PROPORTIONAL DISTRIBUTION OF PREDOMINANT RUMEN BACTERIA BETWEEN THE SOLID AND THE LIQUID PORTIONS OF RUMINAL INGESTA

by

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CHAPTER 1.

GENERAL INTRODUCTION.

That certain bacteria in the rumen of sheep and cattle are attached to solid particles in the ruminal ingesta has been known for many years. In 1942 Baker published direct microscopical evidence that bacteria were attached to cellulose food particles and to starch granules in the rumen. The sites of attachment of these bacteria corresponded to sites of disintegration of the particles when viewed by polarised light. This indicated that at least bacteria attacking solid substrates such as cellulose and starch were attached to particles of ruminal ingesta. Van der Wath (1942) found rumen bacteria attached to particles of chemically pure cellulose and of crushed maize which he suspended in separate compartments of a pure silk bag inside the rumen of sheep. The bacteria associated with the particles of cellulose were mainly Gram negative rods, while clusters of iodophilic cocci were observed in most instances around the maize kernels. The latter organisms were isolated in pure culture and found to be heat-tolerant, short-chain, Gram positive cocci fermenting glucose, maltose, and other soluble sugars as well as starch.

It was thus not surprising that many years later Schwartz et al (1964) obtained evidence which suggested that bacteria metabolising soluble substrates such as glucose also showed marked attachment to solid particles of ingesta. In their experiments measurements were made of the rates of disappearance of glucose and urea from an artificial rumen containing either unstrained ingesta or strained ruminal fluid. The results showed that while the disappearance of urea took place at almost the same rate in both systems, glucose disappeared about five times faster from the unstrained ingesta. This suggested that about 80% of the glucose metabolising activity was removed by straining the ingesta. Since this activity is associated with certain species of rumen bacteria and protozoa it seems that a large proportion of one or both of these groups was removed by straining. On the other hand only a negligible proportion of the bacteria responsible for metabolising urea in the rumen were removed by straining. These findings indicate that some glucose metabolising bacteria are fixed to the food particles and are thus removed, while the ureolytic bacteria are free and therefore not removed by straining.

Because of the difficulties in taking samples of the heterogeneous ruminal ingesta it has been a general practice to use samples of strained

ruminal fluid for experimental purposes, on the assumption that the results obtained were representative of the true situation in the rumen. The fact that there is attachment of rumen bacteria to food particles in the rumen, and that the degree of attachment of different groups appears to vary so greatly, becomes very significant in view of the practice of straining ingesta. Far from being a reflection of the true population dynamics in the rumen the results so obtained would not only give a false indication of the true numbers of various functional groups of rumen bacteria but would also give a totally wrong impression of the relative proportions of the various groups.

This problem could readily be overcome in rate of reaction studies by using unstrained ingesta in the artificial rumen, and straining samples of this before measuring out accurate volumes for chemical analysis. For colony counts used in ecological studies of rumen bacteria, however, the position is quite different in that the solid particles of ingesta clog the syringes and pipettes used, and this interferes with the counting procedure. It was therefore necessary to find methods for determining the proportions of free and attached bacteria in ruminal ingesta, and to use these proportions as factors with which to correct counts made with strained ruminal fluid.

1:a Methods for determining bacteria attached to particles of ruminal ingesta

Two possible ways of approaching the problem of determining the proportions of fixed and free bacteria are:-

(i) By including fixed bacteria in viable counts.

(ii) By determining the proportion of fixed bacteria in situ using radio-isotope - labelled antibodies.

Using a non-specific growth medium and wide-mouth pipettes Bryant & Burkey (1953) obtained viable counts for rumen bacteria in the solid and in the liquid fractions of ruminal ingesta. They found that the former gave a colony count six times greater than that of the latter. This demonstrated that the proportion of bacteria fixed to the particles of the solid fraction was far greater than that free in the liquid portion. However, the method used lacked accuracy since it was unlikely to reveal true values for the numbers of bacteria located in crevices of the food particles. Only by first disintegrating the food particles or in some way dislodging the bacteria would it be possible to obtain an accurate estimation of these attached organisms.

Attempts to include fixed rumen bacteria in total or viable counts by mechanical disintegration of the ruminal ingesta before straining have been

made by very few workers. Warner (1962) homogenised and strained a sample of ingesta seven times and collected the strained fluid. To this he added the washings obtained by suspending any undisintegrated residue in buffer and centrifuging it three times. Microscopical counts on the resulting fluid were about three times higher than those for untreated strained ruminal fluid. Using carbon dioxide under a pressure of 150 pounds per square inch (psi) Munch-Peterson & James (1964) forced ruminal contents through two layers, each 5 mm deep, of glass beads to break up the vegetable matter. These workers reported that statistical analysis showed a significant increase in colony counts as a result of the treatment.

The methods used in the above experiments involved lengthy and drastic treatment of the ingesta. We felt that a simpler and quicker procedure would possibly give higher and more accurate counts. It could thus be used to confirm that a proportion of the rumen bacteria was indeed attached, and to estimate the magnitude of this proportion.

The first procedure tried was that of finely mincing the ingesta under controlled conditions before straining. This was done with the intention of increasing the numbers of strainable food particles carrying the fixed bacteria and possibly at the same time shaking a number of these bacteria free, thereby including both fixed and free bacteria in colony counts. By using a growth medium specific for the functional group of ruminal bacteria metabolising glucose the numbers of viable glucolytic bacteria present in the ruminal fluid strained from ingesta before and after mincing could thus be determined. Counts on ruminal fluid strained from untreated ingesta would give the number of free bacteria. The difference between counts on strained ruminal fluid from untreated and from minced ingesta would show whether bacteria were in fact fixed to the food particles or not, and would give the approximate numbers of fixed bacteria. The proportions of the predominating glucolytic bacteria fixed and free in ruminal ingesta could thus be calculated. Although by this method it might be possible to include food particles plus bacteria in viable counts, the fixed bacteria would probably be counted in units consisting of one or more bacteria attached to the particle. In view of this the possibility was considered of using non-lethal ultrasonic vibrations to free the bacteria individually. This could be done in the presence of a surface active agent to prevent reattachment of the bacteria. Such a method would give a more accurate measure of the numbers fixed and would thus give results more valuable to ecological studies.

Radioisotopes could be used to trace the fixed and the free bacteria by preparing antibodies against bacteria isolated from ruminal ingesta and tagging these antibodies with a radioactive label. After treating ruminal ingesta with this labelled antibody tracing the distribution of the radioactivity in the ingesta would then give a measure of the distribution of the bacteria. Antibodies labelled with a fluorescing radical have been similarly used to detect the presence of certain bacterial strains in ruminal contents (Hobson & Mann, 1957; Davies, 1965). The use of a fluorescing radical is not suitable for locating bacteria on solid particles of ingesta since those bacteria which react with the fluorescing antibodies give the same type of green fluorescence as the coarser particles of hay and other ingesta. This makes it difficult to distinguish these bacteria from the background (Hobson & Mann, 1957). Radicals containing radioactive ^{14}C could be attached to antibodies in the same way as a fluorescing radical. However, coupling of ^{131}I to the tyrosine rings of the serum fraction bearing the antibody is an easier procedure, as it takes place by a simple exchange reaction. Further the gamma-emission of ^{131}I is easier to detect than the beta-emission of ^{14}C, and less effort has to be expended on the preparation of samples for radioactive measurements.

1:b. Outline and significance of the present investigation

1. The present investigation was undertaken to determine the feasibility of three possible methods for determining the proportions of fixed and free bacteria in ruminal ingesta.
2. The intention was to apply each of the three methods to the functional groups of bacteria metabolising glucose and urea in the rumen of sheep conditioned to poor teff hay, glucose and urea. These particular rumen bacteria were chosen because the proportions fixed and free obtained for these organisms by each of the three methods could be checked against the proportions deduced from the rates of disappearance of glucose or urea from unstrained ingesta and strained ruminal fluid in artificial rumen experiments according to Schwartz et al (1964).

The proportions calculated from bacterial counts were based on work done by the author, while data used for calculating the proportions from rates of glucose disappearance were supplied by M. Lochner, F.M.C. Gilchrist and H.M. Schwartz.

3. The first method involved mincing the ingesta to increase the number of bacteria passing into the fluid obtained by straining the ingesta through

cheesecloth. In the development of this method it was necessary to determine the time of mincing for the optimum inclusion of fixed bacteria in the counts without destruction of the bacteria, and to have growth media allowing selective viable counts of specific groups of bacteria. A specific growth medium for glucose metabolisers had been developed by Kistner (1960), but one for the ureolytic bacteria still had to be developed.

4. The second method aimed at detaching fixed bacteria by using ultrasonic vibrations and required the use of non-lethal ultrasonic frequencies in conjunction with viable counts on selective growth medium.
5. The third method was intended to give indirect measurements of the distribution of bacteria in ingesta by determining the distribution in the ingesta of ^{131}I-labelled antibodies against selected species. To perfect this technique it was necessary first to isolate the appropriate rumen bacteria, then to obtain antibodies with specific activity against only these isolates, and thirdly to ascertain that these strains persisted in the rumen for a long enough period to allow such measurements to be made. It was also necessary to show that the distribution of the ^{131}I-labelled antibody in the ingesta was due only to its reaction with the appropriate bacteria.
6. One of the main problems in determining the proportion of fixed bacteria was to know whether all the appropriate fixed bacteria had been accounted for. It was felt that if similar findings were obtained for the proportions of total to free by four different methods then reasonable confidence could be placed in the values obtained.
7. Of the three methods under investigation those involving treatment of the ingesta with mincing or ultrasonic vibrations followed by viable counts would reveal the proportional distribution of the functional groups of glucolytic and ureolytic bacteria. On the other hand the method using ^{131}I-labelled antibodies would indicate the distribution of the individual species within the functional group, the sum of these individual distributions reflecting that of the functional group as a whole, as with the first two methods.
8. The development of a reliable method for determining the proportions of fixed and of free glucolytic or ureolytic bacteria would provide a means of estimating for the first time the degree of attachment of any particular rumen bacteria.
9. These findings would demonstrate that the different results obtained by Schwartz et al (1964) for the disappearance of urea and glucose were in fact

due to a difference in the degree of attachment of the relevant bacteria.

10. The findings would also provide a means of adjusting the results of previous experiments dealing with the numbers of glucolytic and ureolytic bacteria in strained ruminal fluid (Gilchrist & Kistner, 1962; Kistner, Gouws & Gilchrist, 1962; Gilchrist, 1965).
11. A scheme for determining the distribution of any given strain of bacteria in the rumen would thus be established and it would yield results of particular importance to ecological studies in the rumen.
12. Because of the completely different nature of the methods used in the mincing and in the ultrasonic experiments on the one hand, and the radioisotope-labelled antibody experiments on the other, the work was written up in two parts, namely:-

Part I : The use of colony counts to determine the proportional distribution of rumen bacteria:

Chapters 2 and 3.

Part II : Preliminary investigations into the use of labelled antibodies to determine the proportional distribution of rumen bacteria:

Chapters 4, 5 and 6.

PART I

THE USE OF COLONY COUNTS TO DETERMINE THE PROPORTIONAL DISTRIBUTION OF RUMEN BACTERIA

CHAPTER 2

EXPERIMENTAL

2:a. Animal management

Six full-mouth Merino wethers (K4, K7, K8, K10, K11, K12), fitted with permanent rumen fistulae, were housed in separate pens. All were conditioned for at least six weeks to a diet consisting of poor teff hay (Eragrostis tef; 1,200 g) of a similar quality to that used by Schwartz et al (1964) and a mineral trace-element lick (Phosvita, Ruffel; 15 g) given once daily at 8.30 am. A solution of 80 g of glucose (BP) and 10 g of urea (Merck, AR) in 1 l of warm (40°C) water was dosed per fistulam at 9.00 am daily. Once weekly the animals received 1 g of Vitamin A (Hidose A, Agricura). Water was freely available except on sampling days when it was given only after the samples had been taken.

2:b. Sampling

Between 250 and 350 ml of ingesta was withdrawn per fistulam by suction tube into a wide-necked flask surrounded by crushed ice. The flask was immediately stoppered and taken to the laboratory where the contents were used as soon as possible.

2:c. Untreated samples

A portion of the sample of ingesta (± 30 ml) was well squeezed through two layers of cheesecloth, the strained ruminal fluid completely filling a 1 oz bottle which was then stoppered and placed in crushed ice.

2:d. Mincing of ingesta

A 150 ml portion of the sample of ingesta was measured into a 250 ml wide-mouthed flask surrounded by crushed ice and continuously flushed with O_2-free CO_2 (Kistner, 1960), and 1 ml of 10% (v/v) Antifoam B (Dow-Corning) was added. This portion was minced with an Ultra-Turrax mincer (TP 18/2, Janke and Kunkel) fitted with a cutting bead 18 mm in diameter and composed of two square-cross-section blades revolving at 20,000 rev/min 1 mm inside a crown of eight similar teeth at the end of a stainless steel shaft. Mincing was periodically interrupted for 30 sec. to prevent the temperature of the ingesta from rising above 25°C. In the final procedure adopted these interruptions were made every 2.5 min, although intervals of 2 min were used in the developmental stages. The ingesta were then squeezed through two layers of cheesecloth in the same way as the untreated sample, and collected in a 1 oz bottle in crushed ice.

2:e. Ultrasonic treatment of ingesta

For treating the ingesta the ultrasonic pulse generator and transducer units of the Ultrasonic Laboratory Set USL. G300, of Schoeller & Co Electronic Works, Germany, were used. Various units were available for coupling to the generator, enabling frequencies of 20, 300 and 1,000 Kcyc/sec. to be produced. The power outputs of these different units could be adjusted through a range of settings, the resultant output equivalents of which were as follows:

Frequency of transducer (Kcyc/sec)	Power setting		
	1	2	3
	Power output (W/cm^3)		
20 (For 100 ml ingesta)	0.35	0.75	1.25
300 (For 300 ml ingesta)	0.64	1.2	2.1
1,000 (Ditto)	0.7	1.4	2.35

For treatment at 300 and 1,000 Kcyc/sec, 300 ml of the sample of ingesta was placed in a water-cooled vessel, the bottom of which was formed by a very thin stainless steel diaphragm directly coupled to the piezoelectric crystal of the ultrasonic transducer. Immediately after treatment for the required period the ingesta were stirred and a portion was removed for straining. For treatment at 20 Kcyc/sec a magnetostrictive probe was used to treat 100 ml of the sample of ingesta in a 150 ml beaker surrounded by crushed ice, with the tip of the probe lowered to about 1.5 cm below the surface of the ingesta.

In some of the experiments, as indicated in Table 6, 5 ml quantities of 10% ($^{v}/_{v}$) solutions of:

(a) a surface active agent (Tween 80, L. Light & Co); and

(b) an antifoam agent (Antifoam B, Dow Corning)

were added to the ingesta before treatment.

2:f. E_h measurement

The oxidation-reduction potential of the ingesta was measured with a Metrohm 280-E battery operated pH Meter, with a separate millivolt scale. The accuracy of the instrument is given as $\pm$ 10 mV and the amplification was checked by means of an external Weston Standard Cell. The electrode system was:

Unknown solution Pt/K-Cl bridge/Ag/Ag-Cl (Saturated). The potential of the reference half-cell was given by the manufacturer as + 200 mV. The metal electrode was cleaned by immersing just the platinum tip in hot nitric

acid for a few minutes. This treatment was followed by electrolysis in about 100 ml of 1N-HCl solution plus 1 drop of Teepol for 3 min. The platinum electrode was connected to the negative pole of a 6 V dry cell and a graphite electrode served as anode. After rinsing with distilled water the electrode was used immediately to measure the potential. Electrolytic cleaning was carried out between measurements.

2:g. Viable counts of glucose fermenters

Specific viable counts of the glucose metabolising bacteria were made in medium G1 (Appendix I) which was prepared with the anaerobic precuations adopted by Kistner (1960). The counts were made by the method of Kistner (1960) on 1 ml amounts of strained ruminal fluid from untreated and from treated ingesta. The counting bottles were incubated at 39°C for not longer than 20 hr.

2:h. Detection of urea hydrolysers

2:h:(i). Media

Media containing urea and phenol red as pH indicator were used to detect the presence of viable bacteria hydrolysing urea to NH_3 and CO_2. The anaerobic techniques of Kistner (1960) were used in the preparation of these media which had in common a high reducing potential obtained by adding cysteine-HCl and gassing with O_2-free gases. All media also contained Antifoam B. Other ingredients included in the different media were: glucose and cellobiose as energy sources; Bacto Yeast Extract as a source of B vitamins; Bacto Peptone; Bacto Agar; a solution of mineral salts as employed by Hungate (1950) except that $(NH_4)_2SO_4$ was omitted; and volatile fatty acids. The compositions of the salt solutions and the different media are given in Appendix I.

2:h:(ii). Growth in solid media

The procedure used by Kistner (1960) for colony counts of rumen bacteria was adopted for obtaining single colonies of bacteria growing in solid media containing urea as nitrogen source.

2:h:(iii). Growth in liquid media

One-in-ten serial dilutions of strained ruminal fluid were made in the liquid media. A colour change to red after incubation for 24 hr at 39°C was taken as an indication of growth of ureolytic bacteria.

2:i. Counts of rumen protozoa

For counts of rumen protozoa a sample of about 30 ml of ruminal ingesta was taken per fistulam from a sheep. The sample was well mixed and before the heavier particles could settle a 5 ml subsample was taken using a

10 ml graduated pipette with the tip cut off. This 5 ml subsample was added to 20 ml of formol-saline (10% (^{V}/v) formaldehyde in 0.85% (^{W}/v) saline). After mixing well a 1 ml portion of this diluted ingesta was taken, using the same pipette, and added to 19 ml of formol-saline. This gave a final dilution of ruminal ingesta in formol-saline of 1 : 100. A graduated 1 ml pipette delivering a drop of known volume was used to place 1 drop of the well-stirred 1 : 100 dilution onto a 3 x 1 inch microscope slide. All the protozoa in this drop were then counted under a binocular microscope using 40 x magnification. The number counted was divided by the volume of the drop and multiplied by the dilution factor (100) to give the total number of protozoa in 1 ml of the whole ingesta. The weight of two 10 ml portions of the whole ingesta sample was determined, and this allowed the result to be expressed as number of protozoa per gram of ingesta.

CHAPTER 3

RESULTS AND DISCUSSION

3:A. GLUCOSE FERMENTERS

3:A:a. The effect of mincing the ingesta on counts of glucose fermenters

3:A:a:(i). Mincing time for maximum increase in counts

Counts were made of the glucolytic bacteria in strained ruminal fluid obtained from ingesta before and after the ingesta had been minced for different periods of time (Appendix II). These counts were made over a period of two months using the ingesta of one sheep, K8. The counts on untreated strained ruminal fluid fluctuated from one sampling day to the next.

TABLE 1

THE EFFECT ON THE RATIO OF TOTAL TO FREE GLUCOLYTIC BACTERIA OF MINCING RUMINAL INGESTA FROM SHEEP K8 FOR DIFFERENT TIMES

Date (1963)	Time minced (min)	Averages of 8 counts (Appendix II) in strained ruminal fluid (millions per ml)		Increase factor $\left(\frac{B}{A}\right)$
		Untreated ingesta (A)	Minced ingesta (B)	
25.9	1.0	3.8	9.0	2.4
18.11	2.5	52.5	212.5	4.0
3.12	3.0	30.0	163.7	5.5
1.10	4.0	2.0	12.1	6.0
22.10	5.0	45.0	285.0	6.3
18.11	6.0	52.5	333.7	6.4
20.11	7.5	17.5	76.2	4.4
3.12	8.0	30.0	167.5	5.6
20.11	9.0	17.5	75.0	4.3
31.10	10.0	43.7	123.7	2.8
6.11	10.0	136.2	378.7	2.8

Similar fluctuations were observed by Kistner et al (1962) for counts of cellulolytic bacteria in samples taken on different days over a period of four months from one sheep fed lucerne hay. To determine the effect of mincing the ingesta on the counts it was therefore always necessary to do a control count on the untreated ingesta. The increase in the count as a result of mincing was calculated by dividing the count obtained after mincing by the count before mincing, the resulting value being called the increase factor (Table 1). Values equal to, greater than, or less than 1.0 indicated that the count was unchanged, increased or diminished by mincing. A graph (Fig. 1) was constructed of the values obtained for this factor plotted against time of mincing. From this it can be seen that the maximum increase in the count was 6.4 fold after 5.5 to 6 min of mincing. Mincing for a longer time resulted in progressively lower values, indicating the killing off of some of the bacteria. Since the increase factor at 5 min (6.3) was very close to the maximum of 6.4 at 6 min, mincing for 5 min was adopted as the standard procedure, since the shorter time was less likely to be injurious to the bacteria.

3:A:a:(ii). Percentages of glucolytic bacteria fixed and free

The averages of the counts from untreated ingesta and from ingesta minced for different times are listed in Table 1. The counts on ruminal fluid strained from untreated ingesta represent the free portion of the glucolytic bacterial population while the counts on ruminal fluid strained from minced ingesta give the total numbers of free plus fixed bacteria. The difference between the two values gives a figure for the number of fixed bacteria. It is thus possible to calculate what percentages of the total bacteria counted were originally fixed or free (Table 2). At the optimum mincing time 84.3% of the bacteria counted were found to be fixed, while only 15.7% were originally free in ruminal fluid strained from the ingesta of sheep K8 during the last quarter of 1963.

