Culture and Metabolism of the Rumen Ciliate *Epidinium ecaudatum* Crawley

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ABSTRACT

Gutierrez, J. (Agricultural Research Service, Beltsville, Md.) and R. E. Davis. Culture and metabolism of the rumen ciliate *Epidinium ecaudatum* Crawley. Appl. Microbiol. **10**:305–308. 1962.—The rumen ciliate *Epidinium ecaudatum* was cultured for 6 months in the presence of two strains of bacteria, starch, alfalfa, linseed oil meal, and buffered saline. The cultures required daily transfer and addition of fresh substrate. The protozoan degraded starch, soybean oil meal, linseed oil meal, and cottonseed oil meal, and the fermentation end products from the breakdown of starch were acetic and butyric acids with traces of formic, propionic, and lactic acids, carbon dioxide, and hydrogen. The relationship of *E. ecaudatum* to other species of rumen oligotrichs and bacteria is discussed.

Within recent years the ciliates inhabiting the rumen have been shown to contribute to that steady supply of fermentation acids which are absorbed and used as an energy source by the ruminant. The protozoa are useful also as a source of digestible proteins and as an aid in the breakdown of ingested substrates such as starch, cellulose, and plant proteins. Although the population of protozoa in cattle and sheep varies considerably, counts of one million per ml are not unusual. Preliminary observations have indicated that different species of protozoa, besides taking in plant particles, also ingest bacteria as a food source. None of the rumen ciliates has yet been cultured axenically, although the oligotrich *Epidinium ecaudatum* has been cultured in a medium with known bacteria, starch, and ground alfalfa.

Epidinium is a complex rumen ciliate which at times occurs in large populations in ruminants which are fed high starch diets. Sharp (1914) described the neuromotor apparatus in the ciliate, and published his material using the name *Diplodinium ecaudatum* for the protozoan. He recognized that the forms with and without caudal spines belonged to the same species. Bailey (1958) has recently shown that *E. ecaudatum* possesses significant amylase activity, and Oxford (1958) demonstrated that *Epidinium* swallows small clover-starch grains and has a requirement for CO_2. In a recent attempt to establish the interrelation between bacterial associates and *Entodinium* and *Diplodinium* (Gutierrez and Davis, 1959), it was shown that bacterial feeding by protozoa was a common occurrence. In an extension of that finding, growth studies with *E. ecaudatum*, using isolated strains of bacteria, have yielded some preliminary information. This is a report of culture experiments and substrate utilization by *Epidinium*. A part of this work has been published in abstract (Gutierrez, 1959).

Materials and Methods

Rumen samples were removed from an animal which harbored *Epidinium* as the only large oligotrich. The ciliates were collected by sedimentation in 100-ml test tubes and washed free of debris and smaller protozoa, using inorganic salts which contained 0.6% NaCl, 0.01% $CaCl_2$, 0.01% $MgSO_4$, 0.1% KH_2PO_4, and 0.1% $NaHCO_3$. A gas phase of 5% CO_2 in nitrogen was used throughout the experiments. The washed suspension of *Epidinium* was then ground in a tissue homogenizer, and the contents of the ruptured cells were inoculated into a series of tubes containing starch feed extract medium (Gutierrez, 1958). In a parallel control series of tubes, an equal amount of the last-washing liquid adjacent to the protozoa but containing no ciliates was also inoculated. The isotopic techniques used with the C^{14}-labeled amino acids and *Epidinium* suspensions were similar to those previously described (Williams et al., 1961).

