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ROLE OF PROTOZOA IN THE HINDGUT FERMENTATION OF WOOD-EATING TERMITES: *IN VIVO* AND *IN VITRO* STUDIES

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ABSTRACT

ROLE OF PROTOZOA IN THE HINDGUT FERMENTATION OF WOOD-EATING TERMITES: *IN VIVO* AND *IN VITRO* STUDIES

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Studies on the *in vivo* dynamics of the hindgut fermentation of wood-eating termites revealed that acetate dominated the extracellular pool of volatile fatty acids (VFAs) in 6 species of lower termites (*Reticulitermes flavipes*, *Coptotermes formosanus*, *Incisitermes schwarzi*, *Prorhinotermes simplex*, *Schedorhinotermes lamaniances*, and *Zootermopsis angusticollis*.) as well as the higher termite *Nasutitermes corniger*. Small amounts of C_3 to C_5 VFAs were also observed. Estimates of in situ acetogenesis by the hindgut microbiota of *R. flavipes* (20.2 to 43.3 nmol x termite^{-1} x hr^{-1}) revealed that this activity could support 77 to 100% of the respiratory requirements of the termite (51.6 to 63.6 nmol of O_2 x termite^{-1} x hr^{-1}). This conclusion was buttressed by the presence of acetate in *R. flavipes* hemolymph as well as by the ability of termite tissues to readily oxidize acetate to CO_2. About 80% of the acetate produced in the hindgut was derived from cellulose C; the remainder was derived from hemicellulose C. Selective removal of microbes from the hindgut indicated that protozoa were primarily responsible for acetogenesis, but that bacteria also functioned in this capacity.

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In vitro studies with *Trichomitopsis termopsidis*, an anaerobic, cellulolytic protozoan from *Zootermopsis* termites, revealed that putatively axenic cultures (see M. Yamin, 1978. J. Protozool. 25:535-538) contained a methanogenic consort. However, *T. termopsidis* could be cured of methanogenic activity by incubation in the presence of bromoethanesulfonate. Growth of *T. termopsidis* was markedly improved by substituting heat-killed *Bacteroides* sp. strain JW20 (a termite gut isolate) for heat-killed rumen bacteria as a source of bacterial cell material, and JW20 was the best of a number of bacteria tested. Growth was further improved by co-cultivation in the presence of *Methanospirillum hungatii*. *M. hungatii* was the best of a number of H_2-consuming bacteria tested, and under these conditions CH_4 was produced, indicating interspecies hydrogen transfer between the protozoa and *M. hungatii*.

Crude extracts of *T. termopsidis* possessed endo-β 1,4-glucanase and cellobiase activity as well as hydrolytic activity against wood, xylan, starch and protein. Cell extracts also hydrolyzed microcrystalline cellulose albeit in limited amounts; suggesting that cells may lack a "complete cellulase" system. Hydrolysis of cellulose displayed an optima of pH 5.0 and 30°C, and glucose was the sole product liberated from this activity.

With love

To my father who told me "things take time".

To my mother who said "be happy".

And to my brother who told me I could do this.

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INTRODUCTION

TERMITE BIOLOGY

Termites are social insects belonging to the order Isoptera, and are represented by about 2000 different living and fossilized species (48). They are ubiquitous in nature, and can be found on approximately two-thirds of the world's land surface (80). All termites examined to date contain a dense population of microorganisms in their hindgut. In the phylogenetically "lower" termites (families Masto-, Kato-, Hodo-, Rhino-, and Serritermitidae) the microbiota includes unique genera and species of anaerobic protozoa, as well as bacteria. In the "higher" termites (family Termitidae), hindgut microbial populations consist almost exclusively of bacteria, although protozoa have been observed in a few species (39). Although often noted for their economic damage and cost of control (>$750 Million/yr in the U.S. (56)), termites are ecologically important in decomposition processes and nutrient cycling (80), and possibly in the global production of methane (87).

A termite society or colony is comprised of several castes, including workers, soldiers, and reproductives, which are morphologically and functionally different (27). In fact, the termite colony has been used to illustrate evolutionary principles by drawing analogies between the termite society, as a fundamental unit, and an individual organism (48). It is the worker larvae which dominate the colony's nutritive activities in both sheer numbers and in responsibilities, feeding nutrient-dependent castes, such as soldiers,

newly hatched larvae, and the queen. In both higher and lower termites nutrient dependent castes solicit stomodeal food (a mixture of saliva and regurgitated raw food) from workers (60). Lower termites also rely on solicitation of proctodeal food (liquid excretions from the hindgut) from workers, which also serves as a means of transferring the hindgut microbiota to newly hatched larvae, as well as reinfecting recently molted larvae. Additionally, workers are responsible for building the nest and foraging for food. For these reasons most biochemical and microbiological studies of termites have been carried out on the worker caste.

Although frequently cited for their ability to utilize wood as a food source, the diet of termites (depending on the species) can consist of fungal decayed wood, soil, humus, leaves, or even dung (50). While these materials are quite diverse, they are generally rich in cellulose, hemicellulose, and lignin and relatively poor in soluble substrates, including combined nitrogen. Both cellulose and hemicellulose are efficiently dissimilated by termites. Digestion and assimilation (65-99% and 54-93%, respectively) of these polymers are quite high in comparison to other invertebrates (28,79). The extent of lignin decomposition (0-83%) varies widely among species and has been discussed, as well as questioned, by several authors (10,50,61,73). The most convincing evidence for lignin degradation by termites was obtained with the higher termite Nasutitermes exitiosus (13), as determined by $^{14}CO_2$ evolution from ^{14}C-lignin. Unfortunately, it is not known whether the termite itself, or its gut microorganisms, or both mediate this activity.

The alimentary tract of termites is comprised of three main divisions: the foregut, including the crop and gizzard; the midgut; and the hindgut, which includes the paunch, colon, and rectum (60). Both the crop and the gizzard, as well as the mandibles (i.e. the mouth parts) are structurally conserved throughout the entire order of termites (60), suggesting that they are well suited to the needs of the animal. Some higher termites also have a mixed segment at the juncture of the mid- and hindgut, a section bounded on one side of the lumen by midgut tissue and on the other side by hindgut tissue. The hindgut of most termites has a pH of 6-7.5 and is anaerobic (Eo'=-230 to -270 mV) (61) while the foregut and midgut are both aerobic (Eo' > 100 mV) and found to have a pH of 2.0 to 6.8 and 5.5 to 8.0, respectively.

In most insects the main site of nutrient absorption is the midgut (60), and it is likely that termites also depend on this activity to absorb soluble material from ingested food and/or stomodeal and proctodeal solicitations. However, the bulk of the microbiota, in both higher and lower termites, is contained in the bulbous paunch, a region analagous to an anaerobic fermentation chamber. Moreover, Kovoor (44), using termites fed $BaSO_4$ and detecting its location by using x-rays, has shown that in the higher termite Microcerotermes edentatus particulate material takes about 24 hr to move completely through the gut; transit thru the fore- and midgut was rapid (4 hr) and the material was retained the longest in the paunch region. Inasmuch as the hindgut also has absorptive features (60), and both higher (45) and lower (42) termites contain acetate and other volatile fatty acids in this region it is not unreasonable to suggest that termites may represent an exception to the rule, utilizing the hindgut as the main site of nutrient absorption.

TERMITE NUTRITION

As mentioned above cellulose and hemicellulose are both efficiently dissimilated by termites, and represent the primary, ultimate carbon and energy source of these insects. However, very little is known about the mechanism of hemicellulose degradation in termites. Most researchers have focussed their attention on the hydrolysis of cellulose, inasmuch as this polymer comprises the bulk (about 50%) of the dry weight of wood (28). As one might expect, the principle question that has been addressed by investigators has been the contribution of specific gut microorganisms, versus the termite itself, to the overall digestion of cellulose.

At this point a brief discussion is warranted on the structure of cellulose, as well as the enzymatic hydrolysis of this polymer in nature. Native cellulose is water insoluble and its structure is complex, containing both amorphous and crystalline regions (25,31,34). In wood this structure is further complicated by association with other polymers such as lignin and hemicellulose (25). Cellulase activity is responsible for recycling of much of the carbon fixed annually into biomass (34). In vitro the rate of hydrolysis of cellulose appears to be related to its degree of crystallinity as well as its association with lignin (31). Decreasing the crystallinity index (31), by mechanical grinding or by ball-milling, or extracting the lignin, increases the rate of enzymatic hydrolysis. Most work on cellulase activity has focused on the complete cellulase system of fungi, and, to a lesser extent, on bacterial cellulase. At least three major enzymes are associated with the fungal cellulase system: 1,4-β-D-glucan-

4-glucanohydrolase (Cx cellulase or endoglucanase, EC 3.2.1.4), 1,4-β-D-glucan cellobiohydrolase (C1 cellulase or cellobiohydrolase, EC 3.2.1.91), and β-glucosidase (cellobiase, EC 3.2.1.21). A recent review by Erikkson (26) details the biological mechanisms of cellulose hydrolysis. It is important to note that the efficient and complete hydrolysis of cellulose is dependent on the combined and synergistic action of all 3 enzymes. If any one of these enzymes is missing digestion of native cellulose will be severely limited.

To demonstrate the "complete cellulase" system (i.e. C1 cellulase, endoglucanase, and cellobiase) in vitro, workers often use insoluble substrates, such as filter paper, cotton fibers or microcrystalline cellulose, and measure the production of reducing sugar. However, it is not the rate of reducing sugar release from these insoluble substrates that is important, but the extent of hydrolysis (i.e. the percent hydrolyzed). As mentioned above, cellulose is a mixture of amorphous and crystalline regions, and it is the latter regions which are most resistant to enzymatic attack. Ghose et al (34) point out that at least 4% (reducing sugar equivalent) of a 50 mg piece of filter paper must be hydrolyzed to show the presence of a "complete cellulase" system. C1 cellulase activity can be specifically measured by quantitating the release of cellobiose from crystalline cellulose. Endoglucanase is demonstrated by using carboxymethyl cellulose, a soluble derivative of cellulose, and determining the rate of reducing sugar release. Cellobiase is assayed by measuring the production of glucose from cellobiose, although some worker utilize p-nitrophenylglucoside for measuring this activity (i.e. β-glucosidase). The reader is referred to an excellent review by Ghose et al (34) for a more detailed description of methodologies.

Detailed knowledge of the mechanisms of digestion of cellulose by termites has been hampered by a number of factors, including the lack of an available system to rear germ-free termites, as well as the inability to cultivate many of the gut microorganisms *in vitro*. Nevertheless, information which has accumulated over the years has provided some insight into the biochemistry of symbiosis between the termites and their microbiota, and has been recently reviewed by Breznak (9-11) and O'Brien and Slaytor (61).

I. Lower Termites

One of the most frequently cite examples of a biological mutualism is the interaction between lower termites and their cellulolytic hindgut protozoa which enable the insects to thrive on a diet of wood. The obligatory dependence of lower termites upon the hindgut protozoa was first recognized by Cleveland (14-20). Cleveland showed that defaunation (i.e. removal of protozoa) of termites could be achieved by incubating the insects at elevated temperatures (14) or under hyperbaric oxygen (19), and that such termites continued to feed on wood, but starved to death. He also showed that defaunation by either of these methods did not harm the termite itself; defaunated termites could be "reinfected" with their normal protozoan symbionts and would survive on a diet of wood. A similar mutalistic relationship was also shown for the wood-feeding cockroach Cryptocercus punctulatus (22).

Trager (75) was the first to show that extracts of hindgut contents of Zootermopsis termites could degrade crystalline cellulose to glucose, and that little or no cellulase activity was found in other parts of the digestive tract. Hungate (40) confirmed and extended this work by showing that cellulase activity was absent in the hindgut of defaunted termites. More recent work with Mastotermes darwiniensis (77) and Coptotermes lacteus (62) has also found that the hydrolysis of cellulose was principally associated with the hindgut.

As hindgut contents contain both protozoa and bacteria, the definitive role of the protozoa in wood digestion could not be ascertained until the protozoa were obtained in axenic culture. This major achievement was recently accomplished by Yamin (92,83) who

expanded the work of Trager (76) and successfully axenically cultivated 2 anaerobic, cellulolytic protozoa from Zootermopsis sp. termites. One of the isolates, Trichomitopsis termopsidis, was shown to require cellulose for growth; starch, glucose, or a variety of other substrates would not support growth. If defaunted Zootermopsis termites (fed a diet of cellulose) were reinoculated with T. termopsidis, the insects survived as well as those insects reinfected with hindgut contents of normal termites, and significantly longer than non-reinfected controls (85), implying in situ cellulose hydrolysis by T. termopsidis. Additionally, extracts of T. termopsidis contained both endoglucanase and β-glucosidase activity.

Other workers (49,57,58,62,78,81,86) have also presented evidence that the termite itself secretes cellulase components (endoglucanase and cellobiase). Veivers et al (78) found that in M. darwiniensis endoglucanase and cellobiase activity was principally associated with the salivary glands and midgut of the termite. Nevertheless, defaunated M. darwiniensis could not survive on a diet of wood. These workers also found that M. darwiniensis could be defaunated by feeding termites starch, but survive as well as normal termites that were fed wood. The ability to survive on starch was attributed to termite enzymes and hindgut bacteria. However, M. darwiniensis may be a unique example of a lower termite; aside from wood these termites are also known to feed on sugar cane and vegetables (38).

The end-products of cellulose dissimilation in vitro were first determined by Hungate (41,42). Suspensions of hindgut contents from Zootermopsis termites were mixed with powdered cellulose and found to produce principally acetate, carbon dioxide, and hydrogen. By

extrapolating the in vitro rates of hydrogen production to hydrogen production by the intact termite he proposed that acetate served as the major oxidizable energy source for termites. However, attempts to rear defaunated termites on a diet of acetate (24,43) were unsuccessful. Hungate also qualitatively demonstrated acetate in hindgut fluid obtained directly from the termite, and showed that acetate could transverse the hindgut tissue. This work was recently buttressed by Yamin (83,84) who showed that the major products of cellulose hydrolysis by axenic cultures of protozoa were hydrogen, carbon dioxide and acetate.

Although no quantitative in situ studies have been done on the end-products of wood dissimilation in lower (as well as higher) termites, acetate has been implicated as a major end product inasmuch as various studies have shown ^{14}C-acetate fed to termites is readily incorporated into termite long chain fatty acids (8,55), terpenes (70), and cuticular hydrocarbons (7).

While bacterial populations are quite high (10^9 -10^{10}/ml termite) (11 & references therein) in the hindgut of lower termites there is currently no compelling evidence that bacteria are quantitatively significant in cellulose hydrolysis. Microscopic studies have revealed that the bacterial population is morphologically diverse and predominantly found in close association with the paunch epithelium. Bacterial isolates from the hindgut are principally facultative or strict anaerobes and include Streptococcus sp., Bacteroides sp., and Enterobacteriaceae. Various activities have been ascribed to the bacterial component of the paunch region including nitrogen fixation (64), uric acid nitrogen cycling (65), which appears to be an important

symbiotic strategy for nitrogen conservation, and methane production (9,11). Bacteria are also found as endo- and ectosymbionts of hindgut protozoa (see ref. 12), and, in some cases, they provide motility for the eukaryotes (74).

II. Higher Termites

Considering that higher termites constitute about two-thirds of the total known species of termites, it is ironic that less is known about cellulose digestion in this group of termites than in lower termites. High termites may secrete their own cellulase, although it is not known if, in general, they possess the full complement of enzymes required to digest native cellulose. Potts and Hewitt (66) found that about 70% of the endoglucanase activity of Trinervitermes trinervoides was associated with the midgut; about 1/2 of this activity was localized within the gut tissue, an area devoid of microorganisms. Subsequent purification of this enzyme also revealed activity against microcrystalline cellulose, but not to the extent one might expect with a "complete cellulase" system (67). Microcerotermes edantatus also contained most of its endoglucanase activity in the midgut (47). O'Brien et al (62) found that in Nasutitermes exitiosus about 80% of the total endoglucanase activity of the termite gut was associated with the fore- and midgut. These workers further showed that removal of the hindgut bacteria, by feeding termites tetracycline, did not alter this activity. Cellobiase activity was also predominantly associated with the midgut of N. exitiosus, as well as in N. walkeri (57). Other workers have found cellobiase activity either mostly associated with the hindgut (in Termes (Cyclotermes) obesus, 59) or evenly distributed between the mid- and hindgut (Trinervitermes trinervoides, 66).

One of the most informative studies on cellulase activity in higher termites was done with the fungus growing termite, Macrotermes natalensis (52,53). M. natalensis maintains fungal colonies in their

nests on structures derived from chewed, but undigested, plant material (23), and the termites eat both fungal material (i.e. mycelia and conidia) (2) and plant fragments (35). These termites can not survive if the fungal material is first sterilized. Martin and Martin (52,53) showed that in M. natalensis microcrystalline cellulose hydrolysis, endoglucanase, and β-glycosidase activity was predominately associated with midgut homogenates. Isoelectric focusing of these enzyme activities further showed that all of the cellulose hydrolysis activity and part of the endoglucanase activity was acquired from consumption of fungal conidia. β-glucosidase activity appeared to originate from salivary glands, midgut tissue, and fungal conidia. Abu-Khatwa (1) arrived at similar conclusions with studies of M. subhyalinus.

Higher termites generally feed on food material that is in active decay (21), and it is not unreasonable to assume they might also ingest some cellulolytic enzymes as well. As pointed out by Martin (54) the importance of acquired enzymes (i.e. ingested with the food) to higher termite nutrition requires further investigations.

The end-products of wood dissimilation in higher termites have as yet not been quantitated, although in Microcerotermes edantatus (45) a qualitative examination revealed the presence of acetate, propionate, and butyrate.

Although it appears hindgut bacteria may not play an initial role in the digestion of cellulose by higher termites their presence is critical for the termite's survival. Eutick et al (29) showed that feeding N. exitiosus termites the antibiotic metronidazole was lethal to the termite. Limited microbiological studies have shown the hindgut to contain morphologically diverse bacteria, including spirochetes

(3-6,63,66). In a detailed microscopic study Bignell et al (3-6) found that in the higher termites Procubitermes aburiensis and Cubitermes severus bacteria are also contained within the crop, midgut, and mixed segment. Eutick et al (30) found Staphlococcus sp. to be the predominate isolate (10^7 bacteria/ml x termite) from Nasutitermes sp. termites. Pseudomonas sp. (31) and Achromobacter sp. (32) have also been isolated from Nasutitermes, although both studies failed to quantitate the relative numbers of these isolates.

