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Uric Acid and Uricolytic Gut
Bacteria in Termites:
A Strategy for Nitrogen Conservation
presented by

Catherine J. Potrikus

has been accepted towards fulfillment
of the requirements for

a Ph.D. degree in Microbiology

Major professor

Date Sept. 29, 1980

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URIC ACID AND URICOLYTIC GUT BACTERIA IN WOOD-EATING TERMITES: A STRATEGY FOR NITROGEN CONSERVATION

By

Catherine Jane Potrikus

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Microbiology and Public Health

1980

ABSTRACT

URIC ACID AND URICOLYTIC GUT BACTERIA
IN WOOD-EATING TERMITES:
A STRATEGY FOR NITROGEN CONSERVATION

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Uric acid (UA) was present in termites as assessed by paper and thin layer chromatography, by U.V. spectroscopy, by reactivity with uricase, and by gas chromatography coupled to mass spectrometry. Specimens of six species of termites (Reticulitermes flavipes, R. virginicus, Coptotermes formosanus, Marginitermes hubbardi, Paraneotermes simplicicornis, and Cryptotermes cavifrons) contained UA in amounts accounting for between 1 and 45% of the termites' dry weight and for between 4 and 69% of the termites' total nitrogen. Almost all of the UA in R. flavipes was associated with fat body tissue. During 15 months of laboratory captivity, UA in R. flavipes workers increased from 1.7 to 45.5% of the insects' dry weight, apparently at the expense of some endogenous nitrogen and carbon. Extracts of R. flavipes abdominal tissues possessed purine nucleoside phosphorylase and xanthine dehydrogenase activities to synthesize UA, but R. flavipes tissues lacked uricase as well as non-uricase mediated pathways to degrade UA. However, little or no UA was voided in termite feces. Uricolytic bacteria were present in guts of R. flavipes in populations

of up to 6 x 10^4 cells/gut. Of 82 strains isolated under strict anaerobic conditions, most were group N Streptococcus sp., Bacteroides termitidis, and Citrobacter sp. All isolates used UA as an energy source anaerobically, but not aerobically. Streptococcus strain UAD-1 degraded UA incompletely unless formate (or a formicogenic compound) was present as a co-substrate. Formate oxidation to CO_2 (E_o' = -430mV) also provided $2H^+ + 2e^-$ which served to drive uricolysis. Streptococcus UAD-1 degraded UA as follows: 1UA + 1 formate -----> $4CO_2$ + 1 acetate + $4NH_3$. Formate dehydrogenase and uricolytic activities of strain UAD-1 were inducible by growth on UA. B. termitidis UAD-50 degraded UA as follows: 1UA -----> $3.5CO_2$ + 0.75 acetate + $4NH_3$. Uricolytic activity was inducible in strain UAD-50. However, exogenous formate was neither required for nor stimulatory to uricolysis by this strain. Studies of UA catabolism by Citrobacter strains were limited, because only small amounts of UA were consumed by the cells in liquid culture or by resting cell suspensions. Streptococcus UAD-1 and B. termitidis UAD-50 readily evolved $^{14}CO_2$ from [2-^{14}C] UA under anaerobic conditions. Thus, [2-^{14}C] UA was used as a substrate to measure uricolysis by termite gut preparations and by whole termites. Fresh termite gut contents degraded UA anaerobically, but not aerobically, at rates of up to 82 pmoles x gut^{-1} x h^{-1}. No UA was degraded by washed segments of gut tissue. Termite Malpighian tubules transported UA in vitro, and when [2-^{14}C] UA was injected into R. flavipes workers $^{14}CO_2$ was evolved, indicating in vivo transport of UA from the hemolymph to the gut for anaerobic catabolism by the resident microbiota. Products of UA catabolism by gut bacteria may be useable by the termite for nitrogen,

carbon, and energy. Crude extracts of R. flavipes tissue possessed glutamine synthetase activity, suggesting that termites are able to assimilate NH_3, the major nitrogenous product of gut microbial uricolysis. Recycling of UA nitrogen by gut bacteria thus appears to be an important means of nitrogen conservation for termites: the amount of nitrogen liberated by gut uricolysis could permit a population increase of at least 20% annually. Although generally regarded as an insect "waste product", uric acid may be an important reserve material in termites, useable only with the aid of their gut microbiota.

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1980

To my Mother, and in memory of my Father,
for their love, their support, and their trust in me,
even when they did not understand.

ACKNOWLEDGMENTS

I wish to thank the members of my guidance committee, Dr. J. M. Tiedje, Dr. C. A. Reddy, Dr. M. J. Klug, and Dr. D. G. Cochran for their advice and assistance during the course of these studies. My special thanks to Dr. Cochran for his ready replies to letters and phone calls for advice on insect physiology.

I express deep appreciation to some very special friends and colleagues, those with whom I shared the peaks of elation and pits of despair that accompany scientific research: Larry Forney, who kept me sane through countless uric acid assays, and was always a willing (?) listener, a ready critic (in the most favorable sense of the word), and a very special friend; Jon Wegienek, whose friendship likewise has been a source of strength and encouragement; and Bob and Ellen Uffen et al, without whom I never would have quite so happily retained my contact with family life -- thank you for Sunday dinners, a garden plot, kids and pets, and most of all for your sincere friendship, advice, and encouragement.

Finally, and most sincerely, I wish to thank Dr. John A. Breznak, my major professor, for the many, many hours of everything that goes into molding a scientist. Thank you for your patience, your encouragement, your advice and guidance, and particularly for sharing with me your great enthusiasm for scientific research.

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INTRODUCTION

Termites are insects belonging to the order Isoptera. They are social animals whose survival depends on a precisely defined division of labor between various caste members of the colony. The worker caste or functional group dominates the activities of a colony, in part because of their large numbers. Workers are the primary builders and foragers, and they supply nutrients to reproductives, soldiers, and young (nutrient-dependent) larvae. The intricacies of termite survival extend beyond communal interactions, however, and also involve interactions with symbiotic microorganisms. In fact, one of the most oft-cited examples of mutualism in biology is the interaction of evolutionarily "lower" termites* with their cellulolytic hindgut protozoa. The latter organisms enable xylophagous termites to digest cellulose (the major polysaccharide of wood), and the phenomenon lies at the very heart of the termites' carbon and energy nutrition.

Although Cleveland (23,24) long ago recognized the termite's dependency on gut protozoan symbionts, it was Hungate (48,50) who

* The order Isoptera includes five living families: Mastotermitidae, Kalotermitidae, Hodotermitidae, Rhinotermitidae, and Termitidae (103). The phylogenetically more primitive termites, members of the first four families, are referred to as lower termites, whereas the Termitidae are regarded as higher termites.

elucidated the role of protozoa in cellulose metabolism. Hungate demonstrated that suspensions of mixed microorganisms (containing primarily protozoa) from Zootermopsis fermented cellulose to acetate, CO_2, and H_2. Acetate produced in the gut was absorbed through the gut wall, and the termite oxidized the acid to CO_2 and H_2O for energy (50). Hungate's elegant early work has recently been verified by Yamin (107,108) who isolated and maintained the protozoan Trichomitopsis termopsidis from Zootermopsis. By using ^{14}C-cellulose, Yamin (108) showed that pure cultures of T. termopsidis fermented cellulose to acetate, CO_2, and H_2. However, as Yamin (108) points out, this protozoan is not the major cellulolytic species in the termite hindgut. Although progress has been made in studying the intriguing protozoan symbionts from termites, further investigations are needed to fully elucidate their roles in termite nutrition.

In addition to protozoa, an abundant and diverse bacterial population resides in hindguts of lower termites (11). Unfortunately, the procaryotic component of the gut microbiota has been largely ignored. Investigators have only recently begun to elucidate some of the roles termite gut bacteria have in the nutrition of their host (11,12,38,39,91,92). One intriguing role, discussed further below, concerns N_2 fixation by termite gut bacteria. The ability of termites to thrive on relatively N-poor diets prompted investigations for bacteria capable of fixing atmospheric N_2 to supplement the insect's meager N intake. Although these studies proved fruitful, for N_2-fixing bacteria were found in termite hindguts (39,86), the results did not always reconcile the discrepencies that existed between

termite dietary N intake and the presumed N requirements for termite colony maintenance and growth. It appeared that other means for conserving N could be important to wood-eating termites. Thus, after a preliminary investigation which resulted in the isolation and identification of N_2-fixing Enterobacter agglomerans from guts of Coptotermes formosanus termites (see Appendix II), the present study was undertaken to evaluate the role of gut microorganisms in recycling termite nitrogenous excretory compounds. Results presented herein show that gut bacteria conserve termite N by degrading uric acid (a presumed waste product which is synthesized by the termite) to products utilizable by the host. This role of microbes in termite nutrition adds yet another dimension to our growing knowledge of an array of intriguing termite-microbe symbioses.

LITERATURE REVIEW

Insect - Microbe Symbioses

Symbiosis, as defined by deBary (5), is an intimate and relatively stable association of two dissimilar organisms. Buchner (18) and Richards and Brooks (89) elaborated this definition in describing endosymbiosis, wherein one organism is sheltered within the body of another, with the arrangement mutually beneficial to both the host and the symbiont. Symbioses between insects and microbes encompassed by this latter definition embrace a myriad of morphological, physiological, and biochemical interactions, including both intracellular and extracellular microbial habitats within the insect host. Morphological and behavioral aspects of numerous insect - microbe symbioses have been well documented (13,18,58,105). By contrast, the specific roles of microbes in their hosts' nutrition, growth, and reproduction have largely remained speculative, and therefore the importance of the symbionts to their hosts has not, in most cases, been clearly elucidated.

Morphological adaptations between insects and their symbionts are diverse (89). Microorganisms may be harbored in specialized cells (mycetocytes) scattered throughout the insect's fat body tissue; in specific organs (mycetomes) designed to harbor and/or transmit the

symbionts; in Malpighian tubules; in various specialized or non-specialized portions of the digestive tube; or in the reproductive organs. Insects which harbor microbial symbionts may be dependent upon them for survival, or the insect may benefit from the association, but survive in its absence.

The physiological significance of insect endosymbionts can best be studied by removal of symbionts from their host. The symbiont-free, or aposymbiotic, insects are then compared with symbiont-containing counterparts, often under conditions of dietary or environmental stresses. Ideally, the symbionts would also be isolated and cultured, and their in vitro characteristics and physiology correlated with their predicted roles in vivo to affirm the microbes' significance to their host.

Koch (57) has discussed methods frequently used to induce aposymbiosis in insects. Symbionts may be eliminated, or their acquisition by young prevented, by: surface sterilization or physical perturbation of eggs; surgical removal of symbiont-containing mycetomes; incubation of insect hosts at elevated temperatures; treatment with the enzyme lysozyme; or injection or feeding of insects with antibiotics (57). The efficacy of such methods depends upon the individual insect species under investigation and the characteristics of the symbionts and of the symbiosis. Exploitation of aposymbiotic specimens has helped elucidate many intriguing roles of microbial symbionts in insect nutrition (18,57,105).

Roles of microbial symbionts in insect nutrition

Symbionts play significant roles in supplementing nutritionally inadequate diets for their hosts (18,89,105). Richards and Brooks (89)

pointed out a frequent correlation between an insect's food source and the presence or absence of symbionts. For example, insects which thrive on diets of wood, plant sap, or animal blood often harbor symbionts (89). Such diets are deficient in N and/or vitamins. The hypothesized roles for the symbionts are to assist in digestion of the food, or to supplement diets with vitamins or combined N (e.g., amino acids) required for the growth and development of the insect host.

Digestion of wood or cellulose is frequently mediated by microbes found either external to the wood-eating insect (i.e., infesting the wood itself) or harbored within the insect as endosymbionts (37,89). As mentioned in the Introduction, some xylophagous termites rely on cellulose fermentation by their associated gut protozoa for a source of carbon and energy. When Reticulitermes flavipes termites were defaunated (i.e., rid of their protozoa) by incubation at high temperature or under hyperbaric oxygen, or by starvation, they could not survive on a normal diet of sound wood (23,25). However, refaunation, or feeding the termites fungus-degraded wood, prolonged survival indefinitely, implicating the gut protozoa in wood digestion in vivo. Moreover, Hungate (47) demonstrated that cellulase activity in Zootermopsis was confined to the hindgut of normally faunated specimens, and was absent from defaunated termites. These data, and the recent isolation and study of cellulolytic protozoa from termites (107,108), confirm that the protozoa are important to some termites' nutrition.

Symbiotic cellulolytic protozoa are not found in the hindguts of all termites. In the higher termites, devoid of cellulolytic protozoa, cellulase production has been ascribed to gut bacteria (61,71,84,98)

or, in the fungus-growing Macrotermes, to fungal conidiophores (1,68). Evidence for cellulase production by the higher termites themselves has also been presented (1,68,87), but usually in concert with bacterial or fungal cellulolytic activity. It appears, therefore, that most termites are assisted in their digestion of cellulose by symbiotic associations.

Gut protozoa of the wood-eating cockroach Cryptocercus punctulatus ferment cellulose to reducing sugars which are presumably utilizable by the host (99). Trager (99) and Cleveland (27) demonstrated that cellulase activity in normally faunated Cryptocercus was localized in the hindgut, where the protozoa reside, whereas defaunated individuals were devoid of cellulase activity. These results implicate the gut protozoa of Cryptocercus in cellulose metabolism in a manner similar to the role ascribed to termite protozoa.

In contrast to the endosymbiotic cellulolytic protozoa of termites and Cryptocercus, cellulolytic bacteria are important in the nutrition of wood-eating Lamellicorn beetle larvae. The bacteria are ingested and retained with the beetle's food in a specialized sac in the hindgut (104). After digesting the wood, the microbes are themselves consumed by their host (104). Although this relationship is not a permanent endosymbiosis as observed with termites, the role of bacteria in digestion is critical to the beetles. Cellulolytic bacteria likewise assist other species of xylophagous insects in their digestive processes (see 18, 105).

Perhaps more intriguing than their roles in digestion are the roles that insect-associated microbes play in their hosts' N nutrition. Insects whose diets are deficient in vitamins and amino acids may

depend upon symbionts to provide these essential nutrients. Moreover, insects whose diets are N deficient may rely on microbes for N acquisition (e.g., N_2 fixation) or for conservation of combined N. Many terrestrial insects synthesize uric acid as a product of protein and nucleic acid catabolism (19,22,29). Uric acid is a non-toxic nitrogenous compound which can be eliminated with minimal water loss to the insect, and thus it is an ideal excretory waste for insects which must conserve water (19,22,29). However, for insects living on N-poor diets, the loss of N in any form could be detrimental. It would be advantageous if such insects possessed means to conserve uric acid N which might otherwise be lost during defecation. In fact, some insects appear to have evolved strategies for conserving uric acid N. Even cockroaches, which do not normally live on N-poor diets, can accumulate large amounts of uric acid reserves when dietary N is in excess. The potential roles of microbial symbionts in insect N nutrition, and problems in elucidating symbiont roles, are well illustrated by studies with urate-storing cockroaches.

The fat body tissue of insects functions as a site for storage of metabolites, including fats, carbohydrates, and proteins (22). In many insects, this tissue is also the site of uric acid synthesis and storage (29,67). In cockroaches, the purine can account for as much as 30% of the insect's dry weight and 72% of the total body N (31). However, whereas uric acid was once regarded solely as a waste product, and its sequestration in fat body tissue termed "storage excretion" (29,67), recent evidence suggests that the stored purine is in a dynamic state in cockroaches (29).

Urates may provide cockroaches with N reserves utilizable in times

of dietary stress. Haydek (43) first documented the effect of dietary N on the uric acid content of cockroaches. When specimens of cockroaches were fed high protein diets, they accumulated large masses of white crystals, presumably uric acid (43). Cockroaches fed N-free diets of dextrin apparently lost the white deposits (43). In fact, Haydek surmised that insects fed diets containing 79% to 86% protein were killed by the massive deposits which accumulated throughout their bodies and interferred with the function of other organs. Although this conclusion may have been premature, pending further nutritional studies (43,75), cockroaches do appear to store uric acid when dietary N is in excess.

Haydek's findings have been verified and elaborated by Mullins and Cochran. Most cockroaches do not excrete uric acid (28,30,31,77), even under dietary stresses which cause uric acid concentrations to fluctuate significantly (74). When Periplaneta americana were fed diets with a N content greater than the insects' N needs, excess N was stored as uric acid (75), sometimes even at the expense of carbon reserves. Furthermore, insects fed low N diets mobilized their urate stores (76). The rate of uric acid mobilization was related to the level of dietary N, and was always greater in females than in males, presumably because of the females' higher N demand during oothecal production (76). In fact, Mullins and Keil (78) have shown that uric acid synthesized by the male German cockroach [Blattella germanica (L.)] and voided from accessory glands onto spermatophores is incorporated into female oothecae. The amount of spermatophore-associated urates incorporated into the oothecae appears to be related to the female's dietary N, and thus to her need for an

additional exogenous N supply (78). Clearly, uric acid can be more important to cockroach N nutrition than merely serving as a nitrogenous waste.

The mechanism(s) of urate mobilization in cockroaches is not well understood. Symbiotic bacteria, both free-living in the gut and intracellular in fat body mycetocytes, have been implicated in the mobilization and subsequent catabolism of uric acid (16,73). Symbiont-free cockroaches have been useful experimental animals for examining the impact of microbes in cockroach N metabolism. The progeny of cockroaches fed an antibiotic such as Aureomycin for three to four months are aposymbiotic, i.e., freed of their mycetocyte symbionts (15). Brooks and Richards (16) compared urate levels in symbiont-free fat body and normal fat body of B. germanica after transplanting the latter tissue into an aposymbiotic host. The normal tissue, which remained healthy and which contained abundant bacteria-filled mycetocytes, was relatively free of urates 127 days after implant, whereas the symbiont-free tissue accumulated massive amounts of urates during the same period (16). There was no infection of aposymbiotic fat body by bacteria from the implanted tissue. The authors concluded that the accumulation of uric acid was inhibited, or urates were utilized, in tissue containing mycetocyte bacteria (16). The role of the bacteria could not be further defined.

Pierre (83) observed uric acid stores in aposymbiotic cockroaches of the species Leucophia maderae. Moreover, he reported uricase activity (i.e., the aerobic conversion of uric acid to allantoin) in suspensions of isolated mycetocyte symbionts (83). Likewise, Donnellan and Kilby (35) reported that bacteria isolated from the fat body of P.

americana degraded uric acid and allantoin aerobically to glyoxylate and urea; the latter compound was subsequently catabolized to NH_3 and CO_2. However, the work of Pierre, Donnellan and Kilby, and others who have reportedly isolated and studied cockroach mycetocyte symbionts (e.g., see 17) has been challenged by Brooks and Richards (17). As these latter authors point out, the techniques for symbiont isolation were so stringently designed to insure aseptic conditions that the symbionts themselves were undoubtedly killed during the isolation procedures. The cultured "symbionts" were, therefore, contaminants (17). Moreover, studies with aposymbiotic cockroaches were not always valid. As mentioned above, progeny of antibiotic-fed cockroaches were free of mycetocyte bacteria. The parental generation, however, usually retained some symbionts, even during longterm (i.e., four months) antibiotic feeding (15,17). When these insects, rather than their progeny, were used for experimentation, the results did not reflect true aposymbiotic conditions (17). With these criticisms in mind, it appears that the roles of symbionts in cockroach urate metabolism must yet be rigorously investigated. Nevertheless, the presence of bacteria-filled mycetocytes in close proximity to urate cells in cockroach fat body (32), transovarian transmission of the symbionts to oocytes (14), and the possibility that oocyte uric acid is metabolized during embryogenesis (78) further support the contention that mycetocyte bacteria may be involved in cockroach urate metabolism.

Although antibiotic-fed cockroaches may not be truly aposymbiotic, their symbiont population is greatly reduced (15). Studies comparing these insects with normal cockroaches have shown that the symbionts do indeed affect their host's nutrition. "Aposymbiotic" cockroaches,

whether parental antibiotic-fed specimens or their progeny, are much lighter in color than their normal counterparts, grow more slowly and molt less frequently, and produce few, if any, fertile eggs (15,16,66,89). Richards and Brooks (89) caution that these effects may be a result of the antibiotics themselves. Significant, however, was Brooks and Richards' (16) report that implantation of normal fat body into aposymbiotic B. germanica restored the insects' normal coloring and growth.

The effects of aposymbiosis on cockroaches have been ascribed to the lack of essential vitamins and amino acids that are normally supplied by the mycetocyte bacteria. Henry and Block (45) fed [U-^{14}C] glucose to normal and aposymbiotic B. germanica and analyzed the production of ^{14}C-labeled amino acids. Aposymbiotic insects failed to synthesize eight amino acids that were present in normal specimens (45). Brooks and Kringen (14) found that polypeptides present in hydrolyzed lactalbumin, proteose peptone, brewer's yeast, and lyophilized mouse liver enabled symbiont-free B. germanica specimens to grow at a more normal rate than specimens fed on a casein stock diet. However, the authors could not further characterize the growth factor, nor determine the role of symbionts in supplying it to the host (14).

Ludwig and Gallagher (66) found that the fat body of aposymbiotic P. americana contained reduced amounts of ascorbic, folic, and pantothenic acids when compared to normal tissue. They suggested that the lack of these vitamins caused the lighter coloring observed in aposymbiotic specimens (66). Moreover, the authors claimed that symbionts isolated from P. americana synthesized the three vitamins in

vitro (66). Unfortunately, the isolation techniques used by Ludwig and Gallagher are subject to the criticisms of Brooks and Richards (17; see above), and therefore the results should not be accepted as fact without further investigation. The accumulated evidence strongly suggests, however, that mycetocyte symbionts can be crucial to cockroach development.

In contrast to the controversial studies regarding cockroach symbionts, studies with a number of beetle species have proven quite successful in defining roles of microbial symbionts in insect host nutrition. Blewett and Fraenkel (9) and Pant and Fraenkel (81), in extensive studies of larvae of the beetles Stegobium paniceum and Lasioderma serricorne, showed that intracellular symbionts supplied up to six B-vitamins required by their hosts. Pant and Fraenkel's (81) study represents one of a very few wherein the symbionts, in this case yeasts, were isolated and cultured, and subsequently introduced into sterile (i.e., symbiont-free) host insects. The effects of the microbes and their metabolites on the hosts could therefore be clearly established. In the absence of the yeast symbionts, the beetles exhibited requirements for vitamins of the B-complex (9,81). When aposymbiotic larvae inoculated with cultures of the yeast acquired their normal symbiont flora, the vitamins were no longer required for the insects' growth and development (81). Furthermore, a requirement for cholesterol by the symbiont-free beetles was overcome by inoculation of the insects with symbionts (81). It appears that the microbes provide vitamins and cholesterol, or their precursors, to the beetle host.

Pant and Fraenkel (82) also studied symbiosis in the beetle

Oryzaephilus surinamensis L. Larval specimens incubated at elevated temperatures appeared to lose their mycetocyte symbionts (82). Growth of the symbiont-free larvae was retarded, and, as with the species previously investigated (see above), the symbionts were implicated in the synthesis of B-vitamins for their host (82). Although the symbionts were not isolated, and the studies were less extensive than these authors' previous work, the roles of the symbionts appear to be similar. Pant and Fraenkel (81) suggested that vitamins produced by the symbionts within mycetocytes become available to the host when the insects digest their yeast symbionts, or following diffusion of the vitamins into the hemolymph. The latter route was proposed as the more likely (82) in view of Graebner's (40) report of vitamin production and secretion by pure cultures of the Stegobium symbionts.

The beetles studied by Pant and Fraenkel thrive on relatively nutrient deficient diets (e.g., flour, leaves, herbs), supported by their associations with vitamin-producing yeasts. Similarly, blood-sucking insects require supplementation of their B-vitamin deficient diet with products elaborated by gut bacterial symbionts (4,10,18,89,105). In experiments similar to those described above, Brecher and Wigglesworth (10) and Baines (4) demonstrated that a bacterium, Nocardia rhodnii, isolated from guts of Rhodnius prolixus, could supply its host with vitamins of the B-complex that were necessary for the insect's growth. Symbiont-free insects showed retarded and incomplete development, whereas supplementation of their diet with mixtures of B-vitamins could overcome the growth lag (4,10). Furthermore, infection of sterile specimens with N. rhodnii restored the insects' ability to complete development at the same rate, and on

the same vitamin-poor diet, as normal insects (4). Clearly, these symbionts can have a significant influence in supplementing an inadequate diet with essential vitamins.

For insects whose diets consist of N-poor wood or cellulose, microbial symbionts may also have a critical role in N nutrition. Cryptocercus utilizes the microbes harbored in its hindgut as a source of protein (27). The microorganisms are apparently regurgitated into the midgut from the hindgut and digested by their host (27). A similar fate was observed for the cellulose-digesting bacteria associated with Lamellicorn beetles (104), as described above. Tóth (96) suggested that termites might also obtain microbial protein by digesting their own microbiota. He surmised that microbes from the overcrowded hindgut would be forced into the midgut (i.e., regurgitated), and there be digested. However, studies by Kovoor (60) showed that the enteric valve prevents food reflux from the hindgut to the midgut in termites. Gut symbionts could not, therefore, be regurgitated for a source of protein for the termite.

Termites have apparently evolved an alternate means by which to utilize some of their gut microbial protein. The lower wood-eating termites frequently partake in proctodeal feeding, i.e., ingestion of a microbe-laden droplet of the paunch (hindgut) contents of one termite (donor) by another member of the colony (solicitor) (80). By this practice, young larvae and newly molted termites are infected or reinfected, respectively, with their necessary symbionts. Although some of the microbes survive the passage through the midgut to inoculate the paunch, many of the protozoa lyse, and the protein thereby released can be used by the termite. The significance of this

microbial protein to the overall N economy of termites does not appear to be great, however (46); additional N sources must also be available.

Nitrogen Economy of Termites

The ability of xylophagous termites to thrive on N-poor diets of sound wood or cellulose suggests that these insects have evolved an efficient means to acquire and/or conserve N. The diet of termites consists of plant material in various forms. Most of the lower termites feed on dead wood, which may be sound or in various stages of fungal decay (65,106). Sound, woody plant tissues contain only about 0.03 to 0.10% N by weight (34,49,63) and a very high carbon to nitrogen ratio of up to 500:1 (34). The wood N is primarily in the form of proteins, free amino acids and peptides, nucleic acids, and alkaloids (63). Since termites excrete very small amounts of N in their feces (49,65,106), their ability to utilize most of the wood nitrogenous compounds must be acknowledged. Even so, the daily N intake would be very low for most termites. For example, wood consumption by termites in the family Rhinotermitidae is about 20 mg/(g fresh termite x day) (42,106). If the wood consumed contained as much as 0.10% N by weight, the total N intake would be only 0.02 mg/(g fresh termite x day). Although the N needs of termites in their natural environment are not documented, this amount of N would probably not suffice in itself for colony growth and development, particularly during periods of swarming (79).

Mauldin and Smythe (70) discussed the probable sources for N acquisition by termites. Termite N could derive from: (1) dietary N, from ingested food or digestion of symbionts; (2) microbial-mediated

fixation of atmospheric N_2; and (3) utilization of microbial or, as LaFage and Nutting (63) suggest, termite nitrogenous waste products (70). As mentioned above, digestion of symbionts may not offer termites a significant source of N, although the extent of proctodeal feeding by these social insects has not been documented. The symbiotic protozoa of lower termites apparently divide only at post-molt periods (3,56) when they must repopulate the termite hindgut. Moreover, Trager (100) and Hungate (50,53) concluded that individual protozoa live for relatively long periods. Protozoa would not, therefore, provide a ready source of N for the termite by death and lysis.

