SOME STUDIES ON THE MICROFLORA OF THE RUMEN.

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by

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Résumé.

Streptococcus bovis was frequently isolated during preliminary studies on the culture of bacteria from the rumen of cattle. This bacterium was known to occur in the mouth and faeces of cattle and had already been isolated from the rumen, but no studies had been made of the numbers or the frequency of the occurrence of Strep.bovis in rumen contents.

The numbers of Strep.bovis in the rumen of a heifer and a steer, each having a permanent fistula, were usually between 10^5 and 10^7 per ml. and there was little variation in the counts in these animals from day to day or between periods of stall or pasture feeding. A significant increase in the numbers of Strep.bovis occurred during the first two hours after each feed when the animals were stall-fed and then the numbers gradually decreased until the next meal. Strep.bovis was not detected in the feed and it was concluded that the increase in Strep. bovis after feeding was due to multiplication in the rumen.

Rumen contents from freshly slaughtered cattle and sheep from widely separated areas of Scotland and from goats from the Institute herd were also examined. The numbers of Strep.bovis present in the rumen contents of the cattle and goats were of the same order as in the fistula animals. In

sheep the number of Strep.bovis was usually lower than in the cattle. Examination of the rumen contents of calves showed that Strep.bovis unlike most types of rumen bacteria can become established in the rumen in the absence of roughage in the diet. The presence of Strep.bovis in the faeces of cattle was confirmed, and the organism was also isolated from the faeces of the horse.

When rumen liquid collected 2 hr.after feeding was incubated in vitro at 39°C.with the addition of maltose, the numbers of Strep.bovis increased rapidly in 4 hr.. In the presence of starch grains little growth of Strep.bovis occurred in 4 hr. although good growth was obtained after 24 hr. In the absence of added carbohydrate the number of Strep.bovis remained constant. The breakdown of intact starch grains by Strep.bovis was confirmed in pure culture by the estimation of the amount of the starch grains decomposed and by microscopical examination of the decomposing grains.

The formation of iodophilic polysaccharide in the cells of Strep.bovis was demonstrated and shown to occur during growth on media containing starch, dextrin or maltose but not glucose, sucrose, or starch in the presence of glucose.

The isolation in pure culture of a Gram-positive non-sporing rod, possibly a Corynebacterium, and of small numbers of Lactobacilli is described. Bacteria of the coli-aerogenes and Proteus groups were either absent or present in only small numbers in the rumen liquid. Preliminary studies in the isolation of strictly anaerobic bacteria from the rumen were not successful although good growth, in mixed culture, was obtained with cellulose-decomposing bacteria using the method of Sijpesteijn (1948).

GENERAL INTRODUCTION.

The micro-organisms in the rumen of cattle and other ruminants comprise many different types of bacteria and protozoa some of which are now recognised to make an important contribution to the digestion and assimilation of food by the host (Nutrition Abstracts and Reviews, 1947-48). Others occur in such small numbers that their functional significance must be, almost inevitably, negligible while some of the micro-organisms are so unsuited to the environment in the rumen that they are clearly unable to multiply there and consequently their numbers must be derived from some source, external to the rumen, such as the food ingested by the host. The last group of micro-organisms is unlikely to participate in the functions in the rumen or to have any effect upon the balance of micro-organisms and is therefore of little importance in a study of rumen bacteriology. On the other hand, a micro-organism that is of functional significance in the rumen must not, of necessity be assumed to benefit the host. Certain processes performed by the micro-organisms may not, in fact, be advantageous.

Any micro-organism that participates in the chemical reactions which take place in the rumen is important

and its importance is enhanced if it can be shown that its participation is of value to the host. Considerable difficulty has been experienced, however, in assessing the importance of individual types. This is due to the complexity of the population and to the fact that most of the different species of micro-organisms have defied all attempts to isolate them in pure culture, and hence their numbers and their biochemical reactions are not known. While it is unlikely that the significance of an individual type of rumen micro-organism can be completely assessed by means of any single criterion, microscopical methods in some instances have been of great value.

Microscopical methods were used by Ankersmit as early as 1905 to assist in assessing the significance of the spore-forming cellulose-decomposing rods which he had isolated from the rumen. He observed that in fresh rumen contents rods were not associated in large numbers with decomposing cellulose and hence concluded that the spore-forming rods that he had isolated were unimportant. In spite of this early example of the critical value of microscopical methods, they fell into disuse until Henneberg (1922) described cocci associated with decomposing cellulose and starch and drew attention to their

characteristic wine-red to blue staining reaction with iodine (iodophile reaction). He concluded, in agreement with Ankersmit, that spore-forming rods were not the main cellulose-decomposing bacteria in the rumen but he believed that they might be important in the intestine.

Using microscopical techniques Baker and his co-workers observed, in detail, the microbial facies in the caecum of the horse, rabbit and guinea-pig and in the rumen of cattle and sheep. Much of this work has been reviewed by Baker & Harriss (1947-48). Baker (1943) emphasised the importance of iodophiles in the decomposition of cellulose, starch and other carbohydrates in the rumen. He distinguished between those iodophiles that are "fixed" to vegetable particles and those that are unattached or "free". The fixed iodophiles were regarded as being the ones mainly associated with cellulose and starch decomposition. The distinction is rather arbitrary since all "fixed" organisms must have been "free" at some time previously, and ultimately when decomposition has advanced sufficiently they will become "free" again. Also, it seems impossible that the rapid decomposition of cellulose and other material in the rumen could occur unless large numbers of "free"

organisms were present in the rumen contents ready to attack the food immediately after it had been ingested by the host.

Baker further differentiated the iodophilic microflora, according to size, into macro- and micro-types. Micro-iodophile cocci and vibrios were believed to initiate the attack on cellulose (Baker & Harriss, 1947-48) and to be the most important starch decomposers (Baker, 1943; Baker & Harriss, 1947-48).

Various refinements of microscopical methods were applied by Baker to the study of the rumen micro-organisms. Among these were certain staining methods to facilitate the observation of cellulose, starch and of associated bacteria and also the use of polarised light to reveal, by differences in their birefringence, areas of decomposed and undecomposed cellulose and starch. Despite these refinements it must still be admitted that for two reasons the value of microscopical methods as a means of assessing functional significance, is limited. Firstly, organisms in a mixed population can be associated with a particular function by microscopical methods only if this function or some specific result of it can be observed. It is not surprising, therefore, that the methods have been applied mainly and most successfully to cellulose

and starch decomposers that attack a particulate substrate. Secondly, most taxonomically different types of bacteria can not as yet be differentiated by microscopical methods.

Hungate (1947, 1950), Sijpesteijn (1948), Gall & Huhtanen (1951) and other workers have attempted to assess the significance of different types of bacteria in the rumen by relating counts found by cultural methods to total or direct microscopic counts of the rumen micro-organisms. Gall & Huhtanen (1951) have recognised that counts from one animal are inadequate and that for a reliable estimate of the incidence of an organism counts from animals in different geographical locations are necessary. In addition, Gall & Huhtanen considered that a micro-organism, present in the rumen in numbers less than 10^6 per ml.cannot be significant. An estimate of the numbers of bacteria is essential to the proper assessment of the significance of a rumen micro-organism, but unfortunately many errors are involved in counting rumen bacteria and these must be considered before any conclusions can be drawn from the results. Probably the most fundamental error associated with direct counts is caused by the completely unknown number of dead

micro-organisms within the total. Another error, associated with both direct counts and colony counts, is introduced by the number of micro-organisms that are attached to vegetable matter and are impossible to count by either cultural or direct methods. This, as will be realised from the foregoing brief mention of the distinction drawn by Baker between "free" and "fixed" iodophiles must involve in particular any counts of micro-organisms that derive their nutrients directly from vegetable matter. Gall, Stark & Loosli (1947) reduced this error in both direct and viable counts by shaking the rumen contents beforehand to remove bacteria from the feed and obtained counts that were usually greater than those obtained by other workers.

If a staining method is used to make micro-organisms apparent for direct microscopical counts, an error is introduced by the number of organisms that do not stain adequately, since no stain is known that can be taken up by all the different types of micro-organisms of all ages in such a diverse population as that in the rumen. While dark ground illumination overcomes this difficulty it introduces one of its own, namely, the difficulty of distinguishing between debris and micro-organisms. Negative staining with nigrosin has been used by Gall et al. (1947)

and Moir (1951). Williams & Moir (1951) have stated that the nigrosin covers the very small bacteria and thereby renders them uncountable.

The significance of a micro-organism isolated from the rumen can be assessed by comparing the functions that it performs with the functions of the total rumen bacteria (Elsden & Philipson, 1948; Johns, 1951a). This has been done by Johns (1951a, b, c) who found that Veillonella gazogenes, an organism isolated from the rumen contents of sheep, was able to decarboxylate succinate to propionate rapidly at an optimum pH of 6.0. In rumen contents succinate was found to be decarboxylated rapidly to propionate at a similar optimum pH. These and other results suggest that Veillonella gazogenes plays a significant role in the rumen. Johns also showed convincingly that in the same reaction Propionibacteria were without significance. Their reaction rate was slow and their optimum pH was 5.0 - 5.2.

Implicit in the assessment of the significance of a micro-organism is its primary isolation in pure culture. Most of the species of micro-organisms that function in the rumen are closely adapted to their environment and few of them

have been grown in pure culture. The success that has been achieved by Hungate (1947) and Sijpesteijn (1951) has been due chiefly to the closeness with which they copied this environment. Their cultures were buffered with CO_2-bicarbonate and were strictly anaerobic and, in addition, the medium prepared by Hungate contained sterile rumen contents. Further progress may entail a still closer copy of both the physical and chemical aspects of this environment.

The primary object of the present investigation was to determine the conditions of the chemical and physical environment that would admit pure cultivation of micro-organisms that might be important in the rumen so that these bacteria and their reactions could then be studied and their contribution to the processes in the rumen assessed. During preliminary investigations media were used that were known to be suitable for the growth of certain types of micro-organisms which had been isolated from the alimentary tract of animals or which, from a consideration of their growth requirements, might be expected to be capable of growth in the rumen. Attempts were made to cultivate cellulose decomposers under conditions resembling more closely those in the rumen but they were discontinued when the successful experiments of Hungate and

Sijpesteijn were published.

During the course of this preliminary work Strep. bovis was regularly isolated from rumen contents and although it was realised that the small numbers of this organism almost precluded the possibility that it played a significant rôle in the rumen the organism seemed to be sufficiently interesting in itself to warrant further study. This view received support from a similar opinion expressed by Elsden (1945-46) in connection with an organism which was isolated by Van der Wath and which appears to have been Strep.bovis.

A survey of the literature showed that although streptococci had been isolated on several occasions from the rumen of cattle their identification with Strep.bovis was often impossible because of the inadequate methods of classification used by the earlier workers. The predominant streptococcus in the rumen was identified by Kreipe in 1927 as Strep.bovis. There was little information concerning the regularity with which Strep.bovis occurred in the rumen, the possible variation in its numbers and whether it could multiply there or was simply a passenger introduced with the food. An attempt to provide this information was made and is reported in Part 1 of this thesis. Part 2 is concerned mainly with studies of the way in which this

organism functions in the rumen, and in Part 3 the results of attempts to isolate the rumen bacteria are presented.

Part 1

The Incidence of Streptococcus Bovis in the Rumen of Fistula Cattle and in Freshly Slaughtered Cattle, Sheep and Goats and in Calves.

Introduction.

Winslow & Palmer (1910) and Fuller & Armstrong (1913) noted that the predominant streptococcus in cow dung differed from that in the faeces of man or horse by its ability to ferment raffinose and its failure to ferment mannitol. In 1919 Orla-Jensen designated the predominating streptococcus in cow dung Streptococcus bovis and showed that it was a very active decomposer of starch. Ayers & Mudge (1923) isolated the same micro-organism from both the mouth and the faeces of cattle.

Streptococci termed Streptococcus acidi-lactici were isolated from the rumen of cattle by Ankersmit (1905) and from the rumen of sheep by Henneberg (1919). In 1927 Kreipe using Orla-Jensen's classification of the Streptococci found that the predominant streptococcus in the rumen of cattle was Strep.bovis. Kreipe isolated this streptococcus from 24 slaughtered cattle

which came from Germany and Denmark and which had been either stall- or pasture-fed before slaughter. Counts of the organism were not given. A streptococcus which appears to be Strep.bovis was isolated by Van der Wath (1948) from decomposing starch grains that had been suspended in a silk bag in the rumen of sheep. This method of isolation did not allow counts of the organism to be made.

The investigations described in Part 1 of this thesis were concerned with the numbers of Strep.bovis in a heifer and a steer, each having a permanent rumen fistula, and in slaughtered cattle, calves, sheep and goats. The fistula animals were used to trace the numbers of Strep.bovis present in the rumen throughout the day and at different periods of the year. The purpose of using slaughtered animals was to find whether the numbers of Strep.bovis in the fistula animals were similar to those found generally in cattle and other types of ruminants which had been maintained in widely separated areas.

Experimental Methods.

1. Sampling of rumen contents.

Unless otherwise stated the rumen liquid used in the experiments here described was obtained from two animals,

a heifer (Daphne) and a steer (Ernest), each having a permanent rumen fistula. At first the fistula in each animal was closed with a metal tube and screw cap (Watts, 1948), but these fitments were heavy and allowed liquid contents to escape especially while the animals were at grass, so that they were later replaced by rubber fitments (Balch & Johnson, 1948). The rubber fitments consist of a tube which was left in position during sampling, and an inner 'core' that was removed during sampling and afterwards returned to position and fitted tightly by inflation. These fitments were light and allowed only very slight escape of rumen contents.

The normal diet of the animals, when they were stall-fed consisted of 3 lb.concentrates (mainly oats, beans and dried grass) and 10 lb.straw fed at 8 a.m.; and 3 lb.concentrates and 10 lb.hay at 6 p.m.

Samples of rumen contents were taken from well inside the rumen using a small aluminium cup and were transferred to a vacuum flask which was usually filled to capacity (1,100 ml.) and then tightly corked. The rumen contents were strained through butter muslin to remove coarse particles and the resulting rumen liquid was examined immediately. A portion of the rumen

Table 1.

Composition of media used for plates.

Medium	Composition (%)			Water	Other ingredients	Agar (%)	pH (after sterilisation)
	Peptone	Lemco	Yeastrel				
Standard agar	0.5	0.3	-	Tap	-	1.5	7.2
Starch agar	0.5	0.3	-	Distilled	Soluble starch 1%	1.5	6.8 - 7.0
Milk agar	0.5	-	0.3	Distilled	Milk 1% (V/V)	1.5	6.8 - 7.0
Glucose agar	0.5	-	0.3	Distilled	Glucose 1%	1.5	6.8 - 7.0

liquid was preserved for later microscopical examination by the addition of 5% (v/v) formalin in the ratio of 9 ml.of rumen liquid to 1 ml.of formalin giving a final concentration of 2% formaldehyde. Samples from these animals were taken between 10 a.m. and 11 a.m. unless otherwise stated.

2. Enumeration of Strep.bovis.

All counts were made on strained rumen liquid thus excluding all bacteria which were lodged on the larger food particles.

Dilutions of the rumen liquid were prepared in quarter-strength Ringer's solution using a fresh sterile pipette for each dilution (1 ml.to 9 ml.of diluent). Suitable dilutions were plated in duplicate on the required medium and incubated for 3 days at 37°C.in air or in a McIntosh and Fildes type anaerobic jar which had been evacuated and filled with the required gas (carbon dioxide, hydrogen or nitrogen). Strep.bovis grew equally well in air or carbon dioxide and carbon dioxide was used to prevent the growth of obligate aerobes including spore-forming bacilli.

No appreciable difference was found between the colony counts of Strep.bovis on standard, milk, glucose or starch agar (Table 1) but it soon became apparent that

Strep.bovis grew faster and gave larger colonies on both glucose and starch agar than on standard agar. Starch agar was eventually used, firstly, because it was thought that it might be more selective than glucose for Strep.bovis, and secondly, because some indication of the numbers of Strep.bovis could be obtained by flooding a starch agar plate with iodine when, if no starch hydrolysis became apparent, it could be concluded that Strep.bovis was absent.

An attempt to suppress the growth of the few other types of micro-organisms which accompanied Strep.bovis by incubation of the starch agar plates at 45° instead of 37°C. was not successful as some colonies of short rods were also capable of growth at 45°C.

The colonies were counted with a Mattick and Hiscox counting chamber, a 4" diameter hand lens and a tally counter.

3. Isolation and identification of Strep.bovis.

The types of micro-organisms which had grown on the plates were determined as follows :-

A plate in which the colonies were suitably separated (usually one that had been used for the viable count) was divided into segments and from a random pair of opposite segments all the colonies were subcultured to tubes of broth. The species of micro-

organisms present in the rumen liquid in numbers too small to be represented in the dilution of rumen liquid used in the preparation of the plate were not detected by this method of examination.

The fine growth of Strep.bovis in the standard broth which was used at first was difficult to detect and, later, subcultures were always made to glucose broth in which Strep.bovis grew rapidly and gave a dense turbidity. This medium had, however, one disadvantage which was not realised at first. The viability of Strep.bovis in glucose broth was rapidly lost after 24 hr.at 37°C.and, consequently, transfers had to be made within 18 hr. This loss of viability was shown to be due to the sensitivity of Strep.bovis to the acidity produced in the medium (Appendix to Part 1, p.46).

Each subculture was replated on glucose or starch agar and a single well-isolated colony picked to a glucose agar slope. When both the macroscopical and microscopical appearance of the growth was uniform, the characteristics of the organisms were determined and their identity established where possible.

The streptococci were classified according to Sherman (1937). The production of ammonia was determined in the arginine medium of Niven, Smiley & Sherman (1942), instead of

in a 4% peptone broth as given by Sherman (1937). The sugar and other fermentation reactions, the Voges-Proskauer, methyl red and other tests were performed using the methods and media given in most textbooks of practical bacteriology such as Mackie and McCartney (1942) and need not be given in detail here.

The strains of Strep.bovis isolated during the present investigation showed little variation in their characteristics, a description of which is given below:

Morphology. Cocci 0.6 to 1.0 μ in diameter occurring usually in pairs but also in short chains; cells oval or when in chains often flattened at adjacent surfaces.

Growth requirements. In standard broth the organism grew poorly (very slight almost undetectable turbidity in 24 hr.at 37°C.), but growth was markedly enhanced in the presence of glucose. In glucose broth the organism gave visible turbidity at 37°C.after 8 hr. and after 24 hr.it gave a dense turbidity with sediment. In 24 hr.at 37°C.growth was just visible on standard agar but on glucose or starch agar colonies were 1 - 2 mm.in diameter. Good growth was obtained on glucose or starch agar plates under both aerobic and strictly anaerobic conditions; no growth occurred at 15.5°C.but good growth

at 30°C. to 45°C.

Biochemical reactions. Most strains failed to produce any change in litmus milk but a few strains produced slight acid or acid with clot and reduction of the litmus.

All strains failed to grow in glucose broth in the presence of 6.5% NaCl; only very occasional strains grew in 4% NaCl but all strains grew in the presence of 2% NaCl. All strains failed to produce catalase, to reduce 0.1% methylene blue in milk, to liquefy gelatin, to produce ammonia from arginine or indole from peptone; nitrate reduction to nitrite was variable; starch was hydrolysed and aesculin split; the Voges-Proskauer test for acetoin was frequently but not invariably weakly positive and the methyl red test was positive.

Acid was formed from glucose, lactose, sucrose, maltose, raffinose, arabinose, salicin and inulin but not from glycerol or mannitol. About 75% of the strains failed to produce acid from sorbitol.

Precipitin reaction. Using extracts of Strep.bovis prepared according to the technique of Shattock (1949) no precipitin reaction was obtained with Wellcome Brand Group D serum. Control tests with extracts of a typical strain of Strep.faecalis were positive with this serum. The difficulty of preparing sera

with Strep.bovis has been noted by Shattock and in the present study no attempt has been made to obtain Strep.bovis sera.

From the foregoing description it is obvious that the strains isolated from the rumen were typical strains of Strep.bovis according to the classification of Sherman (1937). They do not show the variability in characteristics such as the hydrolysis of starch or the fermentation of inulin and mannitol which was noted in strains from other sources by Abd-el-Malek and Gibson (1947-48), Shattock (1949) and Sherman (1937). The ability to hydrolyse starch rapidly is a constant feature which has been retained even after the organisms have remained in stock laboratory culture in litmus milk for long periods.

4. Quantitative estimation of the total bacterial microflora of the rumen.

An attempt was made to find a method of estimating the total number of 'free' bacteria in the rumen liquid in order that changes in the numbers of Strep.bovis and in the 'total' microflora might be related.

(a) Direct counts. Direct microscopic counts seemed to offer the greatest chance of success. Stained smears were prepared using various dyes but they proved unsatisfactory owing to

the varying affinity of the different types of bacteria for the dyes. 'Negative' staining was then attempted by a method similar to that used by Gall, Stark & Loosli (1947). Samples of rumen liquid of at least 1 litre were obtained. The strained liquid was thoroughly shaken, 9 ml.removed and formalin added to give a final concentration of 2% formaldehyde. The formalised sample was shaken 25 times in 12 seconds, each excursion being 1 ft. Serial dilutions were prepared using 1 ml.of the suspensions and 9 ml.distilled water. Rumen liquid diluted 500 times usually contained the most suitable number of micro-organisms for the count (an average of 10 - 20 bacteria per field) but dilutions of 100 or 1,000 times were also used. 1 ml.of a suitable dilution of the rumen liquid and 0.25 ml. nigrosin (Mackie & McCartney, 1942) were gently mixed and immediately two aliquots of 0.01 ml.were spread over two different areas of 5 sq.cm.etched on a glass slide. The slide was left on a flat surface at room temperature until the smears were dry. The number of micro-organisms in 50 fields on each smear was determined. Difficulty was experienced in deciding which of the small clear areas observed against the dark nigrosin background were bacteria such as small cocci and which were merely small holes or non-bacterial particles in the background of nigrosin. Williams & Moir (1951) using a

Table 2.