One important role of bacteria in higher termite nutrition may be in nitrogen economy (10). Nitrogen fixation, a prokaryotic phenomenon, has been demonstrated by acetylene reduction (37) in Nasutitermes sp. termites (33,68,69) and in Macrotermes ukuzii and Trinervitermes trinervoides (71). French et al (33) isolated nitrogen fixing Citrobacter sp. from N. exitiosus, however, the bacteria were not quantitated. Unfortunately no other attempts have been made to isolate the causative agent of the nitrogen fixation activity in higher termites.

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ARTICLE I

VOLATILE FATTY ACID PRODUCTION BY THE HINDGUT MICROBIOTA OF XYLOPHAGOUS TERMITES

By

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Volatile Fatty Acid Production by the Hindgut Microbiota of Xylophagous Termites†

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Acetate dominated the extracellular pool of volatile fatty acids (VFAs) in the hindgut fluid of *Reticulitermes flavipes*, *Zootermopsis angusticollis*, and *Incisitermes schwarzi*, where it occurred at concentrations of 57.9 to 80.6 mM and accounted for 94 to 98 mol% of all VFAs. Small amounts of C_3 to C_5 VFAs were also observed. Acetate was also the major VFA in hindgut homogenates of *Schedorhinotermes lamanianus*, *Prorhinotermes simplex*, *Coptotermes formosanus*, and *Nasutitermes corniger*. Estimates of in situ acetogenesis by the hindgut microbiota of *R. flavipes* (20.2 to 43.3 nmol · termite^{-1} · h^{-1}) revealed that this activity could support 77 to 100% of the respiratory requirements of the termite (51.6 to 63.6 nmol of O_2 · termite^{-1} · h^{-1}). This conclusion was buttressed by the demonstration of acetate in *R. flavipes* hemolymph (at 9.0 to 11.6 mM), but not in feces, and by the ability of termite tissues to readily oxidize acetate to CO_2. About 85% of the acetate produced by the hindgut microbiota was derived from cellulose C; the remainder was derived from hemicellulose C. Selective removal of major groups of microbes from the hindgut of *R. flavipes* indicated that protozoa were primarily responsible for acetogenesis but that bacteria also functioned in this capacity. H_2 and CH_4 were evolved by *R. flavipes* (usually about 0.4 nmol · termite^{-1} · h^{-1}), but these compounds represented a minor fate of electrons derived from wood dissimilation within *R. flavipes*. A working model is proposed for symbiotic wood polysaccharide degradation in *R. flavipes*, and the possible roles of individual gut microbes, including CO_2-reducing acetogenic bacteria, are discussed.

A classical example of nutritional symbiosis is that which occurs between phylogenetically "lower" termites (families Masto-, Kalo-, Hodo-, and Rhinotermitidae) and their intestinal microbiota, an interaction that enables such termites to thrive by xylophagy. Our understanding of this symbiosis has been presented in several reviews (8–10, 20, 23, 30, 38). To summarize, the carbon and energy nutrition of lower termites is centered on wood polysaccharides (cellulose and hemicelluloses), which constitute about 70% of the dry weight of wood and which undergo up to 99% degradation on passage through the gut of the insect. Most of this degradation occurs in the hindgut, a region analogous to an anaerobic fermentation chamber, and the hindgut microbiota appears to be the driving force of dissimilatory activity. The hindgut microbiota of lower termites includes a heterogeneous population of bacteria, as well as unique genera and species of flagellate protozoa. Although the overall hindgut fermentation probably reflects a concerted interaction between protozoa and bacteria, protozoa appear to dominate this activity inasmuch as they are abundant and are key agents of cellulose hydrolysis. Their presence in the gut is critical to the survival of lower termites.

A scheme for symbiotic cellulose utilization in lower termites was proposed many years ago by Hungate (22), who studied cellulose fermentation by crude suspensions of mixed protozoa obtained from *Zootermopsis* species. According to his model, protozoa ferment wood cellulose to CO_2, H_2, and acetate, and the acetate is subsequently absorbed from the hindgut and oxidized by the termites for energy. The validity of Hungate's model was recently buttressed by Yamin, who found that axenic cultures of hindgut protozoa also formed CO_2, H_2, and acetate from cellulose (56, 57) and that survival of defaunated *Zootermopsis* termites (i.e., with protozoa removed) on a diet of cellulose could be achieved by refaunating the termites with axenic cultures of *Trichomitopsis termopsidis* (58).

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Although acetate appears to be an important oxidizable substrate for termites, as well as an important precursor for the biosynthesis of termite fatty acids (4, 33), terpenes (45), and (along with propionate) cuticular hydrocarbons (5), little is known of the actual concentration of acetate and other volatile fatty acids (VFAs) in termite hindgut fluid. Moreover, no direct measurements have been made of VFA production and oxidation in situ. This is probably due to the small size of most termites, which hampers such analyses. Nevertheless, we felt such information was critical to a better understanding of the termite hindgut ecosystem and the importance of hindgut microbes to termite nutrition. Accordingly, it was to these issues that the present study was directed. *Reticulitermes flavipes* (Kollar), the common eastern subterranean termite, was used for the major portion of this study because (i) it is one of the most abundant termites in the United States (54), (ii) specimens were available locally, (iii) wood polysaccharides are extensively degraded in its gut (18), and (iv) it is a species around which we have developed a substantial data base (8, 11, 12, 40–44, 48, 49).

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MATERIALS AND METHODS

Termites and rumen fluid. *R. flavipes* (Kollar) (Rhinotermitidae) was collected from Janesville, Wis., and from Dansville and Spring Arbor, Mich. Termites were used immediately or were maintained in the laboratory for periods up to a year by incubation in covered, polycarbonate boxes containing slices of commercial Douglas fir lumber interspersed with moist brown paper towels (Scott Singlefold, no. 175). Incubation was at 22 to 26°C, and the paper towels were periodically remoistened with distilled water. *Zootermopsis angusticollis* (Hagen) (Hodotermitidae) was obtained from Dahl Biological Supplies, Berkeley, Calif.; *Coptotermes formosanus* Shiraki (Rhinotermitidae) was kindly supplied by G. R. Esenther (Forest Products Laboratory, U.S. Dept. of Agriculture, Madison, Wis.): *Prorhinotermes simplex* (Hagen) and *Schedorhinotermes lamanianus* (Sjostedt) (Rhinotermitidae), *Incisitermes schwarzi* (Banks) (Kalotermitidae), and the "higher" termite *Nasutitermes corniger* (Motschulsky) (Termitidae) were kindly supplied by B. L. Bentley and G. D. Prestwich (State University of New York, Stony Brook). Worker termites (i.e., externally undifferentiated larvae beyond the third instar) were used for all experiments.

Rumen fluid was obtained by aspiration from a fistulated dairy cow and passed through three layers of cheesecloth before use.

Sampling of VFAs. A "Micro" method was used to sample termite hindgut fluid for VFA analysis. Termites were first chilled to 4°C to immobilize them and then degutted by using fine-tipped forceps (12), a procedure which yielded the entire hindgut along with a short piece of attached midgut. Such preparations were referred to as extracted guts. Extracted guts were dipped into 2 mM potassium phosphate buffer (pH 7.6) to rinse off hemolymph, blotted with paper tissue, and transferred to a slab of dental wax. The bulbous, paunch region of the hindgut (12) was then pierced with a dissecting needle, and the liquidy hindgut contents that issued from the puncture site were aspirated into a glass capillary tube (1-μl capacity; Dade Div. American Hospital Supply Corp., Miami, Fla.). One capillary tube was used per termite, and all sampling was done as rapidly as possible at 2 to 4°C. The height of the column of hindgut contents within each capillary tube was measured by using a dissecting microscope equipped with an ocular micrometer. From this measurement the extracellular fluid volume was inferred (see below). The contents of 2 to 31 capillary tubes were then pooled by quantitative transfer into a small polypropylene centrifuge tube (Brinkmann Instruments, Westbury, N.Y.) containing 10 to 50 μl of BIS (BIS is 2 mM potassium phosphate buffer [pH 7.6] containing 1.0 mM α-methylbutyrate as an internal standard). This resulted in a 20- to 40-fold dilution of hindgut fluid, but yielded volumes that could be more easily manipulated for further processing. Mixtures were then centrifuged at 13,000 × *g* for 20 min at 4°C, and supernatant fluids were used for quantitation of VFAs by gas chromatography.

To determine the extracellular concentration of VFAs in hindgut fluid, it was first necessary to establish a conversion factor for the fraction of extracellular fluid in a given volume of hindgut contents. To do this, we plugged capillary tubes containing various amounts of hindgut contents at the base with paraffin, capped them with a small piece of Parafilm M (American Can Co., Greenwich, Conn.), and centrifuged them at 13,000 × *g* for 60 min with a hematocrit centrifuge. After 60 min, no further compaction of particulate material was observed. The height of the supernatant fluid was then measured as described above and taken to be the extracellular fluid volume. For various termites, the extracellular fluid volume of hindgut contents was (mean ± standard error of the mean): *R. flavipes*, 38.6 ± 7.0% ($n = 9$); *I. schwarzi*, 60.7 ± 3.0% ($n = 4$); and *Z. angusticollis*, 63.2 ± 7.5% ($n = 5$). These conversion factors were used to infer the extracellular fluid volume during routine analyses. All capillary tubes used in this study were first cleaned by immersion in Chromerge (Manostat, New York, N.Y.)–H_2SO_4 solution for 24 h, followed by rinsing with tap and deionized water. The capillary tubes were then oven dried and coated with Sigmacote silicone reagent (Sigma Chemical Co., St. Louis, Mo.). Capillary tubes were calibrated by using a ^3H_2O standard (specific activity, 2.96 × 10^6 dpm/ml).

Termite hemolymph was obtained by gently piercing the cuticle between the third and fourth abdominal tergites and aspirating the clear fluid into a capillary tube. After volume determination, hemolymph samples from nine termites were pooled in BIS for subsequent analysis.

To sample termite feces, we placed 30 termites in a tared polypropylene centrifuge tube in which they were allowed to defecate for 24 h. The termites were

then removed, and the centrifuge tube was reweighed to estimate the fecal mass, after which 50 μl of BIS was added to extract VFAs. Particulate material was removed by centrifugation, and the supernatant fluid was used for analysis.

To estimate the VFA content of different hindgut regions, extracted guts were removed to a slab of dry ice covered with a thin sheet of plastic, whereupon they immediately froze. A razor blade was then used to liberate the posterior portion (rectum) of each hindgut. The bulbous anterior portion (paunch plus colon) of each hindgut was then separated from the midgut by a razor slice just anterior to the enteric valve. Generally, 6 anterior or 30 posterior hindgut regions were pooled in 20 μl of BIS and homogenized with a glass rod. Homogenates were then centrifuged as described above, and supernatant fluids were used for analysis of VFAs.

Rumen fluid was sampled by the Micro method described above, or handled by a "Macro" method as follows. A 200-ml quantity of fluid was clarified by $Zn(OH)_2$ precipitation (37), and 15-ml samples of the clarified liquor were subjected to steam distillation (37). Distillates were neutralized with NaOH and brought to dryness by heating at 80°C. The dry sodium salts of VFAs were dissolved in 2.0 ml of water, acidified with H_2SO_4, and extracted into diethyl ether (40) for subsequent quantitation.

Analysis of VFAs. Quantitation of VFAs in termite preparations, as well as in rumen fluid sampled by the Micro method, was done with a Varian model 2440 gas chromatograph equipped with an H_2 flame ionization detector. Temperature settings were (°C): injector, 175; column, 125; detector, 175. Flow rates were (ml/min): N_2 carrier gas, 30; air, 300; H_2, 30. The glass column (183 by 0.2 cm) was coated with Sigmacote and packed with Carbopack C impregnated with 0.3% Carbowax 20 M and 0.1% H_3PO_4 (Supelco, Inc., Bellefonte, Pa.). Before use, the packed columns were preconditioned with water or H_3PO_4 according to the manufacturer's recommendations (bulletin no. 751B; Supelco, Bellefonte, Pa.). Chromatograms were recorded with a Hewlett-Packard model 3390A reporting integrator, which was also used to calculate quantities of VFAs by reference to the internal α-methylbutyrate standard. This procedure afforded excellent separation and quantitation of C_2 to C_5 VFAs in the range encountered with diluted hindgut contents (0.2 to 5.0 mM; see Fig. 1).

To estimate formate, we homogenized 150 extracted guts in 1.0 ml of 50 mM potassium phosphate buffer (pH 7.6) and removed particulate material by centrifugation. The supernatant fluid was then acidified with H_2SO_4, extracted with diethyl ether, and analyzed by gas chromatography on a column of SP-1220 (Supelco, Inc., Bellefonte, Pa.) (40).

VFA analysis of rumen fluid sampled by the Macro method was done by using gas chromatographic conditions previously described (40).

Mass spectra of acetate were determined by diverting a portion of the compound separated by gas chromatography to a Finnigan model MS/GC mass spectrometer operating at 6×10^{-2} Pa and 70 eV.

Feeding experiments. Incubation vessels and conditions were similar to those described previously (44). However, when ^{14}C-labeled substrates were fed to termites, vessels were modified to be gas tight and contained a piece of tissue wetted with 0.5% H_3PO_4 below the screen platform supporting the termites. For such vessels, the headspace was periodically flushed with air, and $^{14}CO_2$ in the exit air was trapped in phenethylamine (44).

Douglas fir powder (DFP) was the main constituent of food tablets. To prepare DFP, sawdust was first extracted with hot water (12), oven dried, and then ball milled for 120 h. Particles small enough to pass through a standard 180-μm sieve were then pooled as DFP and used for compaction into 100-mg food tablets. DFP was assumed to contain 47% cellulose and 23% hemicelluloses (dry weight basis; reference 18).

To determine the contribution of cellulose versus hemicellulose carbon to acetogenesis in termite hindguts, we amended DFP with 20% (wt/wt) [*U*-^{14}C]cellulose or 10.5% (wt/wt) [*U*-^{14}C]hemicellulose before compaction into tablet form. The specific activity of cellulose or hemicellulose in such tablets was calculated from the amount of radioactivity in weighed portions of tablets and the amount of specific polysaccharide in those portions. The former was determined by complete combustion of tablet material with an elemental analyzer (C. Erba model 1104; Sanda, Inc., Philadelphia, Pa.), followed by measurement of radioactivity liberated as $^{14}CO_2$ (44). The latter was estimated from the amount of ^{14}C-labeled polysaccharide incorporated into tablets and the amount of unlabeled component present as part of the DFP material itself (see above). Termites which had fed on such tablets for 4 days were degutted, and 4 to 6 extracted guts were placed in a small centrifuge tube containing 20 μl of BIS, quickly frozen in dry ice, and then homogenized with a glass rod while thawing. Preparations were centrifuged as described above, and supernatant fluids were used for analysis of [^{14}C]acetate. The legitimacy of this approach was based on the fact that virtually all of the acetate associated with extracted guts was present in the hindgut fluid (see below). For determination of the specific activity of [^{14}C]acetate, samples were first injected into a Varian model 3700 gas chromatograph to quantitate the amount of acetate present. The glass column (183 by 0.4 cm) was packed with Chromosorb W impregnated with 15% SP-1220 and 1% H_3PO_4. Temperatures were (°C): injector, 200; column, 175; flame ionization detector, 200. Flow rates were (ml/min): N_2 carrier gas, 30; air, 300; H_2, 30. $^{14}CO_2$, liberated from [^{14}C]acetate in the flame ionization tower, was then collected by bubbling the gas through 7.0 ml of ethanolamine-methanol, 3:4 (vol/vol). The trapping efficiency of this system was determined to be 88%. The specific activity of [^{14}C]acetate was then calculated from the amount of acetate of acetate present and its radioactivity.

The ability of termites to oxidize VFAs was evaluated by measuring $^{14}CO_2$ evolution from termites feeding on ^{14}C-labeled VFAs. DFP tablets used for such experiments were first moistened lightly with 2 mM potassium phosphate buffer (pH 7.6) followed by 20 μl of buffer containing (nmol): sodium [*U*-^{14}C]acetate, 29.2; sodium [1-^{14}C]propionate, 179.0; or sodium [2-^{14}C]butyrate, 142.0. Carrier-free radioactive compounds were used, and the amounts added were such that each food tablet contained 0.2 to 2.0 μCi. To determine the origin of $^{14}CO_2$ evolution in such experiments, we subsequently removed termites from the incubation vessels, and intact specimens, as well as degutted bodies and extracted guts, were reincubated separately in 5-ml stoppered serum vials containing a filter paper disk moistened with water. $^{14}CO_2$ was collected as previously described (42).

The ability of defaunated or normally faunated termites to survive on a diet of acetate was evaluated by using food tablets consisting of 2 M sodium acetate incorporated into a gel of 10% (wt/vol) agarose. Control tablets consisted of agarose alone and agarose containing 2 M NaCl.

Acetogenic activity in situ. In situ rates of acetogenesis by the termite hindgut microbiota were estimated by a modification of the zero-time-rate method (24). Termites were introduced into an anaerobic glove box (Coy Manufacturing Co., Ann Arbor, Mich.) containing an atmosphere of 90% N_2–10% H_2 and quickly degutted. Groups of six extracted guts were incubated at 23°C in centrifuge tubes containing 45 μl of 2 mM potassium phosphate buffer (pH 7.6). The buffer solution had been boiled and cooled under 100% N_2 before use. At zero time, and at periodic intervals thereafter, triplicate groups of guts were quick frozen in dry ice and processed for VFA analysis as described above, except that the α-methylbutyrate standard was added after the homogenization step. This approach permitted the isolation of acetogenic activity from the respiratory activity of the host (via gut removal and anaerobic incubation), and it also allowed estimation of acetate present within extracted guts as well as that exported to the incubation buffer.

Elimination of gut microbes. Defaunation was accomplished by treating termites with 100% O_2 at 172 kPa (25 lb/in^2) for 2 h at ambient temperature (14). Termites were then reincubated in isolation for 4 days before use. Microscopic examination of gut contents immediately after hyperbaric O_2 treatment revealed that all protozoa were immobile; after 3 days, no protozoa were present.

Bacteria were eliminated from hindguts by feeding termites antibacterial drugs for 4 days, a treatment which reduces specific heterotrophic populations 100-fold (44).

Respiratory gas exchange. Respiratory activity of termites was measured at 23°C by conventional manometric methods (52) and was corrected for H_2 and CH_4 emitted by the termites (see below). Generally, groups of 50 to 80 termites were held in individual reaction vessels, and each determination was made in triplicate. The volume occupied by the termites in the reaction vessels was estimated separately by liquid displacement.

CH_4 and H_2 emission by termites was measured by incubating 30 to 40 termites in 5-ml stoppered serum vials containing air and periodically sampling the headspace gas. CH_4 analysis was made by using gas chromatographic conditions similar to those used for measuring C_2H_4 (40). H_2 analysis was done as described previously by Uffen (51).

