Arch Microbiol (1984) 138:131–139

Archives of
Microbiology

Microbial aspects of the cockroach hindgut *

Diana L. Cruden and A. J. Markovetz

Department of Microbiology, University of Iowa, Iowa City, IA, 52242, USA

Abstract. The cockroach hindgut has a complex, active microbiota, a large portion of which is associated with the chitinous gut wall. It provides a different environment from that of termites and other insects which are dependent on their hindgut microbiota for the digestion of cellulose. The pH of the midgut of *Eublaberus posticus* was not as high as it is in insects with a primarily cellulolytic nutrition. The hindgut of *E. posticus* was highly methanogenic, normal adults producing typically 10–25 μmol of methane per hour. The hindgut contained large amounts of the storage products polyphosphate and poly-β-hydroxybutyrate. Dilution series on nonselective medium yielded 100 times more obligately anaerobic colonies than facultatively anaerobic colonies. The most common facultative isolates were *Klebsiella oxytoca, Citrobacter freundii* and *Enterobacter agglomerans*. Treatment of *E. posticus* with metronidazole caused a dedifferentiation of the different regions of the hindgut. One region of the hindgut is characterized by its visibly black color, a unique microbiota, and electron dense deposits in electron micrographs. Chemical determinations showed high concentrations of ferrous and sulfide ions in the region. X-ray microprobe analysis showed that some of the electron dense deposits consisted of iron, sulfur and lower amounts of copper, aluminium, and chromium associated with ruthenium red staining material. Spectra of other deposits revealed only silicon, which was not associated with ruthenium red.

Key words: Hindgut microbiota – *Eublaberus posticus* – Poly-β-hydroxybutyrate – Polyphosphate – Metal ion deposition – X-ray microprobe – Methanogenesis

Cockroaches are ancient, adaptable and generally omniverous insects. Over the past several years it has emerged that their hindgut microbiota resembles that of the termite and the rumen in complexity.

Eublaberus posticus, the cockroach on which we have concentrated most of our efforts is a large (adult males weigh 2–3 g and females 3–4.5 g) member of the Blaberidae. Its microbiota is concentrated mostly in the hindgut. The hindgut, like that of most insects, is lined with a chitinous cuticle, which in *E. posticus* is 2–3 μm thick and is equipped with long chitinous spines which project up to 100 μm into the lumen. There is a large population of bacteria and protozoa associated with the wall and spines, which resists removal by vortexing. Previous studies have shown that the wall-associated bacteria are different from those of the lumen, and that there are distinct populations in different regions of the colon (Cruden and Markovetz 1981). One region of the colon is characterized by a macroscopically visibly black color which is eliminated when the anaerobic microbiota is eliminated with the antibiotic metronidazole (Bracke et al. 1978; Cruden et al. 1979). Preliminary chemical determinations indicated that the visible black color is correlated with an increased level of sulfide compared with the rest of the gut (Cruden et al. 1979).

The cockroach hindgut microbiotica is anaerobic and methanogenic (Bracke et al. 1979), and contains a number of bacteria which can digest carboxymethylcellulose (Cruden and Markovetz 1979). Products of microbial hindgut fermentations can be transported through the chitinous cuticle into the hemolymph (Bracke and Markovetz 1980).

Several other insect hindguts also have complex microbiota. Studies on termites (Breznak and Pankratz 1977) scarab beetles (*Oryctes*) (Bayon 1980) and cranefly larvae (*Tipula*) (Klug and Kotarski 1980), indicate that these disparate groups also have significant wall-associated populations of bacteria and protozoa. Termites are dependent on the cellulolytic activities of their gut microbiota. Scarab beetles can exist on a pure cellulose diet, although their normal diet is a mixture of dung and plant materials (Bayon 1980), while *Tipula* larvae feed on detritus, with a large fiber and cellulose component, in streams (Klug and Kotarski 1980). Cockroaches (aside from the specialized woodroaches, such as *Cryptocercus*, whose nutrition appears to be very similar to that of termites) on the other hand appear to have no "normal diet", but opportunistically feed, and survive, on whatever is available and can also survive starvation for long periods of time [reviewed in Guthrie and Tindall (1968) and by Bignell 1981)]. In this paper we will examine a number of biochemical and physiological aspects of the cockroach gut which may be traced to microbial activities and provide data useful in comparing this ecosystem with other, better studied, gut systems.

Materials and methods

Insects. Eulaberus posticus were maintained in colonies in the laboratory with access to Purina rat chow (22% protein, 4.5% fat) and water ad lib. "Metronidazole animals" were

* Diana L. Cruden dedicates this paper to the memory of Roger Stanier, with gratitude, admiration, and affection

Offprint requests to: D. L. Cruden

from a subcolony in which metronidazole (Flagyl, Searle) was added to the drinking water at a concentration of 1 mg/ml. *Rumen fluid.* Raw bovine rumen fluid was obtained from an animal equipped with a permanent rumen cannula. Fluid was filtered through cheesecloth and quickly chilled. API-zym analysis (Analytab Products; see Table 2 for the enzymes assayed) and determination of the proportion of Gram-positive cells were performed within 6 h. The rest of the determinations were performed on aliquots which had been stored frozen.

Analysis of gut contents. For analysis of pH, methane production, and API-zym activities of the gut fractions, insects were dissected in an anaerobic chamber. The guts were divided into midgut (between the digestive caeca and malphigian tubules) and hindgut (including the ileum and colon but not the rectum). In some experiments the hindgut was further divided into the paunch, or enlarged region of the colon, and the black band region, which is the macroscopically visibly darker segment of the colon (usually a segment 4–8 mm long in adults) between the paunch and the rectum. In metronidazole animals the black band region was no longer black, but lengths of colon of the appropriate size from the same region were dissected. Gut contents from the fractions were squeezed into small beakers and fractions from four animals were pooled. One milliliter distilled water was added (since the contents were often semi-solid), and the contents gently mixed. The pH was determined in the anaerobic chamber with short range (pH 6.0–8.5) paper, and immediately on removal from the chamber with a pH meter under a stream of N_2. The API-zym strips (Analytab Products) were incubated under aerobic conditions.

For determination of the proportion of Gram-positive cells, samples of diluted hindgut contents from normal and metronidazole-treated *E. posticus* and raw rumen fluid were Gram stained. The total number of bacterial cells and Gram-positive cells were counted in twenty random fields from each sample.

