In Vitro Digestion of Bloat-Safe and Bloat-Causing Legumes by Rumen Microorganisms:
Gas and Foam Production

J. P. FAY, K.-J. CHENG, and M. R. HANNA
Agriculture Canada Research Station
Lethbridge, Alberta T1J 4B1

R. E. HOWARTH
Agriculture Canada Research Station
Saskatoon, Saskatchewan S7N 0X2

J. W. COSTERTON
Department of Biology
University of Calgary
Calgary, Alberta T2N 1N4

ABSTRACT

Leaves of three bloat-safe legumes – birdsfoot trefoil (Lotus corniculatus L.), sainfoin (Onobrychis viciaefolia Scop.), and cicer milkvetch (Astragalus cicer L.) – and of three bloat-causing legumes – alfalfa (Medicago sativa L.), red clover (Trifolium pratense L.), and white clover (Trifolium repens L.) – were incubated with strained rumen fluid or with mixed rumen fluid and solids. Gas released was measured during the early period (0 to 22 h) of this in vitro digestion. Gas volume was greater with a 1:1 (wt/vol) mixture of solid and fluid rumen contents than with rumen fluid alone. It was greater with whole and chewed leaves from the bloat-causing legumes than with whole leaves from the bloat-safe legumes. However, when leaves were homogenized, volumes of gas from bloat-causing and bloat-safe legumes were similar. More gas was released from homogenized leaves than from the same weight of whole leaves. The amount of foam produced on chewed herbage and homogenized leaves of bloat-causing legumes was greater than on those of bloat-safe legumes. These results are consistent with the rate of disintegration and digestion of legumes by rumen bacteria being an important determinant in pasture bloat. Measurement of gas produced early in in vitro digestion may provide a useful bioassay for evaluating the bloat-causing potential of legumes in breeding selections if variability of the method can be reduced.

INTRODUCTION

The danger of frothy bloat in ruminant animals grazing legume pastures is a major limitation to greater use of such species as alfalfa (Medicago sativa L.), red clover (Trifolium pratense L.), and white clover (Trifolium repens L.). These are recognized by agronomists as bloat-causing legumes, as distinct from bloat-safe legumes such as sainfoin (Onobrychis viciaefolia Scop.), birdsfoot trefoil (Lotus corniculatus L.), and cicer milkvetch (Astragalus cicer L.) (25).

The onset of pasture bloat always has been associated with stable foam in the rumen (17, p. 442). Cooper et al. (10) found a fairly close relationship between known bloat potential of 27 legume species and volume of foam formed from extracts of these species. However, the foam volume produced by cicer milkvetch did not fit the pattern. Pounden et al. (23) observed more gas production from alfalfa and Ladino clover than from orchard grass during microbial digestion of fresh material.

Our objective was to measure the rate and volume of gas and foam production of bloat-causing and bloat-safe legumes. Because the possibility of breeding bloat-safe varieties of alfalfa and other bloat-inducing species has

Received November 7, 1979.

1 Fellow of the Consejo Nacional de Investigaciones Científicas y Técnicas de la República Argentina on transfer of work.
been considered in recent years, we also hoped that our work could provide a basis for development of a microbial bioassay. Such an assay would be a useful tool to enable plant breeders to determine the bloat potential of individual plants or breeding lines. Ideally, a microbial bioassay would be rapid, simple, inexpensive, and suitable for assessing small samples.

Because pasture bloat typically occurs about 2 h after feeding (21), we were concerned with the early rate of digestion rather than extended digestion periods, which are widely used for in vitro evaluation of forage digestibility (11, 28).

Recently, Cheng et al. (8) classified rumen bacteria as belonging in three major populations: those free in the rumen fluid, those attached to feed particles, and those adherent to the rumen epithelium. In view of this, our preliminary experiments included an evaluation of two sources of rumen inoculum — rumen fluid and rumen fluid plus solid digesta.

MATERIALS AND METHODS

Plant Material

Trek alfalfa, Lasalle red clover, Merit white clover, Oxley cicer milkvetch, Melrose sainfoin, and Leo birdsfoot trefoil plants were grown in the greenhouse for the experiments with whole and homogenized leaves. Whole leaves were picked manually from vigorous plants in the prebud or bud stages of growth, mixed, and 5 g (fresh weight) were placed in digestion flasks with 315 ml of Dehority's medium (26).

Whole leaves, 5 g in 165 ml of Dehority's medium, were disrupted in a Waring Blender operated at full speed for two 30-s periods. The homogenate was transferred to digestion flasks, and the blender vessel was washed with 150 ml of medium, which also was added to the digestion flask.

