

# Physiological Studies of the Rumen Protozoan *Ophryoscolex caudatus* Eberlein<sup>1</sup>

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## ABSTRACT

WILLIAMS, P. P. (U. S. Department of Agriculture, Beltsville, Md.), R. E. DAVIS, R. N. DOETSCH, AND J. GUTIERREZ. Physiological studies of the rumen protozoan *Ophryoscolex caudatus* Eberlein. Appl. Microbiol. 9:405-409. 1961.—The rumen ciliate *Ophryoscolex caudatus* fermented starch with the production of acetic, butyric, and lactic acids plus CO<sub>2</sub> and H<sub>2</sub>. Cellulose was not significantly metabolized although pectin was rapidly attacked in the Warburg apparatus. The protein sources, cottonseed, soybean, and linseed oil meals, and the amino acids, DL-alanine, DL-valine, and DL-leucine, were utilized by the protozoan, whereas ammonia was demonstrated as an end product of nitrogenous metabolism. Methods for the separation of *O. caudatus* from mixed rumen contents are described.

*Ophryoscolex caudatus* Eberlein is one of the large rumen ciliates which occasionally occurs in high numbers when animals are fed diets which are rich in starch. The physiological functions of the protozoan are of some interest both from the standpoint of cellular metabolism and because of their effect on the host. *O. caudatus* does not usually swallow cellulose materials as does *Diplodinium*, but will ingest starch grains (Hungate, 1955). Little is known of other carbohydrate or nitrogenous nutrients utilized by the protozoan; ammonia production by mixed species of rumen protozoa has been suggested by the work of Warner (1956), and Blackburn and Hobson (1960) have attributed some of the rumen proteolytic activity to the protozoa. The purpose of the present investigation was to gain some knowledge of the nutritional habits of *O. caudatus*, along with information of fatty acid and nitrogenous excretory products.

## MATERIALS AND METHODS

The inorganic salt solution used in the physiological studies of *O. caudatus* contained (w/v): NaCl, 0.5%; MgSO<sub>4</sub>, 0.005%; KH<sub>2</sub>PO<sub>4</sub>, 0.1%; CaCl<sub>2</sub>, 0.005%; and

NaHCO<sub>3</sub>, 0.1%. The salts, except sodium bicarbonate, were dissolved in distilled water and sterilized by Seitz filtration. The sodium bicarbonate was autoclaved separately in a 10% solution and added to the filtered salt solution while the liquid was bubbled with 95% N<sub>2</sub> and 5% CO<sub>2</sub> gas for 15 min at a temperature of 39 C. The salt solution had a pH of 6.9.

*O. caudatus* in counts of approximately 4,000 per ml in rumen samples, removed by stomach tube from a steer 2 hr after the morning feeding, were collected in 1-liter Erlenmeyer flasks. The steer's diet consisted of alfalfa hay, 22%; soybean oil meal, 16%; barley, 61%; and salt, 1%. The rumen liquor was kept in a water bath at 39 C while the mixed protozoan population settled. Fifty milliliters of the sediment were placed in 25 ml of inorganic salt solution in a large rubber-stoppered test tube and flushed with 95% N<sub>2</sub> and 5% CO<sub>2</sub> (Stone and Beeson, 1936), and stored at 39 C until used. The plant debris that floated to the top of the protozoan suspension was removed by pipette and additional salt solution was added to the tube and flushed with 5% CO<sub>2</sub> to maintain anaerobic conditions. The protozoa were allowed to settle out for approximately 5 min and were then examined microscopically. *Entodinium*, *Dasytricha*, and *Isotricha* were observed for the most part in the supernatant; *Ophryoscolex* and a few *Diplodinium* species, which were more dense than either *Entodinium* or *Dasytricha*, and sluggishly motile as compared to species of *Isotricha*, were found primarily in the grayish-white sediment in the bottom of the tube.

