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Cellulose Metabolism by the Termite Flagellate *Trichomitopsis termopsidis*

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The end products of cellulose metabolism by the trichomonad flagellate *Trichomitopsis termopsidis* from the termite *Zootermopsis* sp. were investigated by growing axenic flagellates on [^{14}C]cellulose. The growth of *T. termopsidis* resulted in the release of label into the supernatant fraction of the culture fluid, and >75% was volatile under acid conditions. The label was analyzed for $^{14}CO_2$ and for [^{14}C]volatile compounds by vacuum distillation under acid and alkaline conditions in disposable micro-distillation vessels. The distillate and undistilled culture supernatant fluid were chromatographed on cellulose thin layers to identify the labeled end product. *T. termopsidis* produced $^{14}CO_2$ and [^{14}C]acetate which accounted for 25 to 30% and 55 to 60% of the labeled end products, respectively. The ratio of label in CO_2 to acetate suggests that they are produced in equimolar amounts. No neutral volatile compounds were produced. The remaining unidentified end product (10 to 20%) was not volatile nor extractable into ether. Hydrogen was produced by *T. termopsidis*, and the cells were killed by the drug metronidazole. Enzymatic activities were found which account for these end products: pyruvate:ferredoxin oxidoreductase and hydrogenase. The results indicate that acetate is the end product of *T. termopsidis* cellulose metabolism and is available to the termite for energy metabolism and biosynthesis.

Lower termites depend on their intestinal flagellates, which digest cellulose to end products that the termite absorbs and metabolizes. Over 400 species of termite flagellates have been reported (22), but little is known about the individual species other than their ultrastructure and that many species ingest wood particles. Termite flagellates are killed by oxygen (4, 10, 20) and by the drug metronidazole (24), and they lack mitochondria (6) but possess membrane-bounded organelles (3, 6, 21) similar in ultrastructure to hydrogenosomes (8, 9). Acetate, CO_2, and H_2 were identified by Hungate (10) as end products of cellulose fermentation by mixed flagellates (mainly *Trichonympha* spp.) from the termite *Zootermopsis* sp. The termite flagellates therefore share characteristics of other trichomonads which have been studied in more detail (8, 9).

The termite intestine is a complex habitat where protozoa coexist with bacteria including spirochetes, and no termite flagellate species has been studied in the absence of other living organisms. Bacteria are often found attached to or within flagellates, and their involvement in cellulose metabolism has been suggested (2). The termites *Zootermopsis angusticollis* and *Z. nevadensis* harbor two cellulose-ingesting flagellate genera: the hypermastigote flagellate *Trichonympha*, with three species, and *Trichomitopsis termopsidis*, a trichomonad flagellate. Trager (20) was first to cultivate termite flagellates and obtained cultures of *T. termopsidis* and some multiplication of *Trichonympha sphaerica*. Recently, *T. termopsidis* was axenically cultivated (21), and, although this flagellate is not the principal cellulose-digesting flagellate in *Zootermopsis* (4), it can significantly contribute to the nutrition of the termite when present as the only intestinal flagellate (23). Presumably the end products of cellulose metabolism by this flagellate are absorbed and metabolized by the termite.

This report characterizes the end products of cellulose metabolism by axenic *T. termopsidis*. Flagellates were grown on radiolabeled cellulose, and the label released by the flagellates was analyzed for typical trichomonad end products (8, 9). In addition, the flagellates were examined for other characteristics of trichomonad metabolism, including sensitivity to the antitrichomonad drug metronidazole and enzymatic activities which account for the end products identified.

MATERIALS AND METHODS

Flagellates. Axenically cultivated *T. termopsidis* was used in all experiments. This flagellate was isolated from the intestinal contents of *Zootermopsis* sp. and has been grown for over 3 years in continuous

axenic culture. The anaerobic culture medium contains cellulose, glutathione, fetal calf serum, yeast extract, and heat-killed rumen bacteria, and was prepared as previously described (21) except that the concentration of cellulose was reduced to 0.05% and the amount of autoclaved rumen bacteria was doubled. These changes gave increased yields. A new batch of yeast extract (ICN Pharmaceuticals, Inc.) was toxic at a concentration of 1.0% and was reduced to 0.5%.

