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# A simple gas production method using a pressure transducer to determine the fermentation kinetics of ruminant feeds

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ANIMAL FEED SCIENCE AND TECHNOLOGY

# A simple gas production method using a pressure transducer to determine the fermentation kinetics of ruminant feeds

Michael K. Theodorou*[,a], Barbara A. Williams[a,1], Mewa S. Dhanoa[a], Alex B. McAllan[a], James France[b]

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**Abstract**

This paper describes a simple yet sensitive laboratory procedure that was developed to provide detailed information on the fermentation kinetics of ruminant feeds. In principle, the technique is similar to other in vitro digestibility procedures using ground particulate substrates, anaerobic media and a rumen fluid inoculum. It differs, however, in that incubations are conducted in gas-tight culture bottles, thus enabling gases to accumulate in the head-space as the fermentation proceeds. A pressure transducer connected to a digital readout voltmeter and gas-tight syringe assembly is then used to measure and release the accumulated gas pressures from the incubating culture bottles. By repeating this gas-measurement, gas-release procedure at regular intervals during the fermentation, it is possible to construct gas accumulation profiles for feeds by summation of regression-corrected gas volumes. These profiles are then described using a recently derived growth function developed to characterise gas production profiles. Results obtained establish the pressure transducer as a suitable tool for determining the fermentation kinetics of ruminant feeds and ranking them with respect to their in vitro fermentability.

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## 1. Introduction

Four digestion techniques are currently available to assist in determining the nutritive value of ruminant feeds. Of these, the most frequently used is the rumen liquor, acid pepsin in vitro technique of Tilley and Terry (1963). This method is employed in many forage evaluation laboratories and involves two stages in which forages are subjected to a 48 h fermentation period with rumen fluid followed by 48 h of digestion with pepsin in weak acid. The Tilley and Terry procedure was developed as an end-point digestibility method and thus, unless lengthy and labour intensive time-course studies are made, the technique does not provide information on the kinetics of forage digestion.

Digestibility assays which use enzymes instead of microorganisms have appeared largely as a result of the increased availability of commercially produced fungal enzymes. The method devised by Jones and Hayward (1975), in which ground forage is first treated with acid pepsin, followed by a fungal cellulase, is perhaps the most widely used of the enzymic techniques. Enzymic methods of evaluation are routinely used as end-point digestibility procedures and do not provide information on the kinetics of forage digestion. The main advantage of enzymic methods over their rumen fluid counterparts is that they do not require animals as inoculum donors.

The polyester bag technique has been used for many years to provide estimates of both the rate and extent of disappearance of feed constituents (Quin et al., 1938; Chenost et al., 1970; Mehrez and Orskov, 1977). The method requires fistulated animals for incubation of forage particles suspended in a series of polyester bags in the rumen. Bags are removed sequentially at appropriate times after insertion and their particulate residues are usually subjected to proximate analysis. This technique provides a useful means of estimating rates of disappearance and potential degradabilities of feedstuffs and feed constituents. Owing to its dependence on in situ incubation in the rumen, only small numbers of forage samples can be assessed at any one time. Thus, it is of limited value in laboratories undertaking routine screening of large numbers of samples.

In 1974, Menke and Ehrensvard described an indirect (non-destructive) method for determining the rate and extent of degradation of ruminant feeds in vitro. This was achieved by monitoring the accumulation of fermentation gases above batch cultures inoculated with rumen fluid. The method was elaborated upon later by Menke et al. (1979) when high correlations were reported between gas production values in vitro and apparent digestibilities in vivo. The Menke system differs from other more direct methods of digestibility in that it relies upon an inverse relationship between gas accumulation and degradation of the feedstuff. Fermentations are conducted in large (100 ml capacity) ground-glass syringe barrels containing the feedstuff in an anaerobic medium inoculated with rumen fluid. As the substrate is fermented, gases are produced and the syringe plunger is forced to rise inside the barrel. The rate of fermentation of the feedstuff is determined by simply monitoring the rate of ascent of the plunger. Despite its

obvious attractions, the Menke procedure has not been as widely accepted in forage evaluation laboratories as other digestibility procedures.

