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► **To cite this version:**

Étienne Saur, Jean-François Ponge. Alimentary studies on the Collembolan *Paratullbergia callipygos* using transmission electron microscopy. *Pedobiologia*, 1988, 31 (5-6), pp.355-379. <hal-00507018>

HAL Id: hal-00507018

<https://hal.science/hal-00507018v1>

Submitted on 22 Nov 2010

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Alimentary studies on the Collembolan *Paratullbergia callipygos* using transmission electron microscopy

ETIENNE SAUR and JEAN-FRANÇOIS PONGE

1. Introduction

During the last 30 years much work has been done on the feeding habits of Collembola and other members of the soil fauna, using light microscopy (POOLE, 1959; CHRISTIANSEN, 1964; KNIGHT & ANGEL, 1967; GILMORE & RAFFENSPERGER, 1970; BÖDVARSSON, 1970; MCMILLAN & HEALEY, 1971; ANDERSON & HEALEY, 1972 ; MCMILLAN, 1975; MARSHALL, 1978), but much less by this method on the effect of digestive processes on food material (PONGE & CHARPENTIE, 1981; PONGE, 1984, 1985a, b). KILBERTUS & VANNIER (1978, 1979, 1981) demonstrated with transmission and scanning electron microscopy that fungal, bacterial or algal cytoplasm was the main nutrient source for the collembolan species they studied and that some spores remained viable in their faeces. They considered therefore that Collembola control microbial populations rather than exert a direct influence on ingested plant material. These conclusions are borne out by the experiments of H. GISIN (1949), G. GISIN (1952), TÖRNE (1966, 1967, 1974, 1978), HANLON & ANDERSON (1979), PARKINSON *et al.* (1979), VISSER *et al.* (1981), INESON *et al.* (1982), TOUCHOT *et al.* (1983), TOUCHOT (1984) and HASSALL *et al.* (1986). Preliminary investigations on cleared animals observed under a light microscope (unpublished data) suggested that some collembolan species were able to degrade plant cell walls. There is no evidence from enzymatic studies for cellulase production in the digestive tract of most soil animals, including Collembola (NIELSEN, 1962; ZINKLER, 1971, 1972, 1983). However, in a recent study, ZINKLER & STECKEN (communication to the Int. Coll. Soil Zool., Moscow 1985, unpublished) demonstrated using ¹⁴C-labelled cellulose that digestion of this substrate might occur in the collembolan species *Pogonognathellus flavescens*, even though enzymatic tests failed to show a cellulase activity. The question which arises is how far the actual digestive activity of the animal was concerned, since ZINKLER's experiments were not conducted under aseptic conditions. Electron microscopic studies on the intestinal lumen of starved Collembola failed to reveal any bacterial population (KILBERTUS &

VANNIER 1981), and it is likely that isolated bacteria found in fed animals originated from ingested food. Observations on different groups of soil animals by light microscopy (PONGE, 1984, 1985a, b, and *in litt.*) suggested that Collembola do not possess any intestinal microflora, since bacterial colonies were not found in the zone between the food bolus and the epithelial layer. In contrast, typical bacterial “clouds” could be easily observed in many other groups such as Nematoda (confirmed by ARPIN & KILBERTUS, 1981; SAUR & ARPIN, unpublished), Rotifera, Enchytraeidae, Oribatei (confirmed by STEFANIAK & SENICZAK, 1983) and Diptera larvae (confirmed by SZABÓ *et al.* 1969), even when the guts were empty. This view conflicts somewhat with that of TÖRNE (1967), but a thorough examination of his experimental conditions and inoculation methods would be needed before we can conclude that there is a real disparity between his results and our observations. The present study was conducted to shed light on these questions. Field-collected and laboratory animals fed with natural or pure substrates were studied. The results are presented here in the context of forest soil ecology (within the French PIREN-CNRS-project: “Pedobiological functioning of an acid mull humus in the Orleans forest”).

2. Materials and methods

2.1. *Species description*

Paratullbergia callipygos (BÖRNER, 1902) (= *Tullbergia callipygos*) is a Collembola (Insecta, Apterygota) belonging to the family Onychiuridae and to the subfamily Tullbergiinae. It is one of the largest species in this subfamily (adult length: 1 to 2 mm; GISIN, 1960). Like other Tullbergiinae, this species lacks eyes, pigment and furcula, and has a slender cylindrical body and strongly reduced legs. The body shape and reduction or absence of thoracic and abdominal appendages are anatomical characters which allow it to move through the soil capillary system. Body diameters from 0.1 to 0.2 mm were measured on adult females (the population we studied was strictly parthenogenic).

The gut can be divided into three parts: the foregut or stomodeum (this comprises both pharynx and oesophagus), the midgut or mesenteron and the hindgut or proctodeum (HUMBERT, 1974). Digestive processes are localized in the midgut, i.e. the part derived from endoderm and, unlike the fore- and hindgut, not involved in moulting. The other two regions are only ducts for the food bolus. So our study was focussed on the midgut contents. HUMBERT (1974), working with the collembolan species *Sinella coeca*, recorded a midgut transit time of 8 to 10 hours. Some faeces were also studied for comparison.

The diet of this species is mainly saprophagous, consisting of dead plant material, but many mineral particles are also ingested (MCMILLAN, 1975). In some sites arthropod remains were also seen in the gut (ARPIN *et al.*, 1980).

Paratullbergia callipygos is ubiquitous with regard to soil type. It is found in calcareous as well as in podzolized soils (PONGE, 1980; PRAT & MASSOUD, 1980; HÅGVAR, 1982; WOLTERS, 1983; PONGE, 1983). It is found everywhere in Europe, both in lowlands and mountains (GISIN, 1943, 1944; CASSAGNAU, 1961), but never in great abundance. Most authors consider it a deep soil form but it can also be encountered just under the litter (USHER, 1970; ARPIN *et al.*, 1980; PONGE, 1980; PRAT & MASSOUD, 1980, 1982; PONGE, 1983; HÅGVAR, 1983; WOLTERS, 1983; POURSIN & PONGE, 1984). In reality this species is more tolerant of deep soil conditions than other Collembola.

2.2. Site description

The animals were collected from the experimental site of the PIREN project. This was a mature forest made of 100 to 160 year-old sessile oak [*Quercus petraea* (MATTUSCHKA) LIEBL.] mixed with 30 to 40 year-old beech (*Fagus sylvatica* L.) and hornbeam (*Carpinus betulus* L.), originating from old coppice with standards 100 years ago. Bramble (*Rubus schleicheri* WEIHE) was widespread, as a more or less continuous cover. Holly (*Ilex aquifolium* L.) was occasional. Humus was of the mull type, with a weak F layer (acid variant). Earthworm casts were numerous at the soil surface, beneath and over dead leaves, these being often invaded by white-rot fungi. The soil was a leached brown earth (sandy loam, pH of the A₁ horizon = 4.6) with pseudo-gley (aqualf alfisol in the American classification). The water table was at its highest level (–20 to –50 cm) in March-April.

2.3. Animal experiments

2.3.1. Sampling

Fresh animals were extracted from soil cores by the dry funnel method (Tullgren type; VANNIER, 1970), then collected by flotation on water under a dissecting microscope in September 1985, and April 1986. Some individuals were immediately fixed for ultrastructural study, others were kept in experimental jars. Observations by light microscopy were made on ethyl-alcohol-fixed animals, then mounted in chloral-lacto-phenol. These individuals were collected in May 1984, November 1984 and May 1985.

2.3.2. Feeding experiments

Animals were reared individually in two types of environment:

(1) Long-term experiments (two months at 15°C). Polystyrene circular vessels (35 mm diameter) were partially filled with A₁ horizon sandy loam from the study site which was previously air-dried, then passed through a 1 mm mesh sieve and remoistened with deionized water. Six sets were made each with 6 replicates:

— soil only

— soil + an oak leaf fragment (three months in the litter after autumn leaf-fall)

— soil + beech (same age as above)

— soil + hornbeam (as above)

— soil + living bramble root pieces

— soil + holly root pieces (as above)

Holly roots were killed by cutting them, and then transferred to experimental jars, unlike bramble roots which were still alive and in fact grew well in this environment.

(2) Short-term experiments (24 h at 11°C). Polystyrene vessels (haemolysis tubes 8 mm diameter) were partially filled with a mixture of plaster of Paris and animal bone black prepared with tap water. This technique is commonly used to rear Collembola. Pieces of food were placed at the surface of the plaster substrate and animals were immediately introduced. Collembola were starved for the previous four days, to ensure empty guts. Eight sets were made each with 4 replicates:

— fibrous cellulose Whatman CF 11

— micro-granular cellulose Whatman CC 31

— wheat starch

— filter paper Durieux Nr. 129

— elder pith

— fresh corpses of *Folsomia candida* (another collembolan species reared at the laboratory)

— entire moults from *Paratullbergia callipygos*

— milled mealworm moults

The substrate was not ingested by animals even when food was uneaten, according to TEM observations.

2. 4. Preparation for microscopy

2.4.1. Fixation and embedding

The integument of Collembola is generally not easily wettable. So for correct fixation and easy transport a special technique is needed. Each animal was shaken in a glass tube filled with 3 parts distilled water and 1 part photographic wetting agent (Ilford, or any other without a detergent.). The animals were then drained, transferred to a clean glass plate and covered with a 2%-agar droplet. When the agar has cooled, embedded animals can be easily transported in successive baths without damage, and solvents and fixatives will diffuse freely through the agar.

Animals were pre-fixed in 2.5% glutaraldehyde (buffered with Millonig phosphate up to pH 7.5) for 2 h at 3°C, then fixed in 2% osmium tetroxide (with the same buffer) for one hour at 20°C. After dehydration they were embedded in Epon 812 resin (LUFT, 1961). Polymerization continued for 4 h in a drying-oven at 60°C.

