The fate of water in the rumen

1. A critical appraisal of the use of soluble markers

BY A. C. I. WARNER AND B. D. STACY

CSIRO, Division of Animal Physiology, Ian Clunies Ross Animal Research Laboratory, Prospect, NSW, Australia

(*Received* 30 *August* 1967—*Accepted* 12 *February* 1968)

1. A mathematical treatment is given of the marker technique for studying water balances in the rumen. The treatment is extended to cover non-steady-state conditions and emphasis is placed on the underlying assumptions and practical limitations of the technique.

2. The ^{51}Cr complex of ethylenediamine tetra-acetic acid (^{51}Cr EDTA) was used as a marker in the rumen of sheep.

3. The ^{51}Cr EDTA entered 95–98 % of the water in the rumen. Mixing in the rumen, though adequate, was usually not quite complete even 1·5 h after injection of the dose. The techniques of injecting the dose and of withdrawing the sample were found to be important; some techniques often led to poor mixing.

4. Provided that the time of injection of the dose was suitably selected, reliable estimates of the volume of water in the rumen could readily be made, but reliable estimates of flow rates needed stringent attention to details of technique.

Sperber, Hyden & Ekman (1953) introduced the use of soluble reference substances or markers to study water movements in the rumen, and they described some results obtained with polyethylene glycol (PEG). Hydén (1961) critically examined both the theoretical and the practical aspects of the technique in a paper that had a significant influence on the development of work in this field. Despite the subsequent use of marker substances in various ways (for references, see Warner & Stacy, 1968), no other thorough study has been made of either the theory or the practice of the technique. In the past few years, several new markers have been developed, such as the ^{51}Cr complex of ethylenediamine tetra-acetic acid (^{51}Cr EDTA) (Downes & McDonald, 1964), phenol red (Hecker, Budtz-Olsen & Ostwald, 1964), lithium salts (Ulyatt, 1964), tritium-labelled PEG (Till & Downes, 1965) or chromic oxide (Purser & Moir, 1966). These workers did not test the validity of the markers as thoroughly as did Hydén (1961), nor did they re-examine the mathematical basis of the technique.

Now, Hyden (1961), in most of his theoretical treatment, considered only steady-state conditions, in which it must be assumed that the volume of the water in the ruminoreticulum (rumen volume) is constant and that the rates of inflow and outflow of water are equal and constant. Although he realized that these ideal conditions were rarely if ever precisely attained in practice, Hydén in general did not consider what happened in the absence of a steady state. His treatment was mainly concerned with the estimation of outflow, which is the most difficult quantity to calculate from data of this kind. In the light of later knowledge, Hydén's common practice of injecting the marker into the rumen immediately before feeding and taking samples some time

later is unsatisfactory for general use, since the calculation of results may involve extrapolating a curve past a discontinuity. Also, the precise technique of administering the dose, described as 'spraying', is not clear. More recently, a paper by Ternouth (1967) contains a number of misconceptions about the technique.

Studies at this laboratory have been concerned with events in the rumen associated with feeding or drinking (Warner & Stacy, 1965; Stacy & Warner, 1966; Warner, 1966). For a detailed assessment of this work it was necessary to make a critical study of the marker technique, particularly as applied to non-steady-state conditions. In this paper there is an extended examination of the mathematical treatment of the experimental findings, and attention is drawn to the underlying assumptions and the practical limitations of the technique. In the following paper (Warner & Stacy, 1968), the marker technique is used to follow the fate of water in the rumen during the feeding cycle, i.e. during resting, feeding, drinking, post-prandial and starving periods.

THEORY

Movements of water can be estimated by introducing a soluble marker into the rumen and measuring its concentration at intervals. The marker should be non-toxic both to the animal and to its rumen microbes; it should not be adsorbed on to either particulate matter or tissue cells, and it should be neither absorbed across the rumen wall nor metabolized. Adsorption, absorption and metabolism would lead to an overestimate of the dilution rate of the marker.

The present mathematical treatment assumes instantaneous and complete mixing, even though it is known that in practice mixing is relatively slow and, owing to the continual influx of water in the saliva, never complete. The effects of sampling errors on estimates of volume are inversely related to their effects on estimates of dilution rate; passage of unmixed material out of the rumen to the omasum would have unpredictable effects. In many experimental situations, these effects are small. Ternouth (1967) claimed there were periods in his experiments when the outflow rate reached 50 % or more of the rumen volume per hour; marker introduced during these periods would seem to have very little chance to mix adequately with the rumen contents.

The marker may be administered in two ways, herein referred to as the single-injection technique and the continuous-infusion technique. In a single-injection experiment, a single dose of the marker is given and its concentration in the rumen is measured from time to time. It is often necessary to repeat this process, perhaps several times. In a continuous-infusion experiment, a priming dose is injected to raise the marker concentration to its expected equilibrium value before commencing the continuous infusion of marker at a constant rate. Again, it is often necessary to stop the infusion after a period and study the rate of disappearance of the marker as in the single-injection experiment. While the single-injection technique is the more versatile for investigating rumen function, the continuous-infusion technique allows simultaneous study of water movements elsewhere in the intestinal tract, though its strict use is limited to steady-state conditions (see below). For the sake of completeness, and

in order to expand the brief mathematical treatment given by Hydén (1961), the theoretical basis of the continuous-infusion technique is considered although no experimental results are given in this paper.

In the present work, steady-state conditions are defined by the situation in which the volume of water in the rumen is constant and the net rate of inflow and the rate of outflow of water are equal and constant. Such conditions apply, at least approximately, with some experimental or natural dietary regimes. It may sometimes be justifiable to apply steady-state equations when a complete feeding cycle is considered. However, in general, steady-state conditions do not apply, and it is then necessary to allow for changing volumes and flow rates.

Derivations of many of the following equations are given by Hydén (1961) or by Warner (1966).

Single-injection experiments

Symbols used

Let Q be the dose of marker dissolved in a volume of water U and injected into the rumen at time $t = 0$. Each of the symbols below refers to the value at the instant t. Average values are time averages, thus

$$\bar{X} = \frac{1}{t_2 - t_1}\int_{t_1}^{t_2} X \, dt$$

denotes the average of the parameter X over the time interval from t_1 to t_2. Let

S be the rate of entry of salivary water into the rumen;

A be the rate of absorption (net flux) of water from the rumen into the blood;

F be the net inflow rate of water into the rumen; normally, $F = S - A$, but allowance must also be made for imbibed water entering the rumen during the experiment;

E be the rate of outflow of water to the omasum;

c be the concentration of marker in the rumen;

V be the volume of water in the rumen;

V_1, c_1, V_2, c_2 be the values of V and c at times t_1 and t_2 respectively;

V_i, c_i, V_0, c_0 be the values of V and c immediately before and immediately after injection of the marker.