Other procedures. $^{14}CO_2$ evolution from [*U*-^{14}C]acetate by termite homogenates was measured by using 5-ml stoppered serum vials as reaction vessels. Generally, 10 extracted guts or degutted bodies were homogenized in 0.9 ml of 10 mM potassium phosphate buffer (pH 7.6) and dispensed into the serum vials, and reactions were initiated by the addition of 0.1 ml of 2.5 μM [*U*-^{14}C]acetate (ca. 0.1 μCi). Reactions were terminated by the addition of 0.1 ml of 1 N HCl, and $^{14}CO_2$ in the headspace was trapped as described above.

Measurements of radioactivity and quench corrections were made as previously described (49).

Chemicals. All chemicals used were reagent grade and purchased from commercial sources. Radioactive chemicals were obtained from New England Nuclear Corp., Boston, Mass., except for [*U*-^{14}C]cellulose and [*U*-^{14}C]hemicellulose, which were obtained from ICN Pharmaceuticals, Inc., Irvine, Calif. The ^{14}C-labeled cellulose and hemicellulose were repurified before use by the methods of Rapson (47) and Myhre and Smith (36), respectively.

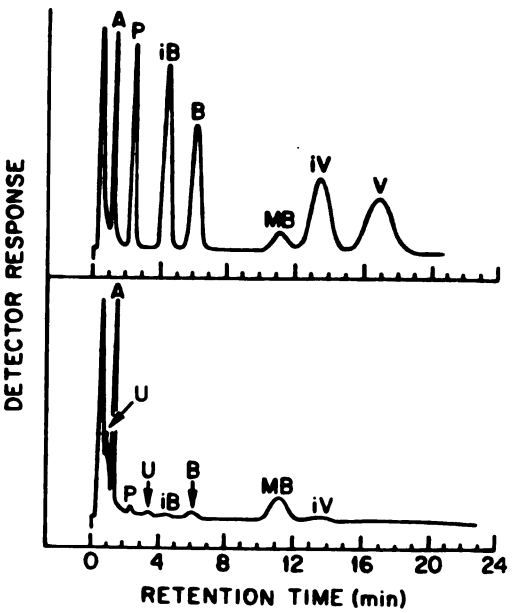

FIG. 1. Gas chromatograms of a standard mixture of VFAs (4 mM each in BIS, top) and a diluted sample of hindgut fluid from *R. flavipes* termites (bottom). Symbols: A, acetate; P, propionate; iB, isobutyrate; B, butyrate; MB, α-methylbutyrate (internal standard); iV, isovalerate; V, valerate; and U, unknown compounds.

RESULTS

VFAs in termite hindguts and feces. Acetate was the major VFA present in the hindgut fluid of *R. flavipes*. The compound was readily detected by gas chromatography (Fig. 1), and its mass spectrum (Fig. 2) was virtually identical to that of authentic acetic acid (16). The mean extracellular acetate pool size was 80.6 mM, which accounted for 94 mol% of all C_1 to C_5 VFAs (Table 1). Small amounts of propionate and butyrate were present, but their concentration frequently fell below the limits of reliable quantitation. Consequently, the values for propionate and butyrate reported in Table 1 were only from those analyses done on more concentrated samples of hindgut fluid (i.e., pooled from >8 termites) which gave detector responses within quantifiable limits. Trace amounts of isobutyrate, valerate, and isovalerate were sometimes observed; however, formate was not detected. No significant differences in VFA

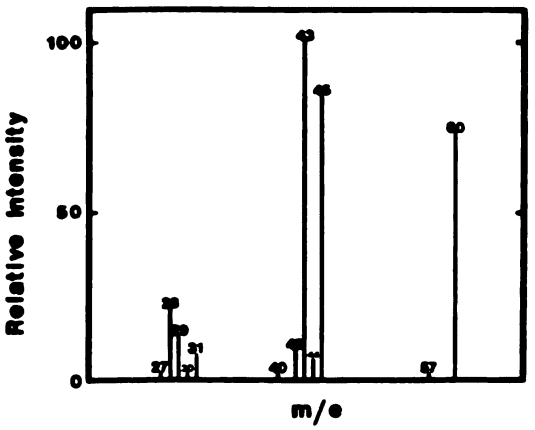

FIG. 2. Mass spectrum of acetic acid from hindgut fluid of *R. flavipes* termites.

content were observed between freshly collected and laboratory-maintained *R. flavipes*, so the data in Table 1 represent a pooled estimate from all determinations. Limited experiments with *Z. angusticollis* and *I. schwarzi* revealed that acetate dominated the VFA pool in the hindgut fluid of these termites as well (Table 1).

Since the Micro method of sampling for VFA analysis entailed manipulation of minute amounts of hindgut fluid, a critical concern was possible errors incurred through sample evaporation or VFA volatilization. However, when bovine rumen fluid was used as a control, results obtained by the Micro sampling method compared favorably with those obtained by a Macro method (Table 1). These in turn agreed well with published values for the VFA content of rumen fluid (24).

As expected, acetate was the major VFA present in homogenates of extracted guts of *R. flavipes* and occurred at a concentration of 18 nmol per gut equivalent (Table 2). Since the volume of a hindgut is about 0.7 μl (48) and consisted of 39% fluid (see above) containing acetate at 81 mM (Table 1), it could be calculated that essentially all of the acetate in extracted guts was present in the hindgut fluid; little existed in intracellular pools of the hindgut microbiota or gut tissue. When extracted guts were sectioned, the anterior region of the hindgut (i.e., paunch plus colon) was found to contain 19 nmol of acetate per gut equivalent, whereas the rectum contained only 0.4 nmol per gut equivalent (Table 2).

Acetate was also the major VFA in extracted guts of three other rhinotermitids examined and in *N. corniger*, and when dissections were performed, the compound occurred mainly in the anterior portion of the hindgut (Table 2). However, when the acetate content of extracted guts was normalized to body weight, a threefold variation was observed, ranging from 1.5 nmol/mg (*N. corniger*) to 4.6 nmol/mg (*R. flavipes*) (Table 2).

In some analyses of hindgut fluid or extracted guts, gas chromatograms revealed minor peaks which did not correspond to those of standard VFAs (Fig. 1, bottom). The compounds responsible for such peaks remain to be identified, although separate experiments indicated they were not acetoacetate, β-hydroxybutyrate, lactate, or ethanol.

Pooled feces, voided by 30 *R. flavipes* workers over a 24-h period, contained only a trace amount of acetate which could not be accurately quantitated.

These data indicated that: (i) acetate was associated with, and probably produced in, the bulbous, microbe-packed anterior region of the hindgut; (ii) virtually all of the acetate present in the gut was metabolized within *R. flavipes* and not voided with feces; and (iii) the short segment of midgut usually attached to extracted guts contained little or no acetate.

TABLE 1. Extracellular pool size of VFAs in termite hindgut fluid

Termite	Method of sampling	VFA concn (mM)[a]		
		Acetate	Propionate	Butyrate
R. flavipes[b]	Micro	80.6 ± 31.5 (n = 16)	2.8 ± 1.0 (n = 8)	2.0 ± 1.1 (n = 5)
Z. angusticollis	Micro	66.2 ± 0.7 (n = 5)	0.9 ± 0.2 (n = 3)	Trace
I. schwarzi	Micro (n = 3)	57.9 ± 3.1	1.4 ± 0.4	Not detected
Control (bovine rumen fluid)	Micro (n = 3)	44.5 ± 3.9	16.7 ± 1.1	6.1 ± 0.6
	Macro (n = 3)	48.8 ± 1.7	14.3 ± 0.3	5.2 ± 0.1

[a] Values are means ± standard error of the means. n, Number of independent determinations.
[b] Trace amounts of isobutyrate, valerate, and isovalerate were also detected. Formate was not observed.

TABLE 2. Acetate content of gut homogenates of worker termites

Termite	Termite fresh wt (mg)	Sample[a]	n[b]	Acetate (nmol) per:[c] Gut portion	mg of body wt
R. flavipes	4.0	EG	11	18.3 ± 3.0	4.6 ± 0.8
		AH	3	19.0 ± 2.6	4.8 ± 0.7
		R	3	0.4 ± 0.2	0.1 ± 0.1
S. lamanianus	3.9	EG	2	7.6	2.0
		AH	2	8.2	2.1
P. simplex	2.8	EG	2	6.5	2.3
		AH	3	5.8 ± 3.4	2.1 ± 1.2
C. formosanus	3.0	EG	4	7.9 ± 0.6	2.6 ± 0.2
N. corniger	2.2	EG	2	3.2	1.5

[a] EG, Extracted gut; AH, anterior hindgut; R, rectum.

[b] *n*, Number of independent determinations.

[c] Mean ± standard error of the mean, or mean of two determinations as indicated.

Acetate production and utilization in *R. flavipes*. Because acetate dominated the VFA pool in *R. flavipes* hindguts, it was of interest to estimate its rate of production in situ, to evaluate the importance of the hindgut microbiota to acetogenesis, and to appraise the termite as a potential user of this metabolite.

By using a modified zero-time-rate method, in situ acetogenesis was estimated to be 20.2 ± 3.4 nmol · termite^{-1} · h^{-1} (equivalent to 5.8 ± 0.1 nmol · mg of fresh weight^{-1} · h^{-1}) (n = 3) for laboratory-maintained termites and 43.3 ± 6.9 nmol · termite^{-1} · h^{-1} (equivalent to 12.4 ± 2.0 nmol · mg of fresh weight^{-1} · h^{-1}) (n = 3) for specimens freshly collected from the field. Results of a representative experiment are depicted in Fig. 3. Rates of acetogenesis appeared linear for the first 2 h of incubation, and regression analyses yielded correlation coefficients ranging from 0.904 to 0.972 for all determinations. Propionate and butyrate were also observed to increase during the incubation period (data not shown); however, their rates of production could not be accurately measured because of their low concentrations.

Measurements of O_2 consumption by *R. flavipes* revealed rates of 51.6 ± 10.7 nmol · termite^{-1} · h^{-1} (equivalent to 0.357 ± 0.074 μl · mg of fresh weight^{-1} · h^{-1}) (n = 6) and 63.6 ± 7.7 nmol · termite^{-1} · h^{-1} (equivalent to 0.440 ± 0.053 μl · mg^{-1} · h^{-1}) (n = 3) for laboratory-maintained and fresh field specimens, respectively (Fig. 3). However, respiratory quotients (RQs) were 1.00 to 1.05 regardless of the origin of the insects.

Defaunation, by treatment of termites with hyperbaric O_2, caused a marked decrease in both the steady-state level of acetate in hindgut contents (1.1 ± 0.4 nmol · termite^{-1}) as well as in its rate of production (3.9 nmol · termite^{-1} · h^{-1}) (Fig. 4). Removal of bacteria with antibacterial drugs also reduced the steady-state level of acetate (8.4 ± 0.7 nmol · termite^{-1}) and its rate of production (6.8 nmol · termite^{-1} · h^{-1}), but to a lesser extent (Fig. 4).

The ability of *R. flavipes* to respire acetate was assessed by feeding them [*U*-^{14}C]acetate and measuring the $^{14}CO_2$ evolved. $^{14}CO_2$ evolution commenced immediately with an initial rate of 0.013 nmol of $^{14}CO_2$ · termite^{-1} · h^{-1} (Fig. 5). $^{14}CO_2$ was also readily evolved from [1-^{14}C]propionate and [2-^{14}C]butyrate, although rates of $^{14}CO_2$ evolution from all VFAs began to decline

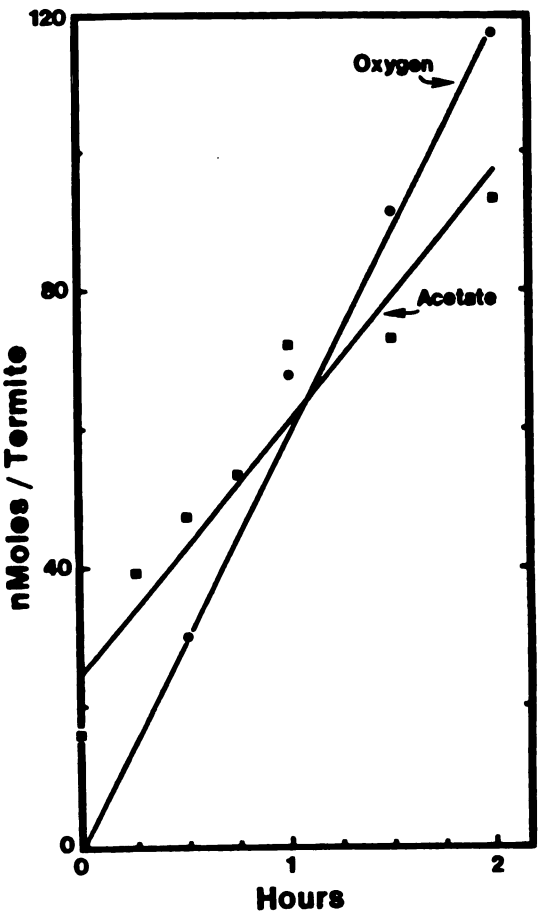

FIG. 3. Rates of in situ acetogenesis by the hindgut microbiota and O_2 consumption by intact worker larvae of *R. flavipes*. Termites were freshly collected from the field before assay.

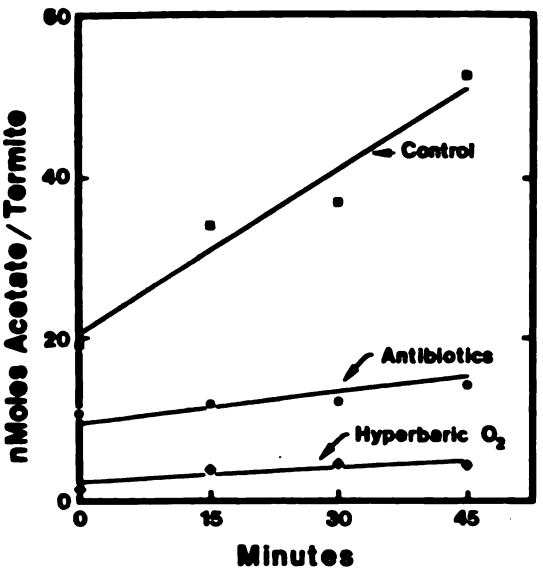

FIG. 4. Effect of defaunation (hyperbaric O_2) or removal of hindgut bacteria (antibiotics) on acetogenic activity of hindguts of *R. flavipes* termites.

between 6 and 24 h of incubation (Fig. 5). This suggests that ^{14}C-labeled VFAs may not have been uniformly distributed throughout the food tablets or that the feeding rate of termites decreased during incubation. In the absence of termites, negligible amounts of $^{14}CO_2$ were evolved from food pellets (Fig. 5). After 52 h,

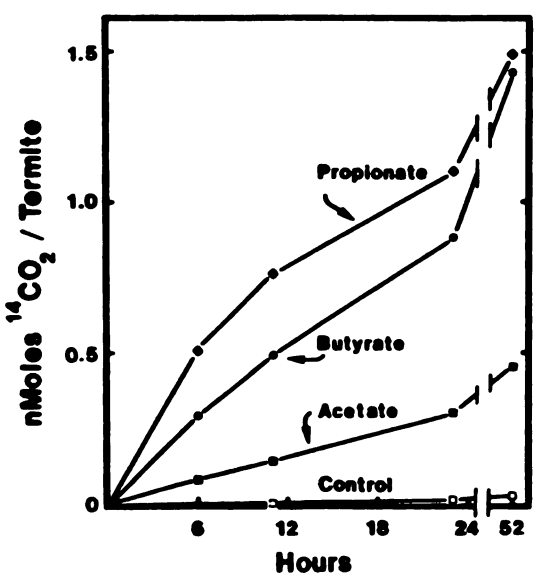

FIG. 5. Evolution of $^{14}CO_2$ by *R. flavipes* termites feeding on [*U*-^{14}C]acetate, [1-^{14}C]propionate, or [2-^{14}C]butyrate. Control vessels contained [*U*-^{14}C]acetate incubated in the absence of termites.

TABLE 3. Origin of $^{14}CO_2$ from *R. flavipes* fed ^{14}C-labeled VFAs

VFA fed to termites[a]	Incubation atmosphere	$^{14}CO_2$ evolved (pmol · termite equivalent^{-1} · h^{-1})[b]		
		Intact termite	Degutted body	Extracted gut
[*U*-^{14}C]acetate	Air	0.82	0.76	0.20
	100% N_2	0.34	0.32	0.10
[1-^{14}C]propionate	Air	1.86	1.63	0.30
	100% N_2	0.87	0.80	0.24
[2-^{14}C]butyrate	Air	3.41	3.03	0.71
	100% N_2	1.65	1.26	0.47

[a] Termites were fed ^{14}C-labeled VFAs for 52 h before assay. The specific activities of the substrates were (dpm · nmol^{-1}): [*U*-^{14}C]acetate, 60,050; [1-^{14}C]propionate, 31,006; and [2-^{14}C]butyrate, 3,419.

[b] Groups of 7 to 10 termite equivalents were used per determination.

termites were removed from the incubation vessels and dissected to determine the origin of $^{14}CO_2$-evolving activity (Table 3). Most of the $^{14}CO_2$ evolution was associated with degutted bodies (i.e., termite tissues only) and was significantly retarded by incubation under N_2. The observation that some $^{14}CO_2$ was evolved under N_2 suggests that dissolved O_2 may still have been present in termite tissues. Only small amounts of $^{14}CO_2$ were evolved from extracted guts and presumably arose from respiratory activity of gut tissue. In separate experiments, termites previously unexposed to [^{14}C]acetate were degutted, homogenized, and incubated in vitro with [*U*-^{14}C]acetate. Under aerobic conditions, 83% of the $^{14}CO_2$ evolved arose from degutted bodies, whereas 17% arose from gut homogenates.

Analysis of hemolymph. The ability of *R. flavipes* tissues to oxidize acetate suggested that acetate produced in the hindgut was absorbed from that site and transported to tissues via the hemolymph. Accordingly, hemolymph was assayed for the presence of this compound. In two separate experiments, acetate was found to be present at concentrations of 9.0 and 11.6 mM, respectively. Propionate and butyrate were not observed, but could have been present in concentrations below the limits of detection (<4 mM in hemolymph).

