THE CULTIVATION OF RUMEN OLIGOTRICH PROTOZOA

II. GROWTH OF ENTODINIA *IN VITRO*

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Rumen oligotrich protozoa, namely some species of *Entodinium*, were successfully cultured *in vitro* on a salt medium containing whole egg protein, proteose-peptone, rice starch, yeast extract, cellulose powder, and white clover juice. Of these constituents proteose-peptone, yeast extract, and cellulose powder could be omitted, but omission of the clover juice resulted in the death of the protozoa. Attempts to replace the clover juice by known compounds were unsuccessful.

Entodinia were maintained for over 10 months on a medium of rice starch and finely ground white clover at population densities of $2-10 \times 10^4$ /ml. On the two media described above populations up to $2-3 \times 10^5$ /ml were obtained by using a simple apparatus. On the latter medium, the ciliates were maintained at a mean density of nearly 2×10^5 /ml for more than 50 days. *Entodinium caudatum*, starting from a single cell, multiplied with the average division rate of approximately once per day during the initial 4 weeks. The clone was maintained for over 3 months with active multiplication. Morphological variation was observed in these clonal cultures.

Many attempts have been made to grow rumen oligotrich protozoa *in vitro*, and several successful results have been reported in maintaining these nutritionally and physiologically fastidious protozoa.

As for the cultivation of *Entodinium* species, KNOTH (1) was able to keep them alive for 5 days outside the host. HUNGATE (2) cultured the organisms for more than 2 weeks in a salt medium containing grass and cellulose. KANDATSU and TAKAHASHI (3) reported that some species of this genus were maintained for 30 days at population densities of $2-5 \times 10^4$ /ml. Their medium contained mineral salts, fresh white clover, vitamin B₁₂, and grass-hay steep liquor fermented previously by rumen bacteria. COLEMAN

(4-6) has maintained *Entodinium caudatum* at a density of 3×10^4 /ml for 9 years by using a medium which consisted of rice starch, dried grass, mineral salts, and rumen fluid. Recently, JARVIS and HUNGATE (7) cultured *Entodinium simplex* for an unspecified time on a medium of ground wheat and grass with buffered saline, obtaining protozoal counts up to 3×10^4 /ml in the presence of protozoal extracts. More recently, COLEMAN (6) reported that *E. simplex* were maintained for over 2.5 years on a salt medium containing wholemeal flour, dried grass, and rumen fluid. The populations obtained by him were more than 6×10^4 /ml.

Thus, the population densities reported have been much lower than those in the rumen, and the media used have been undefined chemically. The purpose of the study reported here was, therefore, to promote the growth of entodinia and to maintain higher populations. In addition, attempts were made to define the culture media chemically.

MATERIALS AND METHODS

Source and counts of rumen protozoa. Rumen protozoa for inoculation were prepared by the method described in the previous paper (8) from the rumen liquor of goats feeding on grass hay and commercial concentrated feed.

The protozoa were counted in the Fuchs-Rosenthal counting chamber after fixing and staining in four volumes of a solution which contained 0.02% Methyl Green, 10% Formalin, and 0.8% NaCl. Only those entodinia which showed no signs of disintegration were counted.

Mineral salt solution. The mineral salt solution contained (g/liter); KH_2PO_4 , 3.0; $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 18.0; NaHCO_3 , 0.5; NaCl, 0.5; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.1.

Antibiotic solution. The antibiotic solution contained, per 100 ml, potassium penicillin G, 100,000 units, and dihydrostreptomycin sulfate, 1.0 g. Unless otherwise stated, this solution (1 ml/100 ml medium) was added in all experiments.

Vitamin mixture. The vitamin mixture contained, per g, vitamin A palmitate, 2,500 I.U.; calciferol, 200 I.U.; thiamine nitrate, 1 mg; riboflavin, 1.5 mg; niacin, 10 mg; pyridoxine-HCl, 1 mg; folic acid, 0.5 mg; calcium pantothenate, 5 mg; cyanocobalamin, 1 μg ; ascorbic acid, 37.5 mg; α -tocopherol, 1 mg.