Enzyme assays. Flagellates were harvested from culture fluid by centrifugation over Ficoll solution (21), except that cells were centrifuged for only 5 min and at 300 × *g*. The harvested cells were washed and suspended in a minimum volume of culture medium buffer plus 0.1% glutathione under anaerobic conditions. Pyruvate:ferredoxin oxidoreductase and hydrogenase activities were measured according to the method of Lindmark and Müller (13). Lactate dehydrogenase activity was determined by the oxidation of reduced nicotinamide adenine dinucleotide (14) by pyruvate. Alcohol dehydrogenase was assayed according to the method of Reeves et al. (18) using either ethanol or 2-propanol as substrate, and either reduced nicotinamide adenine dinucleotide or reduced nicotinamide adenine dinucleotide phosphate as cofactor, in the absence and presence of Triton X-100. Protein measurement (15) was corrected for the presence of glutathione in the flagellate preparation.

Hydrogen. H_2 was analyzed at room temperature on a Perkin-Elmer Vapor Fractometer 154B with a silica gel column and N_2 as the carrier gas. Samples were taken from the gas space above cultures of *T. termopsidis*.

Growth on [^{14}C]cellulose. *T. termopsidis* was subcultured into medium containing radiolabeled cellulose prepared as follows. [^{14}C]cellulose (uniformly labeled, 4.5 μCi/mg; ICN Chemical and Radioisotope Division, Irvine, Calif.) was ground with water in a centrifuge-type tissue homogenizer (Bellco Glass Co., Vineland, N.J.) until, by microscopic examination, some particles small enough for the flagellate to ingest were present (about <10 μm). It was washed 10 times with water by centrifugation at 10,000 × *g* for 5 min and suspended in culture buffer plus 0.1% glutathione under anaerobic conditions. It was dispensed into culture tubes, sealed under nitrogen, and autoclaved.

Standard culture medium was prepared without cellulose. Each tube received 100 μl of a 5×-concentrated suspension of autoclaved rumen bacteria and about 70 μg of [^{14}C]cellulose (about 2.9 × 10^5 cpm) plus the other culture medium components. This medium was inoculated with 1.0 ml of culture fluid from a routine subculture of *T. termopsidis*; the inoculum contained about 500 μg of unlabeled cellulose. Each culture had a final volume of 12.15 ml. Half of the cultures were then kept at 37°C overnight to kill the flagellates, and then these heat-killed flagellate cultures were placed at 27°C with the living flagellate cultures.

Quantitation of end products. Both living and heat-killed flagellate cultures were sampled periodically to determine the amount of label appearing in the supernatant fraction of the medium. A 600-μl sample was withdrawn (with syringe and needle) from each culture, and the cells, cellulose, and rumen bacteria were pelleted by centrifugation for 1 min in a Microfuge B (Beckman Instruments). A 500-μl sample of the supernatant fluid was placed directly in a scintillation vial with 10 ml of scintillation fluid (Aquasol-2, New England Nuclear Corp.) for determination of radioactivity, or the supernatant fluid was further analyzed as described below. The pellet was suspended in the remaining 100 μl of medium, and the flagellates were counted in a 200-μm-deep Fuchs-Rosenthal counting chamber.

[^{14}C]carbon dioxide was measured in either whole-culture fluid or in supernatant fluid. Culture fluid (500 μl) was injected into a 25-ml Erlenmeyer flask containing 100 μl of 50% H_2SO_4 and sealed with a stopper fitted with a disposable center well (no. 882320; Kontes Glass Co., Vineland, N.J.) containing 100 μl of β-phenylethylamine. In some experiments, a 250-μl sample of supernatant fluid was placed in the bottom of the flask, and 100 μl of 50% H_2SO_4 was injected into the flask after stoppering with the center well. The flasks were gently shaken at room temperature for 1 h, and then they were opened and the center wells were cut off into scintillation fluid.

Standardization of this technique with $NaH^{14}CO_3$ (New England Nuclear) resulted in >99% of the $^{14}CO_2$ trapped in the center well. When $^{14}CO_2$ was trapped from supernatant fluid rather than whole-culture fluid, about 15% was lost to the atmosphere during centrifugation and pipetting. Counting efficiencies of the various radiolabeled compounds used were similar in all of the experiments.