Protein was determined by the method of Lowry et al. (1951). Dry weights were determined by drying samples to constant weight at 60° C.

Poly-β-hydroxybutyrate and polyphosphate were determined in the same samples with the method of Poindexter and Eley (1983). Chemical determinations of ferrous and sulfide concentrations in gut fractions were by the methods of Marczenko (1976).

Methane production was measured with a Varian series 2700 gas chromatograph equipped with a spherocarb column, flame ionization detector and Hewlett-Packard 3390 A integrator. Standard curves were constructed with identical tubes into which known amounts of CH_4 were injected. To determine methane production by whole animals, individuals were placed in large test tubes, which were stoppered with black rubber stoppers. After 15 min, the atmosphere in the tubes was sampled through the stopper with a syringe. Total gas volume of the tubes averaged 20 cm^3 for normal sized adults (2.5–3.5 g), and was not corrected for the volume of the insect.

Viable counts. Adult *E. posticus* were dissected in the anaerobic chamber. Midgut and hindgut fractions were weighed, homogenized in 5 ml of anaerobic dilution fluid (Bryant and Burkey 1953), and a series of 10-fold dilutions was made. 0.2 ml aliquots were spread on plates of RCGA (40% rumen fluid-cellobiose-glucose agar) (Bryant and Robinson 1961). Duplicate sets of plates were incubated in the anaerobic chamber and aerobically at 30° C. Plates with between 20–200 colonies were counted after 4 days. Representative colonies from several high dilutions were purified by restreaking on the same medium. Facultative isolates were identified with the API20E system (Analytab products), and anaerobic isolates with the media specified in the VPI manual (Holdeman et al. 1977) (Scott anaerobic media was generally used). Gas chromatography of fermentation endproducts was performed on extracts prepared as in the VPI manual in a Varian gas chromatograph equipped with a capillary carbowax 20 M column, flame ionization detector, and Hewlett-Packard 3390A integrator.

Electron microscopy and X-ray microanalysis. Guts were dissected, vortexed to remove lumen contents, and fixed and embedded as previously described (Cruden and Markovetz 1981).

For X-ray microanalysis, gut fractions were fixed with and without ruthenium red and embedded in Spurr's resin. Sections were cut with a diamond knife and mounted on titanium grids. Specimens were examined (with no poststaining) with a Hitachi H600 transmission electron microscope equipped with STEM and a Kevex 7000 X-ray microanalysis system.

Results

The pH of the gut fractions of normal *Eublaberus posticus* and animals fed metronidazole for an extended period of time is shown in Table 1. The pH of the hindgut is higher than that of the midgut, and within the hindgut, that of the black band region is higher than that of the paunch. Metronidazole treatment, which removes the strictly anaerobic microbes, leads to a lower and more variable pH in the hindgut, and eliminates the difference in pH between the paunch and black band fractions.

The lumen contents of the hindguts of normal and metronidazole-treated cockroaches and raw rumen fluid were Gram stained to determine the proportion of Gram-positive organisms. *E. posticus* hindgut contents contained $20.93 \pm 5.65\%$ Gram-positive cells, while insects treated with metronidazole contained $10.78 \pm 2.63\%$ Gram-positive cells. Raw rumen fluid contained $19.97 \pm 7.39\%$ Gram-positive cells.

Table 2 shows the comparative activities of the 19 enzymes of the API-zym system in the contents from paunch and black band regions of the hindguts of normal and metronidazole-fed *E. posticus*. This determination does not discriminate between enzymes of animal origin secreted into the gut and microbial enzymes. The activity levels of the enzymes from the five pools from control animals were quite constant. In metronidazole-fed animals, however, the activities of several enzymes, notably trypsin-like protease, β-galactosidase, β-glucuronidase, α-glucosidase and β-glucosidase, became very variable. Metronidazole treatment also decreased the difference in activity between the paunch and black band regions of esterase.

Table 3 shows the numbers of organisms from *E. posticus* gut fractions cultivable on plates of the nonselective medium RCGA. Dilutions from the hindgut yielded over 100 times as many colonies on plates incubated anaerobically as aerobically. Numbers of strictly and facultatively anaerobic col-

Table 1. pH of hindgut fractions of *Eublaberus posticus*

	Normal adults			Metronidazole-fed adults		
	midgut	paunch	black band	midgut	paunch	black band
pH paper	n.d.[a]	7.5 ±0.26[b]	7.7±0.1	n.d.	6.9 ±0.3	6.4 ±0.2
pH meter	6.60±0.1	7.47±0.12	8.1±0.21	6.35±0.07	6.75±0.45	6.32±0.50

[a] n.d. = not determined
[b] Five pools of fractions from 4 animals each

Table 2. Enzyme activities in *Eublaberus posticus* hindgut fractions and rumen fluid as determined by the API-zym system

	Alkaline phosphatase	Esterase	Esterase lipase	Lipase	Leucine aminopeptidase	Valine aminopeptidase	Cystine aminopeptidase	Trypsin	Chymotrypsin
E. posticus									
paunch[a]	+++	−	+	±	++	+	±	−	±
black band[a]	+	+	++	−	+++	±	+	±	−
Metronidazole *E. posticus*									
paunch[a]	+++	+	++	−	+++	++	+	0−++++	−
black band[a]	++	+	++	−	+++	+	+	0−++++	−
Bovine rumen fluid	++++	++	+++	−	+++	±	±	+	±

[a] Five pooled fractions from four animals each

Acid phosphatase	Phosphoamidase	α-Galactosidase	β-Galactosidase	β-Glucuronidase	α-Glucosidase	β-Glucosidase	N-Acetyl-β-glucosaminidase	α-Mannosidase	α-Fucosidase
+++	+++	+	++	+	++	++	++	+	+
+++	++++	+	++	±	++	++	+++	±	±
+++	++++	+	+++	+−++++	+−++++	+−++++	++	±	±
+++	++++	+	+−++++	0−+++	+−++++	+−++++	++	±	±
++++	+++	++	++++	++++	+++	+	+++	−	−

Table 3. Numbers of bacteria culturable on RCGA from the midgut and hindgut of *Eublaberus posticus*

