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pH Profiles of the Extremely Alkaline Hindguts of Soil-Feeding Termites (Isoptera: Termitidae) Determined with Microelectrodes

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In three of the four subfamilies of higher termites, the anterior hindgut is characterized by an unusually high pH. This phenomenon is especially prominent among the soil (humus) feeders, which represent more than half of all known termite species, but whose digestive physiology is still largely obscure. In this study, we used microelectrodes to determine axial pH profiles in intact guts of the soil-feeding termites *Thoracotermes macrothorax*, *Crenetermes albotarsalis*, *Noditermes indoensis* and two *Cubitermes* species (all Termitidae: Termitinae). In all species, the pH of the gut contents increased sharply from circumneutral in the midgut to highly alkaline between the midgut–hindgut junction and the first proctodeal dilation (P1). This location coincided exactly with the extent of the mixed segment, a morphologically unique gut region found exclusively among higher termites. The most alkaline pH values occurred in the P1 (pH 11–12.5) and were equal to the highest values ever encountered in biological systems. The second proctodeal dilation (P3) was also alkaline (pH >10), and only in the posterior part of the following segment (P4b) did the gut contents regain a pH close to neutrality. The rectum (P5) was slightly acidic, as was the foregut. Sharp drops of 1–3 pH units were observed between the major gut regions, most notably between P1 and P3, i.e. across the short enteric valve (P2), and also along the P4a. Individual profiles within a species were highly reproducible, as were species-specific differences. The high-resolution profiles presented here show that guts of soil-feeding termites are even more alkaline than reported previously, and they provide sorely needed basic information on microenvironments existing within guts of an extremely important group of terrestrial humivores. Copyright © 1996 Elsevier Science Ltd

Isoptera Humus-feeding Intestinal pH Mixed segment Microsensors

INTRODUCTION

Because termites have most probably evolved from wood-feeding ancestors, the (phylogenetically) lower termites feed primarily on wood—either sound or in various stages of decomposition. Among the higher termites (Termitidae), a diversification in feeding habits has occurred, increasing the spectrum of lignocellulosic food materials to include, for example, grass, dung or leaf litter. However, one of the most successful specializations appears to have been humivory, which evolved independently in several subfamilies and opened up an almost unlimited nutritional resource that is now exploited by more than half of all termite genera [for reviews, see Wood and Johnson (1986), Noirot (1992) and Bignell (1994)].

The digestion of lignocellulose in wood-feeding termites is reasonably well understood. Among lower termites, digestion of lignified plant cell walls is accomplished by a symbiotic association with cellulolytic intestinal protozoa and a variety of prokaryotic microorganisms. The symbiotic fermentation of the carbohydrate fraction of the food yields short-chain fatty acids (SCFAs), which are resorbed and oxidized by the host. In higher termites, other means of cellulolytic activity were acquired (i.e. fungal or host cellulases), resulting in a largely bacterial gut microflora, which is probably still

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responsible for carbohydrate fermentation to SCFAs. Our current knowledge of lignocellulose digestion by wood-feeding termites and the role of microorganisms in this process has been recently reviewed by Breznak and Brune (1994). In contrast, the physiology of digestion in soil (humus)-feeding termites is still largely obscure, despite their immense ecological importance in tropical ecosystems (Wood and Johnson, 1986; Wood, 1988). The diet of soil-feeders appears to consist exclusively of non-cellular organic matter mixed with mineral material (Wood and Johnson, 1986), but virtually nothing is known about the specific nutritive component(s) utilized, the biochemical reactions involved in their metabolism, or the contribution of the gut microflora to digestion.

All higher termites, except the fungus-cultivating Macrotermitinae, share a tendency towards increased length and anatomical diversification of the proctodeal region of the intestine [reviewed by Noirot (1992) and Bignell (1994)]. Besides an increasing number of gut compartments, they usually possess a more or less pronounced asymmetric extension of midgut epithelium along the anterior hindgut, giving rise to a so-called 'mixed segment'. Kovoor (1967) recognized that in wood-feeding Termitinae, this morphological diversification coincided with an alkaline condition in the anterior hindgut, an observation later extended to soil-feeding species (Bignell and Anderson, 1980).

