Factors affecting the **in vitro** digestibility of tropical grasses*

S. J. COWLISHAW**, E. F. UNSWORTH***

COMPENDIO

En el curso de trabajos para establecer una rutina para la determinación in vitro *de la materia orgánica (M.O.D.) de pastos tropicales que estaban evaluándose agronómicamente, se estudiaron en 9 experimentos los efectos de varias variables.*

Los valores M.O.D. no fueron afectados al dar al ovino donador un suplemento de proteína, o al secar las muestras a 65° ó 105°C. Se encontró aconsejable usar licor del rumen de más de un donador, para estandarizar el momento de su recolección en relación con la comida previa, usar un vacío parcial para humedecer las muestras al comienzo del paso 1, y paralizar la fermentación al final sin usar cloruro mercúrico.

Los valores M.O.D. de una muestra de pobre calidad (1,5 por ciento P.C.) fueron aumentados al moler dos veces la muestra, y al incluir 6 mg of N-urea en la solución tampón agregada para el paso 1 con licor del rumen. La prolongación del paso 1 de 48 a 72 horas aumentó la cantidad de M.O. digerida en una diversidad de muestras, y la adición de urea aumentó la tasa de digestión, ocurriendo el aumento más grande en las muestras de calidad pobre. Se propone la ecuación asintótica $Y = A + Be^{-kx}$ para describir la tasa digestión.

La digestión en pepsina ácida por 24 horas aumentó la cantidad de M.O. digerida, pero su continuación por otras 24 horas no tuvo efecto con muestras que contenían hasta 22,6 por ciento de P.C. — Los autores.

Introduction

DURING the period 1965 to 1973 over 100 tropical pasture grass species and strains were introduced into Trinidad and established on the University Field Station. As part of the screening process, the D.M. or O.M. digestibility of dried samples was estimated using the two-stage *in vitro* method originally described by Tilley and Terry (12). The method was modified to suit local humid tropical conditions as a result of a series of trials described here, which tested recommendations made by Minson and McLeod (9), and others (1, 13).

An automated routine system of determining the *in vitro* digestibility of tropical grasses was developed.

Materials and methods

General

Samples of grasses were dried at 100°C in most experiments and milled through the 1.0 mm screen in a 20 cm 'Christy and Norris' hammer mill. In some trials samples were redried and passed through the 0.8 mm screen in a small laboratory mill. Approximately 0.5 g of sample was weighed into each polycarbonate centrifuge tube, and 2 g samples were used to determine

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** Department of Livestock Science, The University of the West Indies, St. Augustine, Trinidad. Present address: 3a Snowdon Road, Bournemouth, Dorset BH1 9HL, U.K.

*** Department of Agriculture, Agricultural and Food Chemistry Division, New Forge Lane, Belfast BT9 5PX, Northern Ireland.

per cent D.M. and O.M. Details of the age and chemical composition of the grass samples are given in Table 1.

Rumen fistulated sheep were kept in pens and given food and water *ad lib*. When the quality of the fresh Pangolagrass was low, a small amount of concentrate was also provided to raise the total dietary protein level to 10 per cent. Food was normally withdrawn 16 h before liquor was collected via a fistula using a vacuum pump, transported in vacuum flasks to the laboratory, and strained through several layers of nylon muslin.

The buffer solution was made up in two parts that were mixed with the rumen liquor just prior to inoculation of the tubes containing the grass samples. Normally 3 tubes/treatment were used as sufficient for the accuracy desired (C.V. <2 per cent). CO_2 was bubbled through the buffer solution and the rumen liquor before and during inoculation of the tubes and, in later experiments, it was passed into each tube as it was filled. All the liquids and the tubes were kept at 38-39°C in water baths or incubators both before and after filling, and the mixture in the tubes was tested to ensure that the pH remained within the limits 6.7-7.0. McLeod* recommended that the filling of tubes be done in 2 stages, the first 25 ml added being entirely of buffer solution with a vacuum applied to make all the grass particles sink before the rest of the buffer and the rumen liquor are added, thus reducing variability between replicates particularly when the sample is very fibrous. For the last 3 experiments this was done in an adapted dessicator jar; inclusion of the rumen liquor caused too much frothing. The tubes were filled from automatic pipettes filled by syphon from flasks immersed in a water bath and stirred by a stream of CO_2 gas.

