Aust. J. Biol. Sci., 1984, **37**, 99–116

The Macropodid Oesophagus. II.* Morphological Studies of Its Adherent Bacteria using Light and Electron Microscopy

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Abstract

The bacterial population attached to the oesophageal lining in herbivorous macropods is described. This feature which occurs in both browsing and grazing macropodid species (*Macropus*, *Setonix*, *Thylogale* and *Wallabia*) is demonstrated by light and electron microscopy. These studies have shown that the oesophagus may support a large and diverse population of bacterial forms, many of which have extracellular fibrous coats or homogeneous capsules. These coats and capsules appear to mediate the attachment of bacteria to the surface epithelium and to each other so that microcolonies or communities are formed. Such aggregations of bacteria are responsible for the greenish coloration of the oesophageal lining of these herbivorous species. Further examination of the bacterial association with oesophageal cells demonstrated that the bacteria did not invade the mucosa but merely colonized the superficial layer of cells. Cells with ruptured cell membranes were invaded by bacteria which appeared to digest the cytoplasmic contents. Sloughing of such cells provided a new surface for bacterial attachment and proliferation. The importance of this bacterial association is discussed in the light of the anatomical studies of the macropodid oesophagus and the known digestive processes of herbivorous macropods. It is proposed that this adaptation may have an important bearing on our knowledge of foregut fermentative digestion in macropods.

Introduction

The morphology of the oesophageal lining in macropodid marsupials has been reported (Obendorf 1984). One of the consistent features of the oesophagus in several herbivorous species is the presence of large epithelial folds, laminations or papillated projections which support an adherent bacterial population. In this paper some observations of these bacteria are presented using light, scanning and transmission electron microscopy. The role of these bacteria is discussed with regard to the digestive processes of these marsupials.

Comparisons are also drawn with similar bacterial populations which attach to the non-glandular linings in the digestive tract of other animals.

Materials and Methods

Animals

All the macropods were collected as described by Obendorf (1984). Each macropodid species was collected from natural free-ranging habitats. The oesophagus of 13 species of macropod (*Macropus agilis*, *M. eugenii*, *M. fuliginosus*, *M. giganteus*, *M. parma*, *M. parryi*, *M. robustus*, *M. rufogriseus*, *M. rufus*, *Petrogale inorta*, *Setonix brachyurus*, *Wallabia bicolor* and *Thylogale billardierii*) were examined with the use of both light and electron microscopes.

*Part I, *Aust. J. Zool.*, 1984, **32**, 415–35.

0004-9417/84/010099$02.00

General Preparation of Oesophageal Tissues

As soon as possible after death, the oesophagus was removed, cut open longitudinally and washed in either 0·9% (w/v) sodium chloride or 0·1 M phosphate buffer. Representative samples of cervical, proximal, middle and distal thoracic, and abdominal oesophagus were removed and prepared for examination by electron microscopy. The remainder of the oesophagus was fixed in cold 10% formol-saline or 10% phosphate-buffered formalin; each fixative was prepared from 4% glutaraldehyde (v/v).

Preparation of Oesophageal Tissues for Light Microscopy

Blocks of oesophageal tissue fixed in formalin fixatives were cut from the five oesophageal regions described above. The tissues were dehydrated through graded changes of ethanol and embedded in paraffin wax. Sections were cut at 5 μm and stained with haematoxylin and eosin (Harris method) (Thompson and Hunt 1966), periodic acid–Schiff (PAS) (Hotchkiss method) (Pearse 1973), Giemsa (May–Grunwald method) (Thompson and Hunt 1966) and Gram (Brown and Hopps 1973) stains. All tissues were examined with a light microscope.

Histochemistry Staining of Ultra-thin Sections for Light Microscopy

Oesophageal tissues from *M. giganteus* were embedded in water-soluble Araldite after dehydration with ethanol. Sections 1–2 μm in thickness were cut on an ultramicrotome using glass knives and mounted on clean glass slides. The slides were then immersed for 2 h in a supersaturated solution of sodium hydroxide in 100% ethanol which had been allowed to stand for 2 days prior to use. This procedure was undertaken to remove the Araldite according to the method of Lane and Europa (1965). Sections were subsequently stained with PAS, Alcian blue, Gram, combined PAS–Alcian blue and PAS–colloidal iron stains using the methods of Munger (1961) and Pearse (1973). Ultra-thin sections cut from blocks embedded in water-insoluble Araldite were also stained by the method of Joen (1965).

Preparation of Oesophageal Tissues for Transmission Electron Microscopy

Selected tissues of *Macropus agilis*, *M. eugenii*, *M. giganteus*, *M. parma*, *M. parryi*, *M. rufogriseus* and *Wallabia bicolor* were pretreated in 0·05% (w/v) ruthenium red in 0·1 M cacodylate buffer (pH 7·2) for 5–10 min and then placed in a solution containing 0·05% (w/v) ruthenium red and 2·5% (v/v) glutaraldehyde in 0·1 M cacodylate buffer overnight at room temperature. The tissues were then replaced in fresh 0·05% ruthenium red in 0·1 M cacodylate buffer for storage at 4°C. After washing three times in cacodylate buffer, for 1 h each, tissues were post-fixed in an aqueous solution of 1% (w/v) osmium tetroxide for 1 h. Tissues were then dehydrated through steps of 30, 50, 70, 90 and two changes of 100% ethanol and subsequently embedded in water-insoluble Araldite. Paired tissues not stained with ruthenium red were also processed at the same time. Gold and silver sections were cut on glass knives using an ultramicrotome. Sections were mounted on 200- and 400-mesh grids, stained with uranyl acetate and lead citrate and examined with a Philips EM 300 transmission electron microscope (TEM) operating at an acceleration voltage of 60 kV. Several ruthenium red and unstained control tissues were also dehydrated with dimethoxypropane according to the method of Muller and Jacks (1975).

Preparation of Oesophageal Tissues for Scanning Electron Microscopy

Selected tissues from all 13 species of macropod were fixed in 2·5% (v/v) gluteraldehyde in 0·1 M cacodylate buffer (pH 7·2) or a mixture of 1% (v/v) gluteraldehyde and 4% (v/v) formaldehyde on 0·1 M phosphate buffer (pH 7·2) at room temperature. After washing twice in buffer for 1 h each and then once in distilled water for 1 h, tissues were finally post-fixed in an aqueous solution of 1% (w/v) osmium tetroxide. The tissues were then processed by two methods. Some tissues were treated with thiocarbohydrazine and osmium tetroxide by the method of Malick and Wilson (1975). They were then dehydrated through 30, 50, 70, 90 and two changes of 100% ethandiol followed by two changes of the transition fluid, absolute ethoxyethanol, critical-point dried using liquid CO_2 and examined with an ETEC Autoscan electron microscope operating at an accelerating potential of 10–30 kV. Other tissues were also dehydrated through ethandiol and ethoxyethanol, critical-point dried and coated with gold using an evaporative metal sputter coating technique. A selected group of tissues were dehydrated using dimethoxypropane according to the method of Muller and Jacks (1975). These tissues were either critical-point dried or placed in a dry air oven at 160°C for 1 h. After sputter coating, these tissues were stored in a sealed desiccator until they were examined in the scanning electron microscope (SEM).

Paraffin-embedded oesophageal tissues of *M. giganteus* were sectioned at 2 μm and placed on 0·5 cm^2 pieces of glass coverslip. After removing the paraffin with xylol, the tissues were rehydrated and immersed in a 1% (w/v) aqueous solution of osmium tetroxide for 1·5 h. The tissues were subsequently washed in distilled water and passed through increasing strengths of ethanol. Once the specimens were fully dehydrated, they were subjected to critical-point drying and then coated with gold using the sputter coating technique. These specimens were viewed in the SEM operating at 20 kV.

Preparation of Oesophageal Smears

Air-dried smears were made of scrapings from the middle thoracic portion of the oesophagus from 6 *M. giganteus*, 4 *M. rufogriseus*, 2 *M. robustus*, 6 *M. rufus*, 4 *W. bicolor* and 4 *T. billardierii*. The smears were stained with Gram stain using the Kopeloff method (Holdeman *et al.* 1977) and examined under oil using a ×100 objective. Some qualitative assessments of the relative proportions of the different morphotypes present were made in each smear. In some instances, a distinction was made between the numerical proportion constituted by a single type of bacteria and the relative dimensional proportion of the total bacteria made up by a particular organism. All organisms were classified on the basis of morphology and Gram-stain reaction.

