CHAPTER 1

INTRODUCTION

1.1 GENERAL

Ruminants are the herbivorous animals that include cattle, sheep and goats. These economically important animals, like other mammals, cannot synthesise intestinal cellulases but they have evolved a rumen and an ectosymbosis with microorganisms which enables ruminants to live on a diet in which the major sources of carbon are complex carbohydrates of the hemicellulose and cellulose group. The ability of ruminants to utilize food sources which are unsuitable for humans and produce meat, milk and hide makes the study and ultimate maximization of their production potential an on-going and vital program.

The rumen association is in a delicately balanced equilibrium requiring the presentation to microbes of fermentable substrates and the removal of end products in a buffered environment. The equilibrium may be disturbed by slight changes in the rumen environment (Hungate, 1966, p 8).

Digestion in the ruminant involves fermentation with the concommitant production of copious quantities of gas, mainly CO_2 and CH_4. Gas production in the rumen of a mature cow is in the order of 60 to 80 litres of rumen gas per day and, since the total rumen volume is only approximately 100 litres, gas must be removed by absorption and/or steady belching (eructation). For reasons that are not fully understood, certain conditions lead to foaming of rumen contents. When this happens, eructation is inhibited and a potentially fatal distension of the rumen by trapped gas (bloat) occurs.

A reduction in the buffering capacity of the rumen resulting in decreased pH and the rapid accumulation of gas in the rumen have been implicated in bloat formation (Blake et al, 1957; Mendal and Boda, 1961; McArthur and Miltimore, 1969; Bryant et al, 1973).

1.2 PURPOSE AND AIM OF THE STUDY

As gas production in the rumen is a necessity for bloat formation then a full understanding of the problem of bloat makes a knowledge of the dynamics of bicarbonate movement in the rumen of sheep and cattle important. This must include reference to the source and routes of rumen CO_2.

A series of experiments were carried out in order to quantify and qualify the movement of bicarbonate-C. The experiments increased in complexity and depth as techniques were estabished. Using a compartmental model approach, continuous infusions were carried out to establish the rate at which carbon dioxide appeared and disappeared from the compartment of physiological interest. This allowed an

adequate description of the dynamics and chemical kinetics of the system. It was hoped that the final model might describe movement of carbon within a system which comprised rumen fluid bicarbonate, rumen gas (CO_2 and CH_4), blood bicarbonate and post ruminal digestive tract bicarbonate.

Lowering rumen pH leads to the release of CO_2 from salivary and rumen fluid bicarbonate. This is generally associated with fermentation which aids in the production of large quantities of gas. Under low rumen pH conditions, an alteration in the dynamics of bicarbonate is expected. The quantification of the bicarbonate system under conditions of low pH was carried out using the same compartmental model approach mentioned above.

In order to avoid the adverse affects of low pH, various feed additives have been tested. The addition of Na-bentonite has been suggested as a means of stabilizing rumen pH and holding it above dangerously low levels. The ability of Na-bentonite to stabilize rumen pH and bicarbonate content was examined.

CHAPTER 2

LITERATURE REVIEW

2.1 COMPOSITION OF RUMEN GASES

The proportions of gases in the mixture of rumen gases under most conditions are 45-70% carbon dioxide, 20-30% methane, 4-7% nitrogen, 0.2% hydrogen, 0.5-1% oxygen and 0.01-0.1% hydrogen sulphide (Kleiber et al, 1943; Cole et al, 1945; McArthur and Miltimore, 1963; Clarke and Reid, 1973; O'Shea, 1973). Traces of ammonia and methylamine may also be present (McArthur and Miltimore, 1961) and Dougherty (1941) reports the presence of carbon monoxide in concentrations up to 0.17%. The composition of the rumen gas pools, however, depends on the feed, the microbial flora, time of sampling in relation to feeding and the inclusion of elements in the feed which alter methane production (for example, Monensin).

Hoernicke et al (1965) showed that grain-fed cattle had a higher CO_2/CH_4 ratio in rumen gas than animals fed orchard grass or lucerne hay. Czerkawski and Clapperton (1968) found a CO_2/CH_4 ratio of 0.76 prior to feeding and 1.80 three hours after feeding. At the same sampling times, nitrogen volume percentage decreased from 36.2% to 6.0%.

2.2 SOURCES OF RUMEN GAS

2.2.1 Carbon dioxide

The highest proportion of rumen gas is made up of CO_2. Measurements of the CO_2 arising from the rumen are about one third that produced in oxidative metabolism in the animal itself (Hungate et al, 1961; Hoernicke et al, 1964). The main sources of rumen gas CO_2 are microbial fermentation, acidification of salivary bicarbonate and bicarbonate entering the rumen from the blood across the rumen wall. Hungate (1966, p84) deals extensively with the microflora responsible for fermentation. The most important rumen bacteria producing CO_2 by fermentative pathways are Butyrivibrio spp., Lachnospira multiparus, Selenomonas ruminantium and Eubacterium spp. The rumen protozoa Isotricha intestinalis and Entodinium spp. also produce CO_2.

2.2.1.1 Fermentation of carbohydrates. -

Figure 2.1 gives the pathways of carbon metabolism in the rumen. The major dietary carbohydrate constituents starch, cellulose and sugars are fermented via hexose, pyruvate or lactate and subsequently CO_2, VFA, methane and energy in the form of ATP. A single scheme of these reactions is given below (Leng, 1970):

$$\text{Hexose} \longrightarrow 2 \text{ pyruvate} + 4H + 2ATP$$

Pyruvate rarely accumulates in the rumen but is fermented immediately as follows:

$$\text{Pyruvate} + H_2O \longrightarrow \text{acetate} + CO_2 + 2H + ATP$$
$$\text{Pyruvate} + 4H \longrightarrow \text{propionate} + H_2 + ATP$$
$$2 \text{ Pyruvate} \longrightarrow \text{butyrate} + 2CO_2 + 2ATP$$

The **stoichiometry** of products and the carbohydrate fermentated can be calculated using the above reactions as a basis and assuming standard microbial biochemical pathways are followed. For a sheep consuming 900 g lucerne chaff/d, VFA production rates were 2.98 moles of acetate, 0.66 moles of propionate and 0.24 moles of butyrate (Leng, 1970). When applied to the above equations, the following values are observed:

(1) 1.49 Hexose + 2.98 H_2O ⟶ 2.98 acetate + 2.98 CO_2 + 2.98 H_2 + 5.96 ATP + 5.96[H]

(2) 0.33 Hexose + 1.32[H] ⟶ 0.66 propionate + 0.66 H_2O + 1.32 ATP

(3) 0.24 Hexose ⟶ 0.24 butyrate + 0.48 CO_2 + 0.48 H_2 + 0.96 ATP

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(4) Hexose ⟶ 2.98 acetate + 0.66 propionate + 0.24 butyrate + 3.46 CO_2 + 4.64 [H] + H_2 + 8.24 ATP

Equation 4 is the summation of equations 1, 2 and 3. According to these pathways, there is a net production of carbon dioxide and hydrogen which may be converted to methane (Section 2.2.2).

2.2.1.2 Fermentation of amino acids -

Another source of CO_2 in the rumen is from microbial fermentation of amino acids. This process denies the animal some of the amino acids it may otherwise have utilized. Studies with glutamate-U-^{14}C showed that in VFA and CO_2, almost half the activity was recovered. With aspartate-U-^{14}C, the value was 55% (Hungate, 1966, p 291).

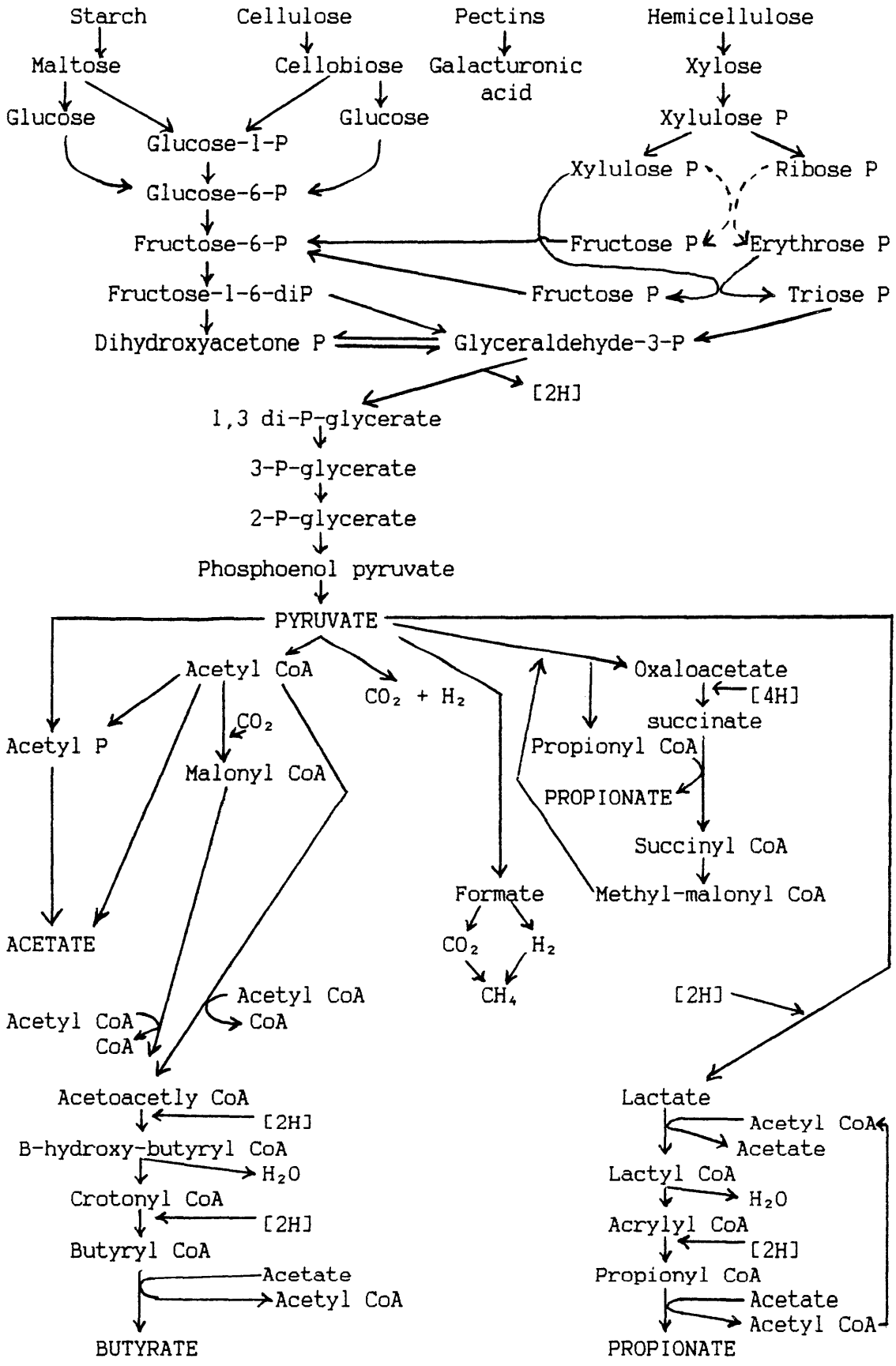


Figure 2.1: Rumen carbohydrate metabolism (Leng, 1970).

The solubility of the protein affects the rate of digestion of the protein in the rumen. Soluble proteins like gelatin and casein are most readily fermented whilst proteins that are more insoluble are less readily degraded in the rumen, for example, lucerne protein (Figure 2.2). Mangan (1972) found that casein had a half life of 5.6 to 21.5 min in the rumen of cattle. However, he also found that ovalbumin, although a soluble protein, had a half life of 180 min. This was due to its cyclic structure, lacking terminal amino or carboxyl groups, which is apparently not easily degraded by bacterial enzymes.

2.2.1.3 Bicarbonate as a source of carbon dioxide -

Salivary bicarbonate is a source of CO_2 gas in the rumen. It was postulated that saliva containing an equivalent of 200 volumes percent of CO_2 secreted by a cow at 60 l/d would deliver the equivalent of 120 l of CO_2 to the rumen in a day (Cole et al, 1945). It has been found that at pH above 6.9, CO_2 is absorbed rather than given off in the rumen (Kay, 1963). Saliva is secreted at pH of about 8.0 where it is in equilibrium with expired air (6% CO_2) and would contain 112 millimols of total CO_2 (CO_3^{2-} + HCO_3^- + H_2CO_3 + CO_2). If it is brought into an atmosphere of 70% CO_2 (as in the rumen) it would tend to absorb CO_2. One litre of saliva would hold 1,360 millimols of CO_2 at pH 8.0 and exposed to 70% CO_2. If the rumen had a pH of 8.0, saliva leaving the mouth would absorb 1,248 (1360-112) millimols (27 litres) of CO_2.

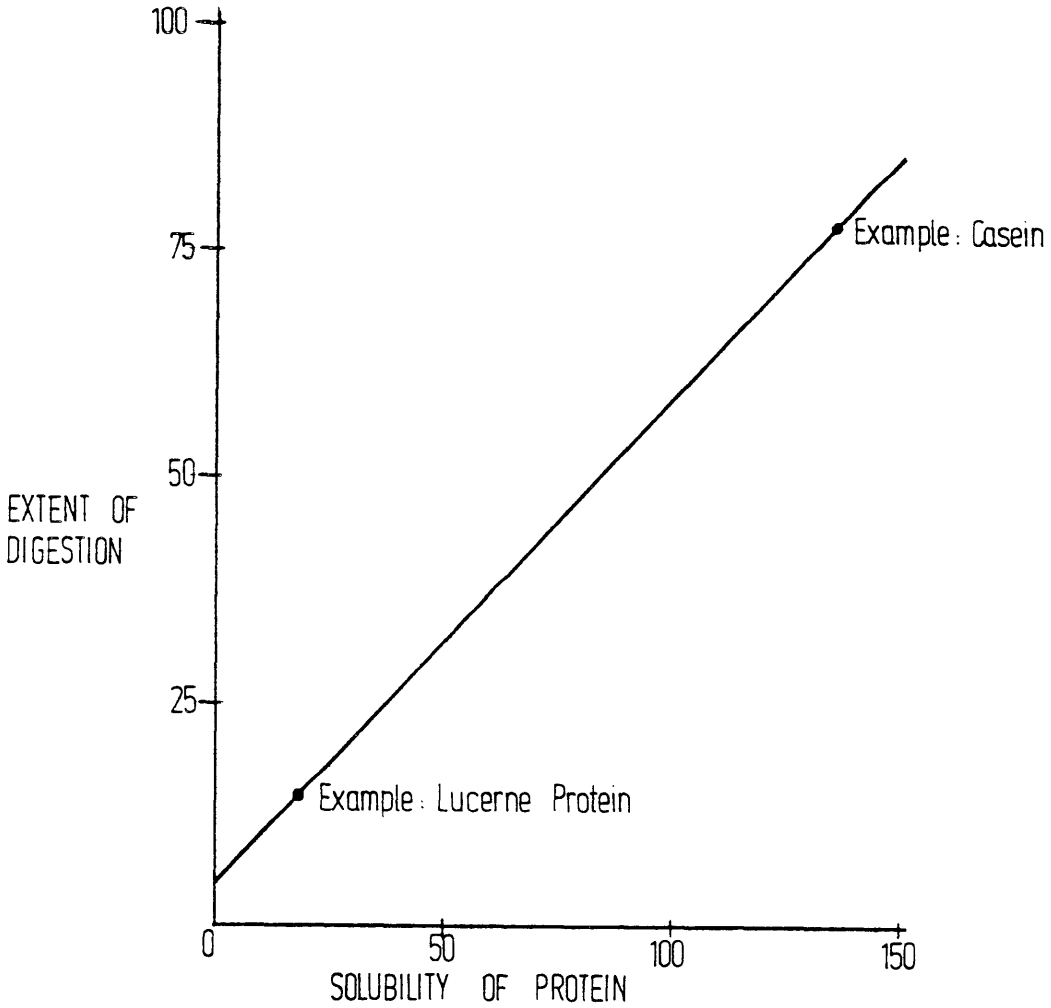


Figure 2.2: The relationship between solubility of protein and the extent of its digestion in the rumen (adapted from Hendrickx and Martin, cited by Hungate, 1966, p287).

However, a decrease in the pH of saliva occurs as it enters the rumen.

According to the Henderson-Hasslebach equation:

$$pH = pK + \log \frac{\text{bound } CO_2}{\text{free } CO_2}$$

where: bound CO_2 = total CO_2 - free CO_2
free CO_2 = undissoc. H_2CO_3 + CO_2

Using this relationship, Cole et al (1945) calculated that the pH of the saliva shifts from 8.0 to 6.91. At this pH, the saliva would only absorb 13 millimols of CO_2 to contain 125 millimols of total CO_2. At 60 litres of saliva per day, this is a total absorption of 17 litres of CO_2 from rumen gas. When rumen pH drops below 6.9, CO_2 is liberated rather than absorbed. Saliva at pH 5.7 in contact with 70% CO_2 will contain 23 millimols total CO_2 per litre. At pH 8.0, it contained 112 millimols (in contact with 6% CO_2). When brought to pH 5.7, 89 millimols (112-23) of CO_2 would be given off. Under these conditions, the 60 litres of saliva daily secreted would contribute 118 litres of CO_2 to the rumen gas (60 X 0.089 X 22) produced daily.

2.2.2 Methane

The process of methane production, methanogenesis, is carried out by Methanobacterium ruminantium (Stanier et al, 1977). The overall reaction is:

$$CO_2 + 4H_2 + Pi \longrightarrow 2H_2O + CH_4 + ATP$$

Figure 2.1 shows the breakdown of formate to CO_2 and H_2 which are subsequently converted to methane using carbon from CO_2 and molecular hydrogen. The regular culture of bacteria that produce methane has

not been regularly achieved (Church, 1976, p 296). Some species such as Ruminococcus albus produce formate but do not degrade it to H_2 and CO_2 (Miller and Wolin, 1973).

Methane is an endpoint of fermentation which has calorific value representing greater than 8% of the gross energy of feeds (Blaxter, 1962). This represents a net loss of energy from the system. For this reason, interest in the inhibition of methanogenesis has developed.

Inhibition of methanogenesis may be achieved with chemicals like carbon tetrachloride, Rumensin (Trade mark, Elanco Products Company, Eli Lilly and Company, Indianapolis, U.S.A.) and unsaturated oils. Inhibition can however decrease microbial growth and provide less protein to the ruminant.

Stoichiometry can also be applied to methane production (Hungate, 1968). From equation 4, if the H_2 is converted to methane:

(5) $$3.45\ H_2 + 0.86\ CO_2 \longrightarrow 0.86\ CH_4 + 1.72\ H_2 + 0.86\ ATP$$

Combining equations 4 and 5 gives:

(6) $$2.06\ Hexose + 0.6\ H_2O \longrightarrow 2.9\ acetate + 0.66\ propionate + 0.24\ CO_2 + 0.86\ CH_4 + 4.64\ [H] + 9.1\ ATP + 0.24\ butyrate$$

By measuring the rate of methane production, an index to the rate of fermentation can be found which can be compared to the amount of food ingested. This is only valid when the ratio of VFA to CO_2 and CH_4 remains constant which does not always occur. There is a tendency for CO_2 to be higher after feeding (Williams et al, 1963; Hungate, 1968).

2.2.3 Hydrogen

Fermentative pathways may yield molecular hydrogen, for instance, in the conversion of glucose to pyruvate (Embden-Myerhof pathway) and in the production of formate from pyruvate. Substrates which can act as sources for bacterial hydrogen gas formation include pyruvate, fumarate, glucose, maltose, and some amino acids (Woods and Clifton, 1937).

The production of pyruvate from glucose is accompanied by the production of NADH. Production of NADH and interconversion with NAD^+ relies on ferredoxin. Unlike the cytochromes which are only found in aerobic cells, ferredoxin is an iron-sulphur protein found in anaerobic bacteria as well as higher plants and animals (Lehninger, 1975, p 488-493). Ferredoxin functions as an electron carrier by undergoing valence changes during the reactions shown below.

NADH may be reoxidised with the production of H_2 (Wolin, 1974):

$$NADH + Fd_{ox} \longrightarrow Fd_{red} + NAD^+$$

$$Fd_{red} + H^+ \longrightarrow Fd_{ox} + H_2 \qquad (Fd = \text{ferredoxin})$$

Hydrogen is normally converted to methane as it is produced but if hydrogen is allowed to accumulate, the reaction will not continue because there is a feed-back switch to the production of electron-sink products such as ethanol, rather than acetate (Ruminococcus albus), succinate (R. flavefaciens) and propionate and lactate (Selenomonas ruminantium). These are produced with a concommitant reoxidation of NADH produced from glycolysis rather than reoxidation with the production of H_2. The removal of H_2 occurs in the presence of methanogenesis.

Bacteria may posses the enzyme formic hydrogen lyase which splits formate to CO_2 and H_2 (Stanier et al, 1977). The H_2 can be used by methanogenic bacteria to produce methane.

Some bacteria and protozoa are able to form hydrogen from reduced pyridine nucleotides (Wolin, 1979). The reverse reaction is strongly favoured however, but if the hydrogen is removed, hydrogen production can continue.

2.2.4 Hydrogen sulphide

Although small in number, bacteria are present in the rumen which can produce H_2S. Some species of the genera Delsulphotomaculum and Desulphovibrio carry out the reaction of reducing sulphate to sulphide (Gottschalk and Andreesen, 1979):

$$SO_4^{2-} + 4H^- \longrightarrow SO_2^{2-} + 4H_2O$$

using the substrates cysteine, cystine and methionine (Cole et al, 1945). The reaction is associated with the conversion of ATP to AMP so that net ATP yield is zero.

It appears possible that H_2S may be used as a source of sulphur by many rumen microorganisms and is assimilated by Bacteriodes succinogens, Ruminococcus flavefaciens and Streptococcus bovis (Hungate, 1966, p 349).

2.2.5 Oxygen and nitrogen

Oxygen and nitrogen enter the rumen pool when food is swallowed during feeding or by diffusion from the blood (Kay, 1963). With swallowing, one would expect a ratio of four nitrogen to one oxygen in

the rumen gas pool. However, Kleiber et al (1943) found the ratio to be seven nitrogen to one oxygen. This may be due to uptake of oxygen during aerobic reactions in the rumen (Church, 1976, p 295). Oxygen may be also derived from oxygen dissolved in drinking water.

Most of the ammonia present is in the liquid phase rather than the gas phase (Church, 1976, p 295).

2.3 RATE OF GAS PRODUCTION

In the literature, the reported data on the rate of gas production in the rumen is highly variable. Hoernicke et al (1965) found a range of 0.2 l/min in fasted cattle to 2.0 l/min following feeding. A gas production rate of 2.0 l/min in cattle has also been found by Hungate et al (1955). Most other researchers quote a figure of about 30 l/hr in cattle (Colvin et al, 1957; Dougherty and Cook, 1962; Kay, 1963). A comparable rumen gas production rate for sheep is 5 l/hr (Church, 1976, p 91). The animal and the feed, its solubility and its rate of fermentation, the time of feeding and pH changes in rumen contents all must play a part in determining gas produced in the rumen (Cole et al, 1945; Church, 1976, p 295).

The relationship of time to feeding has a pronounced effect on the rate of gas production with the rate of gas production increasing after feeding. Cole et al (1942) found maximum rumen gas production occured at one hour after feeding and Colvin et al (1957) found a maximum at three to four hours following feeding. There is a gradual decrease in gas production following the peak period.

Methane production represents a loss of energy to the ruminant. In an attempt to reduce the loss of energy via this route considerable study has been done on predicting methane production (Blaxter and Clapperton, 1965; Hungate, 1968; Leng, 1970; Murray et al, 1976; Murray et al, 1978).

Murray et al (1978) used isotope dilution technique to estimate methane production rates in sheep fed lucerne chaff. They related digestible organic matter intake (DOMI) to methane production in the rumen, and found the following relationship:

$$Y_r = 2.89 + 0.036\ X$$

where: Y_r = methane production in the rumen (l/d)
X = DOMI (g/d).

Hungate (1968) and Leng (1970) used the relationship between VFA production and methane production from balance equations to theoretically predict rumen methane production. The regression equations they found were:

$$Y_{(Leng)} = 0.05 + 1.08\ (\pm\ 0.202)X \text{ (RCV 26.4)}$$
$$Y_{(Hungate)} = 0.06 + 1.24\ (\pm\ 0.248)X \text{ (RCV 28.2)}$$

where: Y = methane production (mol/d)
X = measured methane production (mol/d).

Murray et al (1978) developed regression equations of these theoretical values and their measured values and found that Hungate (1968) overestimated actual methane production by 32%. Similarly, Murray et al (1978) found that the equations developed by Blaxter and Clapperton (1965) based on gross energy (GE) intake overestimated methane production by 10 to 30%.

The overestimation of methane production by Hungate (1968) was due to his assumption that electron generated as reduced co-enzymes were converted to hydrogen. The equation developed by Leng (1970) was not significantly different to the actual values obtained by Murray et

al (1978) since in the stoicheiometry of Leng (1970), only 60 to 66% of the electrons go to hydrogen production.

2.4 LOSS OF GAS FROM THE RUMEN

The major avenues for removal of gas from the rumen are eructation, absorption through the reticulo-rumen epithelium, down the digestive tract and into metabolic pathways within the rumen.

2.4.1 Eructation

Eructation is the most important method for removal of free gas from the rumen. Because of the large volumes of gas produced, the system must be efficient. The movement of gas via eructation involves several stages (Clarke and Reid, 1974):

1. separation, where gas forms into bubbles which rise to the gas space in the dorsal cap.
2. displacement of gas from the dorsal cap into the cardia
3. transfer of gas into the oesophagus when the cardia is open
4. rapid movement of gas up the oesophagus into the pharynx
5. movement of gas into the lungs and expiration of gas in air.

During rumination, some gas is lost directly through the open mouth. Dougherty et al (1965) found 0.5-33% of gas escapes through the mouth directly.

Titchen and Reid (1965) reported two types of reticulo-rumen contractions were involved in eructation. The first is a contraction of the reticulum followed by contraction of the cranial dorsal sac and the caudal ventral sac. It is concerned with the mixing of digesta in the rumen and termed a primary or A sequence. The second contraction type is called a B or secondary sequence and is independent of the

reticulum. It involves contraction of firstly the caudal and cranial regions of the dorsal sac and the main ventral sac. It is this second contraction, a forward moving contraction, which is mostly associated with eructation. Figure 2.3 shows the anatomy of eructation. Eructation is stimulated by gas pressure in the rumen, and is inhibited when neural receptors in the cardial region are covered with digesta, foam or liquids. This may be to prevent these substances getting into the lungs upon belching (Church, 1976, p 77-79). The buildup of foam and the concommitant prevention of eructation leads to bloat in ruminants.

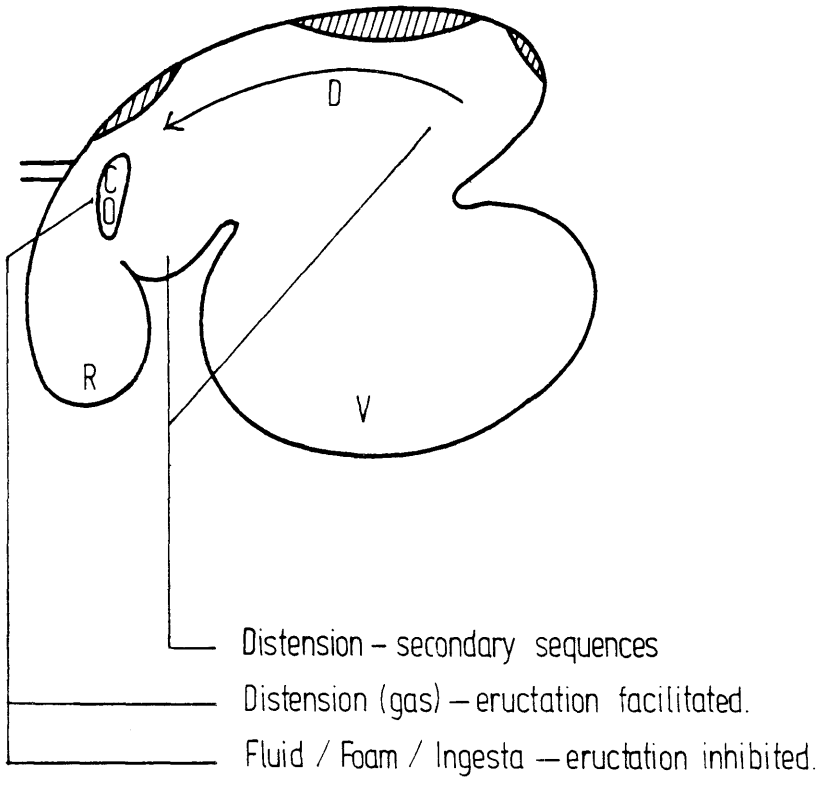


Figure 2.3: Anatomy of eructation showing gas caps ▨ ,
arrow indicates gas movements during secondary sequences.
C = cardia, O = oesophageal groove, D = dorsal sac,
V = ventral sac, R = reticulum. (Akester and Titchen, 1969).

2.4.2 Absorption

Significant amounts of rumen CO_2 and CH_4 may be absorbed from the rumen. Absorption is influenced by changes in gas concentration within the rumen, pH of the rumen fluid, VFA absorption and rumen blood flow.

Using a washed, isolated rumen sac of an anaesthetized sheep, Ash and Dobson (1963) found there was a two-way exchange of CO_2 gas across the rumen epithelium. However, they could not calculate the amounts of CO_2 exchanged. Hoernicke et al (1965) found that up to 86% of CO_2 gas produced in the rumen was absorbed. The amount of CO_2 gas absorbed depends upon CO_2 tension in the rumen (Kay, 1963). Usually, CO_2 gas tension in the rumen is ten times that of blood so this gas tends to diffuse into the blood. Ash and Dobson (1963) found that the exchange of CO_2 gas between rumen and blood was independent of the CO_2 concentration gradient but was influenced by the absorption of VFA anions. Hoernicke et al (1965) found the amount of CO_2 gas absorbed depended partially on the rate at which eructation occured. If CO_2 production is increased and eructation rapid, the gas has less time to diffuse into the blood.

Table 2.1 (adapted from Hoernicke et al, 1965) shows the amount of CO_2 absorbed on different diets and at different times after feeding in two Jersey cows. After feeding, the CO_2 absorbed increases. The amount of CO_2 absorbed during grain feeding was smaller than during hay feeding in spite of the fact that these researchers found that the CO_2 concentration in eructed gas was higher during grain feeding.

The absorption of methane is also shown in Table 2.1. Hoernicke et al (1965) found that after feeding, 23 and 20% of methane produced on hay and grain diets respectively, was absorbed from a total production of 3.90 and 6.31 l/hr on these diets. Murray et al (1976) found mean rumen methane production in sheep fed 800 g lucerne chaff in 24 equal portions/d to be 18.0 ml/min (1.08 l/hr). Of this, 95% of methane was eructated leaving only 5% (0.054 l/hr) to be absorbed compared with 0.54 to 1.15 l/hr found by Hoernicke et al (1965) (see Table 2.1).

Table 2.1: Mean CO_2 and CH_4 absorption from the rumen of 2 cows on hay and grain diets (adapted from Hoernicke et al, 1965).

Feed	CO_2	CO_2	CH_4	CH_4
	No.one hourly collection periods	CO_2 absorbed l/hr	No.one hourly collection periods	CH_4 absorbed l/hr
before feeding				
-hay	5	12.6	12	0.54
grain	6	5.5	14	0.95
1-6 hr after feeding				
-hay	33	15.4	33	0.75
-grain	25	11.5	42	1.15
10-15 hours after feeding				
-hay	18	5.8	50	0.96

Hoernicke et al (1965) gave little consideration to hind gut methane production. They did however comment that methane was detected in expired air which was prevented from mixing with eructed gas. They detected levels of 0.022 and 0.063% (0.4 to 2.0 l/hr) methane in the expired gas. Murray et al (1978) found hind gut fermentation to be about 10% that in the rumen in sheep fed lucerne chaff. Calculations on their measured methane production data show post-ruminal methane production to be between 8.3 and 14.5% of that of the rumen which is a significant contribution to total methane production.

After feeding, the amount of methane absorbed increases but not in proportion to the methane production. With an increasing concentration gradient between methane in the rumen and that in the blood, more methane is absorbed. Absorption of methane is independent of CO_2 absorption.

About half the CO_2 entering the omasum is absorbed (Englehardt and Hauffe, 1974). It may be absorbed in the form of bicarbonate. CO_2 is thought to be absorbed to allow more rapid acidification of gut contents once the digesta reaches the abomasum without CO_2 or bicarbonates in solution being converted to gaseous CO_2.

The CO_2 decreases in concentration as sampling site within the omasum approaches the abomasum (Figure 2.4).

Carbon dioxide absorbed into the blood is excreted via the lungs (Kay, 1963). It may also be converted to bicarbonate and secreted in the saliva and re-enter the rumen via the saliva as bicarbonate.