3:A:b. The effect of mincing strained ruminal fluid on counts of glucose fermenters

It was possible that the increased counts observed after mincing represented an artefact due to the breaking up of clumps of bacteria by the Ultra-Turrax mincer. On the other hand the increased count could have been due to the inclusion of fixed bacteria, but it might have been lower than theoretically possible due to the killing of bacteria by the mincing treatment. To check these

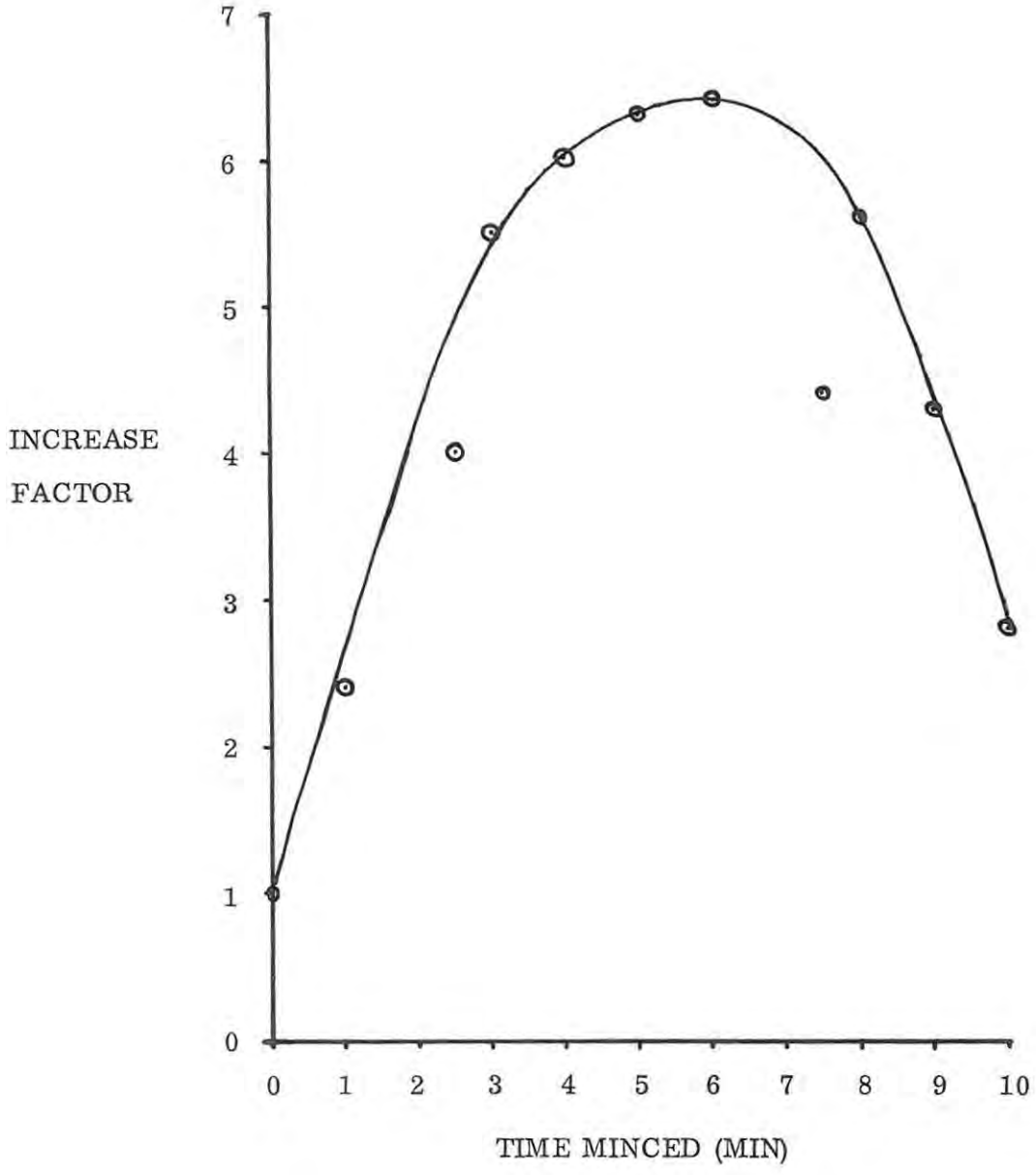

FIG 1. Increase factor of counts as a function of mincing time

TABLE 2

PERCENTAGES OF FIXED AND FREE GLUCOLYTIC BACTERIA CALCULATED FROM THE COUNT AVERAGES IN TABLE 1

Date	Time minced	Averages of counts in strained ruminal fluid (millions per ml)			Distribution of bacteria	
		Untreated ingesta (Free bacteria)	Minced ingesta (Fixed and free)	Difference (Fixed bacteria)	% Free $\left(\frac{A}{B} \times 100\right)$	% Fixed $\left(\frac{B-A}{B} \times 100\right)$
(1963)	(min)	(A)	(B)	(B-A)		
25.9	1.0	3.8	9.0	5,2	42,2	57,8
18.11	2.5	52.5	212.5	160.0	24.7	75.3
3.12	3.0	30.0	163.7	133.7	18.3	81.7
1.10	4.0	2.0	12.1	10.1	16.5	83.5
22.10	5.0	45.0	285.0	240.0	15.8	84.2
18.11	6.0	52.5	333.7	281.2	15.7	84.3
20.11	7.5	17.5	76.2	58.7	23.0	77.0
3.12	8.0	30.0	167.5	137.5	17.9	82.1
20.11	9.0	17.5	75.0	57.5	23.3	76.7
31.10	10.0	43.7	123.7	80.0	35.3	64.7
6.11	10.0	136.2	378.7	242.5	36.0	64.0

possibilities a sample of strained ruminal fluid was divided into two portions, one being inoculated into medium G1 without any further treatment, while the other was minced for 5 min before inoculation.

TABLE 3

THE EFFECT OF MINCING STRAINED RUMINAL FLUID FROM SHEEP K8

Date (1963)	Time minced (min)	Averages of counts (Appendix III) (millions per ml)		Increase factor $\left(\frac{B}{A}\right)$
		Untreated ruminal fluid (A)	Minced ruminal fluid (B)	
7.10	5.0	11.8	12.1	1.025
15.10	5.0	9.9	12.4	1.25

Comparative counts for the strained sample and for the strained and then minced sample (Appendix III) gave increase factors of 1.02 and 1.25 (Table 3). These increases are very small compared to the 6.4 fold increase found after mincing whole ingesta. Thus these result show that the increased counts obtained are in fact due to the incorporation into the strained ruminal fluid of fixed bacteria, and that the counts are not affected by killing of bacteria or by breaking up of clumps or chains of bacteria during the treatment.

3:A:c. The effect of mincing on the E_h and temperature of the ingesta

As a check on the effectiveness of the precautions of flushing the sample with O_2-free CO_2 and cooling it in ice during treatment, the temperature and E_h of ingesta samples were measured just before mincing and again immediately after mincing for 3 or 6 min. The results in Table 4 show that although the temperature rise was as much as 18.5°C, cooling the sample in ice kept the final temperature within acceptable limits. Table 5 shows that the anaerobiosis of the samples was maintained and even slightly improved in some cases. Therefore in future experiments mincing of ingesta was carried out with the sample on ice and gassed with CO_2, and mincing was never continued for more than 3 min without a 30 sec break for cooling.

TABLE 4

CHANGES IN TEMPERATURE OF INGESTA DURING MINCING

Date	Sample volume (ml)	Sample on ice	Time minced (min)	Temperature of ingesta (^{o}C)		
				Before mincing	After mincing	Change
15.11.63	150	+	3	6.5	24.8	+ 18.3
20.10.65	400	-	6	31.5	40.0	+ 8.5
25.10.65	150	-	6	31.5	50.0	+ 18.5

TABLE 5

CHANGES IN OXIDATION - REDUCTION POTENTIAL (E_h) OF INGESTA DURING MINCING

Date	Sample volume (ml)	Sample on ice	Time minced (min)	E_h of ingesta (mV)		
				Before mincing	After mincing	Change
15.11.63	150	+	3	- 310	- 295	+ 15
20.10.65	400	-	6	- 395	- 450	- 55
25.10.65	150	-	6	- 356	- 360	- 4

3:A:d. Ratio of total to free bacteria compared with the ratio of the rates of glucose disappearance in unstrained ingesta and in strained ruminal fluid

Schwartz et al (1964) found glucose disappearance from unstrained ingesta in the artificial rumen to follow very closely that occuring in vivo, but to be very much faster than from strained ruminal fluid in vitro. Moreover the rate of glucose disappearance from ingesta solids, suspended in a volume of buffer equal to that of the ruminal fluid removed, was appreciably greater than that from the fluid. This suggested that the greater part of the glucose-utilizing micro-organisms in the rumen were in fact attached to the food particles, and that the rate of glucose disappearance appeared to be related to the numbers of glucolytic micro-organisms present in the system. Thus if ratios obtained for counts of total/free bacteria were to agree with ratios for rates of glucose disappearance from unstrained ingesta/strained ruminal fluid on samples taken from the same sheep at the same time, then reasonable confidence could be placed in the similar findings obtained by these two completely different methods.

TABLE 6

NUMBERS OF PROTOZOA IN RUMINAL INGESTA FROM EACH OF FIVE SHEEP

Sheep	Counts of protozoa			
	Replicates	Mean	Dilution factor*	Total count per gram ingesta
K4	125 161 99	122	$\frac{100}{0.042 \times 0.98}$	29.6×10^4
K8	101 79 117	99	$\frac{100}{0.042 \times 0.98}$	24.1×10^4
K10	56 66	61	$\frac{100}{0.042 \times 0.98}$	14.8×10^4
K11	116 89 100	102	$\frac{100}{0.042 \times 0.98}$	24.8×10^4
K12	113 118	115	$\frac{100}{0.042 \times 0.98}$	28.0×10^4

* 1 ml Whole ingesta diluted to 100 ml 1 Drop diluted ingesta used for count = 0.042 ml
Density of ingesta = 0.98 g/ml average.

TABLE 7

COUNTS OF GLUCOLYTIC BACTERIA IN INGESTA FROM SHEEP K4 DURING THE COURSE OF AN ARTIFICIAL RUMEN EXPERIMENT WITH WHOLE INGESTA AND WITH STRAINED RUMINAL FLUID

Time after dosing (min)	Counts of glucolytic bacteria (millions per ml)			
	Strained ruminal fluid		Whole ingesta (minced 5 min and strained)	
	Replicates	Mean	Replicates	Mean
0	120 130 150 170	142	270 200 210 230	227
60	150 160 130 -	145	130 220 200 180	182
120	180 150 170 160	165	170 190 280 260	225

The glucolytic population in the rumen of the sheep investigated consisted of both protozoa and bacteria. The contribution of the protozoa to rates of glucose disappearance, however, was likely to be small, since the numbers of protozoa in millions per gram of ingesta (0.148 - 0.296 : Table 6) were found to be a thousand times less than those of the bacteria (100 - 400 million per gram ingesta : Table 8). The bacteria also have the added advantage of a greater surface area to volume ratio to enhance their enzymatic activity. Furthermore, viable counts of glucolytic bacteria (Table 7) taken during the course of an artificial rumen experiment were shown to be the same at 0, 1 and 2 hours. It was thus felt that rates of glucose disappearance from ingesta during the two-hour period directly after dosing could reasonably be taken to be a function of the number of glucolytic bacteria present in the ingesta at the time of sampling.

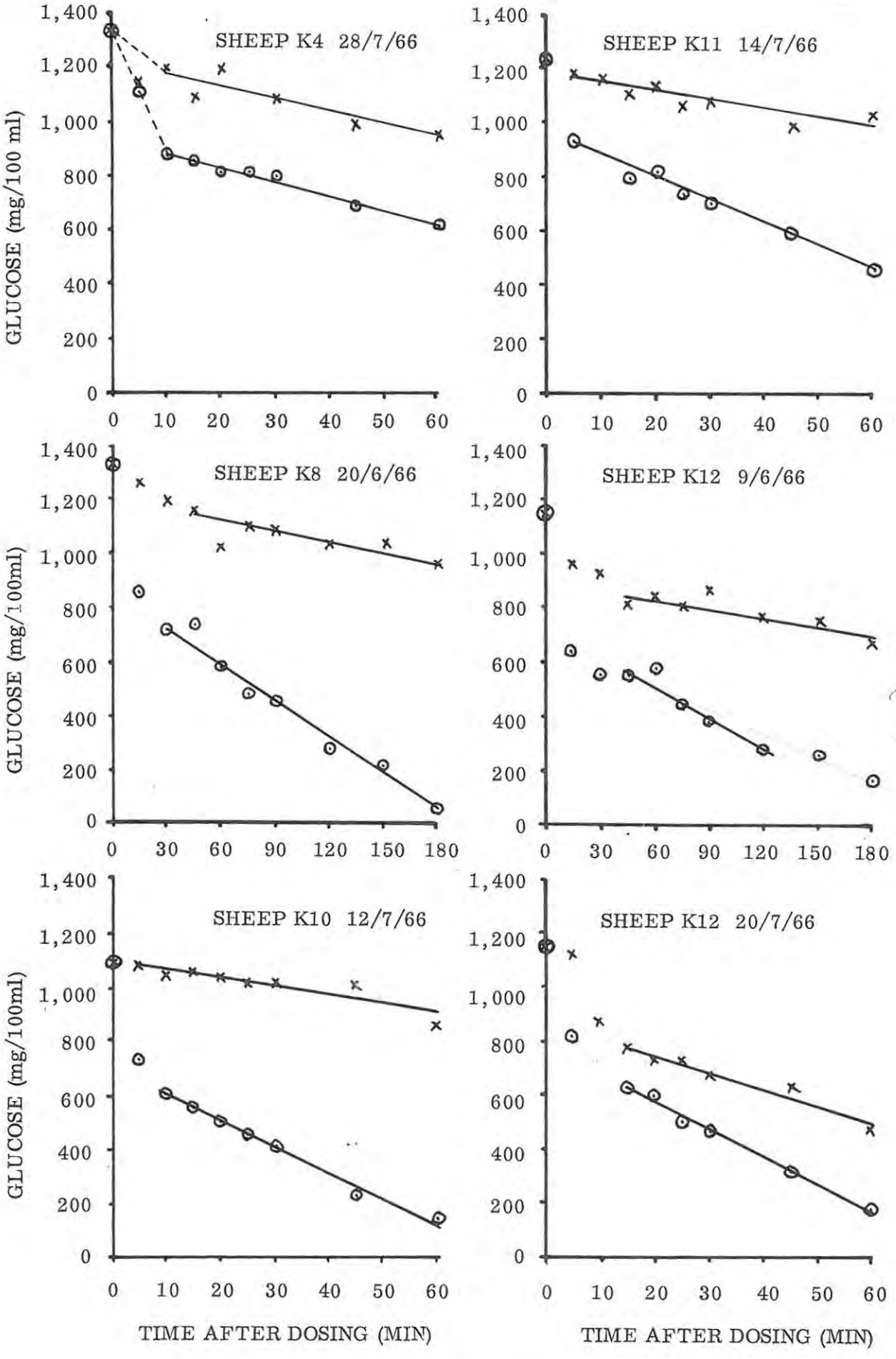

FIG. 2. Disappearance of glucose from whole ruminal ingesta (⊙ - ⊙) and from strained ruminal fluid (x - x) (See Appendix IV).

It was found that glucose disappeared from ruminal ingesta at two rates (Fig. 2, e.g. sheep K4, 28/7/66). The more rapid initial rate was probably a result of glucose storage as it was accompanied by the appearance of polysaccharides stainable by iodine inside the micro-organisms (Schwartz & van Zyl, unpublished). The second stage of glucose disappearance was slower and was most likely determined by the rate of glucose breakdown. This second rate was chosen for the purpose of comparing the rates of glucose disappearance from unstrained ingesta with those from strained ruminal fluid, since constant rates could be obtained for a reasonable length of time (30 - 45 min) during the initial two hour period. Glucose disappearance was plotted against time (Fig. 2) and the rate of disappearance was obtained by determining the slope of the curve where the rate was constant over a period. The ratios (Table 8) for rates of glucose disappearance from unstrained ingesta/strained ruminal fluid (1.4, 3.2, 2.5, 2.1, 3.2, 1.7) were found to agree remarkably well with ratios for total/free glucolytic bacteria (1.6, 2.7, 1.9, 2.1, 3.0, 1.4) obtained for the same samples of ingesta from 5 different sheep conditioned to poor teff hay, glucose and urea. From the data in Table 8 it was evident that the proportion of bacteria fixed was from 28 to 69%, while the free bacteria ranged from 72 to 31%. These percentages for fixed bacteria obtained in the first half of 1966 were lower than those obtained in the last quarter of 1964 (Table 2). This was probably a result of a change in the predominating species of glucolytic bacteria since Gram positive diplococci presumptively identified as Streptococcus bovis (Part II, Table 10) predominated on the earlier occasion, while Gram negative rods predominated in 1966 (van Domselaar, unpublished results). This difference in distribution figures was presumably due to the cocci being attached to a greater extent than the rods, although this could only be confirmed by investigating the distribution in ruminal ingesta of labelled antibodies against isolated strains of these organisms.

3:A:e. The effect of ultrasonic vibration of ruminal ingesta on viable counts of glucolytic bacteria in strained ruminal fluid

Counts (Appendix VII) were obtained from samples of ruminal fluid strained from ingesta before and after ultrasonic treatment for different periods at various frequencies and power outputs. The averages of these counts and the resulting increase factors for each of the various combinations of treatment are set out in Table 9. Surface active agents, Antifoam B and Tween 80, were added in certain experiments at each frequency with a view to preventing dis-

TABLE 8

RATIOS OF TOTAL TO FREE BACTERIA CALCULATED FROM VIABLE COUNTS AND FROM RATES OF GLUCOSE DISAPPEARANCE FROM THE SAME INGESTA

Ingesta from sheep	Date	Rates of glucose disappearance (Appendix IV) given as slopes of curves in Fig 5 (mg %/min)			Mean viable counts of glucolytic bacteria in strained ruminal fluid (Appendix V) (millions/ml)		
		Whole ingesta	Strained ruminal fluid	Ratio	Ingesta minced 5 min	Untreated ingesta	Ratio
	(1966)	(A)	(B)	$\left(\frac{A}{B}\right)$	(C)	(D)	$\left(\frac{C}{D}\right)$
K4	28.7	$\frac{140}{25}$	$\frac{100}{25}$	1.4	227	142	1.6
K8	20.6	$\frac{320}{25}$	$\frac{100}{25}$	3.2	175	65	2.7
K10	12.7	$\frac{240}{25}$	$\frac{95}{25}$	2.5	293	157	1.9
K11	14.7	$\frac{210}{25}$	$\frac{100}{25}$	2.1	427	202	2.1
K12	9.6	$\frac{270}{25}$	$\frac{85}{25}$	3.2	320	107	3.0
	20.7	$\frac{260}{25}$	$\frac{150}{25}$	1.7	390	275	1.4

TABLE 9

THE EFFECT OF DIFFERENT ULTRASONIC TREATMENTS OF INGESTA FROM SHEEP K7 ON THE RATIO OF TOTAL TO FREE GLUCOLYTIC BACTERIA

Transducer output		Date	Time treated	Averages of counts (Appendix VI) in strained ruminal fluid (millions per ml)		Increase factor
Frequency	Power			Untreated ingesta	Ultrasonically treated ingesta	
(Kcyc/sec)	(W/cm^3)	(1964)	(min)	(A)	(B)	$\left(\frac{B}{A}\right)$
1,000	0.7	27.5	5.0	41.0	45.0	1.1
			10.0	41.0	96.0	2.3
		16.6	20.0[A]	15.0	15.0	1.0
			30.0[A]	15.0	11.2	0.7
	1.4	2.6	5.0	48.0	52.0	1.1
			10.0	48.0	52.0	1.1
		20.8	20.0[B]	22.5	12.5	0.6
		9.6	30.0	92.5	91.2	1.0
			60.0	92.5	0	0
	2.35	18.6	5.0[A]	23.4	14.5	0.6
			10.0[A]	23.4	24.1	1.0
300	0.64	25.6	1.0[A]	46.0	49.0	1.1
			5.0[A]	46.0	64.0	1.4
		11.8	5.0[B]	17.5	27.5	1.6
		18.8	12.5[B]	30.0	39.0	1.3
		11.8	20.0[B]	17.5	25.0	1.4
		17.9	30.0	14.0	12.8	0.9

Continued/.....

TABLE 9 - (Continued)

Transducer output		Date	Time treated	Averages of counts (Appendix VI) in strained ruminal fluid (millions per ml)		Increase factor
Frequency	Power			Untreated ingesta	Ultrasonically treated ingesta	
(Kcyc/sec)	(W/cm^3)	(1964)	(min)	(A)	(B)	$\left(\frac{B}{A}\right)$
20	$\pm$ 0.35	25.8	1.0[A]	13.0	15.7	1.2
			5.0[A]	13.0	18.3	1.4
		24.9	10.0[B]	37.5	42.5	1.1
			20.0[B]	37.5	42.5	1.1
		17.9	30.0	14.0	14.0	1.0

A = Antifoam B added; B = Tween 80 added.

dislodged bacteria becoming reattached. These additions appeared to have no marked effects on the results. The increase factors for each power output at 1,000 Kcyc/sec are plotted against the time of treatment in Fig 3. The highest increase factor obtained was 2.3 fold and was obtained after treating the ingesta for 10 min at 0.7 W/cm^3. After treatment for longer than this there was evidence of a killing off of increasing numbers of bacteria with time. This lethal effect was evident much sooner with higher power outputs of 1.4 and 2.35 W/cm^3.

The graphs in Fig 4 show the increase factors obtained plotted against the time of treatment for each of the frequencies 1,000, 300 and 20 Kcyc/sec at the power setting[1] For the sake of comparison the graph of increase factor against time of mincing has been included plotted to the same scale. A higher increase factor was obtained with each increase in frequency, but these never attained the magnitude (6.4) of that obtained by mincing. This tendancy is also demonstrated in Fig 5 in which the maximum increase factors obtained are plotted as a function of the frequency. Extrapollation of this curve shows that at a frequency of about 2,200 Kcyc/sec the number of bacteria freed would approach the value obtained by mincing the ingesta.

