STUDIES ON THE FLORA AND FAUNA OF THE BOVINE RUMEN

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PREFACE

The ruminant, in contrast to other herbivores, has a stomach of four compartments to enable digestion of plant fibre to be carried out. In the rumen, the first and largest of these compartments, there is established a large and diverse population of protozoa and bacteria, all active to some degree in degrading complex carbohydrate material and in converting plant nitrogen into microbial protein. Ingested food is retained and is broken down to a fine consistency before it passes further down the intestinal tract. The main products of this microbial activity in the rumen are the volatile acids, acetic, propionic and butyric, microbial protein and ammonia.

The existence of volatile fatty acids as the end-products of fermentation in the rumen was first demonstrated by Tappeiner in 1883, but their importance was not realized until their absorption from the rumen was demonstrated nearly 60 years later (McAnally and Phillipson, 1942; Barcroft, McAnally and Phillipson, 1944). Acetic, propionic and butyric acids are invariably present and higher acids occur under some dietary conditions. These acids contribute up to about 70% of the total energy requirements of the animal (Carroll and Hungate, 1954), acetic acid being concerned in the synthesis of milk fat (see Kleiber et al., 1952), and propionic acid being mainly glucogenic (Reid, 1950; McClymont, 1951). The precise metabolic function of butyric acid has not been elucidated although it has been shown to be non-glucogenic (Annison et al.,

1963).

Zuntz (1891) was the first to suggest that rumen micro-organisms might convert the non-protein nitrogen of plants to microbial nitrogen which would be available to the host (see McNaught and Smith, 1947) and the conversion of plant protein to rumen microbial protein was demonstrated by McDonald (1948, 1954). The bacteria and protozoa of the rumen are now known to convert plant nitrogen to microbial protein and ammonia; the ammonia is absorbed into the liver, some re-entering the rumen as salivary urea, and the microbial protein is degraded further down the alimentary tract. Weller, Pilgrim and Gray (1962) estimated that, in sheep on a wheaten hay ration, 60-80% of the dietary plant nitrogen, not including the small amount that may be converted to soluble nitrogen, is converted to microbial nitrogen.

A great deal of work has now been carried out on various aspects of rumen metabolism by physiologists, nutritionists, biochemists, bacteriologists and protozoologists. Unfortunately, much of this work has been performed without considering the place of the results in the whole field of rumen metabolism and function. The activity of the microbial population has been neglected too often by physiologists and nutritionists, and only limited knowledge has been obtained of the real role, in the animal, of the many rumen bacteria which have been isolated and studied. Existing knowledge needs to be organised and unified to clarify relationships and to enable the construction

of a comprehensive theory of rumen function (Doetsch, 1957).

Bryant (1959) and Hungate (1960) have both stated that most of the abundant and significant rumen bacteria have been isolated although many less important types still have to be studied. The characterisation of many of the isolated strains is still incomplete. As Hungate (1960, p357) pointed out, "The axenic cultivation, identification, and enumeration of bacteria in a natural habitat such as the rumen is extremely time-consuming. It is little wonder that most ecological investigations are restricted to a few components of the total flora. Development of methods permitting rapid and accurate identification would tremendously stimulate ecological studies. The fluorescent anti-body technique holds promise for rapid identification and enumeration (Hobson and Mann, 1957) but has not yet been developed to the point of usefulness in the solution of rumen ecological problems."

The rumen ciliate protozoa, previously thought of as passengers (Baker and Harriss, 1947) are now known to be of significance in food transformations in the rumen (Oxford, 1955) even though isolated ruminants have been reared in their absence (see Pounden and Hibbs, 1949, 1950). Gutierrez (1955) conservatively estimated that protozoa may perform 20% of the total fermentative activity in the rumen.

The study of the biochemistry of the rumen ciliates has not advanced as far or as rapidly as studies on the rumen bacteria, research being hampered by the difficulty in growing

the ciliates in pure culture. Only a few cultures have been established since Hungate's first successful attempt in 1942 (Hungate, 1942), and none of these cultures has been free of bacteria. As a result of this lack of pure cultures most biochemical studies have been carried out on washed suspensions prepared from rumen liquor. Some of this work has been simplified by the establishment of pure or nearly pure populations of single species or genera of ciliates in isolated animals (see Eadie, 1959; Abou Akkada and Howard, 1960). Because of the difficulty of obtaining suspensions of single species of ciliates it is obviously desirable that pure cultures of rumen protozoa be established. Axenic clones should be the ultimate aim although the work of Coleman (1962) suggests that such cultures are not feasible.

In New Zealand, the domestic ruminant is in an environment different from that for which it has evolved. New Zealand farming practice is based almost entirely on the feeding of pasture and only relatively small amounts of hay and silage are fed. The soluble carbohydrate level of this pasture is low throughout the year while the protein level is high, and in excess of animal requirements (Johns, 1955a). This type of diet is in direct contrast with the high-fibre diet with which the ruminant is capable of dealing, and could result in a rumen population different from those found in other countries.

A considerable amount of work has been carried out in this country on rumen metabolism and function, much of it in

connection with bloat research, but the study of the rumen microbial population has been largely neglected. Those studies that have been made have indicated that New Zealand ruminants may harbour rumen populations different in some respects from those found overseas. Epidinium, an oligotrich ciliate, was shown by Oxford (1958a) to be prevalent in New Zealand cows under certain conditions but is rare and found only in low numbers in cattle overseas. Clarke and di Menna (1961) demonstrated the existence of a yeast population in the rumen of New Zealand cattle but a similar rumen flora has not been found in other countries. Investigations of the role of lactic acid as a metabolic intermediate in the rumen have also indicated a different position in this country. In England, Balch and Rowland (1957) showed that only traces of lactic acid, less than 1 mg./100 ml. rumen liquor, occurred in the rumen of animals on a variety of grain-free diets, and Jayasuriya and Hungate (1959) in the United States, found less than 0.0011% (w/v) in the rumen of a steer on a high grain ration. In this country lactic acid has been shown to reach about 18 mg./100 ml. rumen liquor in cows on a diet of fresh red clover (Mangan, Johns and Bailey, 1959).

Thus, in spite of the large amount of rumen research already carried out in other countries, it is necessary for us to make basic studies on the rumen population of our domestic ruminants. The present work was undertaken to establish the similarities and differences in the flora and fauna of cattle

in this country and overseas, and to provide a basis for more advanced ecological studies. This thesis, therefore reports the results of the following studies: (a) identification of the ciliate protozoa, (b) estimation of protozoal populations, (c) isolation and characterisation of bacteria responsible for some important rumen reactions, (d) manometric estimation of rumen fermentation activity. A section on the growth of ciliates in pure culture is also included.

The work has been divided into several sections but some of the divisions, especially those concerned with the ciliate population, are somewhat artificial and have been created merely to clarify the experimental approach and the results. This has made the discussions difficult to arrange and has led to repetition in some places.

1. THE RUMEN CILIATES

A. IDENTIFICATION OF SPECIES

Introduction

Protozoa occur in the intestinal tracts of many animals, but those found in the first stomach of ruminants and in the gut of the wood-eating roach Cryptocercus punctulatus and of wood-eating termites, exhibit a genuine symbiotic relationship with their hosts (Hungate, 1955). These intestinal protozoa convert cellulose, and other complex dietary materials to substances which are available to the host. The symbionts of the termites and the roach are flagellates and as they may constitute up to one third of the total weight of the host (Katzin and Kirby, 1939) are relatively more important to their hosts than are the rumen protozoa to theirs.

The protozoa of ruminants are mainly ciliates, the flagellates constituting only an insignificant proportion of the total population (see Becker, Schulz and Emmerson, 1929; Warner, 1962b). The two main groups of ciliates, loosely named holotrichs and oligotrichs, are not closely related and belong to two Subclasses of the Class Ciliata within the Subphylum Ciliophora. Table 1 illustrates the position of the families of ruminant ciliate protozoa within the classification of Corliss (1961).

The protozoa of the rumen were first discovered by Gruby and Delafond in 1843. Several reports of their occurrence in

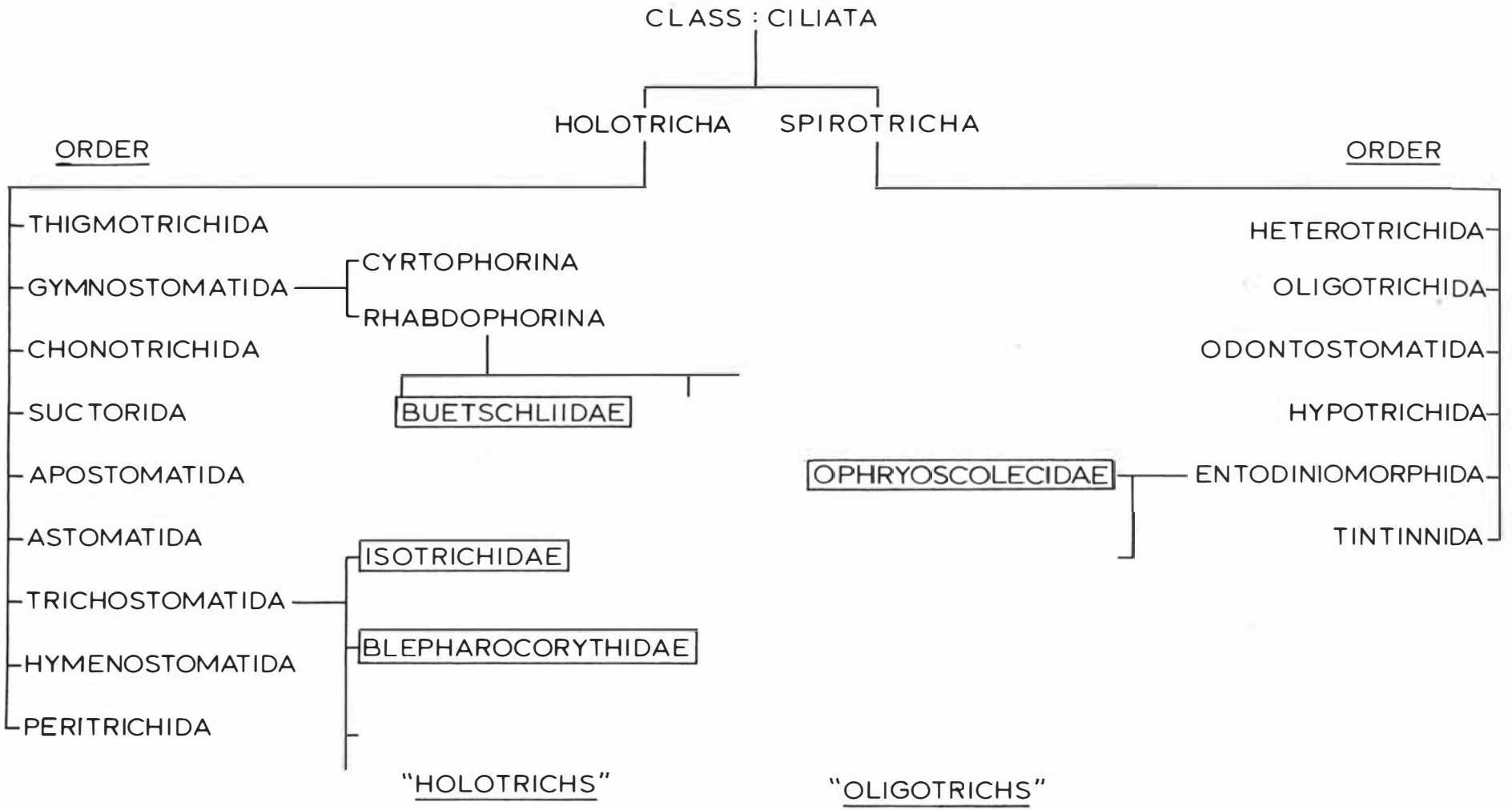


TABLE 1. THE FAMILIES OF RUMEN CILIATE PROTOZOA WITHIN THE SCHEME OF CORLISS (1961).

hay were shown to be erroneous (see Hungate, 1955) and they are now known to be transmitted in saliva (Becker and Hsiung, 1929), and to be restricted to the rumen habitat.

Although the rumen ciliates are not free-living and are not found in animals other than ruminants, there is considerable variation in the composition of the population in different ruminant species. Some ciliates are found in many types of host while others are more restricted in habitat (Dogiel, 1927). Differences in feed and less noticeable differences in the rumen environment affect the composition of the population. There are also regional differences in the genera of protozoa in the rumen of sheep that are not solely the result of differences in the diet of the animals (Coleman, 1963). For all these reasons the qualitative analysis of the population in any ruminant species in a particular environment is of interest.

Since the initial observation on the occurrence of rumen ciliates in 1843 by Gruby and Delafond, many papers have been published on the composition of the protozoan fauna of ruminants. Sharp (1914) gave a complete review of work in the period 1843-1914 and also gave the first detailed account of the organisation of a rumen ciliate.

Some of the more important work since 1914 has included studies on the fauna of goats (Ferber and Fedorowa, 1929; Das-Gupta, 1935), yaks (Dogiel, 1934), sheep (Ferber and Fedorowa, 1929; Bush and Kofoid, 1948), and antelopes (Buisson,

1924; Dogiel, 1932). Various bovine species were studied by Kofoid and MacLennan (1930, 1932, 1933; Bos indicus), Kofoid and Christenson (1934; Bos gaurus), Becker and Talbot (1927; domestic cattle) and Buisson (1923; domestic cattle). The comprehensive monograph of Dogiel (1927) deals with the ophryoscolecid ciliates of cattle, sheep, goats, camels, deer, reindeer and antelope from Asia and Africa. More recently, Lubinsky has published on some entodinia of sheep and goats from Pakistan (Lubinsky, 1957a) and on the ophryoscolecids of the Arctic reindeer (Lubinsky, 1958a,b). The only observations on the fauna of ruminants in New Zealand are those of Oxford (1958a, 1959) on domestic cattle. General accounts of the rumen ciliates have been written by Mangold (1929, 1933), Oxford (1955) and Hungate (1955).

No agreement has been reached on the taxonomy of the rumen ciliates. The main disputes have concerned the generic structure of the group enclosed under Diplodinium (Schuberg, 1888), and the stability of certain characteristics commonly used to delineate species rank. Dogiel (1927) created many sub-genera in the genus Diplodinium and freely used the "forma" division to denote sub-specific types. Kofoid and MacLennan (1932) created several new genera and species eliminating the "forma" rank of Dogiel (1927), and were followed by Bhatia (1936). Oxford (1955) followed Mangold (1929) in ignoring sub-genera within Diplodinium.

Morphological variation has often been observed in

cultures and preparations of rumen ciliates (Poljansky and Strelkow, 1934; Hungate, 1942), and some characteristics used to denote species rank are undoubtedly environmentally plastic (see Lubinsky, 1957a). The use of caudal spination is a notable example. Poljansky and Strelkow (1934) showed that caudal spination was very variable in clones of Entodinium caudatum, and demonstrated that the diet of the host influenced the degree of spination in the ciliate population in vivo.

In spite of the taxonomic inaccuracies which occur when undue "splitting" is carried out, the resulting classification may still be of considerable value. This applies to the classification of Kofoid and MacLennan (1930, 1932, 1933) which although taxonomically inaccurate, is lucid and easy to apply.

In this section the ciliates of the rumen of the New Zealand domestic cow (Bos taurus L.) are briefly described. The oligotrichs are classified according to Kofoid and MacLennan (1930, 1932, 1933), while the holotrichs are classified according to the original authorities.

Materials and Methods

The rumen contents of four rumen-fistulated cows were examined. The cows were stall-fed on either fresh red clover (Trifolium pratense L.) or grass hay from Monday to Friday inclusive. On Saturday and Sunday the animals were turned out to pasture.

Rumen contents were removed through the fistula and strained through gauze into an equal volume of 10% (v/v) formalin. After fixation for 2 hr. the protozoa were washed free of formaldehyde and plant material by centrifuging and suspension in tap water, and stained for 10 min. in 0.5% (w/v) methyl green. The protozoa were then washed twice by centrifuging. Most examinations were made directly on this material.

Some preparations were mounted in 50% (v/v) aqueous glycerol. Skeletal plates were sometimes examined after staining with chlor-zinc-iodide. A few preparations were stained with methyl green, rapidly dehydrated and cleared with graded solutions of alcohol and xylol, and mounted in Euparal. Unstained protozoa were examined by dark-field microscopy.

Terminology

The terminology used here with regard to the orientation of the ciliates is explained by the following extract from Lubinsky (1958a, p820):-

"If the observer orientates the ophryoscolecid ciliates with their oral ends away from him, towards 12 o'clock and with the micronucleus situated to the left of the macronucleus, he will always have the homologous body surfaces directed towards him, the 'left side' of Entodinium and the 'right side' of the higher Ophryoscolecidae by Dogiel's terminology. ..... This side will be called the upper side, a term which denotes its position in relation to the observer

and which has nothing to do with the concept of dorsality and ventrality. The opposite body side will be termed the lower side, and the lateral sides, the right and left sides."

The relation of this system of terminology to the systems of earlier workers is shown in Table 2 which is taken from Lubinsky (1958a).

Table 2.* Designation of body sides of the ophryoscolecid ciliates

Proposed Nomenclature	Nomenclature of Eberlein, Dogiel, and Kofoid		Nomenclature of Stein, Schuberg and Buisson	
	For Entodinium	For Higher Genera	For Entodinium	For Higher Genera
Upper side	Left side	Right side	Ventral side	Dorsal side
Lower side	Right side	Left side	Dorsal side	Ventral side
Right side	Dorsal side	Ventral side	Left side	Right side
Left side	Ventral side	Dorsal side	Right side	Left side

* from Lubinsky (1958a)

The nomenclature of the various structures used in the identification of the ciliates is shown in Plate 1.

The measurements used were as follows. Length was measured from the anterior tip of the body to the anal opening and width from right to left side of the body at the widest point. The length of the macronucleus was the shortest distance between the anterior and posterior tips and the width was measured at the widest point. In the genus Diplodinium the length of the macronucleus was measured parallel to the long axis of the nucleus.

The classification used to generic level was that of Corliss (1961). Kofoid and MacLennan (1930, 1932, 1933) were followed, as far as possible, in the identification of species.

Results

Species belonging to eight genera of the Family Ophryoscolecidae Stein (1859) (Order: Entodiniomorphida Reichenow (Corliss, 1961)) were found in the preparations of rumen liquor which were examined. Four genera of holotrichs belonging to two Orders and three Families were identified.

Descriptions of the species identified are given below. The descriptions of the individual species have been kept as short as was considered consistent with accurate identification. Because of the difficulty of identifying many entodinia, the descriptions of Entodinium species tend to be more complete than those of many of the higher ophryoscolecids. Synonyms according to Dogiel (1927) have been included in the descriptions to facilitate recognition of the species. The measurements of the ciliates are given in microns (μ).

CLASS: CILIATA Perty (1852)

SUB-CLASS: HOLOTRICHA Stein (1859)

ORDER: GYMNOSTOMATIDA Butschli (1889)

FAMILY: BUETSCHLIIDAE Poche (1913)

GENUS: Buetschlia Schuberg (1888)

Small, more or less ovoid in shape; anterior end truncated; mouth at anterior end; body surface uniformly

covered with cilia; nucleus roughly spherical.

Buetschlia parva Schuberg (1888)

Plate 2, Figure 4

The anterior end of the body is truncated. The mouth is in the middle of the anterior end and leads into a conical pharynx.

The measurements of 10 specimens are summarized in Table 3.

Table 3. Dimensions of Buetschlia parva

	$M \pm \sigma M$[1]	σ[2]	C.V.%[3]	Limits of Variation
Length	46.9 ± 1.6	± 5.2	11.1	38 - 57
Width	24.7 ± 0.8	± 2.6	10.5	22 - 30
L/W ratio[4]	1.91 ± 0.08	± 0.26	13.6	1.58 - 2.38

1. Mean ± standard error of mean.
2. Standard deviation.
3. Coefficient of variation.
4. Ratio of body length to width.

ORDER: TRICHOSTOMATIDA Butschli (1889)

FAMILY: ISOTRICHIDAE Butschli (1887)

GENUS: Isotricha Stein (1859)

Body ovoid and flattened; surface covered in cilia in longitudinal rows; mouth at or near end which is to rear during locomotion; many contractile vacuoles.

Isotricha intestinalis Stein (1859)

Plate 2, Figure 2

The mouth lies in an indentation which is lateral, and forward of the end which is to the rear during locomotion.

The measurements of 10 specimens are summarized in Table 4.

Table 4. Dimensions of Isotricha intestinalis

	M ± σ M	σ	C.V.%	Limits of Variation
Length	108.8 ± 6.2	± 19.7	18.1	89 - 149
Width	61.8 ± 3.3	± 10.4	16.8	46 - 78
L/W ratio	1.76 ± 0.03	± 0.1	5.7	1.65 - 1.93

Isotricha prostoma Stein (1859)

Plate 2, Figure 3

The mouth lies near the end which is to the rear during locomotion.

The dimensions of 10 specimens are summarized in Table 5.

Table 5. Dimensions of Isotricha prostoma

	M ± σ M	σ	C.V.%	Limits of Variation
Length	133.8 ± 5.3	± 16.9	12.7	113 - 165
Width	67.4 ± 2.2	± 6.9	10.3	51 - 73
L/W ratio	2.03 ± 0.10	± 0.30	14.8	1.69 - 2.55

GENUS: Dasytricha Schuberg (1888)

A single species.

Dasytricha ruminantium Schuberg (1888)

Plate 2, Figure 1

Similar to Isotricha but smaller and with the cilia arranged in longitudinal spirals. The mouth is at the end which is to the rear during locomotion. There is a single contractile vacuole.

The measurements of 10 specimens are summarized in Table 6.

Table 6. Dimensions of Dasytricha ruminantium

	$M \pm \sigma M$	σ	C.V.%	Limits of Variation
Length	57.5 ± 2.5	± 7.9	13.8	46 - 73
Width	27.4 ± 0.8	± 2.4	8.8	22 - 30
L/W ratio	2.11 ± 0.07	± 0.21	9.9	1.70 - 2.70

FAMILY: BLEPHAROCORYTHIDAE Hsiung (1930)

GENUS: Charon Jameson (1925)

Body lancet shaped; anterior end has knob-like projection; posterior end tapered to a blunt point; cilia at each end, the posterior cilia being plainly divided into two distinct bundles; mouth slightly to rear of anterior tip; macronucleus oval to spherical; a single contractile vacuole.

Charon equi Hsiung (1930)

Plate 2, Figure 5

The body is lancet shaped and has a knob projecting anteriorly. The mouth is a triangular opening on one side of

the anterior projection. The mouth area is surrounded by three zones of cilia. There are two posterior tufts of cilia on either side of the anus. The macronucleus is elongated. There is a single contractile vacuole. This species is larger than C. ventriculi Jameson (1925).

The dimensions of 10 specimens are summarized in Table 7.

Table 7. Dimensions of Charon equi

	M ± σ M	σ	C.V.%	Limits of Variation
Length	38.3 ± 1.6	± 5.2	13.6	33 - 45
Width	14.8 ± 0.5	± 1.6	10.8	13 - 18
L/W Ratio	2.59 ± 0.04	± 0.12	4.6	2.41 - 2.75

SUB-CLASS: SPIROTRICHA Butschli (1889)

ORDER: ENTODINIOMORPHIDA Reichenow (1929; in Doflein and Reichenow, 1929)

FAMILY: OPHRYOSCOLECIDAE Stein (1859)

GENUS: Entodinium Stein (1859)

A single membranelle zone; no skeletal plates; one contractile vacuole; nuclei and caudal spination variable.

Entodinium biconcavum Kofoid and MacLennan (1930)

Plate 3, Figure 7

The body is slightly elongated. The macronucleus is rod-like and lies on the extreme right of the body. The micronucleus lies on the left of the anterior half of the macronucleus. The

vacuole lies on the upper side of the macronucleus immediately adjacent to, and anterior to the micronucleus.

There is a small caudal lobe on the left side. At the level of the posterior end of the macronucleus, the endoplasmic sac leaves the right body side, and there is an anteriorly directed bulge in the sac between the nucleus and the rectum. The area posterior to this bulge, on the right of the rectum, is concave on both the upper and lower surfaces. This biconcavity is very obvious even when the organism is viewed from the upper or lower sides.

The measurements of 10 specimens are summarized in Table 8.

Table 8. Dimensions of Entodinium biconcavum

	$M \pm \sigma M$	σ	C.V.%	Limits of Variation
Length	44.8 ± 1.5	± 4.8	10.7	40 - 51
Width	30.5 ± 0.6	± 1.8	5.9	27 - 32
L/W ratio	1.46 ± 0.03	± 0.11	7.5	1.33 - 1.59
L. macro[1]	25.3 ± 1.2	± 3.7	14.6	20 - 30
W. macro[2]	6.7 ± 0.1	± 0.4	6.0	5 - 8

1. Length of macronucleus.
2. Width of macronucleus.

Entodinium longinucleatum Dogiel (1925)

Plate 3, Figure 2

The body is ellipsoidal with a single, left, caudal lobe. The macronucleus extends for nearly the full length of the body and follows the contour of the right side. Its anterior

end is wider than the posterior end. The micronucleus lies on the left of the anterior quarter of the macronucleus. The contractile vacuole lies slightly anterior to the micronucleus.

The measurements of 10 specimens are summarized in Table 9.

Table 9. Dimensions of Entodinium longinucleatum

	M ± σ M	σ	C.V.%	Limits of Variation
Length	48.8 ± 1.8	± 5.6	11.5	43 - 59
Width	35.1 ± 0.6	± 1.9	5.4	32 - 38
L/W ratio	1.39 ± 0.06	± 0.19	13.7	1.13 - 1.69
L. macro	44.4 ± 1.8	± 5.7	12.8	38 - 51
W. macro	7.3 ± 0.2	± 0.7	9.6	7 - 9

Entodinium bicarinatum Da Cunha (1914)

Plate 3, Figures 3, 4

The body is nearly circular when viewed from the upper side but is flattened and twisted laterally. There are three caudal lobes. The right and left sides of the body are nearly in the same plane. To the left of the mid-line of the body there arises a rib which terminates in a lobe in a plane above that of the rest of the body. The shape and size of this rib and the caudal lobes may vary considerably in different individuals. In some specimens the twisting of the body is different, and the right side of the body and the rib lie in the upper plane. The macronucleus is elongated and relatively short, and lies against the right side at the extreme anterior of the body. The vacuole lies near the upper surface adjacent to the

micronucleus.

The measurements of 10 specimens are summarized in Table 10.

Table 10. Dimensions of Entodinium bicarinatum

	M ± σ M	σ	C.V.%	Limits of Variation
Length	38.7 ± 1.5	± 4.6	11.9	32 - 46
Width	37.7 ± 1.5	± 4.8	12.7	30 - 46
L/W ratio	1.03 ± 0.03	± 0.09	8.7	0.95 - 1.23
L. macro	23.6 ± 1.5	± 4.8	20.4	14 - 30
W. macro	7.8 ± 0.2	± 0.6	7.7	7 - 9

Entodinium rostratum Fiorentini (1889)

Syn. Entodinium rostratum forma rostratum Dogiel (1927)

Plate 3, Figure 6

The body is elongate, with the right surface convex and the left surface nearly flat. The left surface ends in a spine which curves to the right. The right surface terminates in a shorter, broader spine. The macronucleus is elongate and the micronucleus lies on the left of its mid-section. The vacuole is directly anterior to the macronucleus.

The measurements of 10 specimens are summarized in Table 11.

Table 11. Dimensions of Entodinium rostratum

	M ± σ M	σ	C.V.%	Limits of Variation
Length	45.4 ± 1.6	± 5.1	11.2	35 - 54
Width	24.3 ± 0.9	± 2.8	11.5	19 - 27
L/W ratio	1.87 ± 0.05	± 0.16	8.5	1.70 - 2.23
L. macro	28.4 ± 1.6	± 4.9	17.3	19 - 38
W. macro	4.9 ± 0.1	± 0.3	6.1	4 - 5

This organism was at first classified as E. pisciculum Kofoid and MacLennan (1930), but Lubinsky (pers. comm.) suggested that as the left caudal spine was strongly curved, it was better classified as E. rostratum. However, on the basis of the size measurements of the specimens examined here, E. rostratum (Dogiel, 1927, Kofoid and MacLennan, 1930) and E. pisciculum (Kofoid and MacLennan, 1930), and in spite of the absence of the anterior projection found in many specimens of E. pisciculum, this organism may well be E. pisciculum Kofoid and MacLennan.

The measurements of this organism, E. rostratum and E. pisciculum are compared in Table 12.

Table 12. Dimensions of Entodinium rostratum and E. pisciculum

	Entodinium sp.	E. rostratum		E. pisciculum
		Dogiel, 1927	Kofoid and MacLennan, 1930	Kofoid and MacLennan, 1930
Length	45(35-54)	37(27-51)	37(28-41)	47(38-58)
Width	24(19-27)	17(13-23)	20(18-21)	24(22-29)
L/W ratio	1.87 (1.70-2.23)	2.18	1.40 (1.15-1.52)	1.98 (1.76-2.38)

Entodinium indicum Kofoid and MacLennan (1930)

Plate 3, Figure 5

The body is oblong. There are three caudal spines, one each on the left, right and upper surfaces. The rectum opens through a slit-like anus in the base of the upper spine. The macronucleus is small and lies at the anterior end of the right side. The micronucleus lies near the middle of the macronucleus

in a depression in the upper side. The vacuole lies on the upper side of the body, anterior to the macronucleus.