Results

Light Microscopic Findings of Oesophageal Sections

The surface of the stratified squamous epithelium lining the oesophagus of all herbivorous macropods was covered with a dense layer, or plaque of mixed bacteria. These bacteria were closely associated with the superficial layers of epithelium and the desquamating epithelial cells. The thickness of the bacterial plaque appeared to vary between species, between individuals of the same species, and between different sites along the oesophageal lining. In species with type I and type II oesophageal linings the bacterial layer was found over the whole epithelial surface (Obendorf 1984). In most circumstances, the plaque of bacteria was uniform in colour. In species with type III and type IV linings, the bacteria were found predominantly on the surfaces of the papillar and laminar projections. The distribution of the bacteria was related to those parts of the epithelium which had a thick cornified epithelium. Consequently, the bacteria formed wide plaques on the surfaces of papillae where wide layers of cornified cells were present (Fig. 1). The epithelial linings between projections usually contained a thin layer of keratinized cells and maintained a thin bacterial plaque. The bacterial plaque was responsible for considerable variations in the shape and size of individual projections. This was particularly common in type III oesophageal linings, in which, the upper two-thirds of the papillae contained most of the adherent plaque. Occasionally partially sloughed epithelial cells interlaced with bacteria were responsible for the formation of excessively thickened papillae. Some branching Gram-positive filaments were seen intermingled with the layers of keratinized cells in *M. eugenii*. These bacteria were also responsible for distortion of the apical parts of numerous papillae.

In all macropods examined the bacterial plaque was restricted to the surface layers of epithelial cells. Partially sloughed cells showed colonization of all exposed epithelial surfaces. Numerous epithelial cells which remained attached to the epithelium appeared to lose their flattened shape and the compact appearance of their cytoplasm. These cells appeared swollen and contained bacteria interspersed with fragments of cytoplasm. A process of bacterial proliferation on the surface epithelial cells with subsequent desquamation of colonized cells and recolonization of exposed epithelia was observed in all macropodid species with oesophageal plaques.

In sections stained with Gram the bacterial layer was composed of various types of Gram-negative rods and fusiform organisms arranged in short chains and compact pallisades, and Gram-positive cocci, coccobacilli and occasional rods arranged in clusters and chains. Bacteria were aggregated in discrete microcommunities and microcolonies

containing similar types of organisms. In several macropodid species (*M. dorsalis*, *M. fuliginosus*, *M. giganteus*, *M. rufus*, *W. bicolor* and *T. billardierii*) large polyp-like aggregations of Gram-negative rods were seen attached to the oesophageal epithelium. These polyps, which were attached to the epithelium at one end, extended out into the oesophageal lumen as elongated colonies. The rod-like bacteria contained within those colonies were embedded in a matrix material with numerous organisms of various forms clustered about the periphery of the colony. This matrix material stained positively with PAS. Gram-positive cocci and coccobacilli were distributed randomly along the surface of the epithelium. In some species of macropod relatively few Gram-positive bacteria were seen in sections of oesophagus. Macropods with type III and IV linings had bacterial plaques composed almost entirely of Gram-negative bacteria. However, small clusters of Gram-positive cocci were seen on the epithelium at the base of the papillae and laminar plates. Gram-negative fusiforms and rods were encountered in the oesophagus of all macropods. These bacteria were usually oriented such that their long axis was at right angles to the surface epithelium. Closely packed arrays of these organisms produced a bacterial plaque up to 200 μm thick. Long chains of Gram-variable filaments were seen in *M. eugenii*, *M. fuliginosus*, *M. giganteus*, *M. parma*, *M. robustus*, *M. rufus*, *T. billardierii* and *W. bicolor*. These organisms were commonly found intermingled with Gram-positive cocci, randomly dispersed on the oesophageal linings in *M. fuliginosus*, *M. giganteus*, *M. robustus*, *M. rufus* and *T. billardierii*. In *M. eugenii*, *M. parma* and *W. bicolor* (species with a type III papillated oesophageal lining) the filaments were located in large numbers at the base of papillae and on the epithelium between papillae. Filaments in excess of 50 μm, having a portion of the filament attached to the oesophageal epithelium, were seen extending out into the oesophageal lumen. In many instances, the filaments appeared to attach to the epithelium by one end, its long chain coiling through the adjacent bacterial plaque.

Some preliminary observations of the histochemical staining qualities of certain oesophageal plaques containing a diversity of bacterial forms, have confirmed variations in the staining reaction of different microcolonies of organisms. Certain aggregations containing only one recognizable form stained intensely with PAS, Alcian blue and colloidal iron. Mucopolysaccaride stains, used separately and in combination, confirmed that some colonies stained with both PAS and Alcian blue whilst others stained with both PAS and colloidal iron. For paraffin-embedded sections cut at 5 μm it was sometimes difficult to establish whether the bacteria themselves or the spaces between the bacteria were responsible for the observed staining properties.

Light Microscopic Observations of Oesophageal Smears

Several distinct bacterial forms were seen in smears of the oesophageal surface stained with Gram. Gram-negative bacteria were the predominant organisms encountered in all macropods. Smears from the oesophagus of *M. rufus* contained about 40% Gram-positive bacteria as compared with an average of less than 10% for all other macropodid species examined. Gram-negative cocci and coccobacilli were numerically the most populous in all the macropods; however, Gram-negative fusiforms and rods, due to their dimensions, appeared to constitute the greatest mass of microbes present. Fusiforms ranging from 5 to 15 μm in length, with tapering ends and vacuolated cytoplasm, were commonly seen in all macropods while long fusiforms, often in excess of 40 μm, were commonly encountered in oesophageal smears of *T. billardierii* and *W. bicolor*. Gram-negative rods were occasionally seen as individual cells, but were more commonly seen in pallisades and colonies of clumped organisms resembling polyps. In both arrangements, these bacteria were orientated with their long axes parallel. Gram-positive rods were rarely seen in any of the samples. Gram-positive cocci usually in pairs, chains of 3–10 organisms and clusters, were seen in all samples, but were only considered a significant component of the oesophageal bacteria in *M. rufus* and *T. billardierii*. The long Gram-variable filaments

seen in several species of macropod were approximately 2 μm in diameter and of variable length. Coiled filaments in excess of 100 μm were seen. Phase-contrast microscopy showed that they were composed of compact coccoid elements, which in some regions of filaments were so flattened as to form a transversely striated pattern on their surfaces. In most oesophageal smears examined, the filaments made up a small proportion of the bacteria, but in *M. rufus* they constituted a significant proportion of the stained microbes.

Several different sizes of Gram-negative, comma-shaped organisms ranging from 0·5 to 2 μm in length were also seen. These *Vibrio*-like organisms were present in large numbers, usually as single organisms, but occasionally in pairs and compact clusters. In *M. rufus* and *M. robustus* they represented the second most populous group of bacteria after the Gram-negative cocci and coccobacilli. Other organisms only occasionally seen in smears were spirochaetes, filiform organisms and branching rod forms.

Budding blastospores of yeasts were rarely seen in any adult free-ranging macropods. However, clusters of budding septate hyphae were seen attached to desquamated epithelial cells of *W. bicolor* and *T. billardierii*. Large Gram-negative coccobacilli reminiscent of Quin's ovals were recorded in small numbers from oesophageal smears of *M. rufus*.

Aggregations of bacteria into discrete elongated polyps were seen in the oesophageal smears of all macropodid species. These structures were up to 120 μm in length and had a width of 30–50 μm. Several bacterial forms were clustered about their periphery; however, the main body of the polyp was composed of Gram-negative rod forms in parallel rows. Gram-negative cocci, coccobacilli and comma-shaped organisms were frequently clustered about the long striated filaments. Gram-positive cocci in chains and clusters were commonly interspersed between pallisades of Gram-negative fusiforms and rods.

Scanning Electron Microscopic Findings

These studies confirmed the presence of a dense coating of bacteria overlying the oesophageal lining of all the free-ranging herbivorous macropods which were examined. In most instances the closely packed arrangement and thickness of this layer of bacteria prevented any observation of the surface epithelium. In several specimens, only the exposed lumenal extremities of vast carpets of bacteria were seen. The appearance of the complete depth of the bacterial plaque was only apparent in those specimens where adjacent portions of plaque had been removed. Cocci, coccobacilli, fusiform rods, wavy rods, comma-shaped organisms, and long filaments made up of coccoid elements were all recognized as members of the bacterial plaque. Spirochaetes and long filamentous organisms were occasionally found amongst the main types of bacteria. The close associations between the bacteria were frequently mediated by delicate strands or fibres (Fig. 2). The fibres usually took the form of strands radiating from the surface of bacteria, but in certain cases, a fine meshwork of fibres was seen enveloping bacteria (Fig. 3). Some distinct filaments which often branched were also seen in large meshworks of overlying bacteria. On many occasions no visible union between adjacent bacteria was observed despite close and well-ordered associations. Large aggregations of bacteria resembling the polyp-like bacterial colonies described in light microscopic studies were commonly seen emerging from the bacterial plaque (Fig. 4). The surface of these structures was frequently covered with various types of bacteria; however, some rod-like organisms could be seen embedded in the internal parts of the polyps (Fig. 4). The long filaments of coccoid organisms were found on the epithelial surface of many oesophageal specimens. Circumferential bands were very prominent on the surface of the individual coccoid elements. On some bacterial filaments the position of these bands was replaced by wider zones of roughened material (Fig. 5). Coccoid and coccobacillary organisms were frequently attached to the filaments. In the oesophageal specimens of *Macropus parma* long filaments of compact elements were located at the bases of the oesophageal papillae. These bacterial filaments appeared uniformly striated with tuft-like extensions which were positioned evenly in a single row along one side of the filament.