Culture media. For growing entodinia, the following culture media were used:

'Medium A' was prepared by the method of KANDATSU and TAKAHASHI (3), i.e., wild grass hay was steeped in the salt solution (1 g/10 ml), which was incubated anaerobically at 39° with inoculated rumen bacteria. After 24 hr of incubation the liquor was decanted and filtered through two layers of gauze. The filtrate (100 ml), to which fresh white clover (*Trifolium repens*,

5 g) and vitamin B₁₂ (0.045 µg) were added, was used as a growth medium.

'Medium B' was the same as Medium A except that vitamin B₁₂ was omitted and non-fermented grass hay extract (extracted with the salt solution by boiling for 30 min) was used.

'Medium C' differed from Medium B in that whole egg protein (Taiyo Food Co., Ltd., Yokkaichi; 0.2 g/100 ml) and rice starch (Shimada Kagaku Co., Ltd., Niigata; 0.5 g/100 ml) were supplemented.

'Medium D' was composed of whole egg protein 0.2 g, proteose-peptone (Difco) 0.2 g, rice starch 0.5 g, cellulose powder (over 300 mesh; Toyo Kagaku Sangyo Co., Ltd., Tokyo) 0.1 g, yeast extract (Difco) 0.1 g, and white clover juice (fresh leaves were homogenized in a Waring blender and filtered through two layers of gauze) 1 ml, per 100 ml of salts solution.

'Medium E' consisted of rice starch 0.25 g and white clover powder (dried at 70° in an electric oven and finely ground) 0.25 g, per 100 ml of salt solution.

Cultural conditions. All experiments were conducted in 100 (or 200) ml flat-bottomed flasks containing 100 (or 200) ml medium, the surface of which was covered with liquid paraffin to prevent cultures from contact with air and to eliminate gases produced by the organisms. Immediately before inoculation, Na₂S·9H₂O (0.01%, w/v) was added to the medium which had been gassed with N₂ for 5–10 min. Cultivations were made in duplicate at 39°, adjusting the pH to 7.0 every day. For continued cultivations the following growth conditions were employed:

'Condition A.' Additions of white clover were made every 24 hr. Every 48 hr the protozoa were transferred by centrifuging the culture (400×g, 5 min) and re-inoculating the sedimented protozoa into a new flask containing a fresh medium.

'Condition B.' Addition of the substrate was made every 2 days. Every 4 days, the cultures were diluted, *i.e.*, one-half volume of the culture was removed and replaced by an equal volume of fresh medium.

'Condition C.' Cultures were diluted on days when the protozoal count was more than 10,000/ml. When it was less than 10,000/ml, the protozoa were transferred in the same way as in Condition A.

'Condition D.' Experiments were carried out by using a Cellophane tubing hung in a cylindrical vessel. This apparatus is illustrated in Fig. 7A. The protozoa grown inside the Cellophane tubing were transferred every 6 days in the same way as in Condition A, and the mineral salt solution (the outer fluid) and the Cellophane tubing were renewed every day. Substrate was added into the tubing each day.

'Condition E.' Cultures of a single protozoon were started in tubes containing 5 ml of medium and were transferred to larger vessels as the ciliates multiplied; the volume of the cultures was doubled every 4 days by adding an equal volume of fresh medium. Substrate was added on alternate days. (Except where stated, Condition A was employed.)

Fig. 1. Growth of entodinia on various media.

(A) ●, Medium A; ○, Medium A minus vitamin B₁₂; ▲, Medium A minus fresh white clover; ×, Medium A in which fresh white clover was replaced by white clover juice; ■, Medium B.

(B) ○, Medium B; ●, Medium B plus the antibiotic solution, (1%, v/v); □, Medium C; ■, Medium C plus the antibiotic solution; △, the mixture of Medium A and Medium B (1:1, v/v); ▲, the mixture of Medium A and Medium B plus the antibiotic solution. (For details see text.)

Isolation of a single protozoan. A single cell of *Entodinium caudatum* was picked up from the diluted protozoal suspension with a capillary under a microscope. To facilitate the isolation, the protozoa were immobilized by cooling (5–10°). Except when exposed to low temperatures for more than 30 min, most of the protozoa revived satisfactorily when placed in a fresh medium.

RESULTS

Attempts to improve culture medium

In an effort to cultivate the protozoa on chemically defined medium, attempts to improve the culture medium were made. Experiments were initiated with KANDATSU and TAKAHASHI's medium (3), and subsequently, the medium was improved.

