A TWO-STAGE TECHNIQUE FOR THE *IN VITRO* DIGESTION OF FORAGE CROPS

J. M. A. Tilley and R. A. Terry

The Grassland Research Institute, Hurley, Berks.

A simple technique for the determination *in vitro* of the dry- or organic-matter digestibility of small (0·5 g) samples of dried forages is described. It involves incubation first with rumen liquor and then with acid pepsin. Using 146 samples of grass, clover and lucerne of known *in vivo* digestibility (Y), the regression equation $Y = 0{\cdot}99\,X - 1{\cdot}01$ (S.E. $\pm 2{\cdot}31$) has been calculated, where X = *in vitro* dry-matter digestibility. This technique has been used for the study of the digestibilities of plant breeder's material, of the leaf and stem fractions of herbage and of herbage consumed by animals.

INTRODUCTION

Digestibility experiments with herbages have considerable value in the estimation of their nutritive value to ruminants (Blaxter, 4); however, these experiments are tedious and require large quantities of herbage. Further, it is possible only to measure the overall digestibility of a sward and not that of its individual botanical components. Consequently, the plant breeder cannot use digestibility as a criterion during the initial selection of new varieties.

Attempts have been made to develop chemical methods for the assessment of herbage digestibility. While a limited correlation has been found between *in vivo* herbage digestibility and the contents of individual chemical components such as crude protein (11, 22), crude fibre (10, 20) or lignin (18), these relationships cannot be applied equally to all species of forage plants. Thus, Kivimäe (16) has found quite different relationships for timothy (*Phleum pratense*) and red clover (*Trifolium pratense*).

Attention has therefore been turned to laboratory *in vitro* methods, in which feeding stuffs are digested by preparations of micro-organisms or of enzymes which are similar in function to those present in the digestive tract of the ruminant. These methods have been reviewed by Shelton and Reid (27) and Barnett and Reid (3). They vary considerably in their complexity, according to particular experimental requirements. The method described below was evolved after a comparison of several published methods. Brief details have already been given (30, 31); this paper describes the *in vitro* determination of digestibilities for a wide range of herbages whose *in vivo* digestibility had been previously measured in sheep trials (Raymond, Harris and Harker, 25).

METHOD

Principle of method

The method has two main stages; in the first, a small (0·5 g) sample of a dried forage is digested anaerobically with rumen micro-organisms at 38°C in the dark. After initial experiments with dialysis sacs (Huhtanen, Saunders and Gall, 14) and with the continuous gassing of samples (Frens and Stolk, 12), it has been found preferable to digest in glass tubes and to rely on the gas production during the digestion to maintain anaerobic conditions. A relatively large volume of buffer solution (40 ml.) is added; this is usually adequate to maintain the pH level within the usual limits for digestion and to ensure that the final acid concentration does not exceed that found in the animal. The inoculum of micro-organisms is supplied as strained rumen liquor (10 ml). This supplies adequate levels of the accessory factors for efficient digestion, such as valeric acid and trace elements (3), together with protein for bacterial growth. Purified preparations of rumen micro-organisms (washed suspensions: Warner, 33) did not seem to have adequate cellulytic activity; the crude rumen liquor inoculum is preferred as it can be relied upon to give high digestive efficiency.

It has been found that although the process of fibre digestion is complete by the end of 48 h, the conversion of herbage protein into soluble, 'digested', products is not. The greater the content of protein in a herbage, the smaller the proportion that can be converted into these soluble products. The insoluble portion consists of a mixture of unchanged feed protein and microbial protein (35). A second stage of pepsin digestion has been introduced to remove this undigested protein (Tilley, Deriaz and Terry, 31) giving *in vitro* digestibility values similar to those found *in vivo* with sheep.

This method uses simple apparatus; it is reproducible and many samples can be handled in a single experiment. It is therefore suitable for introduction into the routine evaluation of forage samples.

Details of method

Materials

A representative sample of each forage was dried for 6 h at 100°C in a forced-draught Unitherm oven, and ground to pass the 0·8 mm sieve of an 8 in. Christy and Norris laboratory grinding mill (13).

Rumen contents were removed, through a permanent fistula, from a sheep maintained on a diet of hay. The liquor was strained through two layers of muslin into a flask, and CO_2 was passed into the flask to displace air from above the rumen liquor, which was then kept at 38°–39°C until required.