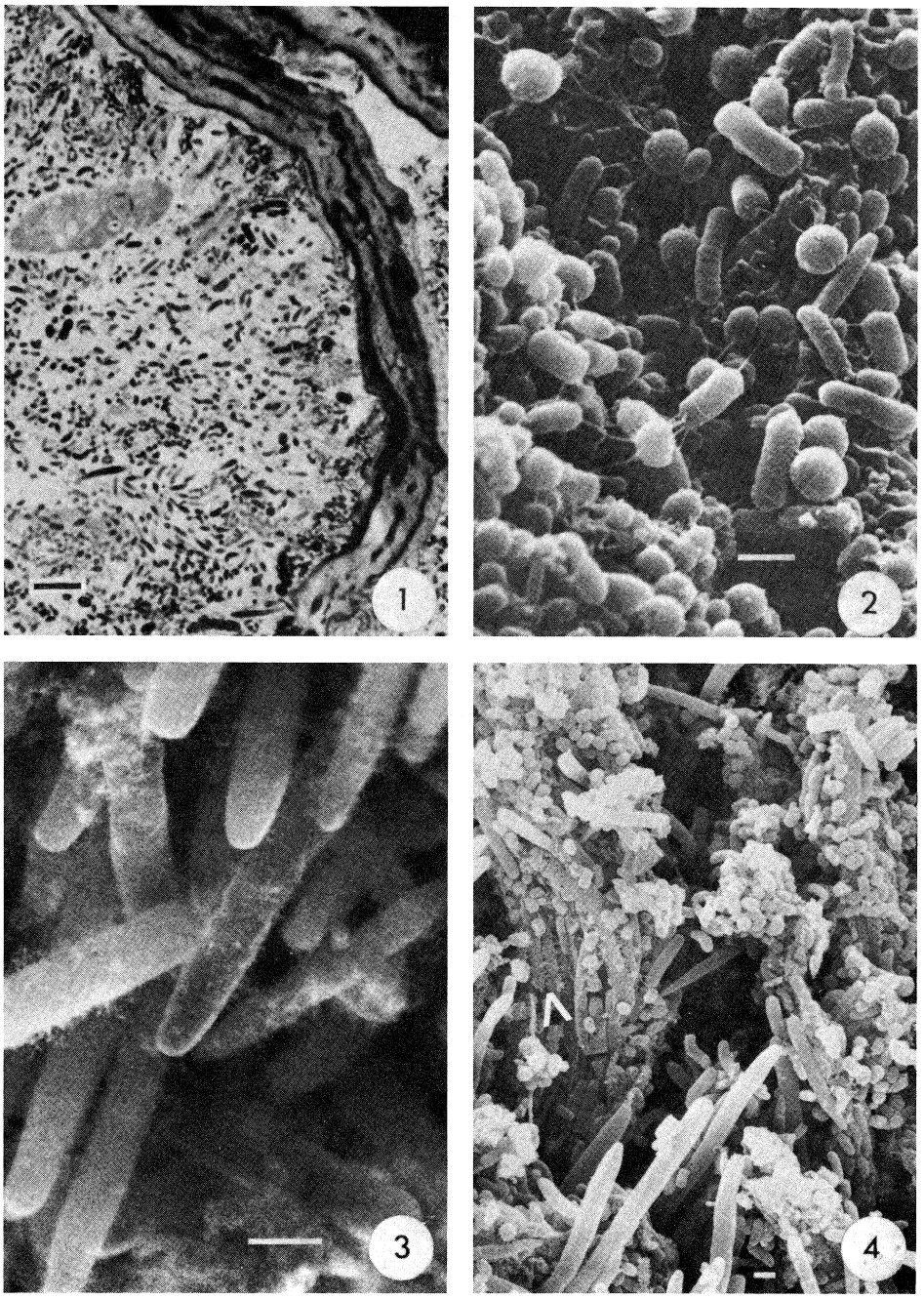


Fig. 1. Transverse section through the outer keratinized layers and the attached bacterial populations in the oesophagus of *Megaleia rufa*. A group of epithelial cells is seen desquamating from the epithelial surface with bacteria attached primarily to its lumenal cell membrane (Joen stain, LM). Scale line in Fig. 1 = 5 μm, in Figs 2–4 = 1 μm.

Fig. 2. Coccoid and coccobacillary organisms attached to adjacent bacteria and to the epithelial cell surface by fine strands. *Macropus parma*, SEM.

In many instances these well-ordered tufts formed attachments with the epithelial cells and other bacteria.

In those oesophageal specimens with thick bacterial plaques, the depth of this layer coupled with certain technical limitations of the SEM materials and methods used, prevented any observations of the association between the bacteria at the bottom of the plaque and the oesophageal lining. The nature of this association could only be investigated in those regions where several epithelial cells had sloughed simultaneously, thereby exposing a new epithelial surface for bacterial colonization. Certain oesophageal specimens from species with type I and II linings were used in these investigations because they maintained smaller populations of bacteria than would normally be expected. In addition, macropodid species with type III oesophageal linings were also found to have much thinner populations of bacteria at the base of the oesophageal papillae than on their sides. Attachment fibres were seen linking bacteria to the cell membrane of epithelial cells. Bacteria attached not only to the lumenal surface of cells, but also to the undersurface of cells which were lifting off the epithelium. Cells partially attached to the epithelium were seen with bacteria apposed to all the exposed epithelial surfaces. Although bacteria were frequently found in close contact with the surfaces of epithelial cells, alteration in the characteristic finely corrugated appearance of the cell membrane as a result of bacterial attachment was rarely observed. Some coccoid and diplococcoid organisms were noted within deep indentations on the cell membranes of oesophageal cells in *Petrogale purpureicollis* (Fig. 6). This association between these bacteria and the epithelial cells appeared to be mediated by a rough granular coating. Epithelial cells with ruptured cell membranes also contained similar bacteria embedded in the cell cytoplasm.

Although some epithelial cells showed evidence of desquamation with intact cell membranes, many of the cells remaining attached to the epithelium had ruptured and torn cell membranes (Fig. 7). Although no evidence of bacteria-induced damage of the cell membranes could be found, bacteria were commonly seen intermingled with the contents of these cells (Fig. 8). Fusiform rods were seen underrunning the cell membrane of ruptured cells, whilst numerous bacterial forms were seen closely associated with the cytoplasmic content of cells. Small pits and depressions relating to the outline of embedded bacteria were seen in the compact roughened material which characterized the internal content of keratinized epithelial cells. Flaps and scrolls of cell membranes lifting up from the cytoplasmic content of damaged cells were seen. Bacteria were located on both the outer and inner surfaces of cell membranes. Sheets of underlying cell membrane, from ruptured cells with the majority of their cytoplasmic content removed, were seen attached to the surface of the epithelial cells beneath.

Oesophageal samples from the same specimen were processed by several methods. Dehydration through ethanol, ethoxyethanol or dimethoxypropane caused no appreciable difference in the appearance of the specimens. Evaporative metal coating was also compared with the method of Malick and Wilson (1975) using thiocarbohydrazine. Although the latter method improved the definition of bacteria and their extracellular fibres, contrast was inferior to the gold-coated specimens.

Paraffin-embedded sections of oesophageal tissue were also processed and viewed with the aid of the SEM. This technique permitted additional observation of the bacterial plaque in transverse section. Numerous bacterial types were demonstrated in colonial groups and pallisades of organisms roughly arranged at right angles to the epithelial surface. In many

Fig. 3. Micrograph of the fine meshwork of material associated with the surface of a fusiform organism at the lumenal extremity of the bacterial plaque. *Macropus parryi*, SEM.

Fig. 4. Micrograph of the polyp-like colonies present above the general surface of the bacterial plaque. Coccoid organisms can be seen attached to the surface of these colonies, some rod-like organisms embedded in a matrix material are also visible (arrow). *Macropus dorsalis*, SEM.

