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THE MICROBIAL ECOLOGY OF THE INTESTINAL MUCOSA.

by M.W. PHILLIPS

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School of Microbiology
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DECLARATION

The candidate

Michael William Phillips

declares that none of the work presented in this thesis has been submitted to any other University or Institution for a higher degree.

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ABSTRACT

As the intestinal mucosa and its mucus layers form the interface between the animal and its intestinal microbiota, this region is potentially of great significance to the host. Nevertheless knowledge of both the conditions existing in this environment, and the muscosal microbiota remains poor. The aim of this thesis was to gain a better understanding of the microbial ecology of the intestinal mucosa from studies of the rodent intestinal tract and its microorganisms.

Initially to define the distribution and composition of the muscosal microbiota an *in situ*, semi-quantitative survey of various sites in the rat intestine was undertaken, using light and electron microscopy. Using similar methods the effects of intestinal perturbation on the mucosal microbiota were also studied. Results from these experiments suggested that perturbation could lead to long term changes in the mucosal microbiota (certain allochthonous spiral-shaped bacteria were able to associate with the colonic mucosa for up to 180 days after treatment).

By using selective media, muscosal scrapings as inocula, and with microaerophilic incubation several mucosal bacteria were isolated in pure culture for the first time. One population of spiral-shaped bacteria, having a distinctive ultrastructure similar to organisms seen in intestinal crypts, was studied in some detail. When given to rodents, lacking mucosa-associated

microorganisms, these isolates were able to colonize intestinal crypts. The distribution of these isolates throughout the intestinal tract was different to that seen in conventional animals and appeared to be influenced by the overall composition of the gut microbiota.

From *in vitro* studies it was found that the mucosal isolates moved most rapidly in viscous solutions. It is suggested that the organisms have adapted to moving in viscous mucus secretions and that this ability is of ecological advantage in the crypt environment. Further experiments demonstrated that the spiral-shaped isolates were obligatory microaerophiles, and were unable to degrade small bowel mucin *in vitro*.

It is considered that the isolation of the muscosal isolates will greatly facilitate further studies of the mucosal environment and its microbiota, and that these studies may also have relevance to the study of intestinal pathogens.

C O N T E N T S

CHAPTER ONE

INTRODUCTION

1. INTRODUCTION

During the process of evolution vertebrate animals have developed complex digestive tracts adapted to efficiently catabolize, and absorb nutrients and water obtained from their environments. The gut has changed from a simple tube to involved systems of connected chambers, each with different physical and chemical parameters. These changes have also created a variety of micro-habitats in the gut suitable for the growth of microorganisms, and as a result the gastrointestinal tracts of most animals contain large and diversified microbiotas.

This thesis is concerned with one component of the gastrointestinal microbiota: those microbes found on or near the mucosal surface. As surface association is a relatively new concept in intestinal microbiology only a small proportion of publications in this field deal with mucosa-associated microbes. Consequently to assess the information relevant to the study of these organisms, it is necessary to examine research undertaken with other components of the gut microbiota. The review is therefore a general appraisal of microbial ecology in the gut with emphasis on the mucosa-associated microbes. Similarly, while emphasis is given to studies of rodents, the microbial ecology of other warm-blooded, mono-gastric animals is also examined.

1.1 HISTORICAL CONSIDERATIONS

Initially the major reason for studying the intestinal microbiota was to understand, and possibly control life-threatening intestinal infections such as cholera,

typhoid and dysentery. While the initial studies of such diseases were often based on microscopy (Wilson and Miles, 1964) methods were quickly developed for the isolation and cultivation of the major pathogenic bacteria, e.g. Salmonella spp., Shigella spp., Vibrio cholerae and Escherichia coli. Each of these organisms was able to grow on relatively simple media when incubated aerobically. From these studies it was found that large numbers of coliform bacteria and enterococci could also be isolated from normal faeces using similar techniques, and it was assumed that these organisms were the main components of the host's normal microbiota. As both pathogens and other facultative anaerobes could be easily isolated from faecal samples using these simple methods, little emphasis was given to examining sites elsewhere in the tract, nor to developing the complex media and growth conditions needed to grow the large variety of microbes seen in microscopic studies, but not isolated in culture. Nevertheless some observations of the normal non-pathogenic members of the gut microbiota were made at this time. Lactobacilli and bifidobacteria (Tissier, 1900; cited by Mitsuoku and Kaneuchi, 1977), spirochaetes (Macfie, 1917; Parr, 1923) and Bacteroides spp. (Distaso, 1911; cited by Eggerth and Gagnon, 1933), were all seen in microscopic preparations of human intestinal samples.

Spirochaetes were the subject of many reports, as this group's status as pathogens was the source of controversy (Parr, 1923). Parr (1923) cites numerous studies

relating the occurrence of spirochaetes with cholera, infant diarrhoea, appendicitis, ulcerative colitis and various dysenteries. In an attempt to clarify the role of these organisms both Macfie (1917)and Parr (1923) undertook comprehensive studies of spirochaetes in both man and a range of animals. Parr (1923) found the organisms in a high proportion (43 of 173) of microscopic preparations of faeces obtained from subjects in Chicago, U.S.A., while Macfie (1917) found these organisms in all subjects in a study conducted in West Africa. Spirochaetes were also reported to be normal members of the faecal microbiota of rats, mice, guinea pigs, but not rabbits, (Parr, 1923) and could also be found in sheep, cattle, goats, pigs, dogs and cats (Macfie, 1917). Macfie (1917) speculated that the organisms were saprophytes rather than pathogens, especially in rats, mice, and guinea pigs where they could be found in over 80% of specimens. Although spirochaetes had been seen in the ileum from an autopsy (Macfie, 1917), Parr (1923) determined, from work with animals, that the caecum was the optimum site for colonization, with few organisms in the colon and very few in the ileum. Metchnikoff (1908) had previously reported that the caeca in some birds contained large numbers of spiral-shaped bacteria, though these were described as being spirilla rather than spirochaetes.

The first description of surface associated microbes in the gastrointestinal tract was that of spirilla present in the fundic glands of cat and dog gastric mucosa

(Solomon, 1896; cited by Palmer, 1954; Kasia et al., 1919). The absence of any pathological consequences arising from the presence of these organisms led most authors to regard the gastric spirilla as being, like the intestinal spirochaetes, saprophytes rather than pathogens.

Despite the observations of tissue association by spirilla, and the widespread occurrence of intestinal spirochaetes, comparatively little attention was paid to these and other non-pathogenic gut microbes in the period leading up to the 1960s. Reliance on aerobic culture meant most of the autochthonous microbiota could not be grown; furthermore a decline in the use of microscopy, meant that the inability of cultural techniques to grow the majority of organisms was not recognized.

A necessary prerequisite to a better understanding of the gut microbiota was the development of techniques for growing fastidious, oxygen-sensitive anaerobic bacteria. Methods were developed early in the century for the growth of anaerobic bacteria: the anaerobic jar of McIntoch & Fildes (1916) and modified by the addition of palladium catalyst (Weiss and Spaulding, 1936, 1937). Although anaerobic jars were important in isolating clinically important organisms such as Clostridium spp. and Bacteroides spp. (Rosenblatt et al., 1973) these developments did not result in a better perception of the nature of the gut microbiota, as the long exposures to oxygen during manipulation would have prevented the more

oxygen-sensitive organisms from growing. Only after procedures had been devised to minimise exposure to air were the major microbial populations present in the gut cultured with any success. Hungate (1950) was the first to use such an approach, in studies of rumen bacteria; rumen contents were inoculated with molten agar, containing rumen fluid, and the agar solidified as a film on the wall of a sealable tube. All operations were conducted under a stream of oxygen-free gas. The tubes were stoppered and then incubated; the rumen bacteria grew on, or in, the agar film. Subsequently these basic techniques have been refined and expanded to constitute a complete *modus operandi* for culturing strictly anaerobic bacteria (Holderman and Moore, 1977). An alternative approach to growing these organisms has been to create an "anaerobic work space": an area large enough to contain reduced media, equipment and space for manipulation, all in an anaerobic atmosphere. This was achieved by building sealed transparent chambers from which air could be removed and replaced by oxygen-free gases (Socronsky *et al.*, 1959; Aranki *et al.*, 1969). Oxygen levels are kept very low by circulating the contents past a palladium catalyst, and by using an entry port for the transfer of materials in and out of the chamber. Implementation of rigorous anaerobic techniques led to increasing numbers of the intestinal microbiota being isolated, and also to a stimulation of interest in this field of microbiology.

The most important contributions to the understanding of gut microbiology, subsequent to the development of new culturing techniques, were made by René Dubos and his co-workers at the Rockefeller University. Dubos and Schaedler (1960, 1962; Schaedler and Dubos 1959, 1962), by using both microscopic and culturing techniques, were able to detail the complexity of the microbiota and note the predominance of strictly anaerobic bacteria in mouse faeces. Furthermore these studies demonstrated different microbiotas in various strains of mice and in groups of mice on different diets. These authors were able to correlate such changes in the faecal microbiota to altered susceptibilities to intestinal infections (Schaedler and Dubos, 1959; Dubos and Schaedler 1960, 1962) and endotoxin (Schaedler and Dubos, 1962). When these studies were extended from investigations of faeces to studies of different regions of the tract, a new dimension was added to gastrointestinal microbiology. Particular populations of microbes were found to preferentially colonize specific sites in the gastrointestinal tract (Dubos et al., 1965). Subsequently it was established by Savage and co-workers that in addition to preferentially colonizing regions of the tract, certain yeasts (Savage and Dubos, 1967) and bacteria (Savage et al., 1968; Savage, 1969) were found in close association with the surfaces of various tissues; many of these organisms were rarely found elsewhere in the tract. The work of Dubos, Schaedler, Savage and co-workers stimulated much interest in the study of the normal

gastrointestinal microbiota and in particular in the concept of tissue association.

1.2 ECOLOGICAL CONSIDERATIONS.

Progression from the study of the faecal microbiota to investigations of regions throughout the tract resulted in gut microbiology becoming increasingly complex. In order to facilitate the interpretation of this new information, Dubos and his colleagues in 1965, and in subsequent publications, sought to understand gut microbiology in terms of ecology theory.

The gastrointestinal tract and its associated microbiota can be considered to be an open ecosystem with continuous inputs of nutrients and microorganisms from the external environment. Ecosystems are composed of habitats and niches; habitats are physical areas or spaces occupied by microbes while niches are the roles of the organisms, i.e. how, over time, the organisms function and survive in the environment (Alexander, 1971; Savage, 1977). The gastrointestinal tracts of monogastric animals contain large numbers of different habitats and have the potential for many and varied microbial niches. Furthermore the complexity of the system is increased by the interactions with the complicated biological system surrounding it: the host animal.

When defining the micriobiota of this open ecosystem distinction needs to be made between microbes originating from exogenous sources, i.e. transients, and organisms present naturally in the gut (Savage, 1977). Dubos *et al*.

(1965) attempted to make such a distinction by describing the "indigenous flora" of the gastrointestinal tract, this being composed of: organisms having co-evolved with the host i.e. autochthonous, and also organisms that establish in all animals of a species, i.e. the "normal flora", and true pathogens able to colonize for long periods. Subsequently Savage (1977) modified the description of the natural gastrointestinal microbiota to conform with the microbial ecology theory put forward by Alexander (1971). Accordingly, all microorganisms in the tract can be described as either autochthonous (indigenous) or allochthonous (non-indigenous or transient).

A number of criteria have been devised to define the term autochthonous when referring to the gut environment; the organisms: a. can grow anaerobically, b. are always found in normal adults, c. colonize particular areas of the tract, d. colonize habitats during succession in infant animals, e. maintain stable population levels as part of the microbiota of normal adults, f. may associate intimately with the mucosal epithelium in the area colonized (Dubos et al., 1965; Alexander, 1971; Savage, 1977). The autochthonous microorganisms colonize habitats, occupy niches and form the interrelationships that constitute the stable ecosystem. Often, lack of information results in the status of gut microbes being doubtful; thus organisms that are ubiquitous in an animal colony are often referred to as being part of the normal gastrointestinal microbiota, even if all the criteria of

autochthony have not been shown. When all the accessible habitats are colonized and the available niches filled, the microbiota is described as being a climax community. In the absence of outside disturbances climax communities are very stable, and resistant to endogenous change, as the organisms best adapted for each habitat and niche are present. Allochthonous organisms can originate from outside the gastrointestinal tract, or from habitats elsewhere in the tract (Savage, 1977), and are normally unable to colonize habitats in the presence of the stable climax community. Only after autochthonous organisms have been displaced from habitats and niches by perturbation, can the allochthonous microbes colonize (Alexander, 1971).

1.3 THE GASTROINTESTINAL MICROBIOTA

1.3.1 Stomach

The environmental conditions present in the stomach are hostile to most microorganisms with rapid clearance of food and long periods of being empty of digesta, and also a low pH (Luckey, 1974). Because of the periods of emptying it is unlikely that there is an autochthonous microbiota in the stomach lumen (Savage, 1977); the organisms isolated from this site are transient, originating from ingested food or from the mouth (Drasar et al., 1969). Autochthonous microbes can be found in the stomachs of some animals such as rats, mice and pigs, but only in close association with the surface of the gastric mucosa. Unlike man the stomachs of these animals are divided into two portions, one of keratinized

stratified squamous epithelial (non-secreting) cells, and another of columnar secreting epithelial cells. The surface of the keratinized region is colonized by large numbers of Gram-positive microbes in: mice (Dubos et al., 1965; Savage, 1969; Roach et al., 1977), rats (Suegara, 1975; Watanabe et al, 1977), and pigs (Fuller et al., 1978; Barrow et al., 1980). Cultural studies indicated that the organisms were predominantly lactobacilli, with streptococci occasionally being isolated (Savage, 1977). Similar types of bacteria have also been observed in an analogous environment in chickens: associated with the surface of the mucosa of the crop (Brooker and Fuller, 1975). When sections of gastric mucosa from the mouse or pig were examined with the light microscope (LM), thick layers of Gram-positive material were seen adjacent to the surface (Dubos et al., 1965; Brooker and Fuller, 1975; Roach et al., 1977). These layers were composed of mainly rod-shaped bacteria with some cocci forms also being present. Scanning electron microscopy (SEM) studies of mouse gastric mucosa revealed that three morphotypes were present: most numerous were short round-ended rods,less numerous were short rods in chains, and less frequently rod or coccoid-shaped forms in filaments (Savage and Blumershine, 1974). The first two of these morphotypes were considered to be lactobacilli, the third was of uncertain identity. A feature of the association between each morphotype and the tissue surface was the occurrence of end-on attachment. A detailed study of neonatal pigs by

Fuller et al (1978) using LM, SEM and transmission electron microscopy (TEM) revealed similar findings; lactobacilli, and less frequently streptococci were found closely associated with the keratinized portion of the stomach, with the rod-shaped organisms attaching to the tissue end-on. The ultrastructural studies often demonstrated the presence of extracellular material, and micro-fibrils extending from the bacterial cells to the keratinized cells and presumably these were involved in the attachment of the organisms to this surface. End-on attachment could be advantageous to the organisms by permitting more to attach to a limited area, and also by facilitating access to stomach contents (Takeuchi and Zeller, 1972; Lee, 1980). While the lactobacilli colonizing the keratinized portion of the stomach fit the criteria for autochthony, the organisms colonizing the surfaces of the secretory portion, yeasts (Savage and Dubos, 1967; Savage, 1969), do not, as this population is only found in some colonies of animals (Savage and Dubos, 1967). The yeast, Torulopsis pintolopesii, can be found in layers only on the surface of the secreting portion, however, if the lactobacilli colonizing the keratinized cells are removed with antibiotics the yeast can also colonize this portion (Savage, 1969). The yeast and lactobacilli share two important features which enable them to exist in the stomach, the ability to grow in acid conditions, and the ability to attach to the mucosa (Artwohl and Savage, 1979; Lee, 1980). However these are not the only abilities that

determine whether organisms can colonize. Kotarski and Savage (1979) demonstrated that strains of Lactobacillus able to attach to stomach tissue in vitro were unable to form associations in mono-associated gnotobiotic mice. In contrast, T.pintolopesii, when given to germ-free mice was able to colonize both secretory and non-secretory surfaces (Suegara et al., 1979). This organism is also able to attach to cells from throughout the rodent gastrointestinal tract in vitro but not in vivo. It appears therefore that nutritional and environmental conditions are also important in determining whether attaching organisms can colonize the tissue surface (Suegara et al., 1979). Similarly the specificity of lactobacilli when colonizing hosts: porcine isolates will not colonize rodent stomachs and visa versa (Tannock et al., 1982), may be due not only to differences in specific attachment but also to different ecological determinants in the various habitats.

As noted previously (1.1), spiral-shaped bacteria have been reported to occur in the fundic glands of a variety of animals. Most reports of this association have been from studies of dogs (Lochard and Boler, 1970) and cats (Weber et al., 1958), though the organisms have also been observed in monkeys (Takeuchi, 1970) and man (Palmer, 1954). The organisms were seen in histological preparations, within the lumens of the fundic glands, and occasionally in the intracellular canaliculi, and cytoplasm of parietal cells (Lochard and Boler, 1970). TEM studies revealed that the organisms had an unusual and variable

morphology. Shadowed preparations of mucosal scrapings showed that the organisms were spiral-shaped with helical ridges running around the length of the cells, and also possessed tufts of polar appendages (Weber and Schmittdiel, 1962). Three morphotypes could be recognised in tissue sections: a. uncoiled, b. loosely coiled, c. tightly coiled,with each having a polar tuft of membrane bound appendages, and a periplasmic fibre coiled helically around the cell (Lochard and Boler, 1970). Lochard and Boler (1970) suggested that the different morphologies were three forms of the same bacterium. Taxonomically the organisms had similarities to members of the families Spirillaceae and Spirochaetaceae but the organisms could be classified as neither (Lochard and Boler, 1970).

1.3.2 The Small Bowel

The microbiota of the small bowel varies from site to site: it is very sparsely populated proximal to the stomach and heavily colonized close to the ileo-caecal junction. From studies on man it appears that the duodenum and jejunum contain very few microorganisms and lack autochthonous populations (Drasar et al., 1969; Mallory et al., 1973). After food intake a variety of bacteria can be found in the lumen at levels of up to 10^4 cfu/ml, however these organisms are likely to have originated from the food, or from further up the tract (Savage, 1977).

Probable explanations for the absence of microbes from the upper small bowel are similar to those suggested for the stomach: long periods of emptying,

active peristalsis, and an adverse chemical environment (Drasar and Hill, 1974). The small bowel is the primary site of catabolism and nutrient absorption. A wide variety of pancreatic enzymes delivered to the duodenum are active in the upper small bowel (Vander et al., 1975). Catabolic enzymes release large amounts of breakdown products such as amino acids and carbohydrates into the lumen of the tract, whilst bile acids facilitate the processing of lipids. The levels of enzyme activity are greatest in the upper regions of the small bowel and decrease towards the caecum. Similarly, due to dilution of bicarbonate ions, the pH decreases from alkaline in the duodenum/jejunum towards neutrality in the ileum; levels of conjugated bile acids follow a similar gradient (Vander et al., 1975). Peristalsis is also more vigorous, and transit of contents more rapid, at the top of the small bowel and less near the ileocaecal junction (Luckey, 1974). Experimental evidence suggests that physical factors are the most important in determining the microbiota in the small bowel. When peristalsis is reduced either as a result of disease (King and Toskes, 1979) or drugs (Lifshitz et al., 1978) large numbers of bacteria can be found in the upper small bowel. In contrast the antibacterial nature of bile acids, identified in vitro, appears to be of questionable significance in vivo (Floch et al., 1972).

Conditions in the ileum, particularly the distal ileum, are more suitable for the establishment of permanent microbial populations; primarily due to the less vigorous

peristalsis, and the concomitant periods of stasis (Savage, 1977). A variety of organisms can be found at this site, often at high concentrations, e.g. lactobacilli at levels of 10^8 cfu/gm (Morotomi et al., 1975). Also present in the ileum are surface-associated microorganisms populating two distinct types of habitats: attached to the epithelial cells, or present in the nearby mucus layers (Lee, 1980). An attached population of segmented filamentous bacteria is found in the ilea of rats, mice, and also dogs (Hampton and Rosario, 1965; Davis and Savage, 1974; Erlandsen and Chase, 1974). The organisms are attached at one end of the cell with the other projecting towards the intestinal lumen. The organisms are composed of variable numbers of segmented sections of variable length. Extensive observational studies suggest the organisms have adapted to the ileal environment by developing a complex attachment mechanism and also an involved life cycle (Chase and Erlandsen, 1976). Attachment enables the organisms to remain near the tissue and to resist peristalsis, however it results in the organisms being irreversibly associated with the epithelial cells that are continually turning over. These cells divide at the base of the crypts of Lieberkuhn and migrate to the tips of the villi, to be sloughed off into the lumen. Total migration time for the cells is 40-50 hours: 20 hours in the crypts and 20-30 hours on the surface of the villi (Lipkin, 1973), where colonization by the filamentous bacteria occurs. The attached bacteria are therefore also sloughed off into the

lumen, and consequently need mechanisms to overcome the cell cycle: a specialized life cycle. The filamentous organisms are first seen as small bodies, termed holdfasts, attached to cells at the base of the villi (Davis and Savage, 1974; Chase and Erlandsen, 1976). As the cells migrate up the villi the bacteria grow, firstly as long rods, and subsequently by dividing into segments. Towards the tip of the villus, distal segments of the bacterium divide, and evolve into intracellular bodies, then into either holdfasts or spores. Both these specialized bodies then serve to perpetuate the organisms: the holdfasts are able to attach to cells at the base of the villi beginning a new cycle, while the spores can facilitate animal to animal transfer (Chase and Erlandsen, 1976).

Holdfasts attach to epithelial cells by inserting into deep invaginations of the basal membrane. The proximal end of the organism is tapered into a conical tip that resides deep in the epithelial cell. Sections of the insertion site reveal a series of electron-dense, and electron-transparent layers. The bacteria have a Gram-positive-like cell wall which is in very close contact with the epithelial membrane; however this membrane remains intact. Immediately adjacent to the eucaryotic membrane, in contact with the bacterium, is a very electron-dense zone 30-50 nm thick, and surrounding this zone in the cytoplasm is a network of filaments (Erlandsen and Chase, 1974). Erlandsen and Chase (1974) have suggested that these

filaments could be modifications of the cells terminal web and that they could facilitate attachment by some contractile mechanism. An alternative explanation for the attachment mechanism has been proposed by Snellen and Savage (1978). These authors noted that the host cells appeared more osmophilic at the attachment sites, in both LM and TEM observations of sections. Furthermore freeze-fracture studies showed this region to have a radically different texture than the surrounding tissue; therefore the authors proposed that the cytoplasm had gelled and that this process was involved in attachment.

The other, less studied, type of habitat in the ileum is the mucus adjacent to the tissue surface (Lee, 1980; Rozee et al., 1982). Mucus-associated populations can be found in the crypts of Lieberkuhn or in the layers overlaying the villi. Erlandsen and Chase (1972) found spiral-shaped bacteria to be common inhabitants of ileal crypts in rats; the organisms had an unusual ultra-structure with polar appendages and periplasmic fibres, and thus resembled the organisms seen in dog and cat fundic glands (Lochard and Boler, 1970). Previously similar microbes had been seen in the small bowel of immuno-suppressed mice (Quastler and Hampton, 1962; Hampton, 1967), however the organisms were intracellular and were not seen in the crypt lumens.

The microbiota found in the mucus layers lining the small bowel has been poorly studied, mainly due to the difficulty in preserving this fragile and easily disrupted

substance. Nelson and Mata (1970) observed that bacteria could be seen embedded in the mucus covering the human jejunal mucosa. In a more detailed study Rozee et al. (1982) developed techniques to retain the small bowel mucus in histological preparations, and found that these layers in mice contained a variety of microorganisms including coccoid, bacillary, and segmented filamentous bacteria,and also protozoa. Information on the status of these organisms as being autochthonous has yet to be obtained, but the authors speculated that blankets of mucus containing large numbers of microbes may be important components of small bowel ecosystems.

1.3.3 The Caecum

The caecum provides an environment suited for the growth of large numbers of microbes. Like the complex stomachs of ruminants, the caecum in most monogastric animals is adapted to act as a fermentation chamber (Wolin, 1981). A major difference between the stomach or small bowel and the caecum, is the slow transit time for contents through the latter region. The contents in the caecum are in stasis for long periods, with peristalsis acting mainly to mix the material rather than removing it to the colon, and consequently the passage rate of contents is slower than the doubling time of bacteria (Savage, 1977). While the contents reaching the caecum have been depleted of: simple carbohydrates, lipids, peptides and amino acids, large amounts of complex carbohydrates unaffected by host enzymes provide large energy and carbon sources for the microbiota (Hoskins, 1981).

The caecum contains very high numbers of bacteria, e.g. up to 10^{10} cfu/gm wet weight of some species (Savage, 1977), and also a great diversity of species. In rodents the type of microbes most commonly seen are fusiform-shaped bacteria: this group is composed of a variety of species of genera such as Fusobacterium, Clostridium and Bacteroides, with similar morphologies (Dubos et al., 1965; Lee et al., 1971). Most of the species occurring in the caecum are autochthonous to this site; the input of bacteria from the ileum being several orders of magnitude less. The vast majority of organisms are strictly anaerobic and obtain energy and carbon from breaking down complex carbohydrates such as pectin and cellulose to oligosaccharides that are fermented, mainly via the Ender Meyerhoff-Parnous pathways, to yield a variety of volatile fatty acids as end products (Smith and Bryant, 1979). Another important source of carbohydrates is host glycoproteins, principally mucin. The ability of organisms to utilize mucin is discussed later in the review.

In addition to the lumen microbes, a variety of surface associated microorganisms have been reported to occur in many animals, often in high numbers. Fusiform-shaped bacteria are the most numerous of such populations in rodents, and can be found in the layers of mucus overlaying the mucosa (Savage and Dubos ,1967). Although seen in close proximity to the tissue surface, attachment probably does not occur; there seems little advantage in attaching, as the absence of vigorous peristalsis and the

mucus' gel-like properties ensure a stable habitat. Another group of organisms also found throughout the mucus layers of the rodent caecum are small spiral-shaped bacteria (Davis et al., 1973; Savage et al., 1971), which, unlike the fusiform bacteria, were rarely seen in caecal contents. Attachment of these organisms to the mucosal surface has not been reported in laboratory rats and mice but has been seen in the Japanese Spinus country rat Tokudaia osimensis osimensis (Kurohmaru et al.,1981).The organisms attach end-on,perpendicular to the surface. A similar association between spiral-shaped bacteria and the mucosa is also found in guinea pigs (Khenhaus and Odell, 1980).

The crypts of Lieberkuhn in the caeca of rats, like the ileal crypts, have been found to contain specialized populations of bacteria. Gustafson and Maunsbach (1971) found large numbers of bacteria in the crypt lumens of conventional rats. Organisms were often packed tightly together and in intimate contact with host cells, but did not penetrate the mucosal barrier. One morphological type was observed, and because of its tapered appearance the authors suggested the bacteria were fusiforms. Subsequently studies demonstrated that the organisms most commonly seen in caecal crypts are spiral-shaped bacteria. Davis et al. (1972) found three morphotypes, two in most animals, one more infrequently. The latter organisms had an ultrastructure very similar to the spiral-shaped bacteria seen in the ileal crypts:

having periplasmic fibres and bipolar tufts of appendages. The other two organisms seen by Davis et al. (1972) and also by Leach et al. (1973) were helically coiled spirochaetes and long, thin spirilla-like bacteria that had an ultrastructure similar to the organisms reported by Gustafson and Maunsbach (1971).

Similar crypt populations have also been found in other animals. Leach et al. (1973) found spirochaetes with a morphology similar to those seen in rodents, in the caecal crypts of dogs. Spirochaetes were also seen in the crypts of guinea pigs, although it was suggested the organisms were involved in Tyzzers disease (Zwicken et al., 1978). However, when larger numbers of these animals were examined these organisms appeared to be part of the normal caecal crypt microbiota (J. O'Rourke, personal communication).

1.3.4 The Colon

Conditions in the colon are very similar to those in the caecum, with a slow passage of highly reduced lumen contents, and a nutrient supply of complex carbohydrates. As the colon is inoculated with large numbers of caecal organisms, and also has similar conditions, the lumen microbiota of both sites is very similar. Likewise many of the mucus associated populations seen in the caecum are also present in the colons of the same host. In rodents, mucus layers populated predominantly by fusiforms and spiral-shaped bacteria are present in both sites (Savage and Dubos, 1968; Savage et al., 1971; Davis et

al., 1973), although the mucus was reported to be thicker, and containing larger numbers of bacteria in the colon (Savage and Dubos, 1968). Moreover the spirochaetes seen in the caecal crypts of guinea pigs, dogs and rats could also be found in the colons of the same hosts (J. O'Rourke, personal communication; Leach et al., 1973).

A number of studies in man indicate that micro-organisms can also colonize the mucus layers adjacent to the lumen colonic mucosa. Both aerobic and anaerobic bacteria have been isolated from this site (Nelson and Mata, 1970; Croucher et al., 1983), while SEM studies have shown a variety of morphological types of bacteria embedded in the mucus gel (Croucher et al., 1983). Of particular interest was the observation of very long (up to 60 µm), tightly coiled, spiral-shaped organisms in the mucus near the mucosal surface.

The presence of bacteria attached to colonic epithelial cells has been reported in several types of animals: dogs (Turek and Meyer, 1978), rhesus monkeys (Takeuchi et al., 1974), man (Lee et al., 1971), and rats (Wagner and Barrnett, 1974). In dogs, monkeys and man the association was between spiral-shaped bacteria and the surfaces of the colonic mucosa directly in contact with the intestinal lumen, while the organisms seen in rats were reported to be coliforms associated with cells in the colonic crypts. The coliforms attached by insertion of one end into a plug-like invagination of the host cell membrane. Considerable specialization of the attached tip

of the bacterium was observed and also striated material was seen between the eucaryotic and procaryotic cells; it was speculated that attachment was mediated by antibody (Wagner and Barrnett, 1974). The mode of attachment of the spiral-shaped bacteria was similar in each of the animals: one end attached to the eucaryotic cell with the organisms projecting perpendicularly towards the intestinal lumen. In dogs the organisms, spirochaetes, appeared to be members of the autochthonous microbiota (Turek and Meyer, 1978). An interesting observation from SEM studies, was that the organisms were often in phase, as though involved in co-ordinated movement (Turek and Meyer, 1978); this type of movement has been reported to occur in spirochaetes that associate with protozoa in the cockroach gut (Cleveland and Grimstone, 1964).