Nitrogen from dietary sources other than sound wood could be provided by fungi associated with the wood, or from the cannibalistic and coprophagous habits of termites (63). Many termites prefer wood that is at least partially degraded by certain fungi (65,90), but preferences vary amoung termite species and with different woods and fungi (6,81). Hungate (49) investigated the potential contributions of certain fungi to the N nutrition of lower termites. Working primarily with Zootermopsis, Hungate found that some fungus-degraded woods supported termite growth and N acquisition whereas others did not (49). When he analyzed the roles of fungi in termite N nutrition, he found that fungal activities actually increased the N content of the wood subsequently consumed by the termites (49). Not only did fungi degrade non-nitrogenous components of the wood, thereby decreasing the carbon to nitrogen ratio, but they also transferred N from the surrounding soil into the wood (49).

Hendee (44) reported a beneficial effect of fungi on termite growth and survival. Zootermopsis angusticolis exhibited better

survival, growth, development, and N acquisition on diets of fungus-degraded woods than on sound woods. Hendee suggested that the fungi offered a source of proteins and vitamins for the termites, and extracellular enzymes from the fungi made wood more suitable for the termite to digest. These conclusions correlated with the results of Hungate (see above) and of Cleveland (24), and have been supported by more recent studies (6,59,90). Moreover, consumption of fungus-degraded wood by Reticulitermes flavipes altered the termites' amino acid (21) and fatty acid (20) composition, suggesting differences in the nutritive value of sound wood and degraded wood (21). Finally, fungi may play a role in detoxifying certain wood components harmful to termites (6,44,90). The ultimate contribution of fungi to termite N nutrition appears to vary depending upon the specific interactions involved for a given termite species (e.g., see 90), but fungi could certainly be important to the insect.

The N content of some termites' diets may directly affect their cannibalistic tendencies. Hendee (44) observed increased cannibalism by Z. angusticolis termites fed low N diets of sound wood compared to termites fed fungus-degraded wood. Similarly, termites fed essentially N-free diets exhibited high rates of cannibalism (2,33). Even termites fed presumably adequate (i.e., natural) diets practiced a regular degree of cannibalism (44), suggesting that cannibalism occurs in the termites' natural habitat. Furthermore, chitinase activity has been found in extracts of termites (98,102). This enzyme may serve a role in recycling N ingested with exuviae and cannibalized termites (72). The consequence of termite cannibalistic behavior may concern not only N (and other nutrients) conservation, but also nest sanitation (65) and

population control (63). Since no quantitative data regarding the extent of cannibalism in natural colonies are available, it is difficult to assess the contribution of this behavior to overall termite N economy.

Coprophagy (i.e., ingestion of fecal material) may contribute to N conservation in termite colonies by mediating a recycling of excreted N. However, the significance of coprophagy to the termite is questionable in view of the low N content of feces. Nitrogen constitutes less than 0.25% of the feces of Zootermopsis (49), and likewise a small percentage of Nasutitermes exitiosus excreta (65); feces of other termites are probably comparable (106). Uric acid, a presumed waste product of termites (72), constitutes an even lesser portion of the excreta. Hungate (49) reported that fecal pellets of Zootermopsis contained only 0.02% uric acid, which accounted for less that 10% of the total N excreted. Since the N content of the termites' diet is so low, these data are not surprising. The fact may, however, reflect the termites' potential to efficiently recycle and conserve their N internally, as suggested by Leach and Granovsky (64) and as discussed further below.

The roles of termite gut symbionts in dietary-related N acquisition have not been clearly established (see 63). Speck et al (95) found that removal of gut bacteria from Nasutitermes nigriceps did not affect the termites' amino acid metabolism. However, Reticulitermes santonensis specimens freed of their protozoa, bacteria, or both, showed reduced incorporation of ^{14}C-glucose into amino acids (95). By contrast, removal of protozoa and most bacteria from guts of Coptotermes formosanus did not alter the insects' protein-bound amino

acid content (69,70). Further studies with C. formosanus revealed that their content of free amino acids was reduced by the removal of gut bacteria and two of three major species of gut protozoa (69). Similarly, incorporation of ^{14}C-acetate into protein-bound amino acids was reduced in the absence of the symbionts (69).

Cleveland (26) postulated that fixation of atmospheric N_2 could be important to termites. Although termites survived when fed N-free diets of filter paper, Cleveland was unable to detect N uptake by termites when he used manometric techniques (26). Likewise, Hungate (49,51) found no evidence for N_2 fixation by termites maintained on a variety of wood, filter paper, or cotton diets. Greene and Breazeale (41) reported the isolation of N_2-fixing bacteria from termites, but did not detail their studies nor pursue their findings.

Tóth (96,97) investigated a number of insects, including termites and aphids, for N_2 fixation by using a "surviving system" technique. He macerated and incubated insect tissue in a N-free organic broth, then measured the increase in combined N in the preparation. Whole termites assayed in this manner caused a 60% increase in the N content of the medium (96,97). Tóth susequently isolated bacteria which similarly appeared to bind N (97). However, the significance of Tóth's results are questionable. Symbiont-free abdominal and thoracic tissues of Kalotermes flavicolis appeared to incorporate more N than did the symbiont-laden hindgut preparations (96). Moreover, the "symbionts" were isolated on medium which contained agar (97), and therefore was not completely free of combined N. The organisms isolated were not identified or quantitated, nor was their origin from the termite hindgut firmly established (97). The importance of the

cultured bacteria to termite N_2 fixation could not be determined.

Tóth's surviving system method for quantitating N_2 fixation was criticized by Smith (94). Using aphids, which Tóth had also reported as being capable of N_2 fixation, Smith (94) measured the ability of insects to take up ^{15}N. No incorporation of ^{15}N by the specimens was detected (94). The sensitivity of Smith's technique would have allowed detection of N uptake equal to 0.03% of the insect's total N (94), a value much lower than Tóth's reported aphid N-binding activity (97). Tóth's results with aphids were, therefore, refuted, and his data for other insects assayed by the surviving system technique were questionable. Although Smith did not assay termites for ^{15}N incorporation, the doubt cast on Tóth's work, in addition to Tóth's own questionable results with termite tissues and "symbionts", suggested that further studies of termite N_2 fixation were necessary.

The potential role of symbiotic gut bacteria in termite N_2 fixation has recently been elucidated (7,12,39,86). The sensitivity of the acetylene reduction assay as a measure of N_2 fixation (85) has permitted quantitation of nitrogenase activity in numerous termite species (7,11,12) and has revealed the potential significance of N_2 fixation to termite development (11). The level of N_2 fixation exhibited by young larvae of Coptotermes formosanus could enable these termites to double their protoplasmic N content in the period of one year (11). Moreover, Breznak et al (12) clearly established that gut bacteria mediated N_2 fixation in termites. These workers found that antibacterial drugs fed to termites abolished the insects' N_2-fixing ability (12). The loss was correlated with the loss of hindgut bacteria, whereas protozoa were not directly affected (12). In

addition, the level of N_2 fixation observed reflected the N content of the termites' diet: on a low N diet of filter paper, N_2-fixing activities were relatively high, compared to lower activities on high N diets (12).

Bacteria mediating N_2 fixation in termites have been isolated and identified (39,86). Potrikus and Breznak (86; see Appendix II) isolated and quantitated N_2-fixing Enterobacter agglomerans from guts of C. formosanus. The bacteria, which fixed N_2 only under anaerobic conditions [the presumed state of the termite hindgut (8,101)], were present in populations of about 200 cells/gut, or about 2.9×10^5 cell/ml of gut fluid (86). The N_2-fixing activity of E. agglomerans could be important to the N economy of C. formosanus (86). N_2-fixing Citrobacter freundii were isolated, though not quantitatively, from three species of Australian termites (39), whereas Enterobacter sp. capable of growth on N-free medium were isolated from a number of other termite species (36). The former isolates fixed N_2 only under anaerobic conditions (39) whereas the latter appeared to be active aerobically (36). However, the medium on which the latter Enterobacter isolates were cultivated contained agar (36), and therefore contained combined N. The authors noted the small size of colonies on the isolation medium (36), suggesting unfavorable growth conditions for the bacteria. It is reasonable to assume that the Enterobacter isolates obtained by Eutick et al (36) were growing aerobically by scavenging the small amounts of N contained in the medium, rather than by fixing atmospheric N_2.

In spite of confirmation of the in vivo ability of termite symbionts to fix atmospheric N_2, and the potential significance of

this activity, not all termites demonstrate high levels of N_2 fixation (7,12). It would seem, therefore, that termites might have yet another means to obtain or conserve N. Indeed, conservation of combined N could be very important to the N economy of a termite colony.

Leach and Granovsky (64) postulated that termite gut symbionts conserve N by catabolizing urates or other nitrogenous wastes elaborated into the hindgut by the insect's Malpighian tubules. If the N was incorporated into microbial protoplasm, it could be recycled to the termite by proctodeal feeding or by direct absorption of nitrogenous compounds released from lysed microbes within the paunch (64). Although studies already discussed have shown that few protozoa die within the termite hindgut, the potential for microbial-mediated N conservation is significant. Protozoan and bacterial metabolites released into the hindgut could be assimilated by the termite directly, as has been hypothesized for the B-complex vitamins synthesized by yeast symbionts of some beetles (see above).

Hungate (49) considered the possibility of microbial recycling of urates, but found that 0.02% of the fecal pellet dry weight of Zootermopsis was uric acid. Thus, he concluded that protozoa could not efficiently metabolize uric acid, and N was therefore excreted and not conserved (49). However, in view of the large amounts of uric acid excreted by truly uricotelic insects (e.g., see 88), the small amount of the purine in termite excreta appears insignificant.

In contrast to Hungate's opinion, Tóth (97) felt that termite gut symbionts could indeed conserve N by catabolism of uric acid. He reported both uricolytic and ureolytic activity in cultures of termite

bacteria incubated in the presence of uric acid and urea, respectively (97). Unfortunately, these microbes were neither identified nor quantitated, and, as with Tóth's purported N_2-fixing termite symbionts, their roles in the insect could not be affirmed. Sebald (93) reported the isolation of uricolytic bacteria from guts of R. lucifugus, but did not study their roles in termite nutrition. Noirot and Noirot-Timothée (80) suggested that bacteria in the mixed segment of higher termites might reutilize nitrogenous wastes. In Cephalotermes rectangularis, urine apparently passes from the Malpighian tubules directly into the mixed segment, where an abundant population of bacteria resides (80). No studies of these bacteria have yet been reported.

Jucci (54) and Moore (72) detected uric acid in termite specimens. However, no quantitative measurements of uric acid in termites were reported, nor was the potential significance of uric acid to termite N nutrition investigated. From the meager evidence presented by Hungate (49) regarding uric acid excretion by termites and from Jucci (54) and Moore (72) regarding detection of uric acid in termites, Moore (72) concluded that uric acid is a nitrogenous excretory waste of termites. Moore (72) as well as Hungate (49) contended that uric acid could not be utilized by termites, either directly or by intervention of microbial symbionts, and therefore the purine was not important to termite N conservation.

STATEMENT OF PURPOSE

Since N acquisition is a problem critical to termite growth and survival, it seemed wise to reconsider the possible significance of microbial-mediated recycling of termite nitrogenous wastes. The paucity of data regarding synthesis, storage, and excretion of nitrogenous compounds by termites severely limits our understanding of termite N nutrition. The potential significance of microbes in conserving termite "excretory" N could not be investigated without more knowledge of termite excretory products and processes. Studies with other insects, particularly cockroaches, to which termites are phylogenetically related (27), have shown that uric acid can serve as an important nitrogenous reserve (see above). The reports of Jucci (54) and Moore (72), who demonstrated that termites do contain uric acid, and the lack of significant amounts of uric acid in termite feces (49), buttressed the contention that uric acid might serve as a nitrogenous reserve material in termites, as it does in cockroaches. Thus, it was felt that a quantitative investigation of the uric acid content of termites and their excreta was necessary as a prelude to investigating the roles of termite symbionts in the N metabolism of their host.

The hypothesized roles of cockroach mycetocyte bacteria in uric

acid catabolism have been difficult to verify (see above). Termites, excepting the species Mastotermes darwiniensis, do not possess mycetocyte symbionts (55). Rather, an abundant population of protozoa and bacteria reside in the readily accessible hindgut (11,36,46,91,93). Sebald's (93) isolation of termite hindgut bacteria capable of fermenting uric acid suggested that the role of termite symbionts in uric acid turnover might be more easily addressed than that of the uncultured cockroach bacteroids. Moreover, preliminary studies with the termite Reticulitermes flavipes revealed significant populations of putative uricolytic bacteria in the insect's hindgut. The microbes grew readily on common laboratory media supplemented with uric acid (see Article II). These results prompted the present investigation of microbial intervention in termite uric acid metabolism and N conservation.

The studies reported here were undertaken to investigate whether the gut microbes of lower xylophagous termites could recycle N within the insect by catabolizing the termite's nitrogenous wastes. The investigations embrace a detailed, quantitative study of uric acid in termites and their excreta; quantitation and identification of uricolytic bacteria from guts of R. flavipes; studies of the metabolism of uric acid (and other purines), including quantitative analyses of the end products of uric acid fermentation, by the isolated symbionts in vitro; measurements of in situ uricolysis by termite gut bacteria; and determinations of the termite's ability to synthesize and transport uric acid, as well as to use the products of gut microbial uricolysis. An understanding of the importance of microbial uricolysis to termite N nutrition may help clarify a problem which has

puzzled biologists for many years, and advance our understanding of the biology of termites. This intriguing insect-microbe symbiosis reveals the potential significance of symbionts to uric acid catabolism in higher organisms.

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ARTICLE I

URIC ACID IN WOOD-EATING TERMITES

By

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Reprinted from Insect Biochem. 10:19-27 (1980)

Insect Biochem., *Vol.* 10, *pp.* 19 *to* 27. 37 0020-1700 80 0101-0019 $02.00 0
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URIC ACID IN WOOD-EATING TERMITES*

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(*Received* 9 *April* 1979)

Abstract—Uric acid (UA) was present in termites as assessed by paper and thin layer chromatography, by U.V. spectroscopy, by reactivity with uricase, and by gas chromatography coupled to mass spectrometry. Specimens of six species (*Reticulitermes flavipes*, *R. virginicus*, *Coptotermes formosanus*, *Marginitermes hubbardi*, *Paraneotermes simplicicornis*, and *Cryptotermes cavifrons*) contained UA in amounts accounting for between 1 and 45% of the termites' dry weight and for between 4 and 69% of the termites' total nitrogen. Almost all of the UA in *R. flavipes* was associated with fat body tissue. Faeces contained less than 0.2% of its weight as UA. During 15 months of captivity, UA in *R. flavipes* workers increased from 1.7% to 45.4% of the insects' dry weight at a linear rate of 2.7% per month. Elemental analyses of termites during this period indicated that synthesis of UA occurred, in part, at the expense of some endogenous nitrogen and carbon. Compounds tentatively identified as inosine and kynurenic acid were also present in termite extracts. Results indicate that termites can store large quantities of UA internally, but they do not void the purine in significant amounts despite the lack of detectable uricase activity in termite tissues.

Key Word Index: uric acid, termite, nitrogen, fat body, uricase, inosine, kynurenic acid, faeces

INTRODUCTION

THE ABILITY of many termites to thrive on nitrogen-poor diets of sound wood or cellulose is intriguing in terms of the insects' nitrogen-economy. HUNGATE (1944) found that bound nitrogen in wood and soil, when acted upon by fungi, was sufficient to support good growth of laboratory colonies of *Zootermopsis* species. However, recent studies (POTRIKUS and BREZNAK, 1977 and references therein) also support the long-held belief that N_2 fixation by gut microbes supplements the intake of dietary nitrogen by some termites. LEACH and GRANOVSKY (1938) had advanced an alternative hypothesis for nitrogen-conservation by termites. They postulated that the nitrogen in uric acid (UA), a presumed waste product of termites, could be recycled by the action of uricolytic gut bacteria.

Two crucial elements of the LEACH and GRANOVSKY hypothesis are: (i) termites form UA (or a similar compound); and (ii) termites contain uricolytic organisms in their hindguts. Although there are a few reports of UA formation by termites (JUCCI, 1921; HUNGATE, 1941; MOORE, 1969), detailed and specific chemical analyses are lacking. In this paper we provide conclusive evidence for the presence of UA in termites and estimate its concentration and distribution in termite tissues and in faeces. A separate communication (in preparation) will document the presence of uricolytic bacteria in termite hindguts.

MATERIALS AND METHODS

Termites and termite faeces. *Reticulitermes flavipes* was collected in Janesville, WI and Spring Arbor, MI and *Coptotermes formosanus* was collected in Lake Charles, LA. Both species were maintained in the laboratory as previously described (SCHULTZ and BREZNAK, 1978). Specimens of *R. flavipes* and *R. virginicus* from Mississippi, and *Paraneotermes simplicicornis* and *Marginitermes hubbardi* from Arizona, were gifts from Dr. M. I. HAVERTY. Samples of *Cryptotermes cavifrons* collected in Florida were kindly supplied by Dr. SYDNEY TAMM.

Faecal pellets were obtained from galleries in infested wood. To insure that extraneous (i.e. non-faecal) material was not included for the analyses, only those pellets which were distinctly prolate spheroid in shape were selected. For collection of fresh excreta, one hundred termites were placed on a disc of stainless steel mesh screen fitted into a 60 ml jar. After 12–24 hr the screen was removed and the adhering faeces, which in this case consisted both of formed pellets and pasty amorphous excrement, were scraped off and processed immediately.

Unless indicated otherwise, all samples for analyses were dried at 85 C for 8 hr, pulverized with a porcelain pestle, and then stored in a vacuum dessicator over $CaSO_4$ until required for assay.

Extraction of uric acid. Routinely 1–5 mg of powdered termite tissue were suspended in 2 ml of 0.6% Li_2CO_3 for 10 min at 60 C, followed by centrifugation at 3000 *g* for 15 min at 25 C. The supernatant fluid was then used immediately for paper (PC) or thin layer (TLC) chromatography; for determination of U.V. absorption spectra; or for enzymatic assay of UA. Experiments showed that all the UA was solubilized by the one extraction.

Faeces were extracted as described above for TLC, but were extracted with hot (85 C) distilled water for 15 min for enzymatic assay of UA. The latter procedure extracted all the UA, but did not solubilize Li_2CO_3-soluble, U.V.-absorbing material in faeces which interfered with the UA assay.

Paper and thin layer chromatography. Samples were co-chromatographed with standards on sheets of Whatman No. 1 qualitative filter paper or on 0.1 or 0.25 mm cellulose MN 300 TL plates (Brinkman) by using ascending solvents in one or two dimensions. From over thirty solvent systems investigated, the three yielding the most satisfactory separations in one dimension, and chosen for routine use, were: (A) isopropanol: H_2O, 10:3 (v/v) (DIKSTEIN *et al.*,

* Journal article No. 8871 from the Michigan Agricultural Experiment Station.

1956); (B) *n*-butanol:acetic acid:H_2O, 12:3:5 (by vol.) (DONNELLAN and KILBY, 1967); and (C) *n*-propanol:NH_4OH:H_2O, 60:26:14 (by vol.) (PATAKI, 1967).

Compounds were detected on chromatograms by viewing under a short wavelength U.V. lamp (Mineralite, Ultra-Violet Products, Inc., San Gabriel, CA) and/or by using the diphenylcarbazone (DPC) spray reagent (DIKSTEIN *et al.*, 1956). When desired, spots visualized by UV light were scraped from TLC plates and eluted from the cellulose with 0.6% Li_2CO_3.

All standards for PC and TLC were prepared in 0.6% Li_2CO_3.

Enzymatic assay of uric acid. UA content of termites and faeces was estimated by measuring the decrease in absorbance at 292 nm after treatment of extracts in 0.1 M glycine buffer (pH 9.4) with 0.015 units of purified uricase (hog liver, Type V; Sigma Chem. Co; Technical Bulletin No. 292-UV). Spectrophotometric measurements were made by using a Varian model 634S double-beam recording spectrophotometer coupled to a Sargent–Welch model SR recorder. All values for UA are reported as % dry weight, unless indicated otherwise.

Gas chromatography and mass spectroscopy (GC–MS). For GC–MS of UA, termites were extracted as described above except that 5 M KOH was used. Tetraethyl derivatives of UA were then prepared (ISMAIL and DAKIN, 1975), extracted into diethyl ether, and separated by GC with a 1.83 m × 2 mm i.d. glass column packed with 3% (w/w) OV-17 on 80/100 mesh Supelcoport (Supelco, Inc., Bellefonte, PA). Nitrogen was the carrier gas (30 ml/min) and operating temperatures were: column, 200 C; injector, 260 C; and flame ionization detector, 270 C. Standards were commercial UA, derivatized as described above, and caffeine, which served as an internal reference.

Appropriate GC fractions were diverted for MS analysis to an LKB 9000 mass spectrometer operating at an ionization potential of 70 eV and a temperature of 250 C.

Total nitrogen and total carbon determinations. Total nitrogen content of termites and faeces was determined by the micro-Kjeldahl method of JOHNSON (1941), except that a 6 hr digestion time was used. Bovine serum albumin was used as a standard. Total nitrogen and carbon were in addition determined by using a C. Erba model 1104 elemental analyzer equipped with a CSI model 208 digital integrator. The combustion temperature was 1050 ± 20°C and the internal standard was cyclohexanone-2,4-dinitrophenylhydrazone. Finally, a few samples were commercially analyzed for total nitrogen by Galbraith Laboratories, Inc., Knoxville, TN.

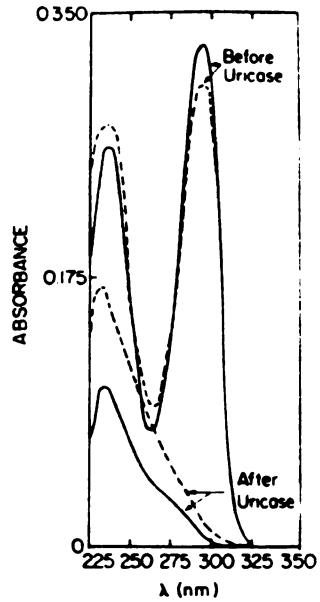

Fig. 1. U.V. absorption spectra of Li_2CO_3 extracts of standard uric acid (———) and of *R. flavipes* workers (– – – – –) before and after exposure to commercial hog liver uricase enzyme for 30 min.

Uricase assay. Whole termites, termite bodies with guts removed, or termite guts were homogenized in 0.5 M potassium phosphate buffer (pH 7.4) and assayed for uricase activity by the method of SCHNEIDER and HOGENBOOM (1952). Gut homogenates were assayed immediately, whereas others were first dialyzed at 4°C against 0.01 M Na borate buffer (pH 10.0) for 36 hr to remove interfering levels of endogenous UA. Mouse liver homogenate was used as a positive control (SCHNEIDER and HOGENBOOM, 1952). Protein was determined by the biuret method (HERBERT *et al.*, 1971) with bovine serum albumin as standard.

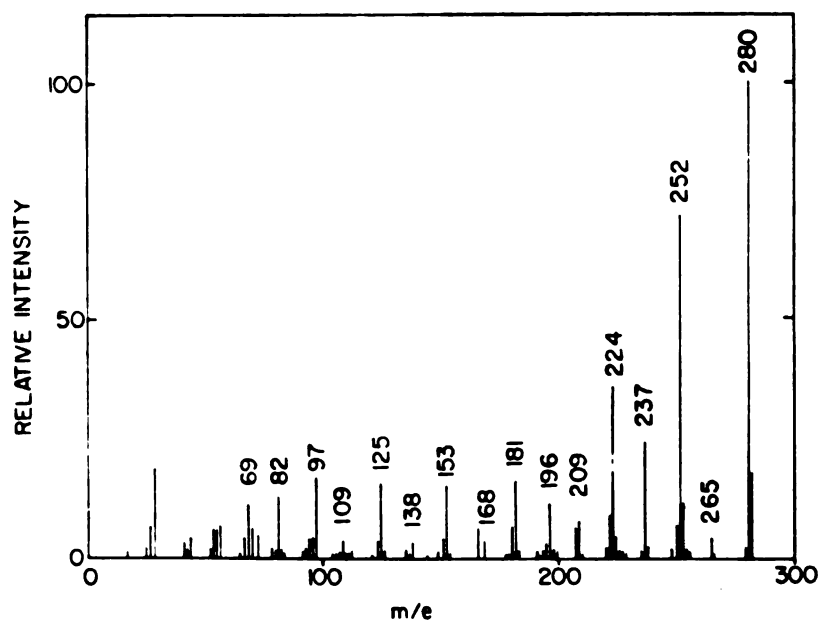

Fig. 2. Mass spectrum of tetraethyl uric acid prepared from workers of *R. flavipes*.

N_2 fixation assay. Live termites were assayed for N_2 fixation activity by methods previously described (BREZNAK *et al.*, 1973).

Chemicals. UA, Li_2CO_3, bovine serum albumin and chromatography standards were obtained from Sigma Chemical Co., St. Louis, MO. All other chemicals were of reagent grade and were obtained from various commercial sources.

RESULTS

Identification of uric acid in R. flavipes

U.V. absorption spectra of Li_2CO_3 extracts of *R. flavipes* workers were similar to that of standard UA, exhibiting maxima at 292 and 236 nm and minima at 262 nm (Fig. 1). Treatment of such preparations with uricase obliterated the 292 nm absorption maximum (Fig. 1).

PC and TLC of *R. flavipes* extracts revealed the presence of a prominent DPC-reactive, U.V. light-absorbing compound whose migration rate was similar to that of standard UA. R_{UA} values for this compound in solvent systems A, B, and C were 1.00, and values ranged from 0.94 to 1.00 in all solvent systems employed. Eluates of the termite compound and standard UA possessed identical U.V. absorption spectra, similar to those depicted in Fig. 1. Likewise, the 292 nm absorption maximum of such eluates was uricase-sensitive.

GC revealed two major derivatives (I and II) present in termite extracts as well as in solutions of standard UA. Retention times of these compounds relative to the caffeine standard were: compound I, 1.30–1.33; compound II, 2.15–2.22. Mass spectra of compounds I and II were virtually identical to each other and to that reported for tetraethyl UA isomers (Fig. 2) (ISMAIL and DAKIN, 1975). The molecular ion was observed at mass/charge (m/e) = 280. Successive losses of 28 mass units each, corresponding to loss of the ethyl groups as ethylene, resulted in peaks at m/e = 252, 224, 196, and 168. Peaks at m/e = 265, 237, 209, and 181 indicated loss of methyl groups which presumably originated from the four ethyl ligands (ISMAIL and DAKIN, 1975). From these data we concluded that termites contain UA.

Uric acid content of R. flavipes *worker termites*

Freshly-collected *R. flavipes* workers contained 1–2.5% UA. However, during laboratory captivity the UA content increased markedly. Data for *R. flavipes* population I (collected in Janesville, WI in June, 1977) are given in Table 1. A more than twenty five-fold increase in UA was observed over the 15 month period of captivity. The relationship between UA content and length of captivity was evaluated by regression analysis and graphed by the method of least squares (Fig. 3; population I). As can be seen, the rate of increase of UA (i.e. the regression coefficient) was approx. 2.7% per month. Data for workers from a separate collection of *R. flavipes* (population II; Janesville, June, 1978) are also graphed in Fig. 3. The regression coefficient for population II revealed that UA increased at a rate of 2.1% per month during the 5½ months of captivity. Samples of workers from a third collection of *R. flavipes* (Janesville; June, 1976) as well as from a population of *C. formosanus*, initially contained 2% UA which increased to 24% after 7 and 14 months, respectively.

