CHAPTER 3

IN VIVO STUDIES ON THE EFFICACY OF ANTI-PROTOZOAL MOLASSES BLOCKS FED TO SHEEP

3.1 Introduction

It has been shown that elimination of ciliate protozoa from the rumen increases protein flow from that organ and thus the amount of protein available for digestion and absorption from the small intestine of the host animal (Bird, 1982, Veira *et al.*, 1983). Oral denching with chemicals proved to be the simplest method of eliminating protozoa from the rumen. However, to be succesful the oral drench had to be given on three consecutive days which could not be applied on a commercial scale in the field.

The present studies were initiated to determine if a molasses block containing an anti-protozoal agent could be used to reduce or completely remove protozoa from the rumen of sheep. The detergents Teric GN9 (Bird *et al.*, 1979) and Alkanate $3SL_3$ (Burggraaf and Leng, 1980) were used because it had been demonstrated that these detergents administered as an oral drench successfully defaunated the rumen. The questions which needed to be answered were:

a) Would a molasses block containing detergent set hard ?

b) Would sheep consume a molasses block containing detergent ?

c) Would the consumption of a molasses block containing detergent defaunate the rumen ?

d) Would the consumption of a molasses block containing detergent have any adverse effects on the animal ?

e)

3.2 General materials and methods

3.2.1 Experimental animals

Twelve Merino wethers were selected from a flock of 35 grazing animals which had been exposed to molasses blocks (control blocks-containing no detergent) for one week. Those selected for use had been eating the blocks (animals with molasses around the mouth). Sheep were transported from the paddock in which they were grazing to the Animal House in the afternoon (14.00 h) and offered a small amount of lucerne. Sheep were allocated to treatment at random and treatments were allocated to pens randomly as shown in Table 3.1. The average body weight of animals for each treatment is shown in Table 3.2.

Table 3.1 Sheep allocation to treatment group and pen

Sheep tag n	Body weight	Treatment	Group	Pen
23	32.8	Control	1	72
54	34.5	A 10%	5	71
150	35.6	T 10%	4	70
289	32.1	Control	1	69
270	36.3	A 5%	3	68
242	39.5	Control	1	67
121	42.8	A 10%	5	66
261	41.5	T 10%	4	65
241	36.3	A 5%	3	64
12	37.0	T 5%	2	63
183	30.6	T 5%	2	62
25	29.4	A 5%	3	61

Table 3.2 Average body weight of the treatment groups

Treatment group	Average body weight (g)
Control (group 1)	34.8
Teric 5% (group 2)	33.8
Alkanate 5% (group 3)	34
Teric 10% (group 4)	38.5
Alkanate 10% (group 5)	38.6

3.2.2 Diet

a. Basal diet

A basal diet consisting of 90% oaten chaff and 10 % barley grain with a supplement of 10 g urea/d was given *ad libitum* at 9.30 each morning. Barley and urea were included in the ration to encourage the proliferation of a large population of protozoa in the rumen. Dry matter and Nitrogen content of ration ingrediients are shown in Table 3.3. Feed given each day was dependent on the amount eaten from the previous day.Animals leaving less than 50g/d were offered an additional 50g/d of feed Animals leaving in excess of 150g/d were offered 50 g/d less than the previous amount offered. Animals had free access to water at all times.

Table 3.3. Dry matter (%) and metabolisable energy contents of the feed and molasses block

	Dry matter (%)	Nitrogen (gN/kg of DM)
Oaten chaff	87.8	96
Barley grain	87.6	115
Block with 5% detergent	74.9	0
Block with 10% detergent	75.7	0
Control block (no detergent)	78.9	0

*) source : ARC/NRC (1975)

b. Molasses blocks

There were 5 types of block, namely, control (C), 5% Alkanate $3SL_3$ (T11), 10 % Alkanate $3SL_3$ (T12), 5% Teric GN9(T21) and 10% Teric GN9 (T22). The composition of each block can be seen in Table 3.4.

Table 3.4 Composition (%) of the molasses blocks used

Ingredient	Control (%)	T 11 and T 21(%)	T21 and T22(%)
Molasses	67	62	57
Lime	8	8	8
Wheat bran	25	25	25
Detergent (Alkanate or Teric)	0	5	10

3.2.3 Collection of rumen fluid

Rumen fluid samples were collected before animals were introduced to the experimental molasses blocks and during the experimental period to determine the

number of protozoa. Rumen fluid samples were taken by a stomach tube from each sheep 3 hours after feeding. A minimum of 20 ml of rumen fluid was collected from each animal. To prevent cross-inoculation, the stomach tube was thoroughly rinsed with water between samples.

Sub-samples of rumen fluid were taken for the assessment of protozoal populations : a sub-sample of 4 ml of each sample of rumen fluid was diluted with 16 ml saline formaldehyde (4%w/w formaldehyde in 0.9% NaCl) solution in a McCartney bottle, giving an overall dilution factor of 5. The concentration of protozoa was determined in this formal saline-preserved sample.

3.2.4 Counting of protozoa

The formal saline-preserved sample was shaken thoroughly to mix its contents and sub-samples were withdrawn for microscopic counting while mixing, using a pasteur pipette and placed in a 0.2 mm deep counting chamber (Hawksley, Sussex, England). The counting chamber consisted of a 4 x 12 squares (each square = 0.1 mm^2). In this study protozoa cells were counted in a volume of 2.4 mm^3. The number of protozoa per ml of original solution was then calculated by using the following formula.

Number of protozoa / ml = n x Df x 1/v x 10^3

n = number of counted protozoa
Df = dilution factor of the sample
v = total volume of the counted area.

In order to check the accuracy of counting, samples with the highest and lowest concentration of cells were sub-sampled and counted ten times. The results were analysed to quantify the variability (Table 3.5).

Table 3.5 Counts of protozoal cells in samples of diluted rumen fluid

Individual counts for sample 1 (lowest)	Individual counts for sample 2 (highest)
37	302
32	311
37	344
29	325
37	340
39	332
37	315
32	304
36	327
33	346
mean 35	325
Standard error 1	5

3.2.5 Statistical analysis

Differences between treatment effects were analysed statistically by one way analysis of variance. All statements of significance imply P< 0.05 unless otherwise stated, while statements of non-significance imply P> 0.05.

3.3 Experiment 1 : Study on the efficacy of anti-protozoal molasses blocks when offered to sheep continuously fed roughage diet

3.3.1 Experimental procedure

The timetable for the procedures used in this experiment is given in Table 3.6. The sheep were individually housed in 1 x 2 m pens in an unheated animal house. Each pen was provided with a feeder, waterer, and molasses block. During the pre-experimental period all sheep were given the control molasses block. It took 22 days for the average daily intake of block to reach 50 g/d. At this level of intake, sheep consuming block which contained 10 % detergent would receive 5 g/d detergent.

Feed and the block intake (estimated from weight loss) were measured daily. The diurnal pattern of block intake for individual sheep was observed by measuring the weight of all blocks four times (9.30 am. 12.30 pm, 4.30 pm and 9.30 pm) on the same day that rumen fluid samples were collected.

Table 3.6 A summary of the experimental procedures

Day 1-22 (May 20-June10)	Molasses block without detergent
Day 16 (June 4)	The 1st collection of rumen fluid samples to determine protozoal numbers before the treatment period
Day 19 (June 7)	The 2nd collection of rumen fluid samples
Day 23 (June 11)	Molasses blocks containing anti-protozoal detergent (Alkanate 3SL3 and Teric GN9) introduced
Day 23 (June 11)	The 1st collection of rumen fluid samples during the treatment period and block intake measured 4 times to determine the diurnal pattern of block intake
Day 24 (June 12)	The 2nd collection of rumen fluid samples and measurement of the pattern of block intake
Day 26 (June 14)	The 3rd collection of rumen fluid samples and measurement of the pattern of block intake
Day 29 (June 17)	The 4th collection of rumen fluid samples and measurement of the pattern of block intake
Day 31 (June 19)	The 5th collection of rumen fluid samples and measurement of the pattern of block intake
Day 37 (June 25)	The 6th collection of rumen fluid samples and measurement of the pattern of block intake
Day 52 (July 10)	The 7th collection of rumen fluid samples and measurement of the pattern of block intake
Day 61 (July 19)	The 8th collection of rumen fluid samples and measurement of the pattern of block intake
Day 61 (July 19)	Weigh animals to finish the experiment

3.3.2 Results

a. Protozoal population density

The total density of rumen protozoa before treatment period was $2.1 \pm 0.3 \times 10^5$ / ml (range 0.98 - 3.8×10^5 /ml). The subsequent mean protozoal population density for each treatment group is presented in Table 3.7. There were no significant changes in the rumen protozoal population in the control sheep during the treatment period compared with the pre-treatment period ($P > 0.05$).

