# INFLUENCE OF SOLUBLE CARBOHYDRATE CONTENT AND PROTEIN LEVEL ON *IN VITRO* CELLULOSE DIGESTION IN ORCHARDGRASS

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ABSTRACT.—Twenty-three forage samples were fermented *in vitro* to determine the effect of soluble carbohydrate content on cellulose digestion. To observe the effect of soluble sugar content on lag period (differential rate in the beginning of cellulose digestion), two groups of samples, one group containing 6% and the other 10.5% soluble carbohydrate, were fermented *in vitro* for six hours. No significant difference could be shown in the extent of cellulose digested between the two groups of forages. Similar analysis also showed there was no significant difference in cellulose digestion (at 12 or 24 hours) due to soluble carbohydrate content.

Nitrogen fertilization significantly increased cellulose digestion at both 12- and 24-hour fermentation times. Initial cellulose content in the nitrated forages was significantly lower than in the nonnitrated forages.

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The animal agriculture in much of southern Illinois and adjoining south central states depends in large measure on grazing ruminants (cattle and sheep). The efficiency of cattle and sheep production is dependent upon the production of high quality forage. Many forage crops have been studied singly and in mixtures in an effort to produce maximum forage yields. In many cases the legumes in legume-grass mixtures are short-lived and thus the establishment of pure stands of grasses is common. Orchardgrass (Dactylis glomerata) is one of the most popular high producing perennial grasses used. Little information is available on the nutritive value of orchardgrass especially under grazing conditions.

Progress in the development of *in vitro* techniques has stimulated interest in the application of this method in the evaluation of forage quality. Recently, many workers have found forage cellulose digestion *in vitro* to be highly correlated with the digestibility of various forage constituents *in vivo* (Baumgardt *et al.*, 1959; Hershberger *et al.*, 1959; Donefer *et al.*, 1960; Le Fevre and Kamstra, 1960; Reid *et al.*, 1960; Baumgardt *et al.*, 1962; Simkins and Baumgardt, 1963). There is little doubt that *in vitro* rumen fermentation techniques offer a method of studying the digestibility and thus nutritive value of forages.

Crampton (1957) suggested level of intake (voluntary consumption) of a forage may be of primary importance in describing its feeding value. This observation was extended and in 1959 Macdonald College workers (Crampton *et al.*, 1959; Donefer *et al.*, 1959) introduced the use of *in vitro* cellulose digestion as a possible means of estimating the total nutritive value of forages.

Donefer *et al.* (1959) observed lag periods in the start of cellulose digestion of 2 to 6 hours between forages. This differential rate of fermentation was reflected in 12-hour cellulose digestion which in turn was highly correlated (r = 0.88) with *ad libitum* consumption *in vivo*. Cellulose digestibility at 24 hours was highly correlated (r = 0.87) with *in vivo* energy digestibility.

Since rumen microorganisms benefit (in terms of cellulose digestion) from a readily available source of carbohydrate, it could be postulated that a close relationship exists between level of soluble carbohydrates in forages and the initiation of rapid *in vitro* cellulose digestion. If a close relationship does exist it may be that level of soluble carbohydrate could be used to predict voluntary intake of the forage.

A series of trials was conducted by Reid *et al.* (1964) to determine the influence of growth phase (first vs. regrowth), stage of maturity, nitrogen fertilization (60 lb. N vs. 240 lb. N) and cutting management (continuous growth vs. interval clipping) on digestibility of Kentucky bluegrass. In Reid's study it was noted that higher levels of nitrogen fertility increased cellulose digestibility *in vitro* and *in vivo* in the first growth phase of grass. It is possible that this effect due to nitrogen is related in part to the amount of nitrogen available to the microorganisms.

In the study reported in this paper *in vitro* fermentation is used to assess the effects of the soluble carbohydrate and nitrogen content of orchardgrass on cellulose digestion.

## Materials and Experimental Procedures

Orchardgrass (Dactylis glomerata) samples used in this investigation were obtained from the Lawrence area of land at the Dixon Springs Agricultural Center, Simpson, Illinois.