To obtain pure samples of *O. caudatus* for experimentation, the following techniques of column sedimentation were employed: (i) the lighter and more active protozoa were drawn off in the supernatant after the protozoa were allowed to settle for about 5 min. The supernatant was replaced with an equal amount of inorganic salt solution. Forms removed by this process included *Dasytricha*, *Entodinium*, and *Isotricha*; (ii) the remaining sediment was resuspended in 50 ml inorganic salt solution. The large *Diplodinium* settled out more rapidly than the *Ophryoscolex*, and removal of the early sediment eliminated this species. The remaining sediment was microscopically exam-

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ined for purity before (iii) dispensing the suspension into a long vertical glass column (dimensions: 146 cm long, 1.7 cm diameter, 270 ml volume) filled with inorganic salt solution. *O. caudatus* settled out in the bottom, and any *Entodinium* and *Dasytricha* remained in the upper portion of the column. The collected protozoa were placed in salt solution containing 1 mg per ml each of dihydrostreptomycin sulfate and procaine penicillin G. One-half milliliter of the protozoan sediment from the column in 12 ml of the antibiotic salt solution gave a protozoan count of approximately 4,000 per ml. Accurate counts were made by bubbling and protozoan suspension rapidly with a 0.1-ml pipette by exhaling through it and then sucking a 0.1-ml aliquot rapidly. Aliquots of 0.01 ml were then counted, averaged, and the total count determined per ml. Methylene blue in 50% (v/v) ethanol was used to stain wet mounts for morphological observations and the oligotrich ciliate was identified using the keys of Dogiel (1927) and Becker and Talbott (1927).

To check the purity of the protozoan suspensions which were used in the metabolism studies, Gram stain smears were made of the washed protozoan suspensions and microscopic observation showed only a few scattered bacterial cells. As an additional precaution against bacterial activity in the Warburg experiments, a control vessel, containing the antibiotic-treated supernatant fluid free of protozoa, was used to measure the amount of gas evolved by any contaminating bacteria. The amount of gas produced by this final washing fluid was always negligible.

Respiration studies using the Warburg apparatus were made with 2 ml of the anaerobic salt solution containing 10,000 to 12,000 *O. caudatus* and 1 mg of dihydrostreptomycin sulfate and procaine penicillin G per ml. From 8 to 10 mg of the particular substrate in 0.2 ml salt solution were provided from the vessel side arm. Metabolic activity was determined by comparison with an endogenous vessel and hydrogen production was measured by exposing the gas to palladium in the center well of the Warburg vessel, while the temperature for all the experiments was 39 C and the gas phase 95% N<sub>2</sub> and 5% CO<sub>2</sub>.

In isotopic tracer experiments to determine substrate utilization, *O. caudatus* were collected and purified by column sedimentation as previously described, and suspended in 5 ml of buffered inorganic salt solution. Cell concentrations ranged from 3,000 to 5,000 organisms per ml. To the suspension was added 0.5  $\mu$ c of the radioactive substrate which was to be tested. A 100- $\mu$ l sample of this original culture was removed and placed on a planchet for counting. The protozoa were then incubated for 1 hr at 39 C and 5% CO<sub>2</sub> and 95% N<sub>2</sub> was used to maintain anaerobic conditions.

After incubation, the cells were washed twice in 10

ml of tap water to remove most of the extracellular radioactivity, and 100  $\mu$ l of the washed cells were placed on a planchet; for a control a planchet was prepared containing a 100- $\mu$ l sample of the final wash supernatant free of protozoa. The planchets were dried under an infrared lamp and 5-min counts were made for each sample using a gas flow counter and scaler; samples were plated at less than 0.5 mg per cm<sup>2</sup> to reduce self-absorption. Background counts were taken also on a 5-min basis, and all counts were recorded as average counts per min. Substrates<sup>2</sup> tested included starch-U-C<sup>14</sup> (specific activity 15.2 mc/mm), DL-leucine-2-C<sup>14</sup> (specific activity 0.05 mc/mm), DL-valine-4-C<sup>14</sup> (specific activity 3.0 mc/mm), and DL-alanine-2-C<sup>14</sup> (specific activity 2.64 mc/mm).

Ammonia production by *O. caudatus* was determined by the Conway (1950) technique. Optical density readings were made on a Beckman quartz spectrophotometer model DU<sup>3</sup> set at 490 m $\mu$  and compared to a standard curve in which (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> was used as a source of NH<sub>3</sub>. Nessler's solution was prepared as described by Umbreit, Burris, and Stauffer (1957).