Acetogenesis from wood polysaccharides. To estimate the contribution of cellulose versus hemicellulose carbon to acetogenesis, we fed DFP amended with [*U*-^{14}C]-labeled polymer to *R. flavipes* and determined the specific activity of acetate in the hindgut fluid (Table 4). Comparison of the specific activity of the food compo-

TABLE 4. Origin of acetate in hindgut fluid of *R. flavipes* termites

Component in food tablet	Sp act (dpm · ng of C^{-1})*		% Contribution
	Food component	Acetate in hindgut fluid	
[*U*-^{14}C]cellulose	0.86 ± 0.12	0.75 ± 0.16	87.2
[*U*-^{14}C]hemicellulose	11.5 ± 1.2	2.66 ± 0.59	23.1

* Mean ± standard error of the mean (n = 3).

nent with that of acetate revealed that about 87% of the acetate was derived from cellulose, whereas 23% was derived from hemicellulose. The sum of these values strongly suggests that acetate was produced only from wood polysaccharides and not from lignin or other wood components. That the sum was greater than 100% suggested that the labeled polymers added to food tablets were more readily converted to acetate than were the corresponding unlabeled polymers in wood, which are complexed with lignin (17).

Survival of *R. flavipes* on acetate. Attempts to prolong the survival of *R. flavipes* on a cellulose-free diet with acetate were unsuccessful. This was true whether defaunated or normally faunated termites were used. In fact, termites feeding on sodium acetate or NaCl (control) generally died faster than did those feeding on unamended agarose.

H_2 and CH_4 emission by *R. flavipes*. Live specimens of *R. flavipes* usually emitted H_2 at rates of 0.4 to 0.9 nmol · termite^{-1} · h^{-1} (equivalent to 0.003 to 0.006 μl · mg of fresh weight^{-1} · h^{-1}), although occasional groups evolved up to 4.2 nmol · termite^{-1} · h^{-1} (equivalent to 0.029 μl · mg^{-1} · h^{-1}). Rates of CH_4 emission were 0.38 ± 0.2 nmol · termite^{-1} · h^{-1} (equivalent to 0.003 ± 0.001 μl · mg^{-1} · h^{-1}) (n = 20). No significant difference in the CH_4 emission rate was observed between laboratory-maintained and freshly collected termites. Thus, on a molar basis, the rate of H_2 or CH_4 evolution was always ≤7% (and usually ≤0.8%) that of O_2 consumption or CO_2 evolution. Interestingly, however, when a group of termites emitting trace amounts of H_2 were fed antibacterial drugs (chloramphenicol, penicillin, and tetracycline), the rates of H_2 evolution increased to 1.7 to 7.0 nmol · termite^{-1} · h^{-1} within 48 h of drug treatment, and CH_4 emission increased from 0.25 to 1.50 nmol · termite^{-1} · h^{-1}.

DISCUSSION

VFAs present in the hindguts of xylophagous termites included C_2 to C_5 representatives (Tables 1 and 2) and were qualitatively similar to those found in other gastrointestinal ecosystems harboring a dense microbiota, such as the large bowel and cecum of vertebrates (1, 13, 35, 50), the rumen (24, 25), and the hindgut of cockroaches (6) and scarabaeid beetles (3). In addition, our results were consistent with those of Kovoor (27), who qualitatively identified acetate, propionate, and butyrate in the hindgut of the higher termite *Microcerotermes edentatus* by using paper chromatography, and those of Hungate (21, 22), who identified acetate in the hindgut fluid of *Z. angusticollis*. Quantitatively, however, the VFA profile in termite hindgut fluid was quite different from that of most other intestinal ecosystems in that acetate accounted for an unusually large fraction (94 to 98 mol%) of all VFAs (Table 1). This was not due to preferential absorption and oxidation by termites of C_3 to C_5 VFAs, because acetate was still the major VFA produced when the oxidative activity of host tissue was circumvented (i.e., by the zero-time-rate method of analysis).

The striking dominance of acetate in the hindgut fluid of lower termites is probably due to several factors, including the relatively insoluble nature of the food of the termites and the rather unique composition of the hindgut microbiota. The major components of wood are insoluble polysaccharides (cellulose and hemicelluloses) complexed with 18 to 35% lignin (17). Although it appears that some lower termites might secrete their own cellulases (53), and may in fact initiate wood glucan decomposition in the fore- and midgut, the majority of cellulolytic activity occurs in the hindgut (where the bulk of wood decomposition occurs) and is of protozoan origin (8–10, 20, 23, 38, and references therein). Moreover, the transit of food from the foregut to the hindgut is rapid (<3 h; 28, 29; Odelson and Breznak, manuscript in preparation). Consequently, most of the food remains in an insoluble, particulate form during passage to the hindgut. Since the protozoan population in termite hindguts is quite large (the hindgut of *R. flavipes* contains 4 × 10^4 protozoa [34] and 3 × 10^6 bacteria [48], and the ratio of protozoa to bacteria is about 1,000 times greater than that of the bovine rumen [25, 55]; in *Zootermopsis* termites the hindgut protozoa account for about one-third of the body weight of the insect [26]), and since most of the termite hindgut flagellates endocytose (and thereby sequester) wood particles as they enter the hindgut, it follows that VFA production in the hindgut should reflect mainly a protozoan fermentation of wood polysaccharides and be dominated by acetate. Acetate is the only detectable VFA produced during cellulose fermentation by mixed suspensions of hindgut protozoa from *Zootermopsis* termites (21, 22), as well as by axenic cultures of these forms

(56, 57), and a similar situation probably holds for cellulolytic protozoa from *R. flavipes*. Consistent with this interpretation is the drastic inhibition of acetogenesis in the hindgut of *R. flavipes* after defaunation (Fig. 4). Nevertheless, bacteria undoubtedly also produce acetate in situ. This inference is based on the moderate suppression of hindgut acetogenesis after *R. flavipes* termites were fed antibacterial drugs (Fig. 4), as well as the recognized ability of heterotrophic bacterial isolates to produce acetate (and C_1 and C_3 to C_5 VFAs) in pure culture (42, 43, 48) and in two-species cocultures (49). However, it is impossible at this time to ascribe the exact quantitative contribution of protozoa or bacteria to acetogenesis in situ, because the sum of the acetogenic activity of defaunated *R. flavipes* and bacteria-free *R. flavipes* is significantly less than the acetogenic activity of control termites (Fig. 4). Either one or both of the following explanations for this observation are possible. (i) The treatment used to remove protozoa or bacteria (exposure of termites to hyperbaric O_2 or to antibacterial drugs, respectively) has some deleterious effect on nontarget organisms; or (ii) acetogenesis in normal *R. flavipes* involves a synergistic interaction between hindgut protozoa and bacteria. Notwithstanding, it seems safe to conclude that protozoa dominate acetogenesis in *R. flavipes* hindguts, whereas bacteria are of secondary importance in this particular activity. Substrates for bacterial production of VFAs could include the small amount of soluble carbohydrate present in the wood itself (30), soluble intermediates secreted by the protozoa (9, 56) or liberated from wood by termite enzymes, or possibly CO_2 and H_2 (see below). True cellulolytic bacteria, i.e., bacteria capable of degrading crystalline cellulose, appear to be quantitatively insignificant in the hindgut of *R. flavipes* (48).

Results of our present studies with *R. flavipes* (family Rhinotermitidae) are consistent with Hungate's (22) model for mutualistic cellulose utilization which was derived from his studies with *Zootermopsis* species (family Hodotermitidae). First, protozoa appear to be primarily responsible for acetogenesis in the hindgut; second, rates of O_2 consumption by *R. flavipes* (52 to 64 nmol · termite^{-1} · h^{-1}) were approximately twice that of hindgut acetogenesis (20 to 43 nmol · termite^{-1} · h^{-1} for laboratory-maintained and freshly collected termites, respectively). Since 2 mol of O_2 is required for complete oxidation of acetate to $2CO_2$ and $2H_2O$, it appeared that 77 to 100% of the energy requirements of the termites could be met by oxidation of the acetate produced by the hindgut microbiota. In support of this interpretation was the demonstration of significant amounts of acetate in *R. flavipes* hemolymph, as well as the ability of termite tissues to readily respire acetate and other VFAs (Fig. 5; Table 3). We do not know why the rates of acetogenesis in the hindguts of freshly collected *R. flavipes* were consistently greater than those of laboratory-maintained specimens. However, it seems likely that the food on which the former were feeding before assay was more readily convertible to acetate (perhaps because it was partially decayed by fungi) than was the sound wood and paper towel mixture fed to laboratory specimens. Nevertheless, the ability of both cellulose and hemicellulose to serve as substrates for acetogenesis by the hindgut microbiota (Table 4) was in line with the high digestibility of these compounds, but not lignin, for *R. flavipes* (18).

Rates of O_2 consumption by *R. flavipes* reported herein (0.357 to 0.440 μl · mg of fresh weight^{-1} · h^{-1}) were similar to those of various other termites, as summarized by Peakin and Josens (39), as well as that determined by LaFage and Nutting (31) for *Marginitermes hubbardi*. By contrast, present values were considerably lower than most of those determined for *R. flavipes* by Damaschke and Becker (summarized in reference 39). The reasons for this discrepancy are not known. Rates of H_2 and CH_4 emissions by *R. flavipes* were also similar to those previously reported for various termites including *R. flavipes* and *R. tibialis* (8, 15, 31, 59). Although emission of such gases by termites might have a significant impact on our atmosphere globally (59), rates of H_2 and CH_4 emission by *R. flavipes* were only about 0.7% that of O_2 consumption. Consequently, overall carbohydrate oxidation in *R. flavipes* closely approximated the classical scheme: $100\ (CH_2O) + 100O_2 \rightarrow 100CO_2 + 100H_2O$. Assuming an oxycalorific equivalent of 5.05 mcal/μl of O_2 consumed (39), our respirometric data indicate that energy flow through normally faunated, feeding workers of *R. flavipes* would be 1.80 to 2.22 mcal · mg^{-1} · h^{-1} at 23°C.

If Hungate's model (22) for symbiotic cellulose degradation in lower termites is fundamentally valid, it must be amplified to accommodate those termite species that evolve relatively little H_2 and CH_4. For example, if symbiotic wood utilization in *R. flavipes* is envisioned to consist mainly of an anaerobic fermentation of glucan ($nC_6H_{12}O_6$) to acetate, CO_2, and H_2 by protozoa (Table 5, reaction A), followed by termite oxidation of acetate (Table 5, reaction B), then an appreciable amount of reducing equivalents, as H_2, should be evolved by termites (Table 5, reaction A + B). In fact, according to reaction A + B, rates of H_2 evolution should be equal to that of O_2 consumption and 66% that of CO_2 evolution, but they are almost always <1% of

TABLE 5. Possible steps in symbiotic dissimilation of glucan ($nC_6H_{12}O_6$) by *R. flavipes*[a]

Designation	Reaction
A	$nC_6H_{12}O_6 + 2nH_2O \rightarrow 2nCH_3COOH + 2nCO_2 + 4nH_2$
B	$2nCH_3COOH + 4nO_2 \rightarrow 4nCO_2 + 4nH_2O$
A + B	$nC_6H_{12}O_6 + 4nO_2 \rightarrow 6nCO_2 + 4nH_2 + 2nH_2O$
C	$4nH_2 + nCO_2 \rightarrow nCH_4 + 2nH_2O$
A + B + C	$nC_6H_{12}O_6 + 4nO_2 \rightarrow 5nCO_2 + nCH_4 + 4nH_2O$
D	$4nH_2 + 2nO_2 \rightarrow 4nH_2O$
E	$nCH_4 + 2nO_2 \rightarrow nCO_2 + 2nH_2O$
F	$4nH_2 + 2nCO_2 \rightarrow nCH_3COOH + 2nH_2O$
B + D or B + C + E	$2nCH_3COOH + 6nO_2 + 4nH_2 \rightarrow 4nCO_2 + 8nH_2O$
A + B + D or A + F + 1.5B	$nC_6H_{12}O_6 + 6nO_2 \rightarrow 6nCO_2 + 6nH_2O$

[a] See text for details.

those values. Furthermore, reaction A + B is inconsistent with the RQ of *R. flavipes*, which was 1.00 to 1.05. Several possibilities exist regarding alternate fates of reducing equivalents produced during glucan decomposition by *R. flavipes*. Interspecies transfer of H_2 to methanogenic bacteria might occur (Table 5, reaction C), and it probably does inasmuch as *R. flavipes* emits CH_4, but the combined reaction A + B + C (Table 5) predicts that CH_4 emission rates should be 20 to 25% that of CO_2 evolution and O_2 consumption, respectively. However, as for H_2 emission, CH_4 emission was <1% of those values, and the RQ predicted by reaction A + B + C is inconsistent with that observed. Consumption of H_2 by aerobic hydrogenotrophic bacteria (Table 5, reaction D) would yield an RQ of 1.00 (Table 5, reaction A + B + D), but implies that O_2 consumption should be threefold greater than hindgut acetogenesis (Table 5, reaction B + D) instead of the observed 1.5- to 2.4-fold. A similar argument can be leveled against the possibility of rapid formation (reaction C) and subsequent oxidation (Table 5, reaction E) of CH_4 (Table 5, reaction B + C + E).

The most likely fate of H_2 is depicted by reaction F (Table 5), i.e., the use of H_2 for the reduction of CO_2 to acetate. The overall dissimilation of glucan by *R. flavipes* is then envisioned to consist of anaerobic fermentation by hindgut protozoa (reaction A), coupled to anaerobic acetogenesis from CO_2 and H_2 by some member(s) of the hindgut microbiota (Table 5, reaction F), followed by aerobic oxidation of acetate by *R. flavipes* tissues (1.5 × reaction B). The sum of these reactions (Table 5, reaction A + F + 1.5B) would be consistent with the RQ and relative rates of O_2 consumption and hindgut acetogenesis of *R. flavipes*. Reaction F is not known to occur in eucaryotes, but is recognized in a few bacterial species such as *Acetobacterium* (2) and *Acetogenium* (32), and in certain species of *Clostridium* (7) and *Eubacterium* (19). Moreover, it is of interest that the cecal microbiota of rats, rabbits, and guinea pigs can effect a total synthesis of acetate from CO_2 and H_2 (46), although the specific bacteria have not yet been identified. Although conclusive proof for the existence of such reactions or organisms in *R. flavipes* hindguts is not yet available, the observed increase in H_2 and CH_4 emission by termites fed antibacterial drugs (see above) suggests that some type of procaryotes, other than methanogens, constitute important "electron-sink" organisms in the hindgut food web.

A working model for symbiotic utilization of wood polysaccharides by *R. flavipes* is depicted in Fig. 6. The central elements of Fig. 6 include a graphic representation of reactions A, F, and B (Table 5). Also depicted in this figure is the participation (to a lesser degree) of other heterotrophic bacteria in acetogenesis, either directly or indirectly through H_2 and CO_2 production (42, 43, 48, 49). As with Hungate's original model (22), the present model (Fig. 6) implies that defaunated termites should be able to survive on a cellulose-free diet if fed acetate. Although this has been tried previously (15, 23), as well as in the present study, negative results have been obtained. Perhaps the feeding of acetate salts to termites does not adequately mimic the in vivo situation in which acetic acid is continuously produced and absorbed from the hindgut. On the other hand, perhaps the cation component of the acetate salts has a deleterious effect on termite survival, as indicated in the present study. However, Fig. 6 suggests a novel way to circumvent the use of acetate salts in such survival experiments, i.e., the present model predicts that defaunated (or normally faunated) termites should be able to survive on a cellulose-free diet longer under an air atmosphere enriched with H_2 and CO_2 than under an atmosphere of air alone. A test of this prediction, as well as a scarch for acetogenic CO_2-reducing bacteria in *R. flavipes* hindguts, is currently under way in our laboratory.

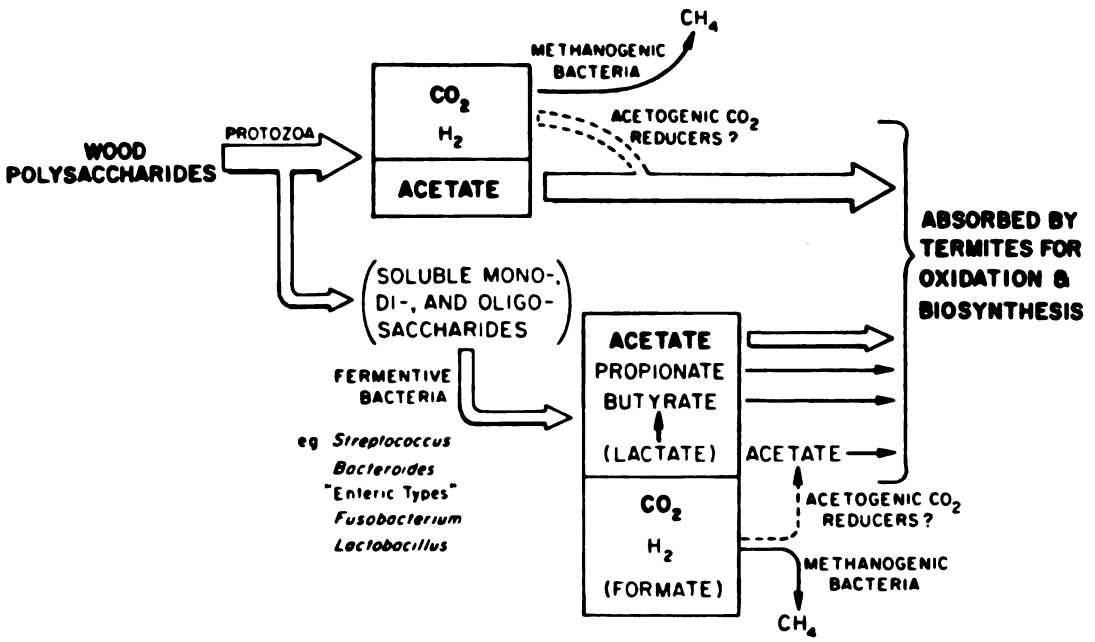

FIG. 6. Proposed working model for symbiotic wood polysaccharide dissimilation in *R. flavipes*. Thickness of arrows represents approximate relative contribution of the respective reactions to the overall dissimilatory pathway. Major products of the hindgut fermentation are indicated in boldfaced type; probable intermediates, which do not accumulate to detectable levels, are indicated in parentheses. (Reprinted from reference 10 by permission from the British Mycological Society.)