2.4.2. Sectioning

Paratullbergia callipygos ingests much mineral material (especially quartz grains) which may be harmful to the diamond knife when sectioning the resin embedded animals. So the technique of DRUM (1963) modified by BOROJEVIC & LEVI (1967) was applied to soil animals. Resin blocks, after they had been trimmed under a dissecting microscope, were soaked in 10% hydrofluoric acid then well rinsed under tap water for 2 h and again oven-dried at 60°C for 12 h. This technique dissolves silica (quartz grains) and silicates (clay minerals) without removal of any other mesenteric component. Dissolved substances leave gaps in the sections, so these must be protected by a membrane before being placed in the vacuum chamber. The sections were cut with a diamond knife. When sectioning the blocks, the animals were orientated so that the guts were cut transversally. No particular region was selected since the midgut is completely full before digestion starts (this process lasts from 8 to 10 h in the collembolan species *Sinella caeca*, with a transit time of no more than 10 min through the hindgut; HUMBERT, 1974). Unfortunately, since the starting time was quite unknown, we had no information on the digestive stage at which the animals were killed. Appropriate orientations were chosen when

sectioning other elements (plant fragments, animal moults, etc...). MAGNAN (1961) and HAGEGE & HAGEGE (1980) were used as reference works on the preparation and examination of sections.

3. Results and discussion

3.1. Vertical distribution and alimentary habits

3.1.4. Sampling

264 animals coming from 60 soil cores were used in this analysis. Samples at the aforementioned station were made on 23/5/84, 21/11/84 and 30/5/84. Soil cores were taken up with the help of a 5 cm diameter pedological corer (VANNIER & ALPERN 1968) and subdivided into 4 strata:

— Zone 1: 0 to 1 cm

— Zone 2: 1 to 3 cm

— Zone 3: 3 to 6 cm

— Zone 4: 6 to 10 cm

3.1.2. Vertical distribution (fig. 1)

To visualize the population depth gradient, population densities per unit of soil volume were calculated (Fig. 1). Considerable variations over the year can be seen to have occurred. Comparing May 1984 with November 1984, the greatest density was in the first zone (0–1 cm) in May whereas it was in the second zone (1–3 cm) in November. The latter date (21/11/84) occurred just after the autumn peak in litter fall. This suggests that freshly fallen litter (mainly oak leaves) was discarded by *P. callipygos*, which then preferred deeper depths. On the other hand, in Spring, this litter is reduced to a thin layer and strongly decomposed by leaching, faunal and microbial consumption (REISINGER & KILBERTUS 1980). VANNIER (in ARPIN *et al.*, 1986) showed that fresh oak litter was unpalatable to several collembolan species.

We must take into account the fact that the 1st zone is just only 1 cm thick, while the other layers are respectively 2, 3 and 4 cm thick. Thus it follows that most animals stay within the 2nd and 3rd zones (i.e. at 1 to 6 cm depth). These results verify that *P. callipygos* has a deep soil habitat, as has already been established by numerous authors (POOLE, 1961; USHER, 1970; ARPIN *et al.*, 1980; PONGE, 1980, 1983; HÅGVAR, 1983;

WOLTERS, 1983; POURSIN & PONGE, 1984). But its distribution fluctuates widely through the year as has been already established by USHER (1970).

Fig. 1 indicates a marked population reduction in Spring 1985. We cannot explain this with certainty but the long winter frost of 1984–85 may have caused it.

3.1.3. Alimentary habits (fig. 2)

In order to study the diet of *Paratullbergia callipygos* in the selected site, 264 individuals were observed by light microscopy. We must notice a problem in directly observing killed animals. Individuals were fixed at any stage of digestion (except if their guts were empty). So the only visible structures were those which have not already been digested. Any dissolved or assimilated materials could not be seen under the microscope. Similarly, liquid components and finely suspended particles (cytoplasm, soil solutions) were not accounted for. We shall see further on that electron microscopy, although not entirely satisfactory, is of some help with this problem. We used light microscopy primarily because it permitted us to treat a large number of animals at the same time and experience has shown that many food particles, so long as digestion is not complete, are still recognizable (PONGE, 1984, 1985a, b).

Gut contents were divided into 7 categories (fig. 2):

— mineral: mineral particles were 2 to 20 μm quartz grains, from the silt part of the A₁ horizon, mixed with clay particles. Amorphous organic matter was present, adsorbed onto clay particles. There were probably also free or aggregated bacteria, either dead or alive.

— organo-mineral mixtures: these consisted of plant debris (recognized by their plant cell wall remains) tightly appressed on mineral matter clumps. This category must be considered as a distinct class because the mixing had probably already occurred before ingestion. Considering the humus type where the animals were living, organo-mineral matter probably came from earthworm casts, where the mixture was already present.

— hyaline plant remains: these were macerated plant tissues, with transparent cell walls still recognizable. Entire cells were no longer present, due to the very fine grinding by the mandibles. It was impossible to discern the origin of most fragments (leaf or root material?), or whether they had been ingested as living or dead matter. Only the fact that fungi and bacteria were all but absent led us to suppose that these plant tissues in general were not previously decayed.

— brown plant remains: these tissues probably originated in the same way as the previous remains, but they had dark-coloured cell walls. We could not deduce whether this change happened before or after ingestion. In addition, we did not know why this browning occurred. If this phenomenon took place before passing through the intestinal tract, perhaps it may have resulted from the tanning of plant cell walls by polyphenol-protein complexes, like those observed by HANDLEY (1954), REISINGER *et al.* (1978), TOUTAIN *et al.* (1981). Except for the brown colour this category did not differ markedly from the previous one.

— organic lysate: this consisted of loosely outlined and never recognizable organic debris. Since we found animal remains in only one of the 264 studied animals, this organic matter probably had a vegetable origin, although no cell structure was identifiable. As before, we cannot be sure that this phenomenon is the result of a digestive process. Nevertheless, one could see, inside clearly distinguishable vegetable patches (assigned to the last two categories), lysis zones very similar to the present category, without any bacteria or fungi. So we hypothesize that it is a true digestive effect, awaiting corroboration by a more sensitive method.

— fungal remains: this category was very rarely encountered. It consisted of melanized (dematiaceous) walls of hyphae or spores. No hyaline wall pieces were found, even though they are clearly discernible in the gut of other Collembola (PONGE & CHARPENTIER, 1981; PONGE, 1985a, b). We conclude that fungi are probably avoided by this species in the site under study.

— void: it was part or all of a mesenteron without any visible contents under a light microscope (and unstained). We shall see later, from electron microscopy, that starved animals do not always have empty guts.

In order to quantify the mesenteric contents, each midgut was arbitrarily divided into ten equal parts, then the category to which every tenth part belonged was observed. The food was generally uniform throughout a large part of each gut.

Table 1 shows that the organo-mineral mixtures were the main food category (40 %). This is not unexpected because of the intense earthworm activity in the depth zone occupied by animals. The second most important category is hyaline plant material (30 %). If we combine all mineral matter categories (46 %), and all pure plant material (37 %), we can see that plant organic matter forms a significant part of the food of this species. This fraction is far higher than its level in the soil. Chemical analyses from this station (unpublished data) produced the following values for total organic matter on May 1984:

— depth zone 1: 5.2 %

— depth zone 2: 4.2 %

— depth zone 3: 3.2 %

— depth zone 4: 2.6 %

Such low values are fairly common for an acid mull humus (TOUTAIN, 1974; BRUN, 1978), and it should be noted that they included humified organic matter, which came into our organo-mineral mixtures. Thus the percentage of plant material in the food of *P. callipygos* cannot be the result of a random foraging activity throughout the soil. The work of MCMILLAN & HEALEY (1971) pointed out that, of four collembolan species dwelling in an ant nest, organic matter was most preferred by *P. callipygos* (94% occurrence in its intestinal gut). This example is somewhat out of line, but it shows that some endogenous Collembola are not primarily geophagous.

The origin of this plant material poses a problem. The marked preference for the A₁ horizon (depth zones 2 and 3 in Spring and Autumn, partly also zone 1 in Spring), led us to suppose that the plant material consisted partly of roots and rhizomes. Breeding experiments (PONGE, unpublished data) produced evidence that root pieces are preferred to leaf pieces when offered at the same time.

Percentages of the different food categories varied throughout the year and at different depths (fig. 2). Differences between sampling dates as well as between depth zones were highly significant [$P(\chi^2 > \text{obs.}\chi^2) < 0.001$]. The most surprising phenomenon is the lowering of pure mineral diets from depth zone 1 to 4. It may be explained by earthworm casting activity which pulls up to the soil surface mineral matter from deeper levels. Thus zone 1 consists of non-incorporated litter together with earthworm casts which are poorer in organic matter, the degree of incorporation increasing with depth. Since zone 1 was preferred in May 1984 and less frequented in November 1984 (fig. 1), we can explain why mineral matter was abundant in Spring and almost absent in Autumn (fig. 2). We wonder whether this food component has some nutritional value. KILBERTUS & VANNIER (1981) demonstrated that the cave collembolan species *Tomocerus problematicus* could not be reared in the laboratory unless clay minerals were included in its diet. Perhaps clay particles play a detoxifier or ion exchanger role during digestive processes as in soil processes.

We cannot give any interpretation of the results concerning organic matter as long as the origin (root or leaf tissues) of the ingested plant debris is unknown. We also need to know whether the observed transformations (browning, lysis) start outside or inside the animals.

The only work comparable to ours is MCMILLAN's (1975) study. This author followed from month to month variations in the gut contents of 60 individuals of *Paratullbergia callipygos* (together with two other species) in a mull-moder humus under beech and chestnut. The aim of his study was to ascertain whether the animals made a choice as to what they ate. He compared their gut contents (after cutting up and passing them through a Millipore filter) to "artificial guts" (glass tubes randomly forced into the soil). Unfortunately MCMILLAN took no more than three categories into account, namely "plant-", "mineral-" and "fungal material". Due to the methodology he employed it is impossible to compare categories such as "mixtures" (they are both mineral and plant material) or "lysate" (unrecognizable or removed by filtration). Nevertheless we noted that our categories "hyaline plant material", "brown plant material" and "mixtures" all increased from May to November 1984, which corresponded with a similar increase in MCMILLAN's "plant material": he showed that this increase was a gradual change and was unrelated to the autumn litter fall. He was unable to explain it. Similar also was the small amount of fungal material in the diet of this species. This was much smaller than it was seen in "artificial guts". Although the methodology of MCMILLAN was questionable (capillary tubes were of the same diameter as the whole animal body and he made no attempt to mimic the cutting up by mouthparts), his results and ours both raise important questions on the subject of collembolan diets and especially of their adaptive value.