Stage 1 fermentation was usually stopped after 48 h by removal from the incubator and centrifuging for 10 min., mercuric chloride ($HgCl_2$) being added as a routine in Experiments 1, 3 and 5 and as a treatment in Experiment 2. As filter sticks were unobtainable in Trinidad, the supernatant liquid was decanted off the residue through a short muslin-covered plastic tube of slightly smaller diameter than the centrifuge tubes. Particles caught on the muslin were washed back into the tube.

After digestion in acid pepsin (50 ml usually for 48 h), tubes were centrifuged and residues washed into tared glass beakers or nickel crucibles for drying and, in later experiments, ashing for 16 h at 550°C.

Details of the treatment of the samples in each experiment are given in Table 2 and below. In all but Experiment 5, standard samples 1 and/or 2 (Table 1) were included, and, in the last 4 experiments, the results from each run were adjusted so that the O.M.D. of samples 1 and 2 milled twice and digested with urea for 48 h in stages 1 and 2 were 60.5 and 35.2 per cent, respectively. Analyses of the variances for the data from all the experiments included values from individual tubes. Where one tube differed by more than 4 digestibility units from the others in that treatment, this datum was omitted.

Table 1.—The age, and per cent ash, crude protein and crude fibre contents of the dry matter of 8 grass samples used in Experiments 1 to 9.

Sample number	Species	Age of regrowth (weeks)	ash %	C.P. %	C.F. %
1	Mixed species (standard)	4-6	12.0	11.1	31.4
2	*Hemarthria altissima*	21	4.5	1.5	39.8
3	*Digitaria decumbens*	2	9.9	22.6	24.1
4	*Digitaria decumbens*	4	10.0	16.5	27.7
5	*Digitaria decumbens*	8	10.8	7.3	36.8
6	*Digitaria decumbens* (Pangola)	2-10	—	—	—
7	*D. milanjiana* subsp *eylesiana*	8	8.8	9.0	30.2
8	*Brachiaria* sp. (Tanner grass)	11	6.8	5.0	—

Experiment 1

The sheep were offered Pangolagrass (5.2-5.6 per cent C.P.) and a protein supplement. Liquor was collected from each sheep and used separately or as a mixture to digest standard grass sample 1. The first stage fermentation was stopped by refrigeration for 30 min at —5°C; both stages lasted for 48 h.

Experiment 2

Samples of rumen liquor were collected at 8.00, 10.15 and 13.30 on two successive Mondays and used to inoculate tubes within 30 min. Fermentation was stopped by cooling or by adding $HgCl_2$. Standard sample 1 was digested in 2 runs for 48 h in each stage.

Experiment 3

Half the residues from the digestion of sample 1 were washed with water and centrifuged a second time after each stage. Half the tubes were flushed with CO_2 between inoculation and insertion of the rubber bungs with bunsen valves.

Experiment 4

Liquor was collected from individual donor sheep after a week on Pangolagrass alone or supplemented with a concentrate. Diets were reversed and the experiment repeated the following week.

* McLeod, M. N. Personal communication 1975.

Table 2.—Treatments applied to grass samples in Experiments 1 to 9

Exp. N°	Grass sample used*	Drying temp (°C)	Times milled	Tubes/ treatment	Vacuum applied	Urea in buffer solution	Length of digestion (h) stage 1	stage 2
1	1	100	1	5	no	—	48	48
2	1	100	1	6	no	—	48	48
3	1	100	1	8	no	—	48	48
4	1	100	1	5	no	—	48	48
5	6	100	1	2	no	—	6, 72	0
6	1	100	1,2	3	no	— & +	48	0, 24, 48
6	2	65, 105	1,2	3	yes	— & +	48, 72	0, 24, 48
8	1, 3, 4, 5	100	2	3	yes	— & +	48	24, 48
9	1 2, 7, 8	100	2	3	yes	+	48, 72	24, 48

* See Table 1 for details

Experiment 5

Pangolagrass was harvested 2.5, 6.5 or 10.5 weeks after being cut back and dressed with 131 kg N/ha. Samples were fermented for 5.5, 12, 24, 36, 48 or 72 h with rumen liquor and buffer solution after which $HgCl_2$ was added and the tubes refrigerated before the residues were dried without a stage 2 digestion.

Experiment 6

Standard sample 1 was milled once or twice, 6 mg of urea-N was added to half the tubes and stage 2 digestion was continued for 0, 24 or 48 h.