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ARTICLE II

NUTRITION AND GROWTH CHARACTERISTICS OF TRICHOMITOPSIS TERMOPSIDIS, A CELLULOLYTIC PROTOZOAN FROM TERMITES

BY

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ABSTRACT

Putatively axenic cultures of Trichomitopsis termopsidis 6057, isolated by M.A. Yamin (J. Protozool. 25:535-538. (1978)) from the hindgut of Zootermopsis termites, apparently contained methanogenic bacteria, inasmuch as small amounts of CH_4 were produced during growth. However, T. termopsidis could be "cured" of methanogenic activity by incubation in the presence of bromoethanesulfonate. Both the cured derivative (6057C) and the parent strain (6057) required $NaHCO_3$ and fetal bovine serum for good growth; the presence of yeast extract in media was stimulatory. Growth of both strains was markedly improved by substituting heat-killed cells of Bacteroides sp. JW20 (a termite gut isolate) for heat-killed rumen bacteria in media as a source of bacterial cell material. Heat-killed Bacteroides JW20 was the best of a number of bacteria tested, and under these conditions H_2 was a major protozoan fermentation product. Growth of T. termopsidis strains was further improved by co-cultivation in the presence of Methanospirillum hungatii. M. hungatii was the best of a number of H_2-consuming bacteria tested, and under these conditions CH_4, but not H_2, was produced, indicating interspecies transfer of H_2 between the protozoa and M. hungatii. Both strains of T. termopsidis used powdered, particulate forms of cellulose (eg. pure cellulose, corncob, cereal leaves) as fermentable energy sources, although powdered wood, chitin, or xylan supported little or no growth. Cells of the cellulose-forming coccus Sarcina ventriculi also served as a fermentable energy source, but these were used poorly as a source of bacterial cell material. The only substantial difference between T.

termopsidis 6057 and 6057C was that the latter grew poorly or not at all with rumen bacteria as a source of bacterial cell material. The improved growth of *T. termopsidis in vitro*, as described herein, should facilitate further studies on the cell biology and biochemistry of these symbiotic, anaerobic protozoa.

INTRODUCTION

One of the most fascinating examples of nutritional symbiosis is the association between phylogenetically lower termites and their hindgut microbiota, which includes dense and diverse populations of flagellated protozoa and bacteria. Early studies by Cleveland (10-13), Hungate (21-23), Trager (40,41), and others (19) indicated that many of the protozoa were cellulolytic and that their presence in the hindgut was critical to termite survival on a diet of sound wood or cellulose. It now seems certain that protozoa are the major (if not sole) agents of wood cellulose hydrolysis in the hindgut of lower termites, and our current understanding of this symbiosis, including the roles of bacteria in termite nutrition, has been presented in several recent reviews (2-4,30).

Despite the central role of protozoa in the nutrition of lower termites, our knowledge of their cell biology and biochemistry has remained meager. This is because for many years pure cultures have not been available. Consequently, many studies have been restricted to the morphology of these intriguing eukaryotes and their physical association with bacteria (5,38). The relatively few biochemical investigations which have been done have either employed mixed species of protozoa harvested directly from the termite hindgut, and which were undoubtedly contaminated to some degree with living bacteria (23), or have employed *in vitro* cultures in which living bacteria were known to be present (41). Recently, however, Yamin succeeded in obtaining axenic cultures of two cellulolytic species: *Trichomitopsis termopsidis* (47) and *Trichonympha sphaerica* (49). He showed that both

of these flagellates grew anaerobically and fermented cellulose to acetate, H_2, and CO_2, although their growth *in vitro* was generally poor. To facilitate further biochemical studies on these protozoa we examined the nutrition and growth characteristics of one of Yamin's isolates, *Trichomitopsis termopsidis*, in an effort to improve its growth rate and yield. The results of that endeavor constitute the substance of this paper. A companion paper reports on cellulolytic and other hydrolytic enzyme activities of *T. termopsidis* (32).

(Portions of this work were presented at the 83rd Annual Meeting of the American Society for Microbiology, 6-11 March 1983, New Orleans, LA, and at the Third International Symposium on Microbial Ecology, 7-12 August 1983, East Lansing, MI.)

MATERIALS AND METHODS

Organisms. Trichomitopsis termopsidis strain 6057 was obtained from M. A. Yamin (Rockefeller University, New York, NY). Bacteroides sp strain JW20, Bacteroides termitidis strain UAD-50, and Streptococcus lactis strain JW1 were previously isolated from the hindgut of Reticulitermes flavipes termites in our laboratory (35, 37). Bacteroides amylophilus strain H18, Bacteroides ruminicola strain 23, Eubacterium limosum strain RF, and Desulfovibrio sp. strain G11 were obtained from M.P.Bryant (U. of Illinois, Urbana, IL). Methanosarcina barkeri strain MS and Methanospirillum hungatii strain JF1 were obtained from R.S. Wolfe (U. of Illinois, Urbana, IL), and Bacteroides fragilis strain V479, Bacteroides ovatus strain V211, and Sarcina ventriculi strain AL-2 were obtained from R.B. Hespell (U. of Illinois, Urbana, IL).

Media and cultivation conditions. Strict anaerobic techniques (20) were employed for preparation of media and for cultivation and manipulation of cells. T. termopsidis was routinely grown under 100 % nitrogen (initial gas phase) in 18 x 150 mm serum tubes equipped with black rubber stoppers and crimped aluminum caps (1) and containing 10.0 ml of medium. The culture medium was slightly modified from that of Yamin (47) and contained (mM, unless indicated otherwise): K_2HPO_4, 10.8; KH_2PO_4, 6.9; KCl, 21.5; NaCl, 24.5; $CaCl_2$, 0.5; $MgSO_4$, 5.3; Pfennig's metal solution (26), 0.1 % (v/v); cellulose powder (Type 20, Sigma Chemical Co., St. Louis, Mo), 0.1 % (w/v);

glutathione (reduced form), 3.2; $NaHCO_3$, 9.5; yeast extract (ICN Biochemicals, Cleveland, Ohio), 0.2-0.4 % (w/v); heat inactivated fetal bovine serum (Gibco Laboratories, Grand Island, NY), 2.5 % (v/v); and heat-killed mixed rumen bacteria (RB; see below), 1×10^{-4} % (w/v). To prepare this medium, a basal solution containing the inorganic salts, cellulose powder, and glutathione was tubed under N_2 in 9.25 ml amounts and heat-sterilized. Remaining components were added to the sterile, cooled basal solution from filter-sterilized or heat-sterilized (i.e. RB) stock solutions. The pH of the medium prior to inoculation was 6.7-6.8, and inoculated tubes were incubated vertically and unshaken at 24-26° C. Additions to, or samples from, the tubes were made by using N_2-flushed, sterile syringes equipped with 22 g or 25 g hypodermic needles (25). Stock cultures of T. termopsidis were maintained by transferring 5 % (v/v) inoculum to fresh medium every 20-30 days. Culture purity was periodically verified by microscopic examination of wet mount preparations and by inoculation of cultures into BHIG broth (see below) amended with 0.3 % (w/v) cellobiose, under which conditions T. termopsidis does not grow.

RB were prepared according to the method of Yamin (47) and were stored under 100 % N_2 at -20° C until needed. The source of RB was a hay-fed, fistulated dairy cow. Stock suspensions of RB contained approximately 1.2×10^{10} cells/ml (equiv. 3.63 mg dry wt./ml). Individual bacterial strains tested as a replacement for RB were prepared in a similar manner, except that cell suspensions of S. ventriculi were also incubated for 5 min in an ultrasonic cleaner bath (Mettler Electronics) to disrupt large aggregates of the cocci.

B. amylophilus and B. ruminicola were grown at 37° C in medium # 10 as described by Caldwell and Bryant (6). Bacteroides sp. JW20, B. fragilis, B. ovatus, B. termitidis, and S. lactis were grown at 30° C in brain heart infusion broth (Difco) supplemented with 0.3 % (w/v) glucose and 0.05 % cysteine-HCl (BHIG, 37). S. ventriculi was grown at 30 C in MYA medium as described by Canale-Parola (7). E. limosum, M. hungatii, and M. barkeri were grown at 30 C in the basal medium as described by McInerney et al (26) under a gas phase of H_2:CO_2, 80:20; Desulfovibrio sp. G11 was grown under the same conditions except that the medium was supplemented with 0.3 % (w/v) Na_2SO_4. Enumeration of bacteria was done microscopically by using a Petroff-Hauser counting chamber.

Nutrition / Growth studies. Nutritional and growth characteristics of T. termopsidis were evaluated by determining their growth rate and/or cell yield, as well as gas production, in response to changes in medium composition or incubation conditions. Three to four replicates were done for each culture condition tested. Cell densities were determined by direct microscopic counts on samples drawn into 50 mm long rectangular glass capillary tubes (i.e. microslides; Vitro Dynamics Inc., Rockaway, NJ). The volume of each microslide was approximately 1.25 µl (Cat.# 5005) or 5 µl (Cat.# 5010), but was determined precisely for each lot by filling representative samples with a standard solution of ^{14}C-toluene and determining the radioactivity present.

Growth of T. termopsidis was also monitored semi-quantitatively by examining cells, directly in the culture tubes, with an inverted microscope. The method was less time-consuming than preforming direct cell counts (see above) and helped insure that accurate cell yield determinations were centered about the stationary phase of growth. This strategy thereby minimized unnecessary sampling of cultures and the risk of exposure of cells to oxygen.

Co-cultivation of T. termopsidis with viable bacteria was done by inoculating modified Yamin's medium with a 3 to 5 % (v/v) inoculum of each of T. termopsidis (eg. a 20-30 day culture containing about 8000 cells/ml) and the bacteria (eg. for M. hungatii a 3-5 day broth culture displaying an O.D. (600 nm) of 0.1).

Analysis of metabolic products. H_2 and CH_4 present in the headspace of T. termopsidis cultures were analyzed by gas chromatography. For H_2 a column of Molecular Sieve 5A (Waters Associates Inc.) was used with thermal conductivity detection (42). For CH_4 a column of Porapak N (Waters Associates Inc.) was used with H_2 flame ionization detection (34). Acetate present in cell-free culture fluids was determined enzymatically by measuring the oxidation of NADH in the presence of the following enzymes: acetylCoA sythetase; pyruvate kinase; myokinase; and lactate dehydrogenase (17). Reducing sugar was determined by the colorimetric ferricyanide assay with glucose as the standard (33). Absorbance readings were determined by using a Gilford spectrophotometer.

Cell free culture fluid was screened for the presence of organic acids other than acetate by using the method of Salanitro and Muirhead (36), and for the presence of ethanol by distillation of clarified culture fluid (29) followed by gas chromatographic analysis of the distillate on a column of Porapak Q with H_2 flame ionization detection (34). Clarified culture fluid was also screened for products of cellulose hydrolysis by using high pressure liquid chromatography as previously described (24).

Other procedures. Methanogenic bacteria were examined microscopically by using a Leitz epifluorescense microscope according to the procedure of Doddema and Vogels (16). Photomicrographs of T. termopsidis were taken by using a Zeiss GFL phase-contrast microscope equipped with a C-35 camera attachment and Tri-X (Kodak Co., ASA 400) film.

Substrates and chemicals. Cereal leaves (Sigma Cat.# C-7141), chitin (Sigma Cat.# C-3387), and Wiley-milled corncob (obtained from R.B. Hespell) were further dry ball-milled for 24-72 hours at ambient temperature to produce fine powder. Douglas fir wood powder was prepared as previously described (31). A crude mixture of cellodextrins (containing cellobiose to cellohexaose) was prepared by acid hydrolysis of cellulose powder followed by column chromatography (27). Coenzyme M was isolated from Methanobacterium bryantii and was a gift from R. L. Uffen. All other chemicals were of analytical reagent grade and were obtained from commercial sources. ^{14}C-toluene calibration standard was obtained from New England Nuclear, Boston,MA.

RESULTS

General growth characteristics and nutrition of T. termopsidis 6057.

T. termopsidis 6057 grew in modified Yamin's medium with a doubling time of 4.5 to 7.5 days and achieved final yields of 3800 to 7600 cells/ml after 30 days incubation. Growth of cells was accompanied by the production of acetate and H_2 (Fig. 1), and presumably CO_2(48). No other organic acids or ethanol were found. Surprisingly, cultures of T. termopsidis 6057 also evolved CH_4. Evolution of CH_4 accompanied that of H_2 during exponential growth, but subsided as cells approached stationary phase. By contrast, H_2 evolution continued during stationary phase (Fig. 1). Consequently, the final concentration of H_2 was generally 7 to 10-fold greater than that of CH_4. The amount of CH_4 evolved was not increased by supplementing the initial gas phase of cultures with 80 % H_2 or by incorporation of up to 5 μM coenzyme M in the medium. The probable origin of CH_4 in cultures of T. termopsidis 6057 is discussed below.

With the culture conditions employed (static, vertical culture tubes), T. termopsidis settled to the bottom and grew on and amongst a soft pellet of cellulose particles and RB. Incubation of cultures horizontally, with or without agitation, was detrimental to growth. Consequently, since agitation was required to resuspend cells uniformly for making direct cell counts, each point on the curves in Fig. 1, 2, and 3 was the mean value of 3-4 independent, and different, cultures. All values were within 5-10 % of the mean.

Figure 1.

Growth of T. termopsidis 6057 in modified Yamin's medium at 25° C. Each point is the mean value of 3-4 independ- ent, and different, cultures.

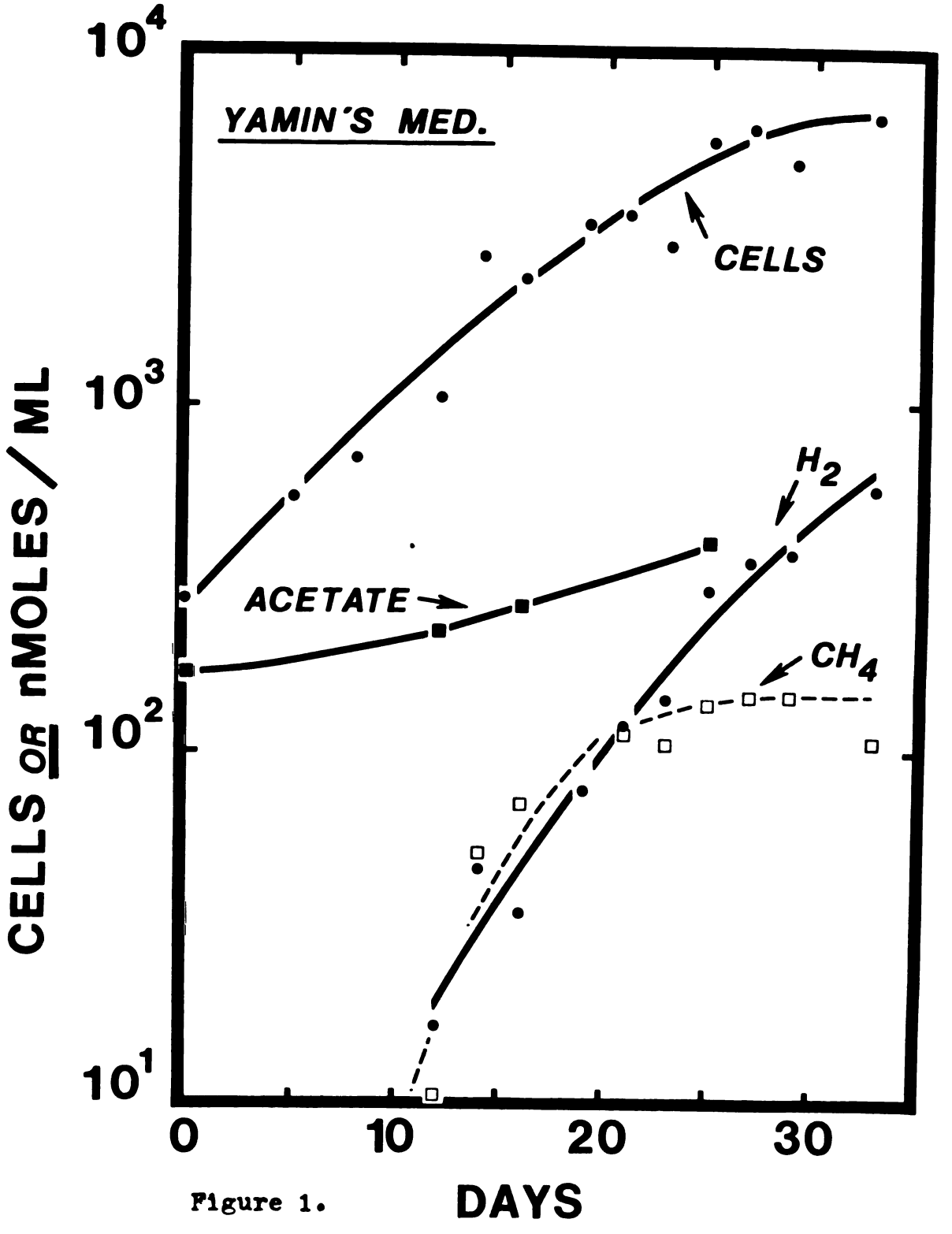
YAMIN'S MED.
CELLS
H_2
ACETATE
CH_4
10^4
10^3
10^2
10^1
CELLS OR nMOLES / ML
0
10
20
30
DAYS

Figure 1.

A significant improvement in growth of strain 6057 was achieved by replacing RB with heat-killed cells of Bacteroides strain JW20, a strain originally isolated from termite guts (37). By using Bacteroides JW20 cells at a concentration of 0.1-0.2 mg dry wt./ml of medium, T. termopsidis grew with a doubling time of 4.0 to 4.5 days and reached final yields of 10,400 to 31,900 cells/ml (Fig. 2). Cell-free filtrates of heat-killed Bacteroides JW20 could not replace the cells themselves. However, when Bacteroides JW20 was used in place of RB little or no CH_4 ($\ll$1 nmole/ml culture) was evolved by protozoan cultures, and H_2 was the only reduced gas formed. Even more striking was the observation that T. termopsidis could be passed through more than 10 transfers in this medium (approx. 1 year) without producing CH_4, but would resume methanogenesis immediately if shifted back to a medium containing RB.

Origin of methanogenesis in cultures of T. termopsidis 6057.

Methane emision by T. termopsidis 6057 was entirely unexpected, inasmuch as methanogenesis is a bacterial phenomenon (45) and Yamin (47) reported the original cultures of T. termopsidis to be free of living bacteria. Nevertheless, our results suggested that cultures of strain 6057 harbored a methanogenic consort, since uninoculated media, as well as heat-sterilized cultures, did not produce methane. However, epifluorescence microscopic examination of living or glutaraldehyde-fixed cultures failed to reveal F420 fluorescent cells (i.e. putative methane bacteria) associated with the protozoa or free in the culture fluid. By contrast, F420 fluorescent cells were readily observed in bovine rumen contents and by examination of pure

Figure 2.

Growth of T. termopsidis 6057 in modified Yamin's medium with heat-killed cells of Bacteroides sp. JW20 in place of heat-killed rumen bacteria. Other conditions were as for Figure 1.