To quantitate and distinguish acid and neutral volatile end products, samples of supernatant fluid were vacuum distilled in disposable micro-distillation vessels in the presence of excess carrier and chaser reagents. For determining acid plus neutral volatile compounds, 250 μl of supernatant fluid was added to 50 μl of glacial acetic acid, 100 μl of propionic acid, 50 μl of syrupy phosphoric acid, and 5 μl of Antifoam (A. H. Thomas Co.). For determining neutral volatile compounds, 250 μl of supernatant fluid was added to 50 μl of 1 N NaOH, 50 μl of 95% ethanol, 100 μl of *n*-butanol, and 5 μl of Antifoam. The distillation vessels were prepared from 15-cm lengths of 6-mm-diameter soft glass tubing. The distilland was pipetted with an elongated Pasteur pipette into a 1.5-cm-diameter bulb blown at one end of the tube. The tubing near the end opposite the bulb was then heated in a flame and pulled out to form a capillary. The tubing was bent near the bulb to about 45°, the bulb was cooled in ice water, and a vacuum line was attached to the open end. When the vacuum reached about 100 torr, the vessel was sealed by flame at the capillary.

Distillation was started by placing the tube end of the vessel into ice water and the bulb into warm water. To prevent bumping, the temperature was slowly increased from lukewarm up to 55°C. Distillation was complete (after 10 to 15 min) when only the viscous phosphoric acid remained in the bulb (acid condition) or a dry residue remained (alkaline condition). The distillation vessel was then broken open, and the distillate and remaining distilland were washed into scintillation fluid with 500 μl of water. To prevent redistil-

lation, care was taken not to warm the distillate-containing portion of the vessel with the fingers before opening.

This method was calibrated with standard labeled compounds (New England Nuclear) prepared in flagellate culture medium (minus cellulose and heat-killed bacteria) at a final radioactivity (counts per minute per milliliter) near that found in labeled supernatant fluid. Under acid conditions, $^{14}CO_2$ (from $NaH^{14}CO_3$) was lost to the atmosphere, but >95% remained in the distilland under alkaline conditions. [2-^{14}C]acetic acid (from sodium [2-^{14}C]acetate) distilled at 91% efficiency from acid distilland, but only 1% distilled from alkaline distilland. [2-^{14}C]ethanol distilled from both acid and alkaline distillands with 95 and 97% efficiency, respectively.

Identification of the volatile end product. Ether extracts of acidified culture fluid, concentrated culture supernatant fluid, and distillates of acidified concentrated supernatant fluid were analyzed by thin-layer chromatography. Samples were spotted on a cellulose thin layer on a plastic support (F1440, Schleicher & Schuell). Standards applied were diethylamine salts of acetic, formic, lactic, propionic, and succinic acids. Samples were also spotted on top of standards. The chromatograms were developed in *n*-propanol–diethylamine–water (80:4:16) and, after drying, were sprayed with Ninhydrin Spray Reagent (Sigma Chemical Co.) and heated to 60°C to detect the diethylamine salts of the acids (5). Spots were cut out and added to 500 μl of water, and then 10 ml of scintillation fluid was added. Standardization with sodium [2-^{14}C]acetate indicated that about 90% of the label was recovered in the acetate spot.

For better recovery, supernatant fluid was concentrated before distillation. A 10-ml sample of culture supernatant fluid was made alkaline to pH > 11 by the addition of 1 N NaOH, and 100 μl of Antifoam was added. The supernatant fluid was lyophilized, and the resulting residue was dissolved in 1.0 ml of water and centrifuged in the Microfuge for 1 min. A 250-μl sample of the resulting supernatant fluid plus 50 μl of phosphoric acid and 0.5 μl of acetic acid was distilled as previously described. The distillate was made alkaline with 10 to 25 μl of diethylamine and was chromatographed as described.

Acetate and formate could not be separated by thin-layer chromatography, but were distinguished by converting acetate to nonvolatile acetohydroxamate using acetate kinase and hydroxylamine (7). Samples of culture supernatant fluid were added to the assay mixture with and without acetate kinase (Sigma), and nonradioactive acetate and formate (0.2 μmol each) were included. After incubation the assay mixtures were acidified with 50 μl of phosphoric acid, and 5 μl of Antifoam was added. The mixtures were then vacuum distilled as described above, and the radioactivity in the distillate and remaining distilland was determined.

