BIOCHEMICAL ASPECTS OF SYMBIOSIS IN CARBON AND NITROGEN METABOLISM IN HIGHER TERMITES

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Fullness in life
contains a balance.
It is both foolish and wise,
aware and unknown,
happy and sad,
hard and soft,
of rock and water
of fields and oceans
of fish and man.

W. E. M. Lands 1986 *Fish and Human Health*
Academic Press, Orlando.

"Termitaria are not the inert tombstones
they appear to be.
They are stationary cows.
They are a vacuum cleaner ...
They are the pithead of a miniature mine ..."

R. Braithwaite 1990 *More than a home for white ants*
Australian Natural History **23**

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Unless otherwise stated, the results
described in this thesis
are from original work planned and carried out
by the author and have not been
presented for another degree.

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PUBLICATIONS

1. Papers

The following papers have been published from original work presented in this thesis and include collaboration with international colleagues.

1 Hogan, M., Veivers, P. C., Slaytor, M. and Czolij, R. (1988) The site of cellulose breakdown in higher termites (*Nasutitermes walkeri* and *Nasutitermes exitiosus*). *J. Insect. Physiol.* **34**; 891-899

2. Veivers, P. C., Mühlemann, R., Slaytor, M., Leuthold, R. H. and Bignell, D. E. (1991) Digestion, diet and polyethism in two fungus-growing termites: *Macrotermes subhyalinus* Rambur and *M. michaelseni* Sjøstedt. *J. Insect Physiol.* **37**; 675-682

3. Williams, C. M., Veivers, P. C., Slaytor, M. and Cleland, S. V. (1994) Atmospheric carbon dioxide and acetogenesis in the termite *Nasutitermes walkeri* Hill. *Comp. Biochem. Physiol.* **107A**; 113-118.

4. Bignell, D. E., Slaytor, M., Veivers, P. C., Mühlemann, R. and Leuthold, R. H. (1994) Functions of symbiotic fungus gardens in higher termites of the genus *Macrotermes*: evidence against the acquired enzyme hypothesis. *Acta Microbiologica et Immunologica Hungarica* **41**; 391-401

5. Anklin-Mühlemann, R., Bignell, D. E., Veivers, P. C., Leuthold, R. H. and Slaytor, M. (1995) Morphological, microbiological and biochemical studies of the gut flora in the fungus-growing termite *Macrotermes subhyalinus*. *J. Insect Physiol.* **41**; 929-940.

2. Abstracts

1. Veivers, P. C. and Slaytor, M. (1992) Termites: Role of symbionts in carbohydrate metabolism. *Abs. 19th Int. Cong. Entomol.* Abstract p. 609.

2. Veivers, P. C. and Slaytor, M. (1994) Similarities and differences between endo-β-1,4-glucanase activities from *Macrotermes* species. *Abs. 12th Congress I. U. S. S. I.* p. 541.

3. Slaytor, M. and Veivers, P. C. (1994) Roles of acetate and oxygen in metabolism in the paunch of the higher termite *Nasutitermes walkeri. Abs. 12th Congress I. U. S. S. I.* p. 355.

ABBREVIATIONS AND CONVENTIONS

Spelling is as in the Macquarie Dictionary and Thesaurus (1991, Herron publications, West End, Qld).

Chemical and enzyme nomenclature and abbreviations are used as in A. L. Lehninger, D. L. Nelson and M. M. Cox (1993) *Principles of Biochemistry* Worth Publishers, New York and as in E. C. Webb (1992) *Enzyme Nomenclature,* Academic Press, San Diego.

AABS: *p*-(*p*-aminophenylazo)-benzene sulphonic acid
ABTS: di-ammonium 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate)
Acetyl CoA: acetyl coenzyme A
ADP: adenosine 5'-diphosphate
ANOVA:analysis of variance
ATP: adenosine 5'-triphosphate
BCA: bicinchoninic acid
BSA: bovine serum albumin
CMC: carboxymethylcellulose
CoA: coenzyme A
cDNA: complementary deoxyribonucleic acid
DTNB: 5,5'-dithiobis[2-nitrobenzoic acid]
DTT: dithiothreitol
dpm: disintegrations per minute
DS: degree of substitution
dwt: dry weight
EDTA: ethylenediaminetetraacetic acid, disodium salt
FAD: flavin adenine dinucleotide
FPLC: fast protein liquid chromatography
GSH: glutathione
h: hour
HIC: hydrophobic interaction chromatography
HPLC: high performance liquid chromatography
IDP: inosine 5'-diphosphate
min: minutes
M_r: relative molecular mass
NAD^+: β-nicotinamide adenine dinucleotide

ABSTRACT

A comprehensive study of carbon metabolism has been carried out on representatives of two genera of higher termites, *Nasutitermes walkeri* and the fungus gardeners, *Macrotermes* spp. Substantial activity of cellulase and its components endo-β-1,4-glucanase and β-glucosidase, was found in workers of *N. walkeri*, *Macrotermes michaelseni* *M. subhyalinus*, *M. bellicosus* and *M. mulleri*. Less activity was found in the soldiers and none in the larvae of the *Macrotermes* spp. In fungal material associated with *Macrotermes* spp. only the fungal nodules had significant enzyme activity. In *N. walkeri* and *M. michaelseni* at least 90% of all enzyme activities was found in the midgut, with the exception of endo-β-1,4-xylanase activity which was not measured in *M. michaelseni*. Low levels of endo-β-1,4-xylanase activity were evenly distributed throughout the gut of *N. walkeri*. Midgut enzyme activity was restricted to the anterior region. The salivary glands contained variable amounts of enzyme activities. Less than 5% of the endo-β-1,4-glucanase activity was located in the hindgut. The endogenous cellulase from *N. walkeri* consisted of multiple β-glucosidase and endo-β-1,4-glucanase components. Elution profiles of fungal endo-β-1,4-glucanase activities on Bio-Gel® P-150 indicate that each *Macrotermes* spp. has a different *Termitomyces* sp. associated with it, consisting of one to three endo-β-1,4-glucanase activities. Endo-β-1,4-glucanase components from termite workers also consisted of one to three enzymes. A comparison of the elution profiles on Bio-Gel® P-60 of endo-β-1,4-glucanase and β-glucosidase activities from fungal and termite material was used to show the absence of fungal enzymes in the termite gut. Similar results were found with fungal and termite material from *M. bellicosus* and *M. mulleri* using Bio-Gel® P-150 chromatography.

The low redox potential in the hindgut of *N. walkeri* workers (-250 mV in the paunch) and the presence of fermentation products, acetate (15 ± 4 nmol termite^{-1}), propionate (0.66 ± 0.19 nmol termite^{-1}), and *iso*valerate (0.38 ± 0.16 nmol termite^{-1}) suggest an anaerobic hindgut. The presence of acetate (7.2 ± 2.9 mM) in the haemolymph indicated that acetate could be absorbed and utilised by the termite. The presence of acetyl CoA synthetase and enzymes of the tricarboxylic acid cycle in termite tissues and hindgut contents indicated that the termites and

NADH: β-nicotinamide adenine dinucleotide, reduced
$NADP^+$: β-nicotinamide adenine dinucleotide phosphate
NADPH: β-nicotinamide adenine dinucleotide phosphate, reduced
OAA: oxaloacetate
PCA: perchloric acid
PDH: pyruvate dehydrogenase
PEG: polyethylene glycol
PFK: phosphofructokinase
PFL: pyruvate-formate lyase
PFO: pyruvate-ferredoxin oxidoreductase
PK: pyruvate kinase
PPO: 2,5-diphenyloxazole
mRNA: messenger ribonucleic acid
NMR: nuclear magnetic resonance
RO: reverse osmosis
RQ: respiratory quotient
SCFA: short-chain fatty acid
SDS: sodium dodecyl sulphate
SE: standard error
Sp. Act. specific activity
PAGE: polyacrylamide gel electrophoresis
s: seconds
TEA: triethanolamine
TCA: tricarboxylic acid cycle
TLC: thin-layer chromatography
TPP: thiamine pyrophosphate
V: volts
W: Watts
wwt: wet weight

bacteria can oxidise acetate to CO_2. Glucose was not detected in the hindgut fluid (less than 0.01 mM) but was present in the mixed segment (0.2 mM) and in the midgut (2 mM). Glycolytic enzymes were present in extracts of termite tissue but no pyruvate dehydrogenase complex activity was present. Thus termites are able to utilise the glucose produced by their endogenous cellulase only as far as pyruvate. Acetate was produced in anaerobically incubated isolated paunches at 32 ± 4 nmol termite^{-1} h^{-1}. Acetate production was stimulated by addition of pyruvate but not by glucose or trehalose, indicating that pyruvate may be the substrate for bacterial metabolism. Glycolytic enzymes, pyruvate dehydrogenase complex activity, enzymes of the tricarboxylic acid cycle and acetyl CoA synthetase were present in the hindgut contents, indicating that aerobic respiration was occurring as well as anaerobic fermentation. No evidence was found for a glyoxylate cycle operating in the gut. The main anaplerotic enzyme activity in the hindgut was PEP carboxykinase. Little activity was found for pyruvate carboxylase in the termite tissue and hindgut contents. Oxygen was utilised in isolated hindgut contents at 7.44 ± 0.59 ($n = 20$) nmol termite^{-1} h^{-1}. Pyruvate stimulated a strong increase in oxygen utilisation but addition of glucose, xylose and acetate (to 60 mM) had little effect on oxygen utilisation.

INTRODUCTION

1.1 General

The abundance of cellulose makes cellulase a most desirable enzyme for a heterotrophic organism to possess. Cellulases are found in many fungi, bacteria (actinomycetes, mycobacteria and true bacteria), protists (Protozoa, ciliates, amoeba and slime moulds), plants and animals, but it is only in animals that the origin of cellulase is controversial. It is clearly established that cellulase is not secreted in vertebrates and that a vertebrate surviving largely on a cellulose diet, such as a ruminant, is dependent on microorganisms in the gut to hydrolyse cellulose and to produce fermentation products some of which can be used by the host. This clear cut story is perhaps the main reason why the presence of cellulase activity in many invertebrates has not been regarded as significant. The near universal distribution of microorganisms in the gut of many invertebrates and the presence of cellulolytic Protozoa in some species has led to the widely held view that cellulose-digesting invertebrates also are dependent on gut microbiota for cellulose utilisation. The most interesting aspect of the association of microbiota with herbivorous vertebrates and invertebrates is the biochemical interdependence, whether it is for the digestion of the cellulose or the production of essential metabolites. In many instances the cellulose content of the diet is complicated by low nitrogen levels, thus presenting a dual problem for the cellulose feeder; a difficult substrate to hydrolyse and a high carbon-nitrogen ratio in the diet. The study of carbon metabolism of cellulose feeders ideally should be studied in conjunction with nitrogen metabolism to investigate fully the relationships between symbionts and their host.

The reasons for the choice of termites as a study group can be found in their diverse, symbiotic microbial populations; the carbon-nitrogen ratio of their cellulose-based diet and their globally important role in the ecosystem. It has been estimated that they consume 3-7 billion tonnes of plant material per year (more than large grazing herbivores) returning carbon to the ecosystem as carbon dioxide, methane or combined with additional nitrogen to provide a nutritious protein source for animal predators such as ants, anteaters and humans (Collins and Wood, 1984). Concerns have been raised over the contribution of termites to atmospheric concentrations of the "greenhouse gases", CO_2 and CH_4

(Zimmerman *et al.*, 1982). The global effects of termite-produced CO_2 and CH_4 have become the focus of a number of ecological studies, especially in the tropics, to estimate the contribution by termites and the effect of land clearing on these levels (Eggleton *et al.*, 1995). Ecological studies have begun to focus on the effect of termite activity on nutrient recycling and soil fertility. In arid north-eastern Australia up to 20% of mineralised carbon and 16% of inorganic nitrogen is due to termite activity (Holt and Coventry, 1990).

1.2 Cellulose and its digestion

Cellulose is composed of linear chains of β-1,4-D-glucopyranosyl residues with cellobiose as the repeating unit. These chains of β-1,4-D-glucopyranosyl residues are organised into fibrils held together by

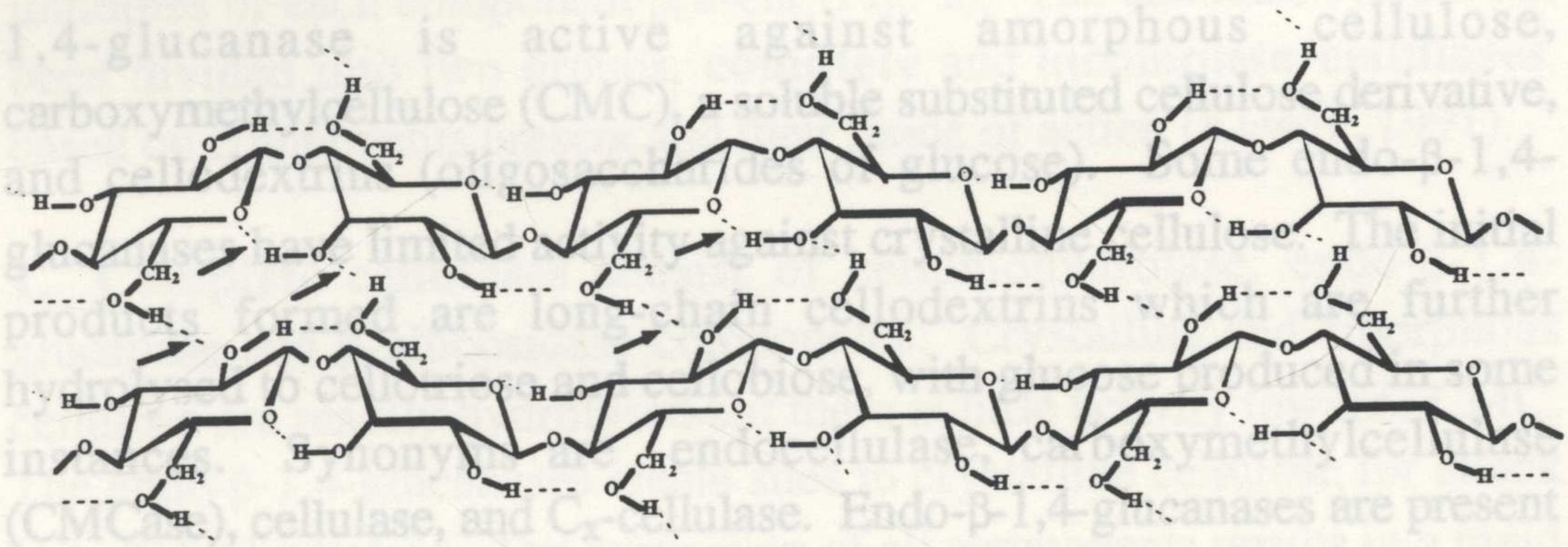

Fig. 1. Cellulose structure showing intrachain and interchain hydrogen bonds (⟶) between β-1,4-glucopyranosyl residues

interchain hydrogen bonding as illustrated in Fig. 1 and are further organised into alternating crystalline regions, which are highly ordered areas with strong hydrogen bonding in three dimensions and amorphous regions, which are less ordered with fewer possibilities for hydrogen bonding. The crystallinity conferred by the intra- and interchain bonding presents an insoluble substrate with poor access for enzyme molecules making it difficult for enzymatic degradation. In wood and other plant tissues lignin and hemicelluloses are interspersed with the cellulose fibrils

increasing the difficulties of enzymatic breakdown (Chang *et al.*, 1981, Krässig, 1985).

Cellulases are a group of hydrolytic enzymes which together can hydrolyse cellulose to glucose. The enzymes of cellulase complexes have been studied mainly in fungi and some bacteria because of the ease of obtaining enzymes from cultures and the potential importance of these cultures in industrial applications such as the production of ethanol from cellulosic wastes. The nomenclature and assays used for different component activities have undergone considerable changes over the years, making interpretation of earlier work difficult. The currently accepted nomenclature of the components and their activities are as follows (Klesov, 1991, Wood, 1985).

Endo-β-1,4-glucanase (EC 3.2.1.4; endo-1,4-(1,3; 1,4)-β-D-glucan glucanohydrolase) randomly hydrolyses internal β-1,4-glucosidic linkages leading to a rapid degree of depolymerisation of the cellulose. Endo-β-1,4-glucanase is active against amorphous cellulose, carboxymethylcellulose (CMC), a soluble substituted cellulose derivative, and cellodextrins (oligosaccharides of glucose). Some endo-β-1,4-glucanases have limited activity against crystalline cellulose. The initial products formed are long-chain cellodextrins which are further hydrolysed to cellotriose and cellobiose, with glucose produced in some instances. Synonyms are endocellulase, carboxymethylcellulase (CMCase), cellulase, and C_x-cellulase. Endo-β-1,4-glucanases are present in all cellulase complexes.

Cellobiohydrolase (EC 3.2.1.91; 1,4-β-D-glucan cellobiohydrolase) hydrolyses terminal non-reducing β-1,4-glucosidic linkages releasing cellobiose or sometimes glucose. Exo-β-1,4-glucanase is active against amorphous and crystalline cellulose, cellodextrins and *p*-nitrophenyl-β-D-cellobioside. Some cellobiohydrolases have limited activity towards CMC. Synonyms are exo-β-1,4-glucanase, exocellulase, avicelase and C_1-cellulase. Cellobiohydrolases have been characterised from only a small group of organisms, mainly fungi.

Exo-β-1,4-glucosidase (EC 3.2.1.74; 1,4-β-D-glucan glucohydralase) hydrolyses terminal non-reducing β-1,4-glucosidic linkages of cellulose

and cellodextrins, releasing glucose. Substrates are amorphous and crystalline cellulose and cellodextrins. A synonym is glucan-β-1,4-glucosidase. This component has been reported from only one source (Wood, 1985).

β-Glucosidase (EC 3.2.1.21; β-D-glucoside glucohydrolyase) hydrolyses terminal non-reducing β-1,4-glucosidic linkages of cellodextrins releasing glucose and non-specific β-linked disaccharides. β-Glucosidases are active against a wide variety of β-1,2; β-1,3; β-1,4 and β-1,6-linked di- and oligosaccharides, including cellobiose, cellotriose and *o*- and *p*-nitrophenyl-β-D-glucopyranosides. The activity against oligosaccharides decreases with increased chain length and it is not active against crystalline cellulose. A synonym is cellobiase.

Cellulases are multienzyme complexes with combinations of two or more of the component enzymes described above and often with multiples of each component present (Fig. 2). The cellulase system has been divided into two groups, **complete** and **incomplete cellulases**, based on components present and their mode of action (Klesov, 1991).

1. Complete cellulases consist of cellobiohydrolase, endo-β-1,4-glucanase and β-glucosidase components. Crystalline and amorphous cellulose are readily hydrolysed but there is little activity towards CMC. The endo-β-1,4-glucanases create sites in crystalline cellulose for attack by cellobiohydrolase. The interaction of all components results in a rapid hydrolysis of crystalline cellulose. Complete cellulases have been found in only a small number of organisms, mainly white rot fungi, brown rot fungi and a few bacteria.

2. Incomplete cellulases are the most common cellulase complexes and consist of endo-β-1,4-glucanase and β-glucosidase components. Amorphous cellulose and CMC are the main substrates with but there is little or no activity towards crystalline cellulose. Incomplete cellulases have been found in soft rot fungi, many bacteria, plants and invertebrates.

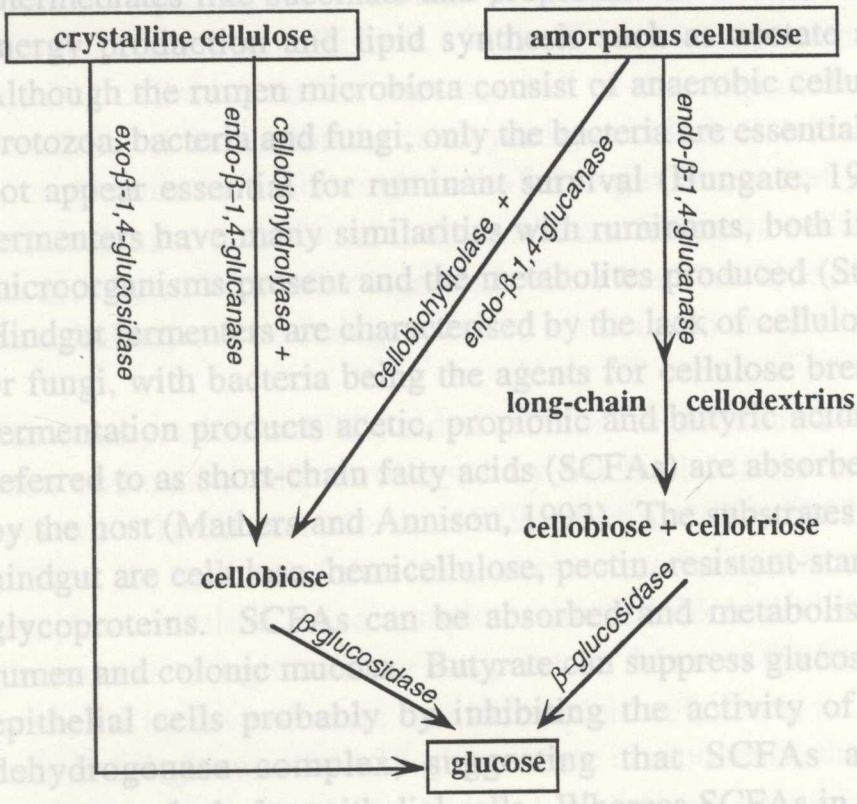

Fig. 2. Mode of action of cellulase components.

Understanding the mode of action of these two groups is crucial to interpreting data on invertebrate cellulases.

1.3 Vertebrates and cellulase

No cellulases have been found in vertebrates: cellulose digestion in herbivorous vertebrates is carried out by symbiotic microbiota. There is a variety of herbivorous vertebrates which includes foregut fermenters (ruminants) such as sheep and cattle, gastric fermenters such as camels and marsupial macropods, and hindgut fermenters such as rabbits, elephants and koalas (Stevens, 1988).

The economic importance of ruminants combined with easy access to rumen contents by surgical fistulae has resulted in a clear understanding of the role of the microorganisms in cellulose digestion and the use of the fermentation products of microbial metabolism by the host animal. The dependence of ruminants on fermentation products for gluconeogenesis means that the products must include gluconeogenic

intermediates like succinate and propionate as well as metabolites for energy production and lipid synthesis such as acetate and butyrate. Although the rumen microbiota consist of anaerobic cellulolytic ciliate Protozoa, bacteria and fungi, only the bacteria are essential; Protozoa do not appear essential for ruminant survival (Hungate, 1966). Gastric fermenters have many similarities with ruminants, both in the types of microorganisms present and the metabolites produced (Stevens, 1988). Hindgut fermenters are characterised by the lack of cellulolytic Protozoa or fungi, with bacteria being the agents for cellulose breakdown. The fermentation products acetic, propionic and butyric acids, collectively referred to as short-chain fatty acids (SCFAs) are absorbed and utilised by the host (Mathers and Annison, 1993). The substrates that reach the hindgut are cellulose, hemicellulose, pectin, resistant-starch and mucin glycoproteins. SCFAs can be absorbed and metabolised by caecal, rumen and colonic mucosa. Butyrate can suppress glucose oxidation in epithelial cells probably by inhibiting the activity of the pyruvate dehydrogenase complex, suggesting that SCFAs are important respiratory fuels for epithelial cells. Whereas SCFAs in ruminants can provide up to 70% of the energy requirements, the production in hindgut fermenters can supply 5-30% of metabolic requirements (Rombeau *et al.*, 1990).

1.3 Invertebrates and cellulase

Many invertebrates feed on plants but the most relevant to the present work on termites are cockroaches, wood borers (beetle larvae), silverfish and snails i.e. those that use dietary cellulose. In advanced invertebrates the gut is a simple structure consisting of salivary glands or accessory digestive glands, a foregut or stomach, a midgut containing secretory and absorptive cells, and a hindgut which can serve as a site for absorption of water and inorganic ions. Caeca or diverticula can form part of the midgut structure. In arthropods, the foregut and hindgut are lined with cuticle which usually precludes any secretion or absorptive processes. Most invertebrates have significant microbial populations which can be distributed throughout the gut, located mainly in the hindgut, or concentrated in specialised regions such as enlarged tracts of the hindgut or fermentation chambers (Buchner, 1965). In some

instances specialised cuticular structures have been elaborated for microbial attachment (Bignell, 1984). The association of microbes in the gut and cellulose in the diet has led to the dogma that no animal, vertebrate or invertebrate, can hydrolyse cellulose without the aid of microorganisms. Although endo-β-1,4-glucanase and β-glucosidase activities have been found in invertebrates living on cellulose diets and not associated with microorganisms, there has been little recognition that the cellulases can be endogenous and are not associated with symbionts.

The concept of microbially aided cellulose digestion in invertebrates has been enthusiastically developed by Michael Martin in a number of reviews (Martin and Martin, 1979, Martin, 1979, 1982, 1983, 1984, 1987). He has stated that invertebrates are incapable of producing a complete cellulase, i.e. a complex with cellobiohydrolase, endo-β-1,4-glucanase and β-glucosidase components. Most of the known cellulases produced by invertebrates are incomplete cellulases, i.e. a complex of endo-β-1,4-glucanase and β-glucosidase components; the exceptions to this will be discussed later. Martin believes that invertebrates living on cellulose are able to do so because cellulose digestion is accomplished, or aided, by bacterial, protozoal or fungal associations. This cellulose digestion may be by symbiotic microorganisms within the gut or by acquiring the necessary enzymes by ingestion. The invertebrate either ingests the substrate already containing cellulases, as in decaying wood, or ingests specific fungal material containing these enzymes. The ingested fungal enzyme, a cellobiohydrolase, complements the host enzymes (endo-β-1,4-glucanase and β-glucosidase), producing a complete cellulase *in situ* that will then enable the enzymatic degradation of crystalline cellulose.

The first demonstration of the role of symbionts in cellulose digestion in invertebrates was that of Protozoa in the wood-feeding lower termites, *Zootermopsis* (*Termopsis*) sp. and *Reticulitermes flavipes* (Cleveland, 1923a). Cleveland initially correlated the presence of Protozoa in the gut of termites with a wood diet. The Protozoa from *Zootermopsis* sp. and *R. flavipes* were then removed by heat treatment. The defaunation resulted in a loss of the termite's ability to live on wood although glucose addition to the diet of defaunated termites did prolong termite lifespan (Cleveland, 1923b, 1924b). As some of the Protozoa (*Trichonympha*

campanula in *Zootermopsis* sp.) were observed ingesting wood fragments and as attempts to cultivate cellulolytic bacteria and fungi were unsuccessful, it was concluded that the Protozoa were responsible for cellulose digestion in the termite (Cleveland, 1924a, 1925b, 1925c). Cleveland (1934) extended his studies to the wood roach *Cryptocercus punctulatus* which he also concluded was dependent on cellulolytic Protozoa. It is interesting to note that in these early experiments cellulase activity was detected in the salivary glands, foregut, midgut and hindgut of *C. punctulatus.* A similar distribution of enzyme activity was found in *Zootermopsis* sp. by Hungate (1938). As cellulolytic bacteria and fungi could not be cultured (Hungate, 1936), the Protozoa were implicated as agents of cellulose hydrolysis. These represent the first reports of endogenous cellulase activity. These early observations of endogenous cellulase activity were considered insignificant by their discoverers when compared to the protozoan cellulase activity in the hindgut. In *Zootermopsis* sp. the activity in the salivary glands, foregut and midgut was ascribed to ingestion of the enzymes by proctodeal feeding rather than by endogenous production, even though the data showed that 44% of the total wood ingested could be digested by the termite with 56% being digested by the Protozoa (Hungate, 1938, 1939, 1943).

Cellulolytic activity due to Protozoa was also demonstrated in *C. punctulatus, R. flavipes* and *Zootermopsis angusticollis*, (Trager, 1932, Trager, 1934) by loss of activity after defaunation. Cellulolytic activity was found in a culture of the protozoan *Trichonympha termopsidis* from *Z. angusticollis.* A single bacterial contaminant which did not have cellulolytic activity was present in these cultures. By using washed suspensions of Protozoa, Hungate (1938, 1939, 1943) demonstrated that acetate, CO_2 and H_2 were the products of hindgut fermentation of cellulose. Acetate was found in the surrounding medium when the ligated hindgut of a large nymph was left overnight in an organic culture medium, indicating that the cuticle-lined hindgut wall was permeable to products of protozoan metabolism. Hungate concluded that the wood is transferred to the hindgut where it is metabolised anaerobically by the Protozoa, producing energy for their needs and metabolic products which are then utilised by the termite. This

established the role of Protozoa as agents of cellulose hydrolysis in invertebrates, providing organic acids to the host.

Final proof that the Protozoa produce cellulase was provided by Odelson and Breznak (1985a, 1985b) and Yamin (1978, 1981, 1979) from work on isolated axenic cultures of *Trichonympha* sp. and *Trichomitopsis (Trichomonas) termopsidis.* Hungate's earlier finding that the products of protozoan fermentation of cellulose were acetate, CO_2 and H_2 were confirmed. The lower rate of hydrolysis of crystalline cellulose by cultured protozoans compared to *in vivo* rates, was considered to be due to the dependence of the Protozoa on pretreatment of the natural cellulose by the termite by physical chewing and termite-secreted enzymes. This is the reverse of the dogma that animals depend on microbes for cellulose digestion.

In other lower termites, endo-β-1,4-glucanase activity has been found not only in the hindgut where the Protozoa reside but also in termite tissues such as the midgut epithelium as in *Hodotermes mossambicus* (Botha and Hewitt, 1979), and in salivary glands and the midgut in *Calotermes flavicollis* (Chararas *et al.*, 1985). All the cellulase activity in the paunch of the lower termites *R. flavipes* (Cleveland, 1924b), *Reticulitermes speratus* (Yamaoka and Nagatani, 1975), *Mastotermes darwiniensis* (Veivers *et al.*, 1983), *Coptotermes lacteus* (Hogan *et al.*, 1988b), is protozoan in origin although cellulase activity is also found in the salivary glands, the foregut and the midgut of these termites.

The involvement of bacteria in cellulose hydrolysis in invertebrates has been studied either in isolated cellulolytic bacteria or in animals fed on ^{14}C-cellulose diets where the production of $^{14}CO_2$ has been measured before and after treatment of the animal with antibiotics.

Bacterial cellulases were presumed to be present in the paunch of termites, especially those of the higher termites which do not contain cellulolytic Protozoa. The failure to isolate cellulolytic bacteria from the hindgut of the lower termites, *Coptotermes acinaciformis, C. lacteus, Cryptotermes primus, Heterotermes ferox, M. darwiniensis, Schedorhinotermes intermedius intermedius*, (Eutick *et al.*, 1978a), *Zootermopsis* sp., *R. flavipes* (Cleveland, 1924b, Dickman, 1931,

Schultz and Breznak, 1978) and the higher termites, *Nasutitermes exitiosus, Nasutitermes graveolus* and *Nasutitermes walkeri* (Eutick *et al.*, 1978a), strongly suggests that cellulose is not hydrolysed by bacteria in these termites. Early claims for the successful isolation of cellulolytic bacteria need to be viewed with caution since the details of surface sterilisation were inadequate or not given and culturing conditions were unsuitable (Dickman, 1931). In other reports on successfully isolated cellulolytic bacteria from the lower termites, *Incisitermes schwarzi, Cryptotermes cavifrons, Neotermes* spp., *Neotermes bosei, Neotermes castaneus, Prorhinotermes simplex, Reticulitermes santonensis,* (Krelinova *et al.*, 1977), *Reticulitermes hesperus* (Thayer, 1976), *Coptotermes formosanus* and *Reticulitermes virginicus* (Mannesmann, 1972) and the higher termite, *Nasutitermes costalis* (Krelinova *et al.*, 1977) no activity could be detected in the gut of the host or else the investigators have not shown that there are significant numbers of the bacteria *in vivo*. For example, a cellulolytic bacterium has been isolated from *N. exitiosus* (French, 1975) but other studies have shown that there is negligible cellulase activity in the hindgut (O'Brien *et al.*, 1979). A similar result was found in the Mexican bean beetle *Epilachna varivestis* (Taylor, 1985). Although cellulolytic bacteria are present in the gut, the numbers are insufficient to account for the high levels of cellulase activity found in the gut indicating that the cellulase activity is not all microbial.

The approach using animals fed on ^{14}C-cellulose diets encompasses the entire metabolism from cellulose to glucose to CO_2 and any transfer of metabolite between host and symbiont and *vice versa* is overlooked. Observations that $^{14}CO_2$ production from dietary ^{14}C-cellulose in the omnivorous *Periplaneta americana* was reduced after feeding antibiotics and the demonstration of the production of $^{14}CO_2$ in cannulated colons led to the suggestion that the hindgut may be an important site of cellulose degradation (Bignell, 1977), even though cellulase activity in the hindgut was known to be negligible with most of the activity being in the salivary glands, crop and midgut (Wharton and Wharton, 1965, Wharton *et al.*, 1965b). Yet Cruden and Markovetz (1979) found significant amounts of cellulase activity in the hindgut of *P. americana* and also isolated several cellulolytic bacteria from the hindgut. Transport of bacterial fermentation products through the colon wall as

demonstrated in *P. americana* by Bracke and Markovetz (1980) could explain one of the benefits of a symbiont population to the roach but does not clarify the role. Similarly, data from Gijzen *et al.* (1994) showing an increase in hindgut cellulase activity and numbers of cellulolytic microorganisms in response to an increase in dietary cellulose can be interpreted simply as a normal response of microbial populations to an increase in nutrients but offers no implications for the host. The decrease in $^{14}CO_2$ production from ^{14}C-cellulose in the desert millipedes *Orthoporus ornatus* and *Comanchelus* sp. (Taylor, 1982) after administering antibiotics was interpreted as indicating that the bacteria were responsible for cellulose breakdown. An alternative explanation is that the bacteria are involved in the fermentation of the products of cellulose breakdown. A similar interpretation is possible for the production of ^{14}C-acetate and ^{14}C-propionate from the incubation of isolated midgut and hindgut segments from the larvae of *Oryctes nasicornis* (Coleoptera: Scarabaeidae) with ^{14}C-cellulose (Bayon, 1980, Bayon and Mathelin, 1980). Even though cellulases could not be demonstrated in gut epithelium or in the gut contents it was concluded that the cellulases were bacterial.

Fungi have also been implicated in cellulose digestion in insects. In the siricid woodwasp *Sirex cyaneus* (Hymenoptera: Siricidae) (Kukor and Martin, 1983) the cellulase activity in the gut is derived from the fungi present in the food source, decaying wood, as shown by the purification of fungal and insect cellulases. The dependence of the larvae on the fungi when feeding on wood was shown by the loss of all cellulase activity, measured enzymatically and by production of $^{14}CO_2$ from ^{14}C-cellulose in the diet, and low survival when the larvae were fed on fungi-free wood or sterilised wood. However, it should be noted that the larvae can survive on an artificial fungus-free diet containing wheat germ and casein. The endo-β-1,4-glucanase activity in these larvae did not appear to be significantly different from the activity in larvae from natural galleries, suggesting that endo-β-1,4-glucanase activity was produced by the insect. The cellulase from insect and fungal material was partially purified by ammonium sulphate fractionation and anion exchange chromatography. However, no data were presented on endo-β-1,4-glucanase activity during the purification, with the exception that "there was some activity towards carboxymethylcellulose" "with various

protein peaks" in the elution profile of the artificially reared larvae which contained no cellulase activity. SDS-gradient gels revealed identical protein bands for the partially purified cellulases from both fungal and insect tissue. A similar study was carried out on the larvae of the balsam fir sawyer, *Monochamus marmorator* (Coleoptera: Cerambycidae) (Kukor and Martin, 1986) and its associated white rot fungus *Trichoderma harzianum.* Unfortunately no data were available on enzyme levels in larvae fed artificial diets as in the previous study. Amylase and endo-β-1,3-glucanase activities were detected in the midgut, suggesting that the larvae could utilise the storage polysaccharides of plant and fungi and the structural polysaccharide of fungi. Endo-β-1,4-glucanase activities were partially purified from the midgut and fungal tissues by ion-exchange chromatography, gel filtration and finally preparative chromatofocusing. The two endo-β-1,4-glucanase activities purified from both fungal culture and insect tissue had similar profiles. Analytical isoelectric focussing of these partially purified fungal and insect endo-β-1,4-glucanases revealed identical protein bands, indicating that the insect endo-β-1,4-glucanases originated in the fungus. From these two studies the presence of fungal cellulases in the gut of the larvae is difficult to dispute but the importance of the ingested enzymes is yet to be confirmed. The ability of the larvae to live on an artificial diet high in storage polysaccharides and the presence of enzymes capable of utilising these substrates as well as fungal mycelia could indicate that the larvae are using the fungi as a food source and that the ingested enzymes are incidental.

The earliest proof of an endogenous cellulase came from studies by Mansour (1934) on the larvae of two wood-eating Cerambicids, *Macrotoma palmata* and *Xystrocera globosa* and on the silverfish, *Ctenolepisma lineata* (Lasker and Giese, 1956). Cellulase activity was detected in guts either free of micoorganisms or from which cellulolytic bacteria could not be cultivated. *C. lineata* could be reared symbiont-free from surface-sterilised eggs and maintained under sterile conditions without any loss of cellulase activity, firm evidence for endogenous cellulase activity. Similarly, endogenous cellulases have been identified in a number of annelids, crustaceans, molluscs and echinoderms (Yokoe and Yasumasu, 1964). Endo-β-1,4-glucanases have been reported and purified (Anzai *et al.*, 1988) from a variety of molluscs (Elyakova *et al.*,

1968, Marshall, 1973), in insects, from the saliva of aphids (Adams and Drew, 1965), from the salivary glands of a number of cockroaches (Wharton and Wharton, 1965) and from the lepidopteran *Philosamia ricini* (Pant and Ramana, 1989) regardless of bacterial population. The firebrat *Thermobia domestica* (Thysanura: Lepismatidae) (Zinkler and Götze, 1987) has activities against crystalline cellulose and CMC and can assimilate 64% of ^{14}C-cellulose in the diet. A large number of roaches (Wharton and Wharton, 1965, Zhang *et al.*, 1993) has been found to have cellulase activity in salivary gland, foregut and midgut tissue.

Chararas *et al.* (1983) purified three cellulase components from the xylophagous larvae of *Ergates faber* (Coleoptera: Cerambycidae) which contains non-essential microorganisms in its gut, in direct contrast to the previously described work by Kukor and Martin (1986) on another Cerambycid larvae. Ammonium sulphate precipitation, gel filtration and preparative electrophoresis were used to purify the cellulases to homogeneity, verified by single protein bands on polyacrylamide gel electrophoresis (PAGE). All three cellulase components were active against CMC and crystalline cellulose. One component was identified as a cellobiohydrolase as it produced cellobiose from crystalline cellulose but it appears to be an endo-β-1,4-glucanase as the main products from crystalline cellulose are cellodextrins and cellobiose.

The reluctance of biologists to accept the evidence that invertebrates can secrete cellulase is clearly shown in the case of the wood-feeding marine isopod, *Limnoria lignorum.* What is of special interest in this cellulolytic organism is that there are no microorganisms in its gut (Boyle and Mitchell, 1978). The original reports (Yonge, 1927) were contemporary with Cleveland's work on cellulolytic Protozoa in termites. As Protozoa were absent, bacteria were declared as the agents, though later careful work (Ray and Julian, 1952) showed that bacteria were absent and that the cellulase activity was located in the midgut caecae. A fungal origin for the cellulase proposed by Meyers and Reynolds (1957) was refuted by Ray and Stuntz (1959). Finally, scanning electron microscopy revealed the complete absence of microorganisms in the gut (Boyle and Mitchell, 1978). A final speculation on the origin of the cellulase is that *L. lignorum* has "acquired useful digestive enzymes from its food" (Martin, 1987).

The demonstration of endogenous cellulases in invertebrates placed the unbelievers on the defensive. They argued that such endogenous cellulases were incomplete cellulases consisting only of endo-β-1,4-glucanase and β-glucosidase components and therefore were not true cellulases. In a review by Michael Martin (1983) 57 reports of endo-β-1,4-glucanase activity originating in salivary gland or midgut tissue from insects from Orthoptera, Isoptera, and Homoptera were given. All of them were incomplete cellulases and therefore not considered as true endogenous cellulases.

The definitive work that leaves no doubt as to the endogenous nature of an invertebrate cellulase that is capable of hydrolysing crystalline cellulose to glucose comes from work by Scrivener (1994a, 1994b, 1989) on the wood-feeding roach *Panesthia cribrata*. The gut of this roach is a straight tube containing a small population of non-essential microorganisms. The roach can survive on a diet of crystalline cellulose, independently of its gut microbiota most of which can be removed by tetracycline treatment (Scrivener *et al.*, 1989). The cellulase complex consists of two major and four minor endo-β-1,4-glucanase and β-glucosidase components (Scrivener and Slaytor, 1994b). Activities are restricted to the salivary glands, foregut and anterior midgut, where fewer than 25% of the bacteria in the gut are found. The cellulase is incomplete but the major endo-β-1,4-glucanase components are weakly active against crystalline cellulose. This, together with the large amount of activity present (13% of soluble protein in the gut), enables the roach to survive on crystalline cellulose, without the need for a complete cellulase. As *P. cribrata* lives in damp, rotten logs, it could be claimed that fungal enzymes were ingested with the wood and thus acquired by the roach. Characterisation of the components of fungal cellulase from the wood source showed they were distinct from those of the roach. Additionally there was no synergism evident between the endo-β-1,4-glucanase components from the fungus and the roach. It was concluded that the roach lives in damp logs to facilitate burrowing (Scrivener and Slaytor, 1994a).

The study by Scrivener *et al.*, (1989) has led to the reluctant acceptance that some insects are capable of producing their own cellulases but it is

still with reservations that "the possibility that no insect digests cellulose without some microbial contribution cannot be totally discounted" (Martin, 1991).

Thus if it is possible for a wood-feeding roach to survive a cellulose diet with an endogenously produced incomplete cellulase, can termites, related to the cockroaches, function similarly?

1.4 Higher termites and cellulase

Termites eat a wide range of food: living vegetation (trees, roots, grasses); fresh, dead vegetation (trees, roots, plant litter, grasses); decomposing vegetation (rotting logs, rotting plant litter, dung); humus (soil organic matter); fungi (cultivated fungi or ingested with rotten material) and special foods (other termites, lichens, nest material, skin from corpses) (Wood, 1978). Those termites feeding on wood have received the most attention.

The higher termites (Termitidae) which constitute 75% of all termite species do not contain Protozoa in the hindgut, only a varied bacterial population (Shewale and Sadana, 1981). The consistency in bacterial numbers and morphotypes (Breznak *et al.*, 1973, Breznak and Pankratz, 1977, Czolij *et al.*, 1985, Eutick *et al.*, 1978a, Krasil'nikov and Satdykov, 1969) does suggest an important function in termite metabolism but not in cellulose digestion as discussed in 1.3.

Although endogenous cellulase activity in termites was detected in lower termites by Cleveland and Hungate, cellulase activity in higher termites was not investigated as the 60 species examined did not contain Protozoa and were believed not to feed on wood. 57 of the 60 species of higher termites examined, including specimens from the genera *Amitermes, Nasutitermes, Macrotermes* and *Thoracotermes,* did not have Protozoa in the gut. Wood fragments were not detected histochemically in the guts of these termites, although hemicellulose and soil particles were found (Cleveland, 1923a). This discrepancy may be due to the choice of species (some such as *Thoracotermes* sp. are soil feeders) or the sampling and preservation of material before the

observations were carried out. Studies by Tracey and Youatt (1957) found cellulase activity in *N. exitiosus,* though no efforts were made to discredit bacterial involvement. The presence of termite-produced cellulase activity in *N. exitiosus* was confirmed by O'Brien *et al.* (1979) and Hogan *et al.*, (1988a) with activity confined to the midgut and the midgut epithelium. Kovoor (1970) found endo-β-1,4-glucanase activity in all sections of the gut of *Microcerotermes edentus,* although extremely long incubation times (20-42 h) were used for the assays. (Potts and Hewitt, 1974a, 1974b) purified a single endo-β-1,4-glucanase component from whole workers of *Trinervitermes trinervoides* that was also active against crystalline cellulose. Activities against CMC and crystalline cellulose were found in the salivary glands, foregut and gut (midgut and hindgut) of *Anacanthotermes ahngerianus* (Mednikova and Tiunova, 1984). Rouland *et al.* (1989) found activities towards CMC and crystalline cellulose in salivary glands and midgut from *Crenetermes albotarsalis.*

The subfamily Macrotermitinae is one of the most interesting of the higher termites partly because they cultivate fungus gardens using *Termitomyces* spp. and also because of the division of labour amongst the workers (McKittrick, 1965). The first discovery of the sponge-like fungus gardens of Macrotermitidae, built from vegetable residues and supporting mycelium with white nodules of conidia and conidiophores was made in 1779 by König (in Sands, 1969). He believed that the fungus was the food of the young termites. Since then the role of the fungus in the lifestyle of the termite has been studied with varied conclusions, summarised by Sands (1969); these include maintenance of the temperature and humidity, an architectural feature or a nutrient source. The complex division of labour and food source is similar in the *Macrotermes* spp. studied to date and is illustrated in Fig. 3 for *Macrotermes bellicosus.* Food is harvested and brought to the nest mainly by old major workers in *Macrotermes subhyalinus* (Badertscher *et al.*, 1983), *Macrotermes michaelseni, M. bellicosus* (Gerber and Badertscher, 1988) and *Macrotermes mulleri* (Garnier-Sillam, 1989). It is then consumed by the young major and the young minor workers, either directly or after being placed on a food store as in colonies of *M. bellicosus* (Collins, 1981b, Thomas, 1987) and *M. mulleri* (Garnier-Sillam, 1989).