Direct counts obtained by 'negative' staining of diluted rumen liquid with nigrosin using 15 smears and counts on 10 fields per smear.

Smears	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
No.of bacteria per field	19	25	23	29	18	21	20	13	17	14	23	24	15	14	23
	24	19	14	20	14	11	12	9	21	17	24	21	17	19	19
	14	17	15	19	14	13	15	15	17	15	28	21	19	18	16
	19	21	19	12	11	17	18	12	24	12	25	28	17	14	22
	15	33	9	11	16	9	20	16	19	10	33	21	15	20	17
	16	17	23	13	13	16	13	23	16	12	24	21	16	24	12
	16	15	19	6	9	19	14	13	24	16	25	25	10	16	11
	24	24	24	15	8	8	16	9	19	19	24	25	11	15	16
	15	37	17	15	11	11	22	25	16	12	38	34	11	18	17
	15	24	21	11	17	17	17	17	20	15	28	21	14	13	22
Total in 10 fields	177	232	184	151	131	142	167	152	193	142	272	241	145	171	175

Analysis of Variance.

Source of variance	Degrees of freedom	Sum of squares	Mean square	Variance ratio	P
Total	149	5278	35.4		
Between smears	14	2364	168.8	7.85	<0.001
Within smears	135	2914	21.5		

Table 3.

Direct counts on a sample of rumen liquid using 10 smears and counting 50 fields.

Smears	1	2	3	4	5	6	7	8	9	10	
No.of bacteria per field	9	21	8	8	10	7	3	13	5	22	
	16	20	15	5	6	13	4	8	6	17	
	23	7	11	9	5	7	4	7	3	13	
	12	7	12	8	9	8	7	5	8	9	
	15	8	2	5	9	7	7	8	4	8	
	8	1	7	9	5	6	6	4	1	5	
	10	7	8	3	7	9	3	5	11	10	
	10	6	7	12	12	12	5	10	8	19	
	10	17	9	11	5	13	12	6	13	16	
	9	6	13	10	7	5	8	8	12	11	
	8	6	8	7	9	8	3	8	17	6	
	15	6	12	12	6	7	13	9	4	13	
	10	5	11	14	9	9	8	5	5	11	
	14	7	10	8	7	14	8	4	12	13	
	14	13	8	8	7	13	8	3	2	12	
	8	12	21	10	4	8	6	4	8	10	
	7	11	21	10	5	17	5	9	1	12	
	7	7	11	8	3	8	9	7	9	6	
	7	7	9	13	1	12	10	11	2	5	
	4	18	13	14	9	10	7	8	3	7	
	3	14	9	14	5	9	7	12	5	11	
	3	16	8	13	10	7	6	8	9	10	
	11	10	8	12	10	16	8	5	5	10	
	12	13	9	9	8	11	5	8	15	10	
	12	2	12	17	12	19	12	3	9	11	
	11	8	10	12	8	8	6	7	4	8	
	10	8	11	10	7	14	9	11	7	14	
	16	4	6	22	6	15	7	7	9	9	
	9	11	12	14	3	10	5	5	8	12	
	15	20	2	7	5	2	9	3	12	11	
	10	8	2	7	18	7	5	15	12	7	
	14	8	10	7	19	10	5	9	5	10	
	11	20	5	9	18	5	5	12	2	9	
	21	11	7	6	11	10	5	10	4	10	
	12	13	7	6	11	13	12	9	10	6	
	9	8	11	10	11	7	9	12	2	18	
	12	15	5	9	10	11	5	7	9	7	
	4	10	1	12	11	7	4	18	7	5	
	13	13	2	8	9	10	4	6	7	10	
	11	13	5	8	8	7	4	8	9	6	
	16	6	1	9	13	8	7	13	8	2	
	10	11	4	7	9	10	6	8	12	7	
	10	9	4	12	15	7	5	9	6	9	
	15	13	8	13	14	4	6	11	6	5	
	10	15	7	20	12	12	7	8	10	3	
	11	12	7	11	10	9	5	7	18	8	
	8	10	6	9	7	9	5	4	2	9	
	10	9	12	22	6	4	4	12	6	3	
	21	8	4	27	6	6	7	6	7	11	
	10	17	15	16	13	11	6	11	13	4	Total
Total in 50 fields	556	527	426	542	440	471	326	406	372	480	4546
Mean ct./field	11.1	10.5	8.5	10.8	8.8	9.4	6.5	8.1	7.4	9.6	9.1
Coefficient of variation	38	45	52	43	44	37	36	40	55	44	46
Count ÷ 10^9/ ml.rumen liquid	22.1	20.5	16.6	21.1	17.2	18.4	12.7	15.8	14.5	19.2	17.8

Analysis of Variance

Source of variance	Degrees of freedom	Sum of Squares	Mean square	Variance ratio	P
Total	499	8768			
Between smears	9	1017	113.0	7.15	< 0.001
Within smears	490	7751	15.81		

similar method noted the impossibility of distinguishing between artefacts and cocci less than 0.5 μ in diameter, but they decided nevertheless that although their counts were low, the method was valid for comparative purposes.

To test the reproducibility of the count a series of 15 smears was prepared from one sample and 10 fields per smear counted (Table 2). The analysis of variance showed that there was a significant difference between the different smears. A second series of 10 smears was prepared from another sample of rumen liquid and 50 fields per smear counted. The counts per field and the analysis of variance are given in Table 3, from which it can be seen that a significant difference occurred again between the smears and also that the coefficient of variation for all 500 fields was 46%. The coefficient of variation ranged from 36 to 55% for the fields in the individual smears.

Direct counts were made on samples of rumen contents that had been collected at intervals over a period of 13 months from both the fistula animals. The mean counts per ml.of rumen liquid on 16 samples from Daphne and 9 samples from Ernest were $(27 \pm 17.7) \times 10^9$ and $(24 \pm 29.6) \times 10^9$ respectively. These figures are of the same order as those found by Gall, Stark &

Loosli (1947) for 10 samples of the liquid fraction of the rumen contents of cattle, i.e. $(76 \pm 10.3) \times 10^9$.

Further doubt was cast on the value of the direct count when it was used in the present investigation to determine the total count before and after *in vitro* incubation of samples of rumen liquid containing added maltose. Thus, the direct count by the nigrosin method did not appear to increase during incubation for 4 hr. although the amount of protein nitrogen (determined by Dr. M.L. McNaught in the Biochemistry Department) increased appreciably :-

Sample No.	Direct count ($\div 10^9$)/ml.		Increase in protein nitrogen (mg.N/100 ml.)
	0 hr.	4 hr.	
1	3.2	3.4	8.77
2	4.3	3.9	9.05
3	4.2	3.4	4.14

Although Gall, Stark & Loosli (1947) and Williams & Moir (1951) have both used direct counts to estimate the total bacteria or changes in the total bacteria in rumen liquid, the method was not adopted for further use in the present investigation for the following reasons :-

1. the difficulty of distinguishing between artefacts and small cocci under the microscope,

2. the amount of variation between counts in different fields in a smear and between different smears from the same sample, and

3. the lack of correlation between the numbers of micro-organisms detected during in vitro incubation of rumen liquid and the increase in protein nitrogen due to bacterial multiplication.

(b) Turbidimetric estimation. The possibility was explored of estimating turbidimetrically the changes of the total population in rumen liquid using a Spekker absorptiometer. The estimations were made on formalised samples of strained rumen contents after vegetable particles had been removed by centrifuging at 2,000 r.p.m.for 5 min. Microscopical observation on the resulting liquid showed that the particulate matter present was almost entirely bacterial. McNaught, Smith, Henry & Kon (1950) had also concluded, from the estimation of the fibre content and microscopical observation of dried rumen bacteria which were prepared from rumen liquid after it had been centrifuged under the above conditions, that the amount of non-bacterial particulate matter was negligible. The turbidity of the centrifuged liquid after it had been diluted 50 times, was estimated in a Spekker absorptiometer using violet filters

Table 4.

The turbidity (as extinctions) of concentrations of three samples of rumen liquid.

Rumen liquid (ml./50 ml.aqueous suspension)	Extinction (E)		
	Sample		
	1	2	3
0.5	0.11	0.09	0.14
0.8	0.17		
1.0	0.22	0.16	0.28
1.2	0.25		
1.5	0.33		0.43
2.0	0.43	0.34	0.56
2.5	0.54		
3.0	0.64	0.50	

and the results were expressed as extinctions.

The colour of the rumen contents varied little except when the proportion of green food was considerable. The rumen liquid, diluted 50 times, was still very turbid but was almost the same shade of grey irrespective of the diet, and the effect of variation in the colour of the rumen liquid on this reading of the Spekker absorptiometer was negligible.

In using this method it was assumed that the regression of the Spekker readings with the variation in turbidity of the rumen liquid was linear. To verify this assumption the Spekker readings of various dilutions of the three samples of rumen liquid were measured by the method described above. All the samples of rumen liquid were obtained from the fistula animals, samples 1 and 2 being taken when the animals were stall-fed and sample 3 from an animal at grass. The results are given in Table 4 and Fig.1. There was a linear relationship between the degree of dilution of each sample of rumen liquid and the turbidity determined with the Spekker absorptiometer.

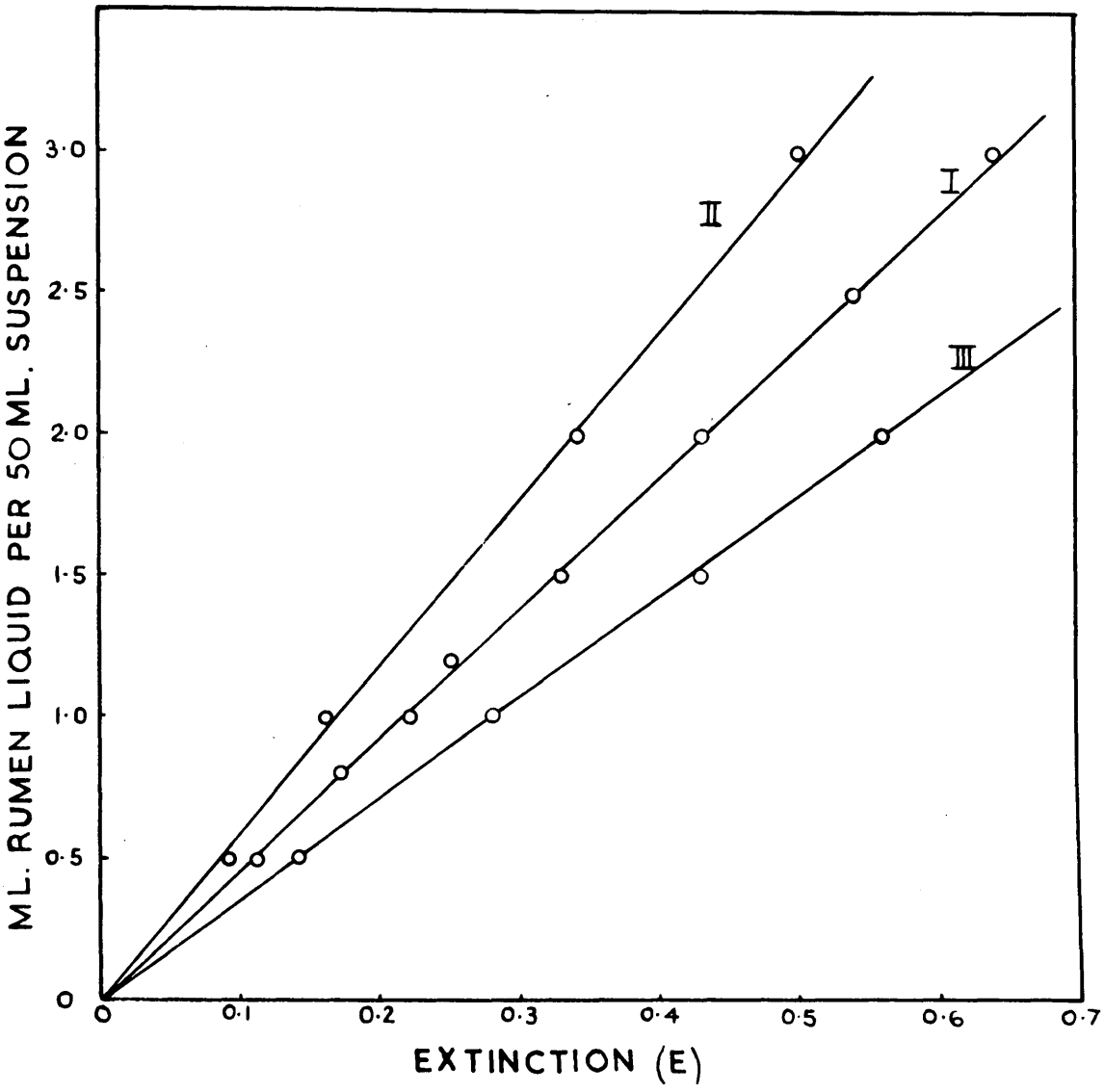

Fig.1.

The effect of dilution on the turbidity of three samples of rumen liquid. The turbidity was measured in a Spekker absorptiometer (p.23).

RESULTS.

1. The Variation Throughout the Year in the Numbers of Streptococcus Bovis in the Rumen Liquid of Two Cattle with Rumen Fistulae.

Samples of the rumen liquid from the two fistula animals were examined at intervals between 20/1/48 and 14/7/51. The observation period included periods when the animals were at grass and when they were stall-fed. The samples from the rumen were usually taken between 10 a.m. and 11 a.m., but occasionally they were taken up to 12 noon. The counts were made almost invariably on starch agar incubated in carbon dioxide and the numbers of Strep.bovis were estimated as described on p.14.

The variation in the number of Strep.bovis per ml. rumen liquid over the whole observation period is shown in Fig.2. Almost all the counts were within the logarithmic range of 5.5 to 7.0 for Ernest and 5.0 to 7.0 for Daphne. One sample from Ernest and three samples from Daphne for which the counts were recorded as less than 1×10^4 were not included in the calculation of the mean count. The geometric mean, maximum and minimum counts were as follows :-

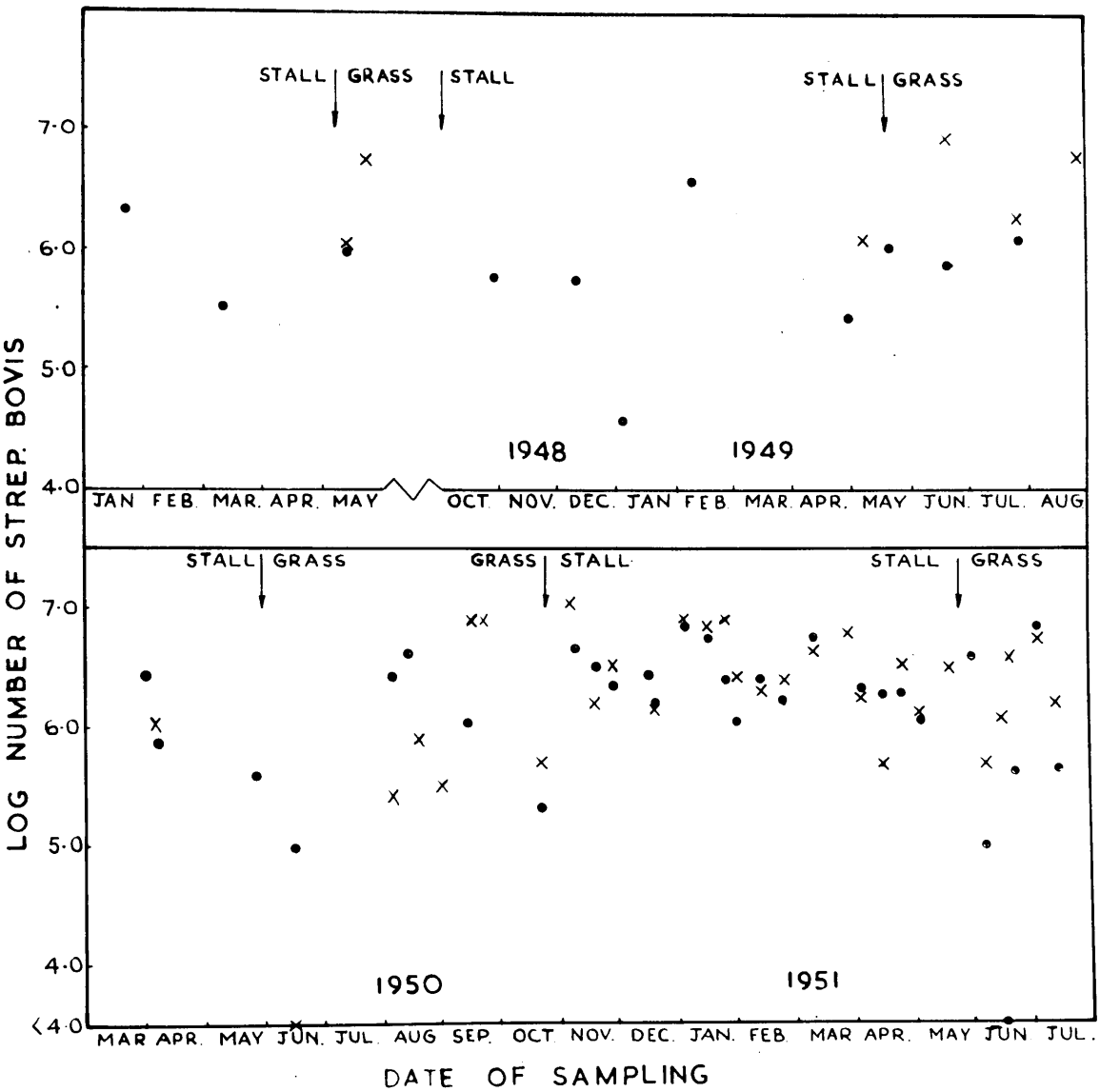

Fig.2.

The numbers of Streptococcus bovis per ml. of rumen liquid from fistula cattle (Jan., 1948 - July, 1951).

Daphne: ● Ernest: x

Animal	No.of samples	Strep.bovis/ml.rumen liquid		
		Geometric mean	Maximum	Minimum
Daphne	59	123×10^4	708×10^4	10×10^4
Ernest	55	219×10^4	1148×10^4	28×10^4

On each of 46 days two samples, one from each animal, were taken consecutively. Ernest had the higher Strep. bovis count on 32 days and Daphne on 14 days. The mean Strep.bovis count for the 46 samples from Ernest was 214×10^4 and from Daphne, 141×10^4, the mean for Daphne being only 68% of the mean for Ernest. Thus, there appeared to be a definite tendency for the numbers of Strep.bovis in the rumen liquid of Ernest to be higher than the numbers in Daphne.

(a) The effect of change of diet on the numbers of Strep.bovis in the rumen liquid. To indicate any seasonal fluctuations in the numbers of Strep.bovis in the rumen, and the effect, if any, of the change of diet from stall- to grass-feeding and vice versa, samples of rumen liquid from the fistula animals were examined at more frequent intervals between 4/8/50 and 14/7/51.

When the animals were stall-fed their normal diet was Ration 1 shown below. This was modified (Rations 2 and 3) during part of the observation period in connexion with experimental work in the Biochemistry Department. The rations, as lb.per day,

Table 5.

The geometric mean and standard error of the counts of Streptococcus bovis in the rumen liquid of fistula cattle on different normal diets.

Period		Daphne				Ernest			
No.	Duration (days)	Diet	No. of samples	No. of Strep. bovis ($\div 10^4$)/ml. G.M.*	S.E.*	Diet	No. of samples	No. of Strep. bovis ($\div 10^4$)/ml. G.M.*	S.E.*
1	83	Grass	12	87	88	Grass	14	145	71
2	50	Stall-fed Ration 1	11	174	38	Stall-fed Ration 1	11	235	101
3	69	" 2	7	275	87	" 3	7	389	118
4	72	" 3	8	178	57	" 2	9	209	64
5	50	Grass	8	63	100	Grass	7	214	70

* G.M. = Geometric Mean

S.E. = Standard error of the mean

were made up as follows :-

Ration	1	2	3
Straw	6	6	6
Hay	10	10	10
Oats	2½	1½	4½
Beans	1¾	4½	1½
Dried grass	1¾	-	-

The differences in soluble carbohydrates and starch equivalent between Rations 1, 2 and 3 were small and were not expected to have any appreciable effect on the microflora.