## RESULTS

*Identification of the protozoa.* The protozoan dimensions were approximately 200 by 80  $\mu$ , depending on the length of time after cell division. The cell was ovoid and terminated posteriorly in a single spine which was longer and broader than the other pre-anal spines. The mouth was observed to be surrounded by an adoral zone of membranelles and a dorsal zone of membranelles covered four-fifths of the circumference of a zone somewhat anterior to the middle of the body. The protozoan was identified as *O. caudatus* Eberlein (Fig. 1).

<sup>2</sup> Supplied by California Corporation for Biochemical Research, Los Angeles, Calif.

<sup>3</sup> Becton Instruments, Inc., Fullerton, Calif.

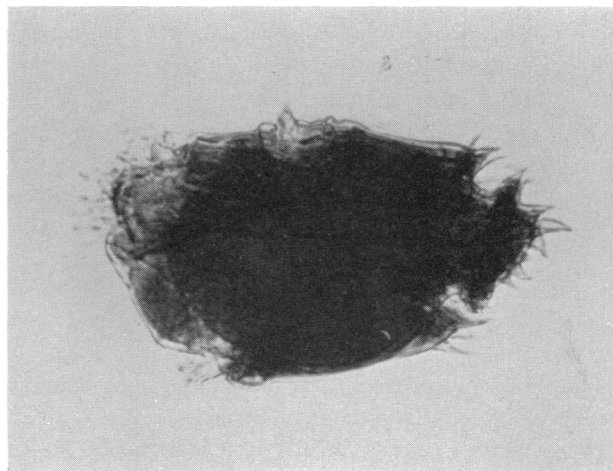


FIG. 1. *Ophryoscolex caudatus* stained with methylene blue. Bright field. Magnification, 343 X.

*Substrates utilized by O. caudatus.* Preliminary Warburg apparatus studies indicated rapid utilization of washed, finely ground rice starch with the production of H<sub>2</sub> and CO<sub>2</sub>. The gas pressure in millimeters Brodie's solution is plotted against time in Fig. 2. The endogenous line in Fig. 2 refers to protozoa exposed to 0.2 ml of inorganic salt solution minus substrate. The adsorption of H<sub>2</sub> with palladium is illustrated by the reduction from 215 mm pressure on the CO<sub>2</sub> and H<sub>2</sub> curve to the 80 mm pressure noted on the CO<sub>2</sub> curve.

The crude substrates, cottonseed oil meal, soybean oil meal, and linseed oil meal, were utilized in Warburg experiments, and gave a total of 225 mm pressure as compared to approximately 150 mm pressure in the endogenous vessel and manometer. The protozoa at the end of the run were filled with the substrates and actively motile. *O. caudatus* did not produce significant amounts of gas from the polysaccharide cellulose

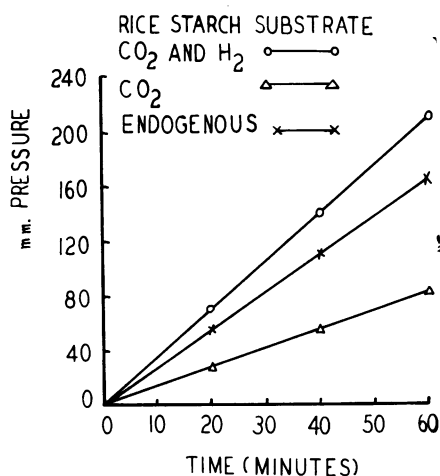


FIG. 2. Gas production from the utilization of rice starch in Warburg experiments by *Ophryoscolex caudatus*. The protozoa were suspended in 2 ml of 0.5% saline buffered with 0.1% NaHCO<sub>3</sub> and the gas phase was 5% CO<sub>2</sub> and 95% N<sub>2</sub>.

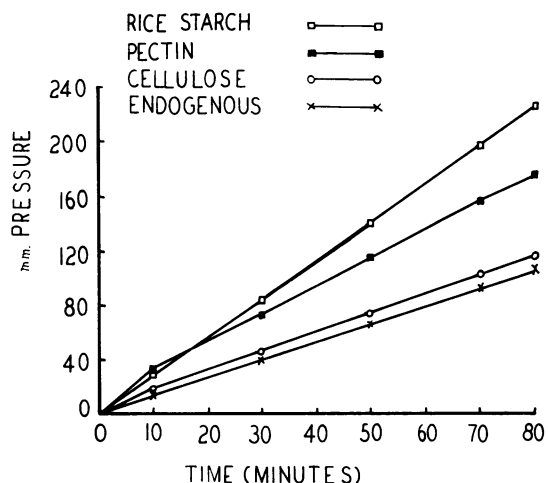


FIG. 3. Comparison of the gas production from the degradation of rice starch, pectin, and cellulose by *Ophryoscolex caudatus*.