FREQUENCY = 1,000 Kcyc/sec

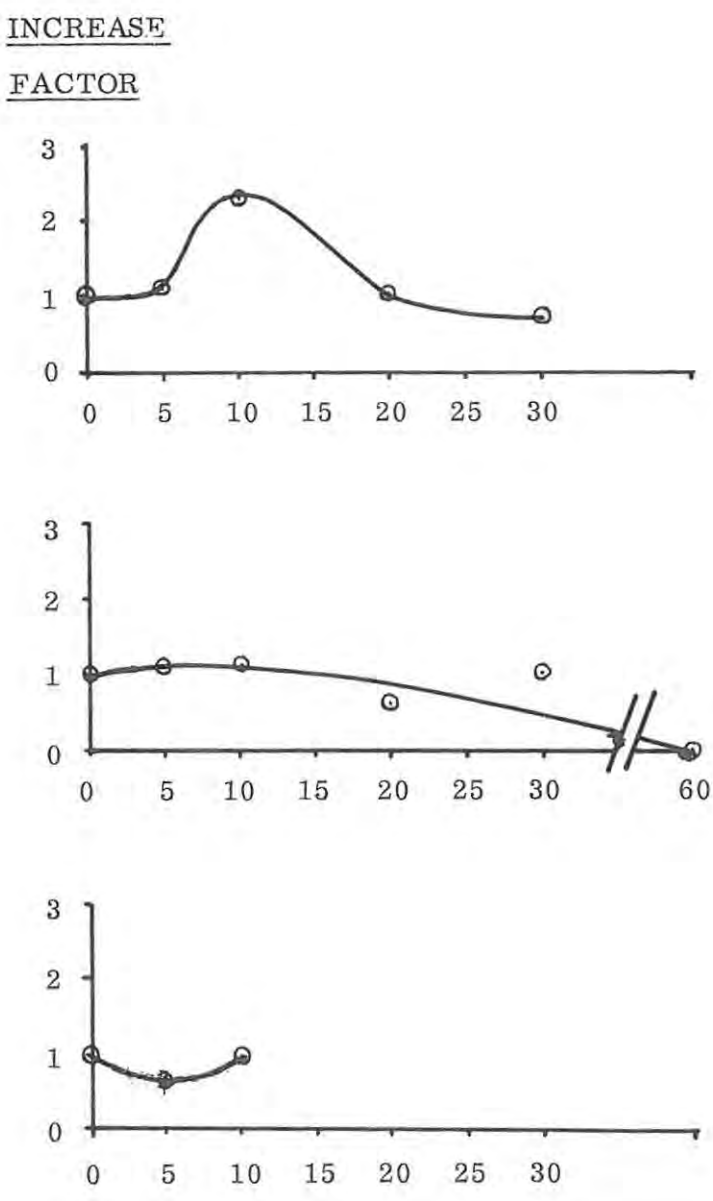

FIG 3. Increase factor as a function of time of ultrasonic treatment of ruminal ingesta at 1,000 Kcyc/sec

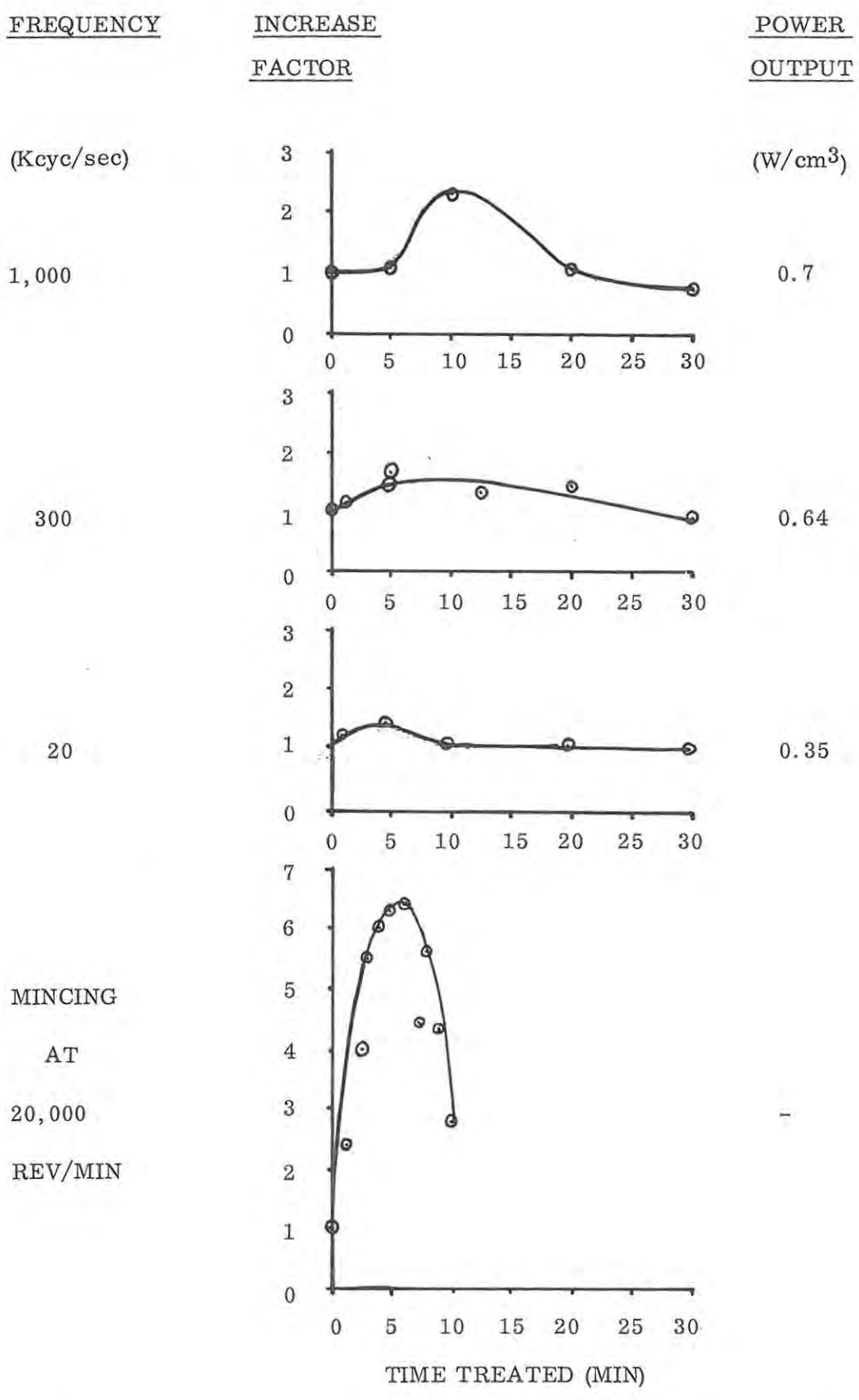

FIG. 4. Increase factor as a function of time of treatment compared at different frequencies and power outputs and with mincing

TREATMENT	MAXIMUM INCREASE FACTOR
Mincing	6.4
1,000 Kcyc/sec	2.34
300 Kcyc/sec	1.57
20 Kcyc/sec	1.41

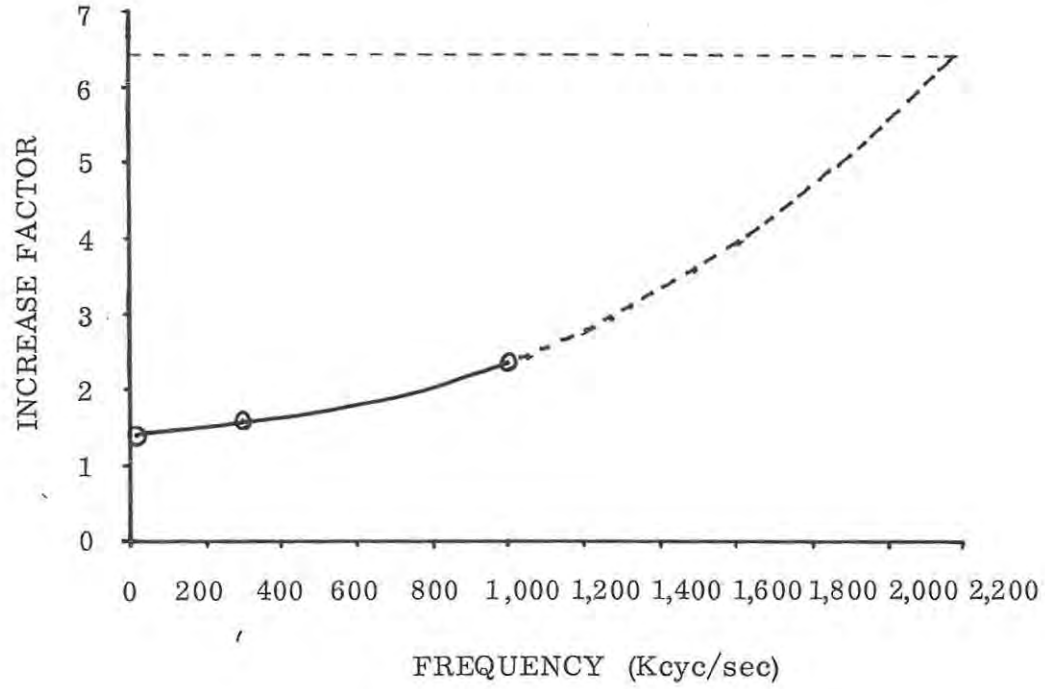

FIG. 5. The maximum increase factor obtained at each frequency plotted against frequency

The lethal effects of ultrasonic treatment are mainly due to the phenomenon of cavitation, which is the formation and collapse of gaseous bubbles in the fluid as a result of the pressure changes set up in the sample. Since at higher frequencies cavitation becomes more difficult to produce, as well as less intense in its effects, and there is more rapid particle acceleration, it is likely that the use of higher frequencies would both reduce the lethal effect and facilitate the release of fixed bacteria. In the absence of accessory units capable of producing frequencies higher than 1,000 Kcyc/sec it was not possible to extend these experiments to test this hypothesis. Part of this hypothesis is however strengthened by the fact that other workers using ultrasonic vibrations to treat bacterial suspensions found that higher frequencies were less lethal than lower ones. Shropshire (1946) found that at a frequency of 9 Kcyc/sec killing of Bacterium coli took place at very low power outputs. Jacobs and Thornley (1954) found that at 1,000 Kcyc/sec there was no lethal effect below a power threshold of between 25 and 50 per cent of the full power output (± 250 Watts), but that at 20.5 Kcyc/sec there was still a very noticeable lethal action at 3 per cent of the full power, and no indication of a threshold.

In none of our experiments using ultrasonic vibrations did the increase factor even closely approach that of the 6.4 obtained by mincing the ingesta. The method of ultrasonic treatment was therefore abandoned in favour of the mincing procedure.

3:B. UREA SPLITTERS

3:B:a. Growth of ureolytic bacteria

Media (Appendix I) used for growing ureolytic bacteria contained between 0.3 and 2 percent of urea, since Gibbons & Doetsch (1959) grew urea hydrolysing rumen bacteria in medium containing 0.3 percent of urea, while the media used in clinical bacteriology for the differential diagnosis of the Enterobacteriaceae contain 2 percent of urea. The media used and the amount of urea in each were as follows:

Media (See Appendix I)	Urea (%)
U 5 through U 8	0.3
U 1, U 15	0.4
U 14, U 16	1.0
U2, U 3, U 4, U 9 through U 13	2.0

As urea is hydrolysed to ammonia and carbon dioxide,

$NH_2 - CO - NH_2 + H_2O = 2\ NH_3 + CO_2$,

and as the formation of ammonia can easily be detected by hydrogen ion indicators, we decided to try and develop a culture medium which would show the presence of urea splitting bacteria by indicating the formation of ammonia in the medium. Phenol red was chosen as indicator as its colour change from yellow to red occurs over the pH range 6.4 - 8.0, and the culture media for rumen bacteria are typically buffered at pH 6.8. Since rumen bacteria are anaerobic the media contained cysteine - HCl as reducing agent and were gassed with O_2-free gases. Both solid and liquid media were developed.

3:B:b. Detection of ureolytic activity

Urea hydrolysis, as indicated by colour change of phenol red occured with all concentrations of urea except once with 2 percent urea in medium U2, which contained no sugars, CO_2, or volatile fatty acids. The lack of activity in U2 might have been due to the lack of a carbon source for the growth of the bacteria.

The colour change tended to be most rapid, intense and persistent with 2 percent urea, particularly in medium U9 which changed in 2 hr. The highest dilution in which colour change occured in the series of dilutions of ruminal fluid in the growth media was 10^{-3}, indicating from 1,000 to 9,000 urea hydrolysers per milliliter of strained ruminal fluid. This constitutes a very low count for a functional group of rumen bacteria since these usually occur in numbers of 10 million per milliliter or higher. It was uncertain whether the low count obtained was due to the fact that ureolytic activity occured only as far as the 10^{-3} dilution, or whether NH_3 production in higher dilutions was marked by the buffering capacity of the medium and/or by the acids produced during fermentation of carbohydrates. Therefore the factors controlling the buffering action and production of acid in liquid medium U5, which gave colour change in the 10^{-1} dilution only, were successively changed. The cellobiose and glucose were each reduced from 0.2 to 0.025% (W/v) in medium U6, since Bryant & Robinson (1961) had shown that these amounts were just sufficient to support the growth of rumen bacteria. This reduction of the carbon and energy source failed to induce a colour change in dilutions higher than 10^{-1}. Reducing also the bicarbonate (U7) resulted in a colour change in dilutions 10^{-1} and 10^{-2} after 72 hr of incubation. Reducing the phosphates in addition (U8) gave a colour change in dilution 10^{-1}

after 5 hr and in 10^{-3} after 72 hr. Keeping the same reduced concentrations of carbohydrates and buffering substances, the urea concentration was increased to 2% in U9. This resulted in a colour change in dilutions 10^{-1} and 10^{-2} after 2 hr, and in 10^{-3} after 24 hr. Leaving out the glucose and cellobiose in lightly buffered media containing 0.4, 1, or 2% urea (U15, U14, U11) failed to induce colour change above dilution 10^{-2}. Thus U9 proved to be the most suitable medium so far developed for demonstrating colour change due to NH_3 production from urea.

3:B:c. Detection of urea hydrolysing colonies

All the solid media which supported growth of urea hydrolysers (U4, U11, U12) were non-specific in that they also supported the growth of bacteria in higher dilutions where there was no colour change. Medium U9 was solidified with agar-agar in an attempt to develop a solid medium in which the urea hydrolysers would show up as pink colonies or as colonies surrounded by a pink halo. The entire agar layer, however, in roll bottles inoculated with dilutions 10^{-1} to 10^{-3} turned pink, probably due to the extremely rapid diffusion of NH_3 throughout the medium. Increasing the phosphate buffer made no difference (U10). Diethylbarbituric acid was added to the medium (U12) in an effort to localise the NH_3 by complexing it with the diethylbarbituric acid in the vicinity of the urea hydrolysing colonies, but with no success. Gibbons & Doetsch (1959) used large colony size as a criterion of ability to metabolise urea, and in this way obtained counts of 100,000 per ml of ruminal fluid for urea hydrolysing bacteria on medium U5 solidified with agar-agar. We found, however, that many large colonies did not produce a colour change when transferred to liquid medium U9, and thus large colony size alone could not be taken as an indication of a ureolytic colony. Since the non-ureolytic bacteria appeared greatly to outnumber the ureolytic bacteria in our samples of ruminal fluid, it has been impossible as yet to count the urea hydrolysers in the same way as the glucose fermenters.

3:B:d. Growth requirements

Attention was therefore paid to counting urea hydrolysers by serial dilution in liquid media. Different growth requirements were first studied to make it possible to encourage the growth of these organisms.

3:B:d:(i). Carbon source

Omitting glucose and/or cellobiose (U2, U11, U14, U15) resulted in little or no growth, while the addition of 0.025, 0.05, or 0.2% of each of these carbohydrates supported growth.

3:B:d:(ii). Growth factors

It was found that a combination of Yeast Extract and acetic, valeric and branch chain fatty acids (U16) could substitute for ruminal fluid as a source of growth factors.

3:B:d:(iii). Carbon dioxide

Gassing with CO_2 (U1, U5 through U16) improved growth and colour change. Improved growth was also found by Gibbons et al (1959) in the presence of CO_2.

3:B:d:(iv). Organic nitrogen

One percent of peptone supported growth in only the 10^{-1} dilution in the presence of glucose but without added Yeast Extract, fatty acids or CO_2.

These preliminary investigations indicated that the ureolytic rumen bacteria could not use urea alone as a source of energy, but also required glucose or cellobiose. They could, however, use urea as a source of nitrogen if Yeast Extract, fatty acids and CO_2 were also provided.

3:B:e. Estimation of numbers of ureolytic bacteria by tenfold dilution series

Estimation of the numbers of ureolytic bacteria in the rumen for determination of the proportions fixed and free could not be done by viable colony counts, since it was not possible to develop a solid medium specific for these organisms. The possibility remained of estimating the numbers of ureolytic bacteria by using tenfold dilution series in liquid growth medium, and testing a number of samples from each dilution for urea splitting activity. Statistical methods are available for determining the most probable number of bacteria from such results. However, Taylor (1962) pointed out that there is an inherent lack of precision in the method, and that some results are so improbable as to cast doubt on the validity of the assumptions necessary in determining the results. In view of this no further attempts were made to estimate the proportions of ureolytic bacteria fixed and free by using bacterial counts.

Single colonies could be isolated from solid media and tested for urea splitting activity. It was therefore decided to leave further investigations until it was possible to use the method of tracing the distribution of isotope - labelled antibodies against strains of ureolytic bacteria in ruminal ingesta.

3:B:f. Significance of the results obtained in Part I of this investigation

The distribution of the functional group of glucolytic rumen bacteria between the liquid and the solid portions of ruminal ingesta was determined,

using differential viable counts of these organisms in strained ruminal fluid from untreated ingesta and from ingesta subjected to high speed mincing. The counts from untreated ingesta gave the numbers of free glucolytic bacteria; those from minced ingesta represented the total numbers of these organisms fixed and free; while the difference between total and free counts gave the numbers fixed. To ascertain whether the counts for minced ingesta did indeed approximate to the total numbers of glucolytic bacteria, the ratios for total/free were compared with the ratios for the rates of glucose disappearance with unstrained ingesta/strained ruminal fluid from the same sample of ingesta, since these rates appear to be related to the numbers of glucolytic bacteria present in these two systems. Ratios obtained for the bacterial counts (1.6, 2.7, 1.9, 2.1, 3.0, 1.4) agreed remarkably well with those for the rates of glucose disappearance (1.4, 3.2, 2.5, 2.1, 3.2, 1.7). Thus reasonable confidence could be placed in the similar findings by these two completely different methods, and the figures obtained for the total counts accepted as giving approximately the correct value. The percentage distribution of the glucolytic bacteria was found to be 28 - 69% fixed and 72 - 31% free. This is the first time that the distribution of any particular group of rumen bacteria between solid and liquid portions of ruminal ingesta has been quantitatively determined.

The count ratios quoted above were lower than that (6.4) obtained in the original mincing experiments 18 months earlier. This change in count ratio was accompanied by a change in the predominating species within the functional group of glucolytic bacteria, from Gram positive diplococci and rods to Gram negative rods, and it would therefore appear that the former were attached to a greater extent than the latter. Thus while the count ratio indicated the proportional distribution of the functional group as a whole, the change in ratio observed indicated a change in the relative numbers of the contributing species, each probably with its set distribution characteristics, rather than a change in the actual distribution of any of these species in the ingesta. The method is thus limited to investigating the proportions fixed and free of a functional group as a whole under given equilibrium conditions in the rumen.

It was not possible to use ultrasonic vibrations to free the attached bacteria as the vibrations produced by the available transducer units were lethal at all power settings tested. Nevertheless indications were obtained that at a frequency of 2,200 Kcyc/sec the number of viable bacteria freed would approach the value obtained by mincing the ingesta.

Estimation of the distribution of the functional group of ureolytic bacteria could not be done by the count ratio method. This was due to the facts that a specific solid growth medium for making colony counts of viable ureolytic bacteria could not be developed, and that counts by serial tenfold dilutions in liquid medium were too inaccurate and time consuming.

PART II

PRELIMINARY INVESTIGATIONS INTO THE USE OF LABELLED ANTIBODIES TO DETERMINE THE PROPORTIONAL DISTRIBUTION OF RUMEN BACTERIA

CHAPTER 4

INTRODUCTION

The composition of the ruminal flora at any given time represents a dynamic balance reached through fierce competition and is dependant on conditions existing in the rumen (Gilchrist & Clark, 1957). Thus the predominating species contributing to a functional group of rumen bacteria fermenting a given substrate tend to change with changing conditions in the rumen. This is likely to happen periodically even on one and the same diet (Gouws & Kistner, 1965). Hence values obtained for the distribution of fixed and free bacteria in ruminal ingesta of a functional group under one set of conditions would be unlikely to apply under any other, even though each of the contributing species probably exhibits a constant degree of attachment. On the other hand values obtained for individual species could be used to ascertain whether their degrees of attachment were indeed constant, and could also be applied more generally to ecological and biochemical studies of the rumen.

The determination of the degree of attachment for individual species could best be done by making use of the specificity of the antigen-antibody reaction, and by determining the distribution between the solid and the liquid portions of ruminal ingesta of ^{131}I labelled antibodies prepared against somatic antigens of predominant strains of rumen bacteria isolated from minced ingesta to ensure the presence of highly attached strains. This technique is essentially a serological one and thus a brief outline of the serology relevant to the present problem is presented here.

For the preparation of specific antibodies of high activity against each of the predominant strains of glucolytic rumen bacteria it was first necessary to isolate these in pure culture. These isolates could then be identified and antisera produced against one representative of each strain. Somatic antigens of the isolated strains were used for the production of antibodies since flagellar antibodies could be prepared against only those of the isolates (vibrios) which possessed flagella. The chance of alterations in the antigenic structure of the isolated strains through frequent serial transfers was minimised by maintaining a large supply of stock cultures on solid CO_2, each stock culture being subcultured from the original pure isolate a minimal number of times. These subcultures were used for making fresh preparations of the antigen as required.

The antigen preparations consisted of heat-killed suspensions of bacteria, since heating destroys the flagellar antigen which would interfere with reactions between the somatic antigen and the somatic antibody. Neither formalin nor ethanol was used in the antigenic preparations since formalin is known to destroy the somatic antigen of certain bacteria (Mackie & McCartney, 1938), and since ethanol brings about the non-specific clumping of the suspended bacteria over a period of time.