The buffer solution was made up according to the formula for 'synthetic saliva' of McDougall (19), adding the $CaCl_2$ last; the solution was thoroughly saturated with CO_2 at 38°C until it became clear.

Pepsin solution was made up by dissolving 2·0 g of 1 : 10,000 pepsin (Chas. Zimmermann & Co. Ltd.) in 850 ml demineralized water. 100 ml of *N* HCl was added and the solution then made up to 1 litre with water.

1st Stage rumen liquor digestion

Duplicate samples of oven-dried forage (0·5 g) were weighed into 80–90 ml glass centrifuge tubes; this capacity allowed space for formation of foam and prevented losses during shaking. The tubes were then stored at 38°C until required.

To each tube 40 ml of the buffer solution were added, followed by 10 ml of the strained rumen liquor. In practice it was found convenient to mix sufficient rumen liquor and buffer solution, in the above proportions, for an experiment; the mixture was stirred, gassed with CO_2, and 50 ml were added to each tube. The space above the liquid in each tube was thoroughly flushed out with CO_2 from a Kipp gas generator, and the tube was then sealed with a rubber cork fitted with a Bunsen gas release valve (Fig. 1). The 4 mm slit in the rubber tube on the valve was cut with a sharp

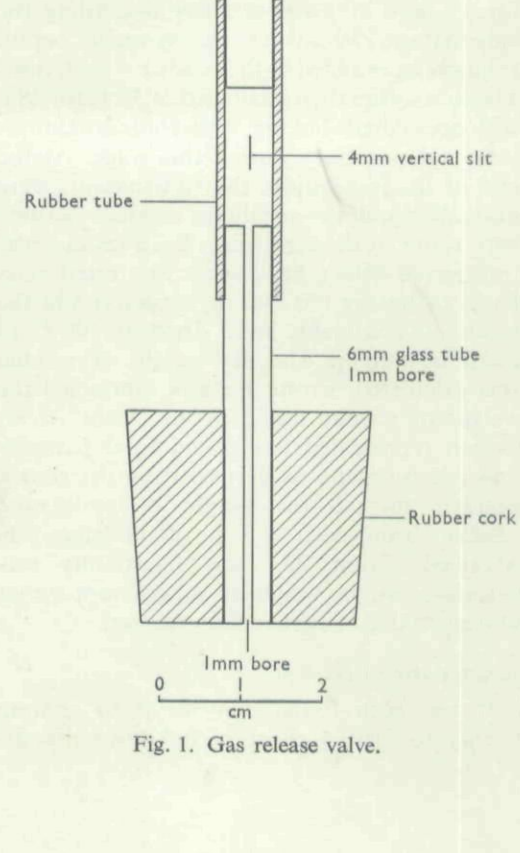

Fig. 1. Gas release valve.

knife; the slit normally remained closed, opening only to release gas from inside the tube. After sealing, the tubes were incubated at 38°C in the dark for 48 h, being shaken 3 or 4 times a day by hand.

During the incubation, the pH was maintained within the limits 6·7–6·9. When the donor sheep was fed on hay, pH correction was not usually required; when grass was fed, the resultant rumen liquor occasionally contained so much undigested food that the total acid production during the experiment exceeded the buffer capacity. In these cases, pH checks were made at 6 h and 24 h after the start of incubation and appropriate adjustments made with N Na_2CO_3.

2nd Stage pepsin digestion

At the end of the first incubation period, bacterial activity was checked by the addition of 1 ml of 5 per cent $HgCl_2$. 2 ml of N Na_2CO_3 were also added to improve sedimentation. Although the tubes could be stored at 1°C they were usually centrifuged immediately for 15 min. at 1,800 g. After discarding the supernatant, 50 ml of freshly-made pepsin solution were added to the residue in each tube. The tubes were then incubated at 38°C for 48 h with occasional shaking. Anaerobic conditions were not necessary during this stage. At the end of the incubation, the supernatants were discarded and the insoluble residues washed with water in the centrifuge. Each residue was transferred with a little water to a tared glass basin or beaker (50–100 ml capacity) and the basins and contents were dried at 100°C to constant weight. The dry weight of residue was calculated. From this was subtracted the weight of residue found in the 'blank' tubes (which represented undigested food particles and micro-organisms derived from the rumen liquor), and so the weight of undigested residue from each 0·5 g of herbage was obtained. From this, the digestibility was calculated as the weight of digestible material in each 100 g of herbage dry matter.