which followed the endogenous curve closely (Fig. 3). The cellulose curve was also representative of that obtained with the soluble carbohydrates: maltose, glucose, galactose, cellobiose, and sucrose. However, the colloidal carbohydrate pectin (previously neutralized with 0.09 N NaOH) was rapidly attacked and gave a pressure increase of approximately 180 mm in 80 min compared to approximately 100 mm pressure in the endogenous vessel.

Since the crude protein sources, cottonseed, soybean, and linseed oil meal, were readily utilized by *O. caudatus*, tracer amino acids were employed to determine if the protozoa could attack a soluble, chemically defined nitrogen source. One-hour incubation periods with C<sup>14</sup>-labeled amino acids showed that *O. caudatus* readily concentrated D-leucine, DL-alanine, and DL-valine within the cells. Starch-U-C<sup>14</sup> was also incorporated in the protozoa. Table 1 gives the magnitude of the radioactivity found in the protozoa in the experiments.

*Metabolic end products.* To determine the fermentation acids of *O. caudatus*, the organisms were collected and purified as described earlier, and placed in two large test tubes containing 70 ml of previously gassed salt solution. To both tubes were added 100 mg washed rice starch and 40 mg each of dihydrostreptomycin sulfate and procaine penicillin G. In one tube, the organisms were killed at the beginning of the experiment by addition of 2 ml of 5 N H<sub>2</sub>SO<sub>4</sub>; the second tube was allowed to ferment the starch for 5 hr at 39 C, at which time the culture was killed by adding 2 ml of 5 N H<sub>2</sub>SO<sub>4</sub>. A count of the number of cells in the experimental and control tubes averaged 3,200 cells per ml in 77 ml total volume.

The contents of the experimental and control tubes were steam distilled and the distillate neutralized by titration with 0.09 N NaOH. The neutralized distillates were evaporated to dryness and used for chromatographic analysis of the volatile acids. The liquid residue from the steam distillation was used for the analysis of the nonvolatile acids. The volatile acids (0.15 meq) were chromatographed on paper in 20- $\mu$ l amounts opposite a spot of mixed known fatty acids, formic,

TABLE 1. Incorporation of labeled amino acids and starch by *Ophryoscolex caudatus*

Substrate	Radioactivity in counts/min/100 $\mu$ l		
	Initial culture	Cells	Supernatant*
DL-Valine-4-C <sup>14</sup>	12,146	1,074	38
DL-Leucine-2-C <sup>14</sup>	6,171	1,798	170
DL-Leucine-2-C <sup>14</sup>	6,790	813	18
Starch-U-C <sup>14</sup>	6,822	207	21

\* Radioactivity of the final wash water in which the protozoa were rinsed. The number of cells contained in 100  $\mu$ l of the buffer solution was approximately 16,000.

acetic, propionic, and butyric, in their sodium salts. The acids were chromatographed ascending in 1% ethylamine-butanol and the paper was treated with 0.04% bromocresol green in ethanol, giving blue spots on a yellow background (Block, Durrum, and Zweig, 1955). The chromatographed volatile portion indicated the presence of two of the lower fatty acids, acetic and butyric acids. The occurrence of lactic acid as a fermentation product of starch-fed *O. caudatus* was determined from the nonvolatile portion of the culture. Only small traces (less than 0.09 meq) of the acid were encountered when analyzed by methods previously described (Gutierrez, 1955).

For the detection of ammonia production, 3 ml of a suspension of *O. caudatus* (3,000 per ml), which had been deprived of substrate for 4 hr in an antibiotic salt solution, were dispensed in test tubes flushed with 5% CO<sub>2</sub> and 95% N<sub>2</sub>. Duplicate tubes, containing 10 mg casein, 10 mg starch, and an endogenous set, were prepared. All the tubes were then incubated at 39 C for 2 hr and 2 ml of the protozoan suspension from each tube were dispensed in the outer well of the Conway dish and analyzed for ammonia by the method of Conway (1950). Experiments with *O. caudatus* revealed that 0.8 μmoles of NH<sub>3</sub> were released from 6,000 protozoa exposed to 6.6 mg of casein after 2 hr incubation at 39 C. With the same experimental conditions, only 0.1 μmole NH<sub>3</sub> was produced by the protozoa from 6.6 mg of starch.

