environmental organizations, recreational organizations, business development groups, railroad commissions, historical groups, etc., concerning the reuse plans being developed by GSA for the BAAP. The reuse ideas expressed to GSA representatives range from restoring the entire 7,400 acres to its pre-settlement prairie status to a multiuse facility that would include prairie restoration, recreation, sustainable agriculture, a USDFRC program and low environmental impact business. The planning process is currently proceeding with a goal of having a preliminary reuse plan available for public comment by early summer 1999. After a period for public comment and refinement, a final reuse plan will be developed which then will require an environmental impact study (EIS) taking approximately 18 months. If the EIS determines that the reuse plan does not negatively impact the environment, then the GSA will begin to implement the reuse plan. At any time in this process a state's elected representative to the congress could sponsor legislation to circumvent the GSA process. In fact, former Congressman Scott Klug attempted to pass legislation to turn over a significant part of the BAAP to the State of Wisconsin. This legislation was not successful. Needless to say the USDA's acquisition of pasture and cropland from the excessed BAAP are critical for the continuation of the USDFRC research programs. We appreciate, are encouraged by, and look forward to continued support from the dairy forage producers; industry representatives and organizations; and the College of Agricultural and Life Sciences, University of Wisconsin - Madison as we seek to acquire the resources needed to maintain this important and productive research program.

This has been an extremely busy and quite remarkable year at the USDFRC. It produced record crop yields, a variety of forage research materials successfully harvested and stored, some major improvement projects completed and an opportunity to acquire the land needed to support our research programs. Many elements contribute to successful programs; the USDFRC farm has an excellent staff of employees that contribute to a productive and successful research program. I thank all of our employees for their efforts this past year.

Table 1. 1998 planting+ and harvesting dates.

	Planting		Harvesting	
Crop	Start	Finish	Start	Finish
Oats	4/6	4/6	7/15	7/16
Soybeans	5/8	5/14	9/22	10/15
Corn	4/20	5/15	9/17	10/26
Corn silage	-	-	8/26	9/21
Alfalfa	8/18	8/18	-	-
Per. Ryegrass	4/24	4/24	-	-

+No alfalfa was spring seeded in 1998.

Table 2. 1998 forage cutting dates.[+]

		Alfalfa		Red clover[‡]		Per. Ryegrass	
Crop	Acres	Start	Finish	Start	Finish	Start	Finish
First	330	5/16	6/2	6/6	6/6	6/30	7/1
Second	330	6/20	7/9	7/11	7/11	8/10	8/10
Third	330	7/23	8/10	8/26	8/26	10/10	10/10
Fourth	148	8/24	8/29	-	-	-	-

[+]Not all alfalfa fields were harvested 4 times, Red clover Acres = 14, Rygrass Acres = 39.

[‡]Red clover was no-till seeded on 8/18/97.

Table 3. 1998 crop yield data.

		Yields			
Crop	Ac	Low	High	Mean	Total
	- - - - - - - - - - - - - - - - bu/acres - - - - - - - - - - - - - - - -				
Oats	50	-	70.6	70.6	3,528
Soybeans	201.6	53.7	68.0	64.7	13,037
Corn grain	259.0	132.3	210.4	181.8	47,095
	- - - - - - - - - - - - tons DM (as is)/acres[+] - - - - - - - - - - - -				
Corn silage	137.6	7.2	8.7	8.1	1,118
		(19.0)	(22.9)	(21.4)	(2,941)
Alfalfa	330.0	4.16	6.61	5.24	1,729
Red clover	14.0	-	6.30	6.30	88
Per. Ryegrass	39.0	-	3.43	3.43	124

[+]Corn silage harvest moisture ranged from about 30% to 41% moisture.

-alfalfa includes hay and haylage DM.

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