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IN VITRO RUMEN FERMENTATION OF ALFALFA HAY. CARBON DIOXIDE, METHANE, VFA AND HEAT PRODUCTION¹

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ALTHOUGH *in vitro* and *in vivo* rumen studies of cellulose digestion and volatile fatty acid production are numerous (Johnson, 1966), a paucity of information is available on the bioenergetics of rumen digestion. From four *in vitro* fermentations, in which the heat of combustion of substrate and the products was determined, Marston (1948) concluded that the heat of rumen fermentation of purified cellulose was 6% of the combustible energy of the cellulose fermented. More recently, Walker and Forrest (1964) incubated whole rumen contents in a novel calorimeter. They found that the rate of heat production of rumen contents 24 hr. after feeding was 1 cal per 1 % solids per 250 ml per hour. The rates of gas and heat production were found to be closely related and directly proportional to the solids contents of the medium.

The purpose of this investigation was 1) to develop an *in vitro* system in which the energetics of rumen fermentations could be studied and 2) to determine the energetics of alfalfa hay digestion by rumen microorganisms *in vitro*.

Experimental Procedure

The large Armsby Respiration Calorimeter (Armsby, 1903 to 1904; Braman, 1933) was selected as the instrument of choice for the *in vitro* rumen fermentation studies because of its sensitivity and availability. Several modifications were required to use the instrument as an incubator-calorimeter. The principal modification consisted of circulating hot rather than cold water through the cooling coils in the inner and outer air spaces. This was necessary to maintain the calorimeter in an adiabatic state since the desired operating temperature of the calorimeter was 39 C and the room temperature was approximately 27 C. In addition, the chamber was modified 1) to introduce the inoculum

at zero time, 2) to determine and adjust the pH of the medium from the outside of the chamber, 3) to maintain the temperature of the chamber at 39.0 ± 0.1 C and 4) to determine and record the temperature of the *in vitro* fermentation. The chamber was checked for air tightness prior to use, a heat blank was determined on the electrical equipment in the chamber, and carbon dioxide and methane recovery experiments were performed. In preliminary trials, the sensitivity of the calorimeter did not appear to be altered by its operation at 39 C.

In order that a measurable quantity of heat would be formed, 10.8 kg air dry alfalfa hay (9.9 kg dry matter) was suspended in a relatively large "artificial rumen" (360 liters), containing the following compounds in grams: KH_2PO_4 , 360; K_2HPO_4 , 720; Na_2SO_4 , 72; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 72; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 36; CaCl_2 , 36; Na_2CO_3 , 842; urea, 389.

In preliminary trials with 100 ml fermentations, it was found that the size of the inoculum could be reduced to 10% (V/V) without significantly altering the cellulose digestibility of alfalfa hay in a 24-hr. period.

The 36 liters of rumen fluid required to inoculate the 360 liters fermentation medium was obtained by "building-up" the inoculum from 0.5 liters to 36 liters in two, successive 24-hr. fermentations. On day 1, 0.5 liter rumen fluid, obtained from two rumen-fistulated sheep fed the same alfalfa hay as that used *in vitro* and squeezed through four layers of cheesecloth, was used to inoculate a 5 liters rumen fermentation medium containing 50 g cellulose (Alphacel), mineral mixture (Hershberger *et al.*, 1959), 5.40 g urea, and a water extract of 150 g alfalfa hay. Carbon dioxide was bubbled through the medium continuously and the pH was maintained between 6.7 and 7.0 by the addition of appropriate quantities of a saturate solution of sodium carbonate. The incubation temperature was 39.0 ± 0.1 C. On day 2, 4 liters of the day 1 fermentation medium

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TABLE 1. PERCENT CELLULOSE DIGESTED *IN VITRO* IN 24 HOURS

Experi- ment no.	Sub- strate	Day 1 ^a	Day 2 ^b	Day 3 ^c	Day 4 ^d
1	Alphacel	66.4	92.4
1	Alfalfa	60.4	55.0	60.3
2	Alphacel	88.9	90.2
2	Alfalfa	56.9	48.5	63.2	56.7
3	Alphacel	68.6	93.2
3	Alfalfa	63.4	60.0

^a Inoculum from two rumen-fistulated sheep fed alfalfa hay.