The measurements of 10 specimens are summarized in Table 13.

Table 13. Dimensions of Entodinium indicum

	M ± σM	σ	C.V.%	Limits of Variation
Length	38.9 ± 1.5	± 4.7	12.1	32 - 46
Width	29.6 ± 0.8	± 2.4	8.1	27 - 35
L/W ratio	1.32 ± 0.05	± 0.15	11.4	1.07 - 1.48
L. macro	27.3 ± 1.5	± 4.9	17.9	19 - 38
W. macro	5.0 ± 0.0	± 0.0	0.0	5 - 5

Entodinium ovinum Dogiel (1927)

Plate 3, Figure 1

The body is a regular oval with a flattened anterior end. The macronucleus is long and rod-like, and extends from the anterior end along three-quarters of the right side. The vacuole lies slightly to the left of the macronucleus, near the upper surface.

The measurements of 10 specimens are summarized in Table 14.

Table 14. Dimensions of Entodinium ovinum

	M ± σM	σ	C.V.%	Limits of Variation
Length	59.8 ± 1.6	± 4.9	8.2	51 - 65
Width	35.0 ± 7.9	± 2.5	7.1	32 - 38
L/W ratio	1.70 ± 0.03	± 0.09	5.3	1.59 - 1.84
L. macro	42.9 ± 1.2	± 3.7	8.6	38 - 49
W. macro	10.1 ± 4.7	± 1.5	14.9	8 - 11

GENUS: Epidinium Crawley (1923)

Body elongate and twisted around the main axis; two membranelle zones, the left zone behind the anterior end of the body; main skeletal complex composed of three twisted plates; macronucleus straight and elongate; two contractile vacuoles; up to five caudal spines.

Epidinium caudatum Fiorentini (1889)

Syn. Epidinium ecaudatum forma caudatum Dogiel (1927)

Plate 4, Figure 1

The body is elongate and tapered posteriorly. There is a single, right, caudal spine.

The measurements of 10 specimens are summarized in Table 15.

Table 15. Dimensions of Epidinium caudatum

	$M \pm \sigma M$	σ	C.V.%	Limits of Variation
Length	124.5 ± 2.7	± 8.4	6.7	113 - 138
Width	49.1 ± 1.6	± 5.2	10.6	38 - 54
L/W ratio	2.55 ± 0.08	± 0.24	9.4	2.30 - 3.13
L. macro	54.0 ± 2.3	± 7.2	13.4	43 - 65
W. macro	14.4 ± 0.5	± 1.6	11.1	12 - 16

Epidinium bicaudatum Sharp (1914)

Syn. Epidinium ecaudatum forma bicaudatum Dogiel (1927)

Plate 4, Figure 2

The body is elongate and tapered posteriorly. There are two caudal spines; a large right spine and a smaller left spine.

The dimensions of the only specimen seen during the survey are given in Table 16.

Table 16. Dimensions of Epidinium bicaudatum

Length	113
Width	59
L/W ratio	1.99
L. macro	57
W. macro	14

Epidinium tricaudatum Sharp (1914)

Syn. Epidinium ecaudatum forma tricaudatum Dogiel (1927)

The body is elongate and tapered posteriorly. There are three spines; one right, one left, and one on the upper surface.

The measurements of 10 specimens are summarized in Table 17.

Table 17. Dimensions of Epidinium tricaudatum

	$M \pm \sigma M$	σ	C.V.%	Limits of Variation
Length	123.2 ± 4.5	± 14.2	11.5	97 - 138
Width	53.9 ± 1.9	± 6.0	11.1	49 - 57
L/W ratio	2.16 ± 0.04	± 0.12	5.6	1.97 - 2.37
L. macro	65.6 ± 4.0	± 12.8	19.5	51 - 89
W. macro	14.8 ± 0.2	± 0.6	4.1	14 - 16

Epidinium quadricaudatum Sharp (1914)

Syn. Epidinium ecaudatum forma quadricaudatum Dogiel (1927)

Plate 4, Figure 4

The body is elongate and tapered posteriorly. There are four spines; one right, one left, and two lateral spines on the upper or lower surfaces.

The dimensions of 10 specimens are summarized in Table 18.

Table 18. Dimensions of Epidinium quadricaudatum

	M ± σ M	σ	C.V.%	Limits of Variation
Length	128.9 ± 4.3	± 14.0	10.9	105 - 151
Width	62.1 ± 1.9	± 5.9	9.5	51 - 70
L/W ratio	2.07 ± 0.06	± 0.18	8.7	1.78 - 2.43
L. macro	73.6 ± 3.9	± 12.4	16.8	59 - 89
W. macro	16.1 ± 0.6	± 2.0	12.4	14 - 19

Epidinium parvicaudatum Awerinzew and Mutafowa (1914)

Syn. Epidinium ecaudatum forma cattaneoi Dogiel (1927)

Plate 4, Figure 3

The body is elongate and tapered posteriorly. There are five caudal spines. The position of the three lateral spines is variable. Some individuals have one spine on the upper surface and two on the lower surface but in other specimens the positions of the spines are reversed.

The measurements of 10 specimens are summarized in Table 19.

Table 19. Dimensions of Epidinium parvicaudatum

	M ± σ M	σ	C.V.%	Limits of Variation
Length	115.8 ± 4.2	± 13.3	11.5	100 - 146
Width	54.8 ± 1.8	± 5.8	10.6	49 - 67

.. Continued over

Table 19. Continued ..

	M ± σ M	σ	C.V.%	Limits of Variation
L/W ratio	2.11 ± 0.04	± 0.13	6.2	1.94 - 2.43
L. macro	69.7 ± 3.8	± 12.0	17.2	54 - 92
W. macro	14.8 ± 0.5	± 1.6	10.8	11 - 16

GENUS: Ostracodinium Dogiel, emended Kofoid and MacLennan (1932)

Two membranelle zones; a broad skeletal plate beneath the upper surface, often bending on the left towards the lower side; two to six contractile vacuoles beneath the left surface.

Ostracodinium mammosum Railliet (1890)

Syn. Ostracodinium dentatum Dogiel (1927)

Plate 5, Figure 4

The body is rectangular and relatively short. The skeletal plate narrows towards the posterior of the body. There are three contractile vacuoles and two caudal lobes. The right lobe is hollowed on the left side.

The measurements of 10 specimens are summarized in Table 20.

Table 20. Dimensions of Ostracodinium mammosum

	M ± σ M	σ	C.V.%	Limits of Variation
Length	94.1 ± 2.2	± 6.9	7.3	89 - 108
Width	56.9 ± 1.4	± 4.3	7.6	51 - 65
L/W ratio	1.66 ± 0.02	± 0.05	3.1	1.56 - 1.74
L. macro	57.0 ± 1.5	± 4.8	8.4	51 - 65
W. macro	11.6 ± 0.4	± 1.3	11.2	11 - 14

Ostracodinium dilobum Dogiel (1927)

Syn. Ostracodinium obtusum forma dilobum Dogiel (1927)

Plate 5, Figure 3

The body is more ellipsoidal than that of O. mammosum. The skeletal plate narrows towards the posterior. There are five contractile vacuoles. The two caudal lobes appear similar to those of O. mammosum but the right lobe is not as scoop-shaped.

The measurements of 10 specimens are summarized in Table 21.

Table 21. Dimensions of Ostracodinium dilobum

	$M \pm \sigma M$	σ	C.V.%	Limits of Variation
Length	105.7 ± 1.2	± 3.7	3.5	103 - 113
Width	60.5 ± 1.6	± 4.9	8.1	54 - 65
L/W ratio	1.76 ± 0.05	± 0.15	8.5	1.54 - 2.00
L. macro	63.4 ± 0.8	± 2.6	4.1	59 - 67
W. macro	11.6 ± 0.2	± 0.5	4.3	11 - 12

Ostracodinium rugoloricatum Kofoid and Maclennan (1932)

Plate 5, Figure 5

The body is elongate and somewhat rectangular. The left edge of the skeletal plate turns and extends a considerable distance under the lower side of the body. There are three contractile vacuoles. The single caudal lobe is a continuation of the right surface and is flattened to make the posterior end appear rounded.

The measurements of 10 specimens are summarized in Table 22.

Table 22. Dimensions of Ostracodinium rugoloricatum

	M ± σM	σ	C.V.%	Limits of Variation
Length	102.8 ± 3.8	± 12.0	11.7	78 - 116
Width	60.7 ± 2.8	± 8.9	14.7	51 - 78
L/W ratio	1.71 ± 0.03	± 0.10	5.8	1.45 - 1.95
L. macro	60.3 ± 2.4	± 7.7	12.8	49 - 78
W. macro	12.4 ± 0.4	± 1.4	11.3	11 - 14

Kofoid and MacLennan (1932, p138) stated "The skeletal plate extends laterally from the macronucleus to the ventral side of the body." In none of the specimens examined was this observed; the plate always tapered towards the posterior end of the body.

GENUS: Eudiplodinium Dogiel, emended Kofoid and MacLennan (1932)

A single species.

Eudiplodinium maggii Fiorentini (1889)

Plate 1; Plate 5, Figure 2

The body is large and roughly triangular, and has two membranelle zones. The macronucleus has a large hook on the left at the anterior end. There is a single narrow skeletal plate. There are two contractile vacuoles.

The measurements of 10 specimens are summarized in Table 23.

All of the specimens examined had a pronounced right caudal lobe tapering to a relatively sharp point from a broad base. This lobe is not indicated in the drawings of either Dogiel

(1927) or Kofoid and MacLennan (1932).

Table 23. Dimensions of Eudiplodinium maggii

	M ± σ M	σ	C.V.%	Limits of Variation
Length	167.6 ± 6.2	± 19.5	11.6	149 - 195
Width	114.4 ± 3.5	± 10.9	9.5	103 - 135
L/W ratio	1.47 ± 0.03	0.11	7.5	1.34 - 1.66
L. macro	86.9 ± 5.3	± 16.7	19.2	59 - 116
W. macro	27.6 ± 1.7	± 5.4	19.6	22 - 38

GENUS: Metadinium Awerinzew and Mutafowa (1914)

Body large and heavy; two membranelle zones and two contractile vacuoles; two skeletal plates, sometimes fused at their posterior ends; macronucleus large and elongated with two or three lobes on the left side.

Metadinium medium Awerinzew and Mutafowa (1914)

Syn. Eudiplodinium medium forma medium Dogiel (1927)

Plate 5, Figure 1

The two skeletal plates are not fused. The macronucleus has three lobes. The posterior end of the body is rounded.

The measurements of 10 specimens are summarized in Table 24.

Table 24. Dimensions of Metadinium medium

	M ± σ M	σ	C.V.%	Limits of Variation
Length	194.7 ± 6.9	± 21.9	11.3	162 - 230
Width	109.3 ± 4.1	± 12.9	11.8	86 - 132

Continued over ..

Table 24. Continued ..

	M ± σ M	σ	C.V.%	Limits of Variation
L/W ratio	1.79 ± 0.04	± 0.12	6.7	1.63 - 2.00
L. macro	117.0 ± 5.6	± 17.6	15.0	89 - 143
W. macro	20.2 + 0.73	+ 2.3	11.4	16 - 23

Metadinium tauricum Dogiel and Fedorowa (1925)

Syn. Eudiplodinium medium forma tauricum Dogiel (1927)

This species is similar to M. medium, but the skeletal plates are fused posteriorly. The posterior lobe of the macronucleus is relatively small.

The dimensions of the two specimens seen are summarized in Table 25.

Table 25. Dimensions of Metadinium tauricum

Length	191, 216
Width	97, 119
L/W ratio	1.98, 1.82
L. macro	113, 132
W. macro	19, 19

GENUS: Diplodinium Schuberg, emended Crawley, emended Dogiel, emended Kofoid and MacLennan (1932)

Two membranelle zones; no skeletal plates; macronucleus has anterior third bent to right at angle of 30-90°; two contractile vacuoles. All species have a longitudinal

cuticular fold, which may be inconspicuous, on the upper left body side.

Diplodinium monacanthum Dogiel (1927)

Syn. Anoplodinium denticulatum forma monacanthum Dogiel (1927)

Plate 6, Figure 1

The body is relatively short and heavy, and slightly tapered posteriorly. There is a single, small, right spine.

The measurements of 20 specimens are summarized in Table 26.

Table 26. Dimensions of Diplodinium monacanthum

	M ± σM	σ	C.V.%	Limits of Variation
Length	75.4 ± 1.1	± 5.1	6.8	67 - 84
Width	49.8 ± 0.8	± 3.7	7.4	43 - 54
L/W ratio	1.62 ± 0.04	± 0.17	10.5	1.35 - 1.69
L. macro	36.6 ± 1.0	± 4.5	12.3	30 - 46
W. macro	20.1 ± 1.8	± 5.8	28.9	16 - 24

Diplodinium diacanthum Dogiel (1927)

Syn. Anoplodinium denticulatum forma diacanthum Dogiel (1927)

Plate 6, Figure 2

Similar to D. monacanthum but with two caudal spines, one on the right and one on the left.

The dimensions of 10 specimens are summarized in Table 27.

Table 27. Dimensions of Diplodinium diacanthum

	M ± σM	σ	C.V.%	Limits of Variation
Length	78.2 ± 3.1	± 9.9	12.7	62 - 89
Width	52.8 ± 1.4	± 4.3	8.1	46 - 62
L/W ratio	1.48 ± 0.04	± 0.12	8.1	1.31 - 1.65
L. macro	38.9 ± 2.6	± 8.1	20.8	27 - 51
W. macro	20.9 ± 1.0	± 3.2	15.3	16 - 24

Diplodinium triacanthum Dogiel (1927)

Syn. Anoplodinium denticulatum forma triacanthum Dogiel (1927)

Plate 6, Figure 3

Similar to D. diacanthum but with a third spine on the upper surface.

The dimensions of 10 specimens are summarized in Table 28.

Table 28. Dimensions of Diplodinium triacanthum

	M ± σ M	σ	C.V.%	Limits of Variation
Length	74.0 ± 1.6	± 4.9	6.6	65 - 81
Width	49.5 ± 1.0	± 3.1	6.3	46 - 54
L/W ratio	1.49 ± 0.04	± 0.12	8.1	1.33 - 1.65
L. macro	38.1 ± 0.8	± 2.5	6.5	35 - 40
W. macro	19.2 ± 0.6	± 1.8	9.4	16 - 22

Diplodinium tetracanthum Dogiel (1927)

Syn. Anoplodinium denticulatum forma tetracanthum Dogiel (1927)

Plate 6, Figure 4

Similar to D. monacanthum but with four caudal spines, one on each body surface.

The measurements of 10 specimens are summarized in Table 29.

Table 29. Dimensions of Diplodinium tetracanthum

	M ± σ M	σ	C.V.%	Limits of Variation
Length	78.8 ± 2.8	± 8.8	11.2	62 - 89
Width	52.4 ± 1.7	± 5.4	10.3	40 - 59
L/W ratio	1.51 ± 0.05	± 0.17	11.3	1.41 - 1.65
L. macro	41.6 ± 1.9	± 6.1	14.7	27 - 51
W. macro	21.1 ± 1.1	± 3.6	17.1	14 - 24

Diplodinium pentacanthum Dogiel (1927)

Syn. Anoplodinium denticulatum forma pentacanthum Dogiel (1927)

Plate 6, Figure 5

Similar to D. tetracanthum but with five caudal spines. The fifth spine is on either the upper or lower body surface.

The dimensions of 10 specimens are summarized in Table 30.

Table 30. Dimensions of Diplodinium pentacanthum

	M ± σ M	σ	C.V.%	Limits of Variation
Length	78.0 ± 2.2	± 6.8	8.7	67 - 89
Width	51.9 ± 1.1	± 3.4	6.6	46 - 57
L/W ratio	1.51 ± 0.03	± 0.10	6.6	1.24 - 1.65
L. macro	36.5 ± 1.7	± 5.4	14.8	27 - 46
W. macro	20.5 ± 0.9	± 2.8	13.7	16 - 24

Diplodinium anisacanthum Da Cunha (1914)

Syn. Anoplodinium denticulatum forma anisacanthum Dogiel (1927)

Plate 6, Figure 6

Similar to D. pentacanthum but with six caudal spines. There are two spines on each of the upper and lower surfaces.

The dimensions of 10 specimens are summarized in Table 31.

Table 31. Dimensions of Diplodinium anisacanthum

	$M \pm \sigma M$	σ	C.V.%	Limits of Variation
Length	73.1 ± 1.9	± 6.0	8.2	65 - 86
Width	50.4 ± 1.8	± 5.6	11.1	35 - 57
L/W ratio	1.46 ± 0.07	± 0.21	14.4	1.28 - 1.85
L. macro	36.4 ± 1.6	± 4.9	13.5	30 - 43
W. macro	19.5 ± 0.79	± 2.5	12.8	15 - 22

GENUS: Eodinium Kofoid and MacLennan (1932)

Two membranelle zones on the same level; no skeletal plates; two contractile vacuoles; macronucleus elongate.

Eodinium lobatum Kofoid and MacLennan (1932)

Plate 7, Figure 5

The body is ellipsoidal and has a small posterior lobe. The anterior vacuole lies in a depression of the macronucleus. The posterior vacuole lies at the posterior end of the macronucleus.

The measurements of 10 specimens are summarized in Table 32.

Table 32. Dimensions of Eodinium lobatum

	M ± σ M	σ	C.V.%	Limits of Variation
Length	45.8 ± 1.6	± 4.9	10.7	40 - 59
Width	26.9 ± 0.9	± 2.8	10.4	24 - 32
L/W ratio	1.70 ± 0.05	± 0.16	9.4	1.48 - 2.04
L. macro	27.1 ± 1.7	± 5.4	24.4	22 - 38
W. macro	6.1 ± 0.3	± 1.1	18.0	5 - 8

Eodinium bilobosum Dogiel (1927)

Syn. Anoplodinium postervesiculatum forma bilobosum Dogiel (1927)

Plate 7, Figure 6

The body is stouter than that of E. lobatum. There are two caudal lobes; the right lobe varies between a lobe and a short spine.

The measurements of 10 specimens are summarized in Table 33.

Table 33. Dimensions of Eodinium bilobosum

	M ± σ M	σ	C.V.%	Limits of Variation
Length	42.2 ± 1.3	± 4.0	9.5	38 - 51
Width	31.0 ± 0.9	± 2.9	9.4	27 - 35
L/W ratio	1.37 ± 0.03	± 0.10	7.3	1.23 - 1.50
L. macro	28.1 ± 1.1	± 3.4	12.1	24 - 32
W. macro	5.6 ± 0.2	± 0.5	8.9	5 - 6

Eodinium postervesiculatum Dogiel (1927)

Syn. Diplodinium postervesiculatum Dogiel (1927)

Plate 7, Figure 7

Similar to E. lobatum, but with the posterior end smoothly rounded.

The dimensions of 10 specimens are summarized in Table 34.

Table 34. Dimensions of Eodinium postervesiculatum

	M ± σ M	σ	C.V.%	Limits of Variation
Length	52.8 ± 1.0	± 3.2	6.1	49 - 57
Width	30.9 ± 0.5	± 1.7	5.5	27 - 35
L/W ratio	1.71 ± 0.04	± 0.11	6.4	1.54 - 1.89
L. macro	28.9 ± 0.8	± 2.4	8.3	24 - 32
W. macro	10.0 ± 0.5	± 1.7	17.0	6 - 12

GENUS: Eremoplastron Kofoid and MacLennan (1932)

Two membranelle zones; macronucleus triangular or rod-like; a single narrow skeletal plate; two contractile vacuoles.

Eremoplastron rostratum Fiorentini (1889)

Syn. Eudiplodinium rostratum Dogiel (1927)

Plate 7, Figure 4

The body is relatively long and has one large caudal spine. The micronucleus lies in the middle of the left edge of the macronucleus.

The measurements of 10 specimens are summarized in Table 35.

Table 35. Dimensions of Eremoplastron rostratum

	M ± σ M	σ	C.V.%	Limits of Variation
Length	52.6 ± 1.8	± 5.8	11.0	43 - 59
Width	29.0 ± 0.7	± 1.8	6.2	27 - 30
L/W ratio	1.81 ± 0.04	± 0.12	6.6	1.59 - 1.97
L. macro	26.2 ± 1.3	± 4.2	16.0	22 - 30
W. macro	7.4 ± 0.3	± 0.9	12.2	6 - 8
L. spine[1]	17.2 ± 0.7	± 2.1	12.2	16 - 22

1. Length of spine.

Eremoplastron bovis Dogiel (1927)

Syn. Anoplodinium neglectum forma bovis Dogiel (1927)

Plate 7, Figure 1

The body is rectangular to ellipsoidal. There is a small caudal lobe. The micronucleus lies in the middle of the left edge of the macronucleus.

The measurements of 10 specimens are summarized in Table 36.

Table 36. Dimensions of Eremoplastron bovis

	M ± σ M	σ	C.V.%	Limits of Variation
Length	84.5 ± 2.5	± 8.0	9.5	73 - 94
Width	47.5 ± 1.5	± 4.8	10.1	38 - 51
L/W ratio	1.79 ± 0.05	± 0.16	8.9	1.59 - 2.00
L. macro	42.9 ± 1.8	± 5.6	13.1	35 - 51
W. macro	13.4 ± 0.3	± 1.1	8.2	11 - 14

Eremoplastron brevispinum Kofoid and MacLennan (1932)

Plate 7, Figure 2

The body is ellipsoidal with two short caudal spines. The macronucleus is rod-shaped. The endoplasmic sac extends into the base of the left spine.

The measurements of 10 specimens are summarized in Table 37.

Table 37. Dimensions of Eremoplastron brevispinum

	M ± σ M	σ	C.V.%	Limits of Variation
Length	80.7 ± 2.8	± 7.1	8.8	73 - 92
Width	51.1 ± 0.8	± 2.4	4.7	49 - 54
L/W ratio	1.58 ± 0.03	± 0.08	5.1	1.49 - 1.74
L. macro	40.1 ± 1.6	± 5.2	13.0	32 - 49
W. macro	12.2 ± 0.7	± 2.3	18.9	8 - 14

Eremoplastron monolobum Dogiel (1927)

Syn. Eudiplodinium neglectum forma monolobum Dogiel (1927)

Plate 7, Figure 3

The body is ovoid to roughly spherical. There is a prominent right lobe, and an inconspicuous short lobe on the left. The macronucleus is a thick rod.

The measurements of 10 specimens are summarized in Table 38.

Table 38. Dimensions of Eremoplastron monolobum

	M ± σ M	σ	C.V.%	Limits of Variation
Length	55.3 ± 1.2	± 3.7	6.7	49 - 59
Width	38.6 + 0.7	+ 2.2	5.7	35 - 40

Continued over ..

Table 38. Continued ..

	$M \pm \sigma M$	σ	C.V.%	Limits of Variation
L/W ratio	1.43 ± 0.04	± 0.12	8.4	1.22 - 1.55
L. macro	23.2 ± 0.9	± 2.9	12.5	19 - 27
W. macro	10.1 ± 0.3	± 0.9	8.9	8 - 11

Discussion

The system of classification of the oligotrichs developed by Kofoid and MacLennan (1930, 1932, 1933) and Kofoid and Christenson (1934), and used here, is probably the most useful scheme available at present. These authors removed the sub-genera created within the genus Diplodinium by Dogiel (1927), and created species of many of the sub-species. In view of the variation within clones of certain oligotrichs noticed by Poljansky and Strelkow (1934), Hungate (1942), the present author (see later) and others, their classification is obviously faulty in places, but remains a useful and practical system.

Ideas on the phylogenetic relationships of the rumen ciliates have changed since the first taxonomic studies were made. Many new ciliates have been discovered and techniques have been improved, and as a result, the taxonomy of the rumen ciliates, particularly the oligotrichs, is in urgent need of revision. Several systems of classification of the oligotrichs are in current use, notably those of Dogiel (1927), Mangold

(1929) and Kofoid and MacLennan (1930, 1932, 1934), and the use of these various systems has led to considerable confusion. Further difficulties have arisen with the discovery of new species, and with new, isolated observations on taxonomy by authors such as Wertheim (1935a, b), Kraschenninnikow (1955) and Zielyk (1961). Because of this lack of order many workers with the rumen ciliates have failed to establish fully the identity of the organisms they were studying. If any studies on the rumen ciliates are to be fully appreciated by other workers in the field, it is important that the name of the ciliate used is fully documented.

Lubinsky in the University of Manitoba, is at present revising the taxonomy of the rumen oligotrichs. He has already published on intra-specific forms of some entodinia (Lubinsky, 1957a) and on the fauna of the Canadian reindeer (Lubinsky, 1958a, b), and has considered evolutionary trends and comparative morphology within the Ophryoscolecidae (Lubinsky, 1957b, c, d).

The fauna identified in the four cows examined here is a typical selection of the ciliates found in ruminants elsewhere. No new species were found which is not surprising in view of the fact that ruminants were introduced to this country only a century ago. The occurrence of Charon equi in the bovine rumen is of interest, especially as it may constitute up to 12% of the ciliates. This is the first reported occurrence of this ciliate outside the colon of the horse (Hsiung, 1930), although Jameson (1925) found the very similar Charon ventriculi in the

rumen of cattle and sheep in England. This is also the first report of the occurrence of a ciliate in both a ruminant and a mono-gastric animal. The relatively high population of Buetschlia (3.4%) in one cow is also worthy of note. Since Schuberg first described this organism in 1888 it has been reported on only a few occasions. Kofoid and MacLennan (1933) reported the occasional occurrence of this ciliate in Bos indicus, and Sharp (1914) implied that it occurred in cattle in California. Becker and Talbot (1927) did not find it in any of 26 cattle in Iowa, and it was not found by Kofoid and Christenson (1934) in a single specimen of Bos gaurus.

The composition of the fauna, depending as it does on the diet of the host, cannot be critically compared with that of ruminants elsewhere. Even in this country the fauna of ruminants undoubtedly varies between areas, although it is probably composed mainly of the species described here. The general similarity of the fauna in different types of bovine the world over is shown in Table 39 where the fauna of Bos indicus and B. gaurus are compared with the fauna of cattle studied here. It is obvious that Entodinium spp. commonly constitute a large proportion of the population.

The absence of members of the genera Polyplastron Dogiel (1927) and Ophryoscolex Stein (1858), not common in Bos indicus or B. gaurus, but common in some circumstances in other countries (see Eadie, 1962a, b), is worthy of note. These organisms are also absent from sheep and the examination of the

Table 39. Approximate percentage generic composition of ciliates in Bos indicus, B. gaurus and New Zealand cattle

Genus	Bos indicus Kofoid and MacLennan (1930, 1932, 1933)	Bos gaurus Kofoid and Christenson (1934)	N.Z. cattle Mean of 4 cows from Table 40
Entodinium	38	52	30
Eodinium	+	+	5
Eremoplastron	6	+	24
Epidinium	1	3	15
Eudiplodinium	3	3	2
Metadinium	2	2	+
Ostracodinium	3	6	2
Diplodinium	9	12	4
Elytroplastron	1	-	-
Ophryoscolex	+	-	-
Polyplastron	-	-	-
Holotrichs	38	21	17

+ = less than 1%

- = not present

fauna of our non-domesticated ruminants, goats, deer, chamois and thar, for these two genera alone, apart from the other recognized genera not found in New Zealand (Enoploplastron Kofoid and MacLennan (1932), Elytroplastron Kofoid and MacLennan (1932), Epiplastron Kofoid and MacLennan (1932) and Diploplastron Dogiel and Fedorowa (1925)), is well warranted. Most of the wild ruminants feed on materials very different from those utilized by our sheep and cattle and may well harbour different ciliate populations.

The measurements recorded here of some of the specimens of both holotrichs and oligotrichs differ slightly from those recorded by others. These differences are small however, and are probably a normal consequence of different diet and environmental conditions (see Lubinsky (1957a)).

B. OCCURRENCE AND POPULATION DENSITY

Introduction

Only a few authors have made quantitative analyses of rumen ciliate populations in cattle although there is a considerable literature on the fauna of sheep. Kofoid and MacLennan (1930, 1932, 1933) and Kofoid and Christenson (1934) gave only the percentage composition of the fauna of *Bos indicus* and *B. taurus* respectively. Becker and Talbot (1927) did not estimate the ciliates in the cattle they examined.

Up to two million ciliates per gram of rumen contents of both cattle and sheep have been reported, but Oxford (1955) pointed out that because of the methods of counting used few such counts were likely to be accurate and discussed the difficulties involved in obtaining an accurate count. Since 1955, however, much work has been carried out on counting techniques, and statistical analysis has shown that present-day techniques are capable of yielding very accurate results (Moir and Somers, 1956; Boyne, Eadie and Raitt, 1957; Purser and Moir, 1959; Warner, 1962b).