Tissue association by spirochaetes in the human colon was clearly demonstrated by Harland and Lee (1967) and subsequently in numerous studies: for example Lee et al., 1971; Takeuchi et al., 1971, 1974; Minio et al., 1973; Sanna et al., 1982; Antonakopoulos et al., 1982). Examination of these reports leads to the conclusion that spirochaetes are not autochthonous to this site, as only a small proportion of tissue samples from normal patients are colonized: for example Takeuchi and Sprinz (1970) found only 3% of normal adults were colonized. In rhesus monkeys, where a very similar association between spirochaetes and the colonic mucosa occurs, the incidence is much greater with 28% of healthy adults colonized

(Takeuchi et al., 1974); although again the organisms could not be classified as being autochthonous. The role and significance of these bacteria in the colonic ecosystem is not understood, however the mode of association has been well studied. In both primates the most numerous tissue-associated bacteria are long loosely coiled Borrelia-like spirochaetes (Takeuchi and Zeller, 1972; Lee et al., 1971), that are found in dense aggregates, with one end embedded into a deep invagination of the host cell's basal membrane (Neutra, 1980). The eucaryotic membrane was intact and, other than a disruption of the microvillus border, there were few alterations to the host cells (Takeuchi et al., 1974). Another spiral-shaped bacterium has been observed associated with the colonic mucosa in both monkeys (Takeuchi et al., 1974; Takeuchi and Zeller, 1972; Neutra, 1980) and man (Takeuchi et al., 1974). These organisms have polar flagella and, like spirochaetes, attach end-on to the surface, however they do not indent the basal membrane, but instead just insert into the microvillus border (Neutra, 1980).

1.4 FACTORS INFLUENCING THE GUT MICROBIOTA

The climax communities found in the gastrointestinal tract are formed as a result of the interplay of numerous factors, and some of the more important are described below.

1.4.1 Motility, pH, and Bile Acids

The importance of these factors in influencing the nature of the microbiota in different regions of the tract have been referred to previously (1.3.1-4.). The slow growth rates of gut bacteria (Gibbons and Kapsimalis, 1967) coupled with rapid flow rates in the stomach and upper or middle small bowel make colonization very difficult, while the slow transit times in the lower ileum, caecum and colon favour bacterial proliferation. Hydrogen ion concentrations of near neutrality generally favour the growth of a diverse range of microorganisms throughout the intestine while the low pH in the stomach selects for aciduric organisms. As previously stated the importance of bile acids in microbial ecology is uncertain (1.3.2). If a regulatory role does exist for bile acids the organisms most likely to be affected are allochthonous species such as the microbes in food and from the mouth or stomach (Savage, 1977; Sakai *et al*, 1980).

1.4.2 Mucus

Mucus has been attributed many functions in the gastrointestinal tract, however despite its ubiquitous nature it remains poorly understood (Allen, 1981). A primary function is the protection of the mucosa from abrasion by food particles (Florey, 1962). Another important role is as an aqueous gel augmenting the epithelial glycocalyx as a site for enzyme action, and also as a sink for breakdown products (Alexander, 1971). The mucus may also protect the epithelial cells from

potentially damaging host enzymes; although easily permeated by H^+ the unstirred mucus gel could prevent larger molecules such as pepsin from reaching the surface (Allen, 1981). A further property suggested is as an antibacterial and antiviral agent (Forstner, 1978).

A function of mucus unrelated to its rheological properties may be as an energy source for gut microbes. *In vivo* studies comparing conventional animals to germ-frees have indicated that considerable degradation of mucin is achieved by the normal microbiota: while little mucin is excreted in conventional faeces, large amounts are found in germ-free stools (Lindstedt *et al.*, 1965; Hoskins and Zancheck, 1968). Although the total microbiota can be shown to degrade mucin the ability of individual species to do so has,in comparison to the degradation of complex plant polysaccharides, been difficult to detect (Salyers *et al.*, 1977; Vercilloti *et al.*, 1977).

Mucin has a complex structure with a central protein core from which oligosaccharide sidechains project, giving the molecule an appearance analogous to a bottle brush (Reid and Clamp, 1978). Degradation begins with cleavage of the outer sugar residues and proceeds stepwise along the oligosaccharide towards the protein core (Roberton and Stanley, 1982). The outer sugars are α-glycosidically linked, the inner residues β-glycosidically linked,and thus degradation requires the sequential actions of a number of specific glycosidases (Hoskins, 1981). Only a small proportion of gut microbes are capable of producing these

extracellular enzymes, e.g. less than 5% of the faecal microbiota in man (Hoskins and Boulding, 1981). However the extracellular nature of the glycosidases means the enzymes can be found throughout the intestinal lumen (Prizont and Konigsberg, 1981) and thus probably benefit the other gut microbes. Degradation needs the presence of a mixed microbiota as most organisms are only able to produce some of the glycosidases necessary; this may explain the ineffectiveness of individual species to degrade mucin in vitro (Roberton and Stanley, 1982).

1.4.3 The Immune System

Interactions between the immune system and the gastrointestinal microbiota are poorly understood, and hence how this host factor influences the composition of the climax community is uncertain.

The large numbers of microbes in close proximity to the permeable mucosal surfaces must result in exposure of the immune system to appreciable amounts of microbial antigens. While such exposure stimulates development of immunologically active tissue in the lamina propria and Peyers patches (Ferguson and Parrott,1972), and also the production of natural antibodies (Walker, 1976), the response of the host immune system to its gut microbiota appears to be minimal. For example, many of the organisms that attach to the tissue and often modify the surface structure of the epithelial cells, do not seem to elicit inflammatory responses (Hampton and Rossario, 1965; Takeuchi and Zeller, 1972; Wagner and Barrnett, 1974). A

possible explanation for this phenomenon has been provided by experiments testing the immunogenicity of autochthonous bacteria. Foo and Lee (1972, 1974) found that serum antibodies to Bacteroides spp., autochthonous to the mouse large bowel, were not increased following parenteral injections of the organisms into its host; in contrast the organisms elicited strong responses when inoculated into other types of animals. When tested there was cross-reactivity between the Bacteroides spp., and foetal mouse intestinal tissue, implying that the organisms were recognized as "self". The bacteria used in these studies were from the lumen of the tract and it is not known whether mucosa associated microbes also avoid immune response by being recognized as host tissue (Lee, 1980).

If the immune system does influence the gastro-intestinal microbiota the most likely mechanism would be the actions of IgA. This type of antibody is transported to the gut by two methods: a. as secretory IgA transferred through the epithelial cells into the lumen of the tract (McNabb and Tomasi, 1981) or b. in some animals, including rodents, serum IgA can be injected into the gut via bile (Jackson et al., 1978). While the effects of this antibody on gut microbes is unclear a number of interactions have been suggested, and include inhibition of attachment, toxin neutralization, prevention of antigens reaching the mucosa, and influencing mucin production (McNabb and Tomasi,1981). Evidence for the first of these mechanisms has come from studies of allochthonous bacteria such as Vibrio cholerae

and enterovirulent Escherichia coli, however it has not been demonstrated with autochthonous microbes (McNabb and Tomasi, 1981).

Another means by which the immune system could influence the gut microbiota has been suggested by Erlandsen and Chase (1972). These authors noted the presence of both the protozoa Hexamita muris, and spiral-shaped bacteria in paneth cells located at the base of crypts in the ilea of rats and suggested these phagocytic cells may play some role in the regulation of the gut microbiota.

1.4.4 Diet

The influence of diet on the gastrointestinal microbiota is the source of some controversy (Savage, 1977). Many studies correlating diet and faecal microbiota in man have been undertaken, however the results have often been contradictory (Savage, 1977). While some differences of faecal biota have been found in people from various cultures fewer dissimilarities have been found to occur between groups within a society (Finegold and Sutter, 1978). Experimental changes in diet usually produce minor alterations in the faecal microbiota; for example, consumption of low residue liquid diets led to little change in the faecal biota of subjects (Holdeman et al., 1976; Attebery et al., 1972). However more substantial changes in the diets of experimental animals can cause considerable changes in the composition of the climax community. When mice were given wheat gluten or casein as

sole protein sources, the numbers of coliforms, enterococci, and slow lactose fermentors in faeces were similar; however the levels of lactobacilli were 5 to 20 times higher in the latter animals (Dubos and Schaedler, 1962). Similarly Brockett and Tannock (1981) found that the ability of lactobacilli to colonize the surface of the gastric mucosa of mice could be affected by the relative proportions of palmitic and oleic acids in the diet, although it was not known if this was a toxicity, or nutritional effect.

Diet can affect the gastrointestinal microbiota, not only by changing the numbers and types of microbes present, but also by influencing the biochemical activities of the microbiota (Brockett and Tannock, 1982). In mice the microbial production of indole, urease, β-glucuronidase, and Volatile Fatty Acids (VFA) in the caecum is different according to the relative amounts of protein, carbohydrate, fat, and fibre in the diet (Brocket and Tannock, 1982).

Fasting has also been shown to affect the composition of the mucosa-associated microbiota in rodents, however it is probable that the changes occurring are largely due to stress, and thus it will be discussed later in the chapter.

1.4.5 Interactions between Microorganisms

By definition the autochthonous microbiota is adapted to occupy habitats and niches in the gut, and therefore prevent other organisms from colonizing.

Possible mechanisms by which organisms interact include: competition for nutrients (Freter, 1962, 1983) and attachment sites (Garland *et al.*, 1982; Freter, 1983), production of conditions more suitable for autochthonous organisms, e.g. by influencing the reduction-oxidation potential, or by producing antagonistic substances, either metabolic end products such as VFA's or specific anti-bacterials such as bacteriocins. One, or a combination of these factors results in a barrier to colonization for allochthonous microbes: the so-called "colonization resistance" (Van der Waaij, 1979). Bacteriocins are produced by some intestinal bacteria such as *Bacteriodes* spp. (Booth *et al.*, 1977), however the small range of organisms affected, and unknown effectiveness *in vivo* makes the role of these substances in the gut environment uncertain.

Toxic metabolic end products have been proposed to be a major regulatory component in the gut. VFA's (Meynell, 1963) and H_2S (Freter, 1983) are toxic to allochthonous bacteria *in vitro*, furthermore toxic concentrations can be found in the large bowel, and can be correlated with resistance to allochthonous colonization (Meynell, 1963; Bohnoff and Miller, 1964; Lee and Gemmel, 1972). The VFA are most inhibitory at low redox potentials (Meynell,1963), and thus the highly reduced conditions present in the normal gut would be expected to enhance any toxic effect.

Recently it has been reported that toxic metabolites, specifically VFA and oxidative-reductive potential do not influence the numbers of aerobic bacteria in rat large bowel (Guiot, 1982). From *in vitro* and *in vivo* studies the author found that the contents of the caecum did not inhibit the growth of *E.coli* embedded in agar blocks containing nutrients, however if the nutrients were omitted very poor growth occurred. The author suggested that the availability of nutrients was the factor governing the ability of the *E.coli* to grow, rather than the presence of inhibitors; that is, nutritional competition postulated previously by Freter (1962). Similarly from studies with continuous-flow culture models of mouse large bowel microbiota, Freter and co-workers (Freter, 1983; Freter *et al.*, 1983; Freter *et al.*, 1983) have concluded that nutritional competition, not growth inhibitory substances, is the major regulator of *E.coli* growth in the large intestine. These authors have also suggested that attachment to host surfaces is an integral component of the ability of organisms such as *E.coli* to colonize the intestinal tract. Conversely an inability to form such associations was postulated to be a major reason why allochthonous microbes are unable to remain permanently in the gut.

1.4.6 Succession

An important aspect of the gut ecosystem is the establishment of the microbiota; a process affected by both host factors and the interactions between microbes.

Studies in rodents have established a definite sequence of colonization by microorganisms, i.e. a succession (Schaedler et al., 1965; Savage et al., 1968). Initially facultative anaerobes and lactobacilli colonize throughout the tract with the latter in high numbers in the stomach. Many of these organisms can be seen associating with the surface of the mucosa (Savage et al., 1968). Within a number of days spiral-shaped bacteria can also be observed associating with the large bowel mucosa (Davis et al., 1973). When animals begin ingesting solid foods, populations of strictly anaerobic bacteria begin to establish. There follows a period of instability as the autochthonous microbes displace many of the initial colonizing populations, however during this period the climax communities form, leading to a stable ecosystem.

1.4.7 Perturbation

Although generally homeostatic, the gastro-intestinal ecosystem can be disrupted by a variety of external influences. Factors known to alter the climax communities in the gut, particularly the microbes associated with the mucosa, include: diet, fasting and stress, changes in motility, gastric secretion, and pH, radiation, hyperbolic stress, chemically induced diarrhoea, food additives and antibiotics. It is probable that stress is a direct cause, or component of, changes induced by some of the above mentioned factors, nevertheless this is often difficult to demonstrate. Fasting produces variable effects, depending on the type of host animal; in some animals such as rodents major changes occur (Tannock and

Savage, 1974) and in others, such as hibernating squirrels (Barnes and Burton, 1970) few differences result. Detailed examination of the gastrointestinal tract in mice demonstrated that while the lumen microbiota generally remained at constant levels, the numbers of coliforms increased. Moreover many of the mucosa-associated populations were disrupted or absent from the fasted animals: the lactobacilli normally associated with the keratinized epithelium were not present, while fewer yeast cells were seen on the secretory component. In the intestines the numbers of ileal filamentous organisms and fusiforms present in large bowel mucus layers were both considerably reduced. A feasible explanation for the changes occurring in the mice, when none occurred in the other fasting animals, may be that the alterations resulted from stress rather than absence of food. Indeed similar changes were seen in rodents stressed by high atmospheric pressures (Merrell et al., 1979) or radiation (Porvaznik et al., 1979). Both studies, which were only on the effects on the small bowel, found that after treatment the incidence of facultative anaerobes rose, while the numbers of filamentous organisms diminished. In animals given 500 rads of γ-radiation the filamentous organisms quickly recolonized, though after a higher dose, 1000 rads, the organisms were still absent after 11 days (Porvaznik et al., 1979).

The effects of altered motility and gastric secretion on the gut microbiota have already been mentioned, however another more common perturbation

resulting from altered physiology is diarrhoea. Studies of diarrhoea in man caused by perfusion (Gorbach et al., 1970) or by chemical purgatives (Levison et al., 1969) have generally revealed only minor changes to the faecal microbiota. In contrast, in experiments where rats were given multiple doses of the purgative $MgSO_4$ over a six hour period, major changes in the faecal microbiota were observed (Leach et al., 1973). Six to eight hours after the final dose of $MgSO_4$ large numbers of spiral-shaped bacteria could be seen in the watery stools; these organisms are rarely seen in normal stools. Leach et al., (1973) suggested that these organisms originated from the mucosal surface and were dislodged by the inflammation, and excessive mucus secretion accompanying diarrhoea. As spirochaetes, and other spiral-shaped organisms have often been seen in the stools of a variety of animals with diarrhoea (Parr, 1923; Pindak et al., 1965; Zymet, 1969), it was further postulated that these organisms were dislodged mucosal populations rather than being the causative agents.

Severe gastrointestinal perturbations can be caused by antibacterial substances; the most studied being antibiotics. Savage and Dubos (1968) demonstrated that penicillin treatment removed the major bacterial populations in the large bowel of mice: the strict anaerobes and lactobacilli, and led to the establishment of a new, penicillin resistant, climax community. Furthermore many of the tissue associated microbiota such as the ileal filamentous bacteria, and the large bowel fusiforms and

spiral-shaped bacteria were also removed by penicillin treatment. When treatment was ceased the original climax communities re-established, although this often took several weeks. Other antibodies such as tetracyclines and kanamycin have similar, but less severe, effects on the gut microbiota (Savage and Dubos, 1968; Savage and McAllister, 1971).

The in vivo effects of penicillin, on the ileal filamentous bacteria, have been studied in detail from SEM and TEM observations (Davis and Savage, 1976). Within 3-5 hours of exposure to the antibiotic the morphology of the organisms changed, and after 10 hours the filaments disappeared from the ileum. Recolonization by these organisms occurred four to five weeks after the cessation of treatment; a period similar to the colonization of neonates by the same organisms.

Another chemical that has been shown to affect the mucosa associated populations of the rodent is the food dye erythrosine (Adams and Conway, 1981). High doses of this substance reduced or removed the lactobacilli in the stomach, the filamentous bacteria from the ileum, and the fusiforms from the large bowel. It was suggested that the food dye could be antibacterial, or alternatively it interfered with attachment of the microbes to the surfaces of the mucosa.

1.5. THE EFFECTS OF THE GASTROINTESTINAL MICROBIOTA ON THE HOST ANIMAL

As a result of advances in the techniques for maintaining and handling germ-free and gnotobiotic animals, the

significance of the normal gastrointestinal microbiota to the host animal has been studied extensively over the last 20-30 years (Gordon and Pesti, 1971; Levenson, 1978).

The microbiota has been shown to influence the host animals in many ways, the most significant being: altering the structure and development of tissues, affecting metabolism and nutrition, and developing resistance against disease. Not unexpectedly, it has been found that the relationship is very complex with both beneficial and deleterious effects to the host. Nevertheless the obvious adaption of host tissues such as the multiple stomachs of ruminants, and the caeca of mono-gastric animals to accommodate large numbers of bacteria indicate that the host probably obtains a net advantage from the association.

The main source of energy for the large bowel microbiota is, as previously noted, complex polysaccharides originating from the diet and host glycoproteins. The major fermentation end products from these compounds are VFA which are absorbed by the intestinal mucosa. While acetate and proprionate add to the host's energy store, it has been suggested that n-butyrate, the major end product, supplies directly up to 70% of the colonic cell's energy requirements (Roediger, 1980). Microorganisms have also been shown to influence the balance of a number of essential growth factors. Vitamin K, while being an essential additive to the diet of germ-free rodents, can often be removed from the diet of conventional animals without deleterious effects as the autochthonous microbiota produce enough of the vitamin for the host's requirements (Gustufson *et al.*, 1962). Other nutritional deficiencies

lessened by the gut biota include, Thiamin, folic acid, pantothenic acid, and choline (Levenson, 1978). In contrast other deficiencies are worsened by the presence of microbes: examples are Vitamins C, A and E, Riboflavin, Niacin and also a number of essential amino acids such as methionine (Levenson, 1978). Despite these deleterious effects it has also been shown that conventional rodents are able to survive starvation more successfully than germ-frees, despite loosing more weight (Levenson, 1978). The microbiota also induces increased metabolic rates and higher colonic temperatures; the reason for these alterations, and also the resistance to starvation, are not well understood (Levenson, 1978).

The morphology and histology of germ-free rodents' intestinal tracts are very different to conventional rodents, particularly in the small bowel. The weight and thickness of this tissue is considerably greater in conventional animals, mainly due to more cells in the lamina propria (Abrams et al., 1963; Abrams et al., 1967). The villi of germ-free rodents are longer and thinner (Abrams et al., 1963), the small bowel longer (Komai and Kimura, 1980) and with more epithelial cells (Savage and Whitt, 1982), nevertheless the surface area is 30% less than that of conventional animals (Gordon et al., 1961). Turnover of epithelial cells is accelerated in conventional animals, with the time taken for cells to transit, from the base of the crypts of Lieberkuhn to the extrusion zones at the tips of the villi, being about half that of germ-free rodents (Abrams et al., 1963; Savage et al., 1981).

Associated with the altered transit times are changes in enzyme activities in the brush border. Alkaline phosphatase activity is two to three times greater (Yolton et al., 1971; Yolton and Savage, 1976; Whitt and Savage, 1981), while disaccharidase activity is also elevated (Whitt and Savage, 1980, 1981). Increased enzyme activity is apparently due to accumulation of these proteins in the slowly migrating cells at the tips of the villi (activity elsewhere on the villi is similar in both types of animals), and also due to the greater number of epithelial cells in the germ-free animals. The small bowel histology of the germ-free animals can be quickly altered to resemble conventional animals by introducing a normal gastrointestinal microbiota (Wells and Balish, 1980; Whitt and Savage, 1980, 1981; Savage et al., 1981).

The differences in the structure of the bowel, seen when germ-free and conventional are compared, are less marked in the large intestine. While the colons of germ-free animals are longer (Komai and Kimura, 1980), and the caecal cells are slightly different shapes (Gustafson and Maunsbach, 1971), the most obvious difference to conventional animals is the size of the caeca: 3 to 4 times normal size (Gordon and Pesti, 1971). Similar enlargement of the caecum can also be obtained by disrupting the microbiota with antibiotics (Savage and Dubos, 1968; Savage and McAllister, 1971). The histology of the enlarged caeca is similar to conventional caecal tissue, however the contents are more hydrated. Explanations for enlargement are: accumulation of a muscle

relaxant (Gordon, 1965) or disruption of water transport (Savage and McAllister, 1971). Introduction of a complex intestinal microbiota into germ-free animals, or ceasing antibiotic treatment in conventional animals, leads to the caecum regaining its normal size; however only some members of the autochthonous microbiota are able to elicit the change (Savage and McAllister, 1971; Tannock and Savage, 1976).

Germ-free animals are acutely sensitive to many intestinal pathogens; for instance while 10^9 viable Salmonella enteritidis provide a 100% lethal dose in conventional mice, as few as <10 organisms are able to kill germ-free animals (Collins and Carter, 1978). Similar results are obtained if the autochthonous microbiota is disrupted by antibiotics (Miller and Bohnoff, 1963). Even less severe alterations in the intestinal climax community such as those caused by stress result in lessened resistance to Salmonella infections (Tannock and Savage, 1974). The means by which the normal gut microbiota protects the host against pathogens is not well understood but it is probably the result of several factors acting together to inhibit the invading organisms, or to stimulate the host immune system (Shedlofsky and Freter, 1974). Changes in histology caused by the presence of microbes, particularly development of the lamina propria, and other components of the immune system, are needed for optimum defence (Carter and Collins, 1974). In addition to providing local immunity, such stimulation can also confer systemic immunity against pathogens such as Salmonella typhimurium

(Roach and Tannock, 1980). Nevertheless as immunologically normal animals given antibiotics are very susceptible to intestinal infections it is clear that the presence of the climax community, or components of it, are very important to host defence. For example Garland *et al*. (1982) found that the ability of *Salmonella enteritidis* to colonize the small bowel of rats and to cause disease, closely correlated with the absence of the segmented filamentous population from this site. Such a correlation could show a protective role for the filamentous population, or be an indicator of changes occurring in the host that alter conditions for colonization (Garland *et al*., 1982).

Many of the mechanisms by which the normal gut micro-organisms could inhibit pathogens from colonizing have already been discussed: nutrient competition, production of toxic end products such as VFA and H_2S, and the creation of a highly reduced environment. A further mechanism suggested by Abrams and Bishop (1966) is that the normal microbiota increased intestinal motility and hence clearance of pathogens. The normal microbiota could also prevent disease even if pathogens are able to remain and multiply in the gut, by preventing the allochthonous population from occupying habitats or niches necessary for a disease aetiology: for example, the filamentous bacteria in the ileum could prevent *Salmonella* spp., colonizing the intestinal lumen, from associating with the mucosal surface (Garland *et al*., 1982).

In addition to the deleterious nutritional effects reported previously the gastrointestinal microbiota have

been implicated in the aetiology of a number of gastro-intestinal diseases. The normal gut microbiota has been shown to cause bacterial overgrowths following changes in small bowel propulsion (King and Toskes, 1979), while members of the microbiota have been shown to be involved in pseudomembranous colitis (Bartlett et al., 1978) and also necrotic enteritis (Egerton and Walker, 1964). It has also been postulated that the microbiota, or members of it, are also involved in the aetiology of diseases such as ulcerative colitis (Jacohina et al., 1975), Chrohn's disease (Axelsson and Justesen, 1977), and cancer of the colon (Moore et al., 1978), however in each case this has yet to be proven.

Improvements in techniques for the culturing and handling of fastidious, strictly anaerobic bacteria made over the last two decades have resulted in a large proportion of the gastrointestinal microbiota being isolated and grown in vitro. The amount of information on the microbiota has therefore increased greatly; moreover interpretation of this data has been improved by applying ecology theory. Nevertheless our understanding of this complex ecosystem remains incomplete. One component of the ecosystem that has been poorly studied is the mucosa-associated microbiota; primarily due to an inability to grow most of these microbes in vitro. The potential importance of this group of microorganisms has been highlighted by the recognition of the role that mucosal association plays in processes which are both beneficial to the host, such as nitrogen metabolism in the rumen (Cheng

et al., 1979), or deleterious such as infectious diseases (Savage, 1980).

The present investigations were undertaken to gain a better understanding of the mucosa-associated microbiota in the rat intestinal tract by determining: the prevalence of tissue association in normal animals, the nature and stability of the associations, and also, if possible, learning more about the microorganisms involved.

CHAPTER TWO

MATERIALS AND METHODS

2. MATERIALS AND METHODS

2.1 BACTERIAL GROWTH MEDIA

2.1.1 Prepared Media

2.1.1.1 Serum agar

Serum agar consisted of: Peptone (10 g), NaCl (5 g), Agar: Oxoid Technical grade (12 g), Distilled water to 1 litre and adjusted to a pH of 7.4.

The medium was autoclaved at 15 psi/15 min and cooled to 55°C. Horse serum (Commonwealth Serum Laboratories: CSL) was added to give a concentration of either 5% or 10%.

2.1.1.2 Horse blood agar

This medium was composed of: Columbia Blood Agar Base; Oxoid (39 g), and distilled water to 1 litre.

The medium was autoclaved at 15 psi/15 min and cooled to 55°C and the horse blood (CSL) was added to give a final concentration of 7.5%.

2.1.1.3 Lysed horse blood agar (LHBA)

The medium contained: Blood Agar base - No.2; Oxoid (40 g) and distilled water to 1 litre.

The medium was autoclaved at 15 psi/15 min and cooled to 55°C and then horse blood (CSL), that had been partly lysed by alternate freezing and thawing,was added to give a final concentration of 7.5%.

2.1.1.4 Campylobacter selective agar (CSA69)

CSA69 was prepared in the same manner as LHBA but with the addition of campylobacter selective supplement - Skirrow, (Oxoid) as per the manufacturers instructions

(1 ampoule per 500 ml of medium), along with the lysed blood when the medium was at 55°C.

2.1.1.5 Serum - egg-yolk agar

The medium had the same composition as serum agar but with 15% sterile egg-yolk suspension added in addition to 5% serum, when the medium was at 55°C.

2.1.1.6 Casein agar, starch agar, and tributyrin agar

Each of these media were made in the same manner as 5% serum agar with the modification of the addition of either 2% casein, 0.1% tributyrin, or 1% starch prior to autoclaving.

2.1.2 Commercially Obtained

The following media was prepared according to manufacturers' instructions: Brain Heart Infusion broth (BHI) and Brain Heart Infusion Agar (BHIA), (Oxoid), MacConkey's Agar (Oxoid), Nutrient Agar (Oxoid).

2.2 EQUIPMENT

The following equipment was used routinely; centrifuges: for use at less than 3,000 g Heka (Anax), for use at greater than 3,000 g Sorvall RC-SB Refrigerated Superspeed Centrifuge (Du Pont Instruments), Spectrophotometer: Unicam SP180 Ultra-violet Spectrophotometer (Pye), Microscopes: Phase Contrast, Riechart; Darkfield, Ziess; Brightfield, Olympus.

2.3 REAGENTS

Unless otherwise stated all chemicals used were high purity analytical reagents supplied by one of the following manufacturers: BDH Chemicals, May and Baker, Merck, Ajax Chemicals and Sigma.

2.4 CELL MAINTENANCE SOLUTIONS

2.4.1 Phosphate Buffered Saline (PBS)

PBS contained NaCl (8 g), K_2HPO_4-anhydrous (1.21 g), KH_2PO_4-anhydrous (0.34 g), dissolved in 1 litre of distilled water. The pH was adjusted to 7.3 and the solution autoclaved at 15 psi/15 min.

2.4.2 Physiological Saline

The solution contained NaCl (8.5 g) in 1 litre of distilled water and was sterilized by autoclaving at 15 psi/15 min.

2.4.3. Motility Buffer (Adler, 1973)

This solution contained the following components: potassium phosphate buffer pH 7.0, 10^{-2} M; and potassium ethylenediaminetetraacetate, 10^{-4} M, in distilled water. The solution was sterilized by autoclaving at 15 psi/15 min.

2.5 OTHER SOLUTIONS

2.5.1 Formal Saline

Formal saline was prepared by adding 10 ml of formalin (40% w/v formaldehyde in water with 10% methanol) to 990 ml of PBS.

2.5.2 Stock Methyl Cellulose Solutions

Appropriate amounts of methyl cellulose (Methocel MC 8000, Dow Chemical Co.) to form 1,2 and 4% solutions (i.e. 1 g, 2 g and 4 g) were added to 30 ml of Adlers Motility Buffer heated to 100°C, to form a slurry and then the remaining 70 mls of buffer added, the solution cooled to 4°C and mixed until all the methyl cellulose was dissolved. The solution was then autoclaved at 15 psi/15 min and cooled to 4°C to resolubilize the gel formed.

2.6 EXPERIMENTAL ANIMALS

2.6.1 Conventional Animals

Conventional Wistar rats and BALB/c mice were obtained from a colony kept at the University of New South Wales (UNSW). The animals were kept in plastic cages with sawdust litter and maintained on a diet of commercial pellets (Mecon) and water. Animals were weaned at 4 weeks of age.

2.6.2 Animals with Restricted Intestinal Microbiotas

Gnotobiotic and Specific Pathogen Free (SPF) Wistar rats and BALB/c mice, and germ-free Wistar rats were obtained from the Australian Atomic Energy Commission Animal Breeding Unit. The gnotobiotic and germ-free animals were maintained in germ-free isolators by germ-free techniques. The gnotobiotic animals were originally derived from germ-free Wistar rats and BALB/c mice; cultures of a Lactobacillus sp. and a Bacteroides sp. were established in rats, and a Lactobacillus sp., a Bacteroides sp, and Fusobacterium sp. were established in mice. These microorganisms were isolated from conventional animals of the same species. The animals were brought to the UNSW in germ-free transport cages, and then transferred to sterilized plastic cages and given presterilized food cubes (Mecon) and water. Mouse cages were covered with Isocaps (Carworth). Test and control animals were kept separately in laminar flow cabinets and away from conventional animals. On arrival and on the day of sacrifice fresh faecal material was suspended in five volumes of PBS, homogenized, and both inoculated onto MacConkey Agar plates and examined by phase contrast microscopy.

