The Cultivation of the Rumen Ciliates *Epidinium ecaudatum caudatum* and *Polyplastron multivesiculatum* *in vitro*

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SUMMARY

Populations of over 400 *Epidinium ecaudatum caudatum*/ml were maintained for over 18 months in the presence of bacteria, in a potassium phosphate-rich medium under 95 % N₂ + 5 % CO₂ with the daily addition of wholemeal flour and dried grass. *Polyplastron multivesiculatum* was also maintained at a population density of 45 to 70/ml under the same conditions, except that it was necessary to add each day a culture of another protozoon such as *Epidinium ecaudatum caudatum*, *E. ecaudatum tricaudatum* or *Eudiplodinium maggii* to give a population density of approximately 100/ml. The epidinia at least were engulfed by the polyplastron up to 10 being taken up each day; the minimum daily requirement for growth was one epidinium/polyplastron. *Polyplastron multivesiculatum* also grew in the absence of other protozoa on a sodium chloride-rich medium, and if these protozoa were inoculated into the potassium phosphate-rich medium in the presence of epidinia, there was a lag of 3 to 5 days before they began to engulf the epidinia. The effect of other variations in the culture conditions is also described.

INTRODUCTION

Although entodinia from the rumen have been successfully cultivated for long periods by several workers including Hungate (1943), Coleman (1960), Tompkin, Purser & Weiser (1966), Jarvis & Hungate (1968) and Coleman (1969), there have been fewer investigations on the growth of the larger rumen entodiniomorphid protozoa. Hungate (1942, 1943) maintained several diplodinia, including *Diplodinium* (*Polyplastron*?) *multivesiculatum*, for over a year at low population densities. Gutierrez & Davies (1962) cultivated *Epidinium ecaudatum in vitro* for 5 months, but obtained populations of only 1200/ml and did not investigate the effect of different cultural conditions on this density. Clarke (1963) maintained *Eremoplastron bovis* in unspecified numbers for 5 months and mixed entodiniomorphs for somewhat longer, while Mah (1964) maintained up to 1000 *Ophryoscolex purkynei*/ml for nearly 3 years, with a mean generation time of 24 h.

The present paper describes the cultivation of *Epidinium ecaudatum caudatum* and *Polyplastron multivesiculatum* in the presence of bacteria in buffered salts medium with wholemeal flour and dried grass as the sources of carbohydrate. Under certain conditions *P. multivesiculatum* had an absolute requirement for the epidinia for growth and died rapidly without them.

Preliminary communications gave the growth requirements of these ciliates immediately after their isolation from the rumen (Coleman, 1970, 1971).

METHODS

Basal mineral salt solutions. 'Caudatum-type' salt solution contained (g/100 ml): K_2HPO_4 , 0.63; KH_2PO_4 , 0.50; NaCl, 0.065; $CaCl_2$ (dried), 0.0045; $MgSO_4 \cdot 7H_2O$, 0.009; CH_3COONa , 0.075. 'Simplex-type' salt solution contained (g/100 ml): K_2HPO_4 , 0.49; KH_2PO_4 , 0.38; NaCl, 0.049; $CaCl_2$ (dried), 0.0035; $MgSO_4 \cdot 7H_2O$, 0.007; $NaHCO_3$, 0.66. 'Dilute simplex-type' salt solution contained (g/100 ml): K_2HPO_4 , 0.31; KH_2PO_4 , 0.24; NaCl, 0.031; $CaCl_2$, (dried), 0.0022; $MgSO_4 \cdot 7H_2O$, 0.0044; $NaHCO_3$, 0.66. 'Hungate-type' salt solution, based on that of Hungate (1942), contained (g/100 ml): NaCl, 0.5; CH_3COONa , 0.15; K_2HPO_4 , 0.1; KH_2PO_4 , 0.03.

Culture media. All experiments were carried out in 50 or 100 ml centrifuge tubes approximately three-quarters filled with salts medium to which was added L-cysteine hydrochloride (neutralized immediately before use; final concentration 0.02 %, w/v) and sometimes 10 % fresh or autoclaved rumen fluid. Ninety-five per cent (v/v) $N_2 + 5$ % (v/v) CO_2 (for caudatum or Hungate-type salts), or CO_2 (for simplex-type or dilute simplex-type salts) was then bubbled vigorously through the medium for 2 to 3 min and the tubes stoppered immediately with a rubber bung.