The herbage in the chewing experiment was taken from the same six cultivars in the field and the greenhouse. Chewed herbage was collected by hand from a rumen-fistulated cow. Previous ingesta were totally removed from the rumen, the animal was offered fresh legumes (leaves plus stems), and the bolus of feed was collected at the cardia. The chewed herbage was kept in plastic bags until weighing, and 15 g were placed into each flask with 315 ml of Dehority's medium.

Microbial Inocula

Fluid and solid rumen contents were obtained from a rumen-fistulated Holstein cow, self-fed cubed alfalfa hay. Solid contents were removed from the upper part of the ingesta. Rumen fluid was aspirated from the rumen and filtered through two layers of cheesecloth before use as inoculum. When a mixture of solid and liquid rumen contents was used as inoculum, it was prepared as follows: 300 g of solid rumen contents and 300 ml of rumen fluid were homogenized in a Waring Blender at full speed for two 30-s periods, squeezed through two layers of cheesecloth into a graduated cylinder, and transferred immediately to incubation flasks. An atmosphere of CO₂ was maintained in all vessels used for the collection and preparation of inocula. Inocula (60 ml) were transferred into 500-ml flasks containing either medium and plant material or medium alone.

Medium and Culture Conditions

Dehority's medium (26) without the addition of carbohydrate was used for all experiments. Incubation was carried out anaerobically (under an initial CO₂ atmosphere) in 500-ml flasks placed in a water bath at 38 C for 22 h. Control flasks lacking either microbial inoculum or plant material were included in all runs. In the control flasks containing plant material alone, NaN₃ (.1%, wt/vol) was added to prevent bacterial growth.

Gas Release Measurements

Gas production in the flasks was measured by a method similar to that described by Forsberg (12). A 50-ml glass syringe (Luer-lock) with a hollow needle inserted through the rubber stopper was used to collect gases produced in each flask. For the first 7 h, flasks were stirred by hand with 20 circular movements every 30 min after which the readings of the syringes were recorded immediately. Between 7 h and 22 h, the flasks were not stirred, and gas volume was recorded every 2 h. When the syringes were full of gas, they were emptied, and recordings were reinitiated from 0 ml.

Foam Formation

Foam was estimated visually 2 to 3 min after flasks were stirred and at the same time intervals
for measurements of gas production. Foam production was rated on a 0 to 5 scale according to the volumes observed: 0, no foam; 1, trace; 2, small amount; 3, medium amount; 4, abundant; and 5, very abundant.

**Dry Matter Determinations**

Dry matter content of plant material was measured routinely. Samples of the same plant material used for digestion assays were weighed and oven-dried in aluminum containers at 90 C for 1 h and then 70 C for 22 h. Dry matter percentage was calculated as the ratio between the weight of plant material after and before the drying process. As saliva was in the chewed material, the dry weight of plants alone in that material was calculated from the following equations:

\[
N = x + y \\
M = Ax + By
\]

where

- \( N \): fresh weight of chewed material placed into each flask
- \( x \): fresh weight of plant material in \( N \),
- \( y \): fresh weight of saliva in \( N \),
- \( M \): dry weight of chewed material placed into each flask,
- \( A \): dry matter per gram of fresh plant material, and
- \( B \): dry matter per gram of saliva.

The \( N \), \( M \), and \( A \) were determined in this laboratory. The 1.02% (wt/vol) for \( B \) was obtained from Bailey and Balch (2).

**Statistical Analyses**

Differences in net gas production among the six legume species and differences between the bloat-causing and bloat-safe group were compared by analysis of variance and Duncan's multiple range test (27). The estimates of foam volume were analyzed by \( \chi^2 \) test (27).

**RESULTS**

**Influence of Type of Rumen Inoculum on Gas Production and Dry Matter Loss of Substrates**

Two types of rumen inoculum were compared for their influence on gas production from digestion of alfalfa leaves (Figure 1). Gas production was more rapid and early with a 1:1 (wt/vol) mixture of solid rumen contents plus rumen fluid than with rumen fluid alone. Gas production from digestion of alfalfa leaves with the solid plus fluid mixture inoculum was also greater, earlier, and faster than from digestion of alfalfa leaves with rumen fluid inoculum alone. Loss of leaf dry matter after 22 h was also greater with the solid plus fluid inoculum than with the rumen fluid inoculum (42.0% against 23.5%). On the basis of these results, a 1:1 (wt/vol) mixture of solid and fluid...
rumen contents was selected as the inoculum for the experiments described here.