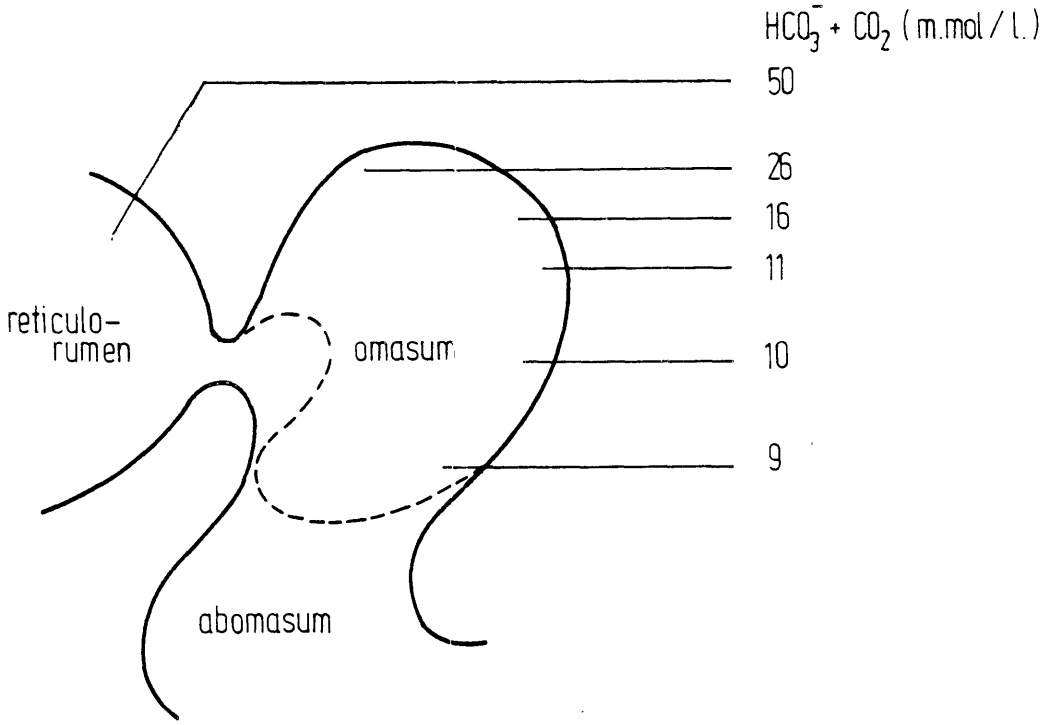
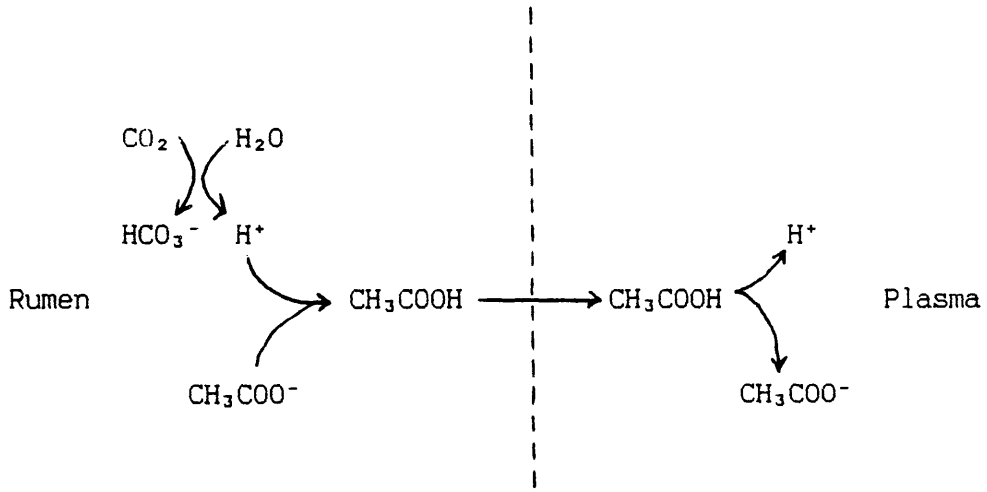


Figure 2.4: Concentration of total CO_2 in omasal contents of slaughtered cows (Ekman and Sperber cited by Englhardt and Hauffe, 1974).

2.4.3 Metabolic pathways

It has been shown that certain rumen microorganisms have the capacity to metabolise CO_2 with CH_4 as one of the products (Virtanen, 1946). Carbon dioxide may also be fixed by succinogenic bacteria (Hungate, 1966, p 343). Using $^{14}CO_2$, van Campen and Matrone (1960) found the major role of CO_2 was as a precursor of propionate and other acids with odd numbers of carbon numbers. The reaction occured through succinate, as a precursor of propionate.

Carbon dioxide is also important in the absorption of VFA through the rumen epithelium. Ash and Dobson (1963) found half of the acetate in the rumen was absorbed in the unionized state at neutral pH thus:



The unionized acetate moves into the plasma down the concentration gradient.

2.5 SALIVA

The release of CO_2 from saliva makes the study of salivary composition and rate of production important. The ruminant also relies upon saliva as a buffering agent against the often excessive acid production in the rumen.

2.5.1 Salivary glands

Figure 2.5 shows the location of the salivary glands and Table 2.2 gives the weights of each gland. The parotid and submaxillary glands make up approximately 70% by weight of salivary tissue.

Table 2.2: The mean weights of salivary glands (g) and body weight (kg) in the sheep and calf (Kay, 1960).

	Sheep	Calf
Mean body weght (kg)	43	102
Both parotid (g)	23.5	65.3
Both submaxillary (g)	18.2	64.0
Both inferior molar (g)	5.9	13.5
Both sublingual (g)	1.3	11.3
Both buccal (g)	6.0	13.1
Labial (g)	10.9	8.9

2.5.2 Salivary characteristics

A summary of the characteristics of the salivary glands of sheep is given in Table 2.3 (Kay, 1960). The parotid secretes rapidly and continuously. The submaxillary secretes about one eighth the volume of the parotid, most of which is secreted during feeding. Due to lower concentrations of Na^+, HCO_3^- and HPO_4^{2-}, submaxillary saliva has a lower buffering capacity than parotid saliva.

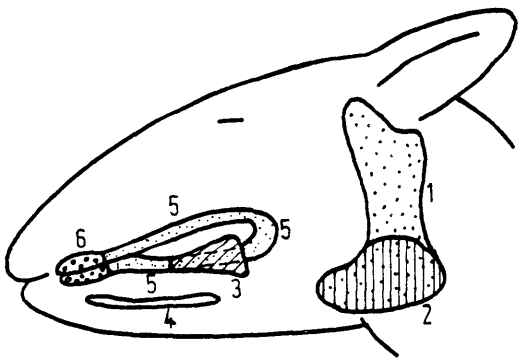


Figure 2.5: Location and relative size of salivary glands in the sheep. 1 = parotid, 2 = submaxillary, 3 = inferior molar, 4 = sublingual, 5 = buccal, 6 = labial. (Kay, 1960).

2.5.3 Composition

The compostition of mixed saliva quoted by McDougall (1948) is given in Table 2.4. This gives the composition of saliva actually reaching the reticulo-rumen. The 'CO_2' content includes bicarbonate which is largely from the parotid gland.

Table 2.3: Summary of characteristics of the salivary glands of sheep (Kay, 1960).

Glands	Cell	Factors governing rate of flow	Flow rate (l/24 hr)	Saliva type
Parotid	Serous	Stim. by mouth, oesoph. & reticulo-rumen.	3-8	Fluid & isotonic. Well buffered.
Inferior molar	Serous	Stim. by mouth, oesoph. & reticulo-rumen.	0.7-2.0	Fluid & isotonic. Well buffered.
Buccal	Mucous	Stim. by mouth, oesoph. & reticulo-rumen	2-6	Mucous, isotonic. Well buffered.
Submaxillary	Mixed	Stim. by feeding.	0.4-0.8	Hypotonic. Weakly buffered.
Sublingual	Mixed	Cont. flow without stimulation.	0.1 (?)	Mucous & hypotonic. Weak buffer.
Labial	Mixed	Small cont. flow.	?	Mucous & hypotonic. Weak buffer.

Table 2.4: Mean composition of mixed saliva for 4 sheep (McDougall 1948).

Content in g/100 ml		Content in mg/100 ml				CO_2 ml/100 ml
DM	Ash	Na^+	K^+	Ca^{2+}	Mg^{2+}	
1.0-1.4	0.7-0.9	370-462	16-46	1.6-3.0	0.6-1.0	117-283

Table 2.5 expresses the composition of saliva from various glands. Composition must be considered in conjunction with flow rate. The parotid gland secretes at a higher rate than other glands, delivering most of the salivary bicarbonate to the rumen. Production rates are discussed in more detail in Section 2.5.4.

The composition of saliva depends upon the rate of flow. Coats and Wright (1957) found that the electrolyte composition of saliva varied with time of secretion following stimulation. After stimulation, the concentration of Na^+, K^+, chloride, bicarbonate and phosphate showed continuous variation for 1-2 min after which a relationship developed between ionic composition and rate of production. Increasing salivary flow was associated with an increased concentration of Na^+ and bicarbonate and a decrease in the concentration of K^+ and phosphate. There was no relationship found between flow rate and chloride composition. Kay (1960) found similar results which are shown in Figure 2.6. Manas-Almendros et al (1982) showed that the level of phosphate in saliva was related linearly to acutely induced changes in blood phosphate concentration.

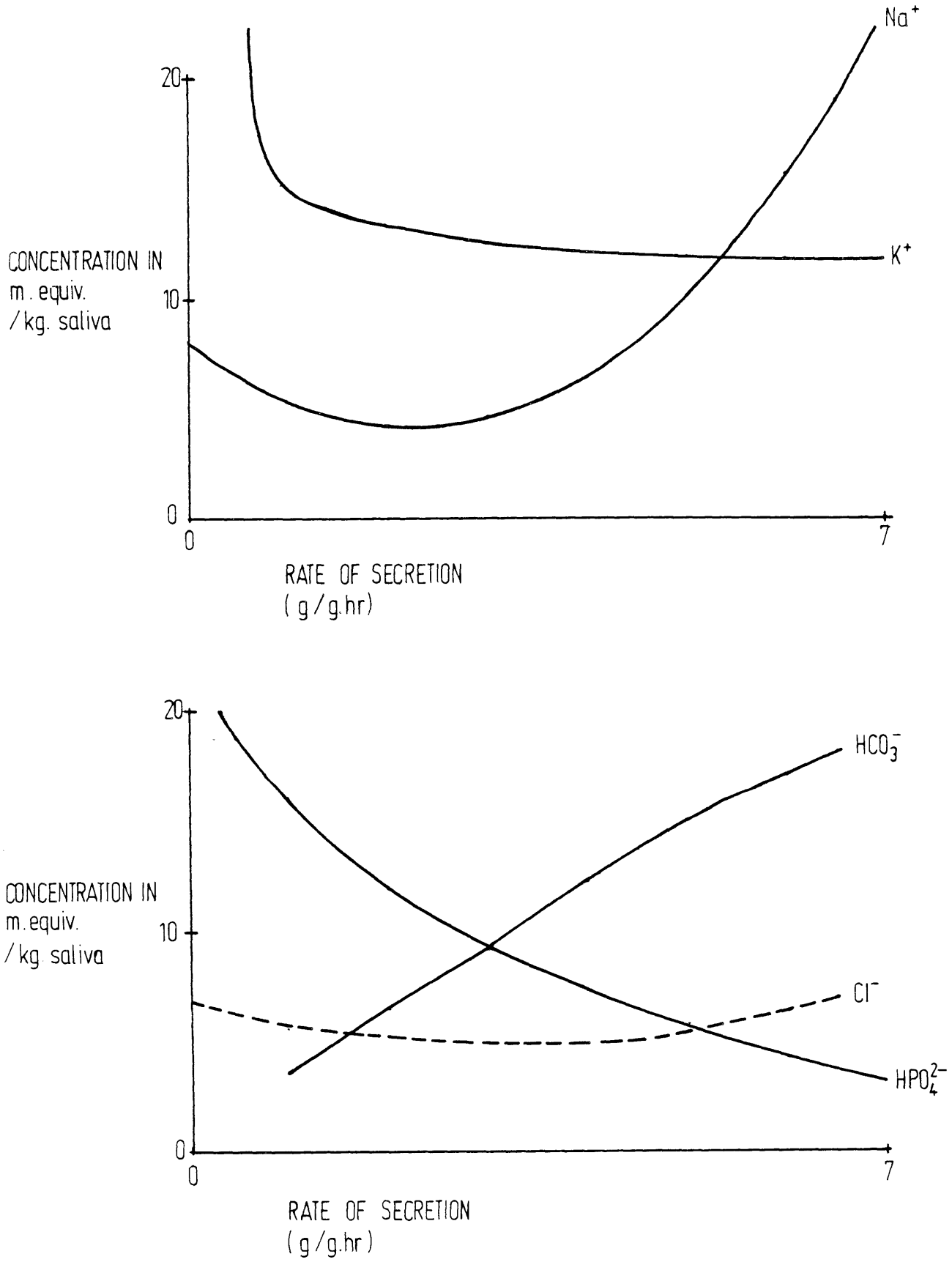


Figure 2.6: Variation in the composition of saliva with time after stimulation, g/g.hr = grams of saliva secreted per gram of salivary gland tissue in one hour (Kay, 1960).

2.5.4 Saliva secretion rate

Large variations exist in the reported values in the literature on the daily secretion rates of saliva in ruminants. McDougall (1948) found a daily secretion rate of saliva of 1310 ml/d as a mean of six sheep. This is much lower than some of the other published results: Kay (1960), 6-16 l and McManus (1961), 8 l. Cattle produce much more saliva: 100 l/d (Mendal and Boda, 1961), 150+ l/d (Church, 1976, p 53-55) and 25-190 l/d (Bailey,1961).

Table 2.5: Composition of saliva from various sources expressed in m.equiv/l.

	Na^+	K^+	HCO_3^-	HPO_4^{2-}	Cl^-	
Submaxillary						
-sheep	15	26	6	54	6	Kay & Phillipson (1959)
-calf	18	33	6	1.5	9	Kay & Phillipson (1959)
Sublingual						
-sheep	30	11	12	0.9	28	Kay & Phillipson (1959)
Labial						
-sheep	39	6	3	5	34	Kay & Phillipson (1959)
Parotid[a,b]	165.5	10	91	32	25	Kay (1960)
Inf. Molar[a,b]	153.5	12	86	36	16.5	Kay (1960)
Residual[a,b]	163.5	10	70.5	27	52.5	Kay (1960)
Submaxillary[b]	9.0	16	9.0	5.0	11.0	Kay (1960)

a = mean of two animals used.
b = anaesthetized sheep used.

Variations occur due to animal difference and experimental conditions. Some researchers collect saliva by parotid cannula which omits saliva from other glands. Oesophageal fistulas have been used

to collect mixed saliva (McManus, 1961) and McDougall (1948) used a sponge strapped into the mouth of the sheep.

McManus (1961) found that collection was affected by the use of anaesthetics. In a comparison of anaesthetised and conscious sheep, he found that anaesthetised sheep produced a mean 3.64 l of saliva per day whilst conscious sheep produced a mean of 6.31 l/d. In a similar study (Wilson, 1964) examined the quantity of saliva secreted from the parotid salivary glands of both anaesthetised and conscious sheep. He found a daily secretion rate of 16.56 g/d for anaesthetised sheep (mean of two sheep). Problems arose with the cannulae of conscious sheep frequently blocking, however, an approximate value of 4.84 l/d of parotid saliva was secreted by conscious sheep. Meyer et al (1964) showed that saliva flow rate varies in relation to time of feeding and feed types. In cattle, Meyer et al (1964) obtained a resting flow rate of 81 g/min from 2 to 14 hr after feeding. This gives a total daily secretion rate of 117 to 183 kg/animal/d.

The volume of saliva varies with the type of diet. Meyer et al (1964) found that for each kg of dry matter of freshly cut lucerne, lucerne pasture and lucerne hay consumed, cattle secreted 2.90, 3.30 and 3.25 kg saliva respectively. Yarns et al (1965) found an increase in saliva produced when animals were changed from a lucerne diet to a ration of Bermuda grass and corn. The more abrasive action of Bermuda grass in the rumen may have been a contributing factor.

For grass and hay diets, Bailey (1966) attempted to correlate rate of swallowing and saliva production. He found that measurements of rate of swallowing could be used to detect the presence of significant changes in secretion rate.

2.5.5 Salivary bicarbonate

In ruminants, bicarbonate is present in sufficient concentrations in saliva to contribute significantly to the total osmolality and buffer capacity of the secretions. The two sources of bicarbonate for saliva are plasma and gland cells. In the metabolism of

carbohydrates, gland cells produce bicarbonate. If the metabolic end product is CO_2, not bicarbonate, rapid formation of bicarbonate by the gland occurs under the action of carbonic anhydrase (Schneyer and Schneyer, 1967):

$$CO_2 + H_2O \xrightarrow[\text{anhydrase}]{\text{carbonic}} H_2CO_3 \quad H^+ + HCO_3^-$$

Coats et al (1958) stimulated the parasympathetic nerve (Moussu's nerve) of anaesthetised sheep with a platinum cathode and found a negative glandular balance of bicarbonate with a rise in salivary bicarbonate during the first 30 seconds of stimulation. This supports glandular contribution to salivary bicarbonate.

Calculations that are derived from gland metabolism show that glands could not provide all the bicarbonate present in saliva. The plasma is also responsible for salivary bicarbonate.

Using carbonic anhydrase inhibitor, Blair-West et al (1980) considered the possible pathways for movement of bicarbonate from blood into saliva. These pathways are shown in Figure 2.7.

Calculations in the appendix of their paper show that 90% of salivary bicarbonate comes via the direct route (i) with 10% of the transfer via molecular CO_2.

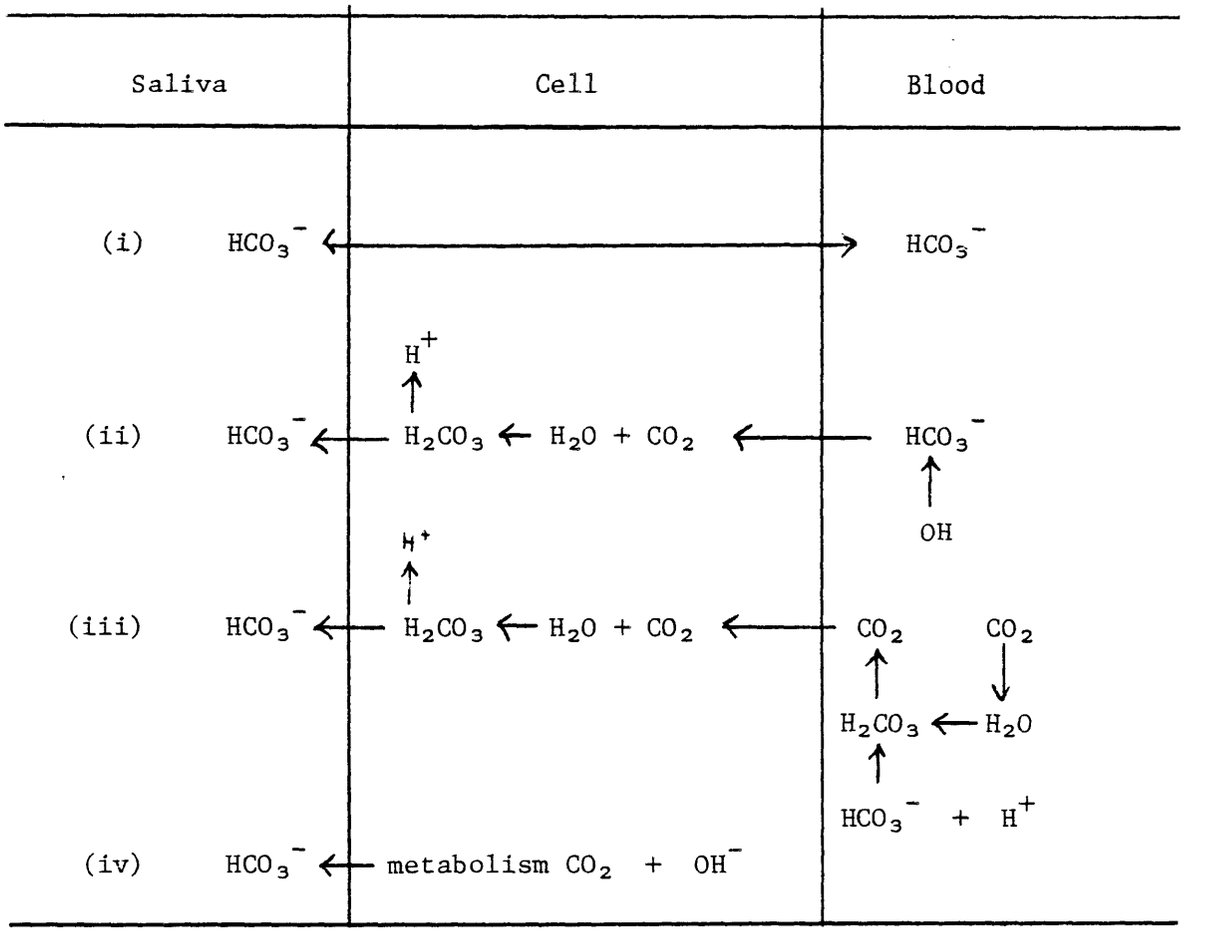


Figure 2.7 : The pathways for movement of bicarbonate from blood into saliva (Blair-West et al, 1980). Arrows indicate movement between blood and saliva.

2.6 BUFFERING IN THE RUMEN

Due to the sensitivity to pH of the rumen microbiota, the rumen needs to be buffered to protect the microflora from rapid changes in pH. The amount and composition of parotid saliva is the most important factor affecting the buffering system of the rumen contents (Kay, 1960; Bailey, 1961; Herod et al, 1978). Other factors are the concentration and rate of absorption of VFA and CO_2, rate of ingestion and fermentation of readily available carbohydrates, rate of passage of food through the alimentary tract and the level of dietary buffers.

Rumen fluid can hold an appreciable amount of bicarbonate in solution. Cole et al (1945) estimated that the total CO_2 in aqueous solution under pH and gaseous conditions similar to the rumen lies between 21.2 and 59.5 mmol/l. Experimentally obtained results with rumen fluid lie within this range (Turner and Hodgetts, 1955a; Emmanuel et al, 1969). Variations in bicarbonate concentration depend on quantity and type of feed, pH of the rumen fluid, intra-ruminal partial pressure of CO_2 (pCO_2) and the method of sampling of the rumen contents (Emmanuel et al, 1969).

Turner and Hodgetts (1955b) studied concentration of bicarbonate in the rumen of sheep on three diets: equal parts of oaten chaff and wheaten chaff (E), equal parts of oaten chaff and lucerne chaff (H) and rich lucerne and ryegrass pasture (F). They obtained bicarbonate concentrations of 19 (E), 27 (H) and 4 (F) m.equiv/l. Sheep on diets H and F which contained much lucerne had rumen contents that were better buffered than sheep fed cereal chaffs (E).

The concentration of CO_2 in rumen fluid also depends on the pCO_2. As the amount of CO_2 gas in the rumen declines, less CO_2 is held in the rumen fluid and there is an exponential decline in pCO_2 concommitant with an increase in pH (Turner and Hodgetts, 1955a). Turner and Hodgetts (1955a) also found that at each pCO_2 level examined, the higher the pH, the higher the concentration of bicarbonate.

Compared to other physiological buffers, bicarbonate has a very good buffering ability. Turner and Hodgetts (1955b) showed the benefit of bicarbonate as a rumen buffer. They added VFA to distilled water to obtain concentrations similar to the VFA concentrations found by Phillipson (1942) for rumen fluid. Acetate at concentrations of 57 to 162 mmol/l gave a pH range of 3.03 to 2.78 without any buffer. They also found that removal of bicarbonate from rumen fluid decreased the buffering capacity by 66% compared with untreated rumen fluid.

Burton (1973) implies the excellent buffering ability of the bicarbonate buffering system in this equation:

$$\frac{-d\phi}{dpH} = B_a \left(1 + \frac{[H^+]}{(K'_1)}\right) + 2.303\,[CO_2]$$

where: ϕ = total CO_2 concentration
B_a = buffering capacity of all non-bicarbonate components.

A change in pH corresponding to a given small alteration in total CO_2 concentration i.e. $\frac{dpH}{d\phi}$ is mainly determined by B_a, the non-bicarbonate buffering capacity since $[CO_2]$ is small in rumen fluid. This implies that bicarbonate concentration can be altered but pH remains fairly stable if the buffering capacity of non-bicarbonate buffer components remains constant.

The ability to minimise pH changes is known as buffering capacity (B) and defined as the number of moles per litre of H^+ required to cause a given change in pH. Emmanuel et al (1969) looked at B values for pH ranges of 4 to 6, 5 to 7 and 6 to 8, and found that although bicarbonate is the most important buffer component, at the pH 4-6 range, VFA were effective buffers. Phosphate also contributed to the buffer system but it was found inadequate in effectively regulating rumen pH in the 6-8 region where it was expected to have most effect.

Assuming VFA, bicarbonate and phosphate were the only important components of the buffering system, Counotte et al (1979) found it was possible to predict the buffering action of the rumen fluid under different feeding conditions.

Concentrates and ground roughage are used in most feedlot conditions. Associated with this feeding regime is a decrease in salivary secretion and lowering of rumen pH (Lawlor et al, 1966). This may result in reduced buffering capacity of the rumen contents and a reduced efficiency of utilization of the ration (Emmanuel et al, 1969). The research of Turner and Hodgetts (1955a,b) confirms that the rumen buffering system is made up of a number of systems. The titration curves of Turner and Hodgetts (1955b) are compounded from the one sinusoidal curve and are more effectively buffered towards the addition of acid than bases.

2.7 BUFFERING MATERIALS IN RUMINANT DIETS.

Feeding of high starch diets may lead to conditions in the rumen which are not optimal for fermentation. In general, a pH of 6.2-6.8 appears to be optimal for a high efficiency of microbial growth (Hespell, 1981). The addition of buffering materials to the diet is a

means by which conditions may be made more favorable for fermentation in the rumen.

A wide range of buffering materials have been examined. The potential of sodium-bentonite (Na-bentonite) buffer as a feed additive has been studied fairly extensively. It is used to improve physical consistency of pelleted feeds. As a clay of volcanic origin with very small particle size, the main mineral of Na-bentonite is montmorillonite. It also contains aluminium silicate and has up to 3% Na. Herod et al (1978) found it was of greatest value when allowed to break down to its molecular components after extended contact with rumen fluid, releasing Al_2O_3 and SiO_2 and sodium ions (Na^+).

There is dispute over the ability of buffering materials to stabilize rumen fluid pH. Generally, the inclusion of buffers in the diet leads to an increase in the pH of rumen fluid in lambs (Colling et al, 1979; Dunn et al, 1979) and cattle (Emery and Brown, 1981; Herod et al, 1978; Galyean and Chabot, 1981) or an increase in the buffering capacity of rumen fluid (Oltjen and Davis, 1965).

There is a range of effective buffers which have been examined. Na-bentonite has been shown to be useful in maintaining higher pH values for rumen fluid (Herod et al, 1978; Colling et al, 1979; Dunn et al, 1979; Galyean and Chabot, 1981). Galyean and Chabot (1981) fed 4 buffer types to steers maintained on an ad libitum cottonseed hull diet. These were McDougall's buffer salts, cement kiln dust, Na-bentonite and clinoptilolite. They found that over the first 3 hr after feeding, McDougall's buffer salts and Na-bentonite held the pH of rumen fluid significantly higher than the other buffer types and at 8 hr after feeding, McDougall's buffer salts maintained rumen fluid pH at a higher level than the other 3 buffer types. The increase in

rumen fluid pH found by Colling et al (1979) was thought to be due to an increase in feed intake in the animals given the Na-bentonite buffer and the trend for increased rumen fluid pH found by Herod et al (1978) was not significant.

Other buffers used in ruminant diets to increase the pH of rumen fluid are sodium bicarbonate and potassium bicarbonate (for example, Emery and Brown, 1961). Emery and Brown (1961) found that these buffers significantly increased the pH of rumen fluid in dairy cattle on a grain diet + buffer compared with cattle on the grain diet alone (pH 5.8 to pH 6.4 respectively).

The effect of buffers on other aspects of ruminant digestion has also been examined. Galyean and Chabot (1981) have found non-significant effects of buffers on osmolality, rumen fluid volume, dilution rate, flow rate or turnover time. They comment however that their dilution rate was high before treatment which does not allow much opportunity of increasing dilution rate with buffering materials.

The reported effects of buffering materials on intake is varied. Herod et al (1978) found a significant decrease in intake, Mendel (1971) and Galyean and Chabot (1981) found non-significant differences in intake and Wise et al (1965) found there was a significant increase in intake when buffering materials were included in the diet.

Wheeler and Noller (1977) examined pH in other portions of the gastrointestinal tract as well as the rumen. They found that on all concentrate diet, limestone and magnesium limestone significantly increased the pH of the rumen, small intestine, colon and faeces.

Ensminger and Gieseking (1942) in a comparison of bentonite and kaolinite *in vitro* found that bentonite, with the higher base-exchange capacity had a higher influence in retarding the hydrolysis of adsorbed protein by proteolytic enzymes. They attributed this to the fact that the protein molecule, when adsorbed, may be orientated such that active groups are inaccessable to the enzyme.

Britton *et al* (1978) complexed bentonite with soyabean meal and urea by adding (w/w) water to the mixture, then dried and ground it. With this complex, they conducted *in vitro* experiments and found that ammonia release from both nitrogen sources was reduced. The slow release of ammonia with the urea-bentonite complex could be useful for extending the time of ammonia production in the rumen. However, this did not prove successful in the animal. When ratios of soyabean meal to bentonite at 3.0:1 and 1.5:1 were compared, animals receiving the 3.0:1 mixture gave better results, thought to be due to greater complexing through the whole tract. Oltjen and Davis (1965) found that the addition of zinc to their buffer mixture gave lower ammonia concentrations than zinc alone. Galyean and Chabot (1981) found non-significant differences in rumen ammonia levels using their buffers.

The effect of dietary buffers on VFA concentration and molar proportions, and its effect on milk production have also been considered. Changes in the proportions of propionate and acetate leading to an increase in the acetate:propionate ratio have been found where buffers are fed (Thomas and Emery, 1969; Herod *et al*, 1978; Colling *et al*, 1979). Bringe and Schultz (1969) found that the inclusion of 5% Na-bentonite in a 3:1 pelleted grain-roughage diet fed to dairy cows led to an increase in the milk fat per cent. In further

studies, Rindsig et al (1969,1970) found an increase in the proportion of acetate in rumen fluid and concommitant increase in milk fat per cent in dairy cows fed 5 and 10% Na-bentonite. They also found a significant increase in milk production in cows fed 5 and 10% Na-bentonite (19.4 and 17.4 kg/cow/d respectively) compared to cows with no supplement (16.0 kg/cow/d). Emery and Brown (1961) found increased milk fat using sodium bicarbonate and potassium bicarbonate buffers which was not due, as they expected, to a decrease in molar percentage in propionate. They thought this may be due to an increase in VFA production which was absorbed too quickly and masked the effect of the buffers. Oltjen and Davis (1965) found a decrease in the acetate/propionate ratio with soyabean meal and buffer but an increase in the acetate/propionate ratio for urea + buffer. In both instances, there was non-significant differences in total VFA concentration. Galyean and Chabot (1981) found non-significant differences in total VFA concentration and molar proportions with their buffering materials.

The reported effects of the inclusion of buffering materials in diets on animal growth are varied although researchers generally find non-significant differences with the use of buffers on liveweight gain (Erwin et al, 1957; Mendel, 1971; Colling et al, 1979) and carcass weight and grade, conformation or dressing perentage (Huntington et al, 1977) or a small liveweight gain (Wise et al, 1965; Briton et al, 1978). Briton et al (1978) used Na-bentonite and the increased gain was thought to be due to better feed efficiency. Wheeler et al (1981) also found increased feed conversion using Na-bentonite but found a reduced average daily gain.

2.8 PROBLEMS ASSOCIATED WITH THE USE OF BUFFERS.

Some disadvantages with the use of buffering materials have been found. An increase in the incidence of bloat has been found when sodium bicarbonate has been included in the diet (Emery and Brown, 1961; Nicholson et al, 1963; Preston et al, 1963; Oltjen and Davis, 1965; Herod et al, 1978). The increase in bloat in cattle found by Herod et al (1978) was not related to the buffering material. Elam and Davis (1962) found that buffers did not appear to increase the incidence of feedlot bloat in steers.

Briggs and Fox (1956) have indicated that Na-bentonite may result in a deficiency in vitamin A. They found that additions of Na-bentonite to poultry diets could result in a destruction of vitamin A. They cite Laughlan and Phillips (1954) who found lower vitamin A stores and a change in liver size with Na-bentonite at 0.5 to 3.5% of the diet. In steers, Erwin et al (1957) found no significant effects of Na-bentonite on hepatic stores of vitamin A and carotene. They believed this may be because Na-bentonite would bind to other pigments which were present in fairly high amounts as lucerne made up a large part of the ration.

2.9 RELATIONSHIP OF RUMEN GAS, pH AND SALIVA WITH BLOAT.

In a bloating situation, one must consider the rate of gas production and degree of accumulation (Clarke and Reid, 1974). Gas is produced in large quantities in the rumen so when combined with foam stabilizing compounds, bloat can result. Foam stabilizers may originate from plant protein material or salivary mucoprotein (Clarke, 1965a; Clarke, 1965b) or as has been more recently been suggested, from protozoa (Leng, 1973). The bacterial polysaccharide glycocalyx

which bacteria use for binding into microcolonies or attachment to surfaces may also act as a foam stabilizing material (Costerton et al, 1978; Cheng and Costerton, 1979). The release of plant foam stabilizing materials involves the penetration and progressive digestion of plant tissue by bacteria so that the rate of release of soluble nutrients and plant foam stabilizers, depends not only on the chemical composition of the plant material, but also its accessibility to colonization by adherent bacteria (Cheng and Costerton, 1979). After observing gas production by Ophryoscolex spp. and other protozoa during feedlot bloat, Kodas (1966) suggests they may contribute to bloat. Jones and Lyttleton (1972) carried out in vitro research on rumen fluid from non-lactating cows and found that soluble protein isolated from protozoa produced rigid foams at pH 5.5 to 6.5 with maximum persistance at pH 5.9 which is close to the optimal pH for maximum foam strength of rumen and plant proteins (Mangan, 1959). At this pH, fermentation has probably been occuring rapidly so gas production would be considerable. They also found that saliva foams were unaffected by pH in the range 4.0-7.0 and although persistant, were not as rigid as leaf foams.