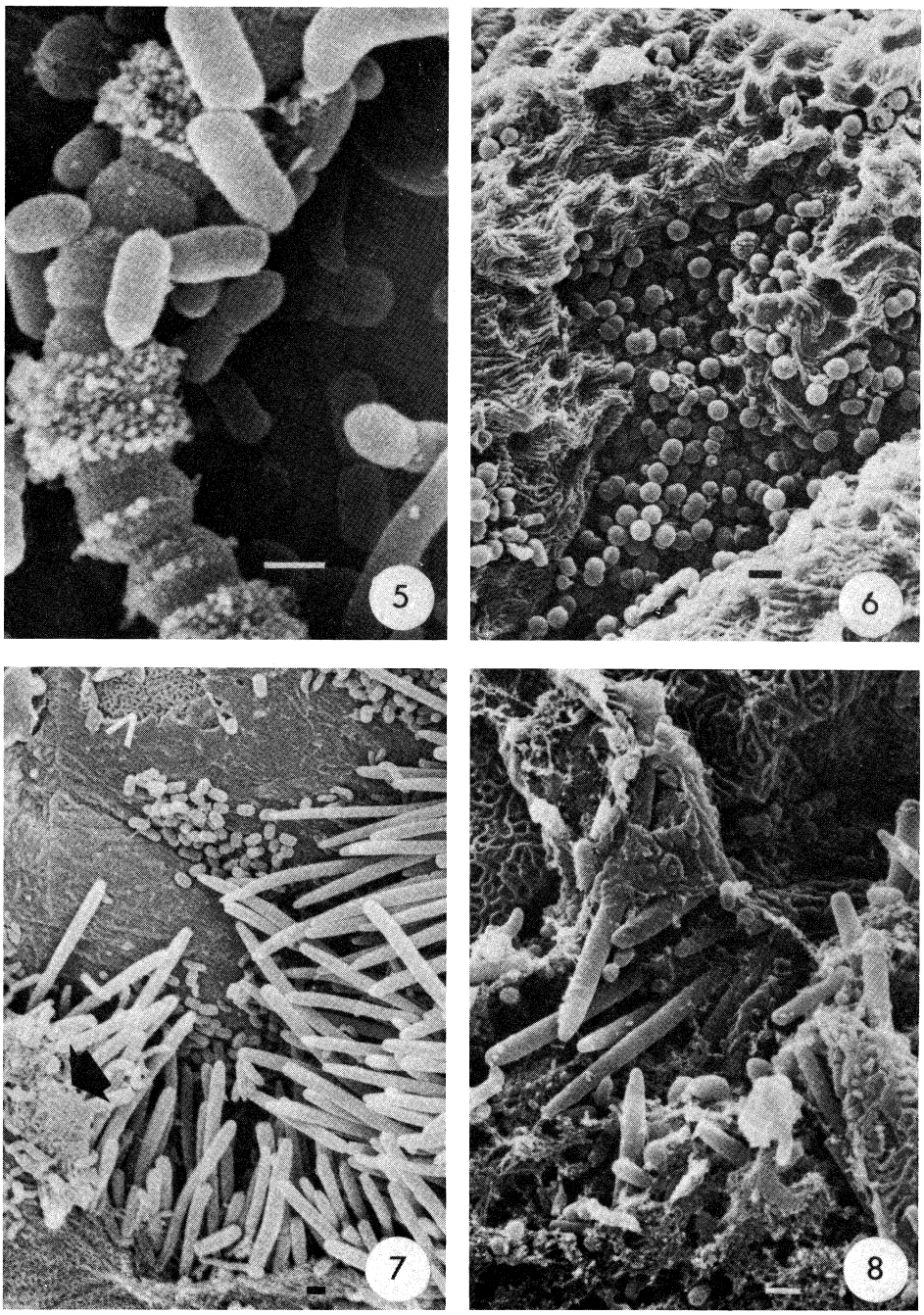


Fig. 5. Filamentous bacteria with thick and thin bands of roughened material around the circumference of individual coccoid elements. *Macropus eugenii*, SEM. Scale line in Figs 5–8 = 1 μm.

Fig. 6. Circular depression formed by coccoid and diplococcoid organism in an intact cell membrane. The arrow illustrates the rough granular material mediating attachment to the cell surface. *Petrogale purpureicollis*, SEM.

cases, the nature of the association between individual bacteria and between bacteria and the epithelial cells was obscured by a smooth coating which covered the bacteria and filled the region of attachment (Fig. 10). Certain radiating strands attaching adjacent bacteria to one another were also seen. Bacteria were both within the cytoplasm of epithelial cells and attached to the exposed surfaces of sloughing epithelial cells (Fig. 9). The matrix within the interior of the polyp-like colonies, described in the light microscopy section, appeared as an extracellular matrix in which rod-shaped bacteria were embedded. Small projections mediating attachment between the filamentous bacterium and neighbouring bacteria were also seen.

Transmission Electron Microscopic Findings

A wide variety of morphologically different bacteria were present in the thick layer of bacterial plaque in the oesophagus. In many regions, distinct meshworks of fibres were located between adjacent bacteria. Aggregations of bacteria with similar cytoplasmic and cell wall morphology were seen in chains and clusters; however, in many instances no fibres mediating attachment were seen. The large aggregates of rod-shaped bacteria embedded within a common matrix material were frequently encountered in ultra-thin sections. These colonies consisted of a central core of bacteria aligned in roughly parallel-sided chains and embedded in a matrix of variable structure and electron density. In some colonies, the bacteria were contained within a loose meshwork of fibres while in others, the matrix was homogeneous and dense. Around the periphery of colonies and occasionally within the colony, other bacteria were found. Some bacteria were seen in small depressions within the more electron-dense outer matrix of the colony. The remaining cell wall of degenerate bacteria were seen in and about this outer matrix. Various envelopes, radiating fibres, meshworks of strands and granular outer coverings were seen on the surfaces of bacteria. In many cases these coatings appeared to mediate interbacterial union. Electron-lucent inclusions or vacuoles with indistinct margins were also recorded in several bacteria making up the bacterial plaque.

The surface epithelial cells were colonized by bacteria morphologically indistinguishable from these found throughout the bacterial plaque. Many of these bacteria were attached to epithelial cells by a fine array of extracellular fibres (Fig. 11). The fibres filled the gap between the bacteria and the epithelial cell. The appearance of the fibres varied according to the type of bacteria attached. Nevertheless, the fibres mediating contact between similar bacteria appeared to be indistinguishable from the fibres mediating contact between bacteria and epithelial cells. Again in certain circumstances, no visible means of attachment between some bacteria and epithelial cells was found.

Bacterial attachment to epithelial cell membranes was not associated with any discernible thinning or distortion in the appearance of these membranes. Although the cells of the outer epithelial layer were generally squamous with compact cytoplasm, numerous cells making up the outermost of the lumenal layers were swollen with open and loose cytoplasm. In many cells, bacteria could also be seen intermingled with the cellular filaments and cytoplasmic components (Fig. 12). Distinct breaks in the continuity of the cell membrane could be seen in some ultra-thin sections, with bacteria of several different types within the cells. Small depressions and pits containing bacteria were seen in the cytoplasmic

Fig. 7. Numerous fusiform bacteria underlying a cell membrane (black arrow). The white arrow illustrates an irregular hole in the underlying cell membrane, with coccobacilli attached to the cytoplasmic surface of the membrane. *Macropus giganteus*, SEM.

Fig. 8. Fusiform rods closely associated with the fibrous cytoplasm of a damaged epithelial cell. Bacteria are seen underrunning a cell membrane and deeply embedded in the cytoplasm. *Setonix brachyurus*, SEM.