The log.counts of Strep.bovis per ml.of the rumen liquid sampled from 4/8/50 to 14/7/51 are shown in Fig.3. The observations were made in five consecutive periods. In Periods 1 and 5 both animals were out in the same field at grass, and in Periods 2, 3 and 4 the animals were housed and were fed the three rations whose compositions are indicated above. The geometric mean counts for each period are given in Table 5. In one sample of rumen liquid from Daphne during period 5 (grass) no colonies of Strep.bovis were detected on the plates from the 10^{-4} dilution of the rumen contents, but for the purpose of comparing the effect of the diet on the number of

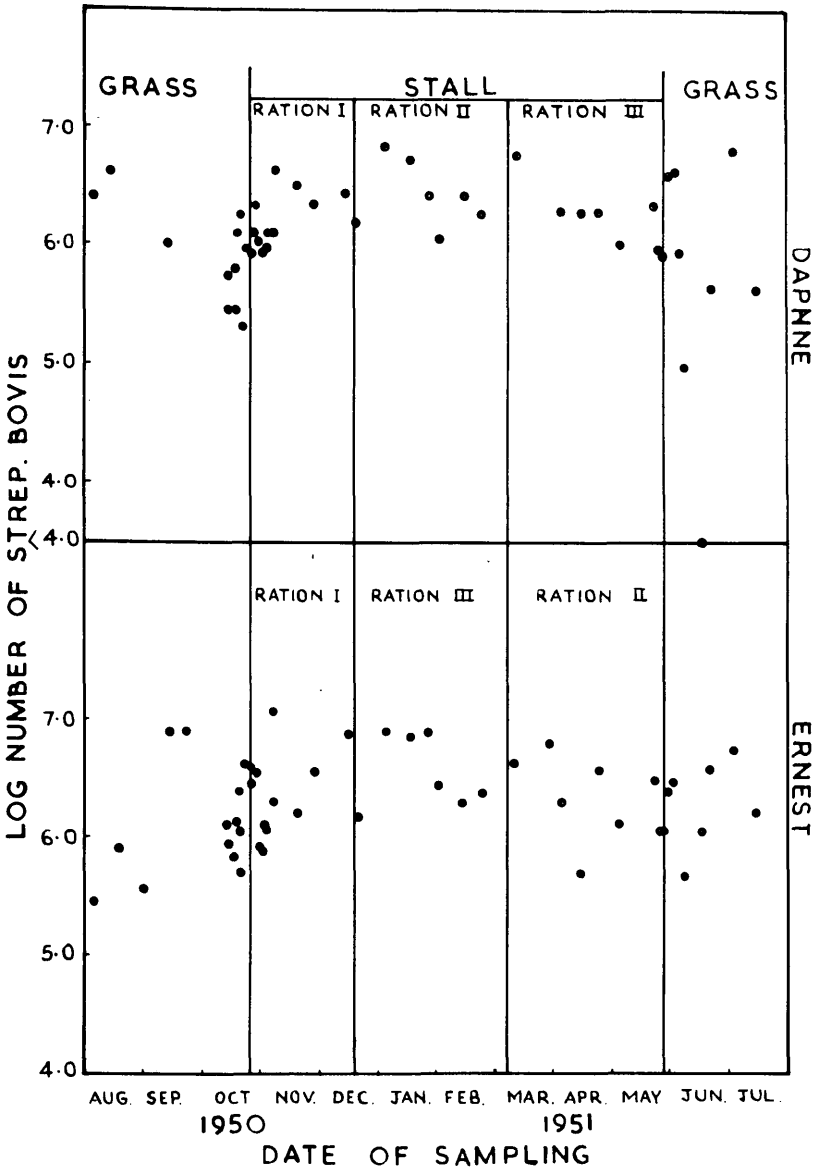

Fig.3.

The numbers of Streptococcus bovis per ml. of rumen liquid from fistula cattle on different diets.

Strep.bovis, the number in this sample was recorded as 1 x 10^4 although it may have been much lower. The numbers of Strep.bovis isolated from Daphne in Periods 1 and 5 tended to be lower than those obtained in the stall-fed rations. However, with the exception just mentioned all the counts fell within the normal range of 10^5 - 10^7 (shown in Fig.2) and this difference had probably no significance. The counts on samples from Ernest did not differ whether the animal was at grass or was stall-fed.

(b) The effect of the diet on the 'total' bacteria in the rumen liquid. During Periods 1 to 5 an estimate of the bacterial population as indicated by the turbidity of the centrifuged suspension of the bacteria (p.23) was obtained, and the results are shown in Fig.4. The variation in the turbidity of the bacterial suspensions at different times showed a surprisingly close correlation between the two animals. As with the Strep.bovis counts, there was no marked trend in the variation in the turbidity of the bacterial suspension during the periods of stall-feeding, but contrary to the Strep. bovis count there was a very definite and immediate rise in the turbidity when the animals went out to grass. This apparently higher level of bacterial content was maintained for at least 46 days.

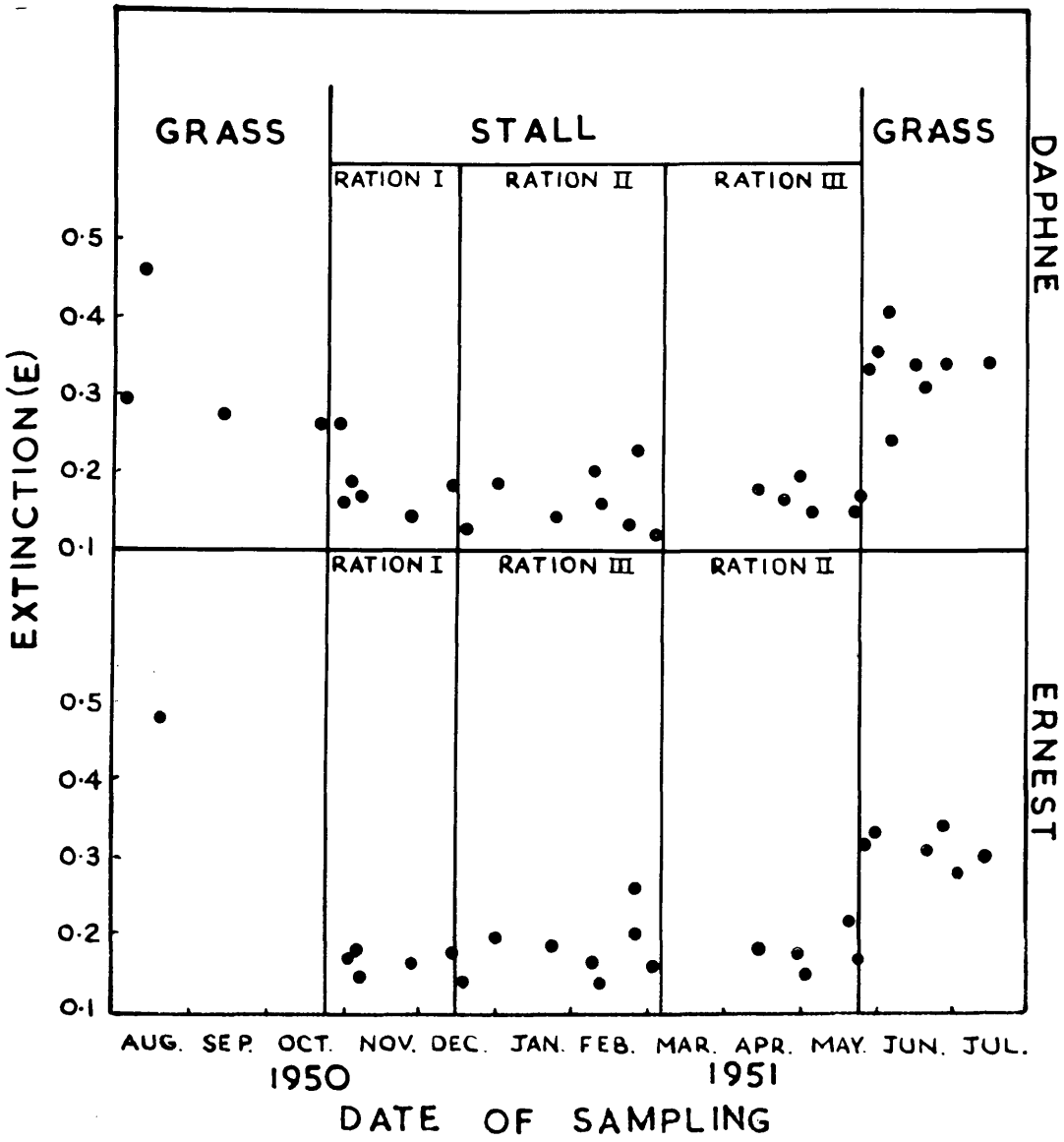

Fig.4.

The turbidity of suspensions of rumen bacteria from fistula cattle on different diets.

These results are in agreement with those obtained by Gall, Burroughs, Gerlaugh & Edgington (1949) who found that direct microscopic counts using nigrosin were higher in rumen contents on 'lush' pasture during late Spring and early Summer than when the animals were stall-fed. However, the possibility could not be overlooked that the differences in the turbidity of the suspensions obtained from the animals on stall and on grass feeds might be due to some non-bacterial factor from the grass. Particulate matter had been shown not to interfere but the possibility remained that colloidal matter had caused the difference. This was investigated in the following way.

100 g.of fresh grass and 15 g.of a typical sample of the stall-fed ration (pts.by wt.; hay 9, oats 2, beans 2, dried grass 2) were each minced up separately with 750 ml.of water. These relative quantities of fresh grass and the stall-fed ration were chosen because the dry matter of fresh grass was approximately only 15% that of the stall-fed ration. The material in the two minced samples was treated in the same way as the rumen contents for the determination of turbidity (p.23). After straining through butter muslin each sample was centrifuged at 2,000 r.p.m.for 5 min., the supernatant fractions were diluted 50 times and their turbidities determined in duplicate. The

results showed that the suspension prepared from grass had a higher turbidity (E = 0.128; 0.132) that the suspension prepared from the stall-feed (E = 0.100; 0.078). It was evident that the difference between the turbidity of the rumen contents of the animals when at grass and when stall-fed may not be attributable entirely to the bacterial fraction of the rumen contents.

Table 6.

The total viable count and the Streptococcus bovis count ($\div 10^4$) per ml. in the rumen liquid of stall-fed animals at different times of the day.

Time of sampling	Counts ($\div 10^4$)/ml.					
	Ernest 4.12.50		Ernest 26.2.51		Daphne 26.2.51	
	Total	Strep.bovis	Total	Strep.bovis	Total	Strep.bovis
8 a.m.	216	185	1270	1210	1500	300
Animals fed						
9 a.m.	457	457	1340	1275	1150	861
10 a.m.	404	404	1360	1360	1050	971
12 noon	230	230	1350	1350	340	295
2 p.m.	128	120	620	620	440	417
4 p.m.	164	164	570	570	230	217
6 p.m.	163	136	650	650	190	180
Animals fed						
7 p.m.	296	250	1310	1230	470	470
8 p.m.	310	310	1270	1270	630	630
10 p.m.	173	173	690	690	400	400

2. The Variation Throughout the Day in the Numbers of Streptococcus bovis and in the 'total' bacteria in the rumen liquid of the two fistula animals.

Samples of rumen contents from the two fistula animals, Daphne and Ernest, when they were stall-fed, were examined at hourly or two-hourly intervals throughout the period beginning immediately before the morning feed, which was given at 8 a.m., and ending 4 hr.after the evening feed, which was given at 6 p.m. When the animals were at grass the samples from the rumen were taken at two-hourly intervals from 8 a.m.to 8 p.m.

The number of Strep.bovis in each sample was determined as described on p.14. The turbidity measurements of the 'total' bacteria (p.23) were determined throughout the day only when the animals were stall-fed. Each sample of rumen liquid was examined microscopically in wet mount with Lugol's iodine for iodophilic bacteria and as heat-fixed smears after staining by Gram's method or with Lugol's iodine.

No difference was detected in the microscopical appearance of the different samples of the rumen liquid collected from the same animal on the same day. The numbers of Strep.bovis and the total viable counts on starch agar for the rumen liquid of stall-fed animals are given in Table 6. With one exception the total count

Table 7

The turbidity of the bacterial suspensions (as extinctions) of rumen liquid of stall-fed animals at different times of the day.

Time	Turbidity (E)		
	Ernest 4.12.50	Ernest 26.2.51	Daphne 26.2.51
8 a.m.	0.162	0.220	0.255
Animals fed.			
9 a.m.	0.210		0.290
10 a.m.	0.190	0.261	0.272
12 noon	0.172	0.245	0.245
2 p.m.	0.140	0.164	0.212
4 p.m.	0.160	0.190	0.181
6 p.m.	0.160	0.225	0.199
Animals fed.			
7 p.m.	0.207	0.300	0.225
8 p.m.		0.304	0.221
10 p.m.	0.160	0.279	0.230

Table 8.

The total viable count and the Streptococcus bovis count ($\div 10^4$) per ml. in the rumen liquid of Ernest when at grass at different times of the day.

Time	Counts ($\div 10^4$/ml.)			
	31.8.50		21.9.50	
	Total	Strep. bovis	Total	Strep. bovis
8 a.m.	430	27	1220	1220
10 a.m.	113	37	980	855
12 noon	49	25	710	710
2 p.m.	72	44	410	410
4 p.m.	49	45	740	690
6 p.m.	38	38	880	740
8 p.m.	64	35	780	730

was due mainly to Strep.bovis and will not be considered further. The definite increase in the numbers of Strep.bovis obtained immediately after feeding is shown more clearly in Fig.5. This increase persisted for at least 1 hr.after the morning meal. The counts then gradually fell. The counts increased again after the evening meal. (The high count recorded for Ernest on 26.2.51 at 8 a.m.suggests that on this occasion the animal had access to food before the sample was taken.) Changes in the 'total' bacteria as determined by turbidimetric measurements (Table 7 and Fig.6) showed trends similar to those observed in the Strep.bovis counts (Table 6 and Fig.5).

The numbers of Strep.bovis in the rumen liquid of one of the animals (Ernest) when at grass were determined at two-hourly intervals on two different days. No definite trend appeared to occur in either series of counts (Table 8 and Fig.7).

This result was to be expected since the animal would graze at frequent intervals and would thus maintain a more constant level of foodstuffs in the rumen than when it was stall-fed.

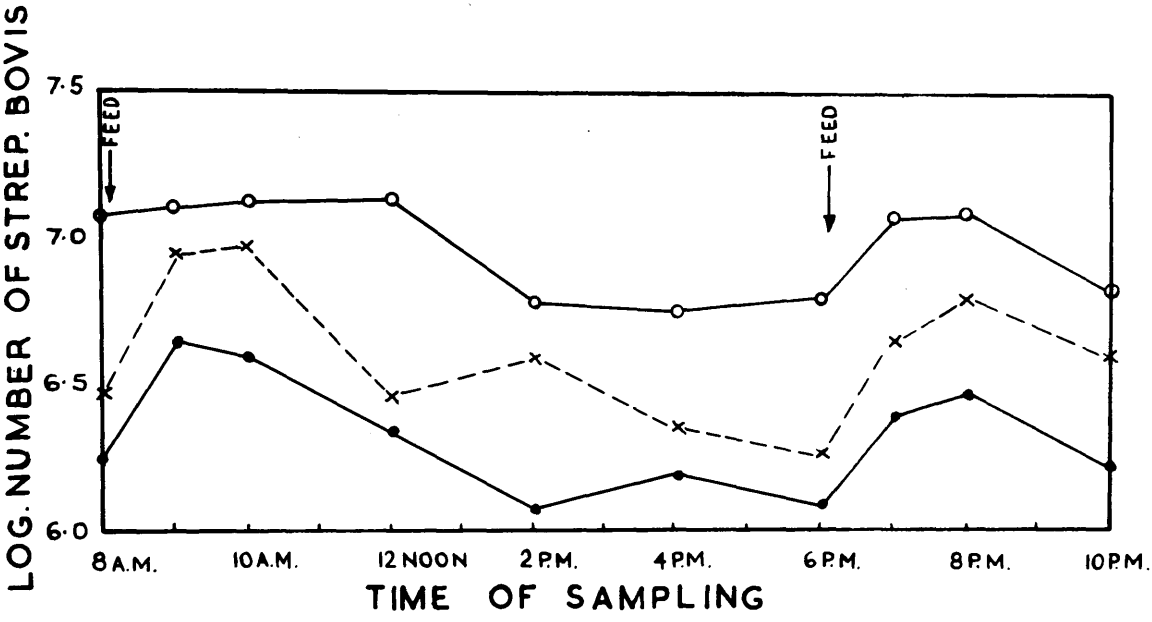

Fig.5.

The variation during the day in the numbers of Streptococcus bovis per ml. of rumen liquid from stall-fed fistula cattle.

Ernest (4.12.50): •——•

Ernest (26.2.51): o——o

Daphne (26.2.51): x---x

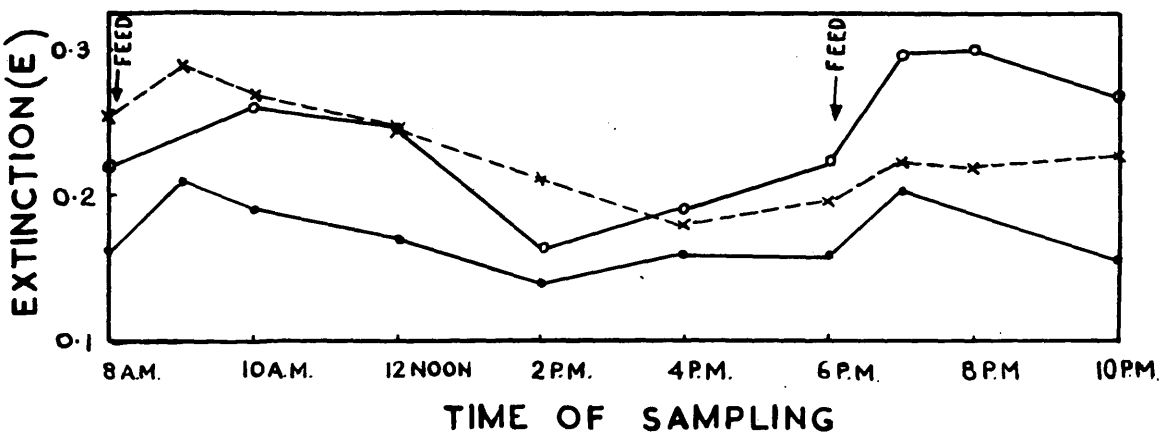

Fig.6.

The variation during the day in the turbidity of suspensions of rumen bacteria from stall-fed fistula cattle.

Ernest (4.12.50): •——• Daphne (26.2.51): x——x
Ernest (26.2.51): o——o

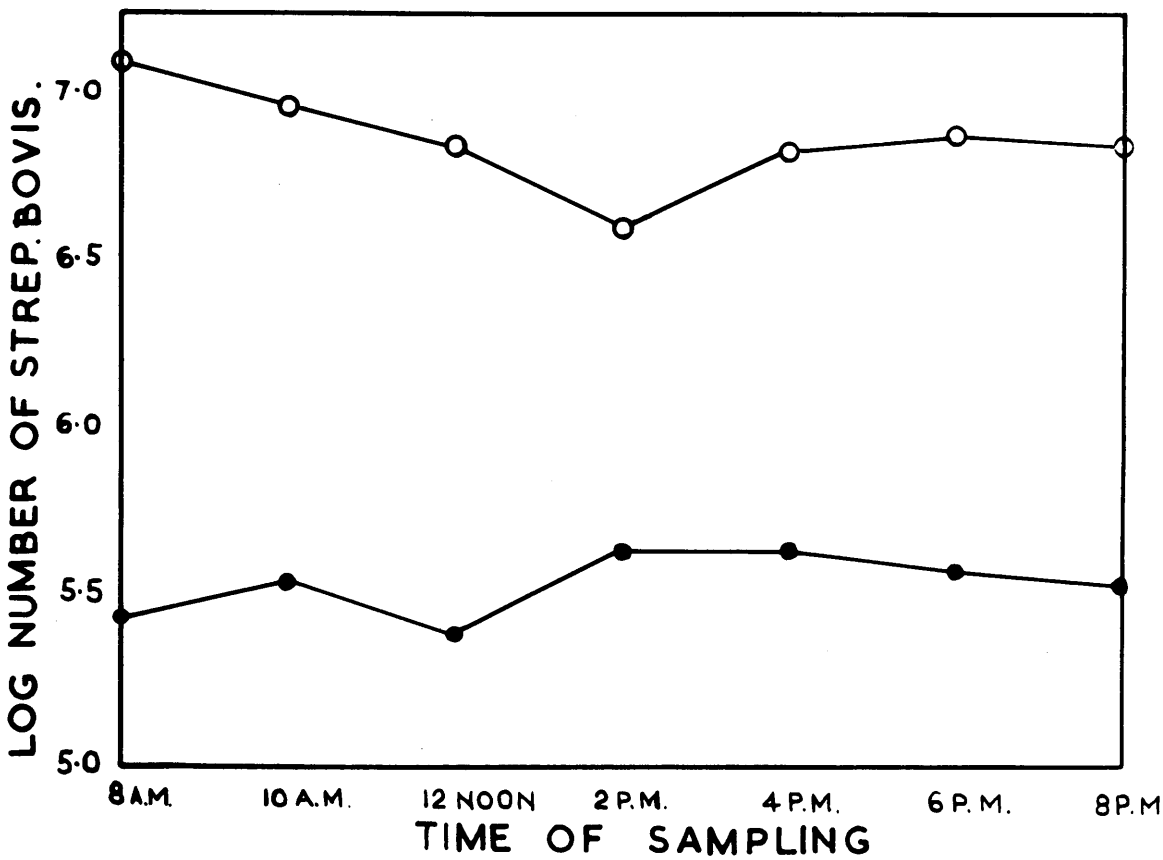

Fig.7.

The variation during the day in the numbers of Streptococcus bovis per ml. of rumen liquid from Ernest when at grass.

31.8.50: ●—● 21.9.50: o—o

Table 9

Examination of the feeds given to the fistula animals.

Expt.	Feed		Diluent (ml.)	Plate count per ml. suspension.	No. colonies examined	Types and no.of colonies of bacteria isolated
1	Concentrates Hay Straw	1.6 g. 26.5 g. 11.9 g.	90 90 90	100×10^3	10	*Streptococcus faecalis* (10)
2	Concentrates	1.0 g.	90	26×10^5	12	*Micrococcus luteus* (6) *Streptococcus faecalis* (4) Strep. sp. (not *bovis*) (1) Lactobacillus sp. (1)
	Hay	19.0 g.	90	43×10^2	19	Micrococcus sp. (19)
3	Dried grass	20.0 g.	100	40×10^2	11	Gram-negative rods (10) Spore-forming bacilli (1)
	Hay	20.0 g.	100	$<1 \times 10^2$	0	-

3. Streptococcus bovis in the feed.

The rise in the numbers of Strep.bovis in the rumen of stall-fed animals immediately after feeding could have been due either to the introduction of bacteria with the food into the rumen or to multiplication following the utilisation of the freshly ingested food by the bacteria or to both causes. As reported by Hastings (1944) considerable numbers of bacteria can be introduced along with the food. It seemed advisable therefore to examine representative samples of the food given to the fistula animals to find whether Strep.bovis was amongst the species ingested with the food. In Experiments 1 and 2 (Table 9) the feeds were well shaken with sterile ¼-strength Ringer's solution, the suspension in Experiment 1 being combined and mixed before plating. Plates were poured with starch agar and incubated in CO_2 at 37°C. In Experiment 3 the grass and hay were cut with sterile scissors into approximately 1 in. lengths, suspended in sterile distilled water, shaken vigorously, allowed to stand for 15 min.and again shaken. Dilutions of the suspensions were plated with glucose agar. A loopful of the undiluted suspensions was also streaked on glucose agar plates. The plates were incubated in CO_2 at 37°C.for 4 days. (Microscopical examination of these suspensions (Gram stain or Lugol's iodine) revealed no micro-organisms.)