Buckingham (1970) showed that the stability of foams produced from red clover protein were affected by pH and temperature. Maximum strength occured at pH of 5.5 and increased rapidly between 40°C and 37°C as temperature decreased. Normal rumen fluid is at a temperature of 39°C and he suggests that the ingestion of cold water may decrease rumen pH sufficiently to give more rigid foams.

When looking at the relationship between bloat and pH, no significant relationships have been found between severity of bloat (McArthur and Miltimore, 1969) and incidence of bloat (Mendel and

Boda, 1961). Using saliva salts, Elam and Davis (1962) found an increase in rumen pH unrelated to bloat. Mendel and Boda (1961) suggest that there is an upset in the buffering capacity of the rumen during bloat because they found that highly susceptible cattle secreted significantly less saliva of higher bicarbonate concentration than low susceptible cattle.

McArthur and Miltimore (1969), investigating the role of rumen pH in pasture bloat during the spring of 1960 and 1961, found no relationship between the severity of bloat and pH. Although the change in rumen fluid pH with time was the same in both years of the experiment, the pH was lower in 1960 with more than 5X greater incidence of bloat in that year. They suggest this is related to foam stability. Foam stability was greatest at rumen pH close to the region of the iso-electric point for 18S protein which has been implicated as the major plant protein foam stabilizing agent. At this pH, foam acts as a mechanical block to normal eructation. In studies on bloat in cattle grazing alfalfa pastures, Blake et al (1957) found pH was lower during bloating periods (6.74) than non-bloating periods (7.09). The administration of detergents to bloating animals has been used to control bloat. It has been found that detergents increase the rumen fluid pH.

A relationship has been found between foam production and methane pool size (Bryant et al, 1973). Using a single injection of tritium labelled methane, it was found that sheep with larger pool size and longest half life suffered most from bloat whilst sheep with smaller pool size and half life had little or no foam. The administration of 3 ml of anti-bloat oil in 30 ml of water to control bloat resulted in a decrease in methane pool size from 1350 ml to 169 ml and a decrease

in the methane half-time from 84 to 14 min. Methane pool size could possibly be used as a gauge of bloat susceptibility.

2.10 LOSS OF BICARBONATE IN FAECES AND URINE.

The few studies that have been carried out on faecal bicarbonate transport suggest that HCO_3^- is secreted from the colon (Johnson, 1981). Thus the concentration of HCO_3^- in faecal water exceeds that in the plasma resulting in alkali faecal contents. Although no figures could be sourced for ruminants, the total CO_2 "equilibration constant" for dogs has been found to be 75 mM (Johnson, 1981).

It has been postulated that the mechanism for HCO_3^- secretion into the colon is a carrier-mediated anion exchange process. This involves the oppositely directed flows of chlorine and HCO_3^- (Johnson, 1981).

The kidney aids in regulating the pH of blood. It does this through the control of extracellular concentrations of HCO_3^- which in turn serves to maintain a constant plasma concentration of HCO_3^-. A fall in extracellular pH due to increased alveolar CO_2 or decreased HCO_3^- concentration is counteracted by the kidney through the excretion of acidic urine and ammonium ions. When there is a tendency toward alkaline extracellular fluid, the kidney excretes Na^+, HCO_3^- and the dissociated forms of other weak acids (White et al, 1959, p695).

Since the residual ash of diets tends to be acidic, HCO_3^- in the renal venous plasma is utilized in the acidification of urine. This aids in stabilizing blood pH.

Renal compensation for situations which would otherwise result in a rise in extracellular pH is accomplished by lowering the concentration of HCO_3^- of extracellular fluid (White et al, 1959, p698). This is possible by the excretion of Na^+ in association with HCO_3^-. This lowers the extracellular fluid concentration of HCO_3^-.

The pH of ruminant urine tends to be alkaline on dry forage diets which are high in Na and K ions although the urine is apt to be slightly acidic for other types of diet (Church, 1976, p118).

CHAPTER 3

GENERAL MATERIALS AND METHODS

3.1 GENERAL

An overview of the materials and methods used in the studies are presented here. Where differences in approach occur between experiments, these are discussed in the appropriate place with any additional procedures involved in the specific experiment.

3.2 ANIMALS

Mature cross-bred Merino wethers of approximately 35 kg live weight were used. Sheep were housed in individual metabolism crates and the room was continuously illuminated. Internal parasites were controlled by regular drenching with anthelmintics (Nilverm, I.C.I., Melbourne, Australia; Razinole, Merck, Sharp and Dohme Aust. Pty. Ltd., Australia; Panacur, Hoeschst Aust. Ltd., Australia).

Diets are described separately for each experiment. Overhead, continuous belt feeders were used in experiments where sheep were fed hourly. Fresh water was available at all times.

Animals were fitted with rumen cannulae (Hecker, 1969) and had had a recovery period of at least two months following surgery. A three pronged probe, permanently positioned in the rumen cannula was used to obtain rumen fluid (probe 1) and gas samples (probe 2) and for infusing solutions (probe 3) into the rumen (Figure 3.1). The sampling probes were wrapped in several layers of nylon stocking to filter out particulate matter.

During experiments where blood samples were being taken, jugular catheters were inserted into one or both jugular veins 12 hr before sampling commenced. This was done by inserting a 13 guage needle into the jugular vein through which a polyethylene catheter (I.D. 1.00 mm; Dural Plastics, Australia) was passed. Catheters were then held in place with surgical tape. Catheters were maintained patent with a sterile heparin-saline solution (9g NaCl/l, 100 i.u. heparin).

3.3 INFUSION SOLUTION TECHNIQUES

Sodium (^{14}C) bicarbonate (2.0 mCi/ml) was obtained from Amersham International Ltd., England. The $NaH^{14}CO_3$ was washed from the ampoule using distilled water which contained approximately 1 mg/ml of sodium bicarbonate carrier and had been made slightly alkali with NaOH. This was stored at -20°C and used as a stock solution from which infusion solutions were prepared.

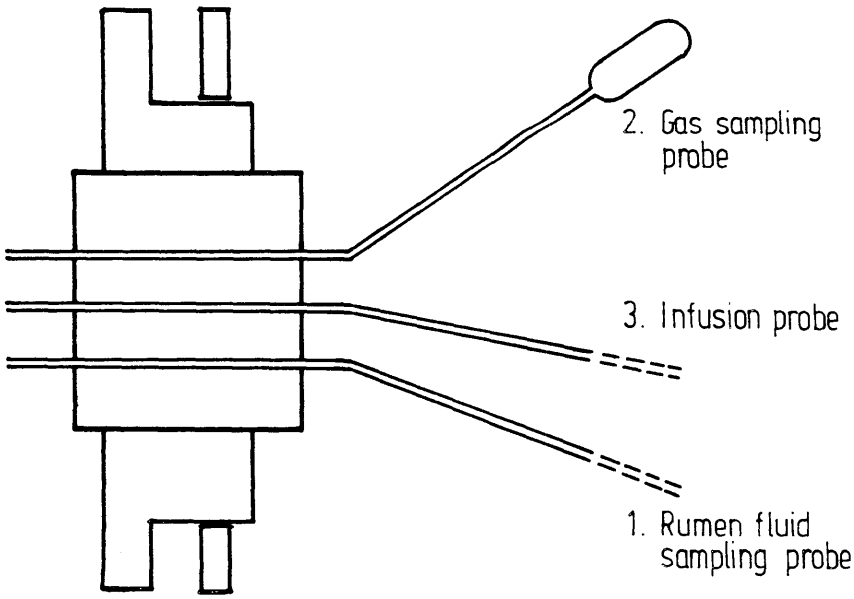


Figure 3.1: Rumen bung with two sampling probes and infusion probe inserted.

$NaH^{14}CO_3$ infusion solutions for intraruminal infusions were prepared with an activity of approximately 800 nCi/ml. Solutions for intravenous infusions were prepared with sterile saline (Travenol Laboratories Pty. Ltd., Sydney, Australia) to give an activity of 2.5 uCi/ml.

Continuous infusions were made using AutoAnalyser type pumps and pump tubing (intraruminal infusion: yellow/yellow (1.4 mm I.D.); intravenous infusion: black/black (0.75 mm I.D.). The rates of infusion of these solutions or amounts injected are given in the chapters dealing with experimental research.

In all studies a subsample of infusion solution was taken during the experiment for determination of radioactivity. Approximately 1.2 g $NaHCO_3$ carrier plus 1.00 ml of infusion solution made to 100 ml with double distilled water which had been made slightly alkali was used for estimation of SR. Three ml of the sample was placed in a McCartney bottle and treated as a rumen fluid or blood sample to isolate ^{14}C-carbon dioxide and determination of the radioactivity of $BaCO_3$ (Sections 3.4.3, 3.4.4 and 3.4.5). Infusion rate of radioactivity (nCi/min) was then calculated as shown in Appendix C.

Single injections of chromium ethylene diaminetetra-acetic acid (Cr-EDTA) were made using a 50 ml syringe of Cr-EDTA which was weighed before and after injection of the required volume into the rumen. The injection probe was flushed with water following injection.

Commercially available rubber bags were found inadequate for holding CO_2 for periods in excess of 24 hr. To avoid leakage of $^{14}CO_2$ into the atmosphere, $^{14}CO_2$ was prepared and stored in plastic lined, aluminium bags (winecask bladders; A.C.I. Liquid Containers,

Melbourne, Australia).

To a four litre bag, 15 g Na_2CO_3 carrier was added along with 5 ml $NaH^{14}CO_3$ (approximately 250 uCi/ml) and the bag was sealed with a rubber bung. To release all the CO_2 from these compounds, 30-35 ml of 10 N H_2SO_4 was injected slowly into the bag. The volume of the CO_2 approximated 3 l. After removal of excess liquid, the gas was infused directly from the bag into the animal using a peristaltic pump.

The activity of the gas infused was determined by taking a sample of the gas into 1 N NaOH and preparing it as for rumen CO_2 gas samples (Section 3.4.6.1). After correction to STP, the $^{14}CO_2$ (g) infused into the animal could be determined (nCi/min) (Appendix C).

Radioactive CH_4 for infusion was prepared by shaking 10 ml CH_4 (at STP) with distilled water that had been degassed by boiling vigourously for 10 min. The solution was then removed into a plastic bottle which was stoppered so that no air remained in the bottle. This was allowed to cool. A syringe fitted with an 18 guage needle was plunged through the stopper to allow equilization of pressure as 10 ml (1 mCi) of $^{14}CH_4$ (Amersham International Ltd., England) was injected. This was storred at 4°C and with occasional agitation, the $^{14}CH_4$ dissolved in about 24 hr. This gave an activity of approximately 333 nCi/ml.

The solution was transferred to 350 ml gas tight metal syringes which were fitted onto a Palmer Injection Pump (Palmer Ltd., England). The $^{14}CH_4$ solution was infused at a rate of 0.10-0.12 ml/min with approximately 0.8 ml/min (yellow/yellow (1.4 mm I.D.) AutoAnalyser tubing) of water through copper tubing (3mm I.D.) into the rumen fluid using a peristaltic pump.

3.4 SAMPLING TECHNIQUES

3.4.1 Measurement of pH

With minimum pressure, approximately 25 ml of rumen fluid was withdrawn for pH measurement (pH meter, Model 292 Mk 2, Pye UniCam Ltd., Cambridge, England) and a combined electrode (Radiometer Electrodes, Copenhagen, Denmark).

3.4.2 Protozoa and VFA samples

Using minimum pressure, 20-25 ml rumen fluid was taken into a disposable syringe after flushing 3 or 4 times. Five ml of this sample was transferred to a McCartney bottle containing 20 ml of formal saline (0.9% (w/v) NaCl in a 10% (w/v) solution of 40% formaldehyde). Samples for protozoa counts were taken using a pasteur pipette whilst mixing and placed into a counting chamber (0.2 ml depth, 9.6 mm^3 volume) (Hawksley, Sussex, England). At least 200 protozoa were counted and the number of squares noted. Using the equation:

$$\text{No. protozoa/ml in undiluted rumen fluid} = \frac{\text{No. counted X 5 X 1000}}{\text{0.2 X No. squares}}$$

the number of protozoa per ml of undiluted rumen fluid was calculated. Protozoa were divided into two groups: small protozoa which were mainly Entodinium spp. (approximately 20-40 um in length) and large protozoa, mainly Epidinium spp. (approximately 108-140 um in length) (Clarke et al, 1982).

The remaining 15 ml rumen fluid was transferred to a McCartney bottle containing 3 drops of concentrated (36 N) H_2SO_4 and shaken gently, then stored at -20°C. These were thawed and centrifuged at

3000 g for 10 minutes. Supernatent samples were then analysed for total VFA and proportions using a gas liquid chromatograph (Model 427, Packard Instrument Company, Illinois, U.S.A.) with an isocaproic internal standard and recording data processor (Model 604, Packard Instrument Company, Illinois, U.S.A.).

3.4.3 Specific radioactivity of rumen bicarbonate

After flushing 3 or 4 times, a 5 ml sample of rumen fluid was withdrawn from the rumen probe into a clean disposable syringe. This was transferred to a McCartney bottle containing a glass vial. One ml of 1 N CO_2-free NaOH was withdrawn from a dispenser which used soda lime (Carbosorb) to maintain a CO_2-free gas space.

The NaOH was transferred to the glass vial and the McCartney bottle sealed tightly. Approximately 1 ml of 1 N H_2SO_4 was injected through a small hole in the cap into the rumen fluid and swirled to facilitate mixing and liberation of CO_2.

After a period of at least 12 and not more than 18 hr, the glass vial was removed and the contents transferred to a small bijou bottle. The carbonate was then precipitated in the form of $BaCO_3$ using 1 ml 5% (w/v) NH_4Cl and 0.4 ml 20% (w/v) $BaCl_2$. The bijou was sealed and shaken.

3.4.4 Preparation of $BaCO_3$ for counting

After a period of at least 30 min, samples for B-radiation (^{14}C) determination were filtered and plated using a graduated funnel (Millipore Corporation, Bedford, Massachusetts, U.S.A.) fitted with a Watman 542 filter paper. The sample was washed twice with double

distilled water and twice with acetone. The planchet was removed and the dry sample of $BaCO_3$ transferred to a pre-weighed scintillation vial (Packard Instrument Company, Illinois, U.S.A.). After drying, the weight of $BaCO_3$ was calculated and the vial sealed.

The sample of $BaCO_3$ was powdered using a Vortex Super Mixer (Lab-line Instruments Inc., U.S.A.). This was achieved by introducing two glass beads (approximately 2 mm diameter) into the vial and mixing. Ten ml scintillation cocktail (xylene containing 3.5% (w/v) Cab-o-sil (Godfrey L. Cabot Inc., U.S.A.), 0.01% (w/v) POPOP and 0.4% (w/v) PPO) was added (Leng and Leonard, 1965).

The $BaCO_3$ was suspended using a rotary stirrer (Dumax, Betts and Co., U.S.A.) and counted in a liquid scintillation counter (Packard Tri-carb, Model 3255, Packard Instrument Company, Illinois, U.S.A.).

3.4.5 Specific radioactivity of blood bicarbonate

Animals were prepared with catheters in jugular veins (Section 3.2). A 5 ml blood sample was taken into a clean disposable syringe and transferred into a McCartney bottle containing a glass vial and treated as for rumen fluid bicarbonate SR (Section 3.4.3).

3.4.6 Gas samples

Rumen gas samples were taken using the probe in Figure 3.1. A sample was withdrawn into a 50 ml glass syringe (Eterna-matic, Santex, Italy) fitted with two-way valve (Figure 3.2). The sample was passed via a moisture trap into a clean disposable syringe.

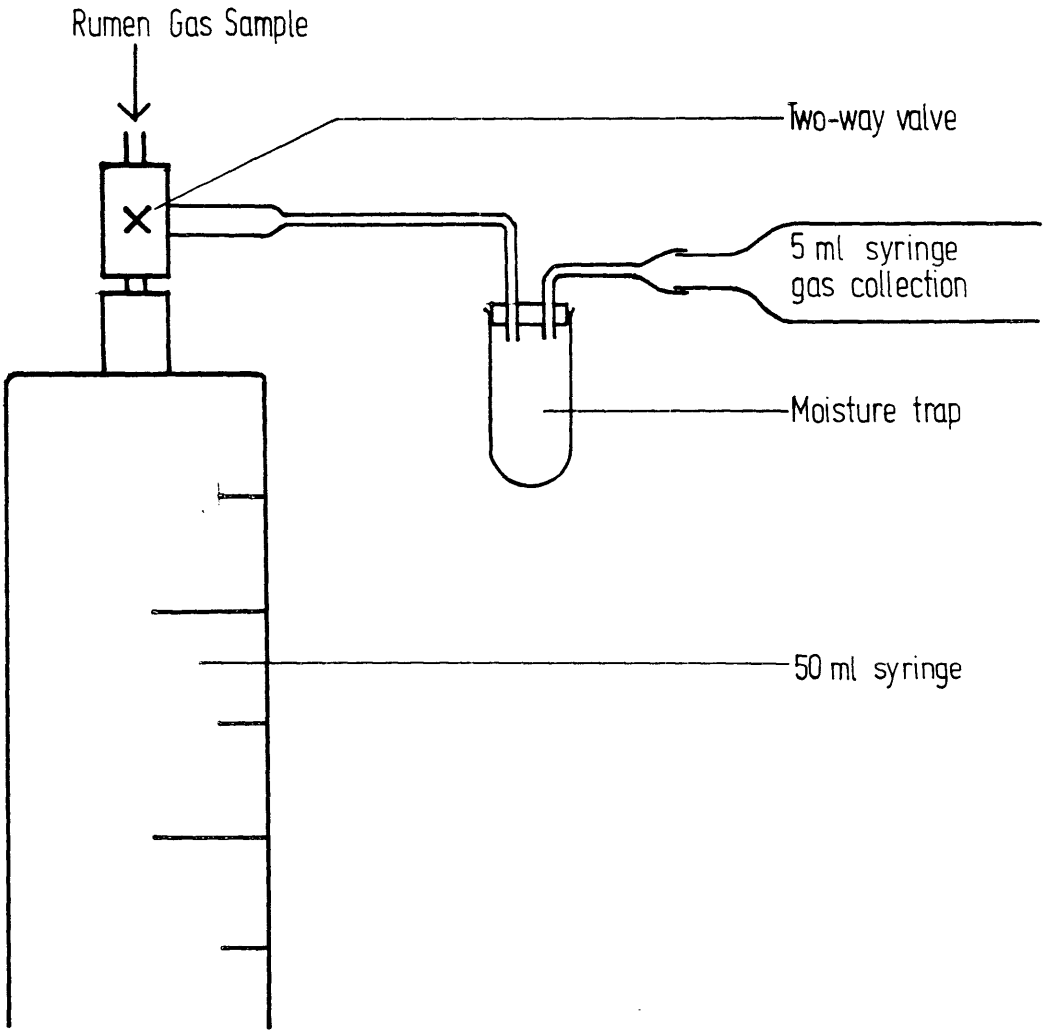


Figure 3.2: Glass syringe used to withdraw gas samples.

3.4.6.1 Gas samples for specific radioactivity -

A 25 ml sample of rumen gas was taken. A 3 ml aliquot of 1 N CO_2-free NaOH was introduced into the syringe via a length of plastic tubing and 18 **gauge** needle. Upon shaking, the plunger was drawn in due to the lowered pressure as CO_2 was absorbed into the NaOH. The liquid portion was then transferred into a small bijou bottle and 2 ml 5% (w/v) NH_4Cl was added and $BaCO_3$ was precipitated with 0.8 ml 20% (w/v) $BaCl_2$. The bijou was sealed and shaken. This was then treated as in Section 3.4.4.

The remaining gaseous portion (largely methane) was oxidized to CO_2 using the apparatus in Figure 3.3. The syringe was fitted with a 26 **gauge** needle and the gas sample was injected into the oxidizer through the rubber sceptum. CH_4 was then oxidized to CO_2 in the oxidizer with a copper oxide catalyst. 1 N NaOH was pumped into the stream of gas leaving the oxidizer and CO_2 was dissolved in the NaOH. This solution was passed through a solution of 5 ml 5% (w/v) NH_4Cl and 2 ml 20% (w/V) $BaCl_2$ to precipitate $BaCO_3$. The sample was then treated as in Section 3.4.4.

3.4.6.2 Rumen gas proportions -

A 10 ml sample of rumen gas was taken and transferred to a 10 ml disposable syringe (Figure 3.2). Slight positive pressure was applied to the syringe as it was removed from the moisture trap, fitted with a 23 **gauge** needle and pushed into a rubber bung. In this way very little atmospheric contamination of gas occured. Samples were analysed within 12 hr of sampling. Samples of approximately 10 ml were analysed using a gas chromatograph (Figure 3.4).

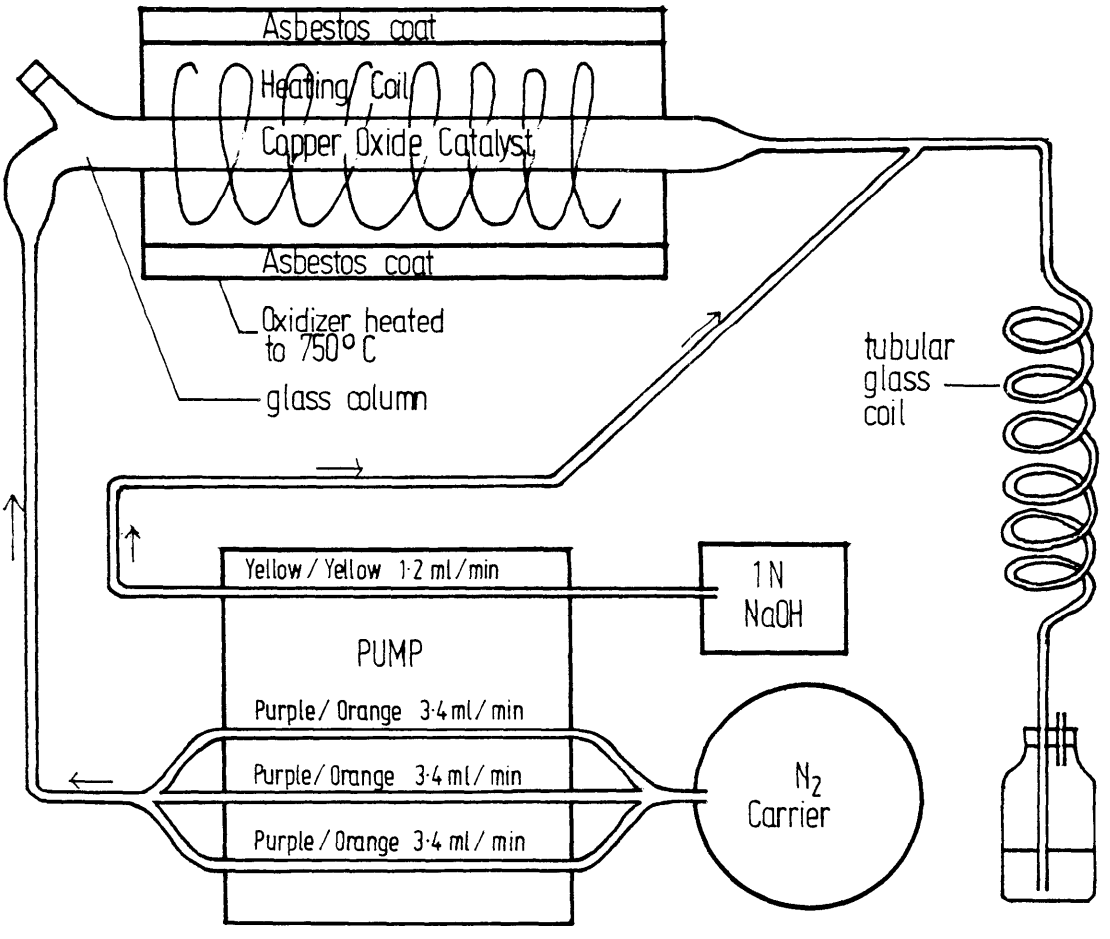


Figure 3.3: The apparatus used for the oxidization of CH_4.

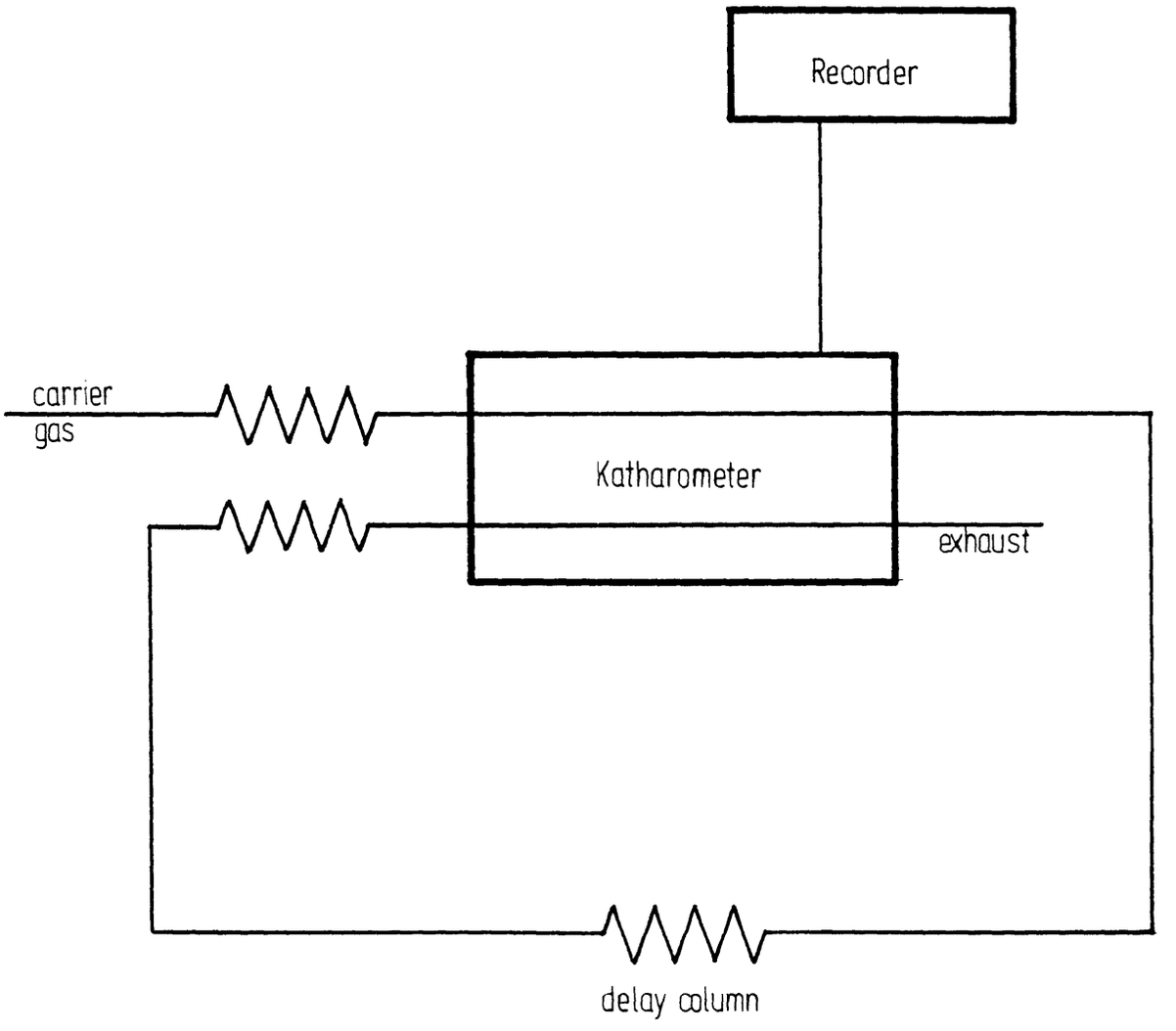


Figure 3.4: Gas chromatograph.

Two separation columns are used in series, the first partially separating O_2, N_2 and CH_4 but full separation of CO_2. The second column completes the separation. The thermal conductivity detector or katharometer (Series 150, Gow MAC Instruments Co., New Jersey, U.S.A.) has tubes containing tungsten filaments arranged in a wheatstone bridge through which the gas passes. The filaments are heated by an electric current (Figure 3.5). Filaments A_1 and A_2 are in the same gas stream and filaments B_1 and B_2 are in another gas stream. Voltage at X and Y will be equal if gases are of the same composition and flow rate. If the gases flowing past filaments A contain an element of greater thermal conductivity than the carrier gas then these filaments will be cooled with respect to filaments B, their electrical resistance will decrease and the voltage at X will decrease and that at Y will increase. These changes are registered on the recorder as peaks for different gases.

The gas factor for each gas was determined using a standard mixture of gases from a pressure pack (Alltech Associates, Illinois, U.S.A.).

$$\text{Gas factor} = \frac{\text{Height of peak X Attenuation}}{\text{Known concentration}} \ \%$$

The proportion of any gas in the sample is calculated as:

$$\text{Proportion} = \frac{\text{Height of peak X Attenuation}}{\text{Gas factor}} \ \%$$

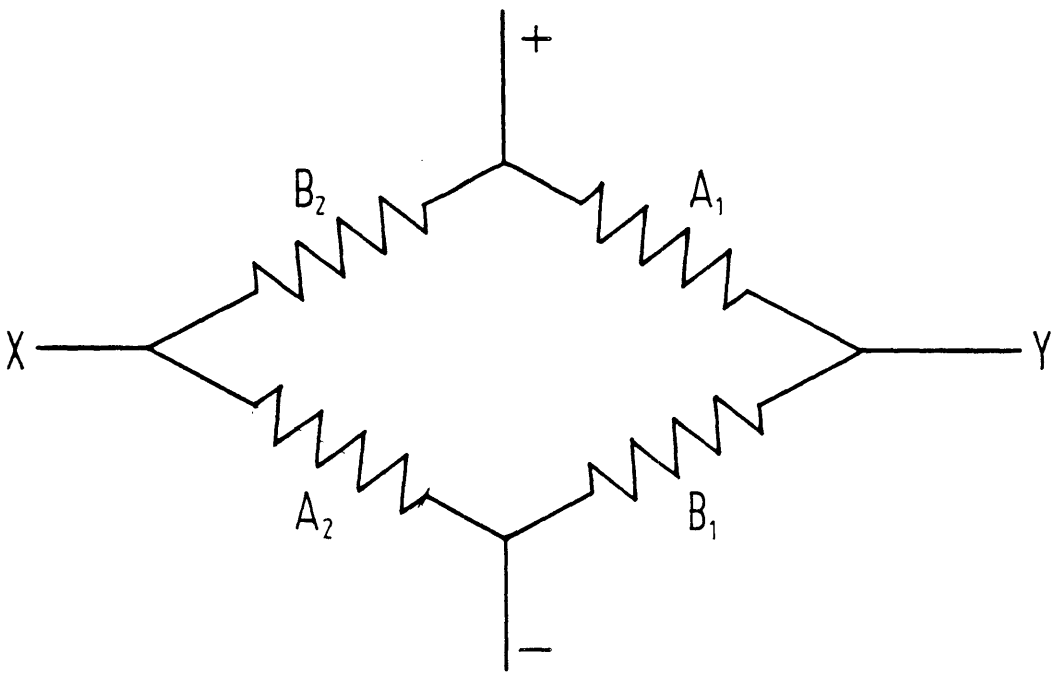


Figure 3.5: Wheatstone bridge contained in the Katharometer.

3.4.7 Blood and rumen fluid bicarbonate concentration

The method of Hinks et al (1966) was used. Five ml rumen fluid was withdrawn from the sampling probe and 5 ml of blood was withdrawn from the jugular vein catheter. One ml samples of either blood or rumen fluid were placed into a cup suspended by a wire in a scintillation vial. The vial was sealed with a rubber bung (Figure 3.6). The vial contained 1.0 ml of approximately 0.045 M $Ba(OH)_2$ plus Thymolphthalein (Thymolphthalein: 0.5 g/l 0.045 M $Ba(OH)_2$). 0.2 ml 1 N H_2SO_4 was injected with a syringe fitted with a 23 guage needle through the bung into the cup. The needle was withdrawn quickly and the vial was swirled gently. After 30 hr, the remaining alkali was titrated using 0.17 M HCl and bicarbonate concentration calculated by difference.

3.5 RUMEN TURNOVER AND VOLUME

A single injection of Cr-EDTA was used to estimate rumen turnover (l/d) and rumen volume (l) according to the methods of Downes and McDonald (1964). The concentration of Cr was determined on rumen fluid supernatent using an Atomic Absorption Spectrophotometer (Model 360, Perkin-Elmer, Connecticut, U.S.A.).