On our part, we consider that the proportion of pure plant material (37%) in the diet cannot result from random foraging, given the make-up of this humus (see above). We can see from our results that *P. callipygos* cannot be classified with either the specialists (VANNIER, 1985) due to its varied and varying diet, or the generalists because it markedly prefers plant to fungal material (not the rule in Collembola).

Another phenomenon, not directly connected to food habits, should be noted. This is the few empty gut animals in the depth zone 1 (fig. 2). Starvation is mostly related to moulting periods in Collembola (SINGH, 1964; THIBAUD, 1968; JOOSSE, 1975). Moulting animals undergo physiological stress with a marked sensitivity to water deficiency (VANNIER, 1973; VANNIER & VERHOEF, 1978). So they must bury themselves in the soil in order to find a suitable and stable humidity, which was borne out by our results.

3.2. Ultrastructural studies

3.2.1. Field animals

Individuals were fixed just after they have been collected in dry funnels as before. Some blocks were

treated with hydrofluoric acid (see above), while others were kept intact in order to show clay and other minerals.

Starved animals may be or not in a moulting period. When it was only a casual food deprivation, we could see in the lumen, at the surface of microvilli, numerous vesicles and fibrillar material (fig. 3). The latter was made up of thin tubules and vesicles ca. 500 Å (50 nm) diameter, apparently produced by epithelial cells at the top of microvilli. This might produce the intestinal mucus (glycocalyx) spread throughout the lumen.

Just before their ecdysis or during the few hours following it, Collembola do not feed. This partly explains the high percentages of empty guts recorded in the literature (POOLE, 1959; ANDERSON & HEALEY, 1972; BÖDVARSSON, 1973). DE WITH & JOOSSE (1971) observed percentages of empty guts in several entomobryid species of 45 to 82%, but only 9 to 20% could be ascribed to pre-ecdysis starvation. Our results (14.7 %) lie within the latter range. It may indicate that, in our species, starving is strictly limited to moulting periods. This feature is worthy of note, since the behaviour of endogenous Collembola is poorly understood. Ecdysis is preceded by the renewal of mesenteron epithelial cells, as established by THIBAUD (1968), LAUGA-REYREL (1977) and HUMBERT (1979a, b). Electron microscopy revealed that several “empty” guts had epithelial remains in their lumen (fig. 4). This seems to be the usual outcome of mesenteron renewal, since we observed numerous “empty” guts in order to follow this process. These remains are probably at least partly digested, when animals are moulting or afterwards. Probably they are the first food the animals have at their disposal before they begin to feed again.

As a rule the food bolus was enclosed in a peritrophic membrane (figs. 5, 6). We could see that its structure was not cuticular by its thinness and absence of characteristic layers. Numerous authors have observed such a structure in insects (Collembola included) and have discussed its role and chemical composition (WATERHOUSE, 1957; MELLO *et al.*, 1971, 1972; KRZYSTOFOWICZ *et al.*, 1973; ROMOSER, 1974, BECKER *et al.*, 1975; ROMOSER & CODY, 1975; ZIMMERMAN *et al.*, 1975; BECKER *et al.*, 1976; LEHANE, 1976; RICHARDS & RICHARDS, 1977).

Plant material was abundant in the mesenteron. Several degradation stages could be seen. Fig. 5 shows barely damaged cell walls which had undergone only grinding by mouth parts. On the contrary, figs. 7 and 8 show attack on secondary walls, with the appearance of electron dense substances. This type of change has been described by authors studying plant litter decomposition by microorganisms (KILBERTUS & REISINGER, 1975; OLAH *et al.*, 1978; REISINGER & KILBERTUS, 1980; SCHWARTZ, 1981). Fig. 9 shows on the left an intermediate

stage of such an attack. Note the breaking of the wall, as a result of the mechanical action of the animal. We could often see accumulations of markedly transformed plant cell walls (figs. 10, 11 on the left). They were very thin in places and had suffered heavy mechanical damage. In extreme cases only a paste of very thin particles remains (fig. 12), without discernible wall structures. This category corresponds probably to the earlier mentioned “organic lysate”, as is the material in figs. 7, 8 and 10. Fig. 5 probably corresponds to “hyaline-” or “brown plant material”. It is still impossible to show whether or not this results from a digestive process, since we do not know anything about the ingested material and the time lag after ingestion. For example, the cell walls represented in figs. 7 and 8 may have undergone a microbial degradation before they were eaten. For this reason we made other observations on laboratory animals (see sect. 3.22.).

“Organo-mineral mixtures” are shown in fig. 6, where quartz grains and clay particles were represented by white spaces due to the hydrofluoric acid treatment. We found greatly altered plant wall remains in these sections (electron dense substances) lying in a paste lacking recognizable structures.

Bacteria were seen in the food bolus but they were never abundant and were always lysed. They occurred singly (figs. 8, 13) or in aggregates (figs. 14, 15, 16). These micro-aggregates closely resembled those present in the soil (KILBERTUS *et al.*, 1977; PROTH, 1978; KILBERTUS, 1980; KILBERTUS & MANGENOT, 1981; SCHWARTZ, 1981), but unlike in the soil forms there were signs of lysis inside the bacteria (figs. 15, 16). Thus bacteria appear to be killed by mesenteron solutions diffusing through the extracellular mucilaginous matrix.

Fungi were rarely observed. Fig. 11 shows a cross section of a fungal hypha which was in a very weakened state. Fragments of arthropod cuticles were also observed in one of these animals (fig. 13, upper part). Only the thin non-chitinous epicuticle remains (RICHARDS, 1911). This layer showed numerous folds and primary grains (MASSOUD & BETSCH, 1973; BETSCH & VANNIER, 1977: compare fig. 43 where cuticle is intact). Some sections of wall remnants were difficult to interpret (fig. 9, right hand).

In order to have a view of the end product of the digestive cycle (5 to 10 h in *Sinella coeca*; HUMBERT, 1974), we have studied some faeces. These were taken from field-collected specimens which had been placed on a soil substrate (A₁ horizon, ground and sifted) at 15 °C, without any other food. This substrate, although close to natural conditions, probably contained a greater quantity of mineral matter and lacked litter, roots and mycelial strands. Faecal content when purely organic appeared to consist of a fine-textured matrix within which were electron dense masses (fig. 17). Lacking information on the ingested substrate it was impossible to ascertain what type of organic matter had been eaten by the animals and to what extent this matter was transformed in the

gut. Faeces with mineral matter show relictual aggregates amongst clay particles (fig. 18). Bacteria had lost their content, remaining only as membranes. These observations, together with those of intestines (figs. 14, 15, 16), show that soil micro-aggregates did not withstand passage through the gut, although protected by their mucilaginous sheaths and clay minerals. The question of the nutritional value of mineral matter may be partly resolved if we remember that such micro-aggregates are very common in soil (KILBERTUS *et al.*, 1977) and may be ingested along with mineral matter. Cuticular remains from arthropods were also observed in one sample of faeces. As previously mentioned, only the epicuticular layer remained, but now we could see the ultrastructure of primary grains reduced to a boundary membrane (figs. 20), which is not yet the case in fig. 13. A transverse section of a chaeta showed the presence of intact cuticular layers beneath the epicuticle, probably as a result of the protective effect of the epicuticle against digestive enzymes in this closed organ.

Since these faeces had been on the soil surface some time before collection, they had been invaded by fungi whose living hyphae were visible in the sections (figs. 19, 18 at the extreme right).

We must be cautious in our conclusions at this point, since there is much we did not know about the state of the ingested food. We could not ascertain whether the animals had caused the changes seen in the plant cell walls; however, we could feel secure that they had caused the destruction of soil micro-aggregates, since none were found intact in the animals or in their faeces. This is probably true for other “geophagous” Collembola.

3.2.2. Laboratory animals

3.2.2.1. Long-term studies with natural food

3.2.2.1 .1. Introduction

These experiments were performed to study the feeding and reproductive behaviour of animals fed on different foods. Ultrastructural studies were made of food, before, during and after transit through the gut. Animals were individually reared for several months on sifted soil from the field site, with or without an added food substrate (see above for methodology).

3.2.2.1.2. Fertility and survival

Mean survival rate was ca. 70% after 3 months, whatever food was offered (bare soil, soil + leaf or soil + root). Oviposition was favoured by the presence of leaf pieces (table 2). Several workers had already shown

that, whatever its quantity, food quality influenced oviposition rates (SNIDER, 1971; BOOTH & ANDERSON, 1979; ZETTEL 1982; ARPIN *et al.*, 1986b). We do not know whether, in natural conditions, litter is necessary for breeding behaviour. It appears to be an alimentary phenomenon, since leaf fragments were not chosen as a substratum for eggs. These were deposited in clusters at the soil surface, along the sides of the jars.

3.2.2.1.3. Food preferences

Roots were often eaten. Bite wounds were numerous, especially on growing apices and cicatrization callus (which were observed on bramble roots). But other tissues, such as epidermis or suberized and lignified older parts, were eaten. Leaves, whatever their position on the soil, had many more wounds on their lower side than on their upper side. The ratio upper side/lower side of the leaf for the number of attacks was:

— 0.15 for oak leaves

— 0.25 for beech leaves

— 0.26 for hornbeam leaves

This phenomenon was more prominent on oak leaves. Penetrability differences may explain it, as was demonstrated by the experiments of SACHELL & LOWE (1967) and HEATH & ARNOLD (1966) on earthworms, and SOMA & SAITO (1983) on woodlice. But other differences between the upper and lower side of the leaf, such as the nature of the mesophyll tissues (palisade parenchyma in the former, spongy parenchyma in the latter) may also account for this choice.