This method was standardized with sodium [2-^{14}C]acetate and sodium [^{14}C]formate (New England Nuclear). Distillation of acetic acid was reduced from 86% (without acetate kinase) to 11% (with acetate kinase). Distillation of formic acid was unaffected after incubation with or without acetate kinase: 80% of the label distilled under both conditions.

Metronidazole. Metronidazole (a gift from M. Müller) was dissolved in 10% dimethyl sulfoxide and was added to cultures to a final concentration of 15 μg/ml. Control cultures received an equal volume of 10% dimethyl sulfoxide (final concentration, 0.1%).

RESULTS

T. termopsidis multiplied in culture medium containing [^{14}C]cellulose, and the label present in the supernatant fraction of the culture fluid increased with time (Fig. 1). At the start of all experiments the cultures had about 3,600 cpm/ml in the supernatant fluid, and the growth of the flagellates resulted in an increase to >8,000 cpm/ml after several weeks. There was a corresponding decrease in the amount of label in the [^{14}C]cellulose-containing pellet. Control cultures containing heat-killed flagellates showed little increase in supernatant label, and the difference between living and control values is regarded as label released by the flagellates.

Some general tests were first performed on the supernatant fluid from log-phase cultures to identify the type of compounds containing label. Lyophilization of supernatant fluid indicated that >75% of the label released by living flagellates was volatile under acid conditions, and no label was volatile under alkaline conditions. Ether extraction of acidified supernatant fluid resulted in extraction of about 55% of the label released by living flagellates, and >96% of this label was volatile under acid conditions. Distillation of acidified supernatant fluid indicated

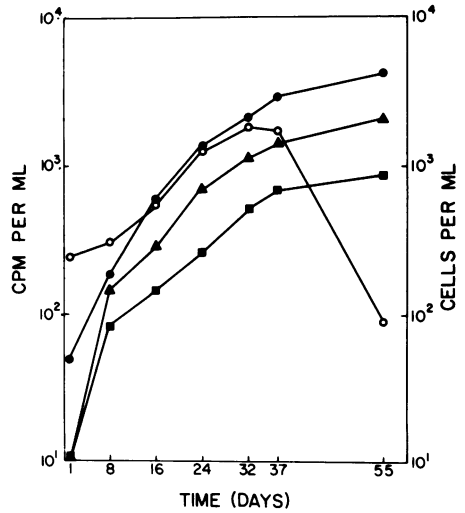

FIG. 1. *Cell numbers* (○) *and net production of supernatant label* (●), $^{14}CO_2$ (■), *and* [^{14}C]*acetate* (▲) *from* [^{14}C]*cellulose by T. termopsidis.*

that 55 to 60% of the label released by living flagellates was volatile acid, and no label distilled from alkaline supernatant fluid.

More precise analyses of the labeled end products in the supernatant fluid revealed that $^{14}CO_2$ and [^{14}C]acetate were present. Label in CO_2 accounted for 25 to 30% of the end products (about 5% of the $^{14}CO_2$ was present in the gas space above cultures). The results of thin-layer chromatography indicated that the label in ether extracts of acidified supernatant fluid and in distillates of concentrated, acidified supernatant fluid was acetate. Only labeled acetate was found when concentrated culture fluid was chromatographed. The acetate kinase reaction converted the distillable label into nondistillable label.

Living flagellate cultures and control cultures were sampled periodically for total label in the supernatant fluid, label in CO_2, label in acetate, and cell numbers. The values for one culture minus the average of four control cultures are shown in Fig. 1. Once detectable in significant amounts and during logarithmic growth, $^{14}CO_2$ and [^{14}C]acetate were present at 25 to 30% and 55 to 60%, respectively, of the labeled end products produced by living cells. When the cells reached stationary phase and began to die, there was a corresponding decline in the production of volatile end products and an increase in label that was neither CO_2 nor acetate (up to 35%).

In addition to analyzing labeled end products of *T. termopsidis*, the cells were examined for other characteristics of trichomonad metabolism. The cells were killed after overnight incubation with metronidazole, and hydrogen was detected at a few percent in the gas space above two active 59-day-old cultures. Activity of hydrogenosomal enzymes involved in acetate, CO_2, and H_2 production was found: coenzyme A-dependent pyruvate:ferredoxin oxidoreductase (EC 1.2.7.1), 528 ± 360 mU/mg of protein, and hydrogenase (EC 1.18.3.1), 1,270 ± 500 mU/mg of protein (n = 3; mean ± standard deviation; 1 U = production of 1 μmol of end product per min). No lactate dehydrogenase or alcohol dehydrogenase activities were detected.