Rumen fluid fractions. Fresh rumen fluid from which the protozoa had been removed was prepared as described by Coleman (1958). Autoclaved rumen fluid (ARF) was prepared as described by Coleman (1969).

Initial isolation of protozoa. This was carried out by picking out single protozoa from crude rumen fluid as described by Coleman (1971).

*Culture of *Epidinium ecaudatum caudatum*.* Coleman (1971) found that in newly established cultures epidinia grew best on dilute simplex-salts under 100 % CO_2 , but older cultures grew better in media equilibrated with gas containing 5 % CO_2 . The standard cultural conditions used for over a year to grow epidinia from the second isolation (see below) are as follows. The protozoa were maintained in 100 ml centrifuge tubes in caudatum salts with 0.02 % L-cysteine, but no rumen fluid, under 95 % $N_2 + 5$ % CO_2 , at 39 °C. Wholemeal flour (0.2 ml of 1.5 %, w/v, aqueous suspension) and ground, dried grass (about 2 mg) were added each day. Twice a week the culture was mixed, half poured into a clean tube and the liquid in each tube made up to the original volume with fresh medium.

*Culture of *Polyplastron multivesiculatum*.* This protozoon was maintained routinely both in the presence and absence of epidinia. In the presence of epidinia *Polyplastron multivesiculatum* was grown in 50 ml centrifuge tubes in caudatum-type salts with 0.02 % L-cysteine, but no rumen fluid, under 95 % $N_2 + 5$ % CO_2 , at 39 °C. Wholemeal flour (0.1 ml of 1.5 %, w/v, aqueous suspension), ground, dried grass (about 1 mg) and 1 ml of a culture of *Epidinium ecaudatum caudatum* (or other protozoa) were added each day. In addition, twice a week the cultures were diluted with an equal volume of fresh medium as above.

In the absence of epidinia *Polyplastron multivesiculatum* was grown in Hungate-type salts, without cysteine or rumen fluid, in 100 ml centrifuge tubes at 39 °C. Wholemeal flour (1 drop 1.5 %, w/v, aqueous suspension) and ground, dried grass (2 mg) were added each day. In addition, twice a week the cultures were diluted with an equal volume of fresh medium as above.

Protozoal counts. The number of epidinia in a culture was estimated by blowing from a pipette 1.0 ml of the culture into 1.0 ml 0.02 M-iodine and counting microscopically all the protozoa in 0.1 ml of the mixture. For *Polyplastron multivesiculatum* cultures, where the numbers were frequently less than 100/ml, a suitable volume of culture was mixed with an equal volume of 0.02 M-iodine in a $4 \times \frac{1}{2}$ inch tube and all the protozoa present counted

under an inverted microscope (Olympus Optical Co. Ltd, Tokyo, Japan – model CK). Only those protozoa which showed no signs of disintegration were counted. To judge the effect of a change in growth medium at least 4 weeks were allowed to elapse before the number of protozoa was estimated. Unless otherwise stated, the number of protozoa was always estimated immediately before the dilution of that culture.

RESULTS

Cultivation of Epidinium ecaudatum caudatum

For the continued maintenance of *Epidinium ecaudatum caudatum* in the presence of bacteria it was necessary to add fresh wholemeal flour to the cultures each day. When no such addition was made all the protozoa died within 48 h. It was not necessary for the maintenance of the protozoa to dilute the cultures frequently with fresh medium. Cultures remained alive for over 4 weeks without the addition of fresh medium provided that wholemeal flour was added each day.