Results

After 24 hr of incubation, the series of agar tubes inoculated with the crushed protozoa debris showed more colonies than the control series, and the strains of bacteria isolated in this manner were gram-positive diplococcus, resembling *Streptococcus bovis*, and a gram-negative rod. The bacterial isolates were then tested for their capacity to stimulate the growth of *E. ecaudatum*. Protozoa washed free of debris and external bacteria were used for the culture experiments, and were provided strains of lactic acid-producing *S. bovis* originally isolated from *Epidinium*. To lessen the amount of lactic acid produced by the food organisms, the daily addition to the cultures of 0.01 ml of a standardized suspension of the lactic acid-utilizing *Peptostreptococcus elsdenii* (Gutierrez et al., 1959) reduced the level of the lactic acid present and aided in maintaining the pH of the cultures between 6.5 and 6.8. The latter organism is a large gram-negative coccus with strong hydrogenase activity, and its use also permitted the

omission of reducing agents such as cysteine hydrochloried from the medium.

Usually, 80 to 100 washed epidinia were inoculated into 5 ml of the culture medium, which contained (in per cent): rice starch, 0.02; alfalfa, 0.003, and linseed oil meal, 0.002. The 5-ml culture flasks supported peak populations of 5,000 to 6,000 protozoa. Figure 1 is a photograph of the culture taken after 3 months of in vitro cultivation. The cultures were maintained in the laboratory for 6 months, and had to be transferred and given fresh substrate twice daily. Transfer was accomplished by replacing half of the old medium with fresh salts solution and adding fresh substrate. The starch and alfalfa must be kept at a low level to limit the growth of the bacteria. After initial inoculation of the cultures with the rapidly growing *S. bovis*, no further addition was made to the cultures. A check of the bacterial population in the protozoan cultures at 3 months demonstrated that *S. bovis* was able to maintain itself in numbers of approximately 500,000/ml of culture fluid. Attempts to grow the protozoa without living bacteria have as yet been unsuccessful.

The effect of different antibiotics on the cultures of *Epidinium* was tested to find antibiotics which would be nontoxic to the protozoa. Chloramphenicol from a fresh solution was added daily to the cultures at a level of 60 μg/ml. A check of the bacteria in the protozoan cultures at 24 hr demonstrated that the bacteria survived the antibiotic treatment, and a cultural count using starch feed extract agar medium gave a count of 6.5×10^6 per ml of protozoan culture fluid. A combination of procaine penicillin G and dihydrostreptomycin sulfate at a level of 20 μg/ml in the cultures also had no deleterious effect on *Epidinium*. The protozoan cultures were healthy and showed numerous dividing cells. Neomycin sulfate at a final concentration 10 μg/ml, and polymyxin B sulfate at a level of 20 μg/ml, had no toxic effect on the protozoa in incubation experiments of short duration (24 hr).

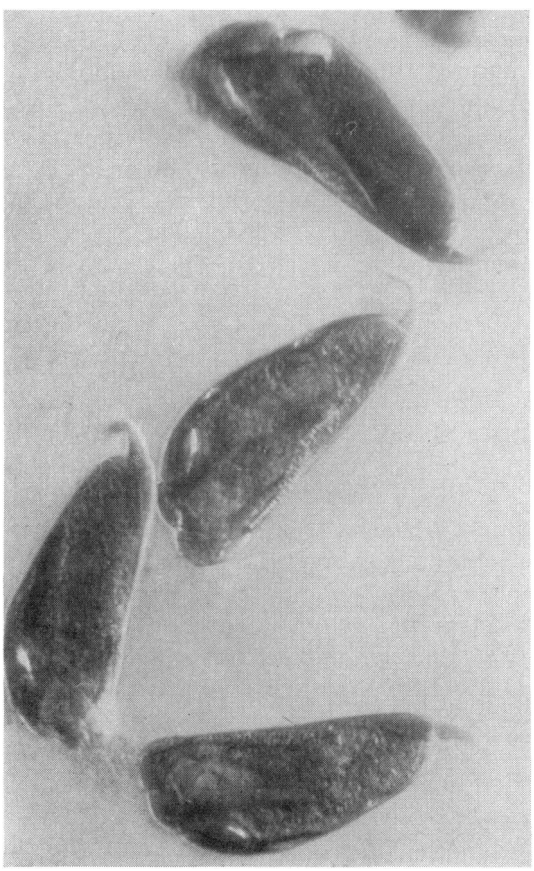

FIG. 1. *Epidinium ecaudatum stained with methylene blue from a 3-month-old culture. Bright field,* 150 ×.

