Chapter 3 Experimental Study

The study presented in this thesis was designed to investigate the effects of the presence or absence of protozoa in the rumen, and of methionine supplement when protozoa are present in the rumen, on ruminal metabolism and microbial production of LCFA.

3.1 Materials

3.1.1 Experimental animals

Twelve first-cross Merino x Border Leicester wethers (2 years old) with body weights of between 35 and 44 kg were used in this study. All animals were surgically prepared with rumina and abomasal cannulas at the commencement of the study.

The ruminal cannula bung was equipped with 2 stainless-steel probes whose inner ends were directed away from one another (Figure 3.1). To the tip of the probe from which ruminal fluid was collected, a small metal cage covered with gauze was attached and directed to the ventral sac of the rumen. The second probe used for the administration of solutions (CrEDTA and, ^{14}C-labelled acetate) was directed to the dorsal position in order to prevent a direct sampling of the infused solutions.

3.1.2 Diet

All sheep were offered a mix of 400 g of oaten chaff + 400 g of lucerne chaff per day (air dry). The oaten chaff was previously spray treated with urea (2 %, w/w) and a mineral mix (2.8 %, w/w) containing the following ingredients: dicalcium phosphate (45.5 %), sodium sulphate (36.4 %), sodium chloride (9.1 %) and trace minerals (Pfizer; 9.1 %).

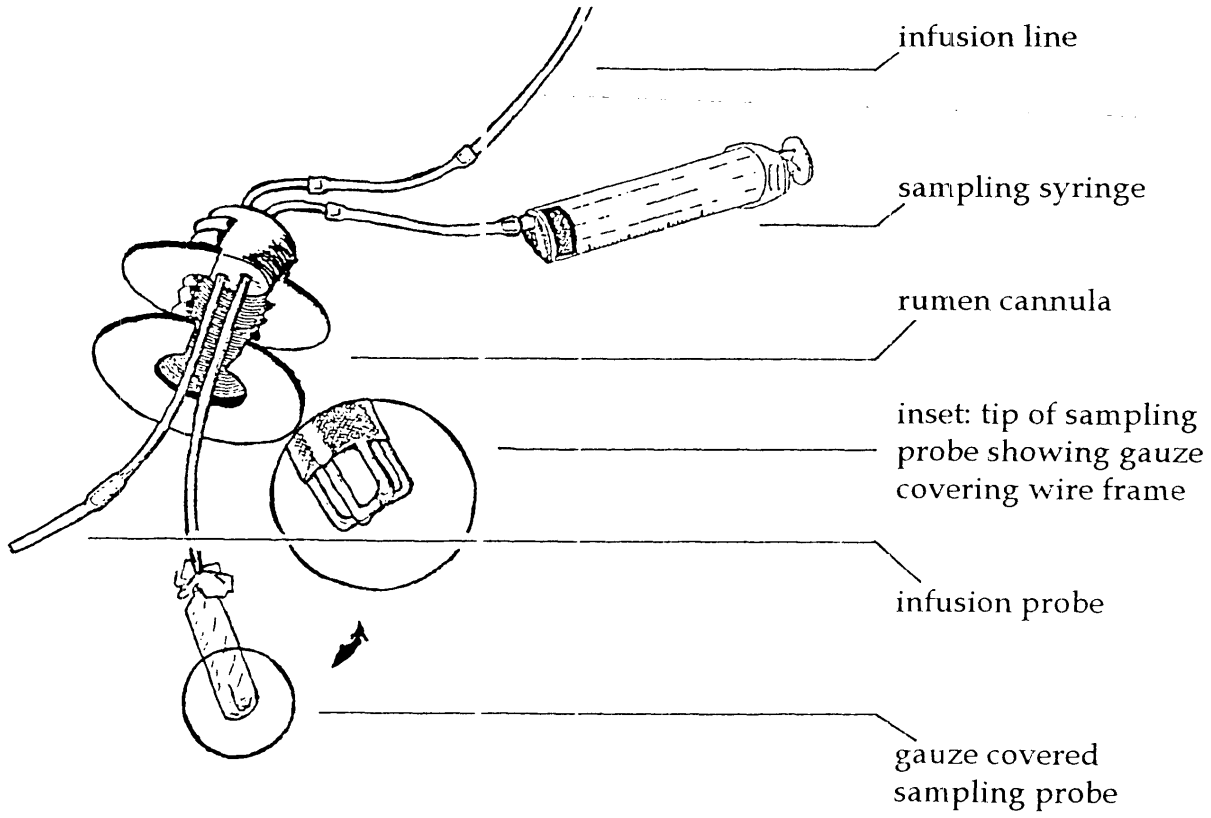

Figure 3.1. Ruminal cannula with its 2 stainless-steel probes. Note that probe carrying infusion solution is directed dorsally, sampling probe is ventrally directed. (Thanks to Jenny Hegarty for artwork.)

3.2 Methods

3.2.1 Defaunation and refaunation procedures

All sheep were initially defaunated using the detergent alkanate 3SL3 (ICI Australia Ltd.; active ingredient sodium lauryl diethoxy sulphate). Approximately 120 ml of alkanate solution (10% w/v) was administered into the rumen through the ruminal cannula on each of 4 consecutive days. Feed was not offered during the treatment, but clean water was freely available. Sheep were allowed at least 2 weeks to recover from treatment prior to the commencement of study.

Eight sheep were refaunated by drenching with 60 ml of ruminal fluid from additional refaunated sheep held on similar feed. This drenching was performed on each of 3 consecutive days. For refaunated animals, ruminal fluid was additionally transferred from one animal to another in order to unify the rumen environments.

3.2.2 Management of animals

Ruminal fluid of defaunated animals was checked to ensure the absence of protozoa at the commencement and completion of the experiment. Defaunated and refaunated groups were housed in separate rooms in individual metabolic crates. The rooms were controlled at a constant temperature of 22 °C and were well ventilated. Animals were adapted to the new environment for at least 7 d before commencement of the study.

3.2.3 Feeding

Oaten chaff impregnated with urea and minerals (400 g) plus 400 g of lucerne chaff was offered as the daily ration to each sheep. The ration was delivered in 24 equal portions at hourly intervals via an overhead automatic-feeding machine. The oaten and lucerne chaffs were evenly mixed during placement on the belt of the machine. Drinking water was available *ad libitum* throughout the experiment. Animals were allowed to become accustomed to the feed provided daily, then twice daily over several weeks, and then hourly for at least 1 week each before the commencement of the study.

3.2.4 Administration of CrEDTA solution

3.2.4.1 Intraruminal infusion of the solution

To measure the flow of digesta to the abomasum, the double marker method of Faichney (1975) was employed. Chromium ethylenediamine tetra-acetic acid (CrEDTA) complex and dietary acid-insoluble ash (AIA) were used to measure the flow of liquid- and particle-digesta, respectively. A solution containing CrEDTA complex (120 μgCr/m) was continuously infused into the rumen via the stainless-steel probe with the tip directed dorsally. Enriched ^{15}N labelled ammonium sulphate was also included in the infusate but analysis arising from this is outside the scope of this thesis. The CrEDTA complex solution was prepared according to the method of Binnerts *et al.* (1968).

The continuous infusion was made intraruminally at a constant, measured rate (approximately 0.4 ml/min) by a peristaltic pump. Infusion solution was delivered through silastic tubing (1.5 mm internal diameter) by the peristaltic pump to the rumen probe. Infusion rate was calculated from the change in mass of the reservoir containing the CrEDTA infusate over periods of 3 - 8 h. Infusions were made for 5 d with samples being collected on days 3, 4 and 5, and ruminal fluid being further sampled over day 6. Samples of ruminal fluid, ruminal and abomasal digesta were collected before and during the infusion. Samples of ruminal fluid were also collected after the infusion to estimate rumen volume, $t_{1/2}$ and ruminal liquid outflow rate of defaunated sheep. Post-infusion samples were not collected for refaunated sheep, so a single injection of CrEDTA was made in this group (*3.2.4.2*).