$D = F/V$ is called the dilution rate, or relative net inflow rate;

$t_{\frac{1}{2}}$ denotes the half-time of marker in the rumen, i.e. the time required for the concentration to fall to half its initial value ($c_1/c_2 = 2$);

θ denotes the mean retention time of marker in the rumen, i.e. the average time spent by a molecule of marker in the rumen.

General equations

Estimation of dilution rate, D. The gradient of the curve relating ln c with t is, at any instant, equal to $-D$.

If, in particular, this curve reduces to a straight line, then D is constant. To estimate the value of D in this case, plot log c (to any convenient base) against t and fit a straight line. Since the error of measuring t is negligible, the correct fitting of this line would

seem to be by the method of least squares. There is, however, a tendency for the error variance of log c to be larger for small values of t. Consequently, though parameters and their confidence limits may be calculated by the least squares method, their interpretation is sometimes doubtful, and their values are not necessarily more reliable than those obtained by fitting the straight line by eye. It can be seen that

$$D = \ln (c_1/c_2)/(t_2-t_1) = 2{\cdot}303 \ (\log c_1 - \log c_2)/(t_2-t_1).$$

It is often convenient to read off the half-time, $t_{\frac{1}{2}}$, from the above graph or, indeed, from the simpler graph of c against t on semi-logarithmic paper, and then

$$D = \ln 2/t_{\frac{1}{2}}.$$

When the points plotted as above do not show a straight line trend, i.e. when D is not constant, it is often sufficient to know the average dilution rate $\bar{D}$. $\bar{D}$ is obtained from the straight line joining the points corresponding to c_1 and c_2 in the same way as in the case when D is constant.

Estimation of volume, V. It can be seen that $V_0 = V_i + U$. Hence, immediately after administration of the dose Q, the amount of marker in the rumen is

$$V_i c_i + Q = (V_i + U)\, c_0.$$

Therefore, $V_i = (Q - Uc_0)/(c_0 - c_i)$.

Two special cases may be considered:

(*a*) There is no marker present in the rumen before administering the experimental dose, i.e. $c_i = 0$. Then

$$V_0 = Q/c_0.$$

(*b*) The dose is administered in negligible volume, i.e. $U = 0$. Then

$$V_i = V_0 = Q/(c_0 - c_i).$$

In practice, c_0 cannot be determined directly, because mixing is slow. It is estimated by back extrapolation of the line fitting points corresponding to several values of c, determined after allowing adequate time for mixing (usually at least 1 h). It is important that the experimental conditions should be such that there is no discontinuity in the curve over the period of extrapolation; that is, conditions in the rumen should be constant, or changing at constant rates, throughout the entire period between administration of the dose and establishment of the curve.

Estimation of flow rates, F and E. Provided that mixing is adequate, the values from marker experiments are sufficient for the determination of the initial volume of water in the rumen (V_i) and the instantaneous dilution rate (D). This is true no matter what movements of water may be taking place in the rumen. However, this does not apply to the flow rates, F and E, in the general case. To calculate flow rates, it is necessary to have one or more parameters constant and to use the appropriate equation as given below; D can be shown experimentally to be constant, V can be shown to be unchanged at particular times but F or E can only be assumed to be constant on the basis of physiological probability. In practice, it has usually been found that the different equations gave results that differed to only a small extent; even for short periods of rapid change in the rumen, differences of more than 30% were uncommon in the

experiments of Warner & Stacy (1968). It should be noted that any net inflow affects the marker concentration, but outflow *per se* has no such effect, so that calculation of outflow is usually less direct than calculation of net inflow, and is usually associated with greater uncertainty.

Consider a period from time t_1 to time t_2. If conditions in the rumen are changing rapidly, this period should be as short as possible, bearing in mind the necessity to know both the initial and the final volumes V_1 and V_2. It is usually only necessary to use special equations to calculate either $\bar{F}$ or $\bar{E}$, according to the assumptions made, and to calculate the other from the equation

$$V_2 = V_1 + (\bar{F} - \bar{E})\,(t_2 - t_1).$$

(*a*) If V is constant, then it can be seen that $\bar{F} = \bar{D}V = \bar{E}$.

(*b*) If D is constant and V varies at a constant rate, $\bar{F} = D\,(V_1 + V_2)/2$.

(*c*) If D and V are both varying, and the rate of change of V is both small and constant, then the equation

$$\bar{F} = \bar{D}\,(V_1 + V_2)/2,$$

though not strictly true, will hold approximately. The errors of this approximation are discussed in the Appendix to this paper.

(*d*) When changes in V are greater, but E may be considered constant throughout the period in question, then it can be shown that

$$E = \frac{V_1 c_1 - V_2 c_2}{\bar{c}\,(t_2 - t_1)}.$$

This equation can be applied to periods of fast eating, $\bar{c}$ being estimated from several measures of c during eating. It can also be used for a period immediately after drinking, taking t_1 as immediately before drinking, t_2 as a time just far enough from t_1 to allow adequate mixing of the water drunk; $\bar{c}$ is the mean marker concentration after drinking (i.e. ignoring c_1): frequently c_2 can be used with little error.

(*e*) If both D and E can be considered constant, then

$$E = D\,(V_1 c_1 - V_2 c_2)/(c_1 - c_2).$$

(*f*) If both E and F can be considered constant, then

$$F = \bar{D}(V_1 - V_2)/\ln\,(V_1/V_2) = (V_1 - V_2)\log\,(c_1/c_2)/(c_1 - c_2)\log\,(V_1/V_2).$$

This equation, introduced by Reid (1965), seems unlikely to have a wide range of practical applications because large changes in volume, which alone can be measured accurately, are unlikely to be accompanied by constant inflow and outflow rates.

Estimation of absorption rate, A. When the average rate of inflow of saliva $\bar{S}$ is known, the average rate of absorption of water from the rumen into the blood $\bar{A}$ can be seen to be

$$\bar{A} = \bar{S} - \bar{F}.$$

Steady-state conditions

Tests for steady-state conditions. Several injections of marker should be made at appropriate intervals. If steady-state conditions apply, the curves for ln c against t,

or their equivalents, should be linear and the separate estimates of D and V should be equal.