Epidinium ecaudatum caudatum was isolated originally in simplex-type salts medium containing prepared, fresh, rumen fluid (10 %, v/v) under 100 % CO₂, because single protozoa picked directly from crude rumen contents grew better in this medium than in caudatum-type salts medium containing 10 % (v/v) prepared, fresh, rumen fluid under 95 % N₂ + 5 % CO₂. However, after 3 months with twice-weekly dilution of the cultures, the population density in the former medium declined slowly from about 1000/ml, and after a further year all the protozoa were dead. Investigations to find the optimal growth conditions carried out 1 to 4 months after the initiation of the culture showed that, at this time, caudatum-type salts medium containing no rumen fluid supported the highest population density and so some stock cultures were transferred to this medium. Despite sporadic improvements in the number of protozoa present, the population density supported by this medium also declined until, regardless of the cultural conditions, all the protozoa from the isolate died about the same time. Throughout the last few months of the life of this isolate, attempts to stimulate growth by variations in the salt concentration (40 to 150 % of standard), CO₂ tension (5 % or 100 % CO₂), type of starch (rice or maize starch or wholemeal flour), presence or absence of 10 % (v/v) autoclaved or prepared, fresh, rumen fluid and in the frequency with which cultures were diluted with fresh medium (twice a week or once a fortnight) were all unsuccessful. The death of this isolate may have been caused in part by an increased susceptibility of the epidinia to the presence of excess starch in the bottom of the tube. If the starch added to the medium during the daily feeding routine was not engulfed immediately by the protozoa, it remained in the bottom of the tube next to the protozoa and was fermented by the bacteria present. Normally protozoa were not harmed by the fermentation products provided that only a small amount of starch was present, but it was difficult to judge the correct amount of wholemeal flour to add each day. If too much was added all the protozoa were frequently dead 16 h later, whereas if too little was added they died of starvation. A second isolate, which was made after the death of the first, did not decline in the same way and 18 months after it was initiated 400 protozoa/ml were present under standard conditions and this population density remained constant for over 6 months.

Clone cultures of *Epidinium ecaudatum tricaudatum* and the cattle form of *E. ecaudatum caudatum* were isolated from a Dexter cow and at the time of writing have been maintained for 3 months on simplex-type salts medium, containing 10 % (v/v) prepared, fresh, rumen fluid, at population densities of 1000/ml and 500/ml respectively.

Morphology

Epidinium ecaudatum caudatum on which all the cultural studies have been carried out was taken from a rumen population (Type B, Eadie, 1962) present in a Clun Forest sheep fed on hay and oats, and measured $109 \pm 9 \mu\text{m} \times 47 \pm 3 \mu\text{m}$ ($n = 10$; range 98 to $120 \mu\text{m} \times 41$ to $52 \mu\text{m}$) when removed from the sheep. The protozoon's caudal spine gradually became smaller during the first few months in culture and disappeared after 4 to 5 months. After 12 months in culture protozoa from the second isolate, grown under standard conditions, measured $118 \pm 14 \mu\text{m} \times 62 \pm 5 \mu\text{m}$ ($n = 10$; range 104 to $148 \mu\text{m} \times 55$ to $72 \mu\text{m}$), showing that in culture the epidinia were less uniform in size. On initial isolation this protozoon had a structure similar to that described for this species by Sharp (1914), except that the single caudal spine, which was curved in the living organism, was shorter, i.e. it was $20.0 \pm 5.6\%$ of the length of the remainder of the protozoon compared with 33 to 50 %. The short spination is characteristic of epidinia present in sheep. Of the two cattle species one was identical with *E. caudatum tricaudatum* (Sharp, 1914) but the other, which had a long straight caudal spine ($42 \pm 8\%$ of the length of the remainder of the protozoon), did not resemble any of the pictures of epidinia published by Crawley (1923), Sharp (1914), Dogiel (1927), or Kofoid & MacLennan (1933), because they were all taken from fixed material in which the spine always appears to be curved regardless of its natural configuration. In the living protozoon the spine extends straight backwards from the posterior end of the organism. The spine was still present with the same configuration after 3 months in culture, although Clarke (1963) found that mixed epidinia grown *in vitro* lost these spines after 7 months in culture. This protozoon is referred to hereafter as the 'cattle form' of *E. ecaudatum caudatum*. The width of the spine at its base was $6.6 \pm 0.7 \mu\text{m}$ in the sheep form and $8.7 \pm 1 \mu\text{m}$ in the cattle form.

Effect of medium constituents

The effects of varying each constituent of the standard medium (see Methods) and of adding other materials are shown below. The actual experiments were carried out with the first isolate of the sheep form but the principal conclusions were confirmed with the second isolate.

Wholemeal flour. The daily addition of wholemeal flour to a final concentration of 0.03, 0.07 or 0.10 mg/ml resulted in protozoal populations of 420, 560 and 800 epidinia/ml respectively. Wholemeal flour was not replaceable by an equal quantity of a purified starch, such as rice starch, for the long-term maintenance of the protozoa, although some epidinia remained alive for up to 20 days in the presence of rice starch as sole source of carbohydrate. *Epidinium ecaudatum caudatum* therefore resembles *Entodinium simplex* (Coleman, 1969) rather than *Entodinium caudatum* (Coleman, 1960) which grew well on rice starch.