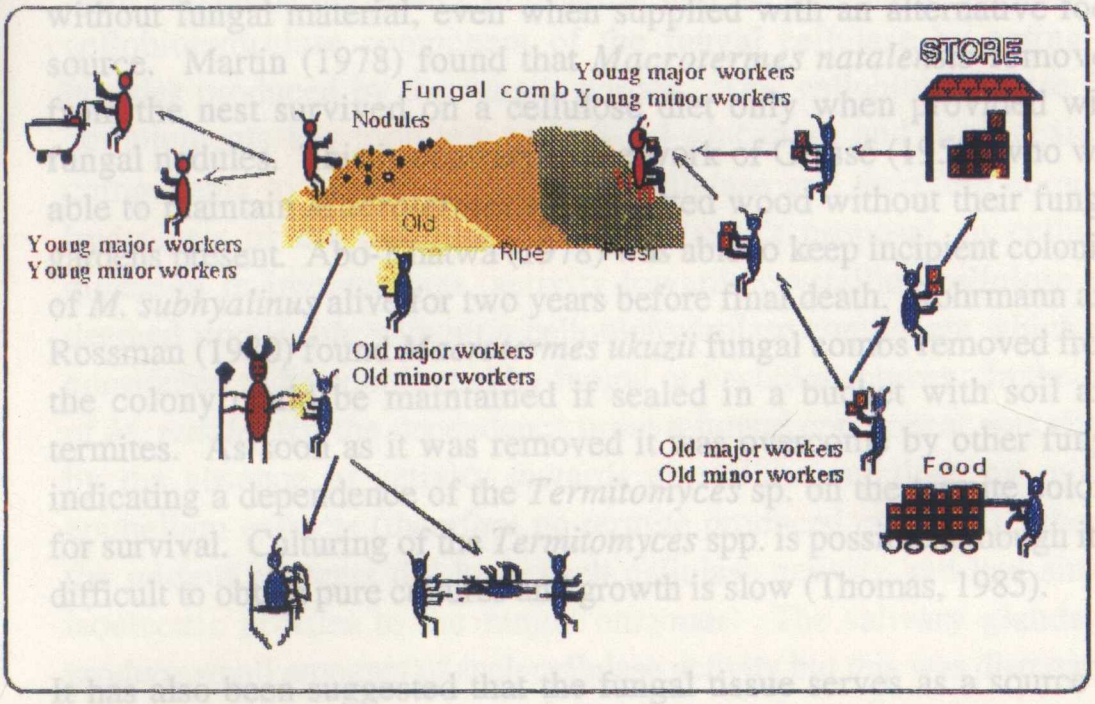

Fig. 3. Relationship between the fungus garden and social responsibilities in categories of termites in *M. bellicosus*. (Redrawn from a diagram supplied by Prof. R. H. Leuthold, University of Bern)

The faeces from the young workers of all species are used to construct the fungal comb (fresh fungal comb) (Sands, 1960; Grassé, 1978,; Badertscher *et al.*, 1983) which supports the growth of the *Termitomyces* sp. The comb ripens (ripe fungal comb) to produce nodules or conidia which are then eaten by the young major and minor workers, which are responsible for feeding the dependent castes. Once the fungal comb has fruited (old fungal comb) it is consumed by the old major and minor workers. The soldiers are fed old fungal comb by the old major and minor workers. Old minor workers are primarily responsible for gallery construction and mound repair (Badertscher *et al.*, 1983, Gerber and Badertscher, 1988). The specificity of the fungal-termite relationship has been shown by experiments where *M. natalensis* can feed and survive on the fungal comb of other species but only its fungus will grow on its comb (Grassé, 1959). The importance of the fungus was first demonstrated by Sands (1956) in

Odontotermes sp., which survived no longer than starved termites when without fungal material, even when supplied with an alternative food source. Martin (1978) found that *Macrotermes natalensis* removed from the nest survived on a cellulose diet only when provided with fungal nodules. This is contrary to the work of Grassé (1959) who was able to maintain *Macrotermes* sp. on rotted wood without their fungal gardens present. Abo-Khatwa (1978) was able to keep incipient colonies of *M. subhyalinus* alive for two years before final death. Rohrmann and Rossman (1980) found *Macrotermes ukuzii* fungal combs removed from the colony could be maintained if sealed in a bucket with soil and termites. As soon as it was removed it was overcome by other fungi, indicating a dependence of the *Termitomyces* sp. on the termite colony for survival. Culturing of the *Termitomyces* spp. is possible although it is difficult to obtain pure cultures and growth is slow (Thomas, 1985).

It has also been suggested that the fungal tissue serves as a source of fixed nitrogen (Rohrmann, 1978) as the nodules eaten by the young workers contain a high percentage of nitrogen (Collins, 1983). No nitrogen fixation by the workers or associated fungal material in the comb or termites has been observed in *M. ukuzii* (Rohrmann and Rossman, 1980), *M. natalensis* or *M. michaelseni* (Collins, 1983), suggesting that the fungus is concentrating the available nitrogen in a very accessible form for the termite. Chitinase activity has been found in the midgut and to a lesser extent in the hindgut, especially in major workers indicating that fungal mycelia can be utilised by the termite, adding to the nitrogen sources. Another function of the fungus garden is the decomposition of lignin, as has been found in *M. ukuzii* (Rohrmann and Rossman, 1980). Staining techniques by Grassé and Noirot, 1958 in (Higashino *et al.*, 1977) also showed that the fungus can break down lignin thus making cellulose more accessible to cellulase.

The relative roles of the fungus gardens and the workers in the overall process of cellulose digestion in the subfamily and particularly in the genus *Macrotermes* have been investigated by several laboratories. Grassé (1959) first suggested that the white spheres or conidia may act as symbionts in the gut of the termite and aid in the breakdown of the cellulose of the ingested wood. This idea has been supported by studies

on *M. subhyalinus* by Abo-Khatwa (1978). Martin and Martin (1978) have expanded the theory to propose that *M. natalensis* acquires the cellobiohydrolase component of the fungal cellulase by eating the nodules produced by the fungus garden. This cellobiohydrolase then complements the termite's endo-β-1,4-glucanase and β-glucosidase components to produce a complete cellulase and therefore complete hydrolysis of the cellulose. Under Martin's proposal on cellulose digestion in invertebrates, activity towards crystalline cellulose was deemed impossible without a cellobiohydrolase component which was considered to be contributed by fungal or bacterial sources. In the case of *M. natalensis*, the dependence upon a fungal component was shown by the absence of activity towards crystalline cellulose by midgut epithelium extracts (therefore no termite produced enzymes), whereas the midgut contents did have such cellulase activity and had similar isoelectric profiles to the fungal enzymes. The salivary glands did produce small amounts of such cellulase activity but this was disregarded as possible contamination of fluid from the salivary reservoirs. Unfortunately this overlooks the important contribution that salivary gland enzymes may make to initiating digestion once they are secreted into the foregut and make first contact with the substrate. In contrast, Rouland and co-workers (1988a, 1988b, 1988c) have investigated the properties of the components of both the fungal and the termite cellulases in *M. mulleri* colonies and concluded that a termite-produced cellobiohydrolase component is synergistically boosted by the endo-β-1,4-glucanase component from fungal nodules. The proportions of the two enzymes present in the termite gut were not given nor were the synergism experiments designed to mimic the *in vivo* ratios, reducing the value of the data presented and leaving doubts over the contribution of fungal enzymes.

1.6 Metabolism and intestinal bacteria in termites

The roles of gut bacteria in symbiosis in the higher termites are not based on the inability of the termite to produce glucose from cellulose. The importance of the bacterial flora to the termite host has been demonstrated in *N. exitiosus* by removing the flora with antibiotics which resulted in the death of the termites (Eutick *et al.*, 1978b). This

poses questions on the roles of the bacteria and the substrates that are utilised for bacterial growth.

Removal of the bacterial flora from *N. exitiosus* resulted in the gut becoming aerobic. Successful reintroduction of the gut flora resulted in the gut again becoming anaerobic, indicating that the flora are responsible for maintaining the redox potential of the gut. Experiments to introduce the pathogen *Serratia marcescens* to *N. exitiosus* were also carried out. Colonisation did not occur when the normal flora were present but did occur in those termites whose flora had been removed by tetracycline treatment, indicating that the flora could exclude foreign bacteria from colonising the gut (Veivers *et al.*, 1982b).

The bacteria can produce essential growth factors such as vitamin B_{12} in *Z. angusticollis*, *C. formosanus*, *R. flavipes*, *R. virginicus* and *Nasutitermes corniger* (Wakayama *et al.*, 1984).

The nitrogen content of plant material varies from 0.03-0.1% nitrogen (dry weight) in wood to 0.5-5% nitrogen in grass and leaf tissues (Cowling and Merrill, 1966). The high carbon:nitrogen ratio of wood presents difficulties in obtaining sufficient nitrogen for growth and metabolism. Termite nitrogen metabolism has been reviewed by Slaytor and Chappell (1994). Fixation of atmospheric nitrogen by gut bacteria is one solution, first suggested by Cleveland (1925a) when he was able to maintain colonies of *Zootermopsis* sp. on a diet of filter paper with only 0.03% nitrogen. The earliest attempt to show nitrogen fixation in termites was by Greene and Breazeale (1937) when cultures of nitrogen-fixing bacteria were isolated from a *Kalotermes* sp. Hungate (1944) concluded from studies on the nitrogen balance in laboratory cultures of *Zootermopsis nevadensis* that no fixation occurred and that nitrogen was obtained from the soil and the wood. The acetylene reduction assay has been used to demonstrate dinitrogen fixation in whole workers from a number of lower and higher termites (Benemann, 1973, Breznak *et al.*, 1973, Collins, 1983, French *et al.*, 1976, Lovelock *et al.*, 1985, Mertins *et al.*, 1973, Prestwich and Bentley, 1981, Prestwich and Bentley, 1982). A review of these studies (Collins, 1983) has been published. The relative fixation rates range from 0.1 to 4.93 $\mu g\ N\ g^{-1}$ (dry weight) h^{-1}. Estimates of the times for the doubling of termite nitrogen ranged from

0.25 to 12.25 years. The use of $^{15}N_2$ by Bentley (1984) in *N. corniger* confirmed the results from the acetylene-reduction method. Degutted bodies did not fix nitrogen but the isolated guts retained some ability to fix nitrogen. The removal of the bacterial flora using antibiotics results in the loss of the nitrogen-fixing activity, implicating bacteria as the agents (Breznak *et al.*, 1973). In recent investigations the ^{15}N natural abundance method has been used to demonstrate nitrogen fixation in *Neotermes koshunensis* (Tayasu *et al.*, 1994). A number of *Enterobacter* sp. have been isolated on a nitrogen-free medium under gaseous nitrogen (Eutick *et al.*, 1978a) from lower and higher termites. *Citrobacter freundii* has been identified as the nitrogen-fixing agent in *M. darwiniensis, C. lacteus* and *N. exitiosus* (French *et al.*, 1976) and *Enterobacter agglomerans* has been isolated from *C. formosanus* (Potrikus and Breznak, 1977). As already mentioned (section 1.4) no nitrogen fixation has been detected in any of the Macrotermitinae studied, in workers or associated fungal material. Similar negative results have been found in many species from rainforest communities (Sylvester-Bradley *et al.*, 1979) where it was assumed that a higher nitrogen content available in the diet made nitrogen fixation unnecessary.

Another possible mechanism for nitrogen conservation is recycling of uric acid, a nitrogenous waste product of termite metabolism, by uricolytic bacteria in the gut. This was first proposed by Leach and Granovsky (1938). Uric acid has been reported from termites (field and laboratory stored termites) in quantities ranging from 1 to 2.5% of the termites' dry weight ((Lovelock *et al.*, 1985, Potrikus and Breznak, 1980b) with a non-physiological increase to 45% occurring in laboratory-stored termites fed on wood impregnated with $(NH_4)_2SO_4$. It is mainly stored in the fat body with small amounts also present in faeces (0.2% termite dry weight). No uricase activity has been reported in termite tissues or in the hindgut (Potrikus and Breznak, 1980b). Anaerobic uricolysis by bacteria isolated from *R. flavipes* produced CO_2, acetate and NH_3 (Potrikus and Breznak, 1980a). The demonstration that isolated Malpighian tubules could take up [2-^{14}C]uric acid from the incubation medium and that [2-^{14}C]uric acid injected into the haemolymph could be recovered as $^{14}CO_2$ provided evidence that some transport of uric acid to the hindgut is possible. ^{15}N from [1,3-^{15}N]uric acid was also assimilated into termite tissue (Potrikus and

Breznak, 1981). The importance of this recycling is illustrated by calculations that the turnover of uric acid would be approximately equal to 30% of the colony biomass, suggesting an important role for the gut bacteria in carbon and nitrogen cycling in the termite.

The role of microorganisms in the digestion of lignin by termites has been critically reviewed by Breznak and Brune (1994) with the conclusion that although there are many technical difficulties in demonstrating lignin degradation, it does occur to a limited extent in *N. exitiosus* (Cookson, 1988). In other species it is either not significant or of questionable significance, but the number of species examined has not been extensive. It is of interest, however, that various monomeric lignin model compounds are readily degraded by a variety of lower and higher termites (Kuhnigk *et al.* 1994, Brune *et al.*, 1995b). An interesting paradox arises from the discovery of lignin-degrading activity in termite guts. Molecular oxygen is required for initial depolymerization of lignin by peroxidases, therefore the concept of an anoxic gut (Bignell and Anderson, 1980, Veivers *et al.*, 1980) does not fit with the presence of lignin degrading activity unless microaerobic sites are present. Although the gut has been measured as anaerobic, it is freely accessible to the atmosphere through an elaborate series of tracheoles which adequately supply the hindgut (Wigglesworth, 1984). Atmospheric N_2 can freely enter and be reduced to NH_3 and atmospheric CO_2 can also enter and be fixed to acetate so that it follows that O_2 must also be able to enter. Exposure of termites to increasing O_2 pressure results in an increase in bacterial numbers (Veivers *et al.*, 1982b). Presumably the normal availability of O_2 in the anaerobic hindgut is rate-limiting for bacterial growth. Facultative bacteria could also act as O_2 scavengers, protecting the obligate anaerobes such as the spirochaetes. In other symbiotic systems involving nitrogenase activity the high amounts of ATP needed are usually generated by oxidative phosphorylation, thus there are few strict anaerobes. Most nitrogen fixing bacteria therefore require microaerobic conditions (Gallon, 1992). The presence of O_2 in the gut has been verified by Brune *et al* (1995a) who were able to present detailed O_2 and pH profiles of the gut in *R. flavipes* and *N. lujae* through the use of microelectrodes. Significant O_2 was present near the epithelium, quickly diminishing to total anoxia in the centre of the

paunch. This presents a strong case for aerobic metabolism occurring in the paunch alongside anaerobic metabolism.

The presence of acetate and other SCFAs in the hindgut of termites has been noted by other workers since Cleveland's and Hungate's work on lower termites. Thus acetate is the major end product (94-98%) of anaerobic microbial fermentation with small amounts of propionate and butyrate also being produced in a number of lower and higher termites, (Kovoor, 1967, Breznak and Switzer, 1986, Odelson and Breznak, 1983). Breznak and Switzer (1986) have demonstrated that gut bacteria capable of synthesising acetate from CO_2 and H_2 could account for one third of the total acetate produced in the hindgut. The importance of acetate in termite metabolism has been demonstrated in the lower termite *R. flavipes* by (Odelson and Breznak, 1983) who calculated that the acetate produced in the hindgut could meet 77 to 100% of the respiratory needs of the termite and that degutted termite bodies could convert [U-^{14}C]acetate to $^{14}CO_2$. Acetogenic bacteria have been isolated and characterised from *N. nigriceps, Pterotermes occidentis,* and *Cubitermes speciosus* (Breznak and Blum, 1991, Kane *et al.*, 1991, Kane and Breznak, 1991). The fact that acetogenesis in freshly collected termites was 50% higher than that of laboratory maintained termites (Odelson and Breznak, 1983), suggests that there is a relationship between CO_2 concentration and the maintenance of efficient acetogenesis. This was discounted by Williams *et al.* (1994) who found acetogenesis in *N. walkeri* to be independent of CO_2 concentration. Incorporation of ^{14}C-acetate into termite lipids has been shown in *R. flavipes* and *C. formosanus* (Mauldin, 1982). ^{14}C-acetate can be incorporated into linoleic acid in *Z. angusticollis* tissue independently of microorganisms (Blomquist *et al.*, 1982). Transport of acetate across the hindgut has been demonstrated in the wood roach *P. cribrata* and the lower termite *M. darwiniensis* (Hogan *et al.*, 1985), indicating that acetate produced by the microorganisms is available for metabolism in termite tissues and it may be a metabolite central to the symbiosis between wood-eating termites and their intestinal microbiota. In a comprehensive study of a number of wood-feeding, grass-feeding, fungus-growing and soil-feeding termites Brauman *et al.* (1992) correlated high rates of acetogenesis with low rates of methanogenesis and *vice versa.* Methanogenesis and hydrogen production are two other

ways of regenerating oxidising power in the anaerobic hindgut but acetogenesis, the more energetically economical process, appears to outcompete these in the hindgut. This is in contrast to ruminants, where methanogenesis is the major process.

What are the normal substrates for bacterial growth in the paunch? Glucose has been detected in the hindgut of *M. subhyalinus* by Veivers *et al.* (1991). French (1975) found isolated bacteria from *N. exitiosus* were capable of utilising cellulose, glucose and a number of other sugars. Contrary to this, (Mannesmann, 1972) noted that glucose was not a major substrate for bacterial growth in the paunch of *N. nigriceps*. Glucose produced in the midgut is more likely to be absorbed by the midgut epithelium rather than reach the bacteria in the hindgut. Schultz and Breznak (1979) used monocultures and cocultures of two facultative bacteria isolated from the hindgut *R. flavipes* to illustrate the interdependence of gut microorganisms. *Streptococcus lactis* produced lactate from glucose; the lactate could be utilised by *Bacteroides* sp. to produce propionate and acetate. This interdependence of gut microorganisms highlights the problem of studying the properties of pure cultures, rather than using the whole termite or the paunch. A better approach to elucidating the biochemical basis for the microbial ecology in the paunch lies in designing experiments (Brock, 1987) that encompass the *in vivo* system.

1.7 Study species

The species selected for this study were the Australian species *Nasutitermes walkeri* Hill which feeds on sound dead wood and the African species *Macrotermes bellicosus* Smeathman, *Macrotermes michaelseni* Sjøstedt, *Macrotermes mulleri* Sjøstedt and *Macrotermes subhyalinus* Rambur which forage mainly on grasses and leaves. The food of these species (wood, grass, and leaves) contains the following structural carbohydrate components: cellulose (30-75%), lignin (20-40%) and hemicelluloses (6-31%) (Szegi, 1988). The nitrogen content of this material varies from 0.03-0.1% nitrogen (dry weight) in wood to 0.5-5% nitrogen in grass and leaf tissues (Cowling and Merrill, 1966). Both genera feed on diets low in nitrogen. The

importance of these termites in their environment can be seen in their consumption and assimilation rates. These vary from 10.6 mg food g termite^{-1} day^{-1} for the closely related *N. exitiosus* (Lee and Wood, 1971) to 90 to 150 mg food g termite^{-1} day^{-1} for *M. bellicosus* (Collins, 1981a) and 28 to 565 mg food g termite^{-1} day^{-1} for other Macrotermitinae. It has been estimated that *N. exitiosus* removes 16.6% of the total estimated annual production of sticks and logs (Lee and Wood, 1971). *M. michaelseni* has harvesting levels of 800 to 1500 kg ha^{-1} which is equivalent to large grazing mammals (Lepage, 1981). When the rates of assimilation are compared, the Macrotermitinae attain almost complete assimilation of food compared to 54% reported for *N. exitiosus* (Lee and Wood, 1971).

Arboreal nests of *N. walkeri* colonies are readily found within the Sydney area (Plate 1). Foraging workers are relatively easy to collect in wood litter or in galleries from the trunks of trees containing the arboreal nests. A nest can be repeatedly sampled providing termites that can be used within hours of collection. The fungus-gardening *Macrotermes* spp. are not found in Australia and therefore work carried out on these species was on laboratory colonies (Plate 2) in Switzerland or in field colonies in Africa (Plates 3a, b and 4a, b).

Plate 1. Arboreal nest of *N. walkeri,* Greenwich, NSW.
(Photograph courtesy of C. M. Williams)

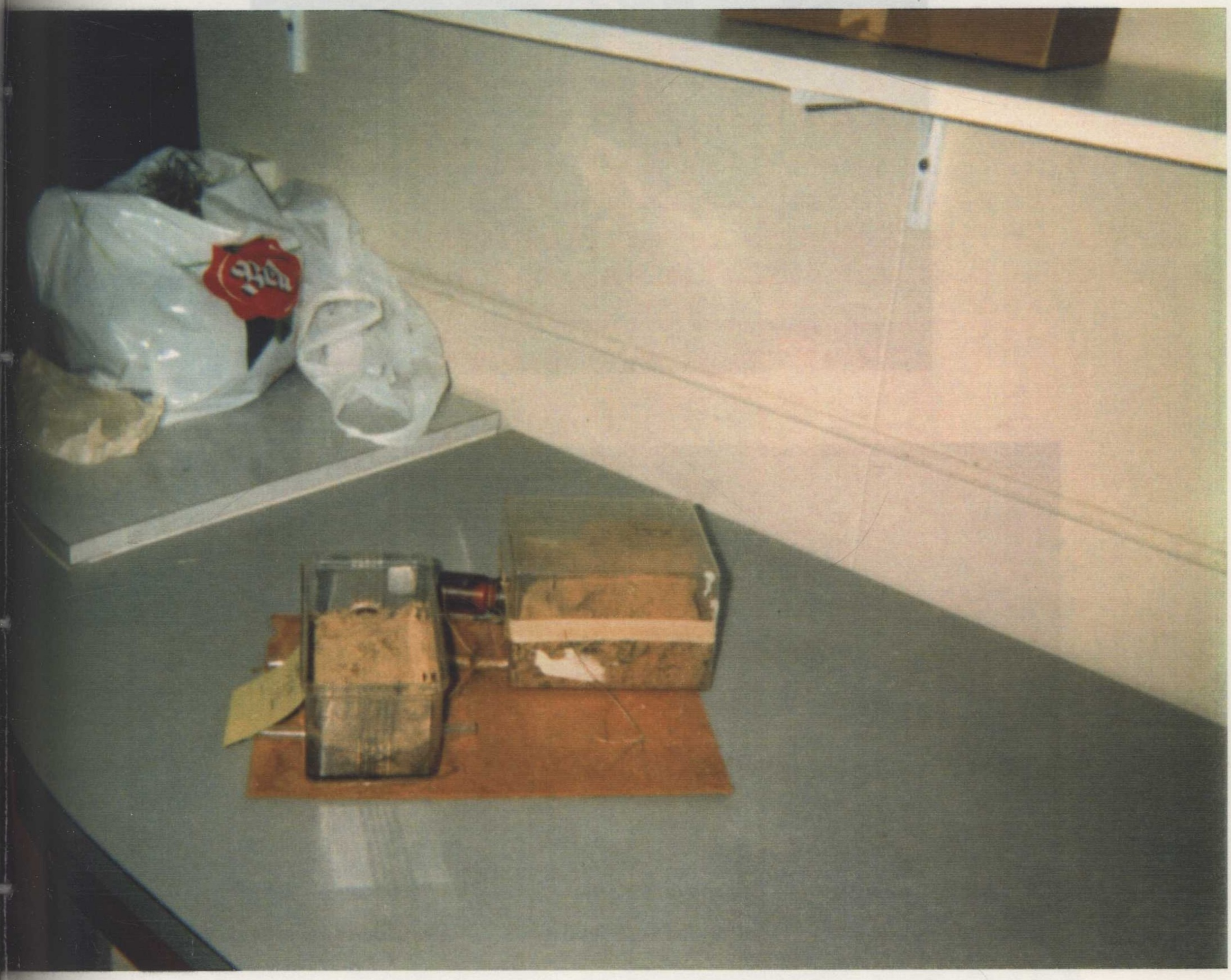

Plate 2. Laboratory colony of *M. subhyalinus,* Bern, Switzerland

a

b

Plate 3. *M. bellicosus*, Abidjan, Ivory Coast

a. Mound showing the associated plant growth. The cocconut trees in the backgound provide the main food for these termites

b. Open mound displaying fungal comb

a

b

Plate 4. *M. mulleri*, Mbalmayo, Cameroon.
a. Mound. Note the clear forest floor where few fallen leaves are present.
b. Workers (old major category) foraging in the open and small soldiers. Taken at 11 pm.

1.8 Aims

The aim of this project is to present a comprehensive study of carbon metabolism in representatives of two genera of higher termites, the *Macrotermes* spp. and *N. walkeri*. In both groups the location of cellulase activity in the gut and the role of symbionts in cellulose digestion have been studied. In the *Macrotermes* spp. an attempt has been made to correlate the complex divisions of labour and the differing food sources in the categories of termites to the overall process of cellulose digestion and nitrogen metabolism in the colony, incorporating the role of the fungus garden.

The lack of activity of pyruvate dehydrogenase complex in termite tissue (O'Brien and Breznak, 1984) means that the termite is unable to convert pyruvate to acetyl CoA for energy purposes (TCA cycle) or for lipid synthesis. It has been suggested that perhaps the bacteria utilise the pyruvate produced, but not used, by the termites (O'Brien and Breznak, 1984). The acetate produced by the microbiota could then be utilised by the termite for energy purposes (TCA cycle) or for lipid synthesis. For this purpose, *N. walkeri* has been used as a model for studying the flow of carbon from cellulose to glucose through to CO_2. Aspects of termite and bacterial metabolism have been investigated, coordinating with other metabolic studies previously carried out on *N. walkeri*, to clarify the roles of the bacterial symbionts in higher termites.

A secondary aim is to have quotes such as the following:

"the digestion of wood by termites depends on the Protozoa in their guts, in a mutually beneficial association" (Stryer, 1995).

"These Protozoa are present in the lower area of the hindgut and are important in the conversion of cellulose to simpler molecular products that can be used by the termites, supplying them with essential carbohydrate. Some termites, such as species belonging to the family Termitidae, do not possess the Protozoa but have intestinal bacteria that perform the same function" (Hadlington, 1987).

removed from textbooks and reference books.

MATERIALS AND METHODS

2.1 Enzymes, substrates and chemicals

Sodium[1-^{14}C]pyruvate (11.0 mCi/mmol) and sodium[1-^{14}C]2-oxoglutarate (51.2 mCi/mmol) were all obtained from New England Nuclear (Boston, MA, USA). Sodium[^{14}C]bicarbonate (60 mCi/mmol) and [U-^{14}C]glucose (280 mCi/mmol) were obtained from Amersham (Amersham, Buckinghamshire, UK).

The following were purchased from the Sigma Chemical Co. (St. Louis, MO, USA): acetohydroxamic acid, acetyl CoA, ADP, ATP, bicinchoninic acid, BSA (Fraction V), cellobiose, chloramphenicol, coenzyme A (CoA), cytochrome *c*, Dalton Mark VII-L molecular weight standards, DTNB, FAD, fructose 6-phosphate, glucose 6-phosphate dehydrogenase, glutathione (GSH, reduced), IDP, liver acetone powder (pigeon), NADH, ovalbumin, PEP, sodium citrate, sodium glyoxylate, sodium malate, sodium oxaloacetate, sodium 2-oxoglutarate, sodium pyruvate, Sigmacell type 20 (microcrystalline cellulose), tetrazolium blue, trehalose, xylan (from larch wood), xylose and xylulose.

Other materials were purchased as follows: hide powder azure and Aquacide from Calbiochem-Behring Corp. (La Jolla, CA, USA); sodium carboxymethylcellulose (DS: 0.7 - 0.8) (CMC) and starch (potato) from British Drug Houses (BDH) (Poole, UK); glucose oxidase, GOD-Perid glucose assay kit, lactate dehydrogenase, malate dehydrogenase, pyruvate kinase, TPP, NAD^+, $NADP^+$ and NADPH from Boehringer Mannheim (Mannheim, Germany).

All other chemicals were of analytical reagent grade.

2.2 Termites

Identification of Australian species was carried out by the late Dr. J.A.L. Watson, Department of Entomology, CSIRO, Canberra.

Identification of African species was carried out by Prof. R. H. Leuthold, Abteilung Neurobiologie, Zoologisches Institut, Universität Bern, and

Dr. P. Eggleton, Department of Entomology, The Natural History Museum, London.

Worker caste termites of *Nasutitermes walkeri* Hill were collected from galleries leading to arboreal nests in suburban Sydney, N.S.W. and used within 3 h of collection unless otherwise stated.

Adult reproductives of *Macrotermes subhyalinus* Rambur and *Macrotermes bellicosus* Smeathman were collected from a coconut plantation at N'Djem near the Centre Suisse de Recherches Scientifiques, Abidjan (Ivory Coast) and used to establish small colonies in Petrie dishes (64 mm diameter). Fungus comb from adult colonies was used for inoculation. Young colonies were later transferred to covered plastic boxes (20 x 10 x 8 cm) connected to a food arena and a water bottle. The colonies were maintained in the laboratory at 27°C and 70-80% relative humidity, fed hay *ad lib.*, and were allowed to grow to about 2000 individuals. Termites were used from these incipient colonies.

Small colonies of *Macrotermes michaelseni* Sjøstedt established as described above from adult reproductives collected near Nairobi (Kenya) were kept for 5-8 years under laboratory conditions in Bern (27°C; 70-80% relative humidity) and fed hay *ad lib.* A mound of *M. michaelseni* was established in a large concrete tank from one of these smaller colonies and kept under similar conditions for several years. Groups of about 2000 termites were removed as required.

Incipient colonies of *M. subhyalinus* and *M. michaelseni* were sacrificed to obtain workers, small soldiers, larvae and fungal material. To facilitate the separation of the young and old workers, green hay was substituted for the normal brown hay and the colonies allowed to feed for 3-4 days prior to sacrifice. During this period the young workers (less than 30 days old), which are solely responsible for the passage of the grass through the gut (Badertscher *et al.*, 1983), were found to contain large amounts of green material which could be clearly seen through the cuticle, whereas the guts of the old workers (more than 30 days old) which feed on old fungal comb remained brown. Three types of fungal comb could be differentiated in the termite mound, namely fresh, ripe and old comb (Badertscher *et al.*, 1983). Fresh comb material, the

primary faeces excreted by young workers, was green-brown in colour (when fed on green hay) and consisted of freshly collected grass as well as conidia from a fungus belonging to the genus *Termitomyces* which is used as a fungal inoculum (Leuthold *et al.*, 1989). Ripe fungal comb was brown and consisted of residual plant material and a well-developed mycelium producing aggregated aerial conidiophores (synnemata) which appeared as white spherical bodies (nodules) on the top of the fungal surface. Old fungal comb material was beige-brown in colour, with a reduced density of mycelium and no nodules as these had been eaten by the young workers or had regressed (Martin, 1987).

Mounds of *Macrotermes bellicosus* and *M. subhyalinus* located in a coconut plantation at N'Djem near the Centre Suisse de Recherches Scientifiques, Abidjan, Ivory Coast, were sampled to provide the four categories of workers as previously described and fungal material. Samples of food source were collected where active foraging was occurring (coconut leaves) and food stores collected from the *M. bellicosus* mound. The food store consisted of special pockets of finely comminuted material that was very moist. Dipteran larvae were often present.

A mound of *M. bellicosus* located on the railway line near Yaounde, Cameroon, was sampled to provide fungal material and the categories of workers as previously described. Samples of food stores were also collected. There was incomplete sampling of all age categories of *M. bellicosus* as the foraging site was not found. Consequently active foragers (old workers) could not be included and material near the mound was used as a food source. Difficulties in opening the mound caused most workers to leave the fungal comb for deeper parts of the mound resulting in poor recovery of young workers.

A mound of *Macrotermes mulleri* Sjøstedt located in the Mbalmayo Forest Reserve near the Humid Forest Station of the International Institute for Tropical Agriculture (IITA), Mbalmayo, Cameroon was also sampled. Young workers and old minor workers were collected when fungal comb was removed. The food store consisted of special pockets of dry leaf material cut into small confetti-like pieces (Grassé and Noirot, 1951). Foraging occurred in the open at night, by major workers

accompanied by large and small soldiers. The major workers (foragers) were collected by returning to the mound at 11 p.m. and removing 'bait' piles of leaves placed near the mound. As no leaf material was found in the proximity of the mound, the leaf material taken from another section of forest was used as bait and as the food source.

All *Macrotermes* spp. were collected from the field or laboratory colonies already mentioned and used fresh, freeze-dried or air-dried.

2.3 Digestive enzymes

2.3.1 Enzyme extracts

0.1 M Acetate buffer, pH 5.5, was used in the preparation of all enzyme extracts and assays unless otherwise specified. All operations were carried out at 0-4°C.

All solutions in Mbalmayo were made with Source Tangui Natural Mineral Water (Société des Eaux Minérales du Cameroun, Douala, République du Cameroun) as no RO (reverse osmosis) or distilled water was available. Details of its chemical analysis are listed in Appendix 6.2.

Workers of *N. walkeri* were immobilised by placing them in Petrie dishes on ice. The termites were decapitated and the cuticle around the anus cut. The whole gut was removed by gently pulling on the cuticle attached to the rectum. The gut was then divided into the following segments; salivary glands, foregut, midgut and hindgut or into salivary glands, foregut, midgut, Malpighian tubules, mixed segment and hindgut. Gut segments (10-20) were homogenised in 1 ml of buffer in a Ten Broeck homogeniser. Supernatants were collected from homogenates centrifuged for 10 min at 9,800 *g* and assayed for enzyme activities.

The midgut contents from 10 *N. walkeri* workers were gently squeezed into 1 ml buffer (contents). The emptied guts (epithelia) were then gently rinsed in 1 ml of buffer (wash) and placed in 1 ml of buffer (epithelia). The gut contents, wash and the epithelia were homogenised

and centrifuged for 10 min at 9,800 *g* and supernatants assayed for enzyme activities.

Homogenates were similarly prepared using whole larvae, worker and small soldier castes of *M. subhyalinus* and *M. michaelseni,* using 10 whole termites in 1 ml buffer.

Additionally, whole guts were removed from young and old major worker termites of *M. michaelseni* and divided into the following segments; salivary glands, foregut, midgut, and hindgut. Gut segments (10-20) were homogenised in 1 ml buffer in a Ten Broeck homogeniser. Supernatants were collected from homogenates centrifuged for 10 min at 9,800 *g* and assayed for enzyme activities. The midgut sections from young and old major worker termites of *M. michaelseni* were removed and the contents washed into 1 ml buffer. The epithelial tissue was placed in 1 ml buffer. These were homogenised and the supernatants were collected after centrifugation for 10 min at 9,800 *g* and assayed for enzyme activities.

Fungal material of *Termitomyces* spp. associated with colonies of *M. subhyalinus* and *M. michaelseni* was also homogenised. Fungal comb (fresh, ripe, and old; 100-200 mg) was homogenised in 1 ml buffer and sonicated for 30 s at 40 W. Nodules (10-20 mg) were similarly prepared in 0.5 ml buffer. Supernatants were collected from homogenates centrifuged for 10 min at 9,800 *g* and assayed for enzyme activities.

Similar preparations were carried out using whole workers of *M. bellicosus* and *M. mulleri* and associated fungal material although homogenisation in a Ten Broeck homogeniser was substituted for the sonication steps.

Dilutions of 1/5, 1/50 and 1/100 were prepared from the homogenates of the worker categories and from the fungal nodules for amylase, cellulase, β-glucosidase and endo-β-1,4-glucanase activities. Dilutions of 1/5 were prepared on homogenates from salivary gland, foregut and hindgut for assaying. Dilutions of 1/10, 1/50 and 1/100 were used for the assay of the midgut homogenates.

2.3.2 Enzyme assays

All assays were carried out at 37°C unless otherwise stated.

Cellulase: Cellulase activity is defined as the rate of hydrolysis of microcrystalline cellulose to glucose by the combined actions of the components present in the cellulase complex. Activity was assayed by incubating 0.1 ml extract with 0.2 ml 2% Sigmacell 20 suspension for 1 h. The glucose produced was estimated using 1.0 ml GOD-Perid reagent modified by preparation in 0.5 M Tris-HCl buffer, pH 7.0 and diluted 1 in 2. Assay tubes were centrifuged at 1000 *g* for 5 min to remove suspended Sigmacell 20 and the absorbance read at 610 nm. A Unit of activity is defined as the production of 1 μmol glucose h^{-1}. Activity is expressed as Units $termite^{-1}$, Units mg (wwt tissue)$^{-1}$ h^{-1} or as specific activity, Units mg $protein^{-1}$.

Endo-β-1,4-glucanase: (EC 3.2.1.4). Activity was estimated by incubating 0.1 ml extract with 0.2 ml of 2% CMC solution for 10 min. Reducing sugars were estimated as glucose equivalents using 2 ml tetrazolium blue reagent (Jue and Lipke, 1985) boiled for 10 min. The absorbance was read at 660 nm. A Unit of activity is defined as the production of 1 μmol glucose equivalents h^{-1}. Activity is expressed as Units $termite^{-1}$, Units mg (wwt tissue)$^{-1}$ h^{-1} or as specific activity, Units mg $protein^{-1}$.

β-Glucosidase: (EC 3.2.1.21). Activity was assayed by incubating 0.2 ml of 2% cellobiose with 0.1 ml extract for 15 min. The glucose produced was estimated using GOD-Perid reagent as described above. A Unit of activity is defined as the production of 1 μmol glucose h^{-1}. Activity is expressed as Units $termite^{-1}$, Units mg (wwt tissue)$^{-1}$ h^{-1} or as specific activity, Units mg $protein^{-1}$.

Amylase: Amylase activity is defined as the rate of hydrolysis of starch to glucose by the combined actions of all of the components present in the amylase complex. Activity was assayed by incubating 0.1 ml extract with 0.2 ml 2% potato starch suspension for 1 h, the glucose produced being estimated using 1 ml GOD-Perid reagent as described above. A Unit of activity is defined as the production of 1 μmol glucose h^{-1}. Activity is

expressed as Units termite^{-1}, Units mg (wwt tissue)$^{-1}$ h^{-1} or as specific activity, Units mg protein^{-1}.

α-Amylase: (EC 3.2.1.1). Activity was estimated using two methods.
a) Extract (0.1 ml) was incubated with a 0.2 ml 1% potato starch suspension for 1 h. The reducing sugars were estimated as maltose equivalents using the tetrazolium blue method (Jue and Lipke, 1985) and measuring the absorbance at 660 nm. A Unit of activity is defined as the production of 1 μmol maltose equivalents h^{-1}. Activity is expressed as Units termite^{-1}, Units mg (wwt tissue)$^{-1}$ h^{-1} or as specific activity, Units mg protein^{-1}.
b) The Phadebas Amylase test utilising an insoluble dye-linked substrate was also used. Extract (0.1 ml) was incubated with 1 ml suspension (1 Phadebas tablet 10 ml^{-1}) for 1 h and the absorbance read at 595 nm. A Unit of activity is defined as the production of an absorbance of 1.0. Activity is expressed as Units termite^{-1}, Units mg (wwt tissue)$^{-1}$ h^{-1} or as specific activity, Units mg protein^{-1}.

α-Glucosidase: (EC 3.2.1.27). Activity was assayed by incubating 0.1 ml extract with 0.2 ml 1% maltose for 30 min, using the GOD-Perid reagent as described above to estimate the glucose produced. A Unit of activity is defined as the production of 1 μmol glucose h^{-1}. Activity is expressed as Units termite^{-1}, Units mg (wwt tissue)$^{-1}$ h^{-1} or as specific activity, Units mg protein^{-1}.

Endo-β-1,4-xylanase: (EC 3.2.1.8). Activity was estimated by incubating 0.1 ml extract with 0.2 ml 1% xylan suspension. Reducing sugars were estimated as xylose equivalents using the tetrazolium blue method (Jue and Lipke, 1985) and measuring the absorbance at 600 nm. A Unit of activity is defined as the production of 1 μmol xylose equivalents h^{-1}. Activity is expressed as Units termite^{-1}, Units mg (wwt tissue)$^{-1}$ h^{-1} or as specific activity, Units mg protein^{-1}.

Protease: Activity was assayed by incubating 0.1 ml extract with 10 mg hide powder azure in 1.0 ml buffer at 30°C for 1 h. One unit of protease activity is defined as the amount of enzyme which produces an absorbance of 1.0 at 595 nm in 1 h.

Standards: Standard curves (0-0.1 or 0-0.2 µmol monosaccharide) were run in parallel with enzyme assays.

Limits of detection: Limits of detection were 5 nmol for glucose, using the GOD-Perid reagent and 10 nmol for glucose and xylose using the tetrazolium blue method.

Protein was determined by the method of Lowry (1951) using BSA as a standard. Limit of detection was 0.5 µg protein.

2.3.3 Localisation of enzyme activity

The foregut, midgut, mixed segment and first proctodeal segment were removed from a *N. walkeri* worker, set in 2% agar made up in 0.1 M acetate buffer, pH 5.5, and frozen in liquid nitrogen. Samples of sections (20 µm), made using a cryomicrotome (American Optical Corporation), were examined by light microscopy to detect the junctions of the foregut and midgut and the midgut and mixed segment. Sections between these junctions were collected in small glass tubes and kept at 4°C until assayed for enzyme activity using the following methods. The experiment was repeated to determine the consistency of the pattern of enzyme distribution although the individual data points were not statistically analysed.

Endo-β-1,4-glucanase: Activity was assayed by incubating 1 x 20 µm (Fig. 2) or 2 x 20 µm (Fig. 1) gut sections, 0.1 ml buffer, and 0.2 ml 2% CMC in buffer in 40°C for 2 h and the reducing sugars produced then estimated as previously described.

β-Glucosidase: Activity was assayed by incubating 2 x 20 µm gut sections, 0.1 ml buffer, and 0.2 ml 2% cellobiose in buffer at 40°C for 2 h and the glucose produced then estimated as previously described.

α-Amylase: Activity was assayed by incubating 9 x 20 µm gut sections, 0.1 ml buffer, and 0.2 ml 2% potato starch suspension in buffer at 30°C for 2 h. Reducing sugars were estimated with tetrazolium blue reagent (Jue and Lipke, 1985) and expressed as maltose equivalents.

2.3.4 Enzyme stability and concentration

To test the stability of enzymes to freeze-drying, whole termites of *M. subhyalinus, M. michaelseni* (all castes and categories as described previously) and fungal material associated with these colonies were weighed into microcentrifuge tubes and freeze-dried. Extracts were prepared and assayed using fresh material or freeze-dried material stored at room temperature or -20°C, prepared and assayed 4-14 days after freeze-drying.

Sucrose and BSA (0 -10% w/v) were tested as preservatives on cellulase activities in extracts of *N. walkeri* which were freeze-dried, resuspended and assayed 7 days later.

Fractions were concentrated using a number of techniques; ultrafiltration in an Amicon® stirred cell (Model 8010, Amicon, Danvers, MA, USA) using pressurised nitrogen and Amicon PM-30 and YM-10 membranes, using Centricon® 30 microconcentrators (Amicon) or by placing solutions in cellulose acetate dialysis tubing and dehydrating with Aquacide®.