Estimation of mean retention time, θ. It can be shown that

$$\theta = 1/D = V/F = V/E.$$

Continuous-infusion experiments

Symbols used

Let R be the rate of infusion of water with the marker and m the concentration of marker in that water. Other symbols used are the same as for the single-injection experiments.

General

For a single-injection experiment, the curve relating ln c and t has a simple physical interpretation, namely that its gradient is $-F/V$. This does not hold for a continuous-infusion experiment. The only useful deduction that can then be made is that when c is constant then F must be constant.

Steady-state conditions

Tests for steady-state conditions. During the course of a continuous infusion, it can only be shown that F is constant, as above. To determine whether V is constant, the infusion must be stopped, and the disappearance of marker from the rumen followed over as long a time as practicable. If then ln c or its equivalent plotted against t is a straight line, then $D = F/V$ is constant, and hence V is constant, assuming conditions are unchanged throughout.

Estimation of flow rates, F and E. It can readily be seen that, at equilibrium,

$$Rm = (F+R)\,c.$$

Hence, $F = R\,(m-c)/c$ and $E = F+R$.

Now, R is usually very small compared with E and F, so that m is very large compared with c. Under these conditions

$$F = E = Rm/c.$$

Estimation of dilution rate, D, and volume, V. The infusion of marker must be stopped and the marker disappearance curve must be used to calculate D and hence $V = F/D$ as for the single-injection experiments above.

Estimation of mean retention time, θ. In a continuous-infusion steady-state system also, it can be shown that

$$\theta = V/F = 1/D.$$

Total daily flow

If it is possible to divide the day into periods such that in each period at least one of V, D, F or E can be considered constant for a time sufficient to allow measurement of the initial volume, then accurate equations as above can be applied. In this case, a single-injection experiment can be carried out and the flow rate estimated for each period, allowing calculation of the total.

In other circumstances, approximate estimates may be made. One single-injection experiment may be extended over the whole day and use made of the approximate equation

$$\bar{F} = \bar{E} = \bar{D}\bar{V},$$

where $\bar{D}$ is determined directly and $\bar{V}$ is deduced from V_0 with correction for any expected deviations. Alternatively, a continuous-infusion experiment may be carried out over the full day and the mean concentration of marker in the rumen, $\bar{c}$, estimated. Then we have the approximate relation

$$\bar{F} = \bar{E} = Rm/\bar{c}.$$

This method is likely to be more accurate than the one single-injection method above, particularly when conditions are far from the steady state (see Appendix). However, the one single-injection method may be preferable in some instances because simpler apparatus and fewer samples would be needed.

Effect of absorption of marker

Markers may not meet the ideal requirement of being non-absorbable across the rumen wall. If it can be assumed that the rate of absorption is proportional to the concentration of the marker in the rumen, with k the constant of proportionality, then it can readily be shown that the results of both single-injection and continuous-infusion experiments would lead to overestimates of flow rates by an amount equal to k; estimates of volume would be unaffected.

EXPERIMENTAL

Sheep. Seven Merino ewes, weighing about 40 kg and fitted with rumen fistulas were used. The cannulas were closed with rubber bungs about 30 mm in diameter with a central hole about 10 mm in diameter, closed with a smaller bung.

The sheep were housed in metabolism crates in an air-conditioned laboratory (temperature range 20° to 25°). To avoid psychological disturbance, all the sheep in the laboratory were usually given food or water at the same time.

Diets. The animals were given 700 g per day of one of two diets which consisted of mixtures of equal parts of lucerne chaff and wheaten chaff (R9) or of lucerne chaff and oats (M2). The ration was supplemented with 10 g NaCl daily. For most of the work reported in this paper, the animals were accustomed to a routine of having food available for about 1 h at noon, and water available for about 1 h some 4 h later. They normally ate all their food in an hour and drank most of their daily intake in the first 5 min or so after water was given to them. The morning period, when the sheep had been some 20 h without food and 16 h without water, was called the 'resting' period; the period between feeding and drinking was called the 'post-feeding' period, and the first few hours following drinking the 'post-drinking' period.

Sampling and dosing. When large samples of rumen contents were required, they were removed by suction, using a plastic tube 25 mm in diameter, with several 8 mm holes in the lower 5 cm. The tube was moved about throughout the rumen and attempts were made to minimize contact with the rumen wall. Large volumes of

material were poured into the rumen through a short-necked plastic funnel making a tight fit in the cannula.

Small volumes were removed from or injected into the rumen by means of a syringe attached to a glass tube 9 mm in diameter passing through the small hole in the rubber bung closing the cannula.

Marker. ^{51}Cr EDTA was used, as described by Downes & McDonald (1964). A few experiments were done initially with the chelate complex prepared by the method of these authors; subsequently the bulk of this work was done with material purchased from the Australian Atomic Energy Commission, Lucas Heights, NSW.

Measurement of radioactivity. Routinely, the rumen contents were centrifuged at 20000 *g* for about 10 min, and the supernatant fraction was assayed for radioactivity in a Packard Auto-Gamma Spectrometer.

RESULTS

Volume of distribution of ^{51}Cr EDTA

Samples of the dry or moist diets R9 and M2 were soaked for various periods either in an aqueous solution of ^{51}Cr EDTA or in centrifuged rumen liquor containing the marker. The weight ratio of sample to solution was about 1 to 4 and the experiments were done both with ground and unground samples of food. After the period of soaking, liquid was expressed from the mixture and the extract was clarified by centrifuging. The supernatant fraction was assayed for radioactivity and the results were compared with the activity of the original solutions. The foods originally contained 0·09–0·11 ml water/g dry weight.

When the ^{51}Cr EDTA solutions had been in contact with the sample for 5 min it was found that, compared with ^{51}Cr EDTA, water had diffused into the food at a faster rate. The radioactivity measurements showed that per g dry weight of food there was 0·15–0·25 ml of unavailable water—unavailable in the sense that this was the calculated volume from which radioactivity was excluded (see Hydén, 1961). As the contact time between feed and marker solution increased, the proportion of unavailable water progressively decreased, and after 3 h the foods contained only 0·05–0·12 ml/g dry weight. When the ^{51}Cr EDTA was dissolved in centrifuged rumen liquor rather than water, it penetrated the food more readily, and the unavailable water was smaller by 0·01–0·05 ml/g dry weight in several parallel experiments, so that after 3 h contact it was 0·04–0·09 ml/g.