Rumen fluid. Although the initial isolation was carried out in the presence of 10 % (w/v) prepared, fresh, rumen fluid this was not essential for the growth of established cultures and population densities obtained with 0, 10 % prepared, fresh, rumen fluid or 10 % autoclaved rumen fluid and the daily addition of 0.07 mg/ml wholemeal flour were 560, 400 and 460 ciliates/ml. The population densities in the presence of the rumen fluids could be raised to that in their absence by doubling the amount of wholemeal flour added each day. As a result of these observations stock cultures were maintained thereafter without rumen fluid. *Epidinium ecaudatum caudatum* therefore differs from both the entodinia studied previously (Coleman, 1960, 1969) where the presence of autoclaved rumen fluid increased the population density almost ten times.

Fig. 1. The effect of salt concentration on the growth of *Epidinium ecaudatum caudatum* from a small inoculum in the presence of 10 % prepared, fresh, rumen fluid. The protozoa were grown on caudatum-type salts medium under 95 % N₂ + 5 % CO₂ (●—●) or on simplex-type salts medium under 100 % CO₂ (x—x), the salt concentration being varied by replacement of the salts solution by water or double strength salts except that the concentration of NaHCO₃ remained unchanged to maintain the pH constant. The protozoa for inoculation were grown on caudatum-type salts medium and added at a population density of 100/ml. The cultures were fed with wholemeal flour and dried grass each day and the number of epidinia estimated after 7 days.

Dried grass. This was stimulatory but not essential for the growth of epidinia, because after 6 months of growth in its absence the population density was 180/ml compared with 560/ml in its presence.

Temperature. *Epidinium ecaudatum caudatum* was even more sensitive to cooling than the entodinia and was killed by exposure for 18 h to a temperature of 30 °C; the normal growth temperature was 39 °C.

Basal mineral salt solutions and CO₂ tension. To determine the optimum salt concentration for growth, epidinia were grown from a small inoculum (60/ml) in simplex- or caudatum-type salts media, the concentrations of which were varied by replacement of some of the complete salts mixture by an equal volume of water or double strength salts medium. However with simplex-type salts medium the concentration of NaHCO₃ (0.077 M), which had to be added to maintain the pH constant at 6.7 under a CO₂ atmosphere, remained unchanged. Fig. 1 shows that, in an experiment which was carried out 3 months after the initiation of the first culture, the optimum concentration of caudatum salts for epidinia grown under 95 % N₂ + 5 % CO₂ was 0.08 M (calculated from the composition of these salts), whereas for simplex salts under 100 % CO₂ it was 0.12 M. Hungate (1955) found a similar effect of partial pressure of carbon dioxide on the optimum salt concentration for the growth of diplomodinia. So, dilute rather than normal simplex-type salts medium was used thereafter for the growth of epidinia under 100 % CO₂, but this change did not prevent the eventual death of the first isolate. Although the results shown in Fig. 1 suggest that growth on caudatum-type salts medium was much poorer than that on simplex-type salts medium, there was no significant difference in the number of protozoa (i.e. about 400/ml) present when they were maintained for over a year in these two media under standard conditions with twice-weekly dilution of the culture.

Fig. 2. The effect of autoclaved rumen fluid on the growth of *Epidinium ecaudatum caudatum* from a small inoculum. The experiment was carried out in 50 ml centrifuge tubes containing dilute simplex-type salts medium inoculated with 1 ml of a standard culture and to which 0.1 ml 1.5 % wholemeal flour suspension and 1 mg dried grass were added each day. ●—●, No rumen fluid; ○—○, + 10 % autoclaved rumen fluid.

Growth from a small inoculum

As the protozoa were normally maintained by twice weekly dilution of the culture with an equal volume of fresh medium, it was of interest to measure protozoal growth when a small inoculum (3 %) was added to fresh medium. For this experiment 1 ml of a standard culture in dilute simplex-type salts medium under 100 % CO₂ was inoculated into 30 ml of the same medium in a 50 ml centrifuge tube and the number of protozoa counted in 1 ml samples removed daily when the cultures were fed. There was no obvious lag before protozoal growth began and over the first six days the mean generation time was 1.1 days (Fig. 2); the presence of autoclaved rumen fluid had no effect on this rate. This mean generation time compares favourably with times of 1.8 and 1.6 days for *Entodinium caudatum* and *E. simplex* respectively (Coleman, 1960, 1969).