2.7 INOCULATION OF ANIMALS

Animals were given inoculations of various solutions and suspensions, either orally or orogastrically through Portex polythene tubing (sizes pp50 for mice and ppl00 for rats) fitted to metal needles (24 gauge and 18 gauge respectively) attached to plastic syringes.

2.8 PREPARATION OF SPECIMENS TAKEN FROM ANIMALS

2.8.1 Faecal Samples

Specimens were examined by phase contrast microscopy within a short period of collection and where fluid, mucus, or solids were present each component was separately observed and recorded. The solid material was suspended in PBS prior to observation, whereas fluid and mucus were examined directly.

2.8.2 Intestinal Specimens

Animals were killed by spinal dislocation. The entire length of intestine was removed and laid out on a clean flat surface. Sections of tissue were dissected for the preparation of intestinal scrapings or for embedding in epoxy resin.

2.8.2.1 Intestinal scrapings

Sections of tissue, 0.5 cm long were removed from the intestines at measured sites. These were slit open and the lumen contents gently removed. The tissue sections were then held in a pair of fine forceps and agitated violently in three separate 200 ml volumes of 0.85% saline contained in beakers. Each specimen was then placed, with the mucosal surface exposed, in a plastic petri dish and was scraped with the flat side of a clean scalpel blade.

The scrapings were then either observed by phase contrast microscopy or used to inoculate media.

2.8.3. Tissue Homogenates

2.8.3.1 Intestinal homogenates

Solutions containing homogenized intestinal tissue were prepared as follows. The intestinal tract was removed from the animal and the ileum, caecum and colon cut into small (<0.2 cm^2) pieces, combined and homogenized with a teflon grinder in 5 volumes of prereduced BHI broth, and then filtered through muslin cloth prior to inoculation.

2.8.3.2 Other tissues

Portions of liver, spleen and mesenteric lymph nodes were asceptically removed from the animal, weighed and then homogenized in BHI broth before being serially diluted and inoculated onto media.

2.9 BACTERIOLOGICAL METHODS

2.9.1 Incubation Conditions

All bacterial cultures were grown at 37°C. Anaerobic conditions were obtained by either incubating organisms in gas jars containing cold catalyst with GasPak H_2-CO_2 generators (BBL Microbiology Systems) or by incubation in an anaerobic chamber of similar construction to that described by Aranki et al. (1969): made of flexible vinyl with, a metal entry port, neoprene gloves, and filled with a mixture of 10% H_2 in CO_2 circulating past palladium catalyst. Microaerophilic conditions were obtained by two methods. Initially, the method of Butzler and Skirrow (1979) was used, i.e. a partial vacuum of 500

mm Hg (66.7 kPa) was drawn in a Brewer gas jar without a catalyst and replaced with 460 mm Hg (61.3 kPa) of nitrogen and 40 mm Hg (5.3 kPa) of carbon dioxide giving a final oxygen concentration of 7% with 5% CO_2. Alternatively,the organisms were grown in gas jars without a catalyst with GasPak H_2-CO_2 generators.

2.9.2 Preparation of Bacterial Suspensions

Suspensions of mucosal isolates and Proteus mirabilis were prepared by washing the surfaces of solid media with BHI broth; for the mucosal isolates LHBA plates with heavy growth were used, BHI agar plates with overnight growth were used for the Proteus sp.

Bacterial suspensions for use in the motility experiments were prepared from biphasic media: agar slopes overlaid with broth. The TSB and campylobacter isolates were initially grown on CSA69 slopes and Serratia marcescens grown on Nutrient Agar. Slopes with 10 ml of medium were inoculated and incubated for either 24 h (TSB and campylobacter-like bacteria) or 15 h (S.marcescens) to obtain surface growth, before peptone water (7.5 ml) was added. The slopes were incubated for a further 24 h (TSB and campylobacter-like bacteria) or 6 h (S.marcescens). The mucosal isolates were incubated in gas jars with microaerophilic atmospheres while the Serratia sp. was incubated aerobically in a shaking water bath (Patton Industries) at 20 oscillations per minute.

2.9.3 Storage of mucosal bacterial isolates

As many of the mucosal isolates were found to lose viability during freeze drying, all isolates were

routinely stored in liquid nitrogen. The organisms were grown on LHBA or CSA69 slopes and incubated until good growth was obtained and then the medium surface was washed with BHI broth containing 10% glycerol. Small aliquots of this dense suspension were dispensed into plastic screw top ampoules and placed into liquid nitrogen.

2.9.4 Biochemical Characterization of Bacterial Isolates

Hydrolysis of casein, starch and tributyrin were tested and interpreted in a manner similar to the methods described by Holderman and Moore (1977) and Roach et al. (1977), though 5% serum agar served as the basal medium. Casein agar plates, Starch agar plates and Tributyrin Agar plates were inoculated with the TSB isolates and incubated for 6 days. Casein hydrolysis was tested by pouring HCl over the surface; areas of clearing in the medium indicated hydrolysis. Similarly hydrolysis of starch was indicated by areas of clearing after the addition of Gram's iodine solution, and tributyrin hydrolysis by zones of clearing around the bacterial growth.

Lipase and lecithinase were tested on egg yolk agar supplemented with 5% horse serum. The medium was incubated for 6 days after inoculation and lecithinase activity indicated by zones of opalescence; lipolysis was seen as an irridescent layer and confirmed by a colour reaction with copper sulphate (Cruikshank et al. 1975). Vibrio cholerae was used as a positive control for each of these tests. Catalase and oxidase tests were carried out as described by Roach and Tannock (1979) from growth on 10%

serum agar plates. In all other tests the organisms were grown on 10% serum slopes to which liquid overlays were added and were then incubated for a further 6 days. Fermentation tests were done with peptone water containing 1% arabinose, cellobiose, galactose, glucose, glycerol, inositol, lactose, maltose, mannitol, raffinose, salicin, sorbitol, starch, sucrose, or xylose. After incubation, the pH of the solutions was determined with a pH electrode. Fermentation was considered to have occurred if the pH was less than 6.0. Bile sensitivity was tested by growth in an overlay of peptone water containing 2% bile salts (Oxoid). The overlays used for gelatin liquefaction, indole production, litmus milk, meat digestion, and nitrate reduction were prepared and interpreted as previously described (Cruikshank et al., 1975). The biphasic tests were found reliable when results for control organisms such as Escherichia coli and Pseudomonas aeruginosa were compared with those obtained by conventional methods.

The production of volatile and nonvolatile fatty acids after 14 days of growth in 1% glucose-peptone water overlaying a 10% serum slope was determined by gas-liquid chromatography. The fatty acids were extracted and prepared by the methods of Holderman and Moore (1977) and analysed with a Perkin Elmer F11 flame ionization gas chromatograph fitted with a glass column containing 10% SP-1000/1% H_3PO_4 on 100/200 Chromosorb WAW (Supelco). Sample peaks were identified by reference to standards prepared according to Holderman and Moore (1977).

2.10 ELECTRON MICROSCOPY

2.10.1 Tissue Samples

Small pieces (1 cm^2) of intestinal tissue were removed, cut along the mid-line of the intestine, rinsed briefly in physiological saline and placed in chilled Karnovsky's combined fixative: 3% formaldehyde, 3% glutaraldehyde (TAAB), in 0.1 M cacodylate buffer (Karnovsky, 1965) containing 0.05% Ruthenium Red (Luft, 1971). The tissues were then cut into thin longitudinal strips (1 mm x 4 mm) and fixed for 4 h and stored in 0.1 M cacodylate buffer. Immediately prior to embedding, specimens were post-fixed in Osmium tetroxide for 4h, in uranyl acetate for 1 h, and dehydrated in an ethanol, acetate series. The tissue blocks were then infiltrated with solutions of 50% acetone and 50% embedding medium, 10% acetone and 90% embedding medium, and finally 100% embedding medium at 60°C for 1h prior to transfer to embedding moulds for curing. The embedding media used were Araldite epoxy resin (Fluka) or Spurrs low viscosity epoxy resin (Polysciences).

2.10.2 Bacterial Cultures

Four-day -old plate cultures were harvested and fixed with Karnovsky's combined fixative with 0.05% ruthenium red. After 4 h, the suspensions were centrifuged, and the pellets were washed with cacodylate buffer and either embedded for sectioning or negatively stained with 2% sodium phosphotungstate containing 0.01% bacitracin. When embedding cultures, the washed pellets were resuspended in a small volume of 3% agar, cut into 1 mm^3 cubes, and embedded in the same manner as tissue specimens.

2.10.3 Preparation of Thick and Thin Sections

Thick sections (0.5 um) and thin sections (60 to 90 nm) were cut on a Huxley Ultramicrotome (LKB Instruments). Thick sections stained with methylene blue containing 1% borax were examined by light microscopy (LM). Thin sections stained with uranyl acetate and lead citrate, and negative stains were examined with a Philips 300 transmission electron microscope (TEM).

2.10.4 Freeze Dried Preparations of TSB

A heavy suspension of bacteria was spread on a cover slip and then plunged into liquid nitrogen. The specimen was then placed in the chamber of a freeze fracture apparatus (Balzers 400) pre-cooled to -170^{o}C and then cooled further to -196^{o}C. The temperature was then raised to -80^{o}C and sublimation allowed to precede for 40 min before the sample was shadowed with platinum/carbon and then carbon. The replica was removed onto water, washed with sodium hypochloride and rinsed with water before being placed on a copper grid and observed with the TEM.

2.10.5 Freeze Fracture Preparations of the TSB

The bacteria, prior to freeze fracture, were fixed in 1% gluteraldehyde for 1 h and then infiltrated with 15% glycerol for 2 h. Freeze fractures were prepared in a freeze fracture apparatus (Balzers 400) with a chamber temperature of -110^{o}C. The specimens were then shadowed and prepared in the same manner as the freeze dried specimens.

2.11 SEMI-QUANTITATIVE ASSESSMENT OF BACTERIAL COLONIZATION OF INTESTINAL CRYPTS

The level of crypt colonization by bacteria was determined by examining thick sections of tissue by bright field LM and assessing the numbers of bacteria, using a semi-quantitative counting system. A suitable (undamaged, undistorted) section was selected at low magnification, and aligned so that the left side was in the centre of the field of view. The section was then observed using an oil immersion objective and an ocular marked with a 7.5 um x 10 um grid. The first twenty intestinal crypts encountered, having an area of greater than 75 um^2, when the section was moved to the left, were assessed for bacterial numbers.

The rectangular grid was orientated so as to cover the area of highest bacterial cell density. The numbers of bacteria were estimated by reference to micrographs with 15, 30 and 45 bacteria per 75 um^2 and each crypt graded on a scale of 0 to 4; 0:having no bacteria present, 1: 1-15 bacteria, 2: 16-30 bacteria, 3: 31-45 bacteria, 4: 46-60 bacteria. For each site, i.e. ileum, caecum or colon, the scores of 20 crypts were totalled and then these scores from each of the replicate animals totalled and averaged. These averaged scores were the basis of comparisons between the colonization of different sites and colonization of the various types of animals.

The results of the colonization experiments were analysed using a Model 1 Two-Factor Analysis of Variance

with Replication (Zar, 1974). The variable in these analyses was the scores for individual intestinal sites, i.e. the score for 20 crypts, while the two factors compared were the tissue sampled (the three levels were ileum, caecum and colon) and the animal group tested (various numbers of levels). Interactions between the two factors were also tested. Where differences were found between levels in a factor, or where interactions occurred, the specific significant differences were determined using the Newman-Keul multiple range test (Zar , 1974).

2.12 ISOLATION OF RAT SMALL BOWEL MUCIN GLYCOPROTEIN

Rat small bowel mucin was isolated using the methods described by Bella and Kim (1972). The mucin was extracted from intestinal scrapings by homogenizing with a saline solution. The solution was dialysed, and then heat treated (50^{o}C) and the supernatant precipitated with centrimide and this material extracted with NaCl solutions. The mucin was then precipitated in ethanol, redissolved in distilled water, dialysed and finally freeze dried.

2.13 ESTIMATIONS OF MUCIN OLIGOSACCHARIDE SUGARS: SIALIC ACIDS AND METHYL PENTOSES

Methyl pentoses were estimated colourimetrically by the method of Dische and Shettles (1948) with rhamnose (Sigma) as the standard. Total (bound and free) sialic acids were estimated colourimetrically using the periodate-resorcinol method of Jourdian et al. (1971), and with n-acetylneuraminic acid (Sigma) as the standard.

CHAPTER THREE

THE MUCOSA-ASSOCIATED MICROBIOTA OF THE RAT: NORMAL AND PERTURBED STATES

3. THE MUCOSA ASSOCIATED MICROBIOTA OF THE RAT: NORMAL AND PERTURBED STATES

Specialized populations of microorganisms associating with the intestinal mucosa have only recently been recognized as important components of the gut ecosystem, and as a consequence have been poorly studied in comparison to the organisms of the intestinal lumen (Lee, 1980). Although the concept of surface association in the gastrointestinal tract was established from observations of rodents (Savage et al., 1967), our knowledge of the organisms associating with the rat and mouse intestine remains fragmentary. While organisms such as the segmented filamentous bacteria present on the ileal villi and the lactobacilli and yeasts seen in the stomach have been extensively studied, other surface associated microbes, such as those found in the intestinal crypts, have been poorly investigated. In the present investigation it was therefore necessary to have a more accurate assessment of the numbers and types of microorganisms associating with the intestinal mucosa of the animals to be studied, before any experimentation with the mucosal microbiota could be attempted. Consequently the first part of the chapter describes a survey of the mucosa associated microbiota of the ileum, caecum and colon, of the animals used predominantly in the studies: the Wistar rat.

Once the numbers and the distribution of the microbial populations associated with the intestinal mucosa had been ascertained it was then possible to begin wider

investigations into the nature of the mucosal ecosystem. One method used previously to study microbial ecosystems has been to observe the effects of experimentally induced perturbations on the climax community: for instance the mucosal environment has been investigated in experiments where the ecosystem was disrupted by agents including antibiotics (Davis and Savage, 1976), radiation (Porvaznik et al., 1979), stress and fasting (Tannock and Savage, 1974), and magnesium sulphate- ($MgSO_4$) induced diarrhoea (Leach et al., 1973),. The remainder of the chapter describes experiments testing the effects of intestinal perturbation on the rat mucosal microbiota. In the present investigation chemically-induced diarrhoea was chosen as a means of disturbing the mucosal microbiota as it had been previously suggested that $MgSO_4$-induced diarrhoea affected the microorganisms present in the intestinal crypts (Leach et al., 1973). Moreover this type of treatment had the advantage that the perturbation resembled events that would occur naturally in most animals, i.e. diarrhoea, and also would indicate the extent to which the host physiology could influence mucosal populations.

3.1.1. Intestinal Mucosa Associated Microorganisms in Normal Rats.

As most of the microorganisms associating with the rodent intestinal mucosa have not been isolated in pure culture it was necessary to use direct microscopic observations, rather than cultural techniques, when assessing these populations. A further advantage of

microscopic observations was that the localisation of the various organisms, with respect to the host tissue and other microorganisms, could be obtained. Fortunately microscopic studies were facilitated by low microbial diversity in the mucosal habitats, and the distinct morphology of many of the associated bacteria. The emphasis of this study was to investigate organisms intimately associated with the host tissue: those either attached to the microvillus border or found within the intestinal crypts. The presence of organisms loosely associated with the tissue, such as those located in the mucus layers between the tissue and the intestinal lumen were noted, but were not specifically studied in this survey.

It was found that the best method of studying the attached, and crypt dwelling mucosal microorganisms was by light and electron microscopic observation of embedded tissue. The occurrence and distribution of organisms was ascertained by examining thick sections with the light microscope; the numbers of bacteria associating with specific tissues were assessed by a semi-quantitative counting system (See Materials and Methods). The ultra-structure and mode of attachment of bacteria were studied by electron microscopy. Intestinal scrapings were examined to determine the presence and type of motility of the mucosa associated bacteria. These preparations were also useful for determining whether organisms were associated with sheets or strands of mucus.

Three sites from the intestine were sampled: the ileum 1-2 cm from the caecum, the tip of the caecum distal from the ileocaecal junction, and the colon 5-6 cm from the caecum.

3.1.1.1. Ileum.

Microorganisms were found attached to the surface of the ileal villi,within the crypts of Lieberkhun, and occasionally in the intervillus spaces. The organisms attached to the villi (Fig.1) had the same ultrastructure and distribution as the segmented filamentous procaryotes reported by other authors (Davis and Savage, 1974; Chase and Erlandsen, 1976).

The majority of the ileal crypts contained microorganisms (Table 1), with two distinct populations being present: thick spiral shaped bacteria (TSB) and very small crescent shaped bacteria (Fig.2). Although both types of organisms were present in all rats the numbers of each varied (Table 1); while similar numbers were found in most rats, in others one type was more prevalent. A similar distribution of organisms occurred in the crypts of individual animals; most colonized crypts contained both organisms however some were found with only one type.

The TSB were found throughout the crypts, with the highest concentrations in the region two-thirds proximal to the base. When large numbers of organisms were present the cells appeared to be in parallel clumps (Fig.2). At the base of the crypts the organisms had no particular

Figure 1. TEM observations of conventional rat distal ileum showing the attachment of segmented filamentous procaryotes to villus epithelial cells.

A. x 5,400, bar = 5 um.

B. x 33,000, bar = 0.5 um.


A

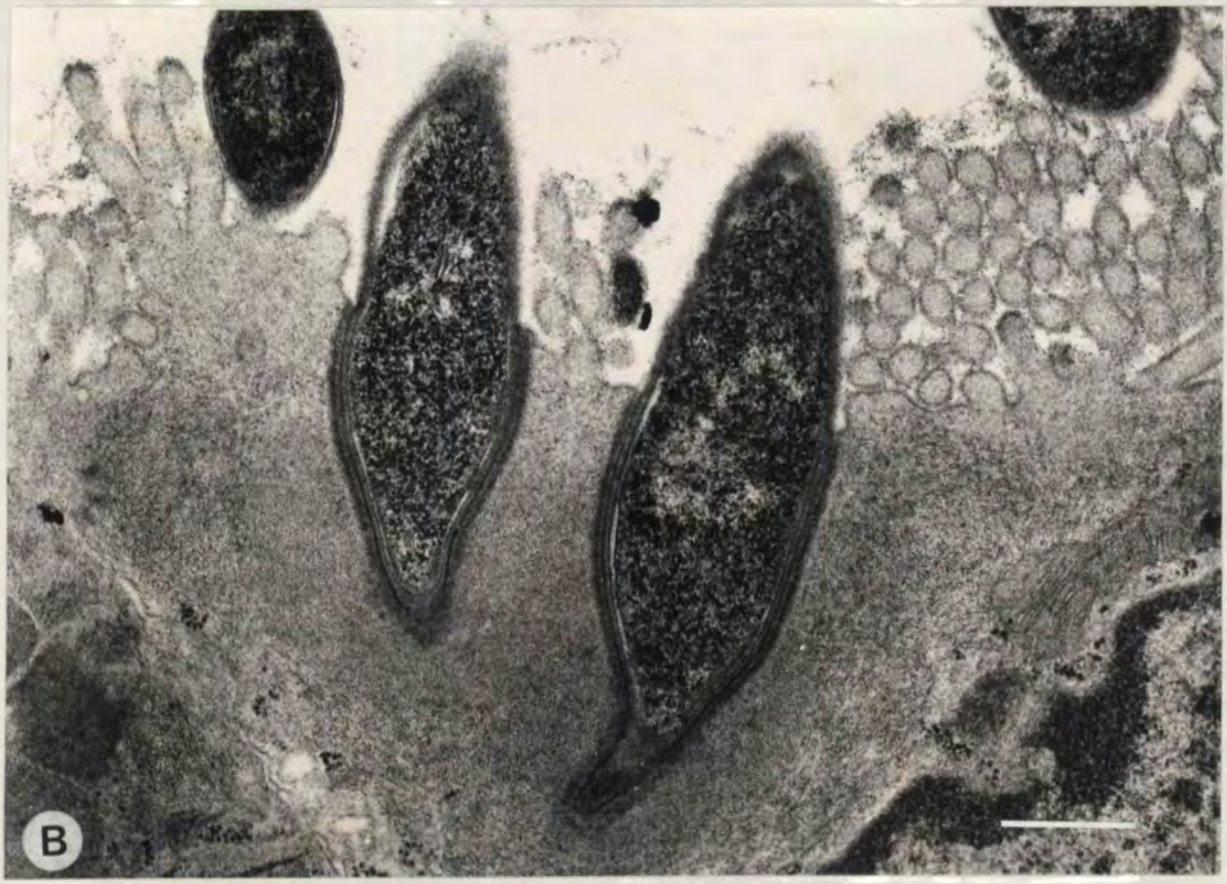

B

Table 1. Semi-quantitative assessment of crypt colonization in the ileum by TSB, crescent-shaped bacteria, and all bacteria.

Animal No..	1			2			3			4			5			6			7			8			9			10		
Population Assessed	S[a]	C[b]	T[c]	S	C	T	S	C	T	S	C	T	S	C	T	S	C	T	S	C	T	S	C	T	S	C	T	S	C	T
No.of crypts colonized (/20)	17	10	19	11	9	15	15	7	15	16	10	18	16	15	18	8	11	15	11	13	15	15	15	19	10	5	12	8	13	15
Semi-quantitative[d] counts																														
0 (0)[e]	3	10	1	9	12	5	5	13	5	4	10	2	4	5	2	12	9	5	9	7	5	5	5	1	10	15	8	12	7	5
1 (1-15)	13	9	15	5	6	8	11	7	11	5	9	3	9	11	8	5	5	7	9	10	12	2	2	3	4	2	5	2	6	6
2 (16-30)	3	1	3	3	2	3	3	0	3	5	1	5	4	3	5	2	3	4	2	3	3	7	10	11	4	2	4	5	4	6
3 (31-45)	1	0	1	2	0	3	1	0	1	3	0	6	2	1	4	1	0	1	0	0	0	3	2	4	2	1	3	1	3	3
4 (46-60)	0	0	0	1	0	1	0	0	0	3	0	4	1	0	1	0	3	3	0	0	0	1	1	1	0	0	0	0	0	0
Score totals[f]	22	11	24	21	10	27	20	7	20	36	11	47	27	20	34	12	23	30	13	16	18	29	32	41	18	9	22	15	23	27

Crypts colonized : S = 63.5%, C = 53.5%, T = 80.5%

Semi-quantitative scores, averages: S = 21.3 $\pm$ 7.1[g], C = 16.2 $\pm$ 7.6, T = 29.0 $\pm$ 8.8

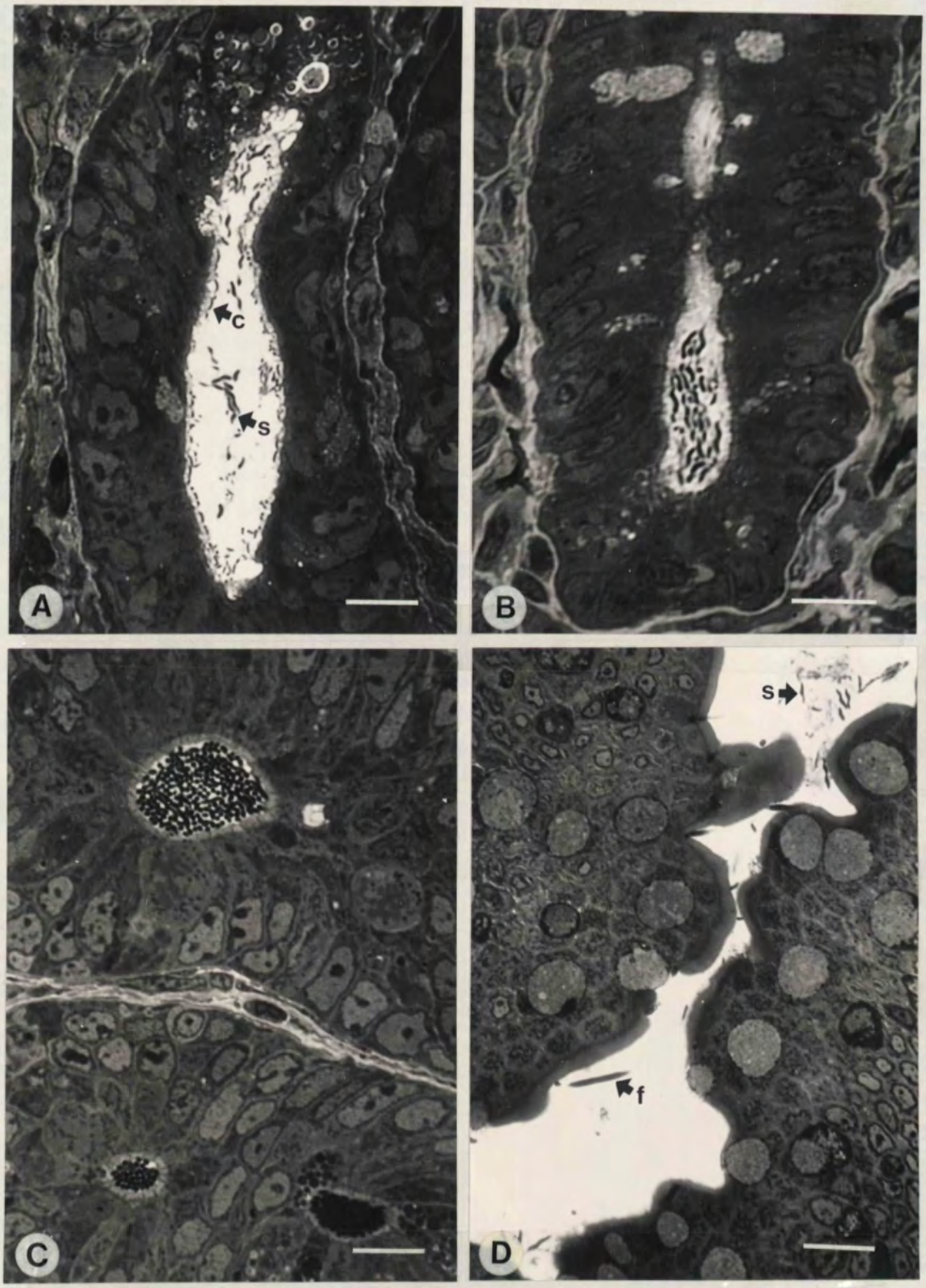
a : TSB
b : crescent-shaped bacteria
c : total of all bacteria
d : for details see Materials and Methods
e : No.of bacteria per 75 μm^2 assessment grid
f : total = No.of scores of 1x1 + No.of scores of 2x2 + No.of scores of 3x3 + No. of scores of 4x4
g : standard deviation

Figure 2. LM observations of conventional rat distal ilea demonstrating mucosa-associated microorganisms.

A. Longitudinal section of a crypt of Lieberkuhn showing two populations, crescent-shaped bacteria (C) and TSB (S) x 1340, bar = 10 um.

B and C. Parallel orientation of TSB in crypts B. Longitudinal section x 1470, bar = 10 um. C. Transverse section, x 1340, bar = 10 um.

D. Intervillus space containing both filamentous procaryotes (f) and TSB (S) x 1340, bar 10 um.


c
s
A
B
C
s
f
D

orientation, however from the middle to the opening of the crypts most cells were orientated parallel to the crypts' longitudinal axis (Fig.2). Occasionally the TSB were seen in the intervillus spaces (Fig.2). The TSB were randomly associated with the surface of the crypt tissue and there was no evidence of attachment. In contrast the crescent shaped bacteria were invariably seen clustered near the surfaces of the cells lining the crypts, with few in the crypt lumen (Fig.2). Like the TSB the crescent shaped bacteria were found in highest concentration in the lower regions of the crypts; they were rarely seen near the openings or the intervillus spaces.

Electron microscopy of the ileal tissue confirmed the presence of only two morphological types of bacteria in the crypts (Fig.3). The crescent shaped bacteria were small (2 x 0.35 µm), with a Gram-negative type cell wall, and were usually orientated horizontally compared to the basal membrane of the host cells. Although a gap of 0.02 µm was generally found between the organisms and the tissue no adhesion mechanism could be seen (Fig.3). The TSB were found to have a very distinctive ultrastructure (Fig.3), which was very similar to that of organisms previously seen in rat ileal crypts (Erlandsen and Chase, 1972) and also in rat caecal crypts (Davis et al., 1972).

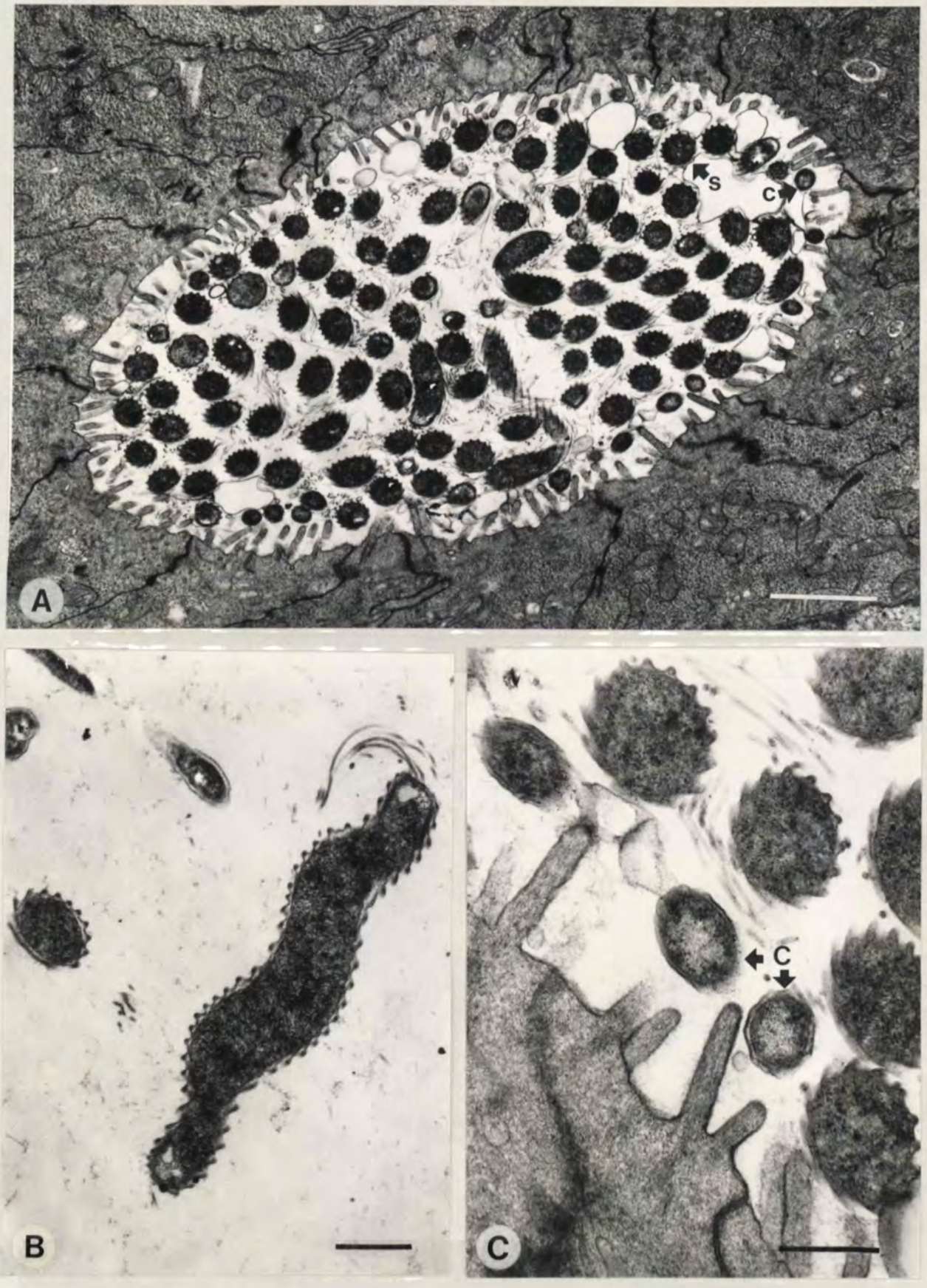
Ileal mucosal scrapings contained four major morphological types of bacteria: the three seen in thick sections, segmented filamentous bacteria, TSB, and small

Figure 3. TEM observations of mucosa-associated microorganisms in the crypts of conventional rat ilea.