2.3.5 Preparation of *N. walkeri* extracts

The salivary glands, foregut and midgut sections of 1000 *N. walkeri* workers were prepared by decapitation, the removal of the hindgut and posterior midgut and as much of the cuticle and fat body tissue as possible without disturbing the salivary glands *in situ*. The tissues were homogenised in 4 ml 0.02 M histidine-HCl buffer, pH 5.8 and centrifuged at 20,000 *g*. The crude extract (3 ml supernatant) was applied to a Bio-Gel P-150 column equilibrated with 0.02 M histidine-HCl buffer, pH 5.8.

2.3.6 Preparation of *Macrotermes* spp. extracts

Supernatants from homogenates of *M. michaelseni* of young and old major and minor worker categories and fungal nodules prepared in Bern for enzyme assays were made 4% wrt sucrose, freeze-dried, transported

to Sydney and stored at 0-5°C. They were redissolved in water and combined to produce an extract equivalent to 30 termites ml^{-1} or 30 mg fungal nodules ml^{-1}. Extracts were centrifuged for 10 min at 9,800 *g* and the supernatants applied to Bio-Gel P-60 and Bio-Gel P-150 columns at 0-5°C.

Supernatants were prepared from homogenates of 200 young major workers and the fungal nodules of *M. bellicosus* and of the fungal nodules of *M. subhyalinus* (30-80 mg nodules ml^{-1}). These were applied to a Bio-Gel P-150 column in Abidjan at room temperature. Fungal nodules were air-dried over silica gel or freeze-dried and then transported to Sydney and stored at 0-5°C. Extracts were prepared as described above using 60 mg nodules ml^{-1} and applied to a Bio-Gel P-150 column at room temperature.

Supernatants were prepared from the homogenates of 60 major workers, 60 minor workers and fungal nodules (60 mg nodules ml^{-1}) from *M. mulleri*. These were applied to a Bio-Gel P-150 column at room temperature in Mbalmayo.

2.3.7 Molecular sieve chromatography

Columns were calibrated using the following protein standards: cytochrome *c*, ovalabumin, BSA (Fraction V) and glucose oxidase.

Bio-Gel® P-60: A Bio-Gel® P-60 (100-200 mesh, Bio-Rad, Richmond, CA, USA) column (40 x 2.2 cm) was equilibrated and the proteins eluted with 0.1 M acetate buffer, pH 5.5, at 0-5°C at a flow rate of 15 ml h^{-1}. Fractions (4 ml) were collected using a LKB Fraction Collector.

Bio-Gel® P-150:

1. A Bio-Gel® P-150 (100-200 mesh, Bio-Rad, Richmond, CA, USA) column (23 x 3.5 cm) was equilibrated at room temperature (25-27°C) in Abidjan, Mbalmayo and Sydney. Fractions were eluted with 0.1 M acetate buffer, pH 5.5, at a flow rate of 15 ml h^{-1}. Fractions were collected using a LKB Fraction Collector in Sydney. In Mbalmayo fractions were manually collected in test tubes, using weight difference to

estimate the volume of the fractions. In Abidjan fractions were manually collected using 2 ml graduated cylinders.

2. A Bio-Gel® P-150 (100-200 mesh; Bio-Rad, Richmond, CA, USA) column (23 x 3.5 cm) was equilibrated at room temperature and the proteins eluted with 0.02 M histidine-HCl buffer, pH 5.8, at a flow rate of 8 ml h^{-1}. Fractions (1-2 ml) were collected using a LKB Fraction Collector.

Bio-Gel® A-0.5 m: A column (900 x 1.8 cm) of Bio-Gel® A-0.5 m (200-400 mesh; Bio-Rad, Richmond, CA, USA) was equilibrated at 0-5°C and the proteins eluted with 0.1 M acetate buffer, pH 5.5, at a flow rate of 10 ml h^{-1}. Fractions (3 ml) were collected using a LKB Fraction Collector.

2.3.8 Enzyme and protein assays

Fractions were assayed for cellulase, β-glucosidase, endo-β-1,4-glucanase, and/or α-glucosidase and amylase activities using previously described assays modified by using 50 µl aliquot or 50 µl appropriate dilutions with 100 µl substrate with 1 ml of reagent added. Protein was detected by measuring the absorbance at 280 nm and by using the bicinchoninic acid (BCA) microassay technique of (Smith *et al.*, 1985). Extract (100 µl or dilution to 100 µl) and BSA standards were added to an equal volume of BCA working reagent in the well of an ELISA tray (Disposable Products, Technology Park, SA, Australia). The tray was incubated at 40°C for 2 h and the absorbance at 595nm measured using a microplate reader (Titertek Multiskan). Limits of detection were 0.02 µg protein.

2.3.9 Ion exchange chromatography

1. A prepacked Mono Q HR 5/5 FPLC anion-exchange column (Pharmacia, Uppsala, Sweden) was equilibrated at room temperature with 0.02 M piperazine buffer, pH 5.5, using a Pharmacia FPLC Controller (model LCC-500, Uppsala, Sweden). Samples were applied to the column in the same buffer using a 10 ml Superloop (Pharmacia, Uppsala, Sweden) at 0.5 ml min^{-1} and then eluted via a two stage linear

gradient from 0 to 0.4 M NaCl in the equilibration buffer over 35 min and then to 1.0 M over 10 min. Fractions (0.5 ml) were collected and assayed.

2. A mono Q column was equilibrated as above at room temperature with 0.02M histidine-HCl buffer, pH 5.8. Fractions from *N. walkeri* crude extract run on Bio-Gel P150 were applied directly to the column and eluted via a two stage linear gradient as above. Fractions rerun were subjected to a two stage linear gradient from 0 to 0.15 M NaCl in the equilibration buffer over 10 min and then to 1 M over 20 min. Fractions (0.5 ml) were collected and assayed.

2.3.10 Hydroxylapatite chromatography

Samples of pooled fractions from Bio-Gel P-150 chromatography were exchanged with 0.01 M Na phosphate buffer, pH 5.8, using new YM-10 ultrafiltration membranes. The concentrate (2 ml) was applied to an Econo-Pac® HTP cartridge (1 ml; Bio-Rad, Richmond, CA, USA) equilibrated at room temperature in the same buffer. Samples were eluted stepwise with 3.0 ml buffer containing the following concentrations of the same buffer; 0.02 M, 0.05 M, 0.16 M, 0.4 M and 1 M. Fractions (1 ml) were collected and assayed.

2.3.11 Hydrophobic interaction chromatography (HIC)

Samples of pooled fractions from Bio-Gel P-150 chromatography were exchanged with 0.1 M Na phosphate buffer, pH 6.5, 2.4 M wrt $(NH_4)_2SO_4$ using new YM 10 ultrafiltration membranes. The concentrate (5 ml) was applied to an Econo-Pac® Methyl HIC cartridge (1 ml; Bio-Rad, Richmond, CA, USA) that was equilibrated at room temperature with the above buffer. Samples were eluted stepwise with 2.0 ml buffer containing the following concentration of $(NH_4)_2SO_4$; 2.4 M, 1.8 M, 1.2 M, 0.6 M, 0.24 M, and 0 M. Fractions (1 ml) were collected and the $(NH_4)_2SO_4$ removed by ultrafiltration as above before assaying.

2.3.11 Chromatofocusing

An extract of fungal nodules (90 mg) was prepared in 0.25 M histidine-HCl buffer, pH 6.2) and applied to a column (40 x 1 cm) of PBE 94 Polybuffer exchanger (Pharmacia, Uppsala, Sweden) equilibrated at room temperature with histidine buffer. Samples were eluted with Polybuffer 74-HCl (Pharmacia, Uppsala, Sweden), pH 3.5 and fractions (2 ml) collected. The absorbance at 280 nm and the pH were recorded and the fractions assayed.

2.3.12 SDS-PAGE

Electrophoresis (SDS-PAGE) was performed at room temperature according to the method of Laemmli (1970). A Tall Mighty Small SE280 (Hoefer Scientific Instruments, San Francisco, CA, USA) vertical slab gel unit was used with a 4% stacking gel and a 12% resolving gel. Protein samples (10 µl) were either reduced by the addition of an equal volume of treatment buffer (0.125 M Tris-HCl, pH 6.8; 4% SDS; 20% glycerol; 10% 2-mercaptoethanol) or prepared non-reduced using treatment buffer without 2-mercaptoethanol. Bromophenol Blue was used as tracking dye. Application volume was 20 µl per well. Electrophoresis was performed at 125 V for 3 h at room temperature. Protein bands were visualised by the silver staining method of Morrissey (1981). A mixture of molecular weight standards consisting of α-lactalbumin, trypsin inhibitor, trypsinogen, carbonic anhydrase, glyceraldehyde 3-phosphate dehydrogenase, ovalbumin, and bovine serum albumin was used to estimate the apparent molecular weights (M_r). These standards were prepared reduced or denatured in an identical manner to the protein samples.

2.4 Gut and haemolymph parameters

2.4.1 Redox and pH

Groups of *N. walkeri* workers (100) were placed in agar micronests ((Eutick *et al.*, 1976)) with a selection of wood impregnated with

thionine, methylene blue, nile blue or phenosafranin (Veivers *et al.*, 1980) covering a E_1^0 range of -312 to +123 mV. At appropriate intervals, termites (10-20) were removed and rapidly degutted. The colours were immediately noted. The presence of dye was checked by exposing the gut contents to air or sodium dithionite and noting any colour changes. The colour of the guts of workers fed on the dyes was recorded and expressed as a percentage ratio of termites with fully oxidised dye (coloured):partially reduced dye (light colour which darkens on exposure to air):fully reduced dye form (colourless).

The pH of gut sections was estimated using two methods. The guts were removed from 10 *N. walkeri* workers, gently rinsed in RO water and divided into the following sections; foregut, midgut, paunch, colon, and rectum. In the first method, the sections were pooled in a drop of water on a glass slide and broken open using a needle. A strip of narrow range indicator paper (Whatman-BDH, Poole, UK; Merck, Darmstadt, Germany) was placed over the suspension and covered with a glass slide. The colour development was noted. pH ranges used were pH 2.5-4.0, 4-6, 6-8, 8-10. Each pH range was carried out on 3 groups of termites (Bignell and Eggleton, 1995). The pH of the rectum was also estimated by testing faeces voided during handling or by encouraging workers to defaecate by abdominal prodding onto a glass slide or onto narrow range indicator paper as above.

In the second method, individual termite guts were removed, rinsed and the gut sections divided as above, with the midgut divided into anterior and posterior sections, and the mixed segment and first proctodeal segment. The contents of individual sections were leaked into a small drop of pH indicator dye in a spotting tile and the colour noted.

2.4.2 Hindgut volume

The volume of the hindgut of workers of *N. walkeri* was estimated by diluting 10 isolated hindguts with ^{14}C-polyethylene glycol 4000 solution (PEG). The hindguts were removed and gently blotted on tissues to remove adhering haemolymph. They were then broken with a dissecting needle and mixed well with 5 µl ^{14}C-PEG containing 11,000

dpm μl^{-1}. Samples (5 µl) were added to 0.5 ml scintillant (Optiphase 'HiSafe' 3; LKB Scintillation Products, Stockholm, Sweden) in 1.5 ml microcentrifuge tubes and counted in a Beckman (Model LS 3800) liquid scintillation counter (San Ramon, CA, USA). Samples were corrected for quenching using [U-^{14}C]glucose as a standard.

2.4.3 Haemolymph volume

The haemolymph volume of workers of *N. walkeri* was estimated using the method of (Wharton *et al.*, 1965a). A Burkard Arnold Hand Microapplicator (Rickmansworth, Herts, UK) was fitted with a 50 µl syringe (Hamilton, Reno, NV, USA) connected by tubing to a needle tip formed by drawing out a 1 µl capillary tube and standardised to deliver 160 nl. The syringe and tubing were pre-filled with paraffin dyed red with Sudan IV; the needle tip was then emptied and refilled with ^{14}C-PEG containing 11,000 dpm μl^{-1}. The border between the PEG and filler was easy to distinguish. Workers (10) were individually secured with adhesive tape to glass slides and by using a dissecting microscope, were injected with 120 nl of PEG through the pleural membrane at the junction of the thorax and abdomen. They were then incubated for 1 h in petrie dishes lined with damp filter paper. Individual haemolymph samples were withdrawn using 1 µl microcapillaries and the amount of haemolymph was estimated using the ratio of the length of the microcapillary filled to the total length of the capillary. The haemolymph was applied to glass fibre discs pretreated with 100 µl of 1 M NaOH and allowed to dry. The remaining termite material was individually homogenised and dissolved in 200 µl 1 M NaOH. Samples (100 µl) were applied to glass fibre discs and dried. Scintillant (10 ml of 0.6% PPO in toluene:triton X-100{1:1}) was added and samples counted in a Beckman (Model LS 3800) liquid scintillation counter (San Ramon, CA, USA). Samples were corrected for quenching using [U-^{14}C]glucose as a standard.

2.5 Metabolic enzymes

2.5.1 Enzyme extracts

Sixty termites (workers of *N. walkeri*) were degutted and the hindguts removed by severing the junction of the mixed segment and first proctodeal segment. The hindguts were ruptured into buffer and carefully rinsed to remove contents. The remaining epithelial tissue was placed in buffer. All were placed in 0.6 ml homogenising buffer. Thus the termites were separated into the degutted body (also containing the foregut, midgut and mixed segment), washed epithelial tissue (containing bacteria attached to the gut wall (Czolij *et al.*, 1985)) and the hindgut contents (containing >99% symbiotic flora and wood particles (Hogan *et al.*, 1988a)). Prepared tissues were sonicated with a Branson B-15 cell disrupter operated for 30 pulses at position 5 and 30% power. After centrifugation the supernatants were removed and applied to Sephadex® G25 (Pharmacia, Uppsala, Sweden) columns to remove pigments, substrates, cofactors and inhibitors. The fraction recovered was termed the extract for the following assays. All operations were carried out at 0-4°C. Enzyme controls were prepared by heating the prepared enzyme extracts to 100°C for 10 min and centrifuging.

2.5.2 Enzyme assays

All incubations were carried out at 25°C.

Enzyme activities are expressed as nmol substrate utilised or product formed h^{-1} $termite^{-1}$.

Assays involving changes in UV/Vis absorbances were carried out by using a Hewlett-Packard HP8452A diode array spectrophotometer interfaced with a Hewlett-Packard Vectra PC. Data were analysed using the HP 89530A UV/Vis software.

Acetyl CoA synthetase (EC 6.2.1.1): An extract was prepared with 0.05 M Tris-HCl buffer, pH 7.5 as the homogenising buffer. The reaction mixture (1 ml) consisted of 0.2 ml extract with CoA (25 units),

20 µmol CH_3COOK, 10 µmol ATP, 10 µmol $MgCl_2$, 0.2 mmol hydroxylamine (pH 7.4) and 10 µmol glutathione (reduced). The reaction was terminated at 30 min by the addition of 1.5 ml 10% $FeCl_3.6H_2O$ in 3.3% TCA in 0.66 M HCl and the hydroxamate produced was measured at 540 nm (Jones and Lipmann, 1955) using acetohydroxamic acid as a standard.

Isocitrate dehydrogenase (EC 1.1.1.4): An extract was prepared with 0.05 M Tris-HCl buffer, pH 7.5 as the homogenising buffer. The reaction mixture (1 ml) contained Tris-HCl buffer pH 7.5 (100 mM), 10 µmol $MgCl_2$, 1.3 µmol $NADP^+$ and 1.7 µmol isocitrate (O'Brien and Breznak, 1984) and was assayed spectrophotometrically at 340 nm. The activity was calculated using a molar extinction coefficient for NADPH of 6,220 M^{-1} cm^{-1} at 340 nm.

Isocitrate lyase (EC 4.1.3.1): An extract of 60-120 termite bodies or guts in 0.6 ml was made with 0.05 M Tris-HCl buffer, pH 7.9 as the sonication buffer. The reaction mixture (1 ml) contained Tris-HCl buffer, pH 7.9 (50 mM), 0.5 µmol $MgCl_2$, 2 µmol cysteine (neutralised) and 10 µmol sodium isocitrate. The reaction (1 ml) was terminated after 30 min with 25% TCA. The glyoxylate produced was estimated using oxalic acid-phenylhydrazine HCl and ferricyanide. The absorbance was measured at 520 nm (McFadden, 1969).

Malate dehydrogenase (EC 1.1.1.37): The enzyme was assayed in both directions.
Malate to OAA: Extract was prepared using 0.1 M Tris-HCl buffer, pH 9.2 as the homogenising buffer. The reaction mixture (1 ml) contained 0.1 ml extract, 0.3 µmol $MgCl_2$, Tris-HCl buffer pH 9.2 (100 mM), 0.3 µmol NADH and 1 µmol malic acid (neutralised with NaOH) (Smith, 1983). The activity was calculated using a molar extinction coefficient for NADH of 6,220 M^{-1} cm^{-1} at 340 nm.

OAA to malate: Extract was prepared using 0.05 M Tris-HCl buffer, pH 7.5 as the homogenising buffer. The reaction mixture (1 ml) contained 0.1 ml extract, Tris-HCl buffer pH 7.5 (100 mM), 0.2 µmol NADH and 0.5 µmol OAA (O'Brien and Breznak, 1984). The activity

was calculated using a molar extinction coefficient for NADH of 6,220 M^{-1} cm^{-1} at 340 nm.

The following enzymes were assayed using 0.1 ml extract prepared with 10 mM TEA buffer, pH 7.6 containing 0.6 mmol EDTA as the homogenising buffer.

Hexokinase (EC 2.7.1.2): A modification of the method of Patni (1971) was used. The reaction mixture (1 ml) contained TEA buffer, pH 7.6 (60 mM), 0.06 mmol $MgCl_2$, 0.03 mmol ATP, 0.4 µmol $NADP^+$ and 0.6 units glucose 6-phosphate dehydrogenase. After initial baseline recording at 340 nm, the reaction was initiated by the addition of 100 µl of 0.15 M glucose. The activity was calculated using a molar extinction coefficient for NADPH of 6,220 M^{-1} cm^{-1}.

Phosphofructokinase (EC 2.7.1.11): A modification of the method of Gottschalk (1982) was used. The assay mixture (1.5 ml) contained Tris-HCl buffer, pH 8.0 (15 mM), 20 µmol $MgCl_2$, 6 µmol KCl, 4.7 µmol ATP (neutralised), 0.6 µmol PEP (neutralised) and 10 µl pyruvate kinase (2 mg ml^{-1}). The reaction was started by the addition of 100 µl 40 mM fructose 6-phosphate and incubated for 15 min. The reaction was terminated by placing the tubes in a boiling water bath for 2 min. After centrifugation, 50-100 µl of supernatant was used to measure the amount of pyruvate present. The reaction mixture contained potassium phosphate buffer, pH 7.0 (100 mM), 0.15 µmol NADH, and 10 µl lactate dehydrogenase (5 mg ml^{-1}). The activity was calculated using a molar extinction coefficient for NADH of 6,220 M^{-1} cm^{-1}.

Xylulokinase (EC 2.7.1.17): The method of (Simpson, 1966) was used. The assay mixture (1.0 ml) contained Tris-HCl pH 7.8 (0.1 M), 0.05M KCl, 1 mM EDTA, 1 mM $MgCl_2$, 0.3 µmol NADH, 0.25 mmol ATP (neutralised) and 10 µl lactate dehydrogenase (5 mg ml^{-1}). The reaction was initiated with 50 µl xylulose (0.01 M). The activity was calculated using a molar extinction coefficient for NADH of 6,220 M^{-1} cm^{-1}.

Pyruvate kinase (EC 2.71.40): The method of (Beutler and Supp, 1983) was used. The reaction mixture (1 ml) contained TEA pH 7.6 (5 mM), 0.05 mmol $MgCl_2$, 0.05 mmol KCl, 5 µmol ADP (neutralised with

KOH), 0.2 μmol NADH and 9 units lactate dehydrogenase. After initial baseline recording at 340 nm, the reaction was initiated with 100 μl PEP (0.1 M, neutralised). The activity was calculated using a molar extinction coefficient for NADH of 6,220 M^{-1} cm^{-1}.

The following enzymes were assayed using 0.1 ml extract prepared with 0.05 M Tris-HCl buffer, pH 8.0 as the homogenising buffer.

Acetyl CoA hydrolase (EC 3.1.2.1): A modification of the method of (Pras *et al.*, 1980) was used. The reaction mixture (1 ml) contained Tris-HCl buffer pH 7.8 (100 mM), 1 μmol acetyl CoA, 0.4 μmol DTNB. The activity was calculated as the reduction of DTNB by CoA to 5-thio-2-nitrobenzoate, using a molar extinction coefficient for DTNB of 13,600 M^{-1} cm^{-1} at 412 nm.

Citrate synthase (EC 4.1.3.7): A modification of Srere (1969) was used. The reaction mixture contained Tris-HCl buffer pH 7.8 (100 mM), 1 μmol acetyl CoA, 0.4 μmol DTNB and 1 mM sodium oxaloacetate. The activity was calculated using a molar extinction coefficient for DTNB of 13,600 M^{-1} cm^{-1} at 412 nm.

Malate synthase (EC 4.1.3.2): A modification of (Dixon and Kornberg, 1962) was used. The 1 ml reaction mixture contained Tris-HCl buffer pH 7.8 (100 mM), 1 μmol acetyl CoA, 20 μmol $MgCl_2$, 0.4 μmol DTNB and 10 μmol glyoxylate. The activity was calculated using a molar extinction coefficient for DTNB of 13,600 M^{-1} cm^{-1} at 412 nm.

2–oxoglutarate dehydrogenase (EC 1.2.4.2): Two methods were used.

1. The UV method used 0.1 M Tris-HCl buffer, pH 7.8 containing 1 mM $MgCl_2$, 0.5 mM EDTA and 5 mM mercaptoethanol as the homogenising buffer. The assay mixture (1 ml) contained potassium phosphate buffer, pH 7.0 (0.02 M), 4 μmol $MgCl_2$, 0.2 μmol thiamine pyrophosphate (TPP), 6.4 μmol cysteine (neutralised), 0.1 μmol CoA, 0.23 μmol NAD^+ and 10 μmol 2-oxoglutarate (Reed and Mukherjee, 1969). The activity was calculated using a molar extinction coefficient for NADH of 6,220 M^{-1} cm^{-1} at 340 nm.

2. A radioassay measuring the formation of $^{14}CO_2$ from [1-^{14}C]2-oxoglutarate was used. For the assay 30 termites were prepared in 0.6 ml of 0.02 M potassium phosphate buffer, pH 7.0. Warburg flasks prepared with 1 ml of the assay mixture used in method 1 above with 0.1 ml extract but containing 10,000 dpm of [1-^{14}C]2-oxoglutarate were incubated for 15 min. The reaction was stopped with 100 µl 20% PCA. The CO_2 trapped in 100 µl 2 M NaOH in the centre well was transferred to glass fibre discs impregnated with 100 µl 10% $(CH_3COO)_2Ba$, dried and placed in scintillation vials containing 5 ml 0.3% PPO in toluene and counted.

2-oxoglutarate dehydrogenase activity was also measured in extracts prepared in the presence of chloramphenicol (25 µg ml^{-1}) to prevent protein synthesis after removal of the hindgut and subsequent exposure of the bacteria to atmospheric oxygen.

Pyruvate dehydrogenase complex (EC 1.2.4.1): Three methods were used to measure the activity of the pyruvate dehydrogenase complex.

1. The UV method of (Reed and Mukherjee, 1969) described for 2-oxoglutarate dehydrogenase (assay 1) was used with sodium pyruvate substituting for the 2-oxoglutarate.

2. Activity was also measured spectrophotometrically by coupling the acetyl CoA produced with *p*-(*p*-aminophenylazo)-benzene sulphonic acid (AABS) through the addition of arylamine acetyltransferase prepared from pigeon liver extract (Tabor *et al.*, 1953). The assay mixture contained 0.2 ml extract, 0.1 µmol CoA, 0.1 µmol pyruvate, 1 µmol TPP, 0.5 µmol NAD^+ and 20 µg AABS. PDH activity was calculated from the decrease in absorbance at 460 nm using a molar extinction coefficient for AABS of 6,500 M^{-1} cm^{-1} (Coore *et al.*, 1971).

3. A radioassay as described for the 2-oxoglutarate dehydrogenase (assay 2) was used with [1-^{14}C]pyruvate substituting for the 2-oxoglutarate.

PDH activity was also measured in extracts prepared in the presence of chloramphenicol (25 µg ml^{-1}) to prevent protein synthesis after removal

of the hindgut and subsequent exposure of the bacteria to atmospheric oxygen.

PDH activity was also investigated in degutted termite bodies incubated for 10-30 min in insect saline (Chappell and Slaytor, 1991) containing [1-^{14}C]pyruvate, using the method described above. Silverfish, bogong moth or woodroach *P. cribrata* tissues were used as positive controls.

Pyruvate-ferredoxin oxidoreductase (EC 1.2.7.1): Termites (30) were dissected and separated into degutted body and hindgut plus epithelium. Tissues were homogenised in 0.3 ml potassium phosphate buffer (0.06 M), pH 6.8, containing 0.04 M EDTA and 0.5 mg ml^{-1} reduced glutathione and sparged with nitrogen. Activity was determined by the formation of $^{14}CO_2$ from [1-^{14}C]pyruvate (Mathers and Annison, 1993). Warburg flasks containing 100 µl 2 M NaOH in the centre well and 0.06 M potassium phosphate buffer, pH 6.8, 0.5 µmol CoA, 25 µmol 2-mercaptoethanol and 0.0625 µmol FAD in the outer well were sealed with Subaseals® and gassed with nitrogen. Extract (0.1 ml) was injected into the flasks, gassed with nitrogen and the reaction started by the injection of 100 µl pyruvate (125 mM solution containing [1-^{14}C]pyruvate, 10,000 dpm). The reaction was terminated after 15 min by the injection of 100 µl 20% PCA. In order to completely evolve all the CO_2 200 µl $NaHCO_3$ was injected and the reaction mixture allowed to stand for 10 min. CO_2 trapped in the centre well was transferred to glass fibre discs, treated as previously described, and counted.

The following enzymes were assayed using extract prepared with 0.01 M Tris-HCl buffer, pH 7.0 as the homogenising buffer.

Malic enzyme (EC 1.1.1.40): Activity was assayed using a modification of Adam (1969) and (Outlaw and Springer, 1983), using $H^{14}CO_3^-$ fixation. The reaction mixture (1.0 ml) contained 0.1 ml extract, 0.1 M Tris-HCl buffer, pH 7.6, 30 µmol Mg_2SO_4, 1 µmol NADPH, 0.5 mg K_2SO_4 and 5 µmol DTT. The reaction was started with 100 µl 0.5 M pyruvate and 100 µl 0.2 M $KH^{14}CO_2$ containing 500,000 dpm. After 30 min incubation the reaction was terminated with 100 µl 20% PCA and sparged with CO_2 for 6 min to remove excess $^{14}CO_2$. Samples from the incubation (0.5 ml) were place in 1.5 ml

microcentrifuge tubes, 1.0 ml scintillant (Optiphase 'HiSafe' 3; LKB Scintillation Products, Stockholm, Sweden) was added and the samples counted.

Malic enzyme (EC 1.1.1.39): Activity was assayed using the method for malic enzyme (EC 1.1.1.40), substituting NADH for NADPH.

Phosphoenolpyruvate (PEP) carboxykinase (EC 4.1.1.49): Activity was assayed using the $H^{14}CO_3^-$ fixation method of Lane (1969). The reaction mixture (1.0 ml) contained 0.1 ml extract, 0.1 M imidazole-HCl buffer, pH 6.6, 1.25 µmol IDP, 1 µmol $MnCl_2$, 2 µmol GSH, 2.5 µmol NADH and 5 Units of malate dehydrogenase. The reaction was started with 100 µl 12.5 mM PEP (neutralised) and 100 µl 0.2 M $KH^{14}CO_2$ containing 500,000 dpm. After 30 min incubation the reaction was terminated with 100 µl 20% PCA and sparged with CO_2 for 6 min to remove excess $^{14}CO_2$. Samples from the incubation (0.5 ml) were place in 1.5 ml microcentrifuge tubes, 1.0 ml scintillant (Optiphase 'HiSafe' 3; LKB Scintillation Products, Stockholm, Sweden) was added and the samples counted.

Pyruvate carboxylase (EC 6.4.1.1): Activity was assayed using a modification of Scrutton (1969), using $H^{14}CO_3^-$ fixation. The reaction mixture (1.0 ml) contained 0.1 ml extract, 0.15 M Tris-H_2SO_4 buffer, pH 7.8, 0.875 µmol ATP (neutralised), 0.083 µmol acetyl CoA, 6.7 µmol Mg_2SO_4, 2.5 µmol NADH and 5 Units of malate dehydrogenase. The reaction was started with 100 µl 0.5 M pyruvate and 100 µl 0.2 M $KH^{14}CO_2$ containing 500,000 dpm. After 30 min incubation the reaction was terminated with 100 µl 20% PCA and sparged with CO_2 for 6 min to remove excess $^{14}CO_2$. Samples from the incubation (0.5 ml) were place in 1.5 ml microcentrifuge tubes and 1.0 ml scintillant (Optiphase 'HiSafe' 3; LKB Scintillation Products, Stockholm, Sweden) added and counted.

Phosphoenolpyruvate-dependent hexose transferase (PTS): Activity was assayed using a modification of the method of (Kornberg and Reeves, 1972). The hindgut contents of 25 workers were prepared as previously described using 1.0 ml 0.1 M NaK phosphate buffer, pH 7.2,

containing 5 mM $MgCl_2$. A bacterial pellet was prepared. The suspensions were centrifuged for 10 s and the supernatant removed. The pellet was washed with 1.0 ml buffer and centrifuged for 10 s and the supernatant removed. These supernatants were then centrifuged for 10 min. The supernatants were discarded and the pellets combined and resuspended in 1.0 ml buffer. After another centrifugation step the pellets were resuspended in 1.0 ml buffer and kept on ice. Immediately before the assay 250 µl bacterial suspension was placed in a small culture test tube and agitated at top speed on a vortex mixer, while 2.5 µl toluene-ethanol (1:9 v/v) was added. The suspension was agitated for 60 s and used immediately in the assay procedure.

The standard PTS assay (Kornberg and Reeves, 1972) was performed by adding 10-100 µg dry weight (determined by multiplying the absorbance of the cell suspension at 680 nm by 0.68) of toluene treated cells to a glass cuvette containing 1 µmol PEP (neutralised), 0.1 µmol NADH and 0.3 µg lactate dehydrogenase to a volume of 1.0 ml. The assay mixture was preincubated at 25°C and monitored for reaction with endogenous carbohydrates. The reaction was started by the addition of 100 µl glucose (50mM) and the absorbance change measured at 340 nm. Activity was calculated from the linear rate using a molar extinction coefficient for NADH at 340 nm of 6,220 M^{-1} cm^{-1}.

2.5.3 Scintillation counting

Radioactivity was measured either in a Beckman (Model LS 3800) (San Ramon, CA, USA) or in a Wallac (Model 1410; Pharmacia, Uppsala, Sweden) liquid scintillation counter. Samples processed by the Beckman counter were corrected for quenching using [U-^{14}C]glucose as a standard; the Pharmacia counter was programmed to correct automatically for quenching and chemiluminescence.

2.5.4 Oxygen utilisation by the hindgut

The hindgut contents (bacteria, solid and soluble components) from 40 *N. walkeri* workers were prepared by rupturing the hindguts into 1 ml

0.1 M potassium phosphate buffer, pH 7.0. The hindguts were carefully rinsed to remove gut contents without the addition of epithelial tissue. Immediately after dissection, the suspension was mixed and transferred to an O_2 electrode (Rank Brothers, Bottisham, Cambridge, UK) calibrated and maintained at 25°C and containing 0.5 ml 0.1 M potassium phosphate buffer, pH 7.0. After an initial rate was established substrates (10-200 µl) were injected into the cell and the rate change measured. Data were acquired using a Maclab/2e data acquisition system (AD Instruments, Castle Hill, NSW, Aust.) with Chart™ v3.3. Substrates used were 0.1 M acetate, 0.2 M alanine, 0.1 M glucose, 0.2-0.05 M pyruvate and 0.1 M xylose.

2.6 Metabolic products

2.6.1 Anaerobic incubations of isolated hindguts

All operations involving anaerobic incubations were carried out in a Kaltec Anaerobic Glove Box in an atmosphere consisting of H_2 (10%), CO_2 (10%) and N_2 (80%) until an untimely and near fatal explosion caused a cessation of these experiments. Samples of 10 paunches were removed from mature worker caste *N. walkeri* and ruptured into 50 µl 2 mM potassium phosphate buffer, pH 7.0, in 1.5 ml microcentrifuge tubes. At zero time, 10 µl of the same buffer or 10 µl of the same buffer with substrate (glucose, trehalose, pyruvate, 30 mM) was added to a final concentration of 5 mM. Each tube was incubated for the appropriate time and the reaction was then stopped by the addition of 10 µl chilled 1 M perchloric acid and 50 µl water chilled to 0-5°C. Samples were centrifuged, and the resultant supernatant precipitated with 10 µl 1 M KOH and recentrifuged. The supernatants were filtered through 4 mm filters (0.45 mm) and kept frozen for acetate estimation using HPLC analysis.

2.6.2 HPLC

The HPLC station consisted of a 2150 pump, a 2151 variable wavelength monitor and a 2152 controller (LKB-Produkter AB,

Sweden), connected to a CI-10 integrator and an SEK plotter printer (LDC Milton Roy, Riviera Beach, FA, USA). Injections were made on-stream through a 7125 syringe loading sample injector (Rheodyne, Cotati, CA, USA) using 25-200 µl syringes (SGE, Melbourne). A Bio-Rad (Richmond, CA, USA) Aminex HPX-87H Organic Acid Analysis (cation-exchange) column (300 x 7.8 mm), fitted with a 5 cm H^+ guard column containing the same resin was used, the solvent being 5 mM H_2SO_4. Chromatography was carried out at ambient temperature. The flow rate was 0.4-0.6 ml min^{-1} and eluates were detected at 190-210 nm.

2.6.3 Preparation of extracts

Dried samples: Whole workers, degutted bodies, dissected guts and gut segments were broken into 50-100 µl 0.1 M NaOH using a needle. Samples were either freeze-dried or dried in a vacuum desiccator over P_2O_5 at room temperature.

Fresh samples: Whole guts were removed from 40-50 *N. walkeri* workers, gently rinsed in water, blotted dry on tissue paper and placed on a glass slide. The fore and midgut sections, the mixed segment and first proctodeal segments and the hindguts were removed and pooled in 1.5 ml microcentrifuge tubes. Guts were broken open carefully using a dissecting needle, taking care to avoid homogenising the gut contents. The samples were centrifuged for 10 min and 10-15 µl supernatant was carefully removed. With the mixed segment preparations only a small volume of fluid was present so the segments were rinsed with 10 µl water. These became the gut fluid samples.

For SCFA analysis, the pellet remaining was washed with 200 µl water and centrifuged for 10 min. The supernatant was removed and the process repeated. The supernatants were pooled and used for determination of total gut SCFA content. All samples were stored at -20°C until used.

2.6.4 Estimation of SCFA using HPLC

The HPLC station previously described was used with the following changes. The solvent was 5 mM H_2SO_4 in 5% acetonitrile and data were acquired using a Maclab/2e data acquisition system (AD Instruments, Castle Hill, NSW, Aust.) with Chart™ v3.3 and Peaks™ v1.0 application programs (AD Instruments, Castle Hill, NSW, Aust.) to record data and integrate peaks.

Dried samples were resuspended in 250 µl distilled water and homogenised. Supernatants were removed after centrifugation and 250 µl transferred to 1.5 ml microcentrifuge tubes, saturated with NaCl, acidified with 50 µl H_2SO_4 and extracted into 1.0 ml diethyl ether and 5 µl acetonitrile. After 5 min centrifugation in a bench centrifuge, the upper organic phase was removed to 1.5 ml microcentrifuge tubes containing 250 µl of 0.1 M NaOH. The samples were thoroughly mixed. After 5 min centrifugation in a bench centrifuge, the upper organic phase was removed and discarded. Acetonitrile (5 µl) was added and the tubes left uncapped to allow residual ether to evaporate before injection. Standards were prepared with each run.

2.6.5 Estimation of SCFA using GC

A column (3 m x 2 mm) packed with 10% SP-1200 with 1% H_3PO_4 on Chromosorb W AW 80/100 (Supelco Inc., Belefonte, PA, USA) was used in a Perkin Elmer gas chromatograph (Model Sigma 3B, Norwalk, CT, USA) with a thermal conductivity device. The following conditions were used: carrier gas, nitrogen; flow rate, 20 ml min^{-1}; column temperature, 115°C; detector temperature, 175°C. The chromatograph was linked to a Perkin Elmer chart recorder (Model 561) and calibrated with appropriate standards. Peak heights were used to quantify samples.

On the day of sampling, fresh samples were acidified with 25% H_3PO_4, centrifuged for 15 min and stored at -20°C until thawed just before use. Hindgut fluid was diluted 1/10 and 10 µl 25% H_3PO_4 added. Total gut content sample (450 µl) was acidified with 100 µl 25% H_3PO_4. A 10 µl sample was used for injection.

2.6.6 Estimation of glucose

GOD-Perid method: Hindgut fluid samples (25 µl) were acidified with 5 µl 20% PCA, centrifuged and the supernatant removed. Glucose was estimated using the GOD-Perid reagent (1 ml).

TLC: Hindgut fluid (20 µl), mixed segment fluid (10 µl), and midgut fluid samples (5 µl) were applied to silica gel 60 plates (553; Merck, Darmstadt, Germany) with glucose standard (0.2-10 nmol) and developed in isopropanol:acetone:0.1 M lactic acid (4:4:2) (Hansen, 1975). The plates were dried and sprayed with GOD reagent to visualise glucose as green spots.

2.6.7 Estimation of pyruvate

Haemolymph was quantitatively removed by 1 µl capillary tube from 50 *N. walkeri* workers into 200 µl 3% PCA. The hindguts were also removed and ruptured into 200 µl 3% PCA. The supernatants were removed after centrifugation and the pellet from the hindgut preparation was washed with 200 µl RO water and the supernatants combined. Samples were neutralised with 1 M KOH, centrifuged and assayed using the method of Lamprecht (19).

2.7 Nitrogen assays

2.7.1 Determination of total nitrogen

Food, foodstore, termites (20), fungal comb and nodules were collected from a field colony of *M. bellicosus* in Abidjan and freeze-dried. Prior to assay, termite samples were homogenised in water and aliquots (0.1 - 0.2 ml) dried in preweighed Pyrex® test tubes (160 x16 mm) at 70°C to provide the dry weight of sub-sample for total nitrogen and uric acid assays. Samples of food, foodstore, fungal comb and nodules were also dried in preweighed test tubes at 70°C to provide dry weight. Food, foodstore, termites (5-10), fungal comb and nodules were collected from a field colony of *M. mulleri* in Mbalmayo and *M. bellicosus* in Yaounde

and dried at 70°C. Two laboratory colonies of *M. bellicosus* from Bern were fed a standard leaf diet and the foodstore sampled every 3 weeks. Material was freeze-dried and transported to Sydney for analysis.

Kjeldahl digestion: A micro method developed by (Chappell and Slaytor, 1993) was used. Samples of 5-10 termites or 25 to 50 mg dwt of food, foodstore, and fungal comb, and 8-10 mg dwt of nodules were added to Pyrex® test tubes (160 x16 mm) containing 1 g of K_2SO_4 and 1 ml of concentrated H_2SO_4 (with 3% tellurium). They were then heated in an electric heating block to 325°C for 2-4 h until clear.

Ammonia estimation: Digests were transferred to 100 ml volumetric flasks with 3 washes of RO water, neutralised with 20 ml 5 M NaOH and made up to volume. The ammonia content was measured using the modified indophenol reagents of (Chaney and Marbach, 1962).

2.7.2 Determination of uric acid

Uric acid was extracted by sonication of samples in 3 ml of 12 mM Li_2CO_3. The homogenate was heated to 60°C for 5 min and then centrifuged at 12,000 *g* for 10 min at room temperature. The extract was assayed for uric acid by the uricase-peroxidase linked oxidation of 4-aminophenazone (Chappell and Slaytor, 1993).

RESULTS

3.0 General

The results presented in this thesis cover two major areas; the first deals with the origin of digestive enzymes, particularly cellulase, in higher termites and the second is a description of the metabolic events in the hindgut of the higher termite *Nasutitermes walkeri*.

The origin of cellulase and other digestive enzymes has been studied in the Australian termite *N. walkeri* and several African *Macrotermes* spp. by measuring activity in different sections of the gut. One of the components of the endogenous cellulase in *N. walkeri*, an endo-β-1,4-glucanase, has been partially purified. The termite-fungal relationship in the *Macrotermes* spp. was investigated by comparing the digestive capabilities of castes with their roles in the colony and by comparing elution profiles on gel chromatography of cellulase components from termites and their associated fungi.

Metabolic studies have been made in *N. walkeri* in order to gain an understanding of the biochemical relationship between the host termite and the symbiotic flora. The approach has been mainly through establishing the presence of key metabolic enzymes in the termite body and the hindgut contents. The activities of the enzymes of glucose, acetate and pyruvate metabolism including the enzymes of aerobic metabolism such as the pyruvate dehydrogenase complex and the tricarboxylic acid cycle have been measured. Evidence is presented that pyruvate is the major substrate used by the bacteria and that significant amounts of oxygen are used in the hindgut which is usually considered to be anaerobic.

3.1 Digestive enzymes in *N. walkeri*

The gut of *N. walkeri* was divided into various sections which were either essentially free of symbiotic flora, namely the salivary glands, the foregut, the midgut, the section of gut containing the Malpighian tubules, and the mixed segment, and the section which housed the symbiotic bacterial population, namely the hindgut. The gut sections are as defined in Fig. 4. These sections were then assayed for the

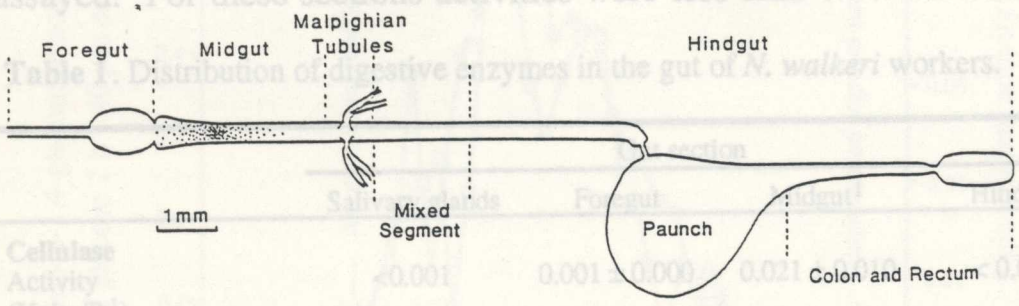

Fig. 4. The gut of *N. walkeri* illustrating the division of the gut into sections used for assays. The stippled section of the midgut represents the distribution of endo-β-1,4-glucanase activity.

following enzyme activities: cellulase, endo-β-1,4-glucanase, β-glucosidase, amylase and endo-β-1,4-xylanase.

The results of assays of digestive enzymes in *N. walkeri* are listed in Table 1. The GOD-Perid assay used to estimate glucose produced from incubations of gut extracts with cellulose, starch and cellobiose is an enzyme-linked assay (glucose oxidase and peroxidase) producing a coloured complex from ABTS. Interference with the functioning of the GOD-Perid assay by enzyme extracts prevented the ready estimation of cellulase, amylase and β-glucosidase activities in the salivary glands by this method. Amylase activity could be detected but not quantified using the Phadebas amylase test. This assay utilises a water-insoluble cross-linked starch polymer carrying a blue dye which is solubilised by amylase. Using the Phadebas test, amylase activity was detected in the salivary glands, foregut and midgut with less than 5% located in the hindgut. Endo-β-1,4-glucanase activity was the only component of cellulase activity which could be readily quantified in the salivary glands where it accounted for 3% of the total activity. More than 90% of cellulase and its components were found in the midgut with small amounts present in the foregut and traces in the hindgut. The highest enzyme activities were found in the midgut with endo-β-1,4-xylanase being the exception. A small amount of protease activity was present only in the midgut (data not shown).

The midgut section was divided in further sections containing the Malpighian tubules and the mixed segment (data not shown) and also assayed. For these sections activities were less than 0.1% for cellulase

Table 1. Distribution of digestive enzymes in the gut of *N. walkeri* workers.