Rumen contents, collected about 20 h after feeding, were incubated with marker for up to 5 h and food particles were collected on sieves with apertures of 1·0 and 0·2 mm. These particles consisted of food material that had spent some time in the rumen and had thus been subjected to fermentation and incrustation with the rumen microbes. Measurements of radioactivity showed that the small particles contained about 0·1 ml unavailable water/g dry weight but the large particles contained 0·4–0·6 ml/g.

Rumen contents were incubated with ^{51}Cr EDTA, in vivo or in vitro and the mixtures were then strained through nylon bolting cloth and centrifuged. The centrifuged

deposit, consisting mainly of microbial cells with some very small food particles, contained 1·6 ml unavailable water/g dry weight after 5 min contact with ^{51}Cr EDTA, 1·1 ml after 1 h and 0·6–0·9 ml in various experiments after 3–5 h. As a consequence of this incomplete entry of ^{51}Cr EDTA into the microbial cells, strained rumen liquor was found to have 97% of the activity of the supernatant liquid from centrifuging after 5 min contact, 98% after 1 h and 99% after 5 h.

Without attempting to obtain a truly representative sample, rumen contents were taken from fistulated sheep; the samples contained 5–6% dry matter. They were mixed with ^{51}Cr EDTA and incubated in vitro at 39°. After intervals of 5, 15, 60 and 300 min they were found to contain, on average, 0·9, 0·7, 0·6 and 0·3 ml unavailable water/g dry weight respectively. Under these conditions, the ^{51}Cr EDTA entered, and could be used to measure, respectively 95, 96, 97 and 98% of the total water in the rumen contents.

It would seem, therefore, that, with the particular diets used in these experiments, water entering the rumen in the food would have no significant effect on marker concentration. The water contained in food particles and microbes already present in the rumen is not measured accurately by this marker technique, but the overall effect of this is small. In subsequent work, no allowance has been made for water in the food, and the volume of distribution of the marker has been called the volume of water in the ruminoreticulum, or briefly the rumen volume.

Table 1. *Repetitive sampling of labelled contents in the rumen of sheep; coefficients* (%) *of variation of marker concentration*

(Doses of marker were given 4 h before or 1 h after feeding, and samples were taken 1·5 and 3·5 h after each dose. At each time samples were drawn from six different positions in the rumen, namely upper and lower anterior, central and posterior. Counting of each sample was continued to give 16000–44000 counts, corresponding to coefficients of variation due to counting of 0·8–0·5%)

Period and time after dosing (h)	Sheep no. and diet*					
	2508 R9 (Expt 1)	2508 R9 (Expt 2)	2523 R9	2520† R9	3605 M2	2606 M2
Resting 1·5	0·8	5·6‡	1·4	5·5	1·6	2·1
Resting 3·5	1·0	1·2	1·3	3·0	1·5	1·9
Post-feeding 1·5	3·4	2·2	1·6	22·3§	7·8	2·2
Post-feeding 3·5	2·3	1·3	4·0§	3·0	7·9§	1·3

* See p. 375.

† Sheep dehydrated (see p. 378).

‡ Most of this variation was due to the sample from the lower posterior rumen, which contained about 12% less marker/ml than the others.

§ Most of the variation was due to the samples from the anterior rumen, which contained about 30% (sheep 2520), 6% (sheep 2523) or 14% (sheep 3605) less marker/ml than the others.

Sampling errors

^{51}Cr EDTA was injected intraruminally and the radioactivity was subsequently measured in several samples withdrawn simultaneously from the rumen. The coefficient of variation between samples removed from six sites in the rumen was determined at several times before and after feeding and the results are given in Table 1.

Sheep on three dietary regimes were used. Two sheep were given the diet R9 and two the diet M2 at noon and all four were given water in the afternoon, so that the first dose of marker was given some 16 h after drinking. A further animal was subjected to partial dehydration at the time of sampling. This sheep (2520) was accustomed at the time to the routine of receiving water in the morning and food in the afternoon; at the start of the sampling experiment it had been without water for 22 h, and by the end of the experiment it had had two meals with no water available. The samples from 2520 were more variable than those from any of the other sheep. Except for this, variation was usually greater in resting samples from sheep given the diet M2 than from those given R9. Variation was usually greater in samples taken 1·5 h after dosing than in those taken 3·5 h after dosing, which suggested that mixing improved even after 1·5 h. The high coefficient of variation found in the samples from sheep 2508 (Expt 2) was due to the sample from one site being conspicuously lower in marker concentration than the others. This indicated that the sample was drawn from a local 'pocket' of relatively unmixed material. In three instances, post-feeding samples showed evidence of similar 'pockets', all in the anterior part of the rumen where saliva enters from the oesophagus.

However, it seems that under favourable conditions, that is, sheep normally hydrated and with no rumen 'pockets', the coefficient of variation of samples from resting sheep was 2·1 % or less, and nearly half this variation was due to the counting error. After feeding, the variation was greater and there was a greater chance of encountering unmixed 'pockets'. Upper and lower parts of the rumen usually yielded samples of similar activity.

Routinely, therefore, material for analysis was obtained by mixing three samples of 2–3 ml each taken from the anterior, posterior and central rumen. Nearly 2000 estimations have been made to determine more than 200 curves; 11 of these curves showed a single point (representing about 0·6% of the estimations) lying well off the line of best fit (deviation of concentration more than 10 % from the expected value), presumably owing to sampling from an unmixed 'pocket'. These values were rejected, but the other points were accepted as they fitted the straight line reasonably well.

Methods of introducing dose

Method 1. The dose, in a small volume (10–20 ml), was poured down a short-necked funnel into the rumen. It was found that there was a considerable danger of losing part of the dose by splashing, or by a sudden increase of pressure in the rumen (e.g. due to bleating or regurgitation). In addition, it was found that a long time, often up to 2·5 h, was needed for adequate mixing. This method was abandoned.

Method 2. A large volume (500–1000 ml) rumen liquor was removed by suction, mixed with the dose (minimal volume) and poured back into the rumen down a funnel. This technique gave very good results for isolated experiments. The quality of the curves of log concentration against time was measured by an arbitrary score chosen for ease of application and described in Table 2 (see also Fig. 1, Table 4). It was noticed there was a tendency for the scatter of points on the graph to increase progressively when several experiments were performed on the same day (reading along rows of

Table 3) or when experiments were done weekly for several months (reading down columns of Table 3 for sheep 2508). It seemed that mixing became less and less satisfactory, possibly owing to progressively less motility of the rumen wall. This method also was abandoned.