A. Crypt with TSB (s) throughout the lumen and crescent-shaped bacteria (c) associated with the mucosal surface x 8,900, bar = 2 um.

B. TSB in transverse and longitudinal section x 25,800, bar = 0.5 um.

C. Crescent-shaped bacteria (c) adjacent to microvilli x 35,900, bar = 0.5 um.


S
C
A
B
C

crescent-shaped bacteria, and in addition, small campylobacter-like spiral shaped bacteria. TSB were seen in the highest numbers, often associated with mucus. Furthermore, in the presence of mucus, these organisms were highly motile by a corkscrew-like motion back and forth over the same one-dimensional path. In the less viscous components of the scrapings the organisms moved less rapidly and with a more random orientation. In preparations left for longer than 20-30 minutes the motility and rotational speed of the TSB decreased, and bundles, of what appeared to be flagella in a helical configuration, could be seen at the ends of the cells. The small crescent shaped bacteria were seen less frequently, were nonmotile, but were also found in aggregates associated with mucus and occasionally in rosette formations. The filamentous bacteria were seen less often and were variable in length and morphology; one end of the organism was tapered, and in longer forms the other end contained refractile bodies. Also seen infrequently were highly motile "S" shaped organisms which, in contrast to the TSB were equally motile in both mucus and saline components of scrapings and were also not found in aggregates.

3.1.1.2. Caecum.

The surfaces of the caecal mucosa in contact with the intestinal lumen were free of intimately associated microorganisms; in contrast 91.5% of the surveyed crypts contained bacteria (Table 2). Although there was some variation in the numbers of organisms in

Table 2. Semi-quantitative assessment of crypt colonization in the caecum.

Animal No.	1	2	3	4	5	6	7	8	9	10
No.of crypts colonized (/20)	20	20	17	19	18	19	18	13	20	19
Semi-quantitative [a] counts										
0 (0) [b]	0	0	3	1	2	1	2	7	0	1
1 (1-15)	5	3	6	1	5	5	2	2	3	3
2 (16-30)	3	4	7	1	7	4	8	5	8	5
3 (31-45)	6	6	3	4	4	2	1	2	4	5
4 (46-50)	6	7	1	13	2	8	7	4	5	6
Score Totals [c]	53	57	33	67	39	53	49	34	51	52

Crypts colonized = 91.5%

Semi-quantitative score, average = 48.8 $\pm$ 10.1 [d]

Table 3. Semi-quantitative assessment of crypt colonization of the colon.

Animal No.	1	2	3	4	5	6	7	8	9	10
No.of crypts colonized (/20)	13@	17@	18@	16@	10@	19	11	15@	18@	13
Semi-quantitative [a] counts										
0 (0) [b]	7	3	2	4	10	11	9	5	2	7
1 (1-15)	13	5	10	4	12	5	9	13	8	8
2 (16-30)	0	7	5	8	5	2	2	2	7	5
3 (31-45)	0	4	3	3	3	2	0	0	2	0
4 (46-40)	0	1	0	1	0	0	0	0	1	0
Score Totals [c]	13	35	29	33	31	15	11	17	32	18

Crypts colonized = 75.0%

Semi-quantitative score, average = 23.4 $\pm$ 8.9 [d]

Animals colonized with attached bacteria, 7/10

a: for details see Materials and Methods
b: No. of bacteria per 75 um^2 assessment grid
c: total = No. of scores of 1x1 + No. of scores of 2x2 + No. of scores of 3x3 + No. of scores of 4x4
d: standard deviation
@: population of attached bacteria present

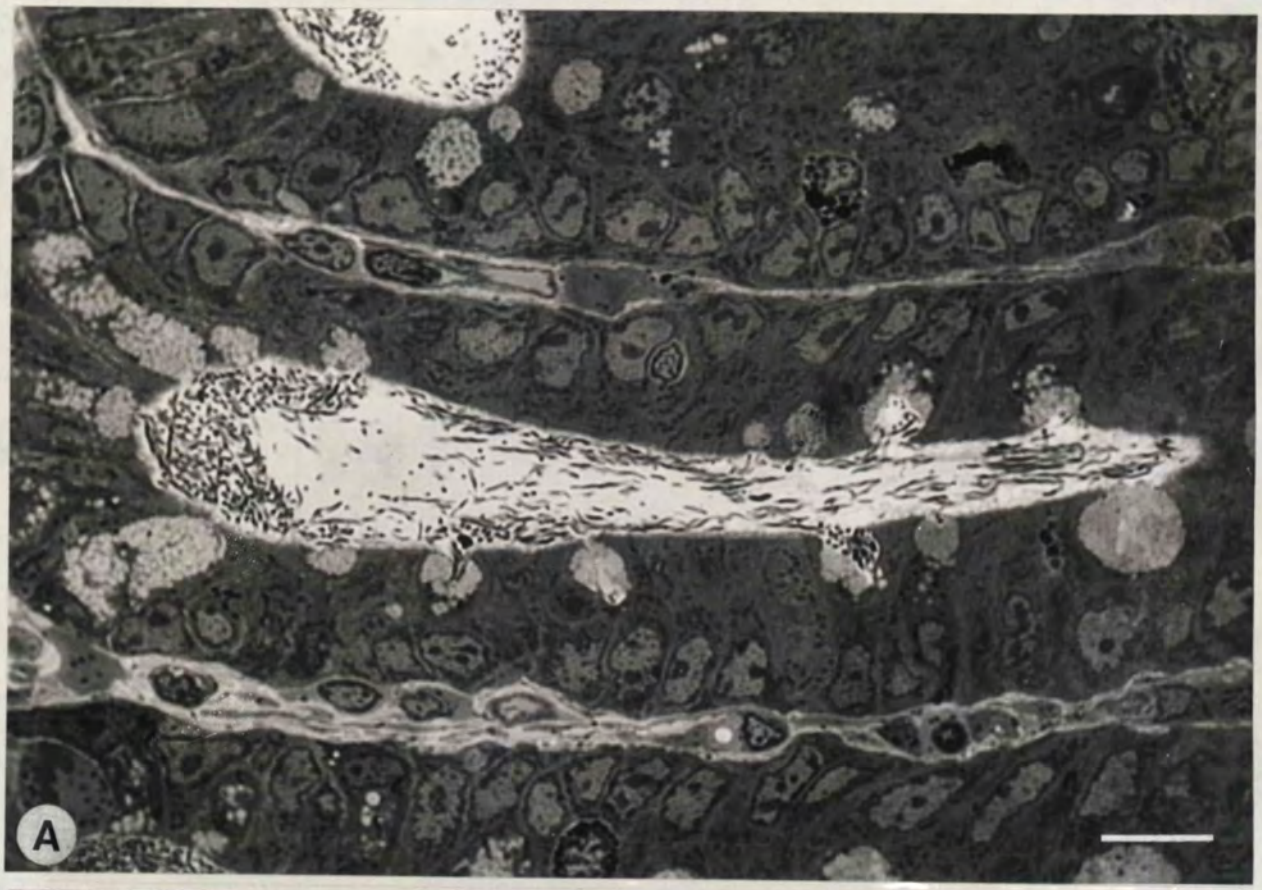
individual caecal crypts it was less than the variation occurring in the ileum or colon. Bacteria were most numerous at the base, and towards the middle of the crypts (Fig.4). Occasionally the openings to the crypts were heavily populated and organisms could be seen in the mucus being expelled into the intestinal lumen (Fig.4). None of the bacteria in the caecal crypts appeared to be attached to the tissue surface. The parallel clumping of bacteria seen in the ileum was also seen in the caecum (Fig.4). Similarly the distribution of organisms was the same as the TSB in the small bowel crypts: most organisms in the lower two-thirds. The orientation of the organisms was the same as the ileal TSB, but in sections the alignment of organisms was more obvious (Fig.4). Although different morphological types of bacteria could be discerned in thick sections it was not possible, with the exception of the TSB, to individually quantitate the various populations. When thin sections were examined by TEM, two major populations were seen: one of loosely coiled, Borrelia-like spirochaetes, and also one rigidly coiled spirillum-like bacteria (Fig.5). Individual crypts usually contained a predominance of one type of bacteria with none or low numbers of the other population. The spirillum-like bacteria were seen more frequently than the spirochaetes. Other bacteria were also seen occasionally in caecal crypts; the TSB were present in 9%, while fusiform bacteria and "S" shaped organisms occurred less frequently.

Figure 4. LM observations of mucosa-associated bacteria in conventional rat caecum.

A. Longitudinal section showing the distribution and orientation of bacteria in a crypt; the base of the crypt is to the left and the opening to the lumen to the right of the micrograph x 1390, bar = 10 um.

B. Clumping of crypt bacteria (C) x 1470, bar = 10 um.

C. Crypt bacteria (B) at the opening to the intestinal lumen x 590, bar 20 um.


A


C
B

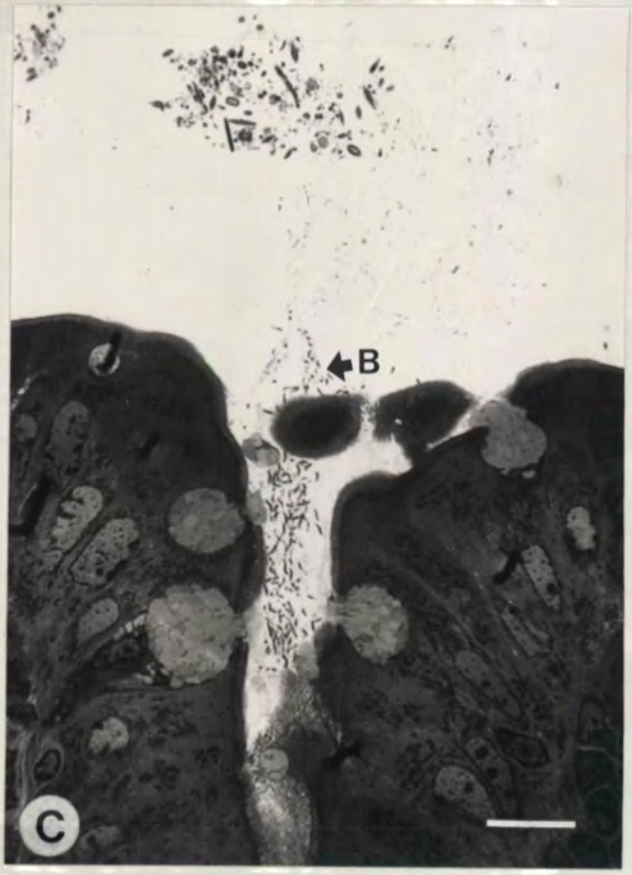
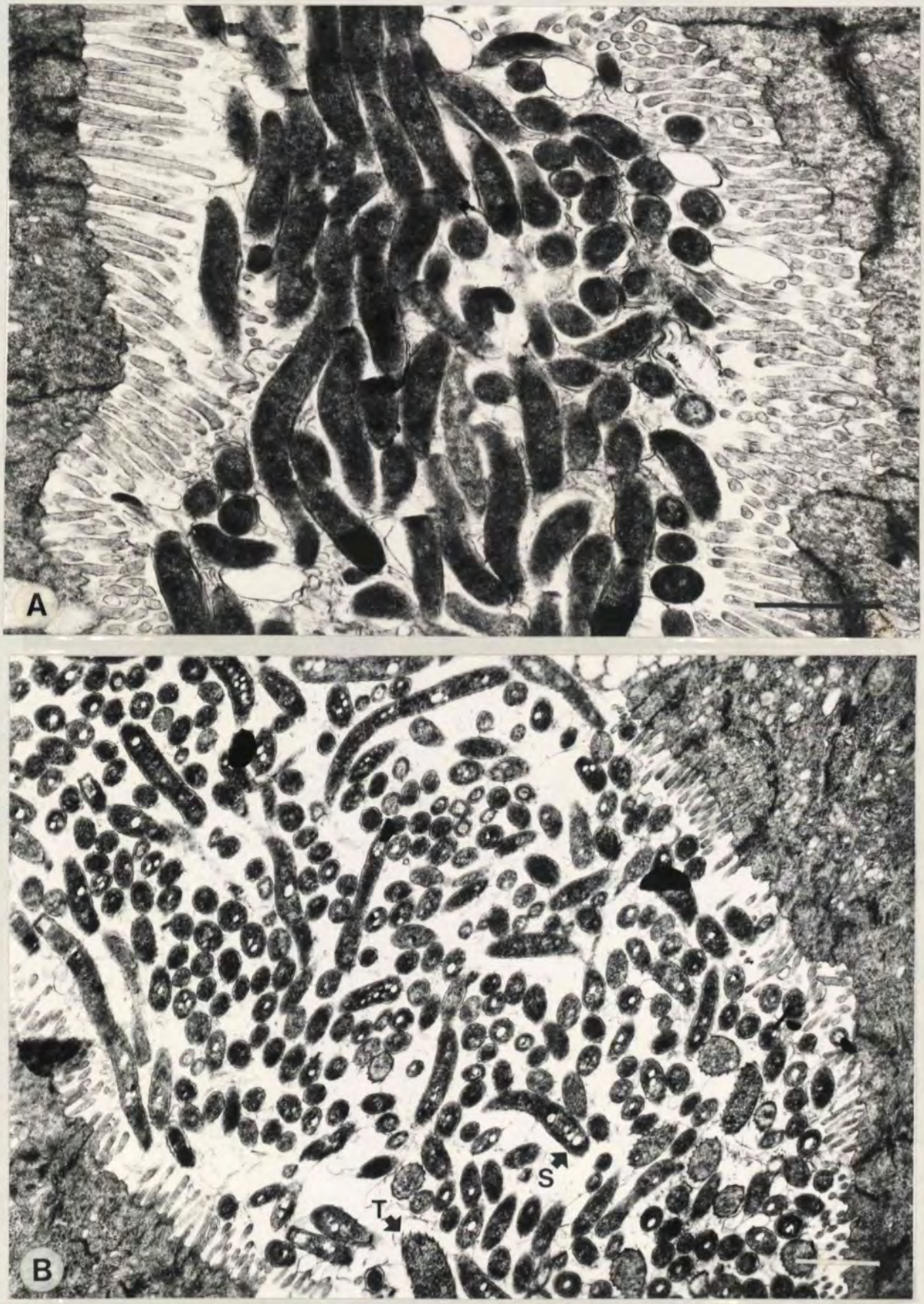

B
C

Figure 5. TEM observations of conventional rat caecal mucosa.

A. Large numbers of Borrelia-like spirochaetes in a crypt x 11,200, bar = 2 um.

B. Crypt containing spirillum-like bacteria (S) and TSB (t) x 15,000, bar = 1 um.


A
B
S
T

Scrapings of the caecal mucosa contained high numbers of bacteria, the most numerous of which were rigid spirillum-like organisms. Also present in large numbers were Borrelia-like spirochaetes; other organisms seen less frequently were "S" shaped bacteria and TSB. All these organisms were highly motile in mucus and less so in the saline components of the scraping; also all moved back and forth over defined one-dimensional paths. Similarly the majority of organisms were found embedded in mucus, the spirochaetes and spirilla in large aggregates, the others singly or in small groups.

3.1.1.3. Colon.

In the colon, as in the caecum, there were no microorganisms intimately associated with the mucosa surfaces adjacent to the intestinal lumen. Fusiform-shaped bacteria were occasionally seen near the surface however there appeared to be no evidence of attachment. The crypts of the colon contained numbers of organisms similar to those found in the ileum, although the composition of the colonic microbiota was more variable than that of either the ileal or caecal crypts (Table 3). In many animals the most frequently seen organisms were thin, loosely coiled, spiral-shaped bacteria that attached to the cells lining the crypt; however, these organisms were completely absent from some of the animals surveyed. In contrast a population of "S" shaped bacteria were found in the crypt lumens of all the animals and thus were the numerically predominant organisms in those animals lacking

the adherent population. Also seen in colonic crypts, although in low numbers, were TSB and fusiform bacteria.

The "S" shaped bacteria and the TSB were found throughout the crypts with the highest concentration in the middle region. The thin, spiral-shaped bacteria and the fusiform bacteria were rarely observed at the base of the crypts and were most numerous from the middle region to the area proximal to the crypt opening. When observed by electron microscopy the adherent population was found to be of one morphological type (Fig.6). The "S" shaped bacteria were also of similar morphologies but it was not possible to determine the homology of the group of organisms.

The thin, spiral-shaped bacteria preferentially attached to goblet cells with very few being found on the surface of columnar epithelial cells. Attachment of the bacteria was at right angles to the tissue surface, with many organisms being able to associate with individual crypt cells (Fig.7). One end of the bacterium was inserted into an invagenated socket in the host's basal membrane. A zone of 0.03 µm separated the procaryotic and eucaryotic cells and this area had a striated appearance suggestive of fibrillar material (Fig.8). The attached tip of the bacterium was modified to a plug-like shape with the membranes considerably thickened. The organisms possessed single, unsheathed, polar flagellar, and these were often seen at the attached, and in the striated zone. These flagella were often curved and presumably, like the

Figure 6. Conventional rat colonic mucosa.

A. LM observation showing crypts with both attached (a) and lumen (l) microorganisms x 1265, bar = 20 um.

B. TEM observation of crypt showing spiral-shaped bacteria attached to a goblet cell x 6,000, bar = 4 um.


a
l
A

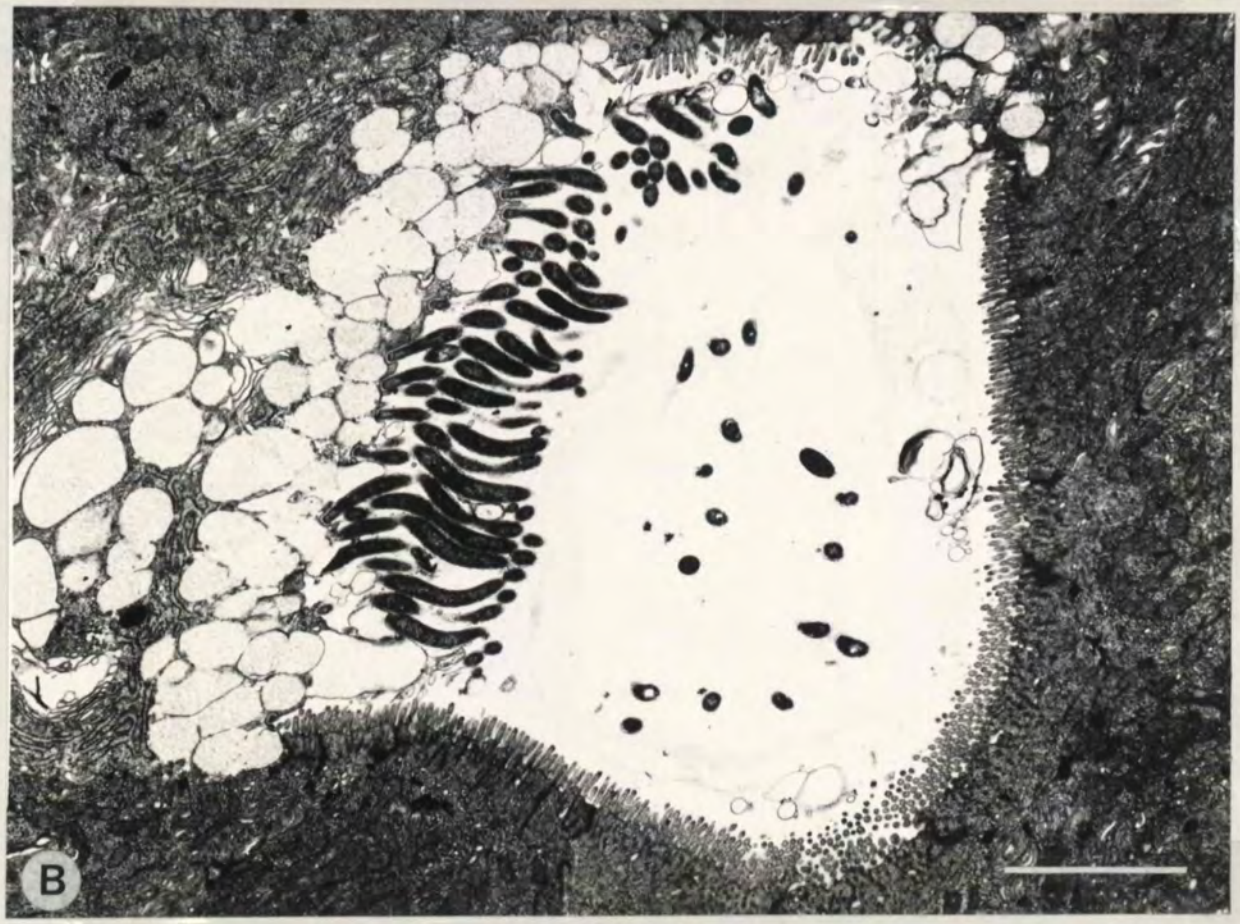

B

Figure 7. Conventional rat colonic mucosa. TEM demonstrating the attachment of spiral-shaped bacteria to a goblet cell, x 25,400, bar = 1 um.

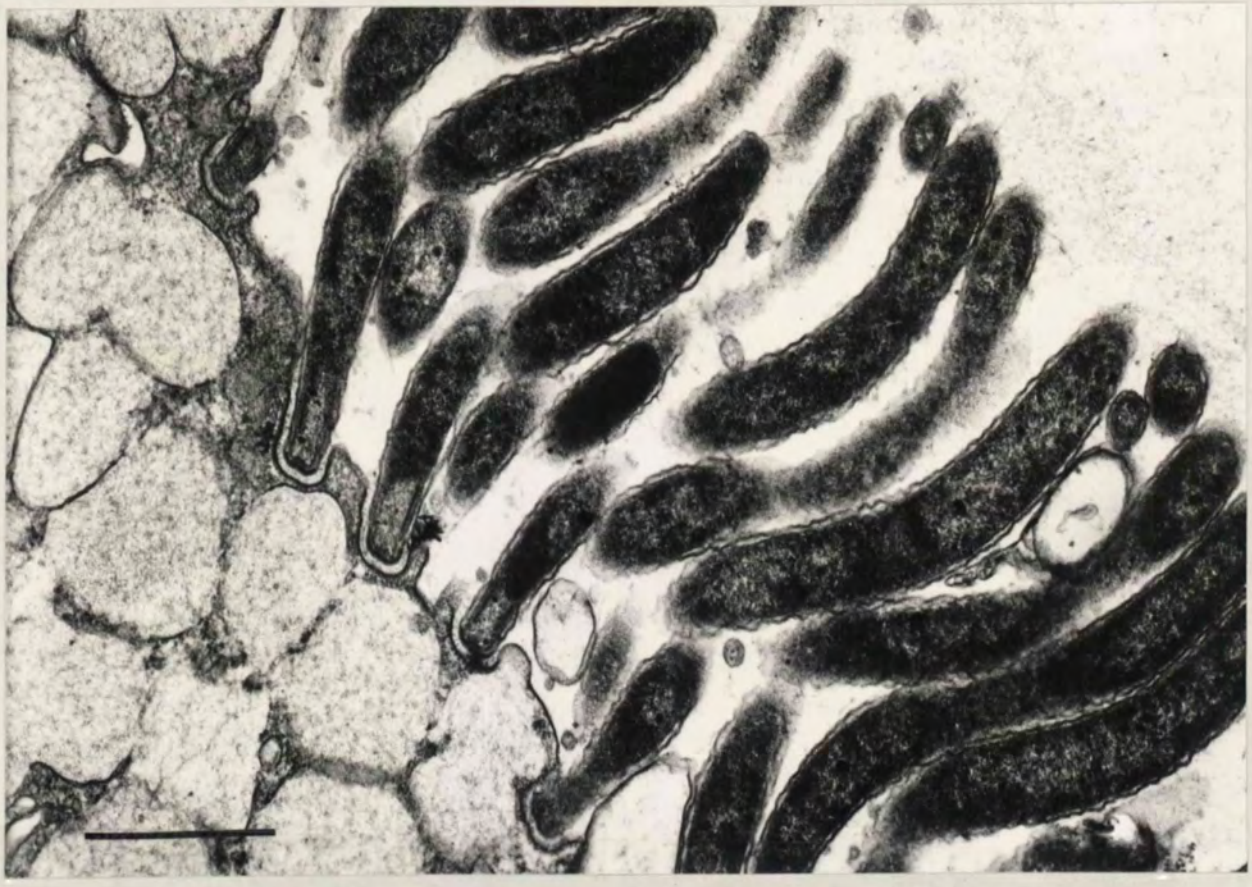


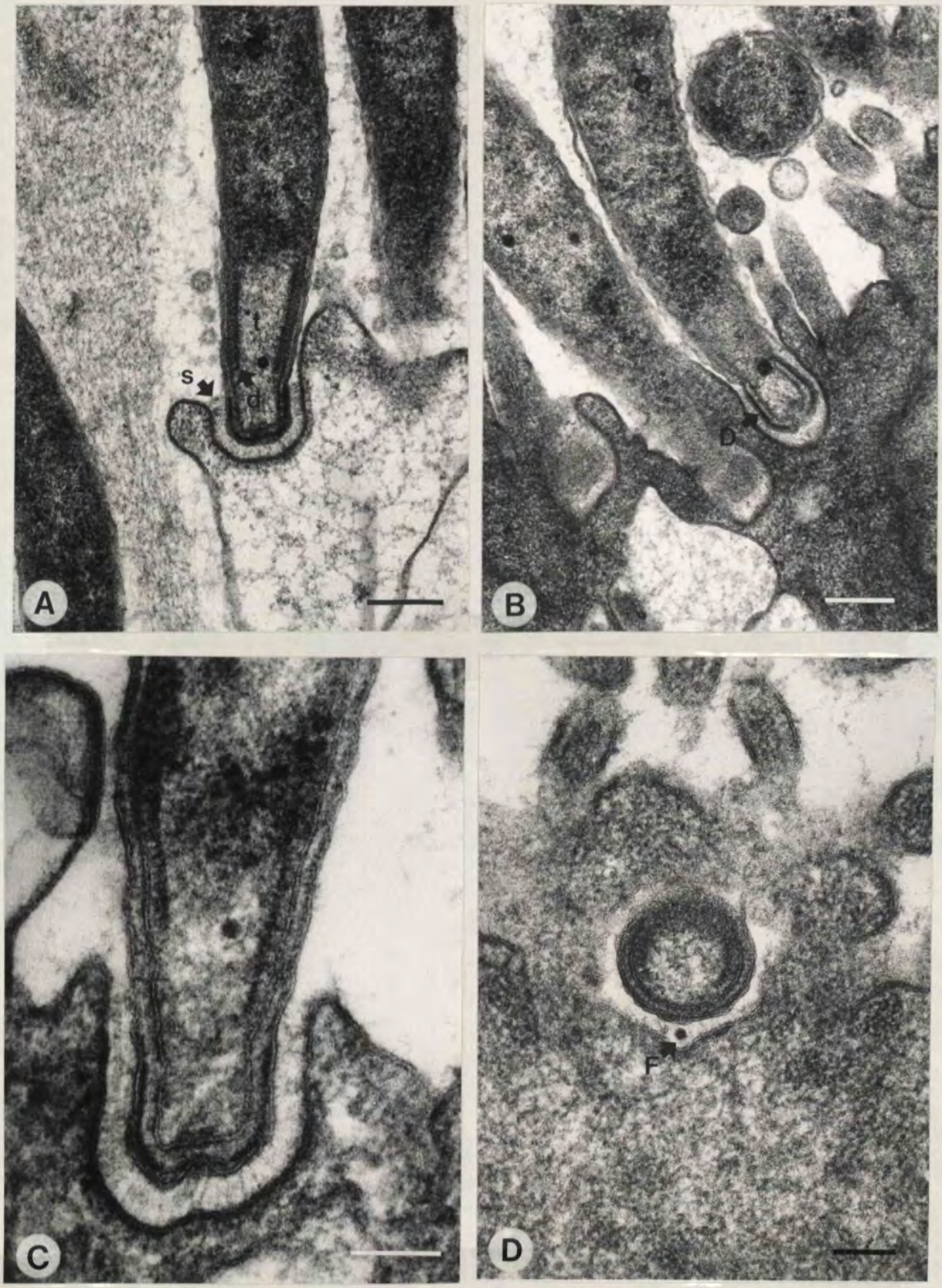
Figure 8. TEM observations of spiral-shaped bacteria attached to rat colonic crypt cells.

A. Attached tip of bacterium showing electron-translucent region (t), striated zone (s) and electron-dense band (d) x 66,400, bar = 0.2 um.

B. Attached tip of bacterium showing electron-dense material (D) on the periplasmic surface of the outer membrane x 61,500, bar = 0.2 um.