The growth of the protozoa on various media with or without antibiotics is shown in Fig. 1. Entodinia, principally *E. caudatum*, *E. longinucleatum*,

Fig. 2. Effect of nitrogen source on the growth of entodinia.

The protozoa were grown on media containing rice starch, glucose, cellulose powder, and white clover juice to which were added (g/100 ml):

(A) ●, whole egg protein (0.2) and proteose-peptone (0.2); ▲, whole egg protein (0.4); ×, proteose-peptone (0.4); ■, none.

(B) ×, whole egg protein (0.2) and proteose-peptone (0.2); ●, whole egg protein (0.2) and casein (0.2); ○, whole egg protein (0.4); ▲, casein (0.4); ■, none.

(C) ▲, whole egg protein (0.1) and proteose-peptone (0.1); ●, white clover powder (1.0); ■, none. (The dotted lines indicate the dilution of the cultures.)

and *E. simplex*, grew well for 30 days on Medium A. Similar results were obtained on Medium A without vitamin B₁₂, but the ciliates failed to grow without fresh white clover. Fresh white clover could be replaced with clover juice. Growth on Medium B was not as good as on Medium A (Fig. 1A). However, entodinia grew better on Medium B supplemented with whole egg protein and rice starch (Medium C) in the presence of antibiotics (Fig. 1B).

Nitrogen source. The ability of various nitrogen compounds to support entodinal growth was studied in the presence of rice starch, glucose, cellulose powder, and white clover juice. The results in Fig. 2A show that the medium containing both whole egg protein and proteose-peptone gave better growth than those containing only one of them. Whole egg protein, which was immersed in boiling water for 1 hr to soften its tissue, supported the growth satisfactorily, but casein (E. Merck) did not (Fig. 2B). This softened egg protein was a little less effective than white clover powder as a nitrogen source for entodinia (Fig. 2C). When the amount of nitrogen compounds was

Fig. 3. Effect of energy source on the growth of entodinia.

The protozoa were grown on media containing whole egg protein, proteose-peptone, and white clover juice to which were added (g/100 ml):

(A) and (B). ■, rice starch (0.4), glucose (0.1) and cellulose powder (0.1); ●, rice starch (0.4) and glucose (0.2); △, rice starch (0.4) and cellulose powder (0.2); ○, rice starch (0.6); ▲, glucose (0.3) and cellulose powder (0.3); ×, none.

(C) ●, rice starch; ○, soluble starch; ▲, dextrin; △, maltose; ■, glucose; □, cellulose powder; ×, none. (each 0.3)

doubled, the protozoa decreased in number, which was followed by the death of the ciliates. This was probably associated with an increase in the number of bacteria, or in the amount of ammonia formed.

Energy source. Figure 3 shows the growth of entodinia on various carbohydrates as an energy source, in the presence of whole egg protein, proteose-peptone, and clover juice. The protozoa grew well on rice starch granules, but not on glucose and cellulose powder (Fig. 3A). Addition of glucose hampered continued cultural maintenance, possibly due to the heavy proliferation of bacteria (Fig. 3B). Glucose, maltose, and dextrin each inhibited the growth of entodinia, but soluble starch permitted some growth, suggesting that the molecular size is important for the uptake of foods by these protozoa. Cellulose powder was ineffective as an energy source (Fig. 3C).

Supplements. As described above, white clover juice is required for the cultivation of entodinia. Subsequent experiments were carried out to test whether clover juice could be replaced by other materials. Figure 4 shows the growth response to various materials when egg protein, proteose-peptone, and rice starch were present. In Fig. 4A, yeast extract showed no growth-stimulating effect, nor did combination of yeast extract and the vitamin mix-

Fig. 4. Effect of supplements on the growth of entodinia.

The protozoa were grown on media containing whole egg protein, proteose-peptone, and rice starch to which were added per 100 ml:

(A) $\blacktriangle$, white clover juice (1 ml) and yeast extract (100 mg); $\circ$, white clover juice; $\bullet$, yeast extract; $\triangle$, none.