The same species were isolated from both the streak and pour plates inoculated with the suspension from the dried grass. No growth in CO_2 was obtained on the streak plate inoculated with the undiluted suspension from the hay or in the pour plates which were prepared from the diluted suspension.

The results of the three experiments are presented in Table 9 where it can be seen that on no occasion was Strep. bovis isolated. This, of course, does not prove that Strep.bovis was entirely absent from the feed but only that if it was present its numbers were smaller than the numbers of the other bacteria that were isolated and which must, therefore, have been introduced into the rumen in larger numbers than Strep.bovis. The fact that these other bacteria were never isolated from the rumen while Strep.bovis was consistently isolated can be explained by assuming either that they all died off more rapidly than Strep.bovis in the rumen or that by multiplication in the rumen Strep.bovis out-numbered the bacterial species isolated from the feed.

The rapidity of the increase in the numbers of Strep. bovis within 1 hr.of feeding does not appear to invalidate the conclusion that this micro-organism was in fact multiplying in the rumen. Johnson, Hamilton, Robinson & Garey (1944) also noted a rise in the plate count of bacteria in the rumen of sheep during

the first hour after feeding and related this with maximum methane production which occurred at the same time. Maximum methane production within 1 hr.after feeding was also reported by Washburn & Brodie (1937), and Monroe & Perkins (1939) found that the pH of the rumen contents of stall-fed animals fell for 1 to 2 hr.after feeding and then rose again until the time of the next meal, but when the animals were at grass no definite trend in the pH of the rumen contents was found. The rapid utilisation of glucose in the rumen has been demonstrated by Elsden (1945-46) who showed that in one sheep 100 g.of glucose was completely utilised within 2 hr. and that within 1 hr. the amount of lactic acid present had risen from 0.5 mM.to a maximum of 2.8 mM.per 100 ml.of rumen liquor. In this connexion it is important to note that Strep.bovis produces lactic acid from glucose (White & Sherman, 1943).

Table 10.

The total number and predominating types of bacteria developing on starch agar in carbon dioxide at 37°C. from the faeces of two bovine animals and one horse.

Sample No.	Animal	Date	Feed	Total bact./g. moist faeces	Predominating types of bacteria
1.	Heifer (Daphne)	4.3.49	Stall	600×10^4	Strep.bovis, Bacillus (terminal spores).
2.	"	12.5.49	Stall	175×10^4	Strep.bovis, Gram-negative rods.
3.	"	24.10.50	Grass	92×10^3	Gram-negative rods, Strep.bovis, Tetracoccus.
4.	Steer (Ernest)	12.5.49	Stall	121×10^4	Strep.bovis.
5.	Horse	27.3.51	Stall	153×10^5	Strep.equinus, Strep.bovis.
6.	"	16.5.51	Grass	149×10^6	Strep.equinus, Strep.bovis.

4. Streptococcus bovis in Faeces.

A small number of samples of faeces from the fistula animals were examined to confirm the presence of Strep.bovis. The samples were collected in a Thermos flask either during or immediately after excretion. A weighed amount of the moist faeces was suspended in quarter-strength Ringer's solution and suitable dilutions of the suspension plated on starch agar and incubated in CO_2 at 37°. The total counts per g.and the predominating microflora on the countable plates are given in Table 10. It can be seen that in the four bovine samples Strep.bovis predominated under the conditions of isolation. As a matter of interest plates were also prepared from the faeces of a non-ruminant, the horse. In these plates the main microflora consisted of colonies of Streptococcus equinus. These colonies were generally rather small but groups of them grew much more vigorously. In the centre of each of these groups was usually a single larger lenticular colony, which proved to be Strep.bovis. By flooding the plate with iodine it was quite clear that each area of stimulation of the growth of Strep.equinus coincided with an area of starch hydrolysis. Microscopical examinations showed that in all the samples of faeces the microflora although still fairly diverse was more simplified than that in the rumen and

contained relatively few rods and predominantly Gram-negative cocci less than 1 μ in diameter. In cattle, some of these cocci were iodophilic, and occasionally large diplococci could be seen that strongly resembled those that occurred in the rumen. The microscopical observations clearly showed that the predominant bacteria in the faeces were not cultivated.

The isolation of Strep.bovis from the faeces of cattle confirms the findings of Orla-Jensen (1919) and others. The source of the organism in the faeces remains to be investigated. It is not known, for instance, whether after leaving the rumen Strep.bovis passes through the intestine without multiplication or whether multiplication occurs in some part of the intestinal tract posterior to the rumen such as the caecum. The isolation of Strep.bovis from the faeces of the horse suggests that multiplication can take place further along the alimentary tract since the horse has no rumen. Moreover, there is reason to believe that unless multiplication occurred further along the tract the number of Strep.bovis surviving after passage through the alimentary canal of either cattle or non-ruminants would be insignificant. Many factors contribute to the digestion of the micro-organisms from the rumen. A particular hazard facing Strep.bovis must be the pH of the abomasum which lies roughly between 2 and 4.1 (Schwarz & Kaplan quoted by Dukes, 1943). In

cattle the contents of the abomasum may remain there for almost 3 hours (Ewing & Wright, quoted by Dukes, 1943) and unless the food exerts a strong protective action against the acid present, a very high proportion of the Strep.bovis cells will be destroyed. (See Appendix to Part 1, p.46.)

5. The Incidence of Streptococcus Bovis in Freshly Slaughtered Cattle, Sheep and Goats, and in Calves.

The number of Strep.bovis has been shown to be usually between 10^5 to 10^7 per ml.of rumen liquid from two fistula cattle at the Hannah Institute. In order to obtain more general information concerning the incidence of this organism in cattle of varying breed, from widely separated areas of the country, and also from different types of ruminants, samples were obtained from the rumen of freshly slaughtered cattle, sheep, goats and calves.

(a) Sampling of rumen contents. The samples from the cattle and sheep were obtained from the Ayr Slaughter House. The rumen, with portions of the intestine, was removed from the animals within a few minutes of slaughter. Using a sterile knife the rumen was slit open and with a sterile long-handled dipper a large sample (ca. 1 litre) of the rumen contents was transferred to a vacuum flask or a flask insulated with sawdust, to prevent any undue lowering of the temperature of the sample before it was examined in the laboratory. Although the samples were frequently much drier than those obtained from the fistulated animals they contained sufficient moisture to give a liquid fraction after straining through muslin in the usual way. This fraction was obtained and examined immediately on arrival at the laboratory.

Table 11.

The examination of rumen contents from slaughtered cattle.

No.	Breed	Point of dispatch to Ayr	Macroscopical description of rumen contents	Strep.bovis count ($\div 10^4$)
1	Aberdeen Angus	Maude, Aberdeen	Liquid, brown, straw present	59
2			"	320
3			*Dry, green	330
4			"	300
5			"	380
6			"	560
7			"	290
8			"	520
9			Liquid, brown	52
10			"	33
11	Aberdeen Angus	Inch, Aberdeen	Dry, brown, frothing	147
12			" " "	84
13	Ayrshire	Ayrshire	Dry, brown	840
14			"	540
15			"	148
16			Liquid, brown	270
17	Galloway x Shorthorn	Newton Stewart Kirkcudbright	Dry, brown	169
18			"	470
19			"	235
20			Liquid, brown	330

* The word 'dry' in Tables 11 and 12 means that the rumen contents contained a very high proportion of solid matter with relatively little liquid.

Table 12.

The examination of rumen contents from slaughtered sheep.

No.	Point of dispatch to Ayr	Macroscopical description of rumen contents	Strep.bovis count ($\div 10^4$)
1	Newton Stewart	Liquid, greenish-yellow	3
2	Kirkcudbright	"	5
3	Dalbeattie	Liquid, brown	27
4	Kirkcudbright	*Dry, brown	42
5	Blairgowrie	Liquid, yellow	58
6	Perthshire	"	< 1
7		Dry, green	380
8		"	2
9	Ayrshire	"	14
10		Dry, greenish-yellow	155
11		"	300

* See footnote to Table 11.

Some of the calves used for this work were experimental animals at the West of Scotland Agricultural College, and, with the kind permission of Dr.George Dunlop, samples from these animals were obtained at the Ayr Slaughter House. The remainder of the calves were at the Hannah Institute and were made available through the collaboration of Dr.K.L.Blaxter. It was not permissible to slaughter these calves so samples were drawn from the rumen by means of a stomach tube and collected in a sterile jar.

With the collaboration of Dr.E.C.Owen rumen contents were taken from goats that had formed part of the Hannah Institute herd. These animals were anaesthetised with chloroform and bled to death. The samples were then obtained in the same manner as from the animals at the Ayr Slaughter House.

(b) Results.

Rumen contents from a total of 20 cattle, 11 sheep, 9 goats and 16 calves were examined. Details of the breed and the source of the cattle and sheep prior to their arrival at the slaughter house are given in Tables 11 and 12. The numbers of Strep.bovis in the cattle varied from 33 to 840 x 10^4 per ml. (log.count 5.52 to 6.92), figures which are of a similar order to those obtained from the fistulated animals. Microscopical examination of the rumen liquid of the cattle showed a diverse microflora in which no iodophilic micro-organisms were detected.

Table 13.

The numbers of Strep.bovis per ml.in the rumen liquid of slaughtered goats.

No.	Strep.bovis count ($\div 10^4$)
1	1288
2	1096
3	214
4	850
5	450
6	3
7	3
8	165
9	122

Table 14.

The numbers of Strep.bovis in the rumen contents of calves.

Group	No.	Age when sampled (days)	Diet	Strep.bovis per ml.($\div 10^4$)
A	1	49	Milk only	7,200
	2	83	" "	59
	3	32	Milk + roughage	154
	4	66	" "	95
B	5	49	Dry skim milk + vitamins A, D and E	2,930
	6	110	As 5 + margarine	96
	7	110	As 6 + sub-optimal straw	1,570
	8	100	As 6 + ample straw	145

Group A: 50 ml.strained rumen contents were given by mouth on the 4th., 15th., and 22nd. day.

Group B: 50 ml.strained rumen contents were given by mouth on the 25th.day except No.5 which received no inoculum.

A large number of 'rosettes' (Baker, 1942b) were seen in the sample from No.17 (Table 11) but otherwise no difference was detected between the microflora in the rumen of the different animals.

Strep.bovis was also present in the rumen contents of sheep, but the numbers per ml.of rumen liquid tended to be lower than in cattle. Microscopical observations showed that the microflora of sheep contained a diversity of morphological types. Some of the sheep (e.g. Nos.3 and 5) contained numbers of large oval and crescent-shaped organisms about 3 to 8 μ wide and 5 to 10 μ long, which were not seen in the rumen contents of the cattle. Large numbers of vibrios, 2 to 3 μ long, and iodophilic protozoa and small cocci (0.4 μ diam.) were seen in the rumen liquid from sheep Nos.7 and 18. In the rumen of goats Strep.bovis varied from 3×10^4 to 1288×10^4 per ml. (Table 13). Goats Nos.6 to 9 were young animals aged 3 to 4 months and the adults Nos.1 to 4 had been treated with thyroxine. Microscopical examination showed a varied microflora but no iodophiles.

The first group of calves to be examined were 6 weeks old at the time of slaughter. They had been fed on a skim milk diet and some had received by mouth rumen contents from adult cattle. The rumen liquid taken at the time of slaughter was plated as usual on starch agar and incubated in CO_2.

It was found that the micro-organisms in 104 of a total of 112 colonies examined were Strep.bovis.

The incidence of Strep.bovis was then examined in more detail in a second group of calves. These animals were taken, when only a few days old, to separate crates and because they were out of contact with adult animals, an inoculum of rumen contents from an adult animal was given to all but one of the calves (No.5) to compensate for the lack of opportunity to acquire the normal microflora of the adult rumen. The age and diet of the calves and the numbers of Strep.bovis in the samples from the rumen are given in Table 14. Strep.bovis was present in the rumen contents of all the calves including No.5 which received no inoculum of rumen contents. The numbers varied from calf to calf and did not appear to be related in any way to the diet. Further details of bacteria isolated from the rumen of these calves are given in Part 3. Microscopical examination of the rumen contents showed that cocci usually 0.6 - 0.8 μ predominated, but that there was frequently a diversity of types of both cocci and rods. Simplification of the microflora was clearly apparent in the contents from the calves which had not received roughage. The macro-iodophiles and protozoa that are characteristic of the rumen contents of adult animals were not detected in the contents from any of the calves, but in all the calves micro-iodophiles were found to be present. This observation appears to contradict

Baker's statement (1942a) that iodophilic micro-organisms occur in animals only after they have eaten vegetable matter.

Summary and Conclusions to Part 1.

1. Strep.bovis counts were determined in rumen contents from two fistula cattle, sampled throughout a period of more than three years. The numbers of Strep.bovis were almost invariably between 10^5 and 10^7 per ml.of strained rumen contents. No variation in the numbers could be attributed to change of season or to change of diet. When the animals were stall-fed the numbers of Strep.bovis increased significantly for approximately 2 hr.after feeding and then decreased until the time of the next feed. When the animals were at grass the Strep.bovis counts did not show any consistent trend during a similar period of the day.

2. Bacteriological examination of the feed of the fistula animals showed that Strep.bovis was not present in appreciable numbers in the feed and it was concluded that Strep.bovis was not a passenger introduced with the feed but maintained itself in the rumen by its ability to multiply there.

3. Rumen contents from cattle and sheep maintained in widely separated areas and from goats maintained at the Hannah Institute were also examined and there can be no doubt that in this country, at least, Strep.bovis is a normal inhabitant in all three types of ruminants. The counts in the cattle and the goats were generally

of the same order as those in the fistula animals but the counts in the sheep tended to be somewhat lower.

4. Examinations by cultural and microscopical methods of rumen contents from calves showed that Strep.bovis, unlike most types of rumen bacteria, can become established in the rumen without roughage having been included in the diet.

5. Strep.bovis was isolated from the faeces of cattle and horses, and a way in which it may arise there was briefly considered.

6. In an appendix to this section the loss of viability of Strep.bovis in glucose broth has been shown to be associated with the acidity of the medium.

Appendix to Part 1.

The Growth of Streptococcus Bovis in Glucose Broth.

Streptococcus bovis grows rapidly and gives a dense turbidity in glucose broth. Nevertheless subcultures to fresh medium frequently failed to grow even when the primary culture had been incubated at 37°C. for only 24 hr. The fact that the cultures in glucose broth had a final pH of 4.5 after 24 hr. suggested that the acidity developed might be the factor limiting the continued growth of the organism but, since the loss of viability was so rapid, the possibility that the inhibition of growth was due to other metabolic products could not be excluded. In order to assess the effect of acidity on the viability of Strep.bovis the following experiments were carried out.

1. A study was made of the total growth (turbidity), the number of viable organisms (plate count) and the pH of the medium during the growth of Strep.bovis in glucose broth in which the initial pH was adjusted to pH 6.8, 6.0, 5.0 and 4.0. Control cultures in broth containing no added glucose were also examined. For convenience standard broth (pH 6.8) was used for this purpose. This differed from the medium used for the glucose broth in containing meat extract ('Lemco') instead of yeast extract ('Yeastrel') (Table 1).

2. The viability of washed suspensions of Strep.bovis was determined in buffer solutions at different pH values.

3. The effect of adding varying proportions of the neutralised cell-free filtrate from a glucose broth culture of Strep.bovis on the growth of the organism in fresh glucose broth was determined.

Table 15.

The growth (log.plate count) of Strep.bovis in glucose broth initially at pH 4.0, 5.0, 6.0 and 6.8 and standard broth initially at pH 6.8, and the pH values during incubation in air at 37°C.

Medium	Incubation time (hr.)	log.plate count	pH of medium
Glucose broth	0	-	6.8
	3	4.78	6.7
	5	6.61	6.7
	7	9.78	5.5
	9	9.08	5.2
	11		4.8
	13	8.78	4.8
	16		4.5
	31	5.94	4.6
Glucose broth	0	-	6.0
	3	4.30	6.0
	5	5.72	6.0
	7	7.42	5.8
	9	8.66	5.2
	11	8.13	5.1
	13		4.8
	16	7.10	4.5
	31	4.07	4.5
Glucose broth	0	-	5.0
	3	2.72	5.0
	5	2.89	5.0
	7	2.85	5.0
	9	2.85	5.0
	11	3.27	5.0
	13	4.17	5.0
	16		5.2
	31	7.41	4.6
Glucose broth	0	-	4.0
	3 to 31		3.8 - 4.0
Standard broth	0	-	6.8
	4	5.53	6.8
	6	6.83	6.8
	8	7.57	6.8
	10	7.80	6.8
	14	7.91	6.8
	17	7.98	6.7
	31	7.71	6.7
	38	6.97	

1. The Effect of the pH of the Medium on the Growth of Strep.bovis.

Immediately before inoculation the pH of the glucose broth was adjusted asceptically using 0.1% (v/v) H_2SO_4 and the total volume of the medium in each tube was adjusted to 6.4 ml. as follows :-

pH of medium	Glucose broth (ml.)	0.1% H_2SO_4 (ml.)	Sterile water (ml.)
6.8	5.0	-	1.40
6.0	5.0	0.26	1.14
5.0	5.0	0.50	0.90
4.0	5.0	1.40	-

The inoculum was a loopful of a 15 hr.culture in glucose broth. The cultures were incubated in air at 37°C. At intervals 0.1 ml.was removed for the viable count and the pH determined by spot test using an external indicator. Tubes of standard broth pH 6.8 (i.e.containing no added carbohydrate) were inoculated and incubated in the same way as the glucose broth. The viable count and the pH of the media are given in Table 15 and Fig.8. The total growth in duplicate glucose broth cultures was determined turbidimetrically using the Spekker absorptiometer and the results are given (as extinctions) in Table 16 and Fig.9.

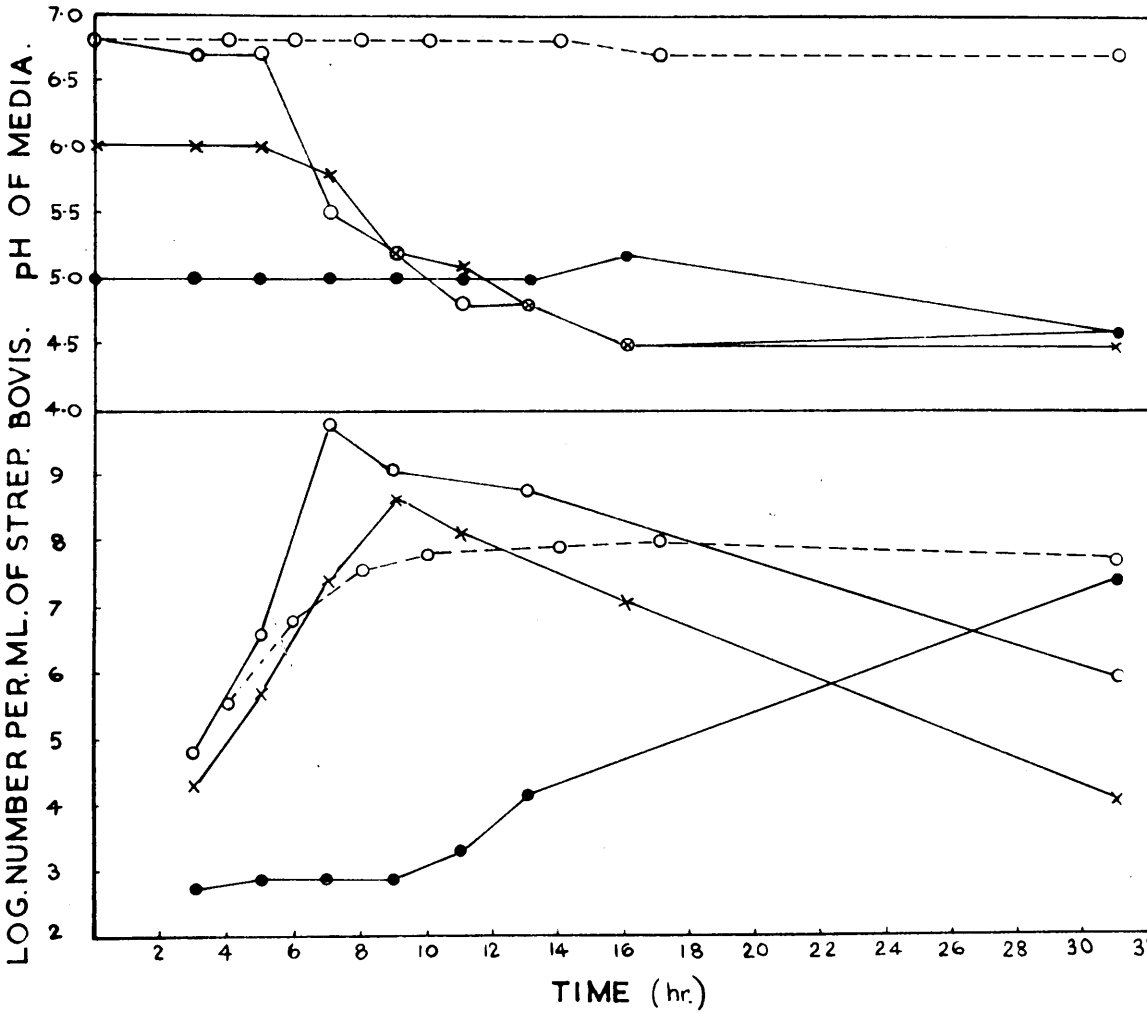

Fig.8.

The growth of Streptococcus bovis in glucose broth at different initial pH and the change of pH of the medium during incubation at 37°C..

Control: Standard broth (no added carbohydrate) pH 6.8.

Glucose broth: (initial pH 6.8) o—o
(initial pH 6.0) x—x
(initial pH 5.0) •—•

Standard broth: (initial pH 6.8) o---o

Table 16.