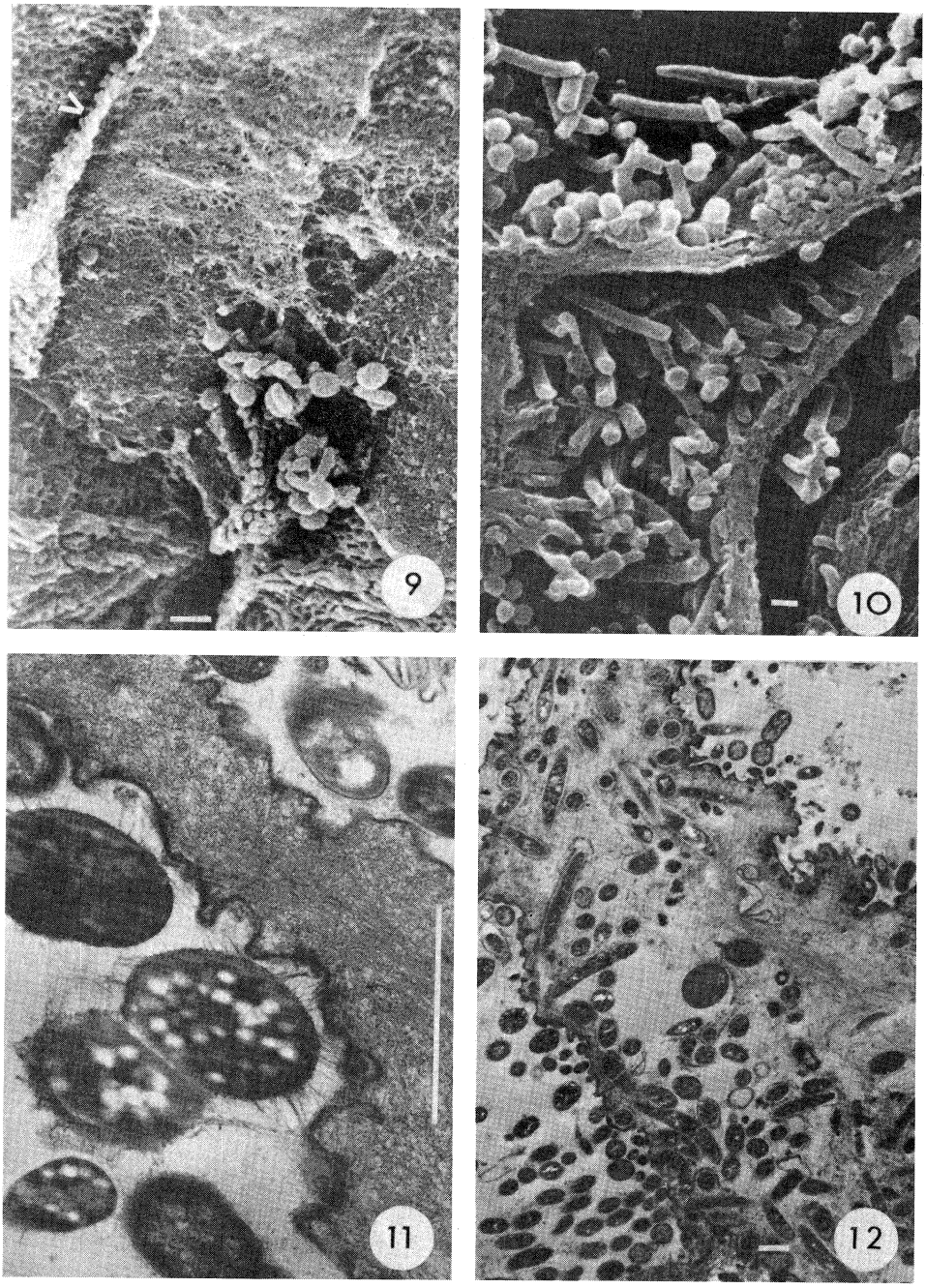
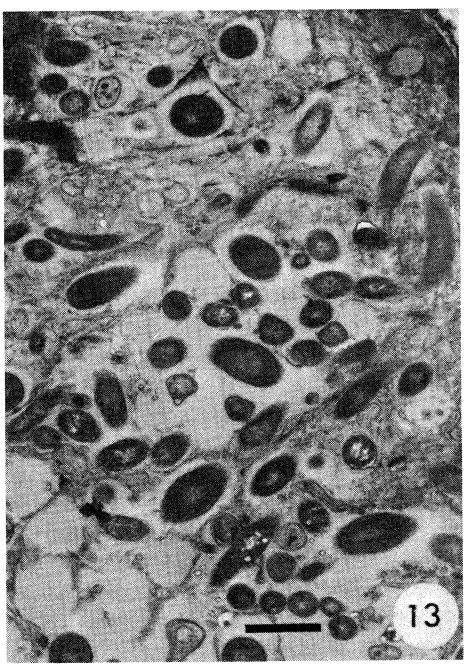


Fig. 9. Section through the superficial desquamating layer showing cells lifting off with evidence of bacterial colonization of the exposed cell surfaces. *Macropus giganteus*, TEM. Scale line in Figs 9–12 = 1 μm.

Fig. 10. Micrograph of an oesophageal epithelial cell with numerous bacteria attached to the exposed cytoplasmic content by strands. The underlying cell membrane of the cell (arrow) is also visible. *Macropus giganteus*, SEM.

material of the breached cells (Fig. 13). The remnant cell membranes of cells invaded by bacteria appeared to remain recognizable and with their characteristic irregularly undulating appearance despite the loss of cellular content or dissociation with other cells (Fig. 14). Empty cells containing large numbers of bacteria were occasionally seen still attached to the undamaged epithelial cells. Remnants of cell membranes were commonly seen within the plaque of bacteria, with bacteria associated with either their internal or external surfaces. Many cell membranes remained attached to either the cell membrane of an intact cell or the membrane of a cell which had also undergone a similar fate. Some cells appeared to be sloughing from the epithelium as flattened squames containing a thick layer of bacteria attached only to their outer epithelial surfaces.



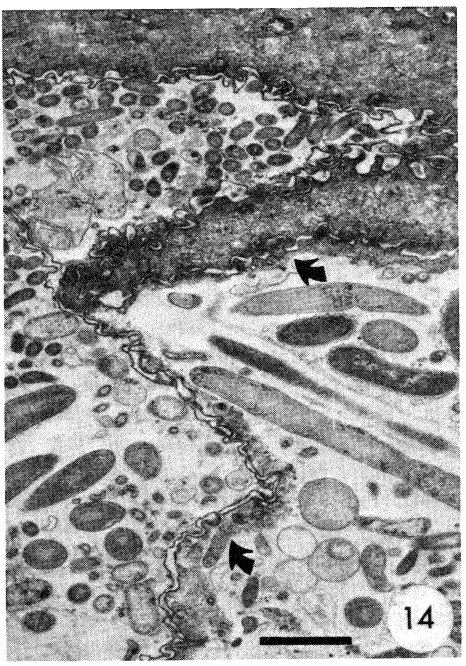


Fig. 13. Numerous types of bacteria contained within pockets or holes in the fibrous content of ruptured epithelial cells; some vacant cavities are also present. *Macropus giganteus*, TEM.

Fig. 14. Micrograph showing the cell membrane or lamina outlines of three epithelial cells containing numerous bacteria. Cytoplasmic components of these cells are restricted to the region under the cell membrane (arrow). These cells maintain contact with one another and to an intact epithelial cell. *Macropus giganteus*, TEM. Scale lines in Figs 12 and 13 = 1 μm.

Tissues stained with ruthenium red appeared similar to unstained control tissues. The definition of the fibres mediating epithelial cell to bacteria and bacteria to bacteria attachment were not enhanced by this staining method. The cell membranes of squamous epithelial cells also failed to stain with ruthenium red. The appearance of all aspects of bacterial associations with epithelial cells was similar in ultra-thin sections dehydrated through ethanol and dimethoxypropane.

Fig. 11. Radiating fibres attaching two electron dense organisms to the cell membrane, no alteration in the shape of the cell membrane has occurred. *Macropus agilis*, TEM.

Fig. 12. Partially sloughed epithelial cell showing bacteria of various shapes and sizes within its open fibrous cytoplasm. Numerous bacteria are also aggregated around the intact cell membrane of this cell. *Macropus agilis*, TEM.

Discussion

The existence of a thick pigmented oesophageal plaque is a striking feature in several species of herbivorous macropod. The fact that this oesophageal plaque is composed of bacteria adherent to the stratified squamous epithelium is not, however, unprecedented. Bacterial attachment to the squamous lining of the oral cavity, the oesophagus and the non-glandular fore-stomach regions is reported in a wide variety of mammals and birds; however, the number and diversity of bacterial forms represented in the oesophagus of macropods far exceeds anything previously reported.

Comparable mixed populations of adherent bacteria are reported from the glandular mucosa of the caecum in rodents (Savage 1972; Savage and Blummershine 1974; Wagner and Barrnett 1974) and in the koala, *Phascolarctos cinereus* (McKenzie 1978). Mixed bacterial populations attached to squamous epithelial surfaces have been demonstrated in the stomach and oesophagus of mice (Savage *et al.* 1968; Savage and Blummershine 1974; Davis 1976; Roach *et al.* 1977) the stomach of rats (Davis 1976), the pars oesophagea of pigs (Fuller *et al.* 1978), the crop of chickens (Fuller 1973; Brooker and Fuller 1975) and the reticulo-rumen of sheep (Bauchop *et al.* 1975) and cattle (McCowan *et al.* 1978). The relationship between mixed adherent bacterial population of the macropodid oesophagus and the squamous epithelial substrate is most analogous to the situation in the reticulo-rumen of cattle and sheep. In both macropods and ruminants, bacteria represented by several different types are attached to and proliferate on the surface squamous cells. The nature of the association between the bacteria and the host epithelium are very similar in both circumstances. Despite the many qualitative similarities between the association and apparent activity of the adherent bacteria of ruminants and macropods, several distinct differences in the structure and function of the respective squamous linings to which they attach are recognized. The adherent bacteria in the ruminant forestomachs lie on a relatively thin epithelium with recognized absorptive functions. McCowan *et al.* (1978) have proposed that the bacteria may play an active role in reducing the layers of keratinized epithelial cells which might otherwise impair the absorption of metabolites from rumenal fermentation. This consideration is not, however, a satisfactory means for explaining the presence of bacteria on the macropodid oesophageal epithelium. Morphological studies conducted on this lining in several herbivorous macropods have shown that although the surface area of the oesophagus is increased in different species by the presence of folds, papillae and laminar plates, the epithelium is comparatively thick with a wide cornified layer (Obendorf 1984). In addition, both the oesophageal papillae and laminar plates contain connective tissue which is restricted to the basal parts of these projections, with the bacteria showing a distinct preference for well-cornified parts. The failure to demonstrate extensive bacterial populations adherent to the oesophageal linings of captive macropods may be related to dietary considerations. Nevertheless, the absence of well-cornified epithelial layers in these animals is thought to be another factor influencing bacterial adherence.