Table 2. *Scoring system for curves relating log marker concentration in rumen contents of sheep and time*

(Straight lines were fitted to the points by eye, the deviation of each point from the line was examined and the line scored as shown. A score of 0 was given when no obvious straight-line trend in the points could be seen or when the limits shown below were exceeded)

	Single points		Mean of all points		
Score	Maximum deviation (log units)	Maximum variation in concentration (%)	Maximum deviation (log units)	Maximum variation in concentration (%)	Correlation coefficient*
4	0·005	1·2	0·005	1·2	0·98–0·99
3	0·010	2·3	0·005	1·2	0·96–0·98
2	0·020	5·0	0·007	1·7	0·90–0·95
1	0·030	7·0	0·014	3·3	0·80–0·90

* Measured for three to five curves in each score group as described on p. 380.

Table 3. *Quality of curves relating log marker concentration in rumen contents of sheep and time, obtained with different methods of dosing*

(Curves were scored as described in Table 2; the better the curve the higher the score. Each experiment consisted of three parts, resting, post-feeding and post-drinking, with a curve for each part)

						Average score		
Sheep	Diet*	Period	Method of dosing†	No. of expts	Overall average score	Resting	Post-feeding	Post-drinking
2508	R9	Month 1	2	4	2·8	3·0	3·0	2·3
		Month 2	2	4	2·8	4·0	2·8	1·8
		Month 3	2	4	1·6	3·8	0·5	0·5
		Month 4	3	3	3·3	3·7	2·7	3·7
2520	R9	Month 1	2	3	1·8	3·3	1·7	0·3
		Month 2	3	2	2·8	3·5	1·5	3·5
All expts (5 sheep)	R9	—	2	22	2·2	3·4	1·9	1·2
			3	6	3·1	3·5	2·3	3·3
All expts (3 sheep)	M2	—	2	14	2·4	2·9	2·3	2·1
			3	4	2·9	3·0	2·8	3·0

* See p. 375. † See p. 378.

Method 3. The dose was mixed with 200 ml water and injected into the rumen through a glass tube attached to a syringe, portions of about 5 ml each being injected at a time into different regions of the rumen, avoiding contact with the rumen wall. As can be seen from Table 3, the progressive deterioration of curves throughout the day, found with method 2, did not occur with method 3. Many subsequent experiments, not included in Table 3 because the routine of presentation of food or water

was different, have confirmed that uniformly good curves can be obtained by this method.

It was noteworthy that the points defining the curve were almost always more uneven in the post-feeding period compared with the resting period. Presumably the mixing of the rumen contents was less efficient after the consumption of food. Ingestion of water diluted the rumen contents so that mixing would have been facilitated and this probably explained why the quality of curves in the post-drinking period was similar to that in the resting period when the dose of marker had been introduced by method 3.

In these experiments, it was noticed that curves from resting sheep given the diet M2 were less satisfactory than those from sheep given R9, but there was less deterioration in the quality of the curves following feeding. When one sheep was changed from M2 to R9, the response changed accordingly.

As exemplified by sheep 2508 and 2520 in Table 3, better results were consistently obtained with some animals than with others.

Table 4. 95 % *confidence limits for volume, dilution rate and net inflow rate of water in the rumen of sheep*

(The values were obtained from the experiments described in Fig. 1)

Score of curve	Calculated from line fitted by least squares method: Value	Lower confidence limit	Upper confidence limit	Coefficient of variation (%)	Estimate from line fitted by eye
		Volume (l.)			
1	5·12	4·74	5·55	8	4·80
2	3·89	3·69	4·11	5	3·82
3	3·13	3·00	3·28	4	3·05
4	5·42	5·34	5·48	1	5·38
		Dilution rate (h^{-1})			
1	0·036	0·015	0·056	57	0·038
2	0·058	0·038	0·078	34	0·063
3	0·068	0·051	0·084	24	0·077
4	0·080	0·075	0·085	6	0·081
		Net inflow rate* (l./h)			
1	0·18	0·08	0·29	58	0·18
2	0·23	0·15	0·30	33	0·24
3	0·21	0·16	0·26	24	0·23
4	0·43	0·41	0·46	7	0·44

* See p. 381.

Confidence limits of results

In this work, it was usual to plot log concentration against time and fit a straight line by eye. To check the scoring system of Table 2, some of the lines were also fitted by the least-squares method. Confidence limits for the dilution rate, D, and the volume, V, were then computed by standard procedures. Calculation of confidence limits for the net inflow rate $F = DV$ presented difficulties, but because it was found (see

Table 4) that confidence limits for V were much narrower than those for D, it was possible to determine approximate confidence limits for F as follows:

Suppose that $D_1 < D < D_2$ and $V_1 < V < V_2$ can each be stated with 95 % confidence. Then the statements $D_1V_1 < DV < D_2V_2$ and $D_1V_2 < DV < D_2V_1$ may be assumed to hold with confidence respectively greater and less than 95 %, being respectively the least and the most favourable combination of the extremes. Now it was found that there was little difference (usually less than 0·03 l./h) between D_1V_1 and D_1V_2, and between D_2V_1 and D_2V_2; the values quoted in Table 4 are the means of D_1V_1 and D_1V_2, and D_2V_1 and D_2V_2 respectively.

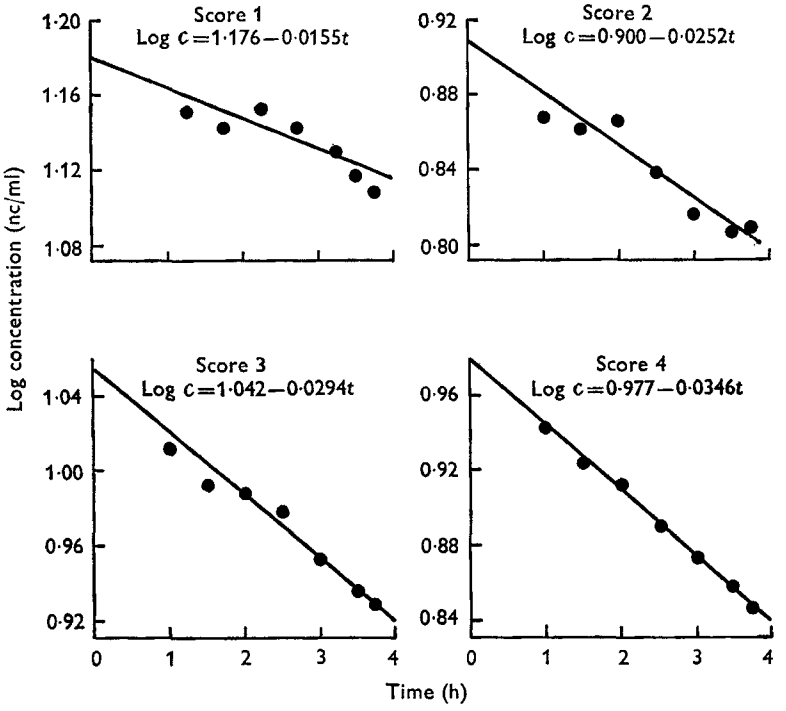

Fig. 1. Curves showing the concentration of marker in the rumen contents of sheep; the curves are representative of the arbitrary scores 1, 2, 3 and 4 (Table 2). The lines were fitted by eye; the equations were derived by the method of least squares (see p. 380). The calculated volumes and flow rates are given in Table 4.

