The Cultivation of the Rumen Ciliate *Entodinium bursa* in the Presence of *Entodinium caudatum*

By G. S. COLEMAN, JUDITH I. LAURIE AND JANET E. BAILEY

Biochemistry Department, Agricultural Research Council,
Institute of Animal Physiology, Babraham, Cambridge CB2 4AT

(Received 19 April 1977)

The rumen ciliate protozoon *Entodinium bursa* has been grown *in vitro* in the presence of bacteria and *Entodinium caudatum* for over a year at population densities of 100 to 200 ml⁻¹. The medium contained potassium phosphate, prepared fresh rumen fluid, cysteine, wholemeal flour (or rice starch), dried grass and a culture of the spineless form of *Entodinium caudatum*. *Entodinium bursa* has an obligate requirement for this protozoon and died within 48 h in its absence. During growth from a 2 % inoculum, the mean generation time of *E. bursa* was 6 h. *Entodinium bursa* engulfed 1.5 to 2.5 *E. caudatum* organisms h⁻¹, and when *E. caudatum* was in excess it developed caudal spines for the first time in 17 years; these spined forms were engulfed much less readily than the spineless organisms.

INTRODUCTION

Although *Entodinium caudatum*, *E. simplex* and *E. longinucleatum* (Coleman, 1960, 1969*a*; Owen & Coleman, 1976) have all been cultivated *in vitro* in the absence of other protozoa, attempts to grow *E. bursa* under the same conditions have been unsuccessful. *Entodinium bursa*, which is the largest *Entodinium* sp. found in domesticated ruminants, engulfs *E. caudatum* *in vivo* (Poljansky & Strelkow, 1938) and we have found that *E. bursa* has an obligate requirement for the spineless form of *E. caudatum* for growth *in vitro*.

Entodinium bursa (Stein, 1858) was subsequently called *Entodinium vorax vorax* by Dogiel (1927) and this practice was followed by other workers, for example Poljansky & Strelkow (1938). We agree with Kofoed & MacLennan (1930) that the latter name is a synonym of the former and have used the original name.

METHODS

Caudatum-type salts medium. This contained [g (100 ml)⁻¹]: K₂HPO₄, 0.63; KH₂PO₄, 0.50; NaCl, 0.065; CaCl₂ (dried), 0.0045; MgSO₄·7H₂O, 0.009; CH₃COONa, 0.075; the pH was 6.7.

Culture medium. The protozoa were maintained routinely in 50 ml centrifuge tubes approximately three-quarters filled with caudatum-type salts medium plus 0.02 % (w/v) freshly-neutralized L-cysteine hydrochloride and 10 % (v/v) prepared fresh rumen fluid (Coleman, 1958). The final pH was 6.7. Nitrogen/carbon dioxide (95:5, v/v) was bubbled vigorously through the medium for 2 to 3 min and the tube was sealed with a rubber bung.

Cultivation of *Entodinium caudatum*. This protozoon, which had been maintained *in vitro* since 1959 and which had no caudal spines, was grown as described by Coleman (1958, 1960, 1971).

Initial isolation of *Entodinium bursa*. Protozoa (1 to 5 individuals) were removed with a micropipette from the rumen contents of a cannulated Clun Forest sheep, fed on hay (800 g daily) and oats (100 g daily), and inoculated into 3 ml of the culture medium contained in a 125 × 12 mm tube. Wholemeal flour (0.2 mg, as an aqueous suspension) and coarse dried grass (2 mg; Coleman *et al.*, 1976) were added immediately and daily thereafter. The tube was gassed with N₂/CO₂ (95:5), sealed with a rubber bung and incubated at 39 °C.