^b Inoculum from alphacel-containing flask, day 1.

^c Inoculum from alphacel-containing flask, day 2.

^d Inoculum from alfalfa-containing fermentation, day 3.

was used to inoculate 40 liters of a similar fermentation medium maintained under similar conditions. On day 3, 36 liters of the day 2 fermentation medium was used to inoculate the 360 liters fermentation medium in a 378 liter stainless steel fermentation tank in the Armsby Calorimeter. Thereafter, the fermentation was followed for 24, 24 and 30 hr., respectively, in Experiments 1, 2 and 3. During the "build-up" period, the activity of the microbial population was monitored by simultaneously determining the digestibility of alfalfa cellulose *in vitro*, using the cellulose-containing, 24-hr. fermentation medium as the inoculum. The fermentation tank was equipped with a cover, a stirring mechanism and a temperature indicating device.

The Armsby Respiration Calorimeter was operated in the usual manner with temperature readings and manual adjustments every 4 min. to maintain the calorimeter in an adiabatic state. The pH of the medium was adjusted to 6.95 every 2 hr. with a saturated solution of sodium carbonate. In Experiment 3, carbon dioxide, methane, hydrogen and heat were determined at 2-hr. intervals.

Cellulose was determined by the method of Crampton and Maynard (1938) as modified by Hershberger *et al.* (1959). Carbon dioxide was determined gravimetrically by soda lime absorption and methane was similarly determined following catalytic combustion. Hydrogen was determined gravimetrically by combusting the hydrogen and absorbing the water on concentrated sulfuric acid. Heat production was determined directly with the Armsby Calorimeter. Volatile fatty acids were determined by the method of Spahr, Holter and Kesler (1963).

Results and Discussion

Normal cellulose digestion was maintained throughout the inoculum build-up and calo-

rimeter test periods (table 1). Rapid agitation of the 40 liters alphacel-containing medium with a mechanical stirrer may have caused the relatively high cellulose digestibility on day 2.

The results of three, 360-liter fermentations are presented in table 2. Volatile fatty acids were not determined in Experiment 1. The average percent alfalfa cellulose digested (61.2) and the average molar percentages of acetic, propionic, butyric and valeric acids, respectively, (61, 24, 11, 4 respectively) were similar to values found *in vivo* by others (Sullivan, 1955; Gray *et al.*, 1951).

The carbon dioxide formed in Experiments 2 and 3 represented 20.2% of the carbon of the products, whereas the carbon lost as methane represented only 5.6% of the carbon

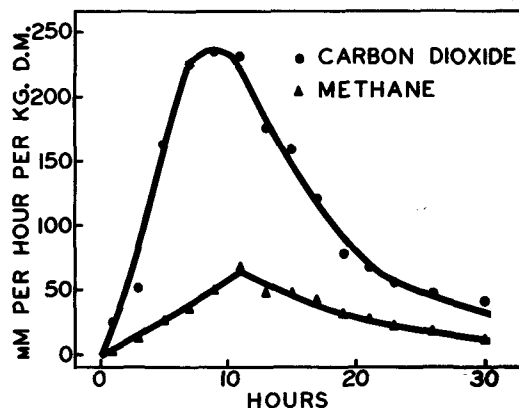


Figure 1. Carbon dioxide and methane formation in Experiment 3.

of the products. The 2.9 g methane formed per 100 g digested carbohydrate was somewhat less than *in vivo* values reported by Armsby and Fries (1915), Kellner (1919) and Kleiber (1961) which were, respectively, 4.8, 4.3 and 4.4 g per 100 g digested carbohydrate respectively. The higher *in vivo* values were probably due to more complete carbohydrate digestion in the gastrointestinal tract than in the *in vitro rumen* fermentation. According to Blaxter (1962) methane production per unit digested carbohydrate is inversely proportional to intake above maintenance.