Results from representative experiments are given in Fig. 1 and Table 4. It can be seen in Fig. 1 that points close to the time of injection were often difficult to fit into an otherwise reasonable straight-line trend. In most experiments, early samples had concentrations of marker lower than expected, and this suggested that mixing was then incomplete and that the samples had been taken from the bulk of the material with a lower concentration than the true average. In a few experiments, the initial samples contained more activity than expected, so it was concluded that they had been collected from 'pockets' of relatively unmixed dose material. When comparing results for lines fitted by eye with those from lines fitted by the least-squares method, it was

found that the former gave lower volumes and higher dilution rates than the latter (Table 4), perhaps because the eye tended to compensate for this change in the error variance.

In five experiments with grade 4 curves and four with grade 3, 95% confidence limits for V averaged $\pm 0{\cdot}12$ l. (maximum $\pm 0{\cdot}16$ l.); for D, $\pm 0{\cdot}008$ h^{-1} (maximum, $\pm 0{\cdot}011$ h^{-1}); and for F, $\pm 0{\cdot}03$ l./h (maximum, $\pm 0{\cdot}06$ l./h). Correlation coefficients between log c and t were all greater than 0·96 (Table 2).

In subsequent work, as a general rule, only curves with scores 3 or 4 have been used.

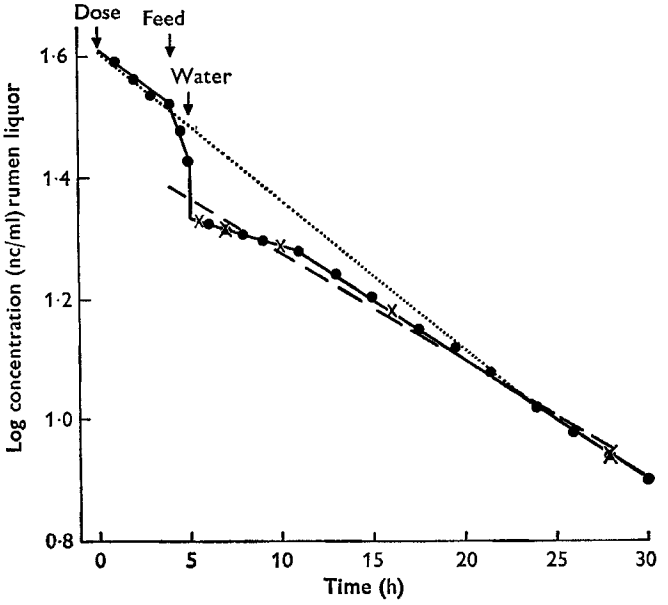

Fig. 2. Effects of a discontinuity in the curve for the concentration of marker in the rumen contents of sheep. The experimental points (●) are joined by the continuous line. Points for a hypothetical experiment, in which it was assumed that the dose was introduced immediately before feeding and samples taken at 1·5, 3·0, 6·0, 12·0 and 24·0 h after feeding, are marked (×) and the interrupted line was fitted to them. The dotted line shows the average dilution rate over the first 24 h of the experiment.

Errors in extrapolation caused by discontinuities in rumen marker concentration curves

Hydén (1961) introduced the dose of marker 'immediately before the morning meal' and established a concentration curve by taking samples at 1·5, 3·0, 6·0, 12·0 and 24·0 h thereafter; food and water were freely available. Hydén took steps to reduce the interfering effects of eating and drinking on the marker dilution rate, but it is instructive to demonstrate what may happen if these effects are not taken into account. The following example shows the errors that would have arisen if Hydén's regime of dosing and sampling had been applied to the present experimental conditions. ^{51}Cr EDTA was given 4 h before feeding, and marker concentration was measured throughout the following 30 h. When food was offered, the 700 g of the diet R9 was

consumed within 1 h; water was then presented, and 1·61. were consumed within 5 min; food and water were then removed. Figure 2 shows the experimental points and the curve joining them. Eating caused a sharp decline in concentration of marker; drinking caused an immediate drop in concentration as the water mixed with the rumen contents. The rate of decline of marker concentration was slow for about 6 h after feeding and drinking, but then returned to about the pre-feeding value. Table 5 shows the calculated initial volume, the average dilution rate, and, assuming constant volume, the average net inflow rate, including the water consumed. Now, if Hydén's schedule

Table 5. *Effects of a discontinuity in the marker concentration curve on the calculation of volume of contents and in flow rate of water in the rumen of the sheep*

(Values calculated from the actual and hypothetical experiments illustrated in Fig. 2)

	Actual experiment	Hypothetical experiment
Resting volume (l.)	5·8	7·9
Average 24 h dilution rate (h^{-1})	0·057	0·041
Average net inflow rate (l./day)	7·9	7·8

of dosing and sampling had been followed, the same experiment would have yielded different results. In Fig. 2 the crosses on the graph mark the hypothetical points that would have been obtained with the alternative technique, and the broken line describes the curve fitted to them. Obviously, the curves are dissimilar and the extent of the difference is reflected in Table 5 where values are compared from the analysis of the two curves. It can be seen that the hypothetical values (from the broken line in Fig. 2) grossly overestimated volume and underestimated dilution rate; on the other hand, the two estimates of average net inflow rate (and hence outflow rate) were essentially the same. Similar results have been found for several other experiments in which the two interpretations of the marker concentration values have been compared.

Urinary excretion of marker

On four occasions, doses of ^{51}Cr EDTA were given once daily to each of three sheep. Equilibrium was reached by the 3rd consecutive day and urine was collected on the 3rd, 4th and 5th days. The daily amount of marker in the urine was found to be (mean and SD) $3{\cdot}2 \pm 0{\cdot}9$ % of the dose.

