The Cultivation of Cellulolytic Protozoa Isolated from the Rumen

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SUMMARY

Conditions are described for the isolation from the rumen and subsequent growth of six species of cellulolytic protozoa: *Enoploplastron triloricatum*, *Eudiplodinium maggii*, *Diploplastron affine*, *Epidinium ecaudatum caudatum*, *Diplodinium monacanthum* and *Diplodinium pentacanthum*. The protozoa were grown in an atmosphere of 95 % nitrogen plus 5 % carbon dioxide, or pure carbon dioxide, in a potassium phosphate-rich medium containing cysteine, sometimes 10 % prepared fresh rumen fluid, and daily additions of powdered dried grass. Population densities of 10 to 6000 protozoa/ml (depending on the species) were obtained in cultures that were diluted with fresh medium twice each week. Extracts of these protozoa digested a [^{14}C]cellulose preparation, liberating soluble ^{14}C -labelled compounds.

INTRODUCTION

One of the most characteristic reactions in the rumen is the breakdown and fermentation of cellulose (Tappeiner, 1884) and ciliate protozoa have long been implicated in the process (Trier, 1926). However, there have been few studies on the growth and metabolism of rumen cellulolytic protozoa. Hungate (1942, 1943) cultured *Eudiplodinium neglectum*, *Eu. maggii*, *Diplodinium multivesiculatum* and *Diplod. denticulatum* in the presence of ground dried grass and demonstrated that extracts of these protozoa produced reducing sugars from cellulose.

We have experienced difficulty in repeating Hungate's work, and so made a fresh study of the conditions required for isolation and maintenance of six species of entodiniomorphid protozoa from the rumen, and for their growth on ground dried grass as sole additional source of organic material, apart from contaminating bacteria.

As some protozoa, e.g. *Entodinium* spp. (Coleman, 1960, 1969*a*), have an absolute requirement for starch grains for growth, even in the presence of ground dried grass, it has been assumed that they cannot obtain sufficient energy for growth by fermentation of cellulose. Thus, any protozoon that can grow on dried grass alone can probably degrade cellulose. Initially this criterion was used to determine if an isolated protozoon was cellulolytic, and subsequently the result was confirmed by the action of extracts on pure cellulose.

METHODS

Basal mineral salts. Caudatum-type salts medium contained (g/100 ml): K_2HPO_4 , 0.63; KH_2PO_4 , 0.50; NaCl , 0.065; CaCl_2 (dried), 0.0045; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.009; CH_3COONa , 0.075. Simplex-type salts medium contained (g/100 ml): K_2HPO_4 , 0.49; KH_2PO_4 , 0.38; NaCl , 0.049; CaCl_2 (dried), 0.0035; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.007; NaHCO_3 , 0.66.

Culture media. All experiments were made in 50 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 % (v/v) prepared fresh rumen fluid (Coleman, 1958) or autoclaved rumen fluid (Coleman, 1960). Nitrogen (95 %, v/v) plus carbon dioxide (5 %, v/v) (for caudatum-type salts medium) or pure carbon dioxide (for simplex-type salts medium) was bubbled vigorously through the medium for 2 to 3 min and tubes were stoppered with rubber bungs.

Dried grass. Lawn mowings were air dried on the bench for 3 days and then ground either in a Lee Attrition Mill (Lee Engineering Co., Milwaukee, Wisconsin, U.S.A.) (when required for addition to cultures with wholemeal flour) or in a ball mill for 16 h (when added as sole source of organic material). The latter material was a fine powder whereas the former was much coarser and contained particles up to 5 mm long.

Initial isolation. One to five identical protozoa were picked out of crude rumen contents with a micropipette and inoculated into caudatum- or simplex-type salts medium (3 ml) plus 10 % prepared fresh rumen fluid contained in a 125 × 12 mm tube. Dried grass (3 mg; in dry form) and, in some experiments, wholemeal flour (0.2 mg; as an aqueous suspension) were then added, and added daily thereafter. Tubes were gassed appropriately, sealed with rubber bungs and incubated at 39 °C. After a week each culture was 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 the same medium. Over this period, the amount of dried grass and wholemeal flour added each day was gradually increased taking care not to add more than was essential for protozoal growth. If too much was added, the protozoa died. After a few weeks, the wholemeal flour could be omitted and powdered dried grass used as sole additional source of food.