	Incubated anaerobically	Incubated aerobically
Midgut	$3.18\times10^{8}\pm4.59\times10^{8}$	$8.77\times10^{7}\pm6.9\times10^{7}$
Hindgut	$5.95\times10^{10}\pm4.1\times10^{10}$	$1.55\times10^{8}\pm2.2\times10^{8}$

onies were more similar in samples from the midgut. No obligately aerobic bacteria were isolated from either hindgut or midgut. The most common colony types in the higher dilutions were purified. Most of the facultatively anaerobic isolates belonged to three species: *Klebsiella oxytoca, Citrobacter freundii*, and *Enterobacter agglomerans*. Their characteristics are shown in Table 4. These three species have also been isolated from *E. posticus* in other studies using selective media [e.g. the *Klebsiella* was very similar to the strain identified as an indole negative variant of *Klebsiella pneumoniae* in the published report of carboxymethylcellulose degradation by cockroach gut microbiota (Cruden and Markovetz 1979), and the *Citrobacter* was similar to strains isolated as uric acid degraders (Becker et al. 1982)]. Members of the genera *Streptococcus* and *Serratia* were also isolated, but were less frequent.

The strictly anaerobic isolates were more varied as well as more numerous than the facultatively anaerobic isolates. They included several members each of a number of genera, including *Fusobacterium, Clostridium, Bacteroides, Eubacterium, Propionibacterium*, and *Peptostreptococcus*. Many of these isolates could not be identified to species using the conventional references (VPI manual and Bergey's Manual).

Methane emission from live *E. posticus* adults varied a great deal from individual to individual and less from day to day. Normal values from healthy animals varied from 10−25 μmol/h · animal. The highest production ever mea-

Table 4. Characteristics of the three most common facultatively anaerobic isolates from the hindgut of *Eublaberus posticus*, as determined by the API 20E system

Characteristic	*Klebsiella oxytoca*	*Citrobacter freundii*	*Enterobacter agglomerans*
ONPG	−[a]	+	−
Arginine	−	−	−
Lysine	+	−	−
Ornithine	−	−	−
Citrate	+	+	−
H_2S	−	+	−
Urea	−	+	−
Tryptophan	−	−	−
Indole	+	−	−
Voges-Proskauer	+	−	−
Gelatin	−	−	−
Glucose	+	+	+
Mannitol	+	+	+
Inositol	+	−	+
Sorbitol	+	+	+
Rhamnose	+	+	+
Sucrose	+	+	+
Melibiose	+	+	+
Amygdalin	+	+	+
+-l-Arabinose	+	+	+
Oxidase	−	−	−
NO_2	+	+	+
Gas	−	−	−

[a] +, positive; −, negative

sured was 165 μmol/h by a female, which consistantly produced very large amounts when assayed periodically for several weeks. Immature *E. posticus* as small as 300 mg also produced methane (up to 5 μmol/h).

When gravid females were isolated shortly before giving birth (*E. posticus* are ovoviviparous), and the young were immediately placed in a clean container (food and bedding were autoclaved before use, but no other attempts were made to keep the environment sterile), they did not begin to produce methane unless fresh fecal material from the main colony was added to the container. When this was done, the young insects began to emit methane within four days, indicating that methanogens were passed from generation to generation by coprophagy.

Electron micrographs of the hindgut of *E. posticus* showed that many of the microbial cells have inclusions resembling typical microbial storage products. Figure 1 is a micrograph of bacteria close to the wall in the posterior paunch area of the colon. Many cells of at least two of the most frequent morphotypes have large inclusions. Other cell types of the hindgut microbiota which typically have inclusions have been illustrated previously (Cruden and Markovetz 1979). Poly-β-hydroxybutyrate is an exclusively prokaryotic product and polyphosphate is accumulated by many bacteria. Gut contents were collected and assayed for these compounds. The amounts present are shown in Table 5, with the amounts present in raw rumen fluid included for comparison. There is significantly more of both PHB and polyphosphate in the hindgut of normal *E. posticus*