Experiment 7

Standard sample 2 of very low quality was dried at 65° or 105°C, milled once or twice, and incubated with or without 6 mg of urea-N in the buffer solution for 48 h, and then for 0 or 48 h in acid pepsin. In run 2 urea was included in all the buffer solution and stage 1 fermentation continued for 48 or 72 h, followed by 24 h in acid pepsin. Standard sample 1 with urea-N was included in both runs.

Experiment 8

Three samples of *D. decumbens* of different ages (Table 1) and standard sample 1, with digestibilities ranging from 55 to 70 per cent, were fermented with or without 6 mg of urea-N in stage 1, and for 24 or 48 h in stage 2.

Experiment 9

Standard samples 1 and 2 were included along with samples 7 and 8 (Table 1) to provide a range in digestibility from 35 to 62 per cent. Urea was included in all the buffer solution, and samples fermented for 48 or 72 h in stage 1, followed by 24 or 48 h in acid pepsin.

Results

Experiment 1 (Table 3)

The D.M.D. of standard sample 1 was the same whichever donor sheep was used to provide liquor for stage 1 fermentation. When mixed liquor was used, there was a significant ($P<0.001$) improvement in D.M.D. There was a significant ($P<0.001$) difference between runs.

Experiment 2 (Table 4)

The addition of $HgCl_2$ to stop fermentation significantly ($P<0.001$) reduced the D.M.D. of sample 1 by 3.2 per cent. There was a significant ($P<0.001$) difference between runs. Collecting liquor at 10.15 resulted in slightly lower D.M.D. values in run 2, the interaction between time of collection and run being significant ($P<0.01$).

Experiment 3

The D.M.D. of sample 1 was 61.2 per cent when washing and centrifuging was done twice at the end of

Table 3.—The *in vitro* dry matter digestibility of a standard grass sample when incubated with rumen liquor donated by one of two sheep

Donor sheep	D.M. digestibility (%)			
	run 1		run 2	Means
A	59.8	—	61.8	60.8
B	59.3	—	61.5	60.4
A+B	61.4	—	63.0	62.2
S.E. (±) of means	—	0.40	—	0.28
means	60.2	—	62.1	—
S.E. (±) of means	—	0.23	—	—

Table 4.—The effects of run, rumen liquor collection time, and the use of $HgCl_2$ to stop stage 1 fermentation on the *in vitro* dry matter digestibility of a standard sample of dried grass

Liquor collection time		D.M. digestibility (%)			
		With $HgCl_2$		Without $HgCl_2$	Means
Run 1	8.00	58.2	—	62.7	60.5
	10.15	58.6	—	60.9	59.7
	13.30	58.6	—	59.9	59.3
Run 2	8.00	57.8	—	58.3	58.0
	10.15	55.5	—	58.1	56.8
	13.30	58.4	—	58.9	58.6
S.E. (±) of means		—	0.48	—	0.34
Run 1 means		58.5	—	61.2	59.82
Run 2 means		57.2	—	58.4	57.83
S.E. (±) of means		—	0.28	—	0.200
8.00 means		58.0	—	60.5	59.24
10.15 means		57.1	—	59.5	58.28
13.30 means		58.5	—	59.4	58.95
S.E. (±) of means		—	0.32	—	0.225
+ or — $HgCl_2$ means		57.86	—	59.79	—
S.E. (±) of means		—	0.200	—	—

each stage and 60.5 per cent when done only once. Flushing the tubes with CO_2 after filling also had no significant ($P=0.05$) effect (D.M.D. 60.9 per cent).

Experiment 4

The D.M.D. of standard sample 1 was 60.7 per cent with and without supplementary protein in the diet of the donor sheep.

Experiment 5 (Table 5)

The data were fitted to a cubic equation ($Y=A+BX-CX^2+DX^3$) and to an asymptotic equation ($Y=A+Be^{-kx}$) in which the D.M.D. (Y) was related to digestion time (X) in hours. The data fitted the asymptotic curves better than the cubic, particularly when X was 72 or more. Digestion of all 3 samples was not completed after 72 h in stage 1, and 116.3, 120.3 and 164.3 h respectively would have been needed to bring 99 per cent of the potentially digestible D.M. of the 3 samples into solution. No estimate was possible of the additional D.M. that would have been digested in acid pepsin.