Sampling from the rumen is difficult and, unless great care is taken in obtaining the sample of rumen contents, the ciliate counts, however accurate the counting technique, may not give a true indication of the rumen population. Considerable stratification of feed material occurs in the rumen of cattle (Pearson and Smith, 1943; Smith et al., 1956) and makes the sampling procedure difficult if comparable samples are required

on different occasions. Difficulties also arise if the rumen material is sieved or strained to remove large particles of plant material. Large organisms are easily trapped in the mat of plant debris even if the pore size of the straining material is large enough to allow their passage.

The errors arising from stratification of the ingesta can be minimized if the animal is on a strict feeding regime and if the sample is always removed at the same time relative to the time of feeding, from the same position in the rumen. A standard sampling time also eliminates errors that might arise if the number of ciliates varies throughout the day. Purser and Moir (1959) and Nottle (1956) have demonstrated a diurnal cycle for Entodinium and Purser (1961) reported a cycle for holotrichs in sheep.

Oxford (1955) commented on the value of protozoa counts and said that (p575) "Since only certain kinds of rumen ciliates seem to have intense fermentative activity the differential count may be rather more important than the total count ....." Both holotrichs and oligotrichs have now been shown to be active fermenters and the first part of this statement can perhaps be disputed, but the differential count certainly remains a most useful assessment of the rumen population in most circumstances. The differential count is easier to perform than the total count provided that large organisms are not lost during removal of plant material from the sample.

Information on the composition and size of the rumen ciliate population in six cows is given in this section.

Differential counts of Epidinium species are presented separately in the following section.

Materials and Methods

The cows used (numbers 294, 90, 35, 30, 29, 2) were rumen-fistulated, and stall-fed, Monday to Friday inclusive, on either fresh red clover (Trifolium pratense L.), grass hay, or red clover hay. On Saturday and Sunday the animals were turned out to pasture. Animals 29 and 30 were identical twins.

All samples were taken before the morning feed after overnight starvation (16-17 hr.). About one litre of rumen contents was removed through the fistula from about six inches below the surface of the ingesta and strained through gauze. The sample was shaken to suspend settled protozoa, and 100 ml. was mixed with an equal volume of 10% (v/v) formalin. This suspension was further diluted, usually by the addition of 10 ml. to 50-100 ml. of 5% (v/v) formalin containing 0.05% methyl green. Occasionally the protozoa were centrifuged from the first formalin preparation and resuspended in 0.05% aqueous methyl green.

Counting cell. The counting cell was based on that used by Adam (1951) and on the MacMaster eelworm cell, and was constructed from glass microscope slides as described by Boyne, Eadie and Raitt (1957). The cell was 1 mm. deep and held approximately 1 ml. of fluid. The sample was added rapidly from a wide-mouth 1 ml. serological pipette to ensure an even distribution of protozoa and to minimize the formation of air bubbles.

Method for total counts. The protozoa in 20 fields were counted, the fields being randomly selected in four rows of five across the slide. The cell was refilled for the duplicate count which was carried out on each of the two samples. Thus, for each daily count, the protozoa in 80 fields were counted and the totals averaged.

Method for differential counts. A sample in formalin was centrifuged and suspended in formalin containing 0.05% methyl green as described above. The ciliates were mounted under a cover glass and the preparation was sealed with nail varnish. 500 ciliates were identified in each sample.

Experimental and Results

The distribution of the ciliate genera in four cows is shown in Table 40. The identical twins, 29 and 30, were used to assess the type of fauna-change occurring with a change of diet. Both cows were sampled when established on a diet of fresh red clover and were sampled a second time two weeks after the diet had been changed to grass hay. The results are included in Table 40. The distribution of the oligotrich species in the same four cows, on the same four occasions, is shown in Table 41.

Total counts of rumen protozoa were made on 167 occasions between November, 1959, and December, 1960. Cow 294 was sampled until September, 1960, but as this animal was not available after that date cow 90 was used until the end of the

experiment. From November, 1959 to May, 1960 inclusive, and in November and December, 1960, the cows were stall fed on fresh red clover. From June to October, 1960, inclusive, both cows were fed on red clover hay.

The results of the total counts, as mean totals for each month, are given in Table 42. The counts ranged from 1.1×10^5 to 1.9×10^6/ ml. rumen liquor. Table 43 shows the mean totals for the two cows on the two diets.

In October and November, 1961, epidinial suspensions could not be obtained from rumen liquor by the method of Oxford (1958a). The total population of oligotrichs appeared to be normal but epidinia could not be separated in large numbers, the few obtained being sluggish and inactive. A short series of counts was carried out to establish the size of the epidinial population. Total protozoa were also estimated. The total ciliate population was found to be abnormally low as were the numbers of Epidinium. The results are shown in Table 44.

Discussion

The errors attached to the method for making total counts were similar to those found by Boyne, Eadie and Raitt (1957). The method of counting used, together with a method utilizing 10 samples from the animal instead of two, and with five fields counted at each filling of the cell instead of 20, were statistically analysed. The analyses showed, for both methods, that dilution and sampling of the initial diluted suspension did not

Table 40. Distribution of ciliate genera in four cows as a percentage of the total ciliates*

Genus	Cow:	294	35	29	30	29	30
	Diet:	Grass Hay		Fresh Clover		Grass Hay	
	Date:	9.8.62	11.9.62	26.2.63		1.6.63	
Entodinium		32.6	33.6	35.4	19.0	8.0	24.2
Eodinium		9.4	5.8	1.6	4.4	14.4	12.8
Eremoplastron		27.2	25.8	22.4	21.4	23.2	32.4
Epidinium		0.9	12.0	22.0	25.4	6.4	3.4
Eudiplodinium		1.9	1.0	2.2	2.0	3.0	4.0
Metadinium		0.2	0.2	-	0.6	0.8	0.4
Ostracodinium		3.4	2.6	1.2	2.2	4.2	3.6
Diplodinium		12.5	3.2	-	0.2	-	0.4
Isotricha		6.0	7.6	4.2	4.2	1.6	5.4
Dasytricha		5.5	7.8	10.8	17.2	23.2	9.6
Buetschlia		0.2	0.2	0.2	3.4	2.4	0.6
Charon		0.2	0.2	-	-	12.8	3.2

* 500 ciliates identified

- = Not seen during the count.

Table 41. Species of ophryoscolecid ciliates in four cows as a percentage of the total oligotrichs*

Organism	Cow:	294	35	29	30	29	30
	Diet:	Grass	Hay	Fresh Clover		Grass	Hay
	Date:	9.8.62	11.9.62	26.2.63		1.6.63	
Entodinium ovinum		6.8	2.6	3.4	3.0	1.6	4.0
E. biconcavum		18.8	19.6	20.0	14.8	3.2	14.8
E. bicarinatum		6.4	3.4	1.2	2.0	1.6	3.2
E. longinucleatum		1.4	2.4	5.8	2.4	4.0	3.4
E. indicum		0.8	3.4	2.8	1.6	2.4	2.0
E. rostratum		2.8	8.4	6.2	0.2	2.4	2.2
Eodinium lobatum		6.8	2.8	1.0	4.2	16.8	8.6
E. bilobosum		2.8	3.0	0.8	1.2	2.4	5.2
E. posterovesiculatum		1.0	0.2	-	-	1.6	1.6
Eremoplastron bovis		20.8	18.0	10.0	11.2	16.0	14.8
E. brevispinum		8.4	6.4	6.2	7.4	15.2	2.8
E. monolobum		3.0	5.2	6.0	4.6	8.8	11.2
E. rostratum		1.0	3.4	4.8	4.6	4.8	12.0
Eudiplodinium maggii		2.2	1.2	3.2	3.2	4.6	4.6
Metadinium medium		0.2	0.2	-	0.2	0.8	0.4
M. tauricum		0.2	-	-	-	-	-
Epidinium caudatum		0.4	4.6	13.6	18.6	4.0	0.8
E. bicaudatum		0.2	-	0.6	-	1.6	-
E. tricaudatum		0.2	0.2	1.4	1.4	0.8	1.2
E. quadricaudatum		0.6	6.0	8.0	12.0	3.2	2.6
E. parvicaudatum		0.2	2.6	3.6	3.6	-	-
Ostracodinium mammosum		0.8	0.6	0.8	2.0	2.0	2.0
O. dilobum		0.6	1.0	0.4	1.4	0.8	1.4
O. rugoloricatum		2.4	1.4	0.2	0.2	1.4	0.8
Diplodinium monacanthum		4.4	2.8	-	0.2	-	-
D. diacanthum		0.6	0.2	-	-	-	0.4
D. triacanthum		2.6	-	-	-	-	-
D. tetracanthum		1.4	0.2	-	-	-	-
D. pentacanthum		0.6	0.2	-	-	-	-
D. anisacanthum		1.6	-	-	-	-	-

* 500 ciliates identified

\- = Not seen during the count.

All totals are X 10^{-5}/ ml. rumen liquor

Month	Diet	Total Ciliates		Range		No. of Counts	
		Cow 294	Cow 90	Cow 294	Cow 90	Cow 294	Cow 90
November, 1959	Fresh clover	3.7	-	2.7 - 5.8	-	12	0
December	"	3.3	-	2.5 - 4.4	-	12	0
January, 1960	"	8.9	-	2.1 -16.4	-	10	0
February	"	11.4	-	6.3 -19.7	-	10	0
March	"	9.1	-	4.0 -13.9	-	16	0
April	"	-	-	-	-	0	0
May	"	3.8	-	2.8 - 4.9	-	18	0
June	Clover hay	2.5	-	1.9 - 3.5	-	21	0
July	"	2.3	-	1.6 - 2.8	-	17	0
August	"	2.3	-	1.4 - 3.2	-	23	0
September	"	2.4	2.2	1.1 - 4.0	1.3 - 3.6	18	11
October	"	-	2.2	-	1.8 - 2.5	0	11
	Fresh clover	-	2.1	-	1.9 - 2.4	0	7
November	"	-	2.3	-	1.9 - 2.7	0	14
December	"	-	2.4	-	1.8 - 2.8	0	12

Table 43. Mean numbers of protozoa in two cows on fresh clover and clover hay diets

	Cow 294 November, 1959 to September, 1960		Cow 90 September to December, 1960	
	Mean No./ml. X 10^{-5}	No. of Counts	Mean No./ml. X 10^{-5}	No. of Counts
Clover	7.3	78	3.3	33
Hay	2.4	79	2.2	22

Table 44. Epidinia and total ciliates in cow 2 during October and November, 1961

All figures are X 10^{-5}/ ml. rumen liquor. Figures in brackets are percentages of total ciliates.

Date	No. of Epidinia	No. of Ciliates
2.10.61	0.10 (9)	1.1
3.10.61	0.18 (12)	1.5
6.11.61	0.17 (9)	1.9
8.11.61	0.09 (10)	0.9
13.11.61	0.06 (7)	0.9

contribute any error. In the 10 sample, 5 field method the main source of variation was in the count itself. The differences between samples contributed an appreciable amount to the total variance. Partly because of stratification in the rumen of cattle there is an uneven distribution of protozoa, and the concentration in any one spot changes with time because of the normal churning action of the rumen. Both of these phenomena may cause considerable variation in duplicate samples unless relatively large samples are taken (e.g., 1 l.). Boyne, Eadie and Raitt (1957) found that in a sheep on a constant diet, day-to-day differences in the population were greater than differences throughout the rumen at any one time and the variations resulting from technique. This situation may not hold for cattle as there is more stratification in the rumen of cattle than in sheep, and hence a greater possibility of variation in the ciliate population in different areas (Warner, 1962b).

The total population of ciliates varied considerably, the highest recorded count (1.97×10^6/ ml) being over 20 times as great as the lowest count (0.9×10^5/ ml.). In the two cows examined over 14 months there were more ciliates present while the animals were feeding on fresh clover than on clover hay, although the mean count for cow 294 on fresh clover is exaggerated by the relatively few high counts recorded in the late summer of 1960. As the counting method was reasonably accurate these high counts recorded in early 1960 were presumably genuine. The ciliate population in the rumen does seem to be able to reach

1-2 x 10^6/ml. rumen liquor.

There are few figures available for the ciliate populations of cattle, most of the counts in the literature being concerned with sheep. Winogradowa-Fedorowa and Winogradoff (1929) and Quinn, Burroughs and Christiansen (1962) reported up to 110,000 and 100,000 ciliates/ml. respectively in the rumen of cattle. Putnam, Gutierrez and Davis (1961) reported only 30,000/ml. in calves.

The increase in ciliate numbers when the animal changes from a hay diet to a diet of fresh clover cannot be ascribed solely to an increase in one type of ciliate as might be expected. The population of Epidinium does rise but the increase is not great. The change in diet causes an increase of most of the ciliate genera. Cows on diets of hay and fresh forage have a similar intake on a dry weight basis so it is hard to explain the population increase following a change from hay to fresh clover unless the increase in certain constituents of the clover is significant for all of the species.

The quantitative examination of the ciliate genera showed that three genera of oligotrichs, Entodinium, Eremoplastron and Epidinium, make up the bulk of the oligotrich population (Table 40). Entodinium and Eremoplastron constituted fairly constant proportions of the total irrespective of the two diets tested, but Epidinium increased, mainly at the expense of Eodinium, when the host's diet was changed from grass hay to fresh clover. Holotrichs averaged 21% (12-40) of the total population, the

proportion being independent of diet. The increase in Dasytricha and the accompanying decrease in Entodinium in cow 29 on 1 June 1963 cannot be explained.

C. SEASONAL VARIATION IN EPIDINIUM

Introduction

Epidinium Crawley (1923), a large oligotrich ciliate, was described by Dogiel (1927) from rumen contents of sheep, cattle, reindeer, camels and goats in Asia and Africa, but was apparently more common in cattle than in the other animals. Many other authors have described Epidinium from bovine rumen contents. Oxford (1958a) found it to be common in cattle in N.E. Scotland but never encountered it in sheep. The genus has a wide geographical distribution in a number of ruminants but does not seem to occur in large numbers. Gutierrez and Davis (1962) though, made cultural studies with epidinia from a cow in which it was the only large ciliate.

The occurrence of Epidinium in the rumen of New Zealand cattle and sheep was first recognized by Oxford (1958a). During studies on the culture and metabolism of these ciliates, Oxford confirmed that they did not seem to ingest cellulose fibres (Dogiel, 1927), but ingested plant starch (Lubinsky, 1957b), and showed that they ingested whole or damaged chloroplasts of red clover (Trifolium pratense L.).

Bloat is a disease characterized by excessive foaming of the rumen contents and by retention of gas in the rumen, and in New Zealand occurs when fresh forage, especially red clover, is fed (Johns, 1954). Mangan (1959) demonstrated that clover chloroplasts, which contain a high concentration of lipids (Neish,

1939), have antifoaming properties, and suggested that they might play a part in the aetiology of bloat. The ingestion of whole chloroplasts by epidinia would lower the lipid concentration in rumen liquor and could effectively lower the antifoaming potential.

Fresh clover, the best forage for provoking the onset of bloat, is characterised by a relatively high starch content (Bailey, 1958c). Grass contains relatively little starch before the flowering stage is reached (Percival, 1952; Bailey, 1958c). Because the amount of starch in clover can vary from time to time, (Bailey, 1958c), and because Epidinium ingests clover starch grains (Oxford, 1958a), Bailey considered that changes in the starch in clover might result in changes in the epidinial population, and suggested (Bailey, 1958a, p832) that "An investigation into the relationship of epidinia activity in the rumen and the changes in the amount of starch in clover in relation to bloat seems worthwhile."

The observation of Oxford (1958a, 1959) that Epidinium was the predominant oligotrich in cows fed on fresh red clover and that it became less than dominant when grass or hay was fed also suggested that this ciliate might influence the onset of bloat.

Epidinia and total ciliates were counted to determine the relative numbers in cows fed on fresh clover and clover hay, and so assess whether they might play a part in the aetiology of bloat by virtue of an increase in numbers. The results of these

counts with an analysis of the numbers of epidinia present on days when bloat did, and did not occur are reported in this section.

Materials and Methods

For the two cows, 294, 90, used during the period November, 1959 to December, 1960, the feeding regime was as described in the previous section. Cow 2 was fed on fresh red clover during the sampling period.

The preparation of samples and the counting technique were also as already described.

Experimental and Results

The populations of Epidinium as both total epidinia and percentages of the total ciliate populations in cows 294 and 90 during the period November, 1959 to December, 1960, are shown in Table 45. Table 46 shows the mean totals of epidinia in the two cows on fresh clover and clover hay diets for the same period. The population figures for Epidinium on "bloat" days are given in Table 47. The means of the counts of "bloat" and "non-bloat" days are shown in Table 48. The mean for "non-bloat" days is the mean of the appropriate epidinial counts while the cows were on a diet of fresh clover. "Bloat" and "non-bloat" days are days on which bloat did, and did not occur, respectively, as measured by visual observation (Johns, 1954).

Table 45. Mean monthly totals of epidinia in the rumen of cows 90, 294

All totals are X 10^{-5}/ ml. rumen liquor. Figures in brackets are percentages of total ciliates.

Month	Diet	Total Epidinia		Range		No. of Counts	
		Cow 294	Cow 90	Cow 294	Cow 90	Cow 294	Cow 90
November, 1959	Fresh clover	1.7 (45)	-	0.9 - 1.9	-	12	0
December	"	1.1 (34)	-	0.7 - 1.5	-	12	0
January, 1960	"	2.5 (28)	-	0.6 - 5.6	-	10	0
February	"	3.5 (31)	-	1.6 - 4.9	-	10	0
March	"	3.6 (40)	-	1.5 - 6.0	-	16	0
April	"	-	-	-	-	0	0
May	"	1.0 (26)	-	0.7 - 1.4	-	18	0
June	Clover hay	0.8 (33)	-	0.6 - 1.2	-	21	0
July	"	0.8 (34)	-	0.5 - 1.0	-	17	0
August	"	0.8 (33)	-	0.5 - 1.2	-	23	0
September	"	0.7 (29)	0.7 (31)	0.4 - 1.1	0.4 - 1.5	18	11
October	"	-	0.8 (36)	-	0.5 - 1.2	0	11
	Fresh clover	-	0.8 (39)	-	0.7 - 1.0	0	7
November	"	-	0.9 (37)	-	0.7 - 1.1	0	14
December	"	-	0.7 (30)	-	0.5 - 0.9	0	12

Table 46. Mean totals of epidinia in cows 294, 90, on fresh clover and clover hay diets for the period November, 1959 to December, 1960

All totals are X 10^{-5}/ ml. rumen liquor.

	Cow 294			Cow 90		
	Mean Total	Range	No. of Counts	Mean Total	Range	No. of Counts
Clover	2.2	0.7 - 6.0	78	0.8	0.5 - 1.1	33
Hay	0.8	0.4 - 1.2	79	0.8	0.4 - 1.5	22

Table 47. Numbers of epidinia in cows 294, 90, before feeding, on days on which bloat occurred

All totals are X 10^{-5}/ ml. rumen liquor.

Cow 294		Cow 90	
Date	No. of Epidinia	Date	No. of Epidinia
11.11.59	1.3	17.10.60	0.7
12.11.59	1.6	2.11.60	1.1
13.11.59	1.6	4	0.8
15	0.9	9	0.9
20	1.2	10	0.8
25	1.2	14	1.0
26	1.3	18	0.7
27	1.8	29	0.9
30	1.9	1.12.60	0.8
7.12.59	1.0	2	0.6
18	1.2	5	0.7
21	0.7	7	0.8
23	0.7	8	0.9
16.2.60	1.6	9	0.5
17	3.5	12	0.6
18.2.60	2.5	13	0.6
		14	0.7
		15	0.6

Table 48. Mean counts of epidinia on "bloat" and "non-bloat" days. Figures in brackets are numbers of counts. Totals are X 10^{-5}/ ml. rumen liquor

	Cow 294	Cow 90
Bloat	1.5 (16)	0.8 (18)
Non-bloat	2.3 (62)	0.8 (15)

Discussion

The observation of Oxford (1958a) that Epidinium may become the dominant oligotrich in cows fed on fresh red clover is largely upheld by the results described here. The counts carried out over a period of 14 months showed that epidinia constituted up to 55% of the total ciliate population although the mean monthly figure was considerably lower. The proportions of the other oligotrichs were not determined during these counts. The more detailed estimations of the fauna of cows 29 and 30 on a red clover diet (Table 40) showed that Epidinium constituted 22% and 25% of the total population, but that Entodinium and Eremoplastron were present in similar or greater numbers. In cow 29 on 26 February 1963, Entodinium and Epidinium constituted 35% and 22% of the population respectively. It appears that Epidinium may, on occasions, be the dominant oligotrich, and in fact the dominant ciliate, in cows on a diet of fresh red clover, but that on some occasions Entodinium and Eremoplastron may be present

in similar or greater numbers.

Oxford's (1958a, 1959) observation that Epidinium was sometimes dominant, was apparently based on observations of ciliates collected by settling in a buffer solution. Only a small percentage of the entodinia was collected during this procedure and the numbers of epidinia were probably over-estimated. It would not be possible to assess whether apparent changes in the population of Epidinium were the result of either an actual change, or a change in the population of other oligotrichs. On the other hand, epidinia in rumen liquor from a hay-fed cow often cannot be collected by settling. The epidinia in cows fed on grass hay are relatively devoid of plant starch grains and storage material, and are apparently not heavy enough to settle rapidly under gravity.

There appears to be no justification for assuming that bloat is caused by removal of chloroplast-contained anti-foaming materials by increased numbers of epidinia. These ciliates may certainly increase in numbers when the host changes from a clover hay diet to one of fresh clover, but the counts made before feeding on days when bloat occurred were not higher than those recorded on "non-bloat" days. This is reflected in the mean counts for "bloat" and "non-bloat" days in Table 48. The mean figure for epidinia in cow 294 on "non-bloat" days is higher than that for "bloat" days. This may be partly because of the high total counts recorded at this time. Bloat occurred on only two of the days when these high counts were recorded (17 February

1960, 3.5 X 10^5; 18 February 1960, 2.5 X 10^5). Epidinia may remove lipids by ingesting whole chloroplasts, and hence increase the foaming potential of rumen liquor, but they certainly do not play a major part in the aetiology of bloat by virtue of an increase in numbers at the time when bloat occurs.

The suggestion of Bailey (1958a) that epidinial activity in the rumen might be related to the amount of starch in clover seems well founded. In the late spring and early summer of 1961, when the weather was very hot and drought conditions prevailed, epidinia contributed only about 10% of the ciliate population (Table 44). During this period the starch content of the clover was probably abnormally low (R. W. Bailey, pers. comm.). Similarly the numbers of epidinia found in cows feeding on fresh clover, good quality clover hay and grass hay were of the same order as the starch content of these materials. Grass hay, which is very low in starch, supported a population containing only a few percent of epidinia, while clover hay and fresh clover, which contain greater amounts of starch (R. W. Bailey, pers. comm.) supported populations containing up to 40% and 55% epidinia respectively.

The diet of the animal influences the total ciliate population as well as that of the epidinia. The total population increased when the cows were fed on fresh red clover following clover hay, and the mean totals for fresh clover were higher than those for clover hay (Table 43). Similarly, during the hot dry weather in late 1961, the decrease in epidinia was accompanied

by a decrease in the total ciliate population. There have been many reports of protozoal population changes following dietary changes by the host. Mowry and Becker (1930) demonstrated a ten-fold increase of protozoa to 2 x 10^6/ ml. rumen liquor in goats following the addition of starch and protein to a diet of hay. Van der Wath (1942) found a seasonal variation in the population of protozoa in sheep. Winter grass supported 10^5/ ml., while the population increased to about 5 x 10^5/ ml. when the sheep grazed summer grass.

2. THE CULTURE OF SOME RUMEN CILIATES

Introduction

Investigations into the biochemistry of rumen protozoa have been hampered by the lack of pure cultures and most studies have been made on washed suspensions prepared from rumen contents. Studies have been confined to those ciliates which, through some dietary or environmental factor, have been dominant, or present in large numbers, in a particular animal (see Eadie and Oxford, 1957; Eadie, 1959; Abou Akkada and Howard, 1960). As a result of this limitation, the biochemistry of many rumen ciliates has yet to be investigated.

In order to make preparations of single species of rumen ciliates available for biochemical studies, many attempts have been made to grow them *in vitro*. The attempts made before 1929 were reviewed by Becker, Schultz and Emmerson (1929). Since then unsuccessful attempts to culture oligotrichs have been made by many workers including Margolin (1930), Westphal (1934), Sugden (1953), Oxford (1958a) and Quinn, Burroughs and Christiansen (1962). The establishment of a culture requires the maintenance of an increasing population of ciliates. A decrease in numbers resulting in the total loss of the ciliates implies a failure to meet all the cultural requirements even if this loss occurs after a period of several weeks. Coleman (1960) demonstrated that some dietary treatments may result in the death of protozoa in cultures after nearly 50 days. The achievement of Kandatsu and

Takahashi (1956; see also 1955a, b) in maintaining Entodinium spp. for 30 days, with some division, did not fulfil all the requirements of a true culture.

The only successful attempts at maintaining rumen oligotrichs in vitro were those of Hungate (1942, 1943), Coleman (1958, 1960) and Gutierrez and Davis (1962). Hungate cultured Eudiplodinium neglectum (Dogiel, 1927; Eremoplastron spp., Kofoid and MacLennan, 1932) for 22 months (Hungate, 1942), and extended the technique (Hungate, 1943) to the culture of Eudiplodinium maggii, Polyplastron multivesiculatum, Diplodinium dentatum (see Kofoid and MacLennan, 1932) and Entodinium caudatum. Coleman (1958, 1960) cultured Entodinium spp., principally E. caudatum, for over 18 months and Gutierrez and Davis (1962) maintained Epidinium ecaudatum Crawley (1923) for six months.

According to Sugden and Oxford (1952), theirs was the first published attempt to maintain holotrich rumen ciliates in vitro. They maintained holotrichs, with division, for one month. Subsequent attempts by other workers have not been as successful. Gutierrez (1955, 1958) maintained isotrichs for only 4 and 6 days, but was able to culture Dasytricha ruminantium for 2 weeks (Gutierrez and Hungate, 1957). Quinn, Burroughs and Christiansen (1962) maintained holotrichs for 7 days on a defined medium in a continuous culture apparatus.

This section describes the successful cultivation of a range of rumen oligotrichs and the establishment of a clone of one species. Unsuccessful attempts to cultivate holotrichs are

also reported.

Materials and Methods

Media

Basal salt solution. The basal solution was modified from those of Oxford (1958a) and Coleman (1958), and contained (g./l.): K_2HPO_4, 0.5; KH_2PO_4, 1.0; NaCl, 3.0; $MgSO_4.7H_2O$, 0.1; $CaCl_2$, 0.008; sodium acetate,$3H_2O$, 1.8. Freeze-dried, clarified rumen liquor, 1.4-2.8 g., or fresh clarified rumen liquor, 100-200 ml., was added. The freeze-dried or fresh rumen liquor was sometimes autoclaved with the basal solution in 1 l. screw-capped bottles. $NaHCO_3$, 1.0 g., and $Na_2S.9H_2O$, 0.1 g. or cysteine-HCl, 1.0 g. were added just before use.

Dried grass. Freshly cut perennial ryegrass (Lolium perenne, L.) or short rotation ryegrass (Lolium perenne x L. multiflorum, L.) was dried at 55° for 2 days (Coleman, 1958). After grinding in a ball mill for 6 hr. the finely divided grass was passed through a 100 mesh (aperture 152 μ) sieve.

Rice starch. Starch was prepared from rice by the method of Whelan (1955).

Casein. Casein (B.D.H.) was dissolved in 1 N NaOH. After adjusting to pH 7.4 and filtering through glass wool, the solution was freeze-dried.

Cellulose. Finely divided cellulose was prepared from absorbent cotton wool. The cotton was digested with 10 N HCl for 24 hr., washed, and ground in a ball mill for 96 hr. The suspension of

cellulose particles was freeze-dried.

Rumen liquor. Clarified rumen liquor was obtained by centrifuging fresh rumen liquor at 10,000 g. for 30 min. The clear solution was freeze-dried, autoclaved with the basal solution or stored for up to one week at -25°. The rumen liquor was obtained from cows feeding on either fresh red clover or red clover hay.

Cultures

Inocula. Protozoa were prepared by the method of Oxford (1958a), but using the salt solution described above, from the rumen liquor of a cow feeding on fresh red clover. The suspension contained the holotrichs, Isotricha, Dasytricha, and Buetschlia, and species of the following oligotrich genera (see Kofoid and MacLennan, 1930, 1932, 1933): Entodinium, Eodinium, Eremoplastron, Epidinium, Eudiplodinium, Metadinium, Ostracodinium, Diplodinium. The bulk of the oligotrichs consisted of Entodinium (20%), Eremoplastron (30%), Epidinium (35%) and Eudiplodinium (10%). The other oligotrichs were present in low numbers.

Cultures. The protozoa were grown in glass-stoppered conical flasks (100 ml.) filled with the basal medium and appropriate substrate materials. The flasks were gassed for 3 min. with CO_2 before addition of the inoculum, and were briefly gassed when any addition or transfer was made.