	Gut section			
	Salivary glands	Foregut	Midgut[1]	Hindgut
Cellulase				
Activity (Units T^{-1})	<0.001	0.001 ± 0.000	0.021 ± 0.010	< 0.001
% of total gut activity	-	4.5	95.5	< 0.1
Sp. Act. (Units mg^{-1})	-	0.02 ± 0.00	0.13 ± 0.06	-
Endo-β-1,4-glucanase				
Activity (Units T^{-1})	0.25 ± 0.02	0.24 ± 0.12	7.67 ± 1.19	0.07 ± 0.07
% of total gut activity	3.0	2.9	93.2	0.9
Sp. Act. (Units mg^{-1})	26.98 ± 4.86	23.67 ± 6.80	146 ± 12	0.78 ± 0.78
β-Glucosidase				
Activity (Units T^{-1})	< 0.001	0.03 ± 0.001	0.41 ± 0.06	0.01± 0.01
% of total gut activity	-	6.7	91.1	2.2
Sp. Act. (Units mg^{-1})	-	0.48 ± 0.05	1.57 ± 0.31	0.01 ± 0.00
Amylase				
Activity (Units T^{-1})	<0.001	0.005 + 0.001	0.013 ± 0.001	<0.001
% of total gut	-	27.8	72.2	-
Sp. Act. (Units mg^{-1})	-	0.79	0.25	-
Endo-β-1,4-xylanase				
Activity (Units T^{-1})	< 0.001	0.001 ± 0.001	0.001 ± 0.001	0.004 ± 0.001
% of total gut activity	3.0	18.6	16.9	61.5
Sp. Act. (Units mg^{-1})	0.02 ± 0.01	0.20 ± 0.11	0.02 ± 0.01	0.09 ± 0.02

1 Midgut includes the mixed segment

n = 3; Units are as defined in Materials and Methods. Results are expressed as mean ± SE.

and less than 1% for β-glucosidase and amylase. Endo-β-1,4-glucanase activity decreased from 2.1% in the segment containing the Malpighian tubules to 1.4 % in the mixed segment.

β-Glucosidase and endo-β-1,4-glucanase activities were easily detected in serial sections (20-40 μm) of the midgut of *N. walkeri*. These

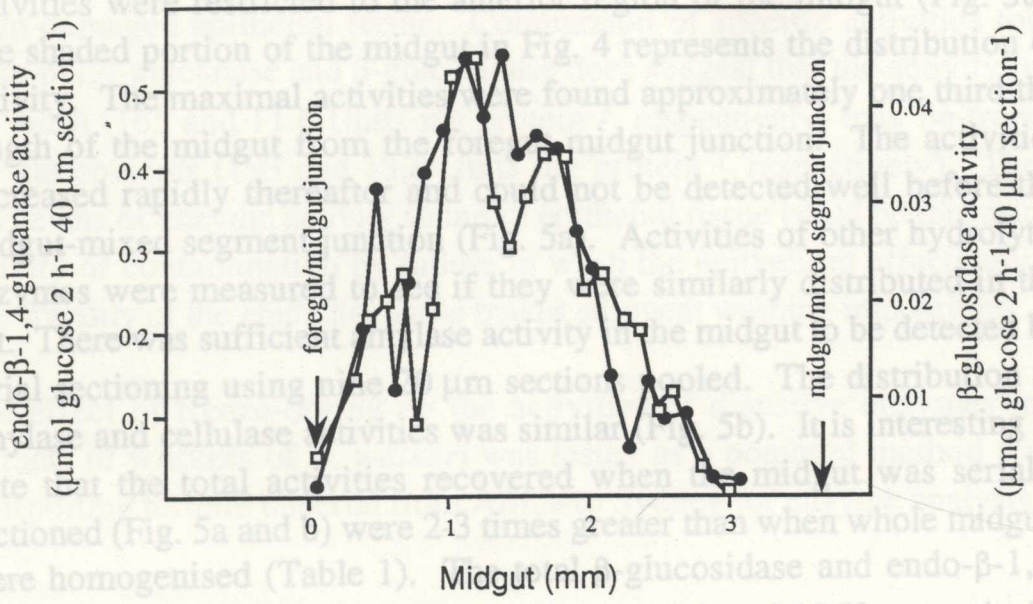

Fig. 5a. Distribution of endo-β-1,4-glucanase (●—●) and β-glucosidase (□—□) activities in sections along the midgut of *N. walkeri.*

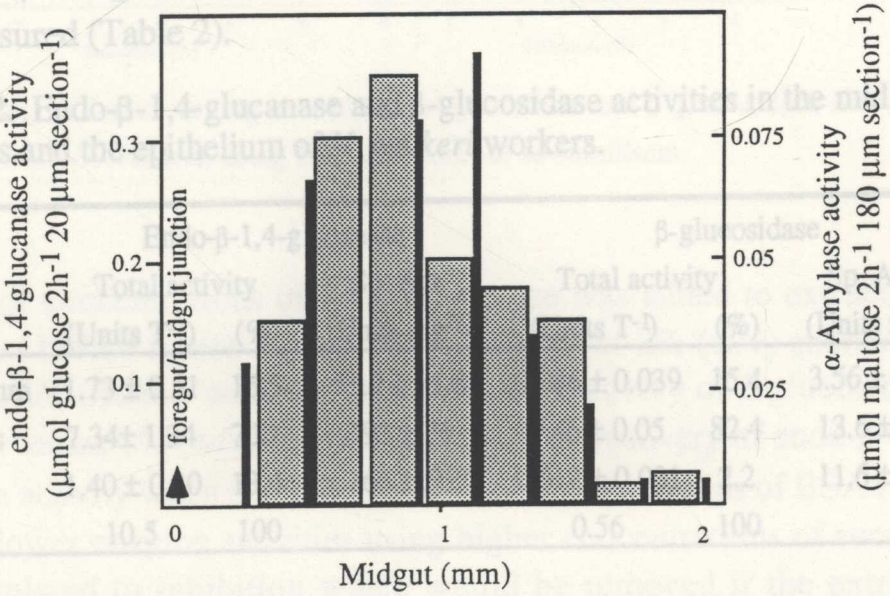

Fig. 5b. Distribution of endo-β-1,4-glucanase (▮) and amylase (▨) activities in sections along the midgut of *N. walkeri.*

activities were restricted to the anterior region of the midgut (Fig. 5a). The shaded portion of the midgut in Fig. 4 represents the distribution of activity. The maximal activities were found approximately one third the length of the midgut from the foregut-midgut junction. The activities decreased rapidly thereafter and could not be detected well before the midgut-mixed segment junction (Fig. 5a). Activities of other hydrolytic enzymes were measured to see if they were similarly distributed in the gut. There was sufficient amylase activity in the midgut to be detected by serial sectioning using nine 20 μm sections pooled. The distribution of amylase and cellulase activities was similar (Fig. 5b). It is interesting to note that the total activities recovered when the midgut was serially sectioned (Fig. 5a and b) were 2-3 times greater than when whole midguts were homogenised (Table 1). The total β-glucosidase and endo-β-1,4-glucanase activities recovered in Fig. 5a were 1.0 and 12 U respectively. There was also a threefold increase in recovery of amylase activity in the sectional experiments. Though the reason for this increased recovery is unknown it has also been noted with carbohydrases from the larvae of *Rhynchosciara americana* (Diptera: Scaridae) (Terra *et al.*, 1979). A 50% higher recovery was found when the distribution of β-glucosidase and endo-β-1,4-glucanase activities between the epithelium and the lumen was measured (Table 2).

Table 2. Endo-β-1,4-glucanase and β-glucosidase activities in the midgut contents and the epithelium of *N. walkeri* workers.

	Endo-β-1,4-glucanase			β-glucosidase		
	Total activity (Units T^{-1})	(%)	Sp. Act. (Units mg^{-1})	Total activity (Units T^{-1})	(%)	Sp. Act. (Units mg^{-1})
Epithelium	1.73 ± 0.51	16.5	53.9 ± 11.8	0.086 ± 0.039	15.4	3.56 ± 0.80
Contents	7.34± 1.34	70.1	193 ± 76	0.46 ± 0.05	82.4	13.6 ± 1.2
Wash	1.40 ± 0.30	13.4	165 ± 56	0.012 ± 0.001	2.2	11.6 ± 0.6
Total	10.5	100		0.56	100	

n = 3
Units are as defined in the Materials and Methods. Total activity is expressed as $termite^{-1}$ (T^{-1}).
Results are expressed as mean± SE.

At least 70% of the activities was present in the gut contents. It is not possible to know unambiguously whether the activity found in the wash fraction should be assigned to the gut contents or whether it has leaked

from damaged epithelial tissue. Comparison of specific activities for endo-β-1,4-glucanase and β-glucosidase does suggest that the activity in the wash (165 and 11.6 Units mg protein^{-1}, respectively) may be from the gut contents (193 and 13.6 Units mg protein^{-1}, respectively). The low levels of enzyme activity found in epithelial tissue could be intrinsic activity or contamination by failure to wash the epithelium effectively. The fragility of the epithelial tissue makes stringent washing impossible.

The effect of freeze-drying on the activities of digestive enzymes in extracts of workers of *N. walkeri* was tested as a potential method for transporting termite material from Africa to Sydney for enzyme analysis.

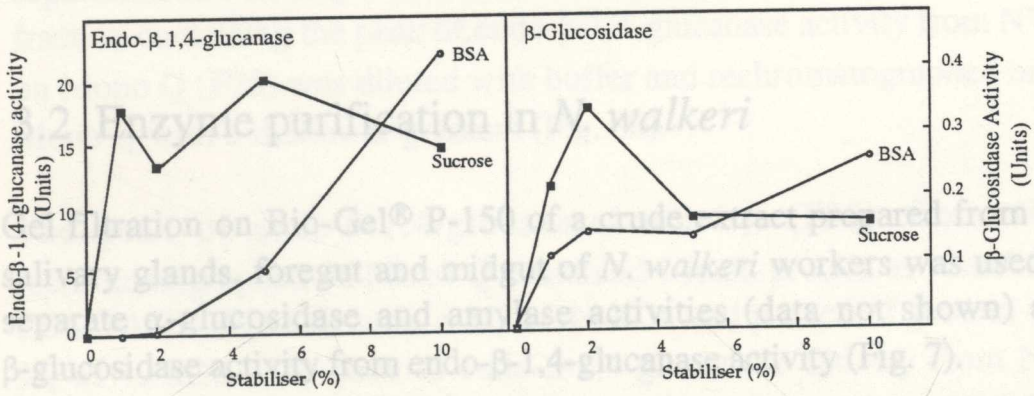

Fig. 6. Effect of freeze-drying on endo-β-1,4-glucanase and β-glucosidase activities in extracts from *N. walkeri*, using BSA and sucrose as stabilisers.

A range of concentrations of BSA or sucrose was added to extracts of *N. walkeri* before freeze-drying. The addition of 2% (w/v) sucrose to extracts of *N. walkeri* workers improved the recovery of β-glucosidase activity whereas 1% (w/v) sucrose improved the recovery of endo-β-1,4-glucanase activity when compared to similar concentrations of BSA (Fig. 6). The lower enzyme activities using higher concentrations of sucrose may be related to inhibition which would be removed if the extracts were subjected to gel chromatography. As BSA would interfere with protein purification steps, 4% sucrose was used in all extracts freeze-dried. All extracts were then stored at 0-5°C.

Attempts to concentrate column fractions using ultrafiltration membranes resulted in loss of endo-β-1,4-glucanase activity. There was a consistent loss when PM-30 membranes were used whereas with YM-10 membranes there was good recovery with new membranes but poor recoveries on reuse of the membrane, indicating some destruction of the pores in the filter which allowed the enzymes to pass through regardless of size. There was no loss of β-glucosidase activity when Centricon 10 microconcentrators were used but less than 30% of endo-β-1,4-glucanase activity could be recovered with about 1% of the activity in the filtrate. No loss of activity occurred when fractions were dehydrated in dialysis tubing packed in Aquacide so this became the method of choice.

3.2 Enzyme purification in *N. walkeri*

Gel filtration on Bio-Gel® P-150 of a crude extract prepared from the salivary glands, foregut and midgut of *N. walkeri* workers was used to separate α-glucosidase and amylase activities (data not shown) and β-glucosidase activity from endo-β-1,4-glucanase activity (Fig. 7).

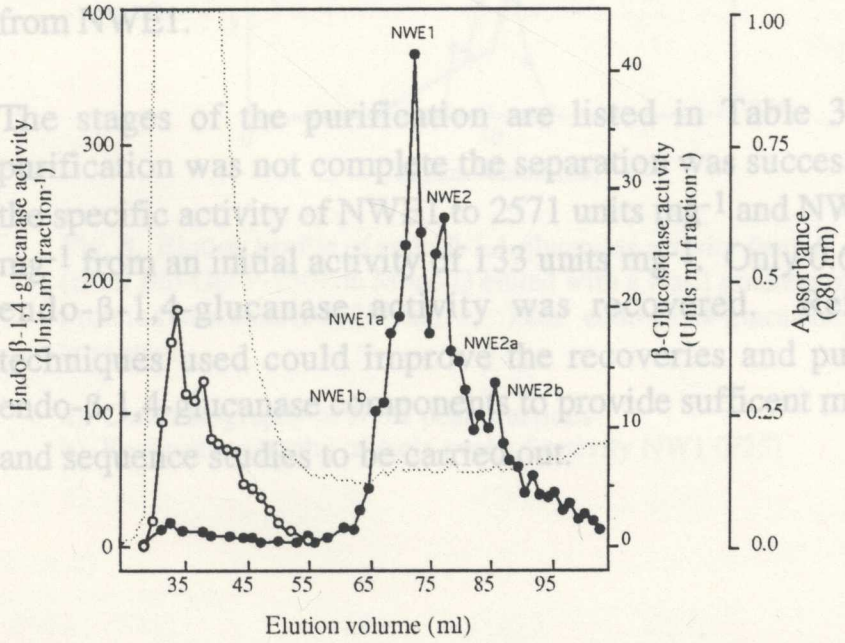

Fig. 7. Elution profile of a crude extract of salivary gland, foregut and midgut tissue from *N. walkeri* on Bio-Gel P-150 showing the protein (A_{280} ····), and the separation of endo-β-1,4-glucanase (●–●) and β-glucosidase activity (○–○) activities.

The 4 peaks of endo-β-1,4-glucanase activity, marked as NWE1 and NWE2, were used on subsequent Mono Q runs . Shoulders of activity were marked NEE1a, NWE1b, NWE2a and NWE2b..

The profile of the β-glucosidase activity indicates a multi-component system with the activity eluting with the excluded proteins. The endo-β-1,4-glucanase activity is also a multi-component system with two major peaks and four minor peaks of activity.

The fractions containing the major peaks of endo-β-1,4-glucanase activity (NWE1 and NWE2) were separately pooled and applied to a Mono Q column. NWE1 was eluted on Mono Q with 0.1 to 0.2 M NaCl (Fig. 8a). Initially NWE1 was partially retained on Mono Q but after thorough cleaning of the column subsequent rechromatographs of unretained material (Fig. 8a) from NWE1 did bind and were eluted under the same conditions as the main fraction of NWE1 (Data not shown). A similar separation on Mono Q was found with NWE2 (data not shown). The fraction containing the peak of endo-β-1,4-glucanase activity from NWE1 on Mono Q (F25) was diluted with buffer and rechromatographed on Mono Q with a shallower gradient (Fig. 8b).

The peak of endo-β-1,4-glucanase activity (F36) from NWE1 rechromatographed on Mono Q contained 1 main protein as visualised on SDS-PAGE (Plate 5) with a M_r of 44 kDa and a trace of protein with a M_r of 42 kDa. The peak of endo-β-1,4-glucanase activity from NWE2 rechromatographed contained 2 proteins as visualised on SDS-PAGE (Plate 5) with M_r of 44 kDa and 42 kDa, indicating incomplete separation from NWE1.

The stages of the purification are listed in Table 3. Although the purification was not complete the separation was successful in increasing the specific activity of NWE1 to 2571 units mg^{-1} and NWE2 to 2024 units mg^{-1} from an initial activity of 133 units mg^{-1}. Only 0.6 and 3.1% of the endo-β-1,4-glucanase activity was recovered. Refinement of the techniques used could improve the recoveries and purity of the other endo-β-1,4-glucanase components to provide sufficent material for kinetic and sequence studies to be carried out.

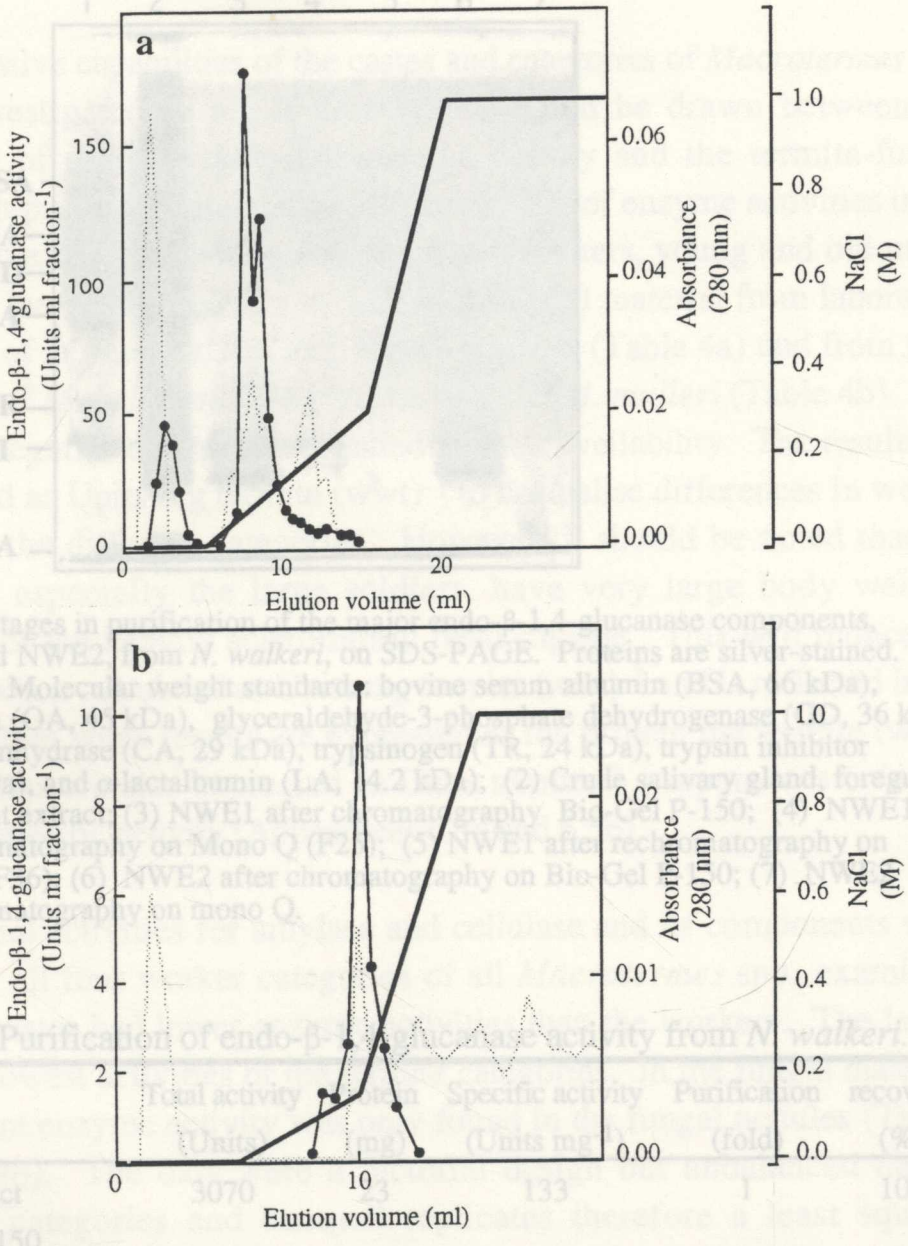

Fig. 8. Elution profile of endo-β-1,4-glucanase activity from *N. walkeri* (from Bio-Gel P-150) on Mono Q eluted with a NaCl gradient of 0 to 1M (—). Protein (Absorbance at 280 nm) (·····) and endo-β-1,4-glucanase activity (•—•) are shown.

a) Chromatography of NW1 peak fractions
b) Rechromatography of main peak of activity NW1 (F25)

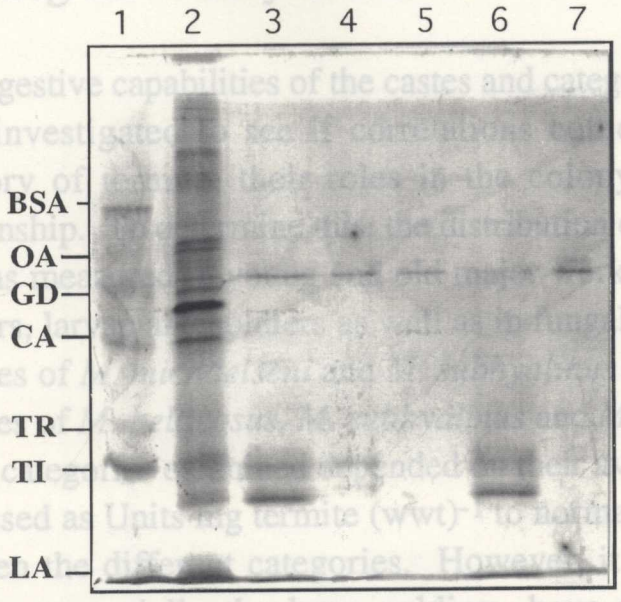

Plate 5. Stages in purification of the major endo-β-1,4-glucanase components, NWE1 and NWE2, from *N. walkeri*, on SDS-PAGE. Proteins are silver-stained. Lane1: (1) Molecular weight standards: bovine serum albumin (BSA, 66 kDa), ovalbumin (OA, 45 kDa), glyceraldehyde-3-phosphate dehydrogenase (GD, 36 kDa), carbonic anhydrase (CA, 29 kDa), trypsinogen (TR, 24 kDa), trypsin inhibitor (TI, 20 kDa), and α-lactalbumin (LA, 14.2 kDa); (2) Crude salivary gland, foregut and midgut extract; (3) NWE1 after chromatography Bio-Gel P-150; (4) NWE1 after chromatography on Mono Q (F25); (5) NWE1 after rechromatography on Mono Q (F36); (6) NWE2 after chromatography on Bio-Gel P-150; (7) NWE2 after chromatography on mono Q.

Table 3. Purification of endo-β-1,4-glucanase activity from *N. walkeri.*

	Total activity (Units)	Protein (mg)	Specific activity (Units mg^{-1})	Purification (fold)	recovery (%)
Crude extract	3070	23	133	1	100
Bio-Gel P-150					
NWE1a	357	0.35	1020	7.7	6.5
NWE1b	387	0.51	758	5.7	7.1
NWE1	967	0.57	1696	12.8	17.7
NWE2	596	0.36	1655	12.4	10.9
NWE2a	328	0.37	886	6.7	6.0
NWE2b	402	0.37	1086	8.2	7.4
Mono Q					
NWE1 (F25)	30	0.047	1034	7.7	1.0
NWE2 (F22)	96	0.040	2024	15.2	3.1
Mono Q (rechromatography)					
NWE1 (F36)	18	0.027	2571	19.3	0.6

3.3 Digestive enzymes in *Macrotermes* spp.

The digestive capabilities of the castes and categories of *Macrotermes* spp. were investigated to see if correlations could be drawn between the category of termite, their roles in the colony and the termite-fungal relationship. To determine this, the distribution of enzyme activities in the gut was measured in young and old major workers, young and old minor workers, larvae and soldiers as well as in fungal material from laboratory colonies of *M. michaelseni* and *M. subhyalinus* (Table 4a) and from field colonies of *M. bellicosus*, *M. subhyalinus* and *M. mulleri* (Table 4b). The actual categories examined depended on their availability. The results are expressed as Units mg termite $(wwt)^{-1}$ to normalise differences in weight between the different categories. However, it should be noted that the soldiers, especially the large soldiers, have very large body weights because of the heavily sclerotinised head capsule. This will reduce the activity when calculated as Units mg termite $(wwt)^{-1}$ and is reflected in the lower values when results are expressed as mg protein mg termite $(wwt)^{-1}$. The data are also expressed as Units $termite^{-1}$ (Appendices 6.4a and 6.4b) or specific activity (Appendices 6.5a and 6.5b).

Substantial activities for amylase and cellulase and its components were found in all four worker categories of all *Macrotermes* spp. examined. Soldier castes had lower enzyme activities than the workers. The larvae had the lowest activities of the termite categories. In the fungal material, significant enzyme activity was only found in the fungal nodules (Tables 4a and 4b). The data were a factorial design but unbalanced due to missing categories and unequal replicates therefore a least squares ANOVA was used to analyse the data. There was some heterogeneity of error but the variation was consistent in most of the species. As the ANOVA is robust the small amount of heterogeneity did not present a problem for the analysis.

Cellulase: No cellulase activity was detected in the larvae of any *Macrotermes* sp. examined (Tables 4a and 4b). Cellulase activity was not determined in *M. mulleri.* The statistical data are tabulated in Appendix 6.6. Cellulase activity in *M. michaelseni* was significantly different between the old minors and all of the categories and soldiers.

Table 4a. Enzyme activities in termite categories and fungal material from laboratory colonies (Bern) of *M. michaelseni* and *M. subhyalinus*.

	cellulase (Units mg tissue^{-1})	endo-β-1,4-glucanase (Units mg tissue^{-1})	β-glucosidase (Units mg tissue^{-1})	amylase (Units mg tissue^{-1})	protein (mg mg tissue^{-1})	wet weight (mg termite^{-1})
M. michaelseni						
Caste						
Old Major Workers	0.006 ±0.001	3.48 ± 0.40	1.35 ± 0.05	0.007 ± 0.001	0.027 ± 0.001	19.3 ± 0.3
Young Major Workers	0.007 ± 0.000	6.11 ± 0.61	2.14 ± 0.39	0.003 ± 0.000	0.030 ± 0.000	21.6 ± 0.4
Old Minor Workers	0.057 ± 0.014	11.3 ± 3.5	4.59 ± 0.49	0.033 ± 0.004	0.038 ± 0.002	11.2 ± 0.3
Young Minor Workers	0.010 ± 0.001	6.08 ± 3.92	2.37 ± 0.49	0.011 ± 0.000	0.038 ± 0.001	11.1 ± 0.2
Minor Soldiers	0.005 ± 0.000	1.39 ± 0.40	0.31 ± 0.02	< 0.000	0.026 ± 0.001	17.6 ± 0.5
Larvae	< 0.000	0.44 ± 0.18	< 0.000	< 0.000	0.028 ± 0.002	8.07 ± 0.26
Fungal Tissue						
Fresh Fungal Comb	< 0.000	0.09 ± 0.02	< 0.000	< 0.000	0.07 ± 0.01	
Ripe Fungal Comb	< 0.000	0.19 ± 0.13	< 0.000	< 0.000	0.011 ± 0.001	
Fungal Nodules	0.046 ± 0.003	19.8 ± 5.2	< 0.000	< 0.000	0.13 ± 0.01	
Old Fungal Comb	< 0.000	0.037 ± 0.018	< 0.000	< 0.000	0.01 ± 0.00	
M. subhyalinus						
Caste						
Old Major Workers	0.005 ± 0.002	3.71 ± 0.78	0.68 ± 0.04	0.002 ± 0.001	0.021 ± 0.001	12.4 ± 0.3 (8)
Young Major Workers	0.019 ± 0.003	10.2 ± 0.3	2.23 ± 0.06	0.013 ± 0.003	0.039 ± 0.001	13.3 ± 0.2 (9)
Old Minor Workers	0.002 ± 0.000	3.67 ± 0.81	1.07 ± 0.17	0.002 ± 0.000	0.031 ± 0.002	8.72 ± 0.35 (9)
Young Minor Workers	0.021 ± 0.001	8.19 ± 0.57	1.95 ± 0.19	0.014 ± 0.001	0.038 ± 0.003	6.51 ± 0.11 (9)
Minor Soldiers	< 0.000	0.84 ± 0.14	0.19 ± 0.00	0.001	0.025 ± 0.002	13.2 ± 0.3 (9)
Larvae	< 0.000	2.71 ± 1.03	0.29 ± 0.26	< 0.000	0.036 ± 0.003	6.03 ± 0.73
Fungal Tissue						
Fresh Fungal Comb	< 0.000	0.085 ± 0.010	0.10 ± 0.01	< 0.000	0.002 ± 0.001	
Ripe Fungal Comb	< 0.000	0.033 ± 0.006	0.07 ± 0.01	< 0.000	0.011 ± 0.005	
Fungal Nodules	0.024 ± 0.001	3.33 ± 1.16	0.98 ± 0.32	0.015 ± 0.004	0.06 ± 0.02	
Old Fungal Comb	< 0.000	0.017 ± 0.006	0.08 ± 0.02	< 0.000	0.014 ± 0.004	

n = 3 unless otherwise indicated
Units are as defined in Materials and Methods. Results are expressed as mean ± SE (n).

Table 4b. Enzyme activities in termite categories and fungal material from field colonies of *M. bellicosus, M. subhyalinus* (Abidjan) and *M. mulleri* (Mbalmayo).

	cellulase (Units mg tissue^{-1})	endo-β-1,4-glucanase (Units mg tissue^{-1})	β-glucosidase (Units mg tissue^{-1})	amylase (Units mg tissue^{-1})	endo-β-1,4-xylanase (Units mg tissue^{-1})	protein (mg mg tissue^{-1})	wet weight (mg termite^{-1})
M. bellicosus*							
Caste							
Old Major Workers	0.013 ± 0.002	4.99 ± 0.26	0.66 ± 0.06	0.006 ± 0.001	4.77 ± 1.16	0.041 ± 0.002	16.4 ± 0.4
Young Major Workers	0.014 ± 0.001	6.39 ± 0.72	0.65 ± 0.03	0.007 ± 0.001	4.90 ± 0.68	0.041 ± 0.002	16.8 ± 0.2
Old Minor Workers	0.023 ± 0.002	7.02 ± 0.83	1.00 ± 0.04	0.009 ± 0.001	6.86 ± 0.65	0.043 ± 0.004	5.83 ± 0.11
Young Minor Workers	0.015 ± 0.001	7.91 ± 0.24	0.69 ± 0.03	0.004 ± 0.001	4.60 ± 0.74	0.048 ± 0.002	6.87 ± 0.05
Large Soldiers	0.002 ± 0.000	1.02 ± 0.12	0.10 ± 0.01	< 0.000	1.19 ± 0.38	0.007 ± 0.002	61.9 ± 1.3
Larvae	< 0.000	1.17 ± 0.10	< 0.000	< 0.000	< 0.000	0.067 ± 0.012	3.79 ± 0.58
Fungal Tissue							
Fresh Fungal Comb	< 0.000	0.013 ± 0.002	0.006 ± 0.000	< 0.000	0.16 ± 0.02	0.006 ± 0.003	
Ripe Fungal Comb	< 0.000	0.002 ± 0.002	< 0.000	< 0.000	0.11 ± 0.02	0.13 ± 0.00	
Fungal Nodules	0.024 ± 0.004	21.8 ± 4.0	0.072 ± 0.029	0.16 ± 0.00	35.5 ± 6.9	0.18 ± 0.01	
Old Fungal Comb	< 0.000	0.024 ± 0.004	0.002 ± 0.002	< 0.000	0.09 ± 0.04	0.015 ± 0.001	
M. mulleri							
Caste							
Major Forager Workers		5.16 ± 0.20	16.8 ± 0.6			0.044 ± 0.001	26.1 ± 0.7
Old Minor Workers		18.9 ± 3.2	10.6 ± 1.9			0.060 ± 0.007	17.7 ± 0.3
Young Minor Workers		14.6 ± 2.7	6.13 ± 1.79			0.058 ± 0.005	13.6 ± 0.5
Fungal Tissue							
Fungal Nodules		9.43 ± 0.64	0.32 ± 0.01			0.45 ± 0.01	
M. subhyalinus							
Old Major Workers	0.020 ± 0.005	3.33 ± 0.42	2.65 ± 0.46	0.031 ± 0.005		0.053 ± 0.005	11.33 ± 1.73
Young Major Workers	0.016 ± 0.001	6.60 ± 1.05	2.42 ± 0.07	0.033 ± 0.001		0.047 ± 0.005	28.10 ± 0.14
Old Minor Workers	0.017 ± 0.000	5.90 ± 0.18	2.21 ± 0.31	0.021 ± 0.003		0.037 ± 0.001	6.87 ± 0.00
Young Minor Workers	0.012 ± 0.002	5.70 ± 0.77	1.49 ± 0.08	0.011 ± 0.001		0.040 ± 0.002	16.73 ± 0.25
Fungal Tissue							
Fungal Nodules	0.029 ± 0.00	6.57 ± 0.99	1.67 ± 0.08	0.11 ± 0.01		0.16 ± 0.00	

n = 3 for all samples except for *M. bellicosus* where *n = 6
Units are as defined in Materials and Methods. Results are expressed as mean ± SE

In laboratory colonies of *M. subhyalinus*, cellulase activity was significantly higher in the young major and young minor workers than in the old major and old minor workers. No activity could be detected in the soldiers. However, in field colonies of *M. subhyalinus*, and *M. bellicosus,* cellulase activity was not significantly different between the worker categories and soldiers.

When the cellulase activities were compared between old major workers of *M. michaelseni, M. subhyalinus* (laboratory and field colonies) and *M. bellicosus*, no significant differences were observed with the exception of *M. bellicosus.* A similar pattern was noted for young major workers and old minor workers. There were no significant differences between soldiers from all species. No relationships were found in young minor workers from all species.

Cellulase activity was not detected in any fungal comb tissue (Tables 4a and 4b). Activity was higher in fungal nodules from *M. michaelseni* than the other *Macrotermes* spp.

Endo-β-1,4-glucanase: Endo-β-1,4-glucanase activity was detected in all termite categories examined, including the larvae (Tables 4a and 4b). The statistical data was tabulated in Appendix 6.7. In *M. michaelseni* endo-β-1,4-glucanase activity in old major workers, old minor workers and soldiers was significantly different from the activity in young major and young minor workers. In laboratory colonies of *M. subhyalinus*, endo-β-1,4-glucanase activity was not significantly different between old major workers, young major workers and old minor workers. In field colonies of *M. subhyalinus*, and *M. bellicosus* no significant differences were found between all categories. In *M. mulleri* the young and old minor workers were significantly different from the major worker (foragers).

When the endo-β-1,4-glucanase activities of old major workers of *M. michaelseni, M. subhyalinus* (laboratory and field colonies), *M. bellicosus* and major forager workers of *M. mulleri* were compared, there were no significant differences. There was a similar pattern in young major workers. Old minor workers exhibited no significant difference between *M. michaelseni, M. subhyalinus* (field colony) and

M. bellicosus only. Young minor workers exhibited no significant difference between all *Macrotermes* spp. with the exception of *M. mulleri.*

Endo-β-1,4-glucanase activity was detected in fungal comb tissue from all species (Tables 4a and 4b). Activity was higher in the fungal nodules from *M. michaelseni* and *M. bellicosus* than in the other *Macrotermes* spp.

β-glucosidase: The statistical data are tabulated in Appendix 6.8. Low to negligible β-glucosidase activity was found in the larvae (Tables 4a and 4b). In *M. michaelseni* β-glucosidase activity between the old major workers and old minor workers was significantly different from that in the young majors workers and young minor workers. The β-glucosidase activity between old majors and soldiers was not significantly different. In *M. subhyalinus*, (laboratory colony) β-glucosidase activity was not significantly different between the young major and young minor workers. However, in field colonies of *M. subhyalinus*, β-glucosidase activity was not significantly different between old major workers, young major workers, and old minor workers. In field colonies of *M. bellicosus* and *M. mulleri* β-glucosidase activity was not significantly different between any categories.

The β-glucosidase activities of the old major workers showed no significant differences. A similar pattern was observed for old minor workers. In young major workers only *M. subhyalinus* (laboratory and field colonies) and *M. michaelseni* showed no significant differences. Little overall relationship was found in the young minor category. Only soldiers showed no significant difference between species.

β-Glucosidase activity was not detected or was detected at low levels in fungal comb tissue from all species (Tables 4a and 4b). Activity was higher in the fungal nodules from *M. subhyalinus* (laboratory and field colonies) and *M. mulleri* with low to negligible activity in *M. bellicosus* and *M. michaelseni.*

Amylase: The statistical data are tabulated in Appendix 6.9. Amylase activity was not detected in the larvae of all *Macrotermes* spp. examined

and only soldiers from laboratory colonies of *M. subhyalinus* exhibited detectable activity (Tables 4a and 4b). *M. mulleri* was not tested for amylase activity. In *M. michaelseni* amylase activity in the old major workers, young major workers and old minor workers were significantly different from the young minor workers. In *M. subhyalinus*, (laboratory colony) activity in the old major workers, old minor workers and soldiers was significantly different to the young major workers and young minor workers. However, in field colonies of *M. subhyalinus*, activity in the old major workers and young major workers was significantly different from the old minor workers and young minor workers. No significant difference was found between categories of workers in *M. bellicosus*.

When the amylase activities of the old major workers of *M. michaelseni, M. subhyalinus* (laboratory and field colonies) and *M. bellicosus* are compared only *M. subhyalinus* (field colony) showed a significant difference. In young major workers only *M. michaelseni* and *M. bellicosus* showed no significant difference. No pattern was observed for old minor workers. In young minor workers, *M. michaelseni* and *M. bellicosus* showed a significant difference to *M. subhyalinus* (laboratory and field colonies). Soldiers showed no significant difference.

Amylase activity was not detected in fungal comb tissue from all species (Tables 4a and 4b). Activity was higher in the fungal nodules from *M. bellicosus* and *M. subhyalinus* (laboratory colony) with low to negligible activity in *M. subhyalinus* (field colony) and *M. michaelseni*.

Endo-β-1,4-xylanase activity was examined only in material from *M. bellicosus* (Tables 4a and 4b). Activity was found in all worker categories and soldiers with no increase in the young categories compared to the old categories. Considerable amounts of endo-β-1,4-xylanase activity were found in fungal comb tissue (when compared to cellulase activity) with high values recorded for the fungal nodules.
The distribution of enzyme activities along the gut of young and old major workers of *M. michaelseni* (Table 5) was examined.

Table 5. Enzyme activities in the gut sections of old and young major workers of *M. michaelseni.*

		Salivary glands	Foregut	Midgut	Hindgut	Total
Old major workers						
Cellulase	Activity (Units termite^{-1})	0.003 ± 0.001	< 0.000	0.233 ± 0.026	< 0.000	0.236 ± 0.026
	% of gut total activity	1.3	< 0.0	98.7	<0.1	100
Endo-β-1,4-glucanase	Activity (Units termite^{-1})	7.06 ±0.17	0.72 ± 0.11	89.3 ± 10.1	2.31 ± 0.25	99.4 + 9.5
	% of gut total activity	7.1	0.7	89.9	2.3	100
β-Glucosidase	Activity (Units termite^{-1})	0.11 ± 0.01	0.06 ± 0.052	7.8 ± 1.3	0.32 ± 0.12	28.3 ± 1.1
	% of gut total activity	0.4	0.2	98.2	1.2	100
Amylase	Activity (Units termite^{-1})	< 0.01	< 0.01	0.17 ± 0.01	< 0.01	0.17 ± 0.01
	% of gut total activity	< 0.1	< 0.1	100	< 0.1	100
Protein	mg termite^{-1}	0.030 ± 0.000	0.010 ± 0.001	0.178 ± 0.005	0.172 ± 0.016	0.389 ±0.020
	% of gut total	7.6	2.5	45.8	44.1	100
Young major workers						
Cellulase	Activity (Units termite^{-1})	< 0.000	< 0.000	0.34 ± 0.04	0.001 ±0.001	0.33 ± 0.04
	% of gut total activity	< 0.0	< 0.0	100	< 0.0	100
Endo-β-1,4-glucanase	Activity (Units termite^{-1})	3.97 ± 0.19	0.19 ± 0.03	37.75 ± 0.14	2.11 ± 0.72	44.0 ± 1.1
	% of gut total activity	9.0	0.4	85.8	4.8	100
β-Glucosidase	Activity (Units termite^{-1})	0.03 ± 0.03	0.03 ± 0.03	5.78 ± 0.33	0.33 ± 0.11	6.17 ± 0.17
	% of gut total activity	0.5	0.4	93.7	5.4	100
Amylase	Activity (Units termite^{-1})	< 0.000	< 0.000	0.47 ± 0.16	0.003 ± 0.003	0.47 ± 0.17
	% of gut total	< 0.0	< 0.0	100	< 0.0	100
Protein	mg termite^{-1}	0.033 ± 0.001	0.009± 0.001	0.23 ± 0.01	0.072 ± 0.004	0.343 ± 0.006
	% of gut total activity	9.5	2.5	67.1	20.9	100

n = 3; Units are as defined in Materials and Methods. Results are expressed as mean ± SE.

The majority of the enzyme activity was located in the midgut in both old major and young major workers, with less than 6% of any enzyme activity located in the hindgut. At least 99% of the cellulase and amylase activities were found only in the midgut of both old and young major workers of *M. michaelseni* (Table 5). The low to negligible activities found in the salivary glands may be related to possible interference in the GOD-Perid glucose assay used. The major site of endo-b-1,4-glucanase activity in old and young major workers was the midgut (86-90%), with 6-9% found in the salivary glands and 2-5% present in the hindgut. An interesting finding is the higher percentage of protein found in the midgut of young major workers (67%) compared to old major workers (46%).

When the distribution of enzyme activity in the contents and epithelium of the midgut was measured the contents contained the highest enzyme activities for all enzymes examined (Table 6).

The effect of freeze-drying on cellulase components in *M. michaelseni* and *M. subhyalinus* was examined to determine if freeze-dried material could be used for assays in Sydney (Table 7a and 7b). Statistically the data were difficult to interpret. ANOVA results are presented in Appendix 6.11 and 6.12. No significant difference was found with protein levels between treatments but significant differences were found between treatments and enzymes. The main conclusion that can be drawn from these studies is that the enzyme activity in fresh material from *M. michaelseni* and *M. subhyalinus* varies significantly with freeze-drying and storage before assaying but the variation is unpredictable. Any studies undertaken with preserved material should be carried out with more extensive controls using fresh material.

Table 6. Enzyme activities in midgut contents and epithelium of *M. michaelseni* workers.

		Contents	Epithelium	Total
Old major workers				
Cellulase	Activity (Units termite^{-1})	0.018 ± 0.001	0.001 ± 0.000	0.019 ± 0.001
	Sp. Act. (Units mg^{-1})	0.038 ± 0.010	0.035 ± 0.005	0.037 ± 0.008
	% of total activity	94.7	5.3	100
Endo-β-1,4-glucanase	Activity (Units termite^{-1})	30.65 ± 3.58	4.72 ± 0.11	35.37 ± 3.58
	Sp. Act. (Units mg^{-1})	63.5 ± 13.0	5.1 ± 2.9	68.6 ± 12.9
	% of total activity	86.7	13.3	100
β-Glucosidase	Activity (Units termite^{-1})	0.019 ± 0.002	0.001 ± 0.000	0.020 ± 0.002
	Sp. Act. (Units mg^{-1})	0.044 ± 0.019	0.032 ± 0.005	0.042 ± 0.016
	% of total activity	95	5	100
Amylase	Activity (Units termite^{-1})	0.013 ± 0.002	< 0.001	0.013 ± 0.002
	Sp. Act. (Units mg^{-1})	0.029 ± 0.008	0.003 ± 0.003	0.027 ± 0.006
	% of total activity	100	< 0.01	100
Protein	mg termite^{-1}	0.53 ± 0.12	0.027 ± 0.005	0.56 ± 0.12
	% of total	95.1	4.9	100
Young major workers				
Cellulase	Activity (Units termite^{-1})	0.29 ± 0.00	0.05 ± 0.00	0.34 ± 0.01
	Sp. Act. (Units mg^{-1})	1.11 ± 0.06	1.08 ± 0.07	1.10 ± 0.05
	% of total activity	85.3	14.7	100
Endo-β-1,4-glucanase	Activity (Units termite^{-1})	28.31 ± 4.44	3.24 ± 0.42	31.55 ± 4.79
	Sp. Act. (Units mg^{-1})	108 ± 10	75.7 ± 3.1	104 ± 9
	% of total activity	89.7	10.3	100
β-Glucosidase	Activity (Units termite^{-1})	0.012 ± .0001	0.003 ± 0.000	0.015 ± 0.001
	Sp. Act. (Units mg^{-1})	0.048 ± 0.004	0.060 ± 0.005	0.049 ± 0.003
	% of total activity	80	20	100
Amylase	Activity (Units termite^{-1})	0.20 ± 0.01	0.03 ± 0.00	0.23 ± 0.01
	Sp. Act. (Units mg^{-1})	0.76 ± 0.02	0.79 ± 0.08	0.77 ± 0.02
	% of total activity	87	13	100
Protein	mg termite^{-1}	0.26 ± 0.02	0.04 ± 0.00	0.30 ±0.02
	% of total gut protein	85.9	14.1	100

n = 3; Units are as defined in Materials and Methods. Results are expressed as mean ± SE

Table 7a. Effect of freeze-drying on the activity of enzymes from termite and fungal material.