Table 5.—Estimated parameter values used in fitting *in vitro* D.M. digestibility data to an asymptotic response curve.*

	Age of regrowth (weeks)		
	2.5	6.5	10.5
D.M. digestibility after 48 h stage 1	57.0	51.6	44.2
D.M. digestibility after 72 h stage 1	60.5	54.4	51.0
Parameter A (asymptote)	64.57	59.51	60.03
S.E. (±)	3.787	2.549	4.680
Parameter B	—64.52	—60.96	—62.70
S.E. (±)	3.540	2.314	3.718
Parameter k	0.04	0.01	0.03
Parameter R (exponent of —k)	0.96	0.96	0.97
S.E. (±)	0.007	0.005	0.005
Residual standard deviations (±)	3.206	2.078	2.400
Time needed to reach 95 per cent of asymptote (h)	75.7	78.5	107.4
Time needed to reach 99 per cent of asymptote (h)	116.3	120.3	164.3

* $Y = A + Be^{-kx}$.

Table 6.—The effects of adding urea to the buffer solution in stage 1 and of digesting in stage 2 for 0, 24 or 48 hours on the O.M. digestibility of a standard sample of dried grass.

Length of stage 2 digestion (h)	O.M. digestibility (%)			
	0	24	48	Means
— urea	52.6	61.5	60.5	58.18
+ urea	55.0	59.8	61.1	58.63
S.E. (±) of means	—	0.68	—	0.395
digestion time means	53.78	60.64	60.80	—
S.E. (±) of means	—	0.483	—	—

Experiment 6 (Table 6)

Digesting for 24 or 48 h in acid pepsin after 48 h in stage 1 significantly ($P<0.001$) increased by 12.9 per cent the amount of O.M. digested, but there was no significant ($P=0.05$) difference between 24 and 48 h for stage 2 digestion of this particular sample. The addition of urea to stage 1 fermentation only had a significant ($P<0.05$) effect when there was no stage 2 digestion.

Experiment 7 (Table 7)

Drying samples at 65° rather than 105°C had no significant ($P=0.05$) effect on the O.M.D. of the poor quality standard sample 2.

Double milling significantly ($P<0.001$) increased O.M.D. by 15.5 per cent when stage 1 lasted for 48 h and there was no stage 2; by 7.2 per cent when stage 1 lasted for 72 h and stage 2 for 48 h.

The addition of urea significantly ($P<0.001$) increased O.M.D. by 10.5 per cent. There was a significant ($P<0.01$) interaction between urea and length of digestion in stage 1. When stage 1 was for 48 h, increasing the length of stage 2 from 24 to 48 h did not increase D.O.M. either in the presence or absence of urea. When stage 2 digestion was for 24 h, 12.1 per cent more O.M. was digested in the presence than in the absence of urea after 48 h of stage 1 digestion, but the difference was only 3.9 per cent after 72 h of stage 1. Increasing stage 1 digestion from 48 to 72 h significantly ($P<0.001$) increased O.M.D. by 19.5 per cent when urea was added, and by 28.7 per cent when it was not.

Experiment 8 (Table 8)

Digesting for 48 h in stage 2 slightly (1.9 per cent) but significantly ($P<0.05$) increased the amount of O.M. digested in all 4 samples. The addition of urea also slightly but significantly increased the O.M.D. of the 4 grasses. The D.O.M. per cent of the 4 grasses differed significantly ($P<0.001$), but there were no significant ($P=0.05$) interactions with other treatments.

Experiment 9 (Table 9)

When stage 1 fermentation was increased from 48 to 72 h, there was a significant ($P<0.001$) increase in O.M. digested, 4.8 per cent when stage 1 lasted for 24 h and 9.5 per cent when it lasted for 48 h, the interaction between stages being significant ($P<0.05$). Digesting for 24 or 48 h in stage 2 had the same effect on O.M.D. The O.M. digestibilities of grass samples 1 and 7 were very similar (mean 63.15 per cent) and significantly ($P<0.001$) greater than that of sample 8 (51.51 per cent), which was greater than that of standard sample 2 (37.74 per cent).

Table 7.—The effects of milling twice, urea in the buffer solution, and digestion for 48 or 72 hours in stage 1 and for 0, 24 or 48 hours in stage 2 on the O.M. digestibility of standard sample 2.