C. Higher magnification of attachment site x 156,700, bar = 0.1 um.

D. Transverse section of attachment site showing the presence of flagellum (F) x 107,460, bar = 0.1 um.


A
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F

flagella of similarly attached spiral shaped organisms (Neutra, 1980), were coiled backwards out of the attachment site. The bacterial cytoplasm in the region proximal to the attachment site was electron-translucent in comparison to the remainder of the cell. Furthermore in this region an electron-dense band was seen lining the inside of the cytoplasmic membrane at a distance from 0.2 µm to 0.5 µm from the end of the cell (Fig.8). Thickening of the outer cell wall was caused by the presence of electron-dense material on the periplasmic surface. The location of this material corresponded to the location of the striated zone on the external surface of the bacterial cell wall (Fig.8).

Colonic scrapings contained considerably fewer bacteria than caecal preparations with most having a spiral shaped morphology. Small "S" shaped bacteria were the most numerous and were found predominantly in the mucus component. These organisms were motile by a corkscrew-like motion and like the other mucosal spiral bacteria moved rapidly in mucus, although rarely back and forth on one-dimensional paths. Long, thin, spiral shaped bacteria were seen infrequently and usually in small clumps of parallel cells; these organisms were never motile. Also seen occasionally in colonic scrapings were TSB and fusiform bacteria.

3.2 CHEMICALLY INDUCED DIARRHOEA

The effects of intestinal perturbation on the mucosal microbiota were tested by giving rats the purgative $MgSO_4$. For comparison the changes in the mucosal biota

caused by two other purgatives, cascara and castor oil, were also recorded. Further experiments, using $MgSO_4$ treated rats, were undertaken to test long-term effects of intestinal perturbation on the mucosal environment.

The animals used for the purgative experiments were adult Wistar rats; these were housed normally and given access to water for the duration of the experiments, however they were fasted for the 30 hours of the treatment. Three hours after the commencement of fasting animals were inoculated, by stomach tube, with 0.5 ml of the test purgative. Inoculations were given at 3, 4 and 5 hours. After resting overnight the animals were given further doses every 90 minutes from 21 to 30 hours. During the periods from 0 to 6 hours and 21 to 30hours faecal samples were collected and observed by phase contrast microscopy. Fluid and mucus components of the faeces were examined directly, whilst the solid components were suspended in a small volume of saline. Gross changes in stools were recorded. One hour after the completion of treatment rats were killed and their intestinal tracts removed. The mucosal populations were assessed by the same methods as used in the study of the normal animals.

3.2.1. Magnesium Sulphate

The numbers and composition of the faecal microbiota changed dramatically over the 30 hours of $MgSO_4$ treatment. Initially, (3-4 hrs), the treatment did not alter the major components of the biota, although small

numbers of spiral-shaped bacteria, normally absent from rat faeces, were observed. The organisms, spirochaetes or long, rigid spirilla, were found mixed with the other faecal biota rather than in aggregates. During this period the faeces had a normal consistency. When treatment was recommenced after an overnight rest, the faeces became increasingly fluid with a large component of viscous mucus. Associated with this change was a further increase in the number of spiral-shaped organisms in the stools (Fig.9B). Moderate numbers of spirochaetes, thin spirilla and TSB, were seen in aggregates, usually associated with mucus. The composition of the faecal biota also changed as the numbers of rod-shaped bacteria decreased while the numbers of cocci increased. Highly motile "S" shaped bacteria were seen in the watery components of these stools. In the later stages of treatment the occurrence of spiral-shaped organisms, other than the "S" bacteria diminished; the mucus, fluid, stools contained mainly cocci and these small spiral bacteria.

When tissue sections from the treated animals were examined their mucosa associated microbiotas were found to be substantially different from that of normal control animals (Table 4). The major change was the diminished numbers of organisms associated with the tissue in the ileum and caecum; in contrast to normal animals the scrapings of ileum from treated animals contained very few TSB, and no filamentous organisms. Similarly both

Figure 9. LM observations of normal conventional rats and those given oral purgatives.

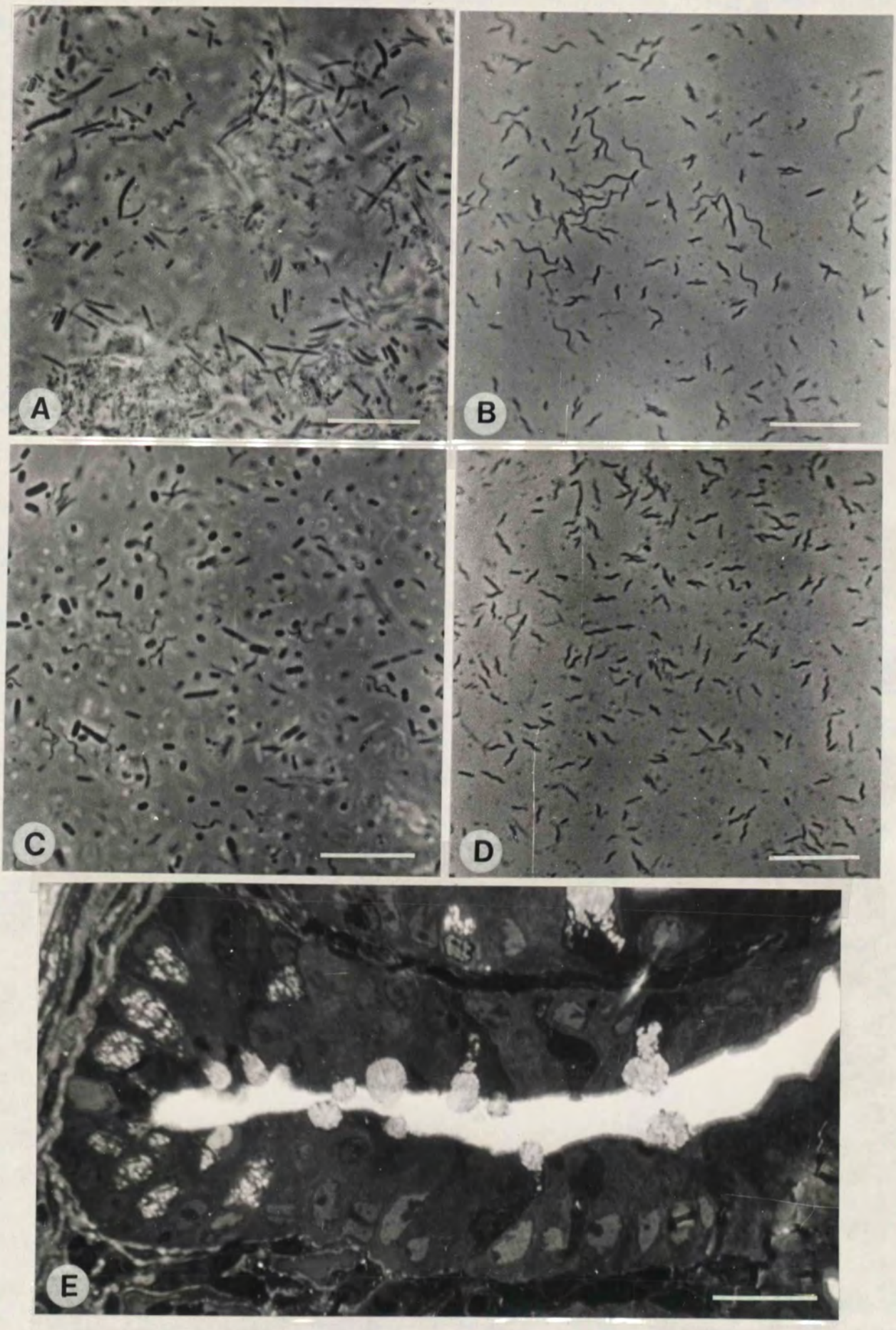
A. Normal faeces x 830, bar = 20 um.

B. Faeces of rats given $MgSO_4$ x 830, bar = 20 um.

C. Faeces of rats given cascara x 830, bar = 20 um.

D. Faeces of rats given castor oil x 830, bar = 20 um.

E. Thick section of a caecal crypt from a rat given $MgSO_4$ x 960, bar = 20 um.


A
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E

Table 4. Effects of various purgatives on the colonization of intestinal crypts.

Tissue	Ileum				Caecum		Colon	
Treatment	Semi-Quant. Count[a]	% Crypts Colonized TSB[b]	CSB[c]	All Bacteria	Semi-Quant. Count	% Crypts Colonized	Semi-Quant. Count	% Crypts Colonized
Normal	26.6[d]±6.9[e]	65	59	76	44.2±7.9	93	23.6±7.5	76
Fasted	27.4 ± 7.5	70	63	80	36.6±9.6	90	20.8±5.3	75
$MgSO_4$	15.6±8.9	5	54	57	7.2±4.2	29	23.2±6.1	69
Cascara	20.6 ± 6.3	58	66	71	49.2+12.1	94	32.0±9.9	90
Castor Oil	30.4±9.5	73	77	85	45.2±10.5	91	29.2±10.6	87

a See Materials and Methods for details of semi-quantitative counting systems.

b Thick spiral shaped bacteria

c Crescent shaped bacteria

d Average score from 5 animals

e Standard deviation (5 animals per treatment).

organisms were absent from tissue sections taken from treated rats (Fig.9E). In contrast the small crescent-shaped bacteria associating with the surfaces of the crypts were present in normal numbers in sections and scrapings. Very few organisms were present in caecal scrapings nor were the crypts of this site found to contain many bacteria. In both types of samples only small numbers of spirochaetes, thin spirilla and TSB were seen.

Mucosal scrapings and tissue sections of the colonic mucosa of the $MgSO_4$ treated rats demonstrated that the crypt biotas were similar to that of control animals. However in two animals small microcolonies, of what appeared to be spiral-shaped bacteria, were seen on the surface of the colonic mucosa in contact with the intestinal lumen. Although a number of such colonies were seen in thick sections the smaller areas assessed with TEM failed to locate this population; these organisms were investigated in later experiments.

3.2.2. Castor Oil

Castor oil was found, like $MgSO_4$, to dramatically alter the composition of the caecal microbiota. The first change seen was the presence of small numbers of the segmented filamentous organisms in the stools: these organisms were never seen in normal faeces. As treatment was continued the overall appearance of the faecal biota changed and the numbers of the predominant populations, fusiforms, rod-shaped bacteria, and cocci all diminished. Upon resumption of purgative treatment (20 hrs+), high

numbers of long flexible rod-shaped organisms were seen, often in large sheet-like aggregates. As the stools became increasingly composed of mucus and oil, increasing numbers of "S" shaped bacteria were observed while the numbers of all other organisms diminished (Fig.9D).

When the intestinal tissue was examined at the completion of treatment the mucosal populations were similar to those of control animals, although a number of small differences were observed. The filamentous organisms were absent from the scrapings and sections of the ileum. No changes were seen in the composition of the ileal or colonic crypt microbiotas. Although the numbers of organisms present in the caecal crypts were comparable to controls, a higher proportion contained TSB.

3.2.3. Cascara

Unlike the other purgatives tested, cascara had little effect on the predominant morphological types of microorganisms in the faecal microbiota over the period of the experiment. The main difference between the faeces of cascara treated rats and normal faeces was the presence of spiral-shaped organisms: spirochaetes, thin spirilla, TSB, and "S" shaped bacteria were all seen frequently (Fig.9C). The appearance of these organisms corresponded to the presence of mucus in the stools and they were usually found in this component.

The scrapings and sections (Table 4) of the ileum showed the mucosal populations to be similar to those of control animals. Caecal scrapings contained high

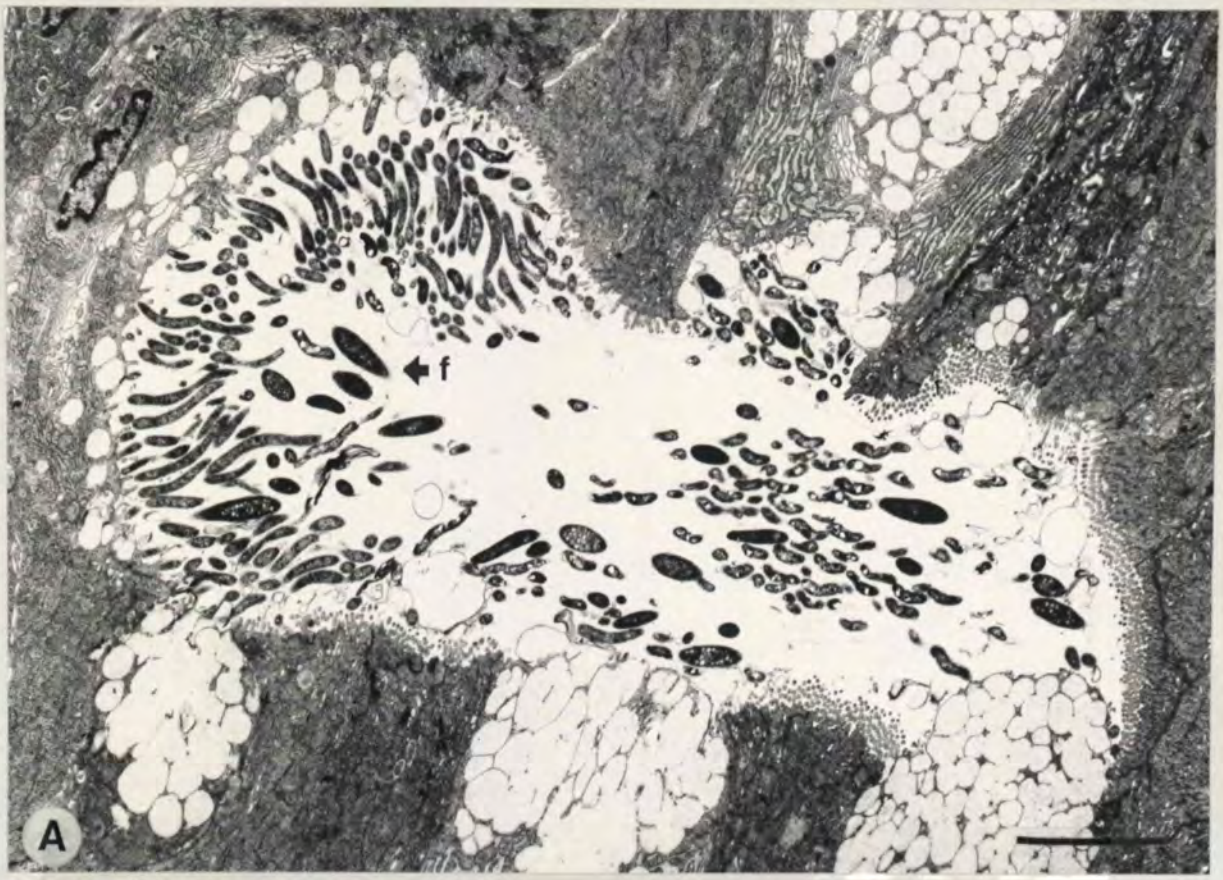
numbers of spirochaetes and thin spirilla, and moderate numbers of TSB, rod-shaped bacteria and fusiforms. All these organisms were found in mixed aggregates suggesting that the rods and fusiforms were from the crypts rather than faecal contaminants. Fusiforms were also seen in some colonic crypts.

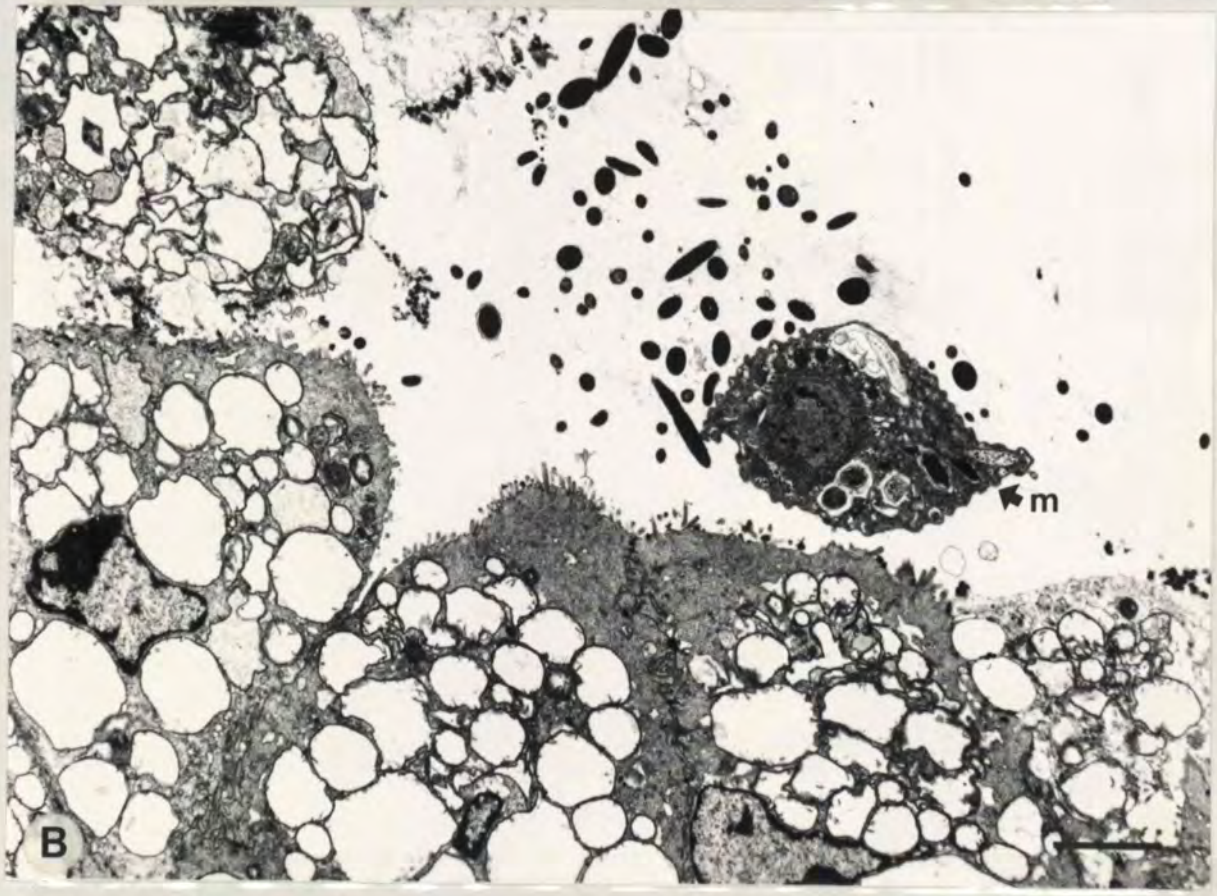
Sections of the caecum revealed both altered mucosal populations and considerable tissue damage. The tissue in direct contact with the intestinal lumen was often vacuolized with considerable numbers of cells being degenerate (Fig.10). Areas of affected tissue were seen to be sloughing away and many dislodged cells were found in the mucus and lumen of the tract. Occasionally macrophages were seen near the tissue and often contained microoganisms (Fig.10). The caecal crypt microbiota was slightly different from the controls, having increased numbers of TSB, as well as fusiforms present. Some vacuolization was also seen on the surface of the colon although not as severe as in the caecum. The crypts were found to have higher numbers of organisms than the controls; primarily as a result of increased levels of thin flexible spiral-shaped bacteria attached to crypt cells, also rod-shaped bacteria were seen in a quarter of the populated crypts (Fig.10A). The results of the scrapings reflected the changes in the crypts, with more thin, flexible bacteria and the presence of rod-shaped bacteria.

Figure 10. TEM observations of intestinal mucosa in rats given cascara.

A. Colonic crypt containing fusiforms (f) x 3860, bar = 5 um.

B. Vacuolized surface of the caecal mucosa. A macrophage (M) containing bacteria is also present x 2730, bar = 5 um.


f
A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3.3. MICROBIAL RECOLONIZATION OF THE MUCOSA AFTER $MgSO_4$ INDUCED DIARRHOEA

Of the three purgatives tested, $MgSO_4$ had the most dramatic effects on the mucosa associated microbiota, with many of the populations normally present in the ileum and caecum removed and the presence of non-indigenous microbes on the surface of the colon in some animals. In many ecosystems a perturbation that results in an alteration in the composition of the microbiota is followed by a succession of populations leading to re-establishment of the original climax community (Alexander, 1971). In the following experiment recolonization of rats following $MgSO_4$ induced diarrhoea was studied and also the fate of the spiral-shaped bacteria colonizing the colonic mucosa was followed. After being treated with $MgSO_4$ over a 30 hr period, rats were returned to a normal diet. Two series of experiments were completed; in the first, rats were killed and the mucosa associated microbiota assessed after: 0, 1, 2,4 and 6 days post-treatment. The second series was undertaken after the results of the first had been obtained, and the mucosal microbiota assessed after: 0, 4, 6, 8, 12, 20, 60 and 180 days. The mucosal microbiota was assessed by observations of embedded tissues; in the first series crypt populations were quantitated as previously described and surface populations noted while in the second only surface populations were studied. The presence of fusiform shaped bacteria in the layers of mucus on the colonic mucosa was determined by LM examination of frozen

sections. The water soluble mucus was retained in the specimen by using the methods of Freter et al. (1981) to section, fix and stain the tissue.

The occurrence of the spiral-shaped organism on the surface of the colon was assessed as in previous studies (Lee et al., 1971; Takeuchi et al., 1974), by LM of thick sections; tissues were dissected and aligned so that a similar length of mucosa was sectioned. The stained sections were examined using an oil immersion objective and the presence of any microorganisms associated with the brush border, noted. Statistical comparisons were made using the X^2 test for Two Independent Samples (Siegel, 1956).

Observations of the ileal and caecal tissues from animals over the six days of the first series of experiments showed that the crypts were rapidly recolonized by the same types of microorganisms as present before treatment: there was no evidence of a succession of populations. Changes in the ileal populations over the period of the experiments are recorded in Table 5. The effects of the purgative on the crypt populations were as previously described: all of the TSB were removed whereas the crescent-shaped bacteria appeared to be unaffected and remained at similar population levels on each of the days. The occurrence of TSB increased rapidly in the days following treatment and within 2-4 days the numbers of these organisms, and hence the total crypt populations, had returned to pretreatment levels. During the period of

Table 5. Recolonization of intestinal crypts following $MgSO_4$ treatment.

Treatment	Ileum				Caecum		Colon	
	Semi-Quant. Count[a]	% Crypts Colonized			Semi-Quant. Count	% Crypts Colonized	Semi-Quant. Count	% Crypts Colonized
		TSB[b]	CSB[c]	All Bacteria				
Normal	28.2[d]±8.6[e]	69	65	84	44.6±7.7	93	35.0±7.5	88
Fasted	29.4 ± 10.0	48	70	81	45.6±12.1	86	25.2±7.6	84
Day 0[f]	15.2 ± 8.7	0	53	53	8.0±4.6	34	24.0±5.5	83
Day 1	20.8 ± 8.3	21	55	59	26.4±14.0	61	24.0±5.7	72
Day 2	25.6 ± 6.8	40	59	74	37.4±9.7	87	28.8±11.4	82
Day 4	34.8 ± 13.3	61	54	79	52.8±14.4	89	21.0±4.0	80
Day 6	31.2 ± 8.8	45	72	89	41.4±7.6	86	22.8±9.3	73

a See Materials and Methods for details of semi-quantitative counting system
b Thick spiral-shaped bacteria
c Crescent-shaped bacteria
d Average score from 5 animals.
e Standard deviation (5 animals per treatment)
f Sampled upon the completion of purgative treatment.

recolonization no organisms, other than the crescent-shaped bacteria and TSB were seen in the ileal crypts. The segmented filamentous bacteria were not seen immediately after cessation of treatment however, like the TSB, this population returned to normal levels after 2-4 days.

The caecal crypts, most of which contained none or low numbers of bacteria after treatment, also returned to pretreatment population levels by 2-4 days (Table 5); similarly no foreign microbes were seen. No major changes were seen in the colonic crypts over the period of treatment (Table 5).

In the normal rat intestine there is only one microbial population intimately associated with the mucosa adjacent to the intestinal lumen: the ileal filamentous bacteria. Other mucosa associated populations such as the fusiform bacteria of the large bowel are found within layers of mucus rather than attached to the mucosa. A feature of many animals after $MgSO_4$ induced diarrhoea was the presence of microorganisms on tissue surfaces normally free of bacteria. In the first series of experiments three other types of microbes were seen closely associating with the mucosal surface: rod-shaped bacteria, very small mycoplasma-like bacteria, and also the small spiral-shaped bacteria seen in the previous purgative experiments.

Immediately following cessation of treatment (day 0) in the first experiment, small numbers of rod-shaped bacteria were seen in close association with the

mucosa in both the ileum and caecum (Table 6). The organisms in the ileum were present on the base of the villi, but not on their upper surfaces while those occurring in the caecum were present in the areas around the necks of the crypts. In both tissues these bacteria were found in small numbers and subsequently were not seen on the following day (day 1). The organisms were not observed in the caecum for the remainder of the experiment, however in the ileum they were seen in one animal on the second day and in a majority of the animals on the fourth and sixth days. Large numbers of rod-shaped bacteria were seen on the lower surfaces of the villi on the latter days (Fig.11). In sections the organisms were present in layers, often many bacteria thick (Fig.11). The cells were well separated from each other and the areas immediately around the organisms were electron-translucent (Fig.12). Bacteria that were in contact with the tissue were randomly orientated (Fig.12), however there appeared to be some interaction between the bacterial cell surface and the host cell glycocalyx. The organisms were small (0.6 µm x 1.5 µm), having a Gram-negative type cell wall with some pleomorphism (Fig.13). The rod-shaped bacteria found in the caecum were of similar size and shape and exhibited the same type of association with the mucosal surface as the ileal organisms (Fig.13).

Mycoplasma-like bacteria were found associated with the ileal mucosa in only 4 animals (Table 6). The organisms were not observed in normal rats nor in animals

Table 6. Colonization of the intestinal mucosa by rod-shaped bacteria and mycoplasma-like bacteria.

Organism		Treatment						
		Controls		$MgSO_4$ Treated				
		Normal	Fasted	0[a]	1	2	4	6
Rod-shaped Bacteria	Ileum	0[b]	0	2	0	1	4	3
	Caecum	0	0	2	0	0	0	0
Mycoplasm-like bacteria		0	0	0	0	0	2	2

a Days post-treatment

b No. animals colonized (5 animals per group).

Figure 11. LM observations of rod-shaped bacteria colonizing the ileum six days after cessation of $MgSO_4$ treatment.

A. Rod-shaped bacteria (b) in the intervillal spaces x 500, bar = 50 um.

B. Rod-shaped bacteria in thick layers on the tissue surface x 350, bar = 50 um.

C. Orientation of rod-shaped bacteria to the tissue surface x 1250, bar = 10 um.

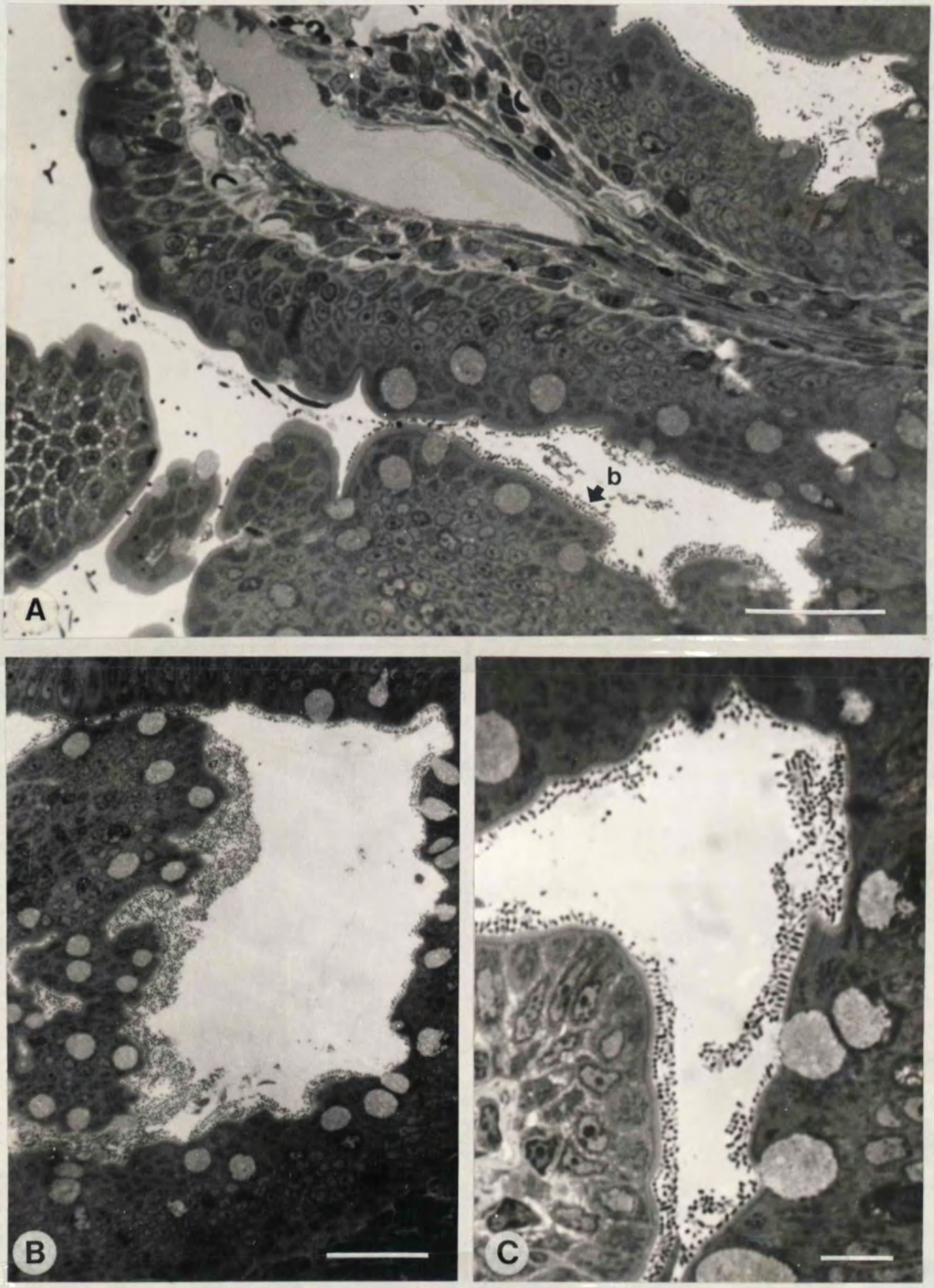
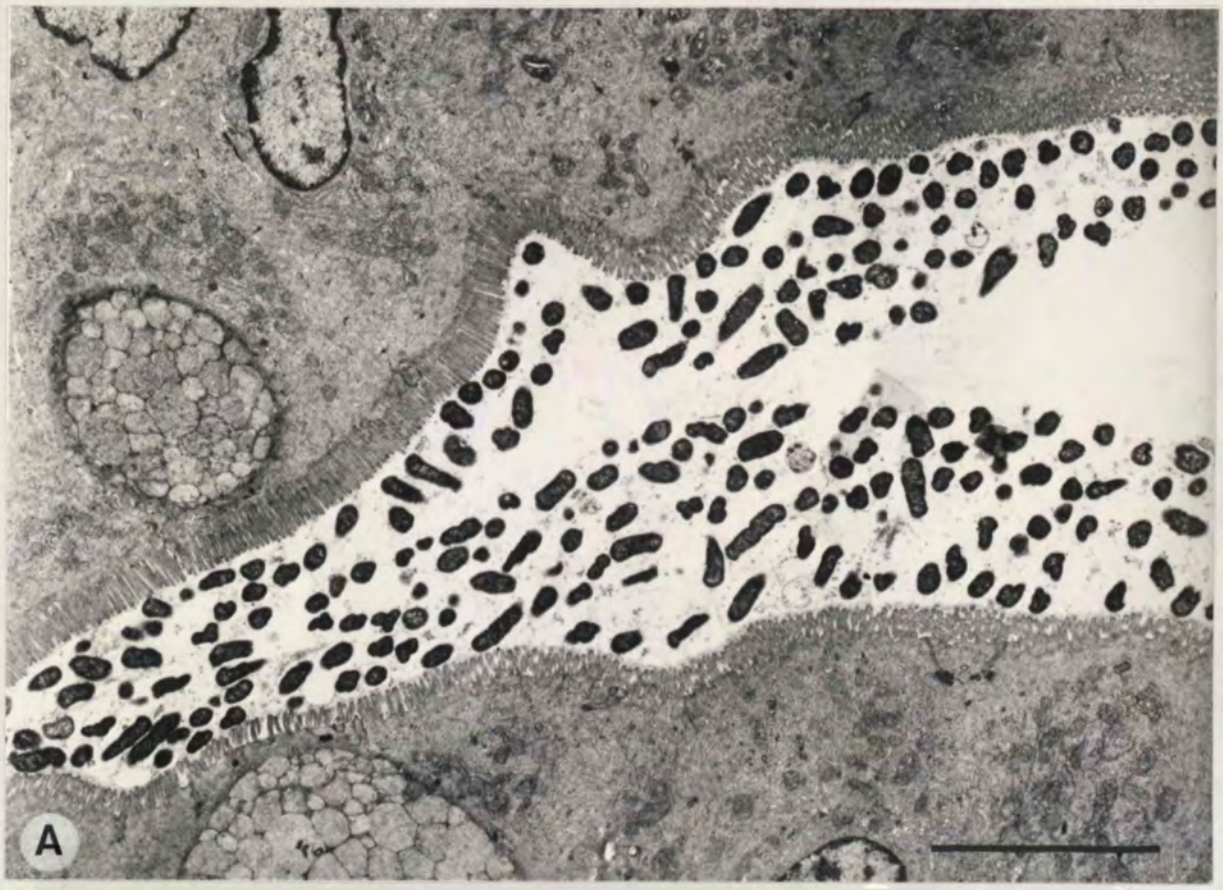

b
A
B
C

Figure 12. TEM observations of rod-shaped bacteria associating with the surface of the ileal villi in animals after $MgSO_4$ treatment.