There are several excellent studies on the gut anatomy of soil feeders [reviewed by Noirot and Noirot-Timothée (1969), Noirot (1992) and Bignell (1994)], especially on the mixed segment (Bignell *et al.*, 1983), and there is also a comprehensive body of data on the pH of major gut regions (Bignell and Anderson, 1980; Bignell, 1994; Bignell and Eggleton, 1995). However, most of these pH measurements have been performed on pooled, disrupted samples of the major gut regions, a procedure necessitated by the use of pH-sensitive paper as an indicator. This approach facilitated surveys of a large number of species and led to important insights into the distribution of elevated pH among the Termitidae, but it also had several shortcomings: (i) pooling and mixing of the contents of relatively large gut segments destroys preexisting pH gradients across the length of the particular segment and yields little or no spatial resolution; and (ii) the buffering effect of the disrupted gut tissue may influence the pH of the sample, thereby introducing artifacts, especially when luminal volumes are small.

Recently, new insights into the microecology of termite hindguts were provided by the use of microelectrodes to measure axial and radial oxygen and pH profiles in guts of several wood-feeding lower and higher termites (Brune *et al.*, 1995a). We have now extended the application of this technique to soil-feeding termites. Herein we present high resolution profiles of pH in guts of several species of soil-feeding termites, as determined with glass microelectrodes inserted into the intact gut. Results reveal the remarkable range of pH in various regions of the gut and resolve the precise location and steepness of pH transition zones.

MATERIALS AND METHODS

Termites

Cubitermes speciosus Sjöstedt, *Thoracotermes macrothorax* (Sjöstedt), *Crenetermes albotarsalis* (Sjöstedt) and *Noditermes indoensis* Sjöstedt were collected in the Mayombe rain forest, Republic of Congo; another unidentified species of *Cubitermes* sp. was from a location near Sarh, Republic of Chad. These species typify the variety of soil-feeding Termitinae from the Ethiopian faunal region. Small nests or nest fragments were brought to the authors' laboratory in containers amended with a small amount of soil from the original collection site, and measurements were performed within 4–6 weeks of collection. Worker caste termites were used for all measurements.

pH measurements

Glass pH microelectrodes were constructed using the design described by Revsbech and Jørgensen (1986) and were equipped with an external casing filled with 1 M KCl to minimize electrical noise (Jensen *et al.*, 1993). No further shielding was necessary. The microelectrodes had tip diameters of 10–30 μm and 90% response times of <10 s, except at pH >10, where response times increased to 20–30 s at pH 12. Electrode potentials were measured against a saturated calomel electrode (Ref 401, Radiometer, Copenhagen, Denmark), which was connected to the measuring chamber via a KCl-filled agar bridge cast in PTFE tubing. Potential measurements were recorded by means of a high-impedance (>10^{14} Ω) electrometer amplifier (Keithley 617), coupled to a chart recorder. Figure 1 illustrates the experimental set up.

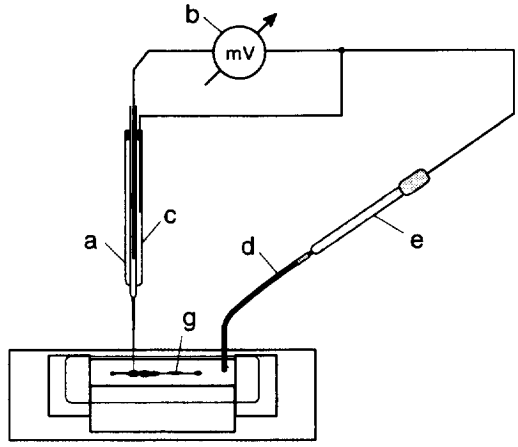

FIGURE 1. Schematic view of the experimental set up used for pH measurements in termite guts: pH microelectrode (a) connected to an electrometer amplifier; (b) and shielded by an external, grounded casing; (c) a salt bridge; (d) connecting the reference electrode; (e) to the microchamber; (f) which contained the gut; (g) embedded in agarose-solidified Ringer's solution. Not drawn to scale. For details, see Materials and Methods.