Digestion length (h)			O.M. digestibility (%)		
	Stage 1	Stage 2	Milled 1	Milled 2	Means
— urea	48	0	26.3	29.8	28.1
	48	24	28.4	31.3	29.8
	48	48	26.4	30.1	28.3
	72	24	36.8	40.0	38.4
+ urea	48	0	28.9	33.9	31.4
	48	24	31.6	35.2	33.4
	48	48	30.8	35.2	33.0
	72	24	38.7	41.0	39.9
S.E. (±) of means			—	0.43 (n.s.)	0.31
— urea means			29.5	32.5	31.14
+ urea means			32.8	36.3	34.41
S.E. (±) of means			—	0.22 (n.s.)	0.151
48/0 means			27.6	31.9	29.74
48/24 means			30.0	33.2	31.60
48/48 means			28.6	32.6	30.63
72/24 means			37.8	40.5	39.12
S.E. (±) of means			—	0.31 —	0.214
Milling means			31.00	34.55	—
S.E. (±) of means			—	0.151 —	—

Table 8—The effects of adding urea to stage 1 and digesting for 24 or 48 hours in stage 2 on the O.M. digestibility of 4 grass samples

Length of stage 2 digestion (h)		O.M. digestibility of grasses (%)*				
		3	4	1	5	Means
— urea	24	66.8	63.2	58.5	53.5	60.5
	48	68.0	65.9	60.7	51.7	61.6
+ urea	24	70.3	63.1	59.6	52.4	61.4
	48	70.4	65.2	60.5	54.5	62.7
S.E. (±) of means		—	—	0.92 (n.s.)	—	0.46 (n.s.)
— urea means		67.4	64.6	59.6	52.6	61.03
+ urea means		70.4	64.2	60.0	53.5	62.01
S.E. (±) of means		—	—	0.65 (n.s.)	—	0.325
24 h means		68.6	63.2	59.0	53.0	60.94
48 h means		69.2	65.6	60.6	53.1	62.10
S.E. (±) of means		—	—	0.65 (n.s.)	—	0.325
Grass means		68.9	64.4	59.8	53.0	—
S.E. (±) of means		—	—	0.46 (n.s.)	—	—

* See Table 1 for details.

Table 9—The effects of digesting for 48 or 72 hours in stage 1 and for 24 or 48 hours in stage 2 on the O.M. digestibility of 4 grass samples

Length of digestion (h)		O.M. digestibility of grasses (%)*				
Stage 1	Stage 2	1	7	8	2	Means
48	24	62.8	62.8	48.2	35.9	52.42
	48	60.5	60.8	50.1	35.2	51.64
72	24	64.2	63.9	52.2	39.6	54.96
	48	64.8	65.5	55.5	40.4	56.53
S.E. (±) of means		—	—	0.95 (n.s.)	—	0.474
48 h means		61.7	61.8	49.2	35.5	52.03
72 h means		64.5	64.7	53.8	40.0	55.75
S.E. (±) of means		—	—	0.67 (n.s.)	—	0.335
24 h means		63.5	63.3	50.2	37.7	53.69
48 h means		62.7	63.1	52.8	37.8	54.09
S.E. (±) of means		—	—	0.67 (n.s.)	—	0.335 (n.s.)
Grass means		63.08	63.22	51.51	37.74	—
S.E. (±) of means		—	—	0.474 (n.s.)	—	—

* See Table 1 for details.

Discussion

Activity of rumen liquor

The activity of the liquor collected from individual sheep on different days may vary (14), although this is not likely if the feed quality and routine are fixed (7). As it is difficult to ensure uniform quality of feed in an environment such as that in Trinidad, where the high rainfall and humidity prevent regular making of good quality hay, it is safer in such circumstances to use mixed liquor from more than one animal, although this will not by itself ensure high activity. As was shown in Experiments 1 and 2, there may be significant differences between runs in which the same treatments and samples are used. In the latter experiment there was also a difference associated with time of collection in one run, but this could have been caused by an unnoticed variation in laboratory routine. Others have not recorded any differences associated with length of time between withdrawal of food and collection of liquor (1).

Checking and correcting for variations in rumen liquor activity between runs was aided by the inclusion of one or two standard samples, depending on the range in digestibilities expected between the samples in the run. If, as happened once, the results for these standards in a run varied by more than 5 digestibility units from expectation, all the results from this run should be rejected.