Net Gas Production by Microbial Digestion of Legumes

Net amounts of gas produced from whole leaves varied ($P<.05$) among the six legume species after 22 h (Figure 2A). Gas production differed ($P<.05$) between sainfoin and alfalfa after only 8 h but not between white clover and birdsfoot trefoil throughout the 22-h digestion. The average gas production of the bloat-causing group exceeded ($P<.05$) that of the bloat-safe group at 7 h and thereafter (Figure 2B).

Only two experiments with the chewed herbage were successful, because cattle generally refused to eat when the rumen ingesta were removed totally. No results are given for birdsfoot trefoil, because the animals consumed this forage only once. Results in Figure 2C,D are means of two experiments. Net gas production in these experiments generally showed trends similar to results from whole leaves, i.e., faster rates of gas production from the bloat-causing legumes than from bloat-safe legumes. Gas production from whole leaves and chewed herbage (leaves plus stems) cannot be compared directly, because lower gas production is expected from stems than from leaves. However, initial rates of gas production were apparently higher with chewed herbage (Figure 2C) than with whole leaves (Figure 2A).

As expected, rates of gas production and total amounts of gas produced from homogenized leaves (Figure 2E,F) were different ($P<.05$) from 2 to 5 h. However, homogenized leaves of birdsfoot trefoil produced a volume of foam similar to that of white or red clovers. Foam volumes from chewed herbage (Figure 3C,D) were similar to foam volume from homogenized leaves.

Initially, whole leaves produced little foam, but chewed herbage and homogenized leaves gave larger volumes of foam at 0 and 1 h. Conversely, foam volumes decreased by 7 h for homogenized leaves and chewed herbage but not for whole leaves. At 22 h, foam volume was essentially zero in all flasks (Figure 3). Control flasks with either plant material (whole leaves or chewed herbage) or with inoculum alone had either no foam or a trace amount of foam. In uninoculated controls of homogenized leaves from all species, foam was abundant at 0 h, decreased rapidly thereafter, and at 1 h only trace to small amounts were recorded. The appearance of trace amounts of foam showed no relationship to the time of digestion nor to the bloat-causing potential of the legumes.

DISCUSSION AND CONCLUSIONS

In legume pasture bloat, gas from microbial digestion of forage remains dispersed throughout the rumen ingesta, giving rise to a characteristic frothy appearance of rumen contents. On the basis of mechanical disintegration of fresh leaves, Howarth et al. (16) proposed a cell rupture theory to explain the bloat-safe nature of cicer milkvetch, sainfoin, and birdsfoot trefoil. According to their theory, cell walls of bloat-safe legumes are more resistant to rupture than cell walls of bloat-causing species. This has been confirmed partially by Cheng et al. (7), who described the sequence of events during invasion and digestion of fresh legume leaves by rumen bacteria. The time required for bacterial penetration through leaf tissue was greater in bloat-safe legumes compared to bloat-causing legumes. Since fermentation of nutrients in the cell sap is a major source of initial gas production (6), differences between legumes in gas production from whole leaves provide additional support for the idea that the rate of bacterial disintegration and digestion of leaf tissue is an important determinant in occurrence of pasture bloat.

Excessive gas production has been ruled out as an immediate cause of pasture bloat (18, 19),
Figure 2. Net gas production (with standard errors) during digestion of legumes in vitro with a 1:1 (wt/vol) mixture of rumen solids and rumen fluids. Net gas production is the volume of gas evolved from flasks containing both inoculum and legumes less the gas volume evolved from flasks with inoculum alone. A and B, whole leaves; C and D, chewed herbage (leaves plus stems); and E and F, homogenized leaves. B, D, and F show means from the bloat-causing species (V) and bloat-safe species (T). O - alfalfa; △ - red clover; □ - white clover; • - cicer milkvetch; ▲ - birsefoot trefoil; ■ - sainfoin.
Figure 3. Relative foam volumes in flasks during in vitro digestion of forage legumes, rated on a scale of 0 to 5: 0, no foam; 1, trace; 2, small amounts; 3, medium amount; 4, abundant; and 5, very abundant. A and B are from whole leaves; C and D from chewed herbage (leaves plus stems); and E and F from homogenized leaves. B, D, and F show means for the bloat-causing group (V) and for the bloat-safe group (T). ○ - alfalfa; △ - red clover; ◇ - white clover; ● - cicer milkvetch; ▲ - birdsfoot trefoil; ■ - sainfoin.
although the problem of gas expulsion certainly must increase as greater volumes are produced. The choice of gas production as a characteristic in the present work was based upon need for a convenient measure of initial rate of leaf disintegration. Gas produced during the initial period of digestion may result from fermentation of sugars and starch (6, 22), pectin (9), or a cell wall component (29). In addition, CO₂ may be released from saliva in rumen fluid in the presence of organic acids (14) from plants or from fermentation.