3.2.4.2 Intraruminal injection of the solution

An intraruminal injection of CrEDTA (2.91 mgCr/ml) was made in the refaunated sheep because inadequate samples were collected after the first infusion period. To ascertain the kinetics of ruminal liquid, the CrEDTA and ^{15}N solution were administered by a single intraruminal injection, 3 d after the intra-ruminal infusion of the solution. The solution was taken into a syringe, weighed and injected via the stainless-steel probe. The injectate was directed to six sites within the rumen and was washed in with 25 ml of warm water, the empty syringe was reweighed and the mass of injected dose calculated.

3.2.5 Intraruminal infusion of ^{14}C-labelled acetate

Production rate of total and individual VFA (acetate, propionate and butyrate) in the rumen were determined by a continuous infusion of 1-^{14}C-acetate into the

rumen according to the method o' Weller *et al.* (1967). A stock solution of 1-^{14}C-acetate was prepared by adding about 1 mCi of the ^{14}C-acetic acid sodium salt (Dupont, Boston, USA) to a base solution containing 10 ml of milli Q water, 100 mg of Na-acetate and 2 drops of 1 M NaOH. A subsample (4 ml containing 0.364 mCi 1-^{14}C-acetate) of stock solution was taken into 1.94 l of milli Q water and made alkaline with 1 ml of 1 M NaOH to be used as the infusion solution.

Ruminal fluid was taken from each sheep before the commencement of tracer infusion and analysed to determine background level of radioactivity. The 1-^{14}C-acetate solution was infused intraruminally for 9 h and a series of ruminal fluid samples were collected over the last 6 h of the infusion (*3.2.6.1*).

3.2.6 Sampling procedures

3.2.6.1 Ruminal fluid

Samples of ruminal fluid were always taken prior to the intraruminal infusion and injection of the tracer solutions to determine the background level of Cr and ^{14}C. Beginning on day 4 of the continuous, intraruminal infusion of the CrEDTA solution (*3.2.4.1*), seven ruminal fluid samples were collected representing 3 hour intervals over the following 48 hours. Approximately 13 ruminal fluid samples were taken over 24 h after the infusion stopped (defaunated sheep) and following the single injection of the solution (refaunated sheep) (*3.2.4.2*), with samples being taken at 60 min intervals in the first 4 h. During the last 6 h of the 9 h continuous, intraruminal infusion of the 1-^{14}C-labelled acetate solution (3.2.5), 7 ruminal fluid samples were collected at intervals of 50 min to be used for VFA analysis (*3.2.8.4*).

Ruminal fluid was withdrawn through the ventrally directed rumen probe into a 20 ml syringe. The first 10 ml was rejected and then 15 ml was flushed back into the rumen 2-3 times before the fluid sample was finally taken. Ruminal fluid required for the analysis of Cr (*3.2.8.2*), NH_3 (*3.2.8.3*) and VFA (*3.2.8.4*) was transferred into a 25 ml McCartney bottle containing 4 drops of 18 M H_2SO_4 and stored at –18 °C prior to analysis. For the enumeration of protozoa (3.2.7), ruminal fluid was placed into a vial containing 16 ml of a solution of formaldehyde in physiological saline and stored at room temperature.

3.2.6.2 *Abomasal digesta*

Samples of abomasal digesta were collected during days 4 and 5 of the intra-ruminal infusion of the CrEDTA solution. Eight abomasal digesta samples were collected over this period (at 10 a.m., 1 p.m., 4 p.m. 7 p.m., 10 p.m., 1 a.m., 7 a.m. and 4 a.m). This ensured diurnal variation in digesta flow did not bias results.

Prior to the collection, the abomasal digesta trapped in the cannula stem were scraped out and discarded. A sample of abomasal digesta was collected by placing a bottle underneath the opened abomasal cannula. Abomasal digesta were then divided into 2 portions. The first portion was centrifuged (9,500 g x 15 m) to obtain "fluid-rich" and "particle-rich" fractions, while a second untreated portion of about equal weight was kept "as sampled". All of these fractions were stored at -18 °C.

3.2.6.4 *Urine*

Urine was collected daily for 7 consecutive days into a tared bucket (changed daily) containing 1 l of water and 20 ml of 9 M H_2SO_4. A subsample of the daily

urine excretion of individual sheep (10% of total volume excreted) was transferred into a small plastic bottle and stored at – 18 °C. The subsamples of urine from each sheep were thawed and bulked for the analysis of allantoin (*3.2.8.6*).

3.2.7 Counting of rumen protozoa

The sample of ruminal fluid (*3.2.5.1*) was thoroughly shaken and pipetted onto a counting chamber (Hawksley, Sussex, England) of 0.2 mm depth and covered with a double thickness coverslide. Protozoa were counted on 48 cells of the counting chamber. Protozoa lying on the triple lines of the chamber were included in the counting. Counts were made with the aid of a light microscope (x 100 magnification).

3.2.8 Laboratory analysis

3.2.8.1 Dry matter (DM) and acid-insoluble ash (AIA)

Samples of ground feed (1 mm sieve), abomasal digesta and particles were weighed into cleaned, tared filter crucibles (porosity 4). To determine their DM (dry-matter) contents, samples were dried at a constant temperature of 80 °C for 48 h.

The AIA (acid-insoluble ash) content of DM was determined according to the method of Choct and Annison (1992). Samples of DM were burnt in an ashing oven at a temperature of 480 °C for at least 8 h. The temperature was taken up

gradually (*i.e.* to 150 °C in the first hour, to 300 °C in the second hour and then to 480 °C). The sample was then transferred into an evaporating dish filled with 4 M HCl, until the sample was wetted from underneath. Three-quarters of the crucible was filled with the acid. The evaporating dish was placed on a hot plate and boiled gently for 15 min. After cooling, the crucible was taken out and the acid containing soluble ash was removed by suction. The sample was rinsed with the acid and distilled H_2O. The sample was dried in an oven at 108 °C for 2 h and transferred into the ashing oven to repeat the procedure of ashing and removing the acid-soluble ash. The sample was dried at 108 °C for 6 h and weighed.

3.2.8.2 *Chromium*

Ruminal and abomasal fluid, as well as mixture of 2 g of abomasal particle-rich samples and 8 g of milli Q (extremely pure distilled) water, were centrifuged (9,500 g x 30 min) to obtain supernatants. The concentration of chromium in the supernatants was determined with an atomic absorption spectrophotometer (Perkin-Elmer, Connecticut, USA; Model 360). Stock standard solution (1000 μgCr/ml) was prepared from K_2CrO_4 (Binnerts *et al.*, 1968).

3.2.8.3 *Ruminal ammonia*

The concentration of NH_3 in ruminal fluid (*3.2.6.1*) was determined by steam distillation. The ruminal fluid was centrifuged (14,000 g x 15 min) and pipetted (5 ml) into a distillation flask. A few drops of universal indicator were added as well as anti-foaming agent. This was made alkaline by adding 5 ml of saturated Na-tetraborate buffer (pH 9). The sample was distilled immediately for approximately 3.5 min, and 30 - 40 ml of distillate was collected into a beaker containing 3 ml of 0.05 M H_2SO_4 and titrated to pH 5 with 0.025 M NaOH

(Autoburette ABU12, Radiometer, Copenhagen, Denmark). Ammonia concentration was adjusted for recovery of NH_3 from an $(NH_4)_2SO_4$ standard. Recovery was typically 99 %.

3.2.8.4 Volatile fatty acids (VFA)

The VFA concentration and proportions of acetic, propionic, butyric, isobutyric, isovaleric and valeric acids were determined in the centrifuged ruminal fluid with a gas liquid chromatograph (Model 427, Packard Instrument Co. USA) according to the method of Erwin *et al.* (1961). Isocaproic acid as an internal standard was added to 0.7 ml of the sample, which was then shaken and injected (1.5 μl) into the chromatograph. A recording data processor (Model 604, Packard Instrument Co., USA) was connected to the chromatograph and integrated the area of eluted peaks.