Diet of donor animals

It is generally recommended that the quality of the roughage on offer to donor animals should be high (13), and a minimum C.P. content of 10 per cent has been suggested*. This may not be necessary when a tropical breed of animal is used, as they utilise the N in low quality roughages more effectively than temperate breeds (5) by recycling of urea through the salivary glands, particularly when there is an adequate supply of dietary energy. This may explain the lack of response in Experiment 4 to the addition of a protein concentrate to a low quality (6 per cent C.P.) roughage diet provided for the donor sheep. Care must be taken to avoid feeding too much soluble carbohydrate, as this may cause the pH of the rumen liquor to fall below 6.8 and decrease digestibility (7, 10).

Number of replications

In early trials up to 8 replicates per treatment were used to measure small differences. In later trials 3 replicates were used rather than the 2 recommended (9, 13) so that an anomalous result could easily be seen, and the remaining 2 replicates were sufficient to give a good estimate of that treatment effect on D.M.D. or O.M.D. The 3 blocks of tubes were filled in turn so that variations in liquor activity or quantity of liquor D.M./tube could be observed. All block variance ratios in these experiments were very small (average 0.88) and non-significant ($P=0.05$).

Urea nitrogen supplementation of stage 1 fermentation

To ensure that there is an adequate amount of N available to the rumen flora for maximum possible *in vitro* digestion of structural carbohydrates, the buffer solution may be fortified with 6 (10) or 10 (6) mg of urea-N. The addition of 6 mg/tube had no effect on the quantity of O.M. in sample 1 digested except when stage 2 was omited (4.9 per cent increase), probably because there was an adequate level of N in the sample (11.1 per cent C.P.). With a low quality (1.5 per cent C.P.) sample, varying the length of stage 2 from 0 to 24 or 48 h had no consistent effect on the response to added urea-N. Increasing the length of stage 1 from 48 to 72 h reduced the effect of urea-N on D.M. digestibility.

In Experiment 8 the poorest sample contained 7.3 per cent C.P. and added urea-N had only a very small effect. Raymond and Terry (10) got no response to the addition of N when the diet of the donor sheep was 9.4 per cent C.P. hay, but there was a response when the diet was 3.1 per cent straw. Alexander and Mc Gowan (1), using temperate type sheep, recorded a negative correlation ($r=0.79$) between response to added N and C.P. content of 21 samples (range 6.1 to 25.0 per cent). The minimum amount of N required in the combined substrate and rumen liquor for maximum digestion of O.M. is not known, but the results show that the adverse effects of low N can be overcome by increasing the length of stage 1 fermentation to 72 h.

Drying temperature

There was no difference in the O.M.D. of a sample dried at 65° or at 105°C. Tilley and Terry (13) reported no difference between freeze-drying and oven-drying (100°C), but repeated re-drying, drying for several days, or drying at more than 100°C caused a loss of D.M. Rapid drying at 100°C and rejection of any unused sample after re-drying was adopted as part of the routine.

Milling of samples

It has been stated (7) that a far higher standard of control of this two-stage technique is necessary for tropical pasture grasses than appears needed for temperate grasses. One probable reason for this is the higher fibre content of tropical grasses, even when young. When milled once through a 1.0 mm screen, the samples often contained quite large particles of fibrous matter, which made accurate sub-sampling very difficult. By regrinding in a small mill these large particles were broken down and digestibility increased, as it was when a smaller screen size was used for tropical grass samples (7, 16) but not with temperate species (12). The coefficient of variation was 1.50 per cent in Experiment 8 (mean O.M.D. was 61.5 per cent) and 1.90 per

* Terry, R. A. Personal communication. 1970.

cent in Experiment 7 (mean O.M.D. was 31.6 per cent) supporting the view (4) that variability was negatively correlated with quality of sample.

Length of stage 1 fermenta

Digestion with rumen microorganisms is necessary in order to bring into solution those fractions of the structural carbohydrate that would be digested by the animal. The length of time required will depend on the per cent crude fibre, how lignified it is, how small the particles are, and the supply of other nutrients needed by the bacteria for maximum activity. The effect of added N has already been discussed.