The need for an evaluation of the nutritive characteristics of ruminant feeds is likely to increase in importance in coming years owing to: (a) advances in plant molecular biology, particularly the development of transgenic plants; (b) crop improvement programmes; (c) environmental and economic constraints concerning the use and/or disposal of crop by-products and residues. In this paper we describe a simple, relatively inexpensive in vitro procedure, using a pressure transducer, to provide precise information on the fermentation kinetics of ruminant feeds. It is the aim of the paper to present the pressure transducer procedure, to define the conditions under which it can work to maximum effect and to show that the information provided is suitable for precise mathematical description.

## 2. Materials and methods

### 2.1. Plant material

The plant material used as substrate (the carbon source for microbial growth) was temperate perennial ryegrass (*Lolium perenne* cultivar 'Melle') harvested (mown and chopped) from grassland lays at the Institute of Grassland and Environmental Research. The ryegrass was freeze dried, ground through a 1 mm dry mesh screen and stored in an airtight polythene container at 4°C until required for gas production studies. Prior to use, the substrate (0.2–2.0 g in 0.2 g increments) was accurately weighed (substrate weight ±0.5%, five replicates) into small aluminium cups and the weights recorded.

### 2.2. Microbial inoculum

Digesta was taken from rumen-fistulated sheep, fed twice daily on good quality Italian ryegrass (*Lolium multiflorum*) hay (1100 g fresh weight per day) and immediately transported to the laboratory in vacuum flasks. In the laboratory, digesta was squeezed through four layers of muslin and the rumen fluid collected in a $CO_2$-filled flask. Solids remaining in the muslin were homogenised anaerobically (under a stream of $CO_2$ gas) for 30–60 s in a bottom-drive blender after addition of an anaerobic buffer (semi-defined medium B minus the plant biomass carbon source—see the section on culture media below) equal in volume to the rumen fluid removed. Buffered fluid from the homogenised digesta was strained through muslin, as above, and combined in equal volumes with the initially collected rumen fluid. This procedure ensured that the resultant inoculum contained attached (fibre-associated) as well as unattached (free-floating) rumen microorganisms. After mixing and during inoculation of culture bottles, the microbial suspension was stirred (with magnetic stirrer and bar) anaerobically under a stream of $CO_2$ gas. Bottles of culture medium were inoculated with 10 ml

of rumen fluid using a 10 ml syringe fitted with a 23 gauge, 1.5 inch needle (Sabre International Products Ltd., UK).

### 2.3. Culture media

Cultures were grown on semi-defined medium B (Lowe et al., 1985), a medium which supports the growth of all major genera of rumen microorganisms (Hazelwood et al., 1986; Lowe et al., 1987). The medium was prepared and inoculated using aseptic, anaerobic techniques (Lowe et al., 1985). Fermentations were conducted in serum bottles (Phase Separations Ltd., Clwyd, UK; nominally of approximately 160 ml capacity, but retailed as 125 ml bottles) sealed with butyl rubber stoppers and aluminium crimp seals (Bellco Glass Inc., Vineland, NJ, USA). Particles of plant biomass and pre-reduced basal solution were added to each bottle using a wide-bore plastic funnel; during these additions, bottles were flushed with $CO_2$ gas. Bottles were sealed and autoclaved, at 121°C for 15 min prior to the addition of components needed to complete the medium (Lowe et al., 1985). When required for gas production studies, the bottles of medium were warmed to 39°C, inoculated with the microbial suspension and incubated without agitation at 39°C until the end of the fermentation period; bottles were shaken after each gas production reading and returned to their position in the incubator.

### 2.4. Gas production measurements

A detachable pressure transducer and LED digital readout voltmeter (Bailey & Mackey Ltd., Birmingham, UK) were used to measure the head-space gas pressure of fermenting cultures. The voltmeter was calibrated by the manufacturer to read units of pressure (psi) and was housed (by us) in a moulded plastic case (length 200 mm, depth 145 mm, height 75 mm; R.S. Components, Northampton, UK). The pressure transducer was originally designed for continuous in-line monitoring of gas pressure and had a range of 0–15 psi, with an accuracy of 0.1 ± 2% (at 25°C). For use in gas production studies, the transducer was modified such that it could be connected to the inlet of a disposable Luer-lock three-way stopcock (Robinet three-way stopcock; Laboratoires Pharmaceutiques, Vycon, BP 7-95440 Ecouen, France). The first outlet of the stopcock was connected to a disposable hypodermic syringe needle (23 gauge × 1.5 inch). The second outlet was connected to a disposable plastic syringe of 5, 10, 20 or 60 ml capacity according to the quantity of gas to be measured (Sterilin Ltd., Teddington, UK).

