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).

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 $\times$ *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 $\times$ *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 $\pm$ standard error of the mean): *R. flavipes*, 38.6 $\pm$ 7.0% (*n* = 9); *I. schwarzi*, 60.7 $\pm$ 3.0% (*n* = 4); and *Z. angusticollis*, 63.2 $\pm$ 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 $\times$ 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 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
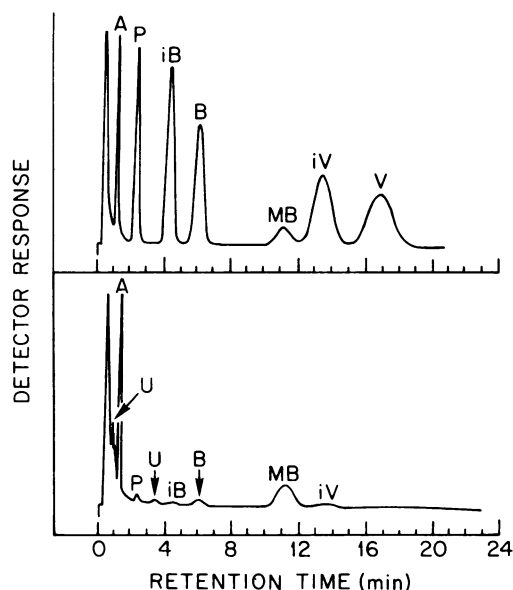

FIG. 1. Gas chromatograms of a standard mixture of VFAs (4 mM each in BIS, top) and a diluted sample of hindgut fluid from *R. flavipes* termites (bottom). Symbols: A, acetate; P, propionate; iB, isobutyrate; B, butyrate; MB, α-methylbutyrate (internal standard); iV, isovalerate; V, valerate; and U,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

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.

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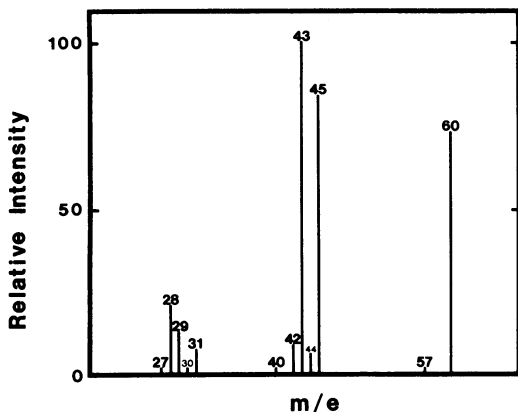
FIG. 2. Mass spectrum of acetic acid from hindgut fluid of *R. flavipes* termites.

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. angusti-collis	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) Macro (*n* = 3)	44.5 ± 3.9 48.8 ± 1.7	16.7 ± 1.1 14.3 ± 0.3	6.1 ± 0.6 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
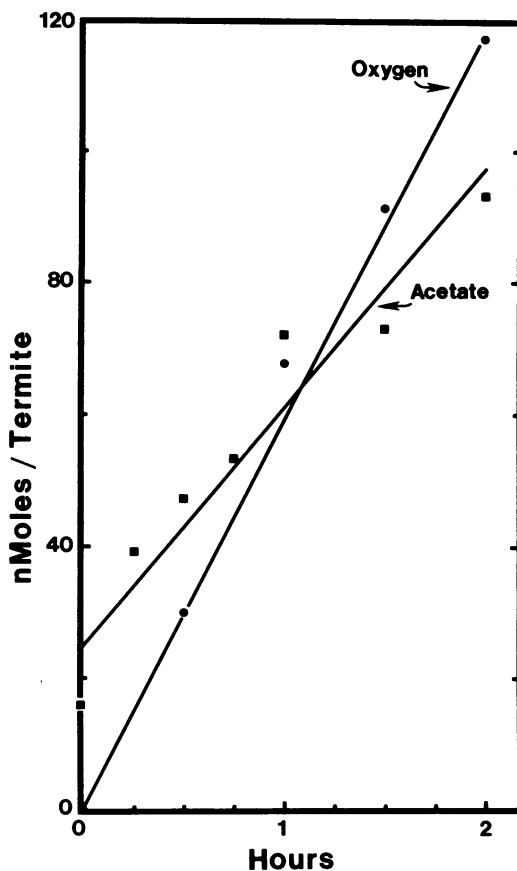