Culture of cellulolytic protozoa. Powdered dried grass (20 mg) was added daily to the contents of each 50 ml centrifuge tube which was then gassed with CO₂ (regardless of the salts medium used) and incubated at 39 °C. Twice a week the culture was mixed, divided into two portions, and 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 (1.0 ml) was added to 0.04 M-iodine (1.0 ml), and all the protozoa present were counted microscopically in 0.1 ml of the mixture on a slide, or in the complete mixture under an inverted microscope (model CK; Olympus Optical Co., Tokyo, Japan). 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.

Identification of protozoa. All protozoa were identified from the descriptions given by Dogiel (1927) and Kofoid & MacLennan (1932), the latter's nomenclature and classification being used where the authors differed.

Preparation of [¹⁴C]cellulose. α-[¹⁴C]cellulose was prepared by the method of Jermyn (1955) from ¹⁴C-labelled perennial rye grass S24 kindly supplied by Dr R. M. C. Dawson and grown as described by Dawson & Hemington (1974). The α-[¹⁴C]cellulose was dissolved in syrupy H₃PO₄ at 4 °C and reprecipitated by adding excess water. This precipitate was washed repeatedly with water on the centrifuge and stored as an aqueous suspension at 4 °C.

Cellulase assay. The incubation mixture contained 0.02 ml [¹⁴C]cellulose (approximately 10⁴ c.p.m.; 70 µg), 0.05 ml 5 % (w/v) NaN₃, 0.03 ml 2 % (w/v) L-cysteine hydrochloride (neutralized immediately before use), 0.6 ml H₂O, and 0.5 ml of either salt solution D (Coleman, 1972) adjusted to pH 6.0, or protozoal broken-cell supernatant fluid prepared in the same salt solution. The reaction was carried out at 39 °C under 95 % N₂ plus 5 % CO₂, and

stopped by immersing the tube in boiling water for 5 min. The mixture was then centrifuged at 4000 g for 20 min, and 0.1 ml of the supernatant fluid plated out for estimation of ^{14}C as described by Coleman (1969*b*).

RESULTS

*Enoploplastron trilorica*tum

Cultivation. *Enoploplastron trilorica*tum was taken originally from a cow with a B-type population (Eadie, 1962, 1967) and grown on simplex-type salts medium containing 10 % prepared fresh rumen fluid with wholemeal flour and dried grass as sources of carbohydrate. Six months after isolation, it grew best on simplex-type salts medium without rumen fluid and with powdered dried grass as sole substrate. It was maintained under these conditions at a population density of 150 protozoa/ml for a further 16 months. In the presence of 10 % prepared fresh rumen fluid, only 50 protozoa/ml were present. After 22 months, the number of protozoa began to decline and the culture died after 2 years: in the presence of wholemeal flour, death occurred after 9 months. Although this protozoon could not be isolated in caudatum-type salts medium, established cultures grew equally well in both media. Attempts to facilitate maintenance of the protozoa by adding dried grass to the cultures only every other day were only partially successful as the numbers present declined steadily until all the protozoa were dead after a month.

Fifteen months after the initial isolation, *Eno. trilorica*tum was introduced into the rumen of a sheep previously defaunated by the method of Abou Akkada *et al.* (1968) and fed on hay (800 g daily) and oats (200 g daily). The protozoon became established at a population density of 50000 protozoa/ml and was the only ciliate present until 6 months later when *Entodinium caudatum* was also found. *Enoploplastron trilorica*tum was then reisolated *in vitro* and at the time of writing (after the original isolate had died) had been maintained on powdered dried grass for over 3 months. The period in the rumen had thus reversed the process that caused the decline and death of the first culture.