Obvious differences were also observed between tree species. Wounds were much more numerous on hornbeam leaves than on the other two species. In addition to obvious penetrability differences, other reasons can be put forward to explain this. These include the differing amount and nature of phenolic compounds (HANDLEY, 1954, 1961; SACHELL & LOWE, 1967; KING & HEATH, 1967; NEUHAUSER & HARTENSTEIN, 1978) and conditioning by microorganisms (BÄRLOCHER & KENDRICK, 1975). The problem of palatability of tree leaves to soil animals is far from resolved, because of many parameters that vary at the same time from one type of litter to another. In addition, there may be an inverse relationship between the amount of ingested food (related to palatability) and the assimilation rate (related to the nutritive value). This was demonstrated by the experiments of SOMA & SAITO (1983) on the woodlouse *Porcellio scaber* feeding on pine needles at different breakdown stages.

3.2.2.1.4. Ultrastructure of mesenteron and faeces contents, as compared with crude material

This study was made on zones free from bite wounds. Leaves and roots, before they had been ingested by animals, had intact secondary walls (figs. 21, 25, 27, 30). The only prominent damage was seen in the cytoplasm (figs. 21, 22) and middle lamella. In the latter case we could see several kinds of attack: a cleared zone in the vicinity of a fungal hypha (fig. 21), disintegration of the middle lamella and the appearance of electron dense substances (fig. 27). Fig. 27 appears to show enzyme diffusion through a simple pit, without degradation of the cellulosic wall. All of this plant material had been recently invaded by fungi (figs. 21, 22, 27), except for holly roots (fig. 30). Most hyphae were still alive and we did not find any bacteria. This indicated an early stage of decomposition (HUDSON, 1968; OLAH *et al.*, 1978; HAYES, 1979).

Beech leaf cells were partly filled with electron dense substances (fig. 25). This feature has often been described on beech, before leaves have been invaded by white-rot fungi (REISINGER *et al.*, 1978; TOUTAIN, 1981; TOUTAIN *et al.*, 1981). These substances were deposited as coatings on the cell wall and nets which spanned the cell lumen (empty of cytoplasm). They probably were polyphenol-protein complexes, which are known to be precipitated after the death of plant tissues (HANDLEY, 1954, MANGENOT & TOUTAIN, 1980).

Ingested material from different feeding regimes showed conspicuous differences in the degree of transformation. Hornbeam leaves (figs. 23, 24) showed significant degradation of cell walls (compare to figs. 21 and 22). Disorganization of the fibrillar structure of cellulose, with appearance of electron dense substances, might indicate enzymatic agency. This has been observed in microbial processes (OLAH *et al.*, 1978; REISINGER & KILBERTUS, 1980). Fungal wall remains could also be recognized (fig. 24, compare to figs. 21 and 22). They resembled those described by BERG & HOFSTEN (1976) on the Hyphomycete *Trichoderma viride* in autolysing cultures: cytoplasm and also parts of the fungal wall had been digested.

A completely different process was observed in beech leaves (fig. 25, 26). In contrast to hornbeam, we did not observe any wall lysis either in the gut or in the faeces (fig. 26). This is probably due to the blocking of cellulose degradation by tannins (HANDLEY, 1954, 1961; BENOIT *et al.*, 1968; LEWIS & STARKEY, 1968; MANGENOT & TOUTAIN, 1980). We should note that electron dense substances were greatly reduced as compared to uningested material. They were present only as a diffuse powdering on cell walls. We may hypothesize that there is digestion and assimilation of tannin substances, as demonstrated in woodlice by NEUHAUSER & HARTENSTEIN (1978).

Since we did not find any microflora in the gut of the individuals studied, we can conclude that the observed effects were animal in origin.

Absence of oak leaf material in guts or faeces compelled us to disregard it as a food category. Nevertheless some oak leaves were ingested, as it was evidenced by the bite wounds found on their lower sides.

Dead or living roots were involved in similar processes; however, their cell walls were always degraded in the gut of *P. callipygos*. In the faeces, bramble roots (fig. 28) had thinner walls, the contents of which were partly diffused outwards and could be seen as electron dense grains (fig. 28). The plant material was mixed with numerous clay particles, probably derived from the soil on which root pieces had been deposited. Fungal hyphae had penetrated into the faecal material (fig. 29), as when soil alone had been ingested (fig. 19). Growing parts of viable hyphae were seen and could be recognized by their darker cytoplasm and thicker walls. Holly roots had not yet been invaded by fungi (fig. 30) but they had undergone autolysis. In the mesenteron of *P. callipygos* a lytic process had taken place, at different stages. Some cell walls were intact and electron transparent (fig. 31, left part) or they might be quite electron dense (fig. 32, 31 on the right).

3.2.2.1.5. Discussion

This laboratory study showed the breakdown of plant cell walls in the midgut of *Paratullbergia callipygos*. It did not offer evidence concerning the chemical nature of the observed processes. We could discern the disruption of the fibrillar structure of the cellulose. Comparison to enzymatic processes in cellulolytic microorganisms suggests that a similar process occurs in these animal guts. In the case of *P. callipygos*, the absence of any active microflora in the digestive tract led us to suppose that the animal was responsible for the cellulose degradation. These results still need to be confirmed by enzyme studies or other suitable techniques, both to assess the chemical nature of the breakdown of cellulose and to ascertain whether this animal is able to assimilate degradation products of such high-molecular mass carbohydrates.

3.2.2.2. *Short-term studies with artificial food*

3.2.2.2.1. Introduction

The techniques described above were used to determine the food value of substances which, unlike roots and leaves which are palatable to these animals, we thought would be rejected in the presence of soil. These included elder pith, insect bodies and moults or chemicals such as purified starch and cellulose. Some of

these substances were never eaten *P. callipygos*. These include exuviae (meal worm as well as its own exuviae) and filter paper. The first example is of interest since it suggests that cuticular remains observed in the gut or faeces probably came from corpses (predatory activity being excluded) and not from moults. But we cannot discard the possibility that a moult may be eaten by chance when incorporated in faecal material and then mixed with other food particles. All other substances were ingested, and were followed during passage through the gut.

3.2.2.2.2. Cellulose

Whatman cellulose, which is purified from cotton fibres, caused some problems, because of its thickness and toughness. When sectioning the material, splits and irregularities appeared, due to vibration of the diamond knife. In spite of the shortcomings, the observations were informative and could be compared with those of BERG *et al.* (1972) and BERG & HOFSTEN (1976), with similar reservations.

Figs. 33 and 36 show the structure of the two Whatman products (respectively CF11 and CC 31). Both were purified from cotton fibres, but the second type (micro-granular cellulose) had been powdered mechanically. We recognized in both cases the thick walls and the small lumen of cotton fibres (BERG *et al.*, 1972). After passage through the digestive tract of *P. callipygos*, we could distinguish several types of alteration, which probably corresponded to successive changes. Fibres were immediately fragmented (fig. 37), then disorganized at the fibrillar level (figs. 34, 38). At this stage, some fragments remained intact (fig. 34). At a more advanced stage the structure of the cellulose was no longer recognizable. It was transformed into an homogeneous paste (fig. 35), with some fibrillae still present in places.

Whatman fibrous cellulose is a specific indicator of the ability of microorganisms to degrade wood cellulose (NILSSON, 1974). This author found that all Whatman cellulose-clearing fungi were capable of forming soft rot cavities in birch wood. Since clearing of cellulose in such agar cultures is probably similar to what we observed directly in the fibres with the help of TEM, we can assume that the observed phenomenon resulted from a true cellulolytic activity. We cannot describe it as a digestive process, unless degradation products are distinguished by special techniques.

3.2.2.2.3. Elder pith

Cell wall ultrastructure exhibited a heavily thickened middle lamella in the meatus zone (fig. 41). In the mesenteron of *Paratullbergia callipygos*, these structures became triangular fragments which corresponded to middle lamella thickenings (fig. 42). The remaining wall parts changed into finely dispersed electron dense

substances. This transformation seemed to be more similar to natural processes where leaf or root cell walls were involved than to cotton-cellulose degradation processes. Thus we see that the type of cellulose or the nature of the substances bound to it strongly influences digestive processes.

3.2.2.2.4. Wheat starch

Fig. 39 shows the ultrastructure of a starch grain. In the digestive tract, grains were broken up and the attack took place along the resulting fracture lines (fig. 40). Separation of particles from the margin and the fuzzy lining of the grain both indicated that diffusion of substances had occurred. In contrast to cellulolysis, amylolysis is widely distributed among soil animals: ZINKLER (1971) extracted an amylase from the collembolan species *Protaphorura subluliginata*, which belongs to the same family as our species.

3.2.2.2.5. Folsomia candida corpses

Cuticular ultrastructure (fig. 43) shows the numerous layers (eight were visible on this animal) of the chitinous cuticle and the thin non-chitinous epicuticle crowning it. This structure was studied by RICHARDS (1951) and BARRA (1977). The epicuticle is thickened just at the site of primary granulation. When fresh corpses were ingested, the only remains that we saw in the mesenteron were epicuticular fragments (fig. 44), with their typical primary granulation. This indicated that a strong chitinolytic activity takes place in the digestive tract of *P. callipygos*, although this species does not appear to ingest its own moults. The small amount of animal remains in the diet of animals from field collections is quite surprising. The distinction between the unpalatability of cuticular remains (exuviae) and the palatability of dead bodies suggests that substances other than chitin (probably the internal tissues) play a role in this preference, even though chitin was easily degraded. This phenomenon was not observed in plant material, since pure cellulose, except in the form of filter paper, was easily eaten.

4. Conclusions

This study showed that the natural diet of *Paratullbergia callipygos*, in a forest acid mull humus, consisted of two main food categories:

— plant material: probably originating partly from the tree and shrub root system, partly from decomposing litter, partly also from faecal pellets.