New substrate was added every 24 hr. Every 24 or 48 hr. the protozoa were transferred by carefully decanting the bulk of the supernatant fluid and pouring the residue into a new flask

containing fresh medium and substrate.

Clonal cultures and cultures of single species. Cultures of single species and clonal cultures were initiated with cells isolated by using a microscope and micropipette. In order to facilitate their isolation the ciliates were immobilized with 0.1% sodium barbitone. Provided that the protozoa were not exposed to the barbitone for more than 3-4 min. they revived satisfactorily when placed in fresh medium. The cultures were started in 5 ml. conical flasks and were transferred to larger flasks as the population of ciliates increased.

Large-scale cultures. An apparatus was designed to produce batches of protozoa suitable for metabolic studies and enzyme experiments. A 5 l. carboy with a flat bottom and fitted with a siphon overflow adjusted to maintain a liquid volume of 2 l. was used as the growth vessel. Constant temperature was maintained by immersing the growth vessel in a thermostatically controlled water bath. Fresh basal medium, at room temperature, was continuously added (900 ml./hr.) with a peristaltic pump (Sigmamotor Inc., Middleport, N.Y., U.S.A.) governed with a Revco "Zeromax" speedchanger (Zeromax Co., Minnesota, U.S.A.). The system was flushed with CO_2 before inoculation and after each addition of substrate which was made manually every 24 hr., the amounts being increased as protozoal numbers increased. At the end of the growth period the protozoa were recovered by low-speed centrifugation.

Microscope technique

The protozoa were identified and counted using the techniques described in Sections 1 and 2.

Experimental and Results

A. THE CULTURE OF OLIGOTRICHS

Preliminary experiments

Many unsuccessful attempts were made to culture the rumen oligotrichs before a successful culture was established in September, 1962. Early attempts were based on the methods of Oxford (1958a) and Coleman (1958) using oligotrich protozoa prepared by the method of Oxford (1958a) and the clover starch-green material substrate of Oxford (1958a). None of the ciliates in any of these cultures survived for longer than 5 days. In subsequent experiments protozoa were prepared by the same method but were not freed of bacteria or holotrichs by incubation with antibiotics or mannose. The protozoa in these cultures survived for 8-9 days. In the experiments immediately preceding the successful establishment of a culture, the basal salt solution already described was substituted for the buffer solutions of Oxford (1958a) and Coleman (1958), and 20% fresh rumen liquor, clarified by centrifugation, was added. Some of the ciliates in these cultures survived for 14 days.

The same conditions were used in the next experiment until the 13th day when the clover starch-green material was

replaced with perennial ryegrass dried at 55° and ground to pass through a 100 mesh sieve. At the time of the addition of the dried grass there were only about 10 organisms surviving in a single flask. By the 17th day there were 60-100 cells present, and the number had increased to 5-6,000 by the 20th day. This culture has been maintained for 14 months and is now a pure culture of Epidinium ecaudatum (Fiorentini 1889).

Observations on cultures

Dried grass. Established cultures from which grass was omitted died within 10 days. The grass could not be replaced by a freeze-dried aqueous extract of fresh grass, although in the presence of this extract a few protozoa persisted for over 20 days.

Rice starch and cellulose. Cultures without starch persisted indefinitely provided that grass or casein was present, but the ciliates were never numerous. The replacement of rice starch with cellulose resulted in cultures containing mainly Eudiplodinium maggii and Epidinium spp. with a few Eremoplastron bovis. The other ciliates died within 10 days.

Casein. Sodium caseinate (4 mg./100 ml.) was added to some cultures and, over a period of several weeks, these cultures produced more organisms than those without casein. There was little short-term difference. These were visual observations and no counts were made. Sodium caseinate, Difco (Nutrose), 4 mg./100 ml., caused death of the ciliates within 4 days.

Rumen liquor. Rumen liquor was essential for the growth and survival of the ciliates; cultures without it survived less than

10 days. Freshly prepared and freeze-dried rumen liquor were equally beneficial. Rumen liquor autoclaved with the basal solution caused gradual loss of the ciliates over periods of up to 60 days. No effect was apparent in the first 20 days, but after this period there was a gradual decrease in the number of cells. Rumen liquor autoclaved undiluted caused death of the protozoa within 30 min. of its addition to the medium.

Yeast Extract, Difco, 5 mg./100 ml., or Casamino acids, Difco, 5 mg./100 ml., added as a replacement for the rumen liquor, caused death of the ciliates within 2 hr.

Antibiotics. Terramycin (Oxytetracycline), viomycin, penicillin G, K benzyl penicillin, neomycin sulphate, streptomycin sulphate and achromycin all killed the ciliates in 2-24 hr. when added to the cultures at a final concentration of 50 µg./ml. Achromycin, 5 µg./ml., controlled bacterial growth, but only for a few hours. At various lower concentrations the other antibiotics were not toxic, but they either did not inhibit bacterial growth or were effective for short periods only. Chloramphenicol, 50 µg./ml., had no obvious effect on the ciliates but bacterial growth was not suppressed enough to warrant its use. Higher concentrations were rapidly toxic.

All the cultures have been maintained without the benefit of antibiotics. Bacterial growth caused only a slight turbidity in the cultures unless the starch was above 0.25 mg./ml.

Oxidation-reduction potential. After gassing, but before inoculation, the redox potential of the culture fluid was

approximately -280 mV. After inoculation and incubation for 48 hr. the potential had dropped to approximately -410 mV.

Rates of division. Division times were obtained by calculating the number of divisions occurring in a given period from the number of ciliates produced. No growth curves were made for the cultures. One culture with casein, dried grass and rice starch (10 mg.) had a mean division time of approximately 21 hr., 500,000 protozoa being produced in 8 days from an inoculum of 1,000 cells. On another occasion a similar culture had a mean division time of 15 hr. over a 9 day period.

Technique for maintaining cultures

The final technique used for routinely maintaining cultures of the ciliates was as follows. The protozoa were grown in 100 ml. glass-stoppered conical flasks. The substrate was suspended in distilled water and 1 ml. was added to the basal medium in each flask. No antibiotics were added. The flasks were thoroughly gassed with CO_2 before addition of the inoculum, and when any addition or transfer was made.

The substrate mixture contained casein (as sodium caseinate), 4 mg., dried grass, 5 mg., and rice starch, 10-20 mg. The amount of starch was varied according to the number of ciliates in the culture and the type of ciliate being cultured. Epidinium spp. were found to metabolize more starch than Eremoplastron spp. Additions of substrate were made every 24 hr. Every 48 hr. the protozoa were transferred by carefully decanting the bulk of the supernatant fluid and pouring the

residue into a new flask containing fresh medium and substrate. Unless care was taken in the initial and final decantations, a considerable number of protozoa were lost. During routine culture the loss of these protozoa tended to keep the numbers constant in spite of the continued division of the organisms.

Cultures established

Epidinium ecaudatum (Fiorentini, 1889) has been maintained for 14 months. The following additional oligotrichs were maintained in mixed culture for over six months, but were lost following a change to autoclaved rumen liquor: Eremoplastron bovis (Dogiel, 1927), Eremoplastron brevispinum (Kofoid and MacLennan, 1932), Entodinium longinucleatum (Dogiel, 1925), Entodinium ovinum (Dogiel, 1927), Eudiplodinium maggii (Fiorentini, 1889).

Additional mixed cultures of the above ciliates together with Eremoplastron monolobum (Dogiel, 1927) and Entodinium biconcavum (Kofoid and MacLennan, 1930) were maintained for several months, most of the ciliate species gradually disappearing until after 10 months only Epidinium and Eremoplastron spp. remained.

A clone of Eremoplastron bovis was maintained for five months before it was accidentally contaminated with epidinia. The clone was grown from a single cell isolated after sodium barbitone treatment.

Morphological variation

Considerable morphological variation took place in the

cultures over a period of several months.

The mixed cultures originally contained only the 1-5 spined members of the "caudatum" group of epidinia, but, after five months none of the epidinia exhibited caudal spination. The pure culture of an Epidinium species which has been cultured for 14 months contains the progeny of Epidinium caudatum, E. bicaudatum, E. tricaudatum, E. quadricaudatum and E. parvicaudatum (see Kofoid and MacLennan, 1933). None of the cells in this culture now have caudal spines so the culture is presumably of Epidinium ecaudatum (Fiorentini, 1889). Variation in the shape of the body also took place. The epidinia originally had the long tapered body typical of the "ecaudatum" group but forms with the shortened appearance of the "hamatum" group of Schulze (1924) were soon evident.

The clone of Eremoplastron bovis also changed in character. Originally started with a single cell measuring about 45 x 80 μ, the clone developed forms measuring 30-50 μ x 55-90 μ, with varying types of caudal lobes. These new forms were similar to Eremoplastron bovis, E. monolobum and E. brevispinum.

Large-scale culture

The large-scale apparatus was used on two occasions to prepare batches of cells suitable for biochemical studies. Two to three gm. (wet wt.) of mixed oligotrichs were grown in 12 days, from an inoculum of 2-5,000 cells on the first occasion. A second batch was grown with an inoculum from the Eremoplastron bovis clone, one gm. (wet wt.) of cells being produced in nine

days from an inoculum of 5,000 cells. This preparation was used for a study of the carbohydrases of the organism (Bailey and Clarke, 1963).

B. THE CULTURE OF HOLOTRICHS

All of the attempts to maintain holotrichs *in vitro* were unsuccessful. In the most successful attempt the protozoa survived for only seven days.

Preliminary experiments were based on the method devised for oligotrich culture, with appropriate changes in the carbohydrate substrates. In most experiments, 0.01-0.1% glucose or sucrose were used in place of rice starch (see Oxford, 1955), and dried grass, 5 mg., and casein, 4 mg., were added as with the oligotrichs.

In some experiments grass was omitted and was replaced with the equivalent amount of a freeze-dried, cold-water extract of fresh grass. Attempts were also made to grow the holotrichs in media with a soluble sugar and rumen liquor, but without grass and casein. In these cultures both the holotrichs and the oligotrichs died within seven days. In cultures containing dried grass the oligotrichs always outlived the holotrichs.

In cultures with 0.1% glucose, death of the holotrichs usually resulted from bursting of the cells (Sugden and Oxford, 1952). When the concentration of soluble sugars was not sufficient to cause the cells to burst, the protozoa steadily declined in numbers. Division was never seen after the third day of incubation.

Discussion

A. OLIGOTRICHS

The conditions of culture described here differed in various aspects from those described by Hungate (1942, 1943), Coleman (1958, 1960) and Gutierrez and Davis (1962).

Rumen liquor was an obligate requirement for the growth of the ciliates. Coleman (1958, 1960) also found rumen liquor to be essential, but Hungate (1942, 1943), and Gutierrez and Davis (1962) were able to maintain their ciliates in its absence. Hungate (1942) showed that ground, dried grass replaced the rumen liquor satisfactorily and routinely added 40 mg. of grass to every 100 ml. of medium. Gutierrez and Davis (1962) added only 3 mg. of alfalfa to 100 ml. of medium in the absence of rumen liquor while the cultures of Coleman (1958) contained 52 mg. of grass per 100 ml. of medium and still had a requirement for rumen liquor. Obviously, in spite of the supposed similarity in the metabolism of rumen ciliates the world over, the protozoa in some situations have different requirements. Further work is needed to show whether rumen liquor and grass provide growth factors or whether they satisfy a complex nitrogen requirement.

Further differences involve the toxicity of autoclaved rumen liquor and the effect of materials such as yeast extract and peptone. Coleman (1960) found autoclaved rumen liquor to be satisfactory, but the autoclaved rumen liquor used in this study was rapidly lethal although dilution of the rumen liquor with

the salt solution before autoclaving reduced the toxicity considerably. With this preparation of rumen liquor the death of the ciliates may have been the result of some essential factor being destroyed during the autoclaving.

The Difco products, Bacto Sodium Caseinate, Bacto Yeast Extract, and Bacto Casamino Acids were also rapidly lethal to the ciliates, even in low concentrations. Hungate (1942) used similar concentrations of beef extract and peptone in his early cultures with variable effects on the protozoa.

The need for antibiotics in cultures apparently depends on the other conditions of culture. Coleman (1958, 1960) used chloramphenicol to suppress some of the bacteria in his cultures and found that the ciliate population dropped when it was omitted but Hungate (1942, 1943) and Gutierrez and Davis (1962) did not find antibiotics essential.

Rather than being essential for maintaining the ciliates in an active state, antibiotics may only help to increase the total yield. Thus Coleman (1960), using chloramphenicol, cultured entodinial populations of 30,000/ml., whereas in the present work, without antibiotics, the ciliates reached only 2,500/ml., and Gutierrez and Davis (1962) and Hungate (1942) achieved populations of only 1,200/ml.

The size of the ciliates being cultured probably determines the final population level to some extent. It has been observed in the present work that there is a high mortality rate among ciliates in a thick layer on the bottom of a flask and the

population is limited by the area in which the protozoa settle. Entodinia are, in general, smaller than the other oligotrichs, and can attain a higher density/unit area by virtue of their small size. This may be one reason why Coleman (1960) was able to culture 30,000 entodinia/ml. Some of the organisms cultured here, and those cultured by Hungate, and Gutierrez and Davis, are considerably larger than most entodinia.

Coleman (1962) was unsuccessful in his attempts at maintaining axenic cultures of entodinia and, also considering the demonstration by Gutierrez and Davis (1959) of ingestion of bacteria by Entodinium and Diplodinium, it now appears that bacteria are essential for the growth of at least some of the rumen ciliates. Metabolic studies with washed suspensions of protozoa, and with protozoa from bacteria-containing cultures, have required the use of antibiotics to suppress bacterial activity. However, since the development by Bailey and Howard (1962) of a specific method for disrupting protozoa, without disruption of associated bacteria, bacteria-free preparations of rumen ciliates are no longer necessary for enzyme studies, although they are still required for manometric investigations.

Many attempts were made to establish cultures from single cells and small numbers of cells following treatment of the protozoa with sodium barbitone, but only one of the cultures was successful. The barbitone did not seem to have any adverse effect on the protozoa and recovery of the cells was complete in all cases. Within 10 min. of placing the cell, or cells, in the

culture flask the ciliates became motile and appeared to be normal. The failure of most of the attempts was probably the result of the difficulties involved in handling small numbers of cells. It was difficult to transfer a few ciliates into fresh medium because of the ease with which the organisms were lost in the grass debris.

Morphological variation in cultures, even short-term cultures, of rumen ciliates has long been established. Poljansky and Strelkow (1934) showed that clones of Eremoplastron bilobum developed forms similar to Eremoplastron bovis and E. monolobum, and that the spination of Entodinium caudatum was variable. Hungate (1942) also noticed variation in cultures of Eremoplastron spp. (Eudiplodinium neglectum) similar to the variation of Eremoplastron bovis noticed in this study. The loss or change in the caudal spination of Epidinium as shown here, was also noticed by Gutierrez and Davis (1962). Morphological variation has also been demonstrated in vivo. Poljansky and Strelkow (1938) showed a change in the spination of Entodinium caudatum when the host's diet was changed.

This morphological variability is a powerful argument against the division of composite groups of protozoa into species, as carried out by Kofoid and MacLennan (1932, 1933). Dogiel (1927) classified the "ecaudatum" group of Epidinium as forms of the single species Epidinium ecaudatum (Fiorentini, 1889) but Kofoid and MacLennan (1933) split the group into separate species. Thus Epidinium ecaudatum (Fiorentini, 1889) forma bicaudatum

(Sharp, 1914) became E. bicaudatum (Sharp, 1914). A similar situation exists with Epidinium hamatum (Schulze, 1924), (Kofoid and MacLennan, 1933). Dogiel (1927) only gave this organism "forma" rank within the species E. ecaudatum, a decision supported by the forms of this organism which developed in cultures containing only the "ecaudatum" group. The Eremoplastron group of Kofoid and MacLennan (1932) also consists of several species originally placed in one species by Dogiel (1927). Further work on the establishment of clones of rumen ciliates is warranted solely from the taxonomic viewpoint. The study of variation in such cultures would undoubtedly clarify phylogenetic relationships within the group.

The large-scale apparatus was useful for producing batches of metabolically active ciliates (see Bailey and Clarke, 1963). With a minimum of effort it was not difficult to produce several grams of cells, a lengthy and time-consuming task using 100 ml. flasks as culture vessels. Much work has still to be carried out on the biochemistry of the rumen oligotrichs, and the ability to produce relatively large batches of cells of pure strains will be useful.

B. HOLOTRICHS

The rumen holotrichs have proved to be more difficult to culture than the oligotrichs. Their ability to use soluble sugars (Oxford, 1951; Masson and Oxford, 1951) suggests that they might be cultured more easily than the oligotrichs. However, their lack of a mechanism for stopping the accumulation of

storage polysaccharide in the presence of an excess of rapidly-fermentable substrate and their tendency to burst as a result of over-accumulation (Sugden and Oxford, 1952; Gutierrez, 1955), limits the amount of substrate that can be provided.

The successful use of a grass extract by Sugden and Oxford (1952) for maintaining both genera of holotrichs for a month suggests that the holotrich requirements are at least as complex as those of the oligotrichs. Dasytricha is unable to ingest starch granules (Sugden and Oxford, 1952) and is probably unable to ingest grass particles. This organism may have an obligate requirement for a grass extract, which will have to be provided in cultures. Isotricha, on the other hand, can ingest small starch grains (Sugden and Oxford, 1952) and should be able to ingest grass particles provided they are small enough.

The failure to supply certain types of bacteria may be the reason for the unsuccessful attempts at culture. Isotricha prostoma (Gutierrez, 1958) and Dasytricha ruminantium (Gutierrez and Hungate, 1957) have been shown to ingest only certain types of bacteria and the bacteria they require may not be found, or may not grow, under the conditions of culture so far used.

A considerable amount of work has been done on the biochemistry of the holotrichs especially since separate genera have been kept isolated in sheep treated by the method of Eadie and Oxford (1957) (see Howard, 1959a, b). The maintenance of these organisms in culture would, however, greatly simplify what work remains, especially with regard to their nutritional requirements.

The cultures of rumen protozoa already established have all been relatively crude from a biochemical viewpoint. Apart from the need to culture holotrichs and to develop techniques to cope with the more difficult of the oligotrichs, the next steps must be towards the development of chemically-defined media. An effective fully-defined medium of the type used by Quinn, Burroughs and Christiansen (1962) must be possible. Such a medium developed for rumen ciliates in this country would have to be based on the composition of rumen liquor and on the composition of fresh grass and clover (see Bathurst, 1953; Ferguson and Terry, 1954) In the absence of a fully synthetic medium a chemically-defined supplement that could replace grass would simplify the actual cultural techniques and would lead to cleaner cultures.

3. THE ISOLATION, IDENTIFICATION AND CHARACTERISTICS OF SOME RUMEN BACTERIA

Introduction

The rumen is an ideal habitat for the growth of bacteria. Aerobes are restricted because of the lack of oxygen, but the constant conditions of pH, temperature, moisture and anaerobiosis, and a semi-continuous food supply encourage a large and diverse population of anaerobes. Assuming constant physical conditions, the flora depends on the type and amount of food entering the rumen. Bacteria capable of degrading complex carbohydrates, soluble sugars, proteins, organic acids, lipids and many other feed materials are found, their quantity and type depending on the diet of the host. The principal metabolic products of this complicated bacterial system are carbon dioxide, methane, and volatile fatty acids. Other end-products of normal metabolism such as hydrogen, and formic, lactic and succinic acids, are merely produced as intermediates and are metabolized in turn.

The real advances in the bacteriology of the rumen have been made since the 1940's when the importance of the rumen volatile fatty acids was realized and Hungate (1947, 1950), Sijpesteijn (1948, 1951) and Gall, Stark and Loosli (1947) published methods of cultivating rumen anaerobes. The methods used since then, and the many species of bacteria isolated, have been reviewed by Bryant (1959).

Physical conditions of culture

The redox potential of rumen fluid is of the order of -300 mV to -400 mV at pH 7 (Horn, Snapp and Gall, 1955; Hungate, 1960; Baldwin and Emery, 1960) although higher values have been reported (Broberg, 1957). Various methods have been employed to attain low redox potentials in culture media, but most workers now use the method of Hungate (1950), together with various reducing agents such as sodium sulphide, cysteine-HCl, thioglycollate and ascorbic acid (see Bryant, 1959). Oxidation-reduction indicators are also used to ensure that a low Eh is maintained. Resazurin (E_o', pH 6.867, -42 mV, Twigg, 1945) is most commonly utilized but indigo carmine (E_o', pH 7, -123 mV) and benzyl viologen (E_o', pH 7, -359 mV) have also been used in studies on the more anaerobic species. Smith and Hungate (1958) found that benzyl viologen had to be decolourized to allow growth of Methanobacterium ruminantium, and used Escherichia coli to lower the redox potential of the medium before inoculation.

Media are usually adjusted to pH 6.8-7.2 before inoculation. The temperature of incubation is that of the rumen, 39°.

Media

The two main techniques of isolation employed are enrichment culture using a liquid medium, and direct isolation on a solid medium. The enrichment technique is of little value apart from being a useful method for isolating a specific organism. Direct isolation with a selective or non-selective substrate has the advantage of allowing enumeration of the

isolated organism. Micromanipulative techniques have been used to isolate viable bacteria directly from rumen contents (Ingram and McGaughey, 1948; Purdom, 1963) but have rather limited application.

Many culture media have been used (see Bryant, 1959). The most generally useful medium consists of a mineral salts solution buffered with phosphate and containing the substrate, a source of nitrogen, a reducing agent and rumen liquor, together with an oxidation-reduction indicator (see Hungate, 1950). Rumen liquor is not required by many rumen bacteria in vitro, but since some important rumen bacteria require it as a source of growth factors not ordinarily available in such preparations as yeast extract, it is usually advisable to add it during the isolation and preliminary studies. For information on the rumen liquor growth factor requirements of some rumen bacteria see Allison, Bryant and Doetsch (1958), Wegner and Foster (1960), Bryant and Robinson (1961, 1962), Caldwell, Bryant and White (1962), and Allison and Bryant (1963).

Criteria for true rumen bacteria

It is important to verify that species of bacteria studied with regard to rumen fermentation are actually functional in the rumen and are not merely passengers. Elsden and Phillipson (1948) and Gall and Huhtanen (1951) were among the first to present criteria for determining the significance of rumen organisms. The essential criteria are discussed in the following extract from Bryant (1959, p126).

"Probably the most important criteria that can be used are that the organism be shown to grow in the rumen and that it have a metabolism compatible with the reactions occurring and the environment present in the rumen. The numbers of a given species present in the rumen as compared to its numbers in the feed and water consumed, along with similar data on other species carrying out the same reactions, are probably the best criteria for use at the present time. The numerical criterion alone can establish that an organism is growing in the rumen and, therefore, that the protoplasm synthesized is available to the host.

While other criteria are of relatively little value in the absence of the numerical criterion the reverse is also true. The organism should be able to attack substrates present in the rumen. These are not necessarily materials present in the feed but may include hydrolytic products of feed constituents such as cellulose, starch, and protein, and fermentation products such as organic acid and hydrogen. It is also possible that considerable amounts of substrate are available in the form of slime and cellular constituents of organisms that die in the rumen. Fermentation products should be compatible with those present in the rumen or metabolized therein. However, some species of rumen bacteria, which present evidence indicates to be of considerable significance, produce <u>in vitro</u> large amounts of ethanol (Bryant and Small, 1956a; Bryant <u>et al.</u>, 1958b), which does not appear to be present or metabolized in

the rumen under usual conditions. The production of ethanol by these cultures is probably due to the artificial environment (see Moorman and Hungate (1963) - author's note) and a study of factors affecting its production might lead to new knowledge of interactions occurring in the rumen. Cultural characteristics such as Eh, pH, and temperature range of growth of the pure culture as well as nutritional requirements should be compatible with the environment present in the rumen. Also the rumen may contain substances that inhibit the growth of species that might otherwise be able to function (Garner, Muhrer and Pfander, 1954; Hoflund et al., 1957)."

Bacteria attacking some common rumen substrates

According to Hungate (1960, p356): "Metabolic processes such as fibre digestion, starch hydrolysis, protein degradation, hydrolysis of urea; production of acetic, propionic, butyric, lactic, formic, and succinic acids, methane, hydrogen and carbon dioxide; and conversion of formate, lactate, succinate, carbon dioxide and hydrogen, all known to occur in the rumen, have been demonstrated in at least one of the pure bacterial cultures."

The range of bacteria responsible for the above reactions comprise many species of more than 20 genera. Many new organisms have been described and new genera created for rumen bacteria include Ruminococcus (Sijpesteijn, 1948), Butyrivibrio (Bryant and Small, 1956b), Succinivibrio (Bryant and Small, 1956a), Succinimonas (Bryant et al., 1958a) and Lachnospira

(Bryant and Small, 1956a). The species of rumen bacteria were described under genera by Bryant (1959). Oxford (1958b) and Bryant (1960, 1963) listed some of the more important bacteria with the substrates they attack and Hungate (1963) discussed the bacteria in their role of symbionts.

Hungate (1960) is of the opinion that most of the important rumen bacteria have been isolated and given, at least, an initial characterization, although he admits that some of the less abundant types have still to be isolated. He also noted (Hungate, 1960) that the axenic cultivation, identification, and enumeration of rumen bacteria is extremely time-consuming and that, as a result, most ecological investigations have been restricted to a few components of the total flora.

The only rumen bacteria to be characterized in New Zealand are Streptococcus bovis (Bailey and Oxford, 1958), Lactobacillus bifidus (Clarke, 1959) and Clostridium bifermentans (Clarke, 1961). A Beggiatoa-like organism described by Jamieson and Loftus (1958) was not grown in culture.

This section reports the results of preliminary investigations of the more important bacterial members of the rumen flora.

Materials and Methods

Isolation of bacteria

Rumen contents were removed from a rumen-fistulated cow and strained through gauze into a 250 ml. conical flask. When full the flask was stoppered and placed in an incubator at

39°. This sample was used to inoculate media within one hour of removal from the animal.

All attempts to isolate bacteria were made under anaerobic conditions by the method of Hungate (1950). Serial tenfold dilutions of the rumen liquor were made in dilution fluid (g./l.: K_2HPO_4, 1.5; KH_2PO_4, 1.5; cysteine-HCl, 1.0; resazurin, 0.01) sterilized by autoclaving. One ml. of each dilution was added to 5 ml. of the appropriate agar medium in 25 ml. Trident vials (Johnsen and Jorgensen, Ltd., London). The vials were tightly closed with sterile rubber stoppers, rolled under a stream of cold water until the agar had solidified, and incubated at 39°.

After 1-7 days, colonies representative of those appearing were picked off into dilution fluid, diluted serially and cultured in roll-tubes. This procedure was repeated until the cultures were considered to be pure as judged by microscopic examination.

The whole operation of medium dispensing, dilution and transfer was carried out in a stream of oxygen-free CO_2. Oxygen was removed by passing cylinder gas over reduced copper turnings at 600°. Resazurin (E_o, pH 0.687, -42 mv.) or indigo carmine (E_o, pH 7, -123 mv.) was added to all media, except where otherwise indicated, and gassing was carried out until the indicator was colourless.

Media

Basal medium. The basal medium consisted of (g./l.) K_2HpO_4, 1.0;

KH_2PO_4, 1.0; NaCl, 2.0; cysteine-HCl, 1.0; Bacto-Tryptose, 2.0; resazurin, 0.01, or indigo carmine, 0.005; in 20% (v/v) clarified rumen liquor. The required substrate was added to this medium. Agar (Davis, N.Z.) was added when required.

Rumen fluid-glucose-cellobiose agar. Rumen fluid-glucose-cellobiose agar (R.G.C.A.) was prepared as described by Bryant and Burkey (1953).

Clarified rumen liquor. Clarified rumen liquor was prepared by centrifuging strained rumen contents at 10,000 g for 30 min. The resultant clear supernate was dispensed in 20 ml. amounts in 1 oz. screw-topped bottles and autoclaved.

Casein. Casein was used as either casein (B.D.H.) or sodium caseinate (Difco; Nutrose).

Cytoplasmic protein. Preparations of cytoplasmic protein were made from red clover (Trifolium pratense, L.) by the method of Lyttleton (1956).

Salivary mucoprotein. Solutions of mucoprotein were prepared from bovine saliva by the method of Lyttleton (1960).

Xylan. Xylan was prepared from wheat flour by the method of Howard (1957).

Pectin. Pectin (Grade 40) was obtained from British Drug Houses Ltd.

Cellulose. Absorbent cotton wool was degraded by immersion in 10 N HCl for 24 hr. The resulting slurry was washed free of chloride and ground for 96 hr. in a ball mill. The product was freeze-dried after removal of the bulk of the water by evapora-

tion at 39°.

Identification of isolates

The routine tests for identification were based on those recommended by Skerman (1959). Final identification was based where possible on Bergey's Manual (Breed et al., 1957).

All soluble carbohydrates were sterilized by filtration through a porosity 5/3 glass sinter, and were stored in 2% (w/v) solutions at -25°. Insoluble carbohydrates were sterilized by autoclaving at 10 lb./10 min. All of the pentoses and hexoses used, with the exception of arabinose, were the D-isomers.