M. michaelseni (laboratory colony)	Treatment and storage	cellulase (Units termite^{-1})	endo-β-1,4-glucanase (Units termite^{-1})	β-glucosidase (Units termite^{-1})	amylase (Units termite^{-1})	protein (mg termite^{-1})
Old Major Workers	Fresh	0.12 ± 0.02	64.9 ± 7.0	25.3 ± 0.6	0.14 ± 0.02	0.50 ± 0.02
	Freeze-dried; room temperature	0.11 ± 0.01	105 ± 20	51.1 ± 3.6	0.11 ± 0.01	0.72 ± 0.04
	Freeze-dried; -20°C	0.17 ± 0.02	58.5 ± 6.5	122 ± 8	0.15 ± 0.01	0.46 ± 0.02
Young Major Workers	Fresh	0.16 ± 0.01	130 ± 8	45.7 ± 8.4	12.1 ± 2.2	0.63 ± 0.03
	Freeze-dried; room temperature	0.09 ± 0.01	213 ± 50	69.5 ± 7.4	18.3 ± 2.6	1.01 ± 0.09
	Freeze-dried; -20°C	0.23 ± 0.01	76 ± 14	116 ± 7	23.2 ± 4.7	0.59 ± 0.02
Old Minor Workers	Fresh	0.23 ± 0.02	130 ± 35	53.2 ± 3.5	0.07 ± 0.01	0.45 ± 0.02
	Freeze-dried; room temperature	0.14	118	60.4 ± 0.2	0.10 ± 0.01	0.48 ± 0.03
	Freeze-dried; -20°C	0.26	32	58.5	0.23 ± 0.04	0.34
Young Minor Workers	Fresh	0.11 ± 0.01	67 ± 44	26.3 ± 5.4	0.12 ± 0.00	0.42 ± 0.02
	Freeze-dried; room temperature	0.09 ± 0.01	52.8 ± 10.3	24.2 ± 2.6	0.11 ± 0.01	0.52 ± 0.03
	Freeze-dried; -20°C	0.13 ± 0.02	9.8 ± 4.5	30.7 ± 1.4	0.13 ± 0.00	0.34 ± 0.01
Minor Soldiers	Fresh	0.009 ± 0.004	23.2 ± 5.5	5.30 ± 0.05	0.005 ± 0.002	0.46 ± 0.04
	Freeze-dried; room temperature	0.016 ± 0.002	23.6 ± 3.3	2.91 ± 0.24	0.028 ± 0.011	0.61 ± 0.02
	Freeze-dried; -20°C	0.014 ± 0.004	14.1 ± 2.4	2.70 ± 0.49	0.016	0.33 ± 0.01
Fungal Nodules	Fresh	0.046 ± 0.003	19.8 ± 5.2	<0.000	0.02 ± 0.00	0.13 ± 0.01
	Freeze-dried; room temperature	0.034 ± 0.012	15.9 ± 2.3	0.002 ± 0.002	0.06 ± 0.02	0.17 ± 0.01
	Freeze-dried; -20°C	0.067 ± 0.014	14.4 ± 3.9	<0.000	0.11 ± 0.03	0.18 ± 0.03

n = 3
Units are as defined in Materials and Methods. Results are expressed as mean ± SE

Table 7b. Effect of freeze-drying on the activity of enzymes from termite and fungal material.

M. subhyalinus (laboratory colony)	Treatment and storage	cellulase (Units termite^{-1})	endo-β-1,4-glucanase (Units termite^{-1})	β-glucosidase (Units termite^{-1})	amylase (Units termite^{-1})	protein (mg termite^{-1})
Old Major Workers	Fresh	0.058 ± 0.022	44.5 ± 8.7	8.14 ± 0.36	0.027 ± 0.02	0.26 ± 0.01
	Freeze-dried; room temperature	0.064 ± 0.003	40.8 ± 4.2	10.09 ± 0.96	0.085 ± 0.004	0.23 ± 0.01
	Freeze-dried; -20°C	0.093 ± 0.026	52.1 ± 2.3	8.07 ± 0.93	0.062 ± 0.024	0.30 ± 0.02
Young Major Workers	Fresh	0.26 ± 0.04	139 ± 4	30.3 ± 1.1	0.18 ± 0.04	0.53 ± 0.01
	Freeze-dried; room temperature	0.18 ± 0.02	126 ± 14	34.1 ± 0.4	0.14 ± 0.01	0.30 ± 0.05
	Freeze-dried; -20°C	0.097 ± 0.011	54.3 ± 2.6	9.80 ± 0.5	0.086 ± 0.017	0.41 ± 0.01
Old Minor Workers	Fresh	0.018 ± 0.005	31.5 ± 4.0	8.75 ± 1.92	0.018 ± 0.006	0.28 ± 0.04
	Freeze-dried; room temperature	0.042 ± 0.003	20.8 ± 5.2	7.97 ± 2.53	0.070 ± 0.024	0.17 ± 0.03
	Freeze-dried; -20°C	0.064 ± 0.004	38.4 ± 5.8	8.67 ± 1.44	0.050 ± 0.003	0.25 ± 0.03
Young Minor Workers	Fresh	0.14 ± 0.01	53.6 ± 5.7	12.65 ± 1.08	0.095 ± 0.01	0.25 ± 0.02
	Freeze-dried; room temperature	0.14 ± 0.02	41.3 ± 10.0	13.37 ± 2.34	0.13 ± 0.02	0.21 ± 0.03
	Freeze dried; -20°C	0.10 ± 0.00	11.3 ± 0.6	3.24 ± 0.60	0.069 ± 0.01	0.25 ± 0.01
Minor Soldiers	Fresh	0.000	11.50 ± 1.94	2.59 ± 0.05	0.018	0.35 ± 0.03
	Freeze-dried; room temperature	0.02 ± 0.02	11.74 ± 0.62	2.24 ± 0.02	0.010	0.21 ± 0.01
	Freeze-dried; -20°C	0.026 ± 0.005	14.00 ± 2.10	1.43 ± 0.02	0.006 ± 0.004	0.24 ± 0.01
Fungal Nodules	Fresh	0.024 ± 0.001	3.33 ± 1.16	0.98 ± 0.32	0.015 ± 0.004	0.06 ± 0.01
	Freeze-dried; room temperature	0.027 ± 0.001	8.50 ± 0.17	0.25 ± 0.12	0.13 ± 0.01	0.10 ± 0.01
	Freeze-dried; -20°C	0.022	12.22	0.28	0.047	0.09

n = 3
Units are as defined in Materials and Methods. Results are expressed as mean ± SE

3.4 Enzyme purification in *Macrotermes* spp.

The endo-β-1,4-glucanase components from the fungal nodules and from old and young major workers of *M. michaelseni* were included in the eluate from Bio-Gel P-60 (Fig. 9) and separated from the β-glucosidase and amylase components (data not shown). By superimposing elution profiles of crude fungal and termite extracts (Fig. 9) it can be seen from the shaded area that there is little overlap on elution from Bio-Gel P-60 between the fungal nodule and the termite enzymes.

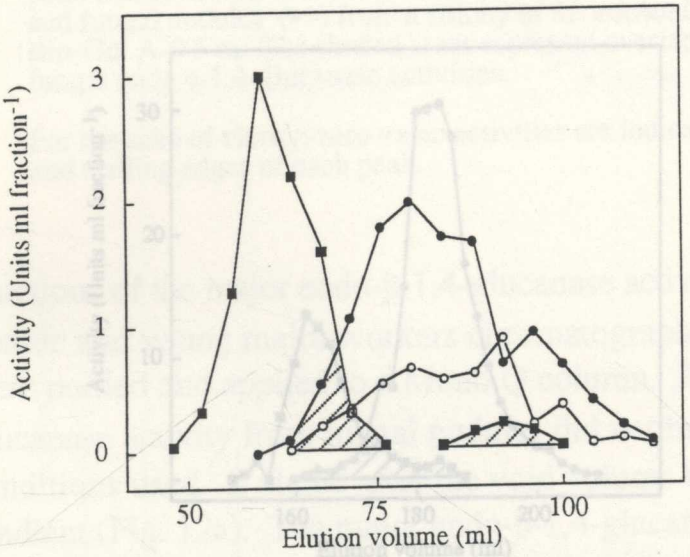

Fig. 9. Profiles of endo-β-1,4-glucanase activities from crude extracts run on Bio-Gel P-60 of young major workers (•—•), old major workers (○—○) and fungal nodules (■—■) from a colony of *M. michaelseni*. The shaded areas represent overlap of termite and fungal endo-β-1,4-glucanase activities.

For the sake of clarity, zero value activities are indicated at the leading and trailing edges of each peak.

The elution profile of endo-β-1,4-glucanase activity from the fungal nodules shows a major, slightly asymmetric, peak and a minor peak containing less than 4% of the total activity recovered (Fig. 9). Only 5% of the total fungal nodule endo-β-1,4-glucanase activity recovered overlaps the termite endo-β-1,4-glucanase activity in the area of the major fungal components, while 4% of the total fungal nodule endo-β-1,4-glucanase activity recovered overlaps the termite endo-β-1,4-glucanase activity in the

area of the minor fungal nodule component (Fig. 9). The elution profiles of the endo-β-1,4-glucanase activities for young major workers and old major workers were similar with both exhibiting a multi-component system with one major peak and several minor peaks (Fig. 9).

Crude extracts of fungal nodules and young major workers applied to a Bio-Gel P-150 column exhibited a similar separation of endo-β-1,4-glucanase (Fig. 10) and β-glucosidase components (data not shown). Overlays of the profiles of termite and fungal nodule endo-β-1,4-glucanase (Fig. 10) show similar differences between the termite and fungal nodule endo-β-1,4-glucanases.

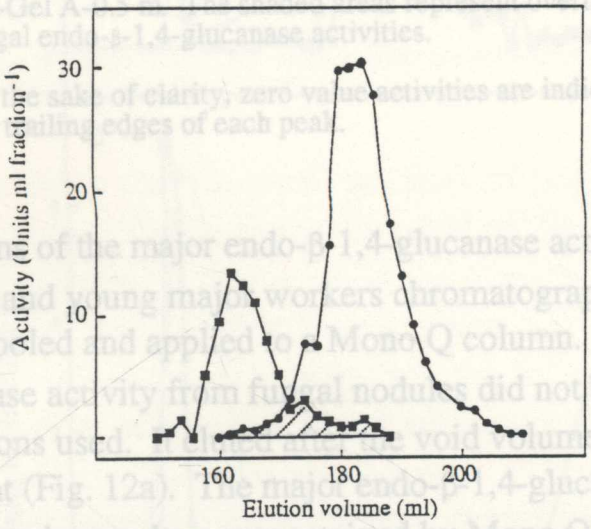

Fig. 10. Profiles of endo-β-1,4-glucanase activities from crude extracts run on Bio-Gel P-150 from young major workers (⊶) and fungal nodules (▪▪) from a colony of *M. michaelseni*.

The shaded areas represent overlap of termite and fungal endo-β-1,4-glucanase activities.

For the sake of clarity, zero value activities are indicated at the leading and trailing edges of each peak.

A crude extract using fungal comb as a source of fungal endo-β-1,4-glucanase activities showed an identical profile on Bio-Gel P-150 to that of fungal nodules (data not shown). Fractions of the major endo-β-1,4-glucanase activities from the fungal nodule and young major workers run on Bio-Gel P-150 were pooled and rechromatographed on Bio-Gel A-0.5 m (Fig. 11). Overlays of the profiles of termite and fungal nodule endo-β-1,4-glucanase activities also showed minimal overlap (Fig. 11).

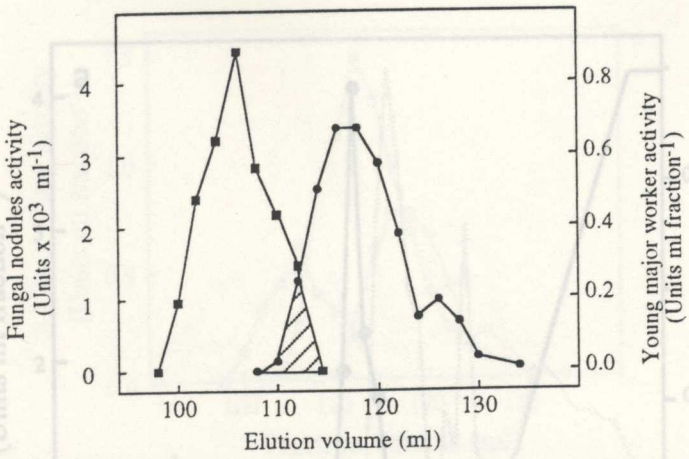

Fig. 11. Profiles of endo-β-1,4-glucanase activities from combined peak fractions (Bio-Gel P-60) from young major workers (•—•) and fungal nodules (■—■) from a colony of *M. michaelseni* run on Bio-Gel A-0.5 m. The shaded areas represent overlap of termite and fungal endo-β-1,4-glucanase activities.

For the sake of clarity, zero value activities are indicated at the leading and trailing edges of each peak.

Fractions of the major endo-β-1,4-glucanase activities from the fungal nodule and young major workers chromatographed on Bio-Gel P-150 were pooled and applied to a Mono Q column. The major endo-β-1,4-glucanase activity from fungal nodules did not bind to Mono Q under the conditions used. It eluted after the void volume but before the salt gradient (Fig. 12a). The major endo-β-1,4-glucanase activity from the young major workers was retained by Mono Q eluting as a major peak and possibly three minor peaks with a small amount (1%) of unretained material (Fig. 12b). Lack of initial material and losses in enzyme activity during purification prevented further purification.

β-Glucosidase components from the fungal nodules and the major workers were excluded from Bio-Gel P-60 (data not shown) but are included in Bio-Gel A-0.5 m (Fig. 13). When the elution profile of the β-glucosidase activities from the young major workers and fungal nodules were compared very little overlap is evident (Fig. 13).

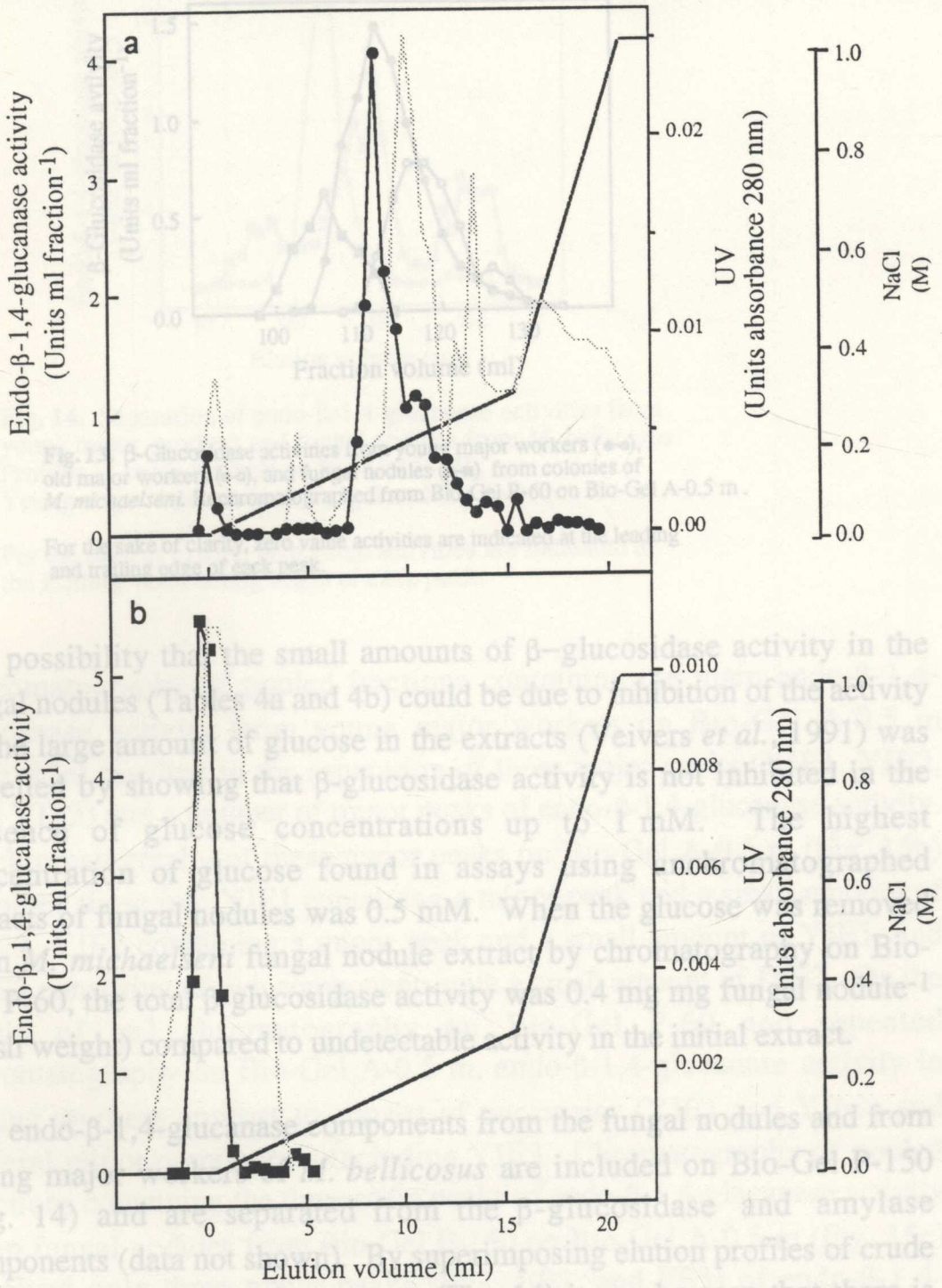

Fig. 12. Elution profile on Mono Q (NaCl gradient, 0-1 M ——) showing endo-β-1,4-glucanase activity and UV absorbance (·····) from young major workers and fungal nodules from colonies of *M. michaelseni.*

a) Combined peak fractions (Bio-Gel P-150) of endo-β-1,4-glucanase activity from young major workers (●—●).

b) Combined peak fractions (Bio-Gel P-150) of endo-β-1,4-glucanase activity from fungal nodules (■—■).

For the sake of clarity, zero value activities are indicated at the leading and trailing edge of each peak.

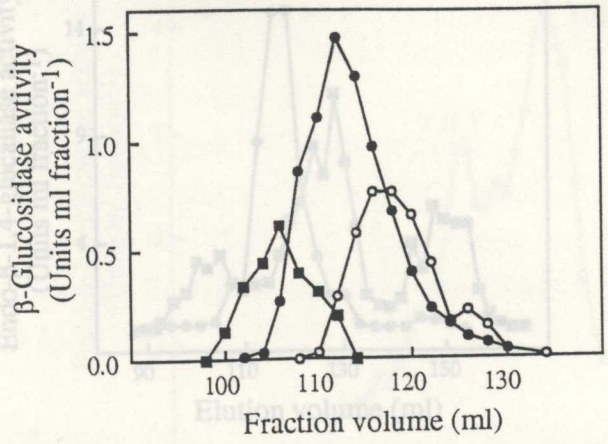

Fig. 13. β-Glucosidase activities from young major workers (●–●), old major workers (○–○), and fungal nodules (■–■) from colonies of *M. michaelseni.* Rechromatographed from Bio-Gel P-60 on Bio-Gel A-0.5 m .

For the sake of clarity, zero value activities are indicated at the leading and trailing edge of eack peak.

The possibility that the small amounts of β–glucosidase activity in the fungal nodules (Tables 4a and 4b) could be due to inhibition of the activity by the large amount of glucose in the extracts (Veivers *et al.*, 1991) was dispelled by showing that β-glucosidase activity is not inhibited in the presence of glucose concentrations up to 1 mM. The highest concentration of glucose found in assays using unchromatographed extracts of fungal nodules was 0.5 mM. When the glucose was removed from *M. michaelseni* fungal nodule extract by chromatography on Bio-Gel P-60, the total β-glucosidase activity was 0.4 mg mg fungal nodule^{-1} (fresh weight) compared to undetectable activity in the initial extract.

The endo-β-1,4-glucanase components from the fungal nodules and from young major workers of *M. bellicosus* are included on Bio-Gel P-150 (Fig. 14) and are separated from the β-glucosidase and amylase components (data not shown). By superimposing elution profiles of crude fungal nodule and termite extracts (Fig. 14) it can be seen that there is little overlap between the major fungal nodule endo-β-1,4-glucanase activities and termite endo-β-1,4-glucanase activities. The profiles indicate a multi-component system with the fungal endo-β-1,4-glucanase eluting as three major peaks and the termite endo-β-1,4-glucanase eluting as one major peak with two minor peaks (Fig. 14).

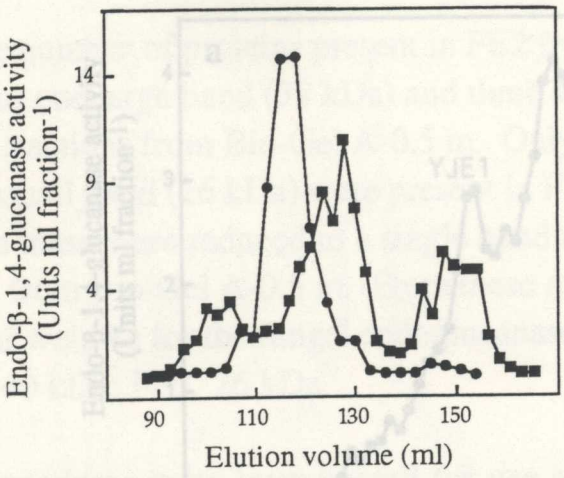

Fig. 14. Separation of endo-β-1,4-glucanase activities from young major workers and fungal nodules from *M. bellicosus*. Profiles of crude extracts on Bio-Gel P-150.
Young major workers (⊷) Fungal nodules (⊷)

For the sake of clarity, zero value activities are indicated at the leading and trailing edges of each peak.

Chromatography of pooled fractions containing the main endo-β-1,4-glucanase activity from young major workers on Bio-Gel A-0.5 m (Fig. 15a) resulted in the separation of three major peaks (YJE1, YJE2, and YJE3) and a number of minor peaks of endo-β-1,4-glucanase activity. Rechromatography of these major peaks on Bio-Gel A-0.5 m (Fig. 15b) resolved these into YJE1 containing a minor peak and a small amount of YJE2, YJE2 containing a minor peak and a small amount of YJE1, and YJE3 consisting of two components with a small amount of JYE2. On the basis of gel chromatography on Bio-Gel P-60 and repeated chromatography on Bio-Gel A-0.5 m, endo-β-1,4-glucanase activity in young workers appears to consist of two major (YJE1 and YJE2) and several minor components including YJE3. Chromatography of pooled fractions containing the three main peaks of endo-β-1,4-glucanase activity from fungal nodules from Bio-Gel P-150 on Bio-Gel A-0.5 m (Fig. 15c) resolved only three major peaks (FE1, FE2, and FE3) of endo-β-1,4-glucanase activity.

The extent of the purification of the endo-β-1,4-glucanase activities from fungal nodules in the two steps of gel filtration, Bio-Gel P-150 and Bio-Gel A-0.5 m (Fig. 15c), was checked with SDS-PAGE using the peak fractions (data not shown). The large number of proteins present in FE1 fractions from Bio-Gel P-150 was reduced to two major bands (40 and 48 kDa) and one minor band (51 kDa) in the peak fractions from Bio-Gel A-0.5 m.

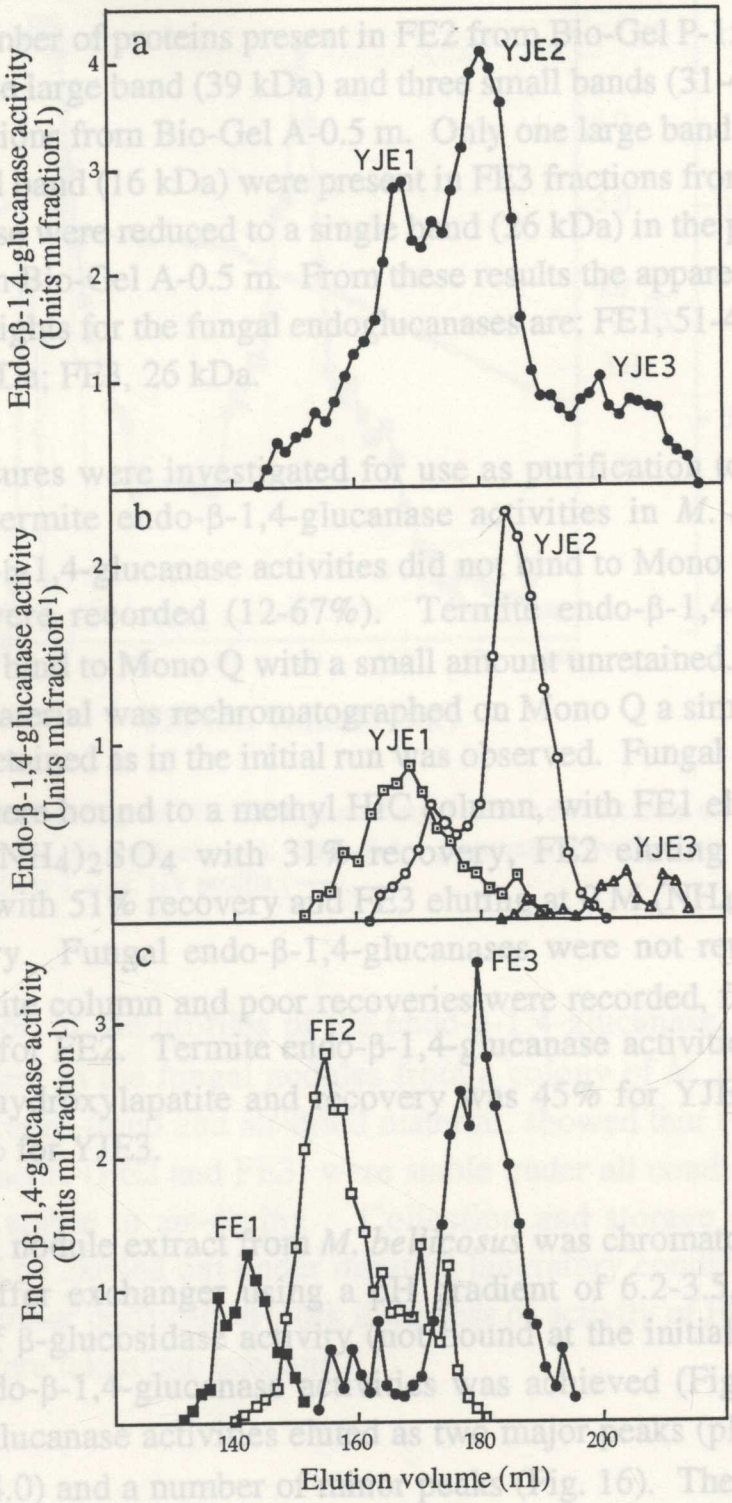

Fig. 15. Separation of endo-β-1,4-glucanase activities from young major workers and fungal nodules from *M. bellicosus.*

a) Chromatography of the major peak of activity of young major endo-β-1,4-glucanase activity from Bio-Gel P-150 on Bio-Gel A-0.5 m.

b) Rechromatography of the major peaks of activity of young major endo-β-1,4-glucanase activities (YJE1 ▫▫ , YJE2 ○○ and YJE3 ▲▲) on Bio-Gel A-0.5 m.

c) Chromatography of the main fungal endo-β-1,4-glucanase activities (FE1■■ , FE2 ▫▫ and FE3 ●●) on Bio-Gel A-0.5 m.

The large number of proteins present in FE2 from Bio-Gel P-150 was reduced to one large band (39 kDa) and three small bands (31-41 kDa) in the peak fractions from Bio-Gel A-0.5 m. Only one large band (26 kDa) and one small band (16 kDa) were present in FE3 fractions from Bio-Gel P150 and these were reduced to a single band (26 kDa) in the peak fractions from Bio-Gel A-0.5 m. From these results the apparent molecular weights for the fungal endoglucanases are: FE1, 51-48 kDa; FE2, 39-40 kDa; FE3, 26 kDa.

Other procedures were investigated for use as purification tools for the fungal and termite endo-β-1,4-glucanase activities in *M. bellicosus*. Fungal endo-b-1,4-glucanase activities did not bind to Mono Q and poor recoveries were recorded (12-67%). Termite endo-β-1,4-glucanase activities did bind to Mono Q with a small amount unretained. When this unretained material was rechromatographed on Mono Q a similar ratio of bound to unretained as in the initial run was observed. Fungal endo-β-1,4-glucanases were bound to a methyl HIC column, with FE1 eluting at 0.6 to 0.24 M $(NH_4)_2SO_4$ with 31% recovery, FE2 eluting at 0.24 M $(NH_4)_2SO_4$ with 51% recovery and FE3 eluting at 0 M $(NH_4)_2SO_4$ with 48% recovery. Fungal endo-β-1,4-glucanases were not retained on a hydroxylapatite column and poor recoveries were recorded, from 1% for FE1 to 36% for FE2. Termite endo-β-1,4-glucanase activities were not retained on hydroxylapatite and recovery was 45% for YJE1, 20% for YJE2 and 6% for YJE3.

Crude fungal nodule extract from *M. bellicosus* was chromatofocused on PBE Polybuffer exchanger using a pH gradient of 6.2-3.5. A partial separation of β-glucosidase activity (not bound at the initial pH of 6.2) from the endo-β-1,4-glucanase activities was achieved (Fig. 16). The endo-β-1,4-glucanase activities eluted as two major peaks (pH 5.5 to 4.7 and 4.25 to 4.0) and a number of minor peaks (Fig. 16). The first eluted peak of endo-β-1,4-glucanase activity (pH 4.5 to 4.7) contained the highest protein concentration (A_{280}) and some β-glucosidase activity. The second main peak and the minor peaks of endo-β-1,4-glucanase activity were low in protein.

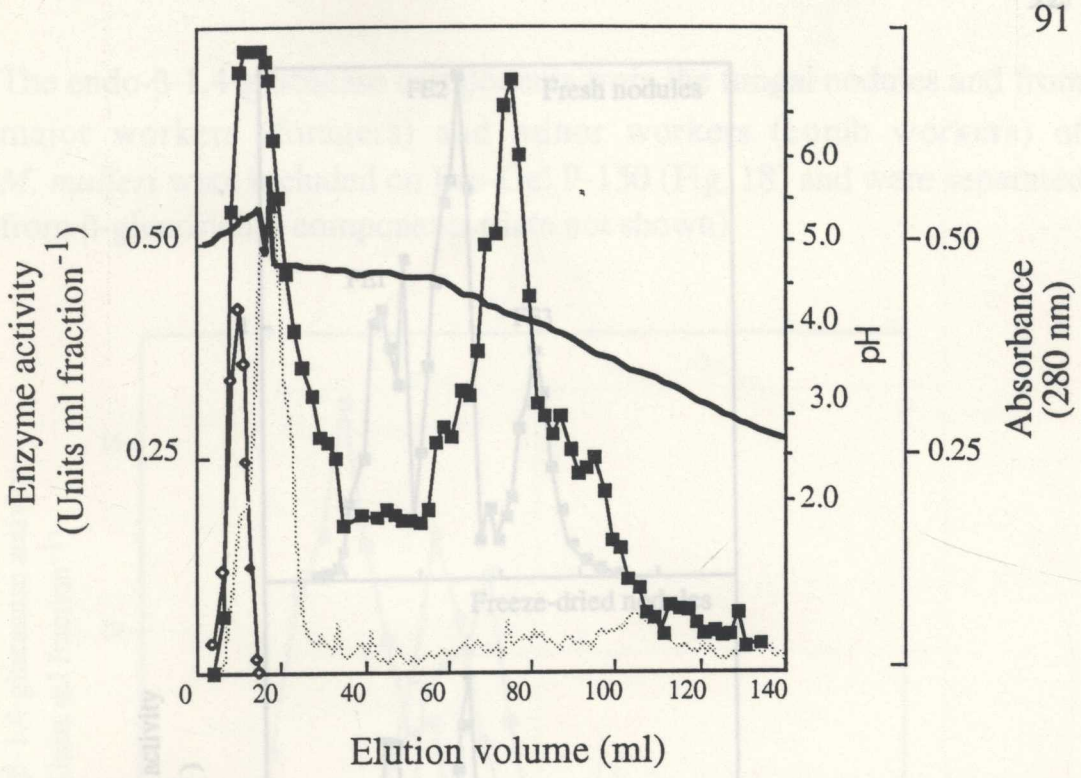

Fig. 16. Chromatofocusing on PBE 94 of crude fungal nodule extract from a *M. bellicosus* colony. pH gradient (—). Endo-β-1,4-glucanase activity (■—■). β-Glucosidase activity (●—●). UV profile (—)

An examination of the profiles of the endo-β-1,4-glucanase activities (Fig. 17) present in the fungal nodules from a colony of *M. bellicosus* using fresh, freeze-dried and air dried material, showed that two of the major components (FE2 and FE3) were stable under all conditions, but FE1 was not stable to air-drying. Collection and storage of fungal nodules could be carried out under minimal laboratory conditions or in the field for further purification work if the loss of activity of FE1 during this process were taken into account.

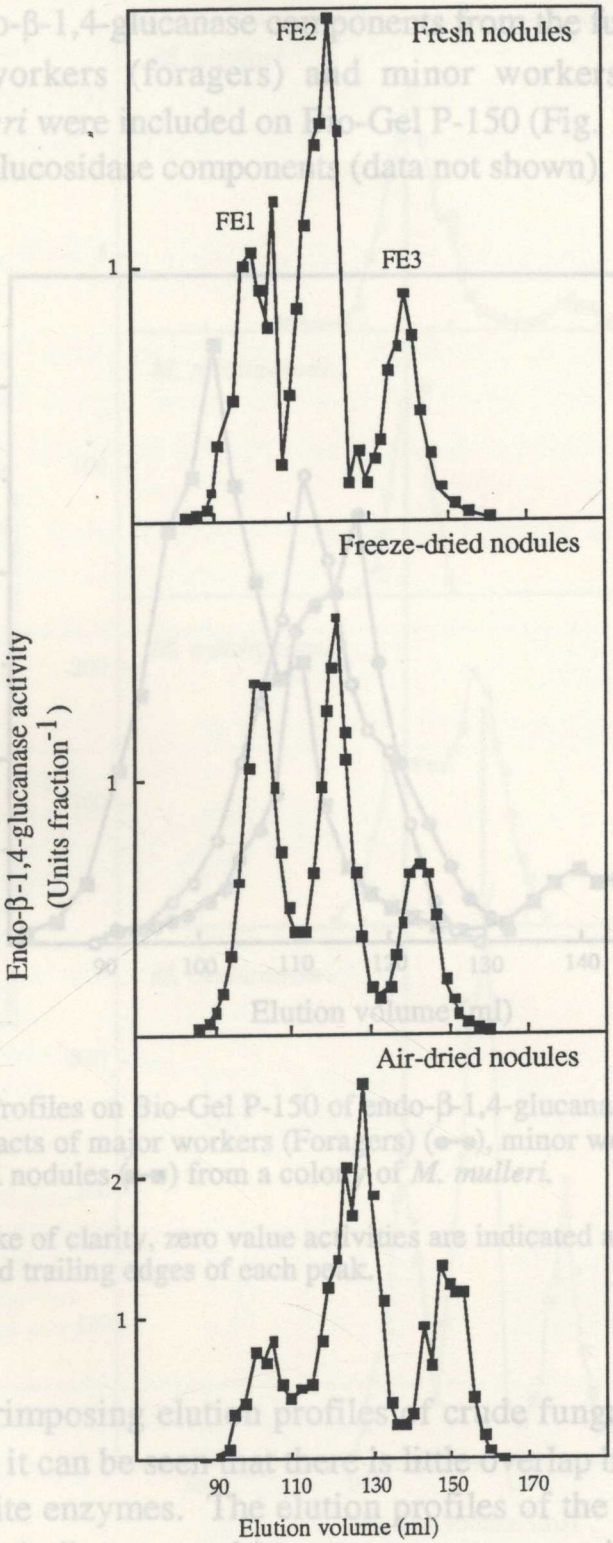

Fig. 17. The effect of different preservation treatments on endo-β-1,4-glucanase activity from fungal nodules from a *M. bellicosus* colony. Elution profiles shown are endo-β-1,4-glucanase activity on Bio-Gel P-150.

For the sake of clarity, zero value activities are indicated at the leading and trailing edges of each peak.

The endo-β-1,4-glucanase components from the fungal nodules and from major workers (foragers) and minor workers (comb workers) of *M. mulleri* were included on Bio-Gel P-150 (Fig. 18) and were separated from β-glucosidase components (data not shown).

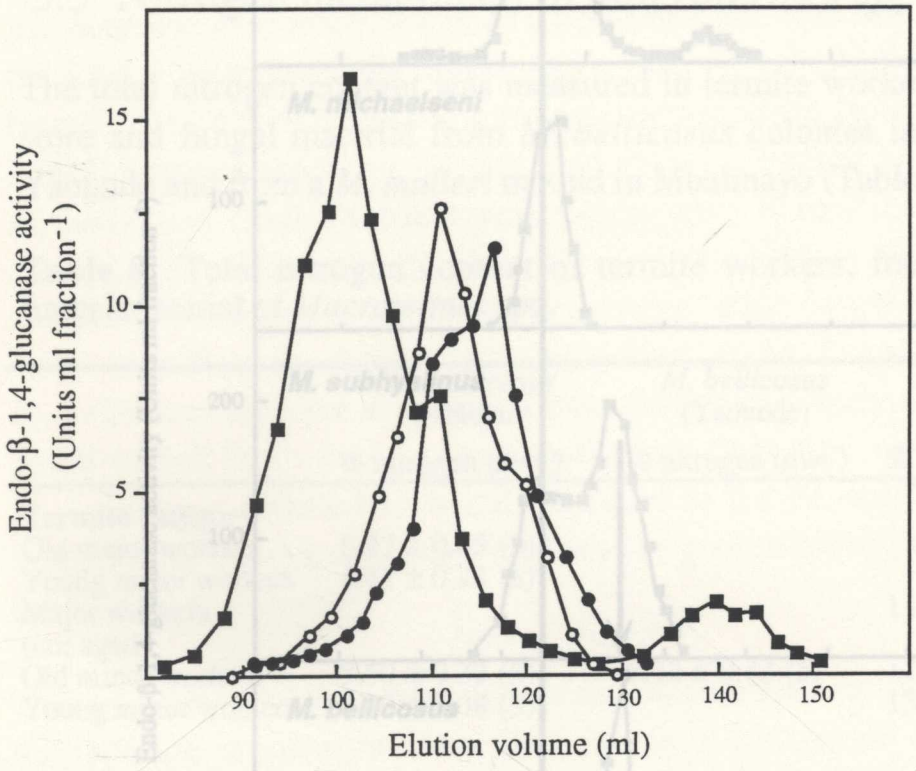

Fig. 18. Profiles on Bio-Gel P-150 of endo-β-1,4-glucanase activities from crude extracts of major workers (Foragers) (●—●), minor workers (comb workers) (○—○) and fungal nodules (■—■) from a colony of *M. mulleri.*

For the sake of clarity, zero value activities are indicated at the leading and trailing edges of each peak.

By superimposing elution profiles of crude fungal and termite extracts (Fig. 18) it can be seen that there is little overlap between the fungal and the termite enzymes. The elution profiles of the endo-β-1,4-glucanase activities indicate a multi-component system with fungal endo-β-1,4-glucanase eluting as one major and one minor peak and termite endo-β-1,4-glucanase eluting as one major and one minor peak (Fig. 18).

A comparison was made of profiles on Bio-Gel P-150 of endo-β-1,4-glucanase activities (Fig. 19) from fungal nodules from colonies of *M. bellicosus, M. michaelseni, M. subhyalinus* and *M. mulleri.*

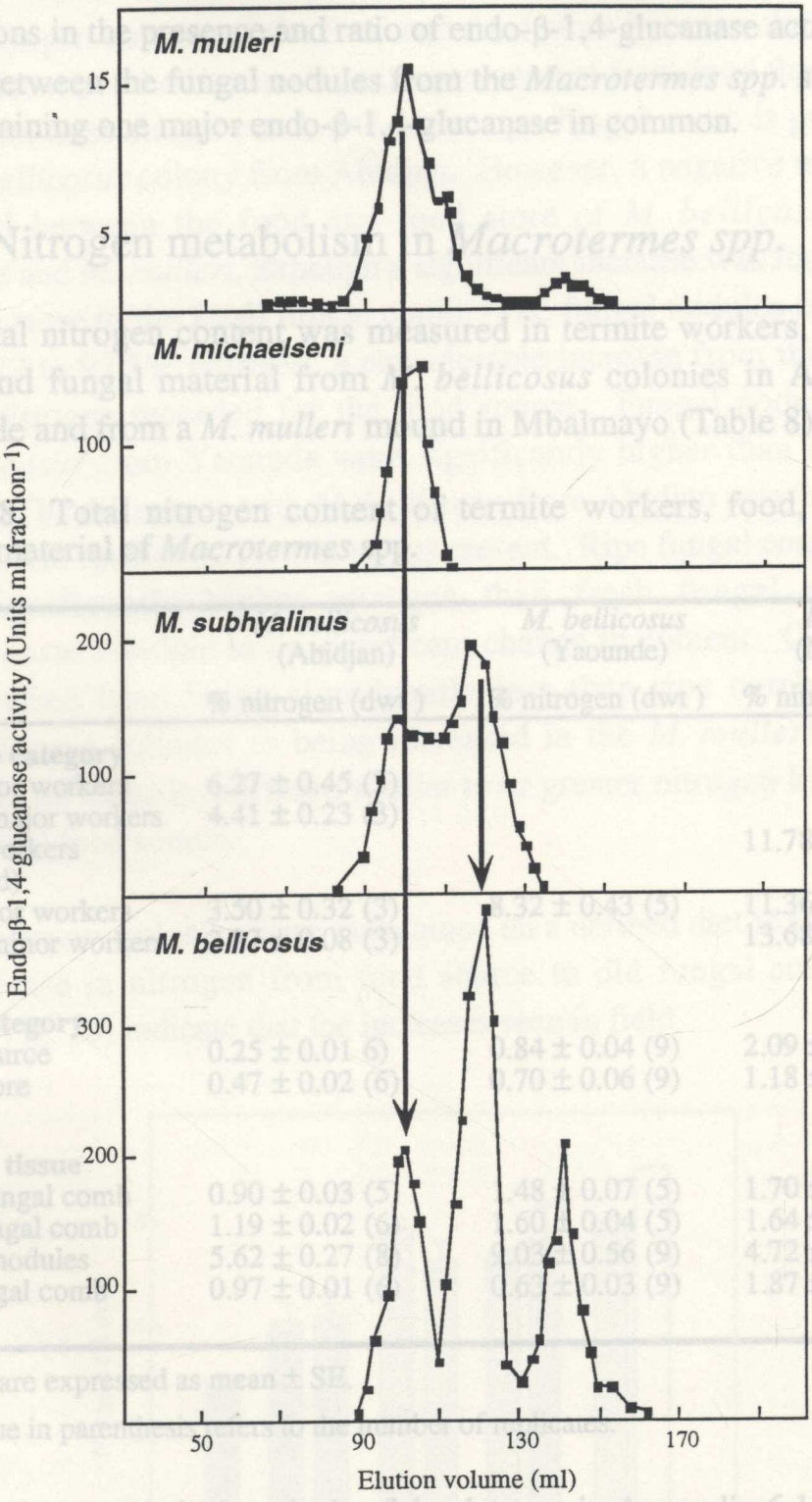

Fig. 19. Endo-β-1,4-glucanase activities in fungal nodules. Elution profile on Bio-Gel P-150 of crude extracts of freeze-dried *(M. michaelseni)* and fresh *(M. mulleri, M. subhyalinus* and *M. bellicosus)* fungal nodules.