Somatic antibodies were produced by injecting rabbits intravenously with heat-killed suspensions of pure cultures of the isolates. The sera containing the corresponding antibodies were obtained by bleeding the animals. An arbitrarily chosen degree of antigen-antibody reaction (the titre) was then determined for each antiserum against its homologous antigen in agglutination reactions. In these the bacterial antigens were sensitised with tenfold dilution series of antibody and allowed to clump together under specified conditions.

Antibodies are mostly associated with the gamma globulin fraction of the serum (Sober & Peterson, 1958 ; Kabat & Meyer, 1961; Morse & Heremans, 1962; Yagi et al, 1963). The gamma globulin fractions of the antisera were therefore isolated by passing the sera through columns of diethylaminoethyl (DEAE) cellulose (Levy & Sober, 1960). The antobodies can readily be labelled with ^{131}I, which becomes coupled to the tyrosine rings (Kabat & Meyer, 1961) of the gamma globulin proteins. Since ^{131}I combines indiscriminately with the tyrosine rings of any protein, labelling only the gamma globulin fraction of the antiserum greatly increases the efficiency of labelling of the antibodies.

With whole bacteria as antigen the antigen-antibody combination takes place at the surface of the bacteria (Heidelberger, 1939). Heidelberger & Kabat (1936, 1937) found that combination at all the antigenic sites on the bacterial surface is complete only when the antibodies are in excess, otherwise the number of antibody molecules combined per bacterium decreases with diminishing concentrations of antibody. The distribution of the antibodies among the suspended bacteria appears to be determined largely by the number of collisions of antibody molecules with antigenic bacteria. An even distribution of labelled antibody was found to be essential for precise quantitative studies such as those contemplated here, and could be assisted and hastened by shaking the vessel containing the antibodies and the bacterial antigen.

The correct concentration of labelled antibody is necessary to ensure an even apportionment of radioactivity among both fixed and free bacteria. If it could be assumed that all the antigenic sites on the surfaces of all the bacteria were available for antigen-antibody combination then the choice would be for high concentrations of antibody. The presence of an excess of antibody would then saturate all the antigenic sites on all the appropriate bacteria. It is probable however that at least some of the antigenic sites will be blocked off on fixed bacteria. Under these conditions there would be an unproportional excess of antibody associated with the free bacteria. Therefore the choice must fall on low concentrations of antibody, resulting in a random distribution of only a few antibodies to each appropriate bacterium. The use of low concentrations of antibody has the additional advantage that agglutination of bacterial antigens does not occur when these are in great excess (Heidelberger & Kabat, 1937). Thus the chance of free bacteria becoming joined to fixed bacteria through antibody linkage will be greatly reduced.

The reaction between the labelled antibodies and their homologous bacterial antigens was carried out at a high temperature (50°C) followed by retention at a low temperature (5°C), since Heidelberger & Kabat (1937) showed that the antigen-antibody reaction is most efficient under these conditions. These workers also demonstrated (1934) that once the antibodies had combined with the antigen the combination could not be uncoupled by washing with physiological saline. The removal of labelled antibody which was uncombined with antigen, or of any which was non-specifically adsorbed onto particles in the ingesta, could thus be effected by repeated washings with saline.

To separate the solid from the liquid portion of ingesta the ingesta were squeezed through a double layer of cheesecloth, since most workers in the rumen field use this method of separation, and results obtained in this way could therefore be compared with those of other workers. The wetted portion of the cheesecloth together with the attached solids was used for determining the radioactivity of the solid portion of the ingesta sample. If the radioactivities of a series of samples are to be compared it is desirable for the geometry of the samples to be similar. The samples were therefore distributed evenly in constant volumes of gelatine and solidified.

^{131}I emits gamma rays which are easily detectable without special processing of the samples of ingesta. The apparatus for recording gamma-radiating

disintegrations consists basically of a well-crystal scintillation detector and photomultiplier tube connected to a scaler with pulse recording and timer circuits. The ^{131}I was used for labelling soon after delivery since the half-life of ^{131}I is only 8 days. This short half-life, together with the fact that the laboratory was licensed for only 1 mc of ^{131}I obtainable every fortnight, meant that information had to be obtained with a series of short, interrupted experiments.

CHAPTER 5

EXPERIMENTAL

5:a. Animal management

5:a:(i). Sheep

The sheep described in Part I were used as a source of ingesta and rumen bacteria for the serological studies.

5:a:(ii). Rabbits

Six-month old white buck rabbits in good condition were used for the preparation of the antisera. These rabbits were housed individually on a covered stoep out of draughts and were given a ration of rabbit bran, green barley and good quality teff hay, and a plentiful supply of fresh water. The rabbits were treated against coccidiosis by adding 0.2% (V/v) of a 33.3% (W/v) solution of sodium sulphadimidine (Sulfadim, Medical and Hospital Supplies) to their drinking water. The treatment continued for 1 week during which no green food was given to ensure that the animals drank the treated water. Since some rabbits drank only the barest minimum of the bitter solution the intake of the drug was insufficient for prophylaxis. It was later found more effective to mix sparingly soluble Sulphamezathine power (ICI) with a little bran and water, and to feed this to the rabbits before giving the rest of the food each day. In this way 0.75 g of Sulphamezathine was given daily for 5 days. For administering the antigen doses the rabbits were confined in rabbit boxes with only their heads free and an ear was rubbed up with a cotton wool swab dipped in 70% alcohol. The marginal ear vein was made to stand out by flicking the ear with a finger and the antigen suspension was injected into the vein using a sterile 26 gge needle. After the injection the wound was pressed firmly with a swab dipped in tincture of iodine until the bleeding stopped. The rabbits were bled ad exitus under anaesthetic for the collection of antiserum. A dose (120 mg/Kg body weight) of pentobarbitone sodium solution (Sagatal, M. & B. Vet, 60 mg/ml) was injected into the ear vein and the rabbit bled from a 16 gge needle stuck into the posterior vena cava, exposed by an incision in the abdomen.

5:b. Counts of glucolytic bacteria in minced ingesta

A sample of ruminal ingesta (± 25 ml) was placed in a thick-walled 50 ml centrifuge tube surrounded by crushed ice and continuously gassed with O_2 - free CO_2. To this was added 0.5 ml of 10% (V/v) Antifoam B. The sample

was minced using an Ultra-Turrax high speed mincer for 5 min, with a 30 sec break after 2.5 min to allow for cooling. The sample was then strained through a double layer of cheesecloth and 1 ml of the resulting ruminal fluid was used immediately to make viable counts in medium G1.

5:c. Isolations

5:c:(i). Procedure

A roll-bottle containing from 2 to 6 well-separated colonies was selected from the 6th or 7th tenfold sample dilutions of the viable count. Each colony was isolated according to the following procedure as described by Kistner & Gouws (1964). The colony was picked from the agar, broken up and suspended in sterile 0.05 M-phosphate buffer of pH 6.8 (Appendix I), and the procedure for viable counts repeated. Three such subcultures were made, with a microscopical examination of about one third of the colonies from each to check the purity of the culture. If no contaminants were found a colony from the third subculture was transferred to a slope of G1 medium and incubated for 20 hr at 39°C. The next morning the bacteria were washed from the slope with 1 ml of buffer, and this was used to inoculate four other slopes which were incubated for 20 hr at 39°C and used as stock cultures.

5:c:(ii). Maintenance

Stock cultures were maintained on medium G1 and kept in solid CO_2. They were subcultured every 6 to 8 months.

5:c:(iii). Inoculum

For bacteriological purposes the stock cultures were inoculated onto slopes of medium G1 and incubated for 20 hr at 39°C. The resulting growth was suspended under anaerobic conditions in 2 ml of buffer, and 0.1 to 0.2 ml portions of the suspension were used for all inoculations.

For the serological work the stock cultures were inoculated onto slopes of medium A1 (Appendix I). The culture was grown and suspended as above and 0.5 ml portions were used for inoculations.

5:d. Characterisations

5:d:(i). Microscopical examination

Preparations for microscopical examination were taken from 20 hr cultures on G1 medium. The shape of the isolates was determined in wet mounts under phase contrast and their Gram reaction in smears stained by Gram's method.

5:d:(ii). Heat resistance

Ability to grow at 45°C was tested in medium G1 without agar.

5:d:(iii). Fermentation tests

The fermentation tests listed in Table 10 were carried out in a lightly buffered medium (Appendix I) of pH 6.8. For each test one of the following substrates was added to the basal medium to a final concentration of 0.5% ($^{W}/v$):- cellulose, xylan, starch, lactose, glycerol, sodium lactate. The lactose was sterilised by Seitz filtration and added to the rest of the medium after this had been heat sterilised in the usual manner. After sterilisation the medium was distributed with anaerobic precautions in 9 ml amounts into sterile, rubber-stoppered, screw-cap, 1 oz bottles flushed with O_2-free N_2 (95% $^{V}/v$) + CO_2 (5% $^{V}/v$). Production of acid from the above substrates by the bacteria was determined by comparing the pH of the inoculated basal medium with that of the inoculated test media. This was done after incubation at 39°C for 14 days for cellulose, and for 7 days for the other substrates. The pH measurements were carried out using a Radiometer pH Meter with a combined glass electrode. A fall in pH of 0.2 units or more was taken as an indication of fermentation of the added substrate.

5:d:(iv). Ammonia from arginine

The production of ammonia from arginine was tested for in a medium similar to the basal medium for fermentation tests but with arginine (0.5% $^{W}/v$) in place of $(NH_4)_2SO_4$, and with Seitz-filtered glucose added to the sterile medium to a final concentration of 0.5% ($^{W}/v$). The isolates were grown both in the test medium and in basal medium, and after 7 days of incubation at 39°C the pH of both sets of cultures was determined as above. A rise in pH in the test medium of at least 0.2 pH units above the value for the same isolate in the basal medium was taken to indicate the production of ammonia.

5:d:(v). End-products of fermentation of glucose

All the curved, rod-shaped bacteria isolated were tested for the production of succinic, butyric and acetic acids from glucose. The cultures were incubated for 3 days at 39°C in medium G1 without agar. The medium was then clarified according to the method of Neish (1952) based on that of Somoygi (1930), and the presence of the above acids was tested for by paper chromatography (Schwartzman, 1960).

5:e. Serological studies

5:e:(i). Preparation of somatic antigens

5:e:(i):1. Cultures - The appropriate stock culture was inoculated onto the surface of a 15 ml flat of medium A1 and grown for 20 hr at 39°C.

5:e:(i):2. Suspensions - Using a 5 ml syringe the culture was washed off the agar surface with a 0.9% (W/v) solution of NaCl (Saline). The resulting bacterial suspension was placed in a tube in a boiling waterbath for 1 hr. Further saline was added until the optical density (OD) of the suspension was equal to that of a number 3 Brown's Standard Tube. This was equivalent to an OD of 1.21 on the Unicam SP 500 Spectrophotometer at 4,700 Å and a slit width of 0.016 mm. The heat-killed suspensions were used immeidately.

5:e:(ii). Preparation of antisera

Heat-killed bacterial suspensions were injected into the marginal ear veins of the rabbits every fourth day in increasing doses of 0.5, 1.0, 2.0 and 4.0 ml. Five days after the last injection the rabbits were bled into beakers. The blood was left at room temperature for the day to clot, and then without disturbing was placed in the refrigerator at 5°C overnight. Next morning the clot was separated from the serum using a glass rod, and the serum was centrifuged at 2,000 rev/min for 20 min to free it from blood cells. The serum was kept in the refrigerator at 5°C.

5:e:(iii). Titration of antibodies

5:e:(iii):1. Dilution of antiserum - Using a 1 ml pipette graduated in units of 0.01 ml and fitted with a robust rubber teat, 0.5 ml antiserum was added to 5.75 ml physiological saline (0.9% W/v aqueous NaCl) in a test tube to give a dilution of 1:12.5. The diluted antiserum was mixed three times by sucking up a portion into the pipette and expelling it vigorously beneath the remaining solution in the test tube. The pipette was rinsed out thoroughly three times along its whole length with saline, drained of the last droplet of rinse, and used with appropriate rinsings for all subsequent steps of the procedure. A series of test tubes in a rack was numbered from 1 to 12, and 0.5 ml saline added to tubes 2 to 12. A 0.5 ml volume of the diluted (1:12.5) antiserum was pipetted into tubes 1 and 2, the pipette was rinsed and the contents of tube 2 were mixed. From tube 2,0.5 ml of diluted antiserum was transferred to the next highest tube in the series, the pipette rinsed, the contents mixed, and the procedure repeated for the following tubes. Finally 0.5 ml of the mixed contents

of tube 11 was discarded. In this way tube 12 contained 0.5 ml saline only, and tubes 1 - 11 contained 0.5 ml each of the following doubling dilutions of antiserum:

Tube	Dilution	Tube	Dilution
1	1 : 12.5	7	1 : 800
2	1 : 25	8	1 : 1,600
3	1 : 50	9	1 : 3,200
4	1 : 100	10	1 : 6,400
5	1 : 200	11	1 : 12,800
6	1 : 400	12	Saline

5:e:(iii):2. Agglutination tests - Using a freshly rinsed pipette 0.5 ml of a heat-killed bacterial suspension containing the homologous antigen was pipetted into tubes 12 to 1 in ascending order to ensure that no trace of antiserum was introduced into the saline in tube 12, since this tube controlled the non-agglutination of the bacterial suspension in the absence of antibody. The contents of each tube were mixed by rolling the tube between the palms of the hands. The composition of the agglutination test and the final dilution of the antibody was :

Tube	Diluted antiserum (ml)	Bacterial suspension (ml)	Final antiserum dilution
1	0.5	0.5	1 : 25
2	0.5	0.5	1 : 50
3	0.5	0.5	1 : 100
4	0.5	0.5	1 : 200
5	0.5	0.5	1 : 400
6	0.5	0.5	1 : 800
7	0.5	0.5	1 : 1,600
8	0.5	0.5	1 : 3,200
9	0.5	0.5	1 : 6,400
10	0.5	0.5	1 : 12,800
11	0.5	0.5	1 : 25,600
12	0.5 Saline	0.5	Control

The contents of tubes 12 to 1 were then transferred in that order using a clean pipette into Dreyer agglutination tubes marked 12 - 1 in a rack. Bubbles were dispelled by shaking and the rack was placed in a covered waterbath for 2 hr

at 50°C. The rack was placed in the refrigerator at 5°C overnight and the titre read next morning after shaking the tubes. Each tube was inspected against a background of artificial light through a 10x lens.

5:e:(iii):3. Titre of antiserum - The control containing antigen suspension and saline in place of antiserum was first examined to establish absence of non-specific agglutination. The titre of the antiserum is the highest dilution of antiserum showing positively detectable granular agglutination, as distinct from a trace of agglutination. The degree of agglutination was recorded by assigning the values of 4 for complete, 3 for all but complete, 2 for more than positively detectable, 1 for positively detectable, and tr for a trace of agglutination.

5:e:(iv). Isolation of gamma globulin (GG) fraction

To separate the GG (Levy & Sober, 1960) the antiserum was first dialysed overnight at 5°C against a flow of 2 l of 0.0175 M-phosphate buffer (Appendix I), pH 6.3, in Visking dialysis tubing (No 20, Union Carbide Corp). The dialysed serum was next passed through diethylaminoethyl (DEAE) cellulose (Cellex D, high capacity, Bio Rad Laboratories) at pH 6.3 in a column 1 x 5 cm for every 2 ml of serum. The GG eluent from the column was collected in 5 ml amounts and the relative protein contents of these samples was determined by measuring their absorbance of UV light at 2,800 Å. Those tubes with a reading of 1.0 or more were kept and combined as the GG sample. The protein content of the GG solution was then accurately determined by the method of Lowry et al (1951). The titre of the GG solution with respect to its homologous somatic antigen was determined by means of agglutination tests as for the antiserum.

5:e:(v). Storage of GG solutions

The GG was stored in screw-cap bottles at 5°C. The stored solutions were kept sterile by the addition of merthiolate (Thiomersal, Schuchardt) to a final concentration of 1 : 10,000.

5:e:(vi). Absorption of GG with liver power

5:e:(vi):1. Preparation of liver power - The liver power used (NF, Nutritional Biochemicals Corp.) was decolourised by extraction with acetone (Coons et al, 1955).

5:e:(vi):2. Absorption - GG was absorbed by stirring in liver power in amounts of 50 mg/ml of GG solution. After standing for 1 hr at room temperature with occasional stirring the supernatant was harvested by centrifugation at 27,000 G in an angle head in the cold.

5.f. Isotope studies

5.f:(i). Radioactivity measurements

5:f:(i):1. Measurement of gamma radiation - Gamma radiation from standard sources (^{137}Cs) and from 5 ml samples was detected by placing these in 15 x 100 mm test tubes in the well of a thallium-activated sodium iodide crystal. The scintillations caused by gamma radiation were recorded by different scaler/timer apparatuses (Philips, Ekco, Tracerlab 1,000, Tracerlab tracer/matic, Packard) to give a measure of the activity in counts per minute (cpm) or counts per second (cps).

5:f:(i):2. To check scaler operation - To check the operation of the detector and scaler a radioactive sample was placed in the detector well and the scaler adjusted to give a counting rate of about 2,600 cpm. A series of 50 1-min counts was then made and the standard deviation (SD) of the results calculated by the formula.

$$SD_x = \sqrt{\frac{1}{N-1}\left[\sum x^2 - \frac{(\sum x)^2}{N}\right]}$$

where N = the number of observations x. Chauvenet's criterion was then applied to determine whether the maximum observed deviations from the mean value fell into an acceptable range. For a series of 50 observations Chauvenet's criterion states that the value of any observed deviation divided by the standard deviation should not exceed 2.57. If all the observed deviations fell within this value then the scaler was assumed to be operating normally. An example of the calculation is worked out in Appendix XVII.

5:f:(i):3. Statistical evaluation of counting accuracy - Since radioactive decay is an essentially random process there will always be some degree of error in the observed counting rate. For practical purposes, in experiments where not very high reliability is required, the standard deviation can be taken as the square root of the count (N).

$$SD = \sqrt{N}$$

The SD can further be expressed as a percentage error (V) of the total count (N).

$$V = \frac{SD}{N} \times 100$$

or $$V = \frac{\sqrt{N}}{N} \times 100 = \frac{100}{\sqrt{N}}$$

or $$N = \frac{10,000}{V^2}$$

Therefore for a standard deviation of 5% the number of counts needed is

$\frac{10,000}{25} = 400.$

For more reliable results, with a confidence limit of 0.95, where 20 out of 21 observations will be within the required % error (P), the number of counts necessary (N) is given by the equation

$$N = \frac{10,000}{V^2} \quad \text{where } V = \frac{P}{2}$$

i.e. $$N = \frac{40,000}{P^2}$$

Therefore for a 5% standard deviation of 0.95 confidence limit,

$$N = \frac{40,000}{25} = 1,600 \text{ counts (Quimby \& Feitelberg, 1963).}$$

In most of the experiments reported in this study the number of counts collected for each sample far exceeded this total.

5:f:(ii). Solutions for ^{131}I labelling

5:f:(ii):1. Borate buffer 2x, pH 7.65 - A distilled water solution of 0.32 M-NaCl and 0.4 M-H_3BO_3 was adjusted to pH 7.65 by the addition of 3.2 M-NaOH to a final concentration of $\pm$ 0.08 M-NaOH.

5:f:(ii):2. Borate buffer 1x, pH 8.0 - Equal volumes of distilled water and borate buffer 2x were combined to give the borate buffer 1x.

5:f:(ii):3. Iodine monochloride (ICl) - Ingredients for 250 ml stock solution were :

KI (Hopkin & Williams, AR)	0.5550	g
KIO_3 (BDH, AR)	0.3567	g
NaCl (Baker, AR)	29.23	g
Conc. HCl (Hopkin & Williams, AR, Sg 1.18)	21.0	ml
Distilled water	229.0	ml

Free iodine was removed by shaking the solution with CCl_4 until the latter remained colourless. The CCl_4 was carefully separated from the solution by means of a separating funnel and any suspended or dissolved CCl_4 was removed by passing a current of air, saturated with water vapour, through the ICl solution. The molarity of the ICl stock solution was determined by adding an excess of KI to an aliquot and titrating the liberated iodine with a standardised thiosulphate solution. For iodination of protein the ICl stock solution was diluted to the required molarity with .2 M-NaCl and kept in ice water until needed.

5:f:(iii). ^{131}I labelling of GG

5:f:(iii):1. Iodine monochloride (ICl) method - The GG solution containing the antibodies was labelled with ^{131}I by the method of Helmkamp et al (1960). The GG solution (5 - 10 ml) was dialysed overnight against 2 - 4 l of 0.2 M-borate buffer, pH 8.0 at 5°C. The protein concentration of the dialysed GG was determined and the volume containing 4 mg of protein was calculated and set aside for iodination. ^{131}I of activity from 0.3 to 0.75 mc (Atomic Energy Board, Pelindaba) was adjusted behind a lead shield to pH 8.0 by the addition of 1 ml borate buffer and a drop or two of 1 N-HCl if necessary, using Neutralit pH paper (Merck) to test the pH. The bottle containing the ^{131}I was immersed in chipped ice to cool and 0.2 ml of ICl (0.0005 M) added. By rapid exchange reaction ^{131}ICl was formed and this in turn reacted with NaOH in the buffer to give HO ^{131}I and NaCl. The resulting solution was jetted vigorously under suction into the GG solution, where coupling of the ^{131}I to the tyrosine residue of the protein took place. Any uncoupled ^{131}I was removed by passing the iodinated GG solution through 2 ml of Dowex 1- x4 resin (50 - 100 mesh, Baker Chemical Co) in a 2 ml glass syringe.

5:f:(iii):2. Chloramine T method - The GG solution was also labelled with ^{131}I by the Chloramine T Method of Bocci (1964). To a volume of serum protein containing 100 mg protein was added 2.1 ml of 0.15 M-phosphate buffer (pH 7.6) containing the carrier-free ^{131}I (0.01 - 6.0 mc). A 0.4 ml volume of Chloramine T solution (BDH, 1.0 mg/ml in 0.15 M-phosphate buffer) was injected into the solution through a finely drawn out glass pipette. After 30 min 2.0 ml of sodium metabisulphite solution (1.25 mg/ml in phosphate buffer) and 0.3 ml of carrier sodium iodide (10 mg/ml in phosphate buffer) were added separately with mixing. Chloramine T and sodium metabisulphite solutions were

used within 1 hr of preparation. The mixture was then passed through a column of Dowex resin to remove uncombined ^{131}I.