Bottles were warmed to an incubation temperature of 39°C and the head-space gas pressure in each was adjusted to ambient pressure prior to and just after inoculation with the microbial suspension. Gas pressure in the head-space was read from the display unit after insertion of the hypodermic syringe needle through the butyl rubber stopper above the culture medium (Fig. 1). The corresponding gas volume was determined by recording the volume of gas displaced into the syringe barrel on withdrawal of the syringe plunger until the head-space gas pres-

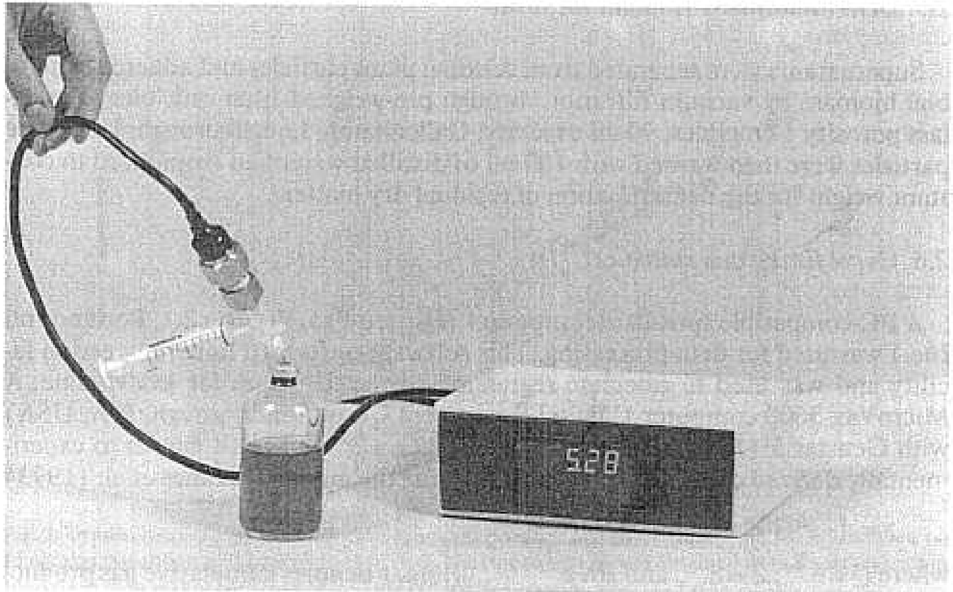

Fig. 1. The pressure transducer assembly and digital display unit in use for the measurement of head-space gas pressure. With the stopcock open between transducer and syringe needle, the needle was inserted through the bottle closure and head-space gas pressure recorded on the digital display. The stopcock was rotated through 180° to open the link to the syringe barrel and the syringe plunger withdrawn until head-space gas pressure was returned to ambient pressure, as indicated by a reading of zero on the digital display unit. The pressure transducer assembly was withdrawn from the bottle, the volume of gas collected in the barrel recorded, the gas discarded, and the bottle returned to the incubator until the next reading.

sure returned to ambient pressure, as indicated by a zero reading on the display unit. Following measurement of pressure and volume, the transducer assembly was withdrawn from the bottle closure, gas in the syringe barrel was discarded and the bottle returned to the incubator until the next reading. The time taken for determination of pressures and volumes was relatively short, amounting to not more than 10–15 s per bottle. Thus, since only a few bottles were removed from the incubator at any one time, it was assumed that the temperature of the head-space gas remained unaltered during the measuring period.

Pressures and volumes were recorded in this way, using five replicate culture bottles per treatment, at 3–24 h intervals during the period of fermentation. Readings were more frequent during the initial 48 h of incubation when head-space gas pressures increased most rapidly, requiring the larger syringes for collection of displaced gas. During the latter part of the fermentation, when relatively small amounts of gas were produced, gas volumes were measured using smaller syringes. It was necessary to record both pressures and volumes in gas production studies in order to correct for possible small differences in head-space volumes between bottles (see Results section).

### 2.5. Determination of residual substrate

Supernatants were separated from residual plant particles and adherent microbial biomass by vacuum filtration through pre-weighed filter crucibles (Sintaglass porosity 1 crucibles, 70 ml capacity; Gallenkamp, Loughborough, UK). The particles were then washed with 100 ml of distilled water and lyophilised to constant weight for the determination of residual dry matter.