Analysis of variance confirmed that there were no significant differences in protozoal populations between treatments ($P > 0.05$). Figures 3.1, 3.2, 3.3 and 3.4 show the changes in the protozoal population density in the rumen of the treatment animals during the experimental period. The protozoal population density measured during the experimental period was expressed as a percentage of the pre-experimental concentration. Although there were non-significant differences between treatment, the protozoal population density in sheep consuming 5 % Alkanate block was always below the pre-experimental population density which suggests that this treatment may have had a depressing effect on the protozoal population (Figure 3.1).

Table 3.7 Protozoal population density (x 10^5/ml)

Treatment group	Means (± SE)of protozoal population density in rumen fluid	
	Pre-experimental period (day 1-22)	Experimental period (day 23- 61)
Control (group1)	2.8 ± 0.81 [a*]	2.6 ± 0.33 [a]
Teric 5% (group 2)	2.4 ± 0.33 [a]	2.1 ± 0.38 [a]
Alkanate 5% (group 3)	2.7 ± 0.46 [a]	1.8 ± 0.24 [a]
Teric 10% (group 4)	1.2 ± 0.18 [a]	1.2 ± 0.21 [a]
Alkanate 10% (group 5)	1.5 ± 0.40 [a]	1.7 ± 0.25 [a]

* Means between columns and within rows with different superscripts differ significantly

Figure 3.1 Protozoal population on 5% Alkanate

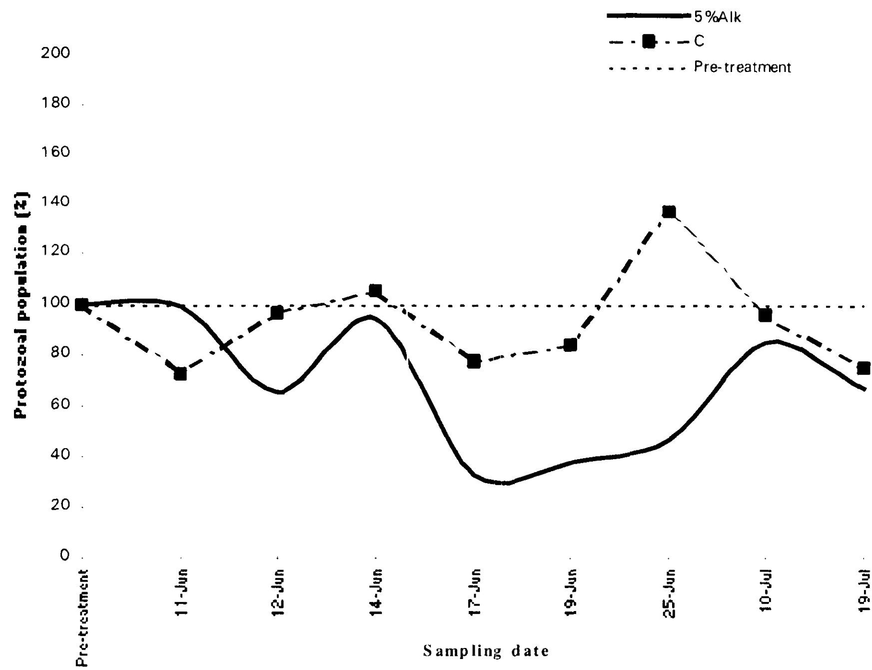


Figure 3.2 Protozoal population on 10% Alkanate

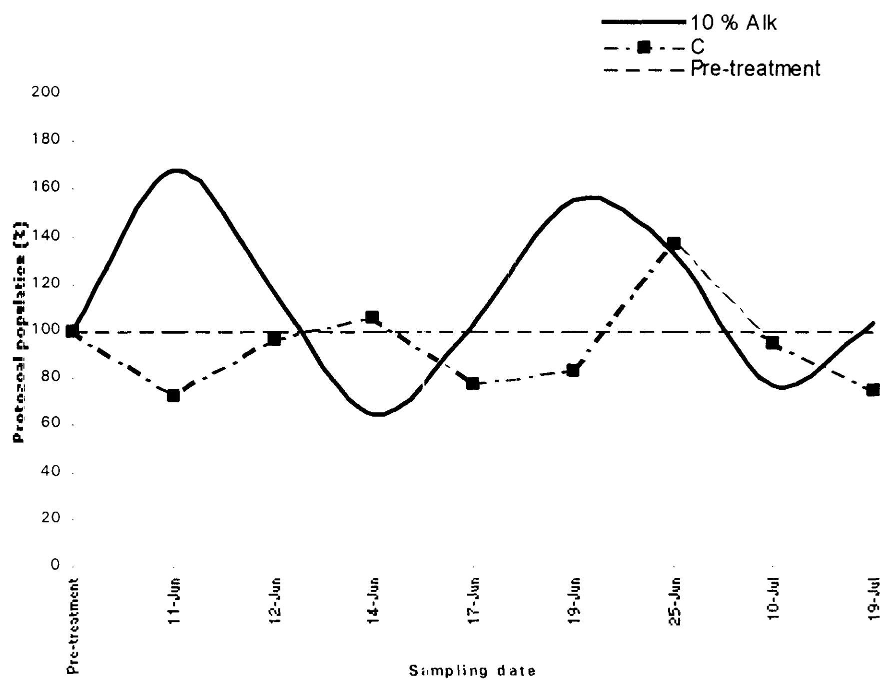


Figure 3.3 Protozoal population on 5% Teric

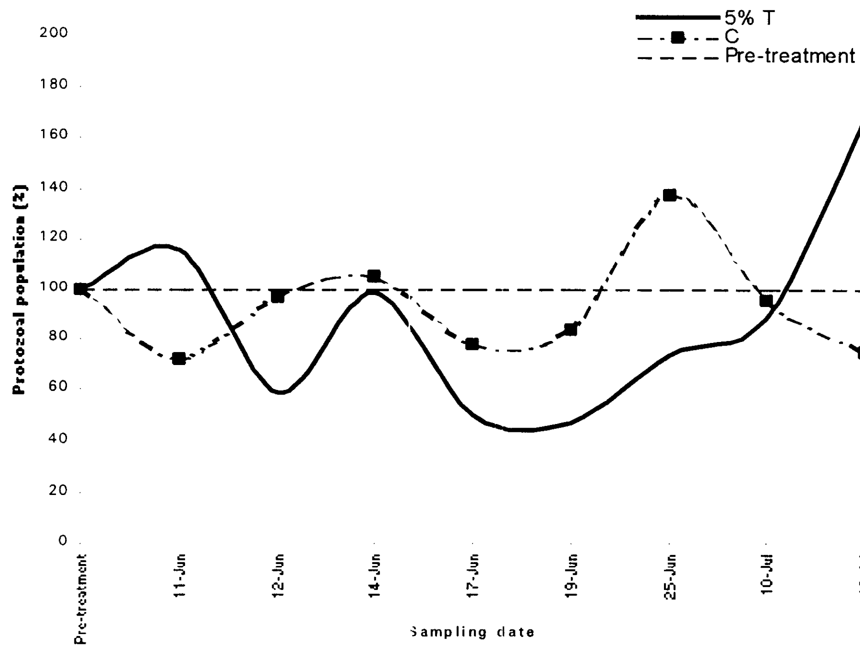
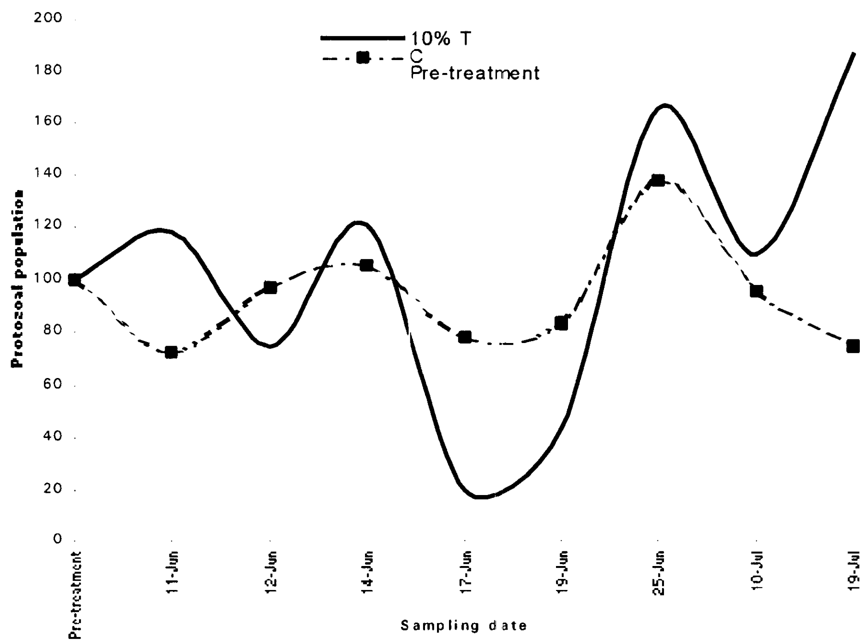