The Lawrence tract, consisting of 40 acres, is of the Grantsburg-Grenada silt loam soil type. After being in corn and winter wheat, the area was seeded in the spring of 1959. The field was cut for hay once in 1959, twice in 1960 and once in 1961. The area became a permanent pasture in the fall of 1961. After being divided into two equal parts, ammonium nitrate was applied at the rate of 209 pounds per acre in the spring of 1962 and 106 pounds per acre in the fall to the north half. In October 1962, soil tests indicated the pH to be 6.57 for the south half and 5.83 for the north half, the latter value apparently a result of fertilization, since the pH prior to dividing the field was 6.30. Lime was applied at the rate of 0.3 ton per acre to the south half and 2.3 ton per acre to the north half. In 1963 fertilizer applications were: 228 pounds ammonium nitrate per acre to the north half on April 12 and 111.6 pounds per acre on August 20; 204.4 pounds of super phosphate per acre on the north half and 207.7 pounds per acre on the south half on August 29.

Orchardgrass samples were collected weekly from two locations (east and west ends) in each half of the field. Forage samples were obtained directly from the grazed area by clipping a four foot square plot. Plots were located at random within each end of the field. Only selected samples taken from the west ends, representing the period from October 29, 1962, to December 30, 1963, were used in the present study. A visual estimate indicates there was very little variation in the forage stand and that 90-95% of the growth was orchardgrass. There was no attempt made to evaluate the extent or type of disease.

The grazing season includes the period from the third week of April to the third week of October. During the 1963 grazing season, "put and take" animals were used to insure an equal degree of grazing on both halves of the field.

Forage samples were dried in a forced draft oven at 60° C, and dry matter content calculated. Macro - Kjeldahl techniques were used to determine protein content. In preparation for sugar extraction and *in vitro* digestion work, forage samples were ground using an intermediate Wiley mill, equipped with a 60-mesh screen.

Two basic approaches were used in evaluating the effect of soluble carbohydrates and protein content of orchardgrass on cellulose digestion. The first involved an extraction and colorimetric determination of the soluble sugars in forage samples. The second phase of this study was the use of an *in vitro* technique to determine cellulose digestibility.

A number of techniques for the extraction of sugars present in forages are reported in the literature. Techniques involving the use of ethanol as the extracting solvent have been commonly used. With this is mind a modification of a method used by Gilmore (1964) was chosen for this study. Following a 6-hour extraction with 70% ethanol and removal of impurities and color pigments, hydrolysis of the di-and oligosaccharides (non-reducing) was carried out enzymatically with a 1% diastase solution. After standing overnight, the colorimetric method for determining sugars, described by Dubois *et al.* (1956), was used.

That phase of the study to be reported in this paper involves three factors: (1) the effect of soluble carbohydrate level on the initial lag period in forage cellulose digestion; (2) the effect of nitrogen fertility on the rate and extent of cellulose digestion; (3) the relationship between level of soluble carbohydrate and extent of cellulose digestion.

With these interests in mind, a careful selection of materials for study was necessary since the sample size, representing each date, was small. Twelve samples, six having around 6% soluble carbohydrate, and six containing around 10% were selected to study lag phase. These selections contained enough forage material to carry out duplicate *in vitro* digestions at 6, 12 and 24 hours. Donefer *et al.* (1959) observed lag periods of 2-6 hours in the start of cellulose digestion. Thus differences in the length of lag phase between forages would be reflected in the 6-hour digestion time. To study the effect of nitrogen fertility eight pairs (2 samples collected on the same date, one from the north half of the field, receiving nitrogen, and one from the south half) were selected. *In vitro* cellulose digestion was determined at 12 and 24 hours. The combined selections (samples from parts 1 and 2) are used to study the third relationship. In all, 23 samples were selected for *in vitro* digestion studies.

Cellulose digestion *in vitro* was determined by the fermentation technique described by Bentley *et al.* (1955), with modifications introduced by Johnson *et al.* (1958), Quicke *et al.* (1959) and Donefer *et al.* (1960). For the sake of completeness and clarity the modified technique used in the present study is described.

Fermentation was carried out in 32 x 200 mm. (125 ml.) pyrex test tubes placed in a water bath thermostatically controlled at 39° C. Tubes were equipped with rubber stoppers fitted with glass inlet and outlet tubes to permit the passage of $CO_2$ throughout the fermentation period.

Approximately 1 gm. of the air-dry test forage was placed in each tube. *In vitro* cellulose digestion was determined on each forage in duplicate for all fermentation times (6-, 12-, 24-hour), within the same fermentation run. The tubes were located throughout the fermentation system at random.

The buffer-mineral solution used in the present study was that used by Baumgardt *et al.* (1962) and was similar to the synthetic saliva suggested by McDougall (1948). The composition is shown in Table 1. Before each fermentation run this solution was bubbled vigorously with $CO_2$ until the pH was approximately 6.9.