### 2.6. Curve fitting and statistics

A PC-compatible spreadsheet program (Quatro-Pro, Version 2.1; Borland Int. Inc.) was used for data processing. This software includes a linear regression facility and was used to calculate regression-corrected values for gas volume. A MicroVax 3600 computer (Digital Equipment Corp., Marlborough, MA, USA) with Genstat 5 (1987) or MLP (Ross, 1987) was used to fit curves to experimentally derived cumulative gas profiles using the model of France et al. (1993)

$$y=A-BQ^{t}Z^{\sqrt{t}}$$

where $Q=e^{-b}$, $Z=e^{-c}$, and $B=e^{bT+c\sqrt{T}}$. Here, $y$ denotes cumulative gas production (ml), $t$ is incubation time (h), $A$ is the asymptotic value for gas pool size (ml), $T$ is the lag-time and $b$ ($h^{-1}$) and $c$ ($h^{-0.5}$) are rate constants.

Estimated values of the four parameters, $A$, $T$, $Q$ and $Z$, were determined from time-course experiments of 120 h duration after subtraction of the mean control profiles for gas produced in inoculated cultures incubated in the absence of substrate. The gas produced within this time approached an asymptote and the fermentation was thus considered to be complete. The model shown above was fitted to each of the five replicate cultures over the range of substrate weights used and the parameter values obtained subjected to regression analysis to detect any substrate weight related trends.

## 3. Results

### 3.1. Performance of the pressure transducer and conversion of head-space gas pressure to fermentation gas volume

When pressure readings ranging from 0 to 10 psi from culture bottles were plotted against their corresponding gas volumes, the linear relationship as shown in Fig. 2 was obtained. This relationship was derived from a total of ten bottles containing from 0.2 to 2.0 g of substrate, increasing stepwise in 0.2 g increments, in 100 ml of medium plus inoculum and read on 13 occasions during a 120 h incubation period. The results established that pressures and volumes, as determined by the transducer procedure, were linearly related over a wide range of pressure readings (typical $r^2>0.97$). However, more scatter was observed with data points collected from the higher transducer readings (over 7.0 psi) than

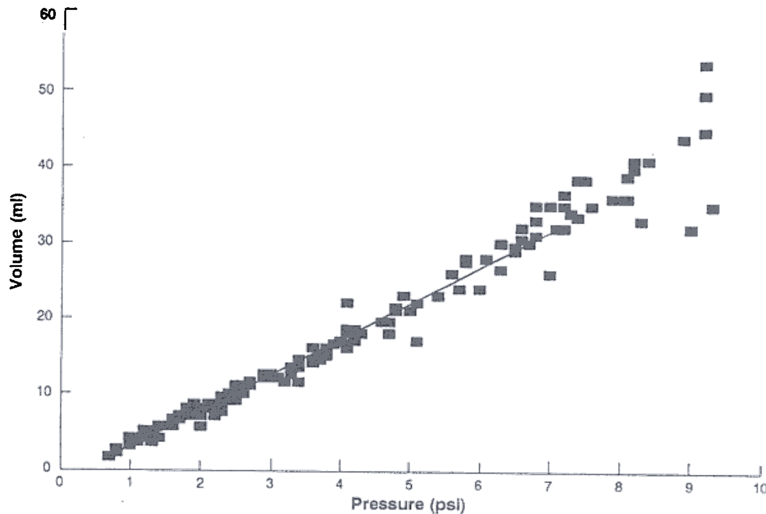


Fig. 2. Typical relationship between head-space gas pressure and gas volume from ten bottles read on 13 occasions during a 120 h incubation period. The line of best fit was obtained from linear regression analysis of data within the range of 0–7 psi ($r^2 > 0.97$).

those from the lower range (Fig. 2). From this we concluded that in all future experiments, transducer readings should be taken at incubation time intervals leading to pressures of less than 7.0 psi in the bottle head-space.

Essentially similar linear relationships to the one shown in Fig. 2 were obtained when pressure readings from individual bottles were plotted against corresponding gas volumes. Parallel curve analysis (and the $F$-test) was used to make comparisons between the lines of best fit from individual bottles or groups of bottles and it was found that the slopes for volume versus pressure were not significantly different. Similar results were obtained in numerous other experiments (not reported here) where different but equally reproducible relationships were obtained in bottles containing different volumes of liquid or in smaller (60 ml) culture bottles.