3.2.8.5 Scintillation counting

A ruminal fluid sample was assayed for ^{14}C radioactivity in a Packard Tri-Carb, Model 3255 (Packard Instrument Company, Illinois, USA). Ruminal fluid (0.2 ml) and samples of diluted infusion solution (0.2 ml, dilution factor of 50) were pipetted into scintillation vials. To each vial, 0.8 ml of milli Q water and 10 ml of liquid scintillation cocktail (LSC: toluene/Triton X [9:4], 0.2% [w/v] POPOP and 0.4% PPO) were added. The sample was shaken gently and allowed to stand at room temperature for at least 24 h before radioactivity was measured.

3.2.8.6 Urinary allantoin

The colorimetric method of allantoin determination of Young and Conway, modified by Chen and Gomez (1992), was applied in this study. Small volumes

(0.5 ml) of diluted urine sample (dilution factor of 40), standard and blank (distilled water) were pipetted into 15 ml tubes, to which were added 2.5 ml of distilled water and 0.5 ml of NaOH (0.5 M). After vortexing, this mixture was placed in a boiling water bath for 7 min and then cooled in iced water. HCl (0.5 M, 0.5 ml) was added, so pH was within the range of 2-3. Phenylhydrazine-HCl solution (0.023 M, freshly prepared, 0.5 ml) was added, and transferred again to the boiling water for 7 min after vortexing. Tubes were immersed immediately in an alcohol/ice bath for several minutes. To each tube, 1.5 ml of concentrated HCl was added, followed by 0.5 ml of 0.05 M potassium ferricyanide (freshly prepared). The potassium ferricyanide was added to tubes at 20 s intervals, until all tubes were treated. Samples were mixed and their absorbance was read at 520 nm after 20 min. The samples were read in turn at intervals of 20 s to ensure equal time had been allowed for colour development in each tube.

3.2.8.7 *Long chain fatty acids (LCFA)*

a. Extraction

The method of Viviani *et al.* (1966) was used to isolate the total LCFA from feed and digesta (Figure 3.2), which were then quantified by titration (Dole and Meinertz, 1960). Because these methods had not been previously used in this laboratory, it was required I set up the apparatus (Figure 3.3) and evaluate the procedures. The method was chosen because:

- it measures total (both free and bound) LCFA;
- it uses low temperatures and an antioxidant to avoid modifying the LCFA profile;
- it excludes non-LCFA lipid components which are of no benefit to the animal;

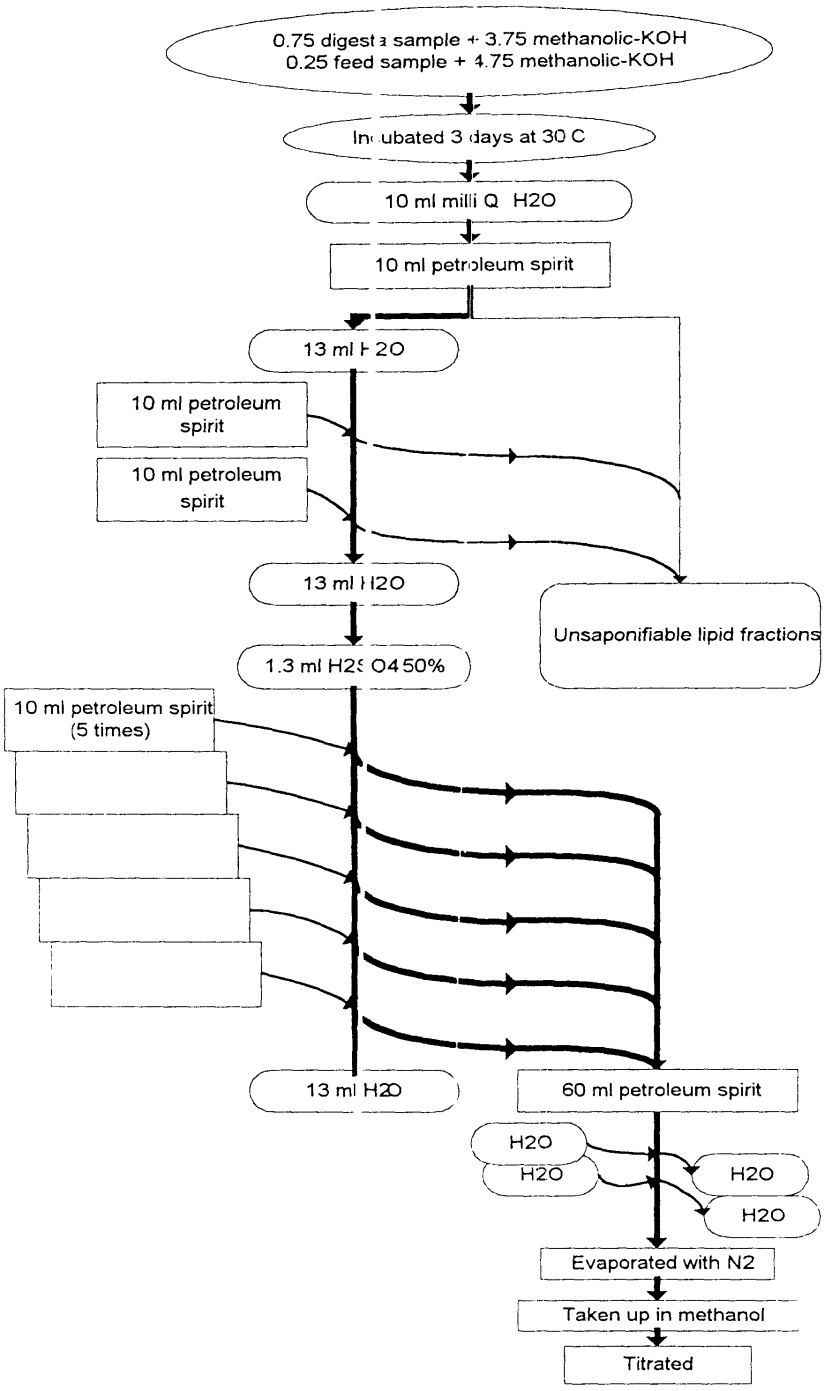

Figure 3.2 Procedure for extracting total LCFA from diet and abomasal digesta (Viviani *et al.*, 1966). Subsamples of final extract were titrated in triplicate.

- it provides an extract ready for methylation and analysis by gas chromatograph.

No other method which could provide all these benefits was found after extensive literature review. The principle of the method is as follows:

1) LCFA are saponified with methanolic-KOH;
2) LCFA soaps are then trapped in an aqueous phase while unsaponifiable materials are removed in the non-polar phase;
3) pH is then reduced to liberate free LCFA;
4) LCFA are absorbed back into a new non-polar solvent leaving other aqueous impurities behind;
5) The quantity of LCFA is determined by titration.

Samples were weighed (0.75 g for abomasal digesta and particles, 0.25 g for feed) into wide-neck McCartney bottles. Methanolic-KOH containing hydroquinone (10 ml of 10 M KOH, 25 ml of methanol, 0.05 g of hydroquinone, 15 ml milli Q water) was added: 3.75 ml for digesta samples and 4.75 ml for feed samples. This mixture was incubated in an oven with a constant temperature of 30 °C for 3 days.