3.6 FEED ANALYSIS

3.6.1 Dry matter and organic matter

Feed samples were milled (1 mm screen) and dry matter (DM) was determined on samples of approximately 5 g by oven drying at 100°C for 24 hr. The samples were ignited for 3 hr at 550-600°C in a muffle furnace to determine organic matter (OM). Samples were duplicated.

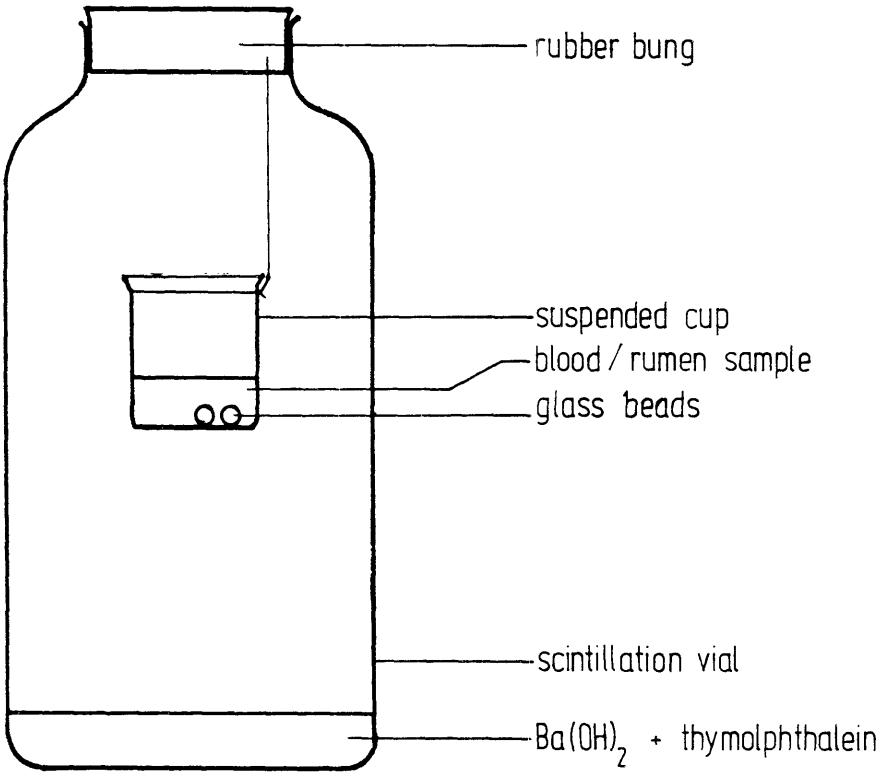


Figure 3.6: The vial with suspended cup for determining bicarbonate concentration.

3.6.2 Total nitrogen

This analysis was carried out on duplicate samples of approximately 0.2 g. Total nitrogen (N) was determined by the micro-Kjeldahl technique using 18 N H_2SO_4 and selenium catalyst in a variable temperature heating block (Model 1007, Tecator Instruments, Sweden). The digestion was continued for 1 hr after clearing. This was followed by steam distillation with collection of ammonia into boric acid (2% w/v) and titrating this mixture to pH 5 (Autoburette ABU 12, Radiometer, Copenhagen, Denmark) with 0.05 M H_2SO_4. The titration value for a reagent was subtracted from sample values and the recovery from a standard checked. Values of 100% $\pm$ 0.5% were acceptable.

The total N result was multiplied by 6.25 to give total crude protein (TCP).

3.6.3 Dry matter digestibilities

Digestibilities were determined using the *in sacco* (nylon bag degradation) method. A sample of approximately 3 g (though accurately weighed) was used. This was placed into a nylon bag weighted with a glass marble. Bags were sealed and suspended by nylon fishing line through the rumen fistula which allowed free movement within the rumen. Bags were removed after 24 hr, dried at 70°C for a further 24 hr and loss of sample determined by the weight difference before and after rumen digestion. Residues were retained for total N determination (Section 3.6.2). This gave an indication of undigested feed protein and microbial protein included in the residue.

All feed sample types were included in each of four sheep and mean values calculated.

3.6.4 Pepsin digestible nitrogen

A dry sample of approximately 1 g was incubated at 39°C for 2 hr in 50 ml of pepsin-HCl solution (2 g pepsin (1:10000) in 1 l 0.01 N HCl). The mixture was filtered (Watman No. 41 paper) and the total N determined on 10.0 ml of the clear solution. This proceedure is the same as Section 3.6.2 except titration is carried out with 0.0075 M H_2SO_4.

This gave an indication of the digestibility of protein beyond the rumen.

3.6.5 Ether extract

The fat content was determined by extraction of all ether soluble substances with anhydrous ether (Petroleum ether) on a dry sample of approximately 5 g which was duplicated. The sample was refluxed with anhydrous ether in a Soxhlet apparatus for 24 hr and fat content determined by weight difference.

3.6.6 Gross energy

A pellet of approximately 1.5 g was burned in a bomb calorimeter (Autobomb, Gallenkamp, England). The increase in temperature of the bomb was measured electronically and the gross energy (GE) calculated.

CHAPTER 4

TRACER DILUTION TECHNIQUES

4.1 INTRODUCTION

The use of radioactive tracers to investigate biological systems has proved extremely useful. The dynamics of a chemical substance within a compartment refers to the physical transfer of the substance into or out of the compartment or metabolism within the compartment (Wrenshall, 1956). The compartmental analysis needed to analyse the data in the experiments presented in this thesis involve both of these processes and the assumptions involved in the techniques are discussed below.

4.2 ASSUMPTIONS

Three conditions are assumed in tracer dilution experiments:

1. When isotopes are injected as a single dose, the substance being studied is assumed to be uniformly distributed at all times. This implies instantaneous and homogeneous mixing (Robertson, 1957). Continuous infusion of isotopes requires complete mixing particularly during measurement of the equilibrium SR reached in the compartment.

2. Steady state dynamics are essential so that for the tracee being studied, the quantity of material entering a compartment of constant size must equal the amount which flows out (Shipley and Clarke, 1972). This is dealt with in some depth by Shipley and Clarke (1972, Ch.10).

3. A further assumption is that the behaviour of the tracer is the same as the tracee, that is, rate constants of labelled and unlabelled material are equal (Robertson, 1957).

4.3 TYPES OF TRACER EXPERIMENTS

4.3.1 Single injection

In the simplest case where no recycling of the tracer out of and back to the same pool occurs, a tracer may be injected into a compartment and the decline in SR with time followed. A plot of $\log_{10}$SR with time is linear. However, in biological systems, recycling generally occurs and the kinetics of the model are described by multi-exponental equations. The equations are of the form (using the terms of White et al, 1969):

$$SR_t = \sum_{i=1}^{n} SRo_i e^{-m_i t}$$

where: SR_t = specific radioactivity at time t
SRo_i = zero time intercept of each exponential component
i = exponential component
m = rate constant of each component
t = time
n = number of components

With the relationships generated, one can obtain compartment size, total rate of flux of tracer, rate of entry and irreversable loss (IL) of tracee and rate of recycling (see Section 4.4).

4.3.2 Continuous infusion

When tracer is injected continuously into a compartment, the equation describing specific activity with time is:

$$SR_t = \frac{F}{Q} \sum_{i=1}^{n} \frac{SRo'_i}{m_i} \left(1-e^{-m_i t}\right)$$

where: F = infusion rate
Q = pool size
SRo'_i = fractional zero time intercepts, for example,

$$SRo'_1 = \frac{SRo_2}{\sum_{i=1}^{n} A_i} \left(\sum_{i=1}^{n} SRo'_i = 1\right)$$

As with single injection, this allows one to calculate total rate of flux, rate of entry and IL of tracee and rate of recycling. More detail may be obtained from Atkins (1969), Shipley and Clarke (1972) or Welch et al (1972).

4.3.3 Linear simultaneous equation solution

An alternative approach is to inject or continuously infuse tracer into each of the compartments under consideration. This allows one to eliminate time as a variable.

For a single injection, the area under a multi-exponental curve is:

$$\int_0^{\infty} (\sum A_i e^{-m_i t})$$

$$\text{i.e.} \sum \frac{A_i}{m_i}$$

This is equivalent to the tracer concentration.

For a continuous infusion, the tracer concentration at plateau is used in the calculation of IL and transfer quotents from primary to secondary pools. These can be used to set up a set of linear simultaneous equations to solve the models (Mann and Gurpide,1966; Depocas and de Freitas, 1970; Nolan and Leng, 1974; Mazanov and Nolan, 1976; Nolan et al, 1976; Nolan and Rowe, 1976; Nagy and Leng, 1980; Norton et al, 1982a; Norton et al, 1982b).

4.4 CALCULATIONS

The method described by Nolan et al (1976) was adopted in the analysis of compartmental systems as described in Section 4.3.3. Thus, calculations shown in following sections are those required for the analysis of the system by linear simultaneous equations.

4.4.1 Irreversible loss

The rate of IL of tracer from a primary pool was calculated as:

$$IL = \frac{\text{Infusion rate of tracer (muCi/min)}}{\text{Plateau SR of primary pool (muCi/mg C)}}$$

4.4.2 Transfer quotients

The proportion of tracee in a secondary pool (i) arising from the primary pool (j) was calculated as:

$$A_{ij} = \frac{\text{secondary pool plateau (muCi/mg C)}}{\text{primary pool plateau (muCi/mg C)}}$$

4.4.3 Linear simultaneous equations

A number of linear simultaneous equations were derived using the IL and transfer quotients from a series of experiments. In each, a continuous infusion was made into a discrete compartment. The derivation of these equations used the assumptions of steady state (influx into the compartment equals outflux) and tracer kinetics being the same as tracee (transfer quotients for tracee were the same as for tracer). A detailed example of constructing these equations is given in Appendix A.

4.5 THE DYNAMICS OF BICARBONATE IN THE RUMEN AND BLOOD POOLS

4.5.1 Introduction

Many researchers have studied the dynamics of CO_2 using radioactive isotopes (for example, Steele, 1955; Corbett et al, 1971; Whitelaw et al, 1972; MacRae et al, 1978; Nagy and Leng, 1980). The last two studies provide examples of rumen fluid bicarbonate and blood bicarbonate being treated as compartments in the model analysis. A search of the literature revealed no examples of rumen CO_2 (gas) being treated as a separate pool as was done in the study presented here.

A variety of isotope dilution experiments and analyses are available as described in the previous sections of this chapter. The present study used continuous infusion techniques which were analysed by developing a set of linear simultaneous equations using IL and transfer quotients (A_{ij}) (Nolan et al, 1976) to obtain the flow of carbon between the various pools being studied.

4.5.2 The system under study

The systems in the studies presented here are shown in Figure 4.1. The compartments that are related in experimental work are total rumen fluid bicarbonate*, total blood bicarbonate, CO_2 gas and rumen CH_4.

Except where stated in chapters dealing with individual experiments, the calculations presented in Section 4.4 (where plateau SR values are used) represent a mean of 10 samples taken when the

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*In the terms 'rumen fluid bicarbonate' and 'blood bicarbonate', bicarbonate includes CO_2, CO_3^{2-}, HCO_3^- and H_2CO_3.

infused isotope had equilibrated with the pool of bicarbonate (usually after 8 hours).

4.6 EFFECT OF DATA ERROR IN THE ANALYSIS OF TRACER DILUTION EXPERIMENTS

Errors in data analysis occur when the assumptions outlined in Section 4.2 are not met, leading to erroneous conclusions from tracer dilution experiments. If mixing is not complete, the kinetics of the tracer may not be that of the tracee giving invalid results. This is more likely to occur in a large compartment where physical mixing is slow. The common error is that plateau is assumed to have been reached before it actually has.

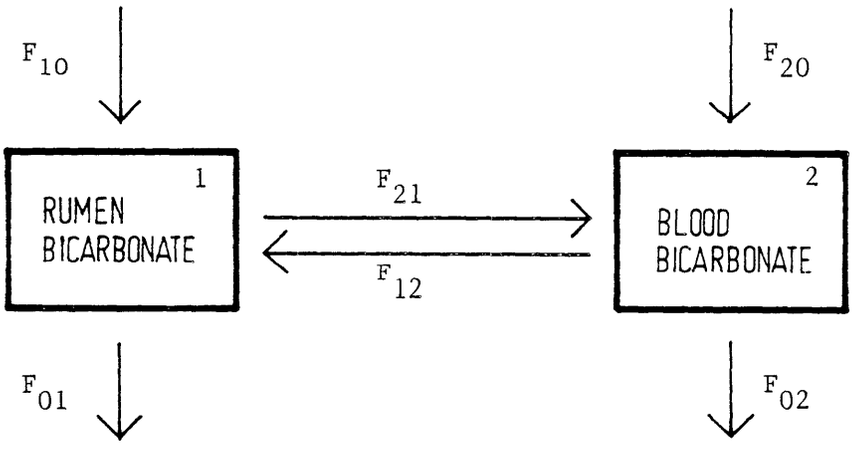
Although endeavours are made to keep animals in steady state, this is, in reality, impossible to obtain with in vivo experiments. Whilst studying the total CO_2 pool in man, Farhi and Rhan (1960) found that there was a continuous change in the CO_2 pool so that steady state was the exception rather than the rule. One must accept conditions that are as close as possible to steady state. To this end, animals are fed portions of the diet at small, regular intervals (usually hourly). Establishment of animals on a diet for some time before experimental work is essential for steady state. Webster (1967) and Brockway and McEwan (1969) in experiments relating O_2 consumption and energy expenditure, suggested that more consistent experimental results are obtained when animals have become adjusted to their environment and the experimental conditions over a long period of time. Brockway and McEwan (1969) suggested that periods of 6 months or more were required for animals to become adjusted to experimental techniques and their environment in order to reduce

errors arising from such conditions.

Further errors arise from sampling error, analytical error and animal difference.

To obtain an unbiased estimate of the plateau SR during a continuous infusion where fluctuations occur in the compartment under study, one must take regular samples over a period covering a normal cycle of variation to obtain a true mean plateau. Computer simulations have been carried out to to analyse the cyclical changes in transfer **quotients** (for example, Myhill, 1967). Rowe (1978) confirmed that the sampling period must be sufficiently long to overcome variation caused by natural fluctuations. Fluctuations in all pools being considered in this study occur due to variations in metabolism of bicarbonate and CO_2, rate of eructation, variations in salivary flow rate and bicarbonate concentration, activity of the animal and fermentation rate.

(a)



(b)

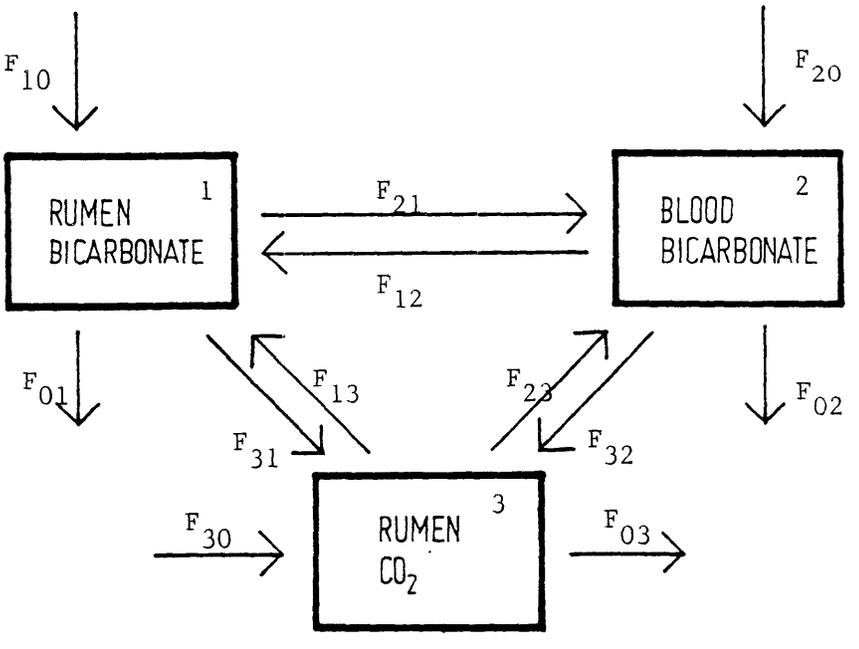


Figure 4.1: General open-compartment models of flows of C between (a) rumen bicarbonate-C (Pool 1) and blood bicarbonate-C (Pool 2); (b) rumen bicarbonate-C (Pool 1), blood bicarbonate-C (Pool 2) and rumen CO_2 (Pool 3); (c) rumen bicarbonate-C (Pool 1), blood bicarbonate-C (Pool 2), rumen CO_2 (Pool 3) and rumen CH_4 (Pool 4). The flows between pools are given for each flow (F_{ij}) as explained in Section 4.4.2.

(c)

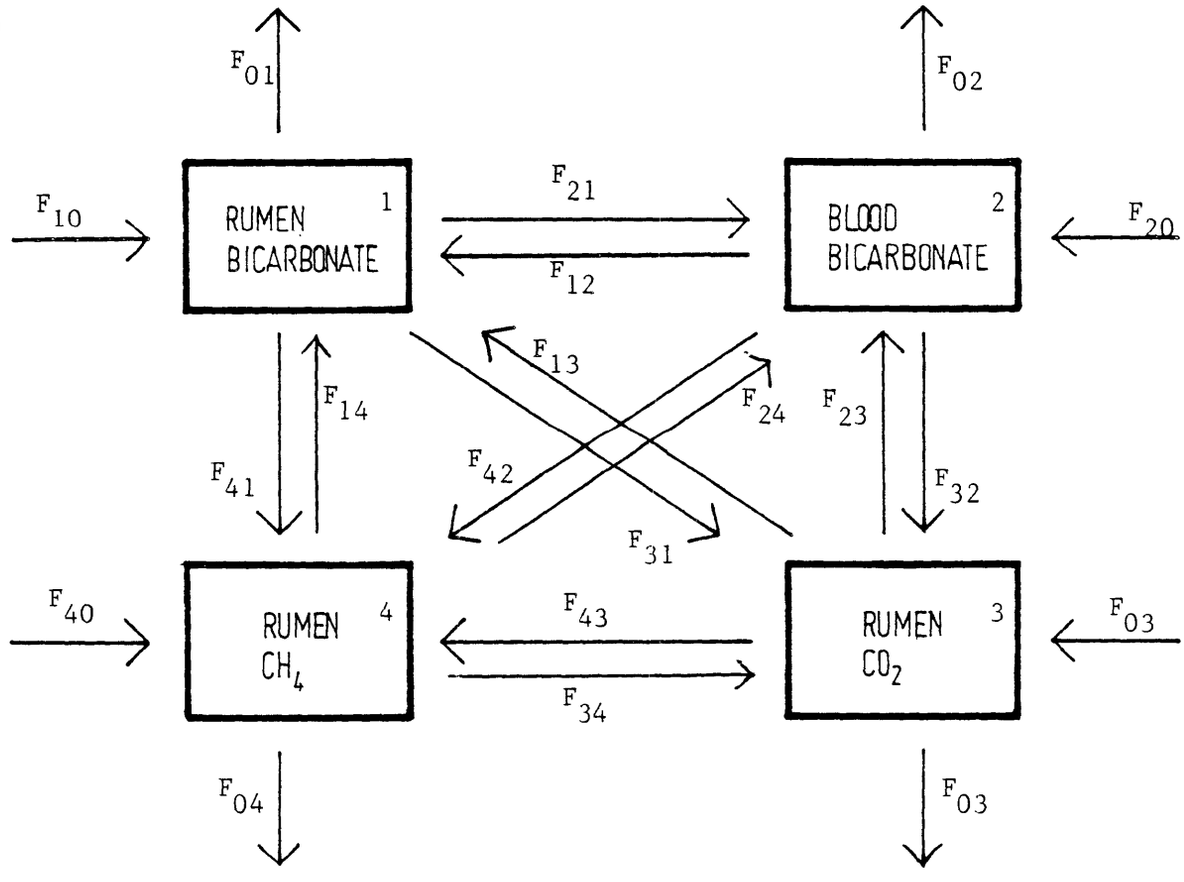


Figure 4.1: continued.

4.7 STATISTICAL ANALYSIS

Tracer infusions were made into each pool under study in each animal giving a separate model for each animal. The accuracy of the infusion rates of tracer infusions (SE) was assumed to be ± 1% of the mean.

$$\text{Standard Deviation (SD)} = \sqrt{\text{sample variance}}$$

$$= \sqrt{S^2}$$

$$\text{where} \qquad S^2 = \sum_i (X_i - x)^2 / (n-1)$$

$$\text{Standard Error (SE)} = \text{Standard Deviation of the mean}$$

$$= \sqrt{\frac{S^2}{n}}$$

The SE of IL was calculated as:

$$SE_{IL} = \sqrt{\frac{IL^2}{(P_{SR})^2} \left[\frac{(SE_{IR})^2}{IR^2} + \frac{(SE_{PSR})^2}{(P_{SR})^2} \right]}$$

where IR = mean tracer infusion rate

P_{SR} = mean SR at plateau

IL = irreversible loss $\frac{(IR)}{(P_{SR})}$

SE_{IR} = Standard error of mean tracer infusion rate

SE_{PSR} = Standard error of mean plateau specific radioactivity

The SE was calculated on the specific ratios of pairs of simultaneous observations in the primary and secondary pools (A_{ij}, Section 4.4.2). Inputs for the model (IL or A_{ij}) were selected at random from a population of normal distribution for which the mean and SE were defined by experimental values. For each set of data generated in this way, a set of results was obtained. The mean and SE of the flow rates into pool i from pool j (F_{ij}) were calculated as explained in Section 4.8 and given as the solution of each model presented.

In the past, many authors have constructed one model solutions for the group of animals under study, thus assuming that all animal error is accounted for in the solution of each set of data from each animal by taking an arithmetic mean and calculating the SD for each flow from:

$$F_{ij}\ (SD) = \frac{1}{n}\sqrt{\sum_{i=1}^{n} (SD_{ij})^2}$$

where: n = number of data sets
SD_{ij} = SD of each F_{ij} in each data set solution.

The grouping of models to form one is discussed further in Chapter 5 using specific examples.

4.8 COMPUTER ANALYSIS

For each set of experimental data, a further 100 sets of data were generated. This was done by using a random standard deviation generator (G05CCF, Numerical Algorithums Group (NAG) software) which selected at random from a population of normal distribution. Each of the 100 sets of data was used to generate a set of linear simultaneous equations. The 100 sets of linear simultaneous equations were then written into matrix form and run through a computer program which allowed one to include only those pathways (F_{ij}) required. The matrix was solved by a least squares solution using a NAG subroutine (F04JAF). The mean and SD for each F_{ij} was calculated as the mean of the 100 F_{ij} generated for each animal.

CHAPTER 5

THE DESTINATION OF BICARBONATE FLOWING OUT OF THE RUMEN IN LIQUID

5.1 INTRODUCTION

The role of salivary bicarbonate in stabilizing rumen fluid pH by neutralizing acids produced during fermentation is well known. When bicarbonate flow to the rumen is insufficient, the pH of rumen contents can fall rapidly especially if fermentation rate is high. This can occur when ruminants consume a diet high in readily fermentable substrates. When high concentrate rations are used, the total production of VFA is extremely high and not enough saliva is secreted to neutralize the increased acid production.

Microbial fermentation in the rumen results in the production of the gases CO_2 and CH_4. CO_2 is the gas of highest proportion in the rumen gas pool and is produced in large quantities from the acidification of salivary bicarbonate, by transport across the rumen epithelium from the blood or from microbial fermentation.

Bloat is a condition of cattle which is due to the trapping of CO_2 and CH_4 in bubbles which form a stable foam in the rumen, inhibiting eructation (Titchen and Reid, 1965). As gas continues to be produced faster than it can be expelled distension of the rumen

occurs and the the conditions of bloat follows. When fermentation rate in the rumen is rapid, rumen fluid pH falls to often quite low levels. It has been shown that the the rigidity of foam produced from rumen fluid reaches a maximum at pH 5.5 to 6.5 (Mangan, 1959; Buckingham, 1970; Jones and Lyttleton, 1972). It is at times of rapid fermentation that there is the largest production of CO_2 in the rumen.

For a full understanding of how these processes occur and are related, a study of the quantitative aspects of bicarbonate and CO_2 production and excretion in the ruminant is essential. The flows of bicarbonate-C between rumen fluid and the blood have been studied by several authors (Cocimano, 1972; Dixon ,1978; MacRae et al, 1978; Rowe, 1978; Cottle, 1980). As yet, it appears that no research has been done which includes rumen CO_2 and CH_4 in a compartmental analysis of the dynamics of bicarbonate-C.

In the studies presented here a series of tracer dilution experiments were carried out to quantify the dynamics of rumen bicarbonate-C. Initial experiments were done to establish techniques and study the relationship between rumen fluid bicarbonate-C and blood bicarbonate-C. With consideration given to the importance of gas production, experiments were carried out to comparmentalize and relate rumen fluid bicarbonate-C, blood bicarbonate-C and rumen CO_2 within animals on diets which contain low to high levels of grain in the diet. This was done to provide a comparison between bicarbonate-C dynamics in animals with high or low rumen fluid pH values. Since CH_4 makes up about 30% of rumen gas and is produced from CO_2-C, experiments were also done to relate CH_4 production to rumen fluid bicarbonate-C, blood bicarbonate-C and rumen CO_2.

The fate of bicarbonate-C in rumen fluid after it passes from the rumen was also studied by continuously infusing $NaH^{14}CO_3$ into the omasum and abomasum. The subsequent buildup of radioactivity in blood bicarbonate-C, rumen fluid bicarbonate-C and rumen CO_2 and CH_4 was followed.

5.2 MATERIALS AND METHODS

5.2.1 Experiment 1

Four mature cross-bred Merino wethers were used (Section 3.2). Animals were fed a diet of 420 g lucerne chaff plus 280 g rolled barley (Table 5.1). Overhead belt feeders were used to deliver hourly, equal portions of the feed. Animals were prepared with jugular vein catheters (Section 3.2). The tracer HCO_3^- infusion solution for infusion into rumen and blood had an activity of 500 nCi/ml and 2.0 mCi/ml respectively. Infusions were made at a rate of approximately 0.80 ml/min and 0.25 ml/min respectively. In each study, $NaH^{14}CO_3$ was infused into the rumen (d 1) and into blood (d 3) (Section 3.3). On experimental days, samples of rumen fluid and jugular blood were obtained at regular intervals (Sections 3.4.1, 3.4.2, 3.4.3, 3.4.4, 3.4.5 and 3.4.6.2).

Table 5.1: Proximate analysis of feed components for experiments 1-5.

	Lucerne chaff	Rolled barley
% Dry matter (DM)	90	90
Organic matter (%DM)	92	94
Ash (%DM)	8	6
Gross energy (MJ/kg DM)	1.5	1.5
% Fat (%DM)	4.8	9.9
N content (%)	3.7	1.5
Crude protein (%) N X 6.25	23	9
%Crude protein (soluble)	36	49
%Crude protein (insoluble)	64	51
Nylon bag		
DM digestibility (%)	65	78
N digestibility (%)	62	68

5.2.2 Experiment 2

The animals and diet described in Section 5.2.1 were used. $NaH^{14}CO_3$ with an activity of 800 nCi/ml and 2.2 mCi/ml respectively were infused on d 1 and d 3 into the rumen fluid and blood respectively at a rate of approximately 0.79 ml/min and 0.25 ml/min respectively and on d 5, $^{14}CO_2$ with an activity of 250 nCi/ml and at a rate of approximately 0.65 ml/min at STP was infused into the rumen (Section 3.3). On experimental days, samples of rumen fluid, jugular blood and rumen CO_2 were taken at regular intervals (Sections 3.4.1, 3.4.2, 3.4.3, 3.4.4, 3.4.5, 3.4.6 and 3.4.7).

5.2.3 Experiment 3

The animals described in Section 5.2.1 were used. They received a diet of 280 g lucerne chaff and 420 g rolled barley (Table 5.1) which was fed in equal portions hourly over a 24 hr period (Section 3.2). Infusions were made on each experimental day: $NaH^{14}CO_3$ was infused into rumen fluid (d 1) and blood (d 3) with an activity of 800 nCi/ml and 2.0 nCi/ml respectively and $^{14}CO_2$ (d 5) and $^{14}CH_4$ (d 7) were infused into the rumen (Section 3.3). CO_2 and CH_4 had an activity of 260 and 200 nCi/ml respectively and were infused at a rate of 0.70 and 0.25 ml/min respectively. Samples of rumen fluid, jugular blood and rumen gas were taken on experimental days at regular intervals (Sections 3.4.1, 3.4.2, 3.4.3, 3.4.4, 3.4.5, 3.4.6 and 3.4.7).

5.2.4 Experiment 4

Two cross-bred Merino wethers were surgically prepared with omasal catheters. A catheter was passed through and attached to approximately half way along the greater curvature of the omasum and extended 3-4 cm into the omasum. After a recovery period of 8 weeks, they were established on a diet of 700 g lucerne chaff (Table 5.1) for 14 d. The diet was fed in equal portions over a period of 24 hr. On the day before the experiment, animals were prepared with a jugular catheter (Section 3.2). On the experimental day, a continuous infusion of $NaH^{14}CO_3$ was made into the omasum with an activity of approximately 80 nCi/ml and at a rate of approximately 0.8 ml/min. This was done using AutoAnalyser pump tubing (yellow/yellow (1.4 mm I.D.)) and a peristaltic pump. Samples of rumen fluid, jugular blood and rumen gas were taken at 15 min intervals for 90 min from the beginning of the infusion period and subsequently at 30 min intervals until plateau was reached. Samples were analysed as previously described.

A single injection of Cr-EDTA was made on d 3 to estimate rumen volume and turnover (Section 3.5).

5.2.5 Experiment 5

Two cross-bred Merino wethers were used. These animals had been prepared with abomasal cannula at least 6 months before the experiment. They were established on a diet of 700 g lucerne chaff (Table 5.1) for 14 d before the experiment. The diet was given in equal proportions at hourly intervals. Animals were prepared with jugular catheters (Section 3.2) and $NaH^{14}CO_3$ was infused continuously into the abomasum at the same rate and activity as in Section 5.2.4.

Samples of rumen fluid, jugular blood and rumen gas were taken at intervals of 15 min for the first 90 min and subsequently at 30 min intervals until plateau was reached for rumen bicarbonate, blood bicarbonate, CO_2 and CH_4. Only four samples of jugular blood could be obtained for each animal. Samples were analysed as previously described.

5.2.6 Calculations

The flows of C (mean $\pm$ SE) for sheep used in experiments 1, 2 and 3 were calculated using the method described in Section 4.8 and the data in Appendix B. In the analysis of 4 pool models, the transfer of C from CH_4 to rumen bicarbonate, blood bicarbonate and CO_2 was found to be zero and a transfer quotient of 0.0 was used in the analysis as shown in Appendix B.

The total concentration of bicarbonate includes HCO_3^-, H_2CO_3, CO_3^{2-} and dissolved CO_2. The concentration of HCO_3^- was calculated using the Henderson-Hasslebalch equation (Segel, 1976, p 83-86). The concentration of H_2CO_3 was found by the difference between total bicarbonate and HCO_3^- and therefore includes dissolved CO_2. When applying the Henderson-Hasslebalch equation to blood bicarbonate parameters, a pH of 7.4 and pK_a' of 6.1 (Altman, 1961, p186) was used. A mean of samples for each animal is given in relevant tables. A pK_a' of 6.19 was used for rumen fluid bicarbonate parameters (Turner and Hodgetts, 1955a).

5.2.7 Statistical analysis

The statistical analysis of the models given in Figures 5.1, 5.2 and 5.3 is explained in Section 4.7.

The statistical methods outlined by Snedecor and Cochran (1967) were used except where noted. An analysis of variance (AOV) was used to test for between animal difference for VFA molar proportions and total concentration and CO_2 and CH_4 proportions and the ratio of CO_2:CH_4.

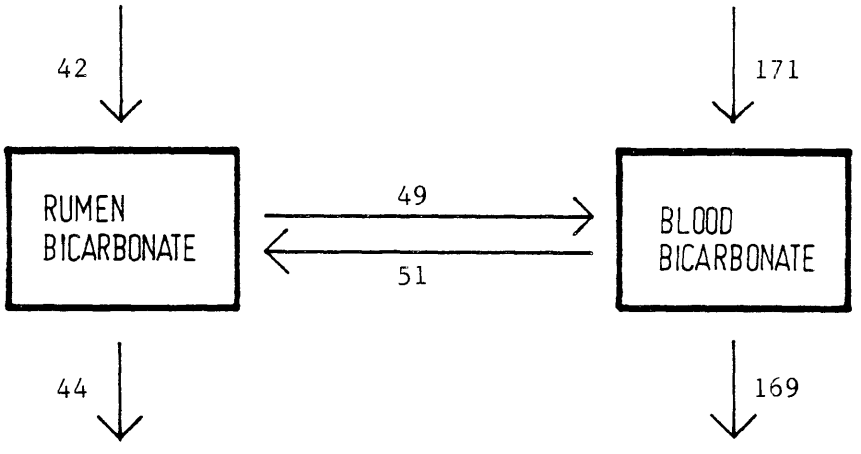
5.3 RESULTS

5.3.1 Statistical analysis

Researchers often combine the results of several models into one general model. Flows are calculated as the arithmetic mean of each flow (F_{ij}) and the SE of each flow as:

$$F_{ij}\ (SE) = \frac{1}{n} \sum_{i=1}^{n} (SE_{ij})^2$$

Figure 5.1 shows this calculation applied to the models in experiment 1. The increase in animal numbers with a general model show means $\pm$ SE which give more confidence in the results through a relatively smaller SE than the means $\pm$ SE of individual models. To demonstrate the variability that occurs with one animal and between animals, the results presented in this thesis give models for each individual animal.