Termites were carefully dissected, and the guts were immediately embedded in a small glass chamber using an insect Ringer's solution solidified with 0.5% agarose (Brune *et al.*, 1995a). Microelectrodes were positioned with a manual micromanipulator (MM33, Märzhäuser, Wetzlar, Germany); and progress of the tip was monitored with a horizontally mounted stereomicroscope. All measurements were carried out at ambient temperatures (22±1°C).

pH calibration

Microelectrodes were calibrated with commercial pH standard solutions (Riedel-de Haën, Seelze, Germany) at intervals of 1 pH unit before, during, and after each set of measurements, observing the necessary temperature corrections. Calibration standards for pH 10–12 were freshly prepared from titrated stock solutions in CO_2-free bidistilled water, which were kept in glass-stoppered bottles to prevent absorption of atmospheric CO_2. The calibration standards were $NaHCO_3$ (pH 10–11), Na_2HPO_4 (pH 11–12) and KCl (pH 12–12.5), at a final concentration of 50 mM, adjusted with standardized 0.1 N NaOH to the respective pH values (Robinson and Stokes, 1968) at intervals of 0.5 pH units.

RESULTS

pH microelectrode calibration

The greatest pitfall in pH measurements at low hydrogen ion concentrations is the instability of highly alkaline buffers due to absorption of atmospheric CO_2. We found commercial standard solutions with nominal pHs of 11 and 12 to be inaccurate by as much as 0.5 pH unit even though the shelflife recommended by the manufacturer had not yet expired, a problem most likely caused by the CO_2 permeability of their polyethylene containers. For this reason, we used freshly prepared alkaline standards, which gave a linear response throughout the pH range observed in this study with a conventional Ross-type combination pH electrode (Model 8103, Orion, Boston, MA, U.S.A.). Our microelectrodes showed similar linearity between pH 4 and 10, with a slope of 55–58 mV per decade, depending on the specific electrode, but at higher pH values their responses (as well as that of other, conventional glass electrodes) became increasingly non-linear (Fig. 2). This is, however, a well-known phenomenon with glass electrodes at extremely low proton activities. The so-called 'alkaline error' increases with pH and alkali metal ion concentration, is dependent on the composition of the pH-sensitive glass, and varies with temperature (Laitinen, 1951; Westcott, 1978). Non-linear calibration curves (polynomial fits) were used to compute the pH from microelectrode potentials, which were determined in small intervals of 1 pH unit across the relevant pH range.

The calibration procedure does not account for a possible underestimation of high pH values due to the presence of small alkali metal ions, but since this error decreases with the size of the potentially interfering ions ($Li^+ > Na^+ > K^+$) (Laitinen, 1951), and since we did not notice any change in response of our microelectrodes in alkaline standards when increasing the KCl concentration to 200 mM, a larger error caused by the increased K^+ concentrations in the alkaline gut regions of soil-feeding Termitinae (Bignell *et al.*, 1983) appears unlikely.

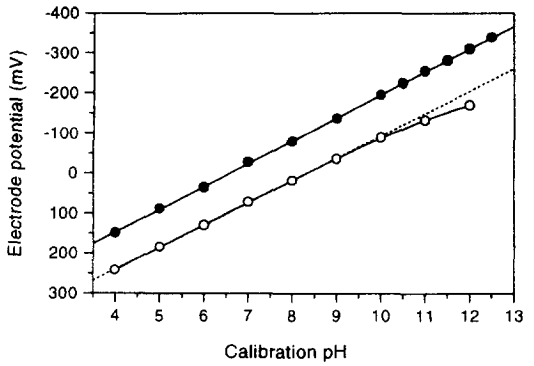

FIGURE 2. Typical calibration curves for a glass pH microelectrode (○) and for a conventional Ross-type glass pH electrode (●) over the whole pH range used in this study. The response of the microelectrodes became increasingly non-linear at alkaline pH (pH >10). The offset between both curves is due to different reference cell potentials.