Figure 3.4 Protozoal population on 10% Teric



b. Block intake

Differences in block intake within the groups in the pre-experimental period (days 1-22) were non-significant, the overall mean (± s.e) being 51 ± 7 g/d. During the experimental period (days 23 -61), sheep showed a clear preference for blocks containing Alkanate. In the first 3 weeks of treatment, intake of the 5 % Alkanate block was greater than of the 10 % Alkanate block ($P<0.05$; mean 218 ± 31 g/d vs. 121 ± 15g/d). Thereafter, intakes of the 5 and 10% Alkanate blocks were similar (148 ± 13 g/d).

The diurnal pattern of block intake for different animals can be seen in Figure 3.5 and Figure 3.6 for pre-experimental period and experimental period respectively. In general, the highest amount of block intake/hour occurred in the morning between 9.30 am and 12.30 am for most animals.

Table 3.8 Block intake (g/d)

Treatment group	Block intake (g/d) ± SE Pre-experimental period (day 1-22)	Experimental period (day 23- 61)
Control (group1)	32 ± 11[a *)]	23 ± 4[a]
Teric 5% (group 2)	64 ± 6.3[a]	31 ± 4[a]
Alkanate 5% (group 3)	80 ±13[a]	190 ±16[a]
Teric 10% (group 4)	43 ± 11[a]	26 ± 4[a]
Alkanate 10% (group 5)	33 ± 11[a]	132 ± 37[a]

* Means within rows and between columns with different superscripts differ significantly

Figure 3.5 Diurnal pattern of block intake during pre-experimental period

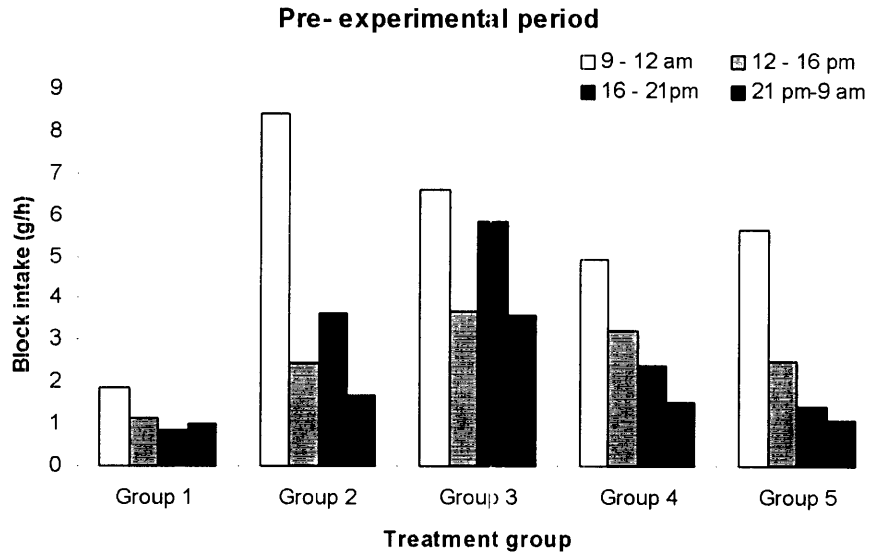
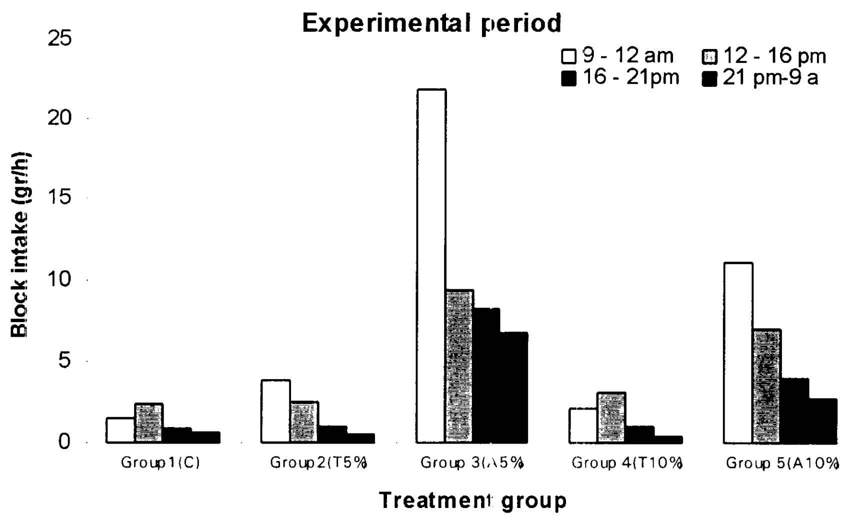


Figure 3.6 Diurnal pattern of block intake during experimental period



3.3.3 Discussion

Alkanate 3SL3 has been shown to be completely effective in removing protozoa from the rumen of lambs (Burggraff and Leng, 1980) and Teric GN9 has also been used to effectively eliminate protozoa from rumen of both sheep and cattle (Bird *et al*., 1978; Bird and Leng, 1978; Bird *et al*., 1979). These results were, however, obtained with an oral drenching regime. The key finding in the present study, was that when Teric and Alkanate were administered in molasses blocks, these detergents were not effective in eliminating protozoa from the rumen of sheep. There are several possible explanations for the failure of the molasses block containing anti-protozoal detergents to remove rumen protozoal populations.

The most obvious reason for the difference between the two detergents was the level of intake of each. The mean daily intake of blocks containing Teric was 28 ± 4 g/d, which was significantly ($p < 0.05$) lower than the mean daily intake of blocks containing Alkanate was 161 ± 26 g/d. The average intake of molasses block in the current experiment suggests that Teric was less preferred than Alkanate. The average daily amount of Teric detergent obtained from the molasses block was 2.05 g Teric/d, considerably lower than the oral dose of 15 g Teric/d used by Bird *et al*. (1979) and thus was unlikely to be successful in defaunating the rumen. This finding leads to the conclusion that when an anti-protozoal is to be administered in a molasses block, it must not reduce the palatability (acceptability) of the block.

On the other hand, the daily intake of Alkanate from the molasses block was 11.4 g/d which was comparable to the daily dose (10-15 g/d) reported to be effective when given as an oral drench (Burggraff and Leng, 1980). However, in the current experiment a daily 11.4 g Alkanate/d from the molasses block did not significantly alter the size of the protozoal population in the rumen.

The drenching method would obviously result in a high concentration of detergent in the rumen immediately after drenching, whereas the dose obtained from the molasses block would be delivered over 24 hours. It is possible that the concentration of Alkanate in the rumen of sheep receiving molasses blocks was too low to be effective.