Entodinium caudatum (approximately 0.1 ml culture) was added initially and thereafter as the protozoa were engulfed by the *E. bursa*. The cultures were examined microscopically each day and care was taken to ensure that between 50 and 100 *E. caudatum* to each *E. bursa* organism were present after the daily feeding. After 7 days the cultures were diluted with an equal volume of fresh medium and after a further week, if the protozoa were growing well, the whole contents of the tube were transferred to a 50 ml centrifuge tube half-filled with fresh medium. Over this period, the amounts of wholemeal flour and culture of *E. caudatum* added daily were gradually increased taking care not to add more than the minimum necessary for protozoal growth.

Culture of Entodinium bursa. Wholemeal flour [1 drop, about 0.05 ml, of 1.5 % (w/v) aqueous suspension], coarse dried grass (2 mg) and *E. caudatum* (1 ml culture; approximately 20000 protozoa) were added daily to the contents of each 50 ml centrifuge tube, which was then gassed with CO₂ and incubated at 39 °C. Twice a week each culture was mixed and divided into two equal portions, each of which was diluted to the original volume with fresh medium.

Protozoal counts. The number of protozoa in a culture was estimated immediately before dilution. A portion of the culture (usually 1.0 ml) was added to 0.04 M-iodine (1.0 ml) and all the protozoa present in 0.1 ml of the mixture were counted microscopically. The size of the organisms was determined microscopically in living cultures with a measuring eye-piece and a 10× objective. All the protozoa in a suitably diluted drop of a well-mixed culture were measured.

RESULTS

Both wholemeal flour and *E. caudatum* had to be added to the culture of *Entodinium bursa* each day, otherwise it died within 48 h. The wholemeal flour could be replaced by an equal amount of rice starch without change in the population density (100 to 200 ml⁻¹) but not by powdered dried grass (Coleman *et al.*, 1976), which suggests that *E. bursa* was not cellulolytic. *Entodinium bursa* grew poorly when the caudatum-type salts medium was replaced by simplex-type salts medium (Coleman *et al.*, 1976), which is equilibrated with CO₂, and the culture died after 2 weeks. The presence of prepared fresh rumen fluid in the medium was also essential for good growth although *E. bursa* survived for 3 weeks in its absence.

The organism

In cultures containing an excess of *E. caudatum*, *E. bursa* measured $80 \pm 6 \times 63 \pm 3 \mu\text{m}$ ($n = 10$; range 75 to 93 × 57 to 69 μm) compared with $88 \pm 12 \times 73 \pm 6 \mu\text{m}$ ($n = 20$; range 71 to 104 × 64 to 82 μm) in the rumen of the sheep from which the protozoa were isolated. The protozoa studied by Dogiel (1927) measured $95 \times 68 \mu\text{m}$ (range 80 to 121 × 52 to 83 μm). In cultures of *E. bursa* that were fed too few *E. caudatum* or in cultures where all the *E. caudatum* had been engulfed, there was more variation in size with the appearance of small forms that measured $69 \pm 7 \times 56 \pm 6$ ($n = 15$). Under these conditions some *E. bursa* organisms failed to divide completely and chains of up to four distorted protozoa were found.

Engulfment of Entodinium caudatum

As *Entodinium bursa* would not grow without *E. caudatum* and as the latter gradually disappeared in the presence of the former, *E. bursa* probably engulfed *E. caudatum*. Vigorously growing *E. bursa* were densely filled with storage material, so it was impossible to see if they contained any *E. caudatum*. We therefore examined protozoa from a culture from which *E. caudatum* had disappeared about 6 h before and in which *E. bursa* was transparent. When *E. caudatum* (Fig. 1) were added, they were slowly engulfed by some of the *E. bursa* and the process was followed microscopically. Figures 2, 4 and 5 show *E. bursa* organisms containing 0, 1 and 2 *E. caudatum* organisms. The *E. caudatum* organism shown in Fig. 4 is apparently still intact and had presumably just been engulfed, while those shown in Fig. 5 have rounded up and were probably in the first stages of digestion.