DISCUSSION

There is little information on the theory and use of marker substances in the rumen, and the aim of the present study was to provide a firmer foundation for this type of work. The rumen in its functional state is a complex continuous-flow system and mathematical treatment of events within the system has in the past usually been restricted to hypothetical steady-state conditions. The theory developed in this paper departs from the usual convention: non-steady-state conditions are dealt with, and care is taken to point out the underlying assumptions and practical limitations of the method. The mathematical treatment opens the way for a fairly detailed interpretation

of the representative curve shown in Fig. 2, and a study of the changing events throughout the feeding cycle is given in the following paper (Warner & Stacy, 1968).

In the past, limitations of equations relating to events in the rumen have not always been clearly stated. Furthermore, the discontinuities in the marker concentration curve caused by eating or drinking have not always been recognized. While Hydén (1961) took steps to reduce the interference arising from eating and drinking, his practice of administering marker before feeding is theoretically unsound and its uncritical adoption could well lead, under other experimental conditions, to serious errors, as is clear from Fig. 2 and Table 5. Ternouth (1967) followed this practice in studying rumen function but made no allowance for the effects of eating or drinking. The results in his table 2 show a puzzling difference between two estimates of rumen volume before feeding, and it seems very likely that the discrepancy was caused by errors of the type just described.

Other aspects of the work of Ternouth (1967) also require comment. The author appears unsure of the theoretical basis of the techniques he was using. Thus, he estimated rumen volume at various times after feeding, but derived his values from experiments on different days or on different animals; he then applied to his estimates a correction that can readily be shown to be mathematically unwarranted and finally used the differences between successive pairs of these values in the calculation of flow rates by the equation of Reid (1965), a procedure liable to considerable error even if, as is doubtful, it could be assumed that for each experimental period the net inflow and outflow rates were constant, as is required for the use of this equation. Ternouth injected doses of marker at times when he claimed that outflows were very large, so that considerable error from incomplete mixing might be expected, and then used a stomach tube to sample the rumen contents. Neither he nor Hecker *et al.* (1964), who also withdrew samples from the rumen via a stomach tube, gave any evidence on the sampling error to be expected, but some unpublished work from these laboratories has shown that samples taken by stomach tube often contain saliva-rich material and sometimes consist of abomasal contents.

So far, all the evidence indicates that ^{51}Cr EDTA is a satisfactory rumen marker. Intravenous administration of large doses of inactive Cr EDTA caused no ill effects in sheep and rats (Downes & McDonald, 1964; Stacy & Thorburn, 1966). Considerable quantities of ^{51}Cr EDTA have been infused into the rumen in the present experiments and also in experiments by Downes & McDonald (1964) and Hogan (1964); no adverse reactions to this marker have been found. This would suggest that the marker is non-toxic to the rumen microbes and in fact no changes in the microbial population have been noticed (A. C. I. Warner, unpublished observations). ^{51}Cr EDTA is readily extracted from feed particles and, as with PEG (Hydén, 1961), a small proportion of the water associated with the feed is unavailable to the marker. It is not adsorbed on to micro-organisms and again, as also found by Hogan (1964), penetration into microbial cells is not complete so that the concentration of ^{51}Cr EDTA in rumen liquor strained through gauze is less than that in the supernatant fraction after centrifuging. Adsorption on to tissue cells does not seem to be significant since the two very dissimilar substances PEG and ^{51}Cr EDTA have essentially the same volume of distribution in the rumen

and recovery in the faeces is virtually complete (Downes & McDonald, 1964; Hogan, 1964). ^{51}Cr EDTA does not appear to be metabolized in the rumen since incubation with rumen liquor did not lead to the production of any chromatographically different compound (Tan & Warner, unpublished). Absorption across the rumen wall is minimal and Hogan (1964) was unable to detect ^{51}Cr EDTA in the enterohepatic circulation or in the bile after intraruminal administration. However, about 3 % of the dose is excreted in the urine; Downes & McDonald (1964) and Hogan (1964) found similar amounts. Since this included marker absorbed from the entire gut as well as any contamination from faeces or rumen liquor splashed in the cage, it seems safe to assume that less than 0·1 % of the amount of ^{51}Cr EDTA in the rumen is absorbed across the rumen wall per hour, so that for a sheep of rumen volume 4 l., inflow and outflow rates would be overestimated by less than 0·004 l./h. No correction for absorption has been applied in this work.

The values in Table 3 show that the scatter of points about the marker concentration curve may be significantly affected by the way in which material is added to or withdrawn from the rumen. Poor mixing was found to accompany methods involving much poking and suction on the rumen wall, particularly when the experiments were prolonged. Poor mixing was also found in one sheep while a small subcutaneous abscess was present near the fistula; the animal became unusually nervous and reacted sensitively when touched near the infected region. After the abscess healed the sheep once again grew docile and satisfactory mixing was found.

Anything that affects the viscosity of the rumen liquor or the amounts of solids suspended therein also affects sampling errors in the rumen and hence the scatter of points about the marker concentration curve. Thus, the all-roughage diet R9 tended to give more satisfactory results than the mixed diet M2 in the resting period (Tables 1, 3) when the rumen liquor from sheep given M2 was noticeably more viscous than liquor from sheep given R9. Similarly, large sampling errors (Table 1) and relatively unsatisfactory curves (Warner & Stacy, 1968) were obtained even with the diet R9 when the sheep had been given two normal meals but no water; in this instance the amount of free liquid in the rumen contents appeared less than usual. During the consumption of food, relatively smooth curves were obtained, despite considerable changes in dilution rate (Warner & Stacy, 1968), presumably because mixing was good due to increased rumen motility (Reid & Cornwall, 1959). However, in the post-feeding period, presumably due to the higher concentration of both solids and solutes in the rumen, larger sampling errors were found and there was a greater tendency to encounter relatively unmixed 'pockets' (Table 1); consequently, in this period the curves were less satisfactory (Table 3). On the other hand, it was found that imbibed water took up to 1 h to mix adequately with rumen contents (Warner & Stacy, 1968), but the post-drinking curves showed little scatter of points (Table 3). Apparently, the more fluid nature of the contents following drinking facilitated mixing of the incoming saliva. Previous experience has shown that less accurate results are obtained from grazing sheep compared with penned animals on dry diets (Hogan, 1964), and that cattle are less satisfactory to work with than sheep since mixing is less adequate (Pearson & Smith, 1943; Bryant, 1964).