— mineral material: present in the faeces of animals which mixed organic matter with mineral matter (mainly

lumbricid worms in this site), in the surrounding soil and in earthworms casts. Some probably originated also from mineral material embedded in roots and ingested by chance when eating cortical tissue. Tunnelling activity by this species was not demonstrated but is possible. Transmission electron microscopy showed that bacteria, both isolated and in aggregates, were associated with mineral matter.

Of interest was the almost complete absence of fungal material, a phenomenon rarely encountered in Collembola. This may be explained partly by the endogenous habitat of this species, although mycelial strands were growing actively through the first 10 cm of the A₁ horizon.

Transmission electron microscopy was rewarding for studying changes in plant material (especially cellulosic walls) in the midgut of a subterranean Collembolan. This ecological group has been poorly studied, although it contains most mull species. Our results corroborate what was hypothesized from light microscopy observations, that is the major transformation of plant cell walls. Absence of any active microflora in the gut of this animal (which nevertheless needs to be further established by axenic cultures) led to the conclusion that this phenomenon was the result of the actions of *Paratullbergia callipygos*. Activation of fungal enzymes, as demonstrated by BÄRLOCHER (1982) in the crustacean species *Gammarus fossarum*, must be rejected as a primary explanation, since pure substrates such as Whatman cellulose gave the same results as feeding regimens. In spite of these new findings, we do not know whether this animal is able to assimilate degradation products. Answer to this question awaits other studies.

The problem of cellulose digestion by soil animals has been discussed for a long time and numerous investigations on their enzyme systems have been carried out (MANSOUR & MANSOUR-BECK, 1933; LASKER & GIESE, 1956; NIELSEN, 1962; ZINKLER, 1971, 1972, 1983). This has resulted in a debate which was complicated by interactions with the soil microflora. Both zoologists (*op. cit.*) and microbiologists (HAYES, 1979; REISINGER & KILBERTUS, 1980) claimed that the group they studied played a leading part in these processes. In fact no cellulases (endo- or exo-glucanases) were isolated from collembolan digestive tracts, but recent results from ZINKLER & STECKEN (unpublished) appear to show that standard enzymological techniques fail to detect cellulase activity even though this activity may be demonstrated by other methods (use of tagged food). This author has produced evidence for the metabolic use of ¹⁴C-labelled cellulose by the Collembolan *Pogonognathellus flavescens*, although he cannot prove that microbial processes are not involved in cellulose digestion. Such techniques will probably be used more and more in the future and they should settle this question.

The source of the plant material ingested by *P. callipygos* is unresolved. In spite of the fact that breeding experiments showed that this species was able to feed on both litter and root material, and on both living and dead roots, we do not know exactly on which plant material it feeds on in a forest soil. Some evidence suggests root material, which disappears very rapidly (PERSSON, 1984). Seasonal variation in materials eaten are probably common, but the large number of observations needed for a satisfactory assessment of this and our choice of an experimental approach prevented us from achieving this goal.

We demonstrated an impact on fungi and bacteria: cells were digested, even when protected by mucosubstances and clay particles (microaggregates). We also showed that fungal cytoplasm disappeared completely when hyphae were present in the ingested plant material. In addition it appeared that part of the wall was also digested to some extent. This species was found to be capable of digesting the chitinous part of the integument of arthropods, and presumably other macromolecules such as starch and polyphenols. In order to more completely understand the digestive abilities of *Paratullbergia callipygos* a more detailed study with pure chemical substances (offered alone or mixed with palatable food substrates) would be necessary. We hope this work will lead to increased interest in soil zoology within the framework of forest science.

5. Acknowledgements

The authors are greatly indebted to Drs. J.A. BARRA, G. KILBERTUS, P. LEVI and R. ROHR (France) for helpful guidance and assistance in TEM techniques and to Pr. K. CHRISTIANSEN (U.S.A.) and Dr. J. FRANKLAND (England) for careful examination of the manuscript and revising of the English language.

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Synopsis: *Original scientific paper*

SAUR, E., & J.F. PONGE, 1988. Alimentary studies on the Collembolan *Paratullbergia callipygos* using transmission electron microscopy.

The food diet of the endogenous Collembolan *Paratullbergia callipygos* was studied in an oak stand on an acid mull humus. Transmission electron microscopy was used, parallel with light microscopy, in order to ascertain the effect of soil-dwelling Collembola upon plant materials and some other food substrates. Plant cell walls proved to be severely damaged when passing through the midgut after they have been broken up by mandibles, unless they were protected by coatings of tannins. Laboratory experiments with known substrates, both natural (dead leaves, dead and living roots) or artificial (pure cellulose fibres, starch, etc.) showed that this species was able to degrade cellulose, the process varying according to the type of cellulose. Degradation of other substrates, such as starch, tannins, fungal cytoplasm and chitin also occurred. The possible role of soil Collembola, especially the Tullbergiinae subfamily, in the fast turn-over of fine roots, was discussed.

Key words: Insecta, Collembola, *Paratullbergia callipygos*, digestive tract, gut content, light, transmission electronic microscopy, food, leaf litter, root, decomposition, degradation, turnover, enzyme.

Legends of figures

Fig. 1. Vertical distribution of *Paratullbergia callipygos* at three different sampling dates. The four zones were 1 = 0–1 cm; 2 = 1–3 cm; 3 = 3–6 cm; 4 = 6–10 cm. Numbers of animals are expressed per unit soil volume.

Fig. 2. Composition of the food bolus at three different sampling dates or at four depth levels. See text for explanation of the different food categories.

Fig. 3. Mesenteron without food contents. Vesicles and fibrillar materials. Bar = 1 μm .

Fig. 4. Renewal of the mesenteric epithelium during pre-ecdysis. Remains of the older epithelium in the intestine lumen. Bar = 1 μm .

Fig. 5. Field animal. Plant cell walls, crushed but non-degraded. Arrow = peritrophic membrane. Bar = 1 μm .

Fig. 6. Field animal. Organo-mineral mixture. Quartz grains and clay minerals as white spots. Bar = 1 μm .

Fig. 7. Field animal. Lysis of secondary plant cell walls. Bar = 1 μm .

Fig. 8. Idem. Arrow = isolated bacteria.

Figs. 9 & 10. Field animal. Plant cell wall remains. Bar = 1 μm .

Fig. 11. Field animal. Plant cell wall remains and fungal hyphae in cross section. Bar = 1 μm .

Fig. 12. Field animal. Paste of very thin particles. Bar = 1 μm .

Fig. 13. Field animal. Isolated bacteria (one of these indicated by an arrow) in a paste of very thin particles. Remains of an arthropod cuticle. Bar = 1 μm .

Fig. 14. Field animal. Micro-aggregate. Bar = 1 μm .

Fig. 15. Field animal. Micro-aggregate. Bar = 1 μm .

Fig. 16. Idem, destroyed bacteria.

Fig. 17. Field animal. Faecal content with non-identified electron dense masses. Bar = 1 μm .

Fig. 18. Field animal. Faecal content with relictual micro-aggregates. Bar = 1 μm .

Fig. 19. Field animal. Faecal content invaded by fungi. Arrow = fungal hyphae. Bar = 1 μm .

Fig. 20. Field animal. Faecal content with cuticular remains. Bar = 1 μm .

Fig. 21. Hornbeam leaf. Invasion of the phloem by fungal hyphae (arrows). Lysis of the middle lamella. Bar = 1 μm .

Fig. 22. Hornbeam leaf. Fungal development at the inside of a cell. Bar = 1 μm .

Fig. 23. Laboratory animal. Degraded plant and fungal wall remains from hornbeam leaves. Bar = 1 μm .

Fig. 24. Idem. Arrow = fungal wall remains. Bar = 1 μm .

Fig. 25. Beech leaf. Electron dense substances at the inside of the cells. Bar = 1 μm .

Fig. 26. Laboratory animal. Plant material coming from beech leaves. Bar = 1 μm .

Fig. 27. Bramble root. Invasion of plant tissues by fungal hyphae (arrows). Simple pit with lysis of the middle lamella. Bar = 1 μm .

Fig. 28. Laboratory animal. Faecal content with plant cell wall remains coming from bramble roots. Bar = 1 μm .

Fig. 29. Idem, but with invasion by fungal hyphae (one of these indicated by an arrow). Bar = 1 μm .

Fig. 30. Holly root. Autolysed plant cells. Bar = 1 μm .

Fig. 31. Laboratory animal. Cell wall remains from holly roots. Bar = 1 μm .

Fig. 32. Idem. Bar = 1 μm .

Fig. 33. Fibrous cellulose Whatman CF11. Cross section of a fibre. Bar = 1 μm .

Figs. 34 & 35. Laboratory animals. Cellulose remains. Bar = 1 μm .

Fig. 36. Micro-granular cellulose Whatman CC31. Section of a crushed fibre. Bar = 1 μm .

Figs. 37 & 38. Laboratory animals. Cellulose remains. Bar = 1 μm .

Fig. 39. Wheat starch grain. Bar = 1 μm .

Fig. 40. Laboratory animal. Attacked starch grain. Bar = 1 μm .

Fig. 41. Elder pith. Meatus between three adjacent cells. Bar = 1 μm .

Fig. 42. Laboratory animal. Wall remains from elder pith. Arrow = fragment of middle lamella. Bar = 1 μm .

Fig. 43. Cuticle of a heavily infected individual of *Paratullbergia callipygos*. Nonchitinous epicuticle with primary grains (one of them indicated by an arrow). Chitinous part with 7–8 layers. Bar = 1 μm .

Fig. 44. Laboratory animal. Cuticular remains of dead bodies of *Folsomia candida*. Arrow = primary grain. Bar = 1 μm .