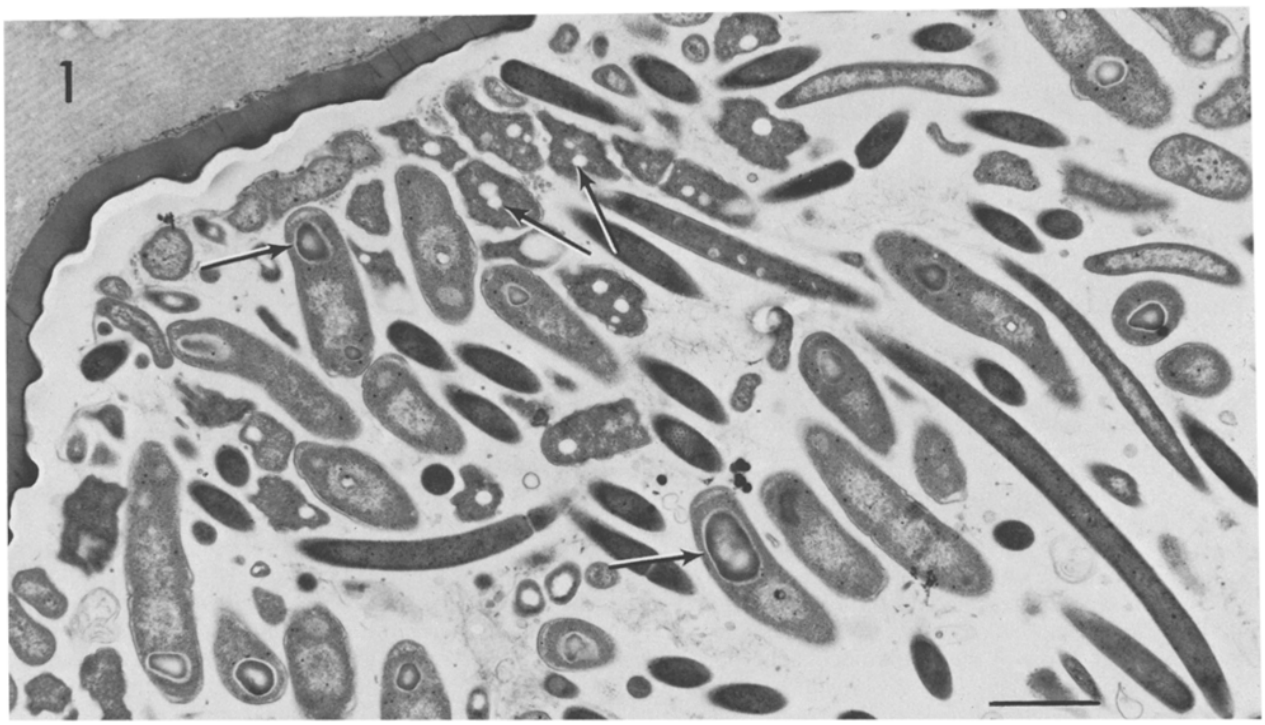

Fig. 1. Micrograph of the posterior paunch region of the colon of *Eublaberus posticus* showing wall-associated microorganisms. The chitinous wall extends across the upper left hand corner. Two predominant bacterial cell types contain large granules of storage material (arrows). Bar = 1.0 μm

Table 5. Poly-β-hydroxybutyrate and polyphosphate contents in *Eublaberus posticus* gut fractions and in rumen fluid

	Protein[a]	PHB[b]	Pn[c]
E. posticus hindgut	0.127	271	1.00
E. posticus midgut	0.120	41.1	0.24
Metronidazole-treated *E. posticus* hindgut contents	0.106	23.2	n.d.[d]
Bovine rumen fluid	0.196	63.6	0.116

[a] mg/mg dry wt.
[b] μg/mg protein
[c] μmol/mg protein
[d] not determined

Table 6. Ferrous and sulfide contents of regions of the hindgut of *Eublaberus posticus*

Gut fraction	Amount of ion (ng/mg [wet wt.] tissue)	
	Fe^{2+}	$S^{=}$
Paunch	86.5 ± 44.6[a]	12.3 ± 5.51
Black band	515.5 ± 101.1	205 ± 28.6
Paunch, metronidazole-fed insect	63[c]	7
"Black band"[b], metronidazole-fed insect	49	8

[a] $N = 5$
[b] No black visible
[c] Sample from one insect

than there is in the midgut, in the hindgut of metronidazole animals, or in bovine rumen fluid.

Gut fractions of *E. posticus* were assayed for the presence of ferrous and sulfide ions. Table 6 shows that the macroscopically visible black color of the black band region of the colon is correlated with high levels of both of these ions. Metronidazole teatment does not significantly alter the levels of these ions in the paunch, but in the black band region (now no longer black) the levels are decreased to approximately the same amounts as are in the paunch. Data for the contents of ferrous and sulfide ions is given for one metronidazole-treated individual. Variability would be expected between individuals, but the amounts of these ions in the black band region were uniformly markedly lower than in normal animals in unpublished qualitative tests on several individuals.

In electron micrographs, the electron dense material characteristic of the black band appears in several different forms. Figure 2a shows a large very electron dense amorphous mass of material. Sometimes it appears as more structured deposits which seem to be associated with particular cell types, either around single cells, as in Fig. 2b, or around microcolonies of cells as in Fig. 2c. The micrograph in Fig. 2c was of a section of a gut prepared with ruthenium red in the fixative. Figures 2a and b are from preparations fixed with glutaraldehyde and osmium without ruthenium red. The electron dense material is present whether or not the preparations are stained with ruthenium red, but it appears to be concentrated in regions which do stain with this compound, which is specific for polyanionic substances such as those which make up the glycocalyces of bacteria in natural environments (McCowan et al. 1978).

Sections of the paunch and black band regions were subjected to X-ray microprobe analysis. Ruthenium red staining regions (there being no other electron dense deposits) of the paunch showed no concentration of elements other than ruthenium and osmium, above the background. Figure 3a shows a micrograph from the black band region, and Fig. 3b shows the X-ray emission spectrum of the electron dense material at the marked point in the micrograph. This is the most typical spectrum obtained of the electron dense deposits. The elements present [other than T_i from the grid (K_α 4.51 keV), Os (L_α 8.91 keV, $L\beta$ 10.35 keV, M_α 1.91 keV) and Ru (L_α 2.56 keV, $L\beta$ 2.68 keV) from the fixation] are Fe (K_α 6.40 keV), S (K_α 2.3 keV), and Cu (K_α 8.0 keV). The ruthenium L peaks overlap the K peaks of chlorine, but its presence was confirmed by the presence of its K_α peak at 19.28 keV (spectrum not shown). Aluminium and chromium were also detected in many deposits. Iron and sulfur were present in all the electron dense deposits of this type, but the presence of copper, aluminium, and chromium was variable. When spectra were obtained of areas in the sections within microbial cells, the gut wall cuticle, animal tissue or areas not visibly electron dense, no peaks other than those of osmium, titanium or ruthenium were detected.

Some sections contain electron dense deposits which have a different appearance in micrographs from the typical black band material. The electron dense material in these deposits often develops small tears during sectioning. This type of deposit, like the more typical type, has only been observed in the black band region of the gut. An STEM image of such a deposit is shown in Fig. 4a. Figure 4b is an X-ray emission spectrum of the deposit showing that silicon (K_α 1.74 keV) is the only element present in significant amounts other than a small amount of titanium from the grid. Figure 4a is an STEM image of the deposit with, superimposed on it, a tracing across the field at the level of the straight line of the quantity of X-rays with the energy of silicon, showing that the silicon is concentrated only in the electron dense deposit. Osmium and ruthenium are not found associated with these deposits. It seems unlikely that contamination with silicon occurred during the fixation procedure (from glass pipettes) or from the pump oil in the electron microscope, since the deposits are found only in the black band region and are localized in apparently undisturbed areas of the preparation, surrounded by bacterial cells.