A. x 6,000, bar = 5 um.

B. x 16,500, bar = 1 um.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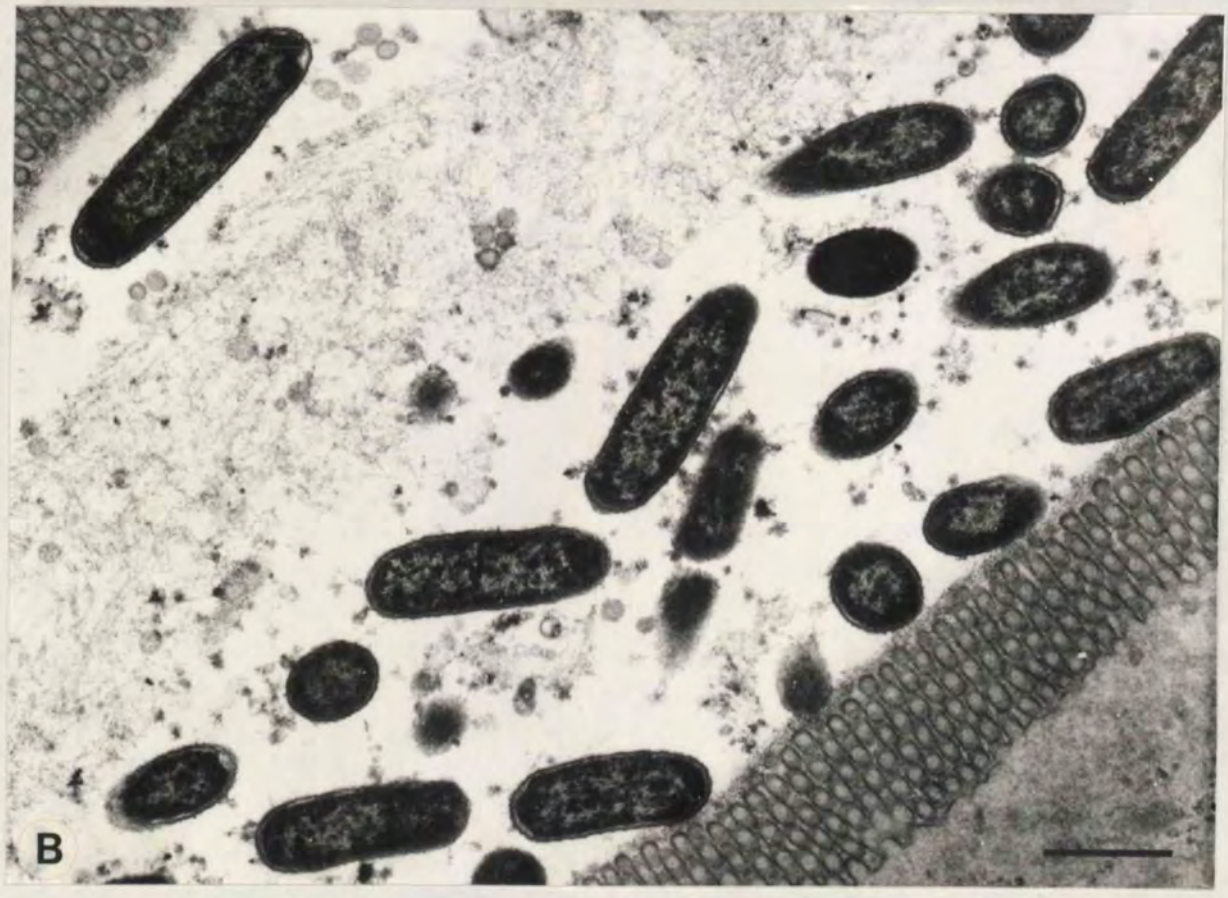
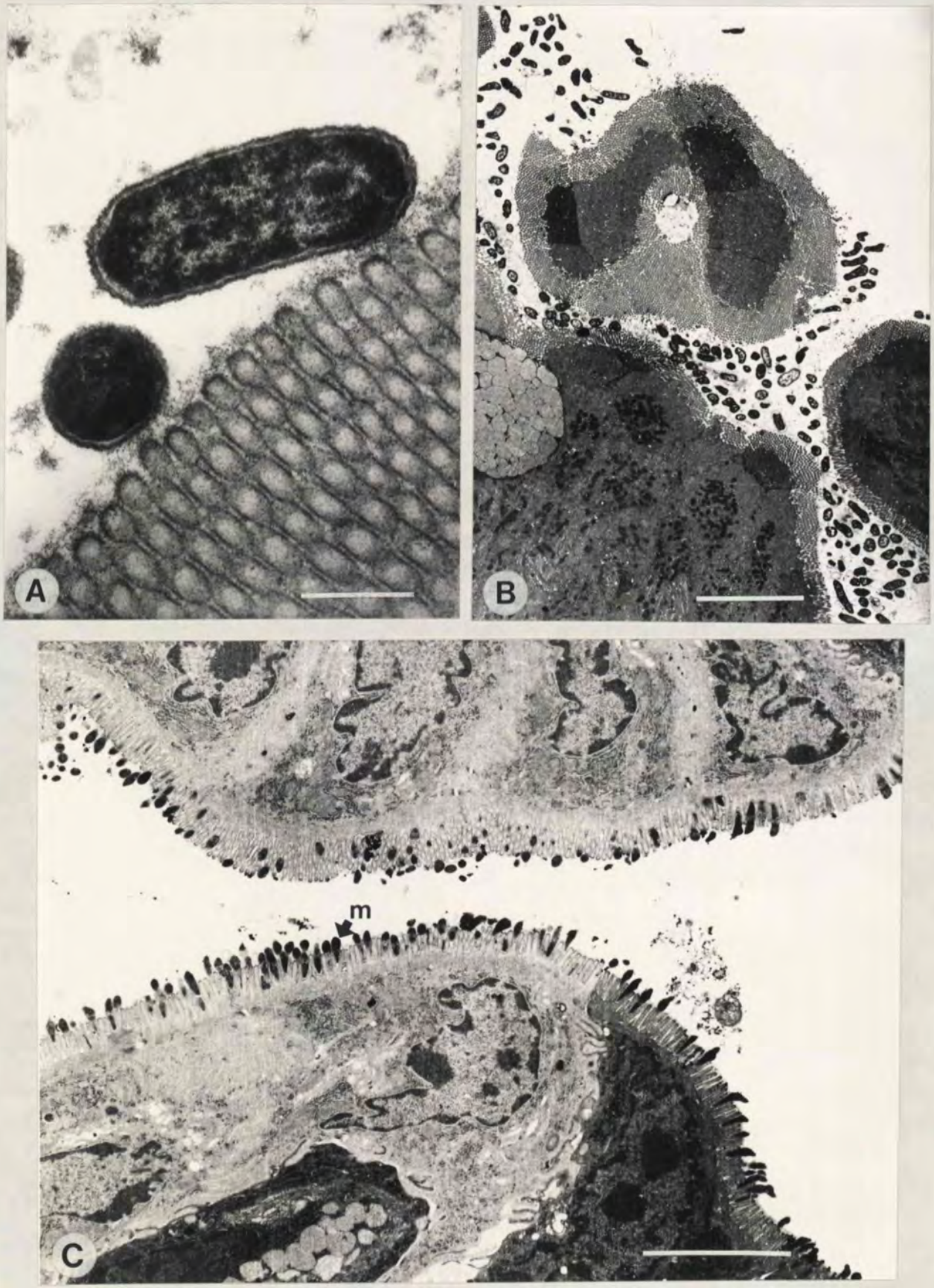

B

Figure 13. TEM observations of microorganisms associating with the intestinal mucosa after $MgSO_4$ treatment.

A. Rod-shaped bacteria in contact with the glycocalyx of an ileal epithelial cell x 39,000, bar = 0.5 um.

B. Rod-shaped bacteria associated with the surface of the caecal mucosa x 3,800, bar = 5 um.

C. Mycoplasma-like bacteria (m) associating with the ileal mucosa x 5,450, bar = 5 um.


A
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m
C

0, 1, 2 days post-treatment; however these were found in 4 of 10 rats on the 4th and 6th days post-treatment. The mycoplasma-like bacteria were not easily seen with the light microscope, but the electron microscope showed them to be present in very high numbers on both the upper and middle surfaces of the villi (Fig.13). The organisms attached to the brush border of the host cells, with none seen further than 3 µm from the tissue surface and at high magnification (Fig.14) were found to be lodged between the host cells' microvilli, with one end in contact with the underlying tissue surface. When the organisms extended beyond the microvilli there was often a pleomorphic swelling of the cell. A zone of 18-25 nm width between the host cell membrane and the membrane of the organism had a striated appearance suggesting the fibrillar material was present. The organisms were very small (0.1-0.2 µm x 0.4-0.8 µm), pleomorphic, and like mycoplasm species possessed a trilaminar membrane (Fig.14).

Neither the rod-shaped bacteria nor the mycoplasma-like bacteria were found associated with the intestinal mucosa of rats in the second series of experiments. In contrast the spiral-shaped bacteria were observed in the initial $MgSO_4$ experiments and consistently in groups of animals given $MgSO_4$: in each of the 13 groups of 5 animals in the recolonization experiments and in 3 of 4 other groups of 5 rats treated with this purgative. The organisms appeared to be distributed on the surface in micro-colonies: areas of heavy colonization adjacent to

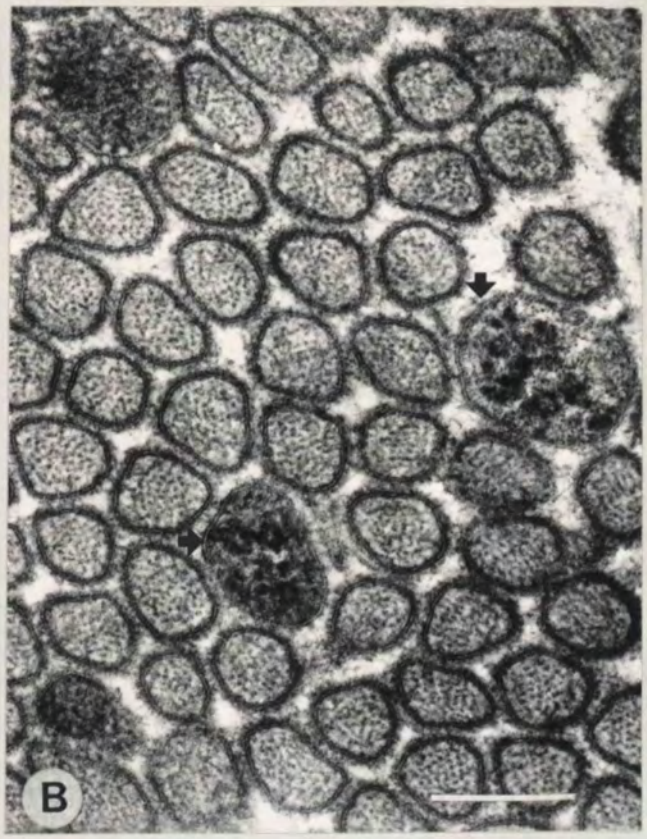
Figure 14. TEM observations of mycoplasma-like bacteria associating with ileal mucosa.

A. Colonization of mucosa adjacent to a filamentous procaryote x 5,900, bar = 2 um.

B. Transverse section of bacteria amongst microvilli showing single trilaminar membranes (arrows) x 95,800, bar = 0.2 um.

C. Longitudinal section of bacterium showing close association with host microvilli x 97,000, bar = 0.2 um.


A


B


C

areas free of bacteria (Cheng, Irvin and Costerton, 1981). Colonization occurred on the mucosa in contact with the lumen but not on the cells lining the crypts (Fig.15). When thick sections of colonic mucosa were examined immediately after the cessation of treatment (Day 0) the spiral-shaped organisms were found in 3 of 10 animals (Table 7). A similar incidence of colonization occurred in animals sampled throughout the 180 days of the two experiments. Surface colonization by the spiral-shaped organisms occurred in 22 (34%) of the 65 treated animals. In contrast, there were significantly fewer ($P<0.01$) normal and fasted control animals colonized; only 1 (4%) of 25 animals. The extent of colonization of the colonic mucosa was variable with some animals having only a few isolated microcolonies while others had large proportions of the surface populated by spiral-shaped organisms.

Electron microscopy showed that the mucosa was populated by one morphological type of bacterium (Fig.15). The organisms were small (0.3 x 3-5 µm), Gram-negative spirals having 2-3 turns and with single flagella-like appendages at each end of the cell (Fig.16). The appendages were membrane-bound continuations of the cell's periplasm and appeared to be sheathed flagella; the membrane was continuous with the cell wall.

The spiral-shaped organisms were rarely found more than 15 µm distant from the mucosa and generally had an orientation perpendicular to the surface epithelial cells (Fig.16). The presence of the organisms often

Figure 15. Spiral-shaped bacteria associating with the colonic mucosa in rats after $MgSO_4$ treatment.

A. 20 days post treatment; bacteria colonizing the surface (arrow) but not the crypts (C). LM, x 930, bar = 20 um., insert; organisms associating with the mucosal surface LM x 2200, bar = 5 um.

B. 60 days post treatment; bacteria associating with the mucosal surface TEM, x 8,100, bar = 2 um.

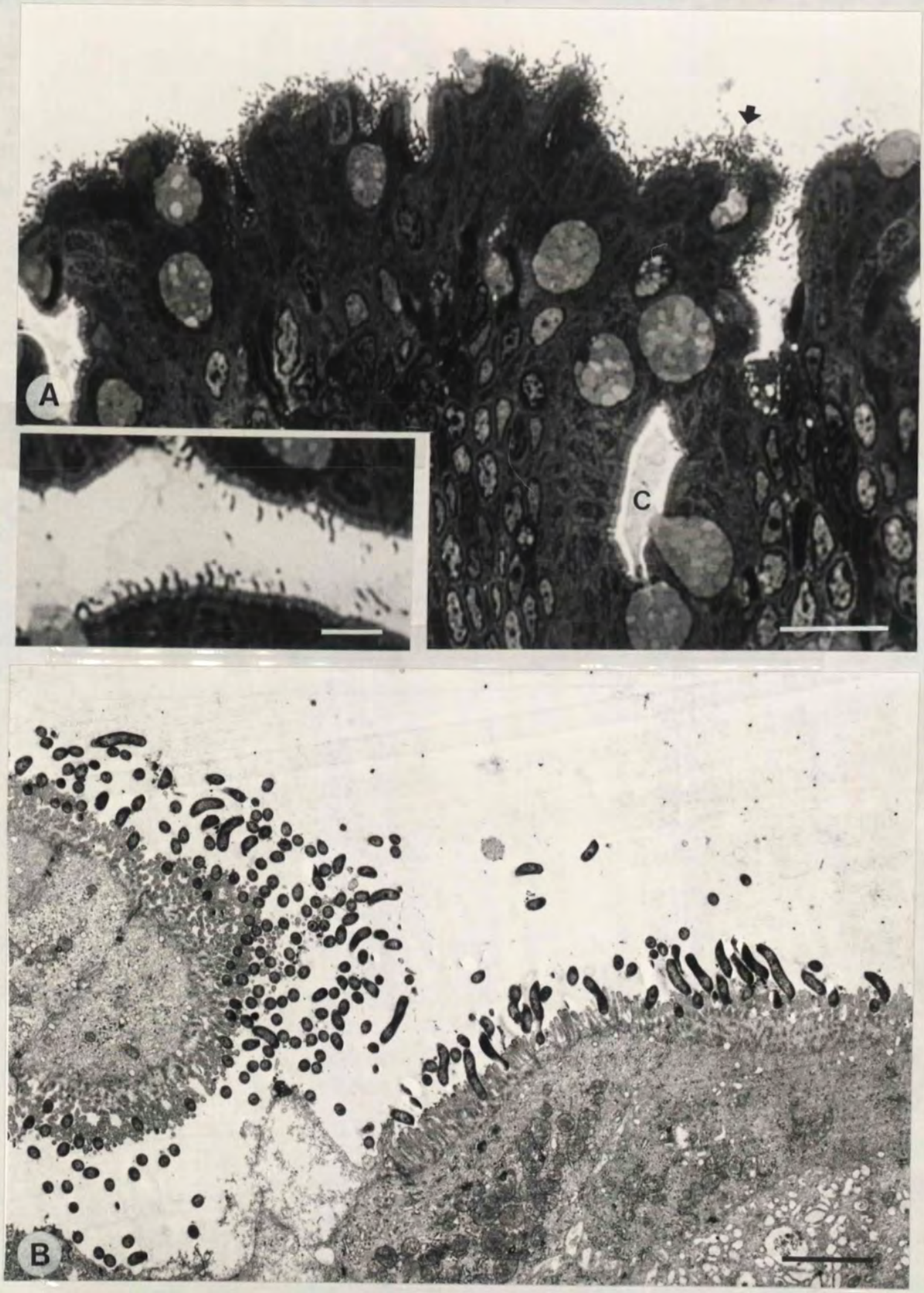

A
C
B

Table 7. Colonization of the colonic mucosa by spiral-shaped bacteria.

	Treatment												
	Controls		Recolonized after $MgSO_4$ treatment										
	Normal	Fasted											
Day sampled	0	180	0	0a	1	2	4	6	8	12	20	60	180
Colonization	1b/10c	0/15	0/10	3/10	2/5	2/5	2/10	4/10	3/5	1/5	1/5	2/5	2/5
Totals	1/25 (4%)			22/65 (34%) : $P<0.01$									

a. Days post treatment

b. No. animals colonized

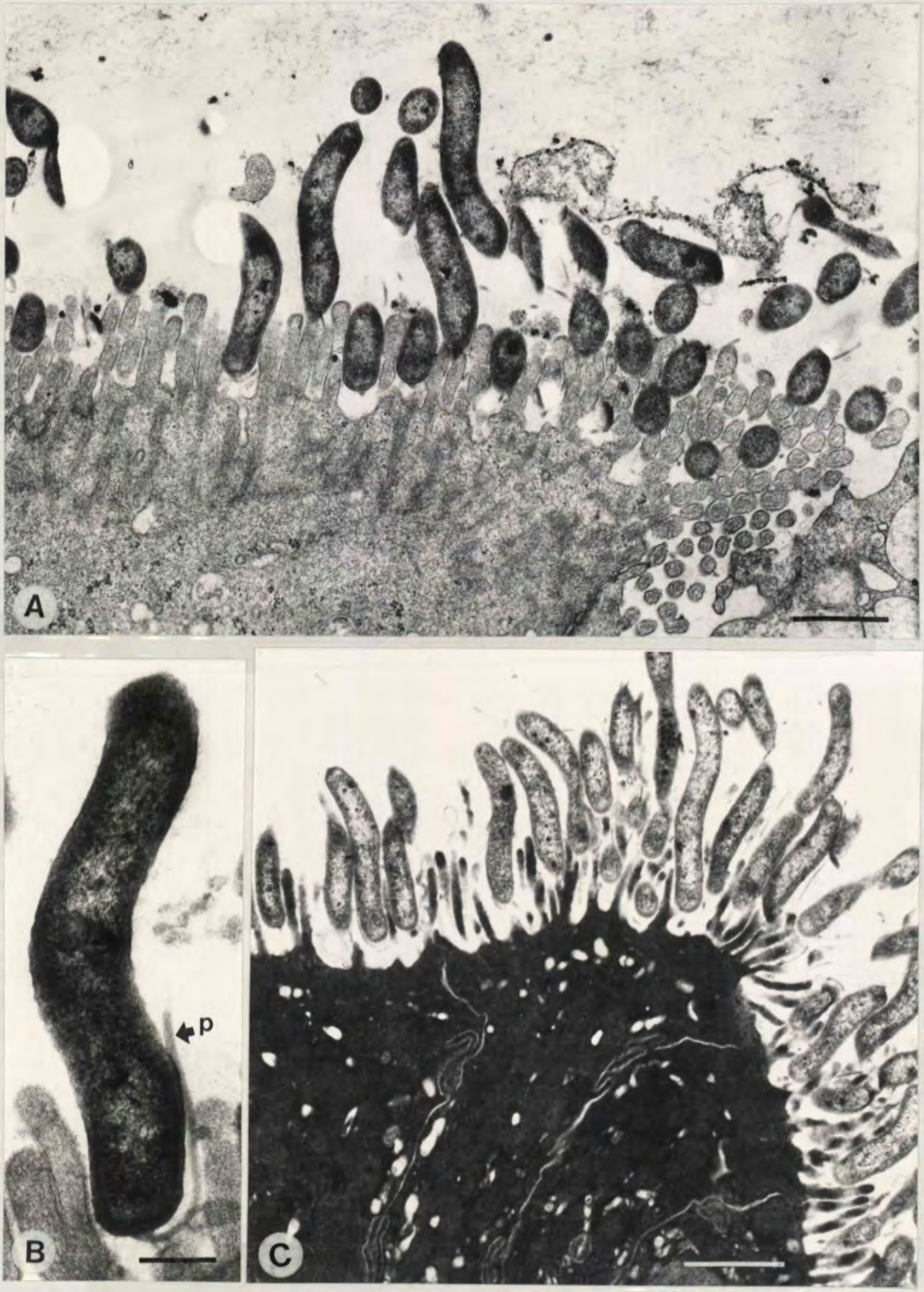
c. No. animals per group

Figure 16. TEM of spiral-shaped bacteria associating with the colonic mucosa in rats after $MgSO_4$ treatment.

A. 60 days post treatment; bacteria inserted into the microvillus border x 33,300, bar = 0.5 um.

B. Higher magnification of the organism showing the spiral morphology and a polar, sheathed flagellum (p) x 68,500, bar = 0.2 um.

C. 6 days post treatment; electron dense cytoplasm in host cells associated with the bacteria x 17,800, bar = 1 um.


A
B
p
C

resulted in the disruption of the microvillus border, with microvilli becoming degenerate or displaced altogether (Figs.16,17). In some animals areas of the host tissue adjacent to the colonized surface were more electron-dense than the surrounding mucosa (Fig.16). Unlike other spiral-shaped bacteria that associate with the mucosa (Lee et al.,1971), the spiral-shaped organisms did not form an invaginated attachment site in the basal membrane of the epithelial cells. Rather, the organisms were generally found in an indentation of the microvillus border and not in direct contact with the epithelial membrane (Fig.16). Frequently the proximal ends of the organisms were seen to be broadened into a curved plate-like structure (Fig.17). The proximal appendage was often found to be in close contact with the tissue as it coiled back around the bacterial cell. Fibrillar material was occasionally observed between the tissue and the organisms or their appendages (Fig.17).

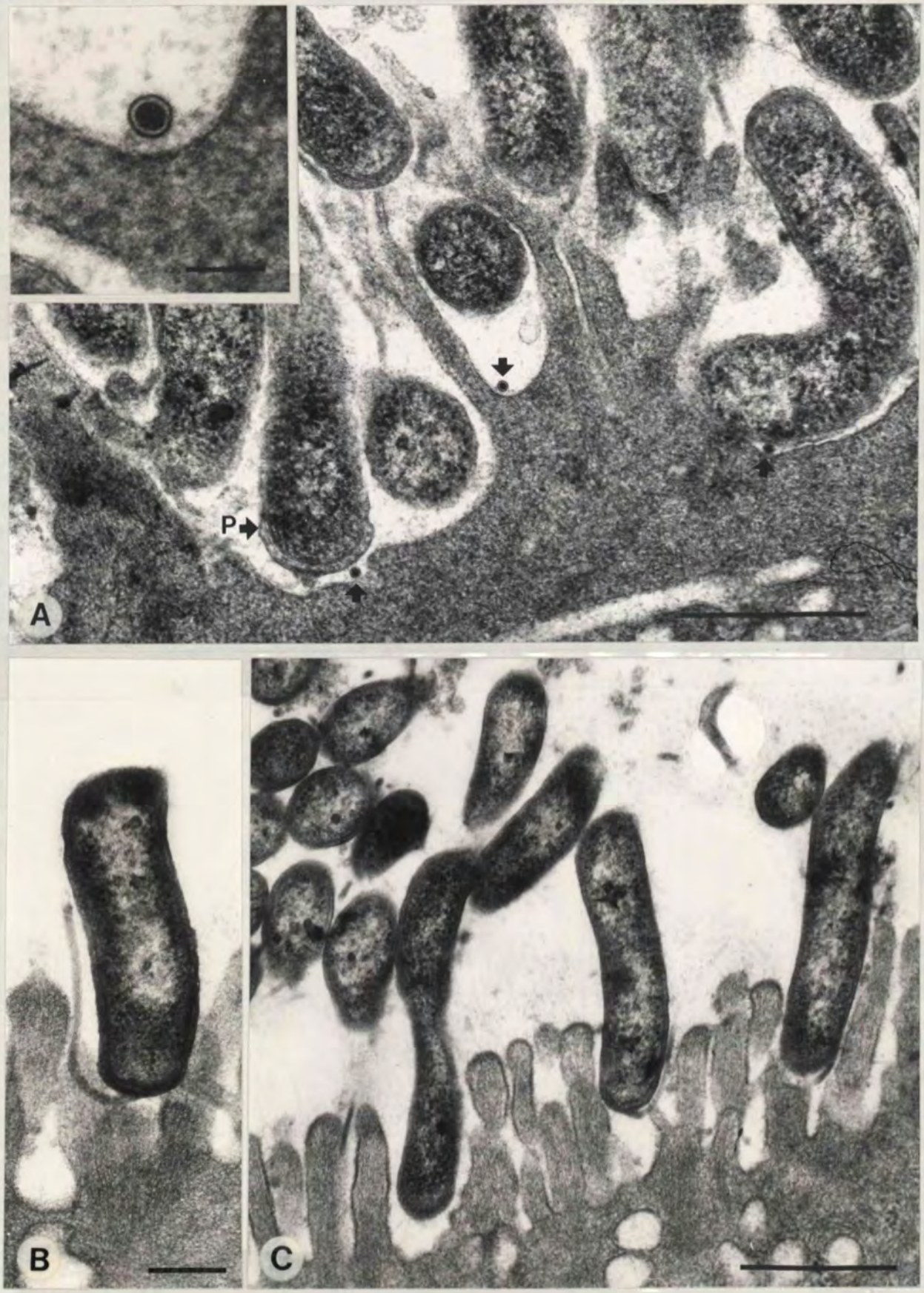
3.4 EFFECTS OF INTRODUCING A CONVENTIONAL INTESTINAL MICROBIOTA INTO SPF RATS

The experiments with $MgSO_4$-treated rats demonstrated that intestinal perturbation could cause long term alterations in the mucosal microbiota. To further investigate the period of instability during the recolonization of the intestinal mucosa, when such alterations appear to occur, an experiment was conducted using SPF rats. Although having a complex lumen microbiota the rats from this colony lacked any mucosa associated

Figure 17. TEM observations of spiral-shaped bacteria associating with the colonic mucosa of rats.