(B) $\blacktriangle$, white clover juice (1 ml), yeast extract (100 mg) and vitamin mixture (100 mg); $\circ$, white clover juice and yeast extract; $\bullet$, white clover juice and vitamin mixture; $\triangle$, yeast extract and vitamin mixture; $\blacksquare$, vitamin mixture; $\square$, none.

(C) $\bullet$, white clover juice (1 ml) and vitamin mixture (10 mg); $\circ$, yeast extract (100 mg) and vitamin mixture; $\blacktriangle$, vitamin mixture; $\triangle$, white clover juice; $\blacksquare$, none.

(D) $\bullet$, white clover juice (1 ml) and copper-chlorophyllin (0.1 mg); $\triangle$, white clover juice; $\circ$, copper-chlorophyllin; $\blacktriangle$, white clover powder (500 mg); $\blacksquare$, none.

ture yield favorable results. The vitamin mixture exerted an inhibitory effect at the concentration of 1 mg/ml, but did not at 0.1 mg/ml (Fig. 4B, C). These results suggest that the vitamins required by entodinia may be produced by co-existing bacteria or may be included in white clover, and that white clover may contain some growth-stimulating factor(s) other than constituents of the vitamin mixture and yeast extract.

Copper-chlorophyllin could not substitute for clover juice. Clover powder, however, was as effective as clover juice (Fig. 4D), suggesting that the growth factor(s) in white clover is (are) not destroyed by drying at 70° for 24 hr.

Growth on improved media. Based on the above results, a new culture medium was prepared (Medium D). This medium contained yeast extract and cellulose powder, although these materials had no obvious effect on entodinia.

Fig. 5. Growth of entodinia on various media.

(A) ●, Medium C with antibiotics; ○, Medium C without antibiotics; ▲, Medium D with antibiotics; △, Medium D without antibiotics.

(B) ▲, Medium D with antibiotics; ○, Medium E with antibiotics; ●, Medium E without antibiotics.

Good growth was obtained on Medium D with antibiotics (Fig. 5A), but the ciliates grew better on a medium of rice starch and white clover powder (Medium E), both with and without antibiotics (Fig. 5B). On Medium E entodinia were maintained at population densities of $2\text{--}10 \times 10^4/\text{ml}$ for over 10 months under Condition B.

Growth on a chemically defined medium

QUINN *et al.* (9) reported that the entire spectrum of rumen protozoa could be maintained for 7 days in continuous culture on a chemically defined medium. To test whether entodinia are able to grow by multiplication for longer periods in medium 4 of QUINN *et al.*, mixed rumen protozoa were inoculated into the medium. The experiments were carried out under Condition C. All the protozoa, inoculated at $4 \times 10^4/\text{ml}$, gradually decreased in number and disappeared from the culture within 6 days. Addition of rice starch (0.5 g/100 ml) prolonged the life of entodinia by a few days, and addition of rice starch and whole egg protein (0.1 g/100 ml) further prolonged protozoal survival by several days, but the protozoa did not grow for a longer period.

Fig. 6. Growth of entodinia in the attempt to increase the division rate.

The protozoa were grown on Medium D (A) or Medium E (B) under Condition C. The dotted lines refer to the dilution of the cultures, and the arrows the transfer of the ciliates.

Growth rate of entodinia

Little accurate information has been obtained on the growth rates of rumen protozoa. The only reported measurement of protozoal division time in the rumen is 15 hr for entodinia, according to the calculation by HUNGATE (10), from the data of WARNER (11). Division rates of approximately once per day have been reported for several types of protozoa, including entodinia, when cultured *in vitro* (12–14). The maximum observed *in vitro* rate is twice per day for *epidinium* (15).

The growth of entodinia on Medium D under Condition C is shown in Fig. 6A. The ciliates obviously grew faster than those under Condition A. The mean division rate was nearly once per 3 days during 25 days. Entodinia on Medium E maintained their number for 15 days when the cultures were diluted each day, indicating a mean division rate of at least once per day (Fig. 6B). During a short time (2–3 days) maximum division rates of 3–5 times per day were frequently observed, but such rapidity did not last long. When the cultures were diluted twice per day the ciliates failed to maintain themselves for 5 days.

Attempts to maintain entodinia at high population densities

As described in the introductory paragraph, the reported population

Fig. 7. Maintenance of entodinia at high population densities.