To each bottle, 10 ml of milli Q water was added, followed by the same volume of freshly distilled petroleum spirit (B.P. 40 - 60 °C). The mixture was shaken and allowed to stand and form two layers with a sharp separation. The petroleum spirit (the upper layer) containing unsaponifiable materials was removed by mild suction. This removal of unsaponifiable substances was repeated twice with the same volume of petroleum spirit (10 ml). The water phase was acidified by addition of 1.3 ml of 9 M H_2SO_4 and 10 ml of petroleum spirit was added to force LCFA from the water phase into fresh non-polar solvent. The mixture was shaken thoroughly, and the petroleum phase

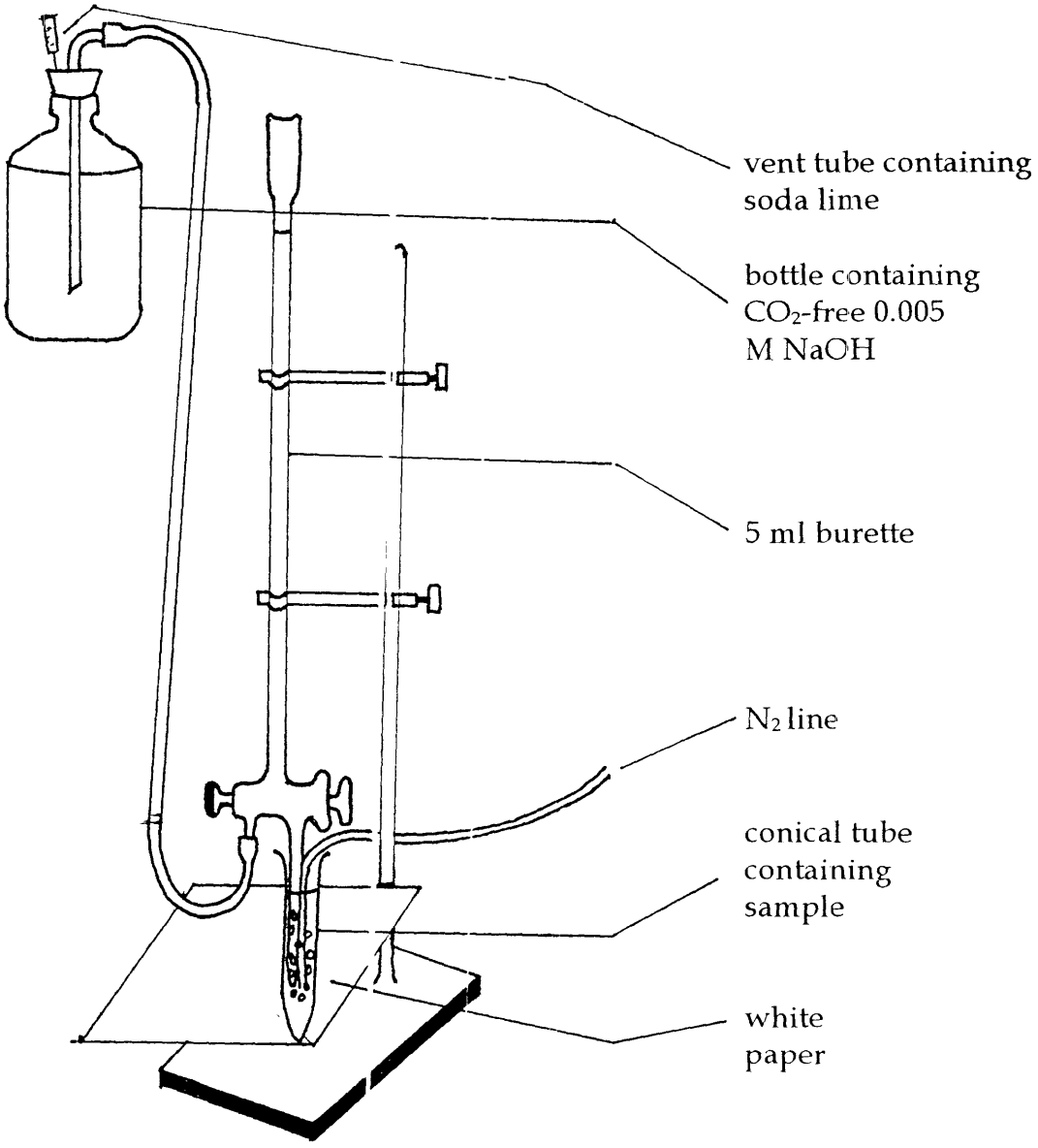

Figure 3.3 Apparatus used for the titration of total LCFA in diet and abomasal digesta. Note: N_2 stream bubbling through sample to exclude atmospheric CO_2.

containing extracted LCFA was removed under suction into another clean McCartney bottle. This procedure of extraction was repeated 5 times with the same volume of petroleum spirit (10 ml). The volume of petroleum in the collecting McCartney bottle was reduced through evaporation with nitrogen gas after each extraction to allow the addition of all of the solvent rinses in one bottle.

The petroleum spirit extract was washed twice with milli Q water and evaporated to dryness with nitrogen gas. Methanol (8 ml) was added to the sample, and this was stored under nitrogen at - 18 °C prior to titration.

b. Titration

Three ml of the sample in methanol were transferred into a conical centrifuge tube for titration with CO_2-free NaOH (0.005 M). Bromothymol blue (0.02% in ethanol) was used as indicator (2 drops). Nitrogen gas was delivered to the bottom of the tube during titration to expel CO_2 (Figure 3.3). Palmitic acid solution (0.3 g crystallised palmitic acid in 100 ml of methanol) was used as the standard. Linearity of the titration over a range of 0 - 60 μmoles palmitic acid is shown in Figure 3.4.

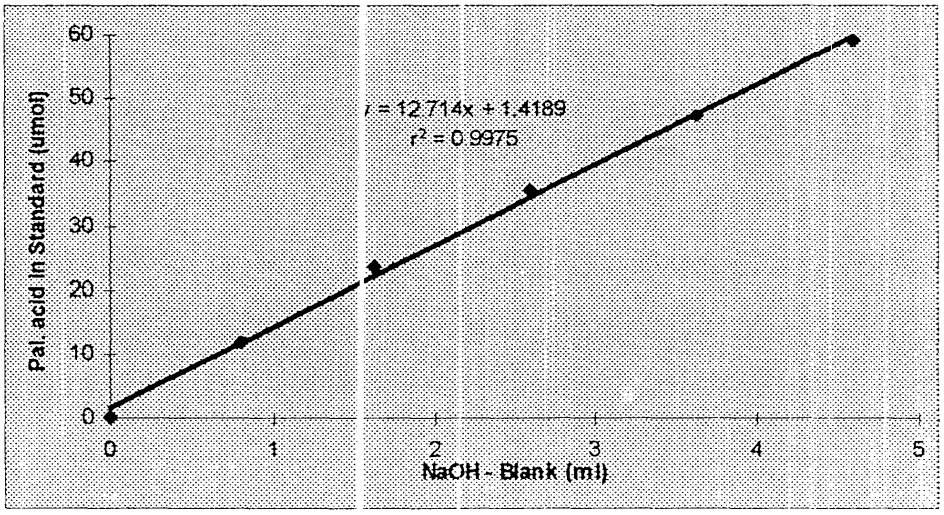

Figure 3.4 Titration of palmitic acid standard with 0.005 M NaOH

3.2.11 Mathematical procedures

3.2.11.1 Calculations

a. Kinetics of rumen fluid

Downes and McDonald (1964) found that under steady-state conditions, the dilution of Cr after a single intraruminal injection of CrEDTA will be first order, and experimental data could be well fitted by the following equation:

$$A_t = A_o \exp^{-kt}$$

where A_t = Cr concentration at time t

A_o = Cr concentration at time zero

k = exponent or rate constant (d^{-1})
= slope of the regression ln [Cr] *vs* time

$$\text{Rumen volume (l)} = \frac{\text{Dose injected } (\mu g \text{ Cr})}{A_o\ (\mu g\ /l)}$$

$$\text{Outflow rate} = \text{Rumen volume} \times k$$

$$\text{Half-time of rumen water } (t_{1/2}), \text{ min} = \frac{\ln (0.5)}{-k} = \frac{0.693}{-k}$$

b. Production rates of VFA

Production rates of total and individual VFA (acetate, propionate and butyrate) are calculated according to the method of Weller *et al.* (1967). It is assumed that acetate, propionate and butyrate are produced in the proportion in which they exist in the ruminal fluid.

$$\text{Total VFA production rate (mmoles/d)} = P = \frac{Q \times R}{\text{Plateau SR}} \times 1440$$

where Q = Infusion rate (g/min)