A. 6 days after cessation of $MgSO_4$ treatment: disruption of the microvillus border caused by the bacteria. The end of one organism is curved into a plate-like structure (P). A number of sheathed flagella can be seen in close contact with the host tissue (arrows) x 67,000, bar = 0.5 um, insert; higher magnification showing fibrillar material between a flagellum and the host membrane x 286,000, bar = 0.05 um.

B and C. Colonic mucosa of a SPF rat 28 days after innoculation with conventional rat intestine. B. x 69,200, bar = 0.2 um. C. x 54,800, bar = 0.5 um.


P
A
B
C

microorganisms: no bacteria in the crypts, attached to the tissue or in layers of mucus near the surface.

The absence of surface-associated bacteria made the mucosal environment of these animals similar to that found in $MgSO_4$ treated rats. The recolonization that occurs in the $MgSO_4$ treated animals was simulated in the SPF rats by exposing them to a conventional mucosal microbiota; each animal was given a 0.5 ml inoculation of homogenized conventional rat intestine. The presence of microorganisms on the tissue surface was detected as previously described, using light and electron microscopy.

After the SPF rats had been treated and left for 4 weeks many, but not all, of the mucosa associated microbes seen in conventional rats were found. In the ileum the numbers of filamentous bacteria on the villi, and the curved bacteria in the crypts, were similar to those in conventional animals; however considerably fewer TSB were found in the ileal crypts. The contents of the caecal crypts resembled those of conventional animals with higher numbers of spirochaetes than spirilla. Similarly the lumen bacteria found in the colonic crypts of conventional animals were present in the crypts of the treated SPF rats. In contrast the thin "S" shaped bacteria, found attached to the cells lining the crypts in most conventional animals, were not seen in any treated rats.

Of the ten treated animals, five were found to have the surface of the colonic mucosa colonized by bacteria; this site was uncolonized in all ten SPF rat

controls. The ultrastructure, distribution, and mode of association of these bacteria was indistinguishable from the spiral-shaped bacteria seen in $MgSO_4$ treated rats (Fig.17)

3.5. DISCUSSION

The absence of a comprehensive description of the rodent mucosa-associated microbiota and the difficulty of culturing many of these organisms were reasons for undertaking an observational survey of the rat intestinal tissue. Also the microbiota of an animal colony cannot be determined by referring to reports of similar animals from other colonies. While many of the microorganisms seen during this survey, such as the filamentous bacteria and TSB from the ileum, and the spiral-shaped bacteria in the caecal crypts, have been described previously others have not: the crescent-shaped bacteria in the ileal crypts and the small spiral-shaped bacteria from the lumens of the colonic crypts. The thin spiral-shaped bacteria attached to colonic crypt cells are most probably the attached population seen at the same site by Wagner and Barrnett (1974) and identified as coliforms.

The occurrence and distribution of the TSB in the ileal crypts was described by Erlandsen and Chase (1972). Variable numbers of these organisms were seen in the crypts with 2-5 bacteria in most and occasionally up to 25-30 packed closely together in others. Although not quantitative, their description of the ileal crypts is similar to the results obtained in the current survey: most crypts containing low numbers of bacteria with a minority containing high concentrations. Although the

crescent-shaped bacteria were found in ileal crypts of all the UNSW rats examined, they were not seen in the animals examined by Erlandsen and Chase (1972) nor have they been reported elsewhere. Preliminary observation of rats from another animal colony in Sydney: (Bosch Animal Unit, Uni. of Sydney) has shown that the crescent-shaped bacteria were present in the ileal crypts. Thus the occurrence of these organisms is not unique to the UNSW colony and suggesting that absence from other colonies may be due to geographical variation. It is also possible that these organisms may be present in some colonies but have not been observed; small size and proximity to the tissue surface make these organisms difficult to see in histological preparations.

The occurrence of the crescent-shaped bacteria in only some colonies of rats suggests that they are not a true autochthonous population. The filamentous bacteria of the ileum and the yeast Torulopsis pintolopesii from the stomach are also found only in a preparation of rat and mouse colonies (Savage, 1972). Savage (1972) has suggested that these organisms were only at an intermediate evolutionary stage on the way to being autochthonous. The crescent-shaped bacteria may also be in a similar pre-autochthonous stage.

The composition of the microbiota of the caecal crypts was found to be similar to that described by Davis et al. (1972): predominantly spirochaetes and thin spirilla, with smaller numbers of TSB. The population density of the crypt populations in the caecum was

considerably higher than in the ileum or colon, furthermore a higher percentage of crypts contained organisms. Factors which may explain these higher populations were: the caecal crypts were considerably larger in size than those of the ileum or colon and in comparison to other sites the crypts appeared to have less of an outflow of mucus.

Although the occurrence of small spiral-shaped bacteria in the mucus layers adjacent to the colon in mice is well documented (Savage et al., 1971), the presence of such bacteria in the colonic crypts of rodents has not been previously reported. As in the ileum two distinct populations occurred, one of attached bacteria and one of organisms occupying the crypt lumen. The lumen population was of "S" shaped bacteria that were very similar to organisms reported to be present in the mucus layers. The ultrastructure and mode of attachment of the thin spiral-shaped bacteria to colonic crypt cells closely resembled that of "coliform" bacteria found at the same site by Wagner and Barrnett (1974). The organisms described by these authors were probably the same as the spiral-shaped bacteria seen in the present study; the reason given for identifying them as coliforms was that "the location of the bacteria within the colon strongly suggest that they are a member of the Enterobacteriaceae and most likely a coliform" is insufficient for such a conclusion. The spiral-shaped bacteria appeared to be highly adapted for association with the host tissue; in addition to the plug-like end of the organisms described by

Wagner and Barrnett other modifications to the cells were seen. The cylindrical electron-dense band: the polar membrane (Beveridge, 1981), at the attached end and the electron-transluscency in this region are associated with this plug-like structure and are possibly there to confer rigidity to this site. The observation that electron-dense material lining the outer membrane of the bacteria corresponds to the appearance of striated material between the tissue and the procaryotes outer surface is interesting. It suggests that material responsible for adhesion is present at the tip of the bacterium rather than being a common outer cell wall component reacting with the eucaryotic membrane as previously suggested (Wagner and Barrnett, 1974).

A feature of the habitats associated with the intestinal mucosa was the low microbial diversity. In contrast to the intestinal lumen which can contain a vast array of different species (Savage, 1977) the mucosal habitats examined commonly contained three or less populations.

Low species diversity normally characterizes areas where one or more ecological factors approach extremes that preclude any microbial colonization (Alexander, 1971). Elucidating the factors responsible for the lack of diversity in the intestinal crypts is difficult as no practicable methods exist for determining accurately the conditions within this microhabitat. Some insight can be gained as to the nature of the environmental

extremes by examining how the organisms have adapted. For example, the highly specialized attachment site and complex life cycle of the segmented filamentous organisms are clearly adaptions to two major obstacles to colonization of the ileum: the rapid flow of intestinal chyme in the small bowel (Luckey, 1974) and the continual turnover of epithelial cells (Abrams et al., 1963). The organisms from the intestinal crypts also appear to be specialized. The crescent-shaped bacteria from the ileum and the thin spiral-shaped bacteria from the colon both attach to the surface of the crypt cells. Moreover, the thin spiral-shaped bacteria, like the filamentous organisms, associate with the tissue by a complex attachment site. The unattached populations are all highly motile and, with the exception of small numbers of fusiforms, all spiral-shaped. These similarities suggest that the organisms are adapted to a common physical factor in the environment. The intestinal crypts are known to be sites of mucus and fluid secretion (Trier and Madara, 1981); thus there would be a considerable outflow of viscous secretions from the base of the crypts towards the intestinal lumen. Attachment is well documented as a means by which microorganisms can resist fluid flow (Lee, 1980), and the results of the $MgSO_4$ experiments are also consistent with this role: in the ileum and caecum the only organisms able to resist the increased fluid flow were the attached crescent-shaped bacteria. For bacteria not attached to the tissue surface an ability to move faster

than the outflow of fluid secretions would also enable them to remain in the crypts. Some evidence for this can be obtained from observation of how the crypt organisms associate with the host. The cumulative effect of goblet cell secretion in crypts would result in an outflow gradient increasing from the base to a maximum flow at the crypt opening, into the intestinal lumen. The alignment of the crypt bacteria reflected this gradient: organisms at the base had no specific orientation however in the middle and towards the tops of the crypts they were often orientated with their axes aligned with that of the crypt. Therefore motility by the bacteria would, in the middle and tops of the crypts, be opposite the flow of secretion. A further indication of adaption to motility in crypts was that these organisms were found predominantly in the mucus component of intestinal scrapings and were highly motile in this substance.

The mucosa-associated populations are well suited to survival on, or near, the tissue surface. In contrast they appear to be less adapted to the conditions in the intestinal lumen: they are rarely seen in this site. Consequently when these populations have been seen in faeces, usually during intestinal disease, they have been implicated as causative agents (Zymet, 1969). Experiments by Leach _et al_. (1973) demonstrated that, organisms with a spiral-shaped morphology appeared in the stools of rats given the chemical purgative $MgSO_4$ and it was suggested these organisms originated from intestinal

crypts. In an attempt to prove this hypothesis the effects of three purgatives on the intestinal crypt microbiota were tested; however, before analysing these results it is necessary to consider how these substances alter conditions in the gut.

Magnesium sulphate has long been characterized as an osmotic purgative (Fondtran, 1967), however it has been proposed recently that the effects of $MgSO_4$ are caused by the release of the hormone cholecystokininpancreozymin (CCK-PZ) (Harvey and Read,1975). This hormone is released from the intestinal mucosa in response to $MgSO_4$ and initiates a wide range of effects, predominantly on the small bowel. The levels of bile, pancreatic juices, intestinal secretions and sodium ions rise in the small bowel. The motor activity in the small bowel is increased so that the colon is presented with an unusually large volume of fluid to reabsorb. The CCK-PZ also impairs water take-up in the colon, although to a lesser degree than in the ileum.

Cascara is a vegetable extract that belongs to a group of purgatives known as sennosides (Todays Drugs, 1969). These substances are degraded by bacteria in the large bowel to release a range of anthroquinones (Hardcastle and Wilkins, 1970). Two major effects of these substances have been described as occurring in the large bowel. Firstly, anthroquinones stimulate Auerbach's plexus and this increases motility in the colon (Todays Drugs, 1969). This group of substances has also been

shown to inhibit active sodium (Na^+) transport across the membranes of intestinal epithelial cells (Phillips et al., 1965).

The active agent in castor oil, ricinoleic acid, is known to be active in the small bowel, with lesser effects occurring in the large bowel (Todays Drugs, 1969). The primary effect is to increase secretions and motility in the small bowel. It has also been shown to affect Na^+ transport across cell membranes; however it does this to a lesser degree than cascara.

The purgative experiments confirmed the hypothesis of Leach, Lee and Stubbs (1973): treatment with either of the purgatives resulted in microbes normally associated with the mucosa being found in the stools. From observations made during these experiments it seemed as though two processes were involved in this result; either the organisms were flushed from the tissue surface or conditions in the intestine changed so that it became suitable for the proliferation of organisms normally associated with the mucosa.

The TSB, spirochaetes, and thin spirilla were all absent from the crypts in $MgSO_4$-treated animals, but present in the intestinal lumen. The ability of the crescent-shaped bacteria to remain in the ileal crypts, despite the effects of $MgSO_4$, is as previously stated, most probably related to their ability to attach to the tissue surface. As it is known $MgSO_4$ stimulates fluid excretion (Harvey and Read, 1975), it can be postulated that the

increased flow overcomes the ability of organisms to remain in the crypts; hence their appearance in the stools. An alternate hypothesisis was that $MgSO_4$ was toxic to bacteria; this seems unlikely as many of the spiral-shaped bacteria in the stools were actively motile and also the purgative did not affect the ileal crescent-shaped bacteria or the colonic crypt populations. In the later stages of $MgSO_4$ treatment the faeces contained large numbers of "S" shaped bacteria. These organisms were not flushed from the colonic crypts, as this tissue was not affected by $MgSO_4$ treatment, but were proliferating in the mucus component of the stools. The same phenomenom was seen with the other purgatives: although neither flushed the bacteria out of the crypts, spiral-shaped bacteria normally associated with the tissue surface were found in the stools. All three purgatives gave stools containing large amounts of mucus and in each the spiral-shaped bacteria were found in this component. Therefore the ability of these organisms to proliferate in the diarrhoeaic stools can therefore be related to the organisms' normal habitat, mucus, being present in large amounts in the intestinal lumen.

The absence of the segmented filamentous bacteria from sections of ileum after $MgSO_4$ and castor oil treatments may indicate the disappearance of these organisms or shearing off of the filament near the surface. Scanning electron microscopy would need to be used to resolve this question as the organisms are difficult to

study in thick sections (Garland et al., 1982). The alterations in crypt populations induced by cascara and castor oil were less pronounced than those caused by $MgSO_4$. The reason for increased numbers of TSB in the large bowel crypts is unclear as it is not known what factors influence the ability of these organisms to compete with the normal crypt biota. The presence of rod- and fusiform-shaped organisms may be due to alterations in the excretion of fluid from the crypts. Cascara has a number of effects on the intestine including changing Na^+ balance, motility,and acting as an irritant (Todays Drugs, 1969; Hardcastle and Wilkins, 1970). Consequently, the amounts and properties of the fluids being produced in the cells lining the crypts may have been changed; either less was excreted or the mucus was of a lower viscosity, thus enabling the less adapted rod-shaped organisms into the crypts. Such changes may also explain the increased number of thin "S" shaped organisms in the colon as they preferentially attach to goblet cells.

The time taken for normal numbers of mucosal populations to re-establish in the intestine following $MgSO_4$-induced diarrhoea was comparatively short: 2-4 days. In previous studies where the mucosa-associated microbiota have been disturbed and allowed to normalize there has been considerable differences in the period taken for various populations to recolonize. Savage and Dubos (1968) found that the populations removed by penicillin treatment recolonized at a rate influenced by the period of treat-

ment; the minimum period for fusiform-bacteria, associated with the colon mucosa, to recolonize was 8 days. After similar antibiotic treatment the segmented filamentous organisms were not able to recolonize the ileum for a period up to 4-5 weeks, even after inoculation of normal ileal homogenates. In contrast the same organisms, after being removed by either 500 rads of γ radiation (Porvaznik et al., 1979) or 35 atmospheres pressure (Merrell et al., 1979), recolonized the ileum within 2-4 days: a period similar to the recolonization after $MgSO_4$-induced diarrhoea. The differences between the periods of recolonization may be due to the extent to which the environment, as a whole, was affected. Antibiotic treatment would appear to cause the greatest disruption; the recolonization of adult animals following such treatment closely mimicked the natural colonization of neonatal animals. The rapid recolonization after $MgSO_4$, γ radiation and hyperbaric pressure suggests that in comparison to antibiotic treatment, less substantial changes occur to the ecosystem, leading to a quick re-establishment of normal conditions.

The most interesting aspects of the recolonization of the $MgSO_4$ treated rats was the presence of allochthonous populations associated with the intestinal mucosa. Of particular interest was the ability of the spiral-shaped bacteria to associate with the colonic mucosa for long periods and their consistent presence in all the groups of rats given this purgative.

In a well-functioning gastrointestinal ecosystem all available habitats and niches are occupied by climax communities of autochthonous microbes (Alexander, 1971). Allochthonous organisms occur as transient populations and are unable to colonize. Only when an ecosystem is perturbed and autochthonous populations displaced, can niches be made available for allochthonous colonization (Alexander, 1971). Clearly $MgSO_4$-induced diarrhoea is a severe perturbation to the intestinal ecosystem and one of its main effects is to dislodge many of the autochthonous populations. However the ability of the spiral-shaped organisms to colonize these animals cannot be explained simply as that of an allochthonous population occupying a vacated habitat, as these surfaces of the colonic mucosa are not colonized by microorganisms in normal animals. Moreover, the spiral-shaped organisms were not excluded from this habitat even after the return of the normal mucosal microflora of the colon. Indeed the organisms appeared to colonize permanently as they were still present six months after cessation of $MgSO_4$ treatment. This suggests the organisms were not occupying habitats or filling niches normally exploited by the normal autochthonous microbiota.

The widespread occurrence of the spiral-shaped organisms suggests this population is endogenous to the intestinal ecosystem, possibly being autochthonous to other habitats in the tract (Savage, 1977). The presence of the organisms in one control animal may represent a low

incidence of natural colonization. Alternatively, the colonization may have resulted from a perturbation in this rat's intestinal ecosystem; a process which can occur in conventionally housed animals (Savage, 1977).

From the results of the colonization experiments it is not clear whether the other populations, the rod-shaped bacteria and the mycoplasma-like bacteria, were also able to associate with the intestinal mucosa for long periods. As these organisms were only seen in one series of experiments they are probably exogenous, rather than endogenous, to the gut. Similarly, allochthonous populations have also been found associated with the ileal mucosa after other intestinal perturbations. Tannock and Savage (1974), observed large numbers of Gram-negative rods and Gram-positive cocci in the intervillial spaces in stressed animals but not in normal controls. Similarly Provaznik et al. (1979) found large numbers of rod-shaped bacteria closely associated with the ileal villi in animals exposed to radiation. In the latter study the allochthonous organisms remained on the tissue for only 2-4 days post-treatment, the period taken for the filamentous bacteria to recolonize after being removed by the radiation.

A possible explanation for the long term association of the allochthonous populations with the intestinal mucosa after perturbation is that a barrier to colonization exists in the conventional animal's intestine which excludes microbes from the surface (Alexander, 1971;

Cheng et al., 1981). Removal of such a barrier during perturbation would enable organisms to reach the surface and proliferate (Cheng et al., 1981). Rozee et al., (1982) have described such a barrier to colonization in the ilea of mice: a thick mucus blanket with a large complement of microorganisms overlaying the tissue surface. These authors have speculated that such a blanket of mucus would preclude pathogens and opportunistic microbes from tissue colonization. Similar layers of mucus are known to exist on the surface of the colon and are usually heavily colonized with specialized populations of fusiforms (Savage et al., 1968) and it is likely that the rat, like the mouse, has layers of mucus on the ileal surface. If so the lack of surface colonization in the colon of rats and the limited colonization of rat ileal vili can be explained by the protective function of these mucus layers. One of the effects of $MgSO_4$ was to disrupt the mucus layers in the colon and it is likely that the layers present in the ileum would also have been disturbed by this strong purgative. In the SPF animals the mucus layers were intact but lacked the specialised populations of fusiforms. Consequently the normal barrier to colonization would be deficient at the site of allochthonous colonization in both types of animals; thus enabling these organisms to reach the surface and proliferate. Presumably when the normal mucosal microbiota returned to the ileum the rod-shaped bacteria and the mycoplasma-like organisms would be displaced. As

there were not autochthonous organisms associating with the surface of the colon, the spiral-shaped bacteria would not be replaced when the normal intestinal microbiota returned. Furthermore if the organisms are autochthonous to sites elsewhere in the tract, as is likely with the spiral-shaped bacteria, normal host defence mechanisms may be ineffective in removing the established population (Cheng et al., 1981). Abilities to resist: antibodies, complement, phagocytic cells, surfactants, and shear forces, may be necessary for the organisms to colonize elsewhere in the tract and could also be advantageous at the new site. Many of the abilities are enhanced when the organisms are in a microcolony surrounded by a glycoprotein glycocalyx (Cheng et al., 1981); such bacterial glycocalyxes could be associated with the spiral-shaped bacteria microcolonies, however, they would be difficult to discern as the organisms colonized the host cells glycocalyx. The absence of competing bacterial populations and the ability to resist host defences would enable the organisms to remain on the tissue surface and to proliferate.

The presence of the spiral-shaped organism in only a proportion of the treated animals is analogous to the colonization of the colon in rhesus monkeys and man by spiral-shaped bacteria and spirochaetes (Neutra, 1980; Takeuchi et al., 1974). These organisms were found by Takeuchi et al. (1974) to be in colons of 62 to 221 healthy monkeys, in 4 of 210 human colon specimens and in 8 of 388 removed appendices. The colonization appeared to be of a

chronic nature however in the human cases, with one exception, and in all the monkeys there were no associated bowel symptoms. The type of association formed by these organisms with the mucosa was similar to that of the spiral-shaped organisms in the rat colon, the microvillus border being heavily colonized with bacteria. The spiral-shaped bacterium designated as the "flagellate" and studied in detail by Neutra (1980) was very similar to the spiral-shaped organism in both ultrastucture and mode of association with the tissue surface. The similarity of the organisms and of the type of association involved makes it likely that the aetiology of colonization in rhesus monkeys and man by spiral bacteria is similar to that occurring in the rat experiments.

The present chapter has described the microbiota intimately associated with the rat intestinal mucosa and the effects of chemically induced diarrhoea on these organisms. The results were obtained by observational means rather than using cultural techniques as few of these microorganisms have been isolated and subcultured on artificial media; the next chapter details attempts to grow some of these microbes.

CHAPTER FOUR

ISOLATION AND CULTIVATION OF MUCOSA-ASSOCIATED BACTERIA

4. ISOLATION AND CULTIVATION OF MUCOSA-ASSOCIATED BACTERIA

Most of the microorganisms isolated from rat and mouse intestinal mucosa have been those from the mucus layers adjacent to the tissue surface. The only crypt bacteria isolated have been spiral-shaped bacteria cultured from rat and mouse caeca (Lee and Phillips, 1978). Consequently an important aim of the present investigation was to isolate and cultivate microbes intimately associated with the mucosa of the rat intestine, particularly crypt dwelling bacteria.

The methods used to isolate these organisms were a modification of the methods used by Lee and Phillips (1978) and were formulated on the basis of parameters chosen as being important in the mucosal environment: the ratio of lumen bacteria to mucosal microbes, the nutritional environment adjacent to the mucosa, and the concentrations of oxygen and carbon dioxide at this site.

As tissue-associated bacteria constitute only a small fraction of the microbiota at most sites in the ileum and large bowel it was necessary to increase the proportion of mucosal microbes in the inoculum prior to culture. This was achieved by a "mechanical enrichment": by using an inoculum of mucosal scrapings taken from tissues agitated in ten changes of saline. Most of the material loosely associated with the mucosa would be removed by the vigorous washing while the organisms in the crypts and attached to the surface would remain. Evidence of this process was the presence of relatively high numbers of mucosa-associated microbes in the intestinal scrapings.

Composition of the gaseous atmosphere needed by the mucosa-associated microbes was also considered an important factor; whilst it could be expected that many mucosal organisms would be strictly anaerobic it was also thought that close proximity to oxygenated eucaryotic cells could result in some of these organisms having other requirements. Consequently isolation of organisms was attempted under a variety of atmospheres: in an anaerobic chamber, using the less rigorous anaerobic conditions of gas jars with Gaspak $H_2^+CO_2$ generating systems, microaerophilically in gas jars without catalyst with Gaspak $H_2^+CO_2$ generators, and aerobically.

A wide variety of media have been used previously to grow microorganisms from the intestinal tract. Many contain complex additions, such as rumen fluid, which attempt to create conditions similar to those of the intestinal lumen. Environmental conditions on the mucosal surface, and particularly in the intestinal crypts, are likely to be quite different from those prevailing in the lumen of the tract. For this reason the medium used initially for isolation experiments was simply, lysed horse blood added to a blood agar base and as this proved successful no further nutritional supplements were added. Although most lumen bacteria were removed by the vigorous washing prior to the preparation of scrapings, the few remaining, often overgrew the culture plates: coliforms in the ileal preparations and fusiforms in those from the large bowel. This overgrowth was inhibited by the addition of

antibiotics such as polymxin B sulphate, penicillin, vancomycin, bacitracin, trimethoprim or colistin. A commercially obtained combination of some of these compounds, Oxoid campylobacter selective supplement SR69 (Skirrow), containing: vancomycin, polymxin B sulphate, and trimethoprim lactate was found tc be very effective in suppressing lumen bacteria while enabling growth of mucosal organisms and hence was used as an additive in most of the isolation media.

Lee and Phillips (1978) were able to isolate spiral-shaped bacteria from rodent caeca using mucosal scrapings as an inoculum, lysed blood agar containing polymxin B sulphate as the medium and anaerobic incubation. By varying the isolation methods as described above: using different atmospheres and CSA69 as the medium other spiral-shaped bacteria have been isolated from the ileum, caecum, and colon of rats and also mice. Microaerophilic incubation of CSA69 plates inoculated with rat ileal scrapings usually resulted in the growth of three types of organisms: lactobacilli, thick spiral-shaped bacteria (RTSB), and small "s" shaped bacteria. TSB were also isolated from mouse ileal scrapings (MTSB). Both TSB isolates failed to grow anaerobically while none of the organisms grew aerobically. Both the TSB's and the "s" shaped bacteria were occasionally isolated from rat large bowel scrapings, however the organisms most commonly isolated from these preparations grew only on the anaerobic plates. The anaerobic isolates, like the spiral-shaped bacteria isolated from rodent caecal mucosa by Lee and

Phillips (1978), were able to survive exposure to oxygen as they could be isolated by manipulation on the bench and incubated in anaerobic gas jars. In addition to the organisms isolated by Lee and Phillips from the caecum: spirochaetes and thin rigid spirilla, a number of small "s" shaped bacteria were also cultured from caecal and colonic scrapings. Some of these isolates resembled the "s" shaped bacteria from the ileum however none would grow micro-aerophilically.

After preliminary studies were made of the various mucosal isolates the TSB were chosen for use in the further investigations of the mucosa-associated microbiota. The ultrastructure of the TSB, when examined by TEM, was found to be very similar to the TSB seen previously in the intestinal crypts of rats, suggesting that they were the crypt dwelling organisms. Also the size and ultrastructure of the TSB made these organisms more easily identified, in histological preparations of tissue, than the other mucosal isolates. Further reasons for studying the TSB were that they appeared not to belong to any existing genera, and also because of their interesting ultrastructure.

4.2 THICK, SPIRAL-SHAPED BACTERIA (TSB) ISOLATED FROM RAT AND MOUSE ILEA

4.2.1 Isolation and Cultivation

The TSB were isolated on CSA69 plates inoculated with intestinal scrapings and incubated microaerophilically. The organisms were fastidious, growing only on media enriched with blood or serum. Optimum growth occurred on plates with a moist surface. If

the condensation present on the surface of the medium after pouring was removed by air drying, the organism grew poorly or not at all. The organisms grew as a fine, spreading film on media with either 1.2 or 3% agar (Roach and Tannock, 1979). The spreading growth became apparent after 2 to 3 days of incubation for the rat isolate and 3 to 5 days for the mouse isolate. Individual spreading colonies were initially present; however, with longer incubation these merged into a continuous film of growth. For subculturing, material was transferred from an isolated focus of growth. After repeated subculture good growth was obtained from both isolates after 2 day's incubation.

Attempts to grow the organisms in broth culture have been unsuccessful; however, large numbers of viable organisms were found to be present in the liquid phase of biphasic cultures. Slopes were inoculated and incubated for 24 hours to obtain surface growth before peptone water was added. After an initial microaerophilic incubation (24 hours) the overlaid slopes were incubated in air. When incubated at 37^{o}C, cultures on solid media lost viability within 6 to 8 days, while those on overlaid slopes remained viable for up to 30 days. The organisms died after 1 to 2 days when kept at 4^{o}C and have not been successfully lyophilized. Long-term retrieval was achieved by storing cultures in liquid nitrogen.

4.2.2. Morphology and Ultrastructure

In 2- to 3-day-old cultures, cells were spiral-shaped, rigid, and Gram-negative. They were 3.5 to 5 µm long and 0.5 to 0.6 µm wide with two to three spiral

turns. The cells were motile by a rapid corkscrew-like motion. In 4- to 5-day-old cultures, cell size and morphology became variable, with longer (5 to 6 μm), shorter (2 to 3 μm), and spherical forms (2 to 4 μm) being present.

When thin sections of the isolates were examined the ultrastructure was found to be very similar to that described previously for TSB in tissue sections (Erlandsen and Chase, 1972; Davis et al., 1972): a coiled protoplasmic cylinder with 9 to 11 periplasmic fibres and a tuft of terminal membrane bound appendages (Fig.18).

Negatively stained preparations revealed that the fibres were arranged concentrically around the length of the cells; thus the fibres did not cross over each other anywhere along the cell. No insertion points nor any basal discs or proximal hooks, characteristic of spirochaetes' periplasmic fibres, could be seen. The terminal appendages, 10-14 of which could be seen in negative stains, were present at each end of the cell and did not, unlike normal flagella, retain a sine wave formation in these preparations. Initial investigations showed, as had a previous study (Davis et al., 1972), no evidence of flagella-like structures where the appendages were inserted; rather they appeared to be the membrane-bound continuations of the cells' periplasm described by these authors (Fig.18C). As the nature of the terminal appendages and the periplasmic fibres, as well as the taxonomic status of the TSB, remained ambiguous further ultrastructural studies were undertaken; firstly by a more extensive study

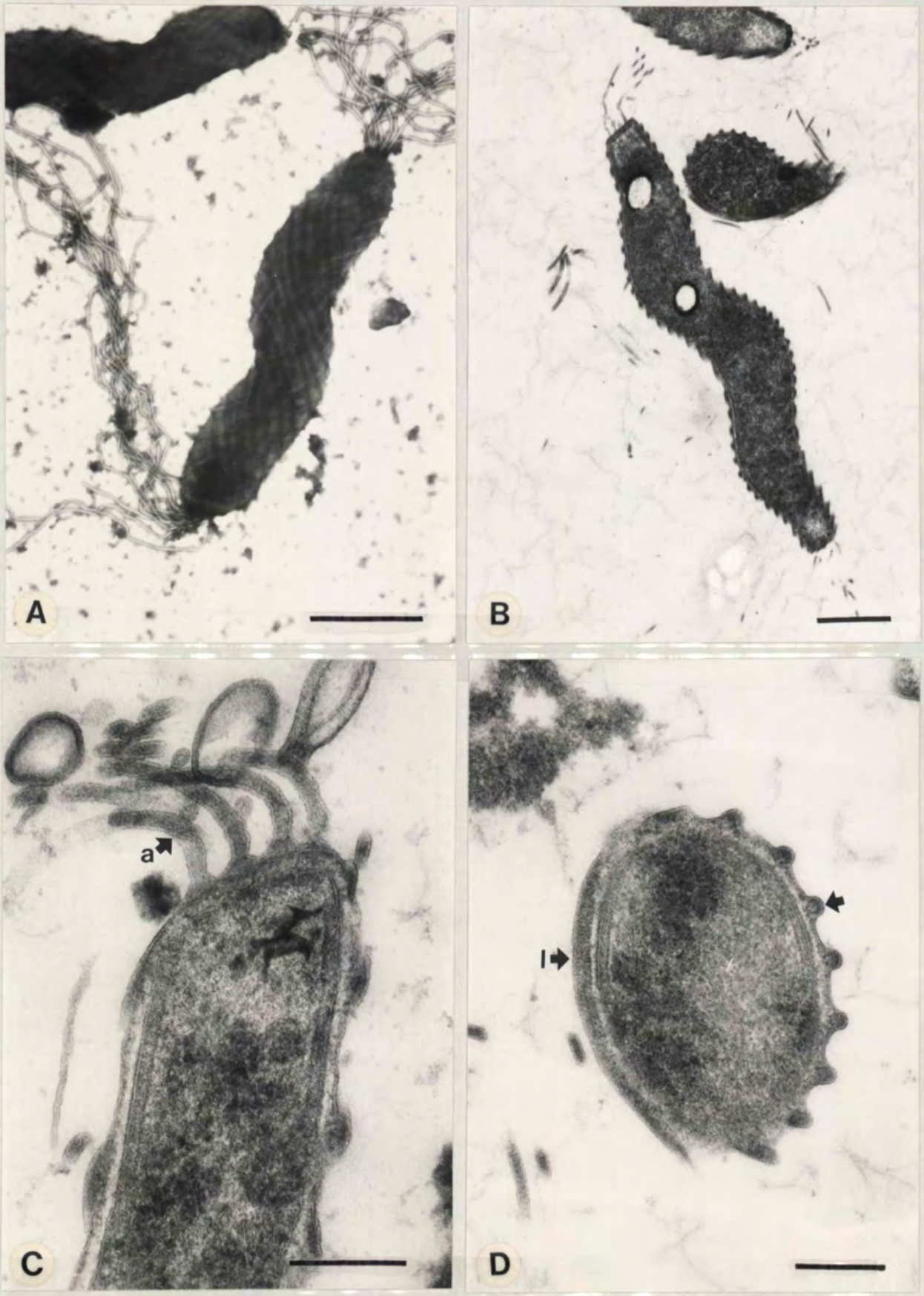
Figure 18. Initial TEM observations of the TSB isolates.