Routine use of method

It has been found convenient to analyse twenty to forty forages of unknown digestibility, together with two standard herbages, in a single experiment. Each standard should be analysed in triplicate or quadruplicate. The two 'standard' samples are selected, of 'high' and 'low' digestibility relative to the unknown materials analysed. The 'mean' digestibility of each of these samples was calculated from its performance in at least 10 different *in vitro* experiments. (These mean values were closely similar to the *in vivo* digestibilities of the standards.) Comparison of the mean digestibility of the standards with the values found in a given experiment enabled an assessment to be made of the digestive efficiency of the particular rumen liquor preparation and pepsin solution used. Appropriate corrections were made if the values for the standard forages were higher or lower than their mean digestibilities.

A graph was constructed for each experiment on which the 'mean' digestibilities of the standard samples were plotted along the Y axis, and the 'found' digestibilities along the X axis. The points for the 'high' and 'low' standards were joined with a straight line, and the correct digestibilities for the unknown samples were estimated as the Y values corresponding to the digestibilities found (X values). For the data in Fig. 2, the standards used had 'mean' *in vitro* dry-matter digestibilities of $75{\cdot}5 \pm 0{\cdot}42$ and $50{\cdot}4 \pm 0{\cdot}48$. The standard error of an individual determination was $\pm 1{\cdot}90$ and $\pm 2{\cdot}31$ respectively as calculated from 23 experiments. The corresponding *in vivo* dry-matter digestibilities were 76·8 and 49·4.

Factors affecting the method

Some variation in digestive efficiency between experiments seems to be unavoidable and wherever possible similar herbages should be compared within the same experiment.

To obtain good digestion it is essential to maintain anaerobic conditions throughout the first stage. Thorough gassing of the solutions and the tubes with CO_2 and attention to the condition of the gas release valves (ensuring that they open only to release fermentation gases) are most important. The pH should be carefully controlled and the inoculum should