(A) The culture apparatus: a, the mineral salts solution (450 ml); b, the culture medium (300 ml); c, Cellophane; d, liquid paraffin; e, an agitating bar connected with a motor.

(B) The protozoa were grown under Condition D on Medium D from a small ($\bullet$, $\circ$) or a large ($\blacktriangle$, $\triangle$) inoculum, or on Medium E ($\blacksquare$).

densities for entodinia maintained *in vitro* are $3\text{--}6 \times 10^4/\text{ml}$. On the other hand, the number of entodinia in the rumen is normally $2\text{--}6 \times 10^5/\text{ml}$ (11, 16). It was of interest to maintain the protozoa *in vitro* at densities approaching the levels in the host.

In attempts to obtain higher densities, entodinia were grown under Condition D, using the culture apparatus illustrated in Fig. 7A. The growth on Medium D from both small ($5 \times 10^4/\text{ml}$) and large ($8 \times 10^5/\text{ml}$) inocula is shown in Fig. 7B. In both cases the protozoa settled at densities of $2\text{--}3 \times 10^5/\text{ml}$ in nearly 10 days, and thereafter the number remained constant for 2 weeks. Entodinia were subsequently grown on Medium E from a low number ($1.5 \times 10^4/\text{ml}$). The protozoa reached $2 \times 10^5/\text{ml}$ on the sixth day, from which time the number was almost constant until the 50th day, when the experiment was stopped.

Single-protozoan cultures

Single-protozoan cultures were initiated with a single cell of *Entodinium caudatum* on Medium E under Condition E. The typical growth is shown in Fig. 8, in which the increase in the total number of the cells is shown. The ciliates multiplied logarithmically during the 4 weeks following inoculation, with an average division rate of approximately once per day, although the

Fig. 8. Increase in the total number of *E. caudatum* from a single cell.

A single cell of *E. caudatum* was inoculated into Medium E and grown under Condition E. (The numbers are the average of four cultures.)

cells during the initial 2 weeks could not be counted by the microscope because of small number. The clone was maintained for more than 3 months at densities of $2-6 \times 10^4/\text{ml}$.

Morphological variation took place in the clonal cultures over a period of several weeks; decrease in the size of cells and/or shortening of caudal spines were observed. Occurrence of such cells increased in percentage as the cultures aged without refreshing the medium, suggesting that unfavorable conditions such as shortage of nutrients and accumulation of waste products may have an influence on the body size and caudal spination. At a glance, these changed cells were undistinguishable from other non-caudated species. When the cells, including the altered ones, were transferred to a fresh medium, normal cells gradually increased in percentage. This suggests that either the change of cells is reversible or the changed cells cannot multiply.

DISCUSSION

The growth of entodinia greatly depends on the ease with which they can ingest nutrients. As emphasized by ONODERA and KANDATSU (17), the protozoa take up foods mainly by engulfing particulate substances. Furthermore, microscopic observation revealed that entodinia were able to ingest lumps of fairly large size by snapping and tearing. Most of the whole egg protein softened by immersion in boiling water may have been ingested in the latter way. On the other hand, casein and proteose-peptone did not support growth of the organisms. This suggests that entodinia do not readily ingest

soluble nutrients, and that the protozoa lose competition with bacteria for foods. Similarly, soluble carbohydrates did not permit protozoal growth, yet larger molecules of soluble starch did slightly. Rice starch granules (*ca.* 5 μ) were most easily ingested. Failure of the protozoa to grow on cellulose powder may be due to the large particle size of the material; or even if ingested, the entodinia may be unable to digest cellulose (2, 18).

It is obvious that bacteria have had no small effect on protozoal growth. As emphasized by RAHMAN *et al.* (19), the relationship between protozoa and bacteria is extremely complex. COLEMAN (5) has suggested that bacterial control rather than elimination is desirable for protozoal maintenance. In most of the cultures in this study bacteria grew to fairly high levels even when antibiotics were present, which may have contributed to the good growth of the protozoa. When bacterial growth was exceedingly vigorous, the ciliates decreased in number and shortly died out. On the contrary, when bacterial growth was extremely poor for some reason, protozoal growth was limited. Studies on the relationship of bacteria to entodinia will be reported elsewhere.