Another problem with pH measurements at extremely alkaline values was that etching of the glass tip led to a slight loss in slope and increased response times within several minutes of exposure. To avoid calibration errors, we routinely rejuvenated the pH-sensitive tip by immersion in acidic buffer (pH 4) for several minutes. In addition, the slope and offset of the calibration curve were carefully monitored during measurements in alkaline gut regions.

Gut pH profiles

The typical morphology of the intestinal tract of soil-feeding Termitinae is depicted in Fig. 3. With all species, the gut was characterized by the same easily-identifiable cardinal points, and the various gut regions could be clearly distinguished. The experimental set up (Fig. 1) enabled accurate positioning of the small electrode tip in any gut region, except in the constrictions between the segments which had extremely limited luminal volumes (e.g. the P2 and the P4a–P4b junction). Inasmuch as the length of the tip (100–150 μm) was significantly larger than its diameter (10–30 μm), the spatial resolution was limited by the dimensions of the pH-sensitive tip. Injury of the gut wall caused by penetration with the electrode tip and leakage of gut fluid upon tip withdrawal were only minor. Immediate embedding of dissected guts in Ringer's solution solidified with agarose prevented their otherwise rapid desiccation and sustained their peristalsis for more than 1 h, which was much longer than the actual time needed to measure a profile. To assess the possibility of artifacts caused by this procedure, the pH in major gut regions was followed and was found to be

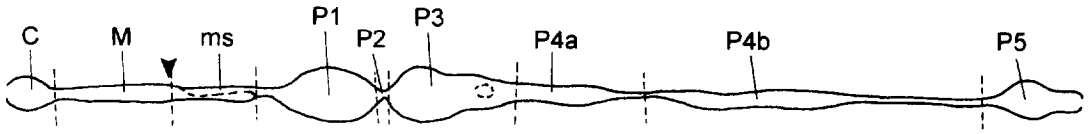

FIGURE 3. Gut morphology of a *Thoracotermes macrothorax* worker, but also representative of the other soil-feeding Termitinae investigated in this study. The gut, which is normally folded in the abdomen, was drawn in its unraveled state to illustrate the various segments: crop (C), mesenteron (M), mixed segment (ms), and the proctodeal segments (P1–5). The insertion point of the Malpighian tubules is indicated by the arrowhead; the cecum (dotted line) is vestigial in *T. macrothorax*. Segment nomenclature follows Holmgren (1909) and is consistent with that of Noirot and Noirot-Timothée (1969) and other authors. Average gut length was 22 mm; not drawn exactly to scale.

stable and independent of the incubation time during this period.

Figure 4 shows typical axial pH profiles for guts of the five different species of soil-feeding termites studied. Individual differences in segment length were minimal and were integrated into the graphical presentation by normalization relative to the cardinal points along the gut axis, indicated in Fig. 3. This enabled us to combine several typical datasets in each graph to illustrate the individual variance of the profiles.