5:f:(iii):3. Ion exchange column - Dowex 1-x4 resin was allowed to stand in contact with 1N-HCl for several hours, washed successively with 20% ($^{W}/v$) NaCl solution and 0.85% ($^{W}/v$) NaCl solution until the filtrate was neutral, and then stored under 0.85% NaCl (Helmkamp et al, 1960).

5:f:(iii):4. Removal of H_2O_2 from ^{131}I solution - H_2O_2 formed by beta-radiation on water containing dissolved oxygen in the ^{131}I solution was removed in some experiments. After adjustment of the ^{131}I to pH 8.0, 0.3 ml of a freshly made 0.05 M-sodium sulphite solution was added to destroy the H_2O_2 present. After 5 min the mixture was placed in a boiling waterbath and aerated with moist air for 15 min to oxidise excess sulphite, while at the same time destroying any newly formed H_2O_2. The solution was then immersed for 2 min in ice and water, the pH adjusted if necessary, and the solution used for iodination of protein as above (Helmkamp et al, 1960).

5:f:(iii):5. Determination of labelling efficiency - The efficiency of the labelling procedures was determined by calculating the percentage recovery of radioactivity in the final GG solution. The volume of the reaction mixture and the activity of a 1 ml sample were determined before passage through the resin column. Then, after unreacted ^{131}I had been removed by the resin column, the eluate was brought up to a known volume and the activity of a 1 ml sample again determined. The second activity value expressed as a percentage of the first gave a measure of the labelling efficiency in terms of the percentage of added activity recovered bound to protein.

5:f:(iv). Treatment of ingesta

5:f:(iv):1. Heat-killing of bacteria - The rumen bacteria in 50 ml (approximately) samples of ingesta were killed by heating the sample to 60°C in a waterbath for 45 min before treating with antibody solutions. This prevented bacterial destruction of the labelled proteins added to the ingesta sample, and also converted the bacteria to their somatic antigen form.

5:f:(iv):2. Pretreatment with non-specific protein solutions - Solutions of unlabelled GG or bovine serum albumen (Fraction V, Nutritional Biochemicals Corp.) were added in the amounts shown in Table 19 (p 65) to 10 ml samples of heat-treated ingesta, and the samples incubated for 10 or 30 min at 50°C before the ingesta were treated with ^{131}I-GG.

5:f:(iv):3. Antigen - antibody reaction - Portions (10 ml) of heat-treated ingesta were measured out into 50 ml centrifuge tubes. To these were added volumes of ^{131}I - GG calculated to give final GG concentrations approximately equal to multiples of 0.2 to 6 times the titre concentrations. The samples were well stirred and incubated in a waterbath at 50°C for 2 hr with shaking every 5 min, and in the final procedure adopted the tubes were then kept at 5°C overnight.

5:f:(iv):4. Separation of solid from liquid ingesta - The ingesta sample was strained through a double layer of cheesecloth in a glass funnel and the liquid strainings were collected in a test tube (15 x 100 mm) labelled "L". The cheesecloth was well squeezed out with gloved fingers and any liquid remaining on the rubber gloves was transferred to the funnel. The area of wetted cheesecloth containing the ingesta solids was cut out with scissors and placed in 5 ml of 0.9% saline in a second test tube marked "S".

5:f:(iv):5. Preparation of samples for ^{131}I measurement - Tubes L and S were centrifuged at 2,500 rev/min for 20 min and the supernatants drawn off into marked test tubes. The deposits were resuspended in saline and recentrifuged at least 3 times, and 5 times in the final procedure; the supernatants being transferred to marked tubes each time. In the early experiments a pipette was used to resuspend the deposists, but later these deposits were resuspended by inserting a syringe needle through the compacted solids and allowing the saline to percolate upwards before breaking up the lump with the needle. After the final wash a coiled wire was used to resuspend the deposits evenly in 5 ml of 20% (w/v) gelatine for measurement of ^{131}I activity. The washings were also measured to determine the amount of uncombined ^{131}I - GG removed from the sample, and to check on the number of washings necessary to remove uncombined ^{131}I - GG activity.

CHAPTER 6

RESULTS AND DISCUSSION

6:a. Isolations

Fourteen strains of bacteria, designated Gl 1-14, were isolated from the 10^{-7} dilution of viable counts of glucolytic rumen bacteria in minced and then strained ruminal ingesta from three sheep (K7, K8, K10) conditioned to a diet of poor teff hay, urea and glucose. Isolates Gl 1-12 were presumptively identified according to the tests recommended by Bryant (1963) together with special tests for ruminal streptococci : growth at 45°C (Hungate, 1957; Davey & Briggs, 1961), hydrolysis of arginine (Davey & Briggs, 1961), and fermentation of lactose (Smith & Shattock, 1962). The tests and the results obtained are shown in Table 10. These isolates comprised Gram positive, short, thick rods characterised as Lactobacillus sp. (Gl 1 and 2); Gram negative curved rods identified as Bacteroides succinogenes (Gl 5 and 8), Bacter. ruminicola (Gl 6 and 9), and Succinivibrio sp., possibly fibrisolvens (Gl 7); and Gram positive diplococci belonging to Streptococcus sp. (Gl 3, 4, 10, 11, 12). Growth at 45°C placed these streptococci among the ruminal streptococci, while failure to produce ammonia from arginine indicated that they were either Strep. bovis or equinus but not faecalis. The production of acid from lactose further indicated that they were bovis rather than equinus. Isolates Gl 13 and 14 were also Gram positive diplococci obtained 16 months after the others for the purpose of discovering whether they would react with antisera prepared against Strep. bovis (Gl 3). Further identification tests were not carried out on these two organisms.

It is interesting to note that two of the isolates were cellulolytic Bacteroides succinogenes since this is the first time that this particular organism has been found in high numbers (more than one million/ml of ruminal fluid) in the rumen of sheep in South Africa, although it has often been found in high numbers in cattle and sheep in the United States of America (Bryant & Burkey, 1953; Bryant & Doetsch, 1954; Hungate, 1947). This could be due either to the fact that most roughages in the United States are fed together with supplements, as in this case, or to the fact that this organism is highly attached to the solid particles of ingesta, and that its presence was revealed in the present studies as a result of mincing the ingesta before straining. The rest of the isolates

TABLE 10

CHARACTERISATION OF ISOLATES

ISOLATE	SHAPE	GRAM REACTION	FERMENTATION TESTS									GROWTH AT 45°C	PRESUMPTIVE IDENTIFICATION
			GLUCOSE	CELLULOSE	XYLAN	STARCH	LACTATE	GLYCEROL	LACTOSE	NH_3 FROM ARGININE	FERMENTATION PRODUCTS FROM GLUCOSE		
Gl 1	Short, thick rods, some in pairs, some elongated	G+	A	O	O	A	O	O	-	-	-	-	Lactobacillus sp.
Gl 2	Ditto	G+	A	O	O	A	O	O	-	-	-	-	Lactobacillus sp.
Gl 3	Diplococci	G+	A	O	A	A	O	O	A	NO	-	+	Streptococcus bovis
Gl 4	Ditto	G+	A	O	A	A	O	O	A	NO	-	+	Strep. bovis
Gl 10	Ditto	G+	A	O	A	A	O	O	A	NO	-	+	Strep. bovis
Gl 11	Ditto	G+	A	O	A	A	O	O	A	NO	-	+	Strep. bovis
Gl 12	Ditto	G+	A	O	A	A	O	O	A	NO	-	+	Strep. bovis
Gl 5	Slightly curved rods with dark metachromatic granules	G-	A	A	A	A	O	A	-	-	SUCCINIC (+ ACETIC?)	+	Bacteroides succinogenes
Gl 8	Ditto	G-	A	A	A	A	O	A	-	-	Ditto	-	Bacter. succinogenes
Gl 6	Slightly curved rods with bipolar granules. Some in pairs and some chains	G-	A	O	A	A	O	O	-	-	Ditto	-	Bacter. ruminicola
Gl 9	Ditto	G-	A	O	A	A	O	O	-	-	Ditto	-	Bacter. ruminicola
Gl 7	Small, curved rods, some in pairs	G-	A	O	O	O	O	O	-	-	Ditto	-	Succinivibrio (fibrisolvens or dextrinosolvens)

G+ = Gram Positive

G- = Gram Negative

A = Acid Production

O = No Acid Production

\+ = Visible Growth

\- = Not Tested

belong to species commonly found in high numbers in the rumen, particularly when dietary supplements containing readily available carbohydrates like sugar or starch are given.

6:b. Titre of serum fractions containing homologous antibody against selected isolates

Rabbit antisera prepared against isolates Lactobacillus (Gl 1) and Streptococcus bovis (Gl 3) were used to study the effect of serum fractionation and aging on the antibody titre obtained when these antigens were agglutinated by their homologous antibodies. From Table 11 and Appendix VII it can be seen that the titre of the antiserum against each isolate was high (1/25,600).

TABLE 11

THE EFFECT OF FRACTIONATION OF SERUM AND AGING ON ANTIBODY TITRE

Antigen	Antibody against	Date	Fraction of serum used	Titre (Appendix VII)
Gl 1[1]	Gl 1	3.12.64	Whole serum	1/25,600
		8.12.64	GG[3]	1/200
		4.6.65	GG	1/200
		9.9.65	GG	1/25
Gl 3[2]	Gl 3	3.12.64	Whole serum	1/25,600
		8.12.64	GG	1/400
		9.9.65	GG	1/200

[1] Gl 1 = Lactobacillus isolated from sheep K7 on 14.4.64

[2] Gl 3 = Streptococcus bovis isolated from sheep K8 on 14.4.64

[3] GG = Gamma globulin.

Processing the antiserum for gamma globulin (GG) resulted in a marked fall in titre. Thus after serum fractionation the antibody titres of the GG fractions of the Gl 1 and Gl 3 antisera were found to be greatly reduced by 128 and 64 times, to 1/200 and 1/400 respectively. This reduction was due partly to dilution since the method used for obtaining the GG dilutes the GG 4-10 fold (Levy & Sober, 1960). Miller & Owen (1960) have pointed out that the antibody activity may be associated not only with the gamma but also with the beta globulin fraction.

By using only the GG therefore, part of the antibody activity might have been lost.

Storage of the Gl 1 and Gl 3 GG solutions at 5°C for 9 months until the end of the present studies caused a further reduction in their titre to 1/25 and 1/200.

6:c. The specificity of antigen - antibody reactions using low titre GG-antibodies

Even with the low titre values found after storage the GG solutions retained their specificity and reacted against their homologous antigens in agglutination reactions while showing no activity against other morphological types of bacteria. This can be seen from the findings of the tests listed in Table 12 and Appendix VII. A GG solution with antibodies against Lactobacillus (Gl 1)

TABLE 12

THE RESULTS OF AGGLUTINATION TESTS BETWEEN DIFFERENT COMBINATIONS OF ANTIBODY AND ANTIGEN

Date	Gamma globulin with antibody against:	Antigen	Titre (Appendix VII)
9.9.65	Gl 1[1]	Gl 1	1/25
		Gl 2[1]	1/50
		Gl 14[2]	0
	Gl 3[3]	Gl 3	1/200
		Gl 13[2]	1/25
		Gl 14	1/50
		Gl 1	0
4.6.65	Control-1a[4]	Gl 1	0
	Control-1b[4]	Gl 1	0

[1] Gl 1, Gl 2 = Lactobacillus isolated from sheep K7 on 14.4.64.

[2] Gl 13, Gl 14 = Gram positive diplococci isolated from sheep K8 on 24.8.56 and morphologically resembling Streptococcus bovis.

[3] Gl 3, = Strep. bovis isolated from sheep K8 on 14.4.64.

[4] Control-1a, Control-1b = Washings from uninoculated solid media.

caused the agglutination of its homologous antigen and of a similar isolate, Lactobacillus (Gl 2), but not of isolate Gl 14 which was a Gram positive diplococcus morphologically similar to Strep. bovis. Similarly a GG solution with antibodies against Strep. bovis (Gl 3) brought about the agglutination of its homologous antigen and of two other Gram positive diplococci (Gl 13 and Gl 14), but not of Lactobacillus.

Furthermore no agglutination occured between Lactobacillus (Gl 1) and antisera Control-1a and Control-1b, prepared against saline washings from uninoculated solid medium A1 used for growing the organisms for antigenic suspensions. This demonstrated that the antigenic suspensions used in the present studies contained no antigenic substances other than those associated with the bacteria.

It is of particular interest that a GG solution with antibodies against heat-killed Strep. bovis (Gl 3), isolated on 14.4.64, agglutinated two other Gram positive diplococci (Gl 13 and Gl 14) isolated 16 months later on 24.8.65 from the rumen of the same sheep, K8. This clearly demonstrates the persistence in the rumen of similar serotypes for at least 16 months. Using fluorescent antibodies prepared against formalin-killed bacteria Margherita et al (1964) failed to find the same serotypes in the rumen after a period of 2 years. This could be taken as an indication that antisera formed to heat-killed bacteria are more species specific than strain specific, and are therefore less sensitive to minor changes in the antigenic composition of a species of bacterium than are sera against bacteria killed with formalin.

6:d. Labelling GG with ^{131}I.

In a number of experiments using the iodine monochloride method of labelling 4 mg amounts of GG with ^{131}I the calculated labelling efficiency was found to be between 0.03 and 4.46% (Table 13 and Appendix VIII). In contrast Helmkamp et al (1960) using the same method found a coupling of approximately 60% of the ^{131}I to protein. It was possible that the very low efficiency obtained was due to the presence of H_2O_2, since the above workers found that where H_2O_2 was present in the ^{131}I solution the percentage incorporation of ^{131}I into protein was negligible. H_2O_2 is formed by the action of beta-radiation on H_2O in the ^{131}I solution and its reducing action on HOI would decrease the iodination yield. It was found, however, that the use of H_2O_2- free ^{131}I gave no significant increase in the labelling efficiency (0.18%).

TABLE 13

THE EFFECT OF VARYING THE LABELLING PROCEDURE ON THE PERCENTAGE OF ^{131}I BOUND TO 4 MG OF GAMMA GLOBULIN

Method of labelling	Volume of ICl used (ml)	Dialysis		Use of H_2O_2-free ^{131}I	Resin for removing unbound ^{131}I	Total activity (counts x 10^{-3}/min) (Appendix VIII)		Labelling efficiency (% of ^{131}I bound to GG) $\left(\frac{A}{A+B} \times 100\right)$
		Volume of buffer (l)	Time (hr)			^{131}I bound & unbound (A + B)	^{131}I bound (A)	
ICl	0.2	2	4	-	Dowex 1-x4 (acid)	316,567	715.9	0.22
						512,325	7,716.9	1.51
						235,040	82.1	0.03
						283,038	837.6	0.29
						318,721	384.1	0.12
						167,860	219.7	0.13
ICl	0.2	2	4	+	Dowex 1-x4 (acid)	277,193	513.4	0.18
ICl	0.2	3	4	-	Dowex 1-x4 (alkaline)	282,023	240.5	0.08
Chloramine T	-	2	4	-	Ditto	268,527	208.5	0.08
ICl	0.2	4	15	+	Ditto	7,921	4.3	0.05
ICl	0.8	4	15	-	Ditto	3,992	28.8	0.72
ICl	0.8	4	15	+	Ditto	3,586	160.1	4.46

The Dowex 1- x4 ion exchange resin used to remove uncombined ^{131}I was next checked and found to be slightly acid. This would affect the binding of the ^{131}I to the GG. The resin was thus washed a number of times in distilled water until neutral and then stored under pH 8 borate buffer. Using the alkaline resin the labelling efficiency obtained was still very low (0.08%). To check whether the fault lay with the reagents used for labelling, an alternative method using Chloramine T was tried. Here again the incorporation of ^{131}I was at the low value of 0.08%. This seemed to indicate that the fault lay with the GG solution rather than with the reagents used in the labelling procedure.

Helmkamp et al (1960) found that low iodination efficiencies were obtained when their protein solutions were dialysed in the Visking cellulose casing used in the present work. They found that during dialysis the casing released material which reduced ICl. This material could, however, be eliminated by sufficiently prolonged (4 hr) dialysis against a minimum of 2 l of buffer in the cold. Thus a number of trials was carried out on GG dialysed in the cold against 4 l of buffer for 15 hr in an attempt to determine at just which part of the procedure contamination of the GG occured. For these trials ^{131}I solutions of lower activity than those in previous experiments could be used as a newly installed Packard Gamma Spectrometer enabled more sensitive measurements to be made of ^{131}I activity with less background interference. The first sample was treated for removal of H_2O_2 and labelled using 0.2 ml ICl. The second sample was not treated for removal of H_2O_2 but the ICl concentration was increased to 0.8 ml. The third sample was treated for removal of H_2O_2 and the ICl concentration used was 0.8 ml. The labelling efficiencies for the three trials were 0.05, 0.72 and 4.46% (Table 13). The values obtained for the first two trials showed no improvement over the values previously obtained. The value (4.46%) obtained for the third trial was still low and indicated that in spite of increased dialysis and the removal of H_2O_2 there was still some factor causing ICl to become unavailable for iodination.

The two most obvious sources of such a factor are contamination from the dialysis casing and the merthiolate added to preserve the GG solutions. This will be the subject of a future investigation, possibly using a molecular sieve material, such as the dextran gel Sephadex (Pharmacia), in place of dialysis. This would serve both to remove all low molecular weight salts from the serum and to avoid any possible contamination from dialysis casing.

Sephadex could also be used in place of the Dowex 1- x4 resin for removing uncombined ^{131}I from ^{131}I - GG solutions under much milder conditions.

For the present, however, even with the low iodination efficiencies obtained, the activity of the ^{131}I - GG solutions was high enough for them to be used successfully in tracer experiments.

6:e. ^{131}I-Antibody-antigen reactions in ruminal ingesta

6:e:(i). The effect of retention at 5°C overnight

Different concentrations of ^{131}I - GG with antibodies against Lactobacillus were allowed to react with the corresponding bacterial antigens in samples of unstrained, heat-treated ingesta. The amount of GG added to the ingesta was such that the final concentration of the GG was a simple multiple of its titre concentration. This multiple was called the titre factor. One series of samples was incubated at 50°C for 2 hr, while a second series, after a similar incubation, was held for a further period overnight at 5°C. The conditions used for the second series were similar to those used to obtain maximum

TABLE 14

THE EFFECT OF RETENTION AT 5°C OVERNIGHT ON THE DISTRIBUTION OF ^{131}I-GAMMA GLOBULIN, WITH ANTIBODY AGAINST LACTOBACILLUS*, IN RUMINAL INGESTA FROM SHEEP K7

Date (1965)	Sub-samples of ingesta (10 ml)	^{131}I - Gamma globulin		Incubation	% Activity retained by particles in ingesta (Appendix IX)	
		Titre factor (X)	Amount used (mg)		Solid fraction	Liquid fraction
25.2	1	1	0.15	2 hr at 50°C	63	37
	2	2	0.3	Ditto	59	41
	3	6	0.9	Ditto	65	35
3.3	4	1	0.13	2 hr at 50°C overnight at 5°C	78	22
	5	2	0.27	Ditto	79	21
	6	6	0.77	Ditto	83	17

* Lactobacillus = Gl 1 isolated from sheep K7 on 14.4.64.

reaction between antibody and antigen in the agglutination tests. From Table 14 and Appendix IX it can be seen that the additional holding overnight at 5°C increased the percentage of activity retained by particles in the solid fraction of the ingesta by approximately 18%, from an average of 62% to 80%, at the expense of the activity retained by particles in the liquid fraction. The magnitude of this effect was approximately the same for increasing amounts of antibody over the range of titre fractors 1, 2 and 6 tested. This indicated that the effect was not due to bacterial antigens in the liquid phase becoming linked to those attached to the solids through agglutination by antibody, since were it so the effect would have increased with increasing antibody concentration. On the other hand this effect could have been due to more efficient combination between antibody and bacterial antigens in the crevices of the food particles. In view of these findings retention at 5°C overnight following incubation at 50°C for 2 hr was adopted as standard procedure.

6:e:(ii). The effect of increasing antibody concentration

When increasing amounts of ^{131}I - GG with antibody against Lactobacillus were added to the ingesta samples it was found that the combined counts of the liquid plus solid portions increased in direct proportion with the increase in added activity until an amount of antibody equivalent to at least six times the titre concentration had been added (Table 15). This indicated that the homologous

TABLE 15

THE EFFECT OF INCREASING AMOUNTS OF ^{131}I -GAMMA GLOBULIN, WITH ANTIBODY AGAINST LACTOBACILLUS*, ON THE RADIOACTIVITY RETAINED BY RUMINAL INGESTA FROM SHEEP K7

Date	Subsamples of ingesta (10 ml)	^{131}I- Gamma globulin		Activity retained by particles in solid & liquid fractions of ingesta (counts/min)	Increase factor $\left(\frac{\text{Activity}}{\text{Activity at 1x titre}}\right)$
		Titre factor (X)	Amount used (mg)		
3.3.65	1	0.5	0.07	1,236	0.4
	2	1.0	0.13	3,426	1.0
	3	2.0	0.27	7,344	2.1
	4	6.0	0.77	18,714	5.5

*Lactobacillus = Gl 1 isolated from sheep K7 on 14.4.64.

bacterial antigen in the test sample was present in sufficient amounts to absorb quantities of antibody equivalent to at least six times its titre. Since amounts of ^{131}I - GG equivalent to only 1 or 2 times titre were needed to provide adequate radioactivity these concentrations were used in future experiments. These concentrations satisfied the requirement for a marked excess of antigen over antibody, thus precluding agglutination and the linking of bacteria in the liquid and the solid phases of the ingesta.