MAH (13) reported on the ingestion of chlorophyll by *Ophryoscolex*, and stimulation of protozoal development by green plants was early noted by TRIER (20). QUINN *et al.* (9) reported that the addition of chlorophyllin or alfalfa chloroplasts seemed to stimulate the feeding activity of *Diplodinium* and entodinia. However, the present experiment indicates that copper-chlorophyllin has no stimulatory effect on entodinal growth, although the effect of chlorophyll was not examined.

Success in maintaining entodinia at high population densities was obtained possibly by virtue of the following factors: (a) Physico-chemical conditions such as pH, Eh, and osmotic pressure were comparatively unchanged throughout the experiment, (b) metabolic products toxic to the protozoa were diluted, (c) since low molecular substances could move in or out through the Cellophane, nutrients were available to the microorganisms over a longer period of time, and sudden growth of bacteria was suppressed, and (d) cultures were continuously stirred at an appropriate speed.

Morphological variation in the culture of rumen protozoa has frequently been reported. For example, the change or loss of caudal spination has been observed in *Entodinium caudatum* (2) and *Epidinium* (21, 22). Since caudal spines are thus variable, spination is indeed a poor taxonomic character, as pointed out by HUNGATE (23).

Entodinia failed to grow without white clover. The attempt to cultivate the organisms for extended periods in the chemically defined medium formulated by QUINN *et al.* (9) was unsuccessful. The investigation as to what components of white clover stimulate the growth will be reported in the near future.

REFERENCES

- 1) M. KNOTH, *Z. Parasitenk.*, **1**, 262 (1928).
- 2) R.E. HUNGATE, *Biol. Bull. Woods Hole*, **84**, 157 (1943).
- 3) M. KANDATSU and N. TAKAHASHI, *J. Agr. Chem. Soc. Japan*, **30**, 96 (1956), in Japanese.
- 4) G.S. COLEMAN, *Nature*, **182**, 1104 (1958).
- 5) G.S. COLEMAN, *J. Gen. Microbiol.*, **22**, 555 (1960).
- 6) G.S. COLEMAN, *J. Gen. Microbiol.*, **57**, 81 (1969).
- 7) B.D.W. JARVIS and R.E. HUNGATE, *Appl. Microbiol.*, **16**, 1044 (1968).
- 8) T. HINO, M. KAMETAKA, and M. KANDATSU, *J. Gen. Appl. Microbiol.*, **19**, 305 (1973).
- 9) L.Y. QUINN, W. BURROUGHS, and W.C. CHRISTIANSEN, *Appl. Microbiol.*, **10**, 583 (1962).
- 10) R.E. HUNGATE, *The Rumen and Its Microbes*, Academic Press, New York (1966), p. 143.
- 11) A.C.I. WARNER, *J. Gen. Microbiol.*, **28**, 129 (1962).
- 12) R.E. HUNGATE, *Biol. Bull., Woods Hole*, **83**, 303 (1942).
- 13) R.A. MAH, *J. Protozool.*, **11**, 546 (1964).
- 14) R.T.J. CLARKE and R.E. HUNGATE, *Appl. Microbiol.*, **14**, 340 (1966).
- 15) J. GUTIERREZ, *J. Protozool.*, **6**, Suppl., 21 (1959).
- 16) A.C.I. WARNER, *J. Gen. Microbiol.*, **28**, 119 (1962).
- 17) R. ONODERA and M. KANDATSU, *Jap. J. Zootech. Sci.*, **41**, 14 (1970), in Japanese.
- 18) B. SUGDEN, *J. Gen. Microbiol.*, **9**, 44 (1953).
- 19) S.A. RAHMAN, D.B. PURSER, and W.J. TYZNIK, *J. Protozool.*, **11**, 51 (1964).
- 20) H.J. TRIER, *Z. Vergleich. Physiol.*, **4**, 305 (1926).
- 21) J. GUTIERREZ and R.E. DAVIS, *Appl. Microbiol.*, **10**, 305 (1962).
- 22) R.T.J. CLARKE, *J. Gen. Microbiol.*, **33**, 401 (1963).
- 23) R.E. HUNGATE, *The Rumen and Its Microbes*, Academic Press, New York (1966), p. 99.