Cultivation of Polyplastron multivesiculatum

Polyplastron multivesiculatum grew well in simplex-type medium in the presence of epidinia provided that wholemeal flour and dried grass were added to the medium each day. It was relatively easy to obtain growth of a single protozoon (picked from crude rumen fluid with a micromanipulator) added to any culture of *Epidinium ecaudatum caudatum*. In preliminary experiments (Coleman, 1971) the epidinia were grown on simplex-type medium under 100 % CO₂ but when it had been found that *P. multivesiculatum* grew better on caudatum-type salts under 95 % N₂ + 5 % CO₂, the epidinia used for the initial isolation were grown under these conditions. Although it was of interest to study the metabolism of *P. multivesiculatum* predatory upon epidinia, it was also important to study this protozoon growing in the absence of other protozoa to determine if its metabolic capabilities were the same when grown under the two different conditions. It was not possible to grow *P. multivesiculatum* in the absence of epidinia just by omitting the latter from the medium because

the former always died, except on the one occasion reported previously (Coleman, 1971). It was very difficult to obtain growth of single *P. multivesiculatum* organisms in any of the media used for entodinia or epidinia (Coleman, 1971) in the presence or absence of rumen fluid, except in Hungate-type salts medium which had been inoculated the previous day with bacteria from crude rumen fluid or an established protozoal culture. Under these conditions approximately 10 % of single protozoal isolates grew and divided. The subsequent maintenance of the cultures was as described under 'Methods'. At all stages it was very important not to add too much wholemeal flour to the cultures. However, even with this precaution the protozoa occasionally all died inexplicably, and no culture was ever maintained for more than a year in the absence of epidinia.

The size of the organism

The protozoa were taken from the rumen of a Clun Forest sheep fed on hay and oats, with a type A population (Eadie, 1962) and measured $175 \pm 19 \mu\text{m} \times 127 \pm 8 \mu\text{m}$ ($n = 10$) when removed from the sheep. The protozoa grown in the presence of the epidinia were always densely filled and measured $205 \pm 13 \mu\text{m} \times 123 \pm 14 \mu\text{m}$ ($n = 10$) after 12 months in culture. *Polyplastron multivesiculatum* grown on Hungate-type salts medium in the absence of epidinia was smaller and measured only $123 \pm 9 \mu\text{m} \times 98 \pm 9 \mu\text{m}$ ($n = 10$). Eadie (1967) also observed that when *P. multivesiculatum* was introduced into a rumen containing protozoa upon which it was predatory (a type B population) it increased in size and became filled with dense material.

Effect of medium constituents on growth in presence of epidinia

The effects of varying each constituent of the standard medium (see 'Methods') are shown below.

Basal mineral salts and CO₂ tension. *Polyplastron multivesiculatum* was isolated initially on simplex-type salts medium under 100 % CO₂, but after 2 months, growth became progressively poorer until 4 months later it was no longer worthwhile to continue with this medium. However, if at any stage the medium was changed to caudatum-type salts medium under 95 % N₂ + 5 % CO₂ the population density increased and at the time of writing *P. multivesiculatum* has been maintained for over a year under these conditions.

Rumen fluid. The addition of rumen fluid to caudatum-type salts medium under 95 % N₂ + 5 % CO₂ did not stimulate protozoal growth and population densities of 63, 49 and 47 protozoa/ml were found without rumen fluid, or with 10 % autoclaved or 10 % prepared, fresh, rumen fluid respectively. *Polyplastron multivesiculatum* was therefore grown without rumen fluid.

Wholemeal flour. The daily addition of wholemeal flour to a final concentration of 0, 0.03, 0.06, 0.1 or 0.25 mg/ml resulted in population densities of 0, 41, 48, 45 and 40 ciliates/ml respectively. This similarity in population densities obtained in the presence of all concentrations of wholemeal flour tested suggested that the requirement of these protozoa for wholemeal flour was met by the lowest concentration and that at the higher concentrations something else was limiting growth. The wholemeal flour was not completely replaceable by rice starch, the protozoa dying after 25 days compared with 6 days in the absence of any added starch. This means that the polysaccharide present in the epidinia added each day would not support even a limited protozoal population.