A mature ewe, fitted with a permanent ruminal canula and maintained on good quality, coarsely ground orchardgrass hay, served as the source of rumen microorganisms for all fermentations. Inoculum preparation was according to a modification of the method described by Johnson *et al.* (1958). Liquor, first expressed from the rumen

TABLE 1. Buffer-Mineral Solution for *In Vitro* Fermentation

| *Component* | *Gm. Per Liter* |
|---|---|
| $NaHCO_3$ | 9.8 |
| $Na_2HPO_4 \cdot 7H_2O$ | 7.0 |
| KCl | 0.57 |
| NaCl | 0.47 |
| $CaCl_2 \cdot 2H_2O$ | 0.053 |
| $MgSO_4 \cdot 7H_2O$ | 0.12 |

contents through several layers of cheesecloth, was discarded. The remaining pressed pulp was extracted with phosphate buffer (2.000 gm. $Na_2HPO_4 \cdot 7H_2O$, 0.436 gm. $KH_2PO_4$ per liter, pH 7.0, prewarmed to 40° C) in the ratio of 454 grams pressed pulp to 375 ml. of buffer. This mixture was re-expressed through cheesecloth and the fluid thus obtained put immediately into prewarmed thermos jugs. This phosphate buffer extract was used as the inoculum.

Inoculum pH values ranged from 7.0 to 7.25 over the fermentation periods. The tubes were inoculated as rapidly as possible (about 10 minutes) with 10 ml. of the phosphate buffer extract. The $CO_2$ supply was adjusted so that moderate bubbling occurred in the tubes. The pH was determined following each fermentation. Even though pH adjustments were not made pH did not drop below 6.4 in 24 hours. It is generally considered cellulose digestion takes place efficiently down to a pH of 6.5 (Barnett and Reid, 1961). The pH level reported here presumably would not have any marked effect on bacterial activity.

At the end of the fermentation period the microbial activity was stopped by addition of 1 ml. of 4N $H_2SO_4$ to each tube.

Cellulose was determined by the gravimetric method of Crampton and Maynard (1938) as modified by Hershberger *et al.* (1955). Where possible all cellulose determinations were carried out in duplicate, i.e., initial cellulose and residual cellulose after fermentation *in vitro*.

To account for variations between *in vitro* fermentation periods a reference forage (fescue) was fermented during each period.

The data were statistically analyzed by the method of least squares. Both simple and multiple regressions were also run on selected data.

## Results and Discussion

A total of 116 orchardgrass samples, collected during a period from October 29, 1962, to December 30, 1963, were analyzed for soluble carbohydrate content. Marked fluctuations in level of soluble carbohydrate content in the forage were observed during the period studied. This observation is in agreement with that of other investigators (Sullivan and Sprague, 1943; Crampton and Jackson, 1944; Barnett and Miller, 1950; Sprague and Sullivan, 1950; Ely *et al.*, 1953; Waite and Boyd, 1953a, b; Waite, 1957; Van Riper and Smith, 1959; and Stoddardt, 1964). Although several trends in forage soluble carbohydrate content due to the effect of ammonium nitrate applications were observed, no significant differences could be shown.

The results of 23 *in vitro* fermentations are presented in Table 2. Percent protein, initial cellulose and soluble carbohydrate for each sample are included. Statistical analysis of the data for the 6-, 12-, and 24-hour fermentations revealed no significant effect on 6-hour cellulose digestion due to the level of soluble carbohydrate in the forage. Similar analysis also showed that there was no effect at 12 or 24 hours although the F value at 24 hours was approaching significance. The mean soluble carbohydrate and *in vitro* cellulose digestibilities and standard deviations are presented in Table 3. Although there was no significant effect of level of soluble carbohydrate on the extent to which cellulose was digested *in vitro* it should be noted that at all time periods greater amounts of cellulose were digested in forage samples containing the higher levels of soluble carbohydrates. It can be concluded from these results that a source of readily available energy was apparently not a limiting factor so far as microbial activity was concerned in the forages studied.