C Flow	SE
F_{10}	4.35
F_{21}	10.48
F_{20}	9.73
F_{12}	5.78
F_{01}	11.80
F_{02}	12.65

Figure 5.1: The two compartment model for experiment 1 giving flows of C (g C/d)(mean ± SE) between rumen fluid bicarbonate-C and blood bicarbonate-C (SE are tabled for each flow (Section 4.5.2) for the combined means of all sheep).

5.3.2 Experiment 1

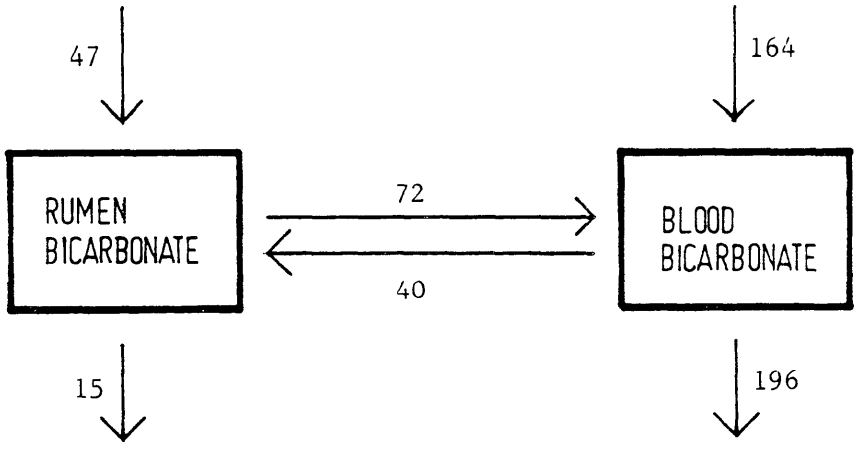
5.3.2.1 Estimates of flow rates of carbon -

The flows of C around the rumen bicarbonate and blood bicarbonate pools in the 4 experimental animals are shown in Figure 5.2. These two compartment models were solved using the data in Appendix B (Table B 1).

5.3.2.2 VFA proportions and rumen fluid pH values -

Mean VFA proportions and total VFA concentration for the experimental period are shown in Table 5.2 and rumen fluid pH values (mean $\pm$ SD) over the experimental period are shown in Table 5.3.

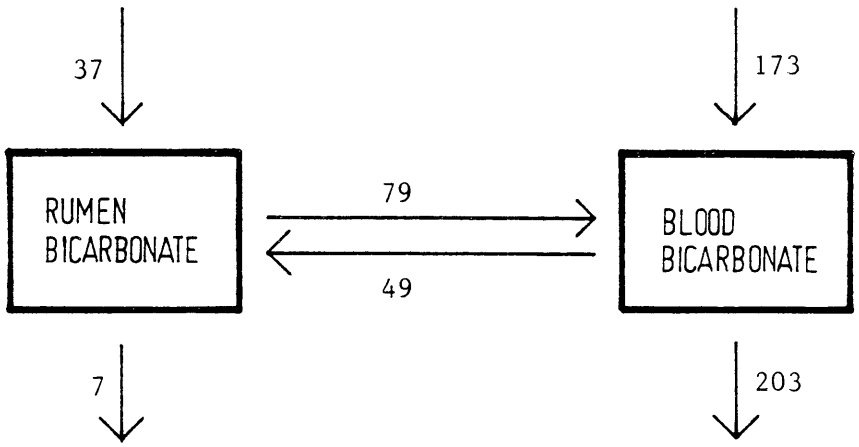
a.



C Flow	SE
F_{10}	8.3
F_{21}	21.9
F_{20}	24.4
F_{12}	9.4
F_{01}	21.8
F_{02}	33.3

Figure 5.2: Two compartment models describing the flow of C (gC/d) between total rumen fluid bicarbonate and blood bicarbonate in sheep fed 420 g/d lucerne chaff and 280 g/d rolled barley (experiment 1):
a. Sheep 78
b. Sheep 187
c. Sheep 60
d. Sheep 65
(SE are tabled for each flow of C (Section 4.5.2) for each sheep).

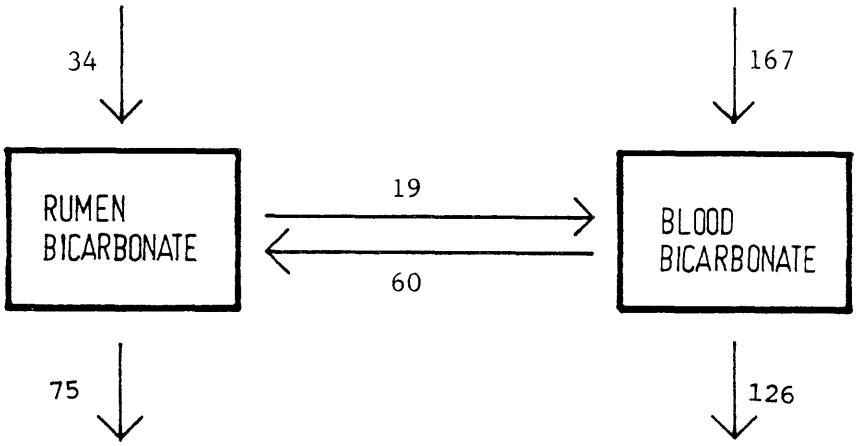
b.



C Flow	SE
F_{10}	5.5
F_{21}	34.8
F_{20}	17.1
F_{12}	5.8
F_{01}	29.8
F_{02}	18.0

Figure 5.2: continued.

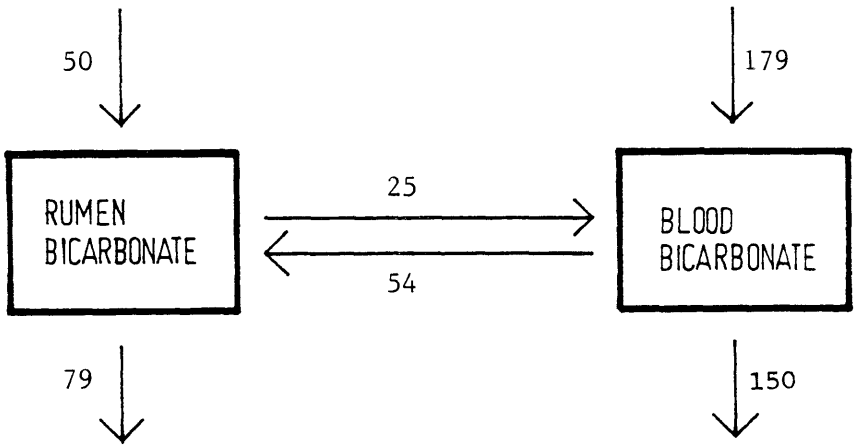
c.



C Flow	SE
F_{10}	11.3
F_{21}	6.5
F_{20}	17.5
F_{12}	17.5
F_{01}	25.1
F_{02}	25.1

Figure 5.2: continued.

d.



C Flow	SE
F_{10}	8.8
F_{21}	4.6
F_{20}	18.2
F_{12}	10.3
F_{01}	15.4
F_{02}	22.4

Figure 5.2: continued.

Table 5.2: Mean VFA proportions and total VFA concentrations in the rumen fluid of sheep used in experiment 1.

Sheep	Acetate	Propionate	Butyrate	Others	Total conc. mM
78	64.4[a*]	18.6[a]	12.7[b]	4.3[b]	73[a]
187	63.7[a]	20.7[a]	11.4[b]	4.3[b]	79[a]
60	67.5[b]	22.1[a]	7.1 [b]	3.4 [b]	95[a b]
65	68.2[b]	17.7[a]	10.2[b]	4.0[b]	128[b]

*Column values with different superscripts differ significantly ($P<.05$)

5.3.2.3 Rumen gas proportions -

The proportions of CO_2 and CH_4 in rumen gas are shown in Table 5.4. There is no significant difference ($P<.05$) between animals in CO_2 and CH_4 proportions or the ratio of CO_2:CH_4.

5.3.2.4 Protozoa numbers -

The numbers of small and large protozoa (mean $\pm$ SD) and the total number of protozoa (mean $\pm$ SD) during the experimental period are shown in Table 5.5.

5.3.3 Experiments 2 and 3

5.3.3.1 Estimates of flow rates of carbon -

Flows of C around the rumen bicarbonate, blood bicarbonate and rumen CO_2 pools for experiment 2 are shown in Figure 5.3 and flows around the rumen bicarbonate, blood bicarbonate, rumen CO_2 and CH_4 for experiment 4 are shown in Figure 5.4. These models were solved as in Section 4.8 using the data in Appendix B (Tables B 2 and B 3).

Table 5.3: Mean rumen fluid pH over experimental period.

Sheep	Mean pH ± SD
1	6.73 ± 0.02
2	6.52 ± 0.07
3	6.75 ± 0.12
4	6.73 ± 0.04

Table 5.4: The proportions of CO_2 and CH_4 in rumen gas and ratio of CO_2 to CH_4 in sheep used in experiment 1.

Animal	CH_4	CO_2	Ratio CO_2/CH_4
78	.31[a]*	.55[a]	1:2.21[a]
187	.27[a]	.54[a]	1:1.90[a]
60	.24[a]	.48[a]	1:2.74[a]
65	.26[a]	.54[a]	1:2.25[a]

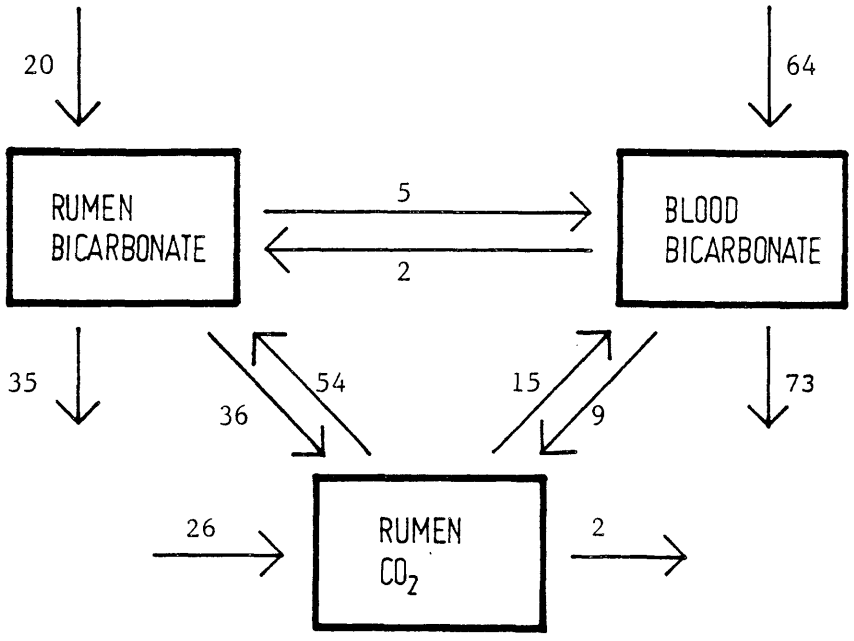
*Column values with different superscripts differ significantly ($P<.05$).

Table 5.5: The number of protozoa per ml (mean ± SD) in the rumen fluid of sheep used in experment 1.

Sheep	Small protozoa[a]	Large protozoa[b]	Total
78	6.04×10^6 $\pm 2.57 \times 10^6$	14.90×10^4 $\pm 13.90 \times 10^4$	6.19×10^6 $\pm 0.24 \times 10^6$
187	1.73×10^6 $\pm 0.50 \times 10^6$	2.70×10^4 $\pm 1.81 \times 10^4$	1.76×10^6 $\pm 0.35 \times 10^6$
60	1.04×10^6 $\pm 0.26 \times 10^6$	0.15×10^4 $\pm 0.16 \times 10^4$	1.04×10^6 $\pm 0.20 \times 10^6$
65	1.00×10^6 $\pm 0.41 \times 10^6$	3.78×10^4 $\pm 1.88 \times 10^4$	1.04×10^6 $\pm 0.17 \times 10^6$

[a] *Entodinium* spp.
[b] *Polyplastron* spp., *Epidinium* spp., *Isotricha* spp., *Eudiplodinium* spp.

a.



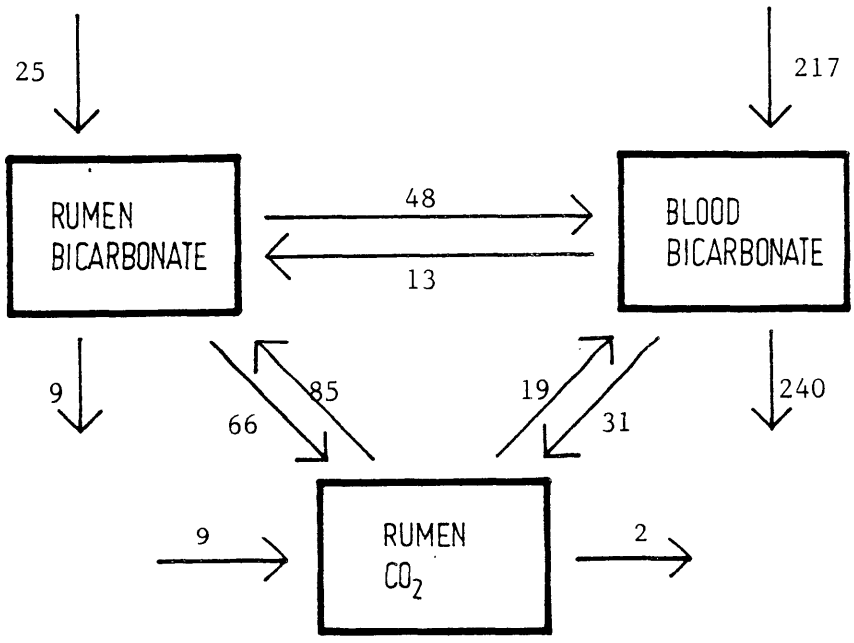
C Flow	SE	C Flow	SE
F_{10}	5.8	F_{31}	7.2
F_{21}	3.7	F_{32}	3.1
F_{20}	13.1	F_{23}	4.8
F_{12}	3.9	F_{02}	17.2
F_{01}	7.7	F_{30}	3.8
F_{13}	12.4	F_{03}	8.6

Figure 5.3: Three pool models describing the flow of C (gC/d) between total rumen bicarbonate, blood bicarbonate and CO_2 compartments in sheep fed 420 g/d lucerne chaff and 280 g/d rolled barley (experiment 2):

a. Sheep 78
b. Sheep 187
c. Sheep 60
d. Sheep 65

(SE are tabled for each flow of C (Section 4.5.2) for each sheep).

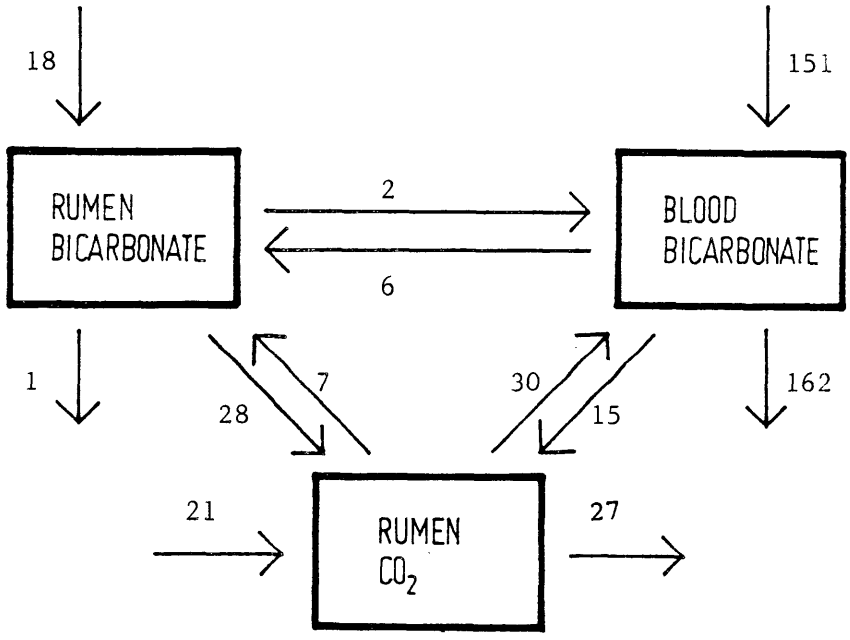
b.



C Flow	SE	C Flow	SE
F_{10}	8.6	F_{31}	15.1
F_{21}	26.8	F_{32}	10.5
F_{20}	29.6	F_{23}	23.2
F_{12}	12.4	F_{02}	39.6
F_{01}	34.3	F_{30}	6.9
F_{13}	29.9	F_{03}	28.9

Figure 5.3: continued.

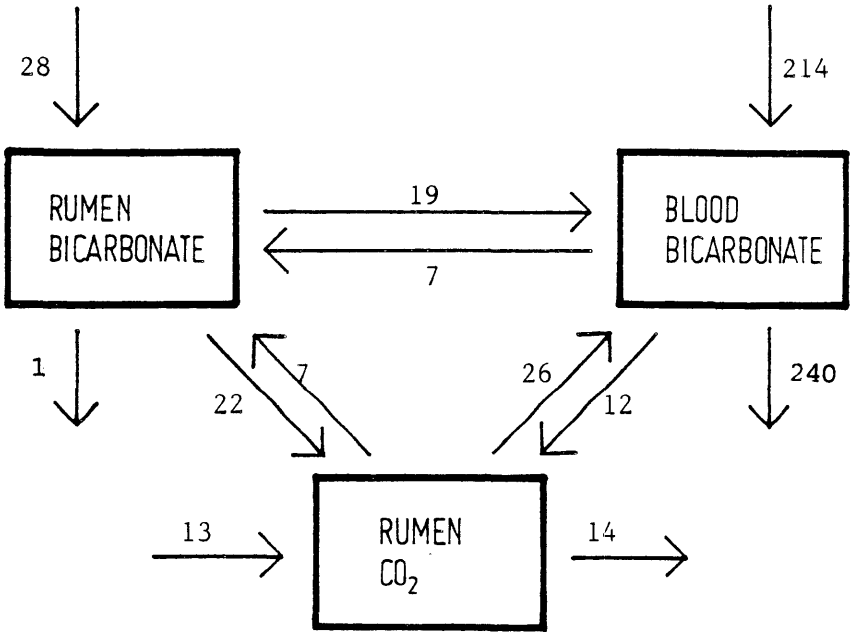
c.



C Flow	SE	C Flow	SE
F_{10}	3.1	F_{31}	13.4
F_{21}	10.4	F_{32}	6.7
F_{20}	33.9	F_{23}	11.8
F_{12}	1.7	F_{02}	40.5
F_{01}	9.9	F_{30}	9.9
F_{13}	3.5	F_{03}	11.8

Figure 5.3: continued.

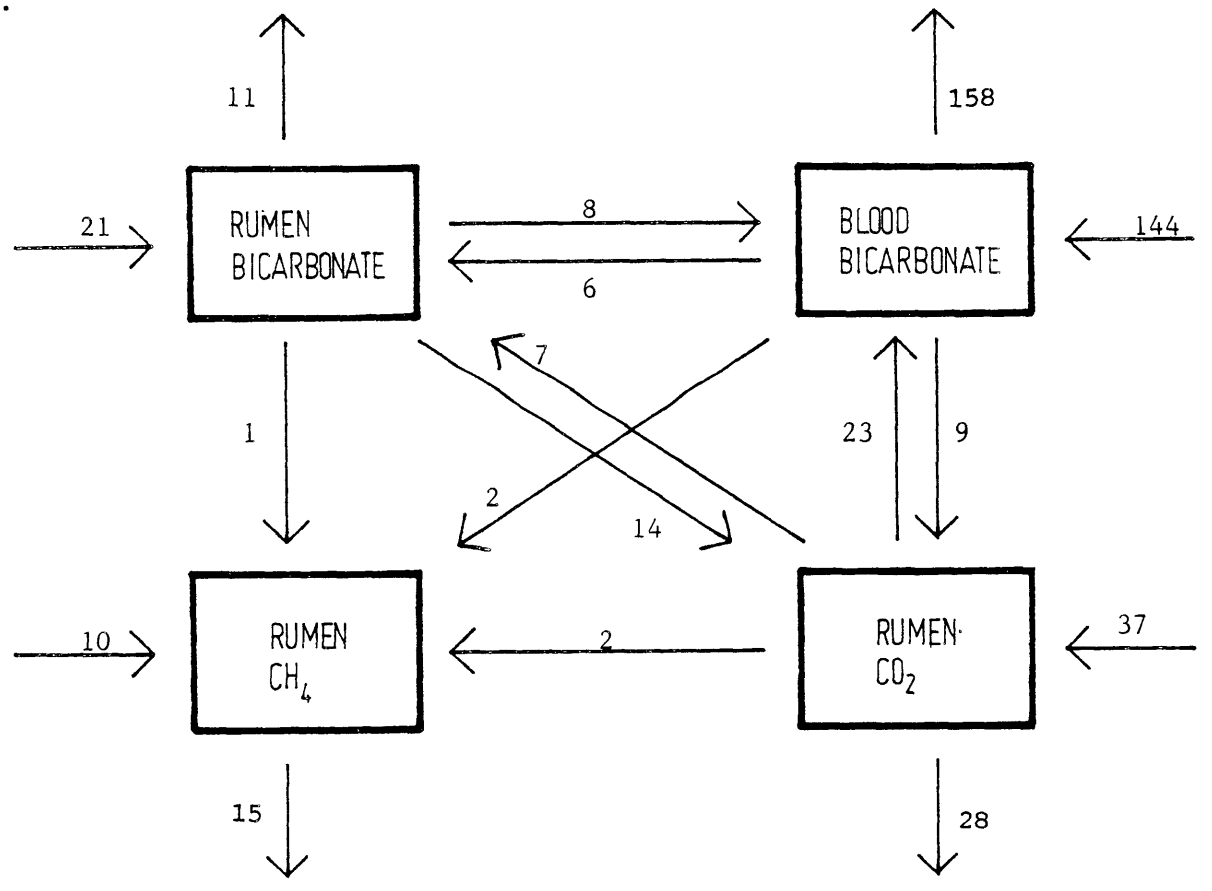
d.



C Flow	SE	C Flow	SE
F_{10}	7.5	F_{31}	14.1
F_{21}	1.6	F_{32}	5.1
F_{20}	15.7	F_{23}	6.6
F_{12}	2.8	F_{02}	17.8
F_{01}	16.8	F_{30}	10.4
F_{13}	2.5	F_{03}	12.6

Figure 5.3: continued.

a.

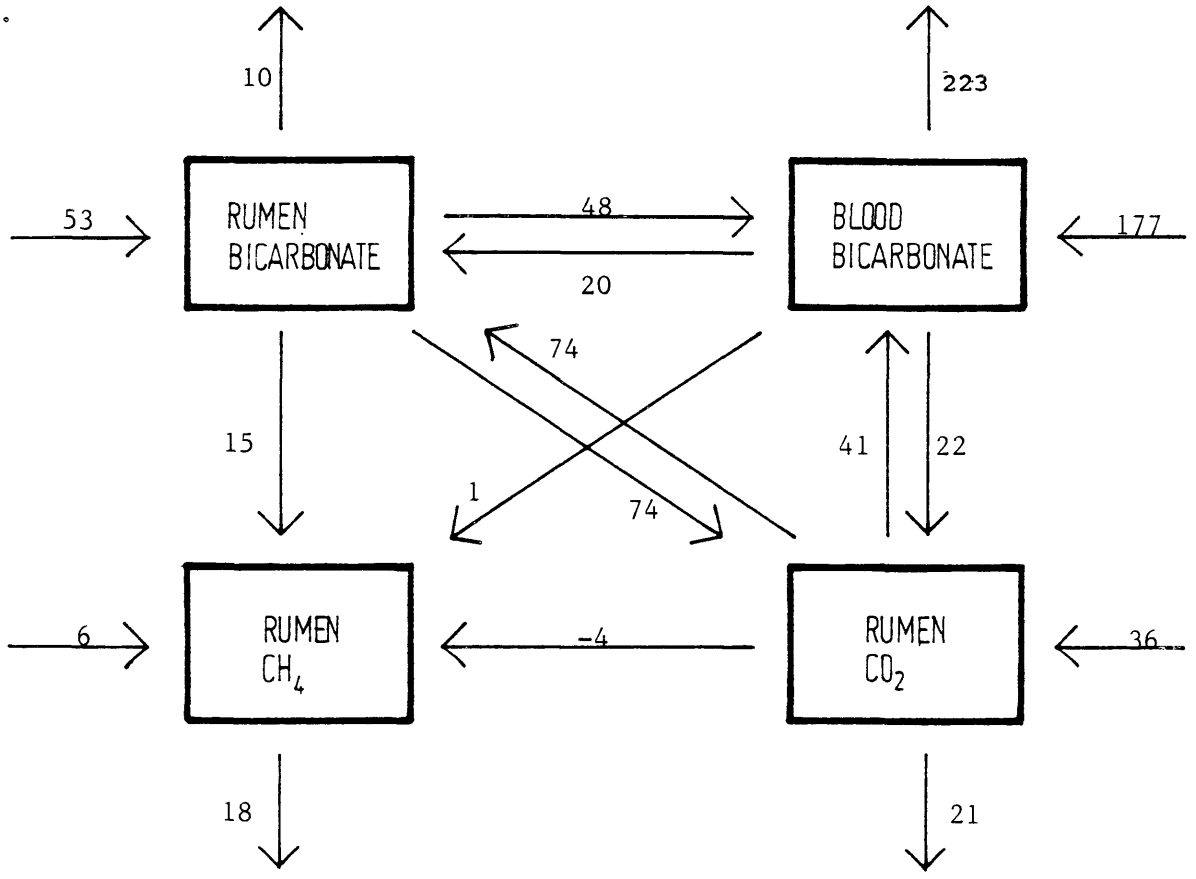


C Flow	SE	C Flow	SE	C Flow	SE
F_{01}	10.8	F_{40}	1.0	F_{32}	3.7
F_{10}	5.7	F_{04}	1.1	F_{43}	0.7
F_{20}	8.4	F_{41}	0.8	F_{42}	0.4
F_{02}	8.9	F_{21}	3.8	F_{13}	2.1
F_{03}	20.3	F_{12}	1.7	F_{31}	8.4
F_{30}	13.5	F_{23}	5.1		

Figure 5.4: Four compartment models describing the flow of C (gC/d) between total rumen fluid bicarbonate, blood bicarbonate, CO_2 gas and CH_4 gas in sheep fed 280 g/d lucerne chaff and 420 g/d rolled barley (experiment 3):
a. Sheep 78
b. Sheep 187
c. Sheep 60
d. Sheep 65

(SE are tabled for each flow of C (Section 4.5.2) for each sheep).

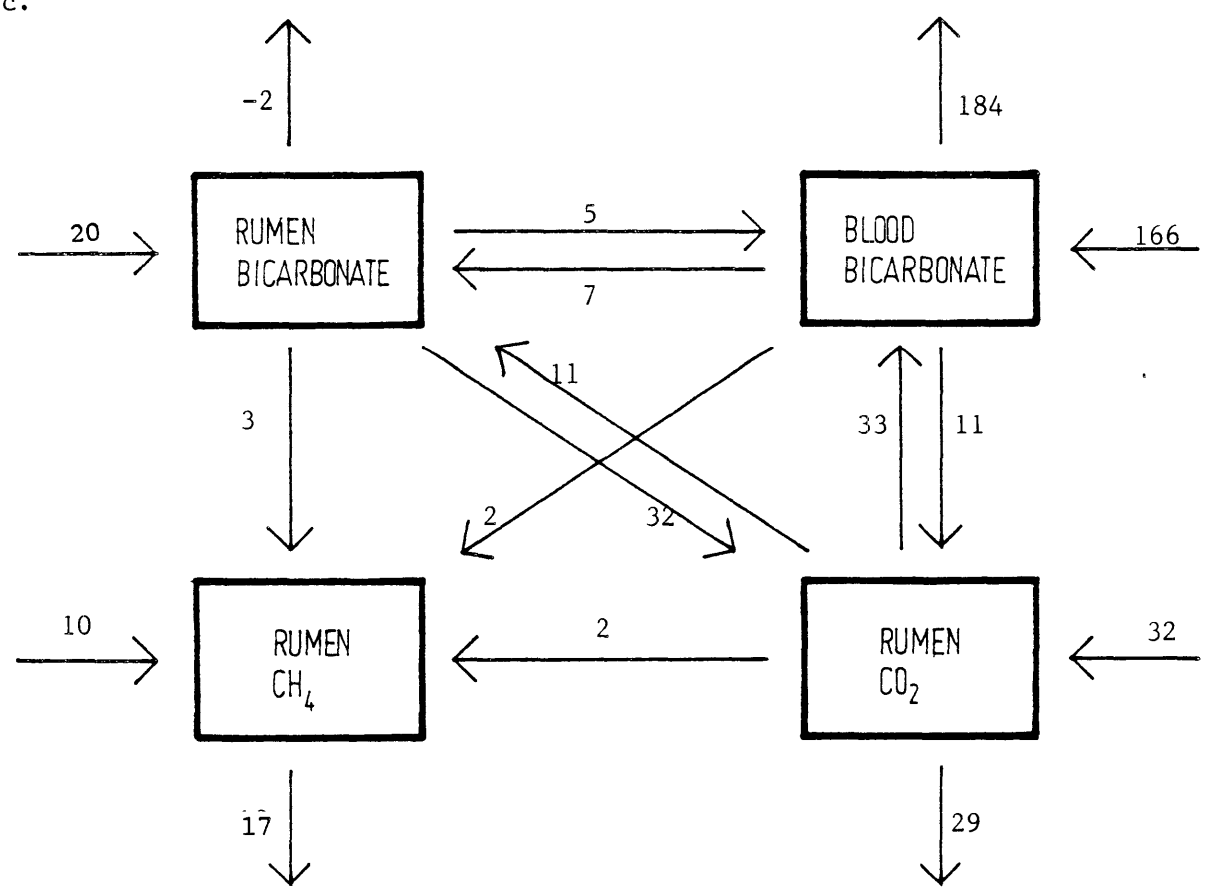
b.



C Flow	SE	C Flow	SE	C Flow	SE
F_{01}	23.4	F_{40}	1.0	F_{32}	4.7
F_{10}	9.2	F_{04}	1.3	F_{43}	1.9
F_{20}	20.0	F_{41}	2.5	F_{42}	0.7
F_{02}	30.6	F_{21}	11.9	F_{13}	17.4
F_{03}	18.1	F_{12}	5.7	F_{31}	10.7
F_{30}	5.3	F_{23}	13.0		

Figure 5.4: continued.

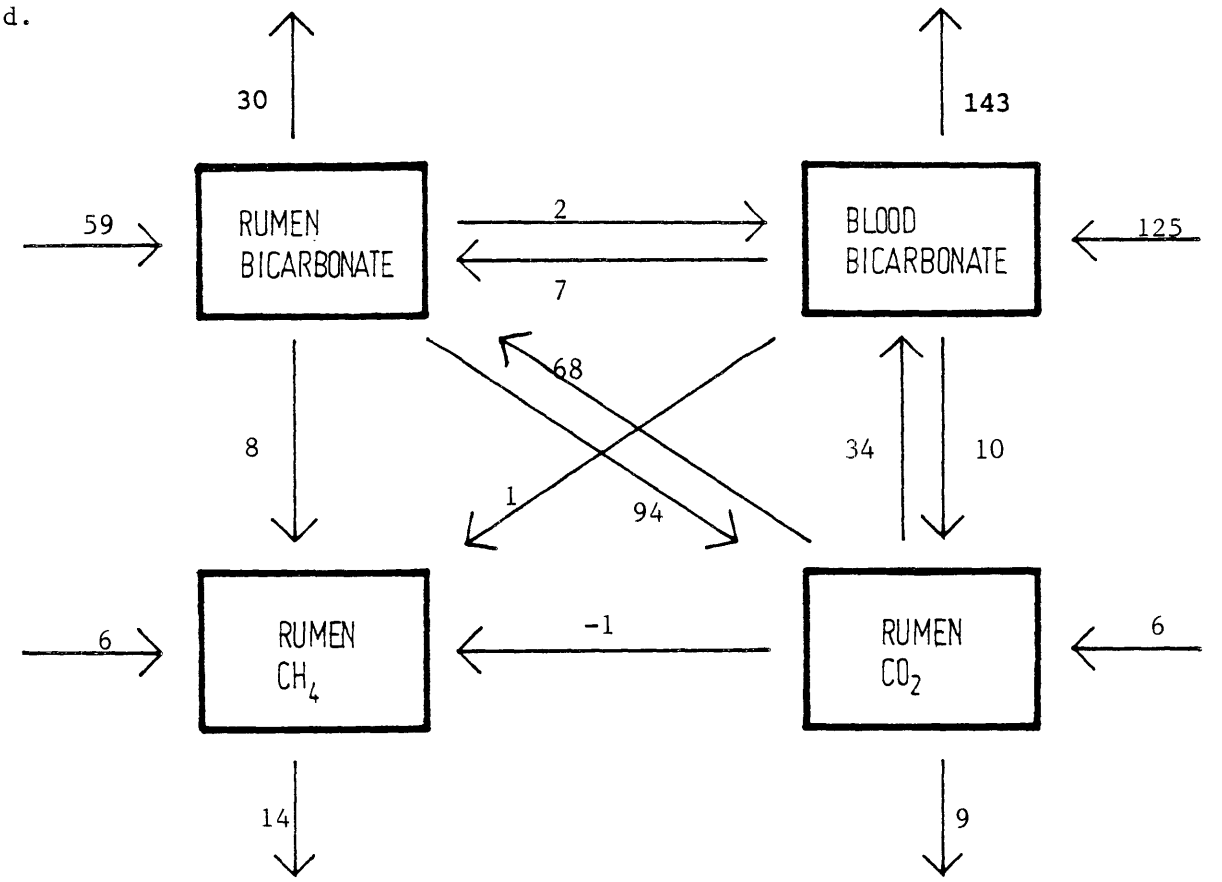
c.