Also during the 3 days drenching program, feed was not offered (Bird, 1989), whereas the molasses blocks were given to animals which were continuously offered feed. The diurnal pattern of block intake (Figure 3.6) indicates that the rate of block intake was highest when the animals consumed the major portion of their daily feed. This would be the period when the detergent would be least effective because of the dilution effects of saliva and fibre and the high rate of adsorption of detergent onto the fibre (Wright and Curtis, 1976).

In addition to reducing the amount of fibre in the rumen, feed restriction will have other effects which may enhance the anti-protozoal activity of the detergent. Firstly, protozoal numbers in the rumen will be reduced when feed is withheld (Hungate, 1966). Secondly, it is possible that under feed restriction conditions that

remaining protozoa will be more susceptible to the detergents. Thirdly, feed restriction is also likely to reduce the turnover of rumen contents so the detergent will remain in the rumen longer compared to the present study.

The Alkanate used in this study was at least 6 years old. The effect of age on the efficacy of the detergent is unknown, but it is possible that some of the anti-protozoal activity may have been lost.

An important practical point to be considered in these studies is the possible effect of defaunating treatment on feed intake. Feed intake is a good parameter of rumen function. In these studies total ME intake was not affected by the intake of detergent (via molasses block) which suggests that the detergent did not have any adverse effects on rumen function. It is apparent that molasses substituted for some of the oaten chaff in the ration.

The diurnal pattern of block intake showed that the rate of block intake was highest when animals consumed the major portion of their daily feed. Therefore if anti-protozoal agents are to be successfully administered with molasses blocks, they will have to overcome the dilution effects of eating and the problem of high levels of fibre in the rumen.

Assuming that a reduction in the amount of fibrous material in the rumen will enhance the effectiveness of detergents as defaunating agents, the efficacy of a period of starvation as suggested by Orpin, (1977c) is examined in the next section.

3.4 Experiment 2: Study on the efficacy of anti-protozoal molasses blocks when feed is withheld for a short period

3.4.1 Introduction

It has been demonstrated that an oral drench of either Teric GN9 or Alkanate $3SL_3$ can successfully defaunate sheep (Bird *et al.*, 1978; Bird and Leng, 1978; Burggraff and Leng, 1980). In the previous study molasses blocks containing Teric GN9 were not consumed in sufficient quantity to provide an effective dose of detergent. In contrast the daily intake of Alkanate 3 SL_3 obtained from the block was more than double the amount used in the oral drench. However, the high intake of Alkanate 3 SL_3 failed to significantly reduce the population of protozoa in the rumen. This result was unexpected and raises the obvious question : Why is Alkanate 3 SL_3 an effective anti-protozoal agent when given as an oral drench but ineffective when administered via a molasses block. The obvious difference between this study and the oral drenching method was the level of feed intake. In the oral drenching program feed was not offered during 3-4 day drenching period. In this study feed was offered *ad libitum* and feed intake was not affected by detergent intake.

Therefore the objective of this experiment was to determine the efficacy of the Alkanate-molasses block in the absence of feed. The detergent Teric GN9 was not tested in this study because daily intake of the molasses block containing the detergent was too low to provide an effective dose.

3.4.2 Materials and methods

a. Experimental animals

The twelve Merino wethers used in the previous experiment were reused without reallocation. The sheep which were in the Teric GN9 treatments were reallocated to the treatments randomly as shown in Table 3.9. The average body weight of animals for each treatment is shown in Table 3.10.

Table 3.9 Sheep allocation to treatment group and pen

Sheep tag n	Body weight	Treatment	Pen
23	34.5	Control	72
54	39.5	A 10%	71
150	37.5	A 10%	70
289	37.5	Control	69
270	41	A 5%	68
242	34.5	Control	67
121	47	A 10%	66
261	45	A 10%	65
241	39.5	A 5%	64
12	43.5	Control	63
183	38.5	A 5%	62
25	36.5	A 5%	61

Table 3.10 Average body weight of the treatment groups

Treatment group	average body weight (g)
Control (group 1)	37.5
Alkanate 5% (group 2)	38.9
Alkanate 10% (group 3)	42.2

b. Diet

b.1 Basal diet

The same basal diet as in the previous experiment consisting of 90% oaten chaff and 10% barley grain with a supplement of 10 g urea/d was given *ad libitum* at 9.30 each morning (dry matter and Nitrogen content are shown in Table 3.3).

b.2 Molasses blocks

Molasses blocks containing two levels of Alkanate 3 SI_3 were tested in this experiment, namely 5% and 10%. The composition of each block is shown in Table 3.4.

3.4.3 Experimental procedures

The present studies were carried out to examine the effectiveness of defaunating treatment using anti-protozoal molasses block when it was accompanied by a short (4 days) period during which the basal diet was withheld. The molasses block and water were freely available at all times.

When tested immediately before the experiment began (day 61), sheep 121 in pen 66 was found to have very low protozoal numbers. This animal was reinoculated with rumen protozoa from other sheep in order to make sure that all sheep had the

number of protozoa at least 1.0×10^5/ml rumen fluid at the beginning of the experiment.

After a ten-day adaptation period to the block containing Alkanate (there were no data taken during adaptation period), rumen fluid collection was begun for protozoal counts on day 71. Animals were denied access to the basal diet on days 74-77 inclusive and rumen fluid samples were collected at 12.30 hours on each of these days. The time- table for the procedures used in this experiment is given in Table 3.11

Table 3.11 A summary of the experimental timetable

Day 61- 70	Adaptation period to molasses block containing Alkanate $3SL_3$ (no data collection)
Day 71	The 1st collection of rumen fluid samples at 12.30 pm
Day 72	The 2nd collection of rumen fluid samples at 12.30 pm
Day 73	The 3rd collection of rumen fluid samples at 12.30 pm
Day 74	Starvation period began at 9.00 am (no basal diet)
Day 74	The 1st collection of rumen fluid samples at 12.30 pm during starvation
Day 75	The 2nd collection of rumen fluid samples at 12.30 pm
Day 76	The 3rd collection of rumen fluid samples at 12.30 pm
Day 77	The 4th collection of rumen fluid samples at 12.30 pm
Day 77	Basal diet was offered at 1 00 pm
Day 78	No data collection
Day 79	The 1st collection of rumen fluid samples at 12.30 pm
Day 80	The last collection of rumen fluid samples at 12.30 pm

3.4.4 Results

a. Protozoal numbers

The average density of rumen protozoa before the basal diet was withheld on day 74 (sample collection on days 71-73) was 2.6 ± 0.25 x 10^5/ml (range 0. 8-3.9 x 10^5/ml) for treated animals and 5.4± 0.7 x 10^5/ml (range 3.9 - 8.5 x 10^5/ml) for control animals. All treated animals were completely defaunated at day 77 (day 4th of feed restriction period), whereas average protozoal population for control animals was 2.6 ± 1.1 x 10^5/ml (range 0.2 - 1.5 x 10^5/ml). The average protozoal populations for animals on each level of Alkanate before and during feed restriction period can be seen in Table 3.12.