A common linear expression, $V = XP + I$, where $V$ is volume (ml), $P$ is pressure (psi), $X$ is slope and $I$ is intercept (bias correction factor), could be used to describe the relationship between volume and pressure for all sets of data derived from bottles of the same size and with the same liquid and head-space volumes. However, to correct for possible small differences between bottles, caused by errors associated with the dispensing of liquids and/or slight manufacturing variations in bottle size, regression equations for individual bottles (with bias correction) were used in gas production studies, to predict gas volumes from their experimentally determined gas pressures. It is to be noted that volume readings are subject to considerable variation in relation to altitude. This is of consequence for comparisons of gas production profiles determined at sites of different elevation where correction factors, given by the ratio of site pressure to standard

sea level pressure, must be used to scale volumes. This can be achieved before curve fitting by scaling down the volume measurements, or after curve fitting by scaling down the estimate of the parameter, $A$, representing gas pool size.

### *3.2. Construction of cumulative gas production profiles and the relationship between substrate concentration and gas accumulation*

In the above section, results from ten bottles, each containing from 0.2 to 2.0 g of substrate, increasing stepwise in 0.2 g increments, were presented to demonstrate the behaviour of the pressure transducer and the relationship between head-space gas pressure and gas volume. These bottles were randomly selected, one from each set of ten × five replicates from a total of 50 bottles. In the following section, the cumulative gas production profiles from all 50 bottles are presented and discussed. The experiment was conducted in order to demonstrate that substrates were degraded to their maximum extent in the culture system employed and that fermentations were not inhibited or limited by (a) nutrient deficiencies (other than a lack of utilisable plant biomass carbon), (b) pH decline or (c) the

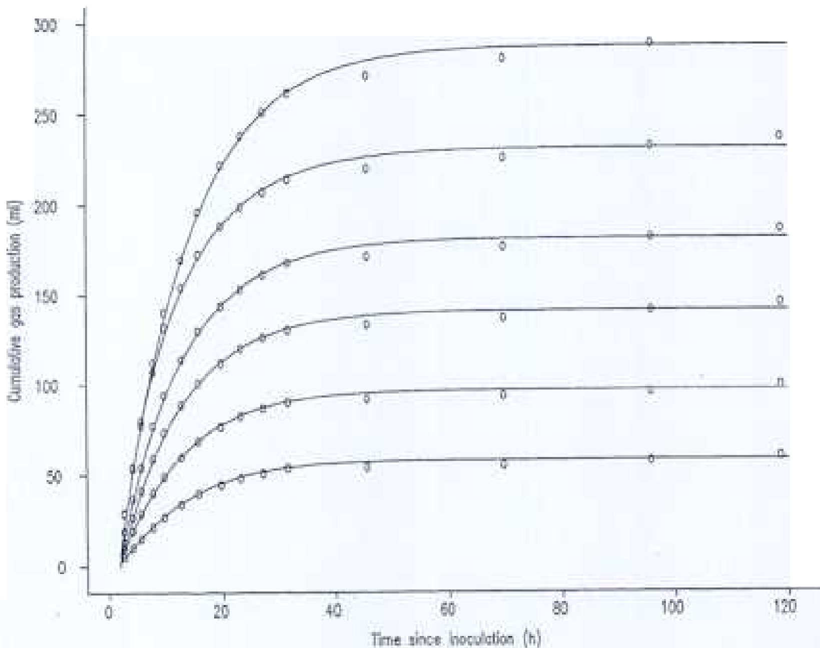


Fig. 3. Cumulative gas production profiles from the fermentation of perennial ryegrass. For clarity of exposition, only gas production profiles from the fermentation of 0.2 g (lowest profile) to 1.2 g (highest profile) of substrate have been presented. The results were obtained using 125 ml serum bottles (five replicates per substrate concentration) containing 100 ml of culture medium plus inoculum. Experimentally determined data points (circles); fitted curves (line) from the model of France et al. (1993).