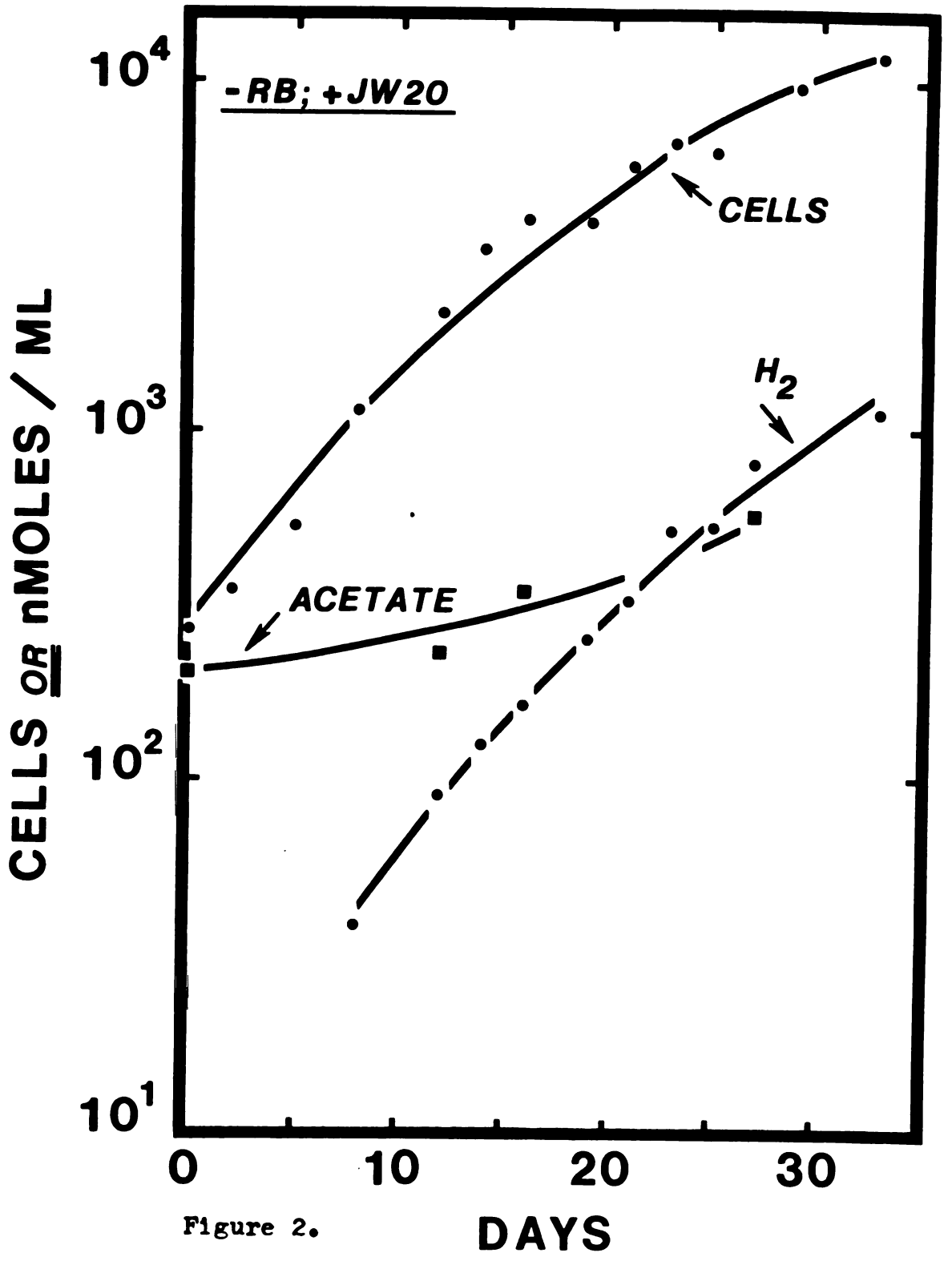
-RB; +JW20
CELLS
H_2
ACETATE
CELLS OR nMOLES / ML
10^4
10^3
10^2
10^1
0
10
20
30
DAYS

Figure 2.

cultures of methane bacteria (M. hungatii and M. barkeri). Moreover, we were unable to successfully enrich for, or isolate, methanogens from cultures of T. termopsidis 6057 by using a variety of substrates (H_2 + CO_2; acetate; formate; or methanol), although methanogens were readily isolated from termite gut homogenates and from putatively axenic cultures of Trichonympha sphaerica (another cellulolytic protozoan isolated from termite hindguts, (49)) by these same procedures (Odelson and Breznak, unpublished).

Methanogenesis by T. termopsidis 6057 could be permanently abolished, however, by growth of cells in the presence of 0.5 μM bromoethanesulfonate (BES), a potent and specific inhibitor of methanogenesis (50). As little as one transfer of cells in medium containing BES was sufficient to accomplish this. The resulting culture was then designated 6057C, the letter "C" denoting the fact that cells were "cured" of their putative methanogenic consort. Strain 6057C grew poorly, however, in culture medium with RB as the source of heat-killed bacteria; cell yields reached only 500-1000 cells/ml. Replacement of RB with heat-killed Bacteroides JW20 improved the growth of strain 6057C in much the same way as it did for the uncured parent strain (6057). With Bacteroides JW20, strain 6057C exhibited a doubling time of 2.6 to 5.2 days and achieved yields of 5,600 to 22,000 cells/ml.

Having established suitable growth conditions for T. termopsidis 6057C, we sought to extend the nutritional studies initiated by Yamin (47) and to compare, where possible, the nutritional characteristics of strain 6057C to those of strain 6057.

Evaluation of soluble medium constituents and heat-killed bacteria.

T. termopsidis 6057C required fetal bovine serum and $NaHCO_3$ for good growth (Table 1). No significant difference in cell yields (at $p \leq 0.05$) was observed by omission of yeast extract. However, cell yields in the absence of yeast extract were significantly lower than that of the control at $p \leq 0.1$ level (Student's T test). Therefore, yeast extract was judged to be stimulatory. Strain 6057C grew poorly or not at all if heat-killed cells of Bacteroides JW20 were omitted from the medium, and heat-killed cells of B. termitidis, B. amylophilus, B. fragilis, B. ovatus, B. ruminicola, or S. lactis, could not replace Bacteroides JW20. Hydrogen production by strain 6057C closely paralleled cell yields (Table 1). Similar results were obtained with strain 6057.

Evaluation of insoluble polysaccarides.

Insoluble polysaccharides, in both purified and natural forms, were tested for their ability to support growth of T. termopsidis 6057C (Table 2). Sigma type 20 cellulose powder supported the best growth and hydrogen production . Whatman microgranular cellulose, powdered corncob, and powdered cereal leaves also supported growth, but to a lesser extent. Powdered chitin and powdered wood supported little or no growth of the protozoa, and xylan appeared detrimental. Soluble intermediates or products of cellulose hydrolysis (i.e. glucose, cellobiose, cellodextrins), or 10 mg discs of cellulose filter paper (Whatman #1), could not replace powdered particulate forms of cellulose as a fermentable growth substrate (data not shown). Similar results were obtained with T. termopsidis 6057.

Table 1

Effect of medium components on growth of T. termopsidis strain 6057C.

	Yield [2]	
Omitted from Medium[1]	Cells/ml	nMoles H_2/ml culture
No Omission (Control)	5600 ± 1100	467 ± 23
Yeast Extract	2700 ± 1400	249 ± 102
$NaHCO_3$	400 ± 400	94 ± 38
Fetal Bovine Serum	<200	0
Bacteroides JW20	<200	0

[1]Growth medium was modified Yahin's medium containing heat-killed Bacteroides JW20 in place of heat-killed rumen bacteria. The initial pH of all media was 6.7 ± 0.2.

[2]Determined 30 days after the second transfer in the medium. Values are the mean ± SEM (n=4).

Table 2

Growth of T. termopsidis strain 6057C on insoluble polysaccharides.

Polysaccharide Tested[1]	Yield[2] Cells/ml	Yield[2] nMoles H_2/ml Culture
Sigma Type 20 Cellulose	6100 ± 1600	601 ± 41
Whatman Microgranular Cellulose	2900 ± 700	396 ± 104
Corncob	3400 ± 900	439 ± 40
Cereal Leaves	1700 ± 100	208 ± 73
Wood	900 ± 300	251 ± 50
Chitin	700 ± 200	83 ± 50
Xylan	0	0
No Substrate	600 ± 600	69 ± 43

[1] Growth medium was modified Yamin's medium containing heat-killed Bacteroides JW20 in place of heat-killed rumen bacteria. Polysaccharides were incorporated in the medium at a final concentration of 0.1 % (w/v).

[2] Determined 30 days following inoculation into test medium. Values are the mean ± SEM (n=4).

Co-culture of T. termopsidis with known H_2-consuming bacteria.

A curious feature of T. termopsidis 6057C, and of strain 6057 growing with heat-killed Bacteroides JW20 in place of RB, was the fact that H_2 appeared to be the only reduced end product formed (eg. see Fig. 2). The final molar ratio of H_2:Acetate was approximately 2:1, and no other volatile fatty acids, lactate, succinate, or ethanol were detected in culture fluid. It seemed likely that some of this H_2 was derived from the oxidation of reduced pyridine nucleotides (eg. NAD(P)H + H^+), a reaction thermodynamically unfavorable at $pH_2 > 10^{-3}$ atm (46). Therefore, on the notion that H_2 accumulation might be suppressing the growth of T. termopsidis, cells were grown in the presence of a variety of potential electron acceptors. However, no stimulation of growth was observed by including in the medium oxaloacetate, fumarate, KNO_3, fructose, triphenyltetrazolium chloride, or neutral red (singly at 4 mM final concentration). Moreover, growth of cells was completely inhibited by as little as 0.01 atm O_2.

Attempts were then made to keep the pH_2 low by co-cultivation of T. termopsidis with anaerobic, H_2-consuming bacteria. Co-cultivation of T. termopsidis 6057C (in modified Yamin's medium containing heat killed Bacteroides JW20) with viable M. hungatii increased protozoan yields to 25,000 to 46,000 cells/ml, but gave little change in doubling time (3.6 to 4.2 days). As expected, CH_4, but not H_2, was evolved, and the final molar ratio of CH_4:acetate was approximately 1:2. Similar results were obtained with strain 6057, which displayed final yields of 58,000 to 75,000 cells/ml and a doubling time of 2.2-5.3 days (Fig. 3). Growth of, and methanogenesis by, M. hungatii was

Figure 3.

Growth of T. termopsidis 6057 in co-culture with Methanospirillum hungatii (Msp). Growth medium and conditions were as for Figure 2.

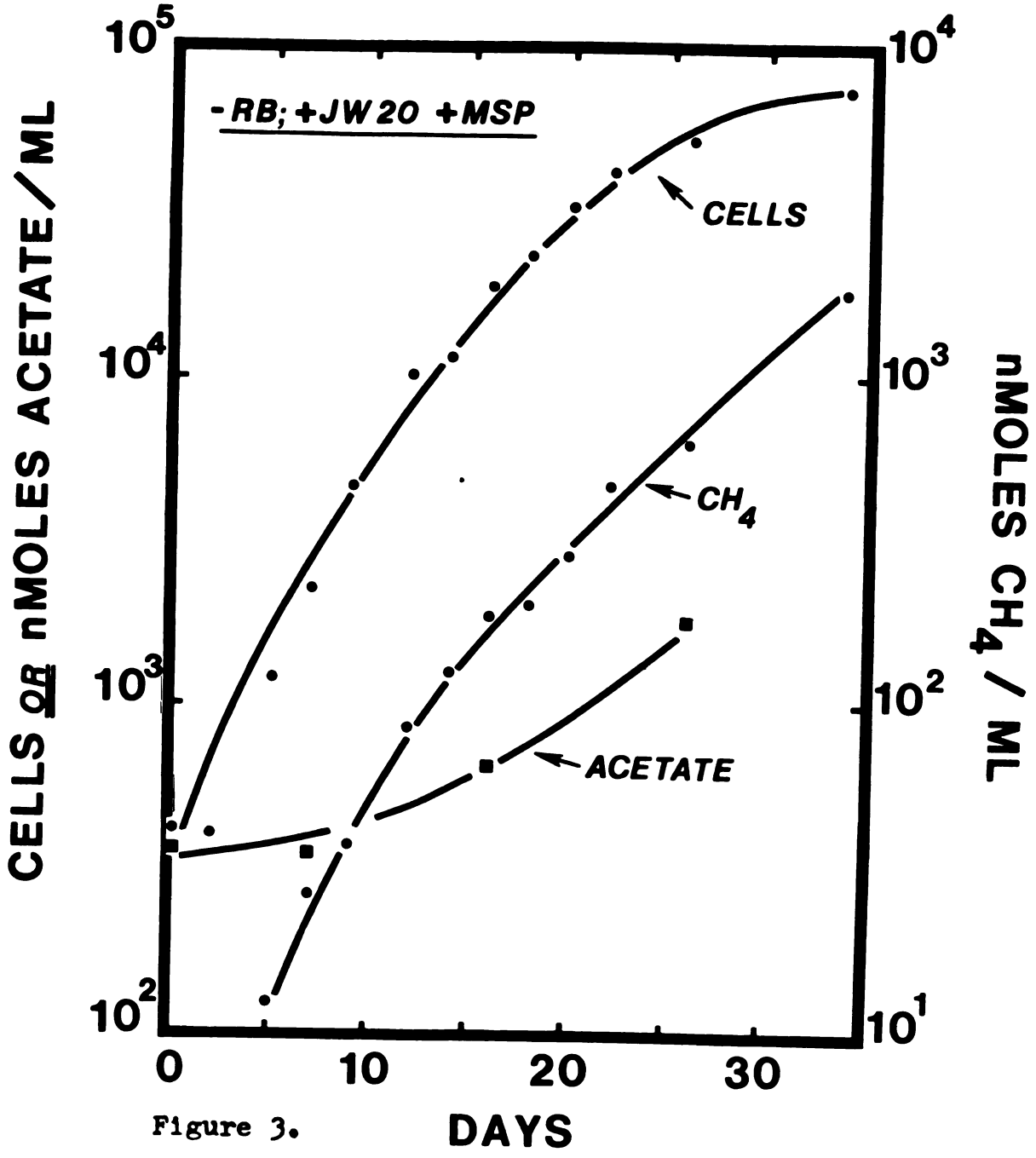
-RB; +JW20 +MSP
CELLS
CH_4
ACETATE
10^5
10^4
10^3
10^2
10^4
10^3
10^2
10^1
0
10
20
30
DAYS
CELLS OR nMOLES ACETATE / ML
nMOLES CH_4 / ML

Figure 3.

dependent on growth of the T. termopsidis strains; and heat-killed cells of M. hungatii did not improve growth of the protozoa. Co-cultivation with M. hungatii did not alter the nutritional requirements of T. termopsidis strains for soluble medium constituents, heat-killed bacteria, or fermentable polysaccharides as described above.

Co-cultivation with Methanosarcina barkeri also improved growth of T. termopsidis strains, but not to the same extent as did M. hungatii, probably because M. barkeri did not scavenge all of the H_2 produced by the protozoa. By contrast, co-culture of T. termopsidis with Eubacterium limosum (an H_2-CO_2-utilizing acetogen (18)) did not improve protozoan growth, and co-culture with Desulfovibrio G11 appeared to suppress growth, possibly because of accumulation of toxic levels of H_2S.

Co-cultures of T. termopsidis with M. hungatii grew best with the reducing agent normally present in modified Yamin's medium (i.e. glutathione). Replacement of glutathione with cysteine-HCl (0.05 %, w/v), a combination of cysteine-HCl and Na_2S (0.025 % each, w/v), or dithiothreitol (1.5 mM) inhibited growth of T. termopsidis in co-culture.

Glucose, cellobiose, or cellodextrins (or an increase in total reducing sugars) was not detected in extracellular fluid during growth of T. termopsidis.

Growth of T. termopsidis on Sarcina ventriculi.

In an effort to identify a single particulate substrate that would serve as both a fermentable energy source (i.e. cellulose) and a source of bacterial cell material, attempts were made to grow T. termopsidis on heat-killed cells of the packet-forming coccus Sarcina ventriculi. S. ventriculi was chosen because it synthesizes cellulose as a uniform, outer layer that accounts for as much as 19 % of the cells' dry weight (8, 9). In co-culture with viable M. hungatii, T. termopsidis 6057C used S. ventriculi simultaneously as a fermentable energy source and as a source of bacterial cell material (Table 3). However, final yields of T. termopsidis under these conditions (1500 cells/ml) were too low to be of practical use. This appeared due mainly to the fact that S. ventriculi was inferior to Bacteroides JW20 as a source of bacterial cell material (Table 3). By contrast, S. ventriculi was entirely effective as a replacement for powdered cellulose alone (in the presence of heat-killed Bacteroides JW20) and significantly ($p \leq 0.05$, Student's T Test) boosted cell yields of T. termopsidis 6057C when added to the complete medium (Table 3). The data also suggested that cell carbon from S. ventriculi could ultimately be used as a substrate for methane formation by M. hungatii. Phase contrast microscopy revealed that T. termopsidis endocytosed packets of the sarcinae; appeared to hydrolyze the outer cellulose layer; then apparently expelled S. ventriculi as single or doublet, denuded (phase dark) cells which accumulated in the medium during growth of the protozoa (Fig. 4).

Table 3

Growth of T. termopsidis 6057C on heat-killed cells of S. ventriculi.

Omitted from Medium[1]	Added to Medium	Yield[2] Cells/ml	Yield[2] nMoles CH_4 ml culture
--	-- (Control)	36,400 ± 10,400	1482 ± 139
Cellulose	SV (1.0 mg dry wt. x ml^{-1})	34,200 ± 7400	1010 ± 134
JW20	SV (0.1 mg dry wt. x ml^{-1})	4300 ± 1200	182 ± 36
Cellulose and JW20	SV (1.0 mg dry wt. x ml^{-1})	1500 ± 1500	109 ± 16
--	SV (0.1 mg dry wt. x ml^{-1})	51,800 ± 2500	1463 ± 86
Cellulose	--	400 ± 200	157 ± 16
JW20	--	200 ± 200	33 ± 12

[1] Protozoa were grown in co-culture with M. hungatii in modified Yamin's medium containing heat-killed Bacteroides JW20 in place of heat-killed rumen bacteria. Symbols: SV, Sarcina ventriculi; JW20, Bacteroides JW20.

[2] Determined after 31 days incubation. Values are the mean ± SEM (n=4).

Figure 4.

Phase contrast photomicrograph of a cell of T. termopsidis 6057C growing in the presence of heat-killed S. ventriculi. Note the cellulose-containing (refractile) sarcinae (black arrows) outside, as well as within, the protozoan, and the phase-dark (presumably cellulose-depleted) sarcinae (white arrows). The latter accumulate in the medium during growth of T. termopsidis.