Discussion

Although bacteria have been isolated from the cockroach gut which can digest carboxymethylcellulose (Cruden and Markovetz 1979), the cockroach is not dependent on the cellulolytic activities of its microbiota for its nutrition, and the environment of the cockroach hingut is very different from that of the termite or scarab beetle. Uric acid, the main excretory product of most terrestrial insects is not excreted by *Eublaberus posticus* or by most other cockroaches, but is stored in large amounts in the fat body of the insect, and its

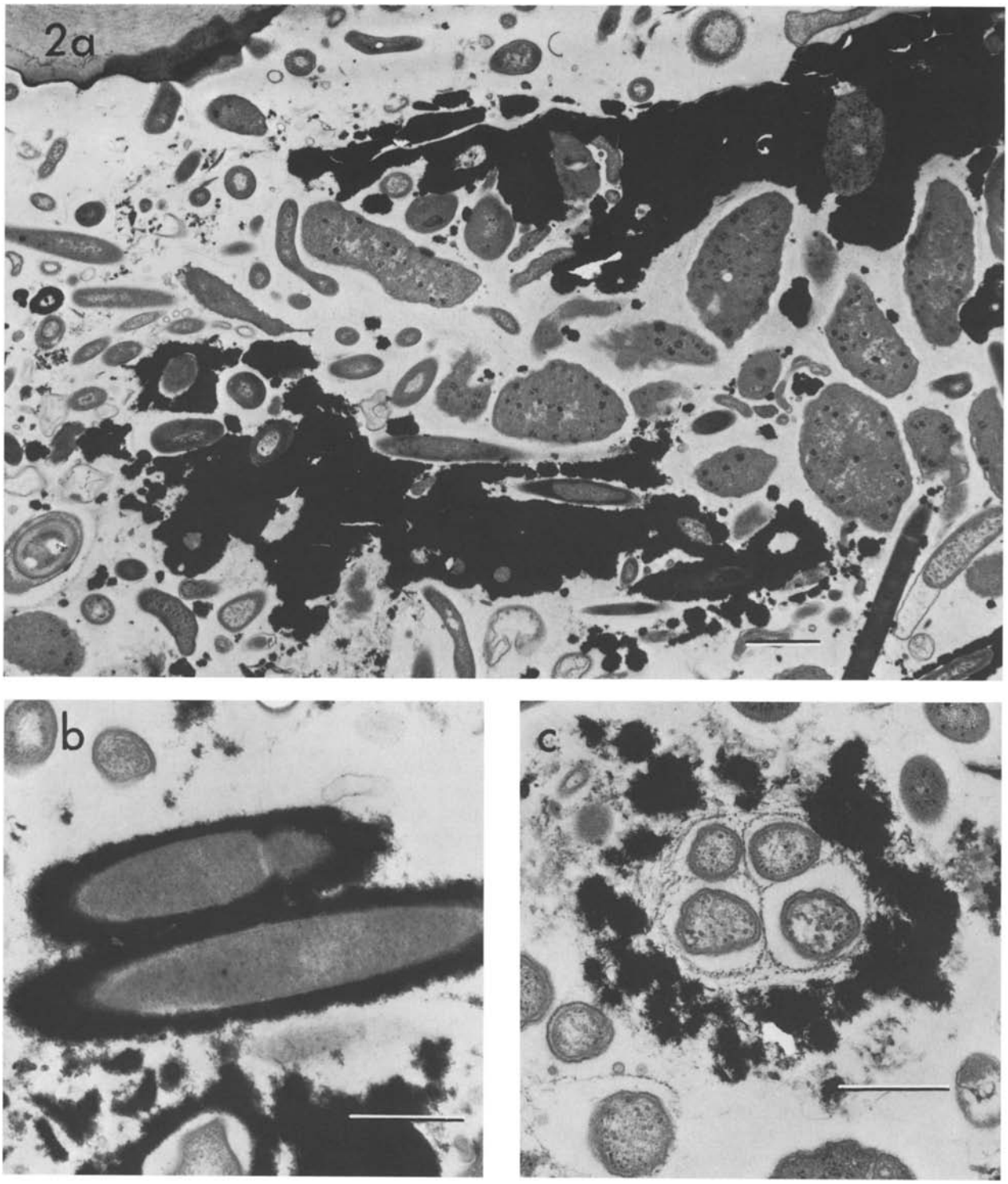

Fig. 2a—c. Micrographs from the black band region of the colon of *E. posticus*. **a** Large electron-dense masses near the hindgut wall. Bar = 1.0 μm. Glutaraldehyde-osmium fixation. **b** Electron dense material around single filamentous cells. Bar = 0.5 μm. Glutaraldehyde-osmium fixation. **c** Electron dense material around a microcolony of cocci. Bar = 0.5 μm. Glutaraldehydehyde-osmium-ruthenium red stain

nitrogen is recycled during periods of starvation. The main nitrogenous compound in the hindgut and feces of most cockroaches is ammonia (reviewed by Mullins 1981). The pH of the midgut of *E. posticus* is near neutrality, which is different from the "precellulolytic" high pH (up to 11) found in the midguts of higher termites (Bignell and Anderson 1980) and scarab beetle larvae (Bayon 1980). The high midgut pH of these insects apparently makes the cullulosic diet more easily digested by their hindgut microbiota. Unlike termites and scarab beetles, the fore- and mid-guts of the opportunistic, omniverous cockroach are very efficient at digesting and transporting most normal nutrients (reviewed by Bignell 1981). What reaches the microbiota in the hindgut are the remains: fiber, unpretreated cellulose, and excretory