Dried grass. Omission of dried grass from the daily feeding resulted in death of the culture in 10 days provided that the epidinia added each day were grown in the absence of grass.

Epidinia. The daily addition of 0, 30, 75 or 150 epidinia/ml (added as a culture of *Epi-*

Fig. 3. A photomicrograph of *Polyplastron multivesiculatum* (p) about to engulf an *Epidinium ecaudatum caudatum* (e). Epidinia were usually taken in anterior end first but the reverse was occasionally seen.

Fig. 4. An epidinium in the process of being engulfed.

Fig. 5. An epidinium inside a polyplastron. Bar markers = 200 μ m.

dinium ecaudatum caudatum) to a culture resulted in *Polyplastron multivesiculatum* populations of 0, 10, 30 and 48/ml respectively. However, the ratio of epidinia added daily to *P. multivesiculatum* organisms present, which was approximately 3 in the above experiment, varied from 0.5 to 4 in different experiments under the same conditions. In each case all the epidinia had disappeared from the medium within 24 h of their addition. Except for the one experiment mentioned above, cultures to which epidinia were not added each day died within a few days (Fig. 6), although a few polyplastrons occasionally survived for 2 weeks. As Eadie (1967) showed that *P. multivesiculatum* was predatory upon protozoa in a type B population it was likely that the epidinia in the present experiments were being engulfed by the polyplastrons. Unfortunately, these protozoa were normally densely filled with storage material and it was not possible to observe epidinia inside them. If, however, the polyplastrons were starved of epidinia for 2 days they became transparent and then on the addition of epidinia to the culture individual polyplastrons could be seen after 30 min of incubation to contain one, or occasionally two, epidinia. Fig. 3 to 5 show three stages in the engulfment of an epidinium by a polyplastron. Further observation on the number of epidinia engulfed was not possible as the polyplastrons became densely filled again. These results show that at least some of the epidinia were killed by engulfment, but it is uncertain if this was the only reason for their disappearance.

The maximum number of epidinia that could be engulfed per day was determined by inoculation of 5 polyplastrons/ml into medium containing 450 epidinia/ml initially. Fig. 6 shows that in 3 days (days 2 to 5) the number of polyplastrons increased from 2 to 42/ml, while the number of epidinia decreased from 700 to 40/ml. Over the last 24 h of this period an average of 33 polyplastrons/ml engulfed or otherwise destroyed at least 380 epidinia/ml, assuming that none of the epidinia divided before they were engulfed. Each polyplastron was therefore capable of killing at least 10 epidinia/day. Once all the epidinia had disappeared, the number of polyplastrons declined, although growth began again if further epidinia were added (Fig. 6).

Fig. 6. The disappearance of *Epidinium ecaudatum caudatum* (x—x) during the growth of *Polyplastron multivesiculatum* (grown previously in the presence of epidinia) (●—●) from a small inoculum in simplex-type salts medium containing 10 % prepared, fresh, rumen fluid under CO₂. At the arrow, when all the epidinia had disappeared, the culture was diluted with an equal volume of fresh medium and divided between two tubes to one of which was added 330 epidinia/ml. Number of polyplastrons: ●—●, without added epidinia; ○—○, with added epidinia.

The specificity of the requirement for epidinia varied with different isolates of *Polyplastron multivesiculatum*. The first isolate, which was grown from a single organism on the first strain of *Epidinium ecaudatum caudatum*, would not grow on *Entodinium caudatum* or *E. simplex* grown *in vitro* (Coleman, 1960, 1969), on mixed entodinia from the rumen or on the second strain of epidinia, and therefore died when the first strain of epidinia died. Experiments carried out during the first few months of the life of this isolate of *Polyplastron* showed that epidinia broken by sonication supported 25 % as many polyplastrons as intact epidinia. Epidinia killed by heating at 60 °C for 5 min, or by the addition of the minimum amount of iodine, supported 50 % of this number for at least 2 weeks under standard conditions. The second isolate of *Polyplastron multivesiculatum*, which was isolated on the second strain of epidinia, would also not grow on the entodinia, but when first tested after a year in culture would grow in the presence of a clone culture of a species of *Eudiplodinium* (probably *Eudiplodinium maggii*). This protozoon, which was grown under the conditions used for *Entodinium simplex* (Coleman, 1969), was engulfed by the polyplastrons and 10 to 20/ml had to be added each day to maintain the polyplastrons. Under these conditions only large polyplastrons, which were the same size as those grown in the presence of epidinia, were found. However, polyplastrons could not be maintained indefinitely under these conditions and although population densities of up to 30/ml were obtained initially these declined after 2 months and after 4 months all the polyplastrons were dead. During this initial period each polyplastron engulfed, or otherwise destroyed, a maximum of nine eudiplodinia/day.