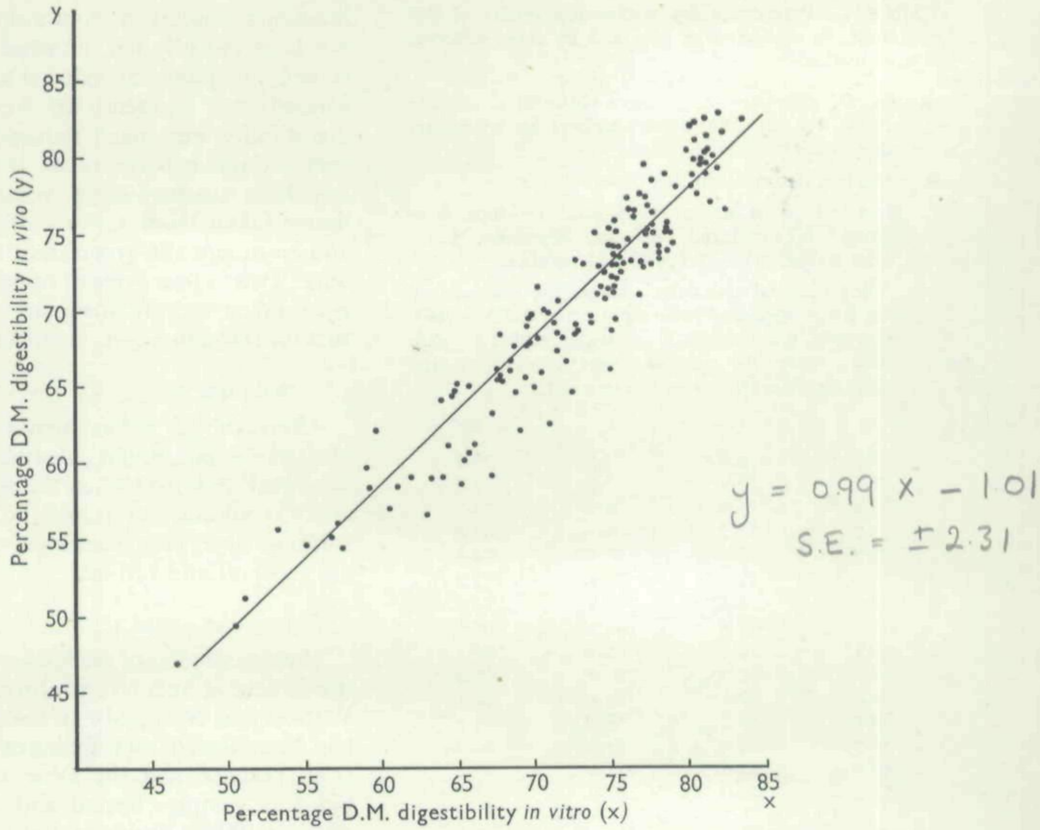

Fig. 2. Comparison of the dry-matter digestibility *in vivo* (y) and *in vitro* (x) of 130 samples of grass and 18 of clover and lucerne.

be kept as near as possible to 38°C. Some cooling during the mixing of inoculum with buffer solution and forage sample is inevitable, but pre-warming of glassware and solutions to 38°C helps to maintain temperature.

Filtration

The intermediate centrifugation after the rumen liquor incubation can be omitted and replaced by direct acidification of the contents of the tubes with (say) 2·2 *N* HCl to *c*. pH 1·5 (*see* Alexander and McGowan, 1). $HgCl_2$ and Na_2CO_3 solutions are not added. The pepsin is in this case added as a 2% solution in water. Centrifuging can also be entirely replaced by filtration at the end of the second stage. Thus residues can be filtered off on tared 12 cm No. 4 filter papers or through porous alumina crucibles. Table 1 shows results obtained by the use of these methods. A similar method is used by Dent (8). Experience has shown that, if the supernatant is not discarded after the first incubation stage, filtration after the second stage will be difficult unless Celite filter aid is added (1, 8). The decision whether to use centrifugation or a filtration method must depend upon the available facilities. The method given in detail above is however more versatile. For instance the final residues are recovered in a form suitable for analysis of digestible carbohydrates (cellulose, hemicellulose, etc.) and for this purpose a modification of the method of Deriaz (9) was used. Centrifuging after the first stage also enables the acid products of rumen digestion to be isolated. Moreover, it may be convenient to halt the analysis at the first stage and to store the tubes in the refrigerator over a period (say

TABLE 1. Percentage dry matter digestibility of S37 cocksfoot, as measured *in vivo* and by three different *in vitro* methods

A. *In vivo* digestibility by sheep (Minson *et al.*, 21; 1959). 1st cut data, expressed here as dry-matter digestibility.

B. Method described in this paper.

C. Method as B except that final residues were filtered off on tared 12·5 cm. Whatman No. 4 filter paper, dried at 100°C and weighed.

D. After the first incubation stage, the contents of the tubes were acidified to pH 1·5 and 2·2 *N* HCl, and 0·12 g pepsin in 5 ml. water added to each tube. After the second stage incubation, the residues were filtered as in method C.

A	B	C	D
76·2	74·4	73·5	73·7
74·2	72·9	72·3	72·0
76·8	73·3	71·9	73·2
75·2	73·1	70·5	69·8
75·5	75·4	72·5	74·9
75·4	72·6	72·9	72·3
72·5	73·4	72·6	74·4
71·5	67·8	66·5	67·3
69·1	70·4	68·3	69·1
64·3	62·0	61·9	63·4
59·0	60·6	59·4	62·1
59·1	59·2	59·2	61·5
54·5	54·6	53·8	56·9
49·4	47·4	48·6	49·2
51·2	48·0	48·0	48·7

a weekend) before commencing the second stage.

Rumen liquor

It has been usual to feed *ad lib.* the sheep from which rumen inoculum is taken. The rumen liquor is then rich in food particles and the weight of 'blank' residue after incubation is about 0·06 g. Since the weight of dry residue from 0·5 g of forage with an *in vitro* digestibility of 80% is only 0·10 g, a large proportion of the undigested residue is derived not from the forage under test but from the rumen liquor inoculum. It has to be assumed, moreover, that the weight of the final residue from the inoculum is the same, whether or not herbage is present during the incubation. This large 'blank' residue is also inconvenient when the digestibility of individual fibre fractions in herbage is being determined. However, it was observed that, when rumen inoculum was taken from animals which had not been recently fed, or when the volume of rumen inoculum was reduced to 1 ml, or when washed cell suspensions were used, fibre digestibility was much reduced and was also very variable between tubes. It therefore seems advisable to use large volumes of rumen liquor taken from actively digesting animals, and to accept the errors that this may introduce. Two to four litres of rumen contents have been taken weekly from an adult sheep by suction through a ¾-in. diameter tube.