TABLE 2.—*In Vitro* Cellulose Digestion—23 Orchardgrass Samples.

| Date of Cutting | Location | Protein (%) | Initial Cellulose (%) | Soluble Carbohydrate (%) | Cellulose Digestion 6-Hour (%) | 12-Hour (%) | 24-Hour (%) |
|---|---|---|---|---|---|---|---|
| 11- 5-62 | N ½ | 21.35 | 25.39 | 6.36 | 24.90 | 38.95 | 56.95 |
| 11- 5-62 | S ½ | 14.00 | 31.42 | 4.91 | ........ | 19.45 | 34.00 |
| 11-12-62 | N ½ | 20.65 | 21.84 | 8.12 | ........ | 22.03 | 49.62 |
| 11-12-62 | S ½ | 16.45 | 27.64 | 6.31 | 16.15 | 23.87 | 39.24 |
| 12-10-62 | N ½ | 16.80 | 25.54 | 6.78 | ........ | 32.47 | 55.02 |
| 12-10-62 | S ½ | 13.83 | 27.13 | 6.39 | 13.88 | 19.60 | 40.29 |
| 1- 7-63 | N ½ | 15.93 | 27.94 | 6.30 | 17.02 | 27.89 | 38.12 |
| 1-21-63 | N ½ | 17.85 | 30.82 | 6.18 | 13.27 | 30.90 | 45.54 |
| 1-21-63 | S ½ | 8.40 | 36.72 | 6.59 | ........ | 11.58 | 23.67 |
| 1-28-63 | N ½ | 16.45 | 32.26 | 6.87 | ........ | 25.84 | 42.33 |
| 1-28-63 | S ½ | 13.13 | 41.30 | 5.67 | 2.31 | 8.60 | 16.11 |
| 4-15-63 | N ½ | 23.28 | 23.86 | 12.79 | ........ | 51.94 | 70.81 |
| 4-22-63 | N ½ | 25.20 | 25.39 | 8.40 | ........ | 54.80 | 69.69 |
| 4-22-63 | S ½ | 15.58 | 26.77 | 10.76 | 12.21 | 25.98 | 51.35 |
| 4-29-63 | S ½ | 11.90 | 26.83 | 10.03 | 14.44 | 19.31 | 49.53 |
| 5-13-63 | N ½ | 20.48 | 30.63 | 8.35 | ........ | 47.19 | 63.35 |
| 5-13-63 | S ½ | 11.90 | 31.36 | 9.67 | ........ | 20.79 | 49.30 |
| 5-20-63 | N ½ | 19.78 | 27.85 | 10.92 | 18.06 | 35.90 | 57.05 |
| 5-20-63 | S ½ | 12.78 | 30.94 | 10.85 | 15.63 | 26.95 | 42.42 |
| 6- 3-63 | N ½ | 22.93 | 27.91 | 10.50 | 22.81 | 41.01 | 53.04 |
| 7-15-63 | S ½ | ........ | 35.50 | 10.16 | 12.55 | 21.29 | 40.20 |
| 8-19-63 | N ½ | ........ | 35.16 | 8.95 | ........ | 20.60 | 35.03 |
| 8-19-63 | S ½ | ........ | 39.69 | 8.55 | ........ | 33.82 | 45.59 |

TABLE 3.—Influence of Level of Soluble Carbohydrates on *In Vitro* Cellulose Digestion at 6, 12, and 24 Hours.

| Level of Soluble Carbohydrates % | | Cellulose Digestion | | | | | |
|---|---|---|---|---|---|---|---|
| | | 6-Hour % | | 12-Hour % | | 24-Hour % | |
| Mean | S[a] | Mean | S | Mean | S | Mean | S |
| 6.2 | 0.8 | 14.6 | 7.3 | 25.0 | 10.3 | 39.3 | 13.3 |
| 10.5 | 0.4 | 15.9 | 4.0 | 28.4 | 8.4 | 49.0 | 6.5 |

[a] Standard deviation.

Other workers have noted various responses in digestibility due to the application of nitrogen fertilizer on several forages. Hall *et al.* (1958) compared utilization of nitrated (120 lb. N per A) and non-nitrated forages at two stages of maturity using *in vitro* cellulose digestion and observed that nitrogen fertilizer at the pasture stage did not affect the utilization of millet or piper sudan, whereas it had a marked negative effect on the utilization of sweet sudan. No differences in utilization were observed at the hay stage. Markley *et al.* (1959) found increased apparent digestibility coefficients *in vivo* of protein and energy of bromegrass and orchardgrass when at least 200 lb. of nitrogen per acre were applied. In *in vitro* work, Reid *et al.* (1964) showed a significant increase in cellulose and protein digestion due to nitrogen fertility. The effect of nitrogen on cellulose digestion *in vitro* can be found in Table 4. Nitrogen application did have the effect of significantly increasing cellulose digestion of orchardgrass at both 12- and 24-hour fermentation times. Reid *et al.* (1964) made the suggestion that

TABLE 4.—Influence of Nitrogen Fertilization on the *In Vitro* Digestion of Cellulose at 12 and 24 Hours.