Table 1
Estimated values for kinetic parameters obtained from the fermentation of perennial ryegrass in Medium B in 125 ml serum bottles inoculated with a microbial suspension prepared from rumen fluid

| Substrate concentration (g per 100 ml culture) | Lag time ($T$) (h) | Fractional rates | | Gas pool size ($A$) (ml) |
|---|---|---|---|---|
| | | $b = -\ln Q$ ($h^{-1}$) | $c = -\ln \sqrt{Z}$ ($h^{-0.5}$) | |
| 0.2 | 1.08 | 0.898(0.0178) | 1.153(0.1287) | 58.0(0.57) |
| 0.4 | 1.60 | 0.908(0.0076) | 1.044(0.0483) | 96.8(0.44) |
| 0.6 | 2.01 | 0.919(0.0113) | 0.969(0.0652) | 141.3(0.93) |
| 0.8 | 1.87 | 0.922(0.0192) | 0.961(0.1120) | 181.3(1.86) |
| 1.0 | 1.95 | 0.936(0.0145) | 0.852(0.0723) | 231.6(1.83) |
| 1.2 | 1.74 | 0.922(0.0113) | 1.003(0.0691) | 288.0(2.21) |
| 1.4 | 1.95 | 0.932(0.0119) | 0.931(0.0664) | 335.3(2.67) |
| 1.6 | 1.82 | 0.924(0.0122) | 1.026(0.0778) | 359.4(32.9) |
| 1.8 | 1.86 | 0.940(0.0116) | 0.917(0.0644) | 425.1(3.84) |
| 2.0 | 1.66 | 0.937(0.0074) | 0.928(0.0413) | 486.0(2.47) |

Values for the $B$ parameter can be calculated from the formula: $B = Ae^{(bT + c\sqrt{T})}$, where $b = -\ln Q$, $c = -\ln Z$. The lag time $T$ is described by the solution of the above equation as described by France et al. (1993).

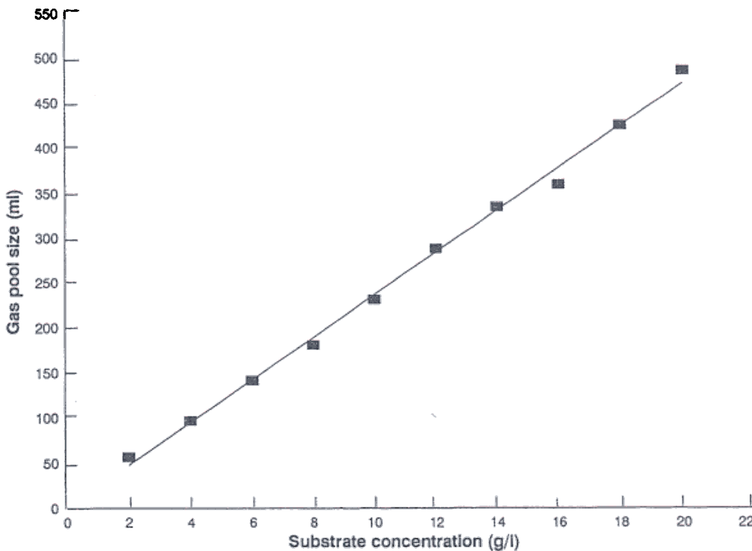


Fig. 4. Relationship between initial substrate weight and the resultant gas pool size. Gas pool sizes for each of the initial substrate weights were the $A$ parameter values in Table 1 determined from curves fitted to experimental data according to France et al. (1993).

accumulation of end-products. In each of the presented gas accumulation profiles (Fig. 3), cumulative gas production was determined by summation of the regression-corrected gas volumes from within each of the five replicate culture bottles

after subtraction of gas which accumulated in the corresponding control cultures, inoculated and incubated without substrate. For the purpose of illustration, only the gas production profiles from substrate amounts ranging from 0.2 to 1.2 g per bottle have been shown in Fig. 3. Curves (the solid lines in Fig. 3) were fitted to the experimental data points (open circles in Fig. 3) and the parameter values generated according to the model of France et al. (1993), as shown in Table 1. The parameter values obtained were then subjected to regression analysis using substrate range as the $X$ variable. Over the range of substrate concentrations used, 0.2–2.0 g per 100 ml of culture medium plus inoculum, the quantity of substrate added to the bottle had no significant effect on the lag-time and fractional rates of gas production. These were found to remain more or less constant (i.e. with zero slope) at about 1.75 h, 0.924 $h^{-1}$ and 0.980 $h^{-0.5}$, for the parameters $T$, $Q$ and $Z$, respectively (Table 1). The amount of gas produced, however, increased linearly with increasing substrate quantity ($r^2=0.99$) throughout the entire range of substrate concentrations used (Fig. 4). From the slope of the line a yield constant of $234.4 \pm 3.59$ ml gas $g^{-1}$ substrate initially offered was determined. By determining the residual dry matter (DM) content in each bottle at the end of the fermentation period, it was calculated that at least 75% of the substrate was lost during the fermentation period. Thus, by relating dry matter loss to gas pool size, the yield of gas produced per gram DM apparently consumed was constant at $312 \pm 4.79$ ml.