Morphology. This protozoon has the three characteristic skeletal plates first described for *Ostracodinium trilorica*tum forma *trilorica*tum by Dogiel (1925). The organism measured $84 \pm 9 \times 51 \pm 7 \mu\text{m}$ ($n = 10$; range 66 to 101 $\times$ 42 to 64 μm) and was therefore slightly smaller than the $100 \times 61 \mu\text{m}$ quoted by Dogiel for protozoa found in the rumen of a cow.

Eudiplodinium maggii

Cultivation. Two separate isolates of *Eudiplodinium maggii* were made: strain CO2, which survived for 30 months; and strain 4L, which was still alive after 26 months. Strain CO2 was initiated on simplex-type salts medium in the presence of wholemeal flour, dried grass and 10 % prepared fresh rumen fluid. After two months, the fresh rumen fluid was replaced by autoclaved rumen fluid and, with the daily addition of 1 mg wholemeal flour (per 30 ml culture) and dried grass, a population density of 700 protozoa/ml was maintained for over a year. Daily additions of 1.5, 3.0 or 7.5 mg wholemeal flour caused a decrease in the number of protozoa present to 460, 420 and 220 per ml respectively. Six months after isolation, the wholemeal flour could be omitted, provided that 10 to 20 mg powdered dried grass was added daily. Over the next 5 months a steady population density of 100 to 150 protozoa/ml could be maintained under these conditions on either salts medium in the presence or absence of rumen fluid.

The second isolate, strain 4L, would not grow on simplex-type salts medium. It was grown initially on caudatum-type salts medium in the presence of 10 % prepared fresh rumen fluid, wholemeal flour and dried grass. After a few months it began to grow poorly

on this medium and, since then, has been maintained on powdered dried grass alone, at population densities of 80 protozoa/ml in the absence of 10 % prepared fresh rumen fluid, and 200 protozoa/ml in its presence. This isolate was unique among the other isolates and species described here in that the population density was, for a period of 4 months, increased by up to 50 % by the daily addition of 10 mg α -cellulose prepared from grass by the method used for [^{14}C]cellulose.

Morphology. The first isolate measured $150 \times 75 \mu\text{m}$ after 18 months in culture and the second $118 \pm 14 \times 78 \pm 9 \mu\text{m}$ ($n = 10$; range 105 to 143×69 to $95 \mu\text{m}$) after 2 years in culture; both had the single slightly curved skeletal plate and hook-shaped macronucleus characteristic of the genus and species. The second isolate was somewhat smaller than *Eu. maggii* described by Dogiel (1927) and by Fiorentini (1889), but as it was larger than the lower limit given by Kofoed & MacLennan (1932) and as the other characteristics were consistent, it has been ascribed to this species.

A third isolate (strain c) that was morphologically similar to *Eu. maggii* measured only $102 \pm 7 \times 62 \pm 8 \mu\text{m}$ ($n = 10$; range 88 to 110×46 to $69 \mu\text{m}$) after 7 months in culture but had increased to $134 \pm 36 \times 73 \pm 10 \mu\text{m}$ ($n = 10$; range 72 to 156×49 to $84 \mu\text{m}$) after a further 4 months. This shows that the species can vary considerably in size, possibly depending on the exact cultural conditions, and accounts for the observation of Kofoed & MacLennan (1932) that small forms are known.

Strain c was grown initially on simplex-type salts medium containing 10 % prepared fresh rumen fluid and powdered dried grass, and never grew well on wholemeal flour. At the time of writing, it had been grown under these conditions for a year at a population density of 150 to 300 protozoa/ml: it grew equally well on simplex- or caudatum-type salts medium and numbers were not affected by the presence or absence of rumen fluid.

Diploplastron affine

Cultivation. *Diploplastron affine* was isolated on simplex-type salts medium containing 10 % prepared fresh rumen fluid and powdered dried grass but, as it grew poorly under these conditions, it was transferred to caudatum-type salts medium without rumen fluid and given daily additions of dried grass. Under these conditions a population density of 150 to 200 protozoa/ml has been maintained for over a year. Addition of 10 % prepared fresh rumen fluid or replacement of the dried grass by wholemeal flour had no effect on the numbers. Attempts to grow *Diplop. affine* from a small inoculum were not always successful, but, when the protozoa did survive, they grew rapidly for a short time and in one experiment divided 3.7 times in 48 h giving a mean generation time of 13 h (Fig. 1).