As the methods for testing nitrate reduction, and hydrogen sulphide and indole production all involved a colorimetric reaction, cultures for these tests were grown in the absence of an oxidation-reduction indicator. Hydrogen sulphide production was tested by the method of Pomeroy (1936). Nitrate reduction was determined by estimating nitrite by the method of Lees and Quastel (1946).

Preservation of cultures

Where possible cultures for stock were kept as stab-cultures in the refrigerator or a dry-ice box at -70°. Transfers were made as required. Attempts were also made to preserve some cultures by freeze-drying.

Cultures for fermentation analysis

Cultures for fermentation analysis were grown in round-bottom flasks closed with a rubber stopper. When an analysis of gas evolution or consumption was required the culture was grown

in the apparatus first used by Elsden and described by Johns (1948) and gassing was carried out with oxygen-free nitrogen.

Washed-cell suspensions

Cells were harvested from young cultures by high-speed centrifugation and were washed twice with phosphate buffer (M/15, pH 6.8) containing 0.01% sodium sulphide. The final suspension was incubated with the substrate under anaerobic conditions in a 50 ml. conical flask.

Analytical methods

Glucose. The fermentation medium was treated with $ZnSO_4$ and NaOH to remove protein and glucose was determined in the clarified solution by the colorimetric method of Nelson (1944).

Glycerol. Glycerol was determined by the periodate/chromotropic acid method of Lambert and Neish (1950). No attempt was made to correct for the small amounts of glucose in some of the fermentation solutions. A solution of glycerol was standardized by titration with periodate (Neish, 1950).

Lactic acid. Lactic acid was determined by the colorimetric method of Barker and Summerson (1941).

Succinic acid. Succinic acid was determined by difference after measuring the lactic acid in the mixed lactate/succinate fraction from a Celite column (Swim and Utter, 1957).

Ethanol. Ethanol was measured by the diffusion method of Conway (1957) using potassium dichromate to oxidise the alcohol to acetic acid (Winnick, 1942). Excess dichromate was titrated with standard thiosulphate.

Volatile acids. Total volatile acids were determined by titration following steam-distillation in a Markham (1942) apparatus. Two ml. of the sample together with 8 g. $MgSO_4$ (Olmstead, Whitaker and Duden, 1929) and 1 ml. 10N H_2SO_4 were added to the apparatus and steam-distilled. Two successive 30 ml. portions of distillate were collected and titrated with standard (0.01-0.04 N) NaOH in a stream of CO_2-free air with phenol red as indicator. The second sample of distillate was used as a control to correct for lactic acid in the original sample (Barker, 1957).

The individual acids, from acetic to valeric, were determined on the gas-liquid chromatograph of James and Martin (1952). Behenic acid usually replaced stearic acid in the column packing, and ethyl cellosolve (B.P. 137°) was used in the vapour jacket. Samples of the evaporated steam-distillate were added to the column as aqueous solutions of the sodium salts. One drop of the solution was added to a 2:1 (w/w) mixture of Celite 545 and $NaHSO_4$ in a Teflon boat which was slid into the end of the column (Elsden, pers. comm.).

Formic acid could not be determined satisfactorily by the above method as it was not completely separated from acetic acid. Its presence in fermentation solutions was shown by paper chromatography using ethylamine/n-butanol and a ninhydrin spray reagent (Burness and King, 1958) and it was measured after column chromatography on Celite (Swim and Utter, 1957). When formic acid was present it was subtracted from the acetic acid

found by gas-liquid chromatography.

Hydrolysis of protein. Protein hydrolysis was determined, after precipitation with trichloracetic acid, by the biuret method of Gornall, Bardawill and David (1949), or by the Folin tyrosine method (Spies, 1957).

Experimental and Results

Fifty-six strains of bacteria belonging to eleven species and ten genera were isolated and identified. Thirty-three of the strains were anaerobic, the remainder being facultative anaerobes. The strains isolated are described below under the headings of the substrates used for their isolation. A summary of their characteristics is given in Table 49. All strains were numbered and given the prefix PC. In the descriptions following, the prefix has been dropped and the strain number quoted.

Casein

Strains 1-4 were obtained from two cows, one on a diet of fresh red clover and the other on grass hay. The medium used was the basal medium with 0.5% (w/v) sodium caseinate and with resazurin as redox indicator. The organisms were isolated in numbers from 2×10^6 - 2×10^8/ml. rumen liquor. Growth was maximum when the indicator was decolourized but was still appreciable when the medium was slightly pink.

In liquid medium the organism grew as gram-positive cocci,

0.5-0.7 μ in diameter, in pairs, clumps and short chains. On solid medium there were fewer chains of cells. Rumen liquor was not required for growth and was replaceable with 0.01% Bacto-Yeast Extract.

Glucose, maltose, mannose, sucrose, lactose, fructose, cellobiose, galactose, raffinose and starch were fermented to a final pH of 4.4-4.7. Xylose, arabinose, glycerol, rhamnose, inositol and dulcitol were not fermented. Gelatin was not liquefied, indole was not produced and nitrate was not reduced. Initial growth on the casein medium was poor and casein was not degraded.

Lactic acid accounted for 60-80% of the end-products from glucose. No gas was produced.

Strains 1, 2, 3 and 4 were identified as variants of Streptococcus bovis.

Organism 5 was isolated on the casein medium from a hay-fed cow at a concentration of 10^5/ml. rumen liquor. It was a gram-positive, non-sporing pleomorphic bacillus, 1.0-1.2 μ x 1.5-4.5 μ, which grew slightly in the presence of air. Luxuriant growth was obtained when resazurin was decolourized.

Only glucose, sucrose and fructose were fermented. Lactose, maltose, xylose, arabinose, galactose, cellobiose, raffinose, starch, mannitol, inositol, dulcitol, rhamnose and lactic acid were not fermented. Gelatin was liquefied, nitrate was not reduced, and hydrogen sulphide and indole were not produced. Casein was not attacked. Propionic acid was the main product

Table 49. Characteristics of some rumen bacteria

	Species	*Streptococcus bovis*	*Streptococcus bovis*	*Corynebacterium acnes*	*Corynebacterium acnes*	*Selenomonas ruminantium*	*Selenomonas ruminantium* var. *lactilytica*	*Butyrivibrio fibrisolvens*	*B. fibrisolvens*	*B. fibrisolvens*	*Ruminococcus albus*	*Ruminococcus flavefaciens*	*Lactobacillus bifidus*	*Butyribacterium* sp.	*Vellionella alcalescens*	*Borrelia* sp.	*Clostridium bifermentans*
		1	2	3	4			5	6	7							
Substrates	Glucose	+	+	+	+	+	+	+	+	+	-	-	+	+	-	+	+
	Galactose	+	+	-	-	+	+	+	+	+	-	-	+	-	-	+	-
	Maltose	+	+	-	+	+	+	+	+	+	-	-	+	+	-	+	+
	Xylose	-	+	-	-	+	+	+	+	+	-	-	+	-	-	+	-
	Arabinose	-	+	-	-	+	+	+	+	+	+	+	+	-	-	+	-
	Lactose	+	+	-	-	+	+	-	-	-	+	-	+	+	-	+	+
	Sucrose	+	+	+	+	+	+	+	+	+	+	-	+	-	-	+	+
	Mannose	+	+	-	-	-	-	-	-	-	+	-	+	-	-	-	-
	Fructose	+	+	+	-	+	+	+	+	-	-	+	+	+	-	+	+
	Mannitol	-	-	+	+	-	-	-	-	-	-	-	-	-	-	+	-
	Cellobiose	+	+	-	-	+	+	+	-	+	+	+	+	-	-	+	+
	Melibiose					-	-						+	-	-	+	
	Glycerol	-	-	-	-	-	+	-	-	-	-	-	+	+	-	-	+
	Starch	+	+	-	-	+	+	+	+	+	-	-	+	-	-	-	+
	Cellulose					-	-	+	-	+	+	+		-		-	-
	Pectin					-	-	+	+	+	-	-		-		-	-
	Xylan			-	-	-	-	+	+	+				-		-	-
	Lactic acid			-	+	-	+	-	-	-				+	+	-	-
	Gelatin	-	-	-	-	-	+	+	+	+	-		-	-	-	-	+
	Casein	-	-	-	-	-	-	+					-	-	-	-	+
End-products	Substrate*	G		G	L	G	L	G			C	C	G	G	L	G	G
	Formate			-		+	-	+			+	+	+			+	
	Acetate			+	+	+	+	+			+	+	+			+	
	Propionate			+	+	+	+	-			-	-	-			-	
	Butyrate			-	-	-	+	+			-	-	-			-	
	Higher acids			-	-	-	-	-			-	-	-			-	
	Lactate	+			-	+	-	+			+	+	+	+		+	+
	Succinate				-	+	-	-			-	+	-			+	
	Ethanol			-	-	-	-	-			+	-	+			-	
	Gas	-		+	+	+	+				+	+	-	-	+	+	+

* G, glucose; L, lactic acid; C, cellobiose

... Continued over

1. Strains 1-4, 7, 9-13, 15, 40-42
2. " 8, 14, 53-56
3. Strain 5
4. Strains 50-51
5. " 36, 44-46
6. Strain 43
7. " 47

of glucose fermentation.

The organism was identified as a variety of Corynebacterium acnes.

Strains 20, 21 were isolated on the casein medium from two cows on a diet of grass hay. Both were gram-positive, obligate anaerobes and occurred in numbers from 10^5-10^7/ml. rumen liquor. In liquid media there were curved rods and spirals of up to six coils, 0.2-0.5 μ x 3-12 μ.

Glucose, lactose, sucrose, maltose, xylose, arabinose, cellobiose, fructose, galactose, melibiose and mannitol were fermented. Starch, cellulose, mannose, glycerol and lactic acid were not fermented. Nitrate was reduced to nitrite. Indole and hydrogen sulphide were not produced, gelatin was not liquefied and casein was not degraded. Rumen liquor was essential for growth and could not be replaced by yeast extract or by acetic, propionic, n-valeric, iso-valeric and iso-butyric acids.

End-products from glucose included succinic, lactic, formic and acetic acids. No ethanol was produced. Succinic acid was equivalent to 25-30% of the carbon in the fermented

glucose. The end-products found accounted for 72% of the glucose used. Some gas was produced but the amount was not measured.

These bacteria were identified as species of Borrelia similar to those described by Bryant (1952).

Clover cytoplasmic protein

Bacteria 7, 8, and 9 were obtained from two cows on a diet of fresh red clover. The medium used was the basal medium with resazurin and 15% (v/v) cytoplasmic protein solution from red clover.

The three strains were identified as variants of Streptococcus bovis.

Salivary mucoprotein

Bacteria 10-15, from three cows, were isolated on the basal medium with resazurin and 3% (v/v) mucoprotein solution and were identified as Streptococcus bovis variants.

Casein and glucose

Five bacteria, 52-56 from four cows, were isolated on the basal medium with casein and 0.5% glucose. Four of the organisms, 53-56, did not degrade casein and were identified as Streptococcus bovis.

Strain 52 was an anaerobic sporing organism similar to Clostridium bifermentans (Clarke, 1961). Growth on the glucose and casein medium was rapid and cooked meat medium was quickly degraded.

Glycerol

Three bacteria, 16, 19 and 22, were isolated on the basal medium with resazurin and 0.5% (w/v) glycerol from three cows. All were gram-positive obligate anaerobes and occurred in numbers from 4×10^6 - 6×10^7/ml. rumen liquor. In liquid medium the cells were rod-shaped or curved and occurred singly and in pairs, with a few short chains. The cells measured 0.8-1.6 μ x 2.9 μ. Rumen liquor was not essential for growth, and could be replaced with yeast extract.

Glucose, lactose, sucrose, maltose, xylose, starch, cellobiose, arabinose, fructose and galactose were fermented by all three strains. Strain 22 also fermented glycerol and lactic acid. Cellulose, pectin, melibiose, mannose and mannitol were not fermented. Hydrogen sulphide was produced. Gelatin was not liquefied, and indole was not produced. Nitrate was not reduced to nitrite.

The end-products produced from glucose by strains 16 and 19 were CO_2 and acetic, propionic and lactic acids in the molar ratio 3:5:6. Traces of succinic and butyric acids and ethanol were also produced by strain 19. Strain 22 produced approximately equal amounts of acetic and propionic acids, toegher with a little CO_2 and a trace of butyric acid, from lactic acid.

Strains 16 and 19 were identified as Selenomonas ruminantium, and strain 22 as S. ruminantium var. lactilyticas (Bryant, 1956).

Rumen fluid-glucose-cellobiose medium

Strain 36 was the only bacteria characterized from this medium, although two other strains resembling Streptococcus bovis and Lactobacillus bifidus were isolated.

The organism was a motile, gram-negative, curved rod, 0.5 μ x 1.5-7.0 μ, with rounded ends. It occurred single, in pairs and in short chains. Rumen liquor could be replaced in the medium by yeast extract.

Glucose, galactose, maltose, cellobiose, sucrose, xylose arabinose, fructose, xylan, starch, cellulose and pectin were fermented. Mannose, mannitol, lactose and glycerol were not fermented. Gelatin was hydrolysed and casein was degraded. Hydrogen sulphide and indole were not produced, and nitrate was not reduced to nitrite.

End-products of the fermentation of glucose were formic, acetic, butyric and lactic acids in the molar ratio of 2:1:2:1. Ethanol and gas were not produced. The organism was identified as Butyrivibrio fibrisolvens (Bryant and Small, 1956b).

Xylan

Four bacteria, 43-46, were isolated on media containing wheat flour xylan (0.5%) as sole carbohydrate source. The four strains were similar to strain 36, Butyrivibrio fibrisolvens, but strain 43 did not ferment cellulose and cellobiose or degrade casein. All the strains except 43, which was from a cow fed on fresh red clover, were isolated from cows on a diet of grass hay.

Cellulose

Four strains of cellulolytic bacteria were isolated in six attempts, 32-34 from a cow fed on grass hay and 35 from a cow fed on fresh red clover. The medium used was the basal medium with resazurin and with enough cellulose to give a faintly turbid suspension. Only colonies surrounded by a clear zone in the cellulose particles were removed for purification and characterization. Two attempts to isolate bacteria at an Eh where indigo-carmine was decolourized did not yield any colonies.

All the organisms were non-motile, gram-positive cocci, 0.6 μ x 1.6 μ, occurring single, in pairs and occasional short chains. There were 10^4-10^6 cells/ml. rumen liquor. Rumen liquor was required for growth in the absence of iso-valeric acid (50 μg /ml.). Yeast extract could not satisfy the requirement.

All four strains fermented arabinose and cellobiose. In addition, 32-34 fermented lactose, sucrose and mannose, and 35 fermented fructose. Glucose, galactose, maltose, mannitol, xylose, glycerol, starch and pectin were not fermented.

In the fermentation of cellobiose, strains 32-34 produced formic, acetic and lactic acids, ethanol and gas. Succinic, propionic and butyric acids were not formed. The proportions of the various end-products varied considerably in different cultures. The gas produced was 80-90% CO_2. These organisms were identified as variants of Ruminococcus albus (Hungate, 1957).

Strain 35 was identified as Ruminococcus flavefaciens, (Sijpesteijn, 1951). The end-products of the fermentation of

cellobiose were formic, acetic and succinic acids in the molar ratio 1:3:2. Very little gas was formed.

Starch

Organisms 37-42, 47, were isolated on the basal medium with 0.2% soluble starch and resazurin. All were isolated from cows on a diet of fresh red clover.

Strains 37-39 were identified as variants of Lactobacillus bifidus. In young cultures the cells were non-motile, gram-positive rods, straight or slightly curved, 0.4-0.8 μ x 1.8-7.0 μ. In older cultures there were many club, branched and Y-forms. There were 10^5-10^7 cells/ml. rumen liquor.

Glucose, lactose, sucrose, maltose, xylose, cellobiose, melibiose, arabinose, mannose, fructose, galactose and glycerol were fermented. Mannitol was not fermented. Nitrate was reduced to nitrite and hydrogen sulphide was produced. Gelatin was not liquefied and indole was not produced.

Formic, acetic and lactic acids and ethanol were produced from glucose in the molar ratio 1:4:10:3.

Strains 40-42 were classified as variants of Streptococcus bovis.

Strain 47 was similar to Butyrivibrio fibrisolvens strain 36, but did not ferment fructose or degrade casein.

Lactic acid

Organisms 17, 30 and 31 were isolated on the basal medium with 1.0% (v/v) 70% sodium lactate at an oxygen tension where

the resazurin was still slightly pink. The three strains were all small gram-negative cocci. They did not ferment soluble sugars but rapidly fermented lactic acid to volatile acids and gas. They were identified as Veillonella alcalescens (syn. V. gazogenes) (Johns, 1951a).

Two isolations were made from cows feeding on fresh red clover. These strains, 48 and 49, were isolated with resazurin fully decolorized, and were identified as Selenomonas ruminantium var. lactilyticas.

Organisms 6, 18, 23-29, were isolated from two cows on a diet of grass hay, over a period of nine months. All the strains except 29 were isolated with resazurin decolorized. Strain 29 was obtained using indigo-carmine but subsequently grew satisfactorily with resazurin decolorized.

All of the isolates were curved rods with rounded ends, 0.3-0.8 μ x 0.9-4.3 μ, occurring single with a few in pairs. In older cultures some of the cells were slightly club-shaped. No branching cells were seen. The cells were gram-positive for the first 18 hr. but in older cultures the staining became irregular. Rumen liquor was essential for growth and could not be replaced by yeast extract, by mixtures of the acids acetic, propionic, n-valeric, iso-valeric and iso-butyric, or by a steam distillate of rumen liquor.

Glucose, maltose, lactose, fructose and glycerol were fermented but the fermentation of lactose was slow. Sucrose, xylose, cellobiose, arabinose, galactose, mannose, raffinose,

mannitol, starch, dulcitol, inositol and rhamnose were not fermented. Hydrogen sulphide was produced. Gelatin was not liquefied and indole was not produced. Nitrate was not reduced to nitrite.

None of the strains were easily maintained as stock cultures and the end-products of the fermentation of glucose and lactic acid were not completely determined before the organisms were lost. Only acetic and butyric acids were demonstrated in lactate cultures, but products other than volatile acids were not determined. Lactic acid was produced from glucose.

The isolates did not show branching but were otherwise similar to Ramibacterium sp. isolated by Bryant et al. (1958c) and were compared with strain L34 obtained from Dr M. P. Bryant. As the branching of cells of Ramibacterium L34 appeared to be a stable characteristic evident in both young and old cultures and on both liquid and solid media, isolates 6, 18 and 23-29 were classified as Butyribacterium sp. similar to B. rettgeri Prevot (Breed et al., 1957).

The end-products of the fermentation of lactate by Butyribacterium rettgeri have been shown to be mainly acetic and butyric acids plus carbon dioxide (Barker, Kamen and Haas, 1945; Pine and Barker, 1954). Glucose is fermented to mainly lactic, acetic and butyric acids plus carbon dioxide (Pine, Haas and Barker, 1954).

Bryant et al. (1958c) did not determine the products of lactate fermentation by Ramibacterium L34, but found formic,

acetic and lactic acids were produced from glucose. L34, obtained from Dr Bryant, was grown on the basal medium with either glucose or lactic acid, and the acidic end-products were analysed. From lactic acid the organism produced approximately equimolar amounts of acetic and butyric acids equivalent to about 70% of the lactate-carbon metabolized. Acetic, butyric and lactic acids were produced from glucose; acetic and butyric in the molar ratio 3:1, and accounting for 40% of the glucose carbon. A small but undetermined amount of carbon dioxide was produced from both glucose and lactic acid. From these results it was evident that the metabolism of lactic acid and glucose by L34 is very similar to that of Butyribacterium rettgeri, both organisms producing the same end-products from both of the substrates.

Two pathways for the formation of propionic acid from lactate have been demonstrated with pure cultures of rumen bacteria. The well-known dicarboxylic acid pathway has been shown to occur in propionibacteria, Veillonella alcalescens (V. gazogenes, Johns, 1951b) and Selenomonas (Bryant, 1956), and the so-called non-randomizing pathway, through acrylate to propionate, has been demonstrated in Peptostreptococcus elsdenii (Ladd and Walker, 1959). Baldwin, Wood and Emergy (1962) in the United States, showed that in cows on various diets, 70-90% of the propionate formed from lactate was produced by the acrylate pathway. P. elsdenii has not been isolated in this country and even if the randomizing pathway is the more prevalent, propioni-

bacteria, Veillonella and Selenomonas are not present in large numbers in the rumen. It is possible that other lactate-utilizing bacteria may be important under New Zealand conditions. For this reason, because of the current interest in the fate of the two main metabolic intermediates, lactate (Jayasuriya and Hungate, 1959; Baldwin, Wood and Emery, 1962, 1963) and succinate (Blackburn and Hungate, 1963), in the rumen and because of the similarities between Ramibacterium and Butyribacterium rettgeri, some additional studies on the fermentation of lactic acid by Ramibacterium were carried out.

B. rettgeri has a requirement for lipoic acid (thioctic acid, BR factor; Kline and Barker, 1950; Kline et al., 1952) in the decomposition of lactic acid (Kline, Pine and Barker, 1963), the organism being unable to attack lactate in the absence of the growth factor. Kline, Pine and Barker (1963) showed that the factor was not required for growth on glucose but that lactate-adapted cells grew better when lipoic acid was provided. Less lactic acid was produced by glucose cultures in the presence of lipoic acid. Both lactate-adapted and glucose-adapted cells used both the lactic acid and the glucose in a lactate/glucose culture containing lipoic acid. Lactic acid was produced from pyruvate, only in the absence of lipoic acid.

Experiments were carried out to test the effect of lipoic acid on the fermentation of lactic acid by Ramibacterium L34. Two culture-lines of L34 were established; L34/g. was cultured in glucose medium, and L34/l. was adapted to lactate. Lipoic

acid (60 µg./100 ml.) was shown to have no effect on lactate fermentation by L34/g. or L34/1., in cultures with glucose or lactic acid or in cultures with both substrates added. Yeast extract (Difco), in the concentration used by Kline, Pine and Barker (1963), also had no effect.

Because L34 could not be grown in the absence of rumen liquor, experiments were carried out with washed suspensions of cells from both lactic acid and glucose media. The cells were incubated under anaerobic conditions for periods of up to 18 hr. with lactic acid, and with and without added lipoic acid. In all experiments the rate of decomposition of lactic acid was low, but there was no detectable increase in utilisation in the presence of lipoic acid.

An experiment where L34 was grown with sodium pyruvate (0.5%) as substrate in the presence of rumen liquor and added lipoic acid, provided more evidence that lipoic acid was not required for lactate-decomposition. Lactic acid was produced from pyruvate even in the presence of added lipoic acid. Butyri-bacterium rettgeri produces lactic acid from pyruvate only in the absence of lipoic acid.

The possibility that L34 could only use one stereo-isomer of lactic acid, and that it produced the other isomer from glucose, was tested by growing the organism on both D(-)- and L(+)-calcium lactate. Growth on the two isomeric forms was similar after 24 and 48 hr., although there was slightly heavier growth on the D(-) isomer after 72 hr.

Several attempts to obtain further isolates of Butyribacterium from the rumen were unsuccessful. Cultures were prepared from the rumen liquor of both cows and sheep on diets of hay and concentrates. On two occasions lipoic acid was added to one of a double series of cultures of rumen liquor dilutions but did not cause an increase in the number of lactate-utilizers isolated, and did not enable Butyribacterium to be isolated.

Discussion

The 56 strains of bacteria isolated and characterized were all typical rumen bacteria and represented most of the types that attack the common rumen substrates. The important rumen bacteria attacking these substrates, together with the bacteria isolated in this study are shown in Table 50. The surprising result was the small number of gram-negative organisms isolated. All the bacteria with the exception of Butyrivibrio fibrisolvens and Veillonella gazogenes were gram-positive, although many of the isolates quickly changed to gram-negative as the culture aged. No Bacteroides species were isolated. As Bacteroides have been shown to carry out several important rumen reactions (Bryant, 1959), and as gram-negative bacteria constitute a large proportion of the rumen flora as shown by microscopic examination, the failure to isolate them was probably the result of an inadequate technique. All are obligate anaerobes and may not have grown under the redox conditions used for most of the isolations.

The other genera which were not found commonly make up

only a small percentage of the total bacterial population. Eubacterium, Lachnospira, Cillobacterium, Succinomonas and Succinovibrio have not been shown to be important rumen bacteria under all conditions as have most of the more common genera. Many of these lesser known bacteria are probably present in the rumen of cows in this country and are outnumbered and outgrown in cultures by the more numerous types. The isolation of the less common rumen anaerobes is often largely a matter of chance unless selective media can be used. A large number of colonies from a culture need to be picked off and cultured to ensure that a bacterium occurring in low numbers is obtained.

The oxygen tension in the medium obviously influences the types of bacteria isolated. Attempts to isolate lactate-decomposing bacteria by means of shake-tubes, without gassing or using reducing agents, always resulted in an overwhelming growth of Veillonella alcalescens. In cultures which incorporated a reducing agent and which had been thoroughly gassed to reduce resazurin, V. alcalescens failed to grow and Selenomonas ruminantium var. lactilyticas and a Butyribacterium species were isolated. This technique of selection by varying the redox potential of the medium was also used by Smith and Hungate (1958) to isolate Methanobacterium ruminantium and is worthy of more extensive application. It is possible that many rumen bacteria only grow or metabolize over a restricted range of Eh.

Several of the bacteria isolated, Ruminococcus spp., Butyribacterium sp. and Borrelia sp., required rumen liquor for

Table 50. Some rumen bacteria and their substrates

\+ attacked by some or all strains

\- not attacked

* species isolated in this study

Numbers in brackets are references given below.

	Cellulose	Xylan	Starch	Pectin	Gelatin	Lactic acid	Glycerol
Bacteroides succinogenes (1, 16)	+	-	-	+	-	-	
Ruminococcus flavefaciens (3)*	+	+	-		-	-	
Ruminococcus albus (2)*	+	+	-		+	-	
Butyrivibrio fibrisolvens (5, 8)*	+	+	+	+	+		
Clostridium cellobioparus (4)	+	+	-			-	-
Clostridium lochheadii (2)	+		+		+		
Clostridium bifermentans (15)*	-	-	+	-	+	-	+
Eubacterium ruminantium (18)	-	+	-		-	-	-
Bacteroides amylogenes (6)	-	+	-		-	-	
Bacteroides ruminicola (7)	-	+	+	+	+	-	-
Bacteroides amylophilus (9)	-	-	+		+	-	-
Succinomonas amylolytica (7)	-	-	+		-	-	-
Streptococcus bovis (2, 10)*	-	-	+		-	-	-
Lactobacillus bifidus (20, 22)*	-	-	+		-	-	-
Selenomonas ruminantium (12, 23)*	-	+	+	-	+	+	+
Succinovibrio dextrinosolvens (21)	-	-	-	+	-	-	-
Lachnospira multiparus (21)	-	-	-	+	-	-	-
Peptostreptococcus elsdenii (10, 13)	-	-	-		-	+	+
Veillonella alcalescens (14)*	-	-	-		-	+	
Ramibacterium sp. (19)	-	-	-	-	-	+	+
Borrelia sp. (17)*	-		-		-	-	-
Propionibacterium acnes (11)*	-	-	+	-	+	-	+

(1) Bryant and Doetsch, 1954; (2) Hungate, 1957; (3) Sijpesteijn, 1951; (4) Hungate, 1944; (5) Bryant and Small, 1956b; (6) Doetsch et al., 1957; (7) Bryant et al., 1958a; (8) Gutierrez et al., 1959b; (9) Hamlin and Hungate, 1956; (10) Gutierrez et al., 1959a; (11) Gutierrez, 1953; (12) Bryant, 1956;

... Continued over

(13) Elsden et al., 1956; (14) Johns, 1951; (15) Clarke, 1961; (16) Hungate, 1950; (17) Bryant, 1952; (18) Bryant, 1959; (19) Bryant et al., 1958c; (20) Clarke, 1959; (21) Bryant and Small, 1956a; (22) Gibbons and Doetsch, 1959; (23) Hobson and Mann, 1961.

growth. Various workers have shown that this requirement can be met by the addition of pure compounds. Thus Bryant and Robinson (1962) and Caldwell, Bryant and White (1962) showed that heme or some other porphyrins could replace the rumen liquor requirement of Bacteroides ruminicola subsp. ruminicola, and Bryant and Doetsch (1955) demonstrated that Bacteroides succinogenes requires either isobutyric, isovaleric or 2-methyl butyric acids, together with a C_5-C_8 straight chain saturated acid, in the absence of certain amino acids. Wegner and Foster (1957, 1960) showed that a large number of rumen bacteria require one or more of the volatile acids, n-valeric, iso-valeric, iso-butyric and 2-methyl butyric for growth. Acetate is also important in the nutrition of some rumen bacteria (Bryant and Robinson, 1962) and has been shown to increase the growth of some strains of Selenomonas ruminantium (Hobson and Mann, 1961; Hobson, Mann and Smith, 1963). Both the Ruminococcus spp. studied here grew well when iso-valeric acid was used to replace rumen liquor in the medium. Allison, Bryant and Doetsch (1962) showed that Ruminococcus flavefaciens incorporates this acid into leucine as it has a limited ability to incorporate exogenous branched-chain amino acids and to synthesize the iso-propyl

group found in these amino acids. Bacteroides succinogenes has been shown to incorporate iso-butyric and valeric acids into phospholipid (Wegner and Foster, 1963).