Quantitative aspects. Washed *E. caudatum* (22000 to 190000 ml⁻¹; prepared as described by Coleman, 1969b) were incubated with unwashed *E. bursa* (4000 ml⁻¹) concentrated

Bar markers in all photomicrographs represent 50 μm .

Fig. 1. The culture form of *E. caudatum*.

Fig. 2. *Entodinium bursa* (b) grown in presence of an excess of *E. caudatum*. Note the short main spine on the *E. caudatum* (c).

Fig. 3. *Entodinium caudatum* with a shorter main caudal spine, taken from the same culture as the protozoa shown in Fig. 2.

Figs 4, 5. Starved *E. bursa* incubated in the presence of a suspension of *E. caudatum* for 1 h. Note the engulfed *E. caudatum* (d) which in Fig. 5 are partially digested.

Fig. 6. The disappearance of washed *E. caudatum* (initial population density, 22 000 to 190 000 ml⁻¹) in the presence of unwashed *E. bursa* (4000 ml⁻¹).

from a vigorously growing culture from which *E. caudatum* had just disappeared. At initial ratios of *E. caudatum*/*E. bursa* of 5.5, 11.0, 12.8, 20.2 and 47.5, the rates of engulfment over the steepest parts of the curves (Fig. 6) were [in units of *E. caudatum* disappearing (*E. bursa*)⁻¹ h⁻¹] 1.58, 2.52, 1.50, 1.52 and 2.16 respectively. During the first 8 h (lowest two ratios) or the first 23 h (three highest ratios) the number of *E. bursa* increased by 31, 40, 78, 58 and 65 % respectively. The finding that the rate of engulfment did not fall off appreciably even when there were less *E. caudatum* than *E. bursa* was not unexpected as all the protozoa were present together at the bottom of the tube.

Specificity of requirement for Entodinium caudatum. Attempts to grow *E. bursa* in the presence of *E. simplex* or *E. longinucleatum* grown *in vitro* (Coleman, 1969*a*; Owen & Coleman, 1976) were unsuccessful. When they, or mixed entodinia taken directly from the rumen, were incubated with a concentrated suspension of *E. bursa*, none disappeared.

To determine if dead *E. caudatum* were engulfed by *E. bursa*, a washed suspension of *E. caudatum* was divided into two parts; one part was treated with 2 % (w/v) glutaraldehyde for 5 min to kill the protozoa which were then washed four times by centrifuging before incubation with *E. bursa*. On incubation of 700 *E. bursa* with 23 500 glutaraldehyde-treated or normal living *E. caudatum* for 23 h, 23 000 *E. caudatum* disappeared from the normal culture and 3100 from the treated culture. In the presence of the dead protozoa many *E. bursa* contained 2 to 4 intact *E. caudatum* suggesting that after fixation with glutaraldehyde, *E. caudatum* could still be engulfed but not digested.

Effect of Entodinium bursa on the size and spination of E. caudatum. When *E. bursa* was grown for several weeks in the presence of an excess of *E. caudatum* (i.e. some were still present when more were added during the daily feeding), the residual *E. caudatum* had caudal spines (Figs 2 and 3). As the culture of *E. caudatum* had been spineless since a few months after it was isolated 17 years ago, the spines had probably grown in response to stimulation by *E. bursa*. This was a gradual process, the length of the main caudal spine increasing from nothing (Fig. 1) through intermediate stages which were often present in the same culture (Figs 2 and 3) until the spine characteristic of the type species was present. These spined *E. caudatum* were engulfed much more slowly than the normal culture form and once present never completely disappeared from a culture. *Entodinium bursa* survived for over 2 months in cultures in which spined *E. caudatum* were present and to which cultured *E. caudatum* were not added each day, but the population density was always low and much less than that of the *E. caudatum* present. This probably corresponds to the condition in the rumen.