In all guts, the pH of the crop (C) was slightly acidic (pH 5–6 or lower), especially in *Crenetermes albotarsalis*, and in general about 1–2 units below midgut pH. The average pH along the length of the midgut was cir-

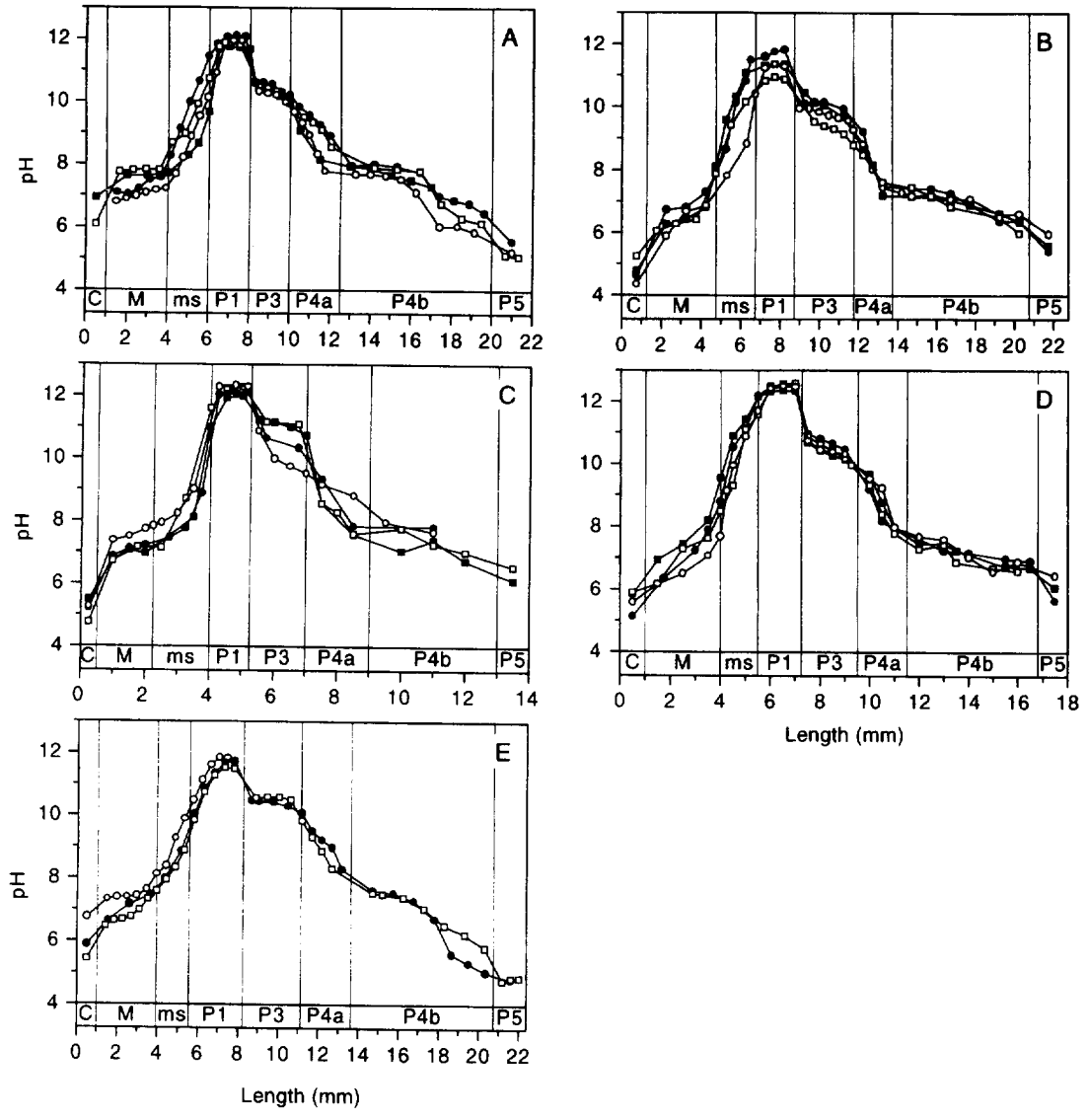

FIGURE 4. Axial pH profiles of the gut contents of several soil-feeding termites. Each graph combines several typical datasets of different individuals. (A) *Thoracotermes macrothorax*, (B) *Crenetermes albotarsalis*, (C) *Noditermes indoensis*, (D) *Cubitermes* sp., (E) *Cubitermes speciosus*. The abbreviations for the gut segments are explained in Fig. 3.

cumneutral (pH 7–7.5), except for *C. albotarsalis* and *Cubitermes* sp., which had slightly acidic midguts; and midgut pH increased slightly from anterior to posterior with all species.