Table 1. Uric acid (UA) and total nitrogen (TN) content of *R. flavipes* workers during captivity[a]

Length of captivity (months)[b]	Average dry weight per termite (mg)[c]	% of dry weight[d] UA	TN[e]	$\frac{\%UA\text{-}N}{\%TN} \times 100$
0	0.81	1.7	11.06	5.1
0.13	0.70	2.9	10.55	9.1
4	0.63	15.1	14.57	34.5
5	—	24.6	14.30	57.3
6	0.63	26.0	14.69	58.8
7.5	0.67	26.1	14.81	58.7
9	0.81	32.4	15.56	69.2
10.5	1.06	31.1	15.41	67.3
14	1.10	39.3	19.74	66.5
15	1.08	45.4	22.87	66.0

[a] Population I. Collected in Janesville, WI in June, 1977.
[b] Zero month refers to termites assayed within 24 hr of collection.
[c] Determined by drying a mass of termites to constant weight and dividing this value by the number of termites in the sample.
[d] Mean of at least two determinations. Standard errors were within 4.0% (UA) or 1.5% (TN) of the mean.
[e] Values for commencement or between 4–6 months captivity were determined with the C. Erba elemental analyzer. Total nitrogen at 0.13 month was determined by Kjeldahl analysis. Other values were determined by Galbraith Laboratories, Inc.
[f] Not determined.

The total nitrogen content of population I workers doubled during the 15 month period (Table 1). Moreover, statistical analysis revealed that the UA and total nitrogen content were highly correlated (Pearson's r = 0.92). However, regression analysis of total nitrogen values yielded the line shown in Fig. 3 (individual data points not depicted). The rate of increase of total nitrogen was only 0.66% per month. If the increase in uric acid nitrogen (UA-N) occurred soley at the expense of exogenous nitrogen, one might expect the total nitrogen to increase at a rate one third that of UA (i.e. 1/3 × 2.7 = 0.9% per month) since one third UA is nitrogen by weight. However, the observed rate of change of total nitrogen (i.e. 0.66% per month) was significantly different from the expected rate at the

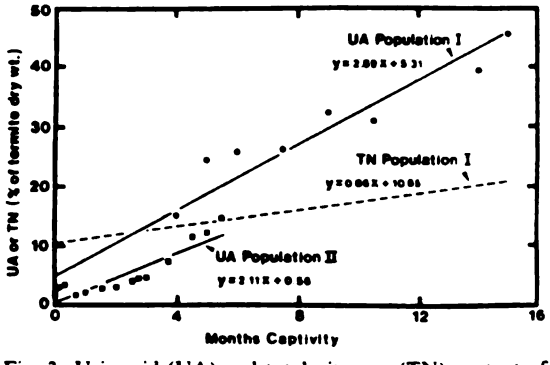

Fig. 3. Uric acid (UA) and total nitrogen (TN) content of *R. flavipes* during laboratory captivity. Coefficients of determination (r^2) for the lines were 0.94 (●——●), 0.83 (■——■), and 0.88 (---).

Table 2. Uric acid (UA) and total nitrogen (TN) content of various termite species

Family	Species	Origin	Approx. length of captivity (months)	Developmental Stage	% dry weight[a] UA	TN[b]	$\frac{\%UA\text{-}N}{\%TN} \times 100$
Rhinotermitidae	*R. flavipes*	Mississippi	1	Larva	3.5	9.35	12.3
				Worker	1.8	10.23	5.7
				Nymph	3.0	7.97	12.7
				Soldier	2.2	10.42	6.9
	R. virginicus	Mississippi	1	Larva	3.0	8.62	11.6
				Worker	1.1	9.99	3.7
				Nymph	1.2	7.23	5.4
				Soldier	1.6	10.82	5.0
	C. formosanus	Louisiana	1	Worker	4.7	10.22	15.2
				Soldier	2.3	7.42	10.5
			3	Alate	5.3	9.53	18.6
Kalotermitidae	*P. simplicicornis*	Arizona	60	Larva	7.8	8.34	31.2
				Pseudergate	8.4	7.35	38.0
				Nymph	5.1	—	—
				Soldier	4.9	10.75	15.2
	M. hubbardi	Arizona	28	Pseudergate	13.6	—	—
				Nymph	9.5	9.04	34.9
				Soldier	11.5	—	—
	C. cavifrons	Florida	12	Worker	7.6	—	—

[a] Mean of at least two determinations. Standard errors were within 4.0% (UA) or 2.0% (TN) of the mean.
[b] Values were determined with the C. Erba elemental analyzer.
[c] UA content of faecal pellets of *P. simplicicornis* and *M. hubbardi* was < 0.01% and < 0.04%, respectively, whereas total nitrogen values were 0.79% and 0.59%, respectively.
[d] Not determined.

0.05 level (Student's *t* test). This suggests that some non-UA-N initially present in the termite was expended during UA biosynthesis. From the data in Table 1, it can be calculated that 26.2% of the initial endogenous non-UA-N was expended over the 15 month period. Also during this period, the contribution of UA-N to the total nitrogen increased from 5% to almost 70% (Table 1).

Assays at the commencement and after 9 months captivity revealed that the N_2-fixing activity of *R. flavipes* was 0.43 and 0.10 μg nitrogen fixed per g fresh wt per day, respectively.

The total carbon content of termites during the first 6 months of captivity of population I revealed a decrease from 50.57% to 46.57% of the dry weight. This was remarkable as uric acid carbon (UA-C) increased from 1.12% of the dry wt to 17.16% during this period (calculated from data in Table 1). Apparently, 40.5% of the initial endogenous non-UA-C, i.e. $[(50.57-1.12)-(46.57-17.16)]/(50.57-1.12)$ was also expended during UA biosynthesis.

While not influencing the foregoing calculations, it may be noted that the average dry wt of *R. flavipes* workers varied between 0.63 and 1.10 mg in a manner that bore no apparent correlation with changes in UA, total nitrogen, or total carbon (Table 1). The variation in the average dry wt during the 15 month period may have been due to reproductive activity in the population (ESENTHER, 1977).

Accumulation of UA by termites was not only detectable by enzymatic analyses, but could also be inferred by visual inspection of live specimens. Accumulation of white, chalky material in fat body tissue accompanied the increase in UA (Fig. 4). Dissection and careful removal of abdominal fat body tissue revealed that it contained from 93% to 95% of the insects' total UA stores (two experiments; pooled tissue from five insects). Little or no UA was associated with excised guts.

Analysis of excreta and nest wood of R. flavipes

R. flavipes faecal pellets selected from nest wood galleries were about 0.5 × 0.25 mm in size and possessed slight longitudinal grooves presumably formed by the termites' rectal papillae during pellet dehydration and expression. Their colour ranged from light tan to moderate brown, with no evidence of a 'salt and pepper' appearance (COCHRAN, 1973). These pellets contained only trace amounts of UA, accounting for less than 0.04% of the pellet dry weight. The total nitrogen content of the pellets was 0.52%. Freshly-voided faeces from *R. flavipes* workers contained only 0.18% UA, despite the fact that the termites used for the experiment contained 15% UA by weight.

Dried milled nest wood contained 0.36% total nitrogen, but no detectable UA.

Distribution of uric acid in various termite species

Specimens of *R. flavipes* collected in Mississippi, as well as those of five other termite species representing the Rhinotermitidae and Kalotermitidae, all possessed UA in amounts ranging from about 1–14% of the insects' dry weight and accounting for 4–38% of the insects' total nitrogen (Table 2). The presence of UA in all specimens was also verified by TLC with solvent systems A and C. No UA was detected in faecal pellets of *P. simplicicornis* or *M. hubbardi*,

Figure 4. Deposition of uric acid in fat body tissue of R. flavipes workers. Specimen A was freshly-collected and contained 1.5% uric acid (w/w), whereas specimen B was from a population held in captivity for 15 months and contained 45.5% (w/w). Note the intense white colouration of fat body tissue of specimen B due to uric acid deposition.

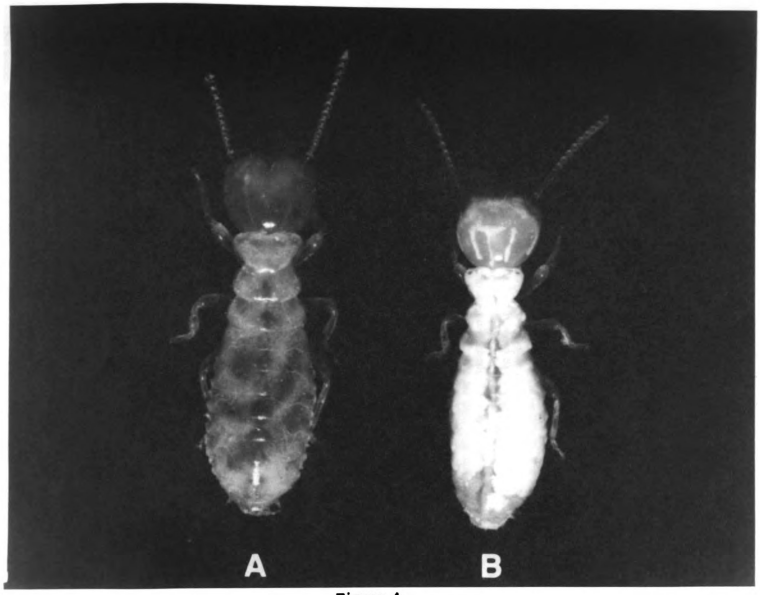

Figure 4.

although such pellets contained about 0.7% total nitrogen (Table 2, footnote [c]).

Assay for uricase activity in R. flavipes *tissues*

No uricase activity was detected in homogenates of whole worker termites, bodies with guts removed, or excised guts. However, commercial uricase (hog liver, type V), when added to termite homogenates, retained full activity even after admixtures were subjected to dialysis (see Materials and Methods). These data indicated that termite homogenates did not contain substances inhibitory to uricase activity. Control homogenates prepared from mouse liver readily degraded UA at a rate of 7.26 nmoles per min per mg protein. This rate was comparable to that reported by SCHNEIDER and HOGENBOOM (1952).

Other compounds present in R. flavipes *extracts*

TLC of *R. flavipes* extracts revealed consistently three compounds in addition to UA that were detectable by U.V. illumination of plates. The compounds, designated T-1, T-2, and T-3, were observed in freshly-collected specimens of *R. flavipes* as well as in specimens containing high amounts of UA. Identification of the compounds was made by comparing them with standards in terms of their: R_f values; visual appearance under short λ U.V. light; and reactivity with DPC spray reagent. T-1 was tentatively identified as kynurenic acid after TLC in two solvent systems. The R_f value in solvent system A was 0.44 while that of standard kynurenic acid was 0.46. Both compounds fluoresced blue–green under U.V. light and were DPC negative. T-2 was tentatively identified as inosine after TLC in six solvent systems. The R_f value in solvent system A was 0.37, compared with 0.39 for commercial inosine. It appeared dark grey under U.V. light and was DPC positive. T-3 gave an R_f value of 0.27 in solvent system A, migrating just ahead of UA (R_f = 0.16). T-3 fluoresced blue-violet under U.V. light and was DPC negative. The identity of T-3 is not known. Kynurenic acid and T-3 were associated primarily with extracted guts of *R. flavipes*, whereas inosine was observed only in extracts of whole termites or bodies from which guts had been removed.

DISCUSSION

Results presented in this paper showed conclusively that xylophagous termites formed UA in amounts ranging from 1–45% of their dry body weight and accounting for 3–69% of their total nitrogen (Tables 1 and 2). While these values seem surprisingly high, they are not unreasonable: MULLINS and COCHRAN (1976) found UA to constitute 8–31% of the dry weight and 4–72% of the total nitrogen of cockroach species they examined. Moreover, as with cockroaches and certain other insects (COCHRAN, 1975), the principle site of UA deposition in termites was fat body tissue. This tissue may also be the site of UA synthesis in termites. In light of significant amounts of UA in *R. flavipes*, it was not unusual to also detect inosine in extracts of this species. Inosine is a precursor for UA synthesis by either the nucleicolytic or uricotelic pathway (COCHRAN, 1975). Inosine, like UA, was not found in the gut. Kynurenic acid, however, was associated primarily with guts of *R. flavipes*. Kynurenic acid may be derived from a side reaction in the biosynthesis of ommochromes from tryptophan (LINZEN, 1974) and, therefore, not be directly involved in termite purine metabolism. Kynurenic acid has also been found in American cockroach excreta (MULLINS and COCHRAN, 1973).

Despite appreciable levels of UA in the termites themselves, faecal analyses revealed that *R. flavipes*, *P. simplicicornis* and *M. hubbardi* did not void significant amounts of this purine. Of the small amount of nitrogen in faecal pellets (approx. 0.6%, w/w) less than 3% of that was attributable to UA-N. Thus, these species, as well as most cockroaches (COCHRAN, 1973, 1976), dispose of their UA by the mechanism of 'storage excretion' (MADDRELL, 1971; COCHRAN, 1975) and constitute exceptions to the general notion that terrestrial insects should void UA (NEEDHAM, 1938).

Freshly-collected *R. flavipes* contained about 2% UA which increased to 45% during 15 months of captivity. It is not yet known what factors cause UA accumulation in termites or whether accumulation of UA to such an extent also occurs in nature. However, it seems likely that termites accumulate UA because their intake of dietary nitrogen exceeds that needed for biosynthesis. This was clearly shown for American cockroaches fed diets high in casein (MULLINS and COCHRAN, 1975a). What was surprising with *R. flavipes*, however, was the fact that appreciable amounts of endogenous non-UA-N and non-UA-C, equivalent to 26 and 41% of the termites' initial total nitrogen and total carbon, respectively, were expended during UA biosynthesis over the 15 month period. This does not necessarily mean that such nitrogen and carbon were incorporated into the UA molecule, although that seems likely. Nevertheless, it must be remembered that whole termites were used for total nitrogen and total carbon assay. Therefore, the origin of such endogenous nitrogen and carbon expended could have been termite tissue, or gut contents, or both.

Expenditure of endogenous nitrogen could not alone have accounted for the increase in UA and total nitrogen, however; obviously exogenous nitrogen must have also been exploited. Two likely sources of exogenous nitrogen were nest wood and termite protoplasm (i.e. via cannibalism). For example, the average dry weight of termites at commencement and after 9 months captivity was 0.81 mg, whereas UA levels were 1.7 and 32.4%, respectively (Table 1). The net increase in UA-N was therefore 0.083 mg per termite at an expenditure of 0.046 mg endogenous nitrogen. If all the latter were incorporated into UA and the balance (i.e. 0.037 mg nitrogen) was derived solely from nest wood (which contained 0.36% nitrogen by weight), wood consumption would have had to have been about 10 mg wood per termite per 9 months or 0.036 mg wood per termite per day. HAVERTY (1976) reported a rate for wood consumption by *R. flavipes* (0.083 mg per termite per day) which could easily have supplied the necessary nitrogen, even assuming that the assimilation of nitrogen has an efficiency of 50% (HUNGATE, 1941; 1944). A similar argument may be made for cannibalism. Considering the average dry weight of *R. flavipes* to be about 1 mg, one can

calculate from the data in Table 1 that the increase in UA-N during 15 months would require 0.12 mg exogenous nitrogen or roughly 0.01 mg exogenous nitrogen per termite per month. Assuming the total nitrogen content of workers to be 10% by weight, cannibalism would have had to have occurred at a rate of at least one worker devoured per ten survivors per month to furnish the required nitrogen for UA synthesis. This level is, in fact, significantly less than that actually observed for *Zootermopsis angusticollis* maintained on artificial diets (HENDEE, 1935), although the extent of cannibalism in natural termite colonies has not been thoroughly documented (LEFAGE and NUTTING, 1978). N_2 Fixation by the gut microbiota might also contribute nitrogen for UA synthesis, although N_2 fixation rates of *R. flavipes* are relatively low compared to some other termite species (BREZNAK, 1975) and were low for the present specimens. Clearly, however, several exogenous nitrogen sources, alone or together, could furnish enough nitrogen to easily account for the observed accumulation of UA during laboratory captivity.

Our findings are consistent with the hypothesis of LEACH and GRANOVSKY (1938) in regard to termites' ability to form UA. However the value, if any, of UA accumulation to termites remains to be determined. In what must be considered a classic study, MULLINS and COCHRAN (1975a, b) showed that UA accumulated by cockroaches (*Periplaneta americana*) on a positive nitrogen balance diet was readily mobilized on negative nitrogen balance diets, and a considerable portion of UA-N was used then for oöthecal production by females. Interestingly, UA was not voided even during periods of extensive mobilization; NH_3 constituted the main nitrogenous compound released. Although *P. americana* is reported to possess uricase (CORDERO and LUDWIG, 1963), DONNELLAN and KILBY (1967) suggested that bacterial symbionts present in the fat body of this species also function in uric acid mobilization. It is not known whether cockroach gut bacteria also carry out uricolysis, but a prominent bacterial flora is indeed present in the alimentary tract of *P. americana* (BIGNELL, 1977; BRACKE *et al.*, 1978). By contrast, we could not detect uricase activity in *R. flavipes* tissues and have not yet thoroughly examined fat body tissue for the presence of endosymbiotic bacteria. However, significant populations of uricolytic bacteria were present in hindguts (POTRIKUS and BREZNAK, manuscript in preparation). It is tempting to speculate that termites store UA as a reserve material but, as suggested by LEACH and GRANOVSKY (1938), require the activity of uricolytic gut bacteria in order to recover carbon, nitrogen and/or energy from the stored purine. If so, the assertion of MOORE (1969), that UA is merely a 'waste product' of termites, may require revision.

Note

Recent, ultrasensitive assays for uricase by the method of FRIEDMAN and MERRIL (1973) also failed to reveal this activity in *R. flavipes* Malpighian tubules or fat body tissue. Control assays, employing single Malpighian tubules from newly emerged adults of *Drosophila melanogaster*, yielded typical positive results (FRIEDMAN and JOHNSON, 1977) whether such tubules were mixed with termite preparations or not.

Acknowledgements—We are indebted to Dr. DONALD G. COCHRAN for advice, encouragement and for comments on the manuscript prior to submission. We thank Dr. RICHARD W. MERRITT for help in photographing Fig. 4 and Drs. MICHAEL I. HAVERTY and SYDNEY L. TAMM for supplying some of the termite specimens. Mass spectrometry of tetraethyl UA was performed at the mass spectrometry facility of Michigan State University, a service supported by National Institutes of Health Grant RR00480. This investigation was supported in part by the Michigan Agricultural Experiment Station of Michigan State University, by National Science Foundation Grants BMS 75-05850 and PCM 77-05732, and by an NSF Predoctoral Fellowship awarded to C. J. POTRIKUS.

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ARTICLE II

URIC ACID-DEGRADING BACTERIA IN GUTS OF TERMITES [*RETICULITERMES FLAVIPES* (KOLLAR)]

By

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Reprinted from Appl. Environ. Microbiol. 40:117-124 (1980)

APPLIED AND ENVIRONMENTAL MICROBIOLOGY, July 1980, p. 117-124
0099-2240/80/07-0117/08$02.00/0
Vol. 40, No. 1

Uric Acid-Degrading Bacteria in Guts of Termites [*Reticulitermes flavipes* (Kollar)]†

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Uricolytic bacteria were present in guts of *Reticulitermes flavipes* in populations up to 6×10^4 cells per gut. Of 82 strains isolated under strict anaerobic conditions, most were group N *Streptococcus* sp., *Bacteroides termitidis*, and *Citrobacter* sp. All isolates used uric acid (UA) as an energy source anaerobically, but not aerobically, and NH_3 was the major nitrogenous product of uricolysis. However, none of the isolates had an absolute requirement for UA. Utilization of heterocyclic compounds other than UA was limited. Fresh termite gut contents also degraded UA anaerobically, as measured by $^{14}CO_2$ evolution from [2-^{14}C]UA. The magnitude of anaerobic uricolysis [0.67 pmol of UA catabolized/(gut × h)] was entirely consistent with the population density of uricolytic bacteria in situ. Uricolytic gut bacteria may convert UA in situ to products usable by termites for carbon, nitrogen, energy, or all three. This possibility is consistent with the fact that *R. flavipes* termites form UA, but they do not void the purine in excreta despite the lack of uricase in their tissues.

Uric acid (UA) is a major nitrogenous excretory product of many terrestrial insects (6, 7), and it is well suited to this purpose. Because of its poor solubility in water, UA can be defecated as a nontoxic solid. Water loss during excretion is thereby minimized (6, 7). However, UA should not be regarded as merely a nitrogenous waste product of insects. For example, *Periplaneta americana* cockroaches store UA internally and use it as a metabolic reserve when placed on nitrogen-deficient diets (24, 25). UA nitrogen mobilized under such conditions is used in part for oothecal production by females (25). Utilization of UA apparently occurs in some other insects as well (7).

Intuitively, one might expect that xylophagous termites, which thrive on nitrogen-poor diets, would have evolved efficient means of conserving combined nitrogen. One strategy to accomplish this was envisioned by Leach and Granovsky (19). These workers hypothesized that UA, elaborated into the termite gut via the Malpighian tubules, is degraded by the hindgut microbiota to a form of nitrogen reusable by the insects. A corollary to their hypothesis is that carbon atoms of UA are also reused. Leach and Granovsky performed no experiments to test their provocative notion, although the abundance of microbes in the termite hindgut (4, 5) makes their suggestion plausible.

A major interest in our laboratory is the role of gut organisms in termite nutrition. We therefore sought to critically test the UA-recycling hypothesis. In a previous paper (28), we reported that termites form UA, but they do not void the purine despite the lack of uricase in termite tissues. We now present data to reconcile this apparent anomaly and which support Leach and Granovsky's hypothesis. Herein we document the presence of uricolytic bacteria in guts of *Reticulitermes flavipes* termites. The present paper deals with the isolation, identification, and nutrition of the bacteria, as well as estimation of their population levels in situ. An accompanying paper (29) concerns the anaerobic metabolism of UA by pure cultures.

† Journal article no. 9312 from the Michigan Agricultural Experiment Station.

MATERIALS AND METHODS

Insects. *R. flavipes* (Kollar) termites were collected in Janesville, Wis., Dansville, Mich., and Spring Arbor, Mich., and were maintained in the laboratory as previously described (31). UA content of termites was determined by enzymatic assay after extraction from tissue with Li_2CO_3 (28).

P. americana L. cockroaches were obtained from the Pesticide Research Center of Michigan State University. They were maintained on a commercial dog food diet and were fed water ad lib.

Media and culture techniques. Strict anaerobic techniques were used for preparation of liquid and solid media (15, 17, 31) unless indicated otherwise.

The composition of culture media is reported as percentage (wt/vol), unless indicated otherwise. Double-layer agar plates were used to isolate uricolytic bacteria and contained 0.1 and 1.0% UA in the bottom and top layers, respectively (1). Isolation media were designated SUA, BHIU, and TYU. SUA was the supplemented uric acid agar of Barnes and Impey (1) with

the following exceptions: 0.09% beef extract (Difco Laboratories, Detroit, Mich.) plus 0.15% peptone (Difco) replaced Lab-Lemco, and 0.1% liver concentrate (Wilson Diagnostics) replaced liver extract. BHIU medium was supplemented brain-heart infusion (BHI) medium (15) containing UA as indicated above. TYU agar medium contained: tryptone (Difco), 1.0%; yeast extract (Difco), 0.1%; salts solution (15) minus $NaHCO_3$, 4% (vol/vol); cysteine·HCl, 0.05%; resazurin, 10^{-4}%; and UA as indicated above. All solid media contained 1.5% agar.

TYU broth medium consisted of TY basal medium into which NaOH-solubilized UA was incorporated. TY basal was similar to TYU agar (above), but lacked UA, agar, resazurin, and cysteine·HCl. For addition to sterile TY broth, UA was solubilized as a 2% solution in 0.5 N NaOH, filter sterilized (filter type GS; 0.22-μm pore size; Millipore Corp., Bedford, Mass.), and added at a final concentration of 0.1%. A predetermined amount of sterile 0.5 N HCl was then added to neutralize the medium before inoculation. TYFU broth medium was identical to TYU, but also contained filter-sterilized fructose at a final concentration of 0.04%. In general, TY-based broth media were prepared in air by heat sterilization and rapid cooling of the TY basal portion, followed soon thereafter by additions as described above and then replacement of the head space atmosphere with O_2-free gas. However, for culture of bacteroides the TY basal portion was prereduced and anaerobically sterilized (15). Culture vessels were then transferred into a vinyl anaerobic glove box (Coy Manufacturing Co., Ann Arbor, Mich.) containing an O_2-free atmosphere of N_2/H_2 (90/10, vol/vol) wherein appropriate additions to the medium were made. The head space atmosphere of culture vessels was replaced with N_2/CO_2 (95/5, vol/vol) after inoculation.

Semi-D basal medium, used to conduct fermentation balances with growing cells, contained: Trypticase (BBL Microbiology Systems, Cockeysville, Md.), 1.0%; salts solution (15) minus $NaHCO_3$, 4% (vol/vol); $MnCl_2 \cdot 4H_2O$, 0.005%; adenine, thymine, guanine, cytosine, and uracil, 5×10^{-4}% each; riboflavin, calcium pantothenate, nicotinic acid, pyridoxine·HCl, and folic acid, 10^{-4}% each; thiamine·HCl, 10^{-5}%; and biotin, 10^{-7}%. A chemically defined medium, D medium, was identical to semi-D medium but lacked Trypticase.

Small volumes (e.g., 10 ml) of broth media were usually contained in 18-mm anaerobe tubes (Bellco Glass, Inc., Vineland, N.J.) equipped with rubber stoppers. Mass culture of cells was done by using aspirator bottles of various sizes and employing O_2-free N_2 in the head space. Aerobic broth culture was as previously described (27).

Isolation of uricolytic bacteria. Removal of termite guts and isolation of bacteria was done as described previously (27, 31), except that gut homogenates were prepared in TY broth and treated in a microblender (Eberbach) for 60 s before dilution and plating. Isolation plates were incubated in an anaerobic glove box and were supplemented with CO_2 (31). After 7 days, presumptive uricolytic isolates were randomly picked from among those colonies surrounded by a clear zone in the otherwise opaque medium (1). Such colonies were easily recognizable, even against a background of 100-fold excess nonuricolytic colonies. Isolates were considered to be pure cultures after three successive passages on streak plates; microscopic observations, including Gram stains of cells, verified this conclusion.

Control experiments used the following inocula: surface washes of intact termites; and homogenates prepared from degutted termite bodies.

Characterization of isolates. General characterization of isolates was done as previously described (27, 31). Acidic fermentation products were determined after anaerobic growth of isolates in BHI containing 0.5% glucose (BHIG). Media for tests with *Bacteroides* isolates were prepared as described by Dowell and Hawkins (11). Spore formation was routinely evaluated by observing wet mounts or malachite green-stained preparations of cells by phase-contrast or bright-field microscopy, respectively. For *Bacteroides* isolates, spore formation was assessed while cells were growing on chopped meat agar slants for 3 weeks (15). In addition, the heat resistance of *Bacteroides* in starch broth was tested (15).

The moles percent guanine plus cytosine (G+C) content of deoxyribonucleic acid (DNA) of *Bacteroides termitidis* was determined by buoyant density analysis. Cells were grown in TY broth supplemented with 1% glucose, 0.024% DL-threonine, and [*methyl*-^{3}H]thymidine (10 μCi/ml) to radioactively label the DNA. DNA was partially purified by the method of Marmur (21), except that only two deproteinization treatments were used, and these were followed by a single precipitation step with isopropanol. The buoyant density of such preparations in CsCl gradients was determined as described by Schildkraut et al. (30). Samples were centrifuged at 20°C in a Beckman model 50Ti rotor at 40,000 rpm for 60 h. Centrifuge tubes were then punctured from the bottom, and fractions (10 drops each) were collected. Fifty microliters of each fraction was applied to trichloroacetic acid-impregnated filter paper squares, which were then washed, dried, and analyzed for radioactivity (12). The refractive index of fractions containing peak radioactivity was measured by using an Abbe 3-L refractometer (Bausch & Lomb, Inc., Rochester, N.Y.)., and from this the moles percent G+C in DNA was calculated (30). DNA from *Escherichia coli* B (ρ = 1.710; 30), prepared as described above but labeled by growth of cells with [*methyl*-^{14}C]thymidine (0.1 μCi/ml culture), was used as an internal standard.