With the exception of one branching rod form from *M. eugenii* the many bacterial types which make up the oesophageal plaque are restricted to the exposed surface layers of epithelium. Most of the bacteria do not cause any appreciable alteration to the cell membranes, although one type of organism did embed deeply into the cell membranes in the oesophageal mucosa of *Petrogale purpureicollis*. No evidence of epithelial damage or bacterial infiltration into the cell layers below the surface squames could be demonstrated. This is consistent with the absence of any oesophageal pathology in all the free-ranging herbivorous macropods examined in this study. In contrast, the yeast, *Candida albicans* is reported to invade and proliferate into the deep epithelial layers in the oesophagus of orphan-reared kangaroo joeys (Obendorf 1980). In these cases, severe mucosal inflammation and reactive hyperplasia of the epithelium were also noted.

The surface epithelial cells appear to either desquamate as intact flattened squames with bacteria attached to their cell membranes or develop tears in their lumenal cell surface

while still attached to the oesophageal epithelium. The actual cause of the break in the cell membrane is not known; however, it may represent a likely outcome of squamous cells which remain firmly joined to the underlying epithelium. The distended appearance of ruptured cells and the presence of open loose cytoplasmic contents suggests that osmotic influences may act once the cells have ruptured. Distended cells with discontinuous cell membranes contain bacteria. Unlike the situation in the rumen it appears that more than one morphological type of bacteria is found within epithelial cells. Numerous types of bacteria are seen attached to both the external and internal sides of cell membranes and to the keratin fibres within the cells. SEM studies reveal indentations and notches caused by bacteria in the fibrous cytoplasm of damaged cells. Distinct spaces are also recognized around bacteria in thin sections. The gap between the bacteria and the adjacent cytoplasm could be associated with shrinkage of the bacteria during processing; however, the absence of compressed keratin fibres around such bacteria suggest that fibres may actually be displaced by a lytic reaction around bacteria. The studies of McCowan *et al.* (1978) also suggest that certain bacteria may be capable of digesting the contents of squamous epithelial cells. Cheng *et al.* (1979) have proposed that the adherent bacteria located in the rumen of cattle may be recycling host tissues initially for microbial replication and growth but ultimately for use as host nutrients. This hypothesis may also help to explain the presence of bacteria within keratinized epithelial cells of the macropodid oesophagus. Utilization of the sulfur-rich keratin by bacteria may represent a means whereby adequate amounts of sulfur are incorporated into the pool of the forestomach microbial populations. The utilization of dietary and recycled urea nitrogen by rumen microbes is known to be dependent upon an adequate intake of sulfur (Hume and Bird 1970). The digestion and use of sulfur derived from superficial epithelial cells by the oesophageal microbes may represent a significant contribution to the available sulfur reserves passing into the stomach and consequently enhance the overall digestibility of the food ingested.

Bacteria are known to adhere tenaciously and often quite specifically to a wide variety of surfaces in numerous environments. Bacterial adhesions are reported in aquatic habitats (Fletcher and Floodgate 1973), in soil (Bae *et al.* 1972) and in numerous associations with mammalian tissues including alimentary tract (Brownlee and Moss 1961), genital tract (Mardh and Westrom 1976) and respiratory tract (Selinger and Reed 1979) mucosa. In many instances, these attachments are achieved by a range of extracellular capsular material which has been considered an example of a glycocalyx (Costerton *et al.* 1978). In this study, attachment of the bacteria to one another and to the various components of epithelial cells is, in many instances, indicated by radiating extracellular fibres. A variety of different types of extracellular material producing cell to cell attachments are reported. Several types closely resemble the glycocalyx material which is reported from bacteria isolated from the forestomach of ruminants (Costerton *et al.* 1974; Cheng and Costerton 1975, 1977; Cheng *et al.* 1976, 1977). Although extracellular fibres are reported in both SEM and TEM studies, the fine fibres seen in TEM photomicrographs are not always demonstrated in the same tissue processed for SEM. This phenomenon may be related to the accelerating potential of electrons bombarding a specimen and the depth to which they penetrate. For thin materials viewed in the SEM at high potentials, electrons will pass through and fail to detect the presence of such materials. Also, the size of individual bacterial fibres are below the limit of resolution for the SEM and are only seen when they are artifactually clumped or aggregated together as a result of dehydration and critical-point drying procedures.

The absence of any visible means of attachment between closely associated bacteria and between bacteria and the epithelial cells prompted the use of several different TEM and SEM processing techniques. Although some subtle qualitative differences were apparent, the specific means whereby these bacteria attach to one another and to the epithelial cells was not ascertained. Similarly the use of ruthenium red, a stain which has been widely used to define the extracellular coatings of various cells including bacteria

(Cagle *et al.* 1972; Fletcher and Floodgate 1973), failed to enhance or specifically emphasize the nature of the adhesions. At the light microscopic level, various microcommunities and microcolonies of the bacterial plaque stained intensely with various mucopolysaccharide stains. The explanation for the differing results in histochemical staining reactions between the light and electron microscopic tissues was thought to be due to the ruthenium red leaching out of the samples during post-fixation and early dehydration steps. Also, the concentration of ruthenium red used in these studies was only one-third that employed by Cagle *et al.* (1972). The differences in the mucopolysaccharide staining reactions of several different groups of bacteria suggested that several carbohydrate-rich substances probably make up the extracellular coating of bacteria.

Bacteria closely resembling the striated filamentous organisms reported in this study have been reported from the oral cavity of cattle (McCowan *et al.* 1979). Bacteria of the genera *Simonsiella* and *Alysiella* were shown to produce asymmetrically positioned bacterial fibres which were implicated in the adhesion to epithelial surfaces. Similar aggregates of asymmetrical bacterial fibres mediating attachment to the oesophageal surfaces were noted in this study. The large polyp-like colonies containing morphologically similar bacteria embedded within a common extracellular matrix have not been previously reported. Nevertheless, the presence of other bacteria adherent to the periphery of this glycocalyx material was similar to the mixed aggregates of bacteria reported by Costerton *et al.* (1978). Such associations between bacteria of different morphological forms was considered as a means whereby bacteria, not capable of adhering to host epithelial cells can become a part of the bacterial plaque. The presence of remnant cell walls of dead bacteria embedded within the glycocalyx of established microcolonies has been reported in other naturally occurring bacterial populations (Costerton *et al.* 1978). These workers suggested that components of dead cells may be utilized by the adjacent living bacteria.

Cytoplasmic inclusions were reported in several bacteria of the macropodid oesophageal plaque. These inclusions were similar to the glycogen granules which were found in the rumen bacteria *Megasphera elsdenii* (Cheng *et al.* 1973).

It now remains to discuss the functional significance of the association between the adherent bacterial populations and the oesophagus of herbivorous macropodid species. This environment has some similarities with the fermentative forestomachs of ruminants or the caecum and colon of Equidae. In the reticulorumen, the maintenance of large and diverse microbial and protozoan populations has been shown to confer distinct nutritional benefits for its vertebrate host, while at the same time providing a stable environment for the growth and replication of its inhabitants (Hungate 1966).

The reasons for the existence of this adherent population of bacteria in macropods are not fully understood, but it is likely that the suitability of this habitat is in part related to factors determined by the host, namely temperature, pH and osmolarity, while at the same time maintaining continuity of nutrition for the growth and regeneration of its inhabitants. A need to control the size and diversity of the populations of organisms would also be necessary.