| Nitrogen Fertility | Level of Crude Protein % | | Level of Soluble Carbohydrates % | | Cellulose Digestion | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | 12-Hour % | | 24-Hour % | |
| | Mean | S[a] | Mean | S | Mean | S | Mean | S |
| + | 19.8 | 2.85 | 7.75 | 1.55 | 36.0 | 10.9 | 54.9 | 9.3 |
| 0 | 13.2 | 2.45 | 7.65 | 2.38 | 24.9 | 6.5 | 37.0 | 12.1 |

[a] Standard deviation.

this effect may be related in part to a limitation in nitrogen availability for cellulose digestion in the *in vitro* growth medium. Other workers (Chalupa *et al.*, 1963) have noted significant stimulatory effects on cellulose digestibility when low levels of urea were added to a rumen fluid phosphate buffer medium. Upon further examination of data obtained in this investigation, it was noted the cellulose content of the nitrogen series was significantly lower than in the non-nitrogen series. This would suggest the possibility the level of cellulose present or other materials associated with various levels of cellulose in forage may be an important factor governing its rate of digestion. The authors question that the levels of cellulose per tube used in this study would cause the differences observed.

It was also of interest to obtain the relationship between level of soluble carbohydrate and extent of cellulose digestion. Correlation coefficients at three *in vitro* digestion times are presented in Table 5. It will be noted rather low correlations were obtained, especially at 6 and 12 hours, between percent soluble carbohydrate and percent cellulose digestion. The correlation improved as fermentation time increased (r = 0.60 at 24 hours). According to Donefer *et al.* (1959) cellulose digestion at 12 hours is closely associated with *ad libitum* consumption and 24-hour cellulose digestion is closely associated with forage digestibility. Bowden (1964), reported simple correlations of 0.60 to 0.69 between soluble carbohydrate content of orchardgrass and 48 hour *in vitro* dry matter digestion. This report appears to agree closely with the present results at a 24-hour digestion time.

Simple correlations between percent nitrogen and percent cellulose digestion were high at all three digestion times (r = 0.72 to 0.87). Also from the correlations made it appears that there was a definite negative association at all digestion times studied between initial cellulose and percent cellulose digestion (r = -.55 to -.78). These results would indicate the nitrogen content

TABLE 5.—Correlation Coeficients Between 3 Forage Components and Cellulose Digestion *In Vitro*.

| Factors Correlated | 6-Hour Digestion r(11 samples) | 12-Hour Digestion r(20 samples) | 24-Hour Digestion r(20 samples) |
|---|---|---|---|
| % protein *AND* % cellulose digestion | 0.72* | 0.87** | 0.77** |
| Initial cellulose *AND* % cellulose digestion | —.78* | —.55* | —.75** |
| % soluble carbohydrate *AND* % cellulose digestion | 0.24 | 0.44 | 0.60** |
| Multiple correlation coefficient | 0.91** | 0.90** | 0.90** |

** P = 0.01
*P = 0.05

and the amount of initial cellulose have a greater effect on digestion of cellulose *in vitro* than the amount of soluble carbohydrate present in the forage, especially early in the fermentation period. It further appears that use of all three dependent variables predicts cellulose digestion more accurately than any one variable alone since a multiple correlation coefficient provided values of 0.90 for all three digestion times. This means that approximately 81% of the variation in cellulose digested can be accounted for by including the variation in soluble carbohydrate, nitrogen and initial cellulose. Bowden (1964) found he could account for approximately 60% of the variation in dry matter digested *in vitro* when water soluble carbohydrate, protein and fructose were included in a multiple regression analysis.

Close examination of Table 2 suggests that there may be other factors involved which influence cellulose digestion *in vitro*. For example, samples collected on November 12, 1962, (N ½) and May 13, 1963, (N ½) have approximately the same protein and soluble carbohydrate content. Even though initial cellulose was higher in the May sample, it was digested to a greater extent in 24 hours. Generally, cellulose digestion values were observed to be higher in forage samples collected during the spring and summer than in those collected from November to January. This would imply that, in addition to the influence of the forage components studied on cellulose digestion, there may be confounding of this response due to season. This, quite possibly, is associated with the degree of lignification in the forage. Kamstra *et al.* (1958) reported a negative relationship between lignin content and cellulose digestion *in vitro*. In the present study, determination of the lignin content of forages, may have been extremely useful in explaining variations in cellulose digestion *in vitro*; however, limited sample precluded this.

## Acknowledgment

The authors wish to express appreciation for the assistance of Dr. H. W. Norton in the statistical analysis and interpretation of the data obtained.

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*Manuscript received May 16, 1967.*