Nutrition and growth studies. Most nutrition studies were done with the growth-limiting TY basal medium (above) to which additions were made. Heterocyclic compounds to be added were solubilized as described above for UA. Exceptions were inosine, purine, and caffeine (which were prepared as 2% stock solutions in water), and xanthine (which was prepared as a 1% stock solution in 0.5 N NaOH). All test substrates were filter sterilized (see above). Where necessary, pH adjustment was made with HCl (above) before inoculation. Some nutritional studies were done with medium D.

Growth of cells was measured turbidimetrically at 660 nm by using a Spectronic 20 colorimeter (Bausch & Lomb). Absorbance readings were converted to cell numbers by means of standard curves relating the

reading to direct microscopic counts (*Streptococcus*) or viable cell counts (*Bacteroides* and *Citrobacter*). Unless otherwise stated, cultures were incubated at 30°C (*Streptococcus*) or 37°C (*Bacteroides* and *Citrobacter*).

Assay for UA, NH_3, and urea in spent growth media was done after removal of cells by centrifugation at 12,000 × *g* for 10 min. UA was assayed spectrophotometrically at 292 nm by using hog liver uricase (uric acid diagnostic kit no. 292-UV; Sigma Chemical Co., St. Louis, Mo.). Urea was assayed by determining urease-dependent NH_3 production with phenol nitroprusside reagent (urea nitrogen diagnostic kit no. 640; Sigma). NH_3 was determined by using the urea assay reagents without urease.

Anaerobic metabolism of UA by minced termite guts. Guts were removed as described above from 100 *R. flavipes* workers and pooled into a small watch glass containing 500 μl of 0.1 M potassium phosphate buffer (pH 7.0). Guts were then minced with a scalpel, and transferred to a 13-mm test tube, and the suspension was made up to 1,500 μl with buffer. The suspension was then subject to agitation in a Vortex blender at high speed for 20 s. Five hundred microliters of such preparations was used per reaction mixture. Individual reaction mixtures (620 μl) contained: potassium phosphate buffer (pH 7.0), 52 μmol; dithiothreitol, 1 μmol; [2-^{14}C]UA, 2 to 3 nmol; sodium formate (optional), 1 μmol; and 30 to 40 minced gut equivalents.

Reactions were performed under 90% N_2/10% H_2 in 5-ml serum vials fitted with stoppers. Suspended from each stopper was a center well assembly (Kontes, Vineland, N. J.; catalog no. K-882320). Reactions were initiated by addition of UA and were terminated after 3 h by injection of 150 μl of 5 N HCl. Phenethylamine (200 μl) was then injected into the center well and allowed to absorb $^{14}CO_2$ for one additional hour, after which time radioactivity was estimated.

Radioactivity measurements. Radioactivity measurements and quench corrections were made as described previously (32).

Microscopy of insect fat body tissue. Fat body tissue was dissected into a primary fixative of 2% glutaraldehyde in 0.1 M potassium phosphate buffer (pH 7.4) and then held at 4°C for 2 h. After primary fixation, tissue was washed three times in glutaraldehyde-free phosphate buffer and postfixed in phosphate buffer containing 1% OsO_4. Specimens were then dehydrated with ethanol, cleared with propylene oxide, and embedded in Epon (20). Sections (1 to 3 μm thick) were cut with a diamond knife and were viewed directly by phase-contrast microscopy or by bright-field microscopy after staining with buffered azure II (18).

Other bacterial strains. For comparative purposes and as positive or negative controls, various known bacterial strains were used. Termite gut heterotrophic bacteria previously isolated on nonselective medium (31) were part of this laboratory's culture collection. *Proteus mirabilis*, *Citrobacter freundii*, *Staphylococcus aureus*, and *E. coli* B were obtained from the culture collection of the Department of Microbiology and Public Health, Michigan State University. *Streptococcus lactis* ATCC 19435, *S. cremoris* ATCC 19257, *S. faecalis* ATCC 19433, and *S. faecium* ATCC 19434 were obtained from the American Type Culture Collection, Rockville, Md. *S. lactis* C2 was obtained from L. L. McKay, Department of Food Science and Nutrition, University of Minnesota, St. Paul.

Chemicals. Heterocyclic substrates were obtained from Sigma Chemical Co. and were of the highest purity available. [2-^{14}C]UA was obtained from Amersham Corp., Arlington Heights, Ill. All other chemicals were of reagent grade and were obtained from various commercial sources.

RESULTS

Isolation and identification of uricolytic bacteria. Over a period of 3 years, uricolytic bacteria were consistently isolated from guts of *R. flavipes*, regardless of the termites' origin or length of captivity (Table 1). Population levels ranged from $<10^2$ to 6.1×10^4 cells/gut, with a grand mean of $3.5 \pm 1.7 \times 10^4$ (excluding the data from experiments 7 and 8). We do not know why so few uricolytic bacteria were detected in experiments 7 and 8 (Table 1). Control experiments (Table 1, footnote *d*) verified that uricolytic isolates were of gut origin. Light microscopy failed to reveal bacteria within termite fat body tissue cells. However, typical mycetocytes (i.e., specialized host tissue cells containing endosymbiotic bacteria) (8) were readily observed in fat body tissue of *P. americana* cockroaches used as a control.

The UA content of termites ranged from 0.4 to 31.1% of the insects' dry body weight (Table 1). As documented previously (28), UA stores of *R. flavipes* generally increase during laboratory captivity, yet no clear correlation existed between the UA content of termites and the number of uricolytic gut bacteria.

Of 117 strains initially isolated, 35 lost the uricolytic phenotype upon subculture from primary isolation plates, and they were not examined further. Of the 82 strains that retained the phenotype as a fairly stable characteristic, most were identified as group N *Streptococcus* sp., *B. termitidis*, and *Citrobacter* sp. One strain of group D *Streptococcus* was obtained.

General physiological and biochemical characteristics of the group N streptococcal isolates are presented in Table 2. Lactate was the major acidic fermentation product (Table 2), suggesting that cells were homolactic. Detailed studies with one isolate (strain UAD-1) verified this notion. When growing anaerobically in semi-D medium containing excess (i.e., 1%) glucose, strain UAD-1 formed (millimoles/100 mmol of glucose fermented): lactate, 162.5; acetate, 21.1; ethanol, 5.2; formate, 5.0; and CO_2, 7.0. Similarly, products from 1% fructose were (millimoles/100 mmol of fructose fermented): lactate, 168.4; ace-

TABLE 1. *Uricolytic bacteria present in guts of R. flavipes*

Expt no.	Termites[a]				No. of uricolytic bacteria/gut[d]	No. of isolates examined	Identification of isolates			
	Origin[b]	Date collected	Length of captivity (mo)[c]	UA content (% of dry wt)			Group N *Streptococcus*	Group D *Streptococcus*	*Citrobacter* sp.	*B. termitidis*
1	J	June 1976	1	ND[e]	3.8×10^4	20	18	0	2	0
2			4	ND	3.9×10^4	23	13	1	9	0
3			10	ND	2.8×10^4	15[f]				
4	J	June 1977	0	1.7	1.8×10^4	20[f]				
5			4	15.1	4.5×10^4	8	0	0	0	8
6			5	24.6	1.5×10^4	3	0	0	0	3
7			10.5	31.1	$<10^2$	0				
8	J	June 1978	0	2.0	$<10^2$	0				
9			8	18.0	3.2×10^4	10	0	0	10	0
10	J	June 1979	0	3.3	0.6×10^4	4	1	0	0	3
11	D	June 1979	0	4.0	5.6×10^4	6	6	0	0	0
12			2	0.4	6.1×10^4	3	3	0	0	0
13	D	August 1979	0	1.7	4.9×10^4	5	5	0	0	0

[a] All termites were workers, except for experiment 6 (brachypterous larvae).
[b] J, Janesville, Wis.; D, Dansville, Mich.
[c] Zero months refers to termites used within 24 h of collection.
[d] Mean value of two to six replicates made with SUA plates. No uricolytic bacteria were detected in surface washes of intact termites or in homogenates of degutted termite bodies.
[e] ND, Not determined.
[f] Isolates lost uricolytic ability upon subculture and were not studied further.

tate, 22.9; ethanol, 13.8; formate, 16.6; and CO_2, 9.1. However, the overall properties of group N isolates did not correspond exactly to those of a known species (9, 13). Uricolytic strains failed to produce NH_3 from arginine (distinguishing them from *S. lactis*), yet showed growth at 40°C, at pH 9.2, and in the presence of 0.3% methylene blue (distinguishing them from *S. cremoris*) (Table 2). Preliminary examination of lactate dehydrogenase activity of strain UAD-1 suggested similarities to that of *S. faecium*, a group D *Streptococcus* (13; E. Garvie, personal communication). The mol% G+C in the DNA of strain UAD-1 was 36.6 (E. Garvie, personal communication), a value in the middle of the range found for the genus *Streptococcus*. Based on these results, we deferred species assignment of the group N isolates.

B. termitidis isolates were acidogenic rods 0.5 × 3.1 μm in size. Their properties corresponded closely to those of known *B. termitidis* (14, 33), although the present isolates did not produce formate as a fermentation product and formed small amounts of acid from lactose in a delayed (5 days) reaction. In addition, all strains produced acid from mannitol, mannose, rhamnose, and trehalose, but not from melezitose. The mol% G+C in the DNA was 35.6 (strain UAD-50).

Citrobacter isolates were indole positive, similar to strains previously isolated on nonselective media (31). These were not assigned to a species.

Nutritional studies. Nutritional studies on the isolates were done to confirm UA utilization; to examine the role of UA as a carbon, nitrogen, and energy source for growth; and to evaluate their nutritional diversity with respect to heterocyclic compounds in general. UA increased the anaerobic cell yield of all isolates when the purine was incorporated into an otherwise growth-limiting medium (i.e., TY medium). These data suggested that energy was derived from anaerobic uricolysis by the isolates. Results with three representative strains are presented in Table 3. Preliminary experiments with *Streptococcus* UAD-1 revealed that small amounts (0.04%) of fructose markedly stimulated UA consumption and cell yields. Consequently, this sugar was frequently added to TY medium for evaluating the utilization of heterocyclic compounds by all strains. Interestingly, diauxic growth of strain UAD-1 (i.e., a primary logarithmic growth phase separated from a small secondary growth phase by 10 to 20 h) resulted when 0.04% glucose was included with UA. Under these conditions UA was consumed only slightly (i.e., 10% of initial) and only during the secondary phase, after all the glucose was depleted. Diauxic growth was not observed when fructose was included with UA.

Anaerobic cell yields of streptococci and bacteroides were at least twice as great with UA as with identical media lacking this purine (Table 3). In addition, NH_3 production was 79 to 100%

TABLE 2. *Characteristics of uricolytic group N Streptococcus isolates from R. flavipes*

Test or substrate	Reaction[a]
Gram reaction	Variable
Morphology	Ovoid; 0.6 to 0.7 μm in diam
Motility	−
Acidic fermentation products	Lf (as)
Final pH (BHIG medium)	4.5
Relation to O_2	Facultative
Catalase	−
Nitrate to nitrite	−
Growth in:	
BHI + 4.0% NaCl	+
BHI + 6.5% NaCl	−
0.1% methylene blue milk	+
0.3% methylene blue milk	+
Growth in BHI at:	
10°C	+
40°C	+
45°C	−(+)[b]
pH 9.2	+
pH 9.6	+
Heat tolerance[c]	−
Hydrolysis of:	
Esculin	+
Hippurate	−
Arginine	−
Hemolysis	α
Precipitin reaction with group N antiserum	+[d]

[a] Symbols: +, Positive reaction; −, negative. Acidic fermentation products: L, lactic; f, formic; a, acetic; s, succinic. Upper-case and lower-case letters refer to major and minor amounts, respectively (15). Parentheses indicate results with some strains.

[b] Two of 46 strains showed growth at 45°C.

[c] 60°C, 30 min.

[d] Only 12 strains tested.

of theoretical (based on UA consumed).

Although *Citrobacter* isolates readily cleared UA-containing plates, their cell yields were not greatly stimulated by UA in broth media (Table 3). Under the latter conditions, only small amounts of UA were consumed by *Citrobacter* strains, yet such UA consumption was accompanied by NH_3 production. Interestingly, fructose suppressed UA utilization and NH_3 production by all *Citrobacter* strains.

UA was not used as an energy source aerobically by streptococci or citrobacters. This conclusion was based on growth yield determinations as well as enzymatic assay of UA consumption.

The ability of isolates to use other heterocyclic compounds anaerobically was fairly limited (Table 3). *Streptococcus* UAD-1 appeared to catabolize allantoin and allantoic acid only slightly, as indicated by a small increase in cell yield and NH_3 production (Table 3), as well as formation of small amounts of urea (Table 3, footnote *d*). Increased growth of this strain on inosine and on fructose plus guanosine apparently resulted from utilization of the ribose portion of the molecules only, since: (i) the final pH of the media (pH 5.0 to 5.1) was more acidic than that of UA-containing media (pH 7.2 to 7.3); (ii) little or no NH_3 or urea was formed; (iii) the purine portion of the ribosides accumulated in (and in the case of guanosine alone, crystallized out of) the medium and was readily identified by thin-layer chromatography; and (iv) hypoxanthine and guanine alone were not used although ribose was. No other purines or pyrimidines tested were used by strain UAD-1.

B. termitidis UAD-50 appeared to use inosine and hypoxanthine; utilization of xanthosine was questionable. Interestingly, xanthine and guanosine inhibited growth of strain UAD-50. *Citrobacter* UAD-25 showed slightly more nutritional versatility than either strain UAD-1 or UAD-50. Utilization of guanosine, hypoxanthine, xanthine, and xanthosine was obvious from the increase in NH_3 production. Inosine utilization was questionable: although cell yields were increased, there was no increase in NH_3 production.

By using a chemically defined medium (medium D), we found that none of the representative strains used UA as sole carbon, nitrogen, and energy source for anaerobic growth. *Streptococcus* UAD-1 would not use 0.1% UA as sole nitrogen or sole energy source with 0.4% fructose or 1.0% Trypticase, respectively, but would use UA as an additional energy source with 0.04% fructose plus 1.0% Trypticase. Two *B. termitidis* strains tested behaved identically to *Streptococcus* UAD-1, except that one strain (UAD-55) used UA as sole nitrogen source in the presence of 0.4% fructose.

Comparative tests with other bacterial strains. Reference strains of group N streptococci (i.e., *S. lactis* ATCC 19435 and *S. cremoris* ATCC 19257) did not use UA when growing anaerobically in TYU or TYFU broth media, nor did these strains form clear zones on TYU agar plates. Tests were also done on *S. lactis* C2, as well as 54 group N streptococci previously isolated from guts of *R. flavipes* (31) and which included *S. lactis*, *S. cremoris*, and strains of unknown species. None used UA anaerobically as judged by lack of clear zone formation on UA-containing plates. Of *Citrobacter* strains isolated previously (31), only indole-positive biotypes cleared UA-containing plates. Previously isolated *Bacteroides* strains (31) were not tested for uricolytic ability, although the characteris-

TABLE 3. *Anaerobic utilization of uric acid and other heterocyclic compounds by growing cells*[a]

Added[b] to TY basal medium	*Streptococcus* UAD-1		*B. termitidis* UAD-50		*Citrobacter* UAD-25	
	Yield[c] (cells/ml × 10^8)	NH_3 produced[d] (μmol/ml)	Yield (cells/ml × 10^8)	NH_3 produced (μmol/ml)	Yield (cells/ml × 10^8)	NH_3 produced (μmol/ml)
No addition	2.0	0.0	0.4	1.2	1.3	7.3
Uric acid	4.2	7.3 (2.3)	1.4	17.3 (4.9)	1.4	12.8 (1.4)
F	5.8	0.2	1.3	0.7	1.9	4.5
F + uric acid	11.5	23.5 (6.1)	2.6	23.3 (6.1)	2.4	5.1 (0.3)
Allantoin	2.3	2.3	0.4	1.3	1.1	7.9
F + allantoin	6.4	2.5	1.4	0.9	ND[e]	ND
Allantoic acid	1.8	0.4	0.4	1.0	1.0	8.3
F + allantoic acid	6.6	3.6	1.4	0.8	ND	ND
Inosine	5.8	0.3	2.1	7.8	3.0	7.0
F + inosine	6.3	0.3	2.4	5.8	ND	ND
Guanosine	—[f]	0.2	0	ND	3.3	18.7
F + guanosine	7.1	0.3	0	ND	ND	ND
Hypoxanthine	1.9	0.0	2.3	8.9	3.0	16.2
F + hypoxanthine	6.1	0.2	2.7	9.1	ND	ND
Xanthine	1.3	0.3	0	ND	1.6	15.7
F + xanthine	4.7	0.4	0	ND	ND	ND
Xanthosine	1.5	0.0	0.5	ND	2.5	25.3
F + xanthosine	5.5	0.0	1.3	4.3	ND	ND
Ribose	3.3	0.0	0.9	1.2	1.3	6.0
F + ribose	7.5	0.0	ND	ND	ND	ND

[a] The following compounds were not used by growing cells: adenine, guanine, purine, caffeine, thymine, cytosine, uracil, and orotic acid.

[b] Final concentration of all heterocyclic compounds was 0.1% except for guanine (0.05%). Fructose (F) and ribose were used at a concentration of 0.04 and 0.05%, respectively. Incubation was under N_2/CO_2 (95/5, vol/vol). Initial pH of media was 7.0 ± 0.2.

[c] Maximum yields achieved between 24 and 48 h (UAD-1) or 36 and 96 h (UAD-50 and UAD-25). A yield of 0 designates <5 × 10^8 cells/ml.

[d] Trace amounts (0.05 to 0.7 μmol/ml) of urea were formed by strain UAD-1 from allantoin and allantoic acid only. Urea was not formed from uric acid by strains UAD-50 and UAD-25. Decomposition of any of the heterocyclics to yield NH_3 or urea did not occur in uninoculated media. Values in parentheses indicate micromoles of uric acid consumed per milliliter during growth. initial concentration of uric acid was 6.1 μmol/ml.

[e] ND, Not determined.

[f] Cell yields were not determined because guanine crystals formed in the medium.

tics of such strains differed significantly from those of *B. termitidis* isolates obtained in the present study.

Anaerobic metabolism of UA by minced termite guts. We attempted to detect anaerobic metabolism of UA in fresh termite gut contents. Since 60% or more of UA carbon is evolved as CO_2 by isolates (29), $^{14}CO_2$ evolution from [2-^{14}C]UA was used as an estimate of uricolysis by minced guts. Results (Table 4) showed that such preparations degraded a significant amount of UA under strict anaerobic conditions. Little or no stimulation of uricolysis was achieved by including formate in reaction mixtures.

The magnitude of uricolysis by minced guts was consistent with the density of uricolytic bacteria in guts (see Discussion).

DISCUSSION

Results presented herein show that uricolytic bacteria are present in guts of *R. flavipes*, generally in populations of 3.5 ± 1.7 × 10^4 cells/gut. This density is roughly 10% that of total cultivable heterotrophs (31). Nevertheless, we believe such populations to be numerically significant, based on arguments developed previously (31) regarding the tiny size of *R. flavipes* guts. Results are consistent with Leach and Granovsky's hypothesis (19), and also with our previous findings (28) that *R. flavipes* termites form UA but do not void the purine despite their lack of uricase. Presumably UA is catabolized by bacteria when it enters the gut, and therefore it is not found in the feces. However, the dynamics of UA mobilization and bacterial uricolysis in situ, as well as the nutritional control of these processes, are important points which remain to be defined.

Uricolytic isolates were most likely derived from the hindgut, although a small portion of midgut usually remains attached to extracted hindguts (5, 31). Previous studies (31) showed that populations of heterotrophs obtained from midgut homogenates were only 1/10 that of the present uricolytic isolates. However, Malpighian tubules of *R. flavipes* empty at the precise junction of mid- and hindgut, i.e., at the enteric valve (26). Thus, some uricolytic bacteria could conceivably colonize the midgut, near the enteric valve, yet still have ready access to UA.

TABLE 4. *Anaerobic metabolism of [2-^{14}C]UA by minced guts of R. flavipes workers*[a]

Added[b] to reaction mixture (nmol)	$^{14}CO_2$ evolved		pmol of substrate ^{14}C evolved as $^{14}CO_2$/gut equivalent
	dpm	pmol	
[2-^{14}C]UA (2.5)	7,601	67.8	2.06
[2-^{14}C]UA (2.5) + sodium formate (1,000)	7,707	68.7	2.08
Control	0	0	0

[a] Termites collected in Spring Arbor, Mich., and held captive for 7 months before assay.

[b] Specific activity of [2-^{14}C]UA was 50 nCi/nmol. Individual reaction mixtures contained 33 gut equivalents. Incubation was at 25°C for 3 h. Control mixture (lacking formate) was terminated with HCl immediately after UA addition.

Three major groups of uricolytic bacteria were associated with *R. flavipes:* group N *Streptococcus* sp., *B. termitidis*, and *Citrobacter* sp. Their generic affiliation was the same as that of major heterotrophs isolated previously on nonselective medium (31). Barnes and Impey (1) found a greater phenotypic diversity of uricolytic bacteria isolated from chicken ceca, although streptococci and bacteroides were among those isolated. Fecal streptococci of human and sheep origin have also been shown to degrade UA (23). Our isolation of uricolytic *B. termitidis* parallels that of Sebald (33), who isolated this species (referred to by her as *Sphaerophorus siccus* var. *termitidis*) from gut contents of *Reticulitermes lucifugus* (14). By contrast, Donnellan and Kilby (10) reported the isolation of an aerobic, uricolytic vibroid bacterium from fat body tissue of American cockroaches. Uricase mediated the first step of uricolysis by the vibroid (10). As reported herein, we have never observed bacteria of any type in fat body tissue of *R. flavipes*. Moreover, ultrasensitive assays for uricase failed to reveal such activity in fat body tissue (28).

Estimates of anaerobic UA metabolism by minced gut preparations suggested an activity of about 2 pmol of UA catabolized/(gut equivalent × 3 h) (Table 4), which is equivalent to 0.67 pmol/(gut × h). Considering that 10^{11} cells of *Streptococcus* UAD-1 or *B. termitidis* UAD-50 can degrade approximately 20 μmol of UA during a 2-h incubation (see Fig. 2 and 3 and Table 2, 3, and 5 of reference 29), it can be calculated that the in situ density of such bacteria would have to be about 0.67×10^4 cells/gut to account for the activity of minced gut preparations. The latter value compares extremely well with results of enumeration studies (Table 1), indicating that the uricolytic activity of fresh gut contents can be completely accounted for by isolates obtained in pure culture.

Uricolytic isolates displayed limited nutritional versatility with respect to heterocyclic compounds other than UA (Table 3). This was especially true for *Streptococcus* UAD-1. Such metabolic specialization of the isolates undoubtedly relates to the ability of their termite host to form UA (28). The ultimate benefit of uricolytic bacteria to *R. flavipes*, however, can only be inferred at the present time. Acetate, a major carbonaceous product of uricolysis by the isolates (29), is also a major product of cellulolysis in the termite gut (4) and it is a source of carbon and energy for the insect (2, 4, 22). On the other hand, the status of NH_3, the major nitrogenous product of uricolysis, is less clear. Ammonia nitrogen could cycle back to the insects directly, or indirectly after assimilation into microbial protoplasm or other microbial metabolites as suggested by Leach and Granovsky (19). Interestingly, such a cycle appears to occur between the UA-forming marine flatworm *Convoluta roscoffensis* (which also lacks uricase) and its intracellular, uricolytic algal symbiont *Platymonas convolutae* (3, 16). In this case, NH_3 formed during uricolysis by *P. convolutae* is apparently used by the alga for synthesis of amino acids, some of which are exported to the flatworm host. Whatever may be the exact flow of UA nitrogen in *R. flavipes*, it seems clear that uricolysis by gut bacteria could be extremely important to nitrogen conservation within the termite colony.

ACKNOWLEDGMENTS

We are grateful to Clifford Sporn for excellent technical assistance and to Donald G. Cochran for helpful discussions. We thank E. I. Garvie for determining the moles percent G+C content in the DNA of strain UAD-1 and for assaying lactate dehydrogenase in that strain.

This work was supported in part by the Michigan Agricultural Experiment Station of Michigan State University and by National Science Foundation grants BMS 75-05850 and PCM 77-05732. C.J.P. held a National Science Foundation predoctoral fellowship during the major portion of this study.

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ARTICLE III

ANAEROBIC DEGRADATION OF URIC ACID BY GUT BACTERIA OF TERMITES

By

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Reprinted from Appl. Environ. Microbiol. 40:125-132 (1980)

APPLIED AND ENVIRONMENTAL MICROBIOLOGY, July 1980, p. 125-132 Vol. 40, No. 1
0099-2240/80/07-0125/08$02.00/0

Anaerobic Degradation of Uric Acid by Gut Bacteria of Termites†

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A study was done of anaerobic degradation of uric acid (UA) by representative strains of uricolytic bacteria isolated from guts of *Reticulitermes flavipes* termites. *Streptococcus* strain UAD-1 degraded UA incompletely, secreting a fluorescent compound into the medium, unless formate (or a formicogenic compound) was present as a cosubstrate. Formate functioned as a reductant, and its oxidation to CO_2 by formate dehydrogenase provided $2H^+ + 2e^-$ needed to drive uricolysis to completion. Uricolysis by *Streptococcus* UAD-1 thus corresponded to the following equation: 1UA + 1formate → $4CO_2$ + 1acetate + $4NH_3$. Urea did not appear to be an intermediate in CO_2 and NH_3 formation during uricolysis by strain UAD-1. Formate dehydrogenase and uricolytic activities of strain UAD-1 were inducible by growth of cells on UA. *Bacteroides termitidis* strain UAD-50 degraded UA as follows: 1UA → 3.5 CO_2 + 0.75acetate + $4NH_3$. Exogenous formate was neither required for nor stimulatory to uricolysis by strain UAD-50. Studies of UA catabolism by *Citrobacter* strains were limited, because only small amounts of UA were metabolized by cells in liquid medium. Uricolytic activity of such bacteria in situ could be important to the carbon, nitrogen, and energy economy of *R. flavipes*.

It is fair to say that much is known about anaerobic degradation of uric acid (UA), but in an extremely limited number of bacteria—primarily *Clostridium acidiurici* and *C. cylindrosporum* (23). However, anaerobic uricolysis is a property fairly widespread among nonspore-forming bacteria (2, 3, 12, 19, 25), and animal intestinal tracts appear to be a rich source of uricolytic organisms (3, 9, 10, 12, 13, 19). Unfortunately, little is known concerning the major products of uricolysis by pure cultures of gut bacteria, let alone the stoichiometry of UA catabolism.

In a companion paper (17) we reported on the isolation, identification, and nutritional characteristics of uricolytic bacteria from the guts of *Reticulitermes flavipes* termites. In this paper we report on anaerobic degradation of UA by representative isolates from the termite gut: a group N *Streptococcus*; *Bacteroides termitidis*; and a *Citrobacter* sp. The purpose of this study was twofold: (i) to increase our understanding of the role(s) of gut bacteria in termite nutrition; and (ii) to contribute to our knowledge of anaerobic purine degradation in general.

MATERIALS AND METHODS

Bacteria, media, and culture techniques. Bacteria, media, and culture techniques are described in the companion paper (17).