When it was first tested, after 15 months in culture, this second isolate of *Polyplastron multivesiculatum* also grew in the presence of a clone culture of a species of *Diplodinium*,

which in culture resembled *Diplodinium monacanthum*. However, population densities of polyplastrons of less than 1/ml were obtained. The diplodinia never disappeared during the 3 months that the culture was maintained, and 200 to 400/ml were always present under standard growth conditions even though fresh diplodinia were not added each day. Although the average size of the polyplastrons under these conditions was $177 \pm 23 \mu\text{m} \times 103 \pm 14 \mu\text{m}$ ($n = 10$) individual protozoa varied in size from $127 \times 88 \mu\text{m}$ to $205 \times 124 \mu\text{m}$, suggesting that only some individuals (the largest) were predatory upon the diplodinia.

The effect of clone cultures of *Epidinium ecaudatum caudatum* (cattle form) and *E. ecaudatum tricaudatum* on the growth of polyplastrons was also investigated. These epidinia, which only became available 18 months after the initial isolation of the polyplastrons and which were grown under the conditions used for *Entodinium simplex* (Coleman, 1969) were used to replace *Epidinium ecaudatum caudatum* (sheep form) under standard growth conditions. These new epidinia had to be added each day to the culture of *Polyplastron multivesiculatum* which was maintained at population densities similar to those obtained previously with *E. ecaudatum caudatum* (sheep form). Protozoa from a clone culture of *Diploplastron affine* (grown on caudatum-type salts medium, without rumen fluid, under 95 % $\text{N}_2 + 5$ % CO_2 , with the daily addition of wholemeal flour and dried grass) did not support growth of polyplastrons and these died at the same rate as if no other protozoa had been added.

Effect of medium constituents on growth in absence of epidinia

Basal mineral salts and CO_2 tension. As mentioned above, *Polyplastron multivesiculatum* only grew in the absence of other protozoa on Hungate-type salts medium under 95 % $\text{N}_2 + 5$ % CO_2 . Attempts to grow *P. multivesiculatum* initially isolated on this medium in caudatum-type salts medium under 95 % $\text{N}_2 + 5$ % CO_2 or on simplex-type salts medium under 100 % CO_2 in the presence or absence of rumen fluid were unsuccessful.

Rumen fluid. The addition of 10 % prepared, fresh, rumen fluid or autoclaved rumen fluid did not increase the population density.

Wholemeal flour. As the population density was usually 1 to 5 protozoa/ml the optimum amount of wholemeal flour added each day was about 0.05 mg/ml. The protozoa were then actively motile in the bottom of the tube, but if more flour was added each day they became very sluggish and were liable to die unexpectedly. In one experiment a few protozoa survived for several months under standard conditions in the absence of wholemeal flour with just the daily addition of dried grass.

Dried grass. Omission of dried grass from the daily regimen resulted in death of the protozoa in 7 to 10 days.

Epidinia. As *Polyplastron multivesiculatum* grew much better with *Epidinium ecaudatum caudatum* it was of interest to determine the effect of epidinia on the growth of *P. multivesiculatum* previously grown without them. A culture of *P. multivesiculatum* (30 ml) grown in Hungate-type salts medium in the absence of epidinia was diluted with an equal volume of caudatum-type salts medium and 10 ml placed in each of two 150×15 mm tubes: the diluted culture contained 2 ciliates/ml. To one of these tubes, and to a similar one containing the same medium, was added 1.0 ml of a culture of epidinia, to give a final population density of 42 epidinia/ml. The protozoa in each tube were then fed with 0.1 ml 1.5 % wholemeal flour and 1 mg dried grass each day and all the *P. multivesiculatum* and *E. ecaudatum caudatum* present in the tube counted under an inverted microscope. Fig. 7 shows that in the presence of epidinia the number of *P. multivesiculatum* did not increase until the protozoa had been in the same culture for more than 3 days (5 days in one experiment) after which