In Experiment 5 the rate of digestion of D.M. was greatest with the youngest sample, and least with the oldest, but the difference between them was less than that between 2 samples of Rhodes grass used by Wilkins (15) in a study of cellulose digestion *in vitro* (see Fig. 1). For 13 forages (16) the cellulose digestibility data for 48 and 72 h incubation also fell between the 2 Rhodes grass curves unless a mixture of glucose, urea and yeast extract had been added to the substrate. This caused a more rapid rate of digestion in the first 48 h but no greater maximum digestion after 6 days. The rate of digestion of the D.M. in a sample of Setaria (7) was similar to that of the better Rhodes grass sample for the first 12 h but, because the maximum digestibility was less, the rate of digestion then became less and, by 72 h, the same amount of D.M. had been digested as in the 2.5-week-old Pangolagrass in Experiment 5. Samples with the same terminal digestibility may be broken down by rumen bacetria *in vitro* at quite different rates depending on the ratio of cellulose to hemicellulose (10). As expected from the above considerations, increasing stage 1 digestion time from 48 to 72 h in Experiments 7 and 9 increased the amount of O.M. digested. The increase was greatest (23.8 per cent) with standard sample 2 of low digestibility and least (4.6 per cent) with the samples of highest digestibility. When samples are finely ground and urea is added to the buffer solution, the probable increase in digestibility brought about by digesting for 72 h, instead of the standard 48 h, will be less that 5 per cent when the 48 h O.M.D. is over 60 per cent. With poor quality samples, a longer stage 1 digestion will give results that are nearer to those likely *in vitro*, because rate of passage of digesta is slower with feeds of low digestibility (15, 16).

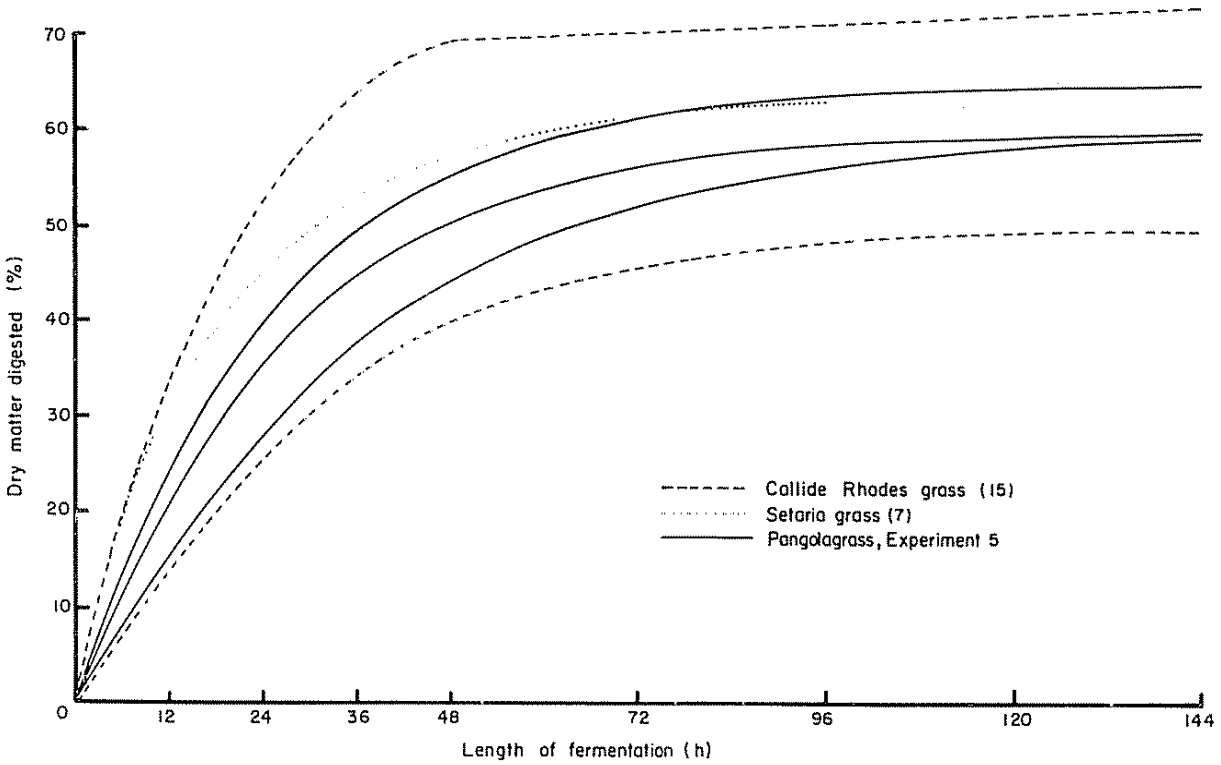

Fig. 1.—*Effect of length of fermentation in rumen liquor on the amount of dry matter digested in 6 samples of tropical grass: data fitted to the asymptotic equation* $Y = A + Be^{-kx}$