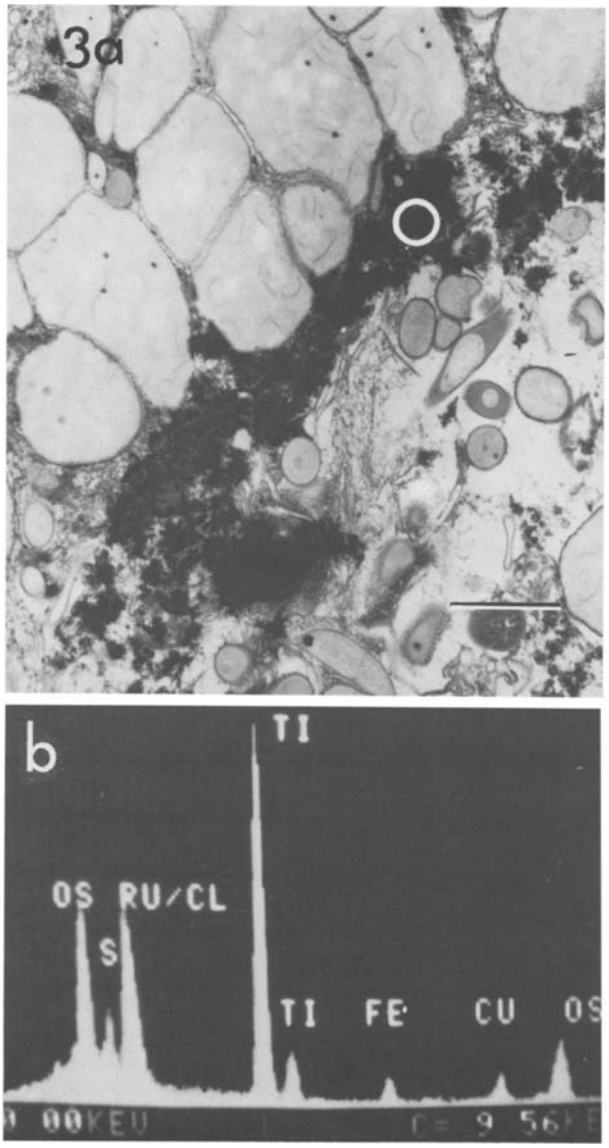

Fig. 3. a TEM image of an area in the black band region. Glutaraldehyde-osmium-ruthenium red fixation. Bar = 0.5 μm. The circled area is the source of the X-ray emission spectrum in **b**

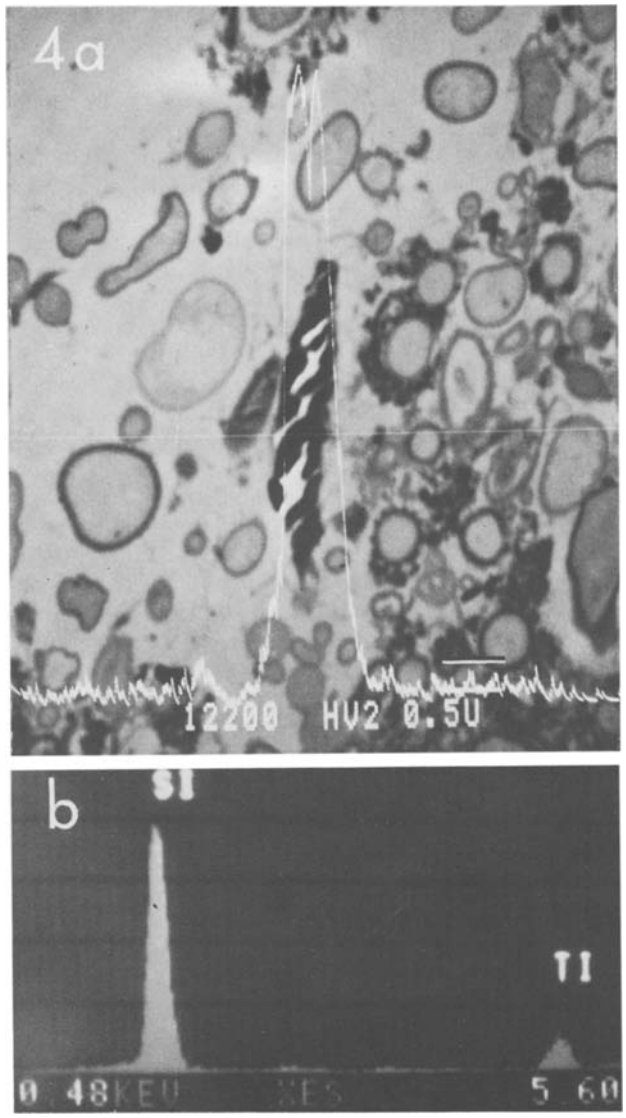

Fig. 4. a STEM image of an electron dense deposit in the black band region. Glutaraldehyde-osmium-ruthenium red fixation. Bar = 0.5 μm. The straight line marks the plane of the superimposed scan for material emitting X-rays at the characteristic energy level of silicon (K_α 1.740 keV). **b** X-ray emission spectrum of the deposit showing that silicon is the only element present, other than a small amount of titanium (K_α 4.51 keV) from the grid

products from the malphighian tubules of the insect. These remains, however, are obviously sufficient to support a large and active population of microbes, which is itself adaptable and able to survive periods of starvation and unbalanced growth conditions.

Elimination of the obligately anaerobic microbiota with metronidazole resulted in a dedifferentiation of a number of biochemical parameters including pH and the concentrations of a number of enzymes. The differences between the paunch and black band regions of the colon in metronidazole animals disappeared, and the differences between midgut and colon were decreased. In normal insects, the pH and enzyme activities of the various gut fractions were quite constant from individual to individual, while between metronidazole treated animals, the amounts of several enzymes varied widely, and the pH values showed larger standard deviations. In metronidazole insects there were generally higher levels of the enzymes involved in the fermentation of sugars than there were in normal animals. Bayon (1980) measured the enzymes of the API-zym series in gut fractions of the scarab beetle. She found a similar pattern to that in *E. posticus*, but several enzymes were completely missing in this insect, including valine and cysteine aminopeptides, phosphoamidase, chymotrypsin and trypsin-like proteases, α-glucosidase, β-glucosidase and α-mannosidase. She assayed epithelium and gut contents separately to differentiate the contributions of host tissue and microbes, but although she states that scarab beetles have an attached wall microbiota, she did not account for its contribution to the epithelial activity, and apparently did not attempt to remove it in her epithelial preparation.

The normal high levels of the typically procaryotic storage products, poly-β-hydroxybutyrate and polyphosphate found in the hindgut of *E. posticus* are also decreased

in metronidazole animals, to levels at or below those found in rumen fluid. There is little literature on storage products in anaerobic bacteria, but in general they are formed in response to an imbalance in nutrients in the environment (Dawes and Senior 1973). Cheng et al. (1973) demonstrated that large numbers of Gram-negative bacteria in the rumina of cows fed a high energy ration of fine particle size contained granules resembling microbial glycogen reserves compared to lower numbers in animals fed a coarser feed. The previously reported high proportion of cells in the process of sporulation in the hindgut of *E. posticus* (Cruden and Markovetz 1981) also is an indication of an unbalanced environment.