The total growth (turbidity) of Streptococcus bovis in glucose broth initially at pH 6.8, 6.0 and 5.0 during incubation at 37°C.

Incubation time (hr.)	Turbidity (E.) at pH		
	6.8	6.0	5.0
4	0.123	0.133	0.118
5	0.135	0.150	0.140
6	0.203	0.170	0.155
7	0.350	0.249	0.178
8	0.445	0.401	0.201
17	0.488	0.485	0.362
24	0.518	0.500	0.340

No growth occurred in glucose broth adjusted to pH 4.0. In glucose broth at pH 6.8 and 6.0 the total growth increased rapidly from about 5 to 8 hr. and continued to increase very gradually. These changes were accompanied by a rapid increase and a subsequent decrease in the numbers of viable bacteria and with a decrease in the pH of the medium. The numbers of viable cocci in standard broth increased at a slightly slower rate and to a lesser extent than in glucose broth. The total growth (turbidimetric measurement) was not determined in standard broth. The numbers of viable organisms in standard broth unlike those in the glucose broth remained at about the same level from the 16th to the 31st hour and during the whole period the pH of the medium fell by only 0.1.

The pH of the cultures initially at 5.0 remained unchanged for 16 hr. Growth was much slower and the total quantity of growth less than that in the cultures at pH 6.0 and 6.8. The results of the turbidity measurements and the viable counts in the cultures at pH 5.0 suggest a slight anomaly, the lag phase as determined by the turbidity measurements being rather less than the lag phase as determined by the viable counts. This difference was probably due to slight variations in the cultures used for the two determinations.

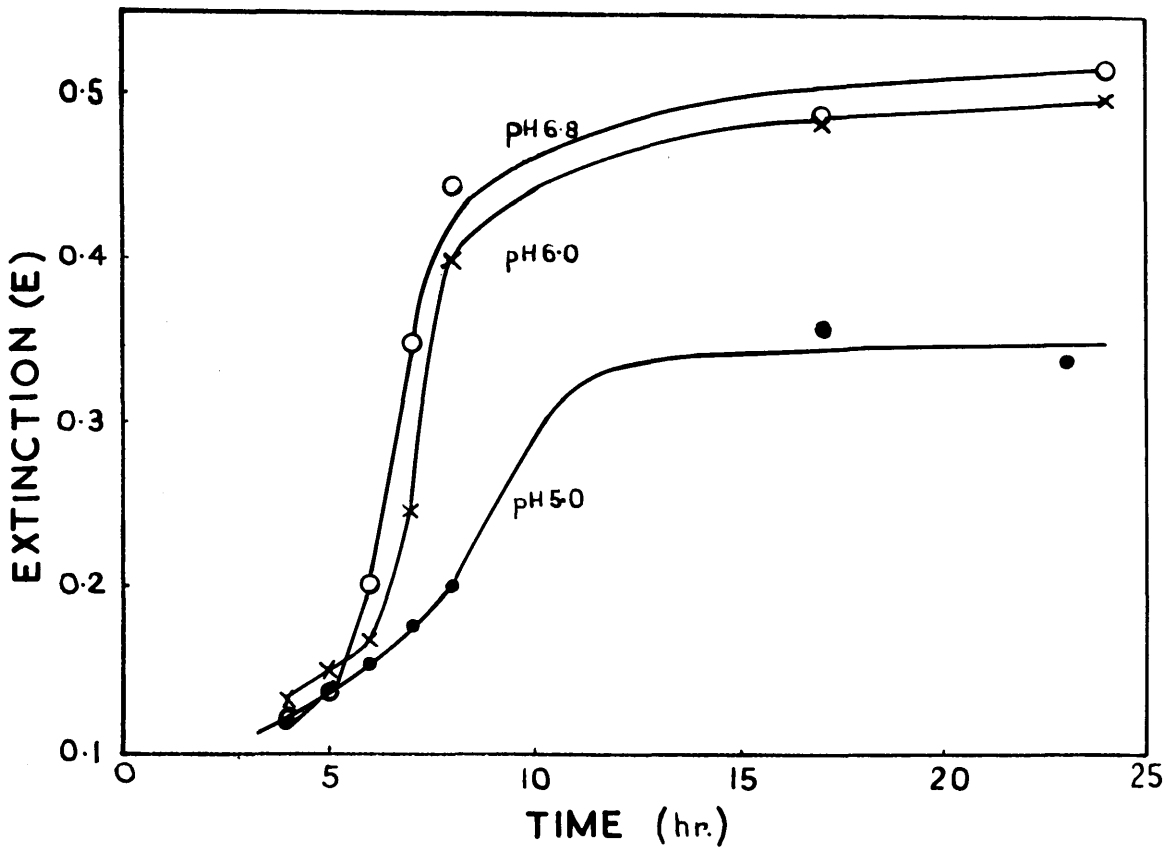

Fig.9.

The effect of initial pH on the growth of Streptococcus bovis in glucose broth. Growth determined by turbidimetry.

Table 17.

The numbers of viable Streptococcus bovis cells (log plate count) per ml. of suspension of Na_2HPO_4-citric acid buffer solutions during incubation at 37°C. in two experiments A and B.

pH of buffer solution	Incubation Time (hr.)	Log. plate count	
		Expt. A	Expt. B
4.0	0	-	6.38
	1	-	6.25
	2	<5.0	3.62
	3	<3.0	-
	4	<2.0	
4.5	2	5.95	-
	3	5.20	-
	4	3.00	-
5.0	0	-	6.59
	2	6.40	6.39
	3	6.72	6.57
	4	6.29	6.35
	5	6.01	6.38
	7½	-	6.22
	10	-	5.92
	21	< 1.00	-
	24	-	0.00
5.5	0	-	6.86
	2	-	6.78
	3	-	6.72
	4	-	6.61
	5	-	6.63
	7½	-	6.36
	10	-	>6.00
	24	-	3.79
6.0	0	-	6.69
	2	7.11	5.98
	3	6.81	5.94
	4	6.67	-
	5	-	5.88
	7½	-	5.62
	10	-	7.31
	21	< 3.0	-
	24	-	3.02
7.0	0	-	6.80
	2	7.08	-
	3	7.04	-
	4	6.98	6.79
	5	7.06	-
	7½	-	6.59
	10	-	6.52
	24	-	3.19
	28	<4.0	-

2. The viability of washed cells of Streptococcus bovis in buffer solutions.

The washed suspensions of Strep.bovis were prepared as follows :- 200 ml.glucose broth (pH 6.8) in a 500 ml. conical flask was inoculated with 0.2 ml.of a 9 hr.glucose broth culture and incubated in air at 37°C.for 14 hr. 10 ml. aliquots of the culture were transferred asceptically to each of 5 sterile centrifuge tubes closed with rubber bungs. The cells were twice spun down and resuspended in $\frac{1}{4}$-strength Ringer's solution. The cells were spun down for a third time and finally resuspended in McIlvaine's 0.2M-Na_2HPO_4 - 0.1-M citric acid buffer solutions at pH 4.0, 4.5, 5.0, 6.0 and 7.0 (Vogel, 1946). The suspensions were incubated at 37°C., and at intervals 1 ml. quantities were withdrawn and plated to obtain the number of viable bacteria. The results (Experiment A) are given in Table 17 and Fig.10. The experiment was repeated (Experiment B) using suspensions of Strep.bovis in buffer solutions of pH 4.0, 5.0, 5.5, 6.0, 7.0 (Table 17 and Fig.10).

The irregularity of the counts at pH 6.0 in Expt.B was almost certainly due to an experimental error and was not shown in Expt.A. The results of experiments A and B show clearly that the loss of viability of the non-proloferating cells of Strep. bovis increased with the decreasing pH of the suspending medium. The effect became pronounced below pH 5.0.

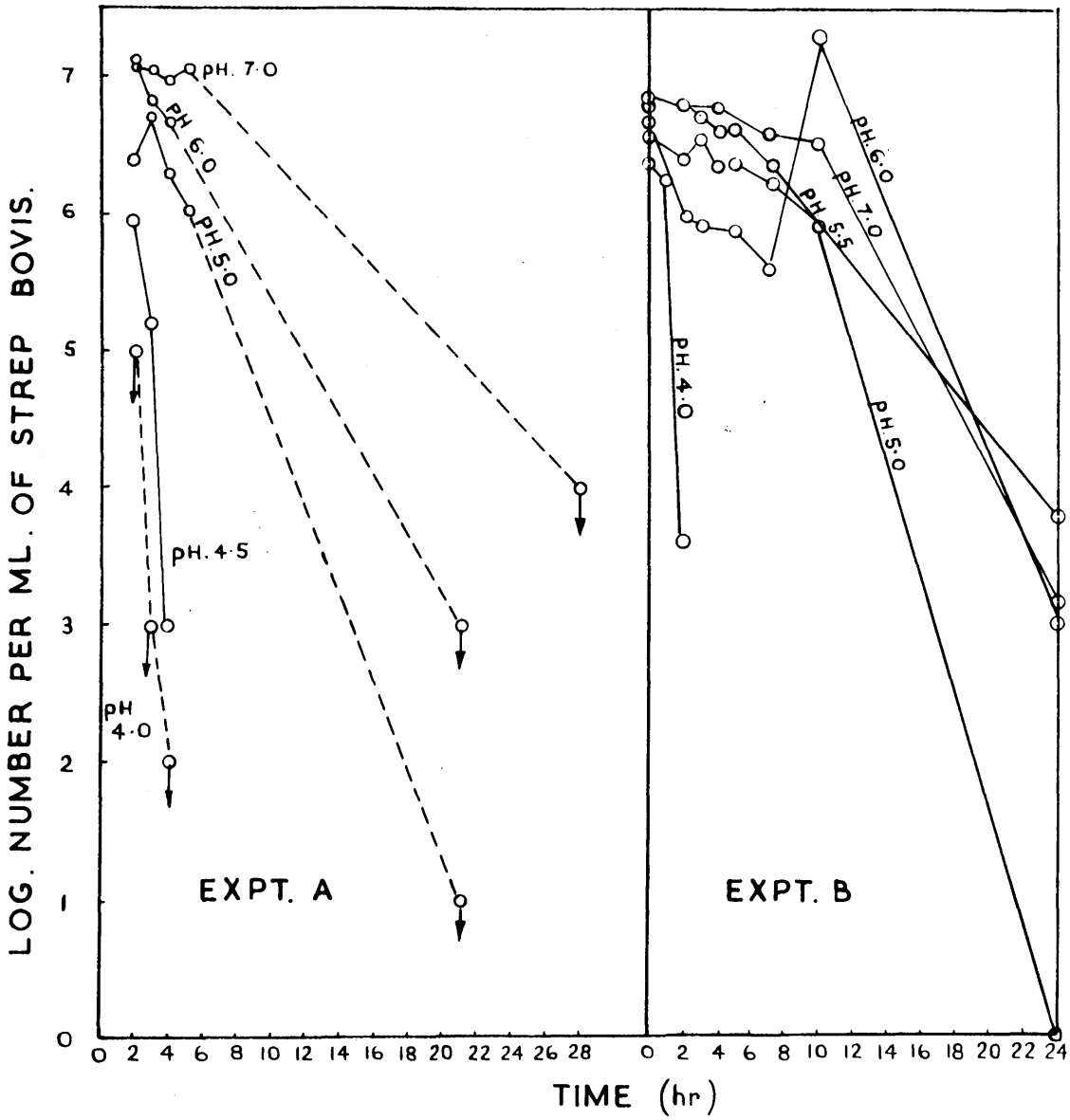

Fig.10.

The viability at 37°C. of washed cells of *Streptococcus bovis* suspended in Na_2HPO_4-citric acid buffer solutions.

↓: no growth on plates prepared from dilution 10^{-1} (pH 5.0), 10^{-3} (pH 6.0), and 10^{-4} (pH 7.0).

Table 18.

The total growth (turbidity) of Strep.bovis in glucose broth and in glucose broth containing varying proportions of the neutralised cell-free filtrate from a glucose broth culture of the same organism, after incubation at 37°C.for 24 hr.

Medium		Turbidity of the culture (E)
Glucose broth (ml.)	Filtrate (ml.)	
0	5	0.621
1	4	0.600
2	3	0.621
3	2	0.635
4	1	0.615
5	0	0.732
5	0	0.705
5	1	0.705
5	2	0.632
5	3	0.693
5	4	0.638
5	5	0.629

3. The Effect of a Cell-free Filtrate on the Growth of Streptococcus bovis.

A sterile cell-free filtrate of a glucose broth culture of Strep.bovis was prepared as follows :-

The cells from a 24 hr.culture in 200 ml.glucose broth were removed by centrifuging and the supernatant was filtered through a Ford's sterilising pad. The filtrate was neutralised asceptically with NaOH and tested for sterility by incubation for 48 hr.at 37°C. The effect of this sterile cell-free filtrate on the growth of Strep.bovis was determined turbidimetrically after inoculation of (a) glucose broth, (b) neutralised filtrate, (c) mixtures in varying proportions of glucose broth and the filtrate followed by incubation at 37°C.for 24 hr. The details of the media and the turbidity measurements (as extinctions) are given in Table 18. The results suggest that the filtrate supported rather less growth than the fresh glucose broth. The difference was slight and certainly not sufficient to account for the loss of viability in the highly acid cultures.

CONCLUSION.

The loss of viability of Strep.bovis in glucose broth cultures appears to be due to the sensitivity of the organism to a pH below 5.0 and not to any appreciable extent to the presence of metabolic products in the medium other than those causing the acidity. At pH values between 5.0 and 7.0 the loss of viability is relatively slow but below 5.0 it becomes extremely rapid.

The pH of the rumen contents of cattle and sheep is usually between pH 6.0 and 7.0 (Smith, 1941) and although it may fall to below 6.0 after feeding (Monroe & Perkins, 1939) a pH below 5.0 has only rarely been recorded (Gall, 1946). There seems, therefore, no reason to suppose that the growth of Strep.bovis in the rumen would be severely checked by the pH of the rumen contents in animals on a normal diet.

Part 2

Investigations Bearing on the Utilisation of Carbohydrate by Streptococcus Bovis in the Rumen.

Many types of soluble carbohydrates added to ordinary peptone-lemco broth are decomposed readily by Strep.bovis. For example, growth becomes rapid on the addition of glucose, maltose or soluble starch, but in the absence of a readily available carbohydrate growth is much slower. It would seem probable therefore that the increase in the numbers of Strep. bovis which occurred in rumen contents in stall-fed animals after feeding resulted from the presence in the feed of a readily utilisable source of carbohydrate. Some studies have now been made of the effect on the numbers of Strep.bovis of adding carbohydrate to rumen liquid in vitro. Such in vitro experiments have the following advantages over experiments in vivo.

(1) Comparative experiments can be made using portions of the same sample of rumen contents.

(2) The sampling error is smaller.

(3) Substances may be added to the rumen contents which might be harmful to the animal if added directly to the rumen.

(4) The conditions can be much more closely controlled.

The important disadvantage of the in vitro method is that changes

which occur in the microflora are not necessarily the same as those which occur in vivo. However, when rumen contents were incubated without any additions no gross differences were detected in the microscopical appearance of the microflora during the first 4 hr. although after incubation for 24 hr. the changes in the microflora were considerable.

The breakdown of maltose and maize starch grains during in vitro incubation of rumen liquid.

1. Methods.

The method of *in vitro* incubation was essentially the same as that used by Pearson & Smith (1943a). Rumen contents from the stall-fed fistula animals were removed between 1 to 2 hr. after the animals had been fed. The sample was strained and centrifuged at 2,000 r.p.m. for 5 min. To reduce aeration this liquid fraction of the rumen contents was filled into 500 ml. flasks up to the neck and the flasks were then covered with a watch glass. The flasks were incubated in a water bath at 39° and the contents mixed by gently swirling them every half-hour during the first 4 hr. Additions to the rumen liquid consisted of 5 g. of either maltose or maize starch grains to the 500 ml. flasks. Control flasks of the rumen liquid were incubated without any addition. Samples were taken after incubation for 0, 2, 4, 6 or 24 hr. for microscopical examination, *Strep. bovis* (plate) count and turbidity measurement.

For microscopical observation heat-fixed smears were stained by Gram's method, with aniline blue (Baker & Nasr, 1947) or with iodine. Wet mounts were also stained with iodine. In all samples many types of micro-organisms were observed so that only predominant types, those noticeably suppressed or favoured

during incubation, are described.

The total number of micro-organisms and the proportion of Strep.bovis viable on starch agar and the turbidity measurements were determined as described in Part 1.

2. Results.

(a) Microscopical examination. The microflora in the rumen liquid after incubation for 4 hr.without added carbohydrate appeared to be unchanged. In the presence of maltose an increase occurred in the numbers of Gram-positive cocci. These were 0.6 to 0.8 μ diam. and were grouped mostly in pairs or short chains. Their increase was readily apparent as the rumen bacteria are mostly Gram-negative. The number of iodophilic bacteria also increased during incubation with maltose. The iodophiles were usually cocci varying in diameter from 0.4 to 1.4 μ and occurring as pairs, short chains, tetrads or clumps. In some incubations, however, iodophilic vibrios, 2.0 to 3.0 μ long, predominated. The iodophilic microflora always formed only a small proportion of the total number of micro-organisms.

Similar but much slower changes in the microflora were observed in the rumen liquid incubated in the presence of starch grains. After incubation for 4 hr.Gram-negative

Table 19.

The Total Viable and Streptococcus bovis counts per ml. of rumen contents incubated in vitro, with and without addition of carbohydrate.

Sample No.	Carbohydrate added	Total count (÷ 10^5) after incubation for 0, 2, 4, 6 and 24 hr.					Streptococcus bovis count (÷ 10^5) after incubation for 0, 2, 4, 6 and 24 hr.				
		0	2	4	6	24	0	2	4	6	24
1	None	21.5		13.5							
	Maltose	21.5		>1000							
2	None	19.3		15.5							
	Maltose	19.3		>1000							
3	Maize starch grains	26.0		36.0							
4	Maize starch grains	26.0		44.0							
5	None	13.5			15.6	2020	13.5			15.6	$<1x10^6$
	Maltose	13.5			7200		13.5			7200	
	Maize starch grains	13.5			20.5	1140	13.5			20.5	1140
6	Maltose	15.0			6500		15.0			6500	
	Maize starch grains	15.0			17.9	1610	15.0			17.9	1610
7	Maize starch grains	1.1		3.2		134	0.3		3.2		134
8	Maize starch grains	7.9		2.9		155	5.9		2.9		155
9	None	66.0			75.0					75.9	
	Maltose	66.0	600	4700	8400		66.0	600	4700	8400	

bacteria still predominated, but Gram-positive bacteria, mostly cocci 0.4 to 0.8 μ in diameter occurring in pairs and short chains and also Gram-negative vibrios 2.0 - 3.0 μ were beginning to accumulate around the starch grains. The few iodophilic bacteria present at this time were also mainly associated with the starch grains. After incubation for 24 hr. the starch grains were obscured by Gram-positive bacteria which included a high proportion of cocci 0.4 μ - 1.0 μ in diameter. It was apparent, however, that large numbers of Gram-negative cocci and vibrios were also associated with the grains. These were not so outstanding, because they stained less intensely than the Gram-positive bacteria.

(b) Plate counts and turbidity measurements. The plate counts during the incubation of rumen liquid in the presence of maltose or maize starch grains or without added carbohydrate are given in Table 19. In the absence of added carbohydrate the total plate count, which consisted entirely of Strep.bovis did not show any appreciable change in 4 or 6 hr. But in the presence of maltose a rapid increase in the numbers of the organism occurred in 2 hr. and continued up to at least 6 hr. No observations were made after longer periods of incubation with maltose. With starch grains, on the other hand, the total plate count (again due entirely to Strep.bovis) showed only a

Table 20.

The turbidity (Spekker readings) of rumen liquid incubated in vitro with 1% maltose.

Sample No.	Turbidity (E) after incubation period (hr.)			
	0	2	4	6
1	0.20	0.29	0.35	0.35
2	0.22		0.385	
5	0.181		0.32	
9	0.17		0.33	

small increase in 6 hr. but increased appreciably in 24 hr., although it was still much lower than that in the presence of maltose after incubation for only 4 hr. Only one sample incubated in the absence of added carbohydrate was examined after 24 hr. (No.5, Table 19). This sample showed a marked increase in the total plate count after 24 hr., but none of the bacteria that were isolated was Strep.bovis. Examination of the plates prepared from lower dilutions other than these used for the counting plate showed that starch hydrolysis was no greater on these plates when prepared after 24 hr. incubation than on the plates prepared initially from the same dilutions. This showed that no appreciable increase in the numbers of Strep.bovis had occurred in this sample.

An increase in the total numbers of bacteria during in vitro incubation of rumen liquid with maltose was shown by the turbidity of the bacterial suspensions (Table 20). Microscopical observation showed that despite the large increase in Strep.bovis Gram-positive cocci were always outnumbered by Gram-negative bacteria. Such growth must consist of bacteria incapable of growth on starch agar plates under the experimental conditions used.

3. Conclusions.

These observations on the effect of maltose and intact starch grains upon the growth of Strep.bovis in rumen

liquid *in vitro* throw some light upon results reported in Part 1. It will be recalled that the numbers of *Strep.bovis* in the rumen liquid of the two fistula animals when stall-fed increased for 1 - 2 hr.after they had been fed. The samples of rumen contents for the *in vitro* experiments were collected 2 - 3 hr.after the animals had fed, i.e.when the increase in *Strep.bovis* had stopped. It is not surprising, therefore, that when these samples were incubated *in vitro*, without any addition, the numbers of *Strep.bovis* did not increase, but that on the addition of maltose a sharp increase resulted, and it was clear that utilisable carbohydrate could be considered to be a factor influencing the amount of growth of *Strep.bovis*. The growth of *Strep.bovis* during the incubation of rumen liquid containing intact starch grains suggested that the organism was able to decompose intact grains but the slowness of the growth almost ruled out any possibility that intact grains had participated significantly in the increase of *Strep.bovis* in the rumen shortly after feeding. It must also be borne in mind that in the rumen starch grains would be embedded in plant cells and would consequently be even less readily available than the starch grains added *in vitro*.