Sample size

Although 0·5 g has proved to be a convenient sample weight, identical digestibilities have been obtained when the weight of sample and the volumes of rumen liquor and buffer solution have been increased to, respectively, 1·5 g, 30 ml and 120 ml.

Drying the samples

The digestibility of the fodder crop is reduced if it is held at high temperatures when dry (*see* Watson and Nash, 34). In the present work it has been found that freeze-dried samples of fresh herbage had the same *in vitro* digestibility as samples heated and dried at either 40°C or 100°C; drying at 100°C had a marked effect on digestibility only if continued for longer than 4 days (Table 2(a)). Reheating previously dried (100°C) and ground samples at temperatures above 100°C for 16 h had no effect except at temperatures of 125°C or more (Table 2 (b)). It was concluded that drying for 1 or 2 days at 100°C was safe.

Grinding the samples

Herbage samples submitted for analysis differ considerably in their fineness of grinding, this being dependent not only on the type of mill and of mill sieve used but also on the moisture content of the sample at the time of grinding. However, when coarsely ground and finely ground samples of the same herbage were compared, their digestibilities were identical (Table 3). Samples need only be ground finely enough to ensure good sampling of the small weights of herbage used. As found by Virtanen (32), Pew (24) and Dehority and

TABLE 2. Effect of drying period and temperature on percentage *in vitro* dry-matter digestibility of grass samples

(a) Effect of period of drying at 100°C on two samples of fresh ryegrass (1 and 2)

	Digestibility	
Period of drying	(1)	(2)
6 h	81·3	82·2
1 day	82·4	81·7
2 days	81·7	82·4
3 days	80·0	81·3
7 days	80·7	—

(b) Effect of 16 h drying at temperatures between 100°C and 145°C on two samples of ground cocksfoot (3 and 4), previously dried at 100°C

	Digestibility of grass sample			
Drying	(3)		(4)	
temperature °C	(i)	(ii)	(i)	(ii)
100	73·5	73·5	56·6	56·6
105	72·8	75·8	55·7	58·7
110	72·9	76·0	55·8	59·0
115	72·7	76·0	55·2	58·4
120	72·8	76·1	53·7	57·4
125	72·4	76·1	54·8	58·3
130	70·3	73·9	47·0	51·0
135	66·3	70·0	45·2	50·3
140	63·8	67·9	34·2	40·2
145	55·2	58·5	26·2	29·8

(i) Digestibility calculated on dry-matter content at indicated temperature.

(ii) Digestibility calculated on original dry-matter content at 100°C

Johnson (7), very fine grinding (ball milling) of herbage greatly increases *in vitro* digestibility. After 3 to 5 hours grinding in the tungsten steel vial of the Spex 800 mill*, the increase was most marked in samples initially of low digestibility (digestibility increased from 47·5 to 74·5) when compared with samples which originally had high digestibility (increased from 74·8 to 78·9). It must be assumed that the effect of fine grinding is to disrupt the cell walls of the plant structure and to enable enzymes to penetrate into regions from which they are normally excluded, perhaps by the protective effect of lignification or of crystallinity in the cellulose structure.

RESULTS

Correlation between in vivo and in vitro

A total of 148 herbages of known *in vivo* digestibility—18 samples of lucerne and clover and 130 samples of grasses—have been examined. The latter included perennial ryegrass (*Lolium perenne*), Italian ryegrass (*L. multiflorum*), H1 ryegrass (*L. perenne* × *multiflorum*), cocksfoot (*Dactylis glomerata*), timothy (*Phleum pratense*), tall fescue (*Festuca arundinacea*) and meadow fescue (*F. pratensis*). The results are shown in Fig. 2; the linear regression equation $Y = 0{\cdot}99X - 1{\cdot}01$ (S.E. $= \pm 2{\cdot}31$) has been fitted to the data, where Y = per cent *in vivo* dry-matter digestibility and X = per cent *in vitro* dry-matter

*Glen-Creston Ltd.