R = Disintegration per min/g infusate

SR = Specific radioactivity of sample (DPM/mmol total VFA)

$$\text{Individual VFA production rate (mmoles/d)} = P \times \text{Molar proportion of individual VFA}$$

The calculation of ATP production by cell-fermenting microbes was made by assuming that fermentation to produce 1 mole of acetate, propionate and butyrate generates 2, 3 or 2 moles of ATP, respectively (Czerkawski, 1986).

c. Outflow of microbial cells from the rumen based on urinary allantoin excretion

The method for predicting microbial cell outflow from the rumen from urinary allantoin excretion of Balcells *et al.* (1991) was applied. These authors suggested the following equation for the relationship between urinary allantoin excretion (y, μmol/kg$^{0.75}$ BW) and purine infused duodenally (x, μmol/kg$^{0.75}$ BW):

$$y = 0.8015x - 43.7$$

The microbial N into the intestine was calculated on the assumptions that nucleic acids N make up 15.2 % of the microbial N (Storm and Ørskov, 1983) and rumen bacteria contain 0.15 g N and 1.10 mmol purines/g (Balcells *et al.*, 1991). Microbial-cell outflow (g DM/d) was then calculated assuming that cells contain 77.7 g N/kg DM cells (Storm and Ørskov, 1983).

d. Estimation of efficiency of cell production

The efficiency of cell production was estimated as g cell DM/mole ATP generated (Y_{ATP}) and g cell DM/kg DM apparently and truly digested in the rumen (g/kg DMADR and g/kg DMTDR, respectively). The DMADR was calculated as the difference between dietary DM intake and DM flow through the abomasum. The DMTDR was calculated as the difference between dietary DM intake and abomasal DM flow corrected for cell DM flow (*3.2.11.1*c).

e. Calculation of abomasal digesta flow

The flow of digesta to abomasum was calculated according to the double marker method of Faichney (1975). Dietary AIA was used as particle digesta marker, while Cr was used as a liquid flow marker.

3.2.11.2 Statistical Analysis

All data collected were analysed with analysis of variance using Minitab spreadsheets. A one way analysis of variance was used initially to test for effects of methionine (in the refaunated group). Where no effect was found, data for all refaunated sheep were pooled and a one way analysis of variance was made to compare defaunated and refaunated sheep.

Chapter 4 Results and Discussion

4.1 Results

There were initially 8 animals in the refaunated group at the commencement of study, 4 of which were intended to receive methionine supplement. Unfortunately, 2 animals (1 of the methionine-supplemented and 1 of the methionine-unsupplemented group) began refusing feed and were removed from the experiment prior to digesta-flow studies being made. An additional animal from each group was excluded prior to the intraruminal injection of CrEDTA, infusion of 1-^{14}C-labelled acetate and the collection of urine samples. Thus, in most cases, comparison of refaunated sheep with or without methionine supplement is based on only 2 sheep per cell. Comparison of defaunated and refaunated sheep is based on 4 sheep per cell.

4.1.1 Rumen environments and fermentation

Methionine supplementation did not significantly affect the rumen volume, half-time of rumen liquid ($t_{1/2}$) or the outflow of rumen liquid ($P > 0.05$; Table 4.1). Rumen volume was, however, significantly larger in protozoa-free sheep which also tended ($P = 0.15$) to have a greater rate of flow of liquid from the rumen. On the other hand, ruminal ammonia concentration tended to be higher in the refaunated group ($P = 0.07$).

Neither protozoa nor methionine affected the concentration or production of total VFA in the rumen (Table 4.2). There was, however, a significantly higher molar proportion of acetate ($P < 0.01$) and a lower proportion of propionate ($P <$

< 0.05) in defaunated sheep. The acetate:propionate ratio was also significantly higher in defaunated animals ($P<0.05$). The molar proportion of other acids was not modified by methionine or protozoa.

4.1.2 Microbial-cell production and efficiency

The apparent efficiency of microbial growth in the rumen was unaffected by the presence of methionine or of protozoa. This was true when efficiency was expressed in terms of YATP, g cells/kg DMADF or g cells/kg DMTDR.

Table 4.1 Rumen environments of defaunated and refaunated sheep consuming 400 g of oaten chaff + 400 g of lucerne chaff.

Parameters	Defaunated				Refaunated				Statistical effects	
					+ M		– M		M	P
Sheep No.	*1*	*2*	*3*	*4*	*7*	*8*	*9*	*10*		
Protozoal counts ($\times 10^{-5}$/ml)	-	-	-	-	7.3	7.9	6.7	8.1		
Rumen NH_3 Concentration (mg N/l)	150	147	142	153	201	150	202	160	ns	= 0.071
Ruminal pH	6.76	6.58	na	na	6.55	6.72	6.74	6.53	ns	ns
Rumen volume (l)	6.6	8.9	7.4	6.4	6.3	4.9	4.5	4.1	ns	< 0.05
Rumen liquid $t_{1/2}$ (min)	414	330	618	522	548	422	426	330	ns	ns
Rumen liquid outflow (l/d)	15.8	27.3	12.0	12.2	7.9	11.6	10.6	12.7	ns	= 0.15

ns = not significant
M = methionine main effect
P = protozoa main effect
na = not available

4.1.3 The flow of nutrients to abomasum

As shown in Table 4.4, the apparent digestibility of dry matter in the rumen (DMADR) was not altered by methionine supplementation, but was significantly higher in refaunated compared with defaunated sheep ($P < 0.05$). The concentrations of total LCFA in the DM of abomasal digesta as sampled and the particle-phase of abomasal digesta were not affected by methionine supplementation. The concentration of total LCFA in the DM of abomasal digesta as sampled also did not differ ($P > 0.05$) between defaunated and refaunated animals. The presence of protozoa in the rumen, however, tended to increase the concentration of total LCFA in particle-phase of abomasal digesta ($P < 0.1$). With the exception of two animals (nos. 6 and 10), the concentration of total LCFA in digesta as sampled was higher than in the particle-phase of abomasal digesta, regardless of methionine supplementation and faunation status of the rumen.

The abomasal flow of DM (g/d) was significantly greater in refaunated than in defaunated animals ($P < 0.05$; Table 4.4). Supplementation of methionine reduced the total flow of LCFA ($P < 0.05$) and the outflow of microbial LCFA produced in the rumen. On the other hand, the presence of protozoa in the rumen increased the daily flow of LCFA and their production in the rumen ($P < 0.01$ and < 0.05, respectively).

Table 4.2 Volatile fatty acid (VFA) concentrations, proportions and production (based on 1-^{14}C-acetate infusion) of defaunated and refaunated sheep consuming 400 g of oaten chaff + 400 g of lucerne chaff.

Parameters	Defaunated				Refaunated				Statistical effects	
					-M		+M		M	P
Sheep No.	*1*	*2*	*3*	*4*	*7*	*8*	*9*	*10*		
Total VFA Concentration (mMol/l)	92.9	83.4	95.2	9[illegible].5	107.1	64.7	76.1	64.5	ns	ns
Molar Proportions (%)										
Acetate	73.7	75.1	75.7	7[illegible].5	71.3	71.3	71.8	69.5	ns	< 0.01
Propionate	18.1	15.1	13.7	1[illegible].6	18.9	18.6	16.9	20.0	ns	< 0.05
Butyrate	6.1	5.5	7.3	[illegible].6	7.2	7.0	7.9	7.0	ns	ns
Isobutyrate	0.5	2.6	1.3	.3	0.7	1.1	1.2	1.3	ns	ns
Isovalerate	0.8	0.7	1.2	.0	1.0	1.1	1.1	1.1	ns	ns
Valerate	0.8	1.1	0.8	0.9	0.9	1.0	1.1	1.2	ns	ns
C2 : C3 ratio	4.1	5.00	5.5	5.5	3.8	3.8	4.2	3.5	ns	< 0.05
VFA Production (mol/d)	3.2	3.4	3.3	3.9	3.9	3.0	2.5	2.4	ns	ns

ns = not significant
M = methionine main effect
P = protozoa main effect

Table 4.3 Microbial growth and efficiency of defaunated and refaunated sheep consuming 400 g of oaten chaff + 400 g of lucerne chaff