## 4. Discussion

The research presented in this paper came from a programme of work concerned with the development of a simple laboratory procedure to assist in the determination of the nutritive value of tropical and semi-tropical crops and crop residues as feedstuffs for ruminants (Theodorou and Brooks, 1990). The method was to be based upon substrate fermentability and make use of the mathematical equations of France et al. (1993) for quantification of gas production profiles. In general, we have found that these equations provide a better description of the gas production data generated by the pressure transducer technique than the exponential function advocated by Orskov and McDonald (1979) and McDonald (1981), as it is capable of describing sigmoidal trends.

The results presented here and those in France et al. (1993) show that a protocol for a simple pressure transducer technique, using fresh microbial inoculum prepared from rumen fluid, is now available. In addition to using the technique in the UK, it has been employed (in collaborative studies with Dr. E. Owen of the University of Reading) in postgraduate research overseas (Sileshi et al., 1994). Experience from our overseas work has shown that the technique can easily be accommodated in laboratories concerned with feed evaluation in tropical and sub-tropical areas. In addition to the ryegrass profiles presented in this study, we have determined gas production profiles from a number of forages, ranging from

perennial ryegrass silage in the UK, to forage crops and crop residues from Ethiopia, Malaysia, Columbia, Indonesia and Syria (Merry et al., 1991; Theodorou et al., 1991, 1993; Longland et al., 1994a,b; Sileshi et al., 1994).

The pressure transducer technique and conditions of culture were developed using exacting, aseptic, anaerobic procedures and the semi-defined medium of Lowe et al. (1985, 1987). However, the medium and its method of preparation were considered too complex for a routine feed evaluation bioassay and have since been modified (Theodorou, 1993) such that they are similar to those used in existing digestibility procedures. Researchers in this laboratory now use the simplified medium and culture conditions almost exclusively for feed evaluation studies. Medium B is still used in some microbiological studies, however, such as those concerned with the fermentation kinetics of axenic cultures of rumen microorganisms growing on particulate substrates (Trinci et al., 1994).

In all our studies we have found that it is imperative to vent culture bottles frequently during the rapid gas production phase to avoid a build up of gaseous components in the head space which ultimately reduce the rate of fermentation of the substrate. Automated systems involving pressure transducers for measuring gas production have been developed (Beaubien et al., 1988; Pell and Schofield, 1993). These are more complex and more expensive than the manual transducer system reported in this paper. Whereas venting of cultures is employed in the automated anaerobic waste fermentation system of Beaubien et al. (1988), cultures are not vented in feed evaluation system of Pell and Schofield (1993).

In our research, we have demonstrated a linear response of gas pool size to substrate weight within the range of 0.2–2.0 g substrate per bottle. Moreover, lag-times and fermentation rates remained constant with increases in substrate weight. Thus, using the culture conditions employed, substrate digestion ultimately ceased owing to the depletion of utilisable carbon in ryegrass. As cultures were carbon-limited and because lags and rates remained constant, it follows that (a) all other nutrients were present in excess throughout the fermentation and (b) volatile fatty acid (VFA) accumulation and pH decline had a negligible effect on fermentation kinetics. Similar substrates (plant biomass generally) are likely to respond in a similar way. In subsequent experiments using the simplified medium and culture conditions reported by Theodorou (1993) and 125 ml serum bottles, 0.5–1.0 g of substrate (i.e. 0.5–1% (w/v) DM) was chosen as an appropriate weight of substrate to ensure ultimate provision of carbon-limitation in time-course incubations.

The work presented in this paper is intended to demonstrate the use of a simple procedure involving a single pressure transducer. The cost of the equipment is not excessive (we pay about £500 at current prices for parts and the transducer units are constructed on-site in the IGER electronics workshop) and our experience has shown that the equipment is remarkably robust. Some of the transducers have been working for at least 4 years without fault (if difficulties are encountered, transducers and/or display units can be replaced with relative ease). In addition to routine screening and feed evaluation, the procedure is suitable for studying some of the more fundamental aspects of plant cell wall digestion, particularly within programmes of plant molecular biology where only small quan-

tities of manipulated materials are available. It is also possible to obtain much useful information on the action of anti-fermentative agents in ruminant nutrition (Longland et al., 1994a,b).

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