#### DISCUSSION

The rapid breakdown of starch by *O. caudatus* indicates that the protozoa may contribute significantly to the digestion of the polysaccharide in the rumen. As was shown in the current experiments not only was the starch ingested by the organism, but the digestion was accompanied by the production of acetic, butyric, and lactic acids, CO<sub>2</sub>, and H<sub>2</sub>. An additional product from the breakdown of casein was ammonia. The quantitative experiments on ammonia production from casein by known numbers of *O. caudatus* show that at least for some of the ruminal protozoa this can be an important end product, and the protozoa probably are a factor in the nitrogenous metabolism of ruminants.

The possession of proteolytic properties by *O. caudatus*, which was demonstrated in the casein and soybean oil meal experiments, indicates that the protozoa are agents which function in the breakdown of ingested food proteins. Ingestion and disintegration of bacterial cells by the protozoa has been demonstrated also (Gutierrez and Davis, 1959; Gutierrez, 1958). A proteinase for the oligotrichs was suggested by Schlottke as early as 1936, and Blackburn and Hobson (1960) have reported the ingestion of stained casein particles with

their subsequent disappearance in some species of oligotrich protozoa. Amino acids can also be assimilated to some extent by *O. caudatus* as was demonstrated in the tracer experiments; a similar phenomenon has been shown for the ciliate *Colpidium campylum* (Hall and Elliott, 1935). Thus the protozoan competes with some species of ruminal bacteria which are capable of attacking amino acids. Bladen, Bryant, and Doetsch (1961) have found the ruminal bacterial species *Bacteroides ruminicola* can break down DL-serine, L-aspartic acid, and L-cysteine with ammonia production, and Dehority et al. (1958) have reported C<sup>14</sup>-labeled valine and proline were metabolized by mixed rumen contents. The amino acid composition of the proteins in the ration probably influences the rate of ammonia production in the rumen since some amino acids are preferentially utilized by the protozoa and bacteria (Lewis and Elsdon, 1955).

*O. caudatus* differs markedly from species of the holotrich group which includes *Isotricha prostoma*, *I. intestinalis*, and *Dasytricha ruminantium*. The latter three species have been shown to rapidly attack soluble carbohydrates such as glucose, fructose, and sucrose with the production of cellular amylopectin, fatty acids, and gases (Heald and Oxford, 1953; Gutierrez, 1955). In the current experiments with *O. caudatus*, glucose and maltose did not give significant gas production in the Warburg apparatus, although the possibility exists that these carbohydrates may be utilized slowly. Manometric experiments with *Entodinium* have shown glucose is not utilized by the oligotrich protozoan (Abou Akkada, Hobson, and Howard, 1959). This points out one of the major differences in the biochemical properties between the oligotrich and holotrich ruminal protozoa, that is the rapid utilization of soluble carbohydrates by the latter group which is not shared by the former.

The major fatty acid end products of starch fermentation in washed suspensions of *O. caudatus* incubated with antibiotics were acetic and butyric acids with traces of lactic acid. The oligotrich protozoan thus differs from the holotrichs *Isotricha* and *Dasytricha* since species of these two genera produce mainly lactic acid plus acetic and butyric acids from the fermentation of glucose (Heald and Oxford, 1953; Gutierrez, 1955). Abou Akkada et al. (1959) have analyzed the end products of starch digestion by the small oligotrich *Entodinium*; the mixture of acids found was formic, acetic, propionic, and butyric plus small amounts of lactic acid. In the ruminal oligotrich protozoa, lactic acid is not an important acidic end product, and this characteristic has aided in making the oligotrichs amenable to culture in vitro (Hungate, 1942; Coleman, 1960). A pH near neutrality was shown to be an important factor in the culture of *Diplodinium*

*neglectum* (Hungate, 1942); culture of holotrich species has been possible only for short periods (Gutierrez, 1955).

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