TABLE 3. Effect of grinding intensity on percentage *in vitro* dry matter digestibility

			Proportion of dry weight of ground sample passing through B.S. mesh sieves					
Sample	Mill*	Screen	>30 mesh	30–60 mesh	60–100 mesh	100–200 mesh	<200 mesh	Digestibility
Lucerne	1	0·8 mm	3	47	28	10	11	61·8
	4	fine	0	29	38	18	15	62·1
S24 ryegrass	1	0·8 mm	0	20	45	21	13	80·3
	2	coarse	0	60	20	17	3	80·4
	3	medium	0	0	55	31	14	80·3
	4	fine	0	0	43	43	14	80·0

*Mill 1 : Christy and Norris 8-in. Laboratory grinding mill 0·8 mm screen.
Mill, 2, 3, 4 : Apex 6-cm cutter type mill with coarse, medium and fine mesh screens, respectively.

digestibility. When only the 18 lucerne and clover samples were examined, the linear regression equation $Y = 0{\cdot}85X + 8{\cdot}37$ (S.E. $= \pm 1{\cdot}42$) could be fitted to the data. However the digestibility range of these samples was only from 55·0 to 77·9 (Y values) and over this limited range the two equations are not significantly different. No appreciable differences could be detected between individual grasses, or between the same grass in different years, and it was concluded that the equation $Y = 0{\cdot}99X - 1{\cdot}01$ could be applied generally to all of these species. The standard error of this relationship ($\pm 2{\cdot}31$) is smaller than that of other general relationships for the prediction of herbage digestibility from chemical composition (see Raymond *et al.*, 26).

Reproducibility

The digestibilities of 46 of the grass samples were measured in duplicate in each of two experiments. Analysis of the results showed that, within an experiment, the standard error of the mean of duplicate measurements was $\pm 0{\cdot}66$ digestibility units. Between experiments, the standard error of the means was $\pm 1{\cdot}18$.

DISCUSSION

There is an obvious dissimilarity between the indigestible residues from *in vitro* digestion and animal faeces, which contain metabolic products in addition to undigested food residues. Apart from this, the failure to predict exactly *in vivo* digestibilities from *in vitro* results reflects not only the inherent analytical errors in the two methods, but also the fact that *in vivo* digestibility is not a constant characteristic of a herbage. It varies according to whether cattle or sheep are used in trials (Cipolloni, Scheider, Lucas and Pavlech, 5), the age and health status of animals (13), the level of feed intake (Ivins, 15) and the manner in which the feed is prepared (Minson, 21). This sets a limit to the accuracy with which *in vivo* digestibility can be predicted from any analysis of herbage. Because the possible variability in *in vivo* digestibility (Ivins, 15) is much larger than that found under the controlled conditions of the *in vitro* digestion (where data are always related to standard samples), it is now considered preferable to report the analytical results as *in vitro* digestibilities, the form in which they are measured. The prediction of *in vivo* digestibility can be made, using the regression equation above, but additional and arbitrary correction may be thought necessary to take account of variability in animal performance.

If considered in terms of *in vitro* digestibility, the precision of results is affected only by the accuracy and reproducibility of the method itself; direct comparisons between samples of closely similar digestibilities become possible. For example, large numbers of individual plants (genotypes) can be examined and significantly different samples chosen for further study in plant breeding programmes (Cooper, Tilley, Raymond and Terry, 6); repeated observations that these differences are maintained over several cuttings or seasons will eventually warrant an animal assessment. This final evaluation with animals is essential, as *in vitro* digestion trials can be a guide only to the potential, rather than to the realizable, value of a feed. Examination of the contributions of the leaves and stems of herbage to the overall digestibility of the plant has also helped to explain the changes in digestibility which accompany maturation (Tilley, 29). The digestibility of herbage consumed by animals at pasture has been determined on samples obtained immediately after ingestion, by means of rumen fistulae with cattle (Tayler and Deriaz, 28), or by oesophageal fistulae with sheep (Lambourne, 17); this digestibility can be compared with that of the herbage on offer and with that of the herbage uneaten. This technique has also been used to study the changes in digestibility of the pastures on offer to a dairy herd during the grazing season (Baker, 2).