FIG. 3. Rates of in situ acetogenesis by the hindgut microbiota and O_2 consumption by intact worker larvae of R. flavipes. Termites were freshly collected from the field before assay.
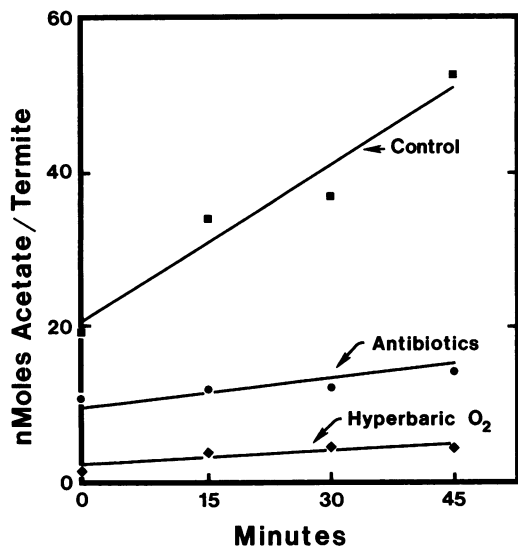
FIG. 4. Effect of defaunation (hyperbaric O_2) or removal of hindgut bacteria (antibiotics) on acetogenic activity of hindguts of *R. flavipes* termites.

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.

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,
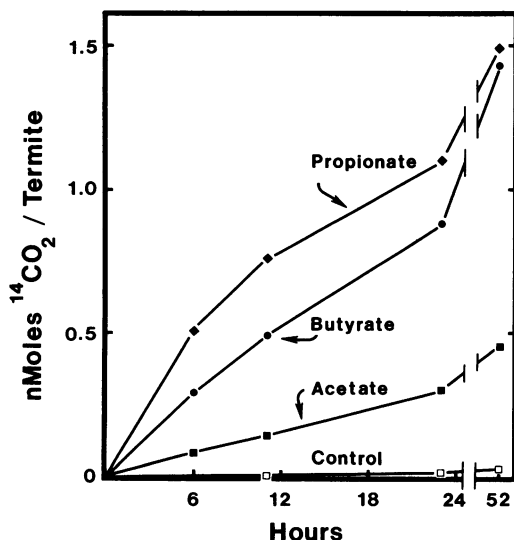
termites were removed from the incubation vessels and dissected to determine the origin of $^{14}CO_2$-evolving activity (Table 3). Most of the $^{14}CO_2$ evolution was associated with degutted bodies (i.e., termite tissues only) and was significantly retarded by incubation under N_2. The observation 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-

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

Component in food tablet	Sp act (dpm · ng of C^{-1})[a]		% 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

[a] 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 La-Fage 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 ($1.5 \times$ 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 search for acetogenic CO_2-reducing bacteria in *R. flavipes* hindguts, is currently under way in our laboratory.
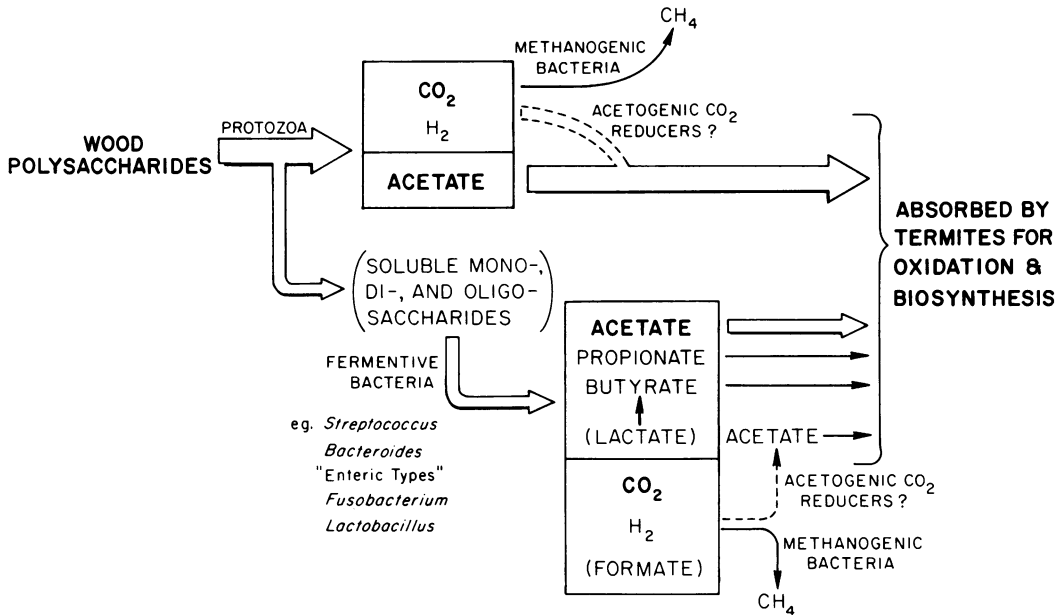
FIG. 6. Proposed working model for symbiotic wood polysaccharide dissimilation in *R. flavipes*. Thickness of arrows represents approximate relative contribution of the respective reactions to the overall dissimilatory pathway. Major products of the hindgut fermentation are indicated in boldfaced type; 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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