Analysis of fermentations by growing cells. Fermentations with growing cells were performed by using a fermentation train (14). UA was assayed spectrophotometrically at 292 nm by using hog liver uricase (uric acid diagnostic kit no. 292-UV; Sigma Chemical Co., St. Louis, Mo.). Samples for UA assay at the end of the fermentation were removed before acidification and clarification (14) of the medium. Cells were removed from such samples by centrifugation at 12,000 × *g* for 10 min, and the supernatant fluid was used for UA assay. Most other substrates and products were assayed as previously described (15, 18). Fructose was assayed enzymatically as described by Bernt and Bergmeyer (4). Urea was assayed by determining urease-dependent NH_3 production with phenol nitroprusside reagent (urea nitrogen diagnostic kit no. 640; Sigma). NH_3 was routinely determined by using the urea assay reagents (above) without urease. However, for fermentation balances, NH_3 was first collected by microdiffusion before assay (7). Oxamate was assayed as described by Valentine and Wolfe (22). Glycine was estimated by visually observing the intensity of glycine spots after one-dimensional thin-layer chromatography (TLC) and staining with ninhydrin. Uninoculated medium served as a control. For TLC of glycine, 0.1-mm-thick cellulose MN 300 layers (Brinkmann Instruments Inc., Woodbury, N.Y.) were used with a solvent system of isopropanol-formic acid-water (40:2:10). Heterocyclic compounds were routinely separated by using one-dimensional TLC with cellulose MN 300 layers (above) and the following solvent systems: *n*-butanol-methanol-water-NH_4OH (60:20:20:1) and isopropanol-water (7:3) (16). Compounds were visualized by illumination with shortwave ultraviolet (UV) light or by spraying plates with Erlich reagent (5).

Oxidation/reduction (O/R) indexes were calculated

† Journal Article no. 9358 from the Michigan Agricultural Experiment Station.

by the method of Neish (14) for sugar fermentations and by the method of Whiteley and Douglas (25) for fermentations involving UA.

Preparation of cell suspensions, cell extracts, and reagents for anaerobic metabolism studies. Cells were grown anaerobically to late logarithmic or early stationary phase in TYU medium. They were then harvested by centrifugation at 6,000 × *g* for 10 min. Cell pellets were washed once with, and suspended in, 0.2 M potassium phosphate buffer to a final density of about 10^{11} cells/ml. The pH of the phosphate buffer was 7.0, except when cells were being suspended for UA metabolism (pH 6.4) or for assay of formate dehydrogenase (FDH) (pH 8.0).

Cell extracts were prepared by first adding lysozyme (0.5 mg/ml, final concentration) to cell suspensions and allowing them to incubate for 30 min at 30°C. Suspensions were then brought to about 4°C and exposed to three bursts (15 s/burst at 30-s intervals) from the probe of a Bronwill Biosonik II sonic oscillator operating at full power. Preparations were then centrifuged at 18,000 × *g* for 10 min, and the resulting supernatant fluids were used as cell-free extracts.

All manipulations of cells and extracts were done in the cold (2 to 5°C) under N_2, with minimum exposure of the preparations to air. Heat-stable reagents (e.g., buffers) were boiled and quickly cooled under O_2-free N_2 before use. Suspensions or heat-labile solutions were held in serum-stoppered bottles, the gas phase of which was vacuum evacuated and flushed at least four times before being sealed.

Metabolism of cell suspensions. Reactions were carried out in double-side-arm Warburg vessels by using conventional manometric techniques (21). This facilitated measurement of gaseous as well as soluble products. Products were sometimes determined from the combined contents of several vessels containing identical reaction mixtures. Individual reaction mixtures (3.0 ml) contained: potassium phosphate buffer, 305 μmol; dithiothreitol, 10 μmol; approximately 10^{11} cells; and a variable amount of substrate as indicated. The pH of phosphate buffer was 7.0 unless UA was the substrate, in which case it was pH 6.4. This assured that when UA solution (0.1 M in 0.5 N NaOH) was tipped into reaction mixtures (at a final concentration of 20 μmol/reaction mixture) the resulting pH would be 7.0. A gas phase of N_2 was used throughout.

Substrates and products were generally assayed as described for fermentations with growing cells (above). $^{14}CO_2$ was trapped in the center well of Warburg vessels which contained NaOH-impregnated filter paper. Filters were then pooled into a 25-mm test tube containing 10 ml of water, 100 mg of nonradioactive $NaHCO_3$, and a drop of cresol red indicator solution. The tube was then sealed with a serum stopper, through which 5 N HCl was injected to acidify the mixture. $^{14}CO_2$ evolved in this way was then swept into phenethylamine-methanol traps for radioactivity determinations (18).

^{14}C-labeled organic acids were separated by silicic acid chromatography before determination of specific radioactivity (14). ^{14}C-labeled ethanol was distilled from clarified fermentation liquors at pH 7.5 (14); radioactivity in such distillates was assumed to be due solely to ethanol.

O/R indexes were calculated as described for fermentation with growing cells (above).

Radioactivity measurements. Radioactivity measurements and quench corrections were made as described previously (18).

Chromatography and spectrophotometry of fluorescent compound FC-1. For TLC of FC-1, cell-free fermentation liquors were concentrated by lyophilization and then redissolved to 1/10 original volume with distilled water or 0.1 M potassium phosphate buffer (pH 7.0). Samples of such material were applied to TLC plates and developed with isopropanol-water (above). FC-1 was subsequently visualized by illumination with UV light. When desired, FC-1 was scraped from plates and eluted from the cellulose MN 300 support with phosphate buffer. Fluorescence spectra were made by using an Aminco-Bowman spectrophotofluorimeter.

FDH. FDH activity of cell-free extracts was determined as described by Wagner and Andreesen (24), except that dithiothreitol replaced dithioerythritol, and benzyl viologen was used as the electron acceptor.

Chemicals. Heterocyclic substrates were obtained from Sigma and were of the highest purity available. Enzymes used in the assay of fructose were obtained from Boehringer Mannheim Biochemicals, Indianapolis, Ind. Radioactive substrates and standards were obtained from New England Nuclear Corp., Boston, Mass. All other chemicals were of reagent grade and were obtained from various commercial sources.

RESULTS

Anaerobic uricolysis by *Streptococcus* UAD-1: growing cells. It was of fundamental interest to determine the overall products of uricolysis by growing cells. However, previous experiments (17) indicated that limited dissimilation of UA occurred unless fructose was included in the medium. Accordingly, 0.04% fructose was included with UA in semi-D medium, and a dual-substrate fermentation was carried out. Products formed were corrected for those formed in a separate fermentation of 0.04% fructose alone. Results (Table 1) did not permit conclusions regarding the precise origin of carbonaceous products, but clearly revealed that significantly greater quantities of CO_2 and acetate were formed during UA fermentation. NH_3 was the major nitrogenous product of UA fermentation. Small amounts of lactate and ethanol may have also been produced from UA. Surprising, however, was the absence of formate in spent fermentation liquors. Thus, when corrected for the formate produced during monosubstrate (0.04% fructose) fermentation, a large negative value was obtained (Table 1). This indicated that, relative to 100 mmol of UA fermented, 133.8 mmol of formate either was not produced or was consumed. It should be noted that batch culture fermentations in media containing limited (i.e., 0.04%) fructose as sole fer-

TABLE 1. *Fermentation of UA and fructose by growing cells of Streptococcus UAD-1*[a]

Product	mmol of product/100 mmol of:	
	Fructose (0.04%)	UA[b]
Lactate	73.7	37.8
Acetate	37.0	92.0
Ethanol	86.5	9.3
Formate	124.4	−133.8
CO_2	26.7	200.4
NH_4		341.3
% C recovery	103.2	76.5
% N recovery		85.3
O/R index	1.0	2.6

[a] Growth medium was semi-D containing either 0.04% fructose alone or 0.04% fructose plus 0.1% UA. Consumption of fructose was 99% in each fermentation, whereas consumption of UA was 35%.

[b] Tabulated data have been corrected for products formed from fermentation of 0.04% fructose alone and then normalized to 100 mmol UA fermented. The following products were not detected: glycine, urea, oxamate, H_2, propionate, butyrate, succinate, fumarate, pyruvate, acetoin, diacetyl, xanthine, hypoxanthine, thymine, uracil, orotic acid, allantoin, and allantoic acid.

mentable substrate were heterolactic, and formate was a significant product (Table 1). This was in marked contrast to the homolactic fermentation pattern of strain UAD-1 with excess (1%) sugars (17). However, these data parallel the observed shift to a heterolactic fermentation when certain group N streptococci are grown at low dilution rates in glucose-limited chemostat cultures (20).

Carbon and nitrogen recoveries were generally low in such dual-substrate fermentations, and O/R indexes were at variance with an expected value of 1.0, even if a more complex basal medium such as TY was used (carbon recovery = 65%, nitrogen recovery = 66%; O/R index = 1.2). Other plausible products of UA fermentation, including reduction products such as xanthine or hypoxanthine, were not detected (Table 1, footnote *b*). However, during a search for the latter by TLC of concentrated fermentation liquors, a compound was observed which fluoresced blue when illuminated by shortwave UV light. This compound, termed FC-1, is discussed further below, but production of FC-1 may account for the relatively low carbon and nitrogen recoveries seen with dual-substrate fermentations.

Anaerobic uricolysis by *Streptococcus* UAD-1: cell suspensions. In an effort to obtain a simpler (i.e., monosubstrate) system for studying UA fermentation by strain UAD-1, UA-grown cell suspensions were incubated in buffer with UA as the sole substrate. However, even cell suspensions required fructose for vigorous dissimilation of UA. By using CO_2 evolution as an index of UA dissimilation, little catabolism of the purine was apparent in the absence of added fructose (Fig. 1). The greater the initial amount of fructose included with UA, the greater were the rate and extent of CO_2 evolution. When initial amounts of fructose and UA were equimolar (20 μmol each), the total CO_2 evolved was much greater than the sum of CO_2 evolved from fructose and UA alone. Little additional stimulation of CO_2 evolution occurred if fructose was increased to 40 μmol in the presence of 20 μmol of UA. These data suggested that some interaction between the substrates was occurring.

To examine the relationship between fructose and UA metabolism more closely, a "titration experiment" was done to determine the amount of fructose needed to effect complete dissimilation of UA (Fig. 2). In the absence of added fructose, about half of the initial UA was consumed. This does not mean that the UA consumed was completely catabolized, merely that it disappeared as uricase-reactive material. Indeed, complete catabolism of the consumed UA seemed unlikely since little CO_2 and NH_3 were evolved. However, addition of increasing initial amounts of fructose resulted in more extensive

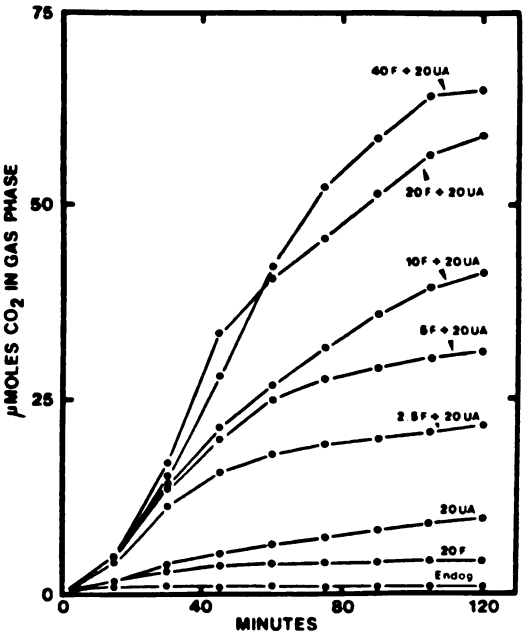

FIG. 1. *CO_2 evolution from fructose (F) and UA by cell suspensions of Streptococcus UAD-1. The numbers indicate the amount (micromoles) of each substrate initially included in each Warburg reaction vessel. Cells were grown in TYFU medium.*

UA consumption and CO_2 and NH_3 evolution. When the initial concentration of fructose was equimolar to UA, complete consumption of UA occurred and theoretical amounts of NH_3 were liberated. Under such conditions CO_2 evolution was also copious.

Since complete UA dissimilation apparently required at least equimolar amounts of fructose, a materials balance was performed in the dual-substrate mode, but with [*U*-^{14}C]fructose. In this way, the partitioning of UA and fructose carbon into products could be evaluated. A control fermentation of fructose alone was essentially homolactic (Table 2, column 3). Moreover, the specific activity of lactate carbon was nearly identical to that of fructose carbon, indicating that no dilution of ^{14}C label occurred (Table 2, footnotes *a* and *b*). For the dual-substrate fermentation, the amount of fructose added was roughly twice that of UA (Table 2, footnote *a*). By determining the specific ^{14}C radioactivity in each carbonaceous product, a materials balance for each substrate was obtained. For ease of evaluation, and since the amount of fructose consumed was almost exactly twice that of UA (Table 2, footnote *a*), the data were transposed to indicate products formed from 100 μmol of fructose and from 50 μmol of UA. Results indicated that CO_2, acetate, and NH_3 were the major products derived from fermentation of UA, although small amounts of lactate and ethanol were also formed. Inherent in this conclusion was the assumption that any dilution of ^{14}C label in soluble products, particularly acetate, occurred in each carbon atom of the molecule (an assumption borne out by further experiments described below). Urea did not appear to be an intermediate in NH_3 and CO_2 production. Urea was never detected as a product of UA fermentation, and UA-grown cells did not evolve CO_2 and NH_3 from urea, although *Proteus mirabilis* control cells did so readily.

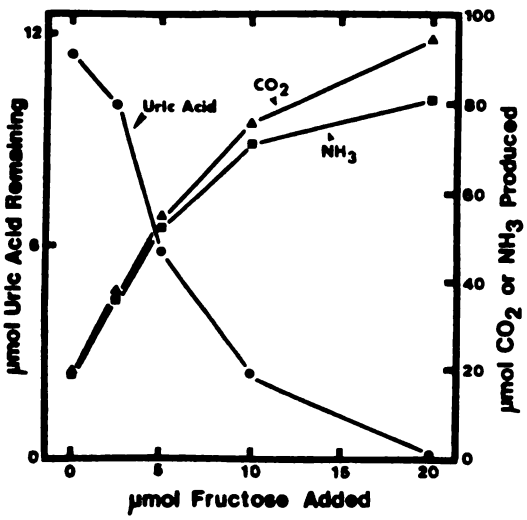

FIG. 2. *Fructose-driven UA dissimilation by cell suspensions of Streptococcus UAD-1. Each reaction vessel initially contained 20 μmol of UA and a variable amount of fructose as indicated. Reactions were terminated after 130 min at 30°C, at which time gas evolution had ceased.*

TABLE 2. *Fermentation of UA and [U-^{14}C]fructose by cell suspensions of Streptococcus UAD-1*[a]

Product[b]	μmol of product per:		
	50 μmol of UA	100 μmol of [*U*-^{14}C]fructose	100 μmol of [*U*-^{14}C]fructose
Lactate	4.2	149.8	145.6
Acetate	19.0	41.0	27.5
Ethanol	5.9	12.9	27.4
Formate	0.0	0.0	35.2
CO_2	157.0	46.4	15.4
NH_3	182.1		
% C recovery		96.8	99.5
% N recovery		91.1	
O/R index		1.0	1.2

[a] Individual reaction mixtures (3.0 ml) contained: 10^{11} TYFU-grown cells (equivalent to 19.5 mg, dry weight); 51.0 μmol of [*U*-^{14}C]fructose (specific activity = 5111 dpm/μmol of carbon) with or without 23.2 μmol of UA; and other reagents listed in Materials and Methods. Incubation was at 30°C for 2 h under N_2. Consumption of substrates in the dual-substrate reaction mixture was 47.0 μmol of [*U*-^{14}C]fructose and 23.2 μmol of UA.

[b] Specific activity (dpm per micromole of carbon) of lactate produced in monosubstrate fermentation was 5,011. Specific activities of products in the dual-substrate fermentation were: lactate, 4,971; acetate, 3,494; ethanol, 3,500; and CO_2, 1,165.

Interestingly, products derived from fructose in the dual-substrate fermentation (Table 2, column 2) were quantitatively similar to those formed in the monosubstrate mode (Table 2, column 3). An exception to this was the lack of formate production in the dual-substrate fermentation, accompanied by an increase in CO_2 from fructose. This observation was similar to that made with growing cells (Table 1). The total amount of CO_2 produced per 100 μmol of fructose in the dual-substrate mode (46.4 μmol; Table 2) was roughly equal to the sum of formate and CO_2 produced in the monosubstrate mode. This suggested that formate oxidation to CO_2 occurred during, and might be important to, UA fermentation.

Formate-driven uricolysis by *Streptococcus* UAD-1. To test the hypothesis that formate oxidation was necessary for extensive uricolysis by strain UAD-1, a titration experiment was

performed with formate by using a protocol similar to that used with fructose. Results indicated that formate, like fructose, also served to drive uricolysis (Fig. 3). Complete consumption of UA, accompanied by liberation of theoretical quantities of NH_3, occurred when the initial quantity of formate was equimolar to that of UA. That formate was oxidized to CO_2 during uricolysis was shown with a companion reaction mixture containing 20 μmol of UA plus 20 μmol of [^{14}C]formate (specific activity = 1,225 dpm/μmol). Approximately 98.7% of the ^{14}C label was recovered as $^{14}CO_2$ at the end of the reaction.

Recognition of the importance of formate to uricolysis prompted a materials balance for a simplified dual-substrate fermentation of UA (i.e., formate plus UA) (Table 3). For ease of interpretation, the raw data were normalized to 100 μmol of UA fermented. Major products of UA fermentation were CO_2, NH_3, and acetate. Almost all (92.4%) of the formate was recovered as $^{14}CO_2$. Thus it is clear that UA contributes both the CH_3 and COOH groups of acetate, supporting the assumption made in evaluating the data of Table 2 (above). However, since radioactivity of acetate was not determined, it is possible that small amounts of formate carbon may have been used for acetogenesis. No evidence of H_2 production was observed.

These data revealed the link between fructose metabolism and uricolysis by strain UAD-1 (Fig. 2). Apparently, fructose metabolism was needed to provide formate, whose oxidation served to drive uricolysis to completion. As anticipated, other formicogenic compounds (e.g., glucose, ribose, and pyruvate) also stimulated uricolysis (data not shown). No stimulation was observed with glycine.

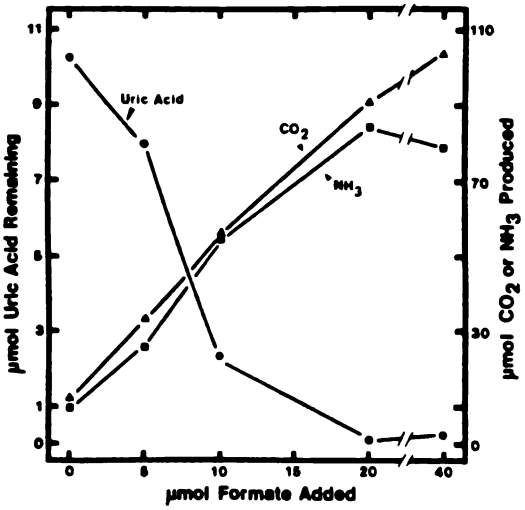

FIG. 3. *Formate-driven UA dissimilation by cell suspensions of Streptococcus UAD-1. Each reaction vessel initially contained 20 μmol of UA and a variable amount of sodium formate as indicated. Reactions were terminated after 130 min at 30°C, at which time gas evolution had ceased.*

FDH activity of *Streptococcus* UAD-1. The importance of formate oxidation to uricolysis prompted a closer inspection of FDH activity in strain UAD-1. In experiments with cell suspensions, $^{14}CO_2$ evolution from [^{14}C]formate was taken as an index of FDH activity. Results (Table 4) indicated that phenotypic expression of FDH required that cells be grown in media containing UA. However, even UA-grown cells did not exhibit FDH unless an exogenous e^- acceptor was present. The e^- acceptor could be a natural one (e.g., UA) or an artificial one (e.g., benzyl viologen) (Table 4). Methyl viologen and methylene blue could also function as e^- acceptors for FDH, but they were not as effective as benzyl viologen (data not shown).

Preliminary experiments revealed FDH activity in cell-free extracts of UA-grown cells. FDH activity (121 nmol of formate oxidized × min^{-1} × mg of $protein^{-1}$) was apparently sensitive to O_2: it could be completely abolished by exposure of extracts to air for 10 min.

Anaerobic dissimilation of heterocyclic compounds by cell suspensions of *Streptococcus* UAD-1. Suspensions of UA-grown cells of strain UAD-1 evolved no significant CO_2 and NH_3 from the following substrates (equimolar fructose also included in reaction mixtures): xanthine, allantoin, allantoic acid, uracil, and orotic acid.

Production of a fluorescent compound FC-1 by *Streptococcus* UAD-1. Growing cells,

TABLE 3. *Fermentation of UA and [^{14}C]formate by cell suspensions of Streptococcus UAD-1*[a]

Product[b]	μmol of product per:	
	125.6 μmol of [^{14}C]formate	100.0 μmol of UA
CO_2	116.0	337.8
NH_3		401.3
Acetate		88.5
% C recovery	100.8	
% N recovery	100.3	
O/R index	0.9	

[a] Reaction conditions similar to those described in Table 2, footnote *a*, except that the substrate mixture was [^{14}C]formate (specific activity = 10,019 dpm/μmol) plus UA. Substrate consumption was: 19.6 of 20.0 μmol of [^{14}C]formate added and 15.6 of 20.6 μmol of UA added.

[b] Specific activity (dpm/micromole) of $^{14}CO_2$ was 2,557. Specific activity of acetate was not determined; all acetate was assumed to be derived from UA.

as well as cell suspensions of strain UAD-1, often secreted a fluorescent compound (FC-1) into the medium. FC-1 production was specific to UA metabolism and was not detected during fermentation of sugars alone. Moreover, control experiments verified that FC-1 was not a spontaneous decomposition product of UA. Conditions which resulted in incomplete catabolism of UA (i.e., fermentation of UA in the absence of a cosubstrate) were those which also favored FC-1 accumulation. For example, in the experiment depicted by Fig. 2, supernatant fluids were obtained from each reaction mixture and a portion of each was applied to TLC plates. After ascending chromatography and visualization with UV light, the FC-1 spot (R_f = 0.15 to 0.20) was most intense from the reaction mixture containing no initial fructose. The intensity of the spot diminished from fluids which contained increasing amounts of fructose until, with 20 μmol of initial fructose, no FC-1 spot was visually observed. This was, of course, the reaction mixture resulting in complete uricolysis (Fig. 2). These data suggested that FC-1 was an intermediate in, or by-product of, uricolysis by strain UAD-1.

When FC-1 was eluted from TLC plates with 0.1 M potassium phosphate buffer (pH 7.0), a fluorescence spectrum of the crude eluate revealed excitation and emission maxima at 308 and 379 nm, respectively. A control spectrum (of material eluted from a comparable position in a TLC lane containing spent fructose fermentation liquor) revealed no significant fluorescence. The identity of FC-1 is not yet known and awaits purification of the compound.

Anaerobic uricolysis by *B. termitidis*. Cell suspensions of *B. termitidis* UAD-50 fermented UA to CO_2, NH_3, and acetate (Table 5). Fructose or formate was neither required for nor stimulatory to UA dissimilation by this strain.

TABLE 4. *Formate oxidation by cell suspensions of Streptococcus UAD-1*

Growth substrate[a]	Added to reaction mixture (μmol)[b]	$^{14}CO_2$ evolved (μmol)
Fructose	No additions	0.05
	UA (20)	0.00
	Benzyl viologen (10)	0.08
Fructose + UA	No additions	0.04
	UA (20)	6.48
	Benzyl viologen[c] (10)	6.96
	Benzyl viologen[c] (10) + boiled cells	0.06

[a] Cells grown in TY basal medium containing 1.0% fructose alone or 0.04% fructose plus 0.1% UA (i.e., TYFU medium).

[b] Reaction mixtures (3.0 ml) contained: 10^{11} cells and 10 μmol of [^{14}C]formate (specific activity = 96,493 dpm/μmol) plus phosphate buffer and dithiothreitol as listed in Materials and Methods. Reaction conditions: pH 7.0; 30°C; 60 min; gas phase, N_2.

[c] Reaction mixture turned dark purple, indicating dye reduction, except when boiled cells were used.

Anaerobic uricolysis by *Citrobacter*. Studies of uricolysis by *Citrobacter* strains were hampered, because isolates exhibited limited consumption of UA in liquid media. This was true not only with TY-based media (e.g., see Table 4 of reference 17), but also with richer media such as BHIU and SUA. These observations were surprising, because *Citrobacter* isolates readily formed prominent clear zones on UA-containing agar plates. That UA was indeed being consumed on such plates was verified by assay for UA in 1-mm-diameter agar plugs, removed at various distances from a single streak line of growth. Plugs from within the clear-zone region (i.e., 0 to 15 mm from the streak line; BHIU plates; 2 days of incubation) contained no UA, whereas plugs external to the clear zone contained UA in amounts identical to those present before inoculation. In addition, plugs removed from clear zones and then inserted into uninoculated plates caused no clearing in the latter. These data argued against a diffusible, UA-degrading enzyme being released from cells on agar media.

The following attempts to increase UA consumption in broth media were unsuccessful: (i) incubation under an atmosphere of 90% N_2/10% H_2 (vol/vol); (ii) incorporation of UA into media as a suspension rather than as an NaOH-solubilized solution; and (iii) incorporation of 0.2% agar into broth media.

Unfortunately, cell suspensions of UA-grown cells showed no degradation of UA at all, even if a cosubstrate (glucose, fructose, ribose, or formate), which was metabolized, was included. This was true whether such cells were derived from broth cultures or were scraped from the surface of BHIU plates which they had completely cleared.

TABLE 5. *Fermentation of UA by cell suspensions of B. termitidis UAD-50*[a]

Product	μmol/100 μmol of UA fermented
CO_2	338.7
NH_3	391.5
Acetate	77.1
% C recovery	98.6
% N recovery	97.9
O/R index	1.03

[a] Reaction conditions were similar to those described in Table 2, footnote *a*, except that UA was the only substrate added and the temperature was 37°C. Cells were grown in TYFU medium before harvest.

DISCUSSION

Major products of anaerobic uricolysis by termite gut streptococci and *B. termitidis* were CO_2, acetate, and NH_3. Limited studies with *Citrobacter* isolates also revealed NH_3 as a major nitrogenous product (17). Mead (12) found that a *Streptococcus faecalis* strain of chicken origin also formed CO_2, acetate, and NH_3 during uricolysis, but no quantitative data were reported. Whole-cell suspensions of rumen bacteria degrade UA to similar products (10), but pure culture studies have not yet been done.

Uricolysis by *B. termitidis* conformed closely to the theoretical equation $1UA \rightarrow 3.5CO_2 + 0.75acetate + 4NH_3$ (Table 5). By contrast, group N streptococci showed incomplete dissimilation of UA unless formate (or a formicogenic compound such as fructose) was present as a cosubstrate in amounts equimolar to that of UA (Fig. 2 and 3). The role of formate appeared to be that of a reductant: formate oxidation to CO_2 by formate dehydrogenase yielded $2\ H^+ + 2\ e^-$ needed to drive uricolysis to completion. Thus, anaerobic uricolysis by strain UAD-1 (Table 3) conformed reasonably well to the following theoretical equations: $1formate + 1UA \rightarrow 4CO_2 + 1acetate + 4NH_3$; or $2H^+ + 2e^- + 1UA \rightarrow 3CO_2 + 1acetate + 4NH_3$.