The numbers of bacteria culturable on nonselective medium confirms the earlier impression that the hindgut of *E. posticus* is highly anaerobic (Cruden and Markovetz 1979; Bracke et al. 1978). Counts on nonselective media of gut fractions from cranefly larvae (Klug and Kotarski 1980) are similar to those from *E. posticus* with the hindgut microbiota more anaerobic than that of the midgut. The hindguts of termites yield proportionately more facultatively anaerobic isolates, mostly streptococci, on nonselective media (Schultz and Breznak 1978), possibly because more oxygen per unit volume could diffuse into the much smaller termite gut than into the cockroach or cranefly larva making it a more suitable niche for organisms resistant to the toxic effects of O_2 and able to consume it. The facultatively anaerobic isolates from *E. posticus* are similar to those isolated from the cricket hindgut (Ulrich et al. 1981), and to some isolated from the termite (Schultz and Breznak 1978). They have been isolated from *E. posticus* on a variety of selective enrichments as well as on nonselective media, and appear to comprise an adaptable population of generalists. We feel, however, that the facultative anaerobes and strict anaerobes isolated on nonselective medium do not begin to give a complete representation of the functioning hindgut microbiota. The large amounts of methane produced, which correlates well with the large numbers of cells with the structure of *Methanospirillum*, the protozoan population, and the large numbers of cells with unique morphology reported earlier (Cruden and Markovetz 1981), are not represented in the isolates from nonspecific medium. Similarly, the organisms we have isolated from spent medium (Cruden and Markovetz 1980) or other dilute media or have carried for several years in mixed culture, often firmly attached to pieces of chitin, are not identifiable with isolates from non-specific medium.

Methane production by the cockroach microbiota (typically 10–25 μmol/h · insect) is very high compared to that reported for other insects. Scarab beetle larvae (which weigh about 20 g) produce from 308–380 nmol/h · insect, (Bayon 1980) and typical values for a termite, *Reticulitermes tibilias*, average up to 31 nmol/day · insect (Zimmerman et al. 1982). There must be a high level of metabolic activity occuring in the cockroach hindgut to produce the amounts of substrates, H_2 and CO_2, necessary to sustain this level of methanogenesis. Hydrogen has been detected in the midgut, but not the hindgut of *E. posticus* (T. E. Gorrell, unpublished experiments), and must be scavenged quickly by the large number of methanogens present, making it possible for otherwise energetically unfavorable reactions in the anaerobic degradation of difficult substrates to proceed.

The characteristic black band in the posterior part of the colon has been observed in several other cockroaches, including *Gromphadorhina*, and both wild and laboratory individuals of *Periplaneta* (this laboratory) as well as other smaller species (B. A. Stay, personal communication). In *Periplaneta*, the only other cockroach examined in detail, the dark color is correlated with a microbiota which in micrographs appears very similar to that in *E. posticus*, and which is eliminated with metronidazole treatment. It remains to be seen how general a phenomenon the black band is. Most published electron micrographs of the hindguts of cockroaches do not include the lumen-wall junction, because of the technical difficulty of obtaining good sections, especially in the regions where the chitinous cuticle is thick and convuluted, and where there are many spines. The dark color of the region is less obvious in the smaller cockroaches, and may not have been noticed in other laboratories.

This region is clearly a unique environment, chemically and microbiologically. The deposition of a variety of metal ions in the black band region appears to be dependent on the presence of its anaerobic microbiota. We have been unable to isolate sulfate reducing bacteria from *E. posticus*, but have isolated bacteria which produce sulfide from organic sulfhydryl compounds from high dilutions of contents of this region (Cruden et al. 1979). Forsberg (1979) reported similar high numbers of rumen bacteria which could produce sulfide from cysteine or methionine. The deposits are correlated with areas which also stain with ruthenium red, i. e., which contain acidic mucopolysaccharides which constitute the glycocalyces elaborated by microbes growing in natural environments (McCowan et al. 1978). Metal deposition has been shown to occur around the cells of *Pedomicrobium*-like bacteria which oxidize manganese and iron in aquatic environments (Ghiorse and Hirsch 1979). The deposition of manganese is dependent on the viability of the cells, while that of iron is apparently a passive process. Some of these deposits resemble the structured deposits in our micrographs. In addition, Beveridge et al. (1983) have recently demonstrated the binding of a number of metals around bacterial cells as sulfides, phosphates and organic condensates in the formation of artificial sediments in vitro. Silicon occurs in the black band region in a different type of deposit. Ruthenium-red staining material does not seem to be involved in these deposits. Bailey et al. (1982) have shown that silicic acid incubated with bacterial protein (alkaline phosphatase) will precipitate around the protein at rumen pH values.

Intracellular mineral concretions have been reported in the epithilial cells of the malphighian tubules of the cockroach *Periplaneta* (Wall et al. 1975), presumably as products of a detoxification mechanism, but they appear in electron micrographs as regular series of concentric rings, and do not at all resemble the much more electron dense and irregular deposits we report in the hindgut lumen. Ballan-Dufrançais (1972) has described similar concretions in the hindgut epithelium of the cockroach *Blatella*, and concluded that they consisted mostly of calcium and magnesium phosphates, with smaller amounts of chloride, potassium and iron in a glycoprotein matrix. Copper, manganese and zinc were not present, and she did not assay for sulfur, silicon or aluminium. We have seen nothing resembling these concretions in the hindgut epithelium of *E. posticus*, and Bignell reports none in the epithelium of *Periplaneta* (1980). The type of epithelium present in the hindgut, including that in the black band region is consistent with a transport

function, despite the presence of the chitinous lining (Bignell 1980), and we have shown that short chain fatty acids are transported from the colon into the hemolymph (Bracke and Markovetz 1980). We do not know whether the metal ions are transported through the gut wall at this point and deposited in the lumen by some microbiologically mediated reactions, or whether they are concentrated there from the food and excretory products from the malphighian tubules. The pH of the gut contents in the black band region is higher than in the rest of the gut, but whether this is a cause or an effect of the deposition of mineral ions in not known.

Acknowledgements. We would like to thank Bill Durbin for technical assistance, Tom Gorrell for the assays of sulfide and ferrous ions, Wes Turner of the University of Iowa Central Electron Microscope Facility for help with the X-ray microanalysis and M. J. Allison of the National Animal Disease Laboratory, Ames Iowa, for keeping us supplied with rumen fluid. This work was supported by Public Health Service grant AI-13990 from the National Institute of Allergy and Infectious Diseases.