C Flow	SE	C Flow	SE	C Flow	SE
F_{01}	7.3	F_{40}	1.6	F_{32}	2.9
F_{10}	3.3	F_{04}	2.8	F_{43}	2.9
F_{20}	6.4	F_{41}	0.6	F_{42}	0.4
F_{02}	7.5	F_{21}	3.3	F_{13}	4.3
F_{03}	9.5	F_{12}	1.5	F_{31}	4.3
F_{30}	4.5	F_{23}	4.9		

Figure 5.4: continued.

d.



C Flow	SE	C Flow	SE	C Flow	SE
F_{01}	10.6	F_{40}	0.7	F_{32}	2.8
F_{10}	5.6	F_{04}	0.9	F_{43}	1.0
F_{20}	4.3	F_{41}	1.4	F_{42}	0.3
F_{02}	5.0	F_{21}	6.4	F_{13}	14.5
F_{03}	9.1	F_{12}	2.2	F_{31}	20.0
F_{30}	7.5	F_{23}	4.4		

Figure 5.4: continued.

5.3.3.2 Rumen fluid and blood pH and bicarbonate concentrations -

The concentration of total bicarbonate, HCO_3^- and H_2CO_3 in rumen fluid is given in Tables 5.6 and 5.7 and for blood in Tables 5.8 and 5.9 for experiments 2 and 3 respectively. These tables also give the proportion of total bicarbonate as H_2CO_3 and rumen fluid pH over the 6 day experimental period.

A comparison of rumen fluid pH and bicarbonate parameters for experiments 2 and 3 shows a decrease in pH, total bicarbonate and HCO_3^- in each animal leading to an increase in the proportion of total bicarbonate as H_2CO_3.

Although a similar trend exists for blood bicarbonate parameters, the decreases are not significantly different.

5.3.3.3 VFA and rumen gas proportions -

Tables 5.10 and 5.11 give the proportions and total concentration of VFA in rumen fluid for the four experimental animals.

The proportions of CO_2 and CH_4 in rumen gas are given in Tables 5.12 and 5.13. An increase in the proportion of CO_2 gas occurred concommitant with the reduction in pH between experiment 2 and experiment 3.

5.3.3.4 Protozoa numbers -

Tables 5.14 and 5.15 give the number of small and large protozoa with the total number of protozoa in rumen fluid over the experimental period. No significant difference was found between animals in each experiment nor within animals from experiment 2 to experiment 3.

Table 5.6: The concentration of total bicarbonate, HCO_3^- and H_2CO_3 and pH in rumen fluid for sheep used in experiment 2.

Sheep	pH	Total bicarb. mM	$[HCO_3^-]$ mM	$[H_2CO_3]$ mM	Proportion as H_2CO_3
78	6.84	37.8	24.9	13.0	.34
187	6.68	28.8	22.7	6.1	.21
60	6.43	34.1	21.1	13.0	.28
65	6.35	25.5	14.5	10.9	.46

Table 5.7: The concentration of total bicarbonate, HCO_3^- and H_2CO_3 and pH in rumen fluid for sheep used in experiment 3.

Sheep	pH	Total bicarb. mM	$[HCO_3^-]$ mM	$[H_2CO_3]$ mM	Proportion as H_2CO_3
78	5.60	24.9	3.9	21.0	.84
187	5.98	23.1	9.9	13.1	.57
60	5.74	20.5	7.8	12.7	.62
65	5.63	18.3	3.8	13.4	.75

Table 5.8: The concentration of total bicarbonate, HCO_3^- and H_2CO_3 and pH in blood for sheep used in experiment 2.

Sheep	pH	Total bicarb. mM	$[HCO_3^-]$ mM	$[H_2CO_3]$ mM	Proportion as H_2CO_3
78	7.4	22.2	18.8	1.1	.048
187	7.4	20.7	19.7	1.0	.048
60	7.4	21.7	18.9	1.0	.048
65	7.4	20.9	19.9	1.0	.048

Table 5.9: The concentration of total bicarbonate, HCO_3^- and H_2CO_3 and pH in blood for sheep used in experiment 3.

Sheep	pH	Total bicarb. mM	$[HCO_3^-]$ mM	$[H_2CO_3]$ mM	Proportion as H_2CO_3
78	7.4	20.8	19.9	0.9	.048
187	7.4	19.1	18.1	1.0	.048
60	7.4	18.9	18.0	0.9	.048
65	7.4	20.1	19.1	1.0	.048

Table 5.10: Mean VFA proportions and total VFA concentrations in the rumen fluid of sheep used in experiment 2.

Sheep	Acetate	Propionate	Butyrate	Others	Total conc. mM
78	67.6[a,b]*	21.3[a]	6.7[a]	5.6[b]	87[a]
187	66.1[a]	21.1[a]	8.6[b]	4.2[b]	99[a,b]
60	69.8[b]	20.2[a]	7.7[a,b]	3.3[a]	114[a,b]
65	66.2[a]	22.3[a]	8.3[b]	3.8[a,b]	130[b]

*Column values with different superscripts differ significantly (P<.05).

Table 5.11: Mean VFA proportions and total VFA concentrations in the rumen fluid of sheep used in experiment 3.

Sheep	Acetate	Propionate	Butyrate	Others	Total conc. mM
78	51.1[a]*	37.7[b]	8.0[a]	2.5[a]	111[a]
187	60.0[b]	30.5[a]	7.6[a]	2.2[a]	105[a]
60	60.0[b]	29.8[a]	7.4[a]	2.6[a]	114[a]
65	50.6[a]	39.1[b]	8.0[a]	2.4[a]	113[a]

*Column values with different superscripts differ significantly (P<.05).

Table 5.12: The proportions of CO_2 and CH_4 in rumen gas and ratio of CO_2 to CH_4 for sheep used in experiment 2.

Animal	CH_4	CO_2	Ratio CO_2/CH_4
78	.24[a]*	.55[a]	1:1.88[a]
187	.27[a]	.57[a]	1:1.82[a]
60	.33[b]	.60[a]	1:2.24[a]
65	.34[b]	.54[a]	1:2.18[a]

*Column values with different superscripts differ significantly (P<.05).

Table 5.13: The proportions of CO_2 and CH_4 in rumen gas and ratio of CO_2 to CH_4 for sheep used in experiment 3.

Animal	CH_4	CO_2	Ratio CO_2/CH_4
78	.24[b]*	.75[a]	1:3.35[a]
187	.24[a]	.74[a]	1:3.14[a]
60	.25[b]	.74[a]	1:3.44[a]
65	.21[b]	.73[a]	1:3.43[a]

*Column values with different superscripts differ significantly ($P<.05$).

Table 5.14: The number of protozoa per ml (mean ± SD) in the rumen fluid of sheep used in experiment 2.

Sheep	Small protozoa[a]	Large protozoa[b]	Total
78	0.64×10^6 $\pm 0.12 \times 10^6$	0.33×10^4 $\pm 0.10 \times 10^4$	0.65×10^6 $\pm 0.01 \times 10^6$
187	1.34×10^6 $\pm 0.38 \times 10^6$	1.32×10^4 $\pm 0.53 \times 10^4$	1.35×10^6 $\pm 0.35 \times 10^6$
60	1.90×10^6 $\pm 0.57 \times 10^6$	2.64×10^4 $\pm 1.77 \times 10^4$	1.92×10^6 $\pm 0.18 \times 10^6$
65	2.63×10^6 $\pm 1.08 \times 10^6$	0.90×10^4 $\pm 0.07 \times 10^4$	2.64×10^6 $\pm 0.98 \times 10^6$

[a] *Entodinium* spp.
[b] *Polyplastron* spp., *Epidinium* spp., *Isotricha* spp., *Eudiplodinium* spp.

Table 5.15: The number of protozoa per ml (mean ± SD) in the rumen fluid of sheep used in experiment 3.

Sheep	Small protozoa[a]
78	$1.93 \times 10^6 \pm 0.72 \times 10^6$
187	$2.40 \times 10^6 \pm 1.22 \times 10^6$
60	$2.11 \times 10^6 \pm 0.92 \times 10^6$
65	$1.83 \times 10^6 \pm 0.81 \times 10^6$

[a] *Entodinium* spp.

5.3.4 Experiment 4

5.3.4.1 The SR of rumen fluid and blood bicarbonate and CO_2 and CH_4 -

The increase in radioactivity with time for rumen HCO_3^-, blood HCO_3^-, rumen CO_2 and CH_4 is shown in Figure 5.5 for the continuous infusion of $NaH^{14}CO_3$ into the omasum.

5.3.4.2 Rumen fluid and blood pH and bicarbonate concentrations -

The concentration of total bicarbonate, H_2CO_3, proportion of total bicarbonate as H_2CO_3 and rumen fluid pH are given in Table 5.16. The same parameters are given for blood in Table 5.17.

5.3.4.3 VFA proportions -

Table 5.18 gives the proportions and total concentration of VFA in rumen fluid for experiment 4.

5.3.4.4 Rumen volume and half-time -

Sheep 166 and 188 had rumen volumes of 3.54 and 4.08 l respectively and a turnover rate of 26.89 and 25.43 hr.

5.3.4.5 Protozoa numbers -

Table 5.19 gives the numbers of small and large protozoa (mean $\pm$ SD) during experiment 4.

Table 5.16: The concentration of total bicarbonate, HCO_3^- and H_2CO_3 and pH in rumen fluid for sheep used in experiment 4.

Sheep	pH	Total bicarb. mM	$[HCO_3^-]$ mM	$[H_2CO_3]$ mM	Proportion as H_2CO_3
166	7.10	35.0	28.5	6.5	.19
188	6.99	29.5	22.7	6.8	.23

Table 5.17: The concentration of total bicarbonate, HCO_3^- and H_2CO_3 and pH in blood for sheep used in experiment 4.

Sheep	pH	Total bicarb. mM	$[HCO_3^-]$ mM	$[H_2CO_3]$ mM	Proportion as H_2CO_3
166	7.4	22.5	21.4	1.1	.048
188	7.4	21.4	20.4	1.0	.048

Table 5.18: Mean VFA proportions and total VFA concentrations for sheep used in experiment 4.

Sheep	Acetate	Propionate	Butyrate	Others	Total conc. mM
166	67.2[a]*	20.6[a]	6.9[a]	5.3[a]	96[a]
188	67.6[a]	20.1[a]	7.3[a]	4.9[a]	105[a]

*Column values with different superscripts differ significantly ($P<.05$).

Table 5.19: The number of protozoa per ml (mean ± SD) in the rumen fluid of sheep used in experiment 4.

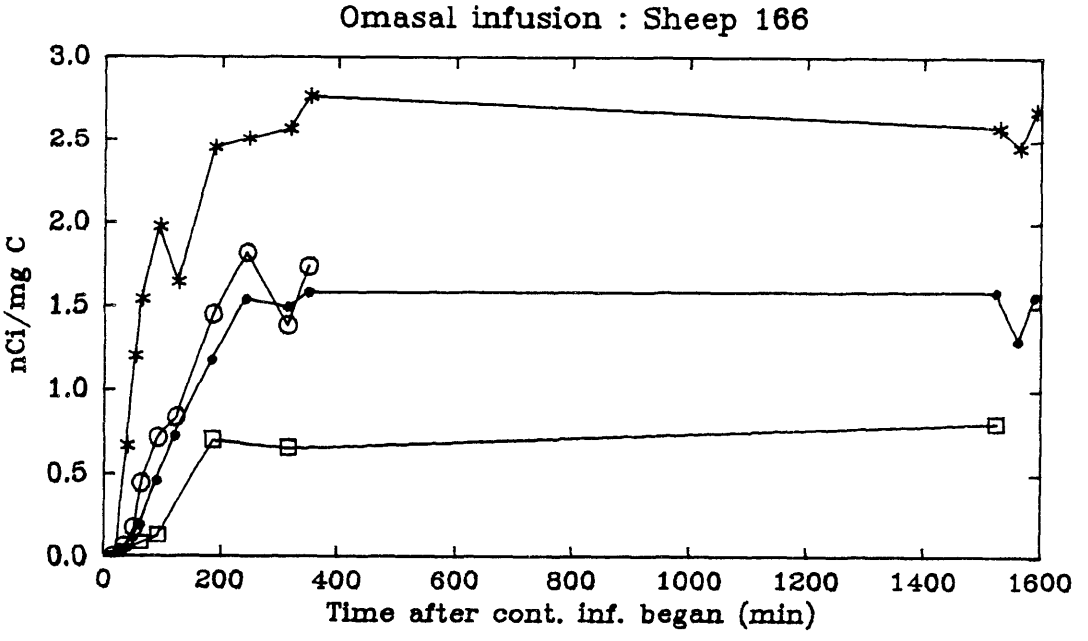
Sheep	Small protozoa[a]	Large protozoa[b]	Total
166	$2.21 \times 10^5 \pm 0.79 \times 10^5$	$2.50 \times 10^4 \pm 1.46 \times 10^4$	$2.46 \times 10^5 \pm 0.94 \times 10^5$
188	$7.82 \times 10^4 \pm 1.15 \times 10^4$	$5.73 \times 10^3 \pm 1.56 \times 10^3$	$8.39 \times 10^4 \pm 0.99 \times 10^4$

[a] *Entodinium* spp.
[b] *Polyplastron* spp., *Epidinium* spp., *Isotricha* spp., *Eudiplodinium* spp.

5.3.5 Experiment 5

5.3.5.1 The SR of rumen fluid, blood bicarbonate and rumen CO_2 and CH_4 -

The curves tracing the SR for rumen bicarbonate, blood bicarbonate, rumen CO_2 and CH_4 are shown in Figure 6.6. The curves follow SR until plateau is reached.



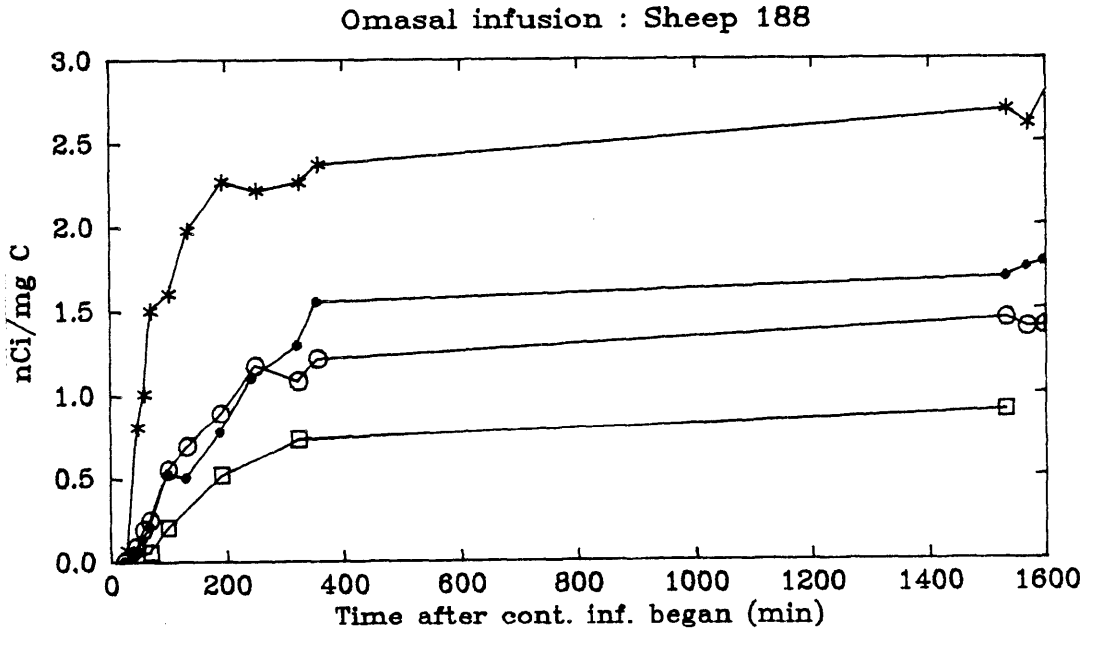


Figure 5.5: The SR of rumen bicarbonate (•), blood bicarbonate (✱), rumen CO_2 gas (○) and rumen CH_4 gas (□) in sheep used in experiment 4 following the continuous infusion of $NaH^{14}CO_3$ into the omasum.

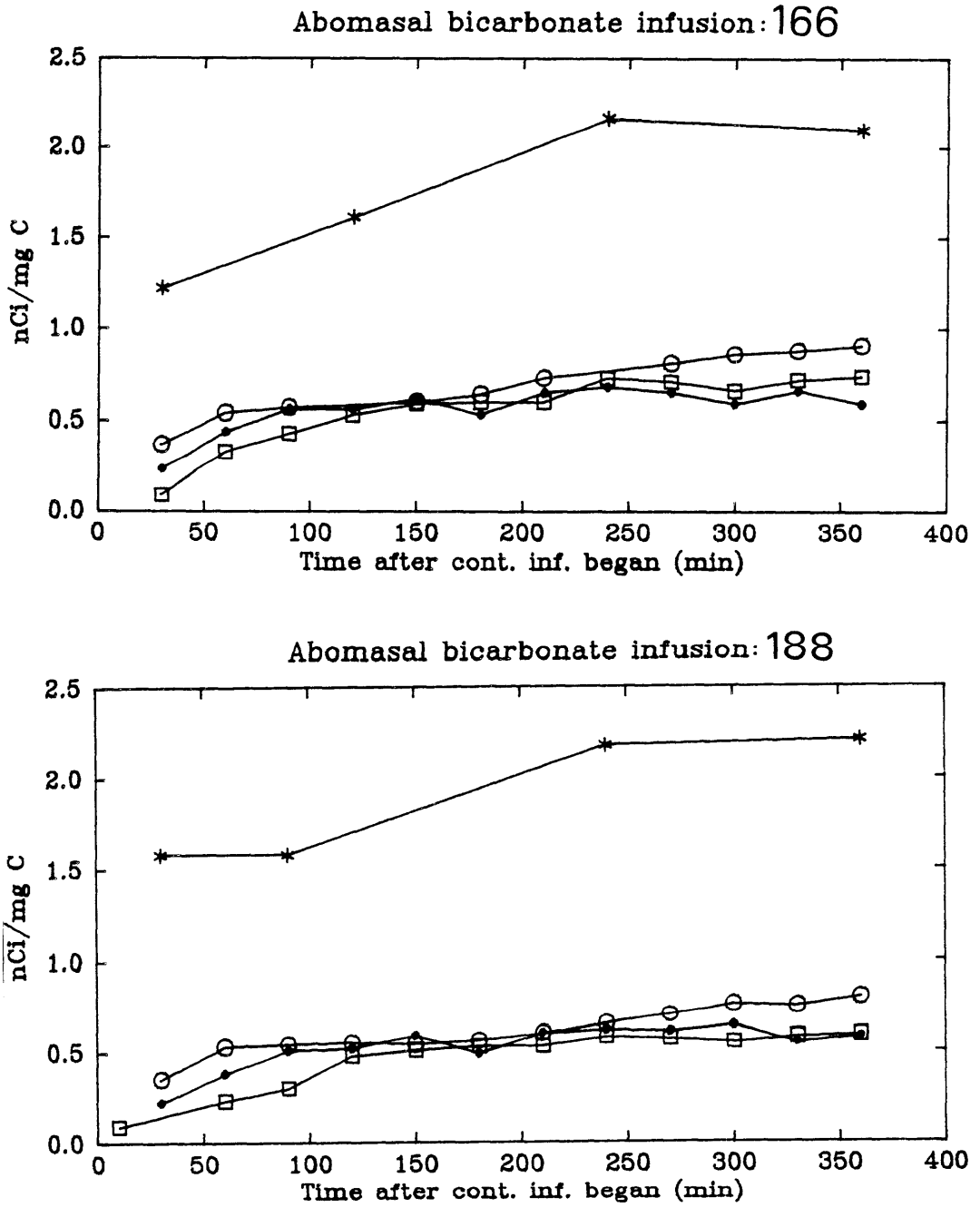


Figure 5.6: The SR of rumen bicarbonate (●), blood bicarbonate (✱), rumen CO_2 gas (○) and rumen CH_4 gas (□) in sheep used in experiment 5 following the continuous infusion of $NaH^{14}CO_3$ into the abomasum.

5.4 DISCUSSION

The movement of C between the pools studied in the sheep in experiments 1, 2 and 3 are shown in Figures 5.2, 5.3 and 5.4 respectively. The most important compartment in regards to the buffering ability of the rumen is the rumen bicarbonate pool. This pool consists of H_2CO_3 + HCO_3^- + CO_3^{2-} and CO_2 in solution (Section 4.5.2). C-flows into the rumen bicarbonate pool comprise CO_2 derived from microbial synthesis, diffusion of bicarbonate across the rumen epithelium from the blood, entrance via saliva and from solution from the CO_2 gas pool. Bicarbonate-C may be lost from the rumen bicarbonate pool by incorporation of C into microbial cells including CH_4 production by methanogenic bacteria, and also absorption into the blood and flow into the omasum in the liquid phase.

The flow of C into the blood bicarbonate pool comprise CO_2 derived from the metabolic processes of the body and transfer of CO_2 (and possibly HCO_3^-) into the blood from the rumen CO_2 and bicarbonate pools. CO_2-C is lost irreversibly from the blood by diffusion into the lungs and expiration.

The rumen CO_2 gas pool is derived from blood bicarbonate and the rumen bicarbonate pool and fermentation of food by microbes. CO_2 is lost by eructation, production of CH_4 and diffusion into the blood. CO_2 absorbed into the lungs equilibrates with blood HCO_3^- and may be excreted via the lungs or converted to bicarbonate and secreted into the saliva.

The increased grain content of the diet in experiment 3 provided the rumen microbes with more readily fermentable carbohydrates which resulted in rumen fluid pH being decreased.

The bicarbonate buffering system functions by neutralizing excess hydrogen ions as indicated below (Segel, 1976):

$$H^+ + HCO_3^- \rightleftharpoons H_2CO_3 \rightleftharpoons CO_2 + H_2O$$

In this way, reductions in rumen fluid pH are buffered.

An increase in the rate of conversion of rumen bicarbonate to CO_2 gas occurred in response to the lower pH conditions of the rumen fluid of sheep in experiment 3. This is the usual response of the bicarbonate buffer system to a challenge to the acid-base status of the rumen. Moreover, the rise in proportion as H_2CO_3 in the rumen bicarbonate pool would lead to an increase in the dissociation of H_2CO_3 to $CO_2 + H_2O$, and then to CO_2 gas in order to maintain the equilibrium of the buffer system.

The lower rumen fluid pH of the sheep in experiment 3 meant that more CO_2 was liberated from saliva and the combination of CO_2 production by microbes and CO_2 originating from bicarbonate resulted in an increase in rumen CO_2 gas. Thus there was a need for increased elimination of CO_2 by eructation, as shown by the large amount of CO_2 leaving the CO_2 pool in sheep in experiment 3 compared with sheep used in experiment 2. Tables 5.12 and 5.13 show the increase in the proportion of CO_2 gas that occurred in experiment 3 when there is a greater rate of fermentation and lower rumen fluid pH.

Figures 5.2, 5.3 and 5.4 demonstrate a fairly wide range of equilibrium constants (k) for transfers between pools. Since a small number of animals were used for each experiment, the SD for each flow is relatively high which reduces the confidence in such models. The values within the models do however give values one could anticipate and provide approximations of the variability that can occur for each

flow.

The lowest SD are found for flows of C into and out of the blood bicarbonate pool that are independent of the other pools. The largest SD for flows of C are found around the rumen CO_2 gas pool. The blood bicarbonate pool is relatively large compared to the rumen CO_2 gas pool. Also, the pH levels of the blood bicarbonate pool, and thus bicarbonate levels are kept within strict limits (Siggard-Andersen, 1974, p 92-131). The smaller pool size and larger turnover rate of the rumen CO_2 gas pool and the fluctuations in the pH of the rumen bicarbonate pool would result in larger variations in the IL and transfer **quotient** on which measured flows of C depend. The larger variation in flows of C for the rumen CO_2 gas pool compared with the blood bicarbonate pool would result in the greater SD found for the flows of C in the rumen CO_2 pool.

Large flows of C occur between compartments although the net flow may be small, for example, in Sheep 65 of experiment 3, a net flow of 26 gC/d from rumen bicarbonate to CO_2 gas occurs although the total flow in that direction is 94 gC/d.

As pH decreases, it is accompanied by a depletion of the bicarbonate concentration of rumen fluid and an increase in H_2CO_3 concentration (Tables 5.6 and 5.7). Bicarbonate concentration was particularly low in Sheep 65 (Table 5.7) at a pH of 5.63. This is consistant with the work of Turner and Hodgetts (1955b) who considered that bicarbonate concentration became so low below pH 5.5 that it was no longer an effective buffer.

The present study shows that the bicarbonate buffering system is a dynamic system which responds markedly to changes in rumen fluid pH in an attempt to maintain the acid-base status of the rumen. Moreover, an understanding of this system is essential if effective means of manipulating the rumen system in order to maintain rumen pH are to be found.

The curves showing the SR of rumen and blood bicarbonate, rumen CO_2 and CH_4 shown in Figure 5.5 for the infusion of $NaH^{14}CO_3$ into the omasum show a rapid transfer of C into the blood. This allows rapid acidification of gut contents entering the abomasum. Bicarbonate is returned to the rumen via saliva where ^{14}C labels rumen bicarbonate and CO_2 and eventually CH_4 by methanogenesis.

Bicarbonate and CO_2 in the omasum may return directly to the rumen under the influence of gut movement rather than the indirect route via blood. The extent to which this may happen cannot be gauged from Figure 5.5 but the rapid increase in blood bicarbonate SR indicates that this indirect route is the major pathway.

The SR curves for the continuous infusion of $NaH^{14}CO_3$ into the abomasum shown in Figure 6.6 are similar to the omasal infusion with blood SR being much higher than the other three pools monitored. This indicates the absorption of bicarbonate and CO_2 from the abomasum. It is however possible that when the acid conditions of the abomasum increase the transfer of bicarbonate to CO_2, CO_2 moves back into the omasum where CO_2 is absorbed rapidly, perhaps in the bicarbonate form. This would allow a rapid increase in the SR of blood bicarbonate. There is little difference in SR between the other pools although the SR of CO_2 is higher than the other 2 pools throughout the experimental period. The SR of CO_2 is also higher than rumen bicarbonate and CH_4

pools during the infusion of $NaH^{14}CO_3$ into the omasum. This indicates the direct transfer of CO_2 from the blood.

The decrease in rumen fluid pH in experiment 3 where a more readily fermented diet was offered was not only due to the lower bicarbonate levels and thus reduced buffering ability of the rumen, but also to the significant increase in VFA concentrations in each animal (Tables 5.10 and 5.11). However, the uptake of VFA from the rumen is accompanied by an uptake of CO_2 (Ash and Dobson, 1963) leading to a production of bicarbonate in rumen fluid. The quantity is however not enough to over-ride the decrease in total bicarbonate when rumen fluid pH is depressed (see Tables 5.6 and 5.7).

An increase in the grain content of a diet leads to a decrease in the number of cellulolytic and an increase in gram positive bacteria (Svendsen, 1975). Along with increased fermentation and rates of passage, there is a rapid generation of reducing power within microbial cells leading to a greater production of ruminal propionate and butyrate and sometimes a higher concentration of total VFA (Prins, 1977). The more readily fermentable diet of the sheep used in experiment 3 gave an increase in the molar proportions of propionate but not butyrate (Tables 5.10 and 5.11). This could have been due to feeding 700 g/d rather than ad libitum (Eadie et al, 1970). Eadie et al (1970) found that when cattle were fed at levels below appetite, there tended to be a reduction in VFA proportions and concentration. So although not of major significance, the less than appetite feeding levels may have masked the effect on butyrate of the more readily digested diet of experiment 3.

There was no effect of rumen pH on the total protozoal numbers in the rumen. It was expected that the extent of depression and period during which low pH conditions prevailed would result in a dramatic decrease in protozoa numbers in experiment 3 (Purser and Moir, 1959; Eadie et al, 1970; Eadie and Gill, 1971). The lack of large protozoa (see Section 3.4.2) could have been due to the low rumen fluid pH of experiment 3.

A comprehensive model of bicarbonate metabolism in the sheep has been reported by Norton et al (1982 a,b). These workers fed sheep two diets: 1000 g/d of pelleted grass cubes (diet A) and 700g/d of pelleted grass cubes plus 300 g/d of flaked barley (diet B). They found that on diet B the major loss of bicarbonate from rumen fluid was directly from the fluid pool rather than through re-cycling into the blood when compared to diet A. This is supported in the present study. In the rumen of sheep with a lower rumen fluid pH (as is the case for sheep on diet B compared to diet A in Norton et al, 1982a) more rumen fluid bicarbonate was transferred to the CO_2 gas pool. Norton et al (1982a) also found that sheep fed diet A had higher rumen fluid bicarbonate concentrates compared to sheep fed diet B. In the present study, it appeared that the decrease in rumen fluid bicarbonate was directly related to the rumen fluid pH i.e. sheep with relatively lower rumen fluid pH had less rumen fluid bicarbonate than sheep with higher rumen fluid pH.

The principles of stoichiometry can be used to predict expected CO_2 production rates using rumen VFA production values. Isotope dilution experiments were not carried out to determine actual VFA production rates, however, values from the literature for sheep suggest rumen VFA production rates of 3.67, 1.07, 0.46 and 5.20 mol/d for acetate propionate, butyrate and total VFA respectively (Church, 1976, p 290).

An alternative approach is to calculate VFA production rates using predictive equations. One such equation is (Weston and Hogan, 1968; cited by Church, 1976, p 291):

$$Y = 0.068X - 1.75$$

where Y = total VFA production (mol/d)

X = total VFA concentration (mmol/l)

Applying this equation to the rumen VFA concentrations found for sheep used in experiments 2 and 3, the predicted mean rumen VFA production rates were calculated as 5.56 (range 4.17 to 7.09) and 5.78 (range 5.39 to 6.00) for sheep used in experiments 2 and 3 respectively. These values are similar to the VFA production rates given for sheep in Church (1976, p 290).

Using stoichiometric principles and VFA production rates of 3.67 mol/d of acetate and 0.46 mol/d of butyrate (see Leng, 1970 and Church, 1976, p290), the amount of CO_2 produced in the rumen can be calculated at approximately 103 l CO_2/d (55.1 g C/d). This value is reasonably close to the actual values found in experiments 2 and 3. In experiments 2 and 3 respectively, isotope dilution studies gave mean rumen CO_2 production rates of 90.46 (range 79.44 to 100.07) and 121.33 (range 97.92 to 160.61) l CO_2/d.

Stoichiometry can also be applied to the production of CH_4. Using the above values of rumen VFA production rates of 3.67 and 0.46 mol/d for acetate and butyrate respectively and assuming all the molecular hydrogen produced is converted to CH_4, rumen CH_4 production rate is 25.76 l CH_4/d.

Actual production rates using isotope dilution techniques (experiment 3) revealed a mean rumen CH_4 production rate of 30.38 (range 25.72 to 33.81) l CH_4/d. The difference between actual and predicted values would probably result from variations in VFA production rates between those predicted for sheep and actual values.

CHAPTER 6

THE INFLUENCE OF NA-BENTONITE ON BICARBONATE DYNAMICS IN SHEEP

6.1 INTRODUCTION

In an attempt to make conditions in the rumen more favorable for fermentation, researchers have examined a variety of buffering materials. The inclusion of a range of buffers in *in vitro* experiments with rumen fluid was carried out by Herod *et al.* (1978). They found that some buffers, particularly sodium bentonite (Na-bentonite) and $NaHCO_3$, were effective in minimizing pH changes in rumen fluid.

An experiment was carried out to examine the response of rumen metabolites, pH and protozoal numbers of sheep to two different Na-bentonites supplemented to high concentrate diets.

A further series of tracer dilution experiments was carried out in order to construct models of bicarbonate dynamics. The models were constructed for sheep on high grain diets with and without added Na-bentonite and at a pre-feeding period when pH was assumed to be high, and in the post-feeding period when pH was assumed to be low.

6.2 MATERIALS AND METHODS

6.2.1 Experiment 6

a) Ten mature cross-bred Merino wethers were used (Section 3.2). All animals were established on a basal diet of 800 g/d of 80% crushed sorghum, 20% oaten chaff, plus, (g/100g total feed) 1.5 urea, 1.0 $Ca_2(PO_4)_2$, 0.5 Na_2SO_4, 0.5 NaCl and 0.5 trace minerals. Two animals (Sheep 1 and 2) received the basal diet alone; Sheep 3, 4, 5 and 6 received (g/100g) 2% Na-bentonite (pH 5.5) and the remaining four sheep (Sheep 7, 8, 9 and 10) received (g/100g) 2% Na-bentonite (pH 9.6). Animals were fed once daily and after a period of 7 d, animals were sampled after consuming all feed offered (approximately 90 min). Samples of rumen fluid were taken at 15 min intervals over a 5 hr period to determine pH, bicarbonate concentration and protozoal numbers per ml (Sections 3.4.1, 3.4.2 and 3.4.7).

b) In a further experiment, 4 animals were fed the basal diet once daily. Samples of rumen fluid were then taken at 15 min intervals after feeding began to determine rumen fluid pH and bicarbonate concentrations (Sections 3.4.1 and 3.4.7). Two animals were then established on basal diet plus 2% Na-bentonite (pH 5.5) and two animals on basal diet plus 2% Na-bentonite (pH 9.6). After a period of 18 d, animals were sampled at 15 min intervals to determine rumen fluid pH and bicarbonate concentration as above.