At the midgut–hindgut junction (i.e. in the mixed segment), the pH began to increase sharply over several units, reaching maximum values in the first proctodeal segment (P1), at times already within its thin, tubular anterior portion. The pH values in the P1 dilation were uniform throughout the dilation (pH 11–12.5), with the most alkaline values recorded in *Noditermes indoensis* and the *Cubitermes* sp.. No radial pH heterogeneity was observed in this or any other gut region.

Across the short enteric valve (P2), indicated in Fig. 4 by the vertical lines separating P1 and P3, the pH dropped sharply about 1–2 units, and then decreased only slightly from anterior to posterior P3.

The transition from the P3 region to the anterior portion of the fourth proctodeal segment (P4a) was quite obvious *in situ*, but was no longer marked by a distinct constriction of the enteric tube once the gut was extended in the microchamber. However, changes in the external texture of the gut wall and of the color of the gut contents clearly marked its location. The P4a was the site of yet another, and even larger, pH drop of 2.5–3 units, which was completed at the constriction separating P4a from the posterior (P4b) portion of this segment. In the P4b dilation, the pH was again relatively constant with only a slight decrease from anterior to posterior. The thin, tubular connection to the rectum (P5), which was acidic (pH 4.8–6) in all tested species, was the site of a last pH drop.

Average values of all data obtained for the major gut regions are summarized in Table 1. The results agree with the observations already made with the individual profiles (Fig. 4). No average values are given for regions of rapid pH transition (mixed segment and P4a). In *N. indoensis*, a species with a well-developed cecum, cecal pH was not significantly different from that of the P3. In all other species, the cecum was vestigial or absent. Fewer determinations were made on the crop and rectum (P5) because the crop was occasionally lost during gut removal and because termites sometimes voided their rectal contents during dissection.

DISCUSSION

It is well known that the midgut contents of certain insect larvae, particularly among the Lepidoptera, Coleoptera and Diptera, can be markedly alkaline [e.g. Waterhouse (1952), Grayson (1958), Dadd (1975), Berenbaum (1980), and Martin *et al.* (1980)]. In higher termites, however, it is always the hindgut that exhibits extreme pH elevations. This feature seems related to phylogeny within the Isoptera since no pronounced alkalinity occurs in (phylogenetically) lower termites. Among the higher termites (Termitidae), unusually alkaline gut regions are absent in the Macrotermitinae, whose simple gut morphology resembles that of the lower termites. However, in all other subfamilies of Termitidae (i.e. Apicotermitinae, Nasutitermitinae and Termitinae), one or more gut regions are pronouncedly alkaline (Bignell and Eggleton, 1995), a feature that generally coincides with (i) a tendency towards increased gut length and number of dilations and (ii) the presence of a mixed segment (Noirot, 1992; Bignell, 1994).

In a comprehensive survey of intestinal pH in 52 species of Termitidae, including genera from all four subfamilies, Bignell and Eggleton (1995) showed that in the Apicotermitinae and Nasutitermitinae, extreme alkalinity is restricted to the P1, whereas in the Termitinae markedly elevated pH values (pH >9) occur also in the P3. From their data, they conclude that regions with elevated pH might have arisen independently several times during evolution of higher termites, and that this phenomenon is principally independent of the nature of a wood- or soil-feeding diet. Their simple method of pH determination, which consisted of blotting pooled, disrupted gut segments on a microscopic slide with high-resolution pH paper, facilitated the screening of a large number of species, but spatial resolution and accuracy was limited. For example, small gut regions were pooled with adjacent

TABLE 1. The luminal pH [mean ± SD (*N*)] in the major gut regions of several soil-feeding termites*