When *E. bursa* was grown from a small inoculum on an excess of *E. caudatum* (ratio

approximately 1/10000 initially), as described below, and the whole culture was fed on rice starch instead of wholemeal flour, large forms of *E. caudatum* occasionally appeared after a few days. In a normal culture, most of the organisms were 40 to 60 μm long whereas in the presence of *E. bursa* they were 45 to 67 μm and 40 % of the protozoa were 60 to 65 μm long. These large *E. caudatum* were too large to be engulfed by *E. bursa* which, as shown above, tended to become smaller ($69 \times 56 \mu\text{m}$) when no *E. caudatum* were available for engulfment. The result was that in these cultures both protozoa were nearly the same size and could only be distinguished by their characteristic shapes.

Growth of Entodinium bursa from a small inoculum. *Entodinium bursa* could be grown from a small inoculum (2 % of a standard culture) added to 100 ml of a standard *E. caudatum* culture (20000 protozoa ml^{-1}). After 72 h all the *E. caudatum* had disappeared and 880 *E. bursa* ml^{-1} were present. From 33 to 55 h, the mean generation time of *E. bursa* was 6 h. Over this time an average of 200 *E. bursa* ml^{-1} engulfed 9600 *E. caudatum* ml^{-1} (assuming no growth of *E. caudatum*) to give a rate of $2.2 \text{ } E. \text{ caudatum } (E. \text{ bursa})^{-1} \text{ h}^{-1}$.

DISCUSSION

These studies provide an explanation for the finding that *E. caudatum* has one long and two short caudal spines when in the rumen of a normal sheep, but loses these spines when grown *in vitro* and will grow indefinitely in the spineless form. Poljansky & Strelkow (1938) showed that in defaunated sheep inoculated with the spined form of *E. caudatum* as the only ciliate protozoon, six forms of it could be identified in the subsequent two months. In some sheep no spined protozoa were present. However, they found, as in the present studies *in vitro*, that 10 to 20 days after the introduction of *E. bursa* only spined forms were present. Probably under all conditions when a spined form of *E. caudatum* divides, the anterior daughter organism has a slightly shorter caudal spine than that present on the original protozoon which becomes part of the posterior daughter. In the absence of *E. bursa*, either *in vivo* or *in vitro*, both daughters divide but as extra metabolic energy is required to produce three caudal spines, this form is at a disadvantage and tends to die out. However, in the presence of *E. bursa*, the forms without spines or with small spines are engulfed preferentially by this protozoon and this gives the spined form an advantage. Spined forms are therefore found when *E. bursa* is present and protozoa with no caudal spines when it is absent.

The obligate requirement of *E. bursa* for *E. caudatum* for growth *in vitro* is the second example of such a requirement among the rumen protozoa. The other is *Polyplastron multivesiculatum* which, for growth in potassium phosphate-rich media *in vitro*, requires an *Epidinium* sp. or protozoa from two other genera, but cannot utilize *Entodinium* spp. (Coleman, Davies & Cash, 1972). However, in sodium chloride-rich medium, *P. multivesiculatum* grows poorly in the absence of other ciliate protozoa. *In vivo*, *E. bursa* is normally found in the presence of the spined form of *E. caudatum* whereas *P. multivesiculatum* is not normally found in the presence of *Epidinium* spp. or other protozoa that it can engulf. Both protozoa will grow in the rumen in the absence of all other ciliates (Poljansky & Strelkow, 1938; Eadie, 1970). In contrast to this requirement for another protozoon only under certain conditions *in vitro*, the free-living protozoon *Didinium nasutum* has an obligate requirement for *Paramecium* for growth *in vivo* (Kudo, 1946). This specificity for a protozoon contrasts with the lack of specificity shown in the engulfment of bacteria by *E. caudatum* and *E. simplex* (Coleman, 1964, 1972).

We wish to thank Dr P. Kemp for inserting permanent rumen cannulae into the sheep used to provide rumen fluid for protozoal cultures and Mr A. L. Gallup for taking the photomicrographs.

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