We do not know the reductive step(s) in uricolysis by streptococci that uses formate as electron donor. However, it may be one which requires low-potential electrons, since formate oxidation exhibits an $E_0' = -0.42$ V (11). If reduced nicotinamide adenine dinucleotide oxidation [$E_0' = -0.32$ V (11)] could function in this regard, one would have expected 5 μmol of fructose (instead of 20) to drive complete dissimilation of 20 μmol of UA (Fig. 2). Likewise, in fermentations of fructose plus UA, one would have expected less lactate production per mole of fructose than from the fermentation of fructose alone (Table 2). However, neither was the case.

To our knowledge, this is the first report of FDH activity in a *Streptococcus*. However, FDH was observed only in cells grown on UA, and then only in the presence of an exogenous electron acceptor (UA or an electron-accepting dye; Table 4). The latter observation is consistent with the apparent absence of an H_2-evolving hydrogenase in streptococci; i.e., cells cannot use protons as an electron sink during formate oxidation. FDH of strain UAD-1 showed a sensitivity to air that is characteristic of the enzyme from other bacteria (1, 8).

Major products of UA degradation by termite gut streptococci and *B. termitidis* (i.e., CO_2, acetate, and NH_3) were the same as those formed by *Clostridium acidiurici* and *C. cylindrosporum* (6). Unlike the clostridia, however, growth of the present isolates was not strictly dependent on purine fermentation (17). Whether the similarity of major products reflects a similar pathway for intermediary metabolism of UA remains to be determined. However, it is noteworthy that xanthine, the first intermediate in uricolysis by clostridia, was not used as a substrate by growing cells of *Streptococcus* UAD-1 or *B. termitidis* UAD-50 (17), nor was it degraded by UA-grown cell suspensions of strain UAD-1.

Based on the results of this study, and as discussed in the accompanying paper (17), we conclude that uricolytic gut bacteria may well be important to the carbon, nitrogen, and energy nutrition of *R. flavipes* as well as other members of the gut microbiota.

ACKNOWLEDGMENTS

We are grateful to Clifford Sporn for excellent technical assistance and to Donald G. Cochran for helpful discussions.

This work was supported in part by the Michigan Agricultural Experiment Station of Michigan State University and by National Science Foundation grants BMS 75-05850 and PCM 77-05732. C.J.P. held a National Science Foundation predoctoral fellowship during the major portion of this study.

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ARTICLE IV

GUT BACTERIA DEGRADE URIC ACID:

A STRATEGY FOR NITROGEN CONSERVATION IN TERMITES

By

C. J. Potrikus and J. A. Breznak

ABSTRACT

Reticulitermes flavipes termites possess purine nucleoside phosphorylase and xanthine dehydrogenase to synthesize uric acid (UA), but lack uricase or any other means to degrade UA. This seemed unusual, because termites do not excrete UA, but was consistent with the low N content of termite feces. However, uricolysis does occur in termites, but it is an anaerobic process mediated solely by the hindgut microbiota. Moreover, ^{14}C-tracer experiments showed that termites transport UA from the site of synthesis and storage (fat body tissue) to the site of degradation (gut microbiota) via Malpighian tubules. NH_3, a major product of uricolysis by the gut bacteria, is a potential N source for termites directly through glutamine synthetase activity present in fat body tissue. Results indicate that uricolysis by the termites' gut microbiota recycles significant amounts of N and appears to be an important means of N conservation for these oligonitrotrophic insects.

INTRODUCTION

The ability of symbiotic microbes to augment the nutrition of their host is well-recognized, and numerous such functions have been ascribed to the associated gut microbiota of termites. Because xylophagous termites are oligonitrotrophic, i.e., their food is low in combined N (13), gut microbes have frequently been implicated in termite N economy. Studies by Hungate (11) indicated that termites, with the aid of fungi, could efficiently sequester the low amounts of combined N from wood and soil. Recent evidence also indicates that N_2 fixation by gut bacteria may contribute to N acquisition by termites (5,24).

In addition to N acquisition, conservation of N should also be important to termite N economy and could be effected by the recycling of nitrogenous wastes (14). Certain termite species form significant amounts of uric acid (UA), but unlike many other terrestrial insects they do not void the purine despite the apparent lack of uricase in termite tissues (25). However, detailed studies of the gut microbiota of the termite Reticulitermes flavipes revealed an abundant population of uricolytic bacteria capable of degrading UA in pure culture to CO_2, NH_3, and acetate (26,27). All of these findings are consistent with the hypothesis of Leach and Granovsky (14) who speculated that recycling of UA by termite gut microbes was important

to the N nutrition of termites.

Little imformation is available regarding the physical and chemical aspects of UA synthesis, storage, mobilization, and degradation in termites (13), or the relevance of gut microbes to these processess *in vivo*. Accordingly, the present study was done to assess the importance of bacterial uricolysis to conservation of N in *R. flavipes*.

MATERIALS AND METHODS

Insects and bacterial strains. Reticulitermes flavipes (Kollar) termites were collected in Janesville, WI, Dansville, MI, and Spring Arbor, MI and were maintained in the laboratory as previously described (29). Worker termites (i.e., externally undifferentiated larvae beyond the third instar) were used for all experiments.

Specimens of Drosophila melanogaster were obtained from the culture collection of T. Friedman of Michigan State University.

Uricolytic bacteria were Streptococcus strain UAD-1 and Bacteroides termitidis strain UAD-50. Both strains were isolated from guts of R. flavipes and were described previously (26).

Preparation of tissues, cell suspensions, and cell extracts. Termite Malpighian tubules were obtained by removing the intestinal tract (24) into a solution of 0.25 M sucrose containing 1.7 mM EDTA (pH 6.9) and dissecting the tubules away from adherent fat body tissue and from their attachment to the gut or rectum.

To obtain fat body tissue, individual termites were degutted and the abdomen was sliced longitudinally with a scalpel. Fat body tissue was then scraped from the abdominal wall into 0.5 ml of sucrose/EDTA solution.

Abdomen extracts were an easily obtainable, but crude, source of fat body tissue enzymes. Termites were decapitated and degutted, and

the abdomens were then transferred to a small tissue homogenizer containing 2.0 ml of 0.1 M Tris (hydroxymethyl) aminomethane (Tris) buffer (pH 8.0) and approximately 10 mg of sand (Mallinckrodt). The mixture was homogenized for 2 to 3 min at 4°C, centrifuged at 27,000 x g for 15 min, and the supernatant fluid was used. Abdomens of 60 termites yielded approximately 16 mg protein/ml of extract.

Minced termite guts were prepared as previously described (26), except suspensions contained five to ten gut equivalents in 500 µl of buffer.

Gut tissue was separated from gut contents as follows: 20 guts were removed under anaerobic conditions (24) and were placed in 500 µl of 0.1 M potassium phosphate buffer (pH 7.0). Each gut was sliced once at the paunch with a scalpel. The suspension was transferred to a 13 mm test tube and vigorously agitated for 3 min by using a Vortex type mixer operating at maximum speed. Gut segments were allowed to settle for 2 to 3 min, and the supernatant fluid (containing the gut contents) was aspirated to a separate tube. Gut segments were washed twice more with 500 µl of buffer as just described. The supernatant fluid from the first wash was pooled with the gut contents, whereas that from the second wash was discarded. The remaining gut tissue segments were finally resuspended in 500 µl of buffer.

Cell suspensions of Streptococcus UAD-1 and B. termitidis UAD-50 were prepared under anaerobic conditions as previously described (27), except 0.1 M potassium phosphate buffer (pH 7.0) was used and cells were resuspended to a density of 10^8 to 10^9 cells/ml.

Assays for enzyme activities. (i) Uricase-mediated UA degradation. Uricase-mediated UA degradation was assessed by measuring

formation of ^{14}C-allantoin from [2-^{14}C] UA as described by Friedman and Johnson (6). In our system, R. flavipes tissue preparations were treated in an ultrasonic water bath (Mettler Electronics Corp.) for 2 min to aid in tissue disruption. Control assays employed Malpighian tubules from newly emerged adults of D. melanogaster as previously described (6).

(ii) Nonuricase-mediated UA degradation. Nonuricase-mediated UA degradation was assessed by measuring $^{14}CO_2$ evolution from [2-^{14}C] UA or conversion of the latter to other ^{14}C-labelled compounds. Reactions were carried out in a manner similar to that described previously (26), except that both aerobic and anaerobic (90% N_2/10% H_2) incubations were used. Samples (100 µl) of some reaction mixtures were removed before acidification with HCl; ammended with 100 µl of a solution containing 5 mM each of nonradioactive UA, xanthine, and hypoxanthine; and 10 µl was then spotted on cellulose MN 300 thin layer chromatography (TLC) sheets (Brinkman). Radioactive [2-^{14}C] UA (250 pmole) was also spotted as a control. TLC sheets were developed in n-butanol:methanol:NH_4OH:H_2O, 90:30:5:30 (by vol). R_f values for the standards in this solvent system were: UA, 0.15; xanthine, 0.21; and hypoxanthine, 0.35. Following development, the TLC sheet was cut into 2 cm-wide vertical strips, and each strip was cut into segments corresponding to the carrier substrate spots, which were located by viewing the chromatograms with short wavelength UV light (Mineralite, Ultra-Violet Products, Inc., San Gabrial, CA). The remainder of each strip was cut into 1.5 cm segments. Each segment was assayed for radioactivity as described above.

(iii) Xanthine dehydrogenase (XDH). XDH was determined

spectrophotometrically by measuring substrate-dependent reduction of NAD to $NADH_2$ (23). Absorbance measurements were made by using a Cary model 219 recording spectrophotometer (Varian Associates, Inc., Palo Alto, CA). Reaction mixtures (1.0 ml final vol) contained (μmole): substrate, 0.15; KCN, 10.0; $NAD(H_2)$, 0.5; Tris buffer (pH 8.0), 60 to 70; and approximately 1.6 mg abdomen extract protein. When inosine was used as substrate, 5 μmole K_2HPO_4 was also included. Reaction mixtures were preincubated for 5 min at room temperature prior to initiation by $NAD(H_2)$ addition. Data were corrected for endogenous NAD reduction. Since KCN was included in the reaction mixtures, $NADH_2$ oxidation was negligible. For calculation of enzyme activity, the molar extinction coefficient of $NADH_2$ at 340 nm was assumed to be 6.22×10^3 (23). One unit of enzyme activity is defined here as that amount whch results in the reduction of 1 μmole of NAD to $NADH_2$ x min^{-1}.

Reaction mixtures containing [2-^{14}C] xanthine were also analyzed for production of ^{14}C-UA. This was accomplished by measuring uricase-dependent conversion of the product to ^{14}C-allantoin. After NAD reduction had been monitored, two 0.4 ml portions were removed from each reaction mixture; 20 μl of uricase enzyme (hog liver, Type V; Sigma Chemical Co., St. Louis, MO) was added to one portion and 20 μl of Tris buffer to the other. After both portions were incubated at room temperature for 2.5 h, 20 μl of each mixture was taken for TLC as previously described (25), except the developing solvent used was isopropanol:HCl:H_2O (65:16.7:18.3, by vol). Allantoin was located on chromatograms by spraying control strips of the TLC sheet with Ehrlich's reagent (1).

(iv) Glutamine synthetase (GS). GS activity was assayed by measuring the production of L-γ-glutamylhydroxamate from hydroxylamine and L-glutamate (synthetase activity) or L-glutamine (transferase activity). Reactions were carried out as described by Rowe et al (28), except that in some cases 0.2 ml of termite abdomen extract (equivalent to approximately 3.2 mg protein) was used and the incubation period was extended up to 60 min. Spectrophotometric measurements were made by using a Varian model 634S double beam spectrophotometer operating at 535 nm. The amount of product formed was determined by reference to a standard curve obtained with L-γ-glutamylhydroxamate in the complete reaction mixture. One unit of enzyme activity is defined here as that amount which results in the production of one μmole of L-γ-glutamylhydroxamate x min^{-1}.

Uricolysis by cell suspensions of termite gut bacteria. Uricolysis by cell suspensions of Streptococcus UAD-1 and B. termitidis UAD-50 were carried out as described previously for metabolism of UA by minced termite guts (26), except 0.1 μmole of Na formate was added to reaction mixtures containing Streptococcus cell suspensions, and incubation was for 60 min at 25°C. Individual reaction mixtures contained 1 x 10^8 to 5 x 10^8 cells.

Uptake of UA by termite Malpighian tubules. The method used to measure UA uptake by Malpighian tubules was modeled after the system described by Sullivan and Sullivan (31). Preparative procedures were carried out aerobically at 4°C. Four or five guts, with Malpighian tubules attached, were removed from R. flavipes and placed intact into 300 μl of Grace's Insect Tissue Culture Medium (Grand Island Biol.

Co., New York) in a shallow depression plate. Twenty nmole of [2-^{14}C] UA and 0.31 mg of ^{3}H-inulin ($\overline{MW}$ = 5200) were added to each well, which was then covered with a glass slide to prevent excessive evaporation. Reactions were incubated at 25°C. Inulin (not generally transported by Malpighian tubules because of its high molecular weight) served as an internal control for tissue integrity. At the end of the incubation periods, reaction mixtures were cooled to 4°C to retard transport activity. Guts were then individually removed, rinsed briefly in 500 µl of sucrose/EDTA solution (above) at 4°C, and the tubules were separated from gut tissues. All sets of tubules (8 tubules/set) from a single well were combined in 50 µl of 0.1 N acetic acid in a 1.5 cc test tube (Eppendorf) at 4°C. The time required for dissection of tubules from five guts was 15 min, and was consistent for each sample, so residual transport activity at 4°C during the preparation period should not have significantly affected the results. Tubule preparations were extracted by sonication (see above) for 10 min, followed by incubation at 4°C for 5 min. Preparations were centrifuged for 5 min at 4°C in an Eppendorf Model 5412 centrifuge (Brinkman), and the radioactivity in a 20 or 100 µl sample of the supernatant fluid was determined.

In vivo uricolysis by R. flavipes termites. *In vivo* uricolysis was assessed by measuring $^{14}CO_2$ evolution from [2-^{14}C] UA injected into termite hemolymph. A solution containing [2-^{14}C] UA (0.5 µmole/ml) and ^{3}H-inulin (78.9 µg/ml) was injected into the abdomen of *R. flavipes*. Injection needles were made from 1.0mm o.d. x 0.8mm i.d. pyrex capillary tubing (Drummond Scientific Co., Broomall, PA) drawn to a very fine point. Termites were immobilized

for injection by cooling them to 4°C and restraining them in a trough (at room temperature) formed by embedding a longitudinally-sliced piece of Teflon tubing (1 mm o.d.) in a sheet of soft clay. Clay was pressed gently against the tubing to hold the termite in position. Injections were made while viewing the termites under a dissecting microscope. The needle was inserted into the ventral side of the abdomen, just beneath the cuticle, between the fifth and sixth sternites. Preliminary tests with $^{3}H_2O$ standards indicated that approximately 0.2 µl of solution could be injected into individual termites without apparent injury to the insects. This volume of solution caused an apparent swelling of the abdomen, but did not result in leakage of fluid from the injection site. The amount of solution injected was thus gauged by observing the extent of abdominal swelling.

Following injection, termites were placed in 5 ml capacity serum vials which were then sealed with rubber stoppers. Usually five termites were placed in a single vial. Vials were placed at 25°C, and at regular intervals each one was flushed with air for 10 min, at a rate of about 20 cc/min. The exit air was bubbled directly into a solution of Aqueous Counting Scintillant (Amersham Corp., Arlington Heights, IL):phenethylamine:methanol (10:3:3, by vol) to trap $^{14}CO_2$. The trapping efficiency of this procedure was determined to be 97.9%. Measurement of ^{14}C-radioactivity was made as described below.

After incubation, termites were removed from vials and cooled to 4°C. Guts were removed, extracted with 0.1 N acetic acid (see above), and the ^{3}H-radioactivity present in extracts was determined. Samples in which ^{3}H-radioactivity appeared in the gut extracts

indicated that the gut may have been pierced during injection and thus were not included in results.

To determine whether $^{14}CO_2$ was evolved from excreta deposited in the vials during the incubation period, vials were resealed after removal of the termites and were incubated at 25°C for one additional hour. After incubation, vials were flushed and $^{14}CO_2$ was trapped as described above.

Other procedures. UA content of termites was determined enzymatically after extraction of UA from tissues with Li_2CO_3 (25). Protein content of termite extracts was determined by the biuret method (9) with bovine serum albumin as standard. Radioactivity measurements and quench corrections were made as previously described. ^{14}C-toluene and $^{3}H_2O$ were used as standards. Enumeration of uricolytic bacteria in guts of R. flavipes was done by quantitative plating on SUA medium as described previously (26).

Chemicals. [2-^{14}C] UA and ^{3}H-inulin were obtained from Amersham Corp.; [2-^{14}C] xanthine from Research Products International, Elk Grove, IL; and ^{14}C-toluene and $^{3}H_2O$ from New England Nuclear, Boston, MA. Scintillation grade phenethylamine was obtained from Eastman Kodak Co., Rochester, NY. Purines, Li_2CO_3, Tris, NAD(H_2), ATP, ADP, L-glutamine, L-glutamate, L-γ-glutamylhydroxamate (L-glutamic acid γ-monohydrate), and bovine serum albumin were from Sigma Chemical Co. All other commercially available chemicals were of reagent grade.

RESULTS

Uric acid synthesis by termite tissue extracts. R. flavipes tissues possessed key terminal enzymes of UA synthesis. Abdomen extracts readily reduced NAD to $NADH_2$ in the presence of xanthine or hypoxanthine, suggesting the presence of XDH. The reaction was dependent upon the presence of substrate and extract in the reaction mixture. No NAD reduction was observed when boiled (5 min) extract was used. XDH activities calculated from the initial linear portion of the reaction were (units x mg protein^{-1}): 3.69×10^{-4} and 9.30×10^{-4} for xanthine and hypoxanthine, respectively.

Purine nucleoside phosphorylase also appeared to be present in abdomen extracts. Inosine served as a substrate for NAD reduction, but only in the presence of added phosphate. However, rates of phosphorylase activity were difficult to determine precisely, due to relatively high endogenous rates of NAD reduction with no added inosine. Since inosine is present in termite extracts (25), it presumably served as substrate when exogenous phosphate was added.

To further confirm the UA-synthesizing ability of termites, and to assure that NAD reduction with xanthine by abdomen extracts represented UA formation, an examination was made for UA as a product of XDH activity. The lack of uricase in termite tissues (see below) and the use of ^{14}C-xanthine as substrate aided this examination. Any ^{14}C-UA formed was converted to ^{14}C-allantoin by reaction with

commercial uricase, and the ^{14}C-allantoin was separated from ^{14}C-xanthine by TLC for determination of radioactivity. With the TLC system used, xanthine and UA spots overlapped so their combined radioactivity was tabulated. However, the critical product (allantoin) was well separated from the substrates (e.g., R_f for allantoin = 0.53; R_f for UA = 0.38) and could be easily assayed. Results are presented in Table 1. Radioactive UA (detected after conversion to ^{14}C-allantoin with commercial uricase) was readily formed from ^{14}C-xanthine by abdomen extracts in the complete reaction mixture. As expected, no ^{14}C-allantoin was formed if this reaction mixture was not treated with commercial uricase. Little ^{14}C-UA was formed if the reaction mixture lacked added NAD or contained boiled extract (Table 1). Lack of UA formation when NAD was omitted strongly suggested that oxidation of xanthine to UA was mediated by XDH rather than xanthine oxidase.

A control reaction mixture containing ^{14}C-UA instead of ^{14}C-xanthine verified that commercial uricase was active under the assay conditions used, since 96% of the ^{14}C-UA was converted to ^{14}C-allantoin (Table 1). This conversion was also accompanied by a marked decrease in the absorbance of the reaction mixture at 292nm (the absorption maximum of UA; data not shown). As expected, reaction mixtures not treated with uricase yielded no ^{14}C-allantoin, supporting our previous conclusion that termite tissues lack uricase (see below).

Lack of uricolytic activity in R. flavipes tissues. R. flavipes termites do not possess uricase. This was first suggested by spectrophotometric assays with crude tissue homogenates (25) and is now

Table 1. ^{14}C-uric acid production from ^{14}C-xanthine by R. flavipes abdomen extracts[a]

Zone on TLC plate	Reaction Mixture[b]							
	Complete		Minus NAD		Complete; boiled extract[c]		Minus ^{14}C-xanthine; plus ^{14}C-uric acid	
	Minus uricase	With uricase	Minus uricase	With uricase	Minus uricase	With uricase	Minus uricase	With uricase
Uric acid + Xanthine	116867	105160	133923	113758	103347	104667	129683	5921
Allantoin	0	5762	0	695	140	325	0	127810
Total Radioactivity	116867	110922	133923	114453	103487	104992	129683	133731

[a]Data are tabulated as ^{14}C disintegrations per minute, and are the mean of two determination.

[b]Complete reaction mixture contained: [2-^{14}C] xanthine; KCN; Tris buffer; NAD; and abdomen extract as indicated in Materials and Methods. Specific activities: [2-^{14}C] xanthine, 48 µCi/µmole; [2-^{14}C] uric acid, 53 µCi/µmole.

[c]100°C; 5 min.

confirmed by more sensitive radiometric assays (Table 2). Neither Malpighian tubule tissue nor fat body tissue converted [2-^{14}C] UA to ^{14}C-allantoin. This was true whether termites used were low in UA stores (i.e., 6.3% UA, dry weight basis) or contained over 20% UA. Control Malpighian tubules from D. melanogaster readily converted ^{14}C-UA to ^{14}C-allantoin (Table 2). Moreover, when tubules from R. flavipes and D. melanogaster were mixed together, the amount of allantoin formed was comparable to that formed by D. melanogaster tubules alone (Table 2). This result indicated that termite tissues (i) did not inhibit uricase activity, and (ii) did not contain an allantoin-degrading enzyme system.

Termite tissue extracts were examined for their ability to degrade [2-^{14}C] UA to compounds other than allantoin. Abdomen extracts failed to evolve $^{14}CO_2$ from [2-^{14}C] UA when incubated aerobically. Moreover, TLC of reaction mixtures incubated with [2-^{14}C] UA for 3 h showed no redistribution of ^{14}C-radioactivity with compared to reaction mixtures lacking termite abdomen extracts (average ^{14}C recovery from TLC plates was 96.1%). From the data of Tables 1 and 2 we concluded that R. flavipes tissues can synthesize UA, but they cannot degrade UA nor can they convert UA to other compounds in significant amounts.

Bacterial uricolysis in guts of R. flavipes. If termites are to recover N from UA in vivo, some nonuricase-mediated degradation of UA must occur. Initial studies with R. flavipes revealed that significant amounts of $^{14}CO_2$ were evolved when minced termite guts were incubated with [2-^{14}C] UA anaerobically (26). We therefore sought to confirm and extend these observations. Minced guts prepared

Table 2. Absence of uricase in tissues of *R. flavipes*[a]

Preparation[b]	UA content of insects (% dry wt.)	^{14}C-Allantoin formed (cpm)
R. flavipes M.T.	6.3	0
F.B.	6.3	0
R. flavipes M.T.	23.6	0
F.B.	23.6	0
D. melanogaster M.T.	N.D.[c]	19,125
D. melanogaster M.T.	N.D.	
+		18,573
R. flavipes M.T.	6.3	

[a] Reaction mixtures (25 µl) contained 4 µmole [2-^{14}C] UA (sp. act. = 57 µCi/µmole). Incubation: 5 min at 26°C.

[b] M.T. = Malpighian tubules; F.B. = fat body tissue. One set of 8 M.T. from *R. flavipes* or a single tubule from *D. melanogaster*, or F.B. from one *R. flavipes* termite was used for individual reactions.

[c] N.D. = not determined.

from laboratory-maintained termites evolved $^{14}CO_2$ from [2-^{14}C] UA at rates of 27.7 pmole $^{14}CO_2$ evolved x gut^{-1} x h^{-1} (Spring Arbor termites) and 82.4 pmole $^{14}CO_2$ evolved x gut^{-1} x h^{-1} (Dansville termites), but only under anaerobic incubation (Table 3). Moreover, virtually all of the uricolytic activity of termite guts was associated with the gut contents: little or no uricolysis was catalyzed by gut tissue alone (Table 3). As expected, uricolysis by gut contents was an anaerobic process (Table 3).

Minced gut preparations from field specimens of R. flavipes also exhibited uricolysis anaerobically, and rates of uricolysis were comparable to those of laboratory-maintained termites (Table 3).

If uricolytic activity of termite gut contents was to be attributed to gut bacteria, pure cultures of such bacteria (26,27) should also be capable of evolving $^{14}CO_2$ from [2-^{14}C] UA. This proved to be the case. Streptococcus UAD-1 and B. termitidis UAD-50 readily evolved $^{14}CO_2$ from [2-^{14}C] UA under anaerobic conditions. Cell suspensions of Streptococcus UAD-1 grown on fructose plus UA evolved $^{14}CO_2$ at rates of up to 249 pmole x $(10^8$ cells$)^{-1}$ x h^{-1}, whereas no $^{14}CO_2$ was evolved by cells grown on fructose alone. B. termitidis UAD-50 cells grown on fructose evolved $^{14}CO_2$ from [2-^{14}C] UA at a rate of 2049 pmole x $(10^8$ cells$)^{-1}$ x h^{-1}, but only after a 30 min lag period. Presumably, the uricolytic enzyme system of strain UAD-50 was induced during this period. Cells of strain UAD-50 grown on fructose plus UA evolved $^{14}CO_2$ at a faster rate (6734 pmole x $(10^8$ cells$)^{-1}$ x h^{-1}), with no apparent lag period. No $^{14}CO_2$ was evolved by either strain when incubated aerobically.

Table 3. $^{14}CO_2$ evolution from [2-^{14}C] uric acid by R. flavipes gut preparations

Origin of specimens[a]	Collection site[b]	Preparation[c]	Gas phase[d]	$^{14}CO_2$ evolution[e] (pmoles / gut equivalent x h)	
Laboratory	D	Minced guts	Air	< 1	(2)
			N_2/H_2	82.4±1.8	(2)
		Gut contents[f]	Air	< 1	(5)
			N_2/H_2	57.1±3.8	(21)
		Gut tissue	N_2/H_2	< 1	(2)
	SA	Minced guts	Air	< 1	(1)
			N_2/H_2	27.7	(1)
Field	D	Minced guts	N_2/H_2	36.1±3.4	(5)
	J	Minced guts	N_2/H_2	29.6±2.0	(3)

[a] Laboratory specimens had been maintained in the laboratory for 9 or 10 months prior to assay. Field specimens were assayed within 24 h of collection.

[b] D = Dansville, MI; SA = Spring Arbor, MI; J = Janesville, WI.

[c] Preparations contained 5 or 10 gut equivalents (minced guts or gut contents) or 20 gut tissue equivalents. Incubation was at 25°C and 80 osc/min for 0.5 to 3.0 hours.

[d] N_2/H_2: 90/10, vol/vol.

[e] Values are given as mean ± standard error where possible. Number in parentheses indicates the number of experiments. Sp. act. of [2-^{14}C] UA = 50 µCi/µmole.

[f] Populations of uricolytic bacteria were approximately 1 x 10^5 viable cells per gut.

These data indicated that UA degradation can occur in termites, but it is an anaerobic process carried out by the gut bacteria and not by the termite tissues.

Transport of UA from termite fat body to the gut. It was important to determine whether termites could transport UA from the site of its synthesis (i.e., the fat body tissue, which lies external to the alimentary tract) to the site of its degradation (the gut microbiota). In most insects, transport of urinary compounds to the gut occurs via the Malpighian tubules. Accordingly, two experimental approaches were used: an in vitro one which measured [2-^{14}C] UA uptake by termite Malpighian tubules; and an in vivo one which measured degradation of [2-^{14}C] UA initially injected into termite hemolymph.