The results of this study coupled with the findings of research in other fields of macropodid digestive function suggest that these requirements are fulfilled. Firstly, the saliva of several herbivorous macropods is recognized for its buffering potential in the neutral to slightly alkaline range. In addition, macropodid saliva is a major source for recycling urea nitrogen back to the digestive tract (Brown and Main 1967; Kinnear and Main 1975). Similar properties of ruminant saliva are recognized as essential for the maintenance of foregut microbial metabolism. Secondly, the results of morphological studies of these oesophageal linings demonstrate that it is only herbivorous macropods which possess the large populations of adherent bacteria. The ultrastructural studies carried out on oesophageal samples from numerous species and individuals indicate that exfoliation of surface epithelial cells along with their adherent bacteria is a consistent feature with subsequent recolonization of newly exposed epithelial surfaces. Thirdly, the nutrition of

the adherent bacterial populations may be provided by soluble components of the ingesta, salivary components and possibly by components of the oesophageal epithelium. The saliva of macropods is known to have significant amylase activity (Forbes and Tribe 1969). This information in conjunction with data indicating that certain herbivorous macropods selectively consume herbage rich in soluble carbohydrate sources (Bailey *et al.* 1971; Griffiths *et al.* 1974) substantiates the suggestion that the oesophageal bacteria are in a favourable position to obtain first use of this nutrient source. The ultrastructural studies of the association between the bacteria and the squamous epithelial cells of the oesophagus also demonstrate that certain organisms may utilize cytoplasmic components of cells.

Despite the gross similarity of the papillated and laminated oesophageal linings with the lining in the forestomachs of ruminants, histological and ultrastructural studies failed to reinforce the analogy (Obendorf 1984). The oesophageal epithelium was thick and composed of numerous layers of cells undergoing keratinization in comparison to the thin stratified epithelium in the rumen. The organization of the oesophageal mucosa in species with papillated and laminated linings consisted predominantly of epithelial tissues in the process of keratinization with the connective tissue cores of these epithelial projections being restricted to their bases. The ultrastructural appearance of the epithelium more closely resembled the protective-type squamous lining typified by mammalian skin or buccal cavity, rather than the absorptive-type squamous lining of the ruminant forestomach. It was concluded that the development of these types of oesophageal linings in herbivorous macropods was not directed towards the function of absorption.

It is proposed that the various types of specialization seen in the oesophagus of herbivorous macropods may represent a specific adaptation on the part of the host to provide additional epithelial lining for the colonization and proliferation of bacterial populations.

In addition to these observational studies on the bacteria, investigations utilizing anaerobic bacteriological techniques and *in vitro* methods simulating conditions within the macropodid upper alimentary tract were also conducted (Obendorf 1981). Most of the adherent bacteria in the oesophagus of captive *M. eugenii* were found to be anaerobes. Members of the genera *Fusobacterium*, *Bacteroides* and *Streptococcus* were the most frequent isolates; however, isolates of *Selenomonas*, *Anaerovibrio*, *Sarcina*, *Peptococcus*, *Peptostreptococcus* and *Eubacterium* were also identified. Facultative anaerobic bacteria classified as enterobacteria, bacilli, streptococci and staphylococci only constituted a very small proportion of the adherent bacteria.

These bacteriological results coupled with the findings of morphological studies of the oesophagus in herbivorous macropods are thought to give some basis to the proposal that the oesophagus may represent a significant source of bacteria for the maintenance of microbial activity in the stomach. Recently there have been several investigations of the structure and function of the stomach in herbivorous macropods which give some credence to this claim. Dellow (1982) states that the food material moves along the length of the stomach as a pulse with differential rates of passage for the fluid and particulate fractions. This directional or tubular flow pattern is recorded in several species of herbivorous macropods (Dellow 1982). By contrast, ruminants with the blind sac development of the forestomach retain ingesta for long periods and have complex reticulo-rumenal movements for circulating and mixing the ingesta. Macropods, in general, do not possess anatomical structures in the alimentary tract to substantially impede the flow of ingesta. Both Langer (1979*a*, 1979*b*) and Hume (1978) have proposed that the evolution of the digestive tract of macropods has been directed towards the utilization of high fibre diets, not necessarily by retaining it longer in the stomach to improve unit digestive efficiency, but by moving it through the stomach foresaking high unit digestibility and thereby maintaining food intake and digestible nutrient supply at a higher level than would otherwise be possible. In this context, the digestive process of macropods is more analogous with the digestion of Equidae. It is suggested that these forms of herbivore may have an advantage over

ruminants when grazing high fibre diets (Langer 1979*c*). Ruminants on high fibre diets are known to reduce their rate of passage of ingesta, retaining food longer within the forestomachs. In this situation feed intake is reduced and may lead to a failure to satisfy the energy requirements of ruminants. Consequently, despite the greater efficiency in unit digestibility, such a process in habitats supporting high fibre plant species may actually disadvantage their energy intake.

In vitro estimations of microbial fermentation in the forestomach of several herbivorous macropods has demonstrated the highly efficient volatile fatty acid and microbial protein production which occurs in this site (Lintern-Moore 1973; Hume 1977; Kennedy and Hume 1978). However, the more readily digested dietary compounds are rapidly fermented in the cranial regions of the stomach and the rates of fermentation and microbial growth are reported to decrease along its length (Dellow *et al.* 1983). The comparatively rapid and directional transit of the stomach ingesta fractions, with their bacterial complements, through the stomach may represent a net loss of microbes to the stomach. Indeed Dellow (1979) concluded independently that in such a circumstance, the relatively short retention time of particulate ingesta and the rapid flow of the fluid phase in the forestomach could be instrumental in precluding the establishment of certain microorganisms that have slow growth and reproduction rates.

In the situation where the oesophagus provides continual replenishment of bacteria for microbial fermentation in the stomach, each intake of food would obtain its complement of bacteria during transit along the oesophagus. In addition, bacteria would be passed into the stomach continually with swallowing of saliva and natural exfoliation of oesophageal cells. This process would ensure that each freshly ingested quantity of herbage acquired oesophageal bacteria, an important consideration in stomachs where flow of ingesta is substantially unimpeded.

Herbivorous macropods generally restrict feeding to nocturnal and crepuscular periods and do not consistently regurgitate food for rechewing. In ruminants, the act of rumination is most advantageous in reducing the size of the herbage, thereby making more damaged plant tissue available for microbial fermentation. It is seen as an important means of maintaining the continuity of fermentation between periods of feeding. Consequently, it would seem highly advantageous for herbivorous macropods to support a large sessile bacterial culture within the oesophagus to act as a continuous source of bacteria for each meal.

Acknowledgments

This paper presents part of the results of the author's Ph.D. Thesis, University of Melbourne. Grateful acknowledgment is made to Drs K. Hughes and J. O'Shea who provided advice and criticism in the preparation of this material. Expert technical assistance in the fields of histology and ultrastructural processing was given by F. Oddi, A. Venestra and J. Wilson. The author warmly thanks and acknowledges their contributions.

References

Bae, H. C., Cota-Robles, E. H., and Casida, L. E. J. (1972). Microflora of soil as viewed by transmission electron microscopy. *Appl. Microbiol.* **23**, 637–48.

Bailey, P. T., Martensz, P. N., and Barker, R. (1971). The red kangaroo, *Megaleia rufa* (Desmarest), in north-western New South Wales. II. Food. *CSIRO Wildl. Res.* **16**, 29–39.

Bauchop, T., Clarke, R. T. J., and Newhook, J. C. (1975). Scanning electron microscope study of bacteria associated with the rumen epithelium of sheep. *Appl. Microbiol.* **30**, 668–75.

Brooker, B. E., and Fuller, R. (1975). Adhesion of lactobacilli to the chicken crop epithelium. *J. Ultrastruct. Res.* **52**, 21–31.

Brown, G. D., and Main, A. R. (1967). Studies on marsupial nutrition. V. The nitrogen requirements of the euro, *Macropus robustus*. *Aust. J. Zool.* **15**, 7–27.

Brown, R. C., and Hopps, H. C. (1973). Staining of bacteria in tissue sections: A reliable Gram stain method. *Am. J. Clin. Pathol.* **59**, 234–40.

Brownlee, A., and Moss, W. (1961). The influence of diet on lactobacilli in the stomach of the rat. *J. Path. Bact.* **82**, 513–16.

Cagle, G. D., Pfister, R. M., and Vela, G. R. (1972). Improved staining of extracellular polymer for electron microscopy: Examination of *Azotobacter*, *Zoogloea*, *Leuconostoc* and *Bacillus*. *Appl. Microbiol.* **24**, 477–87.

Cheng, K. J., Akin, D. E., and Costerton, J. W. (1977). Rumen bacteria: interaction with particulate dietary components and response to dietary variation. *Fed. Proc.* **36**, 193–7.

Cheng, K. J., and Costerton, J. W. (1975). Ultrastructure of cell envelopes of bacteria of the bovine rumen. *Appl. Microbiol.* **29**, 841–9.

Cheng, K. J., and Costerton, J. W. (1977). Ultrastructure of *Butyrivibrio fibrisolvens*: A Gram-positive bacterium? *J. Bacteriol.* **129**, 1506–12.