Large suspensions of *Epidinium* obtained directly from an animal with a large population of the ciliate were washed and used in manometric experiments to determine acid and gas production from starch and plant protein. Penicillin and streptomycin sulfate at a concentration of 1 mg/ml were added to the protozoan suspension to eliminate any activity from the bacteria which might have survived the washing procedures. As an additional safeguard against bacterial activity, gas production was measured in a control vessel containing the supernatant fluid free of protozoa. The gas evolved in these control vessels was always negligible.

In the various experiments, 58,000 to 92,000 organisms were suspended in 2 ml of 0.1% sodium bicarbonate-0.6% saline buffer in each manometer cup; 10 mg of rice starch were added from the side arm of the vessel after initial equilibration. In one of the vessels, 20 mg of palladium black were used in the center well to absorb any hydrogen evolved. The gas production from starch utilization by *Epidinium* is illustrated in Fig. 2. Over half of the gas produced from the utilization of starch by *Epidinium* was hydrogen; the remainder was CO_2 produced in metabolism plus CO_2 liberated from the sodium bicarbonate by acid production of the protozoa.

The crude protein-containing substrates, soybean oil meal, cottonseed oil meal, and linseed oil meal were tested

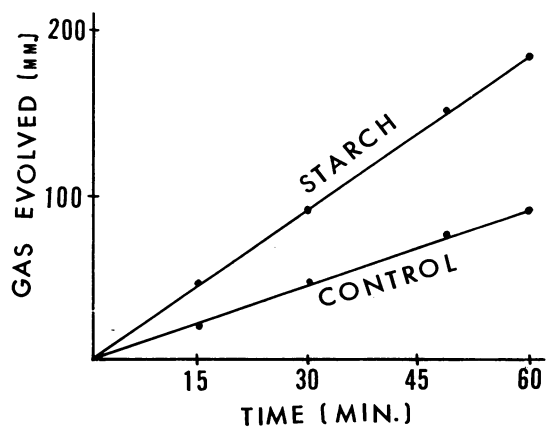

FIG. 2. *Degradation of rice starch by suspensions of Epidinium ecaudatum. Each manometer cup contained washed protozoa suspended in sodium bicarbonate-saline buffer.*

for utilization by *Epidinium* in Warburg respiration experiments. The results of the gas production from the degradation of the three protein sources are shown in Fig. 3. Microscopy showed that the protozoa ingested the plant proteins to a considerable extent. Carbon^{14}-labeled amino acids were also metabolized by washed suspensions of *Epidinium* in short-term (90 min) experiments. The total radioactivity of the protozoa incubated with labeled amino acids is given in Table 1.

To identify the soluble fermentation acids produced from starch by *Epidinium*, suspensions of the ciliates were washed free of debris, and the final harvest of organisms was divided into two equal amounts after the addition of 0.5 mg/ml each of dihydrostreptomycin sulfate and penicillin G. The control culture was killed at the beginning of the experiment with 2 ml of 5 N sulfuric acid, and the second culture was allowed to ferment 100 mg of rice starch for 5 hr before the fermentation was stopped with a similar amount of acid. Butyric acid (0.24 meq), acetic acid (0.09 meq), and traces of formic, propionic, and lactic acid were produced by *Epidinium*, and CO_2 and H_2 production were identified in the Warburg apparatus. In the amounts of fermentation acids formed, *Epidinium* thus differs from the rumen holotrichs, *Isotricha* and *Dasytricha*, which produce mainly lactic acid and lesser amounts of acetic and butyric acids.

TABLE 1. *C^{14}-labeled amino acids concentrated by Epidinium ecaudatum*

Amino acid	Radioactivity*		
	Initial culture	Cells	Supernatant†
DL-Leucine-2-C^{14}	2,052	200	65
DL-Valine-4-C^{14}	1,147	393	92
DL-Alanine-2-C^{14}	2,044	424	84

* Count per min per 100 μg.