Parameter	Defaunated				Refaunated				Statistical effect	
					– Methionine		+ Methionine		Methionine	Protozoa
Sheep no.	*1*	*2*	*3*	*4*	*7*	*8*	*9*	*10*		
Abomasal purine flow (mmol/d)*	10.3	9.3	8.6	10.3	7.7	9.5	8.8	11.4	ns	ns
Microbial cell outflow (g/d)¶	117.9	107.1	98.6	118.2	88.5	109.6	101.4	130.9	ns	ns
Ruminal ATP production (molar/d)‡	9.7	10.0	9.7	11.6	12.1	9.0	7.5	7.5	ns	ns
Efficiency of microbial growth										
Y_{ATP}	12.1	10.7	10.1	10.2	7.3	12.1	13.5	17.6	ns	ns
g cells/kg DMADR	293.9	272.5	258.6	315.4	258.2	368.4	264.9	439.6	ns	ns
g cells/kg DMTDR	227.2	214.1	205.5	239.8	205.2	269.2	209.4	305.3	ns	ns

* = calculated from urinary allantoin excretion
¶ = calculated from abomasal purine flow
‡ = calculated from stoichiometry of estimated production of acetate, propionate and butyrate
ns = not significant
M = methionine main effect
P = protozoa main effect

Table 4.4 Concentration of total LCFA in abomasal digesta and their flow to abomasum in defaunated and refaunated sheep consuming 400 g of oaten chaff + 400 g of lucerne chaff

Parameter	Defaunated				Refaunated						Statistical effects	
					- M			+ M			M	P
Sheep no.	*1*	*2*	*3*	*4*	*6*	*7*	*8*	*9*	*10*	*11*		
Dry matter (DM) intake (g/d)#	733.3	733.3	733.3	733.3	741.9	741.9	741.9	741.9	741.9	741.9		
Total LCFA intake (g/d)	4.2	4.2	4.2	4.2	5.3	5.3	5.3	5.3	5.3	5.3		
DM apparently digested in the rumen (%)	54.7	53.6	52.0	51.1	40.9	46.2	40.1	51.6	40.14	50.14	ns	< 0.05
DM truly digested in the rumen (%)	70.78	68.21	65.45	67.22	-	58.13	54.87	65.27	57.78	-	ns	<0.05
Total LCFA concentration in abomasal digesta												
- digesta as sampled (g/ kgDM)	40.2	34.1	32.4	28.0	32.2	30.6	35.5	36.0	24.1	33.4	ns	ns
- particle digesta (g/kg DM)	24.5	27.2	24.8	23.2	32.3	25.7	29.0	29.5	26.1	32.8	ns	= 0.081
DM flowing to abomasum (g/d)	332.6	340.4	352.3	358.5	438.4	399.1	444.5	359.2	444.2	370.1	ns	< 0.05
Total LCFA flowing to abomasum												
total flow (g/d)	11.8	10.9	10.8	9.6	14.4	13.2	16.2	12.1	10.5	12.2	< 0.05	< 0.01
rumen production (g/d)	7.6	6.6	6.6	5.4	8.8	7.9	10.9	6.9	5.2	6.9	= 0.052	< 0.05
(% of total)	35.8	38.9	39.0	43.9	38.9	40.2	32.8	43.7	50.4	43.3	= 0.059	ns

ns = not significant
M = methionine main effect; P = protozoa main effect
# = DM intake and LCFA were fixed for each group. Small treatment differences occurred due to variable feed moisture

4.2 Discussion

4.2.1 Rumen environments and fermentation

The tendency for the concentration of ruminal ammonia to be lower in defaunated sheep than refaunated sheep in the present study is consistent with many reports as reviewed by Williams and Coleman (1992). Protozoa have a range of deaminase enzymes (Williams, 1989) and they excrete ammonia resulting from deaminating the ingested bacterial proteins and nucleic acids (Smith and McAllan, 1970).

Volatile fatty acids are produced as end-products of ruminal fermentation, and they disappear from the rumen through absorption and outflow (France and Siddon, 1993). The balance between the rate of VFA production in the rumen and the rate of its loss from the rumen determines the concentration of VFA at any given time. The loss of trans-ruminal VFA through absorption is a pH-dependent process (Bergman, 1990; France and Siddon, 1993), but this is unlikely to have confounded estimates of VFA production in this study since ruminal pH was similar in defaunated and refaunated sheep (mean 6.65).

Over a wide range of diets, refaunated animals have been found to have higher ruminal concentrations of VFA compared with defaunated animals (Veira, 1986; Eadie and Gill, 1971; Ushida *et al.*, 1986; Bird, 1982), while this study found that the concentration of VFA was not significantly different between defaunated and refaunated animals. Defaunated animals had a larger rumen volume, however, and would therefore have a larger total quantity of VFA in the rumen at any time. This is consistent with the greater apparent digestibility of DM in the defaunated rumen, but is inconsistent with the apparent lack of effect on VFA production rate estimated from 1-^{14}C labelled acetate. It is possible that

production rates estimated from 1-^{14}C labelled acetate. It is possible that differences in acetate-butyrate interconversion between defaunated and refaunated sheep existed (Leng, 1970) which would have caused errors in isotopic estimates of VFA production based on a single acid. The lack of effect of protozoa on ruminal VFA concentration may also have resulted from the tendency for defaunated sheep to pass more water out of the rumen than did refaunated sheep, therefore losing more VFA in these digesta.

The main VFA produced by protozoa are acetate and butyrate, while propionate is only produced in trace amounts (Hungate, 1966). However, defaunation is not always associated with a decrease in production or molar proportion of acetate or butyrate and an increase in that of propionate (Williams and Coleman, 1992). The pattern of rumen fermentation in defaunated and refaunated animals can not directly be attributed to the effect of protozoa *per se*, because there may be a difference in population between the groups. The lower acetate proportion and acetate:propionate ratio, and the higher propionate proportion of refaunated animals in this study is in agreement with Bird (1982). In his review, Bird (1982) reported that approximately 75 % of the studies where sheep were fed on a roughage:concentrate-based diet, there were lower acetate and higher propionate proportions in defaunated animals. These indicate that defaunation may shift the population of ruminal microbes towards more acetate-producing and less propionate-producing species.

4.2.2 Purine flow and calculated microbial efficiency

4.2.2.1 Effect of defaunation

The similar daily abomasal flow of purine between defaunated and refaunated animals in this study (calculated from the urinary excretion of allantoin) is not in agreement with published studies which constantly report high microbial-protein outflow from defaunated animals (Williams and Coleman, 1992). This calls into question the accuracy of allantoin flux as a marker of microbial-protein flow. This study has the following features which lead the author to expect an increased microbial-protein flow in defaunated sheep should have occurred:

1) For the same dry matter intake, more dry matter was apparently fermented in the defaunated rumen than in the refaunated rumen. This means a greater total mass of fermented organic matter was converted to cells and VFA in the defaunated rumen.
2) There was no effect of protozoa on total VFA production or VFA concentration in the rumen (although the total pool of VFA was greater in the defaunated rumen). It is reasonable to expect the increased organic matter available from fermentation would therefore have been directed into cells or methane.
3) There was no effect of protozoa on methane in this study (data are not included, intellectual property of another researcher). In consequence, it is reasonable the extra fermented organic matter should have appeared as microbial cells.

While urinary allantoin excretion has routinely been used as a marker of protein flow, it is possible that the relative breakdown of microbial protein compared

with nucleic acid, or the relative excretion of allantoin relative to other derivatives (xanthine, hypoxanthine and uric acid) is changed in the absence of protozoa. These possibilities could bias the estimates and invalidate the conclusion. This will be tested by comparison of purine-based estimates of digesta flow with estimates based on ^{15}N enrichment of microbes. This comparison was unable to be completed in the time available.