Table 3.12 The protozoal population density before and during feed restriction (x 10^5/ml)

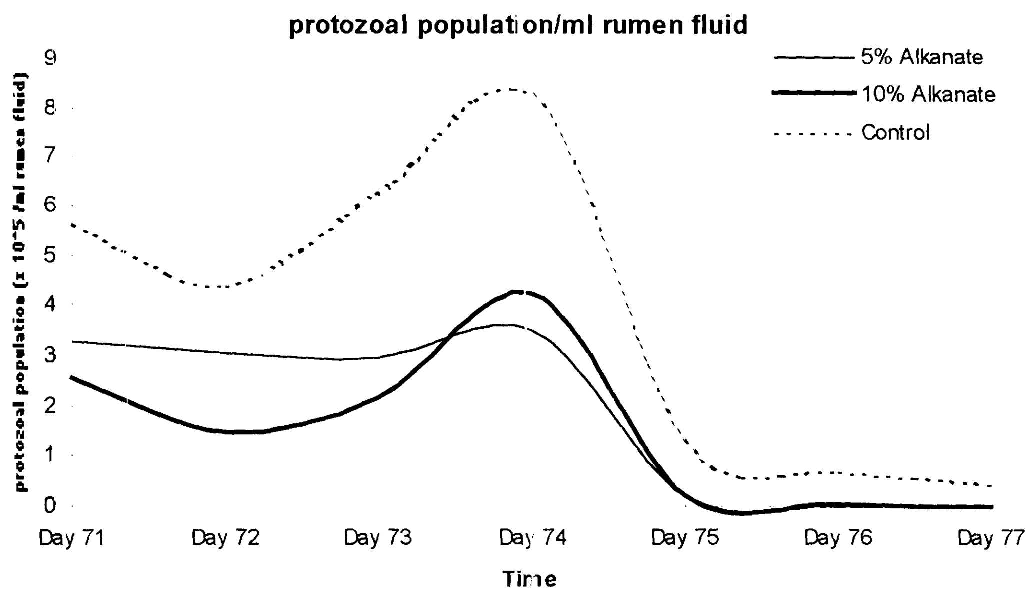
Level	Means ± Se						
	Day -71	Day-72	Day-73	Day-74*	Day-75	Day-76	Day-77
A 5%	3.3±0.69^{b}**	3.1±0.40^{b}	3.0±0.29^{b}	3.5±0.71^{b}	0.2±0.11^{d}	0.02±0.01^{d}	0.00±0.00^{d}
A 10%	2.6±0.76^{b}	1.5±0.39^{b}	2.1±0.80^{b}	4.2±2.41^{b}	0.2±0.19^{d}	0.04±0.04^{d}	0.00±0.00^{d}
Control	5.6±1.80^{a}	4.4±0.53^{a}	6.2±0.96^{a}	8.2±2.96^{a}	1.3±0.40^{c}	0.7±0.22^{c}	0.4±0.20^{c}

* Basal diet withheld as from 9.00 am
** Means within columns and means within rows with different superscript differ significantly

Withholding the basal ration significantly reduced protozoal population in all animals, including control animals. The average protozoal population was significantly lower after feed restriction than before ($P < 0.001$), and Figure 3.7 demonstrates that protozoal population fell rapidly when the basal (roughage) ration was withdrawn.

However, compared with controls, the protozoal population/ml rumen fluids of treated animals was significantly lower during both periods.

Figure 3.7 Protozoal population/ml rumen fluid before and during feed restriction



b. Block Intake

The average block intake during the period of full feeding was 164.4 ± 34.7 g/d and 36.4 ± 9.3 g/d for treated and control animals respectively, whereas corresponding values during the period of feed restriction were 130.5 ± 32.7 g/d and 48.9 ± 12.6 g/d. Although Figure 3.13 shows a dramatic decline in block intake at the beginning of the period of feed restriction for treated animals, the differences in block intake between days 71-74 and days 75-77 were non-significant (P> 0.5) for control group and 5%

Alkanate group, whereas for 10 % Alkanate the difference was highly significant ($P<0.01$).

The level of Alkanate in the molasses block (5 or 10%) significantly affected the amount of block consumed ($P < 0.05$), with the average intake for blocks containing 5% Alkanate being higher than for blocks containing either 10% Alkanate or 0 % (control) (Table 3.13).

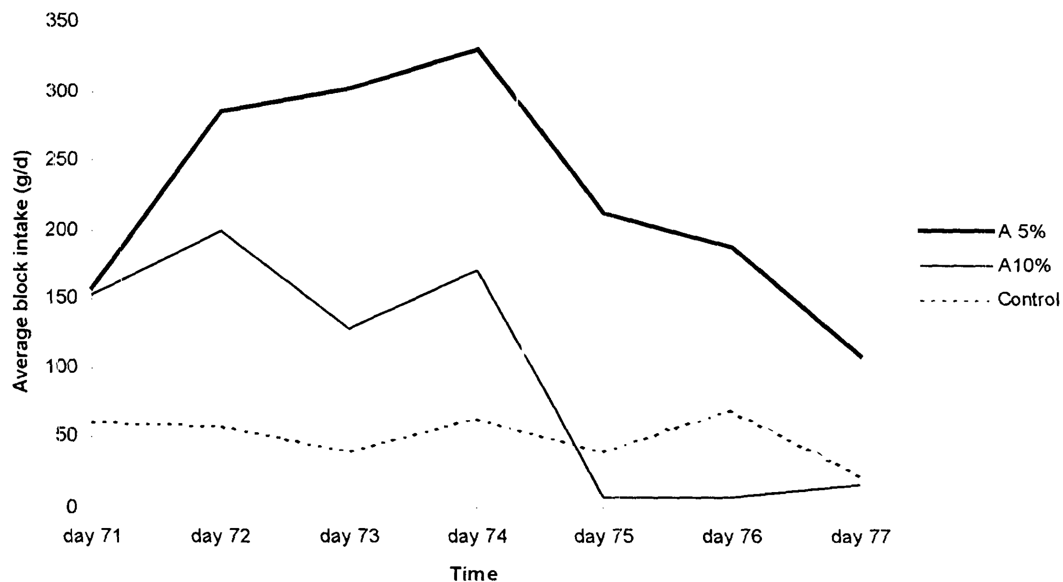
Table 3.13 Daily block intake before and during restricted feeding

	Block intake (Means± SE;g/d)						
Level	Day -71	Day-72	Day-73	Day-74*	Day-75	Day-76	Day-77
A 5%	158±50[a**]	285±76[a]	302±122[a]	331±114[a]	212±91[a]	188±86[a]	109±37[a]
A 10%	153± 71[b]	200±89[b]	128±57[b]	171±65[b]	8±2[c]	7±3[c]	17±13[c]
Control	61 ± 21[d]	57±20[d]	40±10[d]	63±21[d]	40±19[d]	69±42[d]	22±12[d]

* basal diet withheld from this day

** Means within columns and means within rows with different superscript differ significantly

Figure 3.8 Average block intake (g/d)



3.4.3 Discussion

When the basal diet was withheld, the protozoal population in all animals was significantly reduced. However, compared with controls, the protozoal populations in the rumen fluid of treated animals were significantly lower during both periods than in controls ($P<0.01$). It can be concluded that Alkanate 3 SL_3 in the current experiment did significantly reduce the protozoal population in the rumen, and that it completely removed protozoa within one week when the basal diet was withheld for 4 consecutive days. This result suggests that the efficacy of the anti-protozoal activity of the detergent is influenced by the level of feed intake. That is, Alkanate was most effective when the supply of roughage to the rumen was interrupted.

One possible explanation for this result is that feed restriction may have reduced the concentration of feed particles in the rumen, in which circumstance Alkanate $3SL_3$ may have been more effective in abolishing rumen protozoa. Less fibre in the rumen may result in a higher proportion of protozoa in the fluid phase, and less adsorption of detergent onto feed particles. Such an explanation is based on the results of Wright and Curtis (1976), who found that the effective concentration of detergent in rumen fluids was reduced by the adsorption of anti-protozoal molecule onto plant materials.

Moreover, restricted roughage intake is likely to result in less salivation and a concomitant reduction in rumen volume, thereby increasing the concentration of detergent in the rumen. This phenomenon may also explain why a block intake which corresponded to an intake of 11.7 g Alkanate /animal/d did not eliminate protozoa from the rumen in Experiment 1. In contrast, a block intake equivalent to 10.3 g Alkanate/animal/d completely removed protozoa within 3 days in this study.

Another possible explanation for the current result is that restricted roughage may limit the availability of nutrients for protozoa. Warner (1965) reported that starvation for only two or three days can lead to complete loss of some protozoal species, and Potter and Dehority (1973) found that protozoa concentration was decreased at low feeding levels.

Rumen motility is also likely to be reduced in the absence of fibre intake which in turn will reduce the outflow of rumen contents. Therefore the detergent will remain in the rumen for a longer time.

In conclusion, the anti-protozoal molasses block successfully defaunated sheep when it was combined with a short period without access to the basal diet. The result also indicated that even though the Alkanate 3 SL_3 was 6 years old it still retained some anti-protozoal activity. Further work is needed to clarify this situation, and will be undertaken in the next session.