The ability of termite Malpighian tubules to take up [2-^{14}C] UA is depicted in Figure 1. The initial rate of uptake was equivalent to about 0.1 pmole ^{14}C-UA x (tubule set)$^{-1}$ x min^{-1}. After 90 min, radioactivity equivalent to about 3 pmole [2-^{14}C] UA was associated with each set of tubules. Continued incubation up to 10 h did not lead to further accumulation of radioactivity (data not shown). Maintenance of tissue integrity was verified by the exclusion of ^{3}H-inulin from those tubules which were actively taking up UA (Figure 1). Boiled tubules accumulated no ^{14}C-radioactivity.

When [2-^{14}C] was injected into the hemolymph of live termites, $^{14}CO_2$ was readily evolved (Figure 2). Clearly, the UA must have been transported to the gut contents since that is the sole site of uricolysis in termites. The initial rate of $^{14}CO_2$ evolution, which was an estimate of the rate of UA degradation by the gut microbiota in

Figure 1. Uptake of [2-^{14}C] uric acid by Malpighian tubules of *R. flavipes*. Data points represent radioactivity taken up by each set of eight individual tubules. Specific activities: [2-^{14}C] uric acid, 50 µCi/µmole; ^{3}H-inulin, 577 µCi/mg.

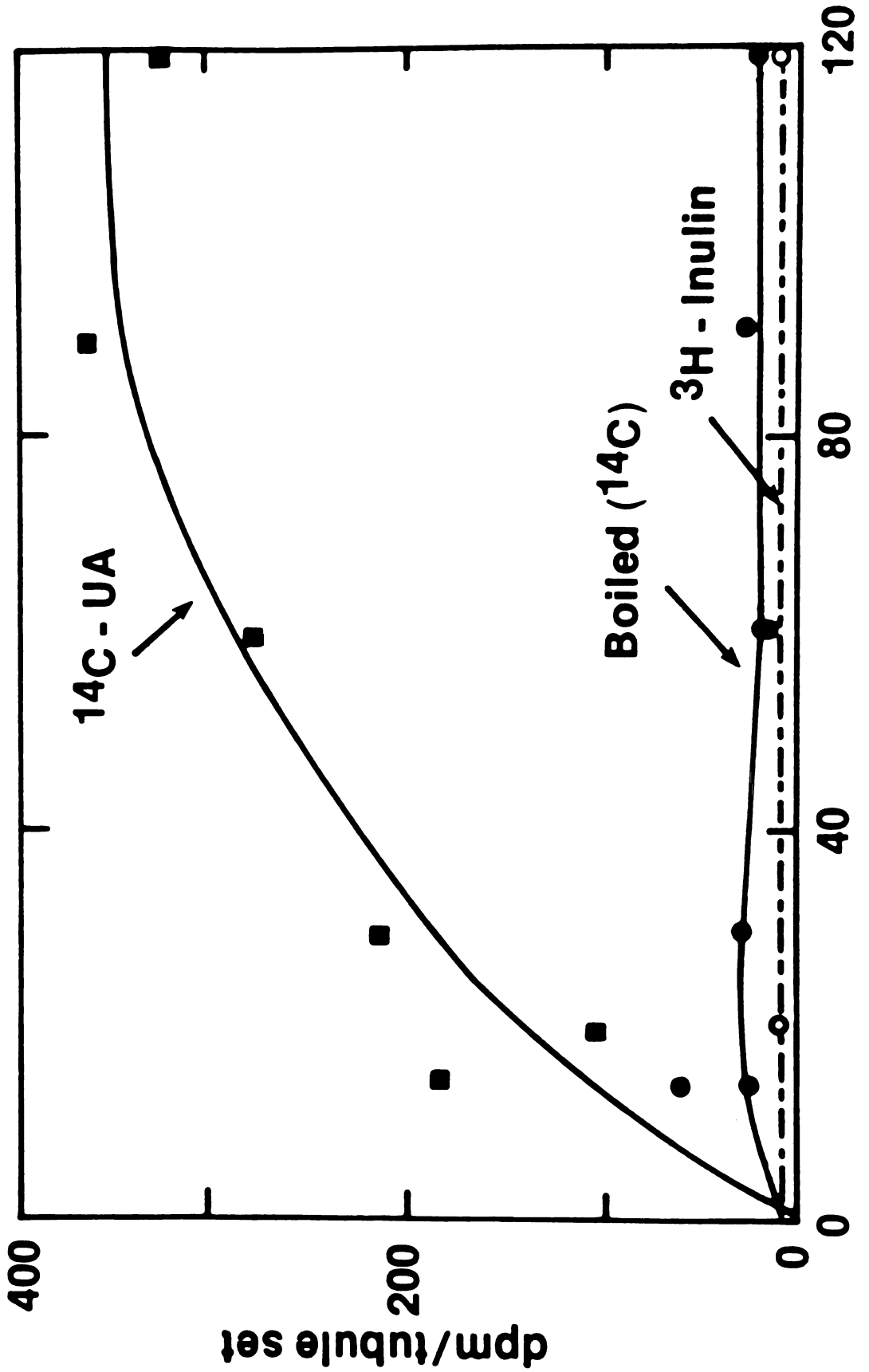
400
200
0
dpm/tubule set
0
40
80
120
Minutes
14C - UA
Boiled (14C)
3H - Inulin

Figure 1.

Figure 2. Evolution of $^{14}CO_2$ from [2-^{14}C] uric acid injected into the hemolymph of R. flavipes. Each point represents the mean of four sample vials, each containing five termites. Bars represent the standard error of the mean. Specific activity of [2-^{14}C] uric acid was 53 µCi/µmole. Termites were laboratory specimens collected from Dansville, MI ten months prior to assay.

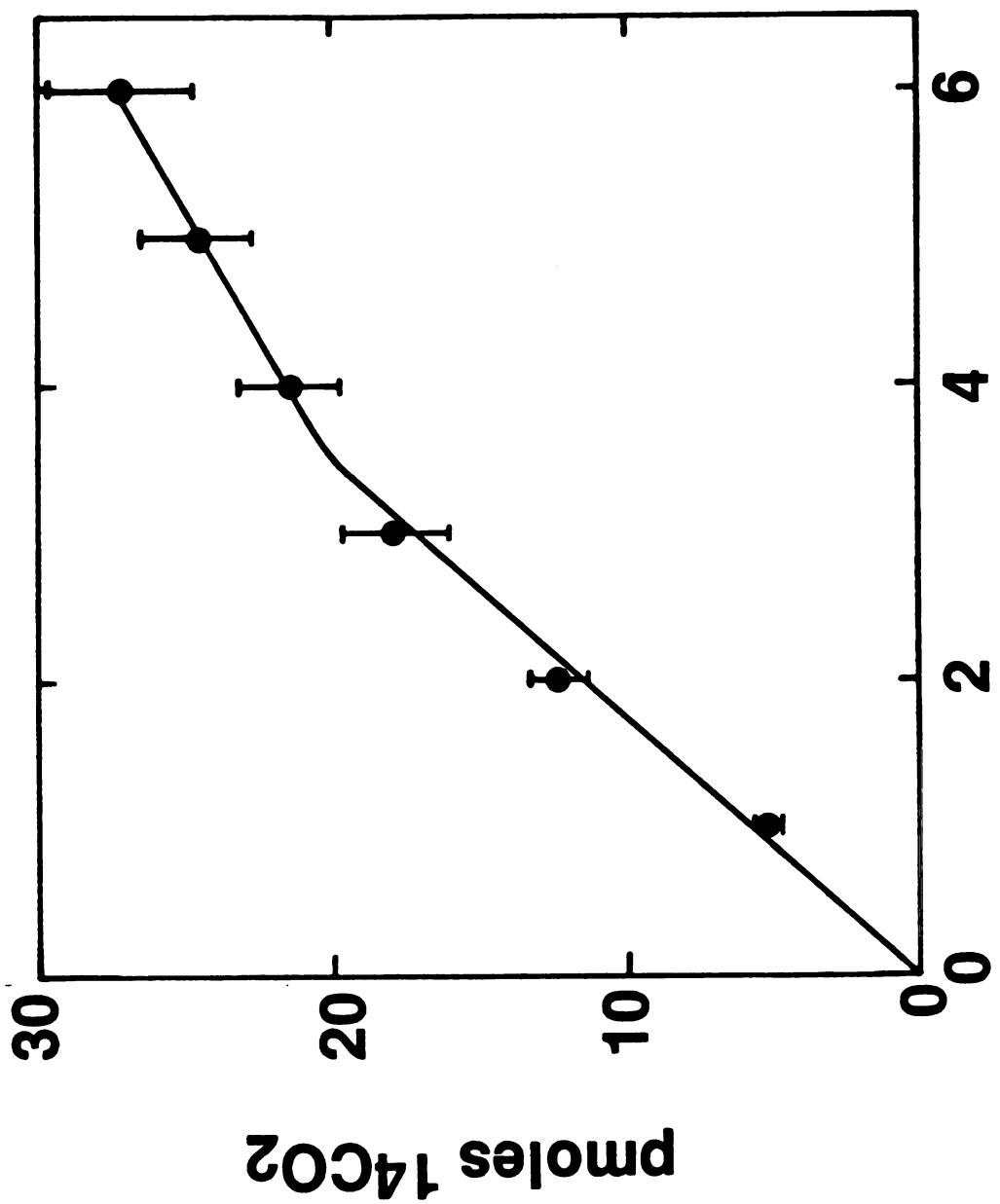

Figure 2.

situ, was about 1 pmole x termite^{-1} x h^{-1}. In separate experiments, initial rates of UA degradation ranged up to 11.7 pmole x termite^{-1} x h^{-1}. Importantly, test vials from which injected termites had been subsequently removed failed to yield $^{14}CO_2$ with further incubation. This argued against the possibility that $^{14}CO_2$ was being evolved from termite excreta.

The amount of $^{14}CO_2$ evolved in an assay vial was proportional to the number of termites held therein. For example, after 2 h of incubation, vials containing one, two, three, and four termites contained 12.5, 35.2, 46.6, and 72.1 pmoles $^{14}CO_2$, respectively. These data verified that UA transport and degradation occurs within each termite in the population.

R. flavipes termites freshly collected from the field (Janesville, WI) also evolved $^{14}CO_2$ when injected with [2-^{14}C] UA. Rates of $^{14}CO_2$ evolution were comparable to those observed with laboratory-maintained termites, and ranged from 5.8 to 9.1 pmoles x termite^{-1} x h^{-1} for the first hour of incubation.

Glutamine synthetase activity in abdomen extracts. Glutamine synthetase activity was present in termite abdomen extracts. Activities were (units x mg protein^{-1}): synthetase, 7.16 $\pm$ 1.46 x 10^{-4}; transferase, 8.45 $\pm$ 0.60 x 10^{-3}. Each value represents the mean $\pm$ the standard error of the mean for four determinations made using samples of a single termite extract preparation. No activity was observed in the absence of added homogenate, ATP, or L-glutamate (synthetase assay), or in the absence of homogenate, L-glutamine, Mn^{++}, or phosphate (transferase assay). Crude extracts which were stored at -20°C for two weeks showed a 10-fold reduction

in transferase activity. Levenbrook and Kuhn (16) reported a similar instability of glutamine synthetase prepared from the Southern armyworm (Prodenia eridania).

DISCUSSION

This paper reveals a new dimension to the interactions of termites with their intestinal microbiota: that of microbial-mediated recycling of a termite nitrogenous excretory product. R. flavipes termites synthesize and store UA, but they cannot degrade the purine by themselves. Instead, they transport UA to the alimentary tract where anaerobic uricolysis occurs via the hindgut microbiota. This allows recycling of the atoms of UA back to the termite. A particularly important consequence of the symbiosis is the conservation of N within the termite colony.

The presence of XDH in termite abdominal extracts was not surprising, since termites can accumulate high levels of UA in fat body tissue (25). XDH-catalyzed reductions of hypoxanthine to xanthine and xanthine to UA are terminal steps in UA synthesis by both the nucleicolytic and uricotelic pathways (3). The fact that the termite enzyme appears to be a dehydrogenase rather than an oxidase correlates with findings made with other insects (3,8,12,23). Moreover, terminal oxidation of $NADH_2$ formed by XDH activity may make UA synthesis by termites a metabolically inexpensive means by which to store nitrogen (3).

Levels of observed XDH activity in crude extracts of R. flavipes were low compared to those observed in D. melanogaster (10,23) and some other insects (8,12). Nevertheless, these levels can fully

account for observed rates of UA accumulation by R. flavipes. For example, XDH activity was 3.69×10^{-4} units x mg protein^{-1} when xanthine was used as substrate. Since the average protein content of termites used for the assays was 0.4 mg x termite^{-1}, this translates to 1.48×10^{-4} units per termite (i.e., 1.48×10^{-4} µmoles UA synthesized x termite^{-1} x min^{-1}) or 6.5 µmoles UA x termite^{-1} x month^{-1}. The highest rate of accumulation of UA we have observed for R. flavipes was 0.027 mg UA (= 0.16 µmole) x termite^{-1} x month^{-1} (25), i.e., a rate only 2.5% that of observed XDH activity. Therefore, XDH activity would appear not to limit UA synthesis in R. flavipes.

The apparent presence of purine nucleoside phosphorylase activity in abdomen extracts was consistent with our previous finding of inosine in extracts of R. flavipes (25) and with studies with other insects (3,4,10).

Novel (i.e., nonuricase-mediated) pathways for uricolysis have been suggested for insects such as Periplaneta americana (18) and Anthonomus (21). However, whereas R. flavipes could synthesize and store UA, they could not alone degrade it. This was concluded from the absence of uricase in termite tissues and from the inability of termite tissues or extracts to effect: (i) a decrease in the 292 nm absorption maximum of UA in solution; (ii) a change in the TLC profile of [2-^{14}C] UA as compared to an untreated [2-^{14}C] UA control; and (iii) $^{14}CO_2$ evolution from [2-^{14}C] UA.

Since termites themselves could not mediate $^{14}CO_2$ evolution from [2-^{14}C] UA, this activity was used to evaluate uricolysis by the gut bacteria in vitro and in vivo. The legitimacy of this approach was validated by the ability of uricolytic bacterial isolates (26,27) to

evolve $^{14}CO_2$ from [2-^{14}C] UA. Uricolysis in R. flavipes was confined to the gut contents and was strictly an anaerobic phenomenon (Table 3). The latter finding was consistent with our previous studies of uricolysis by these bacteria (26,27).

Rates of anaerobic uricolysis by termite gut preparations varied somewhat depending upon the termites used (Table 3), but were significantly higher than rates previously reported (26). Since we do not yet know what factors (e.g., nutritional, environmental) influence the population of uricolytic bacteria in termite hindguts (see 26) or the rates of uricolysis by gut preparations, it is difficult to assess the significance of these differences. Important to note, however, is that gut preparations from both laboratory-maintained termites and freshly collected field samples exhibited high rates of anaerobic uricolysis. Since uricolysis by pure cultures of gut bacteria was an inducible activity (see Results, and 27), the ready evolution of $^{14}CO_2$ from ^{14}C-UA by gut preparations suggests that UA transport to the termite gut is a continuous process.

Since termites do not void UA (25), bacteria presumably degrade any UA elaborated into the gut (see below). Indeed, rates of uricolysis (estimated by manometry) by pure cultures of termite gut bacteria correlated favorably with rates of uricolysis by gut preparations (26,27). Similar comparisons may be made by using rates of $^{14}CO_2$ evolution from [2-^{14}C] UA by gut bacteria and from the data of Table 3. For example, UA-grown cells of B. termitidis UAD-50 evolved $^{14}CO_2$ at a rate of 6734 pmoles x $(10^8 \text{ cells})^{-1}$ x h^{-1}, suggesting that about 8 x 10^5 B. termitidis cells per gut would be necessary to account for the activity observed with gut contents (Table

3). The predicted population level compares well with that determined by plate counts (1 x 10^5 cells/gut; Table 3, footnote f). The rate of $^{14}CO_2$ evolution from [2-^{14}C] UA by Streptococcus UAD-1 (249 pmoles x (10^8 cells)$^{-1}$ x h^{-1}) was rather low compared to rates previously observed (27): perhaps cells used in the present experiment were not fully induced.

Uricolysis by termite gut preparations in vitro would be of questionable significance in vivo unless UA from the fat body or hemolymph could reach the hindgut for microbial degradation. Termite Malpighian tubules can mediate such transport (Figures 1 and 2). R. flavipes possesses eight Malpighian tubules which insert at the proctodeal valve, just anterior to the hindgut (22). Although no detailed studies have been made on the physiology of excretion by termite Malpighian tubules, UA transport would presumably occur in a manner similar to that described for numerous other insects (e.g., see 3,17), i.e., it would pass into the tubules from the hemolymph and be carried to the hindgut by the natural flow of urine through the tubules (17).

The rate of UA transport by termite tubules in vitro [about 400 dpm (= 3.7 pmoles) x (set of tubules)$^{-1}$ x h^{-1} (Figure 1)] would appear to be more limiting to gut uricolysis than rates of microbial uricolysis (see above). In fact, in vitro rates of UA uptake by Malpighian tubules (Figure 1) correlated well with rates of in vivo uricolysis by termites injected with ^{14}C-UA (Figure 2). Moreover, the fact that freshly-collected field specimens of R. flavipes exhibited significant rates of uricolysis suggests that UA synthesis, transport, and catabolism are common processes in the termite's natural

environment.

The hypothesis presented by Leach and Granovsky (14) emphasized N conservation in termite colonies by assimilation of UA-N into microbial protoplasm, which would then remain in the colony as a result of the insects' social habits (14). However, the presence of glutamine synthetase activity in termite tissues suggests that NH_3 produced by gut microbial uricolysis (27) may be used directly by the termite. Glutamine synthetase mediates synthesis of glutamine from glutamate and NH_3 (15,19). Glutamine formed as a product of the reaction may subsequently be used for numerous biosynthetic reactions (15,19). Glutamate is found in R. flavipes (2). Whether the termite competes with its gut microbiota for any free NH_3 produced by uricolysis, and then uses that NH_3 for its own N needs, is not yet known. However, even if UA-N is used directly only by the gut microbes, it could still be conserved within the colony, as mentioned above, by the trophallactic and coprophagous habits of termites (13).

The intricacies of N flow in this regard remain to be defined, but the contribution of bacterial uricolysis to the N economy of termites is undoubtedly significant. For example, the uricolytic activity of guts removed from freshly-collected termites (Dansville) was about 36 pmoles x gut^{-1} x h^{-1} (Table 3). If an average colony of R. flavipes contains 6 x 10^4 worker termites (7), then in the period of one year the gut microbiota could release more than 1.0 g of UA-N. Since the average N content of R. flavipes workers is about 0.1 mg N x $termite^{-1}$ (25), this amount of N is equivalent to more than 10,000 termites, or nearly 20% of the total worker population. Obviously, many variables (nutritional, environmental, hormonal) could effect

these rates. However, the conclusion seems inescapable that gut microbial uricolysis is relevant to N conservation in termites.

ACKNOWLEDGMENTS

We are grateful to D. G. Cochran for advice and encouragement, and to T. Freidman for help with uricase assays and advice on microinjection techniques. This research was supported in part by the Michigan Agriculture Experiment Station of Michigan State University and by NSF grants PCM 77-05732 and PCM 80-12026. C. J. P. held an NSF predoctoral fellowship during a portion of this study.

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APPENDIX I

SUMMARY STATEMENT

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SUMMARY STATEMENT

Based on the results of the present study, a working model is proposed in Figure 1 for the roles of UA and uricolytic gut bacteria in termite N nutrition. Dietary N is incorporated into termite nucleic acids and proteins or into gut microbe cell material. UA, usually considered a nitrogenous waste product of terrestrial insects, is synthesized in termite tissues from the products of nucleic acid and protein catabolism. The terminal steps of UA synthesis are catalyzed by nucleoside phosphorylase (NP) and xanthine dehydrogenase (XDH). Although synthesized and stored in fat body tissue, UA is presumably in a dynamic state within the insect, and therefore also present in the hemolymph. Termite Malpighian tubules transport UA from the hemolymph to the gut, where it is subsequently fermented by the symbiotic hindgut bacteria to CO_2, acetate, and NH_3. Acetate derived from UA enters the large pool of acetate derived from microbial cellulose fermentation and thus serves as a carbon and energy source for the termite. NH_3 is assimilated by the gut microbiota and, through the activity of the termite's glutamine synthetase, by the termite itself. Microbial N may

Figure 1. A strategy for microbial-mediated conservation of combined nitrogen in termites. Solid arrows represent reactions or processes which are known to occur as depicted. Broken arrows depict pathways which are presumed to occur in termites, although the specific enzymes involved have not been investigated. Reactions mediated by termite enzymes are shown in the shaded portion of the figure, whereas reactions mediated by gut microorganisms are shown in the unshaded portion. NP, nucleoside phosphorylase; XDH, xanthine dehydrogenase.

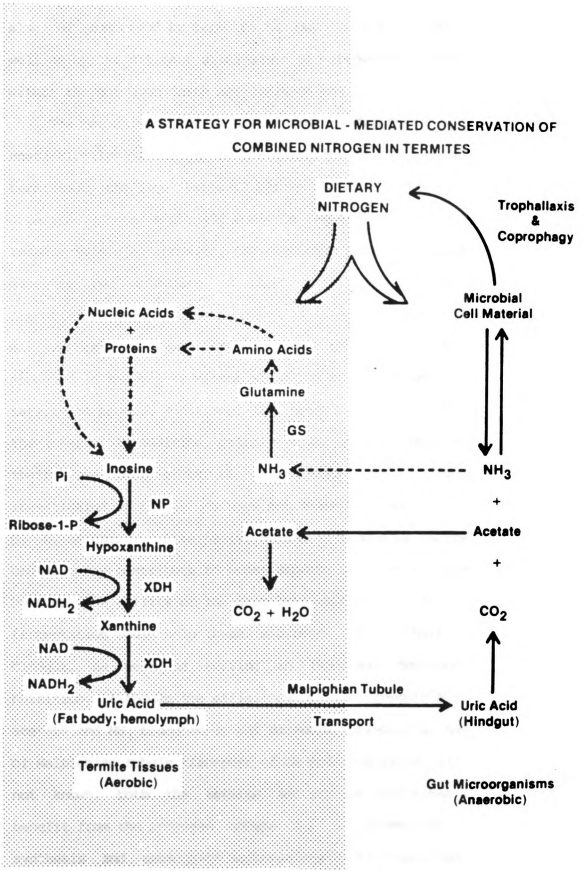
A STRATEGY FOR MICROBIAL - MEDIATED CONSERVATION OF COMBINED NITROGEN IN TERMITES
DIETARY NITROGEN
Trophallaxis & Coprophagy
Microbial Cell Material
Nucleic Acids + Proteins
Amino Acids
Glutamine
GS
Pi
Inosine
NP
Ribose-1-P
Hypoxanthine
NAD
NADH2
XDH
Xanthine
NAD
NADH2
XDH
Uric Acid (Fat body; hemolymph)
NH3
NH3
+
Acetate
Acetate
+
CO2 + H2O
CO2
Malpighian Tubule
Transport
Uric Acid (Hindgut)
Termite Tissues (Aerobic)
Gut Microorganisms (Anaerobic)

Figure 1.

also be conserved by termites through trophallaxis and coprophagy, as well as by the ultimate utilization of nitrogenous compounds released within the gut upon death and lysis of the symbionts.

The mechanisms for N conservation depicted in Figure 1 are energetically favorable to the termite. If inosine is synthesized in termites by the same pathway which has been documented for other organisms, then eight ATP would be required for the production of one inosine molecule. However, the oxidation steps catalyzed by XDH would generate two molecules of $NADH_2$ for each molecule of inosine converted to UA (and thus a potential of six ATP), reducing the cost of UA synthesis to two ATP for each UA molecule. Moreover, acetate produced in the gut by microbial uricolysis could be oxidized by the termite to provide additional ATP, negating the cost of UA synthesis to the insect. In addition, uricolytic gut bacteria generate energy for their own benefit by UA fermentation. The uricolytic bacteria investigated in this study were not dependent upon UA for a source of C, N, or energy. Alternative metabolic processes carried out by these bacteria may contribute to other aspects of termite hindgut ecology. However, the ability of the uricolytic bacteria to acquire energy by UA fermentation could help these microbes retain their niches in the hindgut, as well as serving an important function in termite N nutrition. Although the efficiency of the termite's utilization of acetate and NH_3 produced by gut microbial uricolysis, and the expense of Malpighian tubule transport of UA and termite NH_3 assimilation are not known, both the termite and its gut microbiota would appear to benefit from the proposed scheme for UA metabolism. Clearly, the synthesis and subsequent microbial-mediated catabolism of UA could be

an advantageous strategy for termite N conservation.

APPENDIX II

NITROGEN FIXING *ENTEROBACTER AGGLOMERANS* ISOLATED FROM GUTS OF WOOD-EATING TERMITES

By

C. J. Potrikus and J. A. Breznak

Reprinted from Appl. Environ. Microbiol. 33: 392-399 (1977)

APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 1977, p. 392-399
Vol. 33, No. 2
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Nitrogen-Fixing *Enterobacter agglomerans* Isolated from Guts of Wood-Eating Termites[1]

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Two strains of facultatively anaerobic, N_2-fixing bacteria were isolated from guts of *Coptotermes formosanus* and identified as *Enterobacter agglomerans*. The deoxyribonucleic acid base composition of isolates was 52.6 and 53.1 mol% guanine plus cytosine. Both isolates and a known strain of *E. agglomerans* carried out a mixed acid type of glucose fermentation. N_2 fixation by *E. agglomerans* was inhibited by O_2; consequently, N_2 served as an N source only for cells growing anaerobically in media lacking a major source of combined N. However, peptone, NH_4Cl, or KNO_3 served as an N source under either aerobic or anaerobic conditions. It was estimated that 2×10^2 cells of *E. agglomerans* were present per termite gut. This value was 100-fold lower than expected, based on N_2 fixation rates of *E. agglomerans* in vitro and that of the intact termites. However, low recoveries of *E. agglomerans* may be related to the marked decrease in N_2 fixation rates observed when intact termites or their extracted guts were manipulated for the isolation of bacteria. It was concluded that the N_2-fixing activity of *E. agglomerans* may be important to the N economy of *C. formosanus*.

In 1973 Breznak et al. (7) demonstrated N_2 fixation in termites by using the acetylene (C_2H_2) reduction assay. These workers showed that the activity was associated with the termite gut, could be modulated by the amount of combined N in the diet of the termites and could be abolished by feeding the insects antibacterial drugs, indicating that termite gut bacteria mediated N_2 fixation. It was suggested that N_2-fixing bacteria or their metabolic products might be important as an N source for some termites since the food (wood) of the insects is relatively low in combined N. Benemann (4) also observed N_2 fixation in termites and reported variations in C_2H_2-reducing activity between different groups of *Kalotermes minor*. Recently, Breznak (6) found that C_2H_2-reducing activity of *Coptotermes formosanus* can vary over 200-fold, with high rates being exhibited by young, growing larvae. In fact, it was estimated that the amount of N_2 fixed by young larvae could, over the period of a year, allow the termites to double their N content if the fixation rate remained constant.

These findings and the suggestion that bacterial N_2 fixation might be important to some termites during their development (4, 6, 7) prompted a search for the organisms involved. The results of such an endeavor constitute the substance of the present paper.

(A portion of this work was presented at the 76th Annual Meeting of the American Society for Microbiology, 2-7 May 1976, Atlantic City, N.J.)

MATERIALS AND METHODS

Termites. Formosan subterranean termites, *C. formosanus* Shiraki, were collected in the vicinity of Lake Charles, La., and maintained in the laboratory in the form of termite-infested wood. Externally undifferentiated larvae beyond the second instar (i.e., worker termites; 23) were used. The average fresh weight of workers was 2 mg. The same group of termites was used for all experiments.