For the sake of clarity, zero value activities are indicated at the leading and trailing edges of each peak.

Variations in the presence and ratio of endo-β-1,4-glucanase activities were noted between the fungal nodules from the *Macrotermes spp.* studied with all containing one major endo-β-1,4-glucanase in common.

3.5 Nitrogen metabolism in *Macrotermes spp.*

The total nitrogen content was measured in termite workers, food, food store and fungal material from *M. bellicosus* colonies in Abidjan and Yaounde and from a *M. mulleri* mound in Mbalmayo (Table 8).

Table 8. Total nitrogen content of termite workers, food, foodstore and fungal material of *Macrotermes* spp.

	M. bellicosus (Abidjan)	*M. bellicosus* (Yaounde)	*M. mulleri* (Mbalmayo)
	% nitrogen (dwt)	% nitrogen (dwt)	% nitrogen (dwt)
Termite category			
Old major workers	6.27 ± 0.45 (3)		
Young major workers	4.41 ± 0.23 (3)		
Major workers (not aged)			11.78 ± 0.50 (5)
Old minor workers	3.50 ± 0.32 (3)	8.32 ± 0.43 (5)	11.36 ± 0.64 (5)
Young minor workers	2.87 ± 0.08 (3)		13.68 ± 2.34 (5)
Food category			
Food source	0.25 ± 0.01 6)	0.84 ± 0.04 (9)	2.09 ± 0.08 (9)
Food store	0.47 ± 0.02 (6)	0.70 ± 0.06 (9)	1.18 ± 0.05 (9)
Fungal tissue			
Fresh fungal comb	0.90 ± 0.03 (5)	1.48 ± 0.07 (5)	1.70 ± 0.13 (7)
Ripe fungal comb	1.19 ± 0.02 (6)	1.60 ± 0.04 (5)	1.64 ± 0.07 (5)
Fungal nodules	5.62 ± 0.27 (8)	9.03 ± 0.56 (9)	4.72 ± 0.44 (5)
Old fungal comb	0.97 ± 0.01 (6)	0.63 ± 0.03 (9)	1.87 ± 0.08 (5)

Results are expressed as mean ± SE.

The value in parenthesis refers to the number of replicates.

Results from statistical analysis of the data are in Appendix 6.13. The data series for termite workers is incomplete due to the difficulties in obtaining samples for all age groups from the colonies in Mbalmayo and Yaounde. Considerable differences were found between the species and within species from the different locations. Differences were also noted in the

in the nitrogen content of the food and food store between the species. A significant trend of increasing nitrogen content from food through the food store, fresh fungal comb and to the ripe fungal comb is present in the *M. bellicosus* colony from Abidjan. However, a negative trend was observed between the food and food store of *M. bellicosus* from Yaounde and *M. mulleri*, although a significant increase was found from the food store to the fresh fungal comb. The fungal nodules contained from 4.72 to 9.03% nitrogen, a considerable increase from the 0.25 to 2.09% nitrogen recorded for the food source. Fungal nodules from *M. bellicosus* from Yaounde were significantly higher than the other species. The difference with *M. bellicosus* from Abidjan may be related to a different species of *Termitomyces* present. Ripe fungal comb varied from significantly higher nitrogen than fresh fungal comb in *M. bellicosus* Abidjan to no significant change in content. Old fungal comb varied from being significantly less than ripe comb in both *M. bellicosus* colonies to being increased in the *M. mulleri* colony, though in all cases it contained similar to or greater nitrogen levels than the original food source.

Two laboratory colonies were maintained on a defined diet to investigate the increase in nitrogen from food source to old fungal comb. The results (Fig. 20) indicate that the increases seen in field

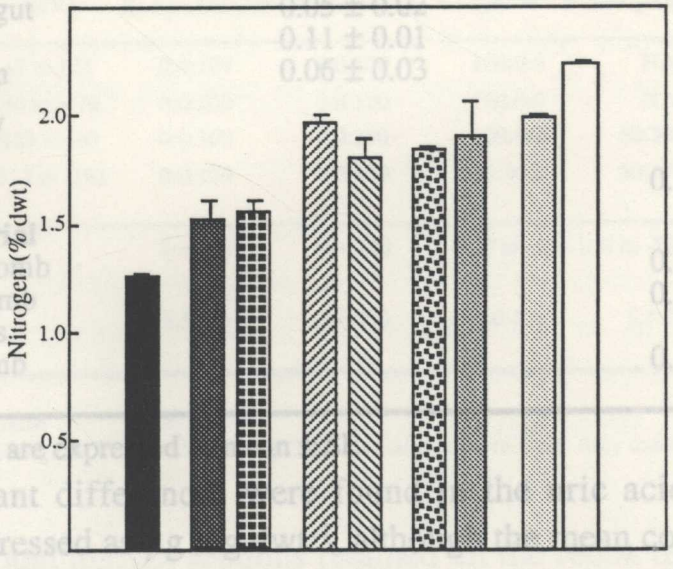

Fig. 20. Nitrogen content (% dwt) in food and fungal material from two laboratory colonies (a and b) of *M. bellicosus* (Bern) maintained on a constant diet. Shown in order of increasing age with SE error bars.

Food ■ Food store colony a ■ b ■

Fresh fungal comb a ▯ b ▯ Ripe fungal comb a ▦ b ▦ Old fungal comb a ▨ b ▨

colonies can be demonstrated in laboratory colonies. Unfortunately the leaf material selected as the constant food source was not tested for nitrogen content prior to the start of the experiment in Bern. It was found to have a high nitrogen content which could mask some of the increases in nitrogen in the steps through the fungal garden.

The levels of uric acid were determined in the workers, food, food store and fungal material from the *M. bellicosus* colony Abidjan (Table 9) to examine possible correlations with the nitrogen content.

Table 9. Uric acid content of termite workers, food, food store, and fungal material from a field colony of *M. bellicosus*.

	Uric acid (μg termite^{-1})	Uric acid (μg mg dwt^{-1})
Termite worker		
Old major	23.40 ± 0.52	6.11 ± 0.63
Young major	24.70 ± 2.67	5.83 ± 0.39
Old minor	16.61 ± 2.66	11.36 ± 1.34
Young minor	21.43 ± 2.70	8.08 ± 3.02
Termite gut section		
Old major		
Foregut-midgut	0.02 ± 0.01	
Paunch	0.14 ± 0.01	
Colon-rectum	0.04 ± 0.05	
Young major		
Foregut-midgut	0.05 ± 0.02	
Paunch	0.11 ± 0.01	
Colon-rectum	0.06 ± 0.03	
Food category		
Food source		0.00
Food store		0.11 ± 0.02
Fungal material		
Fresh fungal comb		0.12 ± 0.03
Ripe fungal comb		0.11 ± 0.01
Fungal nodules		0.00
Old fungal comb		0.19 ± 0.01

n = 3; Results are expressed as mean ± SE.

No significant differences were found in the uric acid content of all workers expressed as μg mg dwt^{-1}, although the mean content did appear higher for the older workers.

In the gut, uric acid was mainly found in the paunch, but this was less than 1% of the total uric acid found in the whole worker. Uric acid was not detected in the food source, but could be measured in the food store and fungal comb. No significant increases were found from food store to ripe fungal comb but a significant increase occurred from ripe comb to old comb (Statistical data in Appendix 6.14).

3.6 Physical characteristics of *N. walkeri*

The haemolymph volume was 1.5 ± 0.5 µl (n = 10) and the total hindgut volume of *N. walkeri* was 1.5 ± 0.4 µl (n = 4) with a gut fluid volume of 0.46 ± 0.06 µl (n = 4). The estimate of gut fluid volume as 30% of the total gut volume is supported by estimates that the bacterial pellet from the hindgut constituted 44% of total hindgut weight (Lo, 1994).

The results from feeding *N. walkeri* workers with redox dyes and from pH estimation are shown in Table 10.

Table 10. pH and redox potentials (E_1^0 mV) of *N. walkeri* worker guts.

		foregut	midgut	paunch	colon	rectum
Redox dye	Range (mV)	% ratio Red:partial:Ox[1]	% ratio Red:partial:Ox	% ratio Red:partial:Ox	% ratio Red:partial:Ox	% ratio Red:partial:Ox
Thionine	+3 to 123	0:0:100	0:0:100	100:0:0	NA	NA
Methylene blue	-50 to + 70	0:0:100	0:0:100	100:0:0	NA	NA
Nile blue	-183 to -63	0:0:100	0:0:100	100:0:0	60:30:10	60:30:10
Phenosafranin	-312 to -192	0:0:100	0:0:100	70:30:0	30:60:10	30:60:10
E_1^0 range (mV)		> + 100	> + 100	-300 to -250-200 to -50		-200 to -50
pH range		5.5-6.0	6.0-8.0	6.0-8.0	5.5-7.0	5.5

n = 20
NA - data not available
[1]% termites containing dye in the reduced fully form: partially reduced form: fully oxidised form.

The foregut and midgut sections retained all the redox dyes tested in the oxidised form, indicating the E_1^0 was in excess of +100 mV. The paunch remained colourless with thionine, methylene blue and nile blue

indicating a redox value less than -183 mV. After feeding phenosafranin the paunch was colourless in 70% of the workers examined and a light pink that darkened on exposure to air in the remaining 30% of workers, indicating a redox range of -300 to -250 mV. The colon and rectum showed some variability in the reduction of the dyes with only 30% showing full reduction of phenosafranin, indicating a redox range of -250 to -200 mV.

Redox dye values are given as the midpoint redox potential at pH 7.0 (Schlossberg and Hollander, 1973). However, these values vary with pH, becoming more positive with a fall in pH below 7.0 (Hewitt, 1950) so the pH of the gut should be estimated in order to apply any necessary corrections to the redox values obtained.

When examining pooled ruptured midgut sections it was found that the epithelial tissue (8.0 - 9.0) had a pH higher than that of the contents (6.0 - 8.0). This caused some problems in interpretation of those results where tissue was included with gut contents so results using individual guts were used where the contents were leaked into a drop of indicator dye. The digestive system begins with an acidic foregut (pH 5.5 - 6.0) and continues with a rapid increase in pH from the anterior midgut to become slightly alkaline (pH 6.0 - 8.0) in the posterior midgut. The pH in the paunch (7.0 - 8.0) drops to 5.5 - 7.0 in the colon, to a final pH of 5.5 for the rectum. The faeces when voided during handling or after stimulation had a consistently low pH of 2.5 - 3.0. The significance of this change from the pH seen in rectal contents (5.5) to voided faeces (2.5) is unclear.

These pH results when correlated with the observed E_1^0 values allow for more accurate conclusions to be drawn on the gut redox value (Hewitt, 1950) . As the foregut is acidic, the effect would be an increase in the E_1^0 value to greater than +100 mV. The midgut E_1^0 value would remain in the +100 mV range; the paunch would remain in the to -300 to -250 mV range; the colon would slightly increase to above -250 to -200 mV; and the rectum would be increased to above -200 to -50 mV.

3.7 Glucose and xylose metabolism in *N. walkeri*

Glucose was estimated in the hindgut of *N. walkeri*, using the GOD-Perid reagent, as 0.07 nmol gut^{-1} (0.15 ± 0.01 mM based on gut fluid volume; n = 4). It was suspected that substances in the gut such as pigments interfered with the assay by absorbing at 610nm thus overestimating the true glucose. Extracts analysed by TLC gave approximate glucose concentrations (based on gut fluid volumes) of midgut, > 2 mM; mixed segment, about 0.2 mM; and hindgut, < 0.01 mM. These estimations were made by visually comparing the intensity of the spots from the extracts with known standards. The production of a higher estimation of glucose in the hindgut extract with the GOD-Perid compared to the TLC method may be due to a contribution to the absorbance at 610 nm by compounds present in concentrated extracts. This would not affect endo-β-1,4-glucanase and β-glucosidase assays in the hindgut as more dilute extracts were used as well as appropriate controls.

No glucophosphotransferase activity could be detected in the hindgut bacterial pellet and there was negligible activity of hexokinase activity in both the gut epithelium and the hindgut contents (Table 11). The key enzymes of glycolysis, PFK and pyruvate kinase have high activity in termite tissues and the hindgut contents (Table 11). Xylulokinase, the enzyme which activates xylulose which is produced from xylose, the pentose produced by xylan hydrolysis, was present in all extracts.

Table 11. Enzymes of glucose and xylose utilisation in *N. walkeri.*

	Enzyme Activity (nmol termite^{-1} h^{-1})		
	Degutted Body	Epithelium	Hindgut contents
Hexokinase	130 ± 17	0.6 ± 0.6	0.6 ± 0.6
Glucophosphotransferase			0.0
Xylulokinase	47.01 ± 7.44	13.44 ± 4.82	30.42 ± 11.17
Phosphofructokinase	664 ± 184	145 ± 25	220 ± 50
Pyruvate kinase	778 ± 15	116 ± 7	126 ± 38

n = 4; Results are expressed as mean ± SE.

3.8 Pyruvate metabolism in *N. walkeri*.

Pyruvate was estimated enzymatically in whole termites prepared by the homogenisation of termites in liquid nitrogen. This technique reduces the effect that the extraction procedure may have on metabolite concentrations. When the values obtained (0.29 ± 0.05 nmol termite^{-1}; n = 7) were compared with the fluid volume of worker of *N. walkeri* (6 µl), a concentration of 0.048 mM was obtained. Pyruvate was also estimated enzymatically in the haemolymph and the hindgut of *N. walkeri*. Values were close to the limits of the assay method with a haemolymph concentration of 0.058 ± 0.029 mM; n = 4. No pyruvate could be detected in the hindgut fluid.

No activity of the PDH complex could be detected in the termite tissue using whole tissue, homogenates and enzyme extracts incubated with [1-^{14}C]pyruvate nor in enzyme extracts using the spectrophotometric assays coupling the acetyl CoA with AABS through the addition of arylamine acetyltransferase and measuring the utilisation of NAD^+. These assays were all functional with silverfish, bogong moth or woodroach (*P. cribrata*) tissues (results not presented). The results using the [1-^{14}C]pyruvate radioassay with enzyme extracts are summarised in Table 12. In contrast, the PDH complex is active in the hindgut indicating that the bacteria can utilise pyruvate aerobically (Table 12). The activity found in the epithelium extract is possibly due to

Table 12. Enzymes of pyruvate metabolism in *N. walkeri*.

	Enzyme Activity (nmol termite^{-1} h^{-1})		
	Degutted Body	Epithelium	Hindgut contents
Pyruvate dehydrogenase complex			
- chloramphenicol	0 (4)	7 ± 4 (3)	46 ± 10 (4)
+ chloramphenicol	N.D.	14 ± 9 (6)	66 ± 18 (6)
Pyruvate-ferredoxin oxidoreductase	1.4 + 0.9 (4)	36 ± 2 (4)[1]	

N.D. - activity not determined; results are expressed as mean ± SE.

The value in parenthesis refers to the number of replicates.

[1]Epithelial tissue and hindgut contents prepared together.

bacteria adhering to the gut wall or not washed completely from the epithelial tissue (Table 12). Exposure of the hindgut contents to oxygen after gut removal during the extraction procedure might stimulate the synthesis of aerobic enzymes, especially the PDH complex (Spiro and Guest, 1991). Extracts prepared in the presence of chloramphenicol, an inhibitor of prokaryotic protein synthesis, did not significantly differ (using *t*-tests) from those prepared in the absence of chloramphenicol (Table 12). Pyruvate can be anaerobically metabolised in the hindgut at 36 ± 2 nmol termite^{-1} h^{-1} by pyruvate-ferredoxin oxidoreductase (PFO) present in the hindgut. A small amount of $^{14}CO_2$ was produced from [1-^{14}C]pyruvate in the body tissue (1.4 ± 0.9 nmol termite^{-1} h^{-1}).

As expected, a fully functional TCA cycle is present in termite tissue (Table 13). The activity of the key enzymes of the TCA cycle in the hindgut contents indicates aerobic metabolism is occurring in some of the hindgut bacteria (Table 13). The epithelial tissue has a very active TCA cycle not consistent with the glycolytic activities. Exposure of the hindgut contents to oxygen after the removal of the gut during the extraction procedure might be expected to stimulate the synthesis of enzymes of aerobic metabolism, especially the TCA enzymes (Spiro and Guest, 1991) so enzyme extracts were prepared in the presence of chloramphenicol.

Table 13. Enzymes of the TCA cycle in *N. walkeri* workers.

	Enzyme Activity (nmol termite^{-1} h^{-1})		
	Degutted Body	Epithelium	Hindgut contents
Citrate synthase	505 ± 81 (3)	309 ± 24 (3)	34 ± 6 (3)
Isocitrate dehydrogenase	143 ± 17 (3)	62 ± 11 (4)	32 ± 9 (3)
2-oxoglutarate dehydrogenase			
- chloramphenicol	707 ± 173 (5)	1439 ± 386 (5)	140 ± 63 (5)
+ chloramphenicol	N.D.	1285 ± 418 (6)	131 ± 53 (6)
Malate dehydrogenase			
- malate to OAA	1146 ± 55 (5)	245 ± 32 (5)	234 ± 31 (5)
- OAA to malate	1530 ± 342 (4)	145 ± 75 (4)	278 ± 37 (4)

N.D. - activity not determined; results are expressed as mean ± SE.

The value in parenthesis refers to the number of replicates.

No significant differences (*t*-tests) were observed between normal extracts and those prepared in the presence of chloramphenicol for the 2-oxoglutarate dehydrogenase assays (Table 13).

The rate of oxygen utilisation in isolated *N. walkeri* hindgut contents (bacteria, wood particles, substrates) was measured, using an oxygen electrode, as a function of the concentration of a range of potential substrates for aerobic bacterial metabolism (Table 14). Acetate and glucose had the least effect on oxygen utilisation while alanine and xylose were effective as potential substrates. The most dramatic effect was with pyruvate which was studied further using a range of concentrations.

Table 14. Effect of metabolites on oxygen utilisation

Substrate	Concentration (mM)	Oxygen utilisation (nmol termite^{-1} h^{-1})
No substrate	-	7.44 ± 0.59 (20)
Acetate	6.91	1.07 ± 0.67 (3)
Alanine	13.33	7.96 ± 1.12 (3)
Glucose	6.67	3.55 ± 0.50 (3)
Pyruvate	6.67	37.4 ± 7.5 (3)
Xylose	6.67	8.71 ± 3.53 (3)

Results are expressed as mean ± SE.

The value in parenthesis refers to the number of replicates.

The oxygen utilisation is presumably the summation of the oxidations of NADH and $FADH_2$ generated by the PDH complex and the TCA cycle. Bearing this in mind, a Michaelis-Menten fit was carried out using the Regression® software package (Blackwell, Oxford, UK) to give an approximation of the maximal rate of oxygen utilisation (Fig. 21). Oxygen utilisation clearly approaches a maximal rate (68.4 nmol termite^{-1}h^{-1}) and increases in pyruvate concentration above 5 mM have little effect on the rate.

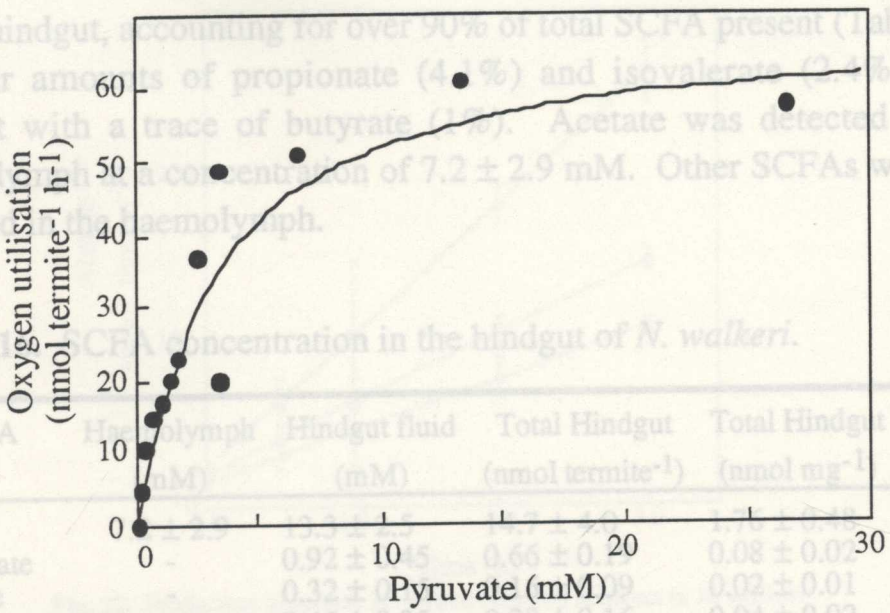

Fig. 21. The effect of pyruvate on oxygen utilisation in gut contents of *N. walkeri.*

The normal anaplerotic enzymes found in animals, pyruvate carboxylase and PEP carboxykinase, were low in activity in termite tissue but PEP carboxykinase was active in the hindgut (Table 15).

Table 15. Anaplerotic enzymes in *N. walkeri.*

	Enzyme Activity (nmol termite^{-1} h^{-1})		
	Degutted Body	Epithelium	Hindgut contents
Pyruvate carboxylase	0.70 ± 0.10	0.14 ± 0.06	0.12 ± 0.03
PEP carboxykinase	1.64 ± 0.51	1.96 ± 0.36	8.36 ± 0.40

n = 3; Results are expressed as mean ± SE.

The key enzymes of the glyoxylate cycle, isocitrate lyase and malate synthase were not detected in termite or hindgut extracts.

3.9 Acetate metabolism in *N. walkeri.*

SCFAs were estimated in *N. walkeri* in the haemolymph (HPLC) and hindgut fluid using HPLC and GC methods. Acetate was the main SCFA

in the hindgut, accounting for over 90% of total SCFA present (Table 16). Smaller amounts of propionate (4.1%) and isovalerate (2.4%) were present with a trace of butyrate (1%). Acetate was detected in the haemolymph at a concentration of 7.2 ± 2.9 mM. Other SCFAs were not detected in the haemolymph.

Table 16. SCFA concentration in the hindgut of *N. walkeri*.

SCFA	Haemolymph (mM)	Hindgut fluid (mM)	Total Hindgut (nmol termite^{-1})	Total Hindgut (nmol mg^{-1})	%
Acetate	7.2 ± 2.9	13.3 ± 2.5	14.7 ± 4.0	1.76 ± 0.48	92.5
Propionate	-	0.92 ± 0.45	0.66 ± 0.19	0.08 ± 0.02	4.1
Butyrate	-	0.32 ± 0.15	0.15 ± 0.09	0.02 ± 0.01	1.0
*Iso*valerate	-	0.45 ± 0.25	0.38 ± 0.16	0.04 ± 0.02	2.4

n = 4; Results are expressed as mean ± SE.

Acetate was measured in the hindgut of *N. walkeri* by HPLC at 12 ± 4 nmol (n = 15) and by GC at 14.7 ± 4.0 nmol (n = 4). The concentration using the estimate of gut fluid volume of 0.46 ± 0.06 µl was thus approximately 27.4 ± 3.4 mM; n = 4. In comparison, the concentration was 13.3 ± 2.5 mM when gut fluid was analysed by GC. There is no obvious explanation for the difference in concentration given by the two methods.

The rate of acetate production was measured in isolated paunches incubated anaerobically in an atmosphere containing 10% CO_2 and 10% H_2. Acetate was produced at a rate of 32 ± 4 nmol termite^{-1} h^{-1} (n = 4) (Fig. 22). Incubation of the paunches in the presence of 5 mM glucose did not increase the rate but the presence of 50 mM glucose increased it to 43 nmol termite^{-1} h^{-1} (Fig. 22). Similar data were obtained with 5 and 50 mM trehalose where the rate increased to 40 ± 6 nmol termite^{-1} h^{-1} (n = 4). However, the rate was dramatically increased to 79 ± 6 nmol termite^{-1} h^{-1} (n = 3) by the addition of 5 mM pyruvate (Fig. 22). A higher concentration (50 mM) did not increase this rate.

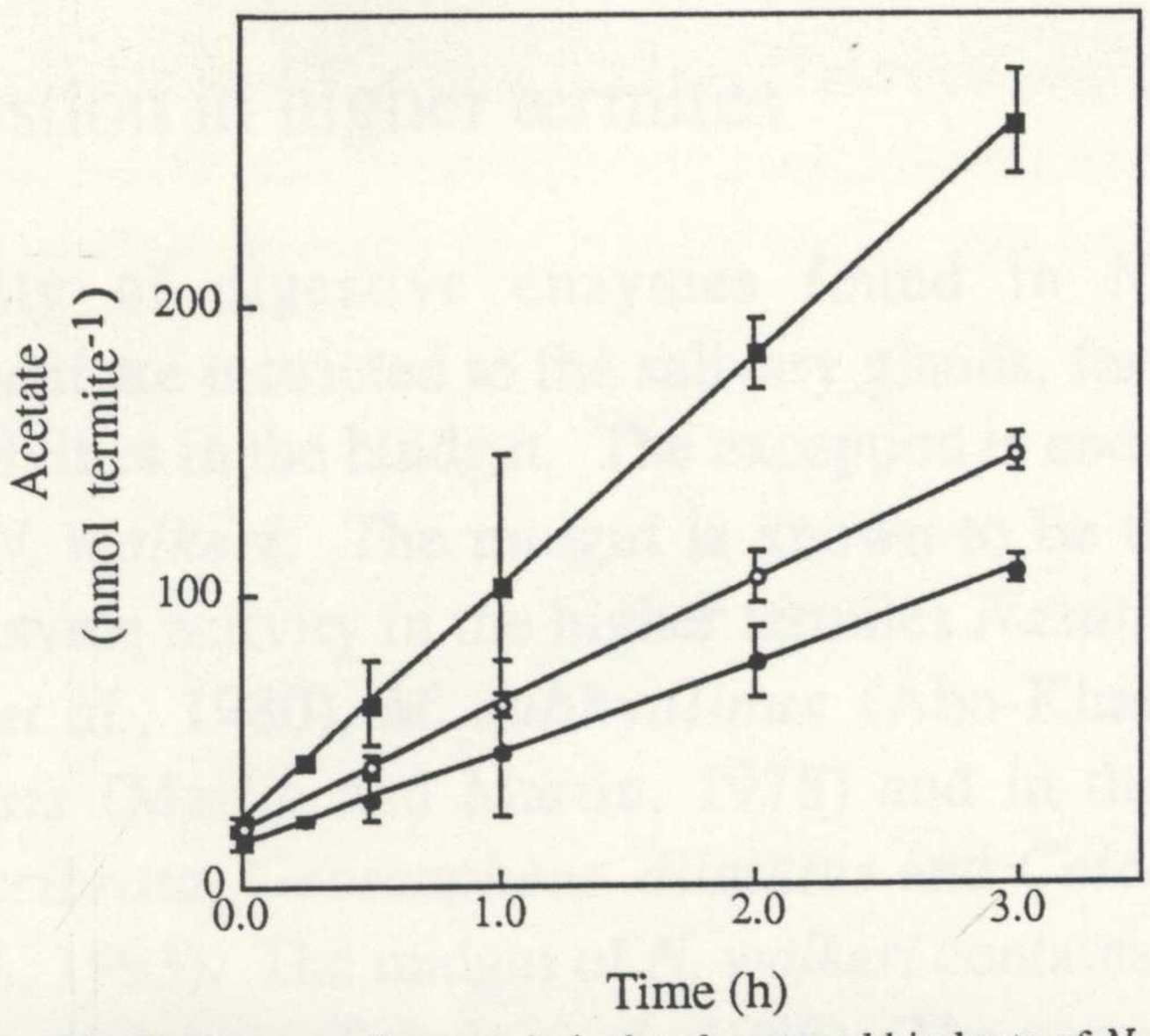

Fig. 22. Production of acetate in isolated ruptured hindguts of *N. walkeri* workers incubated anaerobically with no added substrate (●—●), added glucose (○—○) (50 mM) and added pyruvate (■—■) (5 mM).

An active acetyl CoA synthetase is found in termite body and epithelial tissue with a very high activity in the gut (Table 17).

Table 17. Enzymes of acetate metabolism in *N. walkeri*.

	Enzyme Activity (nmol termite^{-1} h^{-1})		
	Degutted Body	Epithelium	Hindgut contents
Acetyl CoA synthetase	12 ± 2 (4)	43 ± 7 (4)	213 ± 10 (4)
Acetyl CoA hydrolase	97 ± 15 (3)	34 ± 3 (3)	24 ± 6 (3)
Malic enzyme			
+ NADH	0.19 ± 0.10	0.08 ± 0.04	0.71 ± 0.22
+ NADPH	12.92 ± 0.70	0.71 ± 0.03	2.50 ± 0.32

n = 3; Results are expressed as mean ± SE.

Acetyl CoA hydrolase activity is distributed in a reverse pattern with the least activity in the hindgut and the highest activity in the termite body. Low levels of malic enzyme using NADH were present in all tissues but moderate levels of malic enzyme using NADPH were found in the termite tissue with minor values in the hindgut.

DISCUSSION

4.1 Digestion in higher termites

The majority of digestive enzymes found in *N. walkeri* and *M. michaelseni* are restricted to the salivary glands, foregut and midgut with low activities in the hindgut. The exception is endo-β-1,4-xylanase activity in *N. walkeri*. The midgut is known to be the major site of digestive enzyme activity in the higher termites *Nasutitermes exitiosus* (McEwen *et al.*, 1980), *M. subhyalinus* (Abo-Khatwa, 1978) and *M. natalensis* (Martin and Martin, 1978) and in the wood roaches *Panesthia cribrata, Geoscapheus dilatatus* and *Calolampra elegans* (Zhang *et al.*, 1993). The midgut of *N. walkeri* contains less than 0.02% of the total gut bacteria (Schulz *et al.*, 1986). The correlation of the site of the highest cellulase activity, the midgut, with the presence of the lowest bacterial population confirms that the cellulase activity in *N. walkeri* is endogenous. The presence of appreciable bacterial numbers in the midgut of *M. subhyalinus* (Mühlemann *et al.*, 1995) and the symbiotic association of the termite with fungal gardens cannot allow this conclusion to be extrapolated to the *Macrotermes* species studied. However, support for endogenous cellulase activity in these species can be inferred by the presence of endo-β-1,4-glucanase activity in the salivary glands of young and old major workers of *M. michaelseni*. Cellulase is presumably secreted by the salivary glands into the foregut and/or by the anterior midgut epithelium into the midgut as activity is present in small amounts of both tissues. In *N. walkeri* the increase in enzyme activity along the midgut suggests that the enzymes are secreted until the maximal activity is reached. The decline in enzyme activity along the midgut after the peak of activity may be due to a number of factors. The enzymes may be inhibited in the posterior midgut, hydrolysed, rapidly resorbed and/or recycled, or diluted by fluid influx at the mixed segment. The addition of posterior midgut extracts to extracts prepared from an equal number of anterior midguts showed no change in cellulase activity even when preincubated, indicating that the low levels of cellulase activity in the posterior section are not due to inhibition or hydrolysis of the enzyme. A similar pattern of distribution along the midgut has been found in the roaches *P. cribrata* (Scrivener *et al.*, 1989), *G. dilatatus* and *C. elegans* (Zhang *et al.*, 1993). A counter-flow system has been suggested to explain how enzyme activity can be retained in the anterior midgut against the normal posterior flow of food

(Dow, 1981, Terra and Ferreira, 1981). This counter current forms by the addition of water and solutes through the Malpighian tubules coupled with the absorption through the midgut caeca and has been studied in several insects; the fly *Rhynchosciara americana* (Terra and Ferreira, 1981), the mealworm *Tenebrio molitor* (Terra *et al.*, 1985), and the locust *Schistocerca gregaria* (Dow, 1981). Caeca are present in *P. cribrata, G. dilatatus* and *C. elegans* (Zhang *et al.*, 1993) but poorly developed or absent in higher termites (Noirot and Noirot-Timothée, 1969). The deep insertion of the oesophageal valve as described in *Microcerotermes edentatus* (Kovoor, 1968) may provide the necessary conditions to set up the counter flow. During dissections of many thousands of workers of *N. walkeri* it has been noticed by the author that the anterior section of the midgut frequently appears to be enlarged and contains a very dark coloured fluid. This is consistent with observations by Dow (1981) in *S. gregaria* that the anterior caeca become bloated with dark fluid as digestion progresses due to the accumulation of products. When termites are fed dye-impregnated wood such as in the study of pH and redox, the dye accumulates in the anterior midgut contents and tissue, indicating this as the site of absorption.

The low enzyme activities found in the mixed segment (less than 2% of the gut total) and the hindgut (less than 1%) in *N. walkeri* may be from exogenous activity (bacteria), a function of inefficiency in the counter flow system or a natural interruption of the recycling in the counter flow system which allows endogenous enzymes and accumulated by-products (which may be toxic) to move into the hindgut (Dow, 1981). Support for the endogenous nature of endo-β-1,4-glucanase activity in the hindgut was shown in *P. cribrata* by the failure to reduce the activity after antibiotic treatment of the roach to remove the majority of the microbiota (Scrivener *et al.*, 1989). Additionally, the elution profile of the endo-β-1,4-glucanase activity from the midgut was similar to the profile of endo-β-1,4-glucanase activity from the hindgut (Scrivener *et al.*, 1989). As the enteric valve prevents hindgut contents from refluxing into midgut (Noirot and Noirot-Timothée, 1969) hindgut enzymes cannot move forward into the midgut, therefore the hindgut endo-β-1,4-glucanase activity in *P. cribrata* must originate in the midgut. The low activities of cellulase and amylase in the hindgut of *N. walkeri*

and *M. michaelseni* suggest that bacterial involvement in starch or cellulose hydrolysis is minimal.

The presence of endo-β-1,4-xylanase activity in the hindgut of *N. walkeri* does suggest some bacterial involvement in hemicellulose digestion, as is the case with the roach *Periplaneta americana* (Bignell, 1977), although in the roaches *P. cribrata, G. dilatatus* and *C. elegans* (Zhang *et al.*, 1993) hindgut endo-β-1,4-xylanase activity accounts for up to 12% of the total activity. The lower termite *Coptotermes acinaciformis* has 80% of the endo-β-1,4-xylanase activity located in the hindgut (Inoue, unpublished results). In *R. speratus* the small Protozoa are the main agents for xylan digestion in the hindgut (Azuma *et al.*, 1993). In contrast to these results, the endo-β-1,4-xylanase activity purified by Rouland and co-workers (1988d) from workers of *M. mulleri* had similar properties to those from its associated *Termitomyces* sp. leading to the conclusion that the enzyme in these species is acquired by ingesting fungal material. Similar results were found for *M. bellicosus* although of the two endo-β-1,4-xylanase activities purified from the associated *Termitomyces* sp. only one (the one with the lower specific activity) was "apparently acquired". No antibody or sequencing data has been provided to validate these claims.

The site of enzyme secretion cannot be assigned unambiguously. The most likely sites are the midgut epithelium and/or the salivary glands. The enzyme activities present in the midgut have also been detected in the midgut epithelium of *N. walkeri* and *M. michaelseni* although no cellulase activity could be detected in the epithelium of *M. natalensis* (Martin and Martin, 1978) or *P. cribrata* (Scrivener *et al.*, 1989). Noirot (1969) described only one cell type present in termite midgut epithelium indicating that it performs both secretory and absorptive functions. Secretory granules were observed by Bignell *et al.*, (1982) in the midgut epithelium of *Cubitermes severus*. In *N. walkeri* no distinctive secretive cells could be detected in the midgut tissue, though differences were observed between the epithelial cells in the anterior and posterior midgut (Hogan *et al.*, 1988a). The midgut contains the highest levels of β-glucosidase and the histological detection of β-glucosidase in the lumen and the cells of the midgut epithelium of *N. walkeri* (Hogan *et al.*, 1988a) suggests a midgut origin for this enzyme. Maltase and invertase activities in *Blatta orientalis* are only secreted in the midgut whereas amylase is

restricted to the salivary glands (Cornwell, 1968). A number of factors may affect the detection of the site of enzyme secretion. The enzyme may be secreted in an inactive form thus escaping detection by assaying or staining of tissue sections. Interference with the GOD-Perid glucose assay by unknown compound(s) in salivary gland extracts would give false negative or low values for the cellulase and β-glucosidase activities. The fragility of the salivary glands and reservoirs may also result in an underestimation of enzyme activity.

Several roles have been proposed for salivary secretions in insects: lubrication of the food bolus to facilitate passage through to the foregut and midgut (House, 1974), provision of liquid nourishment for the dependent castes in termites (Noirot and Noirot-Timothée, 1969) and the initiation of digestion (Cornwell, 1968). Martin (1978) discounted the cellulase activity in the salivary glands of *M. natalensis* as contamination from the salivary reservoirs and ignored the contribution the enzymes could make to digestion. Considerable variation occurs in the amounts of enzyme activity detected in the salivary glands of a number of termite and roach species. The highest values (expressed as a % of total activity present in the whole gut) have been recorded in the salivary glands of workers of the primitive lower termite *Mastotermes darwiniensis* for cellulase (10%), endo-β-1,4-glucanase (38%), β-glucosidase (37%) and amylase (81%) (Veivers *et al.*, 1982a). This implies a strong contribution to the whole process of digestion or to the initial stages by beginning the hydrolysis of complex substrates such as cellulose and starch. In *M. michaelseni* lower levels of cellulase (0-1%), endo-β-1,4-glucanase (7-9%) and β-glucosidase (<1%) are found in the salivary glands. Cellulase and β-glucosidase activities could not be estimated in *N. walkeri* but low values of endo-β-1,4-glucanase (3%) activity were present in the salivary glands. Low values have also been recorded for the roaches *G. dilatatus* and *C. elegans* (Zhang *et al.*, 1993) and *P. cribrata* (Scrivener *et al.*, 1989). The salivary glands secrete into the foregut which is not known to play a role in enzyme secretion (House, 1974) though it can serve as a site for absorption of free fatty acids and SCFAs in *P. americana* (Bignell, 1981). The size of the foregut varies within the termite genera and is lined with cuticle which may be developed into a heavily sclerotinized gizzard (Noirot and Noirot-Timothée, 1969). Enzyme activity present in the foregut may be from secretion by the salivary

glands or from the midgut by reflux through the oesophageal valve. The latter is known to occur with those termite workers which regurgitate solid food for feeding dependent castes (stomodaeal feeding) (Noirot and Noirot-Timothée, 1969). The endo-β-1,4-glucanase activity in the salivary gland of *Acanthotermes ahngerianus* had a similar pH range of 5 to 7.5 to the activity found in the gut (Mednikova and Tiunova, 1984) suggesting a possible salivary gland origin for the enzyme in the midgut. Generally low enzyme activities are reported for the foregut but in *P. cribrata* (Scrivener *et al.*, 1989) the large foregut accounts for 40-50% of the total activity in the gut of the cellulase and its components. The similarity of the elution profile of endo-β-1,4-glucanase activity from the salivary glands to the profile of endo-β-1,4-glucanase activity from foregut of *P. cribrata* suggests that the foregut activity is salivary gland in origin (Scrivener and Slaytor, 1994a). Further evidence for secretion by the salivary glands may come from as yet unsuccessful attempts to clone the endo-β-1,4-glucanase gene from *P. cribrata* midgut tissue (Scrivener, unpublished results). No expression of endo-β-1,4-glucanase activity has yet been obtained from cDNA libraries prepared from midgut epithelial mRNA though other proteins were expressed. This preliminary result suggests that the enzyme is not expressed in the midgut. This leaves the salivary gland as the only other possibility. No endo-β-1,4-glucanase activity could be histochemically detected in the midgut epithelium of *N. walkeri* (Czolij, unpublished results) but salivary gland tissue was not examined. Comparison of the elution profile of endo-β-1,4-glucanase activity from the salivary glands to that from the foregut and midgut of *M. darwiniensis* also revealed similar profiles, although an extra component with a higher molecular weight was present in the salivary gland extract (Veivers, unpublished data). The present data are insufficient to determine the site of secretion. The midgut appears to be an important secretory site for α- and β-glucosidase activities. It appears that the salivary glands may produce polysaccharidase activities such as cellulase and amylase that initiate the digestive process which is completed in the midgut by α- and β-glucosidase activities produced there. The presence of amylase activity in all termites examined when the diet of these species is low in starch is similar to other findings where enzyme presence cannot be correlated with substrate presence (Rouland *et al.*, 1986a). An unusual example of this is the occurrence of an endogenous endo-β-1,4-glucanase in the carnivorous *Octopus vulgaris*

(Furia *et al.*, 1975) where it is assumed that the endo-β-1,4-glucanase is a genetic remnant carried over from evolutionary change from ancestral herbivory to modern carnivory. This genetic link of cellulase lends a strong support to endogenous production but also suggests that digestive enzymes may be secreted in an unregulated fashion and may be an evolutionary remnant.

The most comprehensive survey of gut pH in higher termites by Bignell and Eggleton (1995) detailed 52 species covering a range of feeding guilds. Data were not available on the foregut. The range of gut pH in *N. walkeri* was similar to the pattern observed for other nasutes. The low pH of the faeces of *N. walkeri* may be related to observations that the faeces of *N. exitiosus* used for carton (nest) construction contain sufficient quantity of humic acids to inhibit microbial growth (Lee and Wood, 1968, Lee and Wood, 1971). The correlation of the gut pH with the enzyme pH optima has been investigated in only a small number of termite species. In a study on *N. exitiosus* by (McEwen *et al.*, 1980) the pH optima of β-glucosidase was found to be 4.5 to 7 for the foregut and midgut activities. Given these conditions, β-glucosidase would have little activity in the foregut which has a pH range of 2 to 2.8. As the midgut pH range is 6.8 to 7.5 the β-glucosidase would be fully active in this gut section. In a similar study on the lower termite *M. darwiniensis* by (Veivers, 1983). the pH optima for β-glucosidase was 4.5 to 5.5 for salivary gland and foregut activities and 5.5 for midgut activity. Given these conditions, β-glucosidase would be 70 to 100% active in the foregut which has a pH range of 3.8 to 4.4. As the midgut pH range is 7.6 to 8.0 the β-glucosidase would only be 20% active in this gut section. Similarly the pH optima for cellulase was 5.0 to 6.5 for salivary gland and foregut activities and 5.5 to 6.5 for midgut activity. When compared with the measured gut pH the enzyme is 30% active in the foregut and 50% active in the midgut Digestive enzyme activities found in termites can thus be correlated with appropriate pH ranges in the gut *in situ* providing optimal conditions for enzyme activity. Subsequent increases in pH after the midgut therefore would have little effect on the rate of cellulose digestion.

The Macrotermitinae, in particular the genus *Macrotermes*, are a group of higher termites which have embraced the art of gardening using species-specific fungi (*Termitomyces* sp.) in specially constructed gardens or combs. It would be expected that all species would cultivate fungus gardens for the same purpose and use a similar mechanism for digesting cellulose. (Martin, 1979) has proposed that the relationship of termite to fungus is based on the termite receiving the "missing" cellulase component by ingesting fungal material containing the necessary enzyme and using this synergistically with its own termite produced component to achieve a complete cellulase system and therefore be able to digest crystalline cellulose.

If the termites need to ingest a cellobiohydrolase to facilitate cellulose digestion as has been proposed for *M. natalensis* (Martin and Martin, 1978) or to ingest fungal endo-β-1,4-glucanase as has been reported for *M. mulleri* (Rouland *et al.*, 1988c) then it would be expected that the termites would eat only fungal material containing the relevant activity. This is not the case for *M. bellicosus, M. subhyalinus*, *M. michaelseni* and *M. mulleri* as the results clearly indicate that all (99%) of the fungal cellulase activity is found in the nodules which are eaten only by the young major and young minor workers (Leuthold *et al.*, 1989). In other studies on *Macrotermes* spp. no separation of worker castes into age groups was carried out, resulting in either solely old major workers (foragers), comb workers which consist of a population of old and young workers or a mix of all age groups and categories. The old major and the old minor worker categories of *Macrotermes* spp. eat old fungal comb (Badertscher *et al.*, 1983) which has insignificant amounts of cellulase activity, earth and some plant material (Badertscher *et al.*, 1983). It would be surprising if these workers had any fungal cellulase components in their gut. Although levels of enzyme activities are lower than in the young workers, cellulase activity is still present, presumably being used to break down cellulose in the plant material as well as any remaining cellulose in the fungal comb material. The soldiers of *Macrotermes* spp. are fed old fungal comb by the workers (Badertscher *et al.*, 1983) thus the cellulase activity found in the soldiers would be expected to be endogenous and active only on cellulose remaining in the

old fungal comb. The larvae are fed liquid food (Sieber, 1983), presumably similar to the secretions from the salivary glands fed to the larvae of *M. subhyalinus* by the young workers (Badertscher *et al.*, 1983). These secretions presumably contain a full complement of nutrients and thus the larvae would have no need for cellulase activity. The absence of cellulase activity in the larvae is also consistent with the absence of cellulase activity from the salivary glands of the young workers found in *M. michaelseni* although it may be related to interference by salivary gland extracts with the GOD Perid assay. However endo-β-1,4-glucanase activity is still present, though in small amounts. If the termites acquire the cellulase from eating the nodules then unexpectedly high cellulase activities should be present in these categories which are fed nodules.