CHAPTER 4

IN VITRO EXPERIMENTS TO EVALUATE VARIOUS ASPECTS OF THE EFFECTIVENESS OF ALKANATE 3 SL_3 AS AN ANTI-PROTOZOAL AGENT

4.1 Introduction

In contrast to Burggraaf's (1980) demonstration of the effectiveness of Alkanate 3 SL_3 in eliminating protozoa from the rumen of lambs, results from experiment 1 demonstrated that Alkanate administered in a molasses block (6.6 - 15.5 g/d) had no significant effect on protozoal number. Two possible reasons for this apparent lack of effectiveness are examined in this study. First of all, the Alkanate 3 SL_3 used in Experiment 1 had been stored for 6 years before it was used and it was thus possible that it might not have been as effective as fresh compound. Secondly, it is possible that the relatively high fibre content in the rumen (in contrast to the drenching method, in which feed is withheld) which resulted from the continuous feeding of the animals may have reduced the effective concentration of Alkanate in the fluid phase.

A series of experiments using the *in vitro* system developed by Willard and Kodras (1967) and modified by Setya Ningrat (1994) were carried out. In order to maintain the consistency of the incubation medium used for *in vitro* study, an artificial rumen fluid (RF^+ medium) was used in all experiments in preference to fresh rumen fluid, which may change over time. The objective of the *in vitro* experiments was to determine if the efficacy of Alkanate 3 SL_3 was affected by either the age of the

detergent used or the fibre content of the test medium. The questions which needed to be answered were :

1) Could RF^+ medium be used to replace fresh rumen fluids in *in vitro* experiments?
2) What level of Alkanate would be optimum to test the effect of fibre on its anti-protozoal efficacy ?
3) Was the Alkanate 3 SL_3 that had been used in the previous *in vivo* experiment less effective than fresh Alkanate 3 SL_3?
4) Was the efficacy of Alkanate 3 SL_3 affected by the fibre concentration in the test medium ?

The above questions are addressed in Experiments 3, 4, 5 and 6 respectively.

4.2 General materials and methods

4.2.1 Source of rumen fluid and protozoa

A single fistulated sheep was used throughout this experiment as a source of rumen fluid and rumen protozoa. The sheep was housed in an individual pen in a slatted-floor animal house. A diet consisting of 500 g lucerne chaff and 500 g oaten chaff was given once per day; the sheep had free access to fresh, clean water.

4.2.2 Incubation medium

Rumen fluid samples were collected from the fistulated sheep using a sampling probe positioned in the dorsal sac of the rumen. The probe was made of a metal cage covered with nylon gauze and connected to the plug of the rumen cannula by a length of plastic tubing. A 50 ml syringe attached to the probe was used to aspirate fluid samples which were then used as fresh rumen fluid samples in Experiment 3. Also this rumen fluid was used in the preparation of a standard rumen fluid medium (see Table 4.1) and for obtaining a concentrated preparation of protozoa which were added to the RF^+ incubation medium.

Table 4.1 Composition of the rumen fluid medium (RF^+) used in all *in vitro* studies (Modified from Hungate, 1969)

distilled H_2O	33 ml
Salt solution A	16.5 ml
Salt solution B	16.5 ml
Rumen fluid*)	33 ml
Peptone	0.1 g
Yeast extract	0.1 g
$NaHCO_3$	0.5 g
VFA solution	1 ml
Resazurin	0.1 ml
Cysteine - HCL	20 mg

*) Rumen fluid was autoclaved at 5 psi for 45 minutes to ensure that all resident protozoa were killed

Salt solution A

	% w/v	g/l
KH_2PO_4	0.3	3
NaCl	0.6	6
$(NH_4)_2 SO_4$	0.3	3
$CaCl_2$	0.03	0.3
$MgSO_4$	0.03	0.3

Salt solution B

KH_2PO_4	0.3	3

Salt solutions A and B were stored at 4° C until used.

4.2.3 Preparation of protozoa inoculum

One litre of rumen fluid was collected over a period of approximately 15 minutes and concentrated rumen protozoa were obtained by the following procedure. The rumen fluid sample was placed in a 50 ml sealed flask to reduce exposure to air, and was held in an insulated container filled with water at 39° C. The rumen fluid sample was centrifuged at 500g for 1 minute in Beckman, Model TJ-6 centrifuge, the supernatant was decanted and the protozoa-rich pellet was resuspended in 50 ml of rumen fluid and stored until used (within 15 minutes) at 39° C. Microscopic examination of the protozoa-rich sample revealed that only a small amount of fibre was present.

4.2.4 Incubation procedures

a. 10 ml RF^+ fluid at 39° C was added to a pre-heated incubation bottle. In Experiment 6, ground fibre was weighed into the incubation bottles before RF^- fluid was added. The protozoal inoculum was taken from storage, and mixed by inversion before 2 ml was added to the incubation bottle. The time was recorded and taken as the start of the incubation period. In experiment 3, an additional 2 ml of fresh rumen fluid was added to the "control" incubation bottles, in order to maintain the same volume as in the RF^+ fluid bottles.

b. Immediately after the addition of the protozoa inoculum the incubation bottle was shaken thoroughly to mix its contents and a sub-sample (1ml) was withdrawn with a "Gilsen" pipette and put into a John's vial containing 4 ml formal saline. The concentration of protozoa cells was later determined in this formal saline preserved sample and the result recorded as the "zero time" count.

c. Except for experiment 3, 1 ml of pre-heated (39° C) Alkanate solution was then added to the incubation bottle. 1 ml of pre-heated (39° C) RF^+ fluid was added to the control bottles to maintain the same volume as in the treatment bottles. This step resulted in a dilution of the protozoa with respect to the zero count of 9%. The incubation bottles were then held at 39° C.

d. After 5 h incubation at 39° C the procedures outlined in b) were repeated to yield a " post-incubation" protozoal count.

4.2.5 Statistical analysis

Differences between treatment effects were analysed statistically by one-way analysis of variance. All statements of significance imply $P < 0.05$ unless otherwise stated, while statements of non-significance imply $P > 0.05$.

4.2.6 Assessment of anti-protozoal activity

a. Measurement of intact protozoal cells

The anti-protozoal activity of the detergent Alkanate $3SL_3$, was assessed by measurement of the intact protozoa cells. This procedure was used to facilitate an observation with a large number of samples in 5 h. The reduction in the number of protozoa over 5 h of incubation was taken as index of anti-protozoal activity. The greater the reduction in protozoal number, the stronger the activity of the tested agent in killing protozoa.

The degree of disintegration of protozoa was measured by counting the number of intact cells present in a sample in a Hawkesley Crystallite Counting Chamber. In order to fix the cells, 1ml of cell solution from every sample was mixed thoroughly with 4 ml formol saline (200 ml formaldehyde, 1800 ml H_2O, and 18 g NaCl). The number of protozoa per ml of original solution can be calculated by using the same

formula as shown in section 3.2.4. The number of intact cells were expressed as a % of the zero time count.

The weakness of this procedure is that the number of intact cells may not give an accurate indication of the number of killed protozoa in the sample because disintegration of the cell is unlikely to occur immediately the cell dies. Therefore, in order to test the reliability of this procedure, the measurement of intact protozoal cells was compared to the measurement of cell motility in the preliminary experiment.

Results presented in Table 4.3 clearly show that cell motility (highest category) in the control incubations (with or without fibre) was maintained over the 5 h incubation period. In contrast no cell motility was observed in the incubations containing Alkanate (no fibre) and cell motility had ceased within 1 h of the start in the incubations containing Alkanate and fibre. An examination of the motility scores taken at 5 h would suggest that the presence of fibre had no effect on the anti-protozoal activity of Alkanate. However, the count of intact cells (Table 4.2) tells a different story. Clearly the addition of fibre has increased the number of intact cells remaining after 5 h incubation. These results provide an early indication that the presence of fibre does reduce the anti-protozoal activity of Alkanate. In contrast, the addition of fibre to the control incubation reduced the cell count. It is unlikely that the fibre killed the protozoa as motility score was not affected by fibre (Table 4.3). Therefore a more likely explanation is that some of the protozoa have attached to the fibre, effectively reducing the concentration of protozoa in the fluid phase (only cells in the fluid phase are counted under microscope). It is apparent from the results of this study that the

intact cell count is a more sensitive assay and consequently this assay was used in all further studies.