On several occasions, it was found that the scatter of points about the marker disappearance curve was increased after other sheep were fed in the presence of an experimental sheep from which food was withheld; the effect was presumably due to psychological stress. Denton (1957) showed that the rate of secretion of parotid saliva in sheep responded to psychic stimuli; such a response would affect marker concentration.

No attempt has been made in this work to compare estimates of rumen volume from marker experiments with more direct measures. A direct measure would involve either emptying the rumen at slaughter at the conclusion of the marker experiment (or at a time corresponding to the start of the marker experiment but on a subsequent day) or emptying through a large cannula before (Tulloh, Hughes & Newth, 1965) or after the marker experiment. Cannulas large enough to permit rapid emptying of the rumen by hand, while satisfactory in cattle (Reid, 1965; Tulloh *et al.* 1965) put undesirable restraints on rumen motility in sheep (J. P. Hogan, unpublished). In general, the volume of water in the rumen is not constant, nor is it the same from day to day at any particular time (Warner & Stacy, 1968). Small movements of water into or out of the rumen might occur during the time needed to complete the emptying process and the procedure of emptying via a cannula might be expected to have some effect on the subsequent physiology of the rumen, even if the effect is not large (Reid, 1965). Consequently, a direct measure of rumen volume would not be expected to be identical with the volume at the start of a marker experiment. This might account for some of the differences found between the two methods by Tulloh *et al.* (1965). Much larger differences were found by Hecker *et al.* (1964) and Purser & Moir (1966), but the markers used by these workers were not adequately tested and may have been unsatisfactory. In the present studies, we have been content to demonstrate that the marker gives a good measure of the volume of the rumen contents in vitro.

The total volume of water in the rumen is slightly underestimated by ^{51}Cr EDTA and by PEG (Hydén, 1961) since both substances enter only 95–98 % of the water in the contents. For the same reason, dilution rates would be slightly overestimated so that flow rates would tend to be unaffected. However, reliable estimates of flow rate can only be obtained under experimental conditions far more stringent than those needed for estimating rumen volume.

Grateful thanks are given to Mr H. Weiler for mathematical and statistical advice, and to Miss G. Wheeler and Mr N. R. Bradbury for technical assistance.

APPENDIX

The error involved in replacing $\overline{xy} = \Sigma(xy)/n$ by $\bar{x}\,\bar{y}$ for any variables may be estimated as follows. Since the correlation coefficient of xy is given by

$$r = \frac{\Sigma xy - n\overline{xy}}{(n-1)\,s_x\,s_y},$$

where s_x, s_y are the standard deviations of x and y, it follows at once that

$$\overline{xy} - \bar{x}\,\bar{y} = [(n-1)/n]\,rs_x\,s_y.$$

However, for the kinds of experiment considered in this work, neither the correlation coefficient nor the standard deviations of the variables will be known with precision. But, even when r is large, it can be seen that

$$\left|\frac{\overline{xy}-\bar{x}\,\bar{y}}{\bar{x}\,\bar{y}}\right| < \frac{s_x}{\bar{x}}\frac{s_y}{\bar{y}}.$$

Now, in marker experiments lasting a full day, for many dietary regimes the coefficients of variation for c (continuous-infusion experiment) and for V seem unlikely to exceed 15–20%; the coefficients of variation of E and, particularly, D may well be greater, but seem unlikely to exceed 50% (Warner & Stacy, 1968). In these conditions, the errors in either of the approximations $\overline{Ec} = \bar{E}\,\bar{c}$ or $\overline{DV} = \bar{D}\,\bar{V}$ would seem highly unlikely to exceed 10% and could well be much less.

If, in addition, $(V_1 - V_2)/(t_2 - t_1)$ is negligibly small and, in the case of a single-shot experiment, $\bar{V}$ can be known with sufficient accuracy or, in the case of a continuous-infusion experiment, $(V_1c_1 - V_2c_2)/(t_2 - t_1)$ is also small, then the probable errors in taking $\bar{F} = \bar{E} = \bar{D}\bar{V}$ (single-injection experiment) or $Rm/\bar{c}$ (continuous-infusion experiment) would seem to be 10% or less. The greatest uncertainty in these calculations would lie in the value of $\bar{V}$ which cannot be reliably ascertained if the volume varies rapidly over a wide range.

REFERENCES

Bryant, A. M. (1964). *N.Z. Jl agric. Res.* **7**, 694.
Denton, D. A. (1957). *Nature, Lond.* **179**, 341.
Downes, A. M. & McDonald, I. W. (1964). *Br. J. Nutr.* **18**, 153.
Hecker, J. F., Budtz-Olsen, O. E. & Ostwald, M. (1964). *Aust. J. agric. Res.* **15**, 961.
Hogan, J. P. (1964). *Aust. J. agric. Res.* **15**, 384.
Hydén, S. (1961). *K. LantbrHögsk. Annlr* **27**, 51.
Pearson, R. M. & Smith, J. A. B. (1943). *Biochem. J.* **37**, 142.
Purser, D. B. & Moir, R. J. (1966). *J. Anim. Sci.* **25**, 509.
Reid, C. S. W. (1965). *Proc. N.Z. Soc. Anim. Prod.* **25**, 65.
Reid, C. S. W. & Cornwall, J. B. (1959). *Proc. N.Z. Soc. Anim. Prod.* **19**, 23.
Sperber, I., Hydén, S. & Ekman, J. (1953). *K. LantbrHögsk. Annlr* **20**, 337.
Stacy, B. D. & Thorburn, G. D. (1966). *Science, N.Y.* **152**, 1076.
Stacy, B. D. & Warner, A. C. I. (1966). *Q. Jl exp. Physiol.* **51**, 79.
Ternouth, J. H. (1967). *Res. vet. Sci.* **8**, 283.
Till, A. R. & Downes, A. M. (1965). *Br. J. Nutr.* **19**, 435.
Tulloh, N. M., Hughes, J. W. & Newth, R. P. (1965). *N.Z. Jl agric. Res.* **8**, 636.
Ulyatt, M. J. (1964). *N.Z. Jl agric. Res.* **7**, 774.
Warner, A. C. I. (1966). *J. gen. Microbiol.* **45**, 213.
Warner, A. C. I. & Stacy, B. D. (1965). *Q. Jl exp. Physiol.* **50**, 169.
Warner, A. C. I. & Stacy, B. D. (1968). *Br. J. Nutr.* **22**, 389.

Printed in Great Britain