The categories that consume the food source as well as fungal nodules high in cellulase activity, the young major and the young minor workers, would be expected to contain high levels of cellulase activity. Statistically no clear pattern emerged with all the species studied and with the enzymes measured. In laboratory colonies of *M. subhyalinus* the young workers, especially the young major workers, have the highest amount of cellulase activity. As they consume a diet of only foraged hay and nodule material, some digestion of the plant material must occur in the gut to supply their energy needs. This is in contrast to the view that foraged material is unaltered by the rapid passage through the gut (Martin and Martin, 1979) before deposition on to fresh fungal comb. The higher levels of digestive enzymes in these young worker castes may compensate for the rapid passage of food through the gut and hence the shorter time available for digestion to be completed. Thus the increased amount of cellulase and its components in the young major workers could be due to ingestion of cellulase from the fungal nodules but is not apparent in all the other *Macrotermes* spp. studied. When endo-β-1,4-glucanase activities are compared, significant differences between old and young categories in *M. michaelseni* are found.. Similar effects are found with β-glucosidase activities and amylase activities. The lack of a clear pattern in enzyme distribution across the categories and across the species may be related to the minimal number of replicates in some species and the difficulties associated with determining the age classification in field colonies. The use of green hay in laboratory

colonies enabled a clear distinction to be made between old and young categories, but this was not possible with field colonies.

The theory of acquired enzymes requires that the ingestion of the fungal nodules should result in all of the fungal enzymes present in termite extracts. There are at least two β-glucosidase activities in the fungal nodules which are distinct from the two in the major workers of *M. michaelseni*. A similar situation exists in *M. natalensis* with two β-glucosidase activities in the fungal nodules and two distinct β-glucosidase activities in the worker castes (Martin and Martin, 1979). The fungal β-glucosidase activities were not detected in the workers. This failure to detect fungal β-glucosidase activity was attributed to limited proteolysis of the fungal enzymes by midgut enzymes. Similarly, Rouland *et al.* (1986b) failed to detect fungal β-glucosidase activity in the worker castes and was able to partially purify one β-glucosidase activity from worker castes of *M. mulleri* and one β-glucosidase activity from the fungal nodules. β-Glucosidase activity in the fungal nodules associated with *M. michaelseni* was obvious only when extracts were resolved chromatographically. The extremely small amount present does not make it surprising that it could not be detected in termite extracts. Therefore it can be concluded that fungal β-glucosidase activities are not present in termite midguts.

The cellulases in both the fungal nodules and the termite workers of *M. michaelseni* are multicomponent but distinct from one another. At least one major and three minor endo-β-1,4-glucanases are present in extracts of the fungal nodules associated with *M. michaelseni*. Osore and Okech (1983) found two cellulases in the fungal nodules of *M. michaelseni* but the use of incorrect assays and very low enzyme recoveries leaves the work uninterpretable. A maximum of only 9% of the fungal activity could contribute to the endo-β-1,4-glucanase activity in major workers of *M. michaelseni* using the partial resolution of the endo-β-1,4-glucanase activities of the fungal nodule and the termite to determine overlap. The rate of feeding by young workers of *M. michaelseni* is not known but workers of the closely related species *M. subhyalinus* consume nodules at 9 mg h^{-1} (Leuthold, unpublished data). If 9% of the fungal enzyme were present in the termite then at this rate of feeding, 0.03% of the total cellulase activity in young major

workers of *M. michaelseni* would be of fungal origin. If a synergism exists between fungal and termite enzymes as suggested in *M. mulleri* (Rouland *et al.*, 1988c), then using this ratio of 2:3 for fungal cellulase to termite cellulase necessary for a maximum synergistic increase of 200% to apply to the cellulase system in *M. subhyalinus* then 20% of the total activity should be due to ingested fungal cellulase from nodules. The amount of fungal cellulase a young worker of *M. subhyalinus* could obtain is less than 2% of its total cellulase so it would take at least 10 days to accumulate the required amount of fungal cellulase. No information is available on the fate of the ingested fungal enzymes but it is unlikely that the activity would be cumulative and stable for such a long period.

A similar pattern of a termite-produced endo-β-1,4-glucanase activity distinct from that in the associated fungus was found in *M. bellicosus.* Three main fungal endo-β-1,4-glucanase activities were partially purified with a similar range of isoelectric points as found by Martin (1979) for three endo-β-1,4-glucanase activities in fungal nodules associated with *M. natalensis.* However, it is not possible to tell from the data presented by Martin (1979) whether these are distinct from those in the termite. At least nine termite endo-β-1,4-glucanase activities were associated with termite salivary gland and midgut tissue in *M. natalensis* (Martin and Martin, 1979).

A similar pattern of a termite-produced endo-β-1,4-glucanase activity distinct from that in the associated fungus was also found in *M. mulleri.* In contrast, Rouland and co-workers (1988b) have shown, using a variety of techniques, the presence of only one endo-β-1,4-glucanase activity in the nodules associated with *M. mulleri* and this activity is present in extracts from unspecified categories of workers along with one termite-produced cellulase.

Thomas (1985) attributed the variable growth characteristics of *Termitomyces* isolates to a difference in species between the termites or in physiological races of the same species. This is also apparent in the pattern of endo-β-1,4-glucanase activities found in the fungal nodules of all *Macrotermes* spp. investigated.

In investigations of cellulase activity in *Macrotermes'* workers and fungus of *M. subhyalinus* (Abo-Khatwa, 1978), *M. natalensis* (Martin and Martin, 1978), and *M. mulleri* (Rouland *et al.*, 1988a, Rouland *et al.*, 1986b), the important question is whether these activities constitute a cellulase capable of degrading crystalline cellulose. Or as Martin (1978), argues, is a cellobiohydrolase of fungal origin essential for breakdown of crystalline cellulose in *Macrotermes* spp.? In *M. natalensis* the evidence for an acquired cellulase component is based on a single step purification, using matching bands to argue the presence of fungal enzymes in termite gut extracts. Surprisingly, the fungus associated with *M. mulleri* has been shown to produce not a cellobiohydrolase but an endo-β-1,4-glucanase (cellulase I_f) which can be detected in the gut (Rouland *et al.*, 1988b) and that the termite produces the cellobiohydrolase (cellulase 11). *In vitro* experiments indicate that it acts synergistically with the cellobiohydrolase of the termite (Rouland *et al.*, 1988c). Several criticisms can be made about this work. No assays were carried out on fresh material to determine the original enzyme activity, loss of activity due to freezing and in the first purification step or the stability of the extracts. In fungal nodules of *M. bellicosus* freeze-drying and air-drying reduced the activity of one of the three endo-β-1,4-glucanase activities present. In the second step in the purification of the termite enzymes only 29% and 19% of the activity was recovered as cellulase I_f and cellulase 11 (Rouland *et al.*, 1988c). All the termites and associated fungal material from the *Macrotermes* spp. studied in this thesis were found to have multicomponent endo-β-1,4-glucanase activities, it is possible that another component could have been lost in the purification process by Rouland (1988c). Rouland and co-workers' most surprising claim is that the termite produces a cellobiohydrolase, an enzyme previously only reported in fungi. The characteristics of the enzyme purified suggest it is actually an endo-β-1,4-glucanase, as it is active against CMC and produced cellobiose and cellotriose from the hydrolysis of crystalline cellulose. It is an endo-β-1,4-glucanase with a high affinity for crystalline cellulose which is referred to as a low endo-β-1,4-glucanase (Okada and Tanaka, 1988). Such enzymes have been characterised from a number of fungi (Wood, 1991). The significance of the fungal cellulase detected in *M. mulleri* workers is difficult to evaluate as the amount present in the termite gut is not stated (Rouland *et al.*, 1988c) and *in vitro* experiments designed to show synergism between

the fungal and termite cellulase systems not only equate endo-β-1,4-glucanase and cellulase activities as production of reducing sugars (expressed as glucose equivalents) from microcrystalline cellulose (Rouland *et al.*, 1988c) but fail to estimate the possible ratio of cellulase components that would be expected *in situ*.

Other termites such as *N. walkeri*, *Coptotermes lacteus* (Hogan *et al.*, 1988b), *Trinervervitermes trinervoides* (Potts and Hewitt, 1974a, Potts and Hewitt, 1974b) and the roach *P. cribrata* (Scrivener and Slaytor, 1994b) produce incomplete cellulases consisting only of endo-β-1,4-glucanase and β-glucosidase activities similar to that found in *Aspergillus niger* (Okada, 1988). All are active against crystalline cellulose. *P. cribrata* (Scrivener and Slaytor, 1994b) compensates for the low activity of its endo-β-1,4-glucanase towards crystalline cellulose by producing very large amounts of the enzyme. The first purification of an endogenous cellulase from a termite has been carried out on *N. walkeri*. One major endo-β-1,4-glucanase activity was purified and the second major endo-β-1,4-glucanase activity was partially purified from *N. walkeri* with similar M_r and specific activities to EG1 and EG2 purified from *P. cribrata* (Scrivener and Slaytor, 1994b). However, *N. walkeri* endo-β-1,4-glucanase did not react with antibodies prepared from EG1 and EG2, indicating little structural similarity between the termite and roach endo-β-1,4-glucanase activities (Scrivener, unpublished data).

In the Macrotermitinae, the possibility exists that the fungus is cultivated as a source of nutrients as found with the leaf-cutting ant *Atta cephalotes* and its cultivated fungus. The hyphae and the staphylae of the fungus are rich sources of nutrients (Quinlan and Cherrett, 1979). The amount of free sugars in the hyphae is comparable to the amount of glucose found in the nodules of *Termitomyces* sp. The combined amounts of reducing sugars in the old fungus comb, eaten by the old workers, is less than half the amount of glucose in the nodules (Veivers *et al.*, 1991). The glucose in the nodules would only supplement to a small extent that produced from cellulose in the young major workers. It can be calculated that these workers, utilising approximately 180 nmol O_2 h^{-1} (Veivers *et al.*, 1991) for the complete oxidation of 30 nmol of

glucose to CO_2 would have to consume approximately 170 µg of dried nodule h^{-1} to obtain this amount of glucose if the workers were only using nodules as a source of glucose. Using data on nodule consumption in *M. subhyalinus* (Leuthold, unpublished), it can be calculated that a young major worker of *M. subhyalinus* would consume 9 µg dried nodule h^{-1} which would be sufficient to supply 5% of its glucose requirements. This is comparable to the fungal-ant relationship in *A. cephalotes* in which only 4% of the respiratory requirements of the colony could be met by fungal consumption (Quinlan and Cherrett, 1979). The combined amounts of reducing sugars in the old fungus comb, eaten by the old workers, is less than half the amount of glucose in the nodules. The rate of consumption of the comb material by *M. michaelseni* is 0.06 g comb (dwt) g $workers^{-1}$ day^{-1} (Darlington, 1986). This corresponds to 0.015 mg comb (dwt) mg termite $(wwt)^{-1}$ day^{-1}. Thus the amount of glucose consumed by old workers eating old comb material would be 0.15 nmol $termite^{-1}$ h^{-1}. This is insignificant in comparison with the amount of glucose which can be produced by the termite's cellulase (0.12 µmol $termite^{-1}$ h^{-1}). However, the possibility exists that the fungal material may contribute to the nitrogen economy of the colony. If the nitrogen content of nodules of *M. michaelseni* is assumed to be in the range of 5-9% dwt, the amount of nitrogen added to the diet would be 3-6 nmol $termite^{-1}$ h^{-1}. As the nodules are fed to developing categories they may be more important in the provision of nitrogen and essential nutrients, similar to the relationship found in *A. cephalotes* where the fungus is of greater importance in the diet of developing larvae than in adults (Cherrett *et al.*, 1989).

The differences in nitrogen estimations of food and food store are possibly due to unsatisfactory sampling because of difficulties in ascertaining the actual food source. An increase in nitrogen on storage in food store may occur in all food storing species but this may have been masked in the Cameroon samples by using non-specific food source. The leaf material supplied to the *M. mulleri* colony was from another forest area and may have been mixed with previous food stores. An increase from food to food store indicates supplementation of the nitrogen content by either microbial action whilst stored or through salivary secretions added by the termite. All species show a characteristic increase in nitrogen from food store to fungal comb which

indicates addition of nitrogen during passage through the guts of the young workers that process the food. Abo-Khatwa (1977) suggested that the uric acid present in fresh fungal comb was added to the comb from termite faeces. An investigation of the uric acid concentrations in material in *M. bellicosus* did not reveal any significant change from food store through the fungal comb, though there was an increase from the food.

The variation in digestive enzyme levels in the four worker categories of laboratory colonies of *M. subhyalinus* and *M. michaelseni* does suggest a more productive role for the young workers, not only in preparing the fungal comb but in the contribution of nutrients to the colony. This is in support of the theory by (Badertscher *et al.*, 1983) that the young workers remain in the colony to process plant material and eat nitrogen-rich nodules and supply nutrients to all developing larvae. However, this cannot be extended to all the Macrotermitinae as no strong relationship between enzyme activity and young and old categories could be statistically established. The termite-fungal relationship in the Macrotermitinae certainly increases the food consumption and utilisation and may be reflected in the increased biomass found in *Macrotermes* spp. (Wood and Thomas, 1989).

4.2 *In vivo* metabolism

An attempt has been made to describe *in vivo* and *in vitro* metabolism in *N. walkeri* at 25°C. In particular, biochemical explanations have been sought primarily to understand the rates of O_2 utilisation and CO_2 production. These rates represent the summation of metabolism in the termite and the gut microbiota. The rates in freshly collected workers as determined by GC are 82 ± 2 nmol O_2 termite^{-1} h^{-1} and 87 ± 2 nmol CO_2 termite^{-1} h^{-1} (Williams et al., 1994). These values have been corrected for H_2 and CH_4. Manometric studies, which are more sensitive than GC methods, produced similar results of O_2 utilisation of 86 ± 2 nmol termite^{-1} h^{-1} and CO_2 production of 88 ± 2 nmol termite^{-1} h^{-1} (Lo, 1994). On the assumption that carbohydrate is the major substrate being metabolised, an assumption which can be justified by the RQ data, O_2 utilisation and CO_2 production indicate that glucose is being utilised *in vivo* at about 15 nmol termite^{-1} h^{-1} (Williams *et al.*, 1994). *In vitro* experiments with cellulase activity isolated from the foregut and midgut of *N. walkeri* indicate that glucose is produced from crystalline cellulose at a comparable rate of 21 nmol termite^{-1} h^{-1} (Schulz *et al.*, 1986). This activity is more than sufficient to produce all the glucose required by the termite.

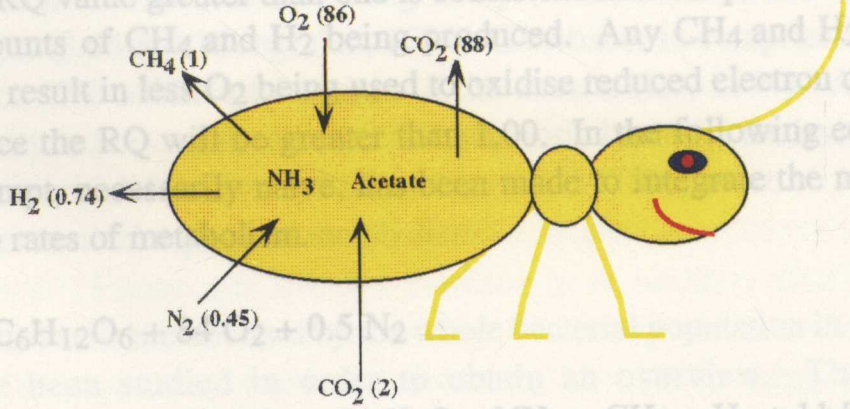

Fig. 23. Happy termite: Normal metabolic fluxes in freshly collected *N. walkeri* at 25°C. Data is expressed as nmol termite^{-1} h^{-1}.

A normal worker caste termite of *N. walkeri*, brought in from the field, is one which consumes a diet largely composed of cellulose and which, per h, utilises 86 nmol of O_2 (Lo, 1994) reduces 2 nmol of CO_2 (Williams *et al.*, 1994) and 0.45 nmol of N_2 (Lovelock *et al.*, 1985). It produces 88 nmol of CO_2 (Lo, 1994), as well as small amounts of CH_4 (~1 nmol) and H_2 (0.74 nmol) (Williams *et al.*, 1994). Normal metabolic fluxes are summarised in Fig. 23.

Before examining the results from *in vitro* experiments, more information can be obtained from the *in vivo* data. The RQ value for freshly collected workers of *N. walkeri* was 1.05 ± 0.01 (n = 5) (Lo, 1994). This RQ is within the range reported for other termites (Cook, 1932, Mühlemann *et al.*, 1995) and similar to that in the wood roach *P. cribrata* (Scrivener *et al.*, 1989). An RQ value greater than 1.00 shows that lignin cannot be metabolised to any great extent, in spite of clear demonstrations that it can be metabolised in termites (Cookson, 1988; Brune and Breznak, 1995). The structure of lignin is complex but is based on coniferyl alcohol and related alcohols The complete oxidation of coniferyl alcohol requires 11.5 O_2 and would give an RQ of 0.87 as shown in the following equation.

$$C_{10}H_{12}O_3 + 11.5\ O_2 \longrightarrow 10\ CO_2 + 6\ H_2O$$

An RQ value greater than one is consistent with the production of small amounts of CH_4 and H_2 being produced. Any CH_4 and H_2 produced will result in less O_2 being used to oxidise reduced electron carriers and hence the RQ will be greater than 1.00. In the following equation, an attempt, necessarily naive, has been made to integrate the measured *in vivo* rates of metabolism.

$$15\ C_6H_{12}O_6 + 84\ O_2 + 0.5\ N_2$$
$$\longrightarrow 89\ CO_2 + 80\ H_2O + NH_3 + CH_4 + H_2 + 11\ [H]$$

This balanced equation (RQ value 1.06) shows a surplus of reducing power, all of which could be used in acetogenesis by the reduction of acetate as shown in the next equation.

$$15\ C_6H_{12}O_6 + 84\ O_2 + 0.5\ N_2 \longrightarrow 86\ CO_2 + 83\ H_2O + NH_3 + CH_4 + H_2 + 1.5\ CH_3COOH$$

This nearly balanced equation - the right hand side has one extra H - shows an RQ value of 1.02, and represents the best fit for the data.

4.3 *In vitro* metabolism

In vitro metabolism has been studied both in termite tissues and in the hindgut. An approximation of the relative contributions of the termite and the gut bacteria to the overall metabolism has been provided by manometric experiments with the degutted body and the ruptured hindgut (i.e. the gut epithelium and the contents containing the bacteria) (Lo, 1994). Incubating these preparations in an insect saline and with substrates such as glucose, pyruvate and acetate, the degutted body utilised O_2 at 34.0 ± 0.6 (n = 5) nmol termite^{-1} h^{-1} and the ruptured hindgut at 31 ± 2.2 (n = 8) nmol termite^{-1} h^{-1}. *In vitro* experiments were designed to explain these values and to gain some insights into the biochemical basis of the symbiosis existing between the termite and its gut microbiota.

The large size of the hindgut in *N. walkeri*, is an indication of its importance. At 3.37 ± 0.4 mg it represents 40% of the total termite weight (8.64 ± 0.12 mg) (Lo, 1994). The bacteria comprise 1.47 ± 0.26 mg or 17% of the total weight of the termite (Lo, 1994). As cellulase activity is not correlated with the symbiotic bacteria, the bacteria which are essential for survival (Eutick *et al.*, 1978b), must be involved in other essential aspects of metabolism.

In this thesis metabolism by the whole bacterial population in the hindgut have been studied in order to obtain an overview. The study of individual bacterial isolates was considered impractical due to the fastidious nature of many of the bacteria present as only a few heterotrophs have been isolated from *N. walkeri* (Eutick *et al.*, 1978a) from the large number that are known to be present (Czolij *et al.*, 1985). Successful isolations from termites have been made for a number of acetogenic and methanogenic bacteria (Breznak and Blum, 1991; Kane

and Breznak, 1991; Kane *et al.*, 1991). A disadvantage of metabolic studies of bacterial isolates is that competition between microbial species for substrates for growth and *in situ* gut conditions such as oxygen concentration is ignored. The contributory functions of the microbial population are best determined by an examination of the system as a whole.

4.3.1 Glucose

Ingested cellulose is hydrolysed to glucose in the foregut and midgut: these are the only regions of the gut where cellulase activity is found in *N. walkeri*.

Body tissues: Glucose produced in the midgut is presumably (see Dow, 1986) taken up by passive diffusion in the anterior midgut where the cellulase activity is predominantly found and is then available to the termite. The maximum rate of glucose production was 21 nmol termite^{-1} h^{-1} (Schulz *et al.*, 1986). A fully functional glycolytic pathway is present in the termite body in *N. walkeri* as in *N. exitiosus* (O'Brien and Breznak, 1984) with hexokinase, PFK and pyruvate kinase in excess of the amount needed to utilise the glucose produced from cellulose breakdown. This is in contrast to studies on the glycolytic enzymes in the fat body of *P. americana* which found unusually low activities of PFK suggesting that in the cockroach the pentose phosphate pathway predominates, not glycolysis (Storey and Bailey, 1978a). This would provide the necessary NADPH for lipogenesis. In glycolysis in aerobic tissues, O_2 would be used in the regeneration of NAD^+. The amount of O_2 needed is equivalent to the amount of glucose oxidised to pyruvate. Thus if 15 nmol glucose are oxidised in *N. walkeri* then 15 nmol of should be used as illustrated in Fig. 24. An *in vitro* estimation of the rate of glycolysis was carried out by Lo (1994) using manometric techniques. Degutted bodies of *N. walkeri* were incubated with F^-, a known inhibitor of glycolysis (Mathews and van Holde, 1990). O_2 utilisation decreased from 35.1 ± 0.9 to 22.9 ± 1.3 nmol termite^{-1} h^{-1} i.e. the *in vitro* rate was approximately 12 nmol termite^{-1} h^{-1} (Lo, 1994).

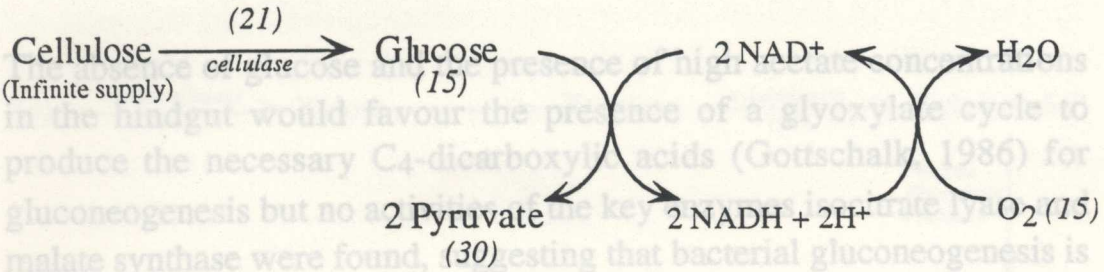

Fig. 24. Production and utilisation of glucose in termite tissues of *N. walkeri*. Data are expressed as nmol termite^{-1} h^{-1}.

The absence of the pyruvate dehydrogenase complex activity in termite tissues, as evidenced by the failure to detect enzyme activity in *N. walkeri* is consistent with the recovery of negligible amounts of $^{14}CO_2$ from degutted bodies of *N. walkeri* incubated with [U-^{14}C]-glucose (Lo, 1994). Metabolism of glucose in termite tissues can not proceed beyond pyruvate.

The low hexokinase activities present in hindgut epithelial tissue suggest that glucose may not be the main substrate for respiration in the tissue.

Hindgut: The presence of low concentrations of glucose in the mixed segment and traces in the hinddgut suggests that some glucose may escape to the hindgut, as has been observed in *P. cribrata* (Scrivener *et al.*, 1989). This may result from inefficiency in the counter flow system or a natural interruption of the recycling in the counter flow system which allows endogenous enzymes and accumulated by-products (which may be toxic) to move into the hindgut (Dow, 1981). Low hexokinase activity (< 1 nmol $^{14}CO_2$ h^{-1} termite^{-1}) was present in the hindgut contents. Glucophosphotransferase activity, a specific transport system in bacterial cells which is normally used to convert glucose into glucose-6-phosphate, could not be detected in hindgut bacterial pellets. These observations indicate that glucose is not the normal metabolic substrate for the bacteria. This conclusion is further supported by the low increase observed in oxygen consumption when glucose is added to aerobic incubations of hindgut contents and the small increase in acetogenesis when glucose is added to anaerobic incubations of isolated intact paunches. Additionally only 1.02 ± 0.10 nmol $^{14}CO_2$ h^{-1} termite^{-1} was produced from ruptured hindguts of *N. walkeri* incubated with [U-^{14}C]-glucose under aerobic conditions (Lo, 1994).

The absence of glucose and the presence of high acetate concentrations in the hindgut would favour the presence of a glyoxylate cycle to produce the necessary C_4-dicarboxylic acids (Gottschalk, 1986) for gluconeogenesis but no activities of the key enzymes isocitrate lyase and malate synthase were found, suggesting that bacterial gluconeogenesis is not necessary.

Substantial activities of the key glycolytic enzymes, PFK and PK are found in the hindgut. The low activities of hexokinase and glucophosphotransferase suggest that they are not involved in glucose metabolism. An alternative explanation is that these activities may be associated with metabolism of other carbohydrates such as xylose.

4.3.2 Xylose

Xylulokinase activity is present in termite and bacterial tissues, complementing the presence of endo-β-1,4-xylanase activity found in the salivary glands, the foregut, the midgut (< 3 nmol h^{-1} $termite^{-1}$) and the hindgut (4 nmol h^{-1} $termite^{-1}$). This is not an unexpected result as the digestion of xylan occurs endogenously and microbially in a number of termite and insects (see 4.1 Digestion in higher termites p.110). Hemicelluloses such as xylan can account for 6-31% of the structural components of wood (Szegi, 1988). *Eucalyptus maculata*, a common source of food for *N. walkeri* contained 34% hemicellulose (Zhang *et al.*, 1993). The digestibility of hemicellulose in the wood roaches *P. cribrata* (Scrivener *et al.*, 1989), *G. dilatatus* and *C. elegans* (Zhang *et al.*, 1993) range from 26 to 64%. Hemicellulose metabolism was found to be responsible for 23% of acetate production in the hindgut of *R. flavipes* (Odelson and Breznak, 1983). In *P. americana* [^{14}C]hemicellulose fed to the insects was extensively degraded (48%) and recovered as CO_2 (Bignell, 1977). Experiments using isolated cannulated colons of *P. americana* demonstrated hemicellulose digestion and transport of the label out of the hindgut, with two unknown compounds as the carriers (Bignell, 1977). Label was also recovered in the haemolymph of *P. americana* as trehalose and unknown compounds after a diet of [^{14}C]hemicellulose.

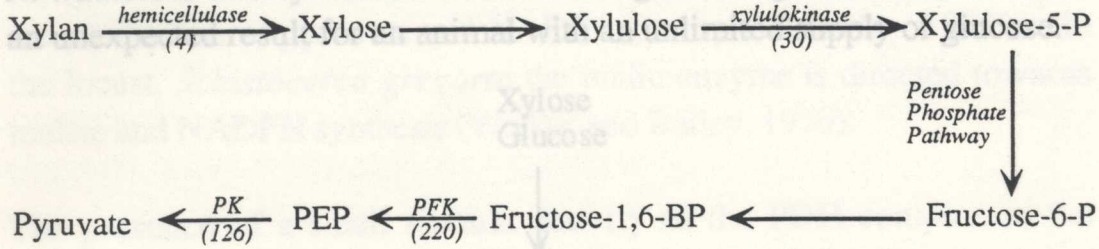

Fig. 25. Proposed scheme for the production and utilisation of xylose in the hindgut of *N. walkeri*. Activities are expressed as nmol termite^{-1} h^{-1}.

The high activities in the hindgut of *N. walkeri* of the glycolytic enzymes, PFK and PK, could indicate that these enzymes are primarily involved in the metabolism of xylose to pyruvate (Fig. 25). The presence of xylulokinase shows that xylose produced by endo-β-1,4-xylanase activity could be metabolised in the hindgut as shown in Fig. 25. The increase in O_2 utilisation when xylose was added to gut contents is a further indicates that xylose can be metabolised.

The presence of xylulokinase shows that the termite can also utilise xylose through the above scheme (Fig. 25). The metabolism of xylose by insect tissue has been demonstrated in *P. americana* (Bignell, 1977). Label from [^{14}C]xylose in the diet was detected in the haemolymph, some as trehalose, from which it had disappeared in 24 h (Bignell, 1977). The importance of xylose relative to cellulose is clearly dependent on the amount of xylan in the diet which varies with the plant material eaten.

4.3.3 Pyruvate

Body tissues: The metabolism of pyruvate is intriguing because of the absence of the pyruvate dehydrogenase complex activity in the termite body. This was first demonstrated in *N. exitiosus*, *R. flavipes* and *C. lacteus* by O'Brien and Breznak (1984) and has been confirmed here in *N. walkeri*. This finding is apparently unique among aerobic organisms. Normally, the PDH complex in animals is inactivated only during starvation. This is a glucose sparing effect so that pyruvate can

be used for gluconeogenesis. Pyruvate metabolism in the body of *N. walkeri* is clearly not directed towards gluconeogenesis (Fig. 26); not an unexpected result for an animal with an unlimited supply of glucose.

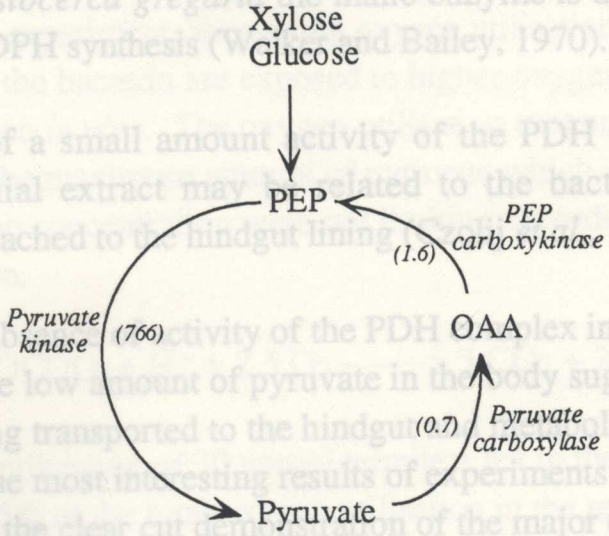

Fig. 26. Pyruvate metabolism in the body of *N. walkeri*. Data are expressed as nmol termite^{-1} h^{-1}.

The activities of gluconeogenic enzymes measured in the fat body of *P. americana* were 5 to 10 times lower than those reported for vertebrates (Storey and Bailey, 1978). An NMR study in larvae of the tobacco hornworm *Manduca sexta* (Lepidoptera: Sphingidae) using 2-[^{13}C]pyruvate found that label appeared directly in alanine and intermediates of the TCA cycle with a minimal level incorporated in glucose even under starvation conditions (Thompson and Lee, 1988). The low level of pyruvate as measured in the whole termite (0.29 nmol termite^{-1}) in comparison to the rate of production (30 nmol termite^{-1} h^{-1}), indicates there must be an efficient mechanism for transporting the pyruvate into the gut though this was not investigated.

The low activity of pyruvate carboxylase, normally the major anaplerotic enzyme in animals, suggests that processes such as amino acid synthesis may not be important in the termite but rather are bacterial processes in the hindgut. The major metabolic activity associated with pyruvate in termite tissues was the malic enzyme (NADP$^+$ dependent), an enzyme normally associated with fatty acid synthesis. Although lower levels of pyruvate carboxylase (mitochondrial) in the fat body of *P. americana*

are correlated with high levels of the malic enzyme ($NADP^+$ dependent) (cytosolic) it was concluded that the malic enzyme was directed towards the production of pyruvate and $NADP^+$ (Storey and Bailey, 1978b). In the locust, *Schistocerca gregaria* the malic enzyme is directed towards malate and NADPH synthesis (Walker and Bailey, 1970).

The presence of a small amount activity of the PDH complex in the hindgut epithelial extract may be related to the bacteria remaining associated or attached to the hindgut lining (Czolij *et al.*, 1985).

Hindgut: The absence of activity of the PDH complex in termite tissues coupled with the low amount of pyruvate in the body suggests that pyruvate is being transported to the hindgut and metabolised there by the bacteria. The most interesting results of experiments on *in vitro* metabolism are the clear cut demonstration of the major enzyme activities associated with the aerobic metabolism of pyruvate in an environment previously considered anaerobic and the extreme sensitivity of oxygen utilisation by the hindgut bacteria to changes in pyruvate concentration. The enzyme activities of aerobic metabolism which have been shown to be present in the hindgut of *N. walkeri* are the dehydrogenases which oxidise pyruvate (the pyruvate dehydrogenase complex), isocitrate (isocitrate dehydrogenase), 2-oxoglutarate (2-oxoglutarate dehydrogenase) and malate (malate dehydrogenase) as well citrate synthase, which governs the entry of acetyl CoA produced by the pyruvate dehydrogenase complex into the tricarboxylic acid cycle. The absence of pyruvate in the hindgut suggests that the availability of pyruvate is perhaps governed by its rate of transport into the hindgut and is rate limiting for metabolism in the hindgut.

The reoxidation of the reduced electron carriers generated in the dehydrogenase reactions would be expected to be carried out by bacterial electron transport chains coupled to oxidative phosphorylation Gottschalk, 1986). The sensitivity of oxygen utilisation to changes in the concentration of pyruvate, but not other obvious metabolic substrates such as glucose and acetate, particularly in the physiological range of oxygen utilisation and possible pyruvate concentration, strengthens the case for pyruvate being the normal substrate for bacterial metabolism. Between 0-2.5 mM pyruvate oxygen utilisation increases to 30 nmol

termite^{-1} h^{-1}. The maximum rate of oxygen utilisation of 68 nmol termite^{-1} h^{-1} at pyruvate concentrations above 5 mM is presumably not physiologically significant but rather is an indication of the total amount of NADH which can be produced in the hindgut bacteria. An obvious criticism of these experiments measuring oxygen utilisation with different substrates is that the bacteria are exposed to higher oxygen concentrations than *in vivo*. The oxygen utilisation measured is used as an indication of the maximum amount of pyruvate which could be oxidised if oxygen concentration were not limiting according to the following equation.

$$CH_3COCOO^- + H^+ + 2.5\ O_2 \text{ -------> } 3\ CO_2 + 2\ H_2O$$

Thus an oxygen utilisation of 30 nnmol termite^{-1} h^{-1} in the hindgut, which is about 35% of the total oxygen utilisation in the termite, would represent 12 nmol of pyruvate being oxidised. This is about 40% of the total amount of pyruvate (30 nmol) produced per h in termite tissues. Fortunately for the preparation of this thesis, radial oxygen gradients have been demonstrated by using fine oxygen electrodes inserted into carefully isolated guts of *N. lujae* and *R. flavipes* (Brune *et al.*, 1995a). In the nasute, *N. lujae,* it was estimated that the peripheral region of the hindgut is up to 30% saturated with oxygen and the paunch region of the hindgut and its bacteria consume 13% of the total oxygen used by the termite (Brune *et al.*, 1995a). As these measurements were carried out with minimal exposure to atmospheric oxygen they reflect more accurately the actual oxygen utilisation by the hindgut bacteria.

Incubation of the ruptured hindgut and its contents in buffer in an oxygen electrode shows a steady consumption of oxygen of 7.44 ± 0.59 nmol termite^{-1} h^{-1}. In these preparations where there is no pyruvate present and no possibility of pyruvate being transported into the gut the oxygen utilisation suggests that an unknown substrate is being oxidised.

Pyruvate can be oxidised anaerobically by anaerobic organisms by two pathways; via pyruvate-ferredoxin oxidoreductase (PFO) or pyruvate formate lyase (PFL) (Kerscher and Oesterhelt, 1982) thus generating energy from pyruvate catabolism, with acetate, methane and hydrogen as the end products. In the hindgut of *N. walkeri* pyruvate-ferredoxin

oxidoreductase (PFO) is sufficiently active to account for all the acetate produced. No attempt was made to assay for pyruvate formate lyase (PFL) activity.

In contrast to termite tissues, PEP carboxykinase is active in the hindgut (Fig. 27). In *Escherichia coli* growing on pyruvate, PEP synthetase

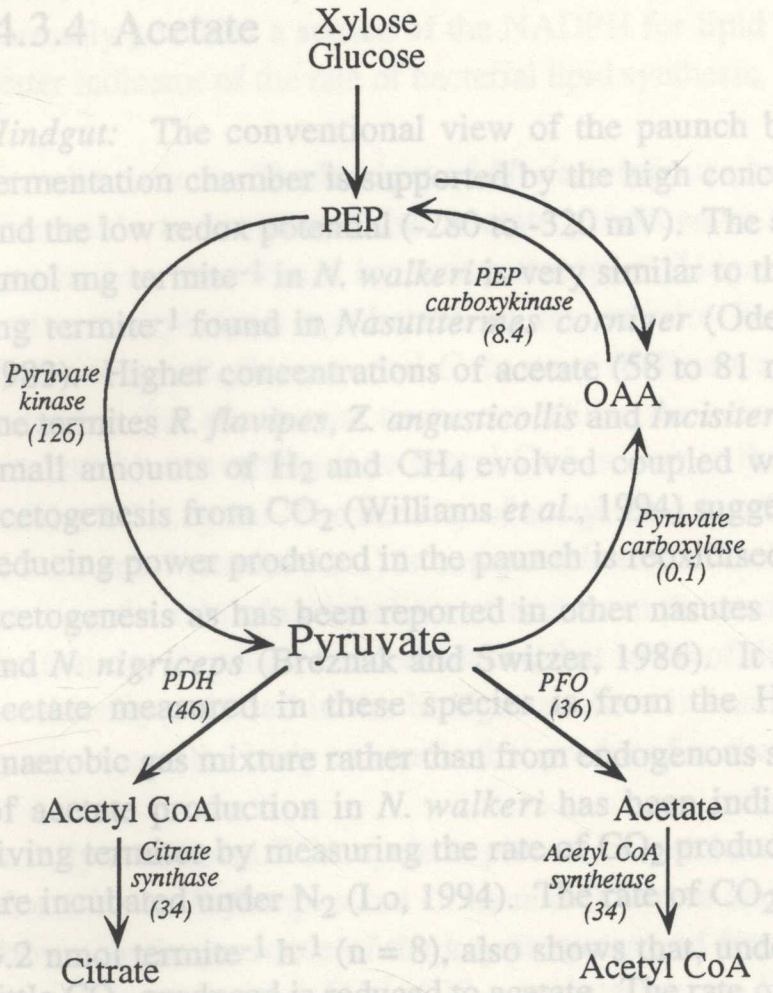

Fig. 27. Pyruvate metabolism in the hindgut of *N. walkeri*. Data are expressed as nmol termite^{-1} h^{-1}.

can produce the necessary PEP from pyruvate for the PEP carboxylase to convert to OAA which can be used in anabolic reactions and in the TCA cycle (Gottschalk, 1986). The PEP carboxykinase may provide the OAA used for the synthesis of amino acids. As the hindgut has high concentrations of amino acids (up to 8 mM) (Chappell, 1992), it is possible that the bacteria may synthesise amino acids as well as fix

nitrogen for the termite. However, free amino acid concentrations did not seem to be affected in *Nasutitermes nigriceps*, *C. formosanus* and *N. exitiosus* when their bacteria were removed by antibiotic treatment (Mauldin *et al.*, 1978, Nazarczuk *et al.*, 1981, Speck *et al.*, 1971).

4.3.4 Acetate

Hindgut: The conventional view of the paunch being an anaerobic fermentation chamber is supported by the high concentration of acetate and the low redox potential (-280 to -320 mV). The acetate pool of 1.76 nmol mg termite^{-1} in *N. walkeri* is very similar to the pool of 1.5 nmol mg termite^{-1} found in *Nasutitermes corniger* (Odelson and Breznak, 1983). Higher concentrations of acetate (58 to 81 mM) were found in the termites *R. flavipes*, *Z. angusticollis* and *Incisitermes schwarzi*. The small amounts of H_2 and CH_4 evolved coupled with the low rate of acetogenesis from CO_2 (Williams *et al.*, 1994) suggests that most of the reducing power produced in the paunch is reoxidised by O_2 and not by acetogenesis as has been reported in other nasutes such as *N. costalis* and *N. nigriceps* (Breznak and Switzer, 1986). It is possible that the acetate measured in these species is from the H_2 and CO_2 in the anaerobic gas mixture rather than from endogenous substrates. The rate of acetate production in *N. walkeri* has been indirectly measured in living termites by measuring the rate of CO_2 production when termites are incubated under N_2 (Lo, 1994). The rate of CO_2 production, 29.7 ± 3.2 nmol termite^{-1} h^{-1} (n = 8), also shows that, under these conditions, little CO_2 produced is reduced to acetate. The rate of CO_2 production is similar to that of acetate production (32 ± 4 nmol termite^{-1} h^{-1} when isolated paunches are incubated anaerobically and close to the expected rate of 30 nmol termite^{-1} h^{-1}) if glucose is being metabolised *in vivo* at 15 nmol termite^{-1} h^{-1}. The increase in the rate of acetate to 79 nmol termite^{-1} h^{-1} when the paunches are incubated in the presence of 5 mM pyruvate is evidence that acetate is derived in the hindgut from pyruvate and perhaps represent maximal rates of paunch metabolism as a result of using non-physiological concentrations of substrates.

The high acetyl CoA synthetase activity in the gut is interesting because acetate does not stimulate oxygen utilisation in isolated hindguts. This finding suggests that the activity may be associated with anaerobic bacteria which would not have a TCA cycle. The acetyl CoA synthetase activity in the gut may be associated with lipid synthesis as lipids represents approximately 10% of a bacterial cell (Gottschalk, 1986). The activity of malic enzyme ($NADP^+$ dependent) which normally provides a source of the NADPH for lipid synthesis may be a better indicator of the rate of bacterial lipid synthesis.

Termite tissues: The hindgut of *M. darwiniensis* has been found to be permeable to acetate so that acetate produced in the paunch can be returned to the termite by active transport (Hogan *et al.*, 1985). An active acetyl CoA synthetase in the termite body of *N. walkeri N. exitiosus, R. flavipes* and *C. lacteus* (O'Brien and Breznak, 1984) indicates that the termite tissue can utilise the acetate produced in the hindgut by converting it to acetyl CoA which is then oxidised to CO_2 via the TCA cycle. The activity of acetyl CoA synthetase in *N. walkeri* (12 ± 2 nmol termite^{-1} h^{-1}) is comparable to that in *N. exitiosus*, clearly limiting the rate of metabolism of acetate in the termite. In *N. walkeri*, the activity provides direct evidence that some of the pyruvate must be aerobically oxidised in the hindgut as there is insufficient acetyl CoA synthetase in the body to handle that produced in the hindgut.

A fully functional TCA cycle is present in termite tissue in *N. walkeri, N. exitiosus, R. flavipes* and *C. lacteus* (O'Brien and Breznak, 1984) as shown by the presence of the key enzymes and from the conversion of [U-^{14}C]acetate and [U-^{14}C]2-oxoglutarate to $^{14}CO_2$ (Lo, 1994). Isocitrate dehydrogenase is normally the rate limiting reaction in the TCA cycle (Mathews and van Holde, 1990) and this was also found in *N. walkeri*. The results of O'Brien and Breznak (1984) suggest that 2-oxoglutarate dehydrogenase is the rate limiting step of the TCA cycle in *N. exitiosus, R. flavipes* and *C. lacteus* This may be an error, as using a more sensitive ^{14}C-assay for *N. walkeri* tissues, one which is free from the interference often associated with NADH assays, shows that this is not the case with *N. walkeri.*

The higher levels of acetyl CoA synthetase in the epithelial tissue coupled with the low hexokinase activity may be a function of the metabolism of acetate, rather than glucose, in the epithelial tissue for energy purposes or they may be contamination from bacterial enzymes. The preference of gut epithelial tissue for SCFAs has been shown in vertebrate systems (Ash and Baird, 1973, Pennington, 1954).