6:e:(iii). Reproducibility of the method used to determine the distribution of ^{131}I-GG in ruminal ingesta

The reproducibility of the method for determining the distribution of ^{131}I-GG in ruminal ingesta was tested. In particular it was felt that the reproducibility of the procedures for separating the liquid and the solid ingesta by straining through cheesecloth, washing, and suspending the particles of ingesta in gelatine prior to counting the radioactivity should be established. Four replicate determinations were made on one sample of ruminal ingesta using one batch of ^{131}I-GG with antibody against Lactobacillus (Gl 1). In these determinations (Table 16 : Appendix X) the particles in the solid fraction of

TABLE 16

REPRODUCIBILITY OF THE METHOD USED TO DETERMINE THE DISTRIBUTION OF ^{131}I-GAMMA GLOBULIN, WITH ANTIBODY AGAINST LACTOBACILLUS*, IN RUMINAL INGESTA FROM SHEEP K7

Date	^{131}I-Gamma globulin		Subsamples of ingesta (10 ml)	% Activity retained by particles in ingesta (Appendix X)	
	Titre factor (X)	Amount used (mg)		Solid fraction	Liquid fraction
25.2.65	2	0.3	1	55	45
			2	59	41
			3	59	41
			4	60	40
			Mean	58 (± 3)	42 (± 3)

*Lactobacillus = Gl 1 isolated from sheep K7 on 14.4.64.

ingesta retained 55, 59, 59 and 60% while those in the liquid fraction retained 45, 41, 41 and 40% of the activity. These results gave mean values of 58% (± 3%) for the former and 42% (± 3%) for the latter. As the replicates agree to within 6% it was felt that the method could be accepted as giving reproducible results.

6:e:(iv). Distribution of ^{131}I-GG with antibodies against Streptococcus bovis in ruminal ingesta of each of four sheep

The distribution of ^{131}I-GG with antibodies against Strep. bovis was tested in ruminal ingesta from each of sheep K11, K10, K8 and K7. Table 17 and Appendix XI show that the distribution of the activity between the particles of the solid and the liquid fractions was about even for K11 and K10, slightly higher in the solid fraction for K8, and shows a tendency to be higher in the liquid fraction for K7. The tests were made twice within a period of ten days. The values obtained for each of sheep K11, K10 and K8 on these two occasions varied only slightly outside the experimental error (± 3%). In the case of sheep K7 very much higher values for activity in the liquid fraction were obtained on the first than on the second occasion. This was probably due to instability in the composition of the ruminal flora. It is known that animals dosed with urea and glucose do in fact tend to display such variations in ruminal flora accompanied by erratic feeding (Gilchrist, 1965). The fact that the food consumption of K7 was lower and more erratic than that of the other three sheep during this period is evidence in favour of this possible explanation. The less marked differences in distribution of activity for ingesta from the other three animals is probably due to a similar effect.

The tests for distribution of activity were carried out at doubling titre factors; 1 and 2 on the first and 2 and 4 on the second occasion. For each single occasion the variation in the values obtained with doubling titre factors was within the experimental error. Thus the results obtained were independant of the concentration of GG added.

6:e:(v). Distribution of ^{131}I-GG with antibodies against Lactobacillus, Streptococcus bovis or washings from uninoculated media in ruminal ingesta of sheep K7

The distribution of ^{131}I-GG with antibodies against two isolates (Lactobacillus Gl 1, and Strep. bovis Gl 3) in the ingesta of sheep K7 was ascertained (Table 18 : Appendix XII). The findings were compared with the

TABLE 17

THE DISTRIBUTION OF ^{131}I-GAMMA GLOBULIN, WITH ANTIBODY AGAINST STREPTOCOCCUS BOVIS*, IN RUMINAL INGESTA FROM EACH FOUR SHEEP

Sheep	Date (1965)	Sub-samples of ingesta (10 ml)	^{131}I-Gamma globulin		% Activity retained by particles in ingesta (Appendix XI)	
			Titre factor (X)	Amount used (mg)	Solid fraction	Liquid fraction
K11	29.3.	1	1	0.14	52	48
		2	2	0.29	50	50
	7.4.	1	2	0.3	52	48
		2	4	0.6	55	45
K10	29.3.	1	1	0.14	48	52
		2	2	0.29	55	45
	7.4.	1	2	0.3	51	49
		2	4	0.6	57	43
K8	29.3.	1	1	0.14	65	35
		2	2	0.29	69	31
	7.4.	1	2	0.3	55	45
		2	4	0.6	59	41
K7	29.3.	1	1	0.14	15	85
		2	2	0.29	21	79
	7.4.	1	2	0.3	46	54
		2	4	0.6	39	61

*Streptococcus bovis = Gl 3 isolated from sheep K8 on 14.4.64

TABLE 18

THE DISTRIBUTION OF ^{131}I-GAMMA GLOBULIN, WITH ANTIBODIES AGAINST LACTOBACILLUS, STREPTOCOCCUS BOVIS, OR WASHINGS FROM UNINOCULATED MEDIA, IN INGESTA FROM SHEEP K7

Date (1965)	Sub-samples of ingesta (10 ml)	^{131}I-Gamma globulin			% Activity retained by particles in ingesta (Appendix XII)	
		With antibody against:	Titre factor (X)	Amount used (mg)	Solid fraction	Liquid fraction
3.3.	1	Gl 1[1]	6	0.77	83	17
	2	Gl 1	2	0.27	79	21
	3	Gl 1	1	0.13	78	22
15.3.	1	Gl 3[2]	2	0.28	34	66
	2	Gl 3	1	0.14	38	62
31.3.	1	Gl 3	2	0.28	33	67
	2	Gl 3	1	0.14	30	70
21.4.	1	Control-1b[3]	-	0.19	27	73
	2	Control-1b	-	0.38	26	74

[1] Gl 1 = Lactobacillus isolated from sheep K7 on 14.4.64.

[2] Gl 3 = Strep. bovis isolated from sheep K8 on 14.4.64.

[3] Control-1b = Washings from uninoculated media

distribution of ^{131}I-GG (Control-1b) known to contain no antibody reacting with rumen bacteria (Table 10). It can be seen that the percentage of ^{131}I activity retained by the solid fraction was much higher when the ^{131}I-GG contained antibodies against Lactobacillus (mean 80, $\pm$ 3%) than when antibodies against Strep. bovis (mean 34, $\pm$ 4%) were present. The average value for the distribution of the ^{131}I-GG with antibodies against Lactobacillus (80% in the solid fraction and 20% in the liquid fraction) was very close to the maximum distribution figure obtained in mincing experiments (84% fixed in the solid fraction and 16% free in the liquid fraction). The distribution values for ^{131}I-GG with antibodies against Strep. bovis fell between the values found in the previous

experiment. ^{131}I-Gamma globulin prepared against washings from uninoculated medium had distribution values very similar to those of ^{131}I-GG with antibodies against Strep. bovis, even though it was known that there were no antibodies against rumen bacteria present in the ^{131}I-Control-1b-GG.

Thus, although there appears to be a significant difference in the distribution of ^{131}I-GG solutions containing antibodies against Lactobacillus and against Strep. bovis, the confidence which can be placed on the measured values is doubtful owing to the apparent non-specific retention of ^{131}I-GG by the ingesta. Although Table 18 shows the percentage distribution of ^{131}I activity retained from GG solutions with and without antibodies it gives no indication of the actual amounts of GG retained. It was possible that the non-specific retention was small in comparison with the retention of GG with antibodies against rumen bacteria, in which case it would have little effect on the values obtained for the distribution of GG solutions with antibodies. If, however, the amount of GG retained non-specifically was high this would seriously influence the results obtained. Thus to enable the distribution of ^{131}I-activity to be used for determining the bacterial distribution it would first be necessary either to prevent the non-specific adsorption of radioactivity, or to keep the non-specific adsorption within controlled and known limits. In the former case the distribution of radioactive immune GG could be calculated directly and in the latter case the distribution could be found after subtracting a blank value for the non-specific adsorption.

6:f. Non-specific adsorption of ^{131}I-GG by ruminal ingesta

6:f:(i). Calculation of amount of ^{131}I-GG non-specifically adsorbed

From the calculations for determining the labelling efficiency, and knowing the amount of protein used for labelling, it is possible to calculate the approximate specific activity of the labelled antibody in counts per minute/µg protein. After incubating, straining, and washing the ingesta sample the amount of GG non-specifically retained can be calculated from the measured activity of the samples (Appendix XIV).

6:f:(ii). The effect of various treatments on the non-specific retention of ^{131}I-GG by ruminal ingesta from sheep K8

It was felt that this non-specific retention was due to the physical adsorption of the labelled GG molecules onto particles in the heat-treated ingesta. To determine the degree of non-specific adsorption of ^{131}I activity ^{131}I-Control-1a-GG was added to samples of heat-treated ingesta and incubated. The ^{131}I-

activities of the solid and the liquid fractions, as well as the activities of the combined washings, were measured, and the activity of the solid plus the liquid fractions expressed as a percentage of the total activity.

In the series of experiments listed in Table 19 (Appendices XIII and XIV) the non-specific retention of activity by ingesta without additional treatments was found to be from 2.5 to 35.2% (6.8 to 112.4 µg protein). Attempts were made to reduce this non-specific adsorption by blocking the protein adsorption sites in the ingesta. Samples of 10 ml of heat-treated ingesta were incubated with GG (5 mg), bovine albumen (250 and 500 mg) before adding the ^{131}I-GG. In all cases there was an increase in the non-specific retention of ^{131}I-GG. This increase was not proportional to the amount of GG or albumen added. Attempts were next made to remove any non-specific factors in the ^{131}I-GG, since Hobson & Mann (1957) found that absorption with liver powder reduced the non-specific reaction of fluorescent antibody in ruminal contents. Absorption of the ^{131}I-GG with liver powder (50 mg/ml) reduced the non-specific adsorption from 12.2 to 7.4%. Addition of GG to both the ingesta and the ^{131}I-GG before incubation reduced the non-specific retention by an amount slightly less than in the previous experiments. Pretreating the ingesta with 100 mg of bovine albumen in place of GG gave a similar slight decrease in non-specific retention.

This persistent and varying retention of ^{131}I-activity led to the suspicion that the non-specific retention might be due not to the adsorption of ^{131}I-GG but possibly to the reaction of free ^{131}I with constituents of the ruminal contents. This would help to explain the observation that pretreatment of the ingesta with protein increased the ^{131}I retention of the ingesta as this would increase the number of sites for iodination by free ^{131}I. Similarly pretreatment of the ^{131}I-GG with protein would remove ^{131}I from the ^{131}I-GG before its addition to samples of ruminal ingesta. To test the effectiveness of the Dowex 1-x4 resin in removing unreacted ^{131}I from the ^{131}I-GG, a sample of the labelled GG was placed in dialysis casing and suspended in borate buffer in the cold overnight. The next morning ^{131}I activity was detected in the buffer. This indicated that the Dowex resin was not altogether effective in removing unbound ^{131}I.

It was felt that here again the use of Sephadex would give a better separation of uncombined ^{131}I. We therefore decided to delay further investi-

TABLE 19

THE EFFECT OF VARIOUS TREATMENTS ON THE NON-SPECIFIC RETENTION OF ^{131}I-GAMMA GLOBULIN (GG) BY RUMINAL INGESTA FROM SHEEP K8

Date (1965)	Subsamples of ingesta (10 ml)		^{131}I-Gamma globulin		% Of added activity retained by particles in solid + liquid ingesta (Appendix XIII)	^{131}I-GG (µg calculated) retained by particles in solid + liquid ingesta (Appendix XIV)
	No.	Absorbed with:	Treatment	Amount used (mg)		
30.8	1	-	-	0.41	35.2	112.4
	2	-	-	0.41	23.4	60.8
23.8	1	-	-	0.43	2.5	6.8
	2	5 mg GG at 50°C for 10 min	-	0.43	3.2	8.8
9.8	1	-	-	0.22	5.0	12.5
	2	250 mg Bovine albumen at 50°C for 10 min	-	0.22	12.6	35.6
	3	500 mg Ditto	-	0.22	11.1	29.8
7.12	1	-	-	0.4	12.2	-
	2	-	Absorbed with 50 mg/ml liver powder after ^{131}I labelling	0.4	7.4	-
5.7	1	-	3.2 ml unlabelled GG added to 5.3 ml of solution containing 3.1 ml labelled GG	0.27	9.2	1.25
	2	1.3 mg GG	Ditto	0.27	7.4	1.02
19.7	1	-	Ditto	0.27	9.2	6.75
	2	100 mg Bovine albumen at 50°C for 10 min	Ditto	0.27	7.1	3.95

gations on reducing non-specific adsorption until supplies of Sephadex could be obtained. In the meantime a few comparative results for the specific retention of ^{131}I-GG with antibodies were required to allow some estimate to be made of the importance of the non-specific retention.

6:g. Specific retention of ^{131}I-GG by ruminal ingesta of sheep K8

The results of four experiments to determine the retention of ^{131}I-GG with antibodies against Lactobacillus and Strep. bovis in the ingesta of sheep K8 are given in Table 20 (Appendices XV and XVI). In the three experiments for Strep. bovis the activity retained varied from 1.1 to 9.3% (4.2 to 18.4 µg protein). For Lactobacillus the activity retained was slightly higher (13.9%; 47 µg protein) and was not significantly changed by pretreatment of the ingesta with 250 mg of bovine albumen. These figures are all within the range found for non-specific retention of ^{131}I-GG activity by ingesta (Table 17) and give no indication of an increased retention due to specific antigen-antibody reaction.

6:h. Significance of the results obtained in Part II of this investigation

The predominant contributing species of the functional group of glucolytic bacteria were isolated from counts made in the original mincing experiments. The Gram positive isolates obtained were probably Lactobacillus sp (2) and Streptococcus bovis (5), while the Gram negative isolates were probably Bacteroides succinogenes (2), Bacter. ruminicola (2), and Succinivibrio fibrisolvens (1). For this preliminary investigation single batches of high titre (1/25,600) antibodies were produced in the serum of rabbits against boiled suspensions of Lactobacillus and Strep. bovis only. These antibodies reacted both against their homologous antigens and against other isolates of the same species, but showed no cross-agglutination with isolates belonging to other species. The antibodies retained their specific activity even when the titre had fallen to low values (1/400; 1/25) after serum fractionation and storage. Furthermore the antibodies against Strep. bovis reacted with strains of the same species isolated from the rumen 16 months later. Ample time was thus available to determine the distribution of rumen bacteria in the ingesta by differential radioactive counts on liquid and on solid portions of ingesta treated with appropriate ^{131}I-labelled antibodies.

The efficiency with which the gamma globulin (GG) bearing the antibodies was labelled with ^{131}I was low (0.03 - 4.46%). Nevertheless the activity of the ^{131}I attached to the GG was sufficiently high for the statistical accuracy

TABLE 20

SPECIFIC RETENTION OF ^{131}I-GAMMA GLOBULIN (GG), WITH ANTIBODIES AGAINST LACTOBACILLUS STREPTOCOCCUS BOVIS, BY RUMINAL INGESTA FROM SHEEP K8

Date (1965)	Subsamples of ingesta (10 ml)	^{131}I-Gamma globulin			% of added activity retained by particles in solid + liquid ingesta (Appendix XV)	^{131}I-GG (µg calculated retained by particles in solid + liquid ingesta (Appendix XVI)
		With antibody against:	Titre factor (X)	Amount used (mg)		
18.8.	1	Gl 3[1]	2	0.25	9.3	17.6
	2	Gl 3	2	0.25	8.7	16.4
27.9.	1	Gl 3	4	0.5	1.2	4.2
	2	Gl 3	4	0.5	1.1	4.4
5.10.	1	Gl 3	4	0.5	4.6	14.0
	2	Gl 3	4	0.5	3.6	18.3
2.8.	1	Gl 1[2]	2	0.43	13.9	47.0
	2 - Absorbed with 250 mg bovine albumen at 50°C for 30 min	Gl 1	2	0.43	12.2	41.0

[1] Gl 3 = Strep. bovis isolated from sheep K8 on 14.4.64

[2] Gl 1 = Lactobacillus isolated from sheep K7 on 14.4.64.

required in counting radioactivity. Preliminary distribution studies were carried out on Lactobacillus and Strep. bovis. The percentage of activity retained by the solid fraction of ingesta was much higher when the ^{131}I-GG contained antibodies against Lactobacillus (mean 80, $\pm$ 3%) than when antibodies against Strep. bovis (mean 34, $\pm$ 4%) were present. The values obtained for Lactobacillus are possibly meaningful, whereas the values for Strep. bovis are doubtful owing to the fact that they are close to those found for the non-specific retention of labelled GG containing no antibodies to rumen bacteria. A start was thus made on decreasing the non-specific adsorption of labelled GG by pretreating the ingesta and/or GG with protein solutions, but with little success. Further work is required to increase the labelling efficiency, possibly using Sephadex to remove interfering substances, and to decrease the non-specific retention of ^{131}I-GG, possibly by pretreatment of the GG and/or ingesta with non-protein materials such as Kieselguhr.

SUMMARY

1. The distribution of the functional group of glucolytic rumen bacteria between the solid and the liquid portions of ruminal ingesta was determined using differential viable counts of these organisms in strained ruminal fluid from untreated ingesta and from ingesta subjected to high speed mincing.

2. The ratios for total/free bacteria were confirmed by the ratios obtained for the rates of glucose disappearance in whole ingesta and in strained ruminal fluid from the same sample of ingesta.

3. This is the first time ever that the distribution of any particular group of rumen bacteria between liquid and solid phases of ruminal ingesta has been quantitatively determined.

4. The count ratios for total/free glucolytic bacteria changed from 6.4 to 1.4 over a period of 18 months; this change being accompanied by a change in predominating species within the functional group of glucolytic bacteria, from Gram positive diplococci and rods to Gram negative rods.

5. The use of ultrasonic vibrations to free the attached bacteria was not successful as the ultrasonic vibrations used were lethal at all power settings tested.

6. Estimation of the distribution of the functional group of ureolytic bacteria could not be done by the count ratio method since a specific growth medium for making viable counts could not be developed.

7. Five predominant species of glucolytic bacteria were isolated and found to be Lactobacillus sp., Streptococcus bovis, Bacteroides succinogenes, Bacter. ruminicola, and Succinivibrio fibrisolvens.

8. Antibodies were produced in rabbits to Lactobacillus and Strep. bovis only.

9. The antisera were fractionated and the gamma globulin fraction bearing the antibodies was labelled with ^{131}I at an efficiency which was low but nevertheless sufficient to give high enough activity for statistical accuracy in counting.

10. Preliminary radioactivity distribution studies showed that the percentage of the activity retained by the solid fraction of ingesta was much

higher when the ^{131}I-gamma globulin contained antibodies against Lactobacillus (mean 80, ± 3%) than when antibodies against Strep. bovis (mean 34, ± 4%) were present.

11. The values for Strep. bovis were found to be close to those for non-specific adsorption of ^{131}I-gamma globulin.

12. Initial attempts failed to decrease the amount of non-specific adsorption of ^{131}I-gamma globulin.

LITERATURE

BAKER, F. 1942. Microbial factors in the digestive assimilation of starch and cellulose in herbivora.
Nature, Lond., 150, 479.

BOCCI, V. 1964. Wirksame merkierung von Serumprotein mit I^{131} unter gebrauch von Chloramin T.
Int. J. appl. Radiat. Isotopes, 15, 450.

BRYANT, M.P. 1963. Identification of groups of anaerobic bacteria active in the rumen.
J. Anim. Sci., 22, 801.

BRYANT, M.P. & BURKEY, L.A. 1953. Cultural methods and some characteristics of some of the more numerous groups of bacteria in the bovine rumen.
J. Dairy Sci., 36, 205.

BRYANT, M.P. & DOETSCH, R.N. 1954. A study of actively cellulolytic rod-shaped bacteria of the bovine rumen.
J. Dairy Sci., 37, 1176.

BRYANT, M.P. & ROBINSON, I.M. 1961. An improved nonselective culture medium for ruminal bacteria and its use in determining diurnal variation in numbers of bacteria in the rumen.
J. Dairy Sci., 44, 1446.

COONS, A.H., LEDUC, E.H. & CONNOLLY, J.M. 1955. Preparation of acetone-dried tissue powders for absorption of conjugates.
J. exp. Med., 102, 51.

DAVEY, L.A. & BRIGGS, C.A.E. 1961. Biochemical properties of Streptococci isolated from the artificial rumen and calves.
Digestive Physiology and Nutrition of the Ruminant, p 102.
Butterworths, London. Ed., D. Lewis.

DAVIES, E.M. 1965. Cellulolytic bacteria in some ruminants and herbivores as shown by fluorescent antibodies.
J. gen. Microbiol., 39, 139.

DIFCO MANUAL. 1963. Ninth edition, pp 170 - 171.
Difco Laboratories, Michigan, U.S.A.

GIBBONS, R.J. & DOETSCH, R.N. 1959. Physiological study of an obligately anaerobic ureolytic bacterium.
J. Bact., 77, 417.

GILCHRIST, F.M.C. 1965. Influence of diet on functional groups of rumen bacteria fermenting carbohydrates and lactate.
Proceedings of the Central African Scientific and Medical Congress, 1963, p. 559. Ed., Snowball, G.J. Pergamon Press, Oxford.

GILCHRIST, F.M.C. & CLARK, R. 1957. Refresher courses in physiology III : The microbiology of the rumen.
J.S. Afr. Vet. and Med. Assoc., 28, 295.

GILCHRIST, F.M.C. & KISTNER, A. 1962. Bacteria of the ovine rumen. I The composition of the population on a diet of poor teff hay.
J. agric. Sci., Camb., 59, 77.

GOUWS, L. & KISTNER, A. 1965. Bacteria of the ovine rumen. IV Effect of change of diet on the predominant type of cellulose-digesting bacteria.
J. agric. Sci., Camb., 64, 51.

HEIDELBERGER, M. 1939. Quantitative absolute methods in the study of antigen-antibody reactions.
Bact. Rev., 3, 49.

HEIDELBERGER, M. & KABAT, E.A. 1934. Chemical studies on bacterial agglutination. I A method.
J. exp. Med., 60, 643.

HEIDELBERGER, M. & KABAT, E.A. 1936. Chemical studies on bacterial agglutination. II The identity of precipitin and agglutinin.
J. exp. Med., 63, 737.

HEIDELBERGER, M. & KABAT, E.A. 1937. Chemical studies on bacterial agglutination. III A reaction mechanism and a quantitative theory.
J. exp. Med., 65, 885.