Ending fermentation

It is important to have full fermentation for the whole period of stage 1 and to stop it completely as soon as the time is expired, otherwise variability increases and the smaller differences cease to be significant statistically. It has been reported (8) that treatment with a partial vacuum of the sample and some of the buffer solution in the tubes before the rumen liquor is added increased digestibility by 3.9 per cent and decreased the S.E. of estimate from ±1.96 to 1.49. In the present study, the effect was not measured, but decantation at the end of stage 1 was much easier.

Mercuric chloride solution stops fermentation at once, but the reduction in per cent D.O.M. when it was used may have been the result of traces of $HgCl_2$ left in the scratched plastic tubes reducing the activity of the acid pepsin in stage 2 (9), although Tilley and Terry (13) state that this will not occur. There was also the danger that these residual traces would reduced the activity of the rumen liquor in subsequent runs using the same tubes. It is therefore recommended that new tubes be used and no mercuric chloride. Rapid centrifuging, and decantation ensured a low coefficient of variation (<2 per cent). Washing the residues in water and a second centrifuging before stage 2 had no effect on digestibility values in Experiment 3 or in trials by Minson and McLeod (9).

Length of stage 2 digestion

When there was no stage 2 digestion in acid pepsin, less O.M. was brought into solution when the sample contained 11.1 per cent C.P. but not when it contained only 1.5 per cent C.P. Thus, for feeds of better quality, digestion in acid pepsin is needed to bring all the digestible protein into solution (11, 12, 13). A period of 24 h was all that was needed to digest this protein in these experiments even with 22.6 per cent C.P. and 70 per cent D.O.M.

Organic matter digestibility

Because some ash is digested and soil contamination is difficult to avoid, it is considered better to express results in terms of organic matter digestibility (13) even though this involves extra work and may make the relationship between *in vitro* and *in vivo* results difficult to determine because of the endogenous excretion of minerals. As *in vivo* digestibility values are affected by a number of factors including level of intake, and the *in vitro* technique is normally used (13) for the rapid comparison of large numbers of samples, it would appear unnecessary in most cases to establish very accurate predictive equations for each species.

Recommendations on laboratory procedures

Liquor should, where possible, be drawn from more than one animal donor and used as quickly as possible. It should be kept in a thermos flask at its original temperature and filtered through a double layer of nylon muslin. The donor sheep should be maintained on a good quality roughage diet free of starch.

As a safeguard, the buffer solution should contain urea and part of it added to each tube initially, followed by partial evacuation of air to thoroughly wet the finely-ground sample. The rest of the buffer solution mixed with the rumen liquor should then be added to the tubes and the bunsen valves inserted.

It is advisable to use 3 tubes per sample and to include 1 or 2 standard samples in each run.

For comparing poor quality samples a stage 1 fermentation period of 72 h should be used. Mercuric chloride should not be used to stop fermentation. A 24 h stage 2 digestion is all that is usually needed to bring all the soluble protein into solution.

Summary

In the course of establishing a routine for the determination of the *in vitro* digestible organic matter (D.O.M.) of tropical grasses being agronomically screened, the effects of several variables were studied in 9 experiments.

D.O.M. values were not affected by giving the donor sheep a protein supplement, or by drying samples at 65° or 105°C. It was found advisable to use rumen liquor from more than one donor, to standardise the time of its collection relative to the previous meal, to use a partial vacuum to wet samples at the start of stage 1, and to stop fermentation at the end without using mercuric chloride.

D.O.M. values of a poor quality sample (1.5 per cent C.P.) were increased by milling the sample twice, and by including 6 mg of urea-N in the buffer solution added for stage 1 fermentation with rumen liquor. Prolonging stage 1 from 48 to 72 h increased the amount of O.M. digested in a range of samples, and the addition of urea increased the rate of digestion, the increase being greatest in the samples of poor quality. The asymptotic equation $Y = A + Be^{-kx}$, to describe the rate of digestion, is proposed.

Digestion in acid pepsin for 24 h increased the amount of O.M. digested, but continuation for a further 24 h had no effect with samples containing up to 22.6 per cent C.P.

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