Table 1. Food categories [%] present in the mesenteron of *Paratullbergia callipygos*

Mineral particles	7.6	}	47.6
Organo-mineral mixtures	40.0		
Hyaline plant material	20.1	}	37.2
Brown plant material	8.1		
Organic lysate	9.0	}	15.2
Fungal remains	0.5		
Void	14.7		

Table 2. Number of individuals which have laid eggs during the course of the 3-months experiment

Soil alone	0
Soil + beech root	1
Soil + holly root	1
Soil + bramble root	0
Soil + oak leaf	5
Soil + beech leaf	4
Soil + hornbeam leaf	4

Note: 6 individuals were reared alone on each food category

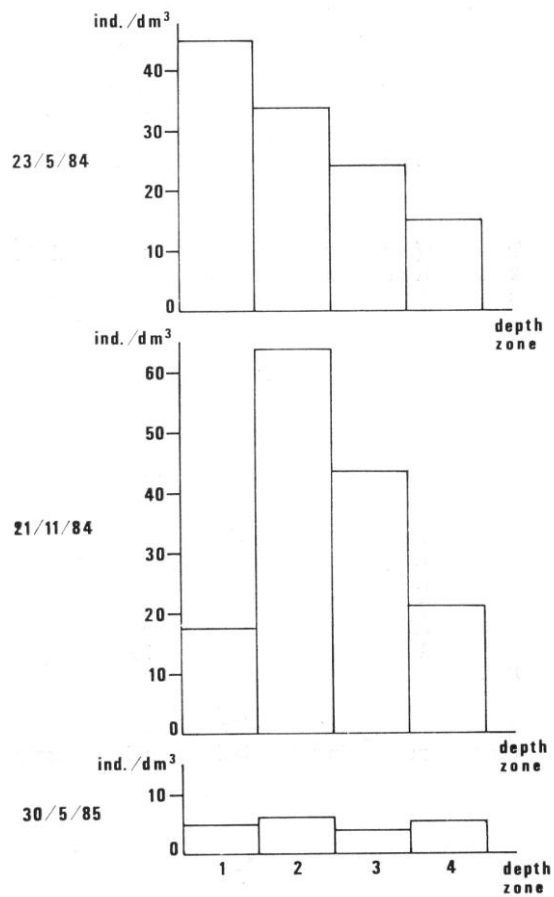


Fig. 1

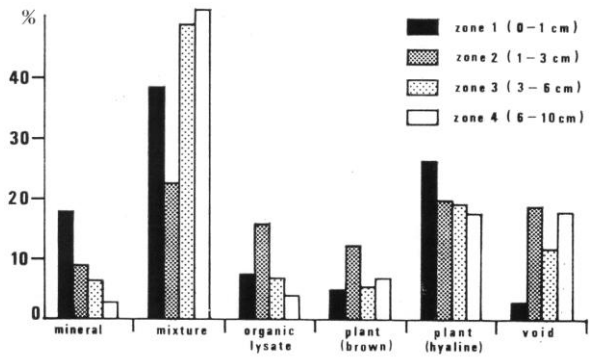
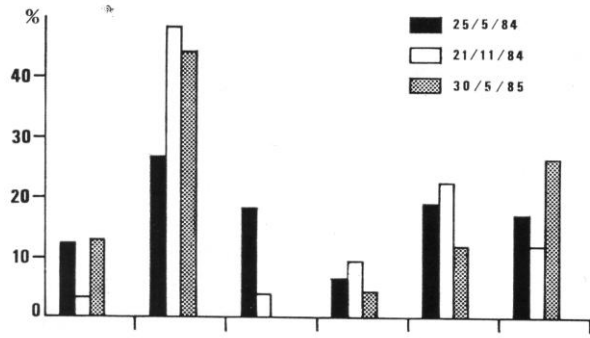
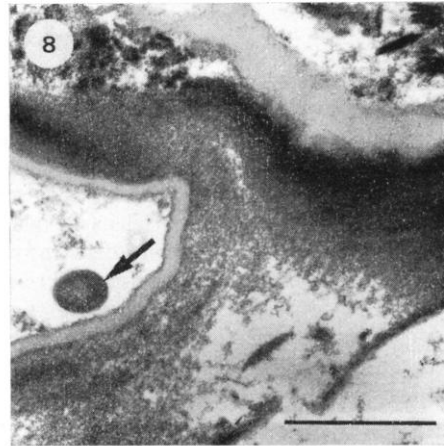
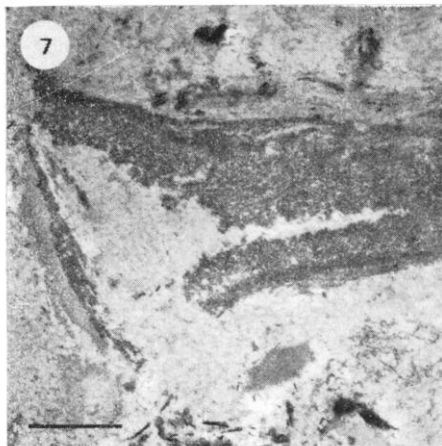
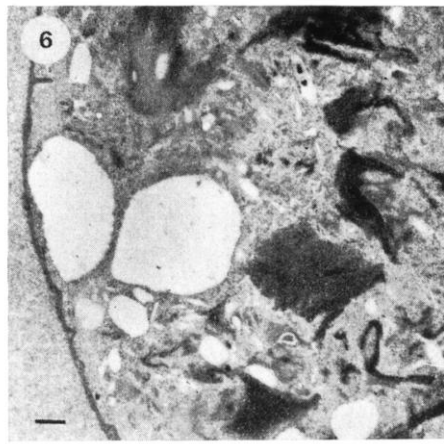
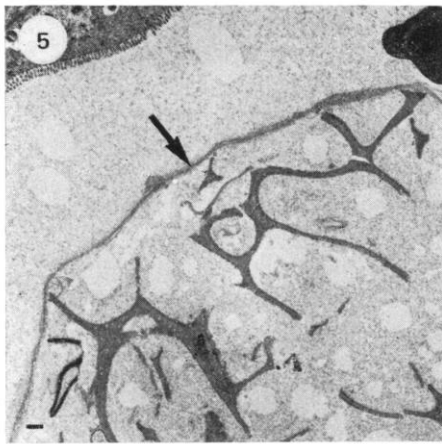
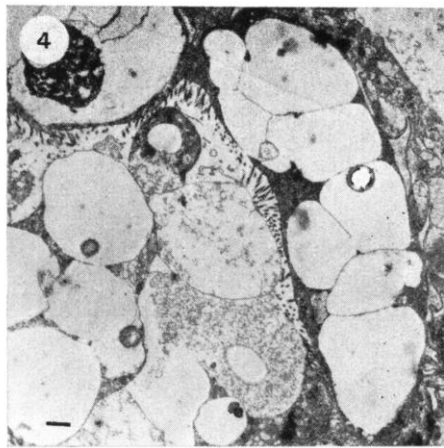
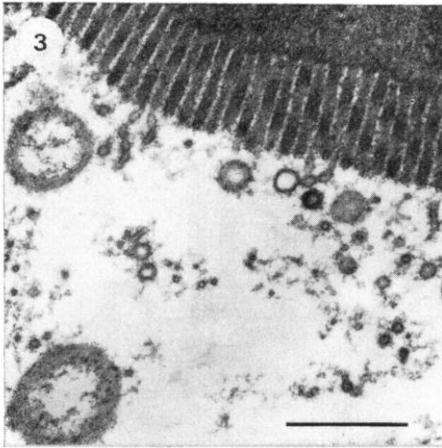
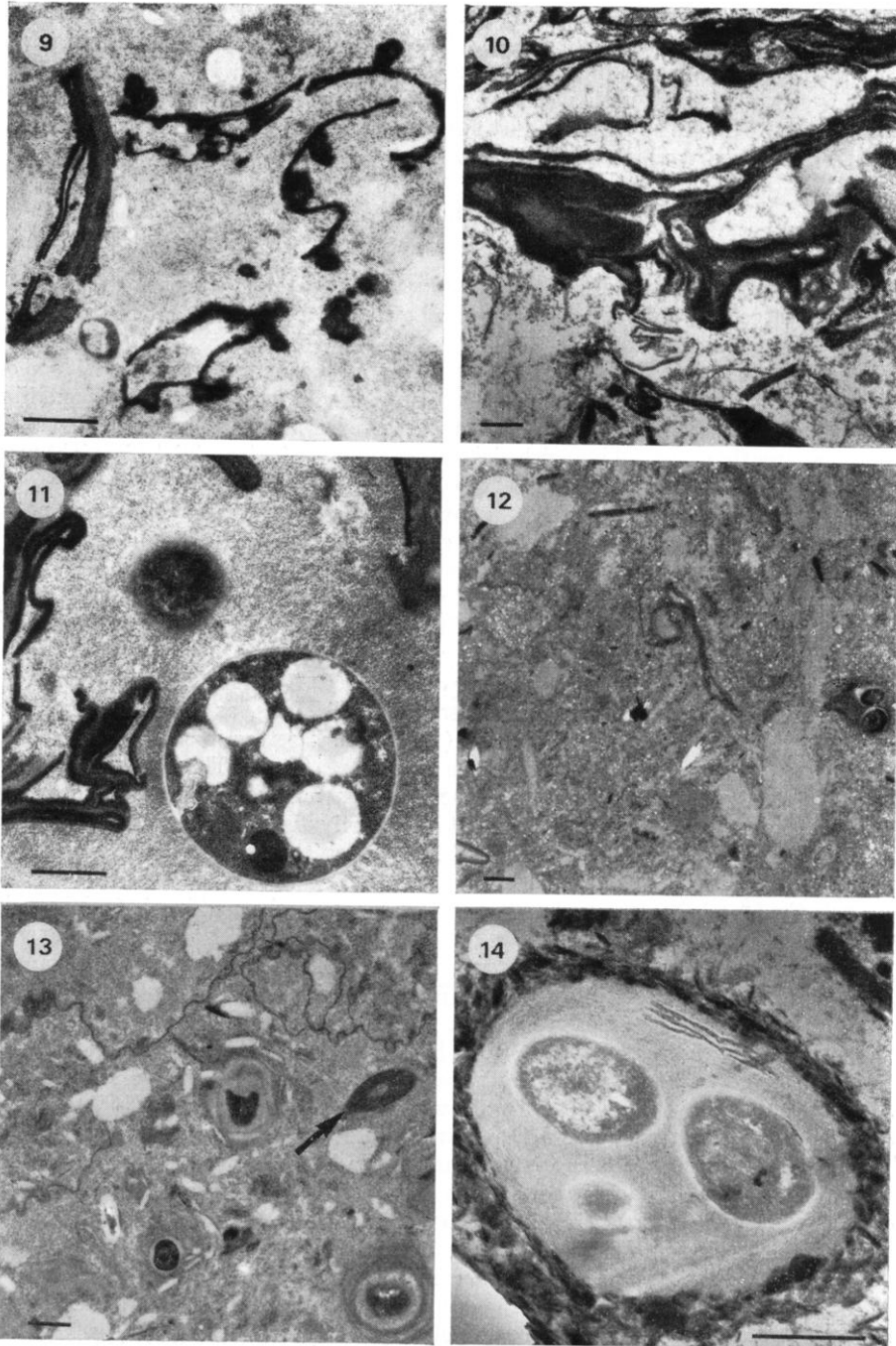