Isolation of *Enterobacter agglomerans*. A successful enrichment medium consisted of equal portions of the following two solutions, which were sterilized separately. Solution 1 contained (milligrams per 100 ml of distilled water): glucose, ribose, glycerol, and mannitol, 800 each; $MgSO_4 \cdot 7H_2O$, 100; NaCl, 2; $FeSO_4 \cdot 7H_2O$, 3; $Na_2MoO_4 \cdot 2H_2O$, 1; $CaCl_2 \cdot 2H_2O$, 13; sodium thioglycolate, 100; and resazurin, 0.2. Solution 2 contained 1% (vol/vol) of a vitamin solution (34) in 0.1 M potassium phosphate buffer, pH 7.0. The medium was prepared under N_2-CO_2 (95:5), using strict anaerobic techniques (22), and was contained in anaerobic culture tubes (Hungate-type; Bellco Glass, Inc., Vineland, N.J.) at a volume of 5 ml/tube. The final pH of the medium was 6.9. Solid medium was prepared by incorporating 1.5% Ionagar no. 2 (Colab Laboratories, Inc.,

[1] Journal article no. 7760 from the Michigan Agricultural Experiment Station.

Chicago Hts., Ill.) and omitting thioglycolate.

Workers of *C. formosanus* were held in a sterile petri dish and irradiated for 15 s with a germicidal lamp (30 W; General Electric Co., Schenectady, N.Y.) positioned 45 cm from the insects. Termites were then transferred to an anaerobic glove box (Coy Manufacturing Co., Ann Arbor, Mich.; 2), and their guts were removed by using sterile forceps. Ten guts were placed in a small tissue homogenizer containing 2.0 ml of sterile enrichment medium and homogenized for 1 to 2 min. A 0.1-ml amount of the homogenate (i.e., 0.5 gut equivalents) was inoculated into 4.9 ml of enrichment medium, and this constituted the 10^{-1} dilution. Thereafter, serial 10-fold dilutions were made up to 10^{-6}. Syringe techniques (26) were used for all dilutions. Tubes were then secured horizontally in a gyratory water bath shaker (model G76; New Brunswick Scientific Co., New Brunswick, N.J.) operating at 176 rpm. The incubation temperature was 30°C.

The highest dilutions developing visible turbidity were tested for C_2H_2-reducing activity (see below). Positive cultures were again serially diluted to 10^{-8} and reincubated. Isolates were obtained from the second 10^{-8} dilution tubes by streaking roll tubes (22) and were considered to be pure cultures after four successive passages in roll tubes.

Other bacterial strains. Known strains of *E. agglomerans*, used for comparative purposes, were CDC 811-74 and CDC 156-74. The former strain belonged to biogroup G2, whereas the latter belonged to biogroup 2 (14). Both strains were obtained from W. H. Ewing, Center for Disease Control, Atlanta, Ga.

Growth studies. Basal medium GSV was used in all growth studies. Its composition was similar to that of the enrichment medium, except ribose, glycerol, mannitol, thioglycolate, and resazurin were omitted, and the glucose concentration was increased to 1%.

For anaerobic cultivation, GSV medium was prepared by boiling individual solutions for 10 min under a stream of O_2-free Ar or N_2, dispensing them into anaerobically maintained vessels, and combining appropriate solutions after heat sterilization. Usually, tubes containing 5 ml of medium were used. However, some experiments also made use of 1-liter Erlenmeyer flasks containing 300 ml of medium and equipped with serum stoppered sampling ports and gas (N_2) inlet and outlet tubes. For aerobic cultivation, media were prepared without anaerobic precautions and dispensed in 50-ml amounts into 300-ml-capacity Nephelo culture flasks equipped with a side arm (12 by 130 mm; Bellco Glass, Inc., Vineland, N.J.). Most cultures were incubated with shaking as described above. Anaerobic cultures in 1-liter Erlenmeyer flasks were vigorously stirred with a magnetic stirrer driving a stirring bar which was included in the culture vessel. The final pH of all media used in growth studies was 7.0 ± 0.1.

Media contained in tubes or Nephelo flasks were inoculated with 0.05% (vol/vol) of a broth culture containing between 1×10^8 and 4×10^8 cells/ml. A 1% inoculum was used for media in 1-liter Erlenmeyer flasks. Growth was measured turbidimetrically and by direct cell counts and in some cases also by viable cell counts and protein determinations. Turbidimetric measurements were made by using a Bausch & Lomb Spectronic 20 colorimeter operating at 660 nm. A Petroff-Hausser counting chamber was used for making direct cell counts. Viable cell counts were determined by diluting samples of culture fluid in nutrient broth (Difco Laboratories, Detroit, Mich.) and spreading 0.1 ml of appropriate dilutions (in triplicate) on plates of nutrient agar (Difco). Plates were then incubated aerobically at 30°C, and colonies were enumerated after 24 h. Protein was assayed with the Folin phenol reagent after treatment of samples with NaOH (19). Crystalline bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) was used as a protein standard.

Analyses of fermentation products. Cells were grown under Ar in GSV medium in which 0.1% NH_4Cl (sterilized separately as a 10% solution) was incorporated. Gaseous products were determined by use of a fermentation "train" (27). At the end of the fermentation, the medium was clarified (27) and the clarified fermentation liquor (CFL) was assayed directly for glucose (31), acetoin and diacetyl (33), and glycerol (28).

Ethanol was qualitatively identified and quantified in neutral volatile distillates (27) of CFL by gas chromatography. A Varian model 2440 gas chromatograph, equipped with an H_2 flame ionization detector, was used. The column was stainless steel (0.125 inch by 5 feet [ca. 0.32 by 152.4 cm]) containing Porapak Q (80/100 mesh; Waters Associates Inc., Milford, Mass.). N_2 was the carrier gas (30 ml/min), and temperatures were as follows: column, 170°C; injector and detector, 205°C each.

Neutral ether extracts (27) of CFL were assayed for 2,3-butanediol by gas chromatography as described above, except that temperatures were: column, 200°C; injector and detector, 250°C each. The limit of detection using this system was 0.5 μmol of butanediol per ml of neutral ether extract.

Organic acids were extracted from acidified CFL with ether (27) and were qualitatively identified and quantified by gas chromatography. A Varian model 1420 gas chromatograph, equipped with a thermal conductivity detector, was used. The column was stainless steel (0.125 inch by 6 feet [ca. 0.32 by 182.9 cm]) and contained 15% SP 1220-1% H_3PO_4 on Chromosorb W AW (100/120 mesh; Supelco, Inc., Belefonte, Pa.). Helium was the carrier gas (25 ml/min), and temperatures were: column, 135°C; injector and detector, 165°C each. Volatile fatty acids were applied to the column as ether solutions. Nonvolatile acids were first methylated and extracted into $CHCl_3$ (20) prior to application. Colorimetric assays were also used for the estimation of pyruvate and oxaloacetate (18) and lactate (3).

Biochemical tests. Most biochemical tests were performed by the methods of Edwards and Ewing (13). Sugar fermentation reactions were evaluated by using phenol red broth base (Difco) in which 1% of the test sugar was incorporated. Sugars were filter sterilized separately as 10% solutions, except esculin, which was sterilized with the broth base.

KCN sensitivity was determined as described by

Holding and Collee (21). Nitrate reduction was tested as described by Lennette et al. (25), using nitrate reduction broth.

Determination of G+C content in DNA. The mole percent guanine plus cytosine (G+C) in deoxyribonucleic acid (DNA) was determined by M. Mandel, using the buoyant density method (30).

C_2H_2 reduction assays. The ability of samples to reduce C_2H_2 to C_2H_4 was taken as presumptive evidence for N_2 fixation (29). C_2H_4 was measured by using flame ionization gas chromatography (29) and stainless-steel column (0.125 inch by 5 feet) containing Porapak N (80/100 mesh; Waters Associates, Inc.). N_2 was the carrier gas (30 ml/min), and the column temperature was 62°C.

C_2H_2-reducing activity in enrichment cultures was measured by injecting 0.3 cm^3 of C_2H_2 into tubes through the rubber septa and then reincubating cultures for 24 h before assaying for the presence of C_2H_4 in the gas phase. For growth studies, assays were performed by injecting culture samples into serum stoppered, Ar-filled vials at a volume of 2.5 to 5% of the vial capacity. C_2H_2 was then introduced in an amount yielding 0.05 atm of C_2H_2 in the gas phase, and samples were incubated for 1 h at 30°C on a reciprocating shaker operating at 88 oscillations/min. The reaction was terminated with 0.4 ml of 50% trichloroacetic acid/ml of sample, and the head space gas was immediately assayed for C_2H_4. O_2 inhibition studies were performed in a similar manner, except that C_2H_2 and varying amounts of O_2 were injected into Ar-filled vials before the introduction of cells. O_2 used in these studies was found to be free from detectable H_2 and CO by gas chromatographic analysis (24).

C_2H_2 reduction assays of intact termites or their extracted guts were performed in a manner similar to that described previously (7).

Other experimental procedures. The Gram stain was performed with Kopeloff reagents (20). Motility of cells was determined by direct observation of wet mounts of broth cultures, using phase-contrast microscopy.

For electron microscopy, cells were negatively stained (5) and examined with a Philips EM 300 electron microscope.

The pH of termite gut macerates was measured by placing 10 guts in 0.05 ml of deionized, glass-distilled water on a sheet of dental wax. Guts were then minced with a scalpel, and the pH of the resulting macerate was measured with a combination pH electrode (model 6020; Ingold Electrodes, Inc., Lexington, Mass.) adapted to a Radiometer pH meter (model 26; The London Co., Copenhagen, Denmark).

RESULTS

Isolation of bacteria. In three independent experiments the highest dilutions of gut homogenate yielding growth in primary enrichments were the 10^{-3} dilutions (two experiments) and the 10^{-2} dilution (one experiment). These also showed C_2H_2-reducing activity and were tentatively judged to contain N_2-fixing cells. From 10^{-3} dilution tubes two isolates of putative N_2-fixing bacteria were obtained, and these (strains C-1 and C-2) were used for further study. For approximating the numbers of N_2-fixing bacteria per termite gut, the three series of diluted gut homogenates were considered as one series (three tubes per dilution) from a single termite population. Consultation of a three-tube most-probable-number table (10) indicated that approximately 2×10^2 N_2-fixing bacteria were present per termite gut.

Numerous additional attempts were made to obtain growth and C_2H_2 reduction activity in primary dilution tubes greater than 10^{-3}. Aerobic enrichments were unsuccessful in this regard, as were the following modifications of the medium: (i) omission of thioglycolate or substitution of this compound with dithiothreitol (0.025%) or glutathione (0.05%); (ii) supplementation of the enrichment medium with 0.05% cholesterol plus 0.3% sodium succinate plus 0.03% yeast extract, 0.3% sodium succinate plus 0.005% casein hydrolysate, 0.1% ethanol plus 0.4% sodium fumarate plus 0.1% Na_2SO_4, or 0.001% yeast extract alone; (iii) substitution of sugars in the enrichment medium with 0.5% sodium pyruvate plus 0.5% sodium formate plus 0.03% glutamine, or 1% sodium lactate plus 0.03% glutamine plus 0.005% serine. Although some of these modified media (i.e., those containing amino acids or yeast extract) yielded visible growth at 10^{-6} dilution, none showed C_2H_2-reducing activity. Subcultures from such tubes, in media containing lesser amounts of combined nitrogen, either yielded no growth or sparse growth, but never C_2H_2 reduction.

General characteristics of isolates. Isolates were gram-negative, nonsporeforming, facultatively anaerobic rods, 0.5 by 1.0 μm in size. Cells were motile, and electron microscopy revealed the presence of peritrichous flagella. Cells grown aerobically on nutrient agar formed colonies that were 2 to 4 mm in diameter, round, and white to cream color.

Biochemical reactions of isolates are shown in Table 1. In addition, sugar fermentation tests indicated that both strains fermented arabinose, dulcitol, esculin, glucose, inositol, maltose, mannitol, rhamnose, salicin, sucrose, trehalose, and xylose. Only strain C-1 fermented lactose. Neither strain fermented adonitol, erythritol, raffinose, or sorbitol. These data indicated that isolates were strains of *E. agglomerans* (14). In accord with the biogroup designations of Ewing and Fife (14), strain C-1 was assigned to aerogenic biogroup G2 (indole negative, Voges-Proskauer negative), whereas

TABLE 1. *Biochemical reactions of strains C-1 and C-2*

Test or substrate	Reaction[a] of strain:	
	C-1	C-2
TSI[b]		
Slant	Acid	Acid
Base	Acid	Acid
Gas	+	−
H_2S	−	−
Urease	−	−
Indole	−	−
Methyl red (37°C)	±	−
Voges-Proskauer (37°C)	−	−
Citrate (Simmons)	+	+
KCN	−	−
Lysine decarboxylase	−	−
Ornithine decarboxylase	−	−
Arginine dihydrolase	−	−
Jordan's tartrate	−	−
Nitrate to nitrite	+	+
Oxidation-fermentation	F	F
Oxidase	−	−

[a] +, Positive reaction; ±, weak positive reaction; −, negative reaction; F, fermentation.
[b] TSI, triple sugar iron agar (Difco).

strain C-2 was assigned to anaerogenic biogroup 2 (nitrate positive, indole negative, Voges-Proskauer negative).

Fermentation products. To buttress the contention that termite isolates were strains of *E. agglomerans* and to learn more about the physiology of the isolates, the products of glucose fermentation by growing cells were compared with those of a known strain of *E. agglomerans* (Table 2). Major fermentation products of all strains were CO_2, H_2, lactate, acetate, ethanol, and succinate. Small amounts of formate, pyruvate, oxaloacetate, acetoin, and diacetyl were also formed. Whereas strains C-1 and CDC 811-74 formed significant amounts of glycerol, strain C-2 formed only trace amounts of this compound. None of the strains formed 2,3-butanediol. Carbon recoveries, based on fermentation products alone, were roughly 90%. Oxidation-reduction indexes were close to 1.0, a value that would be expected from a fermentation of glucose.

G+C content of DNA. The moles percent G+C values in the DNAs of termite isolates were 53.1 (strain C-1) and 52.6 (strain C-2). Values for known strains of *E. agglomerans* were 56.1 (strain CDC 811-74) and 57.1 (strain CDC 156-74). Although the values for termite isolates were slightly lower than those of the two known strains tested, more comprehensive analyses of the DNA base composition of *E. agglomerans* (i.e., the Herbicola-Lathyri bacteria) have revealed values ranging from 52.6 to 57.7 mol% G+C (32). Consequently, we will refer to termite isolates as *E. agglomerans* strains C-1 and C-2 for the remainder of the paper.

Growth studies and C_2H_2 reduction tests. Growth studies were used to verify the N_2-fixing ability of isolates and to characterize this activity. GSV basal medium, containing glucose, N-free salts, and vitamins, was used. Peptone, NH_4Cl, or KNO_3, when added to GSV, served as an N source for cells growing either aerobically, or anaerobically under Ar (Table 3). Cell yields under these conditions ranged from 4×10^8 to 1×10^9 cells/ml, with higher yields being obtained aerobically. No growth occurred in unsupplemented GSV medium aerobically or under Ar (Table 3). However, when

TABLE 2. *Fermentation products of termite isolates and E. agglomerans CDC 811-74*

Product	mmol/100 mmol of glucose fermented		
	Strain C-1	Strain C-2	*E. agglomerans* CDC 811-74
CO_2	103.2	107.3	101.4
H_2	103.0	103.5	101.4
Lactate	26.7	51.7	32.7
Acetate	47.0	32.7	27.3
Ethanol	67.9	71.2	65.9
Succinate	14.0	10.4	15.0
Formate	25.0	7.0	5.7
Glycerol	15.5	0.3	15.1
Pyruvate	4.7	6.0	3.1
Oxaloacetate	0.6	3.9	2.1
Acetoin	0.3	0.2	0.5
Diacetyl	0.5	0.4	0.4
2,3-Butanediol	0.0	0.0	0.0
Carbon recovered (%)	93.4	92.6	86.4
O-R index[a]	1.0	1.0	0.9

[a] O-R, Oxidation-reduction.

TABLE 3. *Specific growth rates[a] of E. agglomerans strains isolated from termites*

Addition[b] to GSV basal medium	Doublings × h^{-1} at initial gas phase					
	Strain C-1			Strain C-2		
	Air	100% N_2	100% Ar	Air	100% N_2	100% Ar
Peptone	1.56	0.47	0.61	1.64	0.59	0.59
NH_4Cl	1.17	0.30	0.40	1.14	0.38	0.47
KNO_3	0.68	0.34	0.44	0.47	0.35	0.43
None	0.00	0.16	0.00	0.00	0.13	0.00

[a] During exponential growth.
[b] Sterilized separately and incorporated at a final concentration of 0.2%.

100% N_2 was used as the initial gas phase, cells exhibited specific growth rates of 0.13 to 0.16 (Table 3) and reached densities of 4 × 10^8 cells/ml. Provision of combined N sources to cells growing under N_2 increased their specific growth rates 1.8- to 4.5-fold (Table 3) and almost doubled the cell yields. Although not shown in Table 3, only cells growing in unsupplemented GSV under N_2 exhibited C_2H_2-reducing activity.

When strain C-2 was grown under N_2 in GSV basal medium, an exponential increase in the optical density of the culture coincided with exponential increases in protein, viable cell number, and C_2H_2-reducing activity (Fig. 1). The latter reached a maximum of 250 nmol of C_2H_4 formed per (h × ml) at the late exponential phase of growth and declined thereafter. It can be calculated from the data in Fig. 1 that the greatest C_2H_2-reducing activity (normalized to viable cell number) occurred at 33 h and was 104 nmol of C_2H_4 formed per (h × 10^8 cells). Formation of C_2H_4 was dependent on the presence of C_2H_2. Almost identical results were obtained with strain C-1.

These data indicated that termite isolates fix N_2 only under anaerobic conditions, in media lacking a major source of combined N, and that C_2H_2 reduction reflected this activity.

O_2 inhibition of C_2H_2 reduction. Because strains C-1 and C-2 could not grow aerobically in unsupplemented GSV medium (Table 3), it was suspected that O_2 inhibited N_2 fixation. To test this, cells growing anaerobically under N_2-fixing conditions (i.e., in unsupplemented GSV under N_2) were placed in vials containing Ar, C_2H_2, and various amounts of O_2. As shown in Fig. 2, as little as 0.01 atm of O_2 almost completely inhibited C_2H_2 reduction by strain C-1. Virtually identical results were obtained with strain C-2. These data indicated that *E. agglomerans* behaves in a manner similar to that of many other N_2-fixing, facultative anaerobes.

Effect of anaerobiosis on C_2H_2 reduction by intact termites and their extracted guts. Intact termites and extracted termite guts were made anaerobic, and their C_2H_2-reducing activity was tested. Some of the anaerobic conditions simulated those encountered in the procedure for the isolation of *E. agglomerans* (Table 4).

Introduction of termites into the anaerobic glove box rendered them unconscious and resulted in a 100-fold decrease of their normal C_2H_2-reducing activity. The activity was not reacquired by reexposure to air, even though the animals regained consciousness (Table 4,

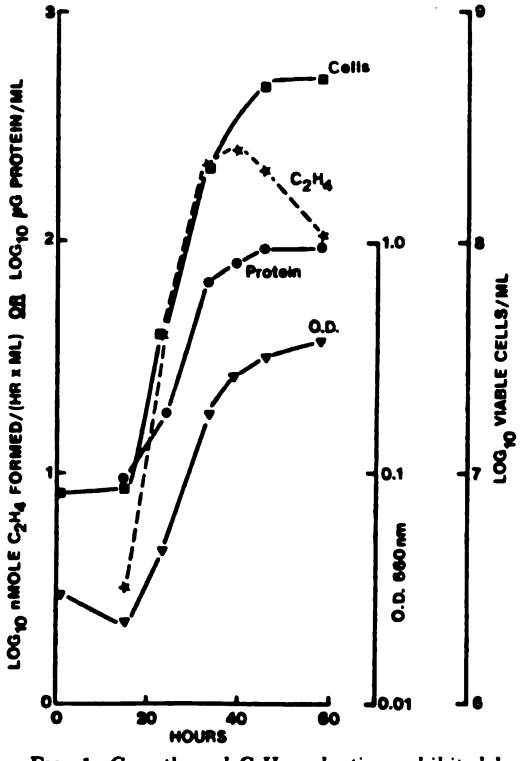

FIG. 1. *Growth and C_2H_2 reduction exhibited by E. agglomerans strain C-2. OD, Optical density.*

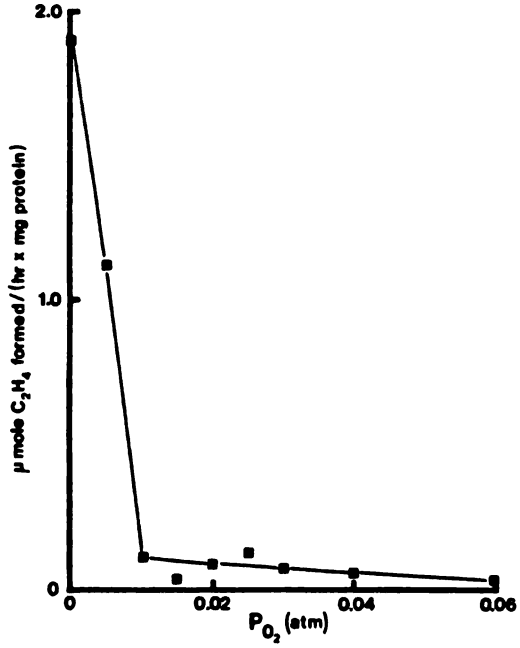

FIG. 2. *O_2 inhibition of C_2H_2 reduction by cells of E. agglomerans strain C-1. Each point on the curve represents the average value of three separate determinations.*

TABLE 4. *Effect of anaerobiosis on C_2H_2 reduction by C. formosanus and their extracted guts*

Expt no.	Specimen[a]	Treatment	Atmosphere[b] in assay vial	C_2H_2-reducing activity[c]
1	IT	Untreated (control)	Air	0.611[d]
2	IT	Admitted into glove box[e] and immediately assayed	Ar-H_2 (90:10)	0.006
3	IT	As for expt 2[e], but reexposed to air prior to assay[f]	Air	0.000
4	IT	Gassed with Ar for 1 min prior to assay[e]	Ar	0.252
5	IT	Gassed with N_2 for 1 min prior to assay[e]	N_2	0.118
6	TG	Guts removed in glove box and immediately assayed	Ar-H_2 (90:10)	0.003
7	TG	As for expt 6, but reexposed to air prior to assay	Air	0.000
8	TG	Guts removed and assayed aerobically	Air	0.000

[a] Groups of 25 specimens were used in each experiment: IT, intact termites; TG, termite guts.
[b] Atmospheres also contained C_2H_2 which was injected at zero time (see text).
[c] Nanomoles of C_2H_4 formed per (hour × 25 specimens).
[d] Equivalent to 12.21 nmol of C_2H_4 formed per (h × g [fresh weight]).
[e] Termites were rendered unconscious by this treatment.
[f] Termites regained consciousness during the 1-h assay period.

experiments 1, 2, and 3). Although H_2 (a known inhibitor of nitrogenase [9]) constituted 10% of the glove box atmosphere, its presence was not alone responsible for the loss of C_2H_2-reducing activity, since even a brief exposure of intact termites to pure Ar or N_2 also had a dramatic inhibitory effect (Table 4, experiments 4 and 5). Extracted termite guts showed a similar response, even if extracted and assayed aerobically (Table 4, experiments 6, 7, and 8).

The loss of C_2H_2-reducing activity by intact termites did not appear to result from a drastic change in the pH of their gut contents during anaerobiosis. Gut macerates of untreated termites had pH's of 6.5 and 7.0 (two separate determinations), whereas those from termites kept under Ar for 1 h had a pH of 6.9.

DISCUSSION

Although *E. agglomerans* has been isolated from a variety of intestinal and extra-intestinal habitats (15, 16), to our knowledge this is the first demonstration of *E. agglomerans* in termite guts and the first quantitative analysis of the fermentation products of this species. Unlike most *Klebsielleae*, the tribe to which the genus *Enterobacter* belongs (11), our strains of *E. agglomerans* did not produce 2,3-butanediol as a major fermentation product. Rather, the products formed (Table 2) were typical of a mixed acid fermentation (12), with a significant production of glycerol by two of the three strains assayed.

It is noteworthy that Aho et al. (1) also documented N_2 fixation by *E. agglomerans*, using isolates obtained from decaying white fir trees. These workers further substantiated the N_2-fixing ability of isolates by demonstration of $^{15}N_2$ incorporation into growing cells. French, on the other hand, recently reported the isolation of N_2-fixing bacteria from Australian termites, but did not state the identity of the isolates, their numbers in guts, or their magnitudes of N_2 fixation (17).

Although *E. agglomerans* was the only N_2-fixing bacterium we isolated from termite guts, we are reluctant to conclude that it is the major N_2 fixer in this habitat, simply because it was isolated from relatively low dilutions of gut homogenate. Calculations based on maximum rates of C_2H_2 reduction by *E. agglomerans* in vitro [104 nmol of C_2H_4 formed per (h × 10^8 cells)] indicated that a population of 2.3 × 10^4 cells/gut would be necessary to account for the activity observed with intact termites [0.611 nmol of C_2H_4 formed per (h × 25 termites); Table 4]. Based on most-probable-number estimates, our isolation attempts yielded 100-fold fewer cells per gut than the expected value, even when aerobic isolation procedures were used or when the enrichment medium was extensively modified. It is significant, however, that removal of termite guts, even under anaerobic conditions, resulted in a 99 to 100% decrease in the C_2H_2-reducing activity of the preparation (Table 4). A 60 to 100% loss in activity occurred even when intact termites were made anaerobic prior to assay (Table 4). Benemann (4) also observed a 90% loss of C_2H_2-reducing activity when *K. minor* was incubated anaerobically. The inhibitory effect of anaerobiosis on C_2H_2-reducing activity of termites may have a bearing on our inability to retrieve *E. agglom-*

erans in greater numbers. Interestingly, recovery of *E. agglomerans* in numbers 100-fold lower than expected paralleled the 100-fold decrease in C_2H_2-reducing activity observed when the insects were made anaerobic.

Low recoveries of N_2-fixing *E. agglomerans* may also result from incomplete dispersion of bacterial aggregates during preparation of gut homogenates. Electron microscopy of guts of *C. formosanus* (8) has revealed the presence of dense bacterial aggregates that adhere strongly to the epithelium. If *E. agglomerans* forms such aggregates, much more vigorous preparatory procedures may be necessary for their dispersal. However, preliminary experiments employing 0.04% Trition X-100 or 0.01% Tween 80 in the homogenizing solution or blending gut homogenates in a microblender assembly were unsuccessful in this regard. Finally, the presence of a true microaerophilic N_2-fixing bacterium in guts, in numbers greater than those of *E. agglomerans*, has not been discounted and is a possibility currently being investigated in our laboratory.

In view of these considerations, we prefer to be conservative at this time and conclude that *E. agglomerans* may be important to the N economy of *C. formosanus*.

ACKNOWLEDGMENTS

We are grateful to Manley Mandel for performing the DNA base composition analyses and H. S. Pankratz for help with electron microscopy. We thank J. M. Tiedje for helpful comments on the manuscript.

The research was supported by the Michigan Agricultural Experiment Station of Michigan State University and by National Science Foundation grant BMS75-05850. C. J. P. is the holder of a National Science Foundation predoctoral fellowship.

ADDENDUM IN PROOF

French et al. (J. Gen. Microbiol. 95:202-206, 1976) recently implicated *Citrobacter freundii* as a nitrogen-fixing agent in guts of Australian termites. However the number of *C. freundii* cells per termite gut was not determined.

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