6.2.2 Experiment 7

Four mature cross-bred Merino wethers were used (Section 3.2). Animals had been established on a diet of 700 g/d of oaten chaff (Table 6.1) for 4 weeks before the experimental period. The sheep were then gradually established on a diet of 800 g/d of 70% pelleted crushed grain (60% crushed wheat, 20% crushed sorghum, 20% crushed maize, plus, (g/100g total diet) 1.5 urea, 1.0 Ca_2PO_4, 0.5 Na_2SO_4, 0.5 NaCl, 0.5 trace minerals and 2 Na-bentonite (pH 5.5 or pH 9.6)) plus 30% lucerne chaff over a period of 7 d and were fed once daily at this level for a further 7 d. The proximate analysis of grain pellets is given in Table 6.1. Two sheep received pellets containing 2% Na-bentonite (pH 5.5) and the other two received pellets containing 2% Na-bentonite (pH 9.6). Feed was pelleted to ensure bentonite ingestion. For this reason the diet varies from that used in Chapter 5 as pelleting could not be carried out on the feed ingredients used in Chapter 5.

Table 6.1: The proximate analysis of feed components for experiment 7.

	Pellets	Oaten chaff
% Dry matter (DM)	90	90
Organic matter (%DM)	97	94
Ash (%DM)	3	6
Gross energy (MJ/kg DM)	1.4	6.3
% Fat (%DM)	3.8	2.4
N content (%)	2.5	0.9
Crude protein (%) N X 6.25	16	6
%Crude protein (soluble)	44	56
%Crude protein (insoluble)	66	44
Nylon bag		
DM digestibility (%)	82	--
N digestibility (%)	52	--

Animals were prepared with jugular catheters (Section 3.2) and a tracer dilution experiment was carried out over a period of 6 d. On d 1, 3 and 5, a continuous infusion of $NaH^{14}CO_3$ into the rumen fluid and blood and $^{14}CO_2$ into the rumen respectively was begun 8 hr prior to sampling (Section 3.3).

Pre-feeding samples of rumen fluid, jugular blood and rumen gas were taken at 30 min intervals for 4 hr before feeding (8 hr after infusions were begun) on d 2, 4 and 6 when rumen fluid pH was assumed to relatively high. Four hr after feeding, a further sampling period was carried out when samples of rumen fluid, jugular blood and rumen gas were taken at 30 min intervals for 4 hr at which time, rumen fluid pH was assumed to be low.

Samples were treated as in Sections 3.4.1, 3.4.2, 3.4.3, 3.4.4, 3.4.5, 3.4.6.

6.2.3 Calculations

The concentration of HCO_3^- and H_2CO_3 in rumen fluid were calculated as in Section 5.2.6.

Flows of C (mean $\pm$ SD) given for experiment 7 were calculated using the data in Appendix B and the methods outlined in Section 4.8.

6.2.4 Statistical analysis

The statistical analysis of the models presented for experiment 7 is outlined in Section 4.7.

Where relevant, analysis of variance (AOV) was carried out to test between animal difference and between treatment differences in experiments 6a and 6b and between pre- and post-feeding periods in experiment 7.

The comparison of regression lines given for experiments 6a and 7 was carried out using the methods described by Snedecor and Cochran (1967, p 432).

6.3 RESULTS

6.3.1 Experiment 6a

6.3.1.1 Total bicarbonate and pH of rumen fluid -

Figure 6.1 gives the effect of total bicarbonate on rumen fluid pH for each group of sheep: Basal diet, basal diet + 2% Na-bentonite (pH 5.5) and basal diet + Na-bentonite (pH 9.6). Each regression curve is derived from the combined results of all animals in each treatment. The Na-bentonite (pH 9.6) treatment is significantly different ($P<.05$) in slope to the Na-bentonite (pH 5.5) and basal diet groups. There was no significant difference ($P<.05$) between the pH 5.5 Na-bentonite and basal diet groups.

6.3.1.2 Protozoa numbers -

Table 6.2 shows the number of small protozoa for sheep in experiment 6a. No large protozoa were detected. No significant difference was found between treatments.

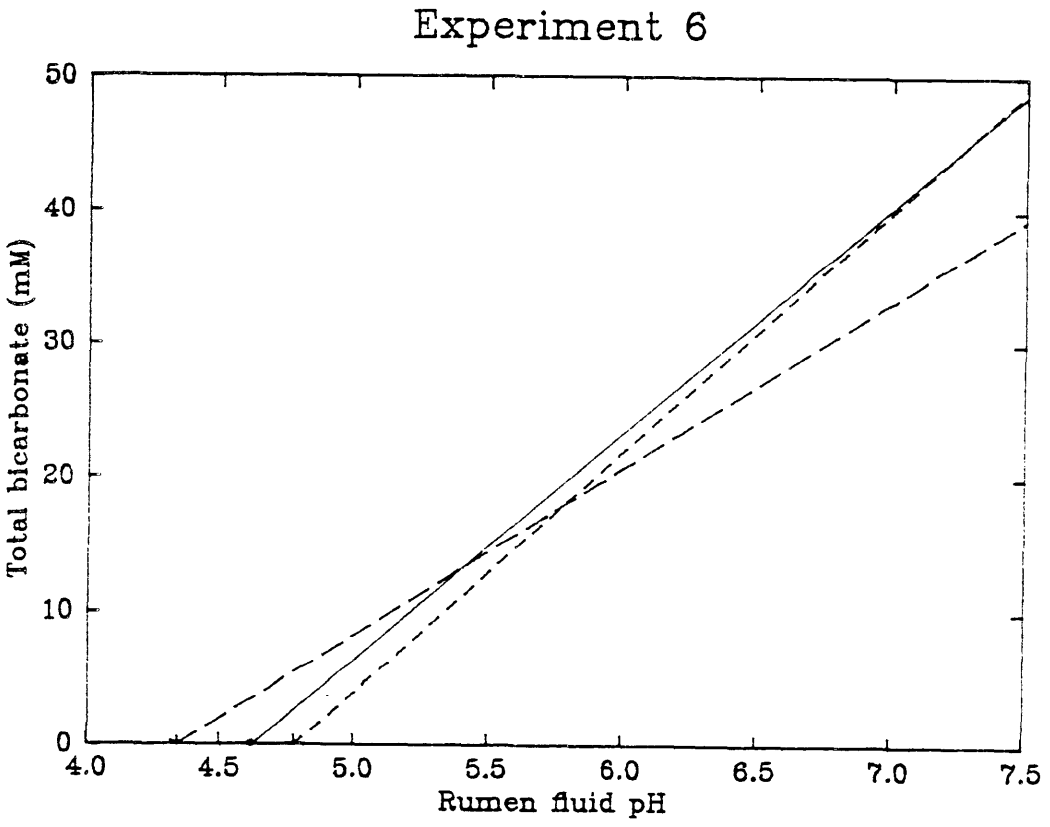


Figure 6.1: The relationship between total bicarbonate and rumen fluid pH for (i) Basal diet (———), (ii) Basal diet plus Na-bentonite (pH 5.5)(- - - - -) and (iii) Basal diet plus Na-bentonite (pH 9.6)(— — —) in experiment 6a. The regression equations were:

i) $y = 17.0x - 78.4$ ($r^2 = 0.92$, RSD = 4.05)
ii) $y = 18.0x - 86.1$ ($r^2 = 0.90$ RSD = 3.27)
iii) $y = 12.4x - 53.9$ ($r^2 = 0.88$, RSD = 4.92)

where : y = total rumen fluid bicarbonate (mM)
x = rumen fluid pH.

Table 6.2: The number of protozoa per ml (mean ± SD) in the rumen fluid of sheep used in experiment 6a.

Sheep	Small protozoa[a]
1	$8.63 \times 10^5 \pm 1.25 \times 10^5$
2	$5.36 \times 10^5 \pm 0.85 \times 10^5$
3	$3.67 \times 10^5 \pm 0.97 \times 10^5$
4	$8.53 \times 10^5 \pm 2.59 \times 10^5$
5	$7.42 \times 10^5 \pm 1.61 \times 10^5$
6	$4.87 \times 10^5 \pm 0.94 \times 10^5$
7	$8.72 \times 10^5 \pm 1.46 \times 10^5$
8	$2.34 \times 10^6 \pm 2.84 \times 10^6$
9	$9.42 \times 10^5 \pm 1.02 \times 10^5$
10	$1.26 \times 10^6 \pm 0.96 \times 10^6$

[a]*Entodinium* spp.

6.3.2 Experiment 6b

6.3.2.1 Total bicarbonate and rumen fluid pH -

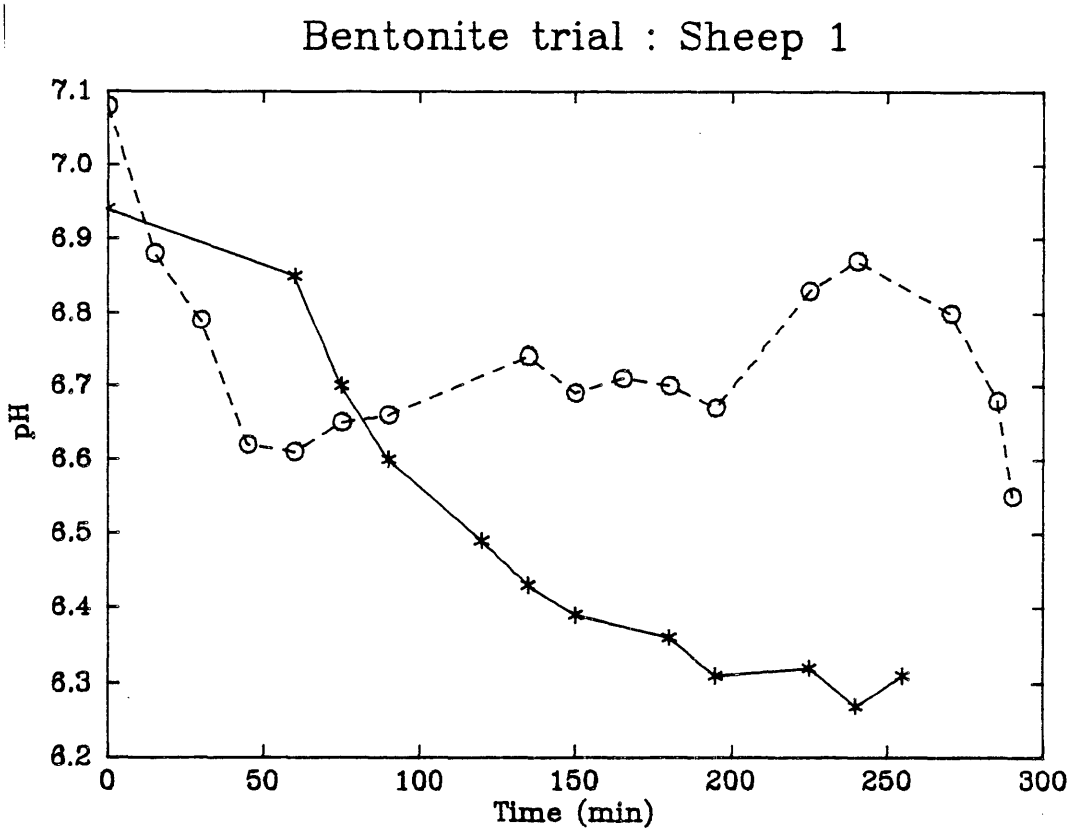
Figure 6.2 shows the change in pH with time for the four sheep with basal diet alone (*) and basal diet plus 2% Na-bentonite (pH 9.6) (o). Figure 6.3 gives the decrease in total bicarbonate with time for sheep on basal diet (*) and basal diet plus 2% Na-bentonite (pH 9.6) (o). In both figures, no difference could be found between the two types of Na-bentonite in its effect on pH or total bicarbonate. However, in all animals , initial pH was higher when Na-bentonite was included in the diet. In all but sheep 1, the initial total bicarbonate was higher with Na-bentonite included in the diet.

The total bicarbonate and pH used for Figures 6.2 and 6.3 are given in Appendix D with the concentration of HCO_3^-, H_2CO_3 and the proportion of total bicarbonate as H_2CO_3.

6.3.3 Experiment 7

6.3.3.1 Estimates of flow rates of carbon -

The flows of C around the rumen bicarbonate, blood bicarbonate and CO_2 pools are shown in Figures 6.4 and 6.5. Flows represent transfer (gC/d) between these pools for each animal at a pre- and post-feeding period.



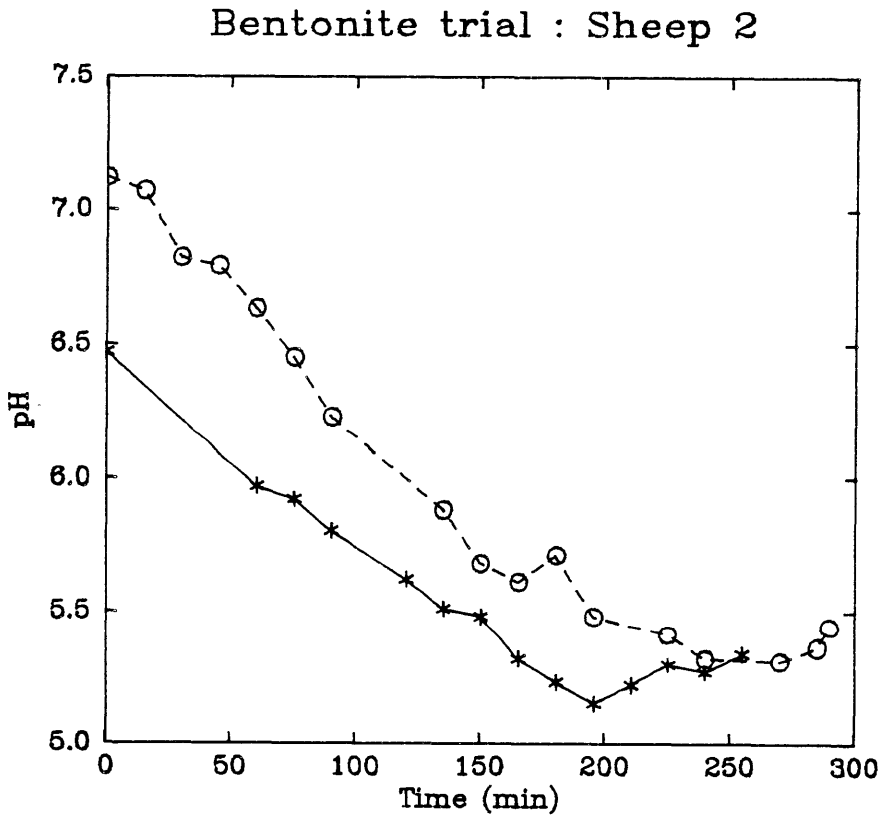
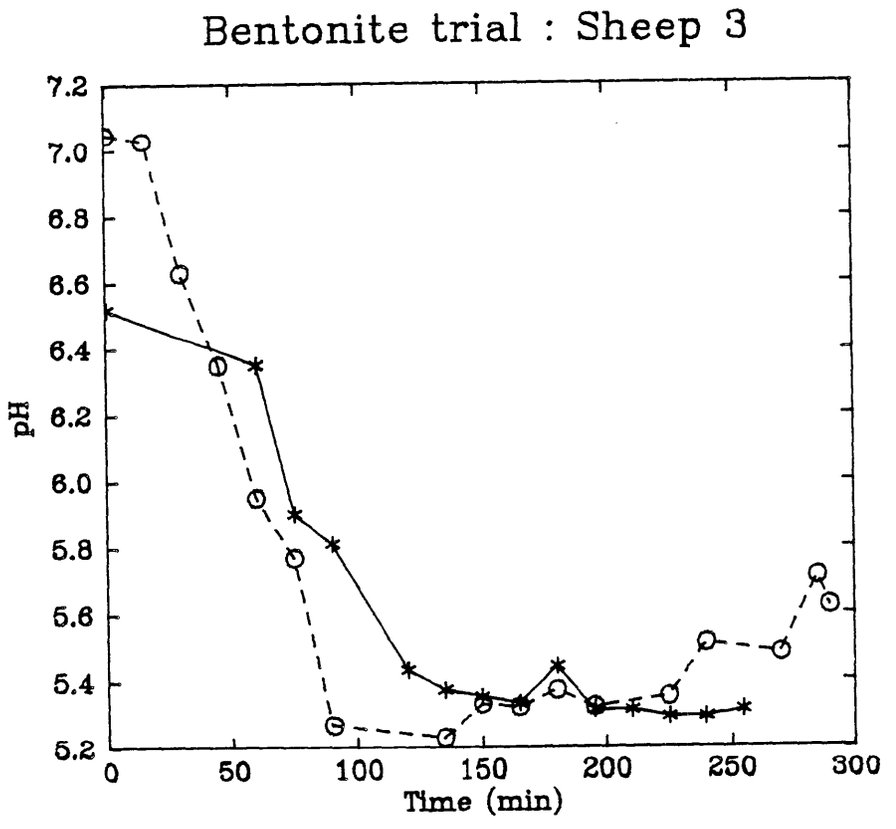


Figure 6.2: The change in pH with time for basal diet alone (*) and basal diet plus Na-bentonite (o) for (a) Sheep 1, (b) Sheep 2, (c) Sheep 3 and (d) Sheep 4 (experiment 6b).



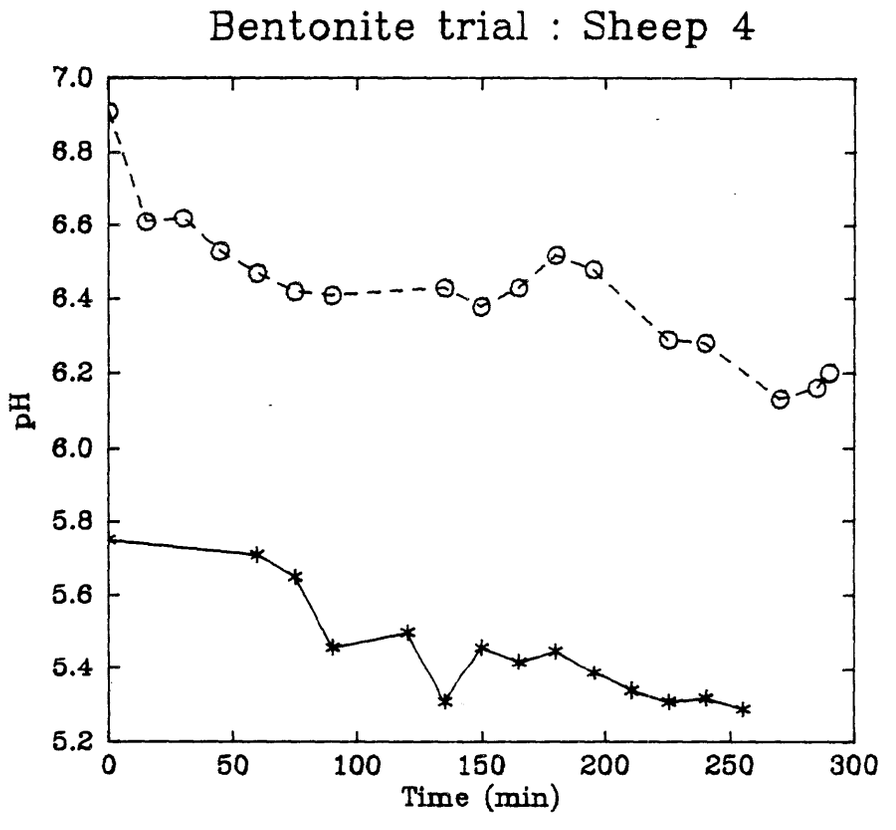
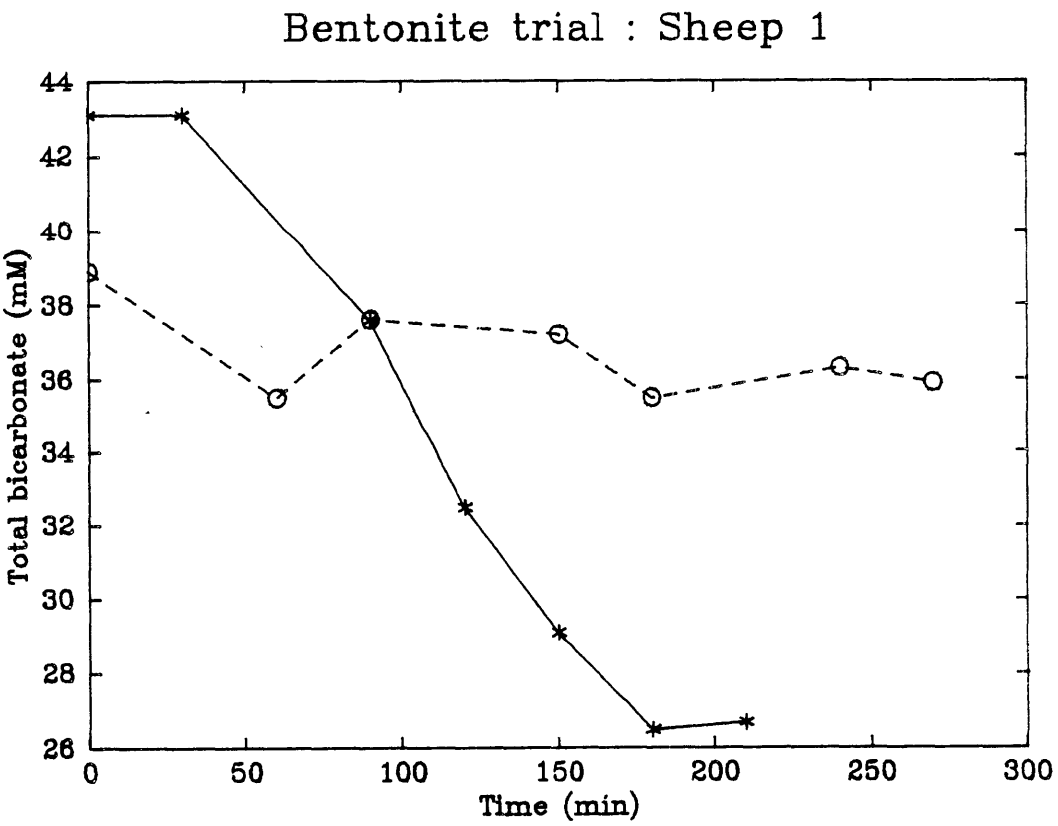


Figure 6.2: continued.



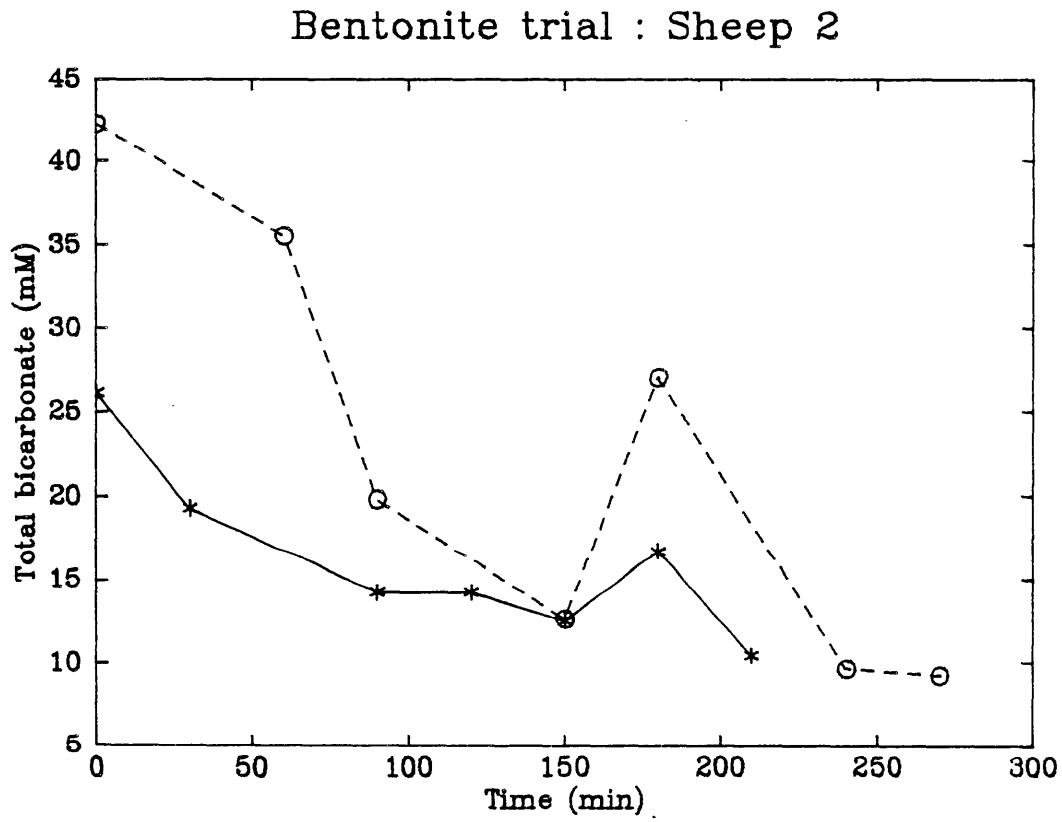
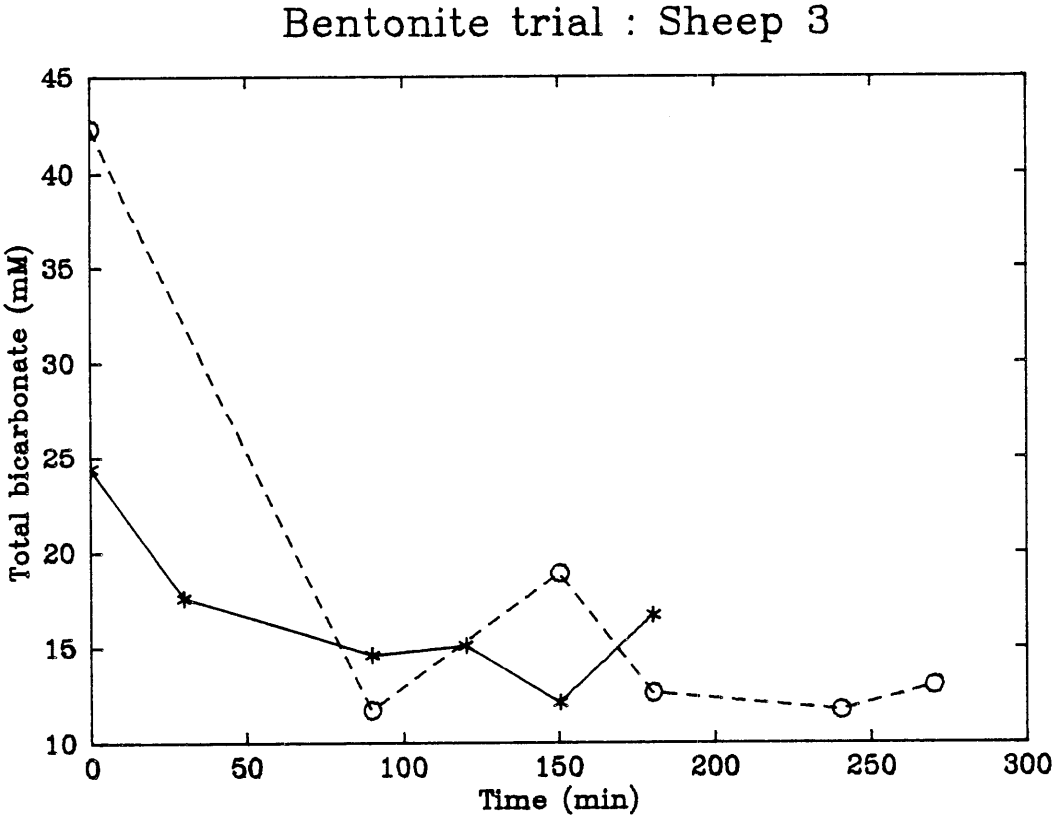


Figure 6.3: The decrease in total bicarbonate with time for the basal diet (*) and basal diet plus Na-bentonite (o) for (a) Sheep 1, (b) Sheep 2, (c) Sheep 3 and (d) Sheep 4 (experiment 6b).



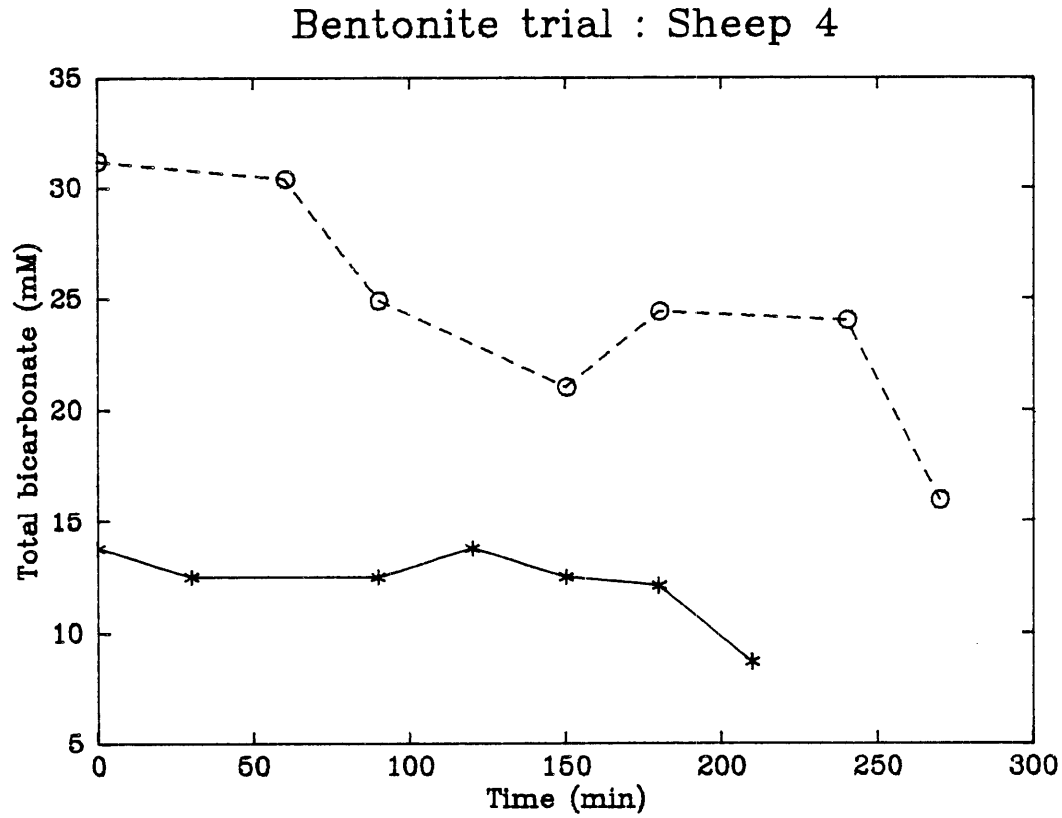
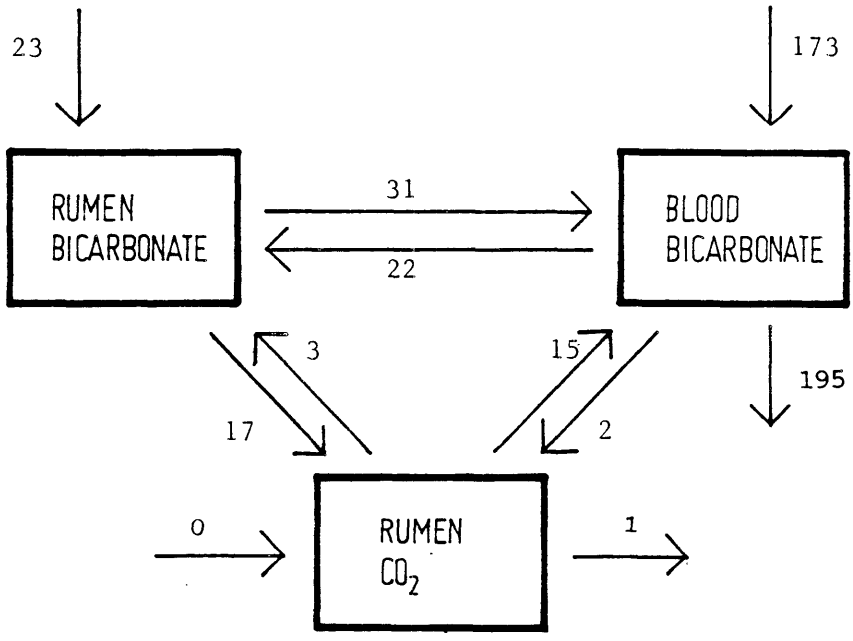


Figure 6.3: continued.