Cheng, K. J., Hironaka, R., Jones, G. A., Nicas, T., and Costerton, J. W. (1976). Frothy feedlot bloat in cattle: production of extracellular polysaccharides and development of viscosity in cultures of *Streptococcus bovis*. *Can. J. Microbiol.* **22**, 450–9.

Cheng, K. J., Hironaka, R., and Roberts, D. W. A., and Costerton, J. W. (1973). Cytoplasmic glycogen inclusions in cells of anaerobic Gram-negative rumen bacteria. *Can. J. Microbiol.* **19**, 1501–6.

Cheng, K. J., McCowan, R. P., and Costerton, J. W. (1979). Adherent epithelial bacteria in ruminants and their roles in digestive tract function. *Am. J. Clin. Nutr.* **32**, 139–48.

Costerton, J. W., Daamgard, H. N., and Cheng, K. J. (1974). Cell envelope morphology of rumen bacteria. *J. Bacteriol.* **118**, 1132–43.

Costerton, J. W., Gessey, G. C., and Cheng, K. J. (1978). How bacteria stick. *Sci. Am.* **238**, 86–95.

Davis, C. P. (1976). Preservation of gastro-intestinal bacteria and their microenvironmental associations in rats by freezing. *Appl. Environ. Microbiol.* **31**, 304–12.

Dellow, D. W. (1979). Physiology of digestion in the macropodine marsupials. Ph.D. Thesis, University of New England, Armidale, N.S.W.

Dellow, D. W. (1982). Studies on the nutrition of macropodine marsupials III. The flow of digesta through the stomach and intestine of macropodines and sheep. *Aust. J. Zool.* **30**, 751–67.

Dellow, D. W., Nolan, J. V., and Hume, I. D. (1983). Studies on the nutrition of macropodine marsupials. V. Microbial fermentation in the forestomach of *Thylogale thetis* and *Macropus eugenii*. *Aust. J. Zool.* **31**, 433–43.

Fletcher, M., and Floodgate, G. D. C. (1973). An electron-microscopic demonstration of an acidic polysaccharide involved in the adhesion of a marine bacterium to solid surfaces. *J. Gen. Microbiol.* **74**, 325–34.

Forbes, D. K., and Tribe, D. E. (1969). Salivary glands of kangaroos. *Aust. J. Zool.* **17**, 765–75.

Fuller, R. (1973). Ecological studies on the lactobacillus flora associated with the crop epithelium of the fowl. *J. Appl. Bact.* **36**, 131–9.

Fuller, R., Barrow, P. A., and Brooker, B. E. (1978). Bacteria associated with the gastric epithelium of neonatal pigs. *Appl. Environ. Microbiol.* **35**, 582–91.

Griffiths, M., Barker, R., and MacLean, L. (1974). Further observations on the plants eaten by kangaroos and sheep grazing together in a paddock in south-western Queensland. *Aust. Wildl. Res.* **1**, 27–43.

Holdeman, L. V., Cato, E. P., and Moore, W. E. C. (1977). 'Anaerobe Laboratory Manual.' (Virginia Polytechnic Institute and State University: Blacksburg, Virginia.)

Hume, I. D. (1977). Production of volatile fatty acids in two species of wallaby and in sheep. *Comp. Biochem. Physiol.* **56A**, 299–304.

Hume, I. D. (1978). Evolution of the Macropodidae digestive system. *Aust. Mammal.* **2**, 37–42.

Hume, I. D., and Bird, P. R. (1970). Synthesis of microbial protein in the rumen. IV. The influence of the level and form of dietary sulphur. *Aust. J. Agric. Res.* **21**, 315–22.

Hungate, R. E. (1966). 'The Rumen and Its Microbes.' (Academic Press: New York and London.)

Joen, S. (1965). Simple method for staining and preserving epoxy resin-embedded animal tissue sections for light microscopy. *Life Sci.* **4**, 1839–41.

Kennedy, P. M., and Hume, I. D. (1978). Recycling of urea nitrogen to the gut of the tammar wallaby (*Macropus eugenii*). *Comp. Biochem. Physiol.* **61A**, 117–21.

Kinnear, J. E., and Main, A. R. (1975). The recycling of urea nitrogen by the wild tammar wallaby (*Macropus eugenii*)—a ruminant-like marsupial. *Comp. Biochem. Physiol.* **51A**, 793–810.

Lane, B. P., and Europa, D. L. (1965). Differential staining of ultrathin sections of epon-embedded tissues for light microscopy. *J. Histochem. Cytochem.* **13**, 579–82.

Langer, P. (1979*a*). Phylogenetic adaptation of the stomach of Macropodidae Owen, 1839, to food. *Z. Säugeteirk.* **44**, 321–33.

Langer, P. (1979*b*). Functional anatomy and ontogenetic development of the stomach in the macropodine species, *Thylogale stigmatica* and *Thylogale thetis* (Mammalia: Marsupialia). *Zoomorphologie* **93**, 137–51.

Langer, P. (1979*c*). Functional gastric anatomy of macropod marsupials. *Am. Rech. Vet.* **10**, 476–9.

Lintern-Moore, S. (1973). Incorporation of dietary nitrogen into microbial nitrogen in the forestomach of the Kangaroo Island wallaby, *Protemnodon eugenii* (Desmarest). *Comp. Biochem. Physiol.* **44**, 75–82.

Malick, L. E., and Wilson, R. B. (1975). Modified thiocarbohydrazide procedure for scanning electron-microscopy: Routine use for normal, pathological or experimental tissues. *Stain Technol.* **50**, 265–9.

Mardh, P. A., and Westrom, L. (1976). Adherence of bacteria to vaginal epithelial cells. *Infect. Immun.* **13**, 661–6.

Muller, L. L., and Jacks, T. J. (1975). Rapid chemical dehydration of samples for electron microscopic examinations. *J. Histochem. Cytochem.* **23**, 107–10.

Munger, B. L. (1961). Staining methods applicable to sections of osmium-fixed tissue for light microscopy. *J. Biophys. Biochem. Cytol.* **11**, 502–6.

McCowan, R. P., Cheng, K. J., Bailey, C. B. M., and Costerton, J. W. (1978). Adhesion of bacteria to epithelial cell surfaces within the reticulo-rumen of cattle. *Appl. Environ. Microbiol.* **35**, 149–55.

McCowan, R. P., Cheng, K. J., and Costerton, J. W. (1979). Colonization of a portion of the bovine tongue by unusual filamentous bacteria. *Appl. Environ. Microbiol.* **37**, 1224–9.

McKenzie, R. A. (1978). The caecum of the koala, *Phascolarctos cinereus* light scanning and transmission electron microscopic observations on the epithelium and flora. *Aust. J. Zool.* **26**, 248–56.

Obendorf, D. L. (1980). Candidiasis in young hand-reared kangaroos. *J. Wildl. Dis.* **16**, 135–40.

Obendorf, D. L. (1981). The lining and inhabitants of the oesophagus of macropods. Ph.D. Thesis, University of Melbourne.

Obendorf, D. L. (1984). The macropodid oesophagus. I. Gross anatomical, light microscopic, scanning and transmission electron microscopic observations of its mucosa. *Aust. J. Zool.* **32**, 415–35.

Pearse, A. G. E. (1973). Histochemistry theoretical and applied Vol. I, 3rd Edn. (J. & A. Churchill, Ltd: London.)

Roach, S., Savage, D. C., and Tannock, G. W. (1977). Lactobacilli isolation from the stomach of conventional mice. *Appl. Environ. Microbiol.* **33**, 1197–203.

Savage, D. C. (1972). Associations and physiological interactions of indigenous micro-organisms and gastro-intestinal epithelia. *Am. J. Clin. Nutr.* **25**, 1372–9.

Savage, D. C., and Blummershine, R. V. H. (1974). Surface-surface associations in microbial communities populating epithelial habitats in the murine gastro-intestinal ecosystem: scanning microscopy. *Infect. Immun.* **10**, 240–50.

Savage, D. C., Dubos, R., and Schaedler, R. W. (1968). The gastro-intestinal epithelium and its autochthonous bacterial flora. *J. Exp. Med.* **127**, 67–76.

Selinger, D. S., and Reed, W. P. (1979). Pneumococcal adherence to human epithelial cells. *Infect. Immun.* **23**, 545–8.

Thompson, S. W., and Hunt, R. D. (1966). 'Selected Histochemical and Histopathological methods.' (Thomas: Chicago.)

Wagner, R. C., and Barrnett, R. J. (1974). The fine structure of prokaryotic-eukaryotic cell junctions. *J. Ultrastruct. Res.* **48**, 403–13.

Manuscript received 27 September 1983, accepted 10 February 1984