Another factor which may have led to this unexpected result is that the lower ruminal liquid flow and therefore outflow of liquid-phase bacteria in refaunated sheep is compensated by the higher flow of particle-phase bacteria so that there was similar abomasal flow of purine between the two groups. The nucleic acid content of particle-phase bacteria (g/100 g DM) is lower than that of liquid-phase bacteria (6 *vs* 5; Merry and McAllan, 1983).

4.2.2.2 Effect of methionine

This study shows that methionine metabolism in the rumen may not have an important effect on the overall ruminal environment and fermentation in the presence of protozoa.

Methionine presented in the rumen may be degraded to yield H_2S, or absorbed passively by ruminal microbes and used as such for protein synthesis, or demethiolated producing CH_3SH (Hegarty, 1989). Therefore methionine is a source of sulphur as well as nitrogen for ruminal microbes. Deficiency in sulphur may reduce the efficiency of microbial growth (Kandylis and Bray, 1987), but this is unlikely to be the cause of lower microbial efficiency (g/kg DMADR) for methionine-unsupplemented animals in this study in light of the high protein content of the diet. In terms of nitrogen, studies have shown that providing a mixture of amino acids as a nitrogen source results in higher microbial growth and efficiency than when NH_3 alone is provided (Maeng *et al.*, 1976; 1989). The effect of individual amino acids on the microbial growth and efficiency, however, is poorly documented.

4.2.3 The abomasal flow of total LCFA

Microbial lipids synthesised in the rumen contribute to the post-ruminal concentration and flow of LCFA (Harfoot, 1981). The contribution of the animal's own lipids rather than microbial lipids to the net gain in LCFA flow across the rumen is negligible (Noble, 1981).

Previous studies of rumen lipid production have frequently been of limited use because the determination of lipid has included non-LCFA fractions and excluded much of the bound microbial LCFA. Microbial lipids contain some LCFA which are in an unesterified form (ULCFA), either free and fully protonated or ionic and so bound to the cationic sites of protein or of carbohydrate molecules (O'Leary, 1962; Viviani *et al.*, 1966). Other microbial LCFA are present in an esterified form (ELCFA). Owing to the acidic conditions in the abomasum (pH 2.00 to 2.5), however, there is no ionic form of ULCFA

present in abomasal digesta (Bauchart, 1993). Even if ULCFA-proteins/carbohydrate complexes were present, all LCFA would have been extracted in the LCFA extraction procedure employed in this study. Viviani *et al.* (1966) found that the recovery of the total ruminal LCFA extracted by this procedure was the same as the sum of the ELCFA plus free and bound ULCFA in microbes when the esterified and unesterified forms were extracted individually.

This study clearly supports previous reports that the amounts of total LCFA leaving the rumen are generally higher than those consumed by animals in the diet (*e.g.* Sutton *et al.*, 1970; Bickerstaffe *et al.*, 1972; Hogan *et al.*, 1972). Total LCFA flowing out from the rumen consist of dietary LCFA which are adsorbed onto particulate matter and microbial LCFA derived from the incorporation of dietary LCFA and *de novo* synthesis. When the amount of LCFA ingested by animals is subtracted from the total LCFA flowing out from the rumen, the value obtained can be interpreted as the quantity of total LCFA derived from *de novo* synthesis by ruminal microbes. In making this calculation, it is assumed that no LCFA are catabolised in the rumen (Bickerstaffe *et al.*, 1972). In this study, approximately 40 % of the total LCFA available for absorption by the animal were of microbial origin.

4.2.3.1 *Effect of defaunation*

Bacterial pool size and amino acid outflow are generally higher in defaunated compared with refaunated animals (Williams and Coleman, 1992). However, it does not follow that a greater LCFA outflow will occur in defaunated ruminants. The full protozoal protein mass is not available for post-ruminal digestion due to sequestration of protozoa in the rumen (Coleman, 1989). Indeed, the flow rate of protozoa is only 20 - 40 % of that of liquid (Williams and Coleman, 1992),

indicating many protozoa are retained and lysed in the rumen. There is, however, a fundamental difference in the ruminal metabolism of protein and of LCFA; this being that protein is degraded within the rumen (mainly by protozoa) but LCFA are not (Bickerstaffe *et al.*, 1972). Consequently, an effect of protozoa on LCFA outflow will be seen only if they synthesise LCFA to a greater or less extent than the bacteria they replace. Protozoal predation of bacteria in the rumen *per se* will not reduce LCFA outflow as it does for amino acid flow for which ruminal deamination is possible.

The finding that protozoa in the rumen significantly increased the ruminal LCFA production and the daily flow of total LCFA to the abomasum indicates a greater *de novo* synthesis of LCFA in protozoa than in the bacteria they replace. This is expected considering that protozoa contain more lipid than do bacteria, and up to 75 % of microbial lipid in the rumen is protozoal in origin (Katz and Keeney, 1969; Keeney, 1970).

In addition to the possible contribution of protozoal cell lipid to the higher flow of LCFA in refaunated animals, some protozoal LCFA may also flow out from the rumen unbound to the protozoal cells. If protozoal cells are lysed in the rumen, their lipids as well as those of engulfed bacteria will be released to the medium. The released lipid will be hydrolysed and the products of hydrolysis (ULCFA) will be taken up by the existing microbes or alternatively will be adsorbed onto particulate matter which flows out from the rumen.

4.2.3.2 *Effect of methionine*

The mode of action of methionine or methionine hydroxy analog (MHA) to affect the synthesis of LCFA or lipids by microbes has been previously studied. *In vitro* study with non-ruminal bacteria (O'Leary, 1959) showed that the

labelled carbon in the methyl group of methionine was incorporated mostly into an "unknown compound", which was later found by the author to be a cyclopropane fatty acid (O'Leary, 1962). While the presence of this LCFA in ruminal microbes has not been shown to date, Patton *et al.* (1970) demonstrated that supplementing methionine hydroxy analog decreased the free ULCFA in ruminal fluid and apparently promoted the formation of an unidentified polar lipid. The consequence of the reduction of free ULCFA will be reduced biohydrogenation of unsaturated LCFA since hydrogenation is dependent on the presence of LCFA in their free form (Hawke and Silcock, 1969). If this is true, the proportion of unsaturated LCFA flowing out from the rumen may be higher in animals receiving methionine. Fatty acid profiles of digesta were not, however, determined.

The present study indicates that the daily total flow of LCFA was significantly reduced by methionine supplement, even though the daily flow of DM or purine was not altered by the supplement. The ruminal production of total LCFA was also lower in methionine-supplemented animals compared with the control animals. Owing to time constraints, the LCFA content of rumen microbes from each group of sheep was not determined.

The lower ruminal production of LCFA associated with methionine supplementation in the present study contrasts with previous observations made by O'Kelly and Spiers (1990) that methionine increased the lipid synthesis by ruminal microbes *in vitro*. Two factors may have contributed to this discrepancy. First, the synthesis of lipids by particle-phase microbes was not accounted for in the study of O'Kelly and Spiers (1990); these microbes may respond differently to methionine supplement compared with liquid-phase bacteria. Second, O'Kelly and Spiers (1990) determined the synthesis of total lipids rather than LCFA (as in the current study).

In contrast to the reports of O'Kelly and Spiers (1990) and in like manner to results of this study, Patton *et al.* (1970) found that supplementing cows with 40 and 80 mg of MHA tended to reduce the concentration of lipid in the rumen, but this was not significant. In that study, it was also found that the methionine hydroxy analog supplement significantly reduced the percentage of stearic acid by inhibiting reduction of oleic and linoleic acids. The digesta-flow study reported here is probably the first to show the suppressive effect of methionine on LCFA synthesis. The reasons why methinone reduced the LCFA outflow are still not known at this stage, and this requires further study.