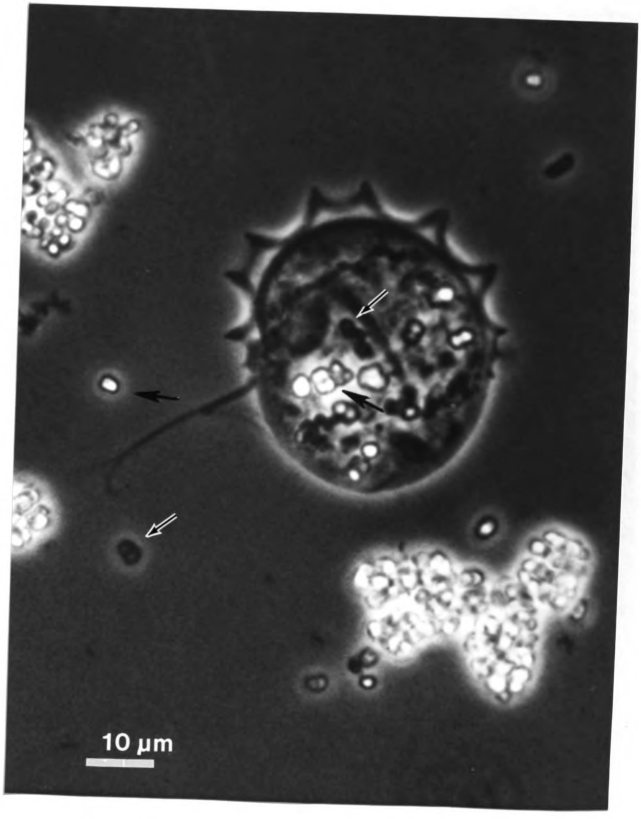

Figure 4.

DISCUSSION

In vitro growth of *T. termopsidis* strains in modified Yamin's medium was markedly improved by replacement of the heat-killed rumen bacteria component with heat-killed cells of *Bacteroides* sp. strain JW20 (a termite gut isolate (37)), and by co-cultivation in the presence of an H_2-consuming methanogen, *M. hungatii*. Under these conditions, the growth yield of *T. termopsidis* was increased from about 7600 cells/ml to 75,000 cells/ml, and the doubling time was decreased from 4.5 days to 2.2 days. Fetal bovine serum and $NaHCO_3$ were found to be required for good growth, and yeast extract was judged to be stimulatory. Nutritional and growth characteristics of *T. termopsidis* 6057 (the parent strain) were similar to those of the strain 6057C (the cured derivative), except that the latter showed little or no growth with mixed rumen bacteria as a source of bacterial cell material.

Our results are consistent with those of Yamin (47) who showed that *T. termopsidis* requires bacteria in media as a nutrient. However, like Yamin found, not all bacteria were nutritionally equivalent. In fact, of a number of *Bacteroides* strains tested in this study, only strain JW20 was capable of supporting growth of *T. termopsidis*. A decided preference for certain bacteria has also been observed for aerobic (39) as well as other anaerobic protozoa (15).

The precise role of bacterial cell material in the nutrition of *T. termopsidis* is not yet known. Our results suggest that even bacteria of the same genus (i.e *Bacteroides*) differ significantly in their intrinsic nutritive value for *T. termopsidis*. However, it may

also be that some bacterial strains are simply more easily endocytosed than others, and are therefore more readily available for intracellular metabolism. Nevertheless, while endocytosis of bacteria may be a necessary prerequisite for their utilization by T. termopsidis, it is not alone sufficient: Sarcina ventriculi cells were readily endocytosed by the protozoa (Fig. 4), and were used as a fermentable source of cellulose, but S. ventriculi was a rather poor replacement for Bacteroides JW20 cell material (Table 3).

Our results confirm and extend Yamin's (47) observation that a particulate source of cellulose is required as a fermentable energy source by T. termopsidis. Powdered celluloses, corncob, or cereal leaves supported growth, whereas chitin or xylan alone did not. Soluble intermediates of cellulose hydrolysis (i.e. cellodextrins) were also ineffective. Surprisingly, wood, the presumed natural substrate for T. termopsidis in situ, was used poorly or not at all. Either one or a number of the following explanations for this observation are possible: (i) heat-sterilization of wood particles adversely affects their utilization by T. termopsidis; (ii) the suitability of wood as a substrate for T. termopsidis depends on physical and/or chemical (i.e. enzymatic) pre-treatment, such as might be afforded by passage through the termite gut; or (iii) most of the wood particles obtained by ball-milling were still too large for efficient endocytosis by T. termopsidis. In this regard it is pertinent to note that mixed cultures of rumen protozoa cannot grow on wheat grain that has been sterilized by heat or by treatment with

ethylene oxide (14). Moreover, it should be emphasized that T. termopsidis must endocytose cellulosic materials to digest them: no growth was obtained if pure cellulose was included in media as 1 cm discs of filter paper.

The apparent presence of methanogenic bacteria in Yamin's original culture of T. termopsidis (strain 6057) was unexpected, but perhaps not surprising. Methane bacteria (identified by their fluorescence when illuminated with ultraviolet light) are commonly observed on and within cells of rumen protozoa (44) and sapropelic protozoa (43), and their presence in termite guts is indicated by the fact that numerous termite species evolve methane (2) and such bacteria can be isolated from termite guts (Reticulitermes flavipes; Odelson and Breznak, unpublished). Moreover, the treatment used by Yamin (47) to render T. termopsidis 6057 putatively "bacteria-free" involved incubation of protozoa in the presence of penicillin and streptomycin. These drugs, while effective against many eubacteria, are ineffective against methanogens which are archaebacteria (1). That we failed to successfully enrich for or isolate methanogens from T. termopsidis cultures, and also failed to observe methanogens by fluorescence microscopy of protozoan cultures, suggests that the methanogens are present in small numbers in cultures of T. termopsidis 6057 and may also require for growth a nutrient provided by the protozoa. Indeed, the long term persistence of methanogens in cultures of T. termopsidis 6057 grown on Bacteroides JW20, during which incubation no methane is detected, leads one to speculate that the endogenous methanogens may be capable of functioning in part as intracellular "energy parasites" similar to chlamydiae (28).

Nevertheless, T. termopsidis 6057 could be completely and permanently cured of methanogenic activity (and presumably of the methanogenic cells as well) by cultivation in the presence of bromoethanesulfonate, a potent and specific inhibitor of methanogenesis (50).

At the present time, we do not know what substrate is used by the endogenous methanogen of T. termopsidis 6057 for methanogenesis, but it is probably H_2 + CO_2, or acetate, or a mixture of these, since these are the only significant products formed by T. termopsidis from cellulose fermentation. In any case, hydrogen was the major or sole reduced end product of strain 6057 or strain 6057C, respectively. This observation suggested that some of the hydrogen produced by T. termopsidis was derived from reduced pyridine nucleotide via NAD(P)H + $H^+ \longrightarrow$ $NAD(P)^+$ + H_2, a reaction thermodynamically unfavorable at $pH_2 > 10^{-3}$ atm (46). Accordingly, this prompted an attempt to improve the growth of T. termopsidis by making cellulose fermentation more favorable energetically (i.e. by keeping the pH_2 low through co-cultivation with H_2-consuming bacteria). This was successful, and M. hungatii proved to be the most effective of all H_2-consumers tested. Under these conditions CH_4, but no H_2, was produced. To our knowledge, this is the first in vitro demonstration of H_2 transfer between protozoa and methanogens, and it suggests that interspecies transfer of hydrogen between protozoa and bacteria is important in "pulling" anaerobic decomposition of cellulose in the hindgut of lower termites.

It is difficult to predict whether T. termopsidis might ultimately prove useful in anaerobic bioconversion schemes designed to produce food, fuels or chemical feedstocks from lignocellulosic substrates. Nevertheless, the improved growth of T. termopsidis in vitro, as described herein, should facilitate further studies on the cell biology and biochemistry of these unique eukaryotes. This is exemplified in a companion paper (32) which reports on studies of cellulase and other hydrolytic enzyme activities of this symbiotic protozoan.

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ARTICLE III

CELLULOLYTIC AND OTHER POLYMER-HYDROLYZING ACTIVITIES OF *TRICHOMITOPSIS TERMOPSIDIS*, A SYMBIOTIC PROTOZOAN FROM TERMITES.

By

D. A. Odelson and J. A. Breznak

ABSTRACT

Crude extracts of the anaerobic, cellulolytic protozoan Trichomitopsis termopsidis possessed endo-β-1,4-glucanase and cellobiase activities, as evidenced by hydrolytic action on carboxymethyl cellulose and cellobiose, respectively. Cell extracts also hydrolyzed microcrystalline cellulose, although the extent of saccharification of this substrate was only 19.4 % of the theoretical maximum, suggesting that cells may lack a complete cellulase enzyme system. Hydrolysis of microcrystalline cellulose displayed optima at pH 5 and at 30° C, and glucose was the sole product liberated. Cellulolytic activities of T. termopsidis appeared to be entirely cell-associated. Hydrolytic activity was also detected against Douglas fir wood powder, xylan, starch and protein, but not chitin. The importance of these enzymes in the nutrition of T. termopsidis is discussed in terms of their natural habitat (the hindgut of wood-eating termites).

INTRODUCTION

Phylogenetically lower termites depend on dense and diverse populations of hindgut protozoa and bacteria in order to thrive by xylophagy, and our current understanding of this nutritional symbiosis has been recently reviewed (4-6,19). Among this heterogeneous microbial community it appears that anaerobic, flagellate protozoa are the major, if not sole, agents of wood cellulose hydrolysis: they are not only abundant in the hindgut, but they also have the ability to endocytose, and thereby sequester, wood particles for intracellular degradation. Major products of cellulose fermentation by mixed and axenic suspensions of the protozoa include H_2, CO_2, and acetate (12,13,21,29,30), the latter compound serving as a major oxidizable energy source for termites (20), as well as constituting an important biosynthetic precursor for the insect (2,3,17,24).

Our knowledge of the cell biology and biochemistry of termite gut protozoa is still in its infancy, having been hampered for many years by the lack of pure cultures for detailed study. However, a major breakthrough was recently made by Yamin, who reported the axenic cultivation of two species (Trichomitopsis termopsidis (28) and Trichonympha sphaerica (30)) and who studied some of their metabolic activities (31).

In a companion paper (21) we reported our studies on the nutrition and growth characteristics of one of Yamin's isolates, T. termopsidis, and our methods for increasing the cell yields of T. termopsidis in vitro. In the present study, we capitalized on the

improved growth characteristics of T. termopsidis to obtain cells for biochemical studies. Herein we report on cellulase and other polymer-hydrolyzing enzyme activities in cell extracts of T. termopsidis.

MATERIALS AND METHODS

Organisms. T. termopsidis strain 6057C was used throughout this study and was obtained by bromoethanesulfonate treatment of strain 6057, as previously described (21). The sources of, and cultivation methods for, Bacteroides JW20 and Methanospirillum hungatii JF1 are given in a companion paper (21).

Cultivation of protozoa. T. termopsidis was grown under anaerobic conditions in modified Yamin's medium containing heat-killed cells of Bacteroides sp. JW20 (0.1 mg dry wt./ml, final concentration) in place of heat-killed rumen bacteria, and in co-cultivation with M. hungatii JF1 as previously described (21). For large volumes, bottles (Wheaton borosilicate glass, Type 400) were used containing 500 ml of medium and were sealed with a butyl rubber stopper. The medium for bottle cultures was prepared in a similar fashion to that used for tube cultures (21), except that glutathione was added to the sterile medium from a separate heat-sterilized stock solution. Growth of co-cultures of T. termopsidis and M. hungatii was monitored by periodic analysis of headspace gas for methane by using gas chromatography (21). Agitation of bottle cultures was completely avoided, or kept to a minimum, as shaking retards the growth of T. termopsidis (21).

Preparation of cell extracts. Bottle cultures of T. termopsidis were harvested when protozoan densities reached 2-3 x 10^4 cells/ml. Generally, the contents of 3 bottles were pooled (ca. 1.5 l total volume), and cells were collected by centrifugation in air-tight centrifuge bottles (Sorvall, cat. no. 03256; equipped with caps, cat. no. 03278) at 1200 x g for 20 min at 10° C. The resulting pellets, consisting mainly of T. termopsidis and unused cellulose particles, were resuspended in a small volume of phosphate-buffered salts solution (see below), pooled, and then layered onto 100 ml of a 20 % (w/v) solution of Ficoll prepared in phosphate-buffered salts. After 30 min, most of the undigested cellulose particles descended into the Ficoll layer. The layer above the Ficoll was then collected by aspiration and re-centrifuged at 1200 x g for 20 min at 10° C. The resulting pellet was again resuspended in phosphate-buffered salts solution, centrifuged as described above, and the final pellet was resuspended in 1-2 ml phosphate-buffered salts solution at 4° C. Cell densities were then determined by direct microscopic counts (21). By using this procedure, recovery of T. termopsidis was approximately 30-50 %. However, the final suspension was highly enriched in T. termopsidis, with only minor contamination from cellulose particles or bacterial cells.

Cell extracts were prepared by either addition of Triton X-100 (2 %, v/v, final concentration) to the cell suspension, or by freezing and thawing. The latter was done by immersion of the cell suspension in a dry ice-acetone bath for 3 min, followed by incubation at room temperature until completely thawed. Both procedures resulted in virtually 100 % lysis of protozoa, as judged microscopically. Cell

extracts were then centrifuged at 12000 x g at 4°C for 20 min and separated into a soluble (i.e. supernatant fluid) and particulate (i.e. pellet) fraction.

All manipulations of cells and cell extracts were performed under O_2-free N_2, by using strict anaerobic techniques (11). The phosphate-buffered salts solution used in the above procedures was prepared under anaerobic conditions and contained (mM): K_2HPO_4, 10.8; KH_2PO_4, 6.9; NaCl, 24.5; KCl, 21.5; and dithiothreitol, 1.0. The final pH of this solution was 7.0.

Enzyme assays. Unless indicated otherwise, all assays were performed at 30°C, and determinations of cellulase activities generally followed the recommendations of Ghose et al (10). "Cellulase" (i.e. the complete enzyme system (10,16)) and endo-β-1,4-glucanase (EC 3.2.1.4) were assayed by measuring release of reducing sugar from microcrystalline cellulose (Sigma Chemical Co.; Type 20, 5.0 %, w/v, final concentration) and carboxymethylcellulose (Hercules Inc., Type 7MF; 0.5 %, w/v, final concentration), respectively, in 0.2 M acetate buffer (pH 5). Reaction mixtures (0.1 ml final volume) were run in triplicate in Eppendorf conical centrifuge tubes (1.5 ml cap.) and were terminated by the addition of 0.025 ml 1 N HCl. Following neutralization with 0.025 ml of 1 N NaOH, samples were centrifuged at 11,310 x g for 5 min and the supernatant fluids were analyzed for reducing sugar by the method of Park and Johnson (22) or Bernfeld (1). In addition, reaction mixtures for the complete cellulase enzyme system were screened for specific products

and intermediates of cellulose hydrolysis (i.e glucose, cellobiose, and cellodextrins) by subjecting supernatant fluids to high pressure liquid chromatography (15). The extent of saccharification of cellulose was estimated by using the prorocedures described above, but with less microcrystalline cellulose in reaction mixtures (0.04 %, w/v, final concentration) as suggested by Johnson et al (14). Cellobiase (EC 3.2.1.21) was assayed by using reaction mixtures similar to those described above, but by measuring release of glucose from cellobiose (1 %, w/v, final concentration) with glucose oxidase (26). In general, reaction mixtures were incubated for (hr): the complete cellulase enzyme system, 24; carboxymethyl cellulase, 0.5-1; and cellobiase, 1-3. One unit of activity was defined as the release of 1 μmole of glucose equivalent per minute.

To determine other hydrolytic activities of T. termopsidis, ball-milled Douglas fir wood powder (20), xylan (Sigma cat. no. X-3875), or ball-milled chitin (21) was substituted for microcrystalline cellulose, and reaction mixtures were assayed for reducing sugar as described above. Protease was assayed by using azocasein as substrate as described by Brock et al (7), and β-amylase was assayed by using potato starch as substrate according to the method of Bernfeld (1).

Estimation of protozoan cell protein. It was virtually impossible to separate T. termopsidis cells completely from bacterial cells (see above), yet still recover enough protozoa to prepare a suitable cell extract. Consequently, enzyme activities are reported

herein as mUnits/10^6 T. termopsidis cell equivalents, determined by direct cell counts of suspensions prior to extract preparation. However, a numerical factor could be calculated for converting protozoan cell equivalents to mg protozoan specific protein by the knowledge that T. termopsidis cells were approximately spheres 40 ± 5 μm in diameter (see Figure 4 of Ref. #21), and assuming: their density was 1.0; their water content was 80 %; and protein constituted 50 % of the cell's dry weight. By using these assumptions, it was estimated that 10^6 cells of T. termopsidis were equivalent to 3.4 mg protein.

Chemicals. Cellodextrins (cellobiose through cellohexaose) were prepared by acid hydrolysis of cellulose followed by column chromatography of the hydrolysate (18). All other chemicals were of reagent grade and were obtained from commercial sources.

RESULTS

Cell extracts of T. termopsidis possessed enzyme activity against carboxymethylcellulose and cellobiose, which indicated the presence of endo-β-1,4-glucanase and cellobiase, respectively (Table 1). Compared to freeze-thaw preparations, extracts prepared by Triton X-100 treatment had greater total amounts of endoglucanase and cellobiase activities, as well as a greater percentage of these activities associated with the soluble fraction of extracts, although both procedures resulted in lysis of protozoan cells. This observation suggested that an appreciable amount of enzyme activity in freeze-thaw preparations remained masked, perhaps because it was associated with membranous subcellular organelles and was inaccessible to the substrates. Cellobiase activity was linear for at least 24 hr (the maximum time tested), however endoglucanase activity decreased significantly after 1 hr, at which time about 2 % degradation of carboxymethylcellulose had occured.

Cell extracts of T. termopsidis also possessed hydrolytic activity against microcrystalline cellulose, although the rate of hydrolysis of cellulose was 3 to 10-fold less than that of carboxymethylcellulose or cellobiose (Table 1). The rate of hydrolysis of microcrystalline cellulose was linear for up to 24 hr, but declined slowly from 24 to 72 hr. By using the saccharification assay system,

Table 1

Cellulolytic enzyme activities of Trichomitopsis termopsidis.

Extract Preparation	Extract Fraction	Enzyme Activity Against (Substrate)[1] Microcrystalline Cellulose	Carboxymethyl cellulose	Cellobiose
TRITON X-100	SOLUBLE	1.60 ± 0.73 (n=4)	22.8 ± 10.5 (n=2)	17.5 ± 1.1
	PARTICULATE	0.88 ± 0.22	5.8 ± 0.8	2.9 ± 0.6
FREEZE THAW	SOLUBLE	1.71 ± 0.22	3.7 ± 0.6	3.5 ± 0.8 (n=3)
	PARTICULATE	1.54 ± 0.28	5.3 ± 1.9	8.7 ± 0.1

[1] Activity expressed as mUnits/ 10^6 cell equivalents. Minimum level of activity detectable (mU): microcrystalline cellulose, 0.02; carboxymethylcellulose, 0.2; and cellobiose, 0.2. Values are the mean ± S.E.M. for 3-6 determinations on a single extract (n=1), except where indicated otherwise.

a maximum of 19.4 % saccharification of microcrystalline cellulose was observed during a 64 hr incubation period. However, most of this saccharification took place during the first 24 hr.