	Crop	Mesenteron	P1	P3	P4b	P5
Noditermes indoensis†	5.17±0.30 (4)	7.20±0.20 (4)	12.28±0.21 (9)	10.71±0.53 (4)	7.52±0.26 (4)	5.99±0.47 (3)
Crenetermes albotarsalis	4.66±0.36 (5)	6.43±0.34 (5)	11.15±0.45 (10)	9.82±0.33 (5)	7.19±0.14 (5)	5.63±0.29 (3)
Thoracotermes macrothorax	6.44±0.44 (3)	7.48±0.42 (5)	11.91±0.11 (10)	10.32±0.15 (5)	7.75±0.13 (5)	5.29±0.25 (3)
Cubitermes speciosus	6.04±0.55 (4)	7.11±0.26 (4)	11.85±0.31 (7)	10.42±0.06 (3)	7.49±0.03 (3)	4.83±0.04 (3)
Cubitermes sp.	5.56±0.32 (5)	7.07±0.37 (5)	12.25±0.31 (9)	10.39±0.17 (5)	7.36±0.20 (5)	6.05±0.40 (3)

*All values were measured at the center of the gut, midway along the length of the particular segment, except where a segment possessed a prominent dilation, whereupon the values are for the center of the dilation.

†*N. indoensis* has a large cecum (an appendix of the P3) whose pH was 10.83±0.62 (*N*=4); in all other species, the cecum was vestigial or absent.

compartments (e.g. the mixed segment with the midgut), so that many of the reported values were, in effect, an average pH of several subsections titrated against each other—with acids and bases derived both from the gut contents and from disrupted epithelial tissues. Hence, that method is not only unable to resolve the exact location of any pH changes, it will also always tend to underestimate pH maxima or overestimate minima. By using microelectrodes, however, we were able to determine the pH in various micro-domains within intact guts (penetrating the gut wall only with the minute electrode tip) while keeping tissue damage and disturbance of local gradients to an absolute minimum. A recent study with wood-feeding higher termites (Brune *et al.*, 1995a) illustrated the superior resolving power achievable with microelectrodes: the luminal pH in the extremely long and thin P1 of *Nasutitermes nigriceps*, when measured with microelectrodes, was much higher (pH >10) than the values obtained for three other *Nasutitermes* species using pH paper with disrupted P1 segments (pH 8.5) (Bignell and Eggleton, 1995). Large discrepancies in pH values of gut contents determined by the microelectrode method vs the blotting method are noteworthy in the data of the present study for P1 and P3 of *Thoracotermes macrothorax* and the *Cubitermes* species (Table 1), which exceed previously published values by 1–2 pH units and now place the pH of the P1 of soil-feeding termites among the highest values recorded for the midguts of certain dipteran and coleopteran larvae (pH 12–12.5) (Bayon, 1980; Martin *et al.*, 1980; Dow, 1984). To our knowledge, these represent the most extreme alkalinities ever measured within biological systems.

The high-resolution pH profiles obtained in this study finally provide unequivocal proof that in soil-feeding Termitinae, the mixed segment is the location of the steep increase of gut pH, which begins at the midgut–hindgut junction and reaches its maximum at the first proctodeal dilation (P1), often in the anterior tubular portion. A previous microelectrode study on wood-feeding termites has shown that the pH increase in the anterior hindguts of *N. nigriceps* (Nasutitermitinae) and *Microcerotermes parvus* (Termitinae) also occurs along the mixed segment (Brune *et al.*, 1995a). It should be interesting to investigate the Apicotermitinae, which are almost exclusively soil-feeding, but where pH elevation seems to be less pronounced in certain species (Bignell and Eggleton, 1995), and where a mixed segment is distinctly developed only in little more than half the genera (Noirot, 1992).