4.2.4 The concentrations of total LCFA in digesta

There was a tendency for the concentration of LCFA in particle digesta of refaunated animals to be higher than that of defaunated animals. It has been demonstrated by many authors (*e.g.* Merry and McAllan, 1983; O'Kelly and Spiers, 1988; Bauchart *et al.*, 1990; Hegarty *et al.*, 1994) that the lipid content of particle-phase bacteria is higher than that of fluid-phase bacteria. Therefore, the changes in the numbers of bacteria associated with both phases of digesta will obviously have an effect on the concentration of LCFA in digesta. It is unlikely, however, that the greater LCFA concentration in particle digesta of refaunated than of defaunated animals is due to the increased numbers of particle-phase bacteria. Orpin and Letcher (1984) found that defaunation increased the number of liquid-phase bacteria but not of particle-phase bacteria.

Gram-negative bacteria are known to contain more lipid than do Gram-positive bacteria (Salton, 1960) and it is possible that the higher LCFA concentration in particle digesta of refaunated sheep was due to a higher proportion of Gram-

negative relative to Gram-positive bacteria. Ushida *et al.* (1986) observed an increase in the number of Gram-negative bacteria but not of Gram-positive bacteria due to defaunation, while other workers (Whitelaw *et al.*, 1972; Hsu *et al.*, 1991b) found that defaunation increased the numbers of both groupings equally.

The difference between defaunated and refaunated groups in the concentration of total LCFA in particle digesta in the abomasum may also be due to the changes in the concentration of LCFA within the bacterial cells. Hegarty *et al.* (1994) found that there was a tendency for both particle- and liquid-phase bacteria to have higher lipid contents when protozoa were present in the rumen, irrespective of the availabilities of nitrogen and sulphur or the kinetics of rumen liquid. The higher LCFA content of particle-digesta DM in refaunated animals could be partly due to the higher lipid content of particle-phase bacteria and a greater flow of these bacteria in refaunated sheep.

In summary, it is postulated that the higher LCFA concentration in the particle phase of abomasal digesta may be partly due to the contribution of protozoa with higher lipid contents compared with bacteria, and/or a shift in the population from mostly fluid to mostly particle attached species, and/or a shift from Gram- positive to Gram-negative species.

4.2.4 The importance of microbial lipids to ruminants

It is clear from the preceding sections that ruminal microbes are able to alter dietary lipids by lipolysis and biohydrogenation. They can also incorporate dietary LCFA as well as synthesise *de novo* their cellular LCFA using shorter chain FA as precursors. Advantages that microbial lipids provide to the host arise from the alteration of ruminal fermentation due to the biohydrogenation

and synthesis of microbial lipids and the added nutritive value of microbial LCFA.

Biohydrogenation of dietary ULCFA and synthesis of lipids by rumen microbes are hydrogen-demanding processes (Czerkawski, 1986). One consequence of this is that the utilisation of the available hydrogen by the undesirable microbial process of methanogenesis can be reduced. A small portion of the available hydrogen (1 - 2 %) could be used for hydrogenating poly-ULFA included in the diet, but the synthesis of microbial lipids could be a further appreciable hydrogen sink (Czerkawski, 1986). Therefore, increasing microbial lipid synthesis may be used as an opportunity to increase the efficiency of feed utilisation by reducing methane production, which is also environmentally desirable.

The nutritive value of microbial lipids has been studied by O'Kelly and Spiers (1990, 1992). These authors found that on the same intake of either low or high quality diets, Brahman cattle obtained and maintained higher body weight compared with Herefords, which was attributed the higher lipids supplied from the rumen of the Brahman cattle Almost all of the bacterial and protozoal lipids will flow out from the rumen since ruminal absorption and catabolism of LCFA are negligible. Approximately three-quarters of the lipids in the rumen of a cow fed on all hay diet is protozoal in origin (Katz and Keeney, 1967; Keeney, 1970), and the protozoal contribution to post-ruminal LCFA, especially polyunsaturated LCFA, may be significant (Williams and Coleman, 1992). When energy is the limiting nutrient for animal growth, enhancing microbial lipid production in the rumen is probably a useful alternative to direct energy supplementation.

Chapter 5 General Discussion

5.1 General Discussion

From the review of the literature and results of this study, it is apparent that protozoa exert a significant effect on the ruminal environment and alter fermentation patterns. A change in the ruminal environment and the pattern of fermentation observed when protozoa are eliminated from the rumen, however, can not be attributed directly to the effect of protozoa *per se*. The rumen is a complex system, and the fermentative digestion occurring in this organ is carried out by a consortium of bacteria, protozoa and fungi. The elimination of one group of microbes creates a niche that will be filled by other organisms.

Williams and Coleman (1992) stated that the most consistent consequence of defaunation was a decrease in ruminal ammonia concentration. This study supports this statement with the ammonia concentration of defaunated sheep tending to be lower ($P = 0.07$) than that of refaunated sheep. The higher ruminal ammonia concentration in refaunated animals may be attributed to higher production of ammonia through degradation of dietary protein and bacteria and to the fact that protozoa do not utilise ammonia as nitrogen source but rather excrete it as an end-product of their metabolism (Nolan, 1993).

The difference in VFA proportions between defaunated and refaunated sheep (*i.e.* higher acetate but lower propionate proportions in defaunated sheep compared with refaunated sheep) may also be linked to the difference in ruminal production of LCFA. It is known that acetate and propionate are utilised as precursors by ruminal microbes for synthesis of their LCFA (Harfoot,

1981). The utilisation of acetate as precursor will result in the formation of even chain length LCFA, while the use of propionate will produce odd chain length LCFA.

Little is known on the microbial response to specific amino acids such as methionine in the rumen. For ruminal microbes, methionine may be utilised as a source of sulphur required for the *de novo* synthesis of other sulphur-containing amino acid (cysteine), incorporated directly during microbial-protein synthesis, or utilised as methyl source for the formation of the methylene bridges of cyclopropane fatty acid (O'Leary, 1959). The higher efficiency of rumen microbial growth in the methionine-supplemented sheep may have been a result of the direct incorporation of this amino acid into microbial protein. The lower ruminal LCFA production associated with methionine supplementation is different from its positive effect on lipid synthesis *in vitro* (O'Kelly and Spiers, 1990). The effect of methionine on *in vivo* ruminal lipid production where particle- and liquid-phase bacteria are both taken into account has not been studied. It is important to include the particle-phase bacteria in any study of lipid/LCFA production because this group have a higher lipid/LCFA content than fluid-phase bacteria.

The most important finding of this study was that the refaunated rumen produced more LCFA than the defaunated one. This is in agreement with the fact that protozoa contain more lipid than do bacteria and that most ruminal lipids are protozoal in origin (Katz and Keeney, 1969; Keeney, 1970). An increase in ruminal LCFA production and supply will benefit the ruminant because it will reduce the need for oxidation of glucose which is necessary for the generation of the co-factor (NADPH) required in LCFA synthesis (Preston and Leng, 1987) as well as increase the proportion of dietary organic matter used by the animal. These authors also noted that the glucose-sparing effect of LCFA

might lead to more amino acids being deposited as protein than being oxidised to meet the glucose requirement.

5.2 Future Research

There have been far fewer studies to investigate microbial LCFA production in the rumen than to study microbial protein (nitrogen) metabolism. Studies in the following areas of microbial lipid metabolism may provide a better understanding of microbial LCFA:

- Particle-phase bacteria. This group has been consistently reported to have higher lipid content than liquid-phase bacteria (Bauchart *et al.*, 1990; O'Kelly and Spiers, 1988; Hegarty *et al.*, 1994). The contribution of each group to post-ruminal digesta LCFA and factors that may alter their relative contributions, however, have not been studied.

- Precursors for LCFA synthesis. Studies on factors such as the availability of precursors (VFA) and methionine considered to have an effect on ruminal lipid and LCFA production should be extended. *In vivo* tracer studies may provide information for a better interpretation on the mode of action of the factors in affecting microbial lipid/LCFA synthesis.

- LCFA as microbial markers. Some of the LCFA synthesised *de novo* by ruminal microbes are branched-chain (Harfoot, 1981). These LCFA are not found in dietary lipids, and thus they are peculiar to microbes. It may, therefore, be possible to use these LCFA as internal microbial markers.

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