Extensive disruption of leaves by homogenization greatly increased the initial rate of gas production compared to that from whole leaves. This increase in rate of gas production was probably due to greater availability of leaf nutrients for initiation of microbial fermentation. Chewed herbage was intermediate between whole leaves and homogenized leaves indicating only partial disruption of leaf tissue by chewing. However, the trend toward differences in gas production among legumes at 6 h were still apparent in chewed herbage. Chewing results in faster onset of digestion compared to intact leaves, but it does not appear to alter the relative susceptibilities of the different legumes to microbial digestion.

Although the in vitro digestion technique with measurement of gas production differentiated between some bloat-causing and bloat-safe species when whole leaves were used, it required an incubation period of at least 7 h to obtain statistically significant differences. Further, not all pairs of legumes could be differentiated according to their known bloat potential. We attribute these limitations of the technique to the high coefficient of variation in the gas volume measurement (60% at 6 h and 31% at 22 h). Nevertheless, trends in different rates of gas production were already evident at 6 h even though longer incubation was required to obtain significant differences. Also, the relative order of gas production from the six species alfalfa > white clover > red clover > cicer milkvetch > birdsfoot trefoil > sainfoin) was similar to rates of dry matter disappearance when these species were digested in vitro (7) or with nylon bags in vivo (15).

The gas production technique as described here would be useful as a bioassay to differentiate between bloat-safe and bloat-causing lines in a plant breeding program if the variability in measurement of gas production can be reduced.

Rumen fluid obtained directly from the rumen or squeezed from total rumen contents has been the traditional source of inoculum for in vitro digestion of forage materials (1, 3, 5, 28). Hungate (17, p. 33) showed that fermentation rate of alfalfa hay was higher in the presence of solid rumen contents than in their absence. Recently, Barry et al. (4) used whole ingesta (solid and liquid) from the rumen in a chopped form as the source of inoculum for in vitro digestion assays. We obtained earlier and faster gas production from alfalfa leaves with a rumen inoculum containing a 1:1 (wt/vol) blended mixture of solid and fluid rumen contents compared to rumen fluid alone. This increase in gas production may occur because the solid rumen contents provide the population of bacteria normally attached to the hay fibers in the cow's rumen (17) and that are, therefore, less numerous in filtered rumen fluid. These food particle-associated bacteria comprise one of the three main bacterial populations of the rumen (8), and Forsberg and Lam (13) have shown that they comprise about 75% of the bacteria in rumen contents. Thus, an inoculum that contains solid rumen contents better approximates the physiological conditions in the rumen, and we suggest that such inocula should be used for in vitro studies of digestion.

In pasture bloat, cell rupture is a prerequisite for microbial access to the readily fermentable nutrients and also for release of foaming agents into the rumen fluid. Howarth et al. (16) suggested that intracellular proteins, assumed to be the principal foaming agents in bloat (20), are released from bloat-safe forages relatively slowly and that they do not reach a sufficiently high concentration to cause frothiness and bloat. In our study, the early appearance of abundant foam with chewed herbage and homogenized leaves, but not with whole leaves, indicates that disruption released foaming agents from the leaves. Also, it appears that the bloat-safe legumes (cicer milkvetch and sainfoin) were less susceptible to mechanical damage by chewing. Reid et al. (24) reported that 24 to 34% of the chlorophyll in red clover was released by chewing, but there is no information of this kind for bloat-safe legumes. The gradual increase in foaminess with whole leaves suggested that bacterial digestion released foaming agents from the leaves. The disappearance of foaminess...
During digestion has been observed previously and is probably from metabolism of the forage proteins (20).

We hypothesize that ability to produce both stable foams and large volumes of gas during digestion by rumen microorganisms is necessary for a legume to be considered bloat-causing. Unless both prerequisites are present simultaneously, a legume will not cause bloat. Thus, even though it gives rise to large amounts of foam during digestion, birdsfoot trefoil would not cause bloat, because it does not give rise to enough gas. Conversely, cicer milkvetch behaves as a bloat-safe legume because it does not give rise to enough foam even when it shows a considerable production of gas during digestion. Possibly, more than these two factors are involved in legume-induced bloat.

In summary, disintegration of leaf tissues is an important event in onset of pasture bloat. Disintegration occurs either from chewing, which causes mechanical disruption, or from microbial digestion. Tissue disruption is a prerequisite for release of foaming agents (leaf proteins) into rumen fluid and for a substantial rate of gas production from fermentation of herbage constituents. Measurement of early gas production from in vitro digestion may be useful as a bioassay of the bloat potential of advanced plant breeding strains, but the technique requires further modification to reduce variability.

REFERENCES