Although we now identified the mixed segment as the region of pH increase in Termitinae, the physiological basis for this phenomenon is still obscure. The same is true for the steep pH drops that occur across the enteric valve (P2), along the P4a, and between the P4b dilation and the rectal ampulla (P5). An interesting clue is provided by Bignell *et al.* (1983), who reported that P1 and P3 region of two soil-feeding Termitinae, *Cubitermes severus* and *Procubitermes aburiensis*, contain large amounts of K^+. Based on their detailed anatomical studies, they proposed that the mixed segment in soil-feeders has functionally replaced the generally reduced Malpighian tubules and serves to secrete copious amounts of a K^+-rich fluid, which irrigates the P1 and P3 and is finally reabsorbed in the P4 region. No further studies exist to support this hypothesis, but for caterpillars there is strong evidence that midgut pH elevation is linked to potassium transport, and a detailed mechanism, driven by a K^+-ATPase in the goblet cells and resulting in a net accumulation of K_2CO_3, has been suggested (Dow, 1984). Similar ion transport events might effect the pH increase along the mixed segment, and since a reabsorption of the K^+-rich fluid would be accompanied by a decrease in luminal pH, this model could also explain the pH drop along the P4a region. Unfortunately, the K^+ contents of the alkaline gut regions of *C. severus* and *P. aburiensis* were determined only for the major compartments, which does not allow testing the correlation with the pH profile; it might be promising to apply K^+-sensitive microsensors to determine the changes in K^+ activity along these regions.

A production of SCFAs by the gut microflora might also contribute to an acidification of the hindgut contents. In wood-feeding termites, large amounts of acetate and other SCFAs are formed in the fermentative degradation of carbohydrates by the microbial hindgut population (Odelson and Breznak, 1983; Breznak and Brune, 1994). However, despite the obvious presence of a large and varied population of bacteria in the various gut regions also of soil-feeding termites [reviewed by Breznak and Brune (1994)], little is known about their physiological activities.

In lepidopteran larvae feeding on tannin-rich leaf material, midgut alkalinity is believed to be an evolutionary adaptation to the potentially detrimental effects of this class of phenolic compounds (Berenbaum, 1980), such as preventing precipitation of digestive enzymes or dietary proteins (Martin *et al.*, 1987; Felton and Duffey, 1991). A similar explanation has been suggested for the alkaline pH in the midguts of detritivorous dipteran larvae (Sharma *et al.*, 1984), whose diet is also rich in phenolic matter. Whereas the major dietary constituents of these animals are living microbial cells or plant cells with little or no lignification of the cell walls, the diet of termites is quite different. It consists of dead, strongly lignified plant cell walls, or, in the case of the humivorous soil-feeders, probably even more recalcitrant, non-cellular organic matter. In such a situation, an alkaline pH would effectively solubilize lignocellulosic and humic residues, and promote autoxidation of polyphenolic constituents and initiate the chemical cleavage of intermonomeric bonds (Stevenson, 1982; Fengel and Wegener, 1984). It is also very important for soil-feeders that most of the soil organic matter is bound in clay mineral aggregates, and an alkaline incubation regimen would efficiently desorb humic compounds from the soil matrix (Stevenson, 1982). An oxidative degradation of aromatic

compounds in wood-feeding termites has been demonstrated recently (Brune *et al.*, 1995b), and since the high rates of oxygen consumption observed with wood-feeder hindguts (Brune *et al.*, 1995a) also occur in the soil-feeding Termitinae (Brune, unpubl. data), aromatic residues must be considered a likely component of their diet.

A further function of the alkaline gut regions in higher termites might be in the digestion of microorganisms, which are normally ingested with the food (soil or decaying wood), analogous to the case of detritivorous dipteran or wood-feeding coleopteran larvae, whose alkaline midguts are highly proteolytic (Rössler, 1961; Dadd, 1975; Sharma *et al.*, 1984). Obviously, additional physiological, biochemical and microbiological studies are needed to clarify the puzzling issues in soil-feeding termites outlined above and to develop a working model of digestion in these remarkable terrestrial insects.

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