It is reasonable, therefore, to attribute the rise in the numbers of *Strep.bovis* in the rumen immediately after feeding

to the presence of soluble carbohydrate in the feed and to suppose that the rate of growth of Strep.bovis decreased when this carbohydrate had been used. Carbohydrates such as glucose and sucrose which can be utilised by Strep.bovis are known to occur in appreciable quantities in grass (Laidlaw & Reid, 1952). After the readily available carbohydrate of the feed has been used the slower decomposition of more complex carbohydrates such as starch grains or cellulose by the rumen microflora will probably be sufficient in itself to maintain the numbers of Strep.bovis in the rumen at a fairly constant level.

The Decomposition of Intact Starch Grains by Streptococcus bovis.

Growth of Strep.bovis has been shown to occur in *in vitro* incubations of strained rumen liquid containing intact maize starch grains, whereas no increase in the numbers of this species occurred in rumen liquid to which no carbohydrate had been added. Since Strep.bovis decomposes soluble starch it seemed likely that it was able also to decompose the intact starch grains even if the rate of decomposition was considerably less with the grains. Microscopical observations had shown that even before growth of Strep.bovis had been detected by plating methods, the starch grains were surrounded by cocci morphologically indistinguishable from Strep.bovis.

The following experiments were, therefore, undertaken to determine to what extent Strep.bovis in pure culture was able to decompose intact starch grains.

1. Experimental.

The dry intact starch grains in 1 oz.McCartney screw-cap bottles were sterilized in the autoclave for 15 min.at 15 lb. pressure. A sample from each batch of grains was examined microscopically after sterilization. The grains appeared to be intact and to have retained their birefringence when examined in polarized light.

20 ml. of sterile broth (0.5% peptone, 0.3% 'Yeastrel', pH 7.4 before sterilization) was added to each bottle containing the sterile grains and inoculated from a broth culture of a stock strain of Strep.bovis and incubated at 37°C. The undecomposed starch was recovered by transferring the culture quantitatively to a weighed Grade 4 sintered glass filter. The grains on the filter were washed thoroughly with water, dried with alcohol and ether and then held over $CaCl_2$ in a desiccator before they were weighed.

In preliminary experiments the cultures rapidly became acid (pH 4.5) and only a very small amount of starch was decomposed. This was probably due to the fact that Strep.bovis is so extremely susceptible to acid (p.52), that the pH of 4.5 would cause the cultures to become sterile. Certainly it was found by the following experiment that the enzyme system involved in the hydrolysis of soluble starch by Strep.bovis was most active between pH 6.0 and 6.5.

> The diastatic activity of a cell-free filtrate of Strep.bovis was determined using soluble starch in buffer solutions of different pH values. Standard broth (30 ml.) containing 0.5% soluble starch was inoculated from a 12 hr. broth culture of Strep.bovis and incubated at 37°C. for 12 hr. A cell-free filtrate was obtained using a Seitz filter. The filtrate was diluted to ten times its volume with sterile distilled water. 1 ml. of the diluted filtrate was added to 3 ml. of McIlvaine's 0.2M-Na_2HPO_4 - 0.1M - citric acid buffer solution (Vogel, 1946). 1 ml. of 0.1% soluble starch

Table 21.

The residual starch (as Spekker readings) in cell-free filtrates of Strep.bovis (strains 80 and 103) incubated at different pH with 1 ml. of 0.1% soluble starch for 3 hr. at 37°C.

pH	Spekker readings	
	Strain 80	Strain 103
5.0	0,20	0.18
5.6	0.18	0.16
6.0	0.19	0.15
6.2	0.16	-
6.4	0.16	0.15
6.6	-	0.17
6.8	0.19	0.18
7.0	0.21	0.25
7.6	0.25	0.28
Control (pH 6.0)	0.34	0.32

was added and the tubes were incubated for 3 hr. at 37°. Buffer solutions at pH 5.0, 5.6, 6.0, 6.2, 6.4, 6.6, 6.8, 7.0 and 7.6 were used.

The starch remaining after incubation was determined colorimetrically by the method of Bourne, Haworth, Macey & Peat (1948). A control tube prepared with buffer at pH 6.0 was boiled to destroy the diastase and then incubated for 3 hr.

The diastatic activities of the filtrates from two strains of Strep.bovis (80 and 103) are given as Spekker readings in Table 21 and Fig.11, and show that the optimum pH for the diastatic activity of the filtrates was between 6.0 and 6.5 and that above pH 6.5 the activity rapidly declined.

In view of these results obtained in the preliminary experiments an attempt was made to control the pH of the cultures by adding phosphate buffer solutions to the medium. A known weight (approximately 1 g.) of the maize starch grains was sterilised in 1 oz.McCartney bottles, and this was followed by the addition under aseptic conditions of 10 ml.double strength broth and 10 ml.M/2 phosphate buffer. The medium was inoculated with 0.2 ml.of a 15 hr.culture of Strep.bovis in unbuffered broth. Replicate cultures were buffered initially at pH 5.3, 5.9, 6.2, 6.6, 7.0, 7.8 and 8.0 and uninoculated controls at pH 5.3, 7.0 and 8.0. The bottles were incubated at 37° and shaken gently each day to redistribute the starch. At intervals up to 8 days, cultures were removed from the incubator and the residual starch determined. Microscopic

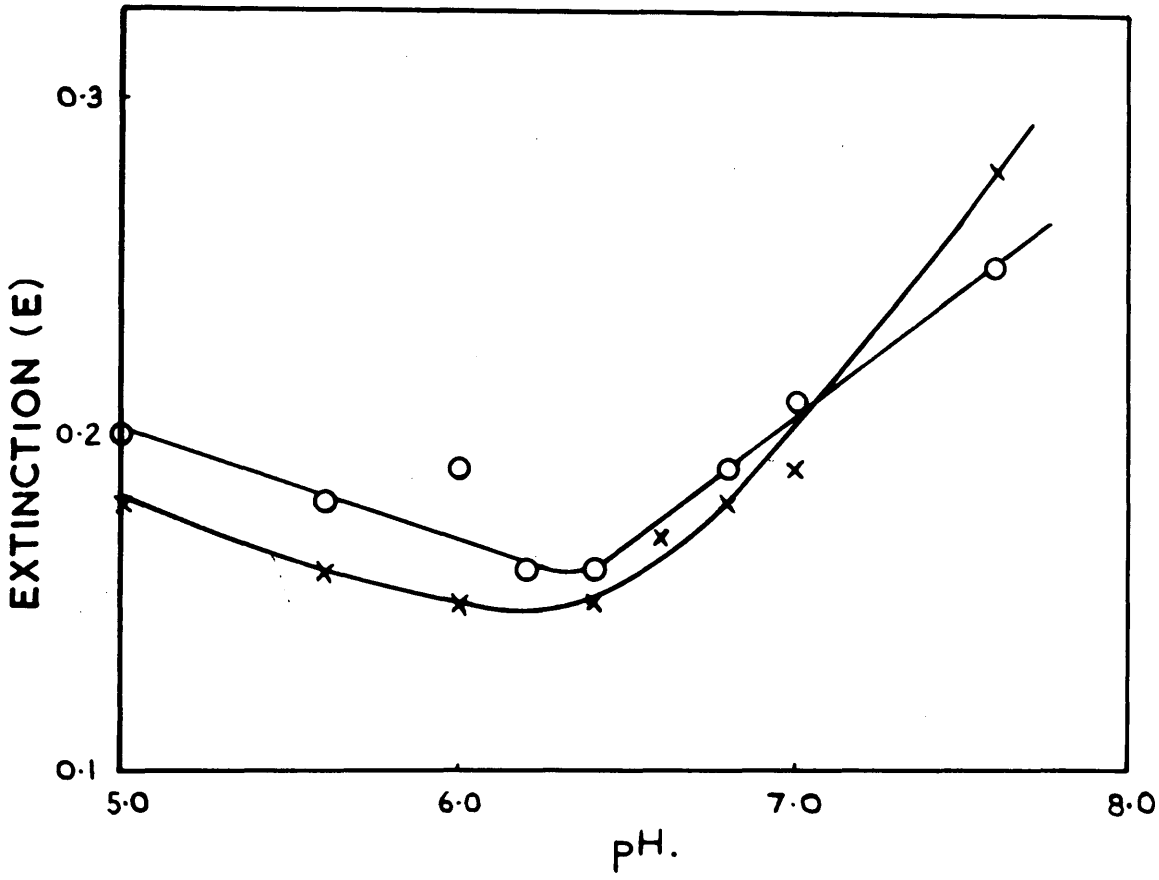

Fig.11.

The effect of pH on the hydrolysis of soluble starch by cell-free filtrates of Streptococcus bovis.

Residual starch was determined by the colorimetric method of Bourne, Haworth, Macey and Peat (1948) after incubation at 37°C. for 3 hr..

Strain 80: -o-o- Strain 103: -x-x-

Table 22.

The decomposition of maize starch grains in buffered cultures of Streptococcus bovis.

Initial pH of culture	Incubation period (days)	Wt. of starch (g.)		% loss in wt. of starch
		Initial	Final	
5.3	0 (control)*	0.99	0.90	9.1
	1	1.00	0.95	9.5
	2	0.99	0.95	4.0
	3	1.02	0.93	8.8
	5	1.03	0.96	6.8
	7	1.01	0.90	7.9
	7 (control)	0.99	0.90	9.1
5.9	1	1.00	0.90	8.0
	2	1.03	0.91	11.7
	3	1.01	0.90	10.9
	5	1.01	0.88	12.9
	7	1.07	0.91	15.0
6.2	1	1.00	0.98	2.0
	2	1.00	0.86	14.0
	3	0.98	0.83	15.3
	7	1.00	0.76	24.0
6.6	1	0.99	0.90	9.1
	2	0.99	0.82	17.2
	3	0.97	0.82	15.5
	5	0.95	0.71	25.3
	7	0.97	0.67	30.9
7.0	0 (control)	0.99	0.92	7.1
	2	0.95	0.81	14.7
	3	0.95	0.76	20.0
	5	0.97	0.67	30.9
	7	0.98	0.47	52.0
	7 (control)	0.99	0.90	9.1
7.4	1	0.95	0.89	6.3
	2	0.97	0.90	7.2
	3	0.97	0.91	6.2
	5	0.98	0.56	42.9
	7	0.99	0.45	54.5
8.0	0 (control)	0.98	0.90	8.2
	5	0.96	0.93	3.1
	8	0.97	0.91	6.2
	8 (control)	0.99	0.91	8.1

* uninoculated control.

examination was also made.

2. Results.

The loss in weight of maize starch in cultures at the different initial pH values is shown in Table 22 and Fig.12.

The loss in weight in the uninoculated controls at pH 5.3, 7.0 and 8.0 would be partly accounted for by the difference in the moisture content of the starch when the initial and final weights were determined. The final weight of starch was determined after drying with alcohol and ether and over calcium chloride while the starch was not dried before the initial weighing. A separate determination of the moisture content of the maize starch grains was obtained later by heating for 3 hr. at 100°C. The loss in weight due to moisture was then 13.7% of the initial weight of the starch and any loss in weight of less than 13.7% must be assumed to be due to moisture.

No growth and consequently no decomposition of starch occurred at pH 8.0. No starch decomposition was observed during incubation for 7 days in broth of initial pH 5.3. In the cultures at the other initial pH values starch was decomposed after varying periods of incubation and after 7 days the quantity of starch decomposed decreased with decreasing initial pH of the cultures. The amount of buffer was in no culture sufficient to keep the pH constant during the whole of the incubation period.

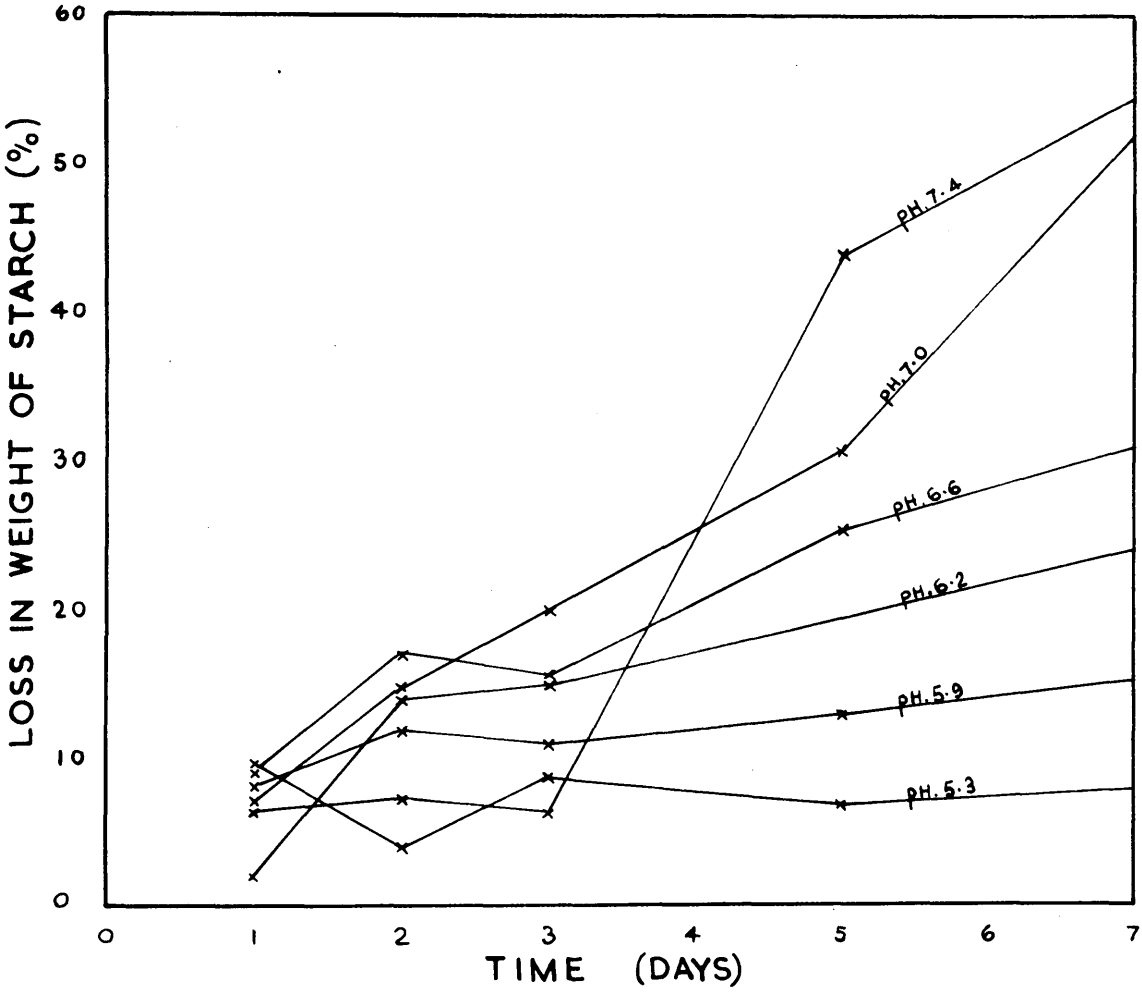

Fig.12.

The effect of the initial pH in buffered broth cultures of Streptococcus bovis on the decomposition of maize starch grains.

Attempts to control the pH with more concentrated buffer caused marked inhibition of the growth of Strep.bovis and even with the concentration of buffer which was used (final conc.M/4) cultures occasionally failed to grow. However, during the first 3 days the fall in pH was slow and during this time there was little decomposition of starch below pH 6.0 and above 7.0. This might be expected, for although the effect of pH upon the production of diastase was not investigated, the activity of the diastase itself has been shown to be greatest at pH 6.0 to 6.5 (p.62). Moreover, the growth of Strep.bovis while extremely rapid between pH 6.0 and 7.0 is much slower at lower pH values (p.52).

The decomposition of the starch could continue only until the pH of the medium had been reduced to the level inhibitory to the action of the diastase so that the higher the initial pH the greater the amount of acid required (and hence the greater the amount of starch decomposed) to bring the pH to the inhibitory level. Thus, the greatest amount of starch was decomposed in the broth in which the initial pH was 7.4. The least was shown in broth in which the initial pH was 5.3.

The decomposition of starch grains from other sources (rice, potato and wheat) was confirmed in similar cultures

Table 23.

The decomposition of starch grains from different sources in cultures of Strep.bovis buffered initially at pH 7.0.

Source of starch	Incubation period (days)	Wt. of starch (g.)		% loss in wt. of starch
		Initial	Final	
Maize	0 (control)*	0.97	0.97	0.0
	2	0.96	0.84	12.5
	4	0.96	0.65	32.3
	6	0.96	0.62	35.4
	6 (control)	0.95	0.95	0.0
Potato	0 (control)	0.94	0.91	3.2
	2	0.92	0.85	7.6
	4	0.93	0.67	28.0
	6	0.92	0.55	40.2
	6 (control)	0.93	0.92	1.1
Rice	0 (control)	0.94	0.93	1.1
	2	0.93	0.77	17.2
	4	0.90	0.68	24.4
	6	0.90	0.59	34.4
	6 (control)	0.90	0.88	2.2
Wheat	0 (control)	0.93	0.92	1.1
	2	0.92	0.86	6.5
	4	0.91	0.63	30.8
	6	0.91	0.57	37.4
	6 (control)	0.92	0.90	2.2

* uninoculated control.

buffered to an initial pH of 7.0 (Table 23 and Fig.13). There was little difference in the amount of each starch decomposed after 6 days although after 2 days the results suggested that the decomposition of potato and wheat starch was slower than that of rice or maize. The moisture content of the starch grains estimated separately was for maize, potato, rice and wheat respectively 13.7, 17.6, 13.0 and 12.4% of the initial weight of the starch.

Microscopical observations showed that the onset of decomposition of the starch grains was preceded by the massing of the cocci round the grains. The first indication of decomposition was the appearance of pitting on the surface of the grains. During decomposition the intensity of the stain with iodine gradually became weaker and birefringence was lost. Finally the grains usually became hollow, the last part to disappear being the outermost and apparently most resistant layer. Evidence to show that starch grains are not homogeneous but have tough layers which are more resistant to attack by bacteria than other parts of the grains is indicated by the work of Baker, Nasr, Morrice & Bruce (1950) and of Baker & Whelan (1950).

An iodophilic reaction was observed in some of the Streptococci particularly in cultures initially at pH 6.2

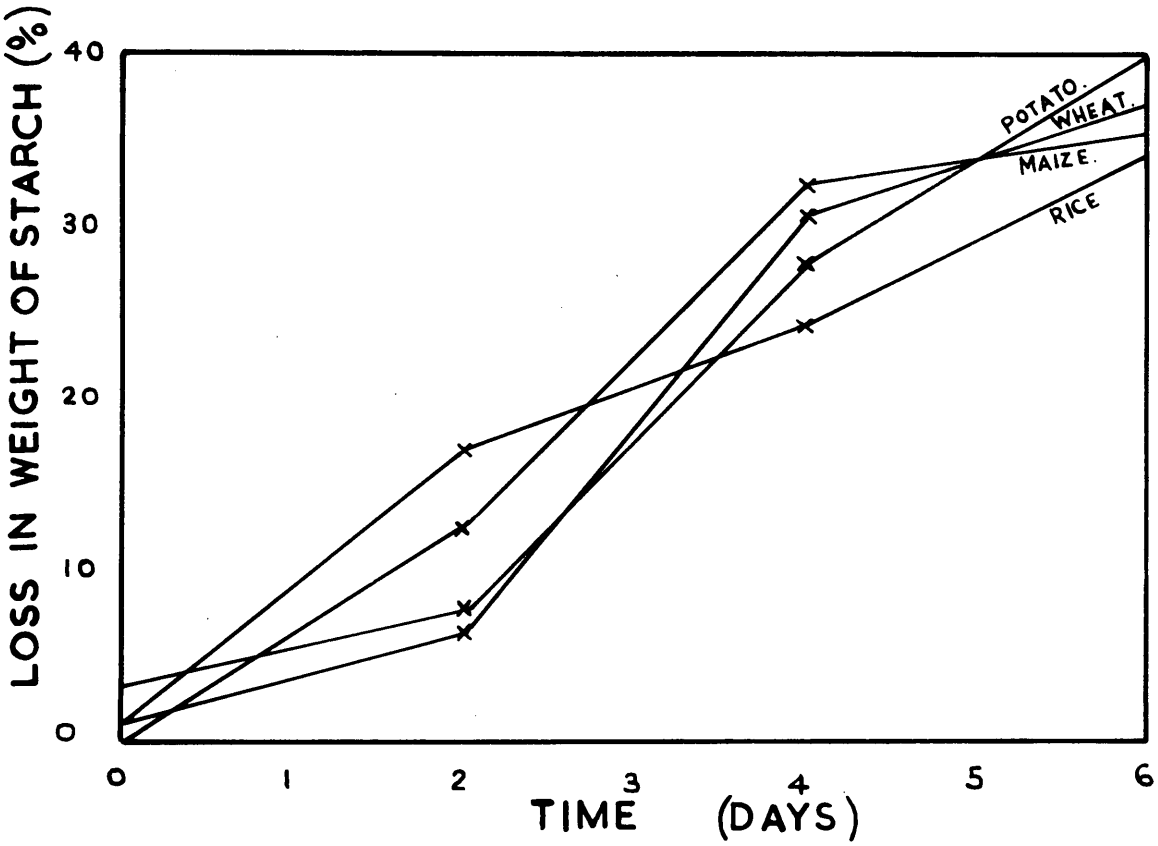

Fig.13.

The decomposition of maize, potato, rice and wheat starch grains in buffered broth (initial pH 7.0) cultures of Streptococcus bovis.

after incubation for 2 days and also after a longer period of incubation in cultures initially at pH 6.6. The absence of iodophilic cocci in the other cultures suggests that the pH range within which iodophilic polysaccharide is stored by Strep.bovis is somewhat narrow.

The Synthesis of Polysaccharide by Streptococcus bovis.