HELMKAMP, R.W., GOODLAND, R.L., BALE, F.W., SPAR, I.L. & MUTSCHLER, L.E. 1960. High specific activity iodination of gamma globulin with iodine - 131 monochloride.
Cancer Res., 20, 1495.

KISTNER, A. 1960. An improved method for viable counts of bacteria of the ovine rumen which ferment carbohydrates.
J. gen. Microbiol., 23, 565.

KISTNER, A. & GOUWS, L. 1964. Cellulolytic cocci occuring in the rumen of sheep conditioned to lucerne hay.
J. gen. Microbiol., 34, 447.

KISTNER, A., GOUWS, L. & GILCHRIST, F.M.C. 1962. Bacteria of the ovine rumen. II The functional groups fermenting carbohydrates and lactate on a diet of lucerne (Medicago sativa) hay.
J. agric. Sci., Camb., 59, 85.

LEVY, H.B. & SOBER, H.A. 1960. A simple chromatographic method for preparation of gamma globulin.
Proc. Soc. exp. Biol. Med., 103, 250.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. & RANDALL, R.J. 1951. Protein measurements with the Folin Phenol reagent. J. biol. Chem., 193, 265.

MACKIE, T.J. & McCARTNEY, J.E. 1938. Handbook of practical bacteriology. Fifth edition. Livingstone, Edinburgh.

MARGHERITA, S.S., HUNGATE, R.E. & STORZ, H. 1964. Variation in rumen Butyrivibrio strains. J. Bact., 87, 1304.

MILLER, H. & OWEN, G. 1960. Immuno-electrophoresis of insulin-binding antibodies. Nature, Lond., 188, 67.

MORSE, J.H. & HEREMANS, J.F. 1962. Immuno-electrophoretic analysis of human insulin-binding antibody and its papain-produced fragments. J. Lab. clin. Med., 59, 891.

MUNCH-PETERSON, E. & JAMES, A.A. 1964. A method of assessing "free living" and "attached" bacteria in rumen contents. Zentralblatt Für Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene. Originale. 194, 358.

NEISH, A.C. 1952. Analytical methods for bacterial fermentations. National Research Council of Canada. Report No. 46-8-3, Second Revision. Saskatoon.

QUIMBY, E.H. & FEITELBERG, S. 1963. Radioactive isotopes in medicine and biology, pp 199 - 203. Lea and Febiger, Philadelphia.

SCHWARTZ, H.M., SCHOEMAN, C.A. & FARBER, M.S. 1964. Utilization of urea by sheep. I Rates of breakdown of urea and carbohydrates in vivo and in vitro. J. agric. Sci., Camb., 63, 289.

SCHWARTZMAN, G. 1960. Separation and identification of sodium salts of C_2 to C_5 volatile fatty acids by paper chromatography. J. Ass. off. agric. Chem., 43, 129.

SHROPSHIRE, R.F. 1946. Turbidimetric evaluation of bacterial disruption by sonic energy. J. Bact., 53, 685.

SMITH, D.G. & SHATTOCK, P.M.F. 1962. The serological grouping of Streptococcus equinus. J. gen. Microbiol., 29, 731.

SOBER, H.A. & PETERSON, E.A. 1958. Protein chromatography on ion exchange cellulose.
Fedn Proc. Fedn Am. Socs exp. Biol., 17, 116.

SOMOYGI, M. 1930. A method for the preparation of blood filtrates for the determination of sugar.
J. biol. Chem., 86, 655.

SOMOYGI, M. 1952. Notes on sugar determination.
J. biol. Chem., 195, 19.

TAYLOR, J. 1962. The estimation of numbers of bacteria by tenfold dilution series.
J. appl. Bact., 25, 54.

VAN DER WATH, J.G. 1942. Studies on the alimentary tract of Merino sheep with special reference to the role of the ruminal microfauna and flora. Thesis for degree of Doctor of Veterinary Science, University of Pretoria. Onderstepoort Library
Acc. No. 12, 604 - P20.

WARNER, A.C.I. 1962. Enumeration of rumen micro-organisms.
J. gen. Microbiol., 28,119.

YAGI, Y.P., MAIER, D., PRESSMAN, D., ARBESMAN, C.E., REISMAN, R.E. & LENZNER, A.R. 1963. Multiplicity of insulin binding antibodies in human sera.
J. Immun., 90, 765.

APPENDICES

APPENDIX I

BACTERIOLOGICAL CULTURE MEDIA

MEDIA FOR GLUCOSE FERMENTERS

G 1

Ingredients for 500 ml:

Bacto Agar (Difco)			7.5 g
Deionised water			294.0 ml
Methylene blue	(BDH)	(0.05% $^{w}/v$)	5.0 ml
$(NH_4)_2\ SO_4$	(Merck)	(5% $^{w}/v$)	5.0 ml
K_2HPO_4	(Merck)	(5% $^{w}/v$)	5.0 ml
$K\ H_2PO_4$	(Merck)	(2% $^{w}/v$)	5.0 ml
NaCl	(Merck)	(10% $^{w}/v$)	5.0 ml
$CaCl_2$	(Merck)	(0.5% $^{w}/v$)	5.0 ml
$MgSO_4 \cdot 7H_2O$	(Merck)	(0.5% $^{w}/v$)	5.0 ml
Antifoam B	(Dow Corning)	(10% $^{v}/v$)	1.0 ml
Cysteine - HCl	(Riedel de Haen)		0.250 g
Clarified ruminal fluid from sheep fed lucerne hay			150.0 ml
Buffer, pH 6.8 :			
	$NaHCO_3$ (Merck)		2.6 g
	O_2 - free CO_2		
Sterilised at 17 psi of steam pressure for 20 min			
Glucose (Merck) (25% $^{w}/v$, sterilised by Seitz filtration)			20.0 ml
Distribution: 9 ml in sterile 1 oz rubber-stoppered, screw-cap McCartney bottles flushed with O_2 - free CO_2			

Phosphate buffer (0.05 M), pH 6.8

Ingredients for 500 ml, using deionised water :

Na_2HPO_4	(Merck)	(M/15)	245.0 ml
KH_2PO_4		(M/15)	245.0 ml
Methylene blue		(0.025% $^{w}/v$)	10.0 ml
Flush with O_2 - free N_2 for 20 min			
Cysteine - HCl			0.250 g

APPENDIX I (CONTINUED)

Sterilised at 17 psi of steam pressure for 20 min

Distribution: 5 ml in sterile 1 oz rubber-stoppered, screw-cap McCartney bottles flushed with O_2 - free N_2.

Phosphate buffer (0.0175 M), pH 6.3

Ingredients for 1 litre, using deionised water :

$NaH_2PO_4 . 2H_2O$	(BDH, AR)	2.074 g
$Na_2HPO_4 . 12H_2O$	(Hopkin & Williams, AR)	1.507 g

Basal medium for fermentation tests

Ingredients for 300 ml :

Deionised water		284.4 ml
Methylene blue	(0.05% W/v)	3.0 ml
$(NH_4)_2SO_4$	(5% W/v)	3.0 ml
K_2HPO_4	(5% W/v)	0.3 ml
KH_2PO_4	(2% W/v)	0.3 ml
NaCl	(10% W/v)	3.0 ml
$CaCl_2$	(0.5% W/v)	3.0 ml
$MgSO_4 . 7H_2O$	(0.5% W/v)	3.0 ml
Cysteine - HCl		0.15 g
Bacto Yeast Extract	(Difco)	3.0 g
Buffer, pH 6.8 :		
$NaHCO_3$		0.081 g
O_2 - free N_2 (95% V/v) + CO_2 (5% V/v)		

Sterilised at 17 psi of steam pressure for 25 min.

MEDIA FOR UREA HYDROLYSERS

The urea, glucose and cellobiose in the following media were sterilised by Seitz filtration and added to the rest of the ingredients after these had been autoclaved at 17 psi of steam pressure for 20 min. Distribution was in 9 ml amounts into sterile 1 oz rubber-stoppered, screw-cap McCartney bottles gassed with appropriate O_2 - free gases.

APPENDIX I (CONTINUED)

Mineral salt solution

Salts (Merck)	Final % (^{W}/v) in deionised water
K_2HPO_4 (Puriss siccum)	0.05
KH_2PO_4 (Sorensen)	0.02
NaCl (GR)	0.1
$CaCl_2$ (GR)	0.005
$MgSO_4 . 7H_2O$ (GR)	0.005

Media

Medium	Ingredients	Final % (^{W}/v) in deionised water
U 1	Urea (BDH, AR)	0.4
	Glucose	0.05
	Cellobiose (BDH)	0.05
	Antifoam B (% ^{V}/v)	0.02
	Bacto Yeast Extract	1.0
	Mineral salts	+
	Methylene blue (BDH)	0.0005
	Phenol red (BDH)	0.001
	Bacto Agar	1.5
	Cysteine - HCl	0.5
	Buffer, pH 6.8 :	
	$NaHCO_3$	0.027
	N_2 (95% ^{V}/v) + CO_2 (5% ^{V}/v)	
U 2	(Formula B 272 Difco Manual, 1963)	
	Urea	2.0
	Antifoam B (% ^{V}/v)	0.02
	Bacto Yeast Extract	0.01
	Phenol red	0.001
	Bacto Agar	1.5
	Cysteine - HCl	0.5
	Buffer, pH 6.8 :	
	KH_2PO_4	0.91
	Na_2HPO_4	0.95
	N_2 gassed	

APPENDIX I (CONTINUED)

U 3	(Formula B 283 Difco Manual, 1963)	
	Urea	2.0
	Glucose	0.1
	Antifoam B (% ^{v}/v)	0.02
	Bacto Peptone	0.1
	Phenol red	0.001
	Cysteine - HCl	0.5
	Buffer, pH 6.8 :	
	KH_2PO_4	0.2
	N_2 gassed.	
U 4	Similar to U 3 but solidified with	
	Bacto Agar.	1.5
U 5	(Gibbons and Doetsch, 1959)	
	Urea	0.3
	Glucose	0.2
	Cellobiose	0.2
	Antifoam B (% ^{v}/v)	0.02
	Bacto Yeast Extract	1.0
	Ruminal fluid (% ^{v}/v)	1.0
	Mineral salts	+
	Phenol red	0.001
	Cysteine - HCl	0.5
	Buffer, pH 6.8 :	
	$NaHCO_3$	0.52
	CO_2 gassed.	
U 6	Similar to U 5 but with	
	Glucose	0.025
	Cellobiose	0.025
U 7	Similar to U 6 but with	
	$NaHCO_3$	0.027
	N_2 (95% ^{v}/v) + CO_2 (5% ^{v}/v) gassed.	

APPENDIX I (CONTINUED)

U 8	Similar to U 7 but with	
	KH_2PO_4	0.005
	K_2HPO_4	0.002
U 9	Similar to U 8 but with	
	Urea	2.0
U 10	Similar to U 7 but with	
	Urea	2.0
	Bacto Agar	1.5
U 11	Similar to U 9 but with no	
	Glucose and no	
	Cellobiose.	
U 12	Similar to U 9 but with	
	Diethylbarbituric acid	0.7
	Bacto Agar	1.5
U 13	Similar to U 3 but	
	N_2 (95% $^{v}/v$) + CO_2 (5% $^{v}/v$) gassed.	
U 14	Similar to U 11 but with	
	Urea	1.0
U 15	Similar to U 11 but with	
	Urea	0.4
U 16	Similar to U 11 but with	
	Urea	1.0
	Volatile fatty acids in place of ruminal fluid:	
	Acetic (Hopkin & Williams, BP)	0.256
	Isobutyric (BDH, LR)	0.00133
	Valeric (BDH, LR)	0.00154
	Isovaleric (BDH, LR)	0.00154.

APPENDIX I (CONTINUED)

MEDIA FOR SEROLOGICAL STUDIES

A 1

Growth medium A 1 was similar to G 1 but with Bacto Yeast Extract (1% $^{w}/_{v}$) in place of the ruminal fluid supplement.

Distribution : 5 ml in sterile 1 oz rubber-stoppered, screw-cap McCartney bottles set in a sloping position or 15 ml in sterile 4 oz McCartney bottles set in a horizontal position.

APPENDIX II

VIABLE COUNTS OF GLUCOLYTIC BACTERIA IN RUMINAL FLUID STRAINED FROM INGESTA FROM SHEEP K8 BEFORE AND AFTER MINCING FOR DIFFERENT TIMES

Date	Time minced	Counts in strained ruminal fluid (millions/ml)			
		Untreated ingesta		Minced ingesta	
(1963)	(min)	Replicates	Mean	Replicates	Mean
25.9	1.0	5 4 5 4 3 2 2 5	3.8	9 10 6 6 13 8 9 11	9.0
18.11	2.5	40 30 70 60 50 60 60 50	52.5	260 170 230 170 220 190 200 260	212.5
3.12	3.0	30 10 70 30 10 30 20 40	30.0	100 120 160 210 200 150 190 180	163.7
1.10	4.0	2 1 1 6 2 1 3 2	2.0	8 5 16 19 27 14 8 -	12.1

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APPENDIX II (CONTINUED)

Date	Time minced	Counts in strained ruminal fluid (millions/ml)			
		Untreated ingesta		Minced ingesta	
(1963)	(min)	Replicates	Mean	Replicates	Mean
22.10	5.0	60 10 50 40 30 60 50 60	45.0	440 320 270 430 190 390 120 120	285.0
18.11	6.0	40 30 70 60 50 60 60 50	52.5	340 390 260 350 360 380 250 340	333.7
20.11	7.5	30 10 0 20 0 20 30 30	17.5	90 40 110 70 70 40 120 70	76.2
3.12	8.0	30 10 70 30 10 30 20 40	30.0	150 150 220 160 170 160 200 130	167.5

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APPENDIX II (CONTINUED)

Date	Time minced	Counts in strained ruminal fluid (millions/ml)			
		Untreated ingesta		Minced ingesta	
(1963)	(min)	Replicates	Mean	Replicates	Mean
20.11	9.0	30 10 0 20 0 20 30 30	17.5	60 50 130 70 90 100 30 70	75.0
31.10	10.0	80 20 60 50 50 30 30 30	43.7	120 140 110 100 120 170 110 120	123.7
6.11	10.0	130 200 130 180 90 110 100 150	136.2	570 370 360 360 390 270 330 380	378.7

APPENDIX III

VIABLE COUNTS OF GLUCOLYTIC RUMEN BACTERIA IN STRAINED RUMINAL FLUID FROM SHEEP K8 BEFORE AND AFTER MINCING

Date	Time minced	Counts (millions/ml)			
		Untreated ruminal fluid		Minced ruminal fluid	
(1963)	(min)	Replicates	Mean	Replicates	Mean
7.10	5.0	22 7 11 10 7 17 10 11	11.8	9 9 11 17 16 12 13 10	12.1
15.10	5.0	10 10 13 13 9 6 7 11	9.9	15 17 12 9 9 7 14 16	12.4

APPENDIX IV

DISAPPEARANCE OF GLUCOSE FROM WHOLE INGESTA AND FROM STRAINED RUMINAL FLUID IN VITRO (DATA SUPPLIED BY M. LOCHNER, F. M. C. GILCHRIST & H. M. SCHWARTZ)

Ingesta from sheep	Date (1966)	Time after dosing (min)	Glucose concentration (mg/100 ml)	
			Whole ingesta	Strained ruminal fluid
K4	28.7	0	1,322	-
		5	1,107	1,131
		10	875	1,196
		15	857	1,086
		20	812	1,187
		25	809	2,168
		30	798	1,074
		45	690	988
		60	616	952
K8	20.6	0	1,320	-
		15	848	1,248
		30	714	1,192
		45	729	1,147
		60	576	1,021
		75	478	1,099
		90	448	1,084
		120	279	1,030
		150	218	1,026
		180	62	962
K10	12.7	0	1,095	-
		5	739	1,091
		10	604	1,045
		15	561	1,059
		20	502	1,036
		25	459	1,023
		30	409	1,018
		45	229	1,011
		60	144	859

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APPENDIX IV (CONTINUED)

Ingesta from sheep	Date (1966)	Time after dosing (min)	Glucose concentration (mg/100 ml)	
			Whole ingesta	Strained ruminal fluid
K11	14.7	0	1,233	-
		5	925	1,180
		10	1,692	1,161
		15	786	1,097
		20	814	1,130
		25	739	1,061
		30	700	1,072
		45	589	983
		60	450	1,019
K12	9.6	0	1,150	-
		15	641	964
		30	557	924
		45	553	814
		60	575	836
		75	442	814
		90	385	862
		120	283	769
		150	265	756
		180	168	663
	20.7	0	1,142	-
		5	818	1,121
		10	1,436	876
		15	623	774
		20	592	735
		25	498	730
		30	461	680
		45	315	626
		60	172	477

APPENDIX V

VIABLE COUNTS OF GLUCOLYTIC BACTERIA IN RUMINAL INGESTA USED FOR IN VITRO DETERMINATION OF GLUCOSE DISAPPEARANCE

Ingesta from sheep	Date	Counts in strained ruminal fluid (millions/ml)			
		Subsamples of in vitro strained ruminal fluid sample		Subsamples of in vitro whole ingesta sample minced 5 min	
	(1966)	Replicates	Mean	Replicates	Mean
K4	28.7	120 130 150 170	142	200 210 270 230	227
K8	20.6	80 60 60 60	65	150 180 200 170	175
K10	12.7	170 120 170 190	157	290 320 270 -	293
K11	14.7	200 210 240 160	202	430 470 430 380	427
K12	9.6	110 80 120 120	107	310 320 320 330	320
	20.7	250 250 300 300	275	470 430 310 350	390

APPENDIX VI

VIABLE COUNTS OF GLUCOSE METABOLISING BACTERIA IN RUMINAL FLUID STRAINED FROM INGESTA FROM SHEEP K7 BEFORE AND AFTER ULTRASONIC TREATMENT FOR DIFFERENT TIMES AT VARIOUS FREQUENCIES AND POWER OUTPUTS

Date	Time treated	Counts in strained ruminal fluid (millions/ml)			
		Untreated ingesta		Ultrasonically treated ingesta	
(1964)	(min)	Replicates	Mean	Replicates	Mean
27.5	5.0	50 40 30 60 30 50 30 40	41.0	40 80 50 0 60 60 20 50	45.0
27.5	10.0	50 40 30 60 30 50 30 40	41.0	40 140 110 70 110 70 110 120	96.0
16.6	20.0	10 10 10 20 30 0 30 10	15.0	10 0 10 10 10 10 40 30	15.0
16.6	30.0	10 10 10 20 30 0 30 10	15.0	10 10 10 20 10 10 20 0	11.2

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APPENDIX VI (CONTINUED)

Date	Time treated	Counts in strained ruminal fluid (millions/ml)			
		Untreated ingesta		Ultrasonically treated ingesta	
(1964)	(min)	Replicates	Mean	Replicates	Mean
2.6	5.0	70 20 60 30 20 60 80 -	48.0	70 40 70 90 50 40 40 20	52.0
2.6	10.0	70 20 60 30 20 60 80 -	48.0	30 100 60 70 30 50 50 30	52.0
20.8	20.0	50 30 20 30 10 0 10 30	22.5	10 10 0 10 20 20 10 20	12.5
9.6	30.0	160 90 100 70 80 30 100 110	92.5	60 70 110 130 90 90 90 90	91.2

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APPENDIX VI (CONTINUED)

Date	Time treated	Counts in strained ruminal fluid (millions/ml)			
		Untreated ingesta		Ultrasonically treated ingesta	
(1964)	(min)	Replicates	Mean	Replicates	Mean
9.6	60.0	160 90 100 70 80 30 100 110	92.5	0 0 0 0 0 0 0 0	0
18.6	5.0	19 26 28 20 19 25 17 33	23.4	11 23 15 14 13 13 13 14	14.5
18.6	10.0	19 26 28 20 19 25 17 33	23.4	30 21 23 25 18 21 33 22	24.1
25.6	1.0	40 50 20 60 40 40 70 50	46.0	60 40 40 60 60 50 50 30	49.0

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APPENDIX VI (CONTINUED)

Date	Time treated	Counts in strained ruminal fluid (millions/ml)			
		Untreated ingesta		Ultrasonically treated ingesta	
(1964)	(min)	Replicates	Mean	Replicates	Mean
25.6	5.0	40 50 20 60 40 40 70 50	46.0	100 50 50 70 40 100 60 40	64.0
11.8	5.0	20 20 10 30 0 20 10 30	17.5	30 20 40 20 40 20 20 30	27.5
18.8	12.5	60 10 30 30 30 40 20 20	30.0	20 30 30 20 40 50 70 50	39.0
11.8	20.0	20 20 10 30 0 20 10 30	17.5	10 20 30 40 40 30 20 10	25.0

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APPENDIX VI (CONTINUED)

Date	Time treated	Counts in strained ruminal fluid (millions/ml)			
		Untreated ingesta		Ultrasonically treated ingesta	
(1964)	(min)	Replicates	Mean	Replicates	Mean
17.9	30.0	16 13 14 13 11 - 13 18	14.0	9 16 10 9 17 17 9 16	12.8
25.8	1.0	17 21 13 14 4 11 13 11	13.0	22 14 33 18 10 13 15 11	15.7
25.8	5.0	17 21 13 14 4 11 13 11	13.0	28 29 17 24 16 12 9 12	18.3
24.9	10.0	30 40 60 20 40 30 40 40	37.5	40 60 50 30 20 30 50 60	42.5

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APPENDIX VI (CONTINUED)

Date	Time treated	Counts in strained ruminal fluid (millions/ml)			
		Untreated ingesta		Ultrasonically treated ingesta	
(1964)	(min)	Replicates	Mean	Replicates	Mean
24.9	20.0	30 40 60 20 40 30 40 40	37.5	50 40 40 50 30 40 30 60	42.5
17.9	30.0	16 13 14 13 11 - 13 8	14.0	16 14 14 14 18 7 14 16	14.0

APPENDIX VII

AGGLUTINATION TESTS OF ANTISERA AND GAMMA GLOBULIN SOLUTIONS (GG)

Date	Antibody	Antigen	Degree of agglutination in final serum dilutions														
			1/3	1/6	1/12	1/25	1/50	1/100	1/200	1/400	1/800	1/1,600	1/3,200	1/6,400	1/12,800	1/25,600	0
3.12.64	Antiserum-Gl 1	Gl 1	-	-	-	4	4	3	2	2	1	1	1	1	1	1	0
3.12.64	Antiserum-Gl 3	Gl 3	-	-	-	4	4	4	4	4	4	4	3	2	1	1	0
8.12.64	GG-Gl 1	Gl 1	-	-	-	4	4	4	4	0	0	0	0	0	0	0	0
8.12.64	GG-Gl 3	Gl 3	-	-	-	4	4	4	4	4	tr	0	0	0	0	0	0
4.6.65	GG-Gl 1	Gl 1	-	-	-	4	4	3	1	tr	0	0	0	0	0	0	0
4.6.65	GG-Gl 3	Gl 1	-	-	-	0	0	0	0	0	0	0	0	0	0	0	0
9.9.65	GG-Gl 1	Gl 1	4	3	2	1	tr	0	0	0	0	0	0	-	-	-	0
9.9.65	GG-Gl 3	Gl 1	0	0	0	0	0	0	0	0	0	0	0	-	-	-	0
9.9.65	GG-Gl 1	Gl 2	3	3	2	2	1	tr	0	0	0	0	0	-	-	-	0
9.9.65	GG-Gl 3	Gl 3	4	4	3	3	2	2	1	0	0	0	0	-	-	-	0
9.9.65	GG-Gl 1	Gl 14	0	0	0	0	0	0	0	0	0	0	0	-	-	-	0
9.9.65	GG-Gl 3	Gl 14	4	3	2	1	1	tr	0	0	0	0	0	-	-	-	0
4.9.65	GG-Gl 3	Gl 13	2	2	2	1	0	0	0	0	0	0	0	-	-	-	0
4.6.65	GG-Control-1a	Gl 1	-	-	-	0	0	0	0	0	0	0	0	0	0	0	0
4.6.65	GG-Control-1b	Gl 1	-	-	-	0	0	0	0	0	0	0	0	0	0	0	0

Key to degree of agglutination: 4 = complete; 3 = all but complete; 2 = more than positively detectable; 1 = positively detectable; tr = trace; 0 = no agglutination; - = not done.