† Activity of the supernatant fluid free of protozoa. The number of protozoa contained in 100 μg was approximately 10,000.

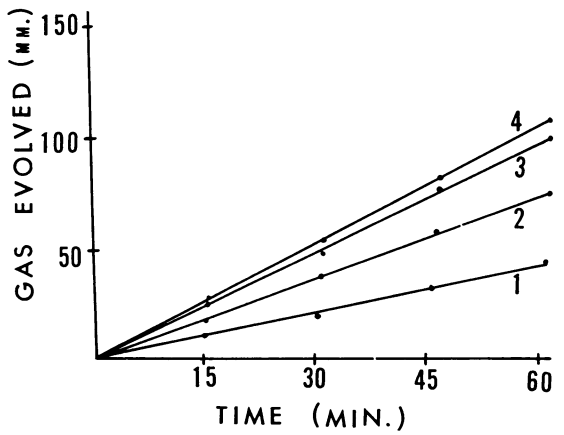

FIG. 3. *Gas production from the breakdown of various substrates by Epidinium ecaudatum. Curves: endogenous (1), cottonseed oil meal (2), soybean oil meal (3), linseed oil meal (4).*

Discussion

Among the cattle ciliates, *E. ecaudatum* appears to be closely related to the genus *Ophryoscolex* in physiological and morphological characteristics. Both genera can readily ingest and break down starch and proteinaceous materials such as soybean oil meal and cottonseed oil meal, but can metabolize soluble carbohydrate such as glucose slowly or not at all (Williams et al., 1961; Oxford, 1958). In both genera, acetic and butyric acids are the major end products of carbohydrate metabolism, with carbon dioxide and hydrogen as the gaseous end products. In cultures, *Epidinium* at times had shortened or caudal spines entirely absent. Oxford (1958) reported organisms with single or multiple tails in the rumen ingesta of cattle. Thus, some aspects of the morphology of *Epidinium* resemble *Ophryoscolex*.

The role of bacteria in in vitro cultures is not entirely clear. Any attempt to free the cultures from bacteria, either by washing techniques or antibiotic treatment, resulted in the gradual decline of the population and the eventual death of the culture. Bacterial feeding has been shown to occur in the genera *Diplodinium*, *Entodinium*, and *Isotricha* (Gutierrez, 1958; Gutierrez and Davis, 1959), and it is probable that bacterial ingestion also takes place in *Epidinium*. Granules of bacterial size have been observed within cells of *Epidinium*, but it is difficult to distinguish between bacteria and reserve polysaccharide granules of the protozoa. Washed *Epidinium* have yielded bacterial colonies when inoculated into agar medium (Oxford, 1958). The bacteria may be beneficial to the protozoa by providing a low oxidation-reduction potential in the cultures; *P. elsdenii* has strong hydrogenase activity (Elsden et al., 1956). Individual epidinia removed from culture flasks often had particles of linseed oil meal visible within the cell. The ingestion of the yellow-hued linseed particles was also easily seen microscopically in washed suspensions of the protozoan which had been provided that substrate in Warburg respiration experiments. The breakdown of proteinaceous plant materials demonstrated by the respiration studies with *Epidinium* indicated findings similar to results obtained in protein degradation experiments with other oligotrichs such as *Ophryoscolex* and *Diplodinium*. The incorporation of C^{14}-labeled amino acids by *Epidinium* also is a property which has been demonstrated for the rumen protozoan *O. caudatus*. The oligotrich species of rumen ciliates help convert plant protein to animal protein in the ruminant.

E. caudatum is one of the hardiest of the rumen protozoa, and lends itself readily to in vitro culture. One of the factors which affected the health of the protozoan cultures to a considerable extent was the number of bacteria in the flasks, but the bacterial population can be controlled by limiting the amount of substrate provided and by the use of suitable antibiotics. Studies of the physiology of rumen ciliates such as *E. ecaudatum* may

reveal more about how they affect the nutrition of the host.

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