The production of starch-like polysaccharide is one of the striking features of the micro-organisms in the rumen, (Henneberg, 1922; Baker, 1943). Recently the synthesis of iodophilic polysaccharide has been demonstrated in cultures and in non-proliferating suspensions of a number of strains of widely differing species of bacteria including Corynebacterium diphtheriae (Hehre, Carlson & Neil, 1947), Neisseria (Hehre & Hamilton, 1948), Acetobacter (Tosic & Walker, 1950), Clostridia (Baker & Nasr, 1949) and Escherichia coli (Monod & Torriani, 1950). The conditions for the production and probably the nature of the iodophilic polysaccharide varied with the different species. Hehre, Carlson & Neil (1947) found that two of nine dextran-producing streptococci were able to synthesise starch-like polysaccharide from glucose-1-phosphate but not from glucose or sucrose.

Van der Wath (1948) claimed to have isolated an iodophilic streptococcus from the rumen of sheep. This streptococcus which appears from Van der Wath's description to be Strep.bovis was isolated from maize starch kernels which were observed to be surrounded by large numbers of iodophilic cocci and by smaller numbers of non-iodophilic bacteria. Van der Wath seemed to assume that the streptococcus isolated and the iodophilic cocci observed on the starch grains were identical and did not demonstrate the in vitro production of iodophilic material in pure culture.

In the present study iodophilic cells of Strep. bovis were observed (p.66) in cultures containing maize starch grains in broth buffered to an initial pH of 6.2 and 6.6. Some studies of the ability of Strep.bovis to synthesise starch-like polysaccharide from different carbohydrates in liquid and on solid media are reported here.

Broth containing 0.3% 'Lemco', 0.5% peptone, 1% carbohydrate (starch, dextrin or glucose) and M/4 phosphate buffer at pH 6.6 was inoculated with 0.2 ml.of a 15 hr. culture of Strep.bovis in standard broth. The cultures, in McCartney bottles, were incubated at 37°C.and one culture was removed on each of ten days for examination for the presence of iodophilic polysaccharide. The culture was

centrifuged and the bacterial sediment and the supernatant liquid examined in dry, unheated smears after staining with Lugol's iodine. No iodophilic polysaccharide was found in the bacteria in any of the cultures of three different strains of Strep.bovis.

To investigate polysaccharide synthesis on solid media plates were prepared from a medium containing 0.3% Lemco, 0.5% peptone, 1.5% agar at pH 7.0 with 1% of the test carbohydrate. These plates were streaked with a broth culture of Strep.bovis, sealed to prevent drying of the medium and examined daily during aerobic incubation at 37°C. The nine strains tested all gave an iodophile reaction when grown in the presence of starch. Three of these strains were also grown on agar containing dextrin, maltose, glucose, sucrose or sodium lactate and on starch and glucose together, but an iodophilic reaction was observed only on dextrin and, with 2 of the strains, on maltose. The strain which failed to show any colour with iodine when grown on maltose gave only a weak reaction when grown with starch or dextrin. The intensity of the blue colour varied with the strain of Strep.bovis. The iodophilic polysaccharide was not usually detected before the fifth day of incubation. When most intense,

the growth of iodine-flooded plates was deep blue and microscopic examination showed large numbers of purple to dark blue cocci. Starch when present in the medium was completely hydrolysed in the area around the growth and was not directly responsible for the blue colour of the cocci. In the presence of glucose and starch together the production of iodophilic material was inhibited and the zones of starch hydrolysis in the medium were smaller than in the absence of glucose.

These results which show that bacterial polysaccharide was formed in media containing starch, dextrin, or maltose but, under the conditions of the experiment, not from glucose, or starch in the presence of glucose, bear a resemblance to results of work by Monod & Torriani (1950) who found that strains of Escherichia coli were able to synthesise iodophilic polysaccharide from maltose according to the following equation

$$n\ C_{12}H_{22}O_{11} \xrightarrow[+\ \text{water}]{\text{amylomaltase}} (C_6H_{10}O_5)_n + n\ C_6H_{12}O_6$$

(maltose) (poly-saccharide) (glucose)

Doudoroff, Hassid, Putman, Potter & Lederberg (1949) found that the reaction was reversed in the presence of glucose, the polysaccharide being gradually broken down and maltose being formed. It would be of interest to determine whether the synthetic processes involved in the formation of iodophilic polysaccharide by Strep. bovis are similar to those in E.coli.

Summary and Conclusions to Part 2.

(1) Samples of rumen contents were collected from the two fistula animals, when they were stall-fed, approximately 2 hr. after the morning feed, i.e. when the increase in the numbers of Strep. bovis (Part 1) had stopped. These samples after straining through muslin were incubated *in vitro* for 4 to 24 hr. with and without the addition of carbohydrate. In the absence of any addition, the numbers of Strep. bovis remained constant; when 1% maltose was added the numbers increased rapidly and when maize starch grains (1%) were added a much slower increase of Strep. bovis occurred. From these results it was concluded that in the samples of rumen liquid the numbers of Strep. bovis were limited mainly by the amount of soluble carbohydrate available and that starch grains could not have caused the increase in the numbers of Strep. bovis in the rumen which occurred shortly after the animals were fed.

(2) The increase in the numbers of Strep. bovis in

in vitro incubations containing starch grains and, also, the observation that cocci resembling *Strep.bovis* formed a high proportion of the total bacteria associated with the decomposing grains prompted an investigation of the action of pure cultures of *Strep.bovis* upon intact starch grains. Starch grains from maize, potato, rice and wheat were found to be decomposed. The optimum pH appeared to be between 6.0 and 7.0. The optimum pH for the breakdown of soluble starch by cell-free filtrates of *Strep.bovis* was between 6.0 and 6.5.

(3) After iodophilic cells had been observed microscopically in some of the pure cultures of *Strep.bovis* containing starch grains an investigation of the synthesis of iodophile polysaccharide was begun. *Strep.bovis* was shown to synthesise iodophile polysaccharide when grown on a solid medium containing either starch, dextrin or maltose but not glucose, sodium lactate, sucrose or starch and glucose together.

Part 3.

Some studies of micro-organisms of the rumen, other than Streptococcus bovis.

The frequency with which colonies of Strep.bovis appeared on nearly all the media containing peptone resulted in the decision to concentrate the work on this organism. The results of studies on other groups of bacteria reported here are realised to be incomplete.

1. Micro-organisms developing under the same cultural conditions as Strep.bovis.

Quite often no colonies of bacteria other than Strep.bovis grew on dilutions which gave well-isolated colonies. The other species that were isolated included micrococci, lactobacilli, spore-forming bacilli and, most frequently, Gram-positive, non-sporing rods. Streptococci other than Strep.bovis were rarely isolated on this medium. From 111 samples of rumen liquid 1,606 colonies were examined. 74% were Strep.bovis, 16% Gram-positive, catalase-positive, non-sporing rods, 1% micrococci, 2% Lactobacilli, 2% spore-forming rods and the remaining 5% consisted of organisms that were either unidentified or failed to grow. The

percentage of colonies of Strep.bovis was higher and of Gram-positive rods lower on the plates containing rumen samples from Ernest (85%, 9%) than from Daphne (64%, 23%). All the species that were isolated were facultative anaerobes.

Lactobacilli.

The few strains of Lactobacilli isolated on starch (or glucose) agar varied in their characteristics; they were not of one species, and were obtained only from one animal (Daphne).

It was thought, at first, that the failure to detect more than a few lactobacilli might have been due to the use of unsuitable media. Accordingly other media were used with the object firstly of cutting out other species and secondly of satisfying better the rather specialised nutritional requirements of this genus. On glucose agar adjusted to pH 4.0 with 0.5% acetic acid or to pH 4.8 with 4% sodium lactate (Davis, 1935) lactobacilli grew in numbers of 6×10^2 to 90×10^2 per ml.rumen liquid. The counts were similar whether the plates had been incubated in an atmosphere of almost pure carbon dioxide or in hydrogen. The particular species of lactobacilli were not fully determined but sufficient information was available to show that they

varied with different samples of rumen liquid. Similar numbers of lactobacilli were isolated on beerwort agar buffered to pH 3.5 and on a medium originally devised for the growth of Acetobacter species and containing sucrose, ethanol and yeast extract and acidified to pH 4.0 with acetic acid. Both the carrot-liver extract-peptone medium (pH 7.0) recommended by Foster, Carey & Frazier (1941) for the growth of lactic acid bacteria including lactobacilli and milk serum agar (British Standards Institution, 1940) modified by the addition of 10% tomato extract supported the growth of Strep.bovis and prevented the detection of the presumably smaller numbers of lactobacilli. No lactobacilli were detected using acidified (pH 4.0) glucose agar in the one sample of rumen liquid from Ernest which was examined, or in the 54 samples plated on starch agar.

Later, during the examination of the rumen contents of calves small numbers of lactobacilli were isolated in addition to Strep.bovis on glucose agar supplemented with 2.5% yeast autolysate (Davis, 1939). Rumen samples from two calves were also plated on a medium containing 'Tween 80'. This substance, a sorbitan mono-oleate polyoxyalkylene derivative (Glassman 1941), had not been available for the earlier work. 'Tween 80' provides oleic acid in a convenient

and non-toxic form and had been used successfully by Keddie (1951) for the enumeration of Lactobacilli on grass and in silage. The medium contained 1% Lemco, 1% peptone, 1% glucose, 20% tomato juice, 5% yeast autolysate and 0.05% 'Tween 80'. The pH was adjusted to 5.4 with sodium acetate-acetic acid buffer solution (final conc.0.2M). In one calf a pure culture of a lactobacillus was obtained on this medium, corresponding to a count of 60×10^6 per ml.rumen liquid, and in a second calf a mixed culture of Strep.bovis and lactobacilli gave a count of 11×10^3 lactobacilli per ml. Lactobacilli in the rumen of calves were not detected on glucose agar containing yeast autolysate.

The 'Tween 80' medium has not yet been used for the detection and estimation of lactobacilli in the rumen of adult cattle, and it is possible that it might reveal the presence of larger numbers of lactobacilli than were obtained with other media. The results obtained so far, however, suggest that the genus Lactobacillus is of no importance in the adult rumen.

Gram-positive non-sporing rods (Type A).

The micro-organism most frequently isolated under the same conditions as Strep.bovis and occasionally present

in larger numbers than Strep.bovis was a Gram-positive, non-sporing, non-motile rod, 0.8 μ wide by 1.4 to 3.0 μ long. The rods occurred singly or in angular or palisade arrangement; they frequently showed metachromatic granules when stained with methylene blue and were slightly pleomorphic some having rounded, swollen ends. This species, Type A, was thus morphologically similar to the Corynebacterium, Microbacterium and other related genera. It differed from the Microbacteria in growing as well in carbon dioxide as in air and in giving good growth at both 37° and 30°, slight growth at 45° and none at 15°. It appeared to be similar to a bacterium isolated by Orla-Jensen (1919) from calf faeces which he provisionally included with the Microbacteria but which differed from the latter in forming inactive- and not d-lactic acid. This point has not yet been investigated with Type A.

Type A produced catalase, failed to hydrolyse starch or liquefy gelatin and produced no change in litmus milk. It reduced nitrate to nitrite and formed acid from glucose, maltose, sucrose and salicin but not from lactose, arabinose, raffinose, zylose, mannitol, inulin, dextrin or pectin. It formed a yellow pigment on potato but usually no pigment

on glucose or milk agar; indole was not formed and the Voges-Proskauer test for acetoin gave a variable but usually weak positive reaction.

The numbers of colonies of Type A developing on the starch agar plates varied considerably from sample to sample (Fig.14). The characteristics of the organisms isolated from the rumen were constant over a long period of observation and no other morphologically similar bacteria were detected. The mean count for 26 samples of rumen liquid from each animal was 111 x 10^4 for Daphne and 35 x 10^4 from Ernest. The organism was obviously present in the rumen in smaller numbers than Strep.bovis and was hence frequently overgrown.

A simple medium was prepared on which Strep.bovis was unable to grow since it fails to utilise ammonia-N. This medium had the following composition: 0.4 g.$MgSO_4 \cdot 7H_2O$, 0.1 g.NaCl, 0.1 g.$CaCl_2 \cdot 6H_2O$, 0.01 g.$FeSO_4 \cdot 7H_2O$, 1 g. $(NH_4)_2SO_4$, 50 ml.M-KH_2PO_4-Na_2HPO_4 buffer solution at pH 7.0, 10 g.starch, water to 1 litre. The counts of Type A from six samples of rumen liquid plated on this medium varied from 8 x 10^4 to 155 x 10^4 per ml.rumen liquid. Of 77 colonies on these plates, examination showed 68 to

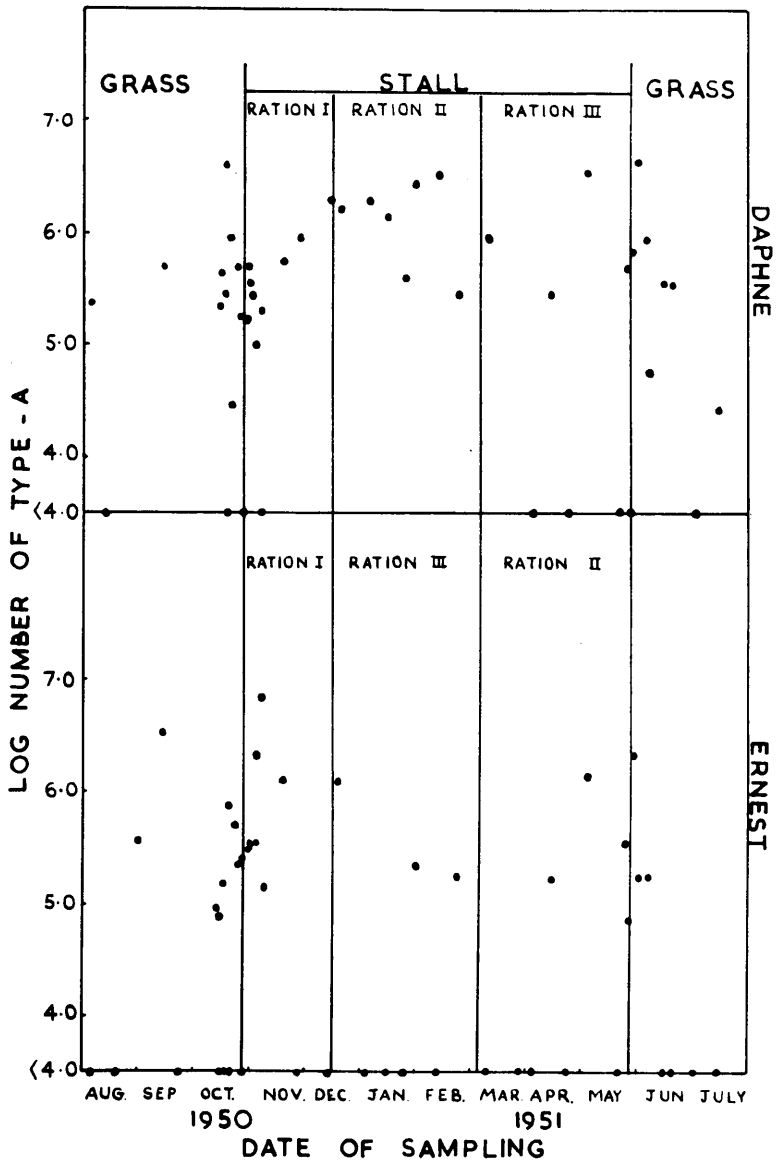

Fig.14.

The numbers of bacterium Type-A per ml. of rumen liquid from fistula cattle on different diets.

be of Type A. The colonies of Type A on this medium were very small and were, therefore, very difficult to transfer to fresh medium. Better growth of Type A was obtained on a lactate medium devised by Bhat & Barker (1947) for the growth of certain anaerobes (see below). This medium had the following composition: 1 g.sodium lactate, 0.3 g.yeast autolysate, 0.05 g.$(NH_4)_2SO_4$, 0.01 g.$MgSO_4.7H_2O$, 0.05 g.K_2HPO_4, 0.002 g.$FeSO_4.7H_2O$, tap water 100 ml.and 1 ml.of a 5% solution of sodium thioglycollate. On this medium incubated at 37° in CO_2 Type A was usually the predominant bacterium although Gram-negative rods giving acid and gas in glucose and lactose (probably Bact.aerogenes) were also present. The numbers of Type A isolated in this medium from samples from Daphne were between 29 x 10^3 and 300 x 10^3 and in samples from Ernest they were between 6 x 10^3 and 19 x 10^3 per ml.rumen liquid. Similar results were obtained when the thioglycollate or the yeast autolysate was omitted and slightly lower counts were obtained at 30° than at 37°. When the lactate was replaced in the medium by glucose as the source of carbon, Strep.bovis again interfered, possibly utilising the organic sources of nitrogen present in the yeast autolysate. On the Bhat and Barker medium incubated in air there was a very mixed microflora

which varied from sample to sample. All the species isolated grew aerobically in the absence of thioglycollate. Their growth was not tested in the absence of oxygen.

From the above results it appears that Type A was a constant inhabitant of the rumen of the two fistula animals although the numbers were very small in relation to the total numbers of rumen micro-organisms and were lower even than those of Strep.bovis. Type A, unlike Strep.bovis, appeared to utilise lactate as a source of carbon and ammonium salts as a source of nitrogen. Type A may belong to the Corynebacterium or a closely related group but differs from many of the saprophytic bacteria of such groups in giving good growth at 37° as well as at 30° and in reducing nitrate to nitrite.

2. Micro-organisms capable of growth on various selective media.

Experimental work was carried out to determine whether some of the commoner facultative aerobic micro-organisms which have been isolated by various workers from the intestine, were present to any extent in the rumen, and also to determine whether certain groups of anaerobes could be isolated from the rumen. In addition, an attempt was

made to cultivate the cellulose-decomposing bacteria of the rumen.

(a) Enterococci. The 'S.F.' medium of Hajna & Perry (1943) containing glucose, peptone, 'Yeastrel' and 0.04% sodium azide and incubated at 45°C.is frequently used to give an estimate of "enterococci". The growth obtained on this medium after inoculation with rumen liquid consisted only of Strep.bovis. Strep.faecalis and other enterococci were not detected.

(b) Coli-aerogenes group. Both Strep.bovis and the bacterium Type A grew on McConkey agar plates and any bacteria of the coli-aerogenes group which may have been present were presumably overgrown and not detected in the 10^{-4} dilution of the rumen liquid. A presumptive positive coliform test was obtained for different samples in 1 ml.of the 10^{-2}, 10^{-3}, 10^{-4} dilutions of the rumen liquid (1 ml.of dilutions of rumen liquid to triplicate tubes containing 5 ml.of McConkey broth). The low dilution in which a positive reaction was observed confirmed the results obtained by the plating method that the coli-aerogenes group of bacteria are not normally present in large numbers in the rumen.

(c) Proteus group. No growth of bacteria of the Proteus

group was obtained on the medium devised by Zarett & Doetsch (1949) for the selective growth of organisms of this group. This medium contained sodium ricinoleate, urea, yeast extract, KH_2PO_4, K_2HPO_4 and NaCl; it had a pH of 6.9 and contained cresol red as indicator. Two samples of rumen liquid from each animal were examined.

(d) Spore-forming bacilli including Clostridia. Rumen liquid was heated to 80°C. for 15 min. to destroy vegetative forms of bacteria and then plated on glucose-peptone-'Yeastrel' agar. From 1 ml. of a 10^{-3} dilution of rumen liquid 1 to 8 colonies developed during aerobic incubation and 0 to 10 colonies developed in carbon dioxide. In cooked meat medium (Mackie & McCartney, 1942) a diverse flora developed, which, on subculture to glucose agar deeps, gave apparently uniform growth throughout the deep and thus failed to indicate the presence of any strict anaerobes.

On agar containing glucose, $(NH_4)_2SO_4$ (no organic nitrogen) and salts no growth of spore-forming bacilli was obtained from 1 ml. of the 10^{-2} dilution of heated rumen liquid during incubation in carbon dioxide. No nitrogen-fixing spore-forming anaerobes of the Clostridium pasteurianum group were detected on pour plates of Winogradsky's medium

(Stephenson, 1930) inoculated with 1 ml. of undiluted heated rumen liquid and incubated at 37°C. in an atmosphere of nitrogen. The few colonies which did develop were of the Bacillus subtilis group or were catalase-positive rods having terminal spores.

(e) Non-sporing anaerobes. Growth of anaerobes unable to utilise glucose but capable of obtaining energy by the simultaneous breakdown of ethanol and salts of lower fatty acids was obtained by Bornstein & Barker (1948) on a medium containing, in addition to ethanol and sodium acetate, yeast extract, sodium carbonate, salts and sodium thioglycollate. When unheated rumen liquid was plated on this medium and the plates incubated in hydrogen or carbon dioxide, colonies of Strep. bovis with a few colonies of 'Type A' and spore-forming rods of the B. subtilis group were obtained. The total count on this medium varied from 33×10^4 to 600×10^4 bacteria per ml. rumen liquid.

Attempts to grow strictly anaerobic bacteria from rumen liquid using the lactate medium described by Bhat & Barker (1947) for the cultivation of Butyribacter, or propionic acid bacteria, e.g. Veillonella species, were not

successful. This medium (p.79) contained sodium lactate, yeast autolysate, salts and sodium thioglycollate. Pour plates were prepared and incubated in the usual way in jars which were evacuated, then flushed out and refilled with carbon dioxide. Later roll-tubes were used instead of plates. Ordinary 6 x 5/8 in. tubes containing 2 ml. medium were placed in boiling water to melt the agar and drive out the air. A rubber bung fitted with suitable tubing was immediately inserted and a rapid stream of carbon dioxide was passed through a sterile cotton wool filter and then through the medium. The tube was inoculated, CO_2 was passed through again and then the bung containing the glass tubing was quickly removed and replaced by a solid rubber bung. Finally, the tubes were rolled in chilled water to give a thin film of medium on the sides of the tube and incubated, bung downwards, at 37°C. The predominating and frequently the only organism on this medium after incubation at 37° was that designated Type A (p.76), a facultative aerobic Gram-positive bacterium. Minute colonies in relatively large numbers (compared with Type A) were visible in some tubes and may have been Veillonella sp. but these have not yet been further investigated. They might equally well have been colonies of

Strep.bovis which while failing to utilise NH_3-N for growth appeared to obtain sufficient organic nitrogen from the yeast autolysate, as for instance in the Bornstein and Barker medium, to develop in the absence of peptone.