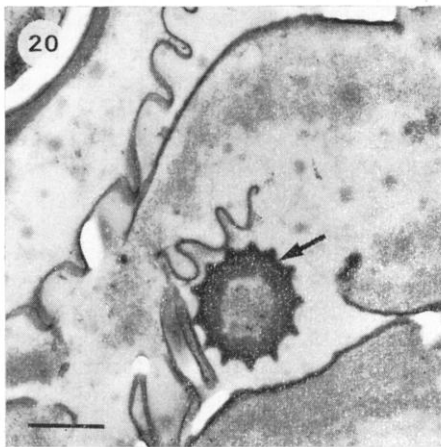
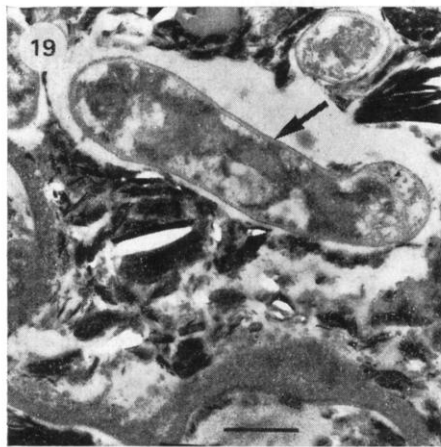
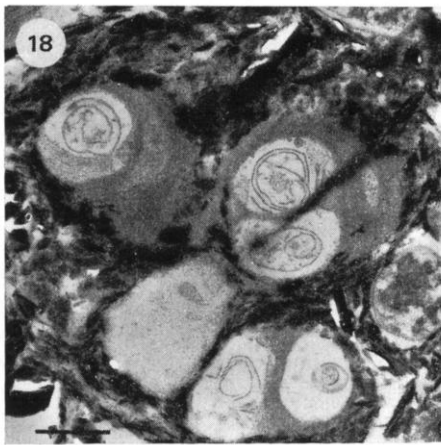
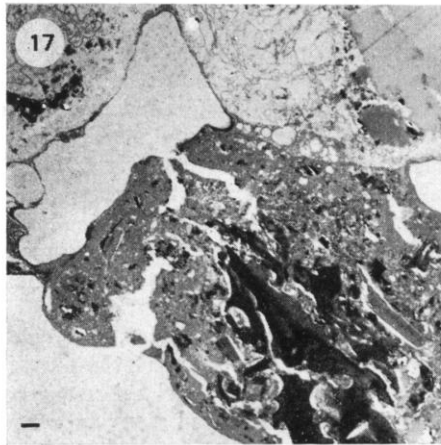
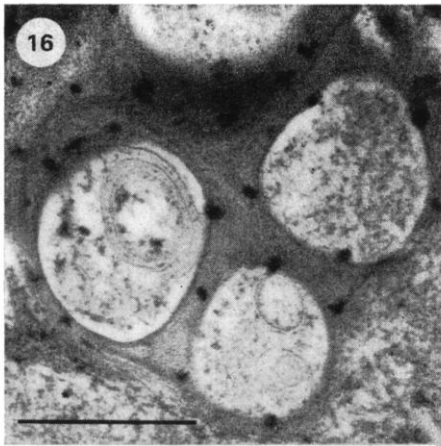
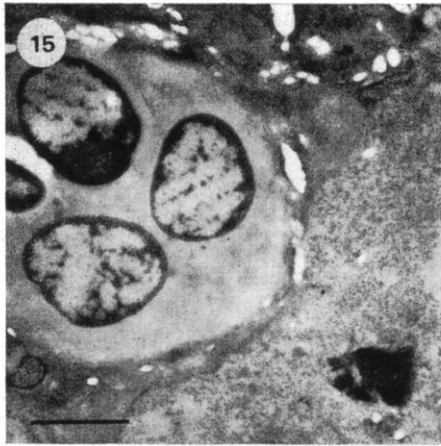
Fig. 2



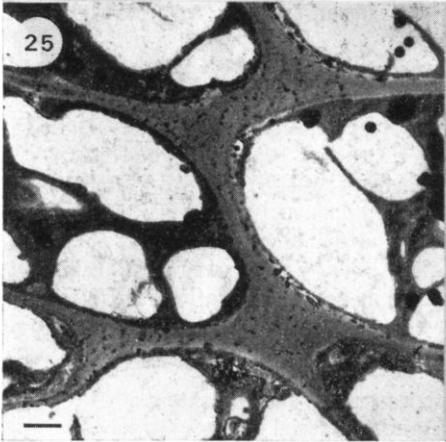
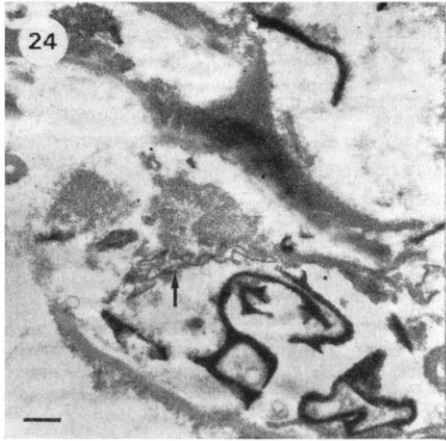
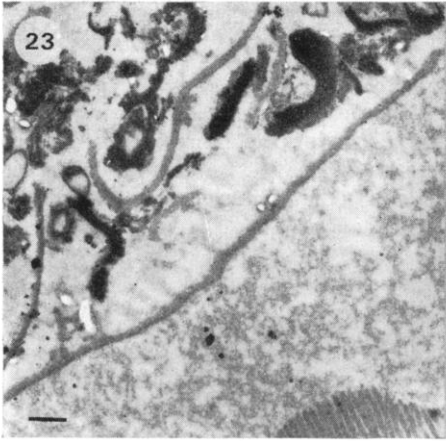
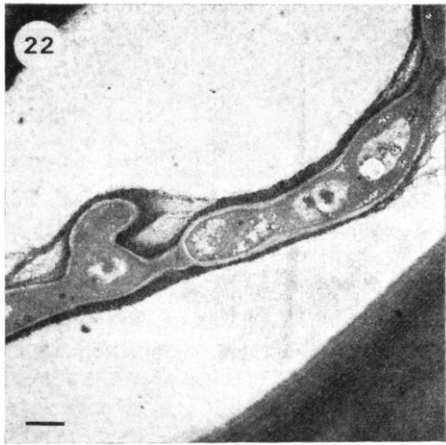
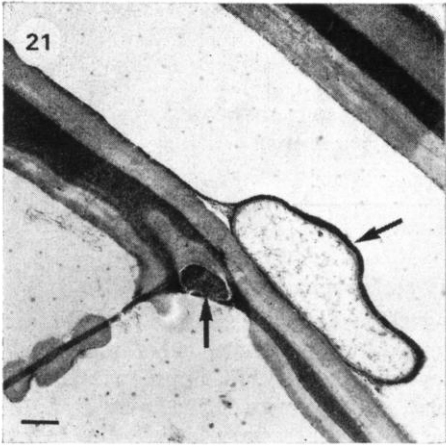
Figs. 3-8