Morphology. This protozoon measured $100 \pm 10 \times 67 \pm 6 \mu\text{m}$ ($n = 10$; range 79 to 117×57 to $79 \mu\text{m}$) compared with $105 \times 59 \mu\text{m}$ for the organisms measured by Dogiel (1927) and is therefore a slightly different shape. However, it has the two skeletal plates characteristic of *Diplop. affine*, which are separated anteriorly but are close together at the posterior end.

A previous isolate, grown for 30 months on caudatum-type salts medium with wholemeal flour (2 mg daily per 30 ml medium) and dried grass, attained population densities of 700 to 1200 protozoa/ml but no attempt was made to grow it on dried grass alone. It measured $87 \pm 8 \times 48 \pm 8 \mu\text{m}$ ($n = 6$; range 80 to 99×42 to $51 \mu\text{m}$).

Epidinium ecaudatum caudatum

Cultivation. Although *Epidinium ecaudatum caudatum* has normally been grown at population densities of 400 to 1000 protozoa/ml on simplex-type salts medium containing

10 % prepared fresh rumen fluid with wholemeal flour and dried grass (Coleman, Davies & Cash, 1972), it has also been isolated and grown on the same medium without wholemeal flour. At the time of writing it has been maintained for over a year on simplex-type salts medium and powdered dried grass only, at population densities of 10 to 20 protozoa/ml.

Diplodinium monacanthum

Cultivation. *Diplodinium monacanthum* was isolated from a sheep with a B-type protozoal population on simplex-type salts medium containing 10 % prepared fresh rumen fluid and daily additions of wholemeal flour and dried grass, and reached a population density of 2100 protozoa/ml. After 2 months, wholemeal flour was omitted from the medium and 4 months later there were 6000 protozoa/ml. Thereafter *Diplod. monacanthum* was maintained either with or without wholemeal flour until the numbers declined and the culture died 13 months after isolation. This was a particularly difficult protozoon to grow and there was a wide variation in population densities of tubes that were treated identically.

Morphology. This protozoon measured $88 \pm 8 \times 58 \pm 6 \mu\text{m}$ ($n = 14$; range 70 to 99×51 to $64 \mu\text{m}$) compared with $76 \times 55 \mu\text{m}$ for the type specimens (Dogiel, 1927), and had no skeletal plate. One month after isolation, only one short caudal spine was present and this tended to disappear in some individuals as cultivation *in vitro* continued. It is therefore possible that one or more spines could have disappeared between the times of isolation and examination but, on the assumption that no change occurred, it is thought to be *Diplod. monacanthum* rather than *Diplod. diacanthum* or *Diplod. triacanthum*.

Diplodinium pentacanthum

Cultivation. *Diplodinium pentacanthum* was grown initially on simplex-type salts medium in the presence of 10 % prepared fresh rumen fluid, wholemeal flour and dried grass and after 2 months had reached a steady population density of 200 protozoa/ml. At this time the protozoon grew better in caudatum-type salts medium (400 protozoa/ml), and omission of the wholemeal flour from the medium with or without rumen fluid increased the numbers to 800 per ml. When the protozoa were grown from a small inoculum (5 to 10 protozoa/ml) in the presence of grass alone, there was a long lag followed by a period of rapid growth during which there were 2.8 divisions of the protozoa in 24 h giving a mean generation time of 8.5 h (Fig. 1).