The 4.8 to 7.8 moles hydrogen formed per 10 kg alfalfa dry matter per 24 hr. is somewhat greater than the 0 to 2 moles hydrogen formed per day in previous large animal calorimeter trials at this station (*unpublished data*). The greater hydrogen formation *in*

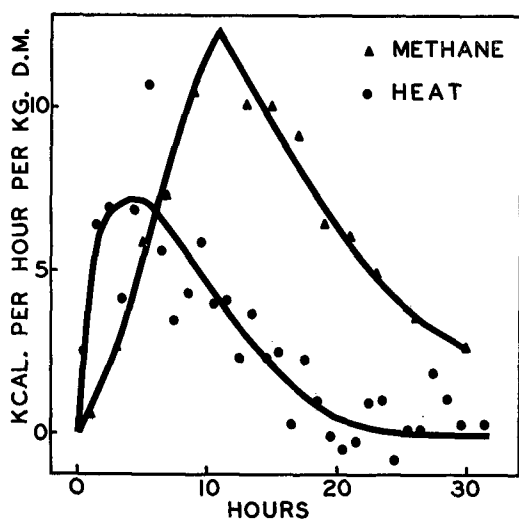


Figure 2. Heat and methane formation in Experiment 3.

vitro than *in vivo* may be related to the ease of hydrogen release from *in vitro* fermentations, or may be due to the presentation of a large quantity of readily available carbohydrates to nutritionally starved microorganisms (Pilgrim, 1948). In Experiment 3, approximately two-thirds of the total hydrogen was formed in the first 2 hours. Hydrogen formation, therefore, appeared to be associated primarily with the initial degradation of readily available carbohydrates or proteins. However, a small, measurable quantity of hydrogen was formed in nine of the 11 remaining 2-hr. fermentation periods.

The carbon dioxide and methane produced

in Experiment 3 as a function of time are presented in figure 1. Carbon dioxide initially increased rapidly, reached a peak at 9 hr., and then declined rapidly thereafter. Methane production increased at a slower rate (figure 1), reached a peak at 11 hr., and declined at a slower rate thereafter.

The average molar ratio of carbon dioxide to methane of 3.52 to 1 was similar to the 3.68 to 1 ratio observed by Markoff (1913) for ruminal gases. It is readily apparent, however, that the molar ratio of carbon dioxide to methane was continually changing during the 30-hr. fermentation period of Experiment 3 (figure 1). The ratio increased to a maximum of 6.5 to 1 at 7 hr. after which it gradually decreased to a minimum of 2.3 to 1 at 24 hours. Up to 14 hr., the carbon dioxide to methane ratio was greater than 3.5 to 1, whereas it was less than 3.5 to 1 thereafter. These data suggest that carbon dioxide and methane would not likely be formed at a constant ratio *in vivo* and that it would be erroneous to estimate ruminal carbon dioxide production from methane formation.

Heat production (figure 2) increased very rapidly at the start of the fermentation, reached a maximum of 7 kcal per kg dry matter per hour in 5 hr. and slowly declined thereafter. No appreciable heat was formed after 24 hours. For the first 6 hr., the energy lost as heat was greater than the energy lost as methane, but the opposite was true thereafter. The average heat of fermentation for three experiments was 83.8 kcal per kg dry matter, whereas the energy lost as methane was 174.1 kcal per kg dry matter. These values

TABLE 2. SUBSTRATE FERMENTED AND PRODUCTS FORMED DURING THREE 360-LITER, 24-HOUR *IN VITRO* FERMENTATIONS

Item	Experiment no.			Mean
	1	2	3	
Substrate fermented				
g Cellulose/kg alfalfa D. M.	187.7	197.3	186.7	190.6
% Cellulose digested	60.3	63.2	60.0	61.2
Products formed/kg alfalfa D.M.				
VFA, moles				
Acetic	...	2.67 (59.9) ^a	2.56 (63.0)	2.62 (61.4)
Propionic	...	1.04 (23.3)	0.98 (23.9)	1.01 (23.6)
Butyric	...	0.53 (11.9)	0.44 (10.9)	0.49 (11.4)
Valeric	...	0.22 (4.9)	0.09 (2.2)	0.15 (3.6)
CO ₂ , moles	2.75	2.81	3.16	2.91
CH ₄ , moles	0.81	0.83	0.83	0.83
H ₂ , moles	0.78	0.54	0.48	0.60
Heat, kcal	74.89	94.71	81.92	83.84

^a Figures in parentheses are molar percentages.

represented energy losses of 1.76 and 3.65%, respectively, of the gross energy of the alfalfa hay.