APPENDIX VIII

ACTIVITY DATA FOR CALCULATING EFFICIENCY OF LABELLING GAMMA GLOBULIN (GG) WITH ^{131}I

GG solution containing bound + unbound ^{131}I			GG solution containing bound ^{131}I only			Labelling efficiency (% ^{131}I bound to GG)
^{131}I activity (counts x 10^{-3}/min/ml)	Volume before ion exchange resin (ml)	Total activity (counts x 10^{-3}/min) (A + B)	^{131}I activity (counts x 10^{-3}/min/ml)	Final volume (ml)	Total activity (counts x 10^{-3}/min) (B)	$\left(\frac{B}{A+B} \times 100\right)$
45,879.3	6.9	316,567	84.2	8.5	715.9	0.22
74,250.0	6.9	512,325	907.9	8.5	7,716.9	1.51
36,160.0	6.5	235,040	10.3	8.0	82.1	0.03
40,434.0	7.0	283,038	104.7	8.0	837.6	0.29
55,916.0	5.7	318,721	48.0	8.0	384.1	0.12
23,980.0	7.0	167,860	27.5	8.0	219.7	0.13
39,599.0	7.0	277,193	64.2	8.0	513.4	0.18
40,289.0	7.0	282,023	30.1	8.0	240.5	0.08
38,361.0	7.0	268,527	24.5	8.5	208.5	0.08
1,131.6	7.0	7,921*	0.5	8.0	4.3	0.05
570.4	7.0	3,992*	3.6	8.0	28.8	0.72
512.3	7.0	3,586*	20.0	8.0	160.1	4.46

* Lower activities of ^{131}I used.

APPENDIX IX

ACTIVITY DATA FOR CALCULATING DISTRIBUTION OF ^{131}I-GAMMA GLOBULIN, WITH ANTIBODY AGAINST LACTOBACILLUS, IN RUMINAL INGESTA FROM SHEEP K7

Sub-sample	Fraction	Count	Time counted (min)	Sample activity (sample-background) (cpm)	Activity retained by particles in ingesta				
					Solid fraction (A) (cpm)	Liquid fraction (B) (cpm)	Liquid + solid fractions (A + B) (cpm)	% Activity in solid fraction $\left(\frac{A}{A+B} \times 100\right)$	% Activity in liquid fraction $\left(\frac{B}{A+B} \times 100\right)$
1	L[1]	2,200	2	325					
	W[2]	3,254	2	852	2,025	1,177	3,202	63	37
	S[3]	2,800	1	2,025					
2	L	4,002	2	1,226					
	W	3,682	2	1,066	3,296	2,292	5,588	59	41
	S	4,071	1	3,296					
3	L	4,068	2	1,259					
	W	4,856	1	4,081	9,816	5,340	15,156	65	35
	S	10,591	1	9,816					
4	L	1,800	2	582					
	W	972	2	168	2,676	750	3,426	78	22
	S	2,994	1	2,676					
5	L	1,536	1	1,218					
	W	1,284	2	324	5,802	1,542	7,344	79	21
	S	6,120	1	5,802					
6	L	2,808	1	2,490					
	W	2,112	2	738	15,486	3,228	18,714	83	17
	S	15,804	1	15,486					

[1]L = Liquid phase [2]W = Washings [3]S = Solid phase

APPENDIX X

ACTIVITY DATA FOR DETERMINING THE REPRODUCIBILITY OF THE METHOD FOR ESTIMATING THE DISTRIBUTION OF ^{131}I-GAMMA GLOBULIN IN RUMINAL INGESTA

Sub-sample	Fraction	Count	Time counted (min)	Sample activity (sample-background) (cpm)	Activity retained by particles in ingesta				
					Solid fraction (A) (cpm)	Liquid fraction (B) (cpm)	Liquid + solid fractions (A + B) (cpm)	% Activity in solid fraction $\left(\frac{A}{A+B} \times 100\right)$	% Activity in liquid fraction $\left(\frac{B}{A+B} \times 100\right)$
1	L	2,954	1	2,179	3,352	2,725	6,077	55	45
	W	2,642	2	546					
	S	4,127	1	3,352					
2	L	4,002	2	1,226	3,296	2,292	5,588	59	41
	W	3,682	2	1,066					
	S	4,071	1	3,296					
3	L	2,911	1	2,136	3,783	2,625	6,408	59	41
	W	2,528	2	489					
	S	4,558	1	3,783					
4	L	3,208	2	829	3,697	2,494	6,191	60	40
	W	2,440	1	1,665					
	S	4,472	1	3,697					

APPENDIX XI

ACTIVITY DATA FOR CALCULATING THE DISTRIBUTION OF ^{131}I-GAMMA GLOBULIN, WITH ANTIBODY AGAINST STREPTOCOCCUS BOVIS, IN RUMINAL INGESTA FROM EACH OF FOUR SHEEP

Date (1965)	Sub-sample	Fraction	Count	Time counted (min)	Sample activity (sample-background) (cpm)	Activity retained by particles in ingesta				
						Solid fraction (A) (cpm)	Liquid fraction (B) (cpm)	Liquid + solid fractions (A + B) (cpm)	% Activity in solid fraction $\left(\frac{A}{A+B} \times 100\right)$	% Activity in liquid fraction $\left(\frac{B}{A+B} \times 100\right)$
29.3	1	L	2,762	1	2,140	2,586	2,367	4,953	52	48
		W	1,698	2	227					
		S	3,208	1	2,586					
	2	L	4,674	1	4,052	5,016	4,951	9,967	50	50
		W	3,042	2	899					
		S	5,638	1	5,016					
7.4	1	L	116,476	1	115,912	144,375	134,274	278,649	52	48
		W	18,926	1	18,362					
		S	144,939	1	144,375					
	2	L	151,649	1	151,085	224,899	182,589	407,488	55	45
		W	32,068	1	31,504					
		S	225,463	1	224,899					

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APPENDIX XI (CONTINUED)

Date (1965)	Sub-sample	Fraction	Count	Time counted (min)	Sample activity (sample-background) (cpm)	Activity retained by particles in ingesta				
						Solid fraction (A) (cpm)	Liquid fraction (B) (cpm)	Liquid + solid fractions (A + B) (cpm)	% Activity in solid fraction $\left(\frac{A}{A+B} \times 100\right)$	% Activity in liquid fraction $\left(\frac{B}{A+B} \times 100\right)$
29.3	1	L	5,100	2	1,928	1,976	2,175	4,151	48	52
		W	2,607	3	247					
		S	2,598	1	1,976					
	2	L	4,283	1	3,661	5,164	4,177	9,341	55	45
		W	4,553	4	516					
		S	5,786	1	5,164					
7.4	1	L	106,410	1	105,846	132,455	125,345	257,800	51	49
		W	20,063	1	19,499					
		S	133,019	1	132,455					
	2	L	186,733	1	186,169	309,083	229,041	538,124	57	43
		W	43,436	1	42,872					
		S	309,647	1	309,083					

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APPENDIX XI (CONTINUED)

Date (1965)	Sub-sample	Fraction	Count	Time counted (min)	Sample activity (sample-background) (cpm)	Activity retained by particles in ingesta				
						Solid fraction (A) (cpm)	Liquid fraction (B) (cpm)	Liquid + solid fractions (A+B) (cpm)	% Activity in solid fraction $\left(\frac{A}{A+B} \times 100\right)$	% Activity in liquid fraction $\left(\frac{B}{A+B} \times 100\right)$
29.3	1	L W S	2,470 3,148 4,856	1 3 1	1,848 ,427 4,234	4,234	2,275	6,509	65	35
	2	L W S	4,346 2,356 4,985	1 2 0.5	3,724 556 9,348	9,348	4,280	13,628	69	31
7.4	1	L W S	102,648 17,799 144,821	1 1 1	102,084 17,235 144,257	144,257	119,319	263,576	55	45
	2	L W S	203,329 33,571 334,298	1 1 1	202,765 33,007 333,734	333,734	235,772	569,506	59	41

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APPENDIX XI (CONTINUED)

Date (1965)	Sub-sample	Fraction	Count	Time counted (min)	Sample activity (sample-background) (cpm)	Activity retained by particles in ingesta				
						Solid fraction (A) (cpm)	Liquid fraction (B) (cpm)	Liquid + solid fractions (A+B) (cpm)	% Activity in solid fraction $\left(\frac{A}{A+B} \times 100\right)$	% Activity in liquid fraction $\left(\frac{B}{A+B} \times 100\right)$
29.3	1	L	3,682	1	3,060	566	3,206	3,772	15	85
		W	3,074	4	146					
		S	2,367	2	566					
	2	L	4,986	1	4,364	1,236	4,713	5,949	21	79
		W	3,885	4	349					
		S	3,716	2	1,236					
7.4	1	L	104,603	1	104,039	103,302	120,665	223,967	46	54
		W	17,190	1	16,626					
		S	103,866	1	103,302					
	2	L	220,748	1	220,184	155,614	246,206	401,820	39	61
		W	26,586	1	26,022					
		S	156,178	1	155,614					

APPENDIX XII

ACTIVITY DATA FOR DETERMINING THE DISTRIBUTION OF ^{131}I-GAMMA GLOBULIN, WITH ANTIBODIES AGAINST LACTOBACILLUS, STREPTOCOCCUS BOVIS, OR WASHINGS FROM UN-INOCULATED MEDIUM, IN INGESTA FROM SHEEP K7

Date (1965)	Sub-sample	Fraction	Count	Time counted (sec)	Sample activity (sample-background) (cps)	Activity retained by particles in ingesta				
						Solid fraction (A) (cps)	Liquid fraction (B) (cps)	Liquid + solid fractions (A+B) (cps)	% Activity in solid fraction $\left(\frac{A}{A+B} \times 100\right)$	% Activity in liquid fraction $\left(\frac{B}{A+B} \times 100\right)$
3.3	1	L	2,810	60	41.5	258.1	53.8	311.9	83	17
		W	2,131	120	12.3					
		S	15,804	60	258.1					
	2	L	3,000	117	20.3	96.7	25.7	122.4	79	21
		W	3,000	280	5.4					
		S	3,000	29	96.7					
	3	L	3,000	200	9.7	44.6	12.5	57.1	78	22
		W	2,438	300	2.8					
		S	3,000	60	44.6					
15.3	1	L	35,099	10	3,499.4	2,045.8	3,995.3	6,041.1	34	66
		W	5,064	10	495.9					
		S	20,563	10	2,045.8					
	2	L	14,907	10	1,480.2	1,098.3	1,771.0	2,869.3	38	62
		W	3,013	10	290.8					
		S	11,088	10	1,098.3					

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APPENDIX XII (CONTINUED)

Date (1965)	Sub-sample	Fraction	Count	Time counted (sec)	Sample activity (sample-background) (cps)	Activity retained by particles in ingesta				
						Solid fraction (A) (cps)	Liquid fraction (B) (cps)	Liquid + solid fractions (A+B) (cps)	% Activity in solid fraction $\left(\frac{A}{A+B} \times 100\right)$	% Activity in liquid fraction $\left(\frac{B}{A+B} \times 100\right)$
31.3	1	L	7,881	60	121.2	65.6	134.4	200.0	33	67
		W	2,803	120	13.2					
		S	4,547	60	65.6					
	2	L	5,274	60	77.7	36.7	86.4	123.1	30	70
		W	2,268	120	8.7					
		S	2,815	60	36.7					
21.4	1	L	28,368	60	465.8	187.9	514.5	702.3	27	73
		W	3,345	60	48.7					
		S	11,691	60	187.8					
	2	L	62,534	60	1,035.2	391.1	1,113.2	1,504.3	26	74
		W	5,701	60	78.0					
		S	23,885	60	391.1					

APPENDIX XIII

ACTIVITY DATA FOR CALCULATING NON-SPECIFIC RETENTION OF ^{131}I-GAMMA GLOBULIN BY RUMINAL INGESTA FROM SHEEP K 8

Date (1965)	Sub-sample	Fraction	Count	Time counted (min)	Sample activity (sample-background) (cpm)	Activity retained by particles in liquid + solid fractions of ingesta (A) (cpm)	Total activity added to ingesta sample (B) (cpm)	% Activity retained by particles in ingesta $\left(\frac{A}{B} \times 100\right)$
30.8	^{131}I-GG 1		37,693	1	37,318			
		L	11,402	1	11,027	13,155	37,318	35.2
		S	2,503	1	2,128			
	^{131}I-GG 2		30,781	1	30,406			
		L	5,252	1	4,877	7,109	30,406	23.4
		S	2,607	1	2,232			
23.8	^{131}I-GG 1		19,491	1	19,091			
		L	1,377	2	288	480	19,091	2.5
		S	1,183	2	192			
	^{131}I-GG 2		19,746	1	19,346			
		L	1,491	2	345	625	19,346	3.2
		S	1,361	2	280			
9.8	^{131}I-GG 1		7,799	1	7,242			
		L	1,773	2	329	364	7,242	5.0
		S	1,185	2	35			
	^{131}I-GG 2		8,788	1	8,231			
		L	1,825	2	355	1,038	8,231	12.6
		S	2,460	2	673			

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APPENDIX XIII (CONTINUED)

Date (1965)	Sub-sample	Fraction	Count	Time counted (min)	Sample activity (sample-background) (cpm)	Activity retained by particles in liquid + solid fractions of ingesta (A) (cpm)	Total activity added to ingesta sample (B) (cpm)	% Activity retained by particles in ingesta $\left(\frac{A}{B} \times 100\right)$
9.8	^{131}I-GG 3		8,344	1	7,787	868	7,787	11.1
		L	1,912	2	399			
		S	2,053	2	469			
7.12	^{131}I-GG 1		308,017	1	308,017	37,596	308,017	12.2
		L	7,908	1	7,908			
		S	29,688	1	29,688			
	^{131}I-GG 2		329,341	1	329,341	24,626	329,341	7.5
		L	5,330	1	5,330			
		S	19,296	1	19,296			
7.7	^{131}I-GG 1		34,614	1	34,179	3,133	34,179	9.2
		L	5,229	3	1,308			
		W	1,692	3	129			
		S	6,393	4	1,696			
	^{131}I-GG 2		35,049	1	34,614	2,566	34,614	7.4
		L	3,096	2	1,113			
		W	1,533	3	76			
		S	3,625	2	1,377			
19.7	^{131}I-GG 1		13,528	1	13,151	1,208	13,151	9.2
		L	1,184	2	215			
		S	2,738	2	992			
	^{131}I-GG 2		10,382	1	10,005	707	10,005	7.1
		L	1,324	2	285			
		S	1,597	2	422			

APPENDIX XIV

CALCULATION OF GAMMA GLOBULIN (GG) RETAINED NON-SPECIFICALLY BY RUMINAL INGESTA FROM SHEEP K8

Date (1965)	Sub-sample	Activity retained by particles in liquid + solid fractions of ingesta (A) (cpm)	Specific activity of ^{131}I-GG (B) (cpm/µg protein)	Amount GG retained by particles in liquid + solid fractions of ingesta $\left(\frac{A}{B}\right)$ (µg protein)
30.8	1	13,155	117	112.4
	2	7,109	117	60.8
23.8	1	480	71	6.8
	2	625	71	8.8
9.8	1	364	29	12.5
	2	1,038	29	35.6
	3	868	29	29.8
7.12	1	37,596	Unknown	-
	2	24,626	Unknown	-
5.7	1	3,133	2,506	1.25
	2	2,566	2,506	1.02
19.7	1	1,208	179	6.75
	2	707	179	3.95

APPENDIX XV

ACTIVITY DATA FOR CALCULATING SPECIFIC RETENTION OF ^{131}I-GAMMA GLOBULIN, WITH ANTIBODIES AGAINST LACTOBACILLUS AND STREPTOCOCCUS BOVIS, IN RUMINAL INGESTA FROM SHEEP K8

Date (1965)	Subsample	Fraction	Count	Time counted (min)	Sample activity (sample-background) (cpm)	Activity retained by particles in liquid + solid fractions of ingesta (A) (cpm)	Total activity added to ingesta sample (B) (cpm)	% Activity retained by particles in ingesta $\left(\frac{A}{B} \times 100\right)$
18.8	^{131}I-GG 1		98,826	1	98,428			
		L	3,943	2	1,573	9,164	98,428	9.3
		S	15,978	2	7,591			
	^{131}I-GG 2		98,606	1	98,208			
		L	4,145	2	1,674	8,564	98,208	8.7
		S	7,288	1	6,890			
27.9	^{131}I-GG 1		190,520	1	190,213			
		L	1,773	2	578	2,204	190,213	1.2
		S	3,866	2	1,626			
	^{131}I-GG 2		200,296	1	199,989			
		L	1,673	2	529	2,308	199,989	1.1
		S	4,172	2	1,779			

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APPENDIX XV (CONTINUED)

Date (1965)	Subsample	Fraction	Count	Time counted (min)	Sample activity (sample-background) (cpm)	Activity retained by particles in liquid + solid fractions of ingesta (A) (cpm)	Total activity added to ingesta sample (B) (cpm)	% Activity retained by particles in ingesta $\left(\frac{A}{B} \times 100\right)$
5.10	^{131}I-GG 1		9,818	1	9,443	433	9,443	4.6
		L	529	1	154			
		S	654	1	279			
	^{131}I-GG 2		15,991	1	15,616	566	15,616	3.6
		L	552	1	177			
		S	764	1	389			
2.8	^{131}I-GG 1		34,873	1	34,475	4,791	34,475	13.9
		La	3,801	2	1,502			
		Lb	3,842	2	1,523			
		S	4,328	2	1,766			
	^{131}I-GG 2		34,625	1	34,227	4,192	34,227	12.2
		La	2,889	2	1,046			
		Lb	2,918	2	1,061			
		S	4,966	2	2,085			

APPENDIX XVI

CALCULATION OF GAMMA GLOBULIN (GG) RETAINED SPECIFICALLY BY RUMINAL INGESTA FROM SHEEP K8

Date (1965)	Sub-sample	Activity retained by particles in liquid + solid fractions of ingesta (A) (cpm)	Specific activity of ^{131}I-GG (B) (cpm/µg protein)	Amount GG retained by particles in liquid + solid fractions of ingesta $\left(\frac{A}{B}\right)$ (µg protein)
18.8	1	9,164	521	17.6
	2	8,564	521	16.4
27.9	1	2,204	530	4.2
	2	2,308	530	4.4
5.10	1	433	31	14.0
	2	566	31	18.3
2.8	1	4,791	102	47.0
	2	4,192	102	41.0

APPENDIX XVII

EXAMPLE OF STATISTICAL CHECK OF SCALER OPERATION

Packard 3003 Gamma Spectrometer adjusted to count approximately 2,600 cpm from a caesium - 137 standard source of gamma radiation.

A series of 50 successive 1 min counts (x) was collected.

2,635	2,651	2,637	2,598	2,609
2,710	2,656	2,603	2,639	2,640
2,592	2,606	2,514	2,658	2,591
2,642	2,678	2,634	2,612	2,785
2,673	2,546	2,729	2,793	2,721
2,706	2,653	2,574	2,804	2,715
2,659	2,559	2,706	2,642	2,685
2,618	2,613	2,735	2,564	2,678
2,643	2,696	2,667	2,738	2,599
2,591	2,561	2,674	2,616	2,611

Σx = 132,459

N = 50

Mean, $\bar{x}$ = 2,649

Standard deviation, SD = $\sqrt{\frac{1}{N-1}\left[\Sigma x^2 - \frac{(\Sigma x)^2}{N}\right]}$

Σx^2 = 351, 104, 637 $\qquad$ $(\Sigma x)^2$ = 17,545,386,681

SD = 63.39

Maximum observed deviations (OD) from mean = + 155, - 135.

Chauvenet's criterion states that for a total of 50 counts the ratio $\frac{OD}{SD}$ should not exceed 2.57.

Now $\frac{155}{63.39}$ = 2.44 and $\frac{135}{63.39}$ = 2.13, therefore all the observations are acceptable and the scaler is operating satisfactorily.

Typed and lithographed by

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