(f) Enrichment cultures in sterile rumen liquid. These cultures were prepared in an endeavour to obtain indications of a suitable method of approach to the problem of the laboratory cultivation of the rumen micro-organisms.

Strained rumen liquid after sterilisation in the autoclave had a pH between 8 & 9 and was adjusted after autoclaving to pH 6.8 using asceptic technique. 80 ml.of medium was placed in 100 ml.conical flasks so that the flasks were filled to the neck to reduce the oxygen tension as much as possible. One series of flasks was set up containing equal quantities of autoclaved rumen liquid and glucose broth and in a second series 0.5% peptone and 0.3% Lemco were dissolved in 80 ml.of the rumen liquid. Additions of maize starch grains or strips of torn filter paper were made to the medium containing undiluted rumen liquid (series 2). All the media were sterilised in the autoclave, boiled and then cooled to 40°C.and immediately inoculated with 1 ml.of fresh strained rumen liquid and incubated at 37°C. The pH of the medium rose

to between pH 8.7 and 9.0 after 7 days in the absence of added carbohydrate and fell to between 4.0 and 5.0 in the presence of glucose or starch.

The development of the microflora was observed by microscopical examination. The microflora was initially very diverse but in the presence of glucose or starch grains Gram-positive cocci usually predominated and were clustered around the starch grains after 24 hr. Decomposition of the starch grains was well advanced by the third day of incubation and in the presence of peptone and 'Lemco' only a few hollowed-out grains remained after three days. A proportion of the cocci associated with the starch grains gave a blue colour with iodine and some iodophilic rods were also observed regularly. No isolations were attempted at this stage but subsequent work (Part 2) has shown that at least a proportion of the cocci must have been Strep.bovis. The number of iodophilic bacteria decreased from the 4th to the 7th day of incubation and the loss of iodophilic properties was most rapid in the absence of added carbohydrate.

Decomposition of filter paper strips in the cultures containing sterile rumen liquid, peptone and 'Lemco' was slow, becoming visible only after 7 days. In the absence

of peptone or in the presence of starch or glucose the decomposition was still slower. The bacteria associated with the decomposing filter paper were mainly Gram-positive rods whereas in the rumen the decomposition of cellulose has been shown to be associated with cocci more frequently than with rods. The pH of the media became alkaline whereas in the rumen the products of cellulose decomposition are mainly acid.

(g) Cellulose-decomposing bacteria. Decomposition of cellulose (filter paper strips) was obtained not only in flasks containing reinforced autoclaved rumen liquid (see p.85) but was also obtained regularly in tube cultures containing half-immersed strips of filter paper when a heavy inoculum (1 ml.fresh rumen liquid to 5 ml. medium) was used. With a smaller inoculum growth of cellulose-decomposing bacteria was only occasionally obtained. Decomposition of the filter paper was very slow and was usually not detected until after incubation at 37° for about 10 days.

Decomposition of filter paper was obtained in broth, sterile rumen liquid or in a simple salt solution (Kellerman & McBeth, 1912) but most cultures of cellulose-

decomposing bacteria died out after a few transfers when incubated in carbon dioxide. The morphology of these mixed cultures did not appear to be different from the morphology of the cultures grown under aerobic conditions. The microflora of the aerobic cultures remained diverse in morphology even after many subcultures. The rate of decomposition was very slow especially in the simple salt solutions though this could be improved slightly by the addition of sodium acetate and especially by the addition of yeast autolysate but was reduced in the presence of 0.05% sodium thioglycollate. It was obvious that these aerobic cellulose-decomposing bacteria were unlikely to be of importance in the rumen.

The results of this preliminary work were very similar to those obtained by Sijpesteijn (1948) in her preliminary studies.

In 1947 Hungate had reported the isolation from the rumen of bacteria capable of decomposing cellulose rapidly and he believed that these bacteria were responsible for most of the cellulose decomposition in the rumen. Full details of the methods used by Hungate were not published until 1950. In the meantime Sijpesteijn (1948) using methods based on Hungate's work had also obtained active decomposition of cellulose in laboratory culture and had succeeded in obtaining

pure cultures although mixed cultures were found to be more reliable if not essential for the maintenance of the viability of these strains. In the present work successful growth of cellulose-decomposing bacteria in large numbers has now been achieved using the methods of Hungate and Sijpesteijn. The medium consisted of a salt solution containing either peptone or sterile rumen liquid, yeast autolysate, sodium thioglycollate, sodium bicarbonate and agar and was saturated with oxygen-free carbon dioxide.

In preliminary trials two different salt solutions (Sijpesteijn, 1948) were used to which 0.25% peptone or 40% autoclaved rumen liquid was added. Both solutions gave much more vigorous decomposition of cellulose in the presence of peptone than in the presence of sterile rumen liquid. This rumen liquid was treated by the method used by Sijpesteijn (1948) to prevent the pH from becoming alkaline during autoclaving. (To the strained rumen liquid 1% of a 25% solution of calcium chloride was added to remove some of the carbonate and, after the removal of the sediment in a Sharples centrifuge at 25,000 r.p.m. for 20 min., the supernatant liquid was sterilised in the autoclave). There was little difference in the vigour of the decomposition with the different salt solutions. The one chosen for

subsequent use had the following percentage composition: K_2HPO_4, 0.06; KH_2PO_4, 0.04; $(NH_4)_2SO_4$, 0.04; NaCl, 0.12; $MgSO_4.7H_2O$, 0.02; $CaCl_2.6H_2O$, 0.02; $FeSO_4.7H_2O$, 0.001; in tap water. Yeast autolysate was prepared by the method of Davis (1935) and sterilised with the salt solutions and peptone. The agar (New Zealand type) as a 2% solution was autoclaved separately. Sodium thioglycollate and sodium bicarbonate were both sterilised by Seitz filtration and added to the medium immediately before use to give a final concentration of 0.05% and 0.4%, respectively.

The sterile medium was used in quantities of either 80 ml.in boiling tubes, or 15 ml.in test tubes, in which strips of filter paper or a complete roll of filter paper placed against the inner wall of the tube had been previously sterilised. Carbon dioxide freed from traces of oxygen by passage through chromous acid solution (Hungate, 1950) and sterilised by passage through sterile cotton wool was passed through the medium before and after inoculation, the culture tube being closed with a rubber bung fitted with suitable inlet and outlet tubes. This rubber bung was then quickly removed and replaced by a sterile solid bung. The medium was allowed to solidify and the tubes incubated at 37°C.

The onset of cellulose decomposition could be readily observed on the filter paper strips or rolls. Signs of decomposition were usually evident in solid medium within 3 days in primary cultures inoculated from a 10^{-2} dilution of fresh strained rumen liquid. The decomposition of the cellulose appeared (1) as colourless holes or 'plaques' from which the cellulose had completely disappeared, (2) as areas of yellow pigmentation usually not showing complete disintegration of the filter paper, and (3) occasionally as areas of orange-yellow pigmentation. The colourless 'plaques' usually predominated and varied in number, often 50 to 100 in an area of about 5 sq.cm. of filter paper in 15 ml. medium in the smaller tubes. This is, of course, an under estimate of the total number of cellulose-decomposing bacteria in the inoculum since it includes only those in contact with the filter paper. Cocci, morphologically indistinguishable, were always associated with decomposition of types 1 and 2 and large rods with terminal spores with type 3.

In liquid media of the same composition the decomposition of the filter paper was even more rapid and in the more active cultures was evident in 1 day, considerable decomposition being obtained in 2 to 3 days. The filter

paper in the liquid cultures usually became bright yellow, thin, and weak and finally after prolonged incubation settled to the bottom of the tube as a deposit of shredded fibres. Although cocci were usually the predominating micro-organisms on the decomposing filter paper, rods and spirochaetes were often abundant in the medium surrounding the filter paper. The cultures in liquid media were transferred to fresh medium as soon as the filter paper began to weaken, usually every 2 to 4 days but after about 5 to 6 transfers the activity of the culture gradually decreased. The same stage of decomposition of the filter paper in the next transfer might then take 10 to 13 days and the following one 15 to 30 days.

The cultures in liquid media remained apparently as mixed after say 10 transfers as in the primary cultures. An attempt was made to reduce the accompanying microflora by washing the decomposing filter paper between transfers. The paper was suspended in Ringer's solution containing sodium thioglycollate and well shaken to give a suspension of the paper fibres. This was centrifuged lightly and the sediment resuspended in fresh Ringer's solution. Five washings in all were used. The resulting suspension was

diluted and then a loopful from each dilution was transferred to fresh liquid medium. It was found that both rods and cocci were still associated in all dilutions showing decomposition of the filter paper and that little or no purification of the microflora had occurred.

Subculture from 'plaques'. An attempt was made to isolate the cellulose-decomposing bacteria by subculture from areas of decomposition of filter paper strips in solid media. The plug of agar was shaken or pushed out of the tubes into a sterile Petri dish and portions of the decomposing cellulose around the 'plaques' were transferred to fresh liquid medium. A few cultures were obtained of the unpigmented growth but the activity of the cultures was rapidly lost and further attempts to obtain pure or less mixed cultures have not yet been made.

Effect of varying the sources of nitrogen. In the absence of peptone cellulose decomposition was less marked but appeared to be increased slightly by increasing the amount of $(NH_4)_2SO_4$. Increase in $(NH_4)_2SO_4$ had no apparent effect on the activity of the cultures in the presence of peptone. Cultures were carried through nine transfers in the absence of peptone but failed to show decomposition of

cellulose at the 10th transfer unless peptone was again added.

Effect of the source of the cellulose. Decomposition of fresh sterile blades of grass (Cocksfoot) was obtained as thin patches and holes in the blades in 6 days with advanced decomposition in 10 days when these were used instead of filter paper strips. 'Cellophane' became yellow and patches of cocci and rods were massed on the surface. Decomposition of dried grass meal was not readily detected microscopically but there appeared to be some action associated with both rods and cocci.

Addition of acetate. The addition of 1 ml. of a molar solution of sodium acetate which had been adjusted to pH 7.0 (the pH of the medium) with acetic acid, increased the rate of cellulose decomposition. As in the absence of acetate, a gradual loss of activity in the cultures occurred after repeated transfers. The micro-organisms associated with the fibres were usually cocci although rods of varying morphology and spirochaetes were present in the culture medium.

Summary and Conclusions to Part 3.

Normal inhabitants of the intestine of animals such as the coli-aerogenes group, enterococci and the Proteus group were shown to be few or absent from the rumen of cattle. Lactobacilli were occasionally encountered on the starch agar plates used to isolate Strep.bovis from the rumen of the adult ruminant. Larger numbers of Lactobacilli were isolated from the rumen of calves using a medium containing 'Tween 80' which appeared to be more suitable for the growth of these lactobacilli than the media used in the examination of the rumen liquid of adult cattle.

A Gram-positive non-sporing rod, designated Type A, and morphologically similar to the Corynebacteria was frequently isolated on the starch agar plates in addition to Strep.bovis. The numbers of Type A were very variable and much lower than those of Strep.bovis. The mean count for 26 samples of rumen liquid from a heifer with a rumen fistula was 111×10^4 per ml. and for samples from a steer it was 35×10^4 per ml.

A few preliminary experiments were made in the cultivation of anaerobes such as Clostridia and Veillonella on solid media but they were unsuccessful.

Rapid decomposition of cellulose was obtained using the methods of Hungate and Sijpesteijn but the development of such methods has been postponed until after the more detailed study of Strep.bovis is completed.

General Discussion.

A Streptococcus capable of hydrolysing starch was persistently encountered during preliminary attempts to obtain pure cultures of bacteria from the rumen of two oxen. This Streptococcus was subsequently isolated on almost every occasion when rumen contents from cattle, sheep, goats and calves were examined by suitable techniques. All the strains conformed to the descriptions of Strep.bovis given by Orla-Jensen (1919) and Sherman (1937) but, unlike those examined by Sherman, they showed only occasional differences in their fermentation reactions and every strain was found to hydrolyse starch. This, more than any other biochemical characteristic, was used in the present investigations to distinguish Strep.bovis from other Streptococci. Shattock (1949) showed by serological methods that Strep.bovis is related more closely to the Enterococci than to the Viridans group, into which it was placed by Sherman. This indicates that Sherman's grouping of the Streptococci is, to some extent, arbitrary; it does not make the identification of Strep.bovis by Sherman's method less reliable.

The numbers of Strep.bovis in the rumen liquid of cattle, sheep, and goats were found, almost invariably,

to be within the range 10^5 - 10^7 per ml. Moreover, in the two oxen which were examined over long periods, the numbers were not significantly altered by the changes from stall to pasture feeding and vice versa. It is probable, however, that the numbers of Strep.bovis would have been higher in animals receiving more carbohydrate, since an increase in the numbers of this organism occurred in vitro when rumen liquid was incubated with added maltose or starch. Hungate, Dougherty, Bryant and Cello (1952) found that the addition of grain or glucose to the rumen of sheep on a diet of hay temporarily increased the numbers of Strep.bovis from less than 10^6 to more than 10^9 per ml. The decrease in numbers which occurred after the maximum value had been reached was probably caused by the pH of the rumen contents which fell to values below 5.0 after the additions of grain or glucose. In the present investigations pH values below 5.0 were found to be rapidly lethal to Strep.bovis in vitro.

Microscopical examination of rumen contents showed that only a small proportion of the rumen micro-organisms consisted of Gram-positive cells of the same shape and size as Strep.bovis. Moreover, the numbers of Strep.bovis determined by viable counts were exceeded at least one thousand-

fold by the numbers of other micro-organisms determined by direct microscopic counts. It must be concluded, therefore, that the metabolism in the rumen of animals on a normal diet at stall or at pasture is affected to only a small degree by Strep.bovis. The activities of the organism may, however, be more important in animals which are receiving higher proportions of soluble carbohydrate or starch in their diet. Hungate et al (1952) observed that the addition of glucose or grain to the rumen caused an increase in the concentration of lactic acid as well as an increase in the number of Strep.bovis. Since Strep.bovis is known to produce lactic acid from glucose (White and Sherman, 1943) it is possible that the increased lactic acid content of the rumen was due to the activity of this organism. On the other hand there may be large numbers of other rumen micro-organisms which are able to produce lactic acid from glucose. In the present investigations the in vitro incubation of rumen liquid with added maltose led to an increase in its turbidity, and Pearson and Smith (1943b) observed corresponding increases of protein nitrogen under similar conditions. The numbers of Strep.bovis were too small to account for these observed

increases and it is clear that multiplication of large numbers of other micro-organisms must have occurred. Little is known about the biochemical reactions of these other organisms, and the need to grow them in pure culture is evident. Until this has been done it will be impossible to assess fully the contribution of Strep.bovis to rumen metabolism.

REFERENCES.

Abd-el-Malek, Y. & Gibson, T. (1947-8). J.Dairy Res., 15, 233.

Ankersmit, P. (1905), cited by Mangold, E. (1929). Handbuch der Ernahrung und des Stoffwechsels der landwirtschaftlichen Nutziere. Berlin: Julius Springer.

Ayers, S.H. & Mudge, C.S. (1923). J.infect.Dis., 33, 155.

Baker, F. (1942 a). Nature, Lond., 149, 582.

Baker, F. (1942 b). Nature, Lond., 149, 220.

Baker, F. (1943). Ann.appl.Biol., 30, 230.

Baker, F. & Harriss, S.T. (1947-48). Nutr.Abstr.Rev., 17, 3.

Baker, F. & Nasr, H. (1947). J.R.micr.Soc., 67, 27.

Baker, F. & Nasr, H. (1949). Nature, Lond., 164, 745.

Baker, F., Nasr, H., Morrice, F. & Bruce, J. (1950). J.Path. Bact., 62, 617.

Baker, F. & Whelan, W.J. (1950). Nature, Lond., 165, 449.

Balch, C.C. & Johnson, V.W. (1948). Vet.Rec., 60, 446.

Bhat, J.V. & Barker, H.A. (1947). J.Bact., 54, 381.

Bornstein, B.T. & Barker, H.A. (1948). J.Bact., 55, 223.

Bourne, E.J., Haworth, N., Macey, A. & Peat, S. (1948). J.Chem. Soc., p.924.

British Standards Institution (1940). British Standard No.895 Microbiological examination of butter.

Davis, J.G. (1935). J.Dairy Res., 6, 175.

Davis, J.G. (1939). Dairy Ind., 4, 360.

Doudoroff, M., Hassid, W.Z., Putman, E.W., Potter, A.L. & Lederberg, J. (1949). J.biol.Chem., 179, 921.

Elsden, S.R. (1945-46). J.exp.Biol., 22, 51.

Elsden, S.R. & Phillipson, A.T. (1948). Annu.Rev.Biochem., 17, 705.

Ewing, P.W. & Wright, L.H., quoted by Dukes, H.H. (1943). The Physiology of Domestic Animals. New York: The Comstock Publishing Co., Inc. p.290.

Foster, E.M., Garey, J.C. & Frazier, W.C. (1941). J.Dairy Sci., 24, 1015.

Fuller, C.A. & Armstrong, V.A. (1913). J.infect.Dis., 13, 443.

Gall, L.S. (1946) (Thesis). Some studies on the rumen micro-organisms of sheep and cattle. Cornell University.

Gall, L.S., Burroughs, W., Gerlaugh, P. & Edgington, B.H. (1949). J.Anim.Sci., 8, 441.

Gall, L.S. & Huhtanen, C.N. (1951). J.Dairy Sci., 34, 353.

Gall, L.S., Stark, C.N. & Loosli, J.K. (1947). J.Dairy Sci., 30, 891.

Glassman, H.N. (1941). Bact.Rev., 12, 105.

Hajna, A.A. & Perry, C. (1943). Amer.J.publ.Hlth., 33, 550.

Hastings, E.G. (1944). Bact.Rev., 8, 235.

Hehre, E.J., Carlson, A.S. & Neill, J.M. (1947). Science, 106, 523.

Hehre, E.J. & Hamilton, D.M. (1948). J.Bact., 55, 197.

Henneberg, W. (1919). Berl.klin.Wschr., 56, 693.

Henneberg, W. (1922). Zbl.Bakt. (Abt.2), 55, 242.

Hungate, R.E. (1947). J.Bact., 53, 631.

Hungate, R.E. (1950). Bact.Rev., 14, 1.

Hungate, R.E., Dougherty, R.W., Bryant, M.P. & Cello, R.M. (1952). Cornell Vet., 42, 423.

Johns, A.T. (1951a). J.gen.Microbiol., 5, 317.

Johns, A.T. (1951b). J.gen.Microbiol., 5, 326.

Johns, A.T. (1951c). J.gen.Microbiol., 5, 337.

Johnson, B.C., Hamilton, T.S., Robinson, W.B. & Garey, J.C. (1944). J.Anim.Sci., 3, 287.

Keddie, R.M. (1951). Proc.Soc.appl.Bact., 14, 157.

Kellerman, K.F. & McBeth, E.G. (1912). Zbl.Bakt. (Abt.2), 34, 485.

Kreipe, H. (1927). Investigations of the Lactic Acid Bacteria of the cow rumen. Dissertation. Kiel.

Laidlaw, R.A. & Reid, S.G. (1952). J.Sci.Fd.Agric., 3, 19.

Mackie, T.J. & McCartney, J.E. (1942). Handbook of Practical Bacteriology. Edinburgh: E.& S.Livingstone.

McNaught, M.L., Smith, J.A.B., Henry, K.M. & Kon, S.K. (1950). Biochem.J., 46, 32.

Moir, R.J. (1951). Aust.J.agric.Res., 3, 322.

Monod, J. & Torriani, A.M. (1950). Ann.Inst.Pasteur., 78, 65.

Monroe, C.F. & Perkins, A.E. (1939). J.Dairy Sci., 22, 983.

Niven, C.F., Smiley, K.L. & Sherman, J.M. (1942). J.Bact., 43, 651.

Nutrition Abstracts and Reviews (1947-48). 17, 1-37.

Orla-Jensen, S. (1919). The lactic acid bacteria. København: Fred.Høst and Søn.

Pearson, R.M. & Smith, J.A.B. (1943a). Biochem.J., 37, 148.

Pearson, R.M. & Smith, J.A.B. (1943b). Biochem.J., 37, 153.

Schwarz, C. & Kaplan, J., quoted by Dukes, H.H. (1943). The Physiology of Domestic Animals. New York: The Comstock Publishing Co., Inc., p.302.

Shattock, P.M.F. (1949). J.gen.Microbiol., 3, 80.

Sherman, J.M. (1937). Bact.Rev., 1, 3.

Sijpesteijn, A.K. (1948). Thesis - Cellulose-decomposing bacteria from the rumen of cattle. University of Leiden.

Sijpesteijn, A.K. (1951). J.gen.Microbiol., 5, 869.

Smith, V.R. (1941). J.Dairy Sci., 24, 659.

Stephenson, M. (1930). Bacterial Metabolism. 2nd.Ed., London: Longmans, Green & Co.

Tosic, J. & Walker, T.K. (1950). J.gen.Microbiol., 4, 192.

Van der Wath, J.G. (1948). Onderstepoort J.vet.Sci., 23, 367.

Vogel, A.I. (1946). A textbook of Quantitative Inorganic Analysis. London: Longmans, Green & Co.

Washburn, L.E. & Brody, S. (1937). Res.Bull.Mo.agric.Exp.Sta., No.263.

Watts, P.S. (1948). Vet.Rec., 60, 4.

White, J.C. & Sherman, J.M. (1943). J.Dairy Sci., 26, 371.

Williams, V.J. & Moir, R.J. (1951). Aust.J.sci.Res., 4, 377.

Winslow, C.E.A. & Palmer, G.T. (1910). J.infect.Dis., 7, 1.

Zarett, A.J. & Doetsch, R.N. (1949). J.Bact., 57, 266.