All cellulolytic enzyme activities measured were proportional to the amount of cell extract used in the reaction mixture; were dependent on the presence of substrate; and were abolished by heating the extract to 100 °C for 10 min. Moreover, control experiments showed that cellulolytic activity was associated only with the protozoa: no cellulolytic activities were detected in separate extracts of heat-killed Bacteroides JW20 or viable M. hungatii which were used in media to culture T. termopsidis.

Inasmuch as protozoan extracts contained small, but significant and variable amounts of bacterial cell protein (and probably fetal bovine serum protein as well), the values reported in Table 1 were normalized to 10^6 protozoan cell equivalents. However, the total (soluble plus particulate) activity of any of the enzymes could be normalized to protozoan cell protein by assuming that 10^6 T. termopsidis cells were equivalent to 3.4 mg protein (see Materials and Methods). Thus, for extracts prepared by Triton X-100 treatment, the specific rates of substrate hydrolysis were approximately (mUnits/mg protein): microcrystalline cellulose, 0.73; carboxymethylcellulose, 8.4; and cellobiose, 6.0 .

Closer examination of the hydrolysis of microcrystalline cellulose by T. termopsidsis extracts revealed apparent optima at pH 5 (Figure 1) and at a temperature of 30 °C (Figure 2). Moreover,

Figure 1.

Effect of pH on the hydrolysis of microcrystalline cellulose at 30° C by soluble (Triton X-100) extracts of *T. termopsidis* (equivalent to 7.3×10^6 cells/ml extract).

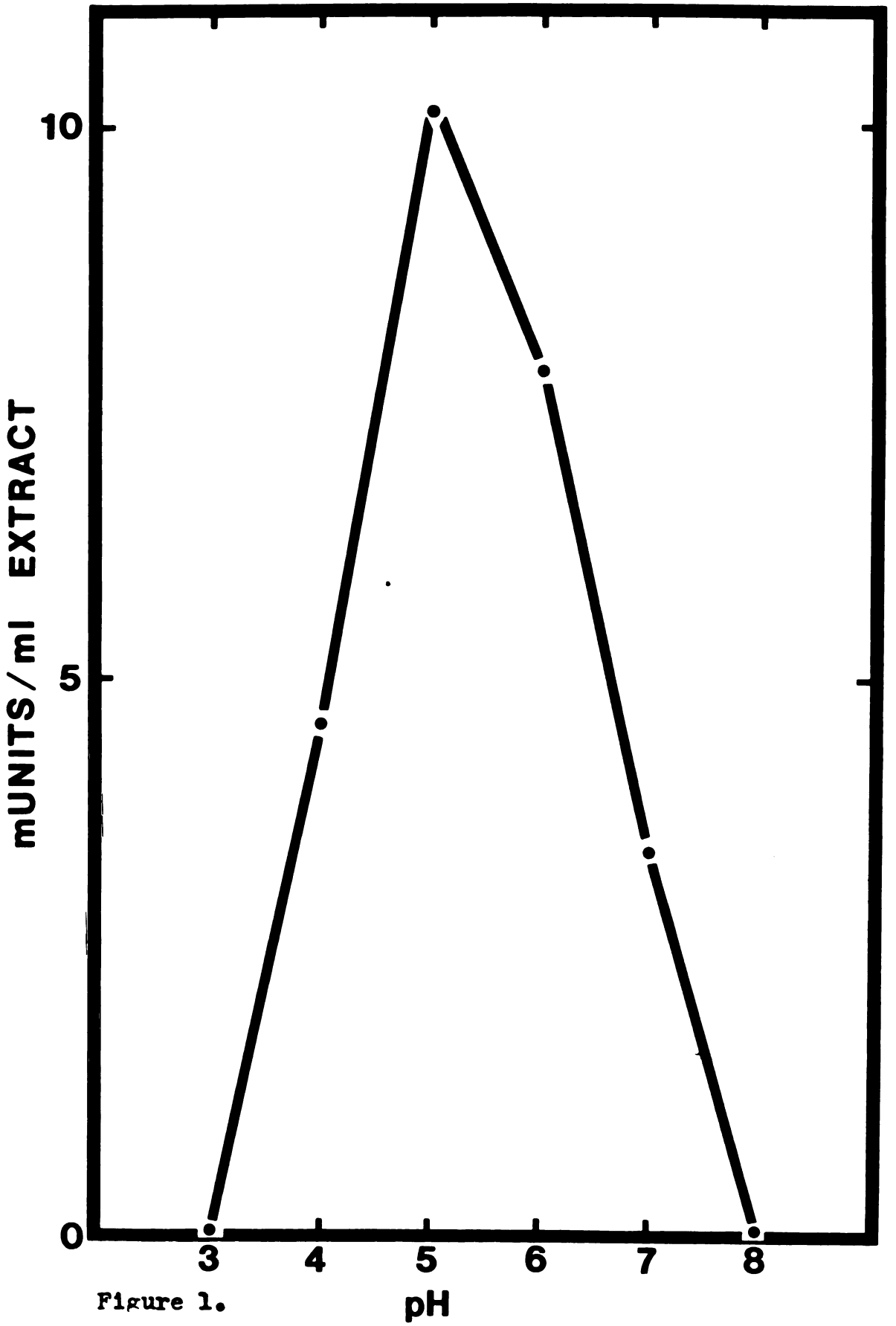

Figure 1.

Figure 2.

Effect of temperature on the hydrolysis of microcrystalline cellulose at pH 5.0 by soluble (Triton X-100) extracts of T. termopsidis (equivalent to 4.0 x 10^6 cells/ml).

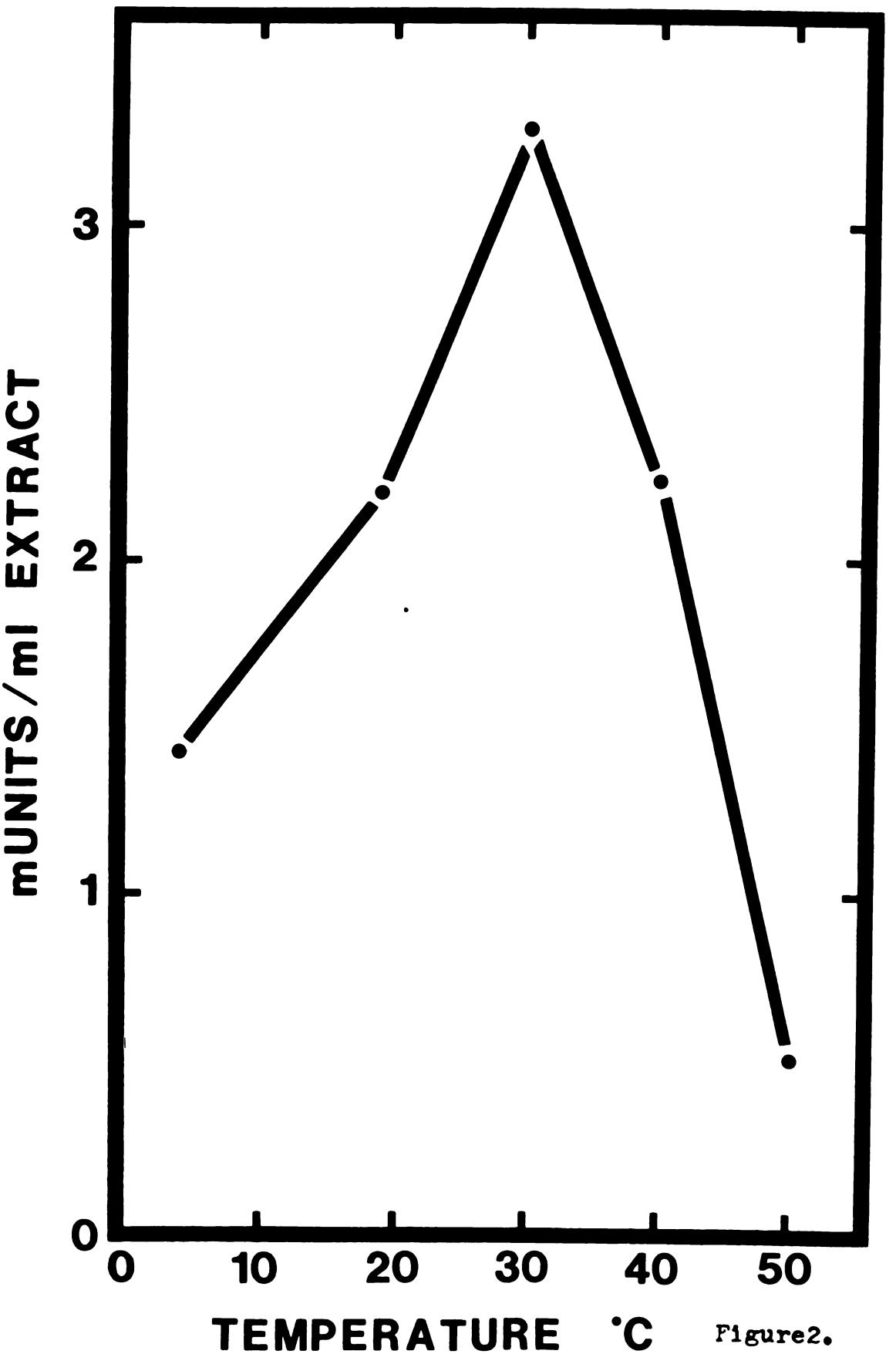

Figure2.

analysis of products formed from microcrystalline cellulose during a 72 hr incubation period revealed that glucose was the only product, accounting for virtually all of the reducing sugar liberated (Figure 3). No cellobiose or other cellodextrins were detected by high pressure liquid chromatography (data not shown).

Other substrates hydrolyzed by extracts of T. termopsidis included Douglas fir wood powder, starch, and xylan (Table 2). Protease activity, as determined by azocasein hydrolysis, was also detected, although no activity against powdered chitin could be demonstrated (Table 2).

Figure 3.

Production of glucose (■) and reducing sugar (●) from microcrystalline cellulose by soluble (Triton X-100) extracts of T. termopsidis (1.1×10^6 cell equivalents per ml of reaction mixture).

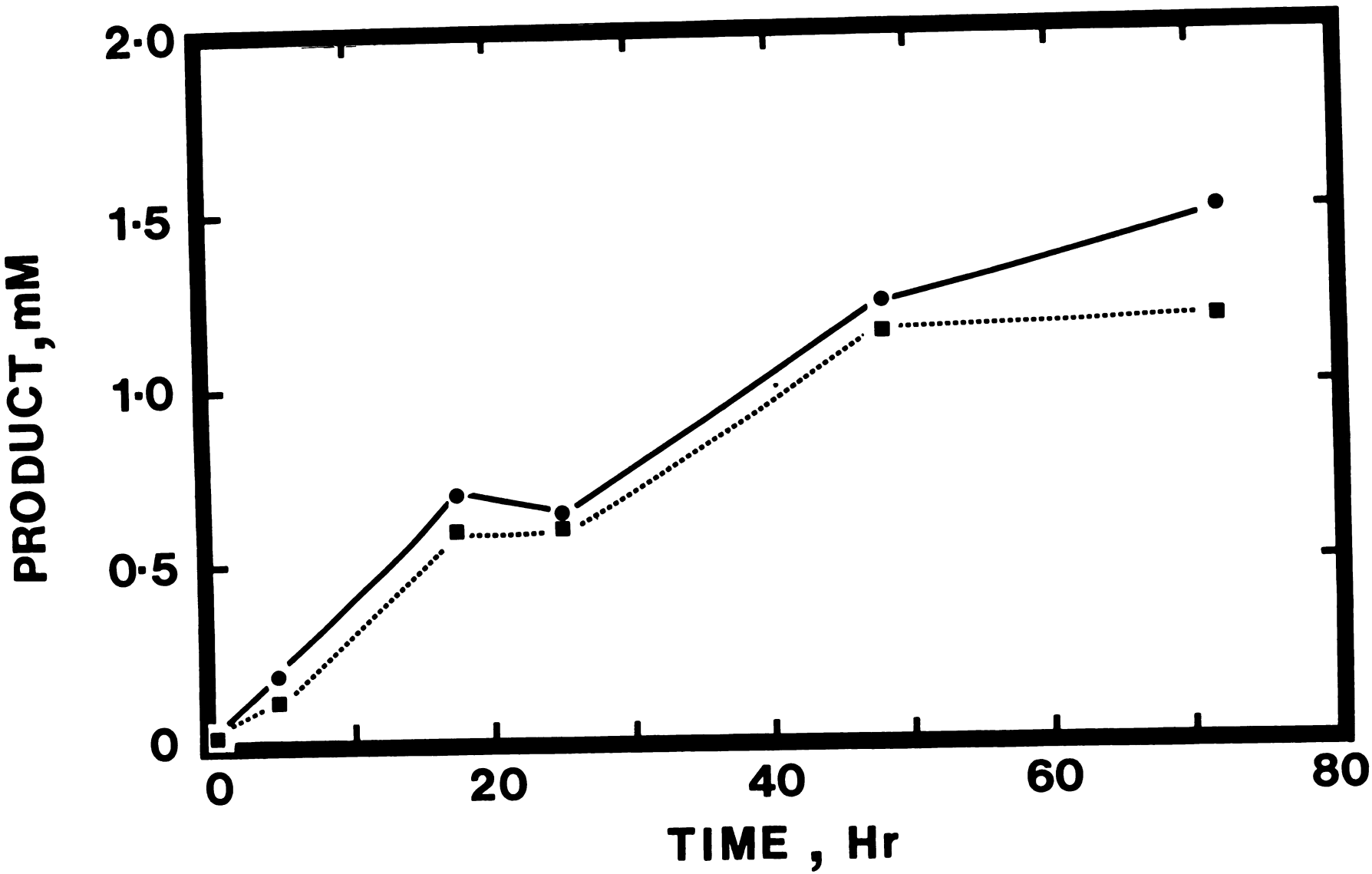
2·0
1·5
1·0
0·5
0
PRODUCT, mM
0
20
40
60
80
TIME , Hr

Table 2

Hydrolytic activities of *Trichomitopsis termopsidis*.

SUBSTRATE	ACTIVITY [1]
Potato Starch	13.8 ± 4.8
Xylan	8.1 ± 1.2
Azocasein	5.3 ± 0.2
Douglas fir powder	1.4 ± 0.2
Chitin	0

[1] Activity expressed as mUnits/10^6 cell equivalents of soluble (Triton X-100) extract, except for azocasein which is ug hydrolyzed/min x 10^6 cell equivalents of soluble (Triton X-100) extract. Values are the mean ± S.E.M. of 6 determinations for n=1.

DISCUSSION

Results presented herein indicate that T. termopsidis possesses at least two enzymes of the cellulase repertoire, endo-β-1,4-glucanase and cellobiase. However, we are reluctant to conclude that T. termopsidis possesses a "complete cellulase enzyme system", which includes additional enzymes such as cellobiohydrolase (EC 3.2.1.91) and which is capable of extensive hydrolysis of crystalline cellulose (10,16). The basis for our reluctance is the fact that: (i) no evidence for cellobiose production was observed by chromatographic analysis of products formed during hydrolysis of microcrystalline cellulose; and, most importantly (ii) the extent of saccharification of microcrystalline cellulose by extracts of T. termopsidis was only about 20 % of the theoretical maximum during an extended (64 hr) incubation.

It may be that saccharification was limited by the intrinsic instability of one or more enzymes of the T. termopsidis cellulase system. However, it may also be that T. termopsidis simply cannot hydrolyze "crystalline" regions of the cellulosic substrate and is restricted to more easily degradable "amorphous" regions (16). Nevertheless our results are consistant with the hypothesis that T. termopsidis possesses enzymes capable of converting microcrystalline cellulose to glucose, a sugar which is undoubtedly used as a

fermentable energy source for the cells. In fact, based on gas production rates of T. termopsidis growing in vitro (eg. see Figure 2 and 3 of the companion paper (21)), it can be estimated that cellulose hydrolysis must occur at a rate of about 1.1 to 1.5 nmoles glucose units liberated/min x 10^6 cells (assuming that the gases are derived exclusively from cellulose decomposition). Our present study revealed that cell extracts hydrolyzed cellulose at rates of about 3 nmoles glucose units liberated/min x 10^6 cells, implying that we had accounted for all of the cellulase activity of the protozoa. This observation also indicates that cellulase activity of T. termopsidis is entirely cell-associated, and probably localized in intracellular food vacuoles. This notion is buttressed by the fact that: no cellulase activity (25) or reducing sugars (21) are detectable in extracellular fluids of actively growing cultures; T. termopsidis endocytoses cellulose particles into food vacuoles (21,28); and T. termopsidis will not grow on large discs of cellulose filter paper, which they are incapable of endocytosing (21).

In view of these considerations, and if T. termopsidis indeed lacks a complete cellulase system, it may be speculated that only limited intracellular degradation of cellulose particles occurs and is followed by exocytosis from cells of particles greatly enriched in their crystallinity. In the natural environment of T. termopsidis (i.e. the hindgut of Zootermopsis termites), such particles might be subsequently endocytosed by other protozoan species that are perhaps more efficient in saccharification, since the overall extent of cellulose dissimilation by termites can be greater than 90 % (8,27).

By the same token, pretreatment of cellulosic material by the termites themselves undoubtedly also enhances its degradability by hindgut protozoa. The grinding and chewing action of the termite's mouthparts reduces the initial substrate particle size, increases substrate surface area, and probably also disrupts the crystalline order of cellulose in much the same way as does ball-milling (a treatment known to enhance the rate of enzymatic hydrolysis (9)). Cellulase secretion by the insect's salivary glands may also augment the action of microbial enzymes (26).

Our results confirm and extend the work of Yamin and Trager (31) who also demonstrated endoglucanase and a β-glucosidase activity in crude extracts of T. termopsidis, but who did not report on the rates and characteristics of hydrolysis of microcrystalline cellulose by such preparations. In addition, our present studies revealed hydrolytic activity against wood particles (the natural substrate of the protozoa in vivo), and noncellulosic polymers that are also present in wood (xylan, starch, and protein). Protease activity may also assist in the digestion of bacterial cells, which are required for in vitro growth of T. termopsidis (21,28) and which are probably grazed upon by these protozoa in vivo.

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