Fig. 7. Effect of the presence (●—●) or absence (×—×) of *Epidinium ecaudatum caudatum* on the growth of *Polyplastron multivesiculatum* previously grown without protozoa on Hungate-type salts medium. The experiment was made in caudatum-type salts medium under 95 % N_2 + 5 % CO_2 inoculated with 10 % of each of the protozoal cultures. ●---●, Number of epidinia in the presence of polyplastrons.

time the number of *P. multivesiculatum* increased from 3 to 81/ml in 3 days with an average mean generation time of 0.6 day. In the absence of epidinia the number declined to one protozoon in 10 ml in the same time. The epidinia were apparently engulfed, as once the *P. multivesiculatum* began to grow (day 4) the epidinia disappeared in 2 days, although their number remained unchanged in the absence of polyplastrons. These results show that *P. multivesiculatum* grown in the absence of epidinia still required epidinia when transferred to caudatum-type salts medium.

Similar results were obtained when the *Epidinium ecaudatum caudatum* (sheep form) was replaced by *E. ecaudatum caudatum* (cattle form) or *E. ecaudatum tricaudatum* and, in one experiment, during the fifth day an average of 2.04, 0.69 and 2.97 individuals respectively of these species of epidinia were engulfed by each polyplastron. However, the actual results obtained varied markedly between experiments and there is thought to be no essential difference in the rate of engulfment of the three epidinia. The addition of *Eudiplodinium maggii* only caused a slight (50 to 100 %) increase in the number of polyplastrons during the 7 days of the experiment; *Diploplastron affine* had no stimulatory effect.

DISCUSSION

The results quoted above provide detailed confirmation of those of Eadie (1962, 1967) in which she showed that in the rumen the protozoa of a type A population (i.e. *Polyplastron multivesiculatum*, *Diploplastron affine* and *Ophryoscolex tricornatus*) were dominant over those of a type B population (i.e. *Eudiplodinium maggii*, *Epidinium* spp., *Eremoplastron* spp. and *Ostracodinium* spp.) and that this was caused by predation of *P. multivesiculatum* on the type B protozoa. However, the present results also show that, under certain conditions, *P. multivesiculatum* has an absolute requirement for growth for at least one type B protozoon/day. The time taken for *P. multivesiculatum* grown *in vitro* in the absence of other protozoa to become predatory upon epidinia was longer than that found *in vivo* (i.e. 3 to 5 days compared with 1 to 2 days), but this lag may have been due in part to placing the

polyplastrons in fresh medium: there was a 2-day lag before growth began in the experiment shown in Fig. 6, where the polyplastrons were grown in the presence of epidinia throughout. Although conditions are very different *in vivo* and *in vitro*, this result suggests that *P. multivesiculatum* growing *in vivo* may be better able to engulf type B protozoa even though it has had no recent contact with them. This is supported by the observation that *P. multivesiculatum* growing *in vivo* in a stable type A population is intermediate in size between this protozoon growing in the presence or absence of epidinia *in vitro* (i.e. 175 μm long compared with 205 or 123 μm). The specificity of the requirement for epidinia compared with entodinia remains unexplained, although it may be related to the observation that the epidinia, but not the entodinia, have many bacteria attached to their surface (G. S. Coleman & F. J. Hall, unpublished observation). It is possible that *P. multivesiculatum* recognizes an epidinium as a bacterium and engulfs it along with the bacteria it takes up as a source of protein, etc.

The finding that the protozoa which support growth of another come from one or two genera only is fairly common in studies with carnivorous ciliates. Claff, Dewey & Kidder (1941) showed that *Bresslaia vorax* and *B. insidiatrix* required *Glaucoma pyriformis* and could not use Tetrahymena, and Dewey & Kidder (1940) found that *Perispira ovum* only utilized euglenoid flagellates. Similarly, *Didinium nasutum* has an absolute requirement for Paramecium and cannot survive in its absence (Kudo, 1946). This specificity for other protozoa by carnivorous protozoa contrasts with the lack of specificity in the engulfment of bacteria shown by the rumen ciliates *Entodinium caudatum* and *E. simplex* (Coleman, 1964, 1972) and by *Colpidium colpoda* (Burbank, 1942) which would grow in the presence of many different bacterial species, although not all were equally effective.

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