References

Bailey CB, Cheng K-J, Costerton JW (1982) Electron micrographic study of precipitates formed by interaction of silicic acid and alkaline phosphatase: contribution to a study of silica urolithiasis in cattle. J Inorg Biochem 17:343–348

Ballan-Dufrançais C (1972) Ultrastructure de l'iléon de *Blatella germanica* (L.) (Dictyoptère). Localisation, genèse et composition des concrétions des minérales intracytoplasmiques. Z Zellforsch Mikrosk Anat 133:163–179

Bayon C (1980) Volatile fatty acids and methane production in relation to anaerobic carbohydrate fermentation in *Oryctes nasicornis* larvae (Coleoptera: Scarabaeidae). J Insect Physiol 26:819–828

Becker GE, Lozada EP, Peterson E (1982) Uric acid degrading bacteria in cockroaches. In: Abstracts of the Annual Meeting of the American Society for Microbiology I 56, p 103

Beveridge TJ, Meloche JD, Fyfe, WS, Murray RGE (1983) Diagenesis of metals chemically complexed to bacteria: Laboratory formation of metal phosphates, sulfides and organic condensates in artificial sediments. Appl Environ Microbiol 45:1094–1108

Bignell DE (1980) An ultrastructural study and stereological analysis of the colon wall in the cockroach *Periplaneta americana*. Tissue and Cell 12:153–164

Bignell DE (1981) Nutrition and digestion. In: Bell WJ, Adiyodi KG (eds) The American cockroach. Chapman and Hall, London, pp 57–86

Bignell DE, Anderson JM (1980) Determination of pH and oxygen status in the guts of lower and higher termites. J Insect Physiol 26:183–188

Bracke JW, Markovetz AJ (1980) Transport of bacterial endproducts from the colon of *Periplaneta americana*. J Insect Physiol 26:85–89

Bracke JW, Cruden DL, Markovetz AJ (1978) Effect of metronidazole on the intestinal microflora of the American cockroach, *Periplaneta americana L.* Antimicrob. Agents Chemother 13:115–120

Bracke JW, Cruden DL, Markovetz AJ (1979) Intestinal microbial flora of the American cockroach *Periplaneta americana L.* Appl Environ Microbiol 38:945–955

Breznak JA, Pankratz HS (1977) In situ morphology of the gut microbiota of wood-eating termites [*Reticulitermes flavipes* (Kollar) and *Coptotermes formosarus* Shiraki]. Appl Environ Microbiol 33:406–426

Bryant MP, Burkey LA (1953) Cultural methods and some characteristics of some of the more numerous groups of bacteria in the bovine rumen. J Dairy Sci 36:205–217

Bryant MP, Robinson IM (1961) An improved nonselective culture medium for ruminal bacteria and its use in determining diurnal variation in numbers of bacteria in the bovine rumen. J Dairy Sci 51:1950–1955

Cheng KJ, Hironaka R, Roberts DWA, Costerton JW (1973) Cytoplasmic glycogen in inclusions in cells of anaerobic gram negative rumen bacteria. Can J Microbiol 19:1501–1506

Cruden DL, Markovetz AJ (1979) Carboxymethylcellulose decomposition by intestinal bacteria of cockroaches. Appl Environ Microbiol 38:369–372

Cruden DL, Markovetz AJ (1980) A thick walled organism isolated from the cockroach gut by using a spent medium technique. Appl Environ Microbiol 39:261–264

Cruden DL, Markovetz AJ (1981) Relative numbers of selected bacterial forms in different regions of the cockroach hindgut. Arch Microbiol 129:129–134

Cruden DL, Gorrell TE, Markovetz AJ (1979) Novel microbial and chemical components of a specific black-band region in the cockroach hindgut. J Bacteriol 140:687–698

Dawes EA, Senior PJ (1973) The role and regulation of energy reserve polymers in microorganisms. Adv Microbial Physiol 19:135–278

Forsberg CW (1979) Sulfide production by some rumen bacteria. Ann Réch Vet 10:347–349

Ghiorse WC, Hirsch P (1979) An ultrastructural study of iron and manganese deposition associated with extracellular polymers of *Pedomicrobium*-like budding bacteria. Arch Microbiol 123:213–226

Guthrie DM, Tindall AR (1968) The biology of the cockroach. Edward Arnold Ltd, London

Holdeman LV, Cato EP, Moore WEC (eds) (1977) Anaerobe laboratory manual. Virginia Polytechnic Institute, Blacksburg, VA

Klug MJ, Kotarski S (1980) Bacteria associated with the gut tract of larval stages of the aquatic cranefly *Tipula abdominalis* (Diptera; Tipulidae). Appl Environ Microbiol 40:408–416

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275

Marczenko Z (1976) Iron; sulfur. In: Chalmers RA (ed) Spectrophotometric determination of elements (English edn). John Wiley & Sons, Inc., New York, pp 305–321, 504–515

McCowan RP, Cheng K-J, Bailey CBM, Costerton JW (1978) Adhesion of bacteria to the epithelial cell surfaces within the reticulorumen of cattle. Appl Environ Microbiol 35:149–155

Mullins DE (1981) Osmoregulation and excretion. In: Bell WJ, Adiyodi KG (eds) The American Cockroach. Chapman & Hall, London, pp 117–149

Poindexter JS, Eley LF (1983) Combined procedure for assays of poly-β-hydroxybutyric acid and inorganic polyphosphate. J Microbiol Meth 1:1–17

Schultz JE, Breznak JA (1978) Heterotrophic bacteria present in hindguts of wood-eating termites [*Reticulitermes flavipes* (Kollar)]. Appl Environ Microbiol 35:930–936

Ulrich RG, Buthala DA, Klug MJ (1981) Microbiota associated with the gastro-intestinal tract of the common house cricket *Acheta domestica*. Appl Environ Microbiol 41:246–254

Wall BJ, Oschman JL, Schmidt BA (1975) Morphology and function of malphighian tubules and associated structures in the cockroach *Periplaneta americana*. J Morph 146:265–306

Zimmerman PR, Greenberg JP, Wandiga SO, Crutzen PJ (1982) Termites: a potentially large source of atmospheric methane, carbon dioxide and molecular hydrogen. Science 218:563–565

Received July 1, 1983/Accepted January 16, 1984