None of the acids, acetic, propionic, n-valeric, iso-valeric or iso-butyric, or yeast extract, or a mixture of growth factors satisfied the requirements of the Borrelia or Butyri-bacterium species. Neither 2-methyl butyric acid nor heme were tested. Bryant (1960) also noted that Borrelia spp. require certain growth factors found in rumen liquor.

Apart from Clostridium bifermentans, which probably is only of minor importance in the rumen, no proteolytic bacteria were isolated on casein. Both bovine salivary mucoprotein and clover cytoplasmic protein were also used as substrates for proteolytic bacteria without success. Proteolytic strains of Butyrivibrio fibrisolvens were isolated on a glucose-cellobiose medium.

Many of the attempts to isolate proteolytic anaerobes from the rumen have been unsuccessful (Appleby, 1955; Gilroy, 1957; Hunt and Moore, 1958; Blackburn and Hobson, 1960b), the organisms isolated being facultative anaerobes. Blackburn and Hobson (1960b), basing their observations on the proteolysis occurring in preparations of whole and fractionated rumen contents (Blackburn and Hobson, 1960a, c), suggested that the proteolytic organisms then isolated represented only a fraction of the proteolytic bacteria of the rumen, and since then many proteolytic anaerobes have been isolated. Blackburn and Hobson (1962) showed

that proteolytic activity was distributed over a wide range of rumen bacteria. This work was extended by Abou Akkada and Blackburn (1963) who found proteolytic rumen isolates of Bacteroides amylophilus, B. ruminicola, Selenomonas, Butyrivibrio, Bacillus, Eubacterium, Clostridium and gram-positive cocci. Similar results were obtained by Fulghum and Moore (1963) who isolated several anaerobic proteolytic bacteria and estimated that these organisms accounted for 38% of the total count on glucose-cellobiose agar. The organisms isolated, Butyrivibrio, Succinivibrio, Selenomonas, Borrelia and Bacteroides, are all organisms which can use substrates other than protein, and it is now apparent that there may be no important rumen bacteria similar to the classical putrefactive bacteria and capable of existing on protein as sole nitrogen and carbon source.

In spite of the large number of proteolytic anaerobes now known to be active in the rumen a considerable proportion of the total proteolysis could be carried out by the facultative anaerobes, in the same way as the euryoxic Streptococcus bovis is active in the degradation of starch. Also, in view of the protozoa shown to be capable of protein degradation (Williams, Gutierrez and Doetsch, 1960; Williams, Davis, Doetsch and Gutierrez, 1961; Gutierrez and Davis, 1962; Abou Akkada and Howard, 1962; Wright, Clarke and Howard, unpublished), the rumen ciliates could contribute appreciably.

There is, perhaps, no need to look further for proteolytic bacteria in the rumen. Butyrivibrio and Bacteroides are nearly

always present in the rumen of sheep and cattle and their activity, together with that of the facultative anaerobes and ciliate protozoa, should be great enough to account for most, if not all, of the observed proteolytic activity.

The breakdown of cellulose in the rumen is apparently carried out mainly by Ruminococcus if Bacteroides succinogenes (Bryant and Doetsch, 1954) is really absent. A more intensive search for this organism is warranted.

Even though the animals used in this investigation were not on high-starch diets, the few types of starch utilizing bacteria isolated was unexpected. Streptococcus bovis, Lactobacillus bifidus, Selenomonas ruminantium and Butyrivibrio fibrisolvens were the main starch-utilizers isolated. Bacteroides ruminicola, B. amylophilus and Succinomonas amylolytica, all of which have been shown to be active starch fermenters (Bryant, 1959) were not found. The presence of Butyrivibrio fibrisolvens may account for a considerable proportion of the bacterial fermentation activity in the rumen. Bryant and Small (1956b) noted that Butyrivibrio are of wide distribution and usually occur in large numbers, and stated (1956a, p20) that "they are of considerable importance in the breakdown of fibrous constituents of cattle feed as shown by the ability of some strains to digest cellulose, and most strains digest xylan ..... Also, the genus Butyrivibrio undoubtedly contributes to the breakdown of starch and protein in the rumen.

Cultural studies suggest that this genus produces a large

portion of the butyric acid found in the rumen. While other organisms producing this acid have been isolated, they have never been found in numbers approaching the numbers of Butyrivibrio."

The main lactate-fermenters appear to be Selenomonas ruminantium and Veillonella alcalescens as Peptostreptococcus elsdenii was not isolated. Butyribacterium is probably important as a lactate-fermenter only in certain circumstances. It was isolated over a period of several months from two cows on a diet of grass hay, but could not be obtained from other animals on a variety of diets.

The metabolism of lactic acid and glucose by Ramibacterium, L34 was shown to be very similar to that of Butyribacterium rettgeri, both organisms producing acetic and butyric acids from both of the substrates, in addition to lactic acid from glucose. Bryant et al. (1958c) were in error in attributing formic acid production to L34. The few analyses made on the end-products produced by Butyribacterium strain 18 indicated its similarity with L34 and B. rettgeri.

The lack of response to lipoic acid by cultures of L34 may have been the result of the presence of rumen liquor in the medium. Lipoic acid participates in many α-keto acid oxidation systems (Reed, 1960), and occurs, largely in association with proteins, in a large number of tissues. It has been found in chloroplast preparations of green plants (Calvin, 1954) and is present in many micro-organisms and animal tissues (Reed et al., 1951; Gunsalus, Struglia and O'Kane, 1952). It is unlikely that

it is not available in rumen liquor. The production of lactic acid during the fermentation of pyruvate suggests however, that L34 does not require lipoic acid for the reduction of lactate. In the presence of lipoic acid B. rettgeri does not produce lactic acid from pyruvate. The studies with washed suspensions confirmed the lack of response to lipoic acid, although the rate of utilisation of lactate was too low to draw any firm conclusions.

Further studies on Butyribacterium from rumen liquor would be worth while. The organism may be important in the decomposition of lactate in the rumen under some circumstances, and is of interest because of its production of butyric acid. The common rumen lactate-utilizers convert lactic acid to propionate.

Most of the bacteria obtained in pure culture were difficult to maintain without frequent sub-culturing. Some strains survived well at -70° but lack of a suitable supply of dry-ice and an efficient storage chest usually resulted in the loss of these cultures. None of the obligate anaerobes tested survived lyophilization. The labour involved in maintaining cultures, even when adequate storage facilities are available, can be excessive, and is one of the main factors contributing to the tediousness of culture isolation and characterization.

The analysis of fermentation solutions is not straightforward when rumen liquor is incorporated in the culture medium. All determinations of the fermentation end-products have to be made by difference, and substances introduced with the rumen liquor

are often fermented along with the substrate under investigation. If sufficient cells can be prepared then washed suspensions are to be recommended in such circumstances.

The analysis of formic acid in the presence of other fermentation products was found to be difficult. Most chemical methods of estimation (Grant, 1947, 1948; Perlin, 1954) were not satisfactory because of the presence of interfering substances. Lactic acid and phenol red, both present in the neutralized distillate following steam-distillation, were shown to interfere with the method of Perlin (1954) which involves oxidation of formate with lead tetra-acetate. The column chromatography methods of Bueding and Yale (1951) and Swim and Utter (1957) are too time-consuming for routine analysis and a precise chemical method would be more suitable. The method based on that of Pirie (1946) and involving a gravimetric estimation of the CO_2 released from formic acid by mercuric acetate is apparently satisfactory in the presence of other fermentation materials (Dr R. D. Batt, pers. comm.), but was not tested.

Succinic acid was not determined quantitatively. The best methods available, both utilizing succinic dehydrogenase, are those of Krebs (1937) and Rodgers (1961). Neither of these methods is really convenient for routine analysis of succinate as both procedures are very lengthy.

4. MANOMETRIC STUDIES ON RUMEN ACTIVITY

Introduction

Hungate (1960, p359) in discussing the microbial ecology of the rumen stated: "The most difficult aspect of the ecological analysis of a habitat is to obtain quantitative information on the extent of the activities. A complete analysis should include not only a measurement of the total activity but also the activity of each component step. The degree of success and completeness in an ecological analysis can be measured by the degree to which the algebraic sum of the activities of the component parts equals the integrated activity of the whole. The measurement of total activity is thus essential for a complete analysis. For many habitats this measurement is difficult, but for the rumen it is relatively easy."

The measurement of fermentation activity in the rumen has many applications other than that of providing ecological information. Walter (1952) suggested that it is useful for evaluating rations and studying the effects of antibiotics and therapeutic agents, as well as for providing general information on ruminant digestion. The method has been used to compare the fermentation rates in bloated and normal cattle (Hungate et al., 1955; Fletcher and Hafez, 1960) and to study the fate of formate (Carroll and Hungate, 1955) and lactate in the rumen (Jayasuriya and Hungate, 1959) and the effects of antibiotics on the rumen flora and fauna (Hungate, Fletcher and Dyer, 1955).

Four methods have been used for measuring the activity of the rumen microbiota. The first method used was that of Barcroft, McAnally and Phillipson (1944) who measured the volatile acid content of the bloodstream of the host, and coupled this with the rate of flow of the blood to obtain an estimate of the total production of fatty acids in the rumen. The second method involves measurement of the total methane formed in the rumen by fermentation, and was used by Hungate et al. (1960).

The other two methods entail in vitro measurements on rumen contents. The zero time rate method of Carroll and Hungate (1954) involves measurement of the change in concentration of either substrate or product, or both, in a sample or rumen liquor incubated in vitro immediately after removal from the animal. The manometric method, first used by Walter (1952) and McBee (1953) uses the total gas production from incubated rumen contents as a measure of the total fermentation activity. The zero time rate method is more precise than the manometric method but is more time-consuming (Hungate et al., 1960a).

Various methods have been used to estimate rumen activity manometrically. Walter (1952) and McBee (1953) both used standard Warburg equipment capable of measuring the gas evolved from a 1-2 ml. sample of rumen liquor. In view of the heterogenous nature of rumen contents, larger samples are really required. This was realized by Hungate et al. (1955, 1959, 1960) and Phillips et al. (1960) who modified the technique to

permit the use of larger samples of rumen liquor. Their technique utilized 160 ml. manometric vessels capable of holding samples of at least 10 g. Mercury was used in the standard Warburg manometers to permit measurement of the large amount of gas produced. Conrad et al. (1958) used 35 ml. samples of rumen liquor. Boda and Johns (1962) used even larger samples and measured the gas evolution from 100-200 ml. of rumen liquor.

Most of the methods used have involved dilution of the sample with an inorganic solution. This dilution of the rumen contents, as Hungate (1960, p360) pointed out, "modifies conditions from those in the rumen, chiefly in the direction of a more rapid and longer maintained activity in vitro, since the fermentation products are diluted." The fermentation may also be reduced because of inhibition of activity following the sudden change of environment and because of the inhibitory effect of substances such as reducing agents which have been added.

Where substrates have been added to the rumen contents under investigation, the materials used have usually been markedly different from normal dietary substances. Most experiments have been carried out with materials such as soluble sugars, soluble starch, prepared cellulose and oven-dried forage, thus contributing further to the artificiality of the system.

In the present work a method similar to those of Boda and Johns (1962) and Conrad et al. (1958) was devised to overcome these limitations. This section reports the results of manometric studies on the fermentation activity of whole and

fractionated rumen liquor under various conditions.

Materials and Methods

Materials

Rumen liquor. Rumen liquor was obtained from rumen-fistulated cows. The animals were stall-fed on fresh red clover (Trifolium pratense L.), red clover hay or grass hay, between 8.30 am and 4 pm, and were starved overnight. When the animals were fed on pasture, they were starved overnight after being taken from the pasture at 4 pm. Samples were always taken just before the morning feed so as to standardize the activity of the rumen contents. The sample was taken from immediately below the fistula at a depth of 6-12 in. The rumen contents were strained through gauze into a quart milk bottle, and the sample was transferred to an incubator at 39° and used within 1 hr.

Clover juice. Clover juice was obtained by extracting fresh red clover in a "Protess" Extraction Plant (E. H. Bentall & Co. Ltd., Maldon, England). It was strained through cheesecloth, divided into 25 ml. portions in 1 oz. screw-capped bottles, and frozen at -25° until required.

Ryegrass juice. Juice from perennial ryegrass (Lolium perenne L.) was extracted and stored under the same conditions as the clover juice.

Clover fibre. This was the fibrous residue from the processing of fresh red clover in the "Protess" extractor.

Buffer for substrate control. As fresh clover juice was in the

pH range 5.6-5.8, phosphate buffer, M/15, pH 5.7, was used to replace clover juice in flasks without substrate.

Soluble sugars. Solutions of glucose, galactose, sucrose and maltose were 0.2 M in M/15 phosphate buffer, pH 5.7.

Clarified rumen liquor. Fresh rumen liquor was centrifuged at low speed in a refrigerated centrifuge to remove large plant material and protozoa. The supernate was then centrifuged at 10.000 g for 30 min. The rumen liquor produced was clear and practically free of bacteria.

Methods

Manometric techniques. Fermentation experiments were carried out in 150 ml. Warburg-type flasks shaken in a water bath at 39°. Gas evolved was collected in 200 ml. manometers, with levelling bulbs connected to the flasks with 4 ft. of thick-walled butyl rubber tubing. The manometers were filled with distilled water containing 0.01% safranin. During equilibration the flasks were gassed with 95% N_2/5% CO_2.

Each flask contained 40 ml. of whole or fractionated rumen liquor. The substrate, or buffer, (10 ml.) was initially placed in a sidearm. The duration of each experiment was usually 1 hr.

Volatile acids. Total volatile acids were determined by steam-distillation, followed by titration with 0.02 N NaOH (see previous section).

Analysis of the individual acids in the steam-distillate was carried out using a gas-liquid chromatograph with an automatic titration detector.

Lactic acid. Lactic acid was determined by the method of either Freidemann and Graeser (1933) or Barker and Summerson (1941).

Total soluble sugars. The total soluble sugars (soluble in 80% (v/v) ethanol) in hay and pasture samples were measured by Dr R. W. Bailey using the method of Bath (1958).

Experimental and Results

A. EXPERIMENTS WITH WHOLE RUMEN LIQUOR

The permeability of various tubing to CO_2

In the preliminary manometric experiments thin-walled rubber tubing was used to connect the flasks and manometers. There was considerable loss of gas from this system and an experiment was carried out to determine the most satisfactory tubing.

Several types of rubber and plastic tubing were used to connect empty flasks and manometers, which were then flushed with CO_2, sealed and left at atmospheric pressure for several hours. There was considerable loss of gas through thin-walled rubber and plastic tubing, but only a slight loss through thick-walled tubing. The results are shown in Table 51. In all subsequent experiments butyl rubber tubing was used to connect the manometers.

The effect of treatment of sample on fermentation rate

The precautions necessary to maintain maximum activity in rumen liquor after removal from the animal were determined by subjecting samples of rumen liquor to various treatments before

Table 51. Permeability of various tubing to CO_2

Type of tubing	Dimensions (in.) Bore	Wall	Gas lost at atmospheric pressure (ml./ft./hr.)
Rubber	0.25	0.06	8.0
Rubber	0.22	0.16	1.5
Silicone	0.09	0.06	7.0
Polyvinyl chloride	0.22	0.06	7.5
Polyethylene	0.22	0.06	8.0
Butyl rubber	0.09	0.16	1.3

measurement of their fermentative ability.

Flask fermentations were carried out with clover juice (10 ml.) as substrate and with rumen liquor (40 ml.) treated as shown below.

(1) The rumen liquor was added to the flasks as quickly as possible with the usual precautions to maintain anaerobiosis, and the flasks were gassed with N_2/CO_2.

(2) As (1), but the flasks were not gassed.

(3) The rumen liquor was cooled to room temperature (18°) on the bench before it was added to the flasks and gassed.

(4) The rumen liquor was vigorously aerated by bubbling for 5 min. before it was added to the flasks and gassed.

The results of a single experiment are shown in Table 52. Provided that the flasks were gassed at the beginning of the experiment and precautions were taken to avoid extensive aeration of the rumen liquor prior to gassing, the fermentation activity was not affected by handling.

Table 52. Effect of treatment of rumen liquor on the fermentation rate

	1. Control-all precautions	2. Flasks not gassed	3. Cooled and gassed	4. Aerated and gassed
Ml. gas in 60 min.	39	24	38	36

Gassing and the oxidation-reduction potential of rumen liquor

Rumen liquor was added to a flask with the usual precautions to exclude air. The flask was then shaken in air for one min. and gassed. The oxidation-reduction potential was measured with a Beckman pH meter (Model 76) before and during gassing which was carried out with the N_2/CO_2 delivered just above the surface of the liquid as if via a side-arm. Intermittent shaking was applied during the gassing. The results of two experiments are shown in Table 53.

Table 53. Effect of gassing on oxidation-reduction potential of rumen liquor

Results given in negative millivolts

Before shaking in air	Flask shaken 1 min. in air	Flask gassed with N_2/CO_2 for (min.) 1	3	4	5	8	10	12
350	220	280	350	370	390	400	400	400
370	260	280	340	370	370	380	390	390

As a minimum potential was reached after gassing for 8-10 min., flasks were gassed for 10 min., with shaking, in all

subsequent experiments.

A sample of rumen liquor left standing on the bench in a flask maintained a potential of -320 mV at the surface, for 10 min. The handling of rumen liquor during sampling and transfer is apparently only sufficient to lower the redox potential slightly.

Daily variation of fermentation rate

Day-to-day variation of the rate of fermentation of clover juice was demonstrated on samples of rumen liquor taken on week days from cow 2 before feeding on red clover hay. The animal was also fed clover hay on Saturdays and Sundays, but samples of rumen liquor were not taken. The rate of fermentation was tested by the usual method.

The results are shown in Table 54. The day-to-day variation was ±5% of the mean recorded rate of 29.6 ml./hr. from 40 ml. rumen liquor.

Table 54. Daily fermentation rate of clover juice

Rumen liquor from cow 2 on clover hay diet

Day:	Thurs.	Fri.	Mon.	Tues.	Wed.	Thurs.	Fri.	Tues.	Wed.	Thurs.
ml. gas /hr.	25	27	32	34	31	29	34	29	30	25

Effect of diet on the rate of fermentation of clover juice

The effect of the diet of the host on the activity of the rumen flora and fauna towards clover juice was studied over a

period of eight months. From June, 1961 to January, 1962, cow 2 was stall-fed on grass hay, clover hay, or fresh red clover, or was fed on mixed clover-ryegrass pasture. Feeding was carried out from 8.30 am to 4.00 pm, and the animal was starved overnight. Rumen liquor samples were taken before feeding. The feeding sequence used over the period June to November, 1961, was grass hay, clover hay, grass hay, pasture, grass hay, fresh clover. The experimental work was discontinued between November, 1961 and mid-January, 1962. During this period the animal was fed on fresh clover or pasture. From mid-January fresh red clover was again fed.

The results of the manometric experiments for the whole period are shown in Figure 1. It is important to realise that the rate determined on any one day is actually influenced by the diet of the previous days, and that it bears no relation to the diet of the day on which it was recorded. The figure has been drawn to stress this point.

Determinations of total soluble sugars were made on representative samples of the grass and clover hay and pasture fed during the experimental period. No determinations were carried out on the fresh red clover but the total soluble sugars of leaf and stem would probably constitute 16-20% of the dry weight (Bailey, 1958b).

The mean figures for gas evolution by rumen liquor, for the four diets, together with the total soluble sugar concentrations for samples of those diets are shown in Table 55.

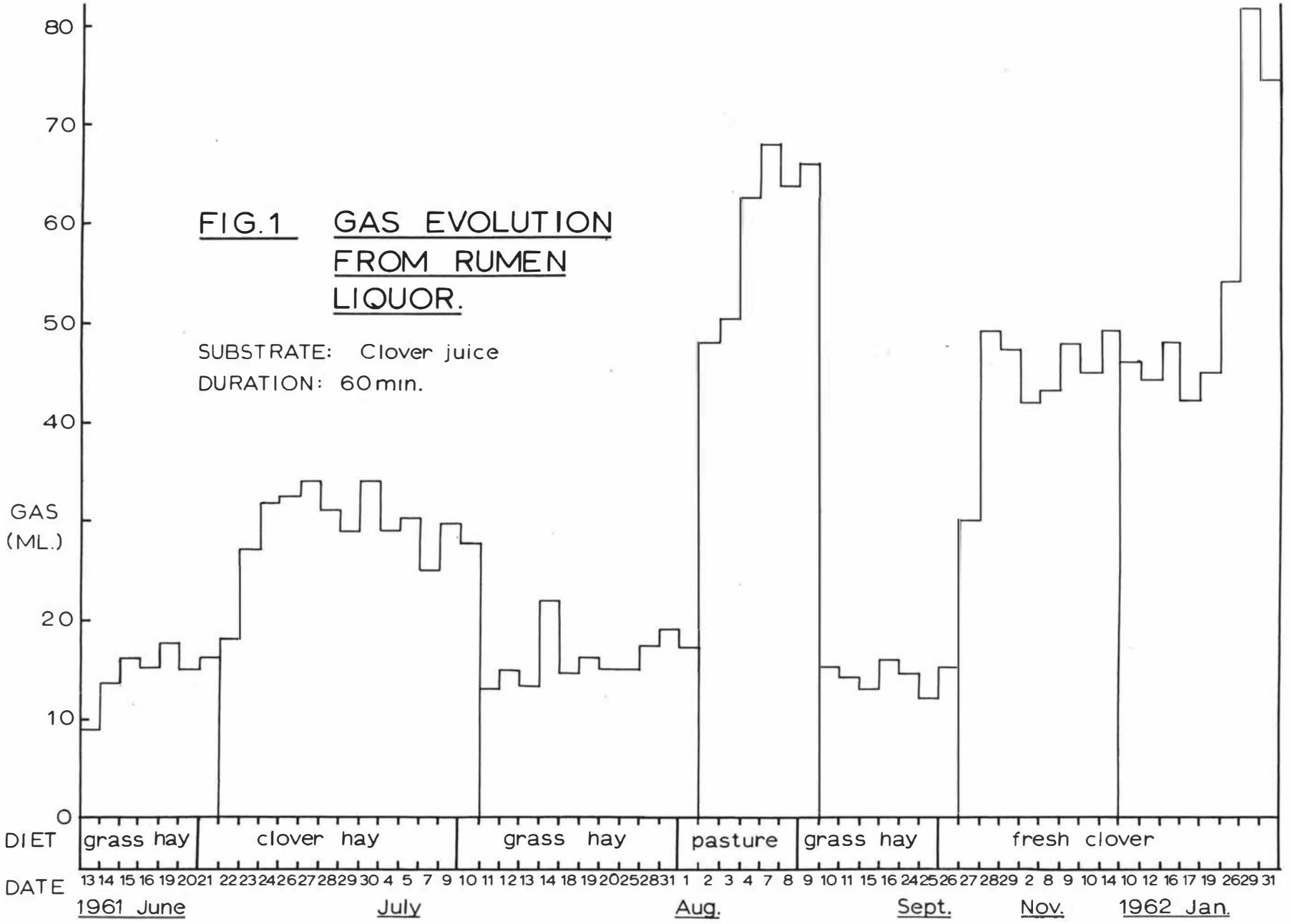


FIG.1 GAS EVOLUTION FROM RUMEN LIQUOR.
SUBSTRATE: Clover juice
DURATION: 60 min.
GAS (ML.)
80
70
60
50
40
20
10
0
DIET
grass hay
clover hay
grass hay
pasture
grass hay
fresh clover
DATE
13 14 15 16 19 20 21 22 23 24 26 27 28 29 30 4 5 7 9 10 11 12 13 14 18 19 20 25 28 31 1 2 3 4 7 8 9 10 11 15 16 24 25 26 27 28 29 2 8 9 10 14 10 12 16 17 19 26 29 31
1961 June
July
Aug.
Sept.
Nov.
1962 Jan.

Table 55. Dietary soluble sugars and fermentation rate

Diet	Mean gas evolved (ml./hr.)	Total soluble sugars of dietary material (% dry wt.)
Grass hay	16	4.8
Clover hay	30	5.2
Mixed pasture	56	16.9
Fresh clover	52	16-20 (Bailey, 1958b)

Fermentation of soluble sugars

As previous workers have used soluble sugars as substrates for measurement of rumen activity (Walter, 1952; McBee, 1953), glucose, galactose, maltose and sucrose were occasionally compared with the clover juice substrate. 10 ml. of a 0.2 M solution of each sugar was used per flask. Sucrose and glucose caused the most active fermentations but the gas evolved was always less than that evolved from clover juice. The results are shown in Table 56.

Table 56. Gas evolution from clover juice and soluble sugars

Diet	Gas evolved (ml./hr.)				
	Clover juice	Glucose	Sucrose	Galactose	Maltose
Clover hay	34	18	19	8	7
"	31		22	11	
"	29	14	16		
"	49	20	22	11	9
Grass hay	15		12		
"	13		9	5	
"	22		8	2	
"	20	5		2	1
Mixed Pasture	48	18	21	8	
"	68	23	22	12	8

Fermentation of clover fibre

Experiments were carried out to compare the rates of fermentation of fibre with those for the fermentation of juice. Conrad et al. (1958) and Boda and Johns (1962) reached different conclusions regarding the relative rates.

The Warburg-type flasks were used and the fibre was weighed and poked into the flasks so that it was sitting on top of the centre well. After gassing and equilibration it was mixed with the rumen liquor by shaking. In each experiment unwashed fibre was compared with fibre washed in warm and cold water and with clover juice. The rumen liquor was from a cow feeding on fresh red clover. Different batches of fibre were used for each experiment. The results of four experiments are shown in Table 57.

Table 57. Gas evolution from clover fibre and juice

Gas, ml./hr; fibre, 2g.; juice, 10 ml.; rumen liquor, 40 ml.

Fibre 1	Fibre 2	Fibre 3	Juice
38	14	12	39
34	9	9	36
34	11	10	32
25	6	4	25

1. Untreated fibre
2. Fibre washed 1½ hr. in cold running water
3. Fibre washed in two changes of water at 45°

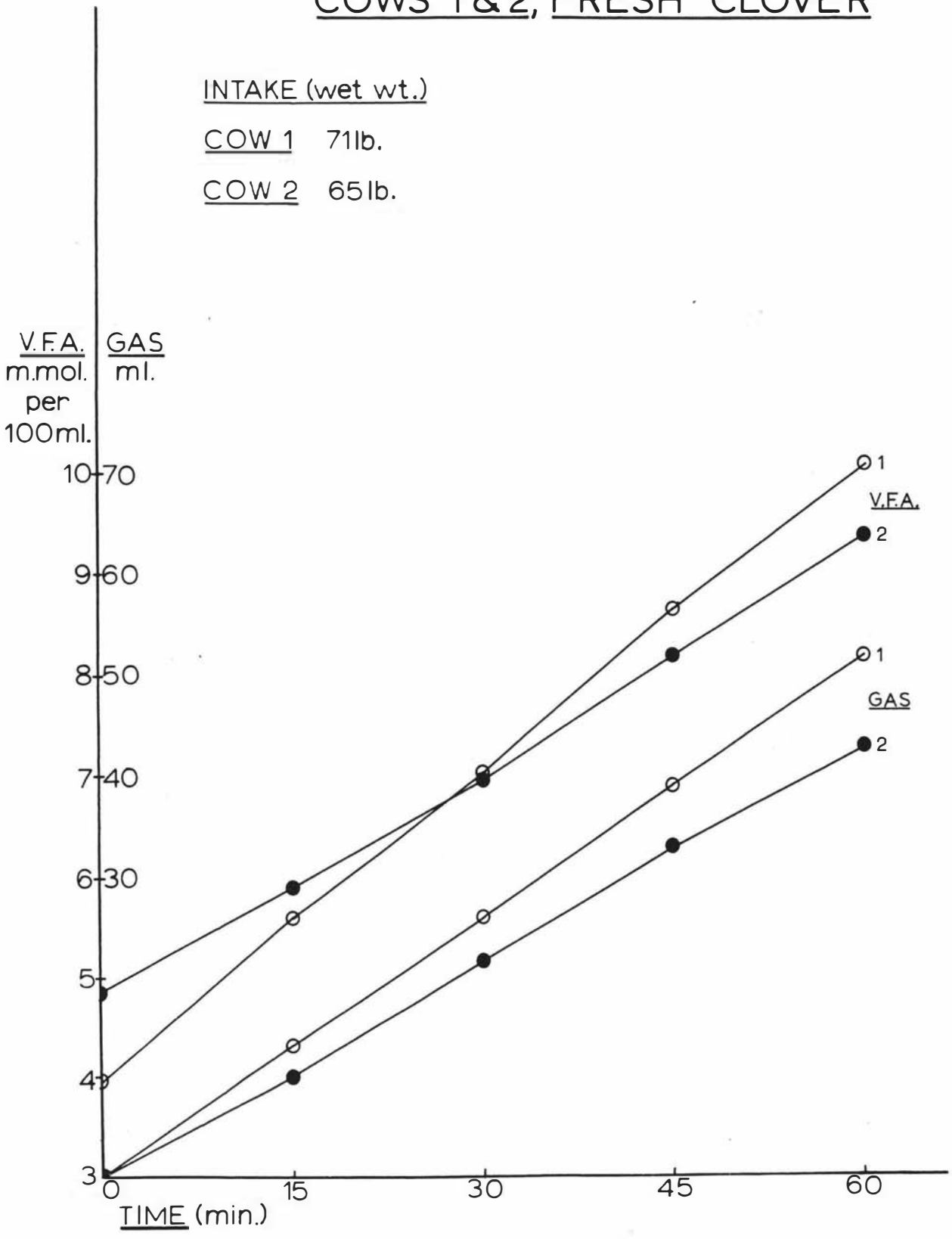
The influence of intake on the fermentation rate

As a high basal fermentation rate might result in an immediate, rapid utilisation of added substrate, the total food intake of an animal on any one day might be expected to influence the fermentation rate, before feeding, on the day following. Food present during all or most of the overnight starvation period would keep the rumen population at a high level of actual or potential metabolic activity. Substrate added under these conditions could be more rapidly fermented than substrate added to the rumen liquor of a cow whose rumen was devoid of food material for most of the overnight starvation period. This theory was tested by comparing the rates of fermentation shown in Figure 1 with the food intakes for the preceding days but no correlation was found. The animal's intake of food bore no relation to the fermentation rate on the following day.