In all studies cell motility in the control was observed at the beginning and end of the incubation period to confirm that the study had been conducted with viable cells.

b. Measurement of cell motility

Cell motility was estimated by observing protozoa under a microscope at 40 x magnification. The fresh samples were kept at 39° C and the chamber was kept warm (39° C) during the observation. Cell motility was categorised by assessment of the number of non-motile cells. Protozoal movement was categorised as follows :

1 = many protozoa moving
2 = some protozoa moving
3 = less than 10 cells moving in each viewing field
4 = cells stationary - but cilia moving
5 = no internal or external movement of cilia (all presumed dead)

Table 4.2 Number of intact protozoal cells in 2.4 mm^3 of the microscopic counting chamber during 5 hours of incubation in different treatments.

Treatment	Number of intact cells				
	0 h	1 h	2 h	4 h	5 h
Control1	289	275	284	261	259
Control 2	339	237	256	258	208
C + fibre 1*)	307	314	264	245	177
C + fibre 2	338	280	233	213	209
Alkanate 1	356	147	32	0	0
Alkanate 2	234	127	24	0	0
A + fibre 1/1	343	245	127	122	17
A + fibre 1/2	266	163	185	68	25
A + fibre 2/1	317	258	229	253	185
A + fibre 2/2	411	195	255	296	176

*) fibre 1 = 0.2 g fibre/ml
fibre 2 = 0.4 g fibre/ml

Table 4.3 Number of non-motile cells (nm) and protozoal movement categories (ctg) in 2.4 mm^3 of the microscopic counting chamber during 5 hours of incubation in different treatments.

Treatment	0 h		1 h		2 h		4 h		5 h	
	nm	ctg	nm	ctg	nm	ctg	nm	ctg	nm	ctg
Control1	1	1	nc	1	2	1	nc	1	nc	1
Control 2	2	1	nc	1	1	1	nc	1	nc	1
C + fibre 1*)	5	1	12	1	2	1	6	1	3	1
C + fibre 2	2	1	1	1	1	1	5	1	5	1
Alkanate 1	42	5	nc	5	nc	5	d	5	d	5
Alkanate 2	65	5	nc	5	nc	5	d	5	d	5
A + fibre 1/1	54	4	nc	5	nc	5	nc	5	nc	5
A + fibre 1/2	70	3	nc	5	nc	5	nc	5	nc	5
A + fibre 2/1	62	3	nc	5	nc	5	nc	5	nc	5
A + fibre 2/2	65	4	nc	5	nc	5	nc	5	nc	5

*) fibre 1 = 0.2 g fibre/ml
fibre 2 = 0.4 g fibre/ml
nc = > 100 non-motile cells
d = All disintegrate

4.3 Experiment 3 : Comparison of RF^+ medium and fresh rumen fluid

4.3.1 Experimental procedures

Fresh rumen fluid was collected from the fistulated sheep prior to the experiment (see section 4.2.4). To compare the efficacy of RF^+ medium as compared to fresh rumen fluid, the concentration and motility of protozoal cells were determined every hour for 5 hours of incubation. Five replicate tubes were used. Procedures used to prepare samples for this experiment are described in 4.2.4.

4.3.2 Results

There was non-significant ($P>0.05$) difference in average of protozoal numbers between RF^+ medium and fresh rumen fluid at each of the five different times examined (Table 4.1). The results of the motility observations in this assay showed that all samples had a motility score of 1 throughout the 4 h of incubation. Only 1 to 2 protozoa were presumed dead throughout the 4 hours of incubation. There was no significant difference in the number of non-motile protozoal cells between two test media ((Table 4.2). Protozoal motility in RF^+ medium was comparable to that of fresh rumen fluid (Control).

Table 4.4 Number of intact protozoal cells in 2.4 mm^3 of the microscopic counting chamber during 4 hours of incubation in either RF^+ medium or fresh rumen fluids.

Samples	Number of intact cells				
	Means ± SE				
	0 hours	1 hours	2 hours	3 hours	4 hours
RF^+ medium	93 ± 3^{a} *)	87 ± 11^{a}	90 ± 6^{a}	89 ± 7^{a}	84 ± 5^{a}
Fresh RF	87 ± 5^{a}	80 ± 7^{a}	81 ± 4^{a}	79 ± 10^{a}	80 ± 5^{a}

*) means within columns with different superscripts differ significantly (P< 0.05)

Table 4.5 Number of non-motile protozoal cells in one field of the microscopic view during 4 hours of incubation in either RF^+ medium or fresh rumen fluids.

Samples	Number of non-motile cells				
	Means ± SE				
	0 hours	1 hours	2 hours	3 hours	4 hours
Artificial RF	0 ± 0^{a}	1 ± 0.4^{a}	0 ± 0^{a}	0.4 ± 0.2^{a}	1 ± 0.3^{a}
Fresh RF	0 ± 0^{a}	0.2 ± 0.2^{a}	0.8 ± 0.4^{a}	0.6 ± 0.4^{a}	0.2 ± 0.2^{a}

*) means within columns with different superscripts differ significantly (P< 0.05)

4.3.3 Discussion

The percentage of intact cells (ie. final N° / initial N°) in RF^+ medium and fresh rumen fluid after 4 hours incubation were 91 % and 91.5 % respectively and the difference was non-significant (P>0.05). From the results of the measurement of protozoa motility over 4 hours of incubation, it also appears that RF^+ medium was completely normal in its ability to sustain protozoa (100 % protozoal moving). These two results led to the conclusion that RF^+ medium could be reliably used in *in vitro*

experiments as an alternative to fresh rumen fluid. RF^+ medium was used in these *in vitro* experiments in preference to fresh rumen fluid because the latter may change over time.

4.4 Experiment 4 : Examination of the level of Alkanate required to kill protozoa in the *in vitro* test system

4.4.1 Experimental procedures

The minimum level of Alkanate required to kill from the test media was established by studying the effects of 4 concentrations of Alkanate on cell disintegration. The concentrations of Alkanate chosen for the study were : 0.04 g/ml (I), 0.03 g/ml (II), 0.02 g/ml (III) and, 0.01 g/ml. Four replicate tubes were used for each Alkanate level and the means of the reduction in the number of intact protozoa for each level were plotted. The incubation procedures described in section 4.2.4 were followed. At time zero, 1 ml of the respective Alkanate standards was added to the incubation tubes, giving a final Alkanate concentration in the incubation tubes of 0.0032, 0.0024, 0.0016 and 0.0008 g/ml.

Incubation procedures were followed as described in section 4.2.4. Cell disintegration was monitored by counting intact cells at zero and 5 hours of incubation. The "zero hour" readings were taken immediately before Alkanate was added and the second reading was made 5 hours later. The number of intact cells was counted in 2.4 mm^3 of the counting chamber.

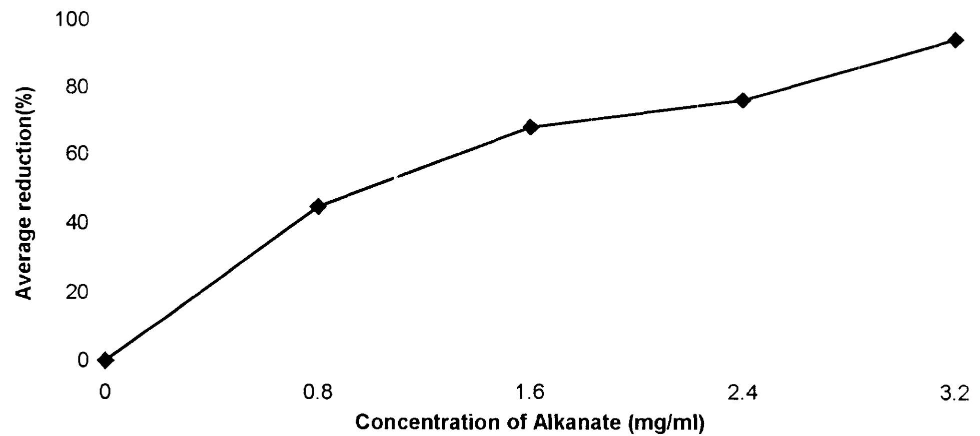
4.4.2 Results

The results of experiment 4 are presented in Table 4.6, and the percentage reduction in intact rumen protozoa as a response to different concentrations of Alkanate is illustrated, in Figure 4.1. It was found that a level 0.0032 g Alkanate/ ml of incubated fluid was sufficient to kill 95 ± 1.26 % of protozoa originally present.