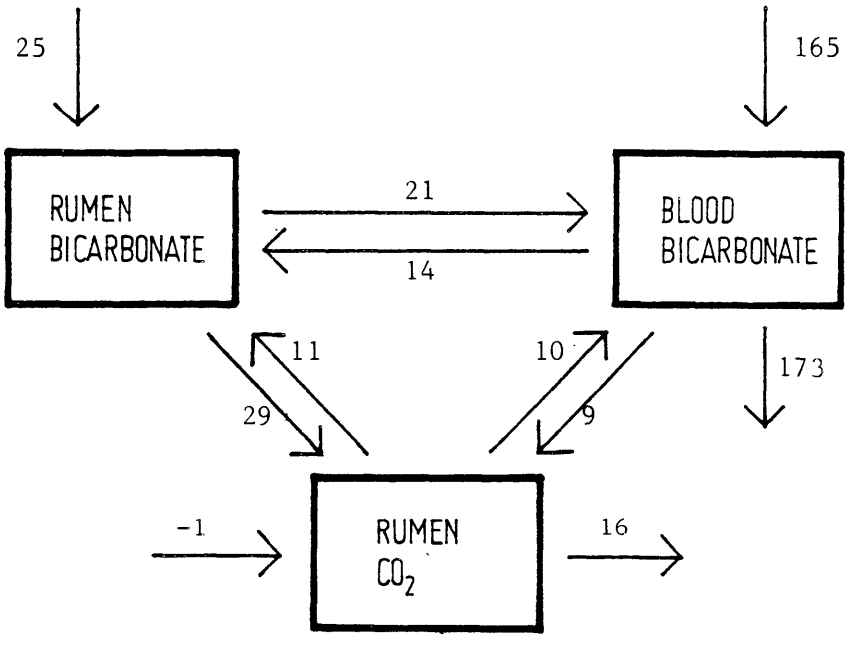
a.



C Flow	SE	C Flow	SE
F_{10}	2.6	F_{32}	2.0
F_{21}	5.8	F_{23}	1.4
F_{20}	16.0	F_{02}	19.3
F_{12}	3.3	F_{30}	1.6
F_{13}	0.3	F_{03}	3.6
F_{31}	3.4		

Figure 6.4: Three pool models describing the flow of carbon (gC/d) between the rumen fluid and blood bicarbonate and CO_2 gas compartments for:
a. Sheep 78 (Basal diet + Na-bentonite: pH 5.5)
b. Sheep 60 (Basal diet + Na-bentonite: pH 5.5)
c. Sheep 187 (Basal diet + Na-bentonite: pH 9.6)
d. Sheep 65 (Basal diet + Na-bentonite: pH 9.6)
in the pre-feeding period (experiment 7)(SE are tabled for each flow of C (Section 4.5.2) for each sheep).

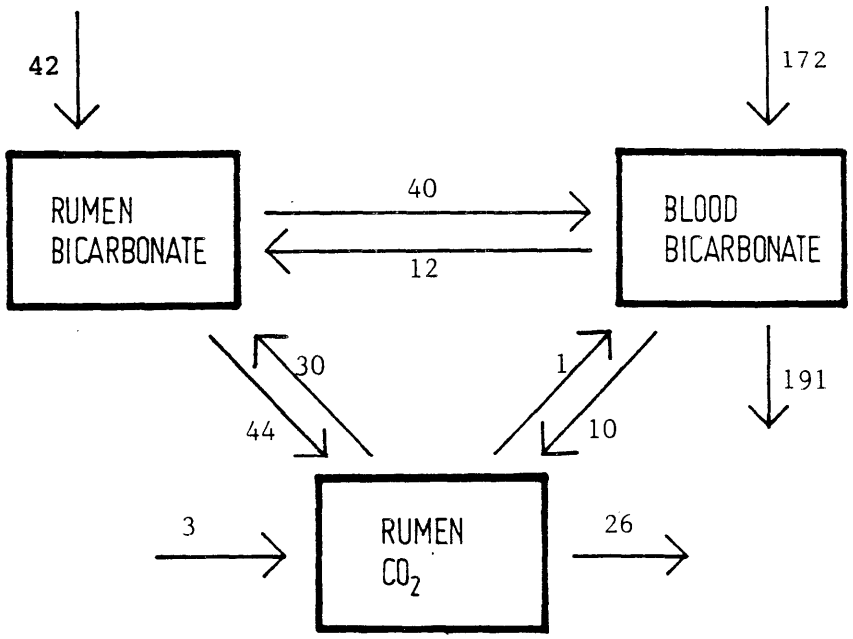
b.



C Flow	SE	C Flow	SE
F_{10}	4.6	F_{32}	3.8
F_{21}	3.9	F_{23}	1.4
F_{20}	9.9	F_{02}	11.2
F_{12}	5.5	F_{30}	3.4
F_{13}	0.9	F_{03}	2.6
F_{31}	3.3		

Figure 6.4: continued.

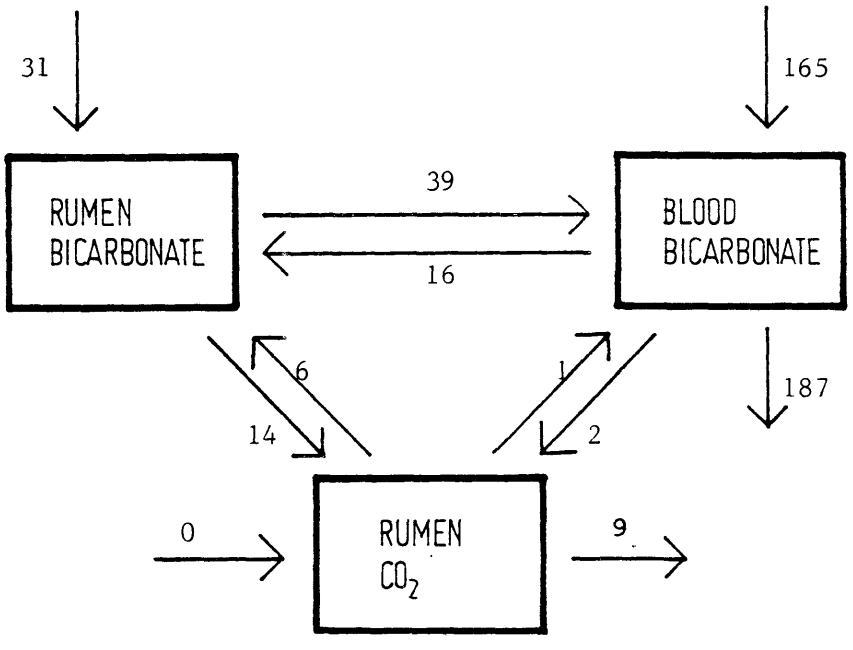
c.



C Flow	SE	C Flow	SE
F_{10}	4.8	F_{32}	4.3
F_{21}	6.5	F_{23}	5.3
F_{20}	8.8	F_{02}	9.6
F_{12}	4.3	F_{30}	5.0
F_{13}	12.2	F_{03}	6.7
F_{31}	10.4		

Figure 6.4: continued.

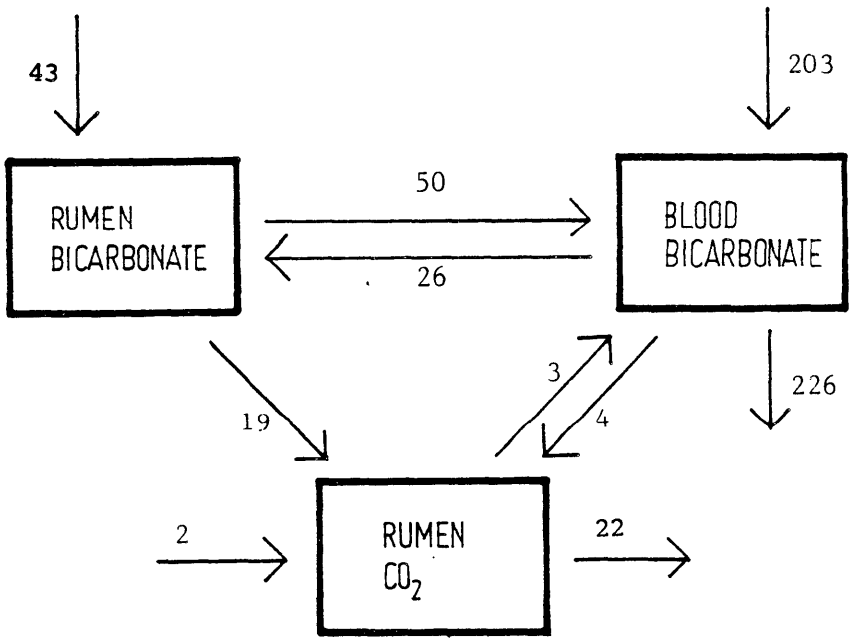
d.



C Flow	SE	C Flow	SE
F_{10}	4.0	F_{32}	1.3
F_{21}	6.7	F_{23}	2.8
F_{20}	12.0	F_{02}	12.4
F_{12}	3.2	F_{30}	1.4
F_{13}	3.6	F_{03}	3.4
F_{31}	3.2		

Figure 6.4: continued.

a.



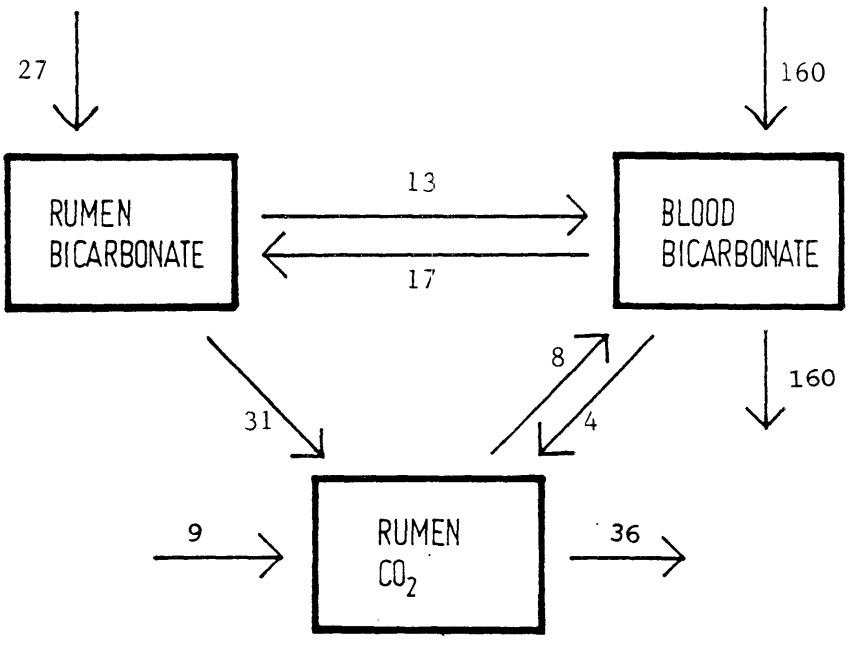
C Flow	SE	C Flow	SE
F_{10}	3.3	F_{32}	2.3
F_{21}	5.1	F_{23}	1.3
F_{20}	16.9	F_{02}	20.4
F_{12}	3.0	F_{30}	2.2
F_{31}	2.7	F_{03}	3.1

Figure 6.5: Three pool models describing the flow of carbon (gC/d) between the rumen fluid and blood bicarbonate and CO_2 gas compartments for:

a. Sheep 78 (Basal diet + Na-bentonite: pH 5.5)
b. Sheep 60 (Basal diet + Na-bentonite: pH 5.5)
c. Sheep 187 (Basal diet + Na-bentonite: pH 9.6)
d. Sheep 65 (Basal diet + Na-bentonite: pH 9.6)

in the post-feeding period (experiment 7)(SE are tabled for each flow of C (Section 4.5.2) for each sheep).

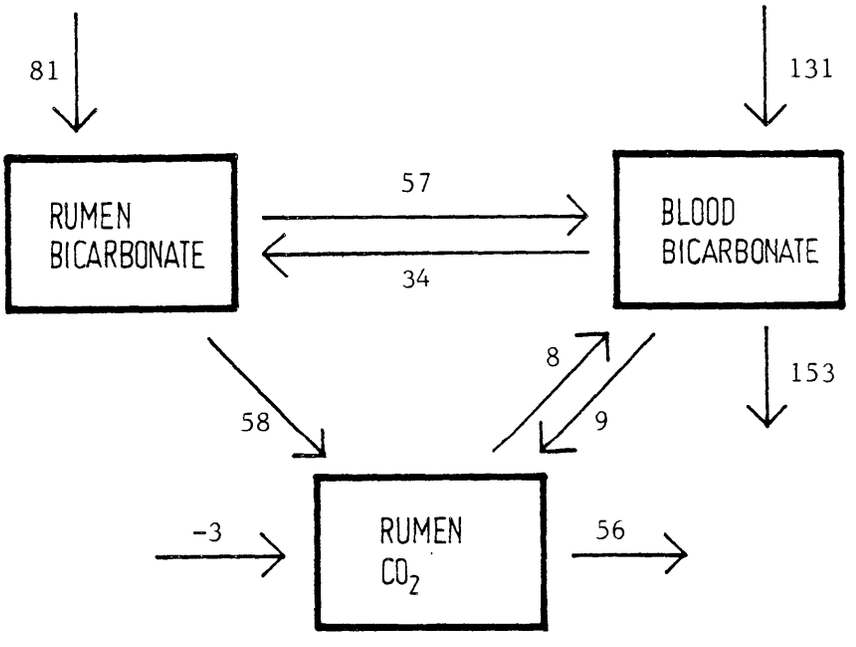
b.



C Flow	SE	C Flow	SE
F_{10}	3.1	F_{32}	3.3
F_{21}	2.7	F_{23}	2.1
F_{20}	10.9	F_{02}	12.8
F_{12}	3.0	F_{30}	3.1
F_{31}	3.2	F_{03}	3.4

Figure 6,5: continued.

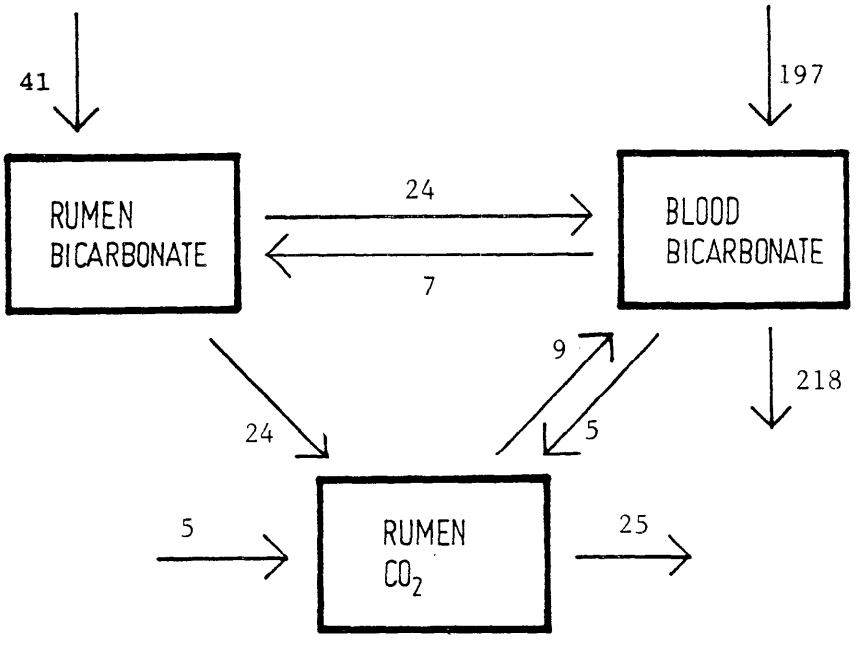
c.



C Flow	SE	C Flow	SE
F_{10}	5.1	F_{32}	2.4
F_{21}	2.2	F_{23}	1.1
F_{20}	4.6	F_{02}	7.7
F_{12}	4.5	F_{30}	1.6
F_{31}	6.1	F_{03}	6.6

Figure 6.5: continued.

d.



C Flow	SE	C Flow	SE
F_{10}	2.1	F_{32}	0.8
F_{21}	2.5	F_{23}	1.3
F_{20}	9.3	F_{02}	11.0
F_{12}	0.7	F_{30}	1.9
F_{31}	2.2	F_{03}	2.4

Figure 6.5: continued.

6.3.3.2 Rumen fluid pH and bicarbonate concentration -

Figures 6.6 and 6.7 show the change in pH and total bicarbonate respectively during the 5 day experimental period. These were constructed using the data given in Appendix D which also includes the concentration of HCO_3^-, H_2CO_3 and proportion of total bicarbonate as H_2CO_3.

There is an increase in pH over the experimental period for both the pre- and post-feeding periods although the rate of increase is greater for the pre-feeding period. No significant difference ($P<.05$) occurs between the two types of Na-bentonite in either the pre- or post-feeding periods.

6.3.3.3 VFA and gas proportions -

Mean VFA proportions and total VFA concentration for the pre- and post-feed periods are shown in Table 6.3. No significant difference ($P<.05$) was found between animals in the pre-feed period or the post-feed period for VFA. A significant increase ($P<.05$) in propionate and significant decrease ($P<.05$) in acetate occured. There was no change in total VFA concentration.

The proportions of CO_2 and CH_4 in rumen gas are shown in Tables 6.4 and 6.5. An increase in the proportion of CO_2 gas and ratio of CO_2/CH_4 occurred in each animal between the pre- and post-feeding periods.

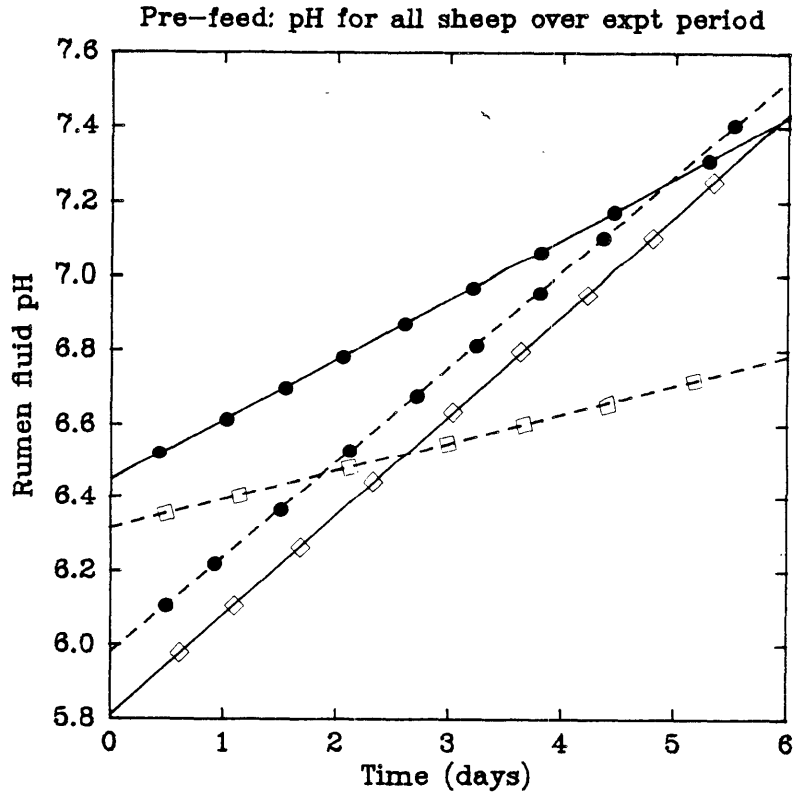


Figure 6.6: The relationship between rumen fluid pH and time for the pre-feed periods of sheep used in experiment 7 given Na-bentonite (pH 5.5)(———) and Na-bentonite (pH 9.6)(- - - -). The regression equations were:

a. Sheep 78 —●—● y = 0.18x + 6.42 (r^2 = 0.92, RSD = 0.134)
b. Sheep 60 —□—□— y = 0.27x + 5.81 (r^2 = 0.95, RSD = 0.148)
c. Sheep 187 —●—● - y = 0.26x + 5.98 (r^2 = 0.97, RSD = 0.119)
d. Sheep 65 -□-□- y = 0.08x + 6.32 (r^2 = 0.77, RSD = 0.014)

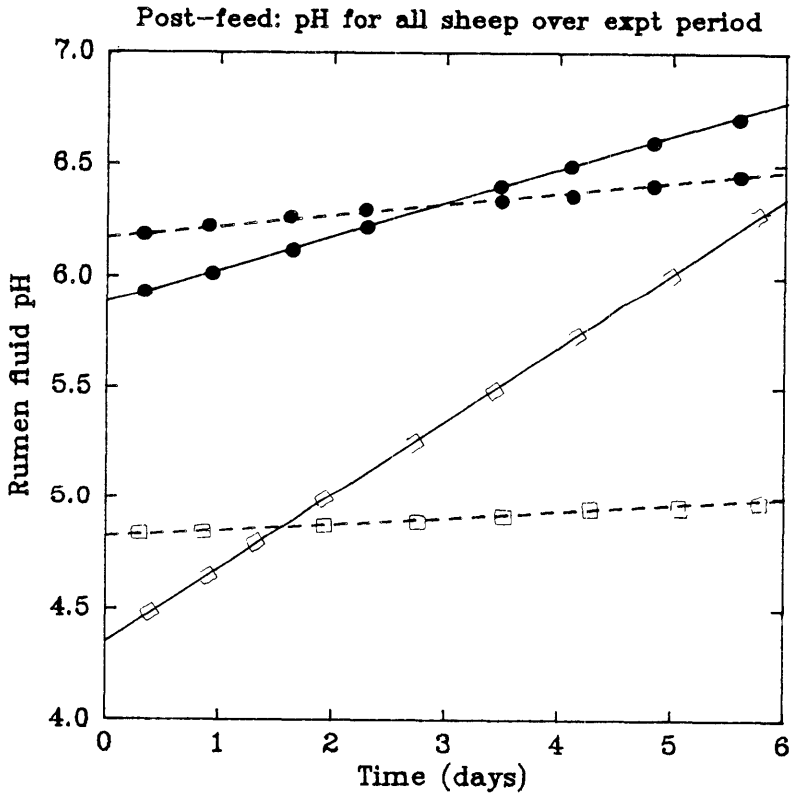


Figure 6.7 The relationship between rumen fluid pH and time for post feeding periods for sheep used in experiment 7 given Na-bentonite (pH 5.5)(———) and Na-bentonite (pH 9.6) (- - - -). The regression equations were:

a. Sheep 78 —●—●— $y = 0.15x + 5.88$ ($r^2 = 0.96$, RSD = 0.079)
b. Sheep 60 —□—□— $y = 0.33x + 4.35$ ($r^2 = 0.79$, RSD = 0.389)
c. Sheep 187 -●- -●- $y = 0.05x + 6.17$ ($r^2 = 0.94$, RSD = 0.096)
d. Sheep 65 -□- -□- $y = 0.03x + 4.82$ ($r^2 = 0.42$, RSD = 0.699)

Table 6.3: Mean VFA proportions and total VFA concentrations for all sheep used in experiment 7.

Sample period	Acetate	Propionate	Butyrate	Others	Total conc. mM
Pre-feed	63.2[a]*	22.1[a]	9.0[a]	4.9[a]	94[a]
Post-feed	50.8[b]	27.4[b]	8.6[a]	3.6[b]	117[a]

*Column values with different superscripts differ significantly ($P<.05$).

Table 6.4: The proportions of CO_2 and CH_4 in rumen gas and ratio of CO_2 to CH_4 for sheep used in experiment 7 (pre-feed).

Animal	CH_4	CO_2	Ratio CO_2/CH_4
78	.36	.57	1:1.57
60	.35	.54	1:1.53
187	.35	.64	1:1.85
65	.18	.56	1:3.07

Table 6.5: The proportions of CO_2 and CH_4 in rumen gas and ratio of CO_2 to CH_4 for sheep used in experiment 7 (post-feed).

Animal	CH_4	CO_2	Ratio CO_2/CH_4
78	.19	.49	1:2.55
60	.27	.63	1:2.37
187	.22	.59	1:2.69
65	.20	.70	1:3.36

6.4 DISCUSSION

In the animals in experiment 7, following feeding, there was an increase in fermentation rate. The increased VFA production and lowering of rumen fluid pH resulted in increases in the transfer of bicarbonate from rumen bicarbonate to the CO_2 gas pool and from the CO_2 gas pool to blood bicarbonate. This, with the increase in CO_2 lost through eructation (Figures 6.4 and 6.5) and the increase in the proportion of CO_2 gas (Table 6.4) after feeding, demonstrate the large release of gas with which the ruminant must cope. Manipulation of the rumen contents to increase rumen fluid pH during rapid fermentation may ameliorate any detrimental effect by giving the animal greater buffering ability in the rumen fluid and also holding pH above the range in which bloat foam is most stable.

The ability of Na-bentonite to create a higher buffering of rumen contents can be seen in the results given in Figures 6.2 and 6.3. The maintenance of higher rumen fluid pH is assisted by a higher initial bicarbonate level. Over a longer term (experiment 7), the pre-feeding pH and presumably total bicarbonate in rumen fluid of the sheep, showed a gradual increase over the experimental period of 5 days. The same trend also occured for the rumen fluid pH in the post-feeding period although to a lesser extent. Since experiment 7 was conducted over a 5 day period only, the maximum level to which initial pH and bicarbonate could rise was not determined.

The mode of action of Na-bentonite in increasing rumen fluid pH is not clear. It has been suggested that when grazing cattle were fed Na-bentonite, there was an increase in salivary flow rate (Gibbs, pers. comm., 1983). This is yet to be shown experimently , but if this is the case, then Na-bentonite itself may not be acting as a

buffer, but its effects are brought about by increasing the salivary buffer components in the rumen fluid.

The relationship between total bicarbonate and pH of rumen fluid (Figure 6.1) indicates that in the region where bicarbonate is the important buffer component of the rumen system, the inclusion of Na-bentonite (pH 9.6) in the diet results in an increase in rumen fluid pH at the same level of total bicarbonate concentration. Therefore, although Na-bentonite may be increasing the buffer components reaching the rumen via saliva, it is not solely the increase in total bicarbonate concentration of rumen fluid (Figures 6.2 and 6.3) that is responsible for the increase in rumen fluid pH. This however is not proof that Na-bentonite is acting as a buffer itself.

The ability of Na-bentonite to adsorb enzymes, peptides and proteins is well known (Theng, 1979, p157-226). If the enzymes specific to fermentation or the co-factors required in the fermentative pathways are adsorbed, then there may be a slowing down of fermentation rate and hence acid production which would lessen the potential for a rapid drop in rumen pH and the lower levels reached.

The reason for the increased total bicarbonate in the rumen fluid of animals fed Na-bentonite is not apparent from the models shown in Figures 6.4 and 6.5 which only compare two different Na-bentonites i.e. pH 5.5 and pH 9.6. No significant difference ($P<.05$) could be found between these two types of Na-bentonite for flow rates between compartments.

When compared with the models for sheep used in experiment 3, the models depicting Na-bentonite included in the diet of sheep in experiment 7 show a greater amount of CO_2 gas irreversibly leaving the rumen. This appears to arise partially from a reduction in the net movement of CO_2 from the CO_2 gas pool into the blood bicarbonate pool. This would be advantageous to the animal where lower rumen fluid pH results in a large amount of CO_2 because the gas could be removed from the rumen by eructation rather than remaining within the rumen fluid.

With an increase in fermentation and rapid production of VFA, rumen fluid pH decreases. Under these conditions, the increased production of propionate and decreased production of acetate in Section 6.3.3.3 are also apparent in the comparison of pre- and post-feeding periods. No significant difference ($P<.05$) was found between the two Na-bentonites. However, a comparison between animals at higher rumen fluid pH (experiment 2) and the relatively lower rumen fluid pH (experiment 3) showed an increase in total VFA concentrations. This increase was larger than the increase between the pre- and post-feeding periods of experiment 7.

Although no significant difference was found between treatments for protozoal numbers in experiment 6a due to the large variation, the sheep receiving Na-bentonite (pH 9.6) tended to have a larger number of small protozoa. This could be due to the better buffering ability in the rumens of animals 7-10. Since protozoal numbers are dramatically and adversely affected by low rumen fluid pH (Purser and Moir, 1959), a rumen which was buffered more effectively would allow greater numbers of protozoa to survive.

The assumption that pH is relatively high prior to feeding and decreases following feeding has been recorded (Figure 6.2). It was also noted that there was quite a variation in rumen fluid pH between sheep at the same time relative to feeding (Figure 6.2). In experiment 7, it was desired to consider the effect of Na-bentonite and pH on bicarbonate and CO_2 dynamics. A large resource commitment is required for isotope dilution studies and this limits the number of experiments which can be carried out. In order to evaluate any effect of Na-bentonite and pH on carbon dynamics and considering the variation in rumen fluid characteristics between animals, it was decided to complete isotope infusions within one sheep. This gave a comparison between rumen parameters at relatively high rumen fluid pH (pre-feeding) and low rumen fluid pH (post-feeding). The validity of applying isotope dilution kinetics to such a method is perhaps questionable. In an attempt to satisfy the assumption of steady state (see Section 4.2), animals are generally fed at short, regular time intervals.

Rumen pH varies diurnally depending on feeding patterns; the pre-feeding period is characterised by a relatively stable plateau of higher rumen fluid pH. Following feeding, a rapid drop in rumen fluid pH occurs. The rate of decrease in pH generally levels out at about 4 hr following feeding and remains relatively stable. This is evident in Figure 6.2. Rumen pH then remains at a low level for several hours before rising again.

An alternative approach to the one chosen could have been to alter the diet in two separate isotope experiments to manipulate rumen fluid pH. It is possible to manipulate rumen fluid pH by feeding different diets. A high grain diet can be fed in order to decrease rumen fluid pH. However, in an attempt to create steady state

conditions, animals are maintained on a diet, fed at short regular intervals for a period of several days prior to experimentation. During this period, there is a gradual increase in mean rumen fluid pH (see Figures 6.6 and 6.7). The aim of experiment 7 was to compare bicarbonate dynamics at relatively high and low rumen fluid pH within each animal. Any effect of pH on carbon dynamics would be highlighted by a large difference between low and high pH levels. If the situation mentioned above exists, with rumen pH gradually increasing, the effect of rumen fluid pH on carbon dynamics could possibly be masked.

Although steady state conditions were not maintained in experiment 7, the above reasons of gradual increases in rumen fluid pH with time and relatively stable plateaus of pH at pre-feeding and 4 hr following feeding were thought sufficiently valid to use the method selected. A future approach which could be considered by researchers is to develop mathematical solutions for the non-steady state situation.

CHAPTER 7

GENERAL DISCUSSION AND CONCLUSIONS

7.1 EXPERIMENTAL TECHNIQUES

In the studies presented here, isotope dilution techniques have been used to quantitatively assess the movement of C between specific compartments that contain CO_2 (H_2CO_3, HCO_3^- and CO_2) in sheep. In using these techniques, it is essential that the basic assumptions of steady state and adequate mixing of the isotope occur within the pools of tracee being considered. However, the large numbers of analyses, and therefore large amount of time involved with trials of this nature meant that a maximum of four animals could be used for each tracer experiment. There was considerable variation between animals in the values obtained for the flow rate of C between specific pools, and the SE associated with that flow. Therefore, although a compartment modelling approach gives a satisfactory estimate of flow rate between compartments, the use of a small number of animals could reduce the confidence placed in the results.

Variations in the plateau SR over a sampling period also contributes to the error associated with model analysis. Largest variation was found in the rumen CO_2 gas pool and the smallest variation with the blood bicarbonate pool. This is probably due to

the blood bicarbonate pool being a relatively large pool with a relatively finely controlled pH and bicarbonate levels whereas the CO_2 gas pool is small and has a rapid throughput of CO_2. A pool with a rapid turnover rate such as the CO_2 gas pool, and a pool size that continuously fluctuates through timed eructations and in which infusions of tracer are made at a constant rate, is more likely to have larger variations in IL and transfer quotient leading to a greater SE associated with that flow rate.

7.2 CONCLUSIONS

When ruminants are fed a diet high in concentrate, the concomitant increase in fermentation rate results in an increase in the production of CO_2 by microbes and a decrease in the concentration of bicarbonate and pH of rumen fluid.

The present study shows that at a relatively high rumen fluid pH, bicarbonate (perhaps as CO_2) is largely absorbed. However, an increase in the acidity of rumen fluid results in an increase in the amount of CO_2 moving through the liquid phase into the CO_2 gas pool. A decrease in the pH of rumen fluid also resulted in an increase in the amount of CO_2 absorbed into the blood from the rumen CO_2 gas pool.

When bicarbonate in the liquid phase moves from the rumen into the omasum, it is mostly absorbed into blood rather than moving back into the rumen as CO_2. It is possible that some bicarbonate moves back from the omasum into the rumen, however, the amount appears to be small.

The inclusion of a Na-bentonite supplement in the diet of sheep led to an increase in rumen fluid pH which was partially due to an increase in the concentration of bicarbonate in the rumen fluid resulting in a more stable rumen environment.

7.3 FUTURE RESEARCH PRIORITIES

Further studies are required to evaluate quantitatively similar compartmental models in cattle. This would give the researcher an experimental basis with which to work when evaluating the implications of rapid gas production and accumulation in the rumen of cattle on the occurence of bloat. Preliminary studies examining the rate of flow of C between the rumen fluid bicarbonate, blood bicarbonate and rumen CO_2 gas pools under high and low rumen fluid pH have shown similar trends to those in the sheep as reported in this thesis. These studies do however need to be done in more detail.

Future research regarding Na-bentonite should take two directions:

a) A detailed examination of the exact mode of action of Na-bentonite in raising rumen fluid pH and the effect of Na-bentonite on other aspects of rumen function.

b) The use of Na-bentonite in stabilizing rumen fluid pH in cattle in the field under conditions where rapid gas production, and possibly bloat can occur. This must also encompass a study to find the best means of administering Na-bentonite to the grazing animal.