Hungate et al. (1959) found the ratios of the weight of rumen contents to body weight of different ruminant species did not increase with decreasing body size as might be expected if the energy requirements of mammals correspond approximately to the $\frac{3}{4}$ power of their weight and if the rumen fermentation supplies the major part of these requirements in ruminants. They suggested that in small ruminants the total fermentation should be greater, in proportion to their weight, than in larger ones, and that the demand for extra energy could be met by greater food consumption and a greater fermentation rate per unit of dry matter.

The difference in fermentation rates between two animals

FIG.2 FERMENTATION RATES
COWS 1 & 2, FRESH CLOVER



was studied in a series of experiments on the identical twin cows, 1 and 2. This pair of twins was selected because at the time of the experiment, cow 1 was considerably smaller than its twin, had a smaller rumen volume, and usually had the higher intake. After overnight starvation the rumen contents of cow 1 always appeared to be the least frothy of the two, suggesting that the fermentation of ingested foodstuff was the more complete.

The intake of fresh redclover by the two cows was measured and the rate of fermentation of clover juice determined on six occasions. Table 58 shows the gas produced in each experiment and the intake of fresh clover on the preceding day. Cow 1 usually had the higher intake, and always had a higher fermentation rate. On two occasions the production of volatile acids was also measured. In both experiments the level of volatile acids in the rumen liquor of cow 1, before the addition of substrate, was lower than that in cow 2 but was higher after 1 hr. Figure 2 shows the gas and volatile acid produced in one of the experiments.

B. EXPERIMENTS WITH FRACTIONATED RUMEN LIQUOR

Estimates of the contributions of the bacteria and protozoa to the total rumen fermentation have indicated that the protozoa are responsible for about 20% of the total activity (Hungate, 1960). These estimates have been derived mainly from figures for the metabolic activity of single cells *in vitro* (Gutierrez, 1955).

Table 58. Gas evolution from clover juice by rumen liquor from cows 1 and 2 compared with intake of fresh clover on preceding day

Date	Cow 1		Cow 2	
	Gas ml./hr.	Intake lb./wet wt.	Gas ml./hr.	Intake lb./wet wt.
10.1.62	52	90	46	71
11.1.62	51	67	39	70
12.1.62	50	85	45	86
16.1.62	52	n.r.	48	n.r.
17.1.62	49	96	42	82
18.1.62	52	71	44	65

n.r. = not recorded

Experiments were carried out to assess the activity of the bacterial and protozoal fractions of rumen liquor. The manometric system was the same as previously described, and clover juice was used as substrate. The methods of preparing the fractions are described with the appropriate experiments.

The effect of the suspending medium on protozoal activity

Manometric experiments were carried out with protozoa suspended in both Oxford's (1958a) and Coleman's (1958) buffers. These preliminary experiments showed that protozoa suspended in the buffer of Oxford had a lower fermentation rate than protozoa in Coleman's buffer. Further work was carried out to determine the cause of this effect.

Mixed protozoa were prepared from the rumen liquor of a clover-fed cow by the method of Oxford (1958a) using Coleman's

(1958) buffer with 0.02% sodium sulphide. Equal volumes of the washed protozoa suspension were centrifuged, and the protozoa were suspended in clarified rumen liquor, Coleman's buffer with 0.02% sodium sulphide, and a mixture of buffer and rumen liquor (1:1). The suspensions were added to the large Warburg flasks and their rate of fermentation of clover juice determined. Measurements were made of gas evolution and volatile acid and lactic acid production. The results of one experiment are given in Table 59. The suspension of protozoa in rumen liquor produced more gas and acids than the other two suspensions. The amounts of end-products produced by the buffer/rumen liquor suspension were not appreciably lower but repeated experiments confirmed the difference.

Cysteine-HCl and sodium sulphide were compared as reducing agents in Coleman's buffer. The conditions of the experiment were the same as before but cysteine-HCl (0.1%) was used to replace sodium sulphide in one preparation of buffer. The results of one experiment are shown in Table 60. More end-products were produced by the protozoa in the cysteine buffer than by those in the buffer with sodium sulphide.

The effects of two levels of sodium sulphide and cysteine-HCl were compared with rumen liquor in a series of experiments. Equal numbers of protozoa were suspended in Coleman's buffer with 0.01% or 0.02% sodium sulphide or cysteine-HCl, or in clarified rumen liquor. The results are shown in Table 61. The protozoa in the cysteine buffer had a fermentation rate very

Table 59. End-products of the fermentation of clover juice by protozoa suspended in various media

	Na_2S*	Rumen liquor	Buffer*/Rumen liquor
Gas (ml.)	19.5	29	26
V.F.A. (m. mol./100 ml.)	1.3	1.9	1.8
Lactic acid (m. mol./100 ml.)	0.95	1.02	0.98

* Coleman's (1958) buffer with 0.02% sodium sulphide.

Table 60. End-products of the fermentation of clover juice by protozoa in Coleman's (1958) buffer with cysteine or sodium sulphide

	Na_2S (0.02%)	Cysteine-HCl (0.1%)
Gas (ml.)	16	26
V.F.A. (m. mol./100 ml.)	1.1	1.9
Lactic acid (m. mol./100 ml.)	1.02	1.57

similar to those in the rumen liquor and both preparations showed higher activity than the sulphide suspension. The protozoa in the buffer with 0.02% sodium sulphide produced only 60-70% of the gas and volatile acids produced by the rumen liquor suspension. There was a 4-8% reduction in gas and volatile acids in the presence of 0.01% sulphide. Lactic acid production was slightly decreased in both the systems with sodium sulphide.

Lower concentrations of sodium sulphide were not tested as concentrations less than 0.01% did not give sufficiently good reducing conditions.

Table 61. End-products of the fermentation of clover juice by protozoa suspended in various media

End-products produced in 60 min.	Protozoa suspended in Coleman's (1958) buffer with			Protozoa suspended in clarified rumen liquor
	Sodium sulphide 0.01%	Sodium sulphide 0.02%	Cysteine HCl 0.1%	
Gas (ml.)	1. 30	22	32	30
	2. 31	20	33	32
	3. 24	17	26	26
Volatile acids (m. mole)	1. 2.9	2.3	3.0	3.0
	2. 2.9	2.0	3.1	3.0
	3. 1.9	1.2	1.9	1.8
Lactic acid (m. mole)	1. 0.96	0.87	1.05	0.98
	2. 0.74	0.69	0.88	0.87
	3. 0.51	0.50	0.54	0.61

The potentials of three suspensions as used in the experiments in Table 61 were measured to determine whether the inhibitory effect of sodium sulphide was the result of a higher oxidation-reduction (redox) potential. The usual precautions to exclude oxygen were taken and the measurements were made with a Beckman pH meter (Model 76). The redox potential of the cysteine preparation (-310 mV) was higher than the sodium sulphide

and rumen liquor suspensions which were the same (-350 mV). The reduced fermentation rate in the suspensions with sodium sulphide was obviously not the result of less reducing conditions.

In all later experiments the buffer contained 0.01% sodium sulphide.

Experiments to check the inertness of the clarified rumen liquor showed that no gas was evolved when the rumen liquor was incubated with clover juice.

The contribution of the bacteria and protozoa to the total fermentation

The gas production from clover juice by the protozoal and bacterial fractions of rumen contents was determined with the manometric apparatus. Two methods of fractionation of the rumen liquor were used. In some experiments the protozoa were centrifuged at low speed from 100 ml. of rumen liquor, washed once with Coleman's (1958) buffer, and suspended in 100 ml. of clarified rumen liquor. The supernate, containing all the bacteria from the original 100 ml., was made to 100 ml. with clarified rumen liquor. In the second method, 100 ml. of rumen liquor was centrifuged at low speed for 2 min. The top 50 ml. of liquid was carefully removed. This fraction contained all the bacteria from 50 ml. of rumen liquor except those few sedimented with the protozoa. The residual fraction of 50 ml. contained the bacteria from 50 ml. and the protozoa from 100 ml. of rumen liquor. The calculation of the gas evolved by the protozoa was made according to the equation

$$P = \frac{A - B}{2}$$

where A = gas evolved by bacteria/protozoa fraction

B = gas evolved by bacterial fraction

P = gas evolved by protozoa

This calculation makes the reasonable assumption that the bacteria sedimented with the protozoa are few in number and do not contribute significantly to the fermentation.

The results of experiments using both of these methods are given in Table 62. Regardless of the diet of the host, the protozoa produced from 42-57% of the total gas produced. The percentage of the total gas production by the protozoa in the method using a combined bacteria/protozoa fraction was not higher than that in the other method as might be expected if the assumption about sedimented bacteria made in the calculation were incorrect.

Two further experiments where carbon dioxide was liberated from the rumen liquor before and after fermentation with H_2SO_4, gave 39% and 45% for the protozoal contribution to the gas production.

The amounts of volatile acids produced by the two fractions were also in agreement with the gas production, although the percentage acid from the protozoal fractions was a little higher than the percentage gas. The results of determinations on volatile acid production carried out on the first four clover hay experiments in Table 62 are shown in Table 63.

Table 62. Gas evolved from clover juice by the protozoa and bacteria of rumen liquor (ml./hr.)

	Diet of Cow						
	Grass Hay	Clover Hay					Fresh Clover
Unfractionated	14	28	24	30*	23*	22*	44
Protozoa	7	15	13	14	9	9	20
Bacteria	6	12	11	14	13	12	23
Recombined	12		23	29			42
% Total gas by protozoa	54	56	54	50	41	43	47

* Method utilizing a bacteria/protozoa fraction

Table 63. Volatile acids produced from clover juice by the protozoa and bacteria of rumen liquor (m. mole/100 ml.)

Whole Rumen Liquor	Protozoa	Bacteria	% Total Acid
2.1	1.2	1.0	54
2.0	1.2	0.9	57
2.4	1.4	1.0	58
	1.8	1.0	64

The effect of lithium on the protozoa

While Mr M. J. Ulyatt was investigating the use of lithium compounds as "marker" substances for estimating rumen volume, the inertness of lithium towards the rumen microbial population was checked manometrically.

Experiments were conducted with rumen liquor and clover juice. It was found that Li^+ (250 µg./ml.), added as either LiCl or $Li_2SO_4.H_2O$, caused a reduction in the fermentation end-products of about 10%. Further experiments with rumen liquor divided into bacterial and protozoal fractions showed that lithium reduced the fermentation rate of the protozoa and that the bacteria were unaffected. No further experiments were carried out. More complete experimental evidence of the inhibitory effect is to be presented by Ulyatt (pers. comm.).

Discussion

The manometric method of estimating rumen activity used in this study differed in some aspects from those used by other workers (see Walter, 1952; McBee, 1953; Hungate et al., 1955, 1960), but proved to be reliable and gave reproducible results. Hungate et al. (1955, 1960) also used Warburg-type fermentation flasks capable of handling rumen samples of 10 g. or more, but measured the amounts of gas evolved with mercury manometers. The constant-pressure manometers used here permitted the measurement of large quantities of gas, were accurate, and were less complicated. The only weakness in the system was the need to connect the flasks and manometers with flexible tubing. Both rubber and some types of plastic tubing were shown to be permeable to CO_2, but the use of thick-walled butyl rubber tubing allowed only a small amount of gas to escape. As the experiments were conducted over a short period and as each experiment contained

adequate controls to compensate for differences between each treatment, the loss of gas (5 ml./hr. at atmospheric pressure from an atmosphere of pure CO_2) was not considered significant.

The methods of handling the samples and gassing the flasks were also satisfactory. The reducing conditions in the system were comparable to those in the rumen, and the fermentation activity was of the same order as that found by other workers (see Boda and Johns, 1962).

Only a few attempts were made to measure the total gas production, including that from the bicarbonate of rumen liquor, by terminating the experiments with the addition of acid. Preliminary trials of this technique demonstrated the impossibility of obtaining quantitative gas release, especially with rumen liquor from pasture and clover-fed cows, because of the copious formation of a very stable foam. The foam produced during the normal course of fermentation did not interfere with gas evolution provided that the flasks were vigorously shaken. Only comparative results were required and, as Hungate (1955) stated, all of the fermentation products cause an increase in gas pressure, the acids produced liberating carbon dioxide from the bicarbonate of rumen liquor.

Clover juice was used as substrate for the fermentations to eliminate the use of more artificial substrates. Where substrates have to be added to fermentations <u>in vitro</u> they should be materials closely resembling or similar to those eaten by the animal. Soluble sugars, soluble starch and hemicellulose

were used by Walter (1952), and McBee (1953) used glucose, cellulose and hemicellulose. Even though the fermentation of these pure materials has been shown to vary with the host's diet, there can be little correlation between the fermentation of these materials *in vitro* and the activity of the whole rumen population, *in vivo*, towards normal dietary materials. The use of more "natural" substrates such as fresh plant juice and fibre, as used by Conrad *et al.*, (1958) and Boda and Johns (1962), gives results with more direct application to the fermentation *in vivo*.

The analysis of the fermentation of clover juice carried out over a period of several months on rumen liquor from a single cow on a series of diets (Fig. 1) gave results similar to those expected. The materials in the juice of clover, probably soluble sugars and starch, constitute the bulk of the rapidly fermentable substrate in the fresh plant and the fermentation rates found for each diet bore a direct relation to the soluble sugar content of that diet. Grass hay contained the lowest level of soluble sugars of the four diets used (Table 55) and consistently produced a rumen population with a poor ability to ferment the soluble constituents of clover juice. The finely-divided plant material present in the juice was undoubtedly efficiently fermented, but the end-products would only be produced at a relatively slow rate. The clover hay used during the experiment contained more soluble sugars than the grass hay and resulted in a rumen population that fermented clover juice at a faster rate.

The rumen liquor obtained while the animal was on the pasture and fresh clover diets produced the most vigorous fermentations, the rates for pasture being higher than the rates for most of the clover samples. The low response to clover juice by the rumen liquor from the cow when fed on clover was probably the result of the drought conditions prevailing at the time. The soluble sugar and starch contents of the clover were probably low during this period (R. W. Bailey, pers. comm.). The sudden rise in the fermentation rate in late January, 1962, must have been the result of an increase in the rapidly fermentable constituents of clover. The weather was warm over the whole of January however, and there was no marked change in the rainfall pattern during the last week of the month. Rain totalling 3.60 in. fell on 5 days prior to 15 January 1962. There was no further rain until 22 January, 1.15 in. falling on 3 days between then and the end of the month.

Data on the population changes which follow a dietary change can also be obtained from Figure 1. If only the rapidly fermentable constituents of the diet are considered, then the four dietary materials used may be listed in the order of increasing complexity, grass hay, clover hay, and pasture and fresh clover. In Figure 1 it can be seen that two to four days are required for the microbial population to reach maximum efficiency in fermenting a more complex diet. A change of diet in the other direction, from complex to less complex, causes an immediate drop in the fermentation rate. Hence, in this case,

the fermentation rate immediately dropped on changing the diet from pasture to clover hay, or from clover hay to grass hay. Obviously, a change to a diet poorer in a particular constituent does not require an increase in the population attacking that constituent. The existing population is more than sufficient to cope with that portion of the new diet. The adaption of the flora and fauna to the more resistant materials following a dietary change might, of course, take longer than the two to four days required for easily fermentable materials.

The amount of food eaten by the animal had no effect on the fermentation of clover juice on the following day. The easily fermentable materials would have disappeared from the rumen after overnight starvation and the micro-organisms concerned with the fermentation would not be fully active metabolically. Similarly the before-feeding fermentation rate could not be expected to correlate with the subsequent fermentation *in vivo*.

It is important that observations such as these are made on single animals, and that the fermentation rates of a group of animals are not directly compared. Hungate *et al.* (1955) found no correlation between the before-feeding rate and the subsequent bloating of a series of animals, but if bloat was related to the fermentation rate then animal variation could invalidate any analysis made by comparing the rates for different animals.

The magnitude of the fermentation of clover juice by rumen liquor from the cow feeding on pasture or fresh clover was

similar to that found by Boda and Johns (1962). In this investigation, 0.7-1.2 ml. of gas per minute were produced from 40 ml. of rumen liquor and 10 ml. of clover juice. Boda and Johns (1962), using a similar ratio of rumen liquor and clover juice, obtained 0.7 ml. of gas per minute from 40 ml. of rumen liquor, from the same cow (No. 2) used in this investigation.

Conrad et al. (1958) obtained very low yields of gas from the fermentation of alfalfa juice (1.8 ml./hr. from 35 ml. of rumen liquor and 5 g. alfalfa juice). Their conclusion that the plant substances reponsible for the initial rapid gas production obtained with green alfalfa were closely associated with the fibre fraction and not with the juice, was based on this low fermentation rate compared with that for fibre. Boda and Johns (1962) contradicted this statement and found the initial rapid gas production was associated with the plant juice. The experiments carried out here also demonstrate that the rapidly fermentable materials of clover are closely associated with the juice fraction.

Conrad et al. (1958) based their conclusions on results from animals fed on 1 lb. of grain and 12-16 lb. of hay daily. From the results shown here in Figure 1, the microbial population in their animals would not be adapted to ferment the alfalfa juice at a maximum rate. If rumen liquor from alfalfa-fed animals had been used the amount of gas produced from the juice would have been considerably increased, and different conclusions might have been reached. This was suggested by Boda and Johns

(1962, p196) who said that "it is probable that the particular feeding régime can modify the rumen microbial population sufficiently to change the relative capacity of the micro-organisms to ferment plant fibre."

Walter (1952) noticed a similar effect. A sheep fed on oat straw had decreased ability to ferment glucose and starch compared with its ability when on a diet which included barley. Further evidence was supplied by Freer and Campling (1963) who showed that cotton thread, suspended in the rumen, was not digested by cows on a diet of concentrates. The thread was rapidly digested by cows on diets of hay and dried grass.

The results from the fermentation of soluble sugars are of little value except for comparison with the results of other workers. Walter (1952), for example, also found that more gas was usually evolved from sucrose than glucose, and that gas production was greatest from plant material (alfalfa, presumably fresh). The work of McBee (1953) cannot be compared as he reported gas evolution as changes in pressure in the mercury manometers.

In the experiments with the identical twin cows, 1 and 2, cow 1 was shown to have a greater rate of fermentation than its twin, and usually had the greater intake. This is in accordance with the observation of Hungate et al. (1959) that an increased intake and a smaller rumen must result in an increased fermentation rate per unit of dry matter. Cow 1 certainly had a smaller rumen than cow 2 at the time of the experiment. Boda and Johns

(1962) however, found that cow 2 had the higher fermentation rate.

The inhibitory effect of sodium sulphide on the rumen ciliates was unexpected considering the wide use of this substance as a reducing agent in preparations of rumen protozoa. The failure of some attempts to culture rumen ciliates may have been the result of sodium sulphide toxicity. Oxford (1958a), for example, used 0.08% sodium sulphide in his experiments on the culture of epidinia and the ciliates survived for only eight days.

Where sodium sulphide is used as the reducing agent in metabolic studies on the rumen protozoa, care must be taken to use 0.01% or less. Several workers have used concentrations in excess of this. Abou Akkada and Howard (1960, 1962) used 0.02% sodium sulphide in their studies on the metabolism of Entodinium, and also in studies on the decomposition of pectic substances by rumen ciliates (Abou Akkada and Howard, 1961).

The contribution of the protozoa to the production of volatile acids in the rumen (50-65%) was two to three times that calculated by Gutierrez (1955) from observations on the fermentation of glucose by washed suspensions of holotrichs. The figures for the production of acid were supported by the similar figures obtained by measuring gas evolution.

The calculation of Gutierrez (1955) on the total volatile acids produced by Dasytricha and Isotricha are not applicable to the rumen protozoa of the cows used in this study. Gutierrez estimated that, in 100 kg. of rumen contents, 77 mg. of acid

would be produced in 24 hr. by a concentration of one isotrich/ml., and that one dasytrich/ml. would produce 2.0 mg. of acid. Using rumen counts of 3,000 isotrichs and 5,000 dasytrichs/ml. he calculated that 240 g. of fermentation acids were produced by the holotrichs each day. The same method of calculation applied to the holotrich populations of the clover-fed cows examined in this study (30-50,000 dasytrichs/ml.; 15-25,000 isotrichs/ml.) suggests that the holotrichs alone, produce up to 3 kg. of acid every 24 hr. Assuming, as did Gutierrez, that the oligotrichs although outnumbering the holotrichs only produce an equal amount of acid, then the total protozoal contribution to volatile acid production in the rumen is 6 kg. If the bacteria produce an equal quantity, then 12 kg. of fermentation acid is produced in the rumen every 24 hr. This figure is out of all proportion to the 2.2 kg of acid reported by Armsby and Moulton (1925) and Carroll and Hungate (1954), and to the average production of four lactating cows estimated at 4.3 kg. by Hungate, Mah and Simesen (1961). The production figures of 2.35 μ mM/hr./isotrich and 0.62 μ mM/hr./dasytrich given by Gutierrez (1955) are obviously far too great for the protozoal populations encountered here. It should be noted that Gutierrez obtained his estimates of the fermentation ability of the holotrichs from endogenous fermentations and not from the fermentations of soluble sugars.

Further work on the activity of single species of protozoa should be carried out (see Hungate, 1960). There should be a

reasonable correlation between the summation of the contributions by individual protozoa and the contribution calculated by observing the protozoa as a whole. Any work of this nature would need to be accompanied by an investigation of the actual production of fermentation acids in the rumen under the appropriate dietary conditions.

SUMMARY

Studies were made on the composition and size of the bacterial and protozoal populations in the rumen of New Zealand cows and manometric experiments were carried out on the activity of these organisms in rumen liquor. Studies were also made on the *in vitro* cultivation of rumen ciliates.

1. The fauna of the four cows examined was mainly composed of a selection of ciliates similar to those in ruminants elsewhere. No new species were found but the holotrichs *Charon equi* Hsiung and *Buetschlia parva* Schuberg were found in all of the animals. *C. equi* had not previously been described outside the colon of the horse and is the first ciliate to be found in both a ruminant and a mono-gastric animal. In one cow it constituted 1% of the total ciliate population. *B. parva* has only been reported on a few occasions. The rest of the holotrich population was composed of species of *Isotricha* and *Dasytricha*.

The oligotrich population consisted of *Entodinium*, *Eodinium*, *Eremoplastron*, *Epidinium*, *Eudiplodinium*, *Metadinium*, *Ostracodinium* and *Diplodinium* (all according to Kofoid and MacLennan).

2. The total number of ciliates present in the rumen varied between 1.1×10^5 and 1.9×10^6/ml. over a period of 14 months. The mean total for two cows on a diet of fresh red clover

(Trifolium pratense L.) was 5.4 X 10^5/ml. rumen liquor. The mean for the same two cows on a diet of clover hay was 2.3 X 10^5/ml. The dominant oligotrichs in two cows eating fresh clover were Entodinium, Epidinium and Eremoplastron. Oligotrichs constituted from 75-85% of the population. In four cows on a diet of grass hay the dominant oligotrichs were Entodinium, Eremoplastron and Ecdinium. Oligotrichs constituted 60-89% of the total population.

Epidinium sometimes became the dominant ciliate in the rumen of cows on a diet of fresh red clover but Entodinium and Eremoplastron were often present in similar or greater numbers. There was no relation between the numbers of epidinia in the rumen and the occurrence of bloat, although the numbers increased as dietary starch increased.

3. Various oligotrich protozoa were cultured in vitro, some of them for the first time. Eremoplastron bovis, E. brevispinum, Entodinium longinucleatum, E. ovinum, E. biconcavum and Eudiplodinium maggii were maintained in mixed culture, with division, for over six months and Epidinium ecaudatum was grown in pure culture for over 14 months. A clone of Eremoplastron bovis was established after isolation of a single cell immobilized with sodium barbitone. Considerable morphological variation was noticed in these cultures. Attempts to culture holotrichs were unsuccessful.

The ciliates were grown in a mineral salts medium with rumen liquor, dried grass and rice starch. Sodium sulphide

(0.01%) was added as a reducing agent and air was replaced with CO_2. No antibiotics were added.

Sodium sulphide was shown to inhibit the metabolism of rumen ciliates. Gas and volatile acids were reduced by 30-40% when the protozoa were suspended in 0.02% sodium sulphide. There was a 4-8% reduction in the presence of 0.01% sulphide.

4. Anaerobic and euryoxic bacteria were isolated from the rumen on media with the following substrates: protein, cellulose, xylan, starch, glycerol, lactic acid, glucose, cellobiose. The organisms isolated were all similar to previously isolated rumen bacteria. A curved rod producing butyric acid from lactate, and similar to Ramibacterium, was classified as Butyribacterium sp. No Bacteroides spp. could be isolated. Butyrivibrio fibrisolvens was isolated on several occasions and is possibly responsible for a considerable proportion of the activity against cellulose, hemi-cellulose, starch, pectin and protein. The only other proteolytic organism isolated was Clostridium bifermentans. Other genera isolated were Streptococcus, Selenomonas, Corynebacterium, Ruminococcus, Lactobacillus, Veillonella and Borrelia.

5. Studies on the fermentation activity of rumen liquor were made using a large-scale manometric method. All experiments were carried out with rumen liquor removed from the animal before feeding after overnight starvation. Clover juice was usually used as substrate. The rate of fermentation varied

from day to day and depended on the diet of the host. Hay diets produced a flora and fauna with poor ability to ferment the easily fermentable constituents of clover juice.

6. Manometric experiments with rumen liquor divided into bacterial and protozoal fractions showed the protozoa to be responsible for about 40-65% of the gas and volatile acid produced from clover juice in one hour.

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PLATES 1-7

All figures are from upper views of whole mounts stained with methyl green and drawn with a Zeiss Drawing Attachment.

PLATE 1

Semi-diagrammatic View of Eudiplodinium maggii Fiorentini (1889) from the Upper Side

a, membranelles; b, membranelle zone; c, inner lip; d, outer lip; e, oesophagus; f, skeletal plate; g, ectoplasm; h, endoplasm; i, boundary layer; j, rectum and anus; k, caudal lobe; l, lateral cuticular groove; m, contractile vacuole; n, macronucleus; o, micronucleus; p, excretory pore; q, outer furrow; r, operculum.