4.4 Conclusions

The demonstration of endogenous cellulase in the higher termites, *N. walkeri* and *Macrotermes* spp. is not a novel discovery but the purification of one of the major endo-β-1,4-glucanase of *N. walkeri* is. Like cellulases in other organisms, that in *N. walkeri* is composed of multiple β-glucosidase and endo-β-1,4-glucanase activities. Insufficient endo-β-1,4-glucanase was recovered to allow for the protein to be sequenced. The production of antibodies to the protein could be used in studies to localise the site of enzyme production in the tissue and to determine relationships to other termite and roach endo-β-1,4-glucanases. The present study has provided the purification scheme for the large scale preparation of the endo-β-1,4-glucanase components for future work.

The investigation of digestive enzymes in the Macrotermitinae and the associated *Termitomyces* sp. did not support the concept of the termite acquiring fungal enzymes in order to carry out digestion of cellulose. In the majority of species investigated there was no correlation between the young worker categories and high enzyme activities as would expected from feeding habits. As the young workers eat the fungal nodules which contain high levels of enzyme activity it would have been expected that they would have higher levels of enzyme activities due to the acquired enzymes. This was not so. A comparison of profiles on Bio-Gel P-150 of endo-β-1,4-glucanase activities from termite and fungal material showed there was little evidence of overlap of activities of the fungal and termite enzymes, dismissing the possibility that the fungus is an important source of endo-β-1,4-glucanase activities in the termite. The fungal cellulases associated with the different *Macrotermes* spp. contained at least one common endo-β-1,4-glucanase activity and were

usually multicomponent activities. Lack of material prevented the complete purification of these endo-β-1,4-glucanase activities. The purification procedure developed would be suitable for the production of pure endo-β-1,4-glucanase components for the production of antibodies. Antibodies could be used to settle the debate of enzyme acquisition by identifying whether fungal enzymes are present in the gut of the termite and whether they make any significant contribution to cellulose digestion in the Macrotermitinae. This could be supported by further work on nitrogen metabolism. In the limited study on nitrogen metabolism an increase in nitrogen content was found in the fresh comb in comparison to the food source. In *M. bellicosus* an increase in nitrogen content was also found in the food store in comparison with the food. In both steps the nitrogen could be added by the termite as salivary secretions or it could be added during passage through the gut or it could be fixed by the microbiota. Further work is needed on the exact form of this nitrogen supplementation.

The most significant results from the metabolic studies are the clear demonstrations of the enzymes of aerobic respiration in the hindgut. These results which are complementary to the demonstration of an O_2 gradient in the hindgut are the first demonstrations that the bacteria are taking advantage of the termite's respiratory system and show that the hindgut is the site of significant amount of aerobic metabolism and is not just an anaerobic fermentation chamber.

This study has been successful in satisfying the broad outline of integrating carbon and nitrogen metabolism in higher termites. As in any research field, for every success there are failures and for every problem solved more arise to challenge the researcher. No doubt this thesis will provide the basis for a number of challenging avenues for future research in the amazing jigsaw puzzle of termite metabolism.

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APPENDIX

6.1 Taxonomic Index

6.2. Mineral content of Source Tangui Natural Mineral Water

(Société des Eaux Minérales du Cameroun, Douala, République du Cameroun)

Calcium	32 mg/ml
Magnesium	21 mg/ml
Potassium	10 mg/ml
Sodium	1 mg/ml
Bicarbonate	217 mg/ml
Sulphate	2 mg/ml
Chloride	1 mg/ml
Nitrate	absent

6.3. pH indicators - properties and colours in the gut of *N. walkeri*

Indicator dye			Gut section						
	pH range	Colour[1]	Foregut	Midgut (anterior)	Midgut (posterior)	Mixed segment	Paunch	Colon	Rectum
Bromocresol green	3.8 - 5.4	Y - B	B	B	B	B	B	B	B
Methyl red	4.2 - 6.2	R - Y	Y - O	Y	Y	Y	Y	Y	O - Y
Bromocresol purple	5.2 - 6.8	Y - P	G - P	P	P	P	P	G - P	G - P
Bromothymol blue	6.0 - 7.6	Y - B	G - Y	G - BG	B	B	G	G - Y	Y
Phenol red	6.8 - 8.4	Y - R	Y - Pk	P - O	R	R	O - Y	P - Y	Y - O
Cresol red	7.2 - 8.8	Y - R	Y	Y - R	P	P	R - Y	Y	Y
pH range Indicator paper			4.2 - 7.2 5.5	6.0 - 8.4	6.0 - 8.8 8.0 - 9.0	6.0 - 8.8	6.0 - 8.4 7.0 - 8.0	4.2 - 7.2 7.0	4.2 - 6.8 5.5

[1] Y=Yellow; B=Blue; R=Red; O=Orange; P=Purple; G=Green; BG=Blue-green; Pk=Pink

6.5a. Specific activity of enzymes in termite categories and fungal material from laboratory colonies (Bern) of *M. michaelseni* and *M. subhyalinus* .

	cellulase[1] (Units mg $^{-1}$)	endo-β-1,4-glucanase (Units mg $^{-1}$)	β-glucosidase (Units mg $^{-1}$)	amylase (Units mg $^{-1}$)
M. michaelseni				
Caste				
Old Major Workers	0.25 ± 0.04	132 ± 18	51.22 ± 3.62	0.28 ± 0.07
Young Major Workers	0.25 ± 0.01	207 ± 22	72.08 ± 12.26	0.10 ± 0.01
Old Minor Workers	0.50 ± 0.03	295 ± 87	120 ± 10	0.88 ± 0.14
Young Minor Workers	0.26 ± 0.04	159 ± 98	62.90 ± 13.91	0.31 ± 0.02
Minor Soldiers	0.019 ± 0.008	53.18 ± 14.99	11.76 ± 0.83	0.011 ± 0.004
Larvae	0.0000	14.96 ± 5.22	< 0.000	< 0.000
Fungal Tissue				
Fresh Fungal Comb	0.002 ± 0.001	1.44 ± 0.41	< 0.000	< 0.000
Ripe Fungal Comb	0.001 ± 0.001	18.80 ± 13.16	< 0.000	0.007 ± 0.005
Fungal Nodules	0.36 ± 0.01	151 ± 28	< 0.000	0.17 ± 0.01
Old Fungal Comb	0.00	3.70 ± 1.85	< 0.000	< 0.000
M. subhyalinus				
Caste				
Old Major Workers	0.22 ± 0.08	174 ± 28	32.23 ± 0.22	0.10 ± 0.06
Young Major Workers	0.49 ± 0.06	263 ± 6	57.50 ± 0.67	0.34 ± 0.07
Old Minor Workers	0.062 ± 0.013	121 ± 33	35.44 ± 3.21	0.054 ± 0.014
Young Minor Workers	0.56 ± 0.07	214 ± 5	50.66 ± 2.21	0.38 ± 0.01
Minor Soldiers	< 0.00	32.95 ± 4.20	7.57 ± 0.58	0.056
Larvae	< 0.00	75.84 ± 27.15	7.81 ± 7.01	< 0.000
Fungal Tissue				
Fresh Fungal Comb	< 0.00	48.76 ± 10.71	53.70 ± 9.80	0.046 ± 0.02
Ripe Fungal Comb	< 0.00	3.10 ± 0.53	6.31 ± 1.02	0.005 ± 0.005
Fungal Nodules	0.40 ± 0.07	47.80 ± 10.75	14.95 ± 2.71	0.23 ± 0.02
Old Fungal Comb	0.01 ± 0.01	1.28 ± 0.51	5.99 ± 0.08	< 0.000

n = 3

[1]Unit are defined in the Materials and Methods. Results are expressed as Units mg protein^{-1} ± SE

6.5b. Specific activity of enzymes in termite categories and fungal material from field colonies of *M. bellicosus, M. subhyalinus* (Abidjan) and *M. mulleri* (Mbalmayo).

	cellulase[1] (Units mg $^{-1}$)	endo-β-1,4-glucanase (Units mg $^{-1}$)	β-glucosidase (Units mg $^{-1}$)	amylase (Units mg $^{-1}$)	endo-β-1,4-xylanase (Units mg $^{-1}$)
M. bellicosus*					
Caste					
Old Major Workers	0.32 ± 0.06	122 ± 8	15.93 ± 0.98	0.01 ± 0.00	109 ± 23
Young Major Workers	0.36 ± 0.06	163 ± 24	16.53 ± 1.51	0.17 ± 0.01	7.41 ± 1.03
Old Minor Workers	0.57 ± 0.09	162 ± 12	23.80 ± 1.34	0.21 ± 0.01	163 ± 18
Young Minor Workers	0.34 ± 0.03	166 ± 7	2.15 ± 0.15	0.01 ± 0.00	95.23 ± 13.4
Minor Soldiers	0.40 ± 0.27	52.5 ± 7.5	34.36 ± 9.74	0.17 ± 0.15	671 ± 237
Larvae	< 0.00	18.98 ± 2.16	< 0.00	< 0.00	
Fungal Tissue					
Fresh Fungal Comb	0.016 ± 0.002	2.34 ± 0.49	0.99 ± 0.06	0.07 ± 0.02	27.41 ± 3.23
Ripe Fungal Comb	0.003 ± 0.001	0.13 ± 0.13	< 0.00	< 0.00	8.59 ± 1.79
Fungal Nodules	0.14 ± 0.3	133 ± 30	0.41 ± 0.17	0.92 ± 0.10	187 ± 32
Old Fungal Comb	0.013 ± 0.01	1.56 ± 0.19	0.12 ± 0.12	0.02 ± 0.02	5.79 ± 2.93
M. mulleri					
Caste					
Major Forager Workers		117 ± 2	14.66 ± 0.56		
Old Minor Workers		309 ± 22	9.32 ± 1.07		
Young Minor Workers		249 ±27	7.48 ± 1.60		
Fungal Tissue					
Fungal Nodules		20.78 ± 1.13	0.72 ± 0.02		
M. subhyalinus					
Caste					
Old Major Workers	0.42 ± 0.16	66.46 ± 16.17	39.42	0.62 ± 0.18	
Young Major Workers	0.35 ± 0.01	140 ± 16	52.25 ±3.72	0.71 ± 0.05	
Old Minor Workers	0.47 ± 0.01	161 ± 0	60.54 ± 10.33	0.58 ± 0.10	
Young Minor Workers	0.31 ± 0.04	140 ± 14	36.71 ± 0.49	0.41 ± 0.02	
Fungal Tissue					
Fungal Nodules	1.16 ± 0.01	232 ± 32	59.11 ± 2.80	0.68 ± 0.05	

n = 3

6.4a. Enzyme activities (Units termite^{-1}) in termite categories and fungal material from laboratory colonies (Bern) of *M. michaelseni* and *M. subhyalinus*.

	cellulase (Units T^{-1})	endo-β-1,4-glucanase (Units T^{-1})	β-glucosidase (Units T^{-1})	amylase (Units T^{-1})	protein (mg T^{-1})
M. michaelseni					
Caste					
Old Major Workers	0.12 ± 0.02	64.89 ± 7.05	25.28 ± 0.61	0.14 ± 0.02	0.497 ± 0.025
Young Major Workers	0.16 ± 0.01	130 ± 8	45.72 ± 8.39	0.07 ± 0.01	0.63 ± 0.03
Old Minor Workers	0.23 ± 0.02	130 ± 35	53.18 ± 3.52	0.39 ± 0.06	0.45 ± 0.02
Young Minor Workers	0.11 ± 0.01	64.89 ± 7.05	25.28 ± 0.61	0.12 ± 0.00	0.42 ± 0.02
Minor Soldiers	0.01 ± 0.00	23.19 ± 5.50	5.30 ± 0.05	<0.01	0.46 ± 0.04
Larvae	<0.01	3.65 ± 1.69	<0.01	<0.01	0.23 ± 0.03
Fungal Tissue					
Fresh Fungal Comb	<0.01	0.09 ± 0.02	<0.01	0.01 ± 0.01	0.07 ± 0.01
Ripe Fungal Comb	<0.01	0.19 ± 0.13	<0.01	0.01 ± 0.01	0.011 ± 0.001
Fungal Nodules	0.05 ± 0.00	19.83 ± 5.21	<0.01	0.02 ± 0.00	0.13 ± 0.01
Old Fungal Comb	<0.01	0.04 ± 0.02	<0.01	<0.01	0.01 ± 0.00
M. subhyalinus					
Caste					
Old Major Workers	0.06 ± 0.02	44.53 ± 8.67	8.14 ± 0.36	0.03± 0.02	0.26 ± 0.01
Young Major Workers	0.26 ± 0.04	139 ± 4	30.30 ± 1.09	0.18 ± 0.04	0.53 ± 0.02
Old Minor Workers	0.02 ± 0.00	31.46 ± 4.01	8.75 ± 1.92	0.02 ± 0.01	0.28 ± 0.06
Young Minor Workers	0.14 ± 0.01	53.61 ± 5.68	12.65 ± 1.08	0.10 ± 0.01	0.25 ± 0.03
Minor Soldiers	<0.01	11.50 ± 1.94	2.59 ± 0.05	0.018	0.35 ± 0.04
Larvae	<0.01	16.20 ± 6.79	1.89 ± 1.67	<0.01	0.22 ± 0.06
Fungal Tissue					
Fresh Fungal Comb	<0.01	0.085 ± 0.010	0.10 ± 0.01	0.01 ± 0.01	0.002 ± 0.001
Ripe Fungal Comb	<0.01	0.033 ± 0.006	0.07 ± 0.01	<0.01	0.011 ± 0.005
Fungal Nodules	0.02 ± 0.00	3.33 ± 1.16	0.98 ± 0.32	0.02 ± 0.00	0.06 ± 0.02
Old Fungal Comb	<0.01	0.017 ± 0.006	0.08 ± 0.02	<0.01	0.014 ± 0.004

n = 3 unless stated

Units are defined in the materials and methods. Results are expressed as Units termite^{-1} or Units mg fungal tissue^{-1} ± SE

Appendix 6.4b. Enzyme activities (Units termite^{-1}) in termite categories and fungal material from field colonies of *M. bellicosus*, *M. subhyalinus* (Abidjan) and *M. mulleri* (Mbalmayo).

	cellulase[1] (Units T^{-1})	endo-β-1,4-glucanase (Units T^{-1})	β-glucosidase (Units T^{-1})	amylase (Units T^{-1})	endo-β-1,4-xylanase (Units T^{-1})	protein (mg.T^{-1})
M. bellicosus						
Caste						
Old major workers	0.21 ± 0.03	81.90 ± 4.35	10.89 ± 1.08	0.11 ± 0.02	76.64 ± 19.97	0.68 ± 0.03
Young major workers	0.23 ± 0.02	107.62 ± 12.07	10.96 ± 0.55	0.12 ± 0.01	82.50 ± 11.44	0.68 ± 0.04
Old minor workers	0.13 ± 0.01	40.90 ± 4.82	5.86 ± 0.21	0.05 ± 0.01	39.99 ± 3.80	0.25 ± 0.02
Young minor workers	0.10 ± 0.01	53.61 ± 1.62	4.68 ± 0.21	0.03 ± 0.00	31.18 ± 5.00	0.32 ± 0.01
Large soldier	0.12 ± 0.01	63.26 ± 7.34	6.23 ± 0.45	0.03 ± 0.02	74.84 ± 23.70	0.42 ± 0.15
Larvae	<0.01	4.65 ± 0.38	<0.01	<0.01		0.25 ± 0.04
Fungal tissue						
Fresh fungal comb	0.001 ± 0.00	0.013 ± 0.002	0.006 ± 0.000	<0.001	0.16 ± 0.02	0.006 ± 0.003
Ripe fungal comb	<0.001	0.002 ± 0.002	<0.001	<0.001	0.11 ± 0.02	0.13 ± 0.00
Fungal nodules	0.024 ± 0.004	3.92 ± 0.71	0.072 ± 0.029	0.16 ± 0.01	35.53 ± 6.93	0.18 ± 0.01
Old fungal comb	<0.001	0.024 ± 0.004	0.002 ± 0.002	<0.001	0.09 ± 0.04	0.015 ± 0.001
M. mulleri						
Caste						
Major workers		134 ± 4	16.85 ± 0.64			1.15 ± 0.02
Old minor workers		352 ± 55	10.65 ± 1.88			1.13 ± 0.11
Young minor workers		198 ±38	6.13 ± 1.79			0.79 ± 0.09
Fungal tissue						
Fungal nodules		9.43 ± 0.64	0.32 ± 0.01			0.45 ± 0.01
M. subhyalinus						
Caste						
Old major workers	0.212 ± 0.016	36.37 ± 2.60	28.41 ± 0.96	0.33 ± 0.01		0.62 ± 0.14
Young major workers	0.46 ± 0.03	186 ± 30	68.01 ± 2.36	0.93 ± 0.03		1.32 ± 0.13
Old minor workers	0.12 ± 0.00	40.56 ± 1.22	15.17 ± 2.11	0.14 ± 0.02		0.25 ± 0.01
Young minor workers	0.21 ±0.03	95.06 ±11.8	24.85 ± 0.93	0.28 ± 0.01		0.68 ± 0.02
Fungal tissue						
Fungal nodules	0.029 ± 0.001	6.57 ± 0.99	1.67 ± 0.08	0.11 ± 0.01		0.16 ± 0.00

n = 3

[1] Units are defined in the materials and methods. Results are expressed as Units termite^{-1} or Units mg fungal tissue^{-1} ± SE

6.6 Least squares ANOVA for cellulase in *Macrotermes* spp.

Source	Model DF	Reduced DF	Seq S	MS	F	p
spec	3	3	.0087073	.0029204	61.8	< 0.0001
type	4	4	.0080798	.0020200	43.01	< 0.0001
spec*type	12	11+	.0069258	.0006296	13.406	< 0.0001
Error	53	54	.0025361	.000047		
Total	72	72	.026249			

Tables of means with significance levels ($p < 0.001$)

	Old major	Young major	Old minor	Young minor	Soldier	ALL
M. bellicosus	.041235a	.04055a	.043168a	.047935a	.006952	.03520
M. subhyalinus (Abidjan)	.020301a	.016479a	.017075a	.01242a	-	.01652
M. subhyalinus (Bern)	.004774ab	.019054b	.001946a	.02099b	.0000a	.00935
M. michaelseni	.006516a	.007301a	.057	.009818a	.000497a	.01622
ALL	.022812	.025772	.033571	.02782.003358		.02317

	M. bellicosus	*M. subhyalinus* (Abidjan)	*M. subhyalinus* (Bern)	*M. michaelseni*	ALL
Old major	.041235	.020301a.004774a		.006516a	.022812
Young major	.04055	.016479a.019054a		.007301a	.025772
Old minor	.043168	.017075a.001946a		.057a	.033571
Young minor	.047935	.01242	.02099	.009818	.02782
Soldier	.006952a	-	.0000a	.000497a	.003858
ALL	.035204	.016523	.009353	.016226	.023178

6.7 Least squares ANOVA for endo-β-1,4-glucanase activity in *Macrotermes* spp.

Source	Model DF	Reduced DF	Seq S	MS	F	p
spec	4	4	443.962	110.9905	23.037	< 0.0001
type	4	4	560.800	140.2000	29.099	< 0.0001
spec*type	16	13+	283.542	21.8100	4.527	< 0.0001
Error	55	58	279.444	4.8180		
Total	79	79	1567.748			

Tables of means with significance levels ($p < 0.001$)

	Old major	Young major	Old minor	Young minor	Soldier	ALL
M. bellicosus	4.988a	6.391a	7.015a	7.907a	1.024	5.355
M. subhyalinus (Abidjan)	3.331a	6.605a	5.903a	5.698a	--	5.337
M. subhyalinus (Bern)	3.481a	6.111a	11.297	6.079a	1.388	5.642
M. michaelseni	3.711ac	10.218b	3.673ac	8.193ab	0.843c	5.443
M. mulleri	5.156	--	18.888a	14.578a	--	12.874
ALL	4.309	7.096	9.145	8.530	1.066	6.264

	M. bellicosus	*M. subhyalinus* (Abidjan)	*M. subhyalinus* (Bern)	*M. michaelseni*	*M. mulleri*	ALL
Old major	4.988a	3.331a	3.481a	3.711a	5.156a	4.309
Young major	6.391a	6.605a	6.111a	10.218a	--	7.096
Old minor	7.015a	5.903a	11.297	3.673a	18.888	9.145
Young minor	7.907a	5.698a	6.079a	8.193a	14.578	8.530
Soldier	1.024a	--	1.388a	0.843a	--	1.066
ALL	5.355	5.337	5.642	5.443	12.874	6.264

6.8 Least squares ANOVA for β-glucosidase activity in *Macrotermes* spp.

Source	Model DF	Reduced DF	Seq S	MS	F	p
spec	4	4	39.5393	9.8848	92.759	<0.0001
type	4	4	19.2559	4.814	45.174	<0.0001
spec*type	16	13+	24.3906	1.8762	17.606	<0.0001
Error	56	59	6.2873	.1066		
Total	80	80	89.4731			

Tables of means with significance levels (p<0.001)

	Old major	Young major	Old minor	Young minor	Soldier	ALL
M. bellicosus	.6635a	.6507a	1.0053a	.6897a	.1013	.6067
M. subhyalinus (Abidjan)	2.6477a	2.4197a	2.2075ab	1.4866b	-	2.1891
M. subhyalinus (Bern)	1.354	2.143a	4.5947	2.3667a	.3063	2.1529
M. michaelseni	.6795ac	2.2327d	1.2827ab	1.947bd	.19c	1.3083
M. mulleri	.7467a	-	.5687a	.4423a	-	.5526
ALL	1.1344	1.5588	1.7521	1.2705	.1691	1.2232

	M. bellicosus	*M. subhyalinus* (Abidjan)	*M. subhyalinus* (Bern)	*M. michaelseni*	*M. mulleri*	ALL
Old major	.6635a	2.6477	1.354	.6795a	.7467a	1.1344
Young major	.6507	2.4197a	2.143a	2.2327a	-	1.5588
Old minor	1.0053a	2.2075	4.5947	1.2827a	.5687a	1.7521
Young minor	.6897a	1.4866b	2.3667c	1.947bc	.4423a	1.2705
Soldier	.1013a	-	.3063a	.19a	-	.1691
ALL	.6067	2.1891	2.1529	1.3083	.5526	1.2232

6.9 Least squares ANOVA for amylase in *Macrotermes* spp.

Source	Model DF	Reduced DF	Seq S	MS	F	
spec	3	3	.00311457	.0010382	103.795	<.0001
type	4	4	.00083663	.0002092	20.911	<.0001
spec*type	12	11+	.00234622	.0002133	21.324	<.0001
Error	42	43	.00043010	.00001		
Total	61	61	.00672752			

Tables of means with significance levels (p < 0.001)

	Old major	Young major	Old minor	Young minor	Soldier	ALL
M. bellicosus	.006521a	.007018a	.009021a	.004122ab	.000439 b	.006019
M. subhyalinus (Abidjan)	.030743a	.033019a	.02124b	.016538b	-	.025762
M. subhyalinus (Bern)	.002239a	.013361b	.001768a	.014527b	.001a	.008425
M. michaelseni	.007388a	.003123a	.033223a	.011298	.000288	.011047
ALL	.011286	.012352	.01537	.01165	.000453	.011084

	M. bellicosus	*M. subhyalinus* (Abidjan)	*M. subhyalinus* (Bern)	*M. michaelseni*	ALL
Old major	.006521a	.030743	.002239a	.007388a	.011286
Young major	.007018a	.033019	.013361	.003123a	.012352
Old minor	.009021a	.02124	.001768	.033223	.01537
Young minor	.004122	.016538a	.014527a	.011298(b)	.01165
Soldier	.000439(b)	-	.001a	.000288a	.000453
ALL	.006019	.025762	.008425	.011047	.011084

6.10 Least squares ANOVA for protein in *Macrotermes* spp.

Source	Model DF	Reduced DF	Seq S	MS	F	P
spec	4	4	.00429898	.0010747	34.965	<0.0001
type	4	4	.00472747	.0011819	38.45	<0.0001
spec*type	16	13+	.00343468	.0002642	8.595	<0.0001
Error	55	58	.0017828.00)0307			
Total	79	79	.01424392			

Tables of means with significance levels ($p < 0.001$)

	Old major	Young major	Old minor	Young minor	SoldierALL	
M. bellicosus	.041235a	.038895a	.043168a	.047935a	.007811	.035809
M. subhyalinus (Abidjan)	.052725a	.046959	.036665b	.040483(a)b	-	.044894
M. subhyalinus (Bern)	.02658a	.029624ab	.038322a	.037957b	.026084b	.031706
M. michaelseni	.021144a	.03882b	.031069ab	.038354b	.025392a	.030956
M. mulleri	.044082	-	.060461a	.057619a	-	.054054
ALL	.037834	.038639	.042464	.045047	.016766	.037431

	M. bellicosus	*M. subhyalinus* (Abidjan)	*M. subhyalinus* (Bern)	*M. michaelseni*	*M. mulleri*	ALL
Old major	.041235a	.052725	.02658b	.021144b	.03882a	.037834
Young major	.038895ab	.046959a	.029624b.03382ab		-	.038639
Old minor	.043168a	.036665ab	.038322ab	.031069b	.060461	.042464
Young minor	.047935ab	.040483a	.037957a	.038354a	.057619b	.045047
Soldier	.007811	-	.026084a	.025392a	-	.016766
ALL	.035809	.044894	.031706	.030956	.054054	.037431

6.11 ANOVA on the effects of freeze-drying on enzyme activities from termite and fungal tissue of *M. michaelseni.*

```
MTB > glm c3 = c8 + c9 + c8*c9

Factor   Levels Values
type         10      1     2     3     4     5     6     7     8     9    10
method        3      1     2     3

Analysis of Variance for prot

Source        Model DF   Reduced DF      Seq SS
type                 9            9   0.1338696
method               2            2   0.0025607
type*method         18          10+   0.0044644
Error               33           41   0.0068813
Total               62           62   0.1477760

+ Rank deficiency due to empty cells, unbalanced nesting or collinearity.
  No storage of results or further analysis will be done.


MTB > glm c4 = c8 + c9 + c8*c9

Factor   Levels Values
type         10      1     2     3     4     5     6     7     8     9    10
method        3      1     2     3

Analysis of Variance for cello

Source        Model DF   Reduced DF      Seq SS
type                 9            9   0.02224640
method               2            2   0.00080396
type*method         18          10+   0.00528875
Error               34           42   0.00379583
Total               63           63   0.03213494

+ Rank deficiency due to empty cells, unbalanced nesting or collinearity.
  No storage of results or further analysis will be done.


MTB > glm c5 = c8 + c9 + c8*c9

Factor   Levels Values
type         10      1     2     3     4     5     6     7     8     9    10
method        3      1     2     3

Analysis of Variance for endo

Source        Model DF   Reduced DF      Seq SS
type                 9            9     5767.83
method               2            2      702.32
type*method         18          10+     7318.05
Error               31           39      453.04
Total               60           60    14241.25

+ Rank deficiency due to empty cells, unbalanced nesting or collinearity.
  No storage of results or further analysis will be done.


MTB > glm c6 = c8 + c9 + c8*c9

Factor   Levels Values
type         10      1     2     3     4     5     6     7     8     9    10
method        3      1     2     3

Analysis of Variance for gluco

Source        Model DF   Reduced DF      Seq SS
type                 9            9    215.4390
method               2            2     19.9124
type*method         18          10+    31.9136
Error               34           42     6.3802
Total               63           63   273.6452

+ Rank deficiency due to empty cells, unbalanced nesting or collinearity.
  No storage of results or further analysis will be done.


MTB > glm c7 = c8 + c9 + c8*c9

Factor   Levels Values
type         10      1     2     3     4     5     6     7     8     9    10
method        3      1     2     3

Analysis of Variance for amyl

Source        Model DF   Reduced DF      Seq SS
type                 9            9   0.0302500
method               2            2   0.0024008
type*method         18          10+   0.0110783
Error               29           37   0.0071390
Total               58           58   0.0508681

+ Rank deficiency due to empty cells, unbalanced nesting or collinearity.
  No storage of results or further analysis will be done.
```

6.11 *cont.*

```
MTB > glm c3 = c8 + c9 + c8*c9

Factor   Levels Values
type         10      1     2     3     4     5     6     7     8     9    10
method        3      1     2     3

Analysis of Variance for prot

Source        Model DF   Reduced DF     Seq SS
type                 9            9  0.1338696
method               2            2  0.0025607
type*method         18          10+  0.0044644
Error               33           41  0.0068813
Total               62           62  0.1477760

+ Rank deficiency due to empty cells, unbalanced nesting or collinearity.
  No storage of results or further analysis will be done.


MTB > glm c4 = c8 + c9 + c8*c9

Factor   Levels Values
type         10      1     2     3     4     5     6     7     8     9    10
method        3      1     2     3

Analysis of Variance for cello

Source        Model DF   Reduced DF     Seq SS
type                 9            9  0.02224640
method               2            2  0.00080396
type*method         18          10+ 0.00528875
Error               34           42  0.00379583
Total               63           63  0.03213494

+ Rank deficiency due to empty cells, unbalanced nesting or collinearity.
  No storage of results or further analysis will be done.


MTB > glm c5 = c8 + c9 + c8*c9

Factor   Levels Values
type         10      1     2     3     4     5     6     7     8     9    10
method        3      1     2     3

Analysis of Variance for endo

Source        Model DF   Reduced DF     Seq SS
type                 9            9    5767.83
method               2            2     702.32
type*method         18          10+    7318.05
Error               31           39     453.04
Total               60           60   14241.25

+ Rank deficiency due to empty cells, unbalanced nesting or collinearity.
  No storage of results or further analysis will be done.


MTB > glm c6 = c8 + c9 + c8*c9

Factor   Levels Values
type         10      1     2     3     4     5     6     7     8     9    10
method        3      1     2     3

Analysis of Variance for gluco

Source        Model DF   Reduced DF     Seq SS
type                 9            9   215.4390
method               2            2    19.9124
type*method         18          10+    31.9136
Error               34           42     6.3802
Total               63           63   273.6452

+ Rank deficiency due to empty cells, unbalanced nesting or collinearity.
  No storage of results or further analysis will be done.

MTB > glm c7 = c8 + c9 + c8*c9

Factor   Levels Values
type         10      1     2     3     4     5     6     7     8     9    10
method        3      1     2     3

Analysis of Variance for amyl

Source        Model DF   Reduced DF     Seq SS
type                 9            9  0.0302500
method               2            2  0.0024008
type*method         18          10+ 0.0110783
Error               29           37  0.0071390
```

6.12 ANOVA on the effects of freeze-drying on enzyme activities from termite and fungal tissue of *M. subhyalinus*.

```
  COLUMN    NAME       COUNT   MISSING
A C1                    63
A C2                    63
  C3        prot        65        30
  C4        cello       65        29
  C5        endo        65        32
  C6        gluco       65        32
  C7        amyl        65        35
  C8        type        65
  C9        method      65

CONSTANTS USED: NONE


MTB > glm c13 = c18 + c19 + c18*c19

Factor   Levels Values
C18           5    1    2    3    4    5
C19           3    1    2    3

Analysis of Variance for C13

Source     DF      Seq SS      Adj SS      Adj MS      F      P
C18         4 0.00163809 0.00163809 0.00040952  32.44  0.000
C19         2 0.00070324 0.00070324 0.00035162  27.86  0.000
C18*C19     8 0.00021298 0.00021298 0.00002662   2.11  0.066
Error      30 0.00037867 0.00037867 0.00001262
Total      44 0.00293298


MTB > glm c14 = c18 + c19 + c18*c19

Factor   Levels Values
C18           5    1    2    3    4    5
C19           3    1    2    3

Analysis of Variance for C14

Source     DF      Seq SS      Adj SS      Adj MS      F      P
C18         4 0.00188280 0.00188280 0.00047070  81.16  0.000
C19         2 0.00001898 0.00001898 0.00000949   1.64  0.212
C18*C19     8 0.00033147 0.00033147 0.00004143   7.14  0.000
Error      30 0.00017400 0.00017400 0.00000580
Total      44 0.00240724


MTB > glm c15 = c18 + c19 + c18*c19

Factor   Levels Values
C18           5    1    2    3    4    5
C19           3    1    2    3

Analysis of Variance for C15

Source     DF      Seq SS      Adj SS      Adj MS      F      P
C18         4     231.154     231.111      57.778  50.03  0.000
C19         2      38.204      35.303      17.651  15.28  0.000
C18*C19     8      97.630      97.630      12.204  10.57  0.000
Error      29      33.493      33.493       1.155
Total      43     400.481


MTB > glm c16 = c18 + c19 + c18*c19

Factor   Levels Values
C18           5    1    2    3    4    5
C19           3    1    2    3

Analysis of Variance for C16

Source     DF      Seq SS      Adj SS      Adj MS      F      P
C18         4     15.1088     15.0947      3.7737  78.16  0.000
C19         2      4.7406      4.5984      2.2992  47.62  0.000
C18*C19     8      5.2305      5.2305      0.6538  13.54  0.000
Error      29      1.4002      1.4002      0.0483
Total      43     26.4800


MTB > glm c17 = c18 + c19 + c18*c19

Factor   Levels Values
C18           5    1    2    3    4    5
C19           3    1    2    3

Analysis of Variance for C17

Source     DF      Seq SS      Adj SS      Adj MS      F      P
C18         4   0.0010160   0.0009593   0.0002398   1.54  0.221
C19         2   0.0000771   0.0000996   0.0000498   0.32  0.729
C18*C19     8   0.0013264   0.0013264   0.0001658   1.07  0.417
Error      25   0.0038908   0.0038908   0.0001556
Total      39   0.0063104
```

6.12 Cont.

```
MTB > glm c3 = c8+c9+c8*c9

Factor   Levels Values
type         10      1     2     3     4     5     6     7     8     9    10
method        3      1     2     3

Analysis of Variance for prot

Source          Model DF   Reduced DF      Seq SS
type                   9            9  0.02490442
method                 2            2  0.00016642
type*method           18        10+0  0.00218552
Error                 34           42  0.00262000
Total                 63           63  0.02987636

+ Rank deficiency due to empty cells, unbalanced nesting or collinearity.
  No storage of results or further analysis will be done.


MTB > glm c4 = c8+c9+c8*c9

Factor   Levels Values
type         10      1     2     3     4     5     6     7     8     9    10
method        3      1     2     3

Analysis of Variance for cello

Source          Model DF   Reduced DF      Seq SS
type                   9            9  0.00477500
method                 2            2  0.00002747
type*method           18        10+0  0.00034907
Error                 35           43  0.00018000
Total                 64           64  0.00533154

+ Rank deficiency due to empty cells, unbalanced nesting or collinearity.
  No storage of results or further analysis will be done.


MTB > glm c5 = c8+c9+c8*c9

Factor   Levels Values
type         10      1     2     3     4     5     6     7     8     9    10
method        3      1     2     3

Analysis of Variance for endo

Source          Model DF   Reduced DF      Seq SS
type                   9            9     417.590
method                 2            2      13.024
type*method           18          10+     193.627
Error                 32           40      47.942
Total                 61           61     672.182

+ Rank deficiency due to empty cells, unbalanced nesting or collinearity.
  No storage of results or further analysis will be done.


MTB > glm c6 = c8+c9+c8*c9

Factor   Levels Values
type         10      1     2     3     4     5     6     7     8     9    10
method        3      1     2     3

Analysis of Variance for gluco

Source          Model DF   Reduced DF      Seq SS
type                   9            9     23.4039
method                 2            2      4.8881
type*method           18          10+      5.9829
Error                 32           40      2.2305
Total                 61           61     36.5054

+ Rank deficiency due to empty cells, unbalanced nesting or collinearity.
  No storage of results or further analysis will be done.


MTB > glm c7 = c8+c9+c8*c9

Factor   Levels Values
type         10      1     2     3     4     5     6     7     8     9    10
method        3      1     2     3

Analysis of Variance for amyl

Source          Model DF   Reduced DF      Seq SS
type                   9            9  0.0241761
method                 2            2  0.0040582
type*method           18          10+  0.0166551
Error                 29           37  0.0044868
Total                 58           58  0.0493762

+ Rank deficiency due to empty cells, unbalanced nesting or collinearity.
  No storage of results or further analysis will be done.
```

6.13 ANOVA on nitrogen content of food, food store, fungal and termite material from *Macrotermes* spp.

```
MTB > onew c51 c53

ANALYSIS OF VARIANCE ON C51
SOURCE      DF       SS        MS        F        p
C53          4    3.50668   0.87667   573.97   <0.0001
ERROR       25    0.03818   0.00153
TOTAL       29    3.54487

                                        INDIVIDUAL 95 PCT CI'S FOR MEAN
                                        BASED ON POOLED STDEV
LEVEL      N      MEAN      STDEV      ---+---------+---------+---------+---
  2        6    0.2517    0.0340      (*)
  3        6    0.4727    0.0381           (*)
  4        6    0.8930    0.0415                (*)
  5        6    1.1868    0.0493                     (-*)
  6        6    0.9692    0.0297                (*)
                                        ---+---------+---------+---------+---
POOLED STDEV =    0.0391                0.30      0.60      0.90      1.20

                  .001 lsd .0629
                   all means significantly different


MTB > copy c1-c3 c67-c69;
SUBC> use c3=1.


MTB > onew c67 c68

ANALYSIS OF VARIANCE ON C67
SOURCE      DF       SS        MS        F        p
C68          2     58.73     29.36     26.91   <0.0001
ERROR       14     15.28      1.09
TOTAL       16     74.00

                                        INDIVIDUAL 95 PCT CI'S FOR MEAN
                                        BASED ON POOLED STDEV
LEVEL      N      MEAN      STDEV      --+---------+---------+---------+----
  1        6     5.621     0.652           (---*----)
  2        6     9.029     1.360                          (---*----)
  3        5     4.723     0.987      (----*----)
                                        --+---------+---------+---------+----
POOLED STDEV =    1.045                4.0       6.0       8.0       10.0

                  .001 lsd (n1=n2=6) = 1.7952

                 5.621 a     9.029       4.723 a


species 1 only different termite types

MTB > onew c71 c73

ANALYSIS OF VARIANCE ON C71
SOURCE      DF       SS        MS        F        p
C73          3     34.80     11.60     8.94    0.001
ERROR       19     24.64      1.30
TOTAL       22     59.44

                                        INDIVIDUAL 95 PCT CI'S FOR MEAN
                                        BASED ON POOLED STDEV
LEVEL      N      MEAN      STDEV      ---+---------+---------+---------+---
  7        6     8.554     1.459                          (-----*------)
  8        6     7.779     0.722                     (------*------)
  9        6     7.773     1.396                     (------*------)
 10        5     5.159     0.643      (------*-------)
                                        ---+---------+---------+---------+---
POOLED STDEV =    1.139                4.5       6.0       7.5       9.0

                  .001 lsd = 2.55

                 8.554 a   7.779 a   7.773 a     5.159
```

6.13 cont.

c2 = source
c1 = data

```
MTB > glm c31=c32+c33+c32*c33

Factor   Levels Values
C32           3      1      2      3
C33           4      3      4      5      6

Analysis of Variance for C31

Source      DF      Seq SS      Adj SS      Adj MS        F       P
C32          2      6.5109      6.5410      3.2705   136.15   0.000
C33          3      5.4816      5.5057      1.8352    76.40   0.000
C32*C33      6      3.0025      3.0025      0.5004    20.83   0.000
Error       66      1.5854      1.5854      0.0240
Total       77     16.5805

MTB > table c32 c33;
SUBC> means c31.

 ROWS: C32       COLUMNS: C33

             3         4         5         6       ALL

  1     0.4727    0.8930    1.1868    0.9692    0.8804
  2     0.7042    1.4750    1.6054    0.6332    0.9800
  3     1.1812    1.6979    1.6388    1.8704    1.5408
ALL     0.8252    1.3677    1.4589    1.0433    1.1363

  CELL CONTENTS --
            C31:MEAN

MTB > copy c1-c3 c41-c43;
SUBC> use c3=2:6.
MTB > glm c41=c42+c43+c42*c43

Factor   Levels Values
C42           3      1      2      3
C43           5      2      3      4      5      6

Analysis of Variance for C41

Source      DF      Seq SS      Adj SS      Adj MS        F       P
C42          2     16.1589     14.6955      7.3477   289.70   0.000
C43          4      5.7186      5.6699      1.4175    55.89   0.000
C42*C43      8      6.8325      6.8325      0.8541    33.67   0.000
Error       87      2.2066      2.2066      0.0254
Total      101     30.9167


MTB > table c42 c43;
SUBC> means c41.

 ROWS: C42       COLUMNS: C43

             2         3         4         5         6       ALL

  1     0.2517    0.4727    0.8930    1.1868    0.9692    0.7547
  2     0.8387    0.7042    1.4750    1.6054    0.6332    0.9456
  3     2.0923    1.1812    1.6979    1.6388    1.8704    1.6827
ALL     1.1620    0.8252    1.3677    1.4589    1.0433    1.1424

  CELL CONTENTS --
            C41:MEAN

MTB > copy c41-c43 c51-c53;
SUBC> use c42=1.                                  spec 1 only
MTB > onew c51 c53                                M. bellicosus

ANALYSIS OF VARIANCE ON C51
SOURCE     DF         SS         MS         F         P
C53         4    3.50668    0.87667    573.97     0.000
ERROR      25    0.03818    0.00153
TOTAL      29    3.54487
                                          INDIVIDUAL 95 PCT CI'S FOR MEAN
                                          BASED ON POOLED STDEV
 LEVEL       N      MEAN      STDEV       ---+---------+---------+---------+---
     2       6    0.2517     0.0340       (*)
     3       6    0.4727     0.0381               (*)
     4       6    0.8930     0.0415                              (*)
     5       6    1.1868     0.0493                                      (-*)
     6       6    0.9692     0.0297                                (*)
                                          ---+---------+---------+---------+---
POOLED STDEV =    0.0391                    0.30      0.60      0.90      1.20
MTB > copy c1-c3 c67-c69;
SUBC> use c3=1.
MTB > prin c67-c69
```

```
ANALYSIS OF VARIANCE ON C67                              source = 1
SOURCE     DF        SS        MS         F        p     (nodules)
C68 Spec.   2     58.73     29.36     26.91    0.000     M. bellicosus (A)
ERROR      14     15.28      1.09                        M. bellicosus (W)
TOTAL      16     74.00                                  M. muelleri
                                          INDIVIDUAL 95 PCT CI'S FOR MEAN
                                          BASED ON POOLED STDEV
 LEVEL       N      MEAN      STDEV       --+---------+---------+---------+----
     1       6     5.621      0.652              (---*----)
     2       6     9.029      1.360                                  (---*----)
     3       5     4.723      0.987        (----*----)
                                          --+---------+---------+---------+----
POOLED STDEV =     1.045                   4.0       6.0       8.0      10.0
```

6.14 ANOVA on uric acid of food, food store, fungal and termite material from *Macrotermes* spp.

```
ANALYSIS OF VARIANCE ON C22
SOURCE     DF        SS         MS         F        p
C25         5   0.11210    0.02242     21.86    0.000
ERROR      18   0.01846    0.00103
TOTAL      23   0.13056

                                        INDIVIDUAL 95 PCT CI'S FOR MEAN
                                        BASED ON POOLED STDEV
 LEVEL      N      MEAN      STDEV     -----+---------+---------+---------+-
     1      4   0.00000    0.00000     (----*----)
     2      4   0.11240    0.03276                (----*----)
     3      4   0.12411    0.05810                  (----*----)
     4      4   0.11106    0.02857                (----*----)
     5      4   0.18858    0.02981                          (----*----)
     6      4   0.00000    0.00000     (----*----)
                                        -----+---------+---------+---------+-
POOLED STDEV =   0.03202                0.000     0.070     0.140     0.210
```

```
ANALYSIS OF VARIANCE ON C12
SOURCE     DF        SS         MS         F        p
C15         3     58.37      19.46      2.26    0.158
ERROR       8     68.76       8.60
TOTAL      11    127.13

                                        INDIVIDUAL 95 PCT CI'S FOR MEAN
                                        BASED ON POOLED STDEV
 LEVEL      N      MEAN      STDEV     ------+---------+---------+---------+
     7      3     6.111      1.098       (--------*---------)
     8      3     5.828      0.684      (---------*--------)
     9      3    11.355      2.326                (--------*---------)
    10      3     8.083      5.225          (---------*---------)
                                        ------+---------+---------+---------+
POOLED STDEV =   2.932                    4.0       8.0      12.0      16.0
```