Brassica crops and fodder maize have been examined (Dent, 8), together with a number of tropical and sub-tropical forages. In addition, the proportions of carbohydrate fractions digested from grasses at different maturity stages have been examined.

In these and other ways the versatility of this simple technique has been demonstrated, and problems have been examined which purely chemical methods of analysis have been unable to solve and which could not be studied with animal digestibility trials.

ACKNOWLEDGEMENTS

The authors would like to thank Dr. William Davies and Mr. W. F. Raymond for their help and encouragement in this work. Dr. A. T. Cowie of the National Institute for Research in Dairying kindly performed the rumen fistulation operation. Mr. C. D. Kemp and Miss S. M. Green have given valuable advice over the statistical interpretation of data. The authors' thanks are also due to Dr. D. J. Minson and Mr. C. E. Harris who, together with Mr. Raymond, provided the samples of known *in vivo* digestibility. The co-operation of Mr. R. H. Alexander of the West of Scotland College of Agriculture and Mr. J. Dent of the National Institute of Agricultural Botany in the development of the method are gratefully acknowledged.

REFERENCES

1. ALEXANDER, R. H. and MCGOWAN, Mary. 1961. A filtration procedure for the *in vitro* determination of digestibility of herbage. *J. Brit. Grassl. Soc.* Vol. 16, pp. 275–6.
2. BAKER, R. D. 1963. Personal communication. See also, Grassland Research Institute 1963 *Experiments in Progress*, No. 15. Hurley: G.R.I.
3. BARNETT, M. J. F. and REID, R. L. 1961. Reactions in the rumen. London: Edward Arnold (Publishers) Ltd., pp. 252.
4. BLAXTER, K. L. 1960. The utilization of the energy of grassland products. *Proc. 8th Int. Grassl. Congr.* pp. 479–84.
5. CIPOLLONI, Mary A., SCHNEIDER, B. H., LUCAS, H. L. and PAVLECH, Helen M. 1951. Significance of the differences in digestibility of feeds by cattle and sheep. *J. Anim. Sci.* Vol. 10, pp. 337–43.
6. COOPER, J. P., TILLEY, J. M. A., RAYMOND, W. F. and TERRY, R. A. 1962. Selection for digestibility in herbage grasses. *Nature, Lond.* Vol. 195, pp. 1276–7.
7. DEHORITY, B. A. and JOHNSON, R. R. 1961. Effect of particle size upon the *in vitro* cellulose digestibility of forages by rumen bacteria. *J. Dairy Sci.* Vol. 44, pp. 2242–9.
8. DENT, J. 1962. Personal communication.
9. DERIAZ, R. E. 1961. Routine analysis of carbohydrates and lignin in herbage. *J. Sci. Fd Agric.* Vol. 12, pp. 152–60.
10. DIJKSTRA, N. D. 1954. What has the State Agricultural Experiment Station at Hoorn contributed to research into the feeding value of roughage? *Neth. J. agric. Sci.* Vol. 2, pp. 273–97.
11. FORBES, R. M. 1950. Protein as an indicator of pasture forage digestibility. *J. Anim. Sci.*, Vol. 9, pp. 231–7.
12. FRENS, A. M. and STOLK, K. 1957. De invloed van het ruwvoederrantsoen op het cellulose—afbrekend vermogen van de penslora in een kunstpens. *Versl. RijkslandbProefst.*, *'s Grav.* Vol. 63, Part 2, pp. 1–12.
13. GRASSLAND RESEARCH INSTITUTE. 1961. Research techniques in use at the Grassland Research Institute, Hurley. Bull. 45, Commonw. Bur. Past. Fld. Crops, Farnham Royal, C.A.B.
14. HUHTANEN, C. N., SAUNDERS, R. K. and GALL, L. S. 1954. Fiber digestion using the minature artificial rumen. *J. Dairy Sci.*, Vol. 37, pp. 328–35.
15. IVINS, J. D. 1960. Digestibility data and grassland evaluation. *Proc. 8th Int. Grassl. Congr.*, pp. 459–61.