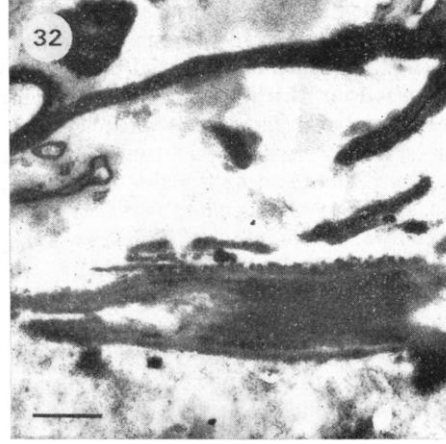
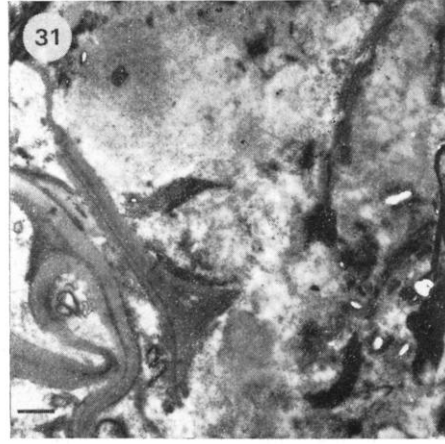
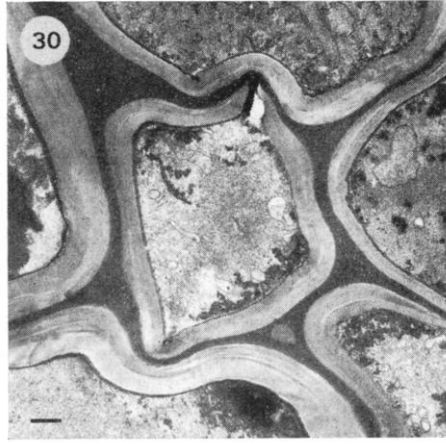
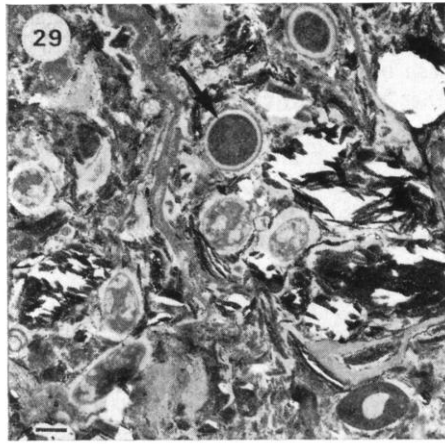
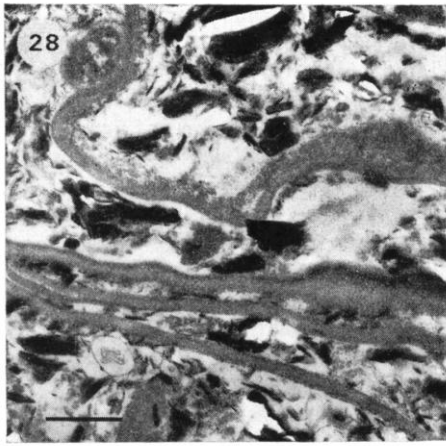
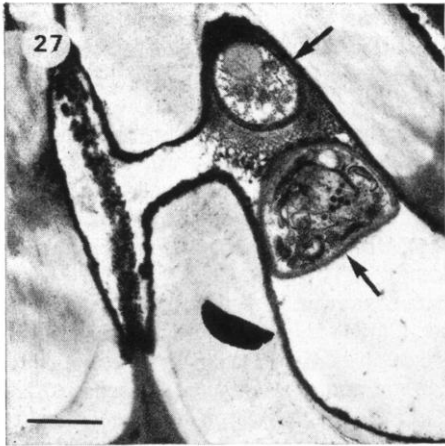
Figs. 9-14



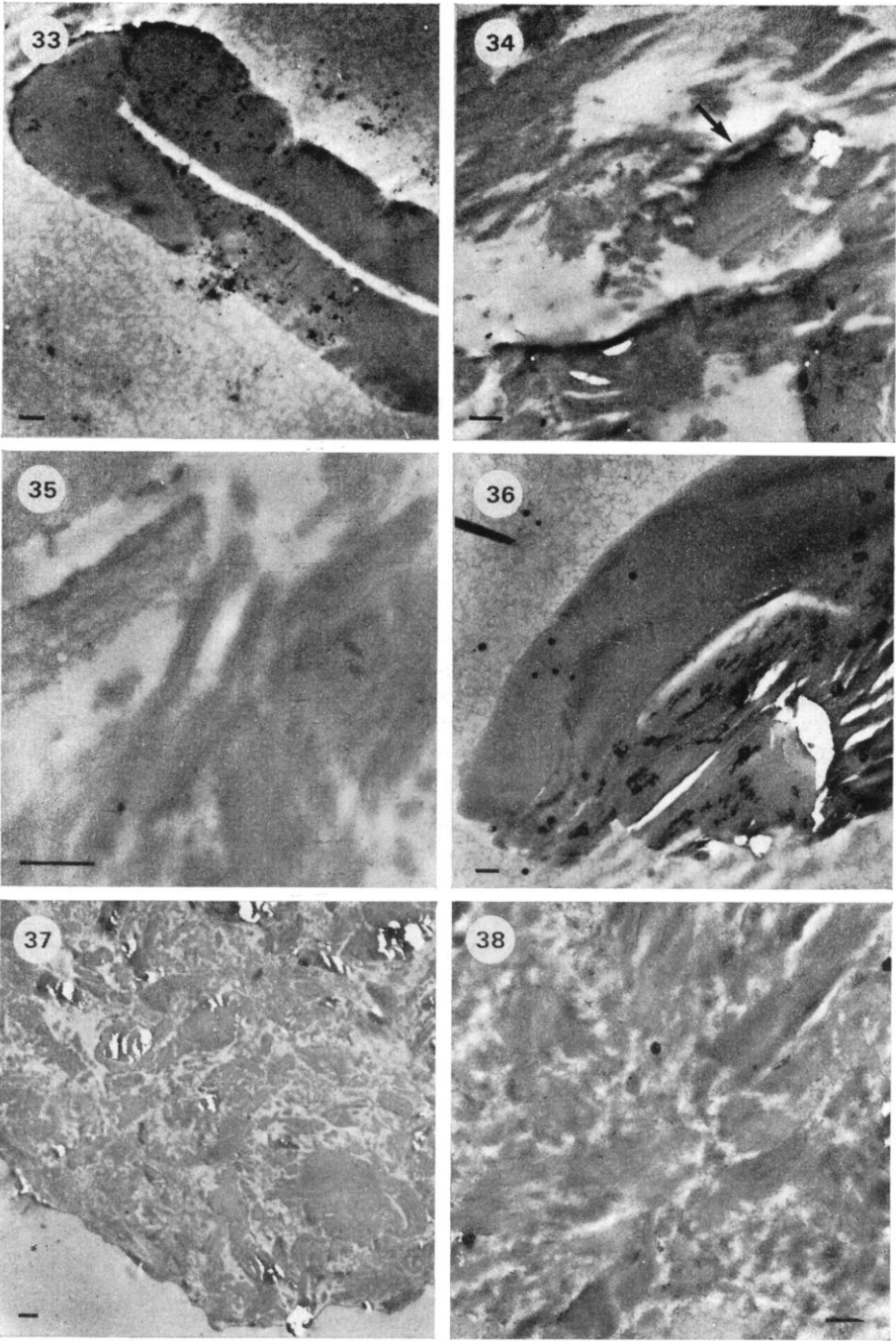
Figs. 15-20



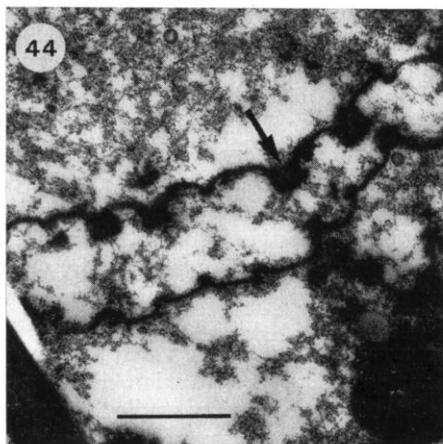
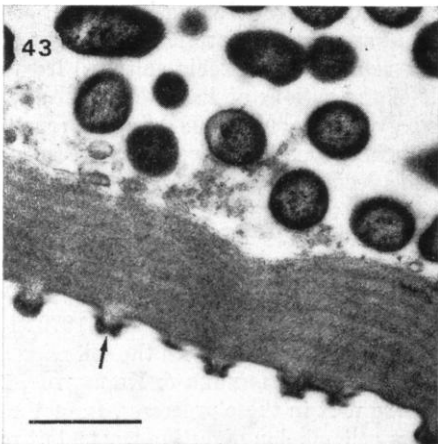
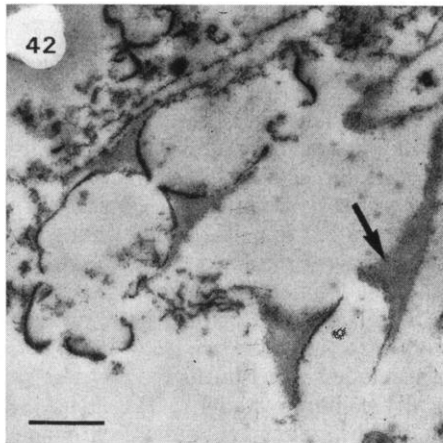
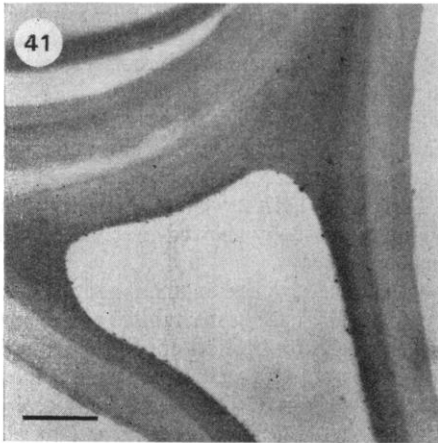
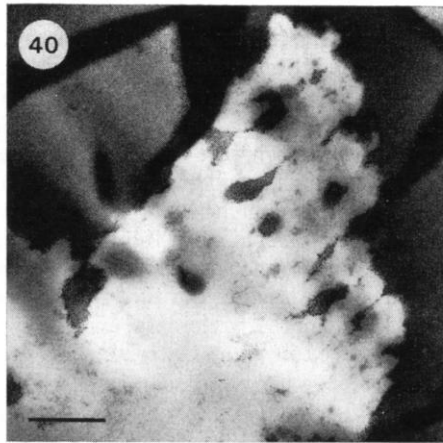
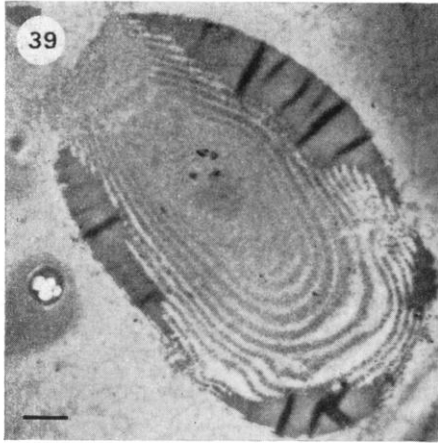
Figs. 21-26



Figs. 27-32



Figs. 33-38



Figs. 39-44