DISCUSSION

This study has shown that acetate and CO_2 are the principal end products of cellulose metabolism of axenic *T. termopsidis*. The ratio of the label in CO_2 to acetate, about 1:2, suggests an equimolar production of the two compounds and is similar to one type of fermentation of cellulose primarily by *Trichonympha* spp. (11). The end-product analysis also indicated that 10 to 20% of the supernatant label from living flagellates was not volatile, was not extractable into ether, and did not migrate during thin-layer chromatography with any other typical trichomonad end product. The amount of this label increased when cell numbers began to decline. It is probably not a fermentation product, but may be an intermediate hydrolysis product (oligomer) of cellulose released by living cells, or a result of the action of cellulase from lysed cells. Its identity was not determined because it made up only about 5 to 10% of the total label in the sample (because of the high initial supernatant label). Hungate (11) also found that acetate and CO_2 accounted for only 70 to 75% of the carbon from cellulose fermentation mainly by *Trichonympha* spp., and no glucose could be detected; however, Trager (20) had indirect evidence for glucose production by *T. termopsidis*. That the termite flagellates may release other products is suggested by the observation that bacteria, spirochetes, and commensal flagellates in the termite intestine are attracted to the cellulose-digesting flagellates, and epibiotic and endosymbiotic bacteria are very common among the termite flagellates (2). Perhaps some fermentable products are released from the cellulose-digesting flagellates which may feed intestinal bacteria and commensal flagellates or even provide some nutrients to the termite.

T. termopsidis is similar in many respects to other trichomonads in ultrastructure and metabolism. The flagellates produced acetate and CO_2, which are also end products of other trichomonads (8). *T. termopsidis* produces H_2 and contains organelles (21) similar in ultrastructure to hydrogenosomes, and the cells possessed activities of two enzymes involved in hydrogenosomal pyruvate metabolism at specific activities similar to other trichomonads (13, 14). Like other trichomonads, the flagellates were killed by metronidazole, which indicates that they possess a low Eh system capable of activating the drug (17). Whereas other trichomonads can grow on soluble sugars such as glucose (8, 9), *T. termopsidis* cannot (21), but it phagocytizes cellulose and hydrolyzes it via cellulase (23) and cellobiase (unpublished data) to glucose. The glucose is then metabolized to acetate, CO_2, and H_2.

The results presented here indicate that acetate is the end product of cellulose metabolism by *T. termopsidis*, available to the termite for oxidation and biosynthesis. Hungate (11) identified acetate in intestinal fluid of termites and showed that acetate could cross the wall of the termite intestine. Mauldin et al. (16) demonstrated that termites fed [^{14}C]acetate incorporate label into amino acids, and other workers (1) have shown that [^{14}C]acetate injected into termites is incorporated into cuticular hydrocar-

bons. Acetic acid is also the principal organic acid present after intestinal contents of the termite *Reticulitermes flavipes* are incubated with [^{14}C]cellulose (19). Considering that acetate is a important and central metabolic intermediate and that termites cannot survive without their flagellates, the production of acetate by a mutualistic intestinal flagellate links cellulose with the energy metabolism of the termite. Acetic acid is also the principal organic acid resulting from rumen fermentation that is absorbed by the ruminant and oxidized and used for biosynthesis (12).

T. termopsidis is the first cellulose-digesting termite flagellate to be studied in the absence of other organisms. Recently, several other cellulose-digesting flagellates have been cultivated axenically, including *Trichonympha sphaerica* from *Zootermopsis* sp. (manuscript in preparation). End-product analysis indicates that this hypermastigote flagellate produces acetate, CO_2, and H_2 from cellulose, and the ratio of CO_2 to acetate is similar to that mainly of mixed *Trichonympha* spp. from *Zootermopsis* sp. (11) and of *Trichomitopsis termopsidis*. Thus, acetate is the major usable end product of cellulose metabolism by both cellulose-digesting flagellate genera in this termite.

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