Morphology. Two weeks after isolation this protozoon had five small caudal spines and generally resembled *Diplod. pentacanthum*, but after 4 months in culture only the largest ventral spine was present in some individuals and no spine of any size in others. It therefore resembled *Diplod. monacanthum* or *Diplod. anacanthum*. At 4 months the protozoon measured $100 \pm 15 \times 62 \pm 9 \mu\text{m}$ ($n = 10$; range 85 to 117×54 to $71 \mu\text{m}$) when grown on powdered dried grass alone and $89 \pm 22 \times 65 \pm 8 \mu\text{m}$ ($n = 10$; range 64 to 100×56 to $80 \mu\text{m}$) when grown on wholemeal flour and dried grass. This organism was therefore in the size range of *Diplod. monacanthum* ($92 \times 55 \mu\text{m}$; $n = 10$; range 60 to 124×38 to $72 \mu\text{m}$; Kofoid & MacLennan, 1932) rather than that of *Diplod. pentacanthum* ($77 \times 55 \mu\text{m}$; $n = 10$; range 67 to 84×51 to $60 \mu\text{m}$; Kofoid & MacLennan, 1932).

Cellulolysis

The ground dried grass that was added to the cultures each day was not completely degraded, so there was always more plant material, contaminated with bacteria, than protozoa in the pellet at the bottom of each tube. Unfortunately it was not possible to

Fig. 1

Fig. 1. Growth of *Diploplastron affine* (○—○) and *Diplodinium pentacanthum* (●—●) from a small inoculum on caudatum-type salts medium, in the absence of fresh rumen fluid, with the daily addition of powdered dried grass and without dilution of the culture.

Fig. 2

Fig. 2. Release of soluble ¹⁴C-labelled material (expressed as c.p.m. in medium) from [¹⁴C]cellulose (2300 c.p.m.) by supernatant fluids prepared from a culture of *Eudiplodinium maggii*. A protozoa-rich fraction (○, ●) and an equal volume of grass (△, ▲) from the culture were sonicated and then centrifuged (7000 g, 20 min); and the resulting supernatant fluids, either untreated (○, △) or treated by immersion in boiling water for 5 min (●, ▲) were assayed for cellulase activity.

separate protozoa from grass completely by differential centrifugation, although grass could be prepared free of protozoa. The protozoa-rich fraction and an equal volume of grass were each suspended in portions of salt solution D, adjusted to pH 6.0, and sonicated (Coleman, 1969*b*) until all the protozoa in the former had disintegrated. Both sonicates were then centrifuged at 7000 g for 20 min and the clear supernatant fluids were assayed for cellulase activity, that from the sonicated grass serving as a control.

Figure 2 shows a typical curve for the release of soluble ¹⁴C-labelled compounds into the medium from [¹⁴C]cellulose by *Eudiplodinium maggii* (strain 4L). Similar results were obtained for all the protozoal species except *Diplodinium monacanthum*, which died before the assay was developed.

DISCUSSION

The method we have used to isolate and grow the rumen cellulolytic protozoa differed from that of Hungate (1942, 1943) in that we inoculated the protozoa into medium containing 10 % prepared fresh rumen fluid and 0.02 % cysteine as reducing agents. We found it necessary to add fresh substrate to the cultures each day. The rumen fluid could be omitted from established cultures but it was essential for the survival of small numbers of protozoa inoculated directly from the rumen. Although Hungate used anaerobic conditions, his salt solutions must have had a high redox potential until this was decreased by bacterial

metabolism, which suggests that his protozoa were less sensitive to oxidizing conditions than those we have used. Even using the method described above, individual protozoa taken from the rumen frequently failed to grow. As with other ciliate protozoa (Coleman, 1960, 1969*a*), the population densities of the cellulolytic protozoa were always smaller *in vitro* than *in vivo*.

Unfortunately the dried grass on which the protozoa were grown contained other materials apart from cellulose and it is therefore not certain that the protozoa were utilizing only cellulose for growth. Nevertheless the results confirm and extend those of Hungate (1942, 1943) and suggest that extracts of all species of rumen protozoa that live on dried grass alone will digest cellulose. Although the enzyme was obviously not coming from bacteria attached to the grass in the culture but was associated with the protozoa, it is still uncertain if it was of protozoal origin or was derived from bacteria present in the endoplasm.

Experiments to determine the properties of the cellulase and studies with the electron microscope on the relationship of bacteria and cellulose fibres inside the protozoa are being completed.

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