Table 4.6 The number (and % reduction) in intact protozoa in 2.4 mm^3 at 0 and 5 hours of incubation at 0, 0.01, 0.02, 0.03 or 0.04 g Alkanate/ml.

Alkanate conc.	means ±SE of intact protozoa/2.4 mm^3		% reduction in intact protozoa during incubation
	0 hours	5 hours	
0	354 ± 17	337 ± 20	5 ± 2.55
0.8 mg/ ml	296 ± 24	157 ± 11	45 ± 7.09
1.6 mg/ml	339 ± 6	108 ± 14	68 ± 4.14
2.4 mg/ ml	369 ± 41	82 ± 7	76 ± 3.94
3.2 mg/ ml	348 ± 25	18 ± 3	95 ± 1.26

Figure 4.1 Average reduction (%) of intact protozoal cells as influenced by the Alkanate concentration



4.4.3 Discussion

Before the efficacy of Alkanate could be tested *in vitro* it was necessary to establish the minimum concentration of Alkanate required to significantly reduce (>90%) the number of intact protozoa cells. The dose-response results obtained in this study (Fig. 4.1) indicated that a concentration of 3.2 mg/ml of Alkanate reduced the number of intact protozoa cells by 95 ± [illegible].2% after 5 hours incubation. This result established the maximum concentration of Alkanate to be used in the *in vitro* studies.

4.5 Experiment 5 : Examination of the anti-protozoal activities of old compared with fresh Alkanate 3 SL_3

4.5.1 Experimental procedures

The anti-protozoal activity of fresh Alkanate and the Alkanate (old) which was used in Experiment 1 were compared at 3 different concentrations. Based on the findings from Experiment 4, the concentrations of Alkanate used were 0.01 g/ml, 0.02 g/ml and 0.03 g/ml. The same procedures were used as in Experiment 4.

4.5.2 Results

The results are presented in Table 4.4. There was a non-significant difference (P > 0.01) in reduction (%) of protozoal cells between the old and the fresh Alkanate tested at all concentrations.

Table 4.7 The reduction (%) in intact protozoa in 2.4 mm^3 during incubation period at 0.01, 0.02 or 0.03 g old or fresh Alkanate/ml

	The reduction (%) in intact protozoa means± SE	
Level	old Alkanate	fresh Alkanate
0.01 g/ ml	59.4 ± $2.5^{a*)}$	40.2 ± 5.2^{a}
0.02 g/ ml	73.5 ± 4.3^{a}	75.9 ± 1.2^{a}
0.03 g/ ml	87.3 ± 2.5^{a}	92.8 ± 1.2^{a}

*)means within rows with different superscripts differ significantly (P< 0.05)

4.5.3 Discussion

The mortality rate of protozoa increased progressively with concentration of Alkanate which confirmed the ability of this compound to act as an anti-protozoal agent. The results of the comparison of old and fresh Alkanate (Table 4.7) showed non-significant difference (P > 0.01) in reduction of protozoal count at all Alkanate concentrations tested. There is thus no reason to doubt the effectiveness of the "old"

Alkanate used in the *in vivo* experiments (Experiments 1 and 2). This result removes one of the explanations advanced (see section 3.3.3) for the failure of Alkanate in molasses blocks to completely defaunate the rumen.

4.6 Experiment 6 : Assessment of anti-protozoal activities of Alkanate $3SL_3$ in an *in vitro* test system using different fibre concentrations

4.6.1 Experimental procedures

Based on the findings from the previous *in vitro* experiment (sections 4.3 and 4.4), RF^+ medium was used instead of fresh rumen fluid as the incubation medium and a concentration of 0.04 g Alkanate/ml was used to assess the anti-protozoal activities of this detergent at two different levels of oaten chaff fibre, ie., 0.02 g/ml and 0.04 g/ ml.

A sample of the same oaten chaff used in *in vivo* experiments (chapter 3) was ground in a mill using a 1mm screen. The 0.2 g and 0.4 g of finely ground fibre was placed in each Mc Cartney bottle, 10 ml RF^+ and 2 ml concentrated protozoa were added and the contents were mixed gently by inversion. One ml of Alkanate (0.04 g/ml) was then added to each "test" tube and an equal volume of RF^+ was added to each "control", and the tubes were incubated in an oven at 39° C. The incubation procedures described in section 4.2.4 were followed.

Sub-samples were withdrawn for microscopic counting (see section 3.2.4) two times, at 0 and 5 hours of incubation The zero hour reading was made before

Alkanate was added to the incubation tube to yield an overall concentration of 0.0032 g/ ml. Due to low numbers of protozoa in the original inoculum used in this experiment, the number of intact cells was counted in volume of 4.8 mm^3 of the counting chamber. Changes in the number of protozoa after 5 hours are expressed by reduction percentage or mortality percentage, that is, the difference between protozoa count at 0 hours and protozoa count at 5 hours divided by protozoa count at 0 hours times 100 %.

4.6.2 Results

The results of this experiment are presented in Table 4.6. The addition of Alkanate (final concentration 3.2mg/ml) reduced the number of intact protozoan cells by 84 % after 5 hours of incubation which was significantly lower ($P<0.05$) than the 3% reduction that occurred in the control incubation.. The number of intact cells in the Alkanate treated tubes after 5 hours incubation was signicantly lower ($P<0.05$) than the number of intact cells remaing in the tubes containing fibre and Alkanate.The number of intact cells remaining in the incubation tubes were the same for both levels of fibre treatments.

The addition of fibre to the incubation solution significantly reduced ($P<0.05$) the intact cell count after 5 hours of incubation The cell count was the same for both levels of fibre treatments(Table 4.8).

Table 4.8 The reduction (%) in intact protozoa in 4.8 mm^3 during incubation period at 0.04 g Alkanate/ml in 0.02 or 0.04 g fibre/ ml

Treatment	% Reduction of intact protozoal cells
	Means ± Standard error (SE)
Control	$3.0 \pm 2.0^{a*)}$
C + 0.2 g fibre	15.7 ± 3.8^{b}
C + 0.4 g fibre	11.0 ± 4.4^{b}
Alkanate	84.0 ± 2.9^{c}
A + 0.2 g fibre	51.7 ± 6.3^{d}
A + 0.4 g fibre	50.2 ± 5.9^{d}

*)means with different superscripts differ significantly (P< 0.05)

4.6.3 Discussion

This experiment appears to support the earlier supposition that the doses of Alkanate in the molasses block were not sufficient to remove rumen protozoa, due to the presence of feed particles or fibre in the rumen. The presence of fibre in the rumen fluids significantly reduced the effectiveness of Alkanate. It was demonstrated in this *in vitro* experiment that the effectiveness of 0.04 g Alkanate/ml to kill rumen protozoa was reduced when fibre of oaten chaff was added into RF^+ medium at both level 0.2 g and 0.4 g fibre (P< 0.001). This reduction of the effectiveness was probably due to the adsorption of the active molecules onto fibre (Wright and Curtis, 1976).

The result also demonstrated that the number of protozoa was significantly reduced in the presence of fibre alone compared with control sample. The Count of protozoa in the control was reduced by only 3% in 5 hours, whereas reduction in samples containing 0.2 g and 04 g fibre was 15 % and 11.5 % respectively. Because

only a fluid sample was examined, this reduction may have been due to the sequestration of protozoa onto fibre, so it could not be detected under microscope. Therefore, in test medium with Alkanate+ fibre the actual % of killed protozoa may be lower than the % reduction of protozoal number that could be detected under microscope.