In experiments 2 and 3, the fermentation products (carbon dioxide, methane, hydrogen, heat and volatile fatty acids) were determined quantitatively and the quantity of substrate fermented was calculated from the carbon of the products (table 2). The energy changes associated with the *in vitro* fermentation of alfalfa are summarized in table 3. The values obtained by Marston (1948) for cellulose degradation and the calculated values from Wolin's (1960) theoretical rumen fermentation balance are included for comparative purposes. Marston (1948) calculated the heat of fermentation as the difference between the heat of combustion of substrate (cellulose) and products (volatile fatty acids, methane and microorganisms). The values for Wolin were calculated from the thermodynamics of

TABLE 3. HEAT CONTENT OF PRODUCTS AS PERCENT OF HEAT CONTENT OF SUBSTRATE FERMENTED

Product	Marston	Wolin ^a	Expt. 2	Expt. 3
Volatile fatty acids	84 ^b	74.5	79.6	74.3
Methane	..	19.1	10.2	11.0
Heat	6	6.4	5.5	5.1
Hydrogen	2.2	2.1
Microorganisms	10	...	2.5	7.5

^a Calculated from Wolin's (1960) theoretical rumen fermentation balance.

^b Average of four experiments in which cellulose was the substrate; this value includes the energy of methane. (Marston, 1948).

Wolin's (1960) theoretical rumen fermentation, i.e., the fermentation of glucose to acetate, propionate, butyrate, methane, carbon dioxide and water. In Experiments 2 and 3, the heat of fermentation was determined directly with the Armsby Calorimeter and the energy remaining in the microorganisms was calculated by difference.

The theoretical fermentation balance of Wolin (1960) appears to provide a good estimate of the percent substrate converted to volatile fatty acids but overestimates methane production. The present results substantiate Marston's (1948) earlier estimates of the heat of rumen fermentation *in vitro*. The experimentally determined heat production, 5.3% of the energy of the substrate, was slightly lower than the value (6%) reported by Marston (1948). The difference may be due to differences in substrates, techniques or experimental error. The present results do

confirm, however, that 5 to 6% of the energy of the substrate fermented is lost as heat. Heat production may not be a total loss, however, since the heat of fermentation may contribute to the maintenance of body temperature.

Summary

The Armsby Respiration Calorimeter was modified to function as an incubator-calorimeter at 39 C and used to determine the fermentation products (heat, carbon dioxide, methane, hydrogen) of alfalfa hay by rumen microorganisms *in vitro*. The 36 liter inoculum for the 360 liter *in vitro* fermentation was obtained by "building-up" the inoculum from 0.5 liter rumen fluid to 36 liters rumen fermentation medium in two successive 24-hr. *in vitro* fermentations of 5 and 40 liter, respectively.

In Experiments 2 and 3, 74.2% of the carbon in the substrate fermented was converted to volatile fatty acids, 20.2% to carbon dioxide and 5.6% to methane. In Experiment 3, the mean molar ratio of carbon dioxide to methane was 3.5 to 1, but the ratio varied from 6.5 to 1 to 2.3 to 1. A measurable quantity of hydrogen was formed during each fermentation period. Since, in Experiment 3, approximately two-thirds of the hydrogen was formed in the first 2 hr., it was postulated that hydrogen formation was associated primarily with the initial degradation of readily available carbohydrates or protein. Twenty-four hour heat, carbon dioxide and methane production curves were presented with maxima occurring at 5, 9 and 11 hr., respectively. In three experiments, the mean energy lost as heat of fermentation was 83.8 kcal per kg dry matter or 1.76% of the gross energy of alfalfa; whereas the energy lost as methane was 174.1 kcal per kg dry matter or 3.65% of the gross energy of alfalfa. In Experiments 2 and 3, the heat content of the products (volatile fatty acids, methane, heat, hydrogen, microorganisms) represented 77.0, 10.6, 5.3, 2.1 and 5.0%, respectively, of the heat content of the carbohydrate fermented.

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