16. KIVIMAE, A. 1960. Estimation of the digestibility of grassland crops from their chemical composition. *Proc. 8th Int. Grassl. Congr.*, pp. 466–70.
17. LAMBOURNE, L. J. 1963. Personal communication. See also, Grassland Research Institute, 1963. *Experiments in Progress*, No. 15. Hurley: G.R.I.
18. LANCASTER, R. J. 1943. Metabolism trials with New Zealand feeding stuffs. IV. The relative significance of lignin, cellulose and crude fibre in the evaluation of foods. *N.Z. J. Sci. Tech.* Vol. 25A, pp. 137–51.
19. MCDOUGALL, E. I. 1948. Studies on ruminant saliva. I. The composition and output of sheep's saliva. *Biochem. J.* Vol. 43, pp. 99–109.
20. MCMEEKAN, C. P. 1943. A note on the relationship between crude fibre and the digestibility of organic matter. *N.Z. J. Sci. Tech.* Vol. 25A, pp. 152–3.
21. MINSON, D. J. 1962. The effect of grinding, pelleting and wafering on the feeding value of roughages—a review. Contribution No. 84, Animal Research Institute, Canada Dept. Agric. Ottawa, pp. 1–84.
22. MINSON, D. J. and KEMP, C. D. 1961. Studies in the digestibility of herbage. IX. Herbage and faecal nitrogen as indicators of herbage organic matter digestibility. *J. Brit. Grassl. Soc.* Vol. 16, pp. 76–9.
23. MINSON, D. J., RAYMOND, W. F. and HARRIS, C. E. 1960. Studies in the digestibility of herbage. VIII. The digestibility of S37 cocksfoot, S23 ryegrass and S24 ryegrass. *J. Brit. Grassl. Soc.* Vol. 15, pp. 174–80.
24. PEW, J. C. 1957. Properties of powdered wood and isolation of lignin by cellulytic enzymes. *TAPPI*, Vol. 40, pp. 553–8.
25. RAYMOND, W. F., HARRIS, C. E. and HARKER, Vivian G. 1953. Studies on the digestibility of herbage. I. Technique of measurement of digestibility and some observations on factors affecting the accuracy of digestibility data. *J. Brit. Grassl. Soc.* Vol. 8, pp. 301–20.
26. RAYMOND, W. F., TILLEY, J. M. A., DERIAZ, R. E. and MINSON, D. J. 1960. Herbage composition and nutritive value. *Soc. Chem. Ind. Monograph. No. 9*, pp. 181–90.
27. SHELTON, D. C. and REID, R. L. 1960. Measuring the nutritive value of forages using *in vitro* rumen techniques. *Proc. 8th Int. Grassl. Congr.* pp. 524–28.
28. TAYLER, J. C. and DERIAZ, R. E. 1963. The use of rumen-fistulated steers in the direct determination of nutritive value of ingested herbage in grazing experiments. *J. Brit. Grassl. Soc.* Vol. 18, in press.
29. TILLEY, J. M. A. 1961. Etudes *in vitro* de la digestion des fourrages. (In English). *Annales de la Nutrition de l'Alimentation*, Vol. 15. pp. 267–72.
30. TILLEY, J. M. A. and TERRY, R. A. 1961. Tecnica "*in vitro*" para la determinacion de la digestibilidad de los forrages. *Publ. 6 Inst. Nac. Tecn. Agropec.* Buenos Aires, pp. 1–5.
31. TILLEY, J. M. A., DERIAZ, R. E. and TERRY, R. A. 1960. *Proc. 8th Int. Grassl. Congr.* pp. 533–7.
32. VIRTANEN, A. I. 1946. Fermentation of wood dust by cellulose bacteria. *Nature, Lond.* Vol. 158, p. 795.
33. WARNER, A. C. I. 1956. Proteolysis by rumen microorganisms. *J. gen. Microbiol.* Vol. 14, pp. 749–62.
34. WATSON, S. J. and NASH, M. J. 1960. The conservation of grass and forage crops. Edinburgh: Oliver and Boyd, pp. 758.
35. WELLER, R. A., GRAY, F. V. and PILGRIM, A. F. 1958. The conversion of plant nitrogen to microbial nitrogen in the rumen of the sheep. *Brit. J. Nutr.* Vol. 12, pp. 421–9.

(*Received for publication 22 February 1963*)