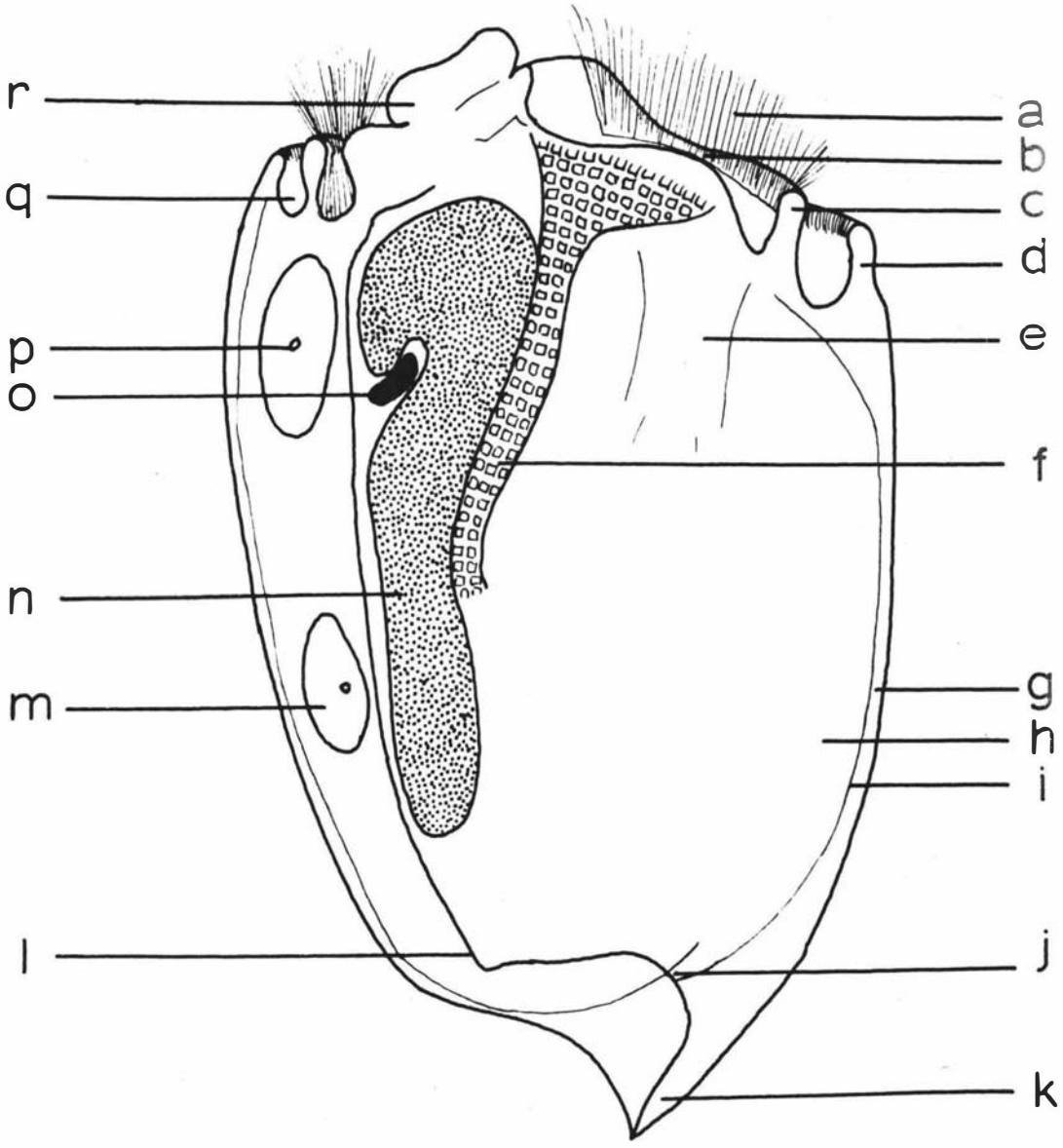
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PLATE 2

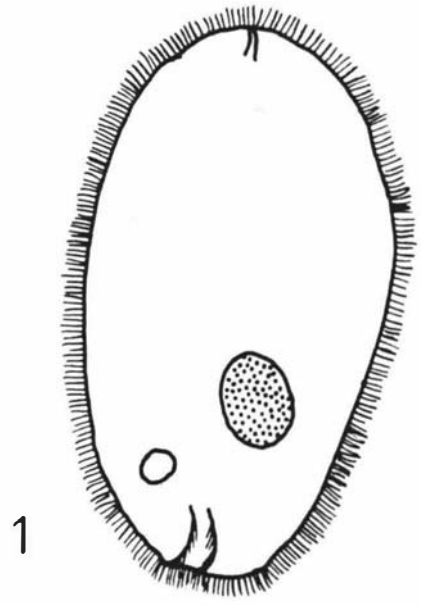
Fig. 1 *Dasytricha ruminantium* Schuberg (1888)

Fig. 2 *Isotricha intestinalis* Stein (1859)

Fig. 3 *Isotricha prostoma* Stein (1859)

Fig. 4 *Buetschlia parva* Schuberg (1888)

Fig. 5 *Charon equi* Hsiung (1930)



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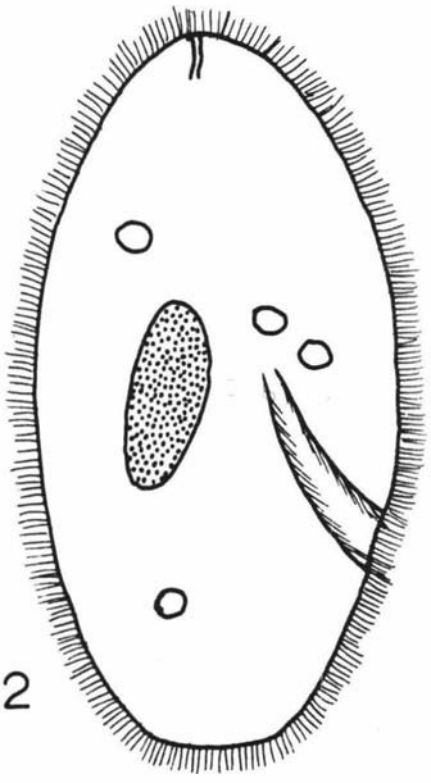

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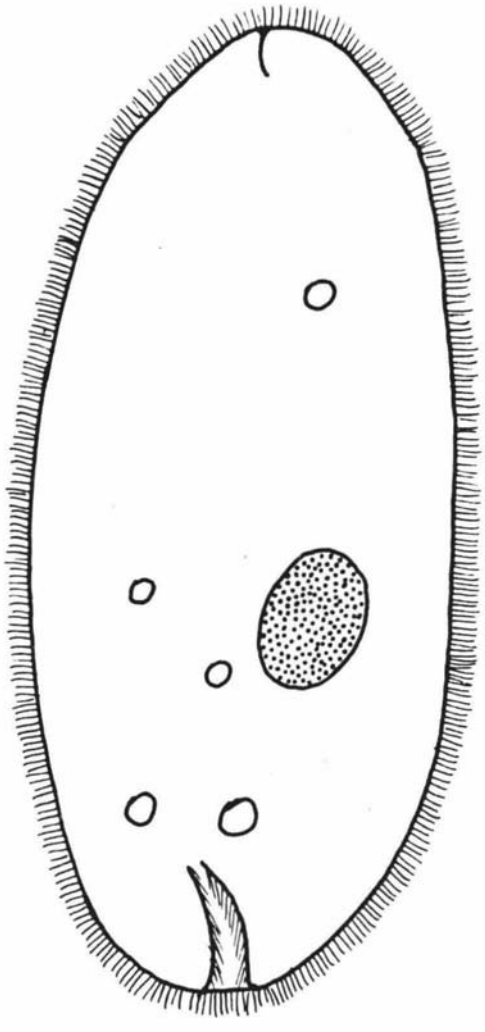

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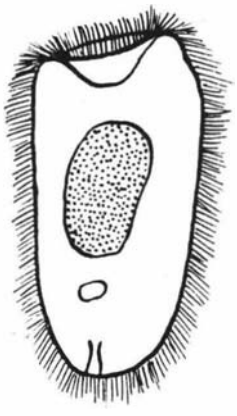

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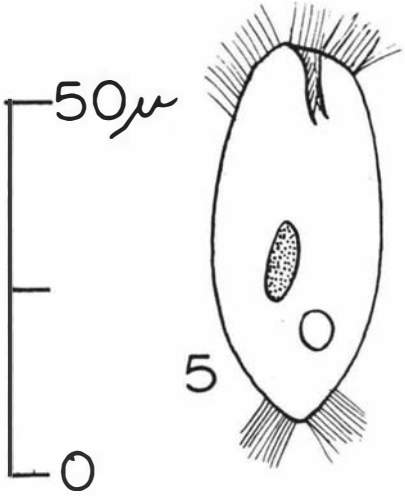

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PLATE 3

Fig. 1 *Entodinium ovinum* Dogiel (1927)

Fig. 2 *Entodinium longinucleatum* Dogiel (1925)

Fig. 3 *Entodinium bicarinatum* Da Cunha (1914)

Fig. 4 *Entodinium bicarinatum* Da Cunha (1914)

Fig. 5 *Entodinium indicum* Kofoid and MacLennan (1930)

Fig. 6 *Entodinium rostratum* Fiorentini (1889)

Fig. 7 *Entodinium biconcavum* Kofoid and MacLennan (1930)

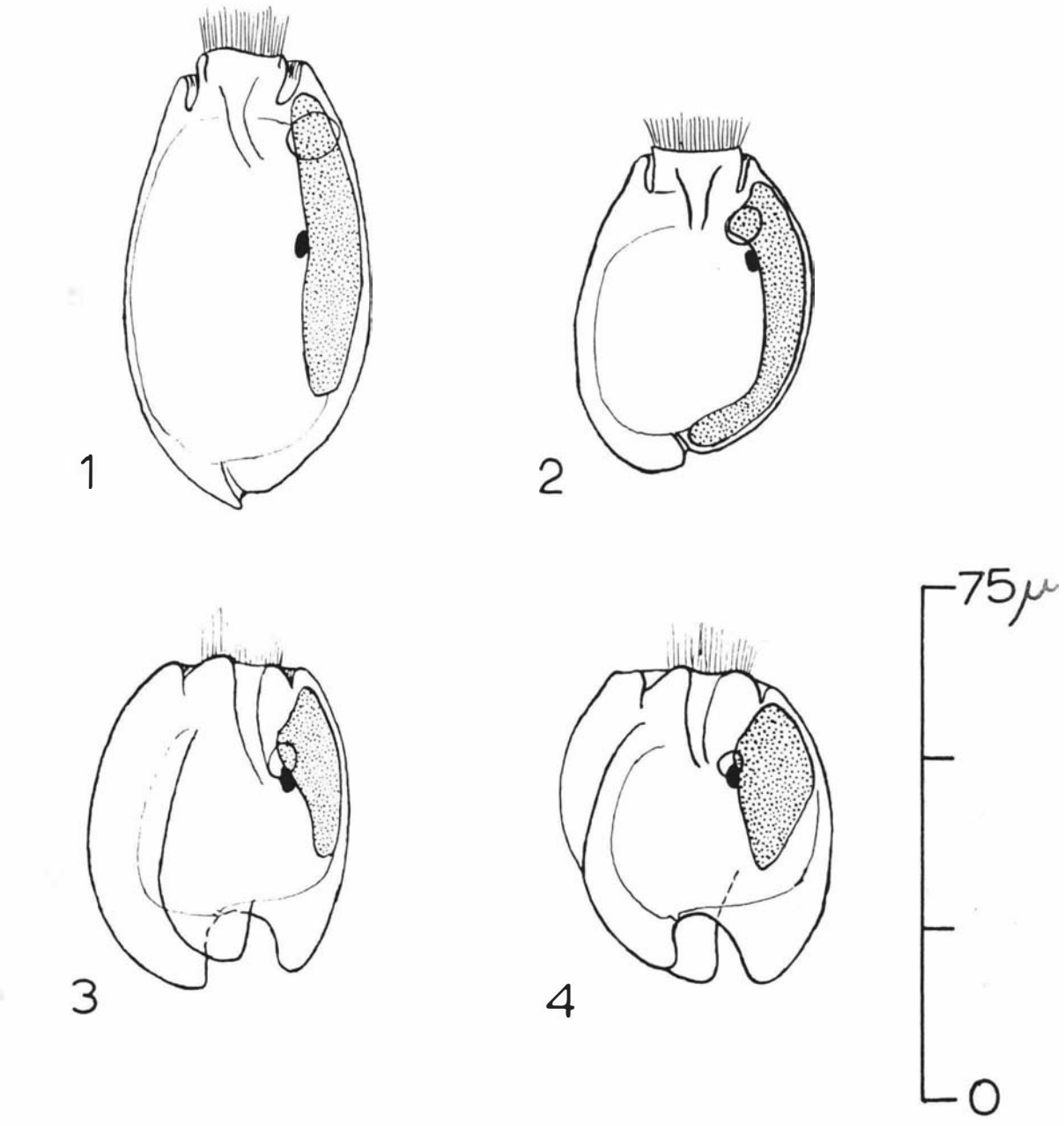
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PLATE 4

Fig. 1 Epidinium caudatum Florentini (1889)

Fig. 2 Epidinium bicaudatum Sharp (1914)

Fig. 3 Epidinium parvicaudatum Awerinzew and Mutafowa (1914)

Fig. 4 Epidinium quadricaudatum Sharp (1914)

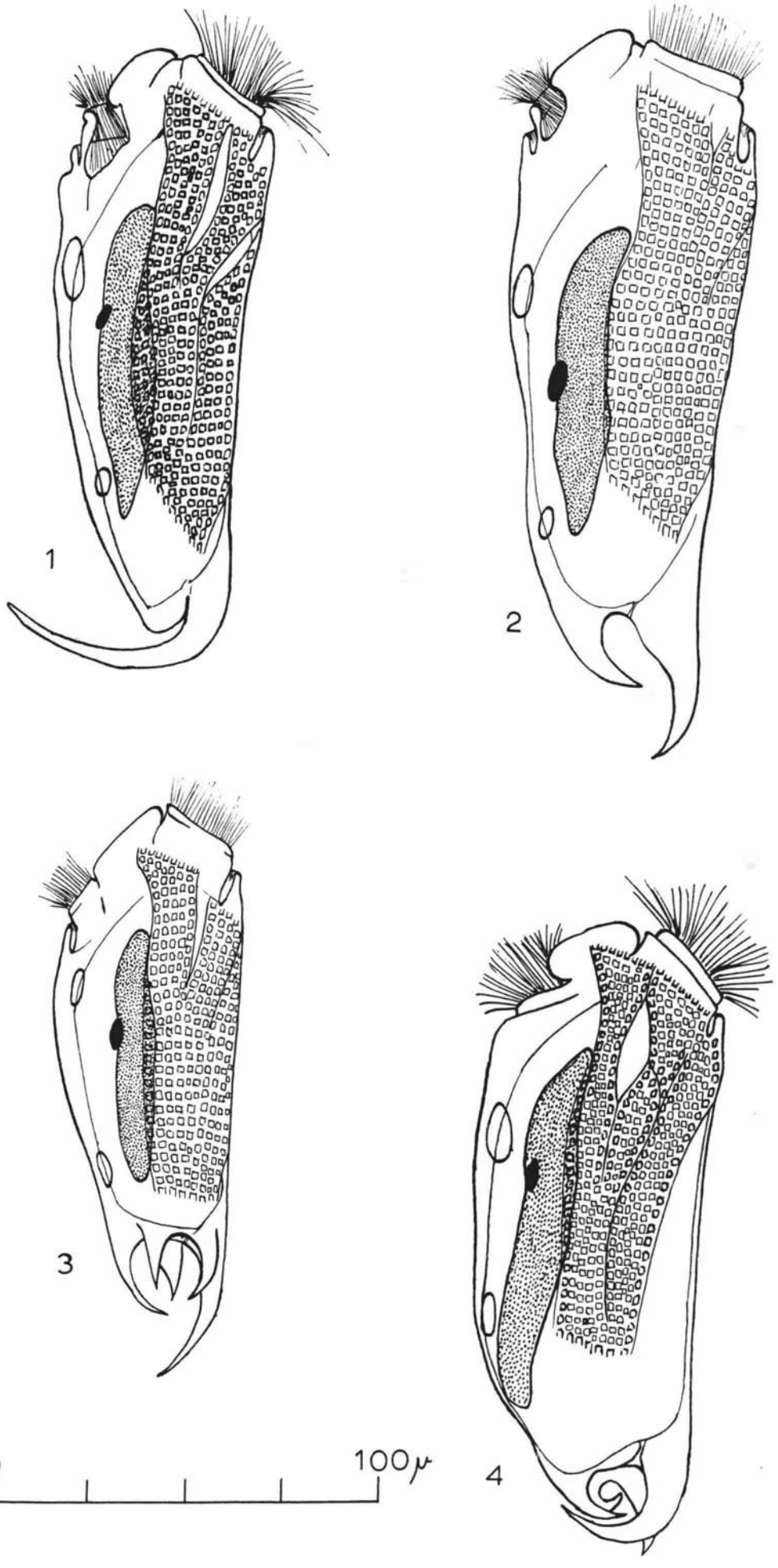
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PLATE 5

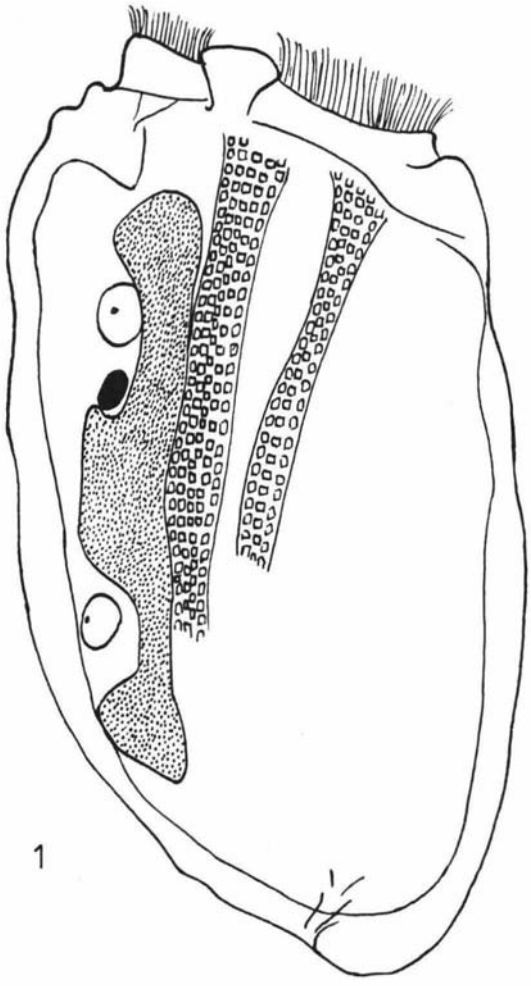
Fig. 1 *Metadinium medium* Awerinzew and Mutafowa (1914)

Fig. 2 *Eudiplodinium maggii* Fiorentini (1889)

Fig. 3 *Ostracodinium dilobum* Dogiel (1927)

Fig. 4 *Ostracodinium mammosum* Railliet (1890)

Fig. 5 *Ostracodinium rugoloricatum* Kofoid and MacLennan (1932)



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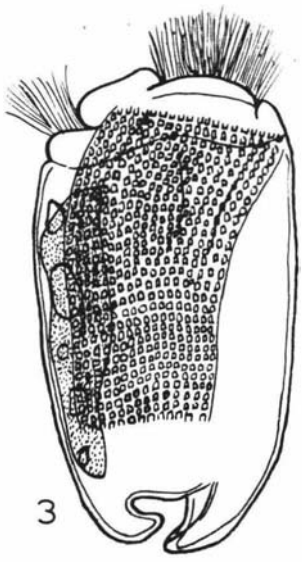

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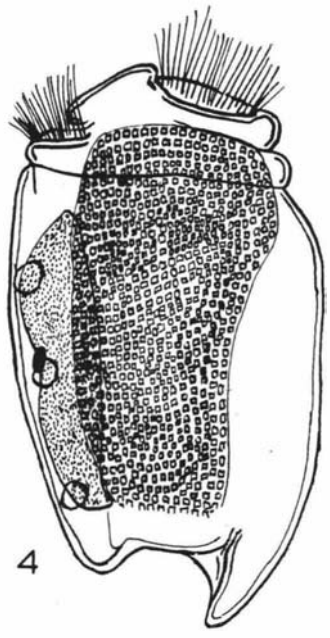

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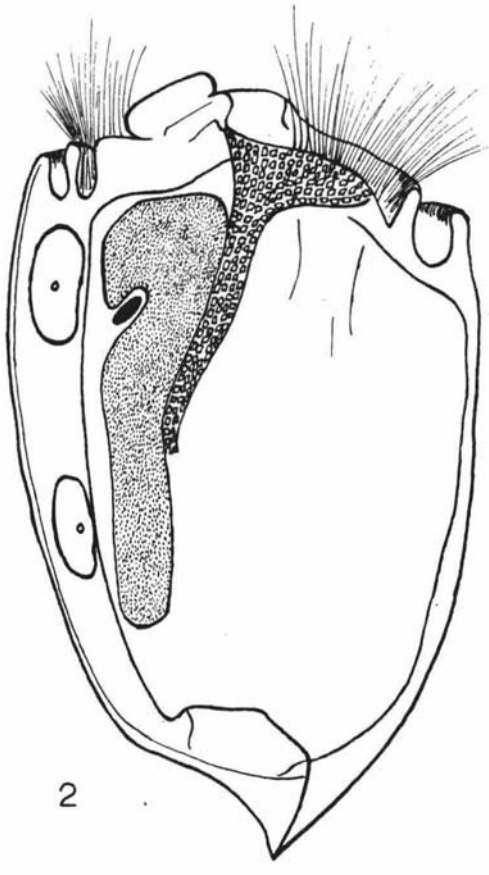

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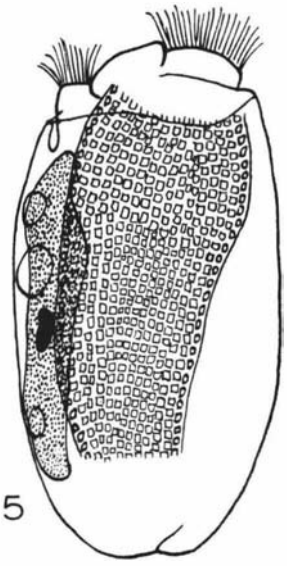

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PLATE 6

Fig. 1 *Diplodinium monocanthum* Dogiel (1927)

Fig. 2 *Diplodinium diacanthum* Dogiel (1927)

Fig. 3 *Diplodinium triacanthum* Dogiel (1927)

Fig. 4 *Diplodinium tetracanthum* Dogiel (1927)

Fig. 5 *Diplodinium pentacanthum* Dogiel (1927)

Fig. 6 *Diplodinium anisacanthum* da Cunha (1914)

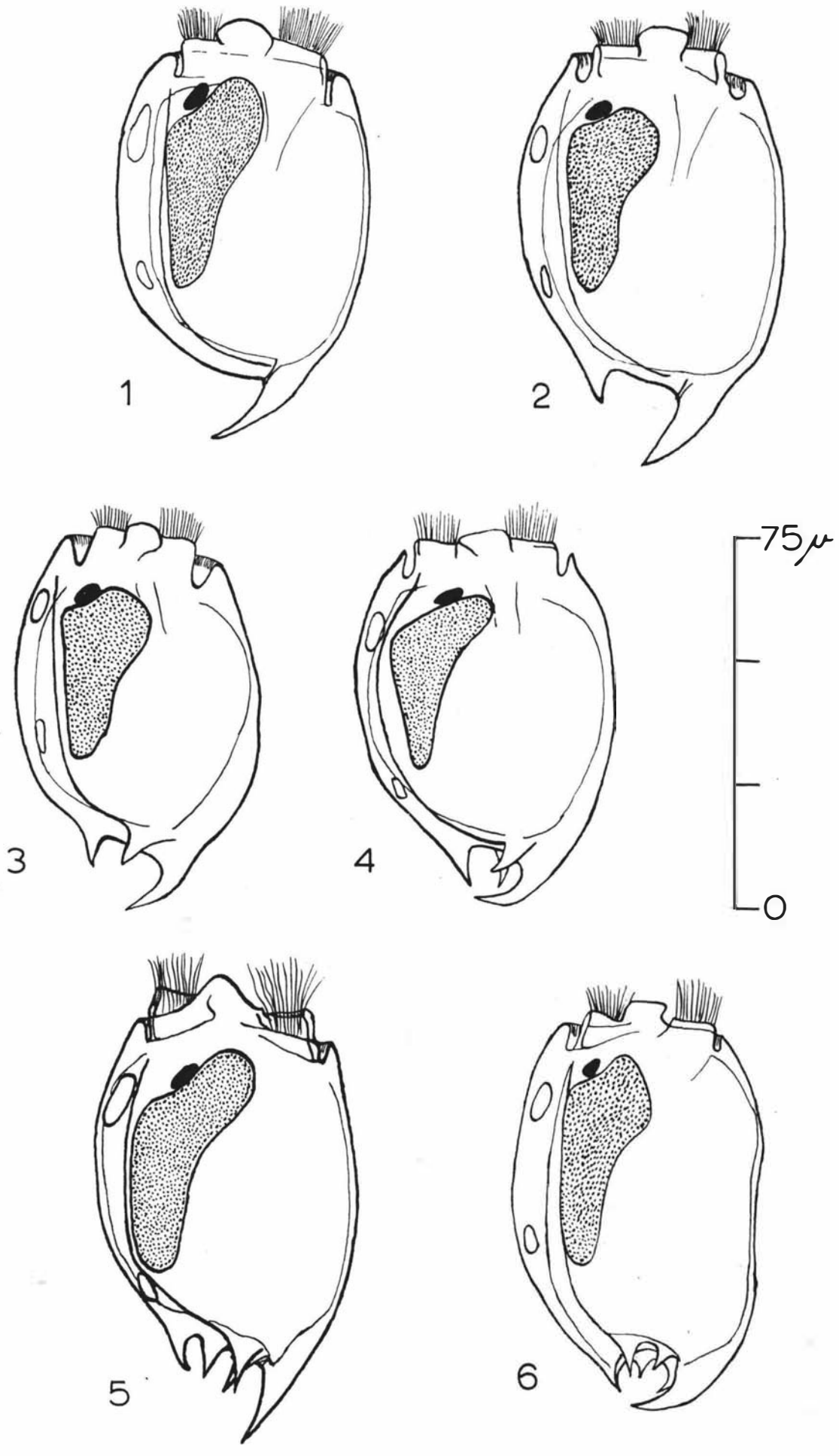
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PLATE 7

Fig. 1 *Eremoplastron bovis* Dogiel (1927)

Fig. 2 *Eremoplastron brevispinum* Kofoid and MacLennan (1932)

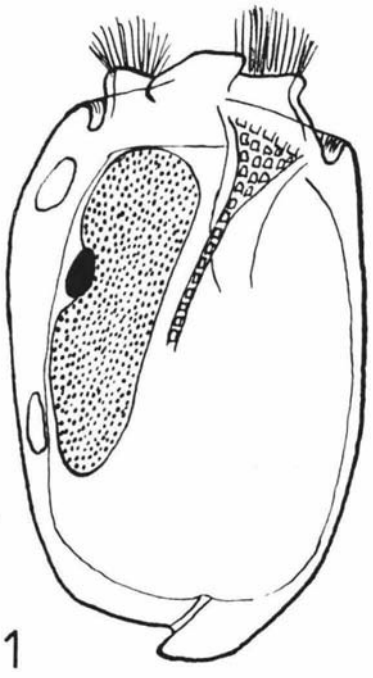
Fig. 3 *Eremoplastron monolobum* Dogiel (1927)

Fig. 4 *Eremoplastron rostratum* Fiorentini (1889)

Fig. 5 *Eodinium lobatum* Kofoid and MacLennan (1932)

Fig. 6 *Eodinium bilobosum* Dogiel (1927)

Fig. 7 *Eodinium posterovesiculatum* Dogiel (1927)



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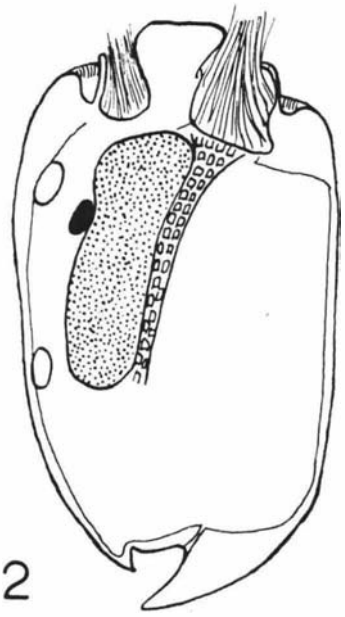

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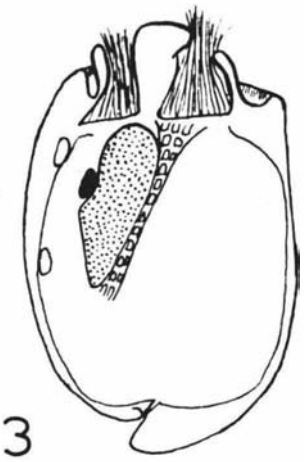

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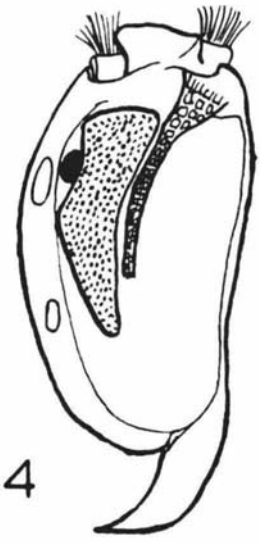

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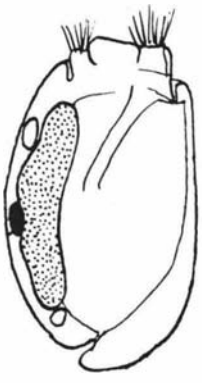

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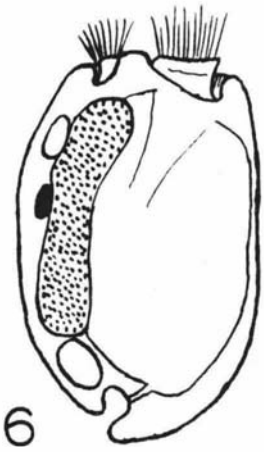

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