A. Negative stain x 19,600, bar = 1 um.

B. Thin section x 25,000, bar = 0.5 um.

C. Thin section of polar region showing membrane bound appendages (a) x 42,900, bar 0.5 um.

D. Transverse section of TSB showing periplasmic fibres in cross section (arrow) and in longitudinal section (l) x 33,200, bar = 0.5 um.


A
B
a
C
l
D

of thin sections and also by examining negatively stained preparations of disrupted cells. In both studies no consistent differences were seen between the rat and mouse isolates; consequently no distinction is made when describing the results in the following sections.

4.2.2.1. Thin sections

Examination of sections from both the initial taxonomic study and the subsequent investigations revealed a number of features, predominantly associated with the terminal appendages and the periplasmic fibres, that characterized the TSB isolates.

The polar region of the isolates, adjacent to the insertion of the terminal appendages, was electron-translucent mainly as a result of an absence of ribosomes (Fig.19); also present in this region was an electron-dense band lining the cytolasmic membrane and similar to the polar membrane reported to occur in the other polarly flagellated bacteria (Coulton and Murray, 1978). The band extended from close to the insertion of the polar appendage to 0.3-0.4 μm distal from the site and consisted of a layer 8-10 nm wide, 20 nm distant from the cytoplasmic membrane. Fibrils were present in the space between the two membranes and had periodicy of 10 nm (Figs.19C).

As previously stated, the terminal appendages were sheathed by a continuation of the cell wall. The sheath was loosely arranged at the base of the appendages and occasionally an electron-dense core with a maximum diameter of 12-13 nm was seen within the sheath. Further from the cell the membrane became more closely associated

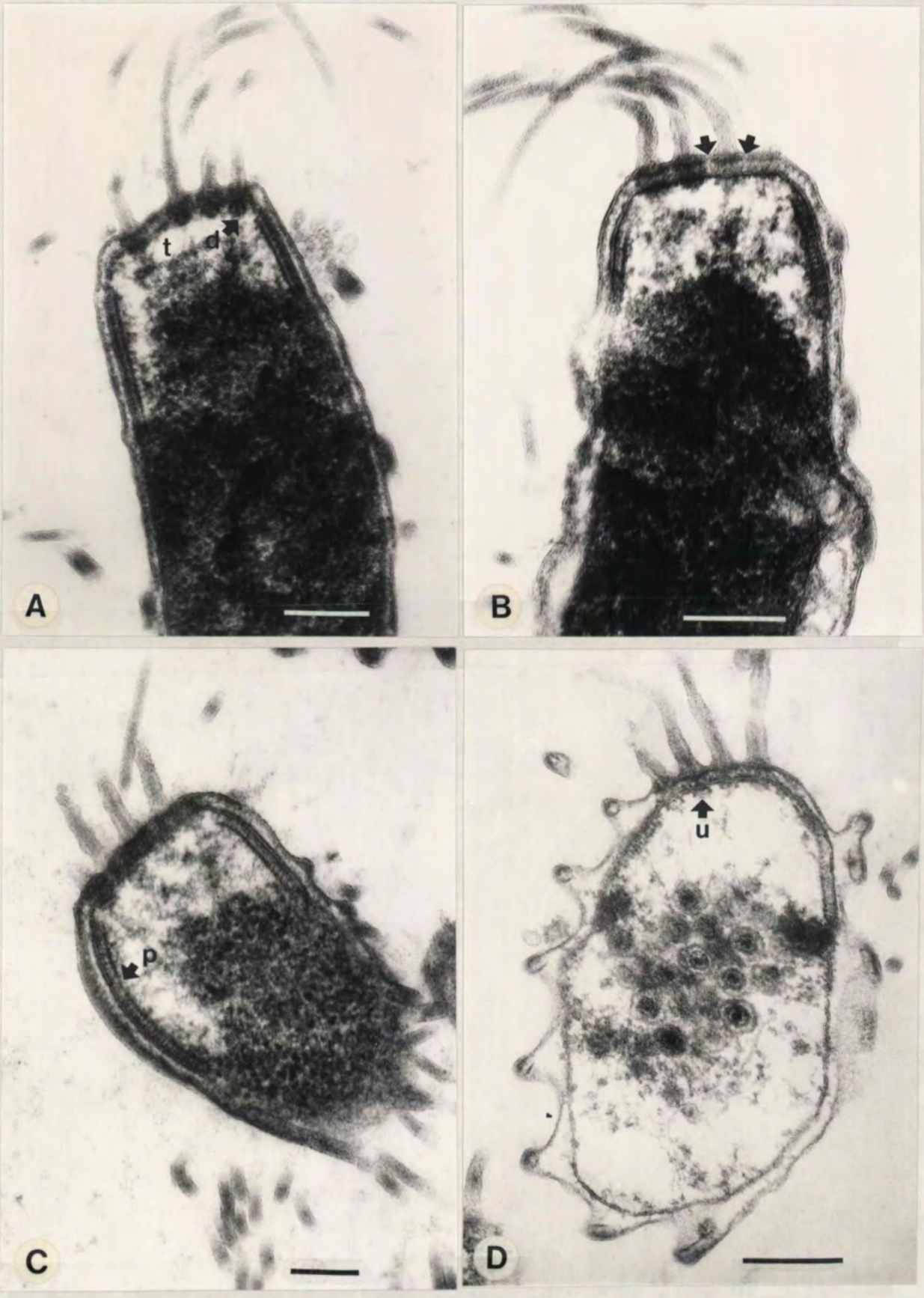
Figure 19. TEM observations of the polar region of the TSB isolates.

A. The region adjacent to the insertion of the polar appendages is electron-translucent (t), and electron-dense material (d) can be seen below the insertion of the appendages x 76,700, bar = 0.2 um.

B. Electron-dense bands of 50-70 nm. (arrows) can be seen associated with the insertion of the appendages x 88,500, bar = 0.2 um.

C. An electron-dense band: the "polar membrane" (p) associated with the cytoplasmic membrane x 117,400, bar = 0.1 um.

D. Small "U" shaped membranes (u) can be seen on the inside of the cytoplasmic membrane in a spherical body x 87,600, bar = 0.2 um.


t
d
A
B
p
C
u
D

with the core and hence the diameter of the appendage decreased from the base to the tip.

Visualization of the insertion site of the polar appendages was difficult as this region was generally more electron-dense than the surrounding material, and also the thickness of the sections (50-70 nm) meant that components were often superimposed; nevertheless some features were discernable. Where the core of the appendages entered the periplasm there was a flat, electron-dense band below the level of the cell wall, having a diameter of 65-70 nm (Fig.19B). Further electron-dense material was present below the cytoplasmic membrane, often in the form of an inverted, flattened "U"-shape, with a diameter of 41$\pm$2 nm. In some sections there was evidence of a membrane-like component in this material; these were most apparent in spherical bodies (Fig.19D).

Examination of many sections indicated that the periplasmic fibres were not solid filaments but were hollow tubules. In angled sections these appeared oval shaped (Fig.20B), however in fortuitous cross-sections they were circular (Fig.20A) with an overall diameter of 28-32 nm; the thickness of the tubule wall was approximately 5 nm.

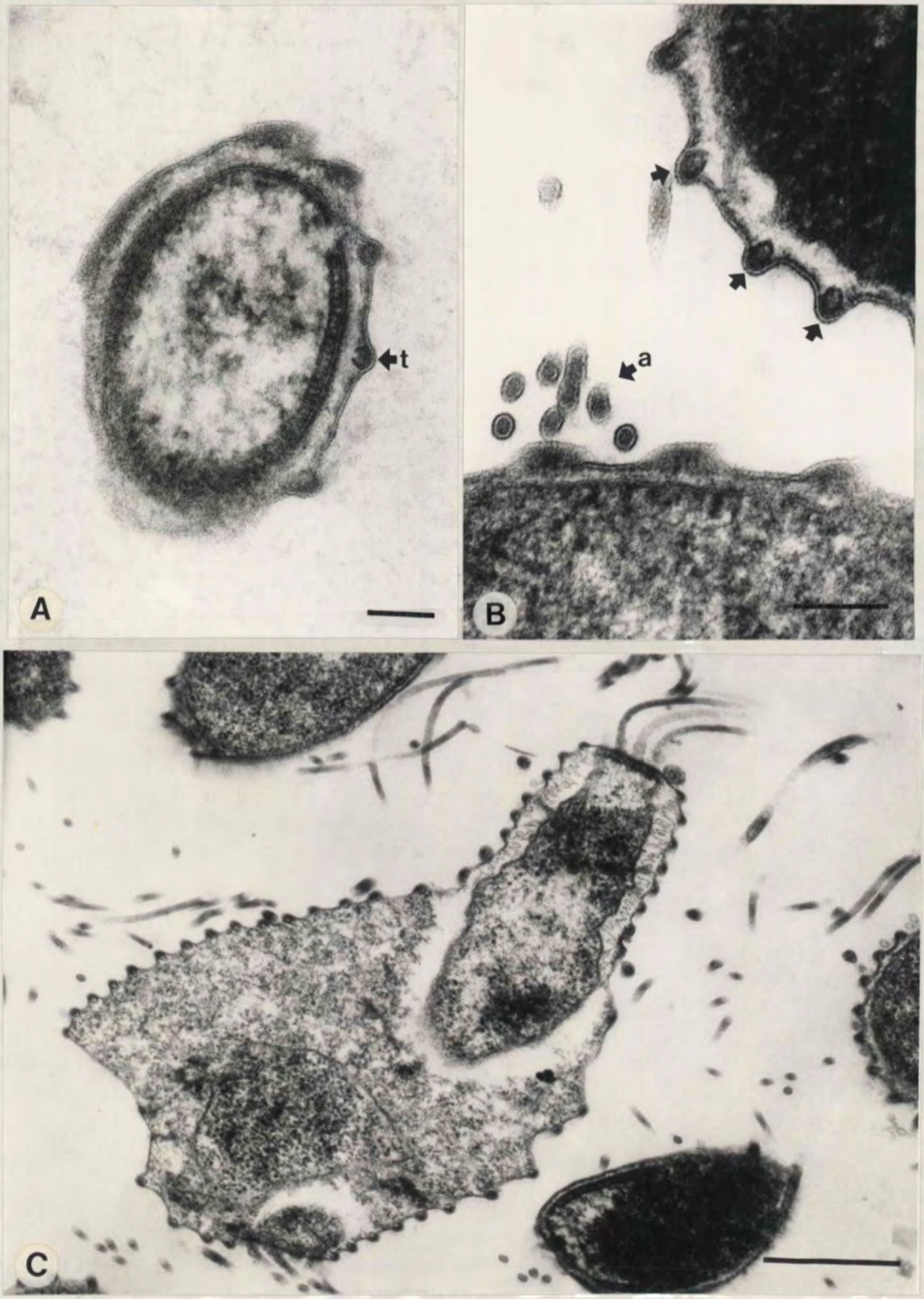
In some sectioned preparations the tubule wall was discontinuous (Fig.20A), however it is not known whether this was its structure or a preparation artifact. A characteristic of the TSБ isolates was the occurrence of spherical bodies. When examined by EM these bodies were seen to be TSB in various stages of degeneracy. Two types of spherical bodies could be seen: those with disrupted

Figure 20. TEM observations of the TSB isolates.

A. Transverse section showing a circular, but discontinuous periplasmic tubule (t) x 123,900, bar = 0.1 um.

B. Section showing oval shaped periplasmic tubules (arrows) and sheathed appendages (a) x 169,400, bar = 0.1 um.

C. A spherical body, having a disrupted cytoplasmic membrane x 48,800, bar = 0.5 um.


t
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cytoplasmic membranes (Fig.20C) and those with disrupted cell walls (Fig.21). Where the cytoplasmic membrane had ruptured, the cytoplasm filled the periplasm, bloating the surrounding cell wall; often only one end of the cell was affected (Fig.20C). Interestingly, although losing its spiral shape, the appearance of the cell wall was similar to normal organisms: the tubules remained attached to the inner surface and the ridges in the cell wall remained, as did the insertion sites of terminal appendages.

In organisms where the cell wall had ruptured the cytoplasm was contained in spheroplasts bound by the cytoplasmic membrane (Fig.21A). Although tubules were not present on the disrupted cell walls, the ridges in the surface remained and were lined with a semi-circle of electron-dense material, with a width of 4-5 nm (Fig.21B,C). As such a band was not observed between the cell wall and the tubules in sections of intact bacteria it is possible that this material was a residue of the tubule wall.

4.2.2.2 Negatively stained preparations

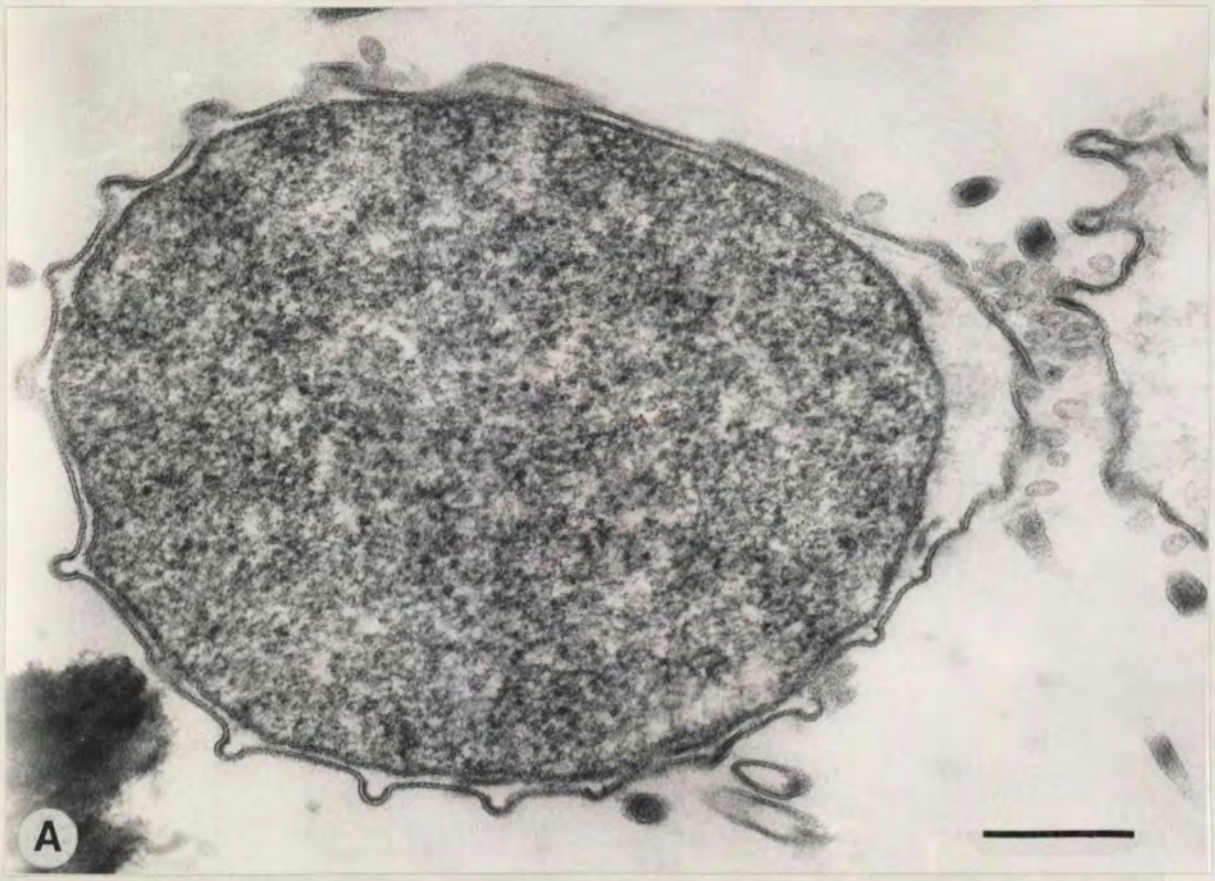
In order to investigate the internal organisation of the TSB, experiments were undertaken to disrupt the cell wall and also the cytoplasmic membrane without destroying the tubules or flagella. The cell wall was removed by resuspending centrifuged cultures in 0.2% Teepol solutions for 10 min before preparation for TEM. Such treatment resulted in the periplasmic tubules unravelling from the protoplasmic cylinder (Figs.22,23). A number of observations were made of the tubules: a. the tubules were not inserted into the protoplasmic cylinder at

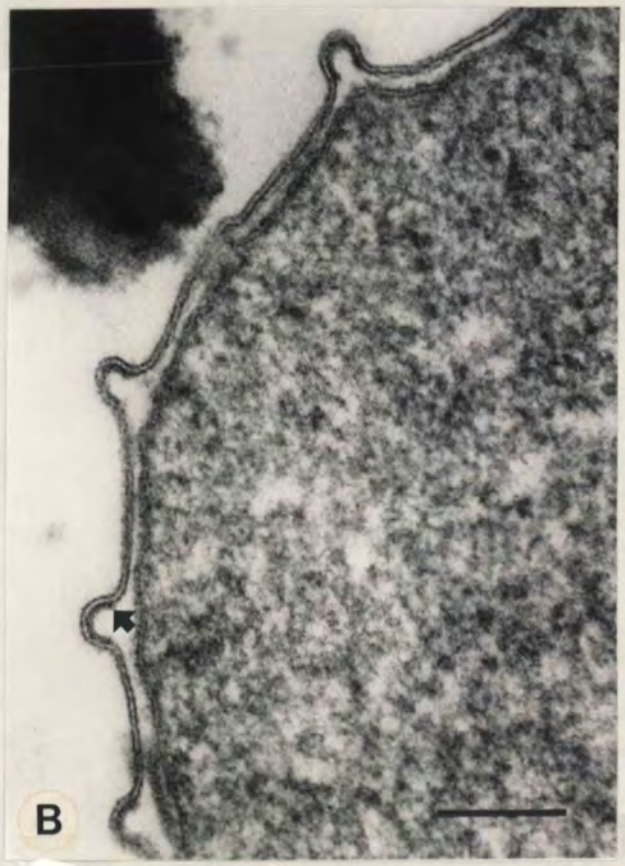
Figure 21. TEM observations of TSB spherical bodies: those with disrupted cell walls.

A. x 101,400, bar = 0.2 um.

B. While tubules are absent from the periplasmic space: electron-dense material (arrow) remains on the inside of the cell wall, x 169,400, bar = 0.1 um.

C. Although disrupted, the cell wall retains ridges, lined with electron-dense material (arrow) x 87,600, bar = 0.2 um.


A


B

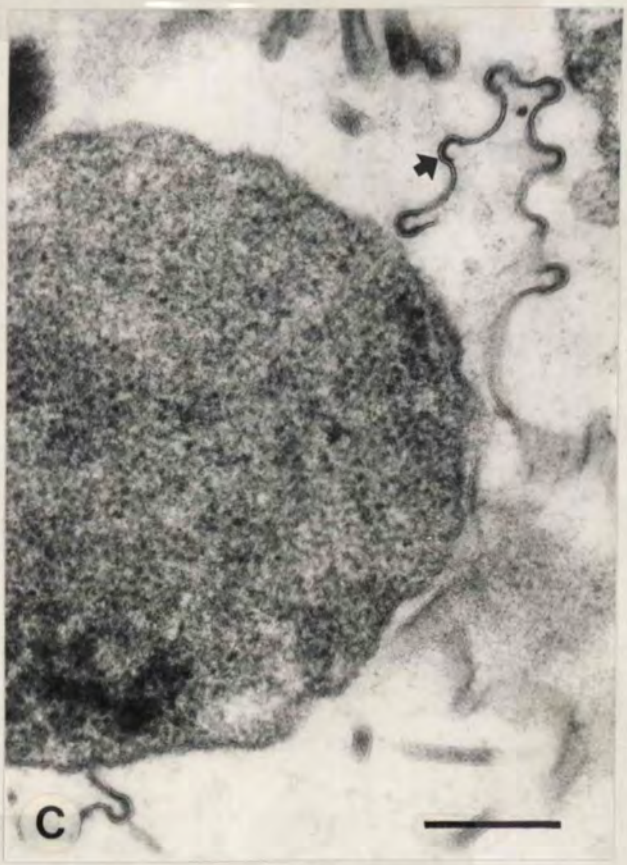

C

Figure 22. TEM observations of TSB isolates treated with Teepol and negatively stained.

A. Disruption of cell wall and release of periplasmic tubules x 32,100, bar = 0.5 um.

B. Demonstration of no continuity of periplasmic tubules and terminal appendages x 56,100, bar = 0.5 um.

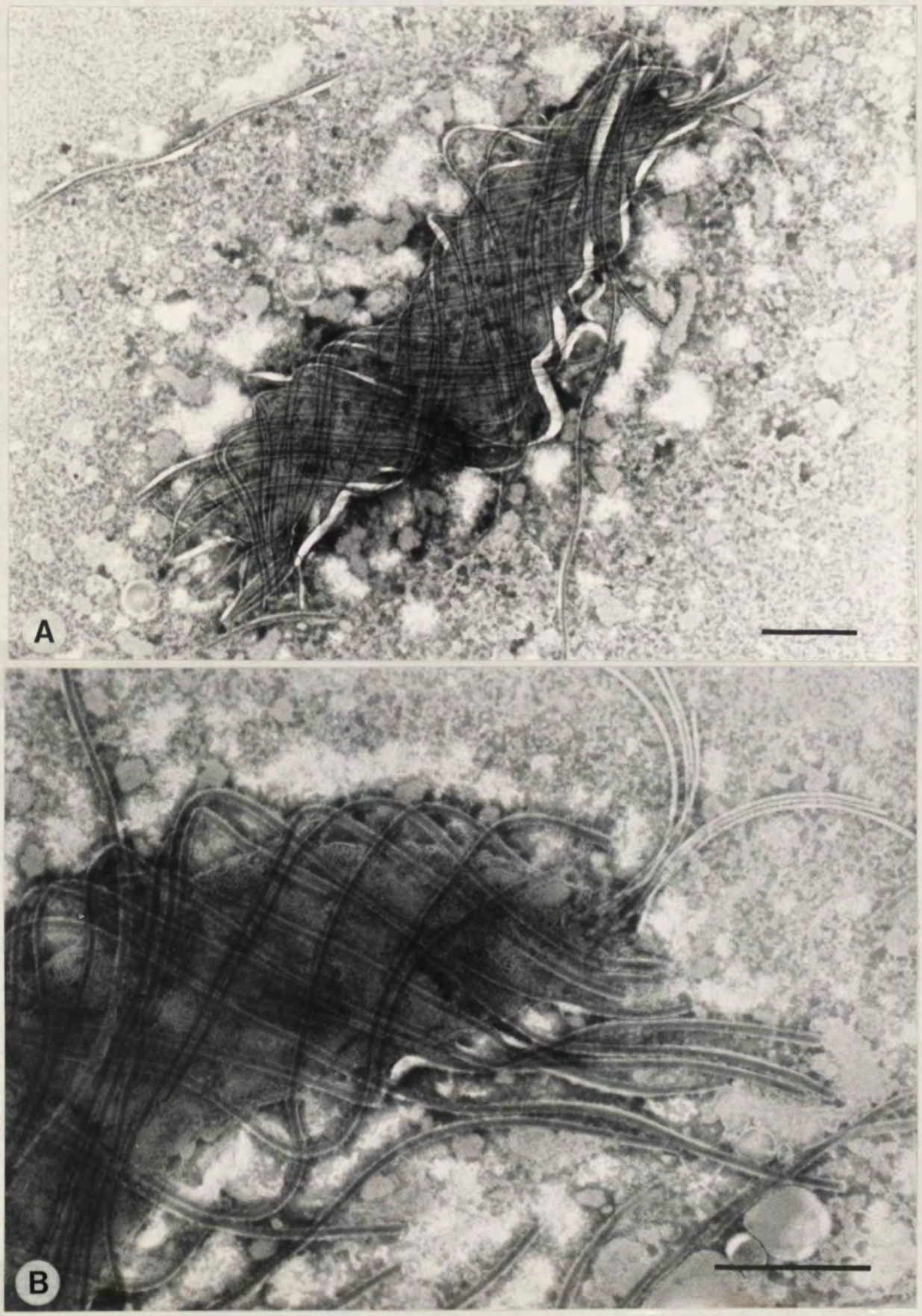
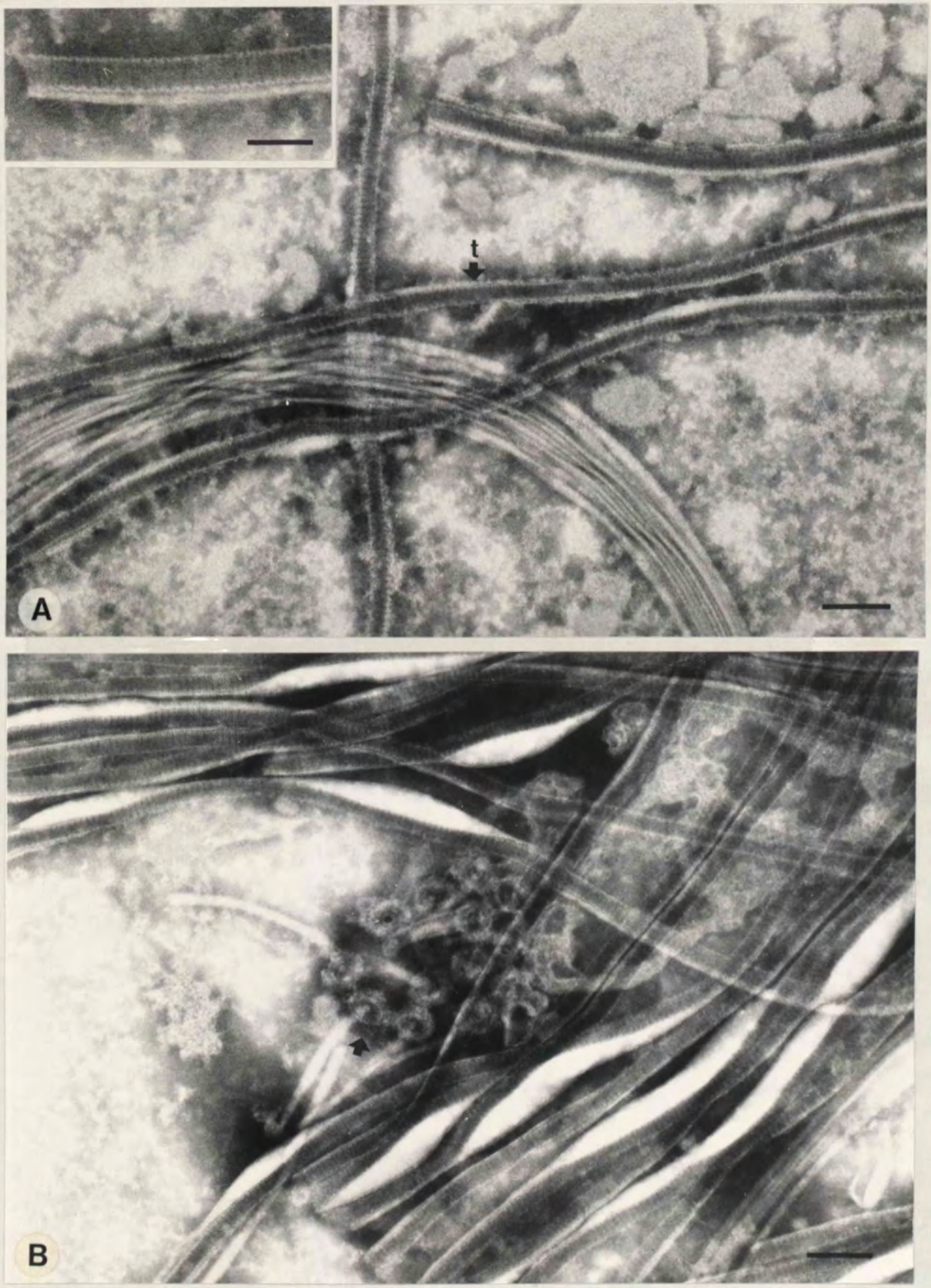

A
B

Figure 23. TEM observations of negatively stained TSB preparations.

A. The striated appearance of the periplasmic tubules (t) can be seen in this Teepol treated sample x 117,410, bar = 0.1 um; insert; the periodicy of the bands was approximately 9 nm. x 240,600, bar = 0.05 um.

B. Circular bodies (arrow) associated with appendages in sample treated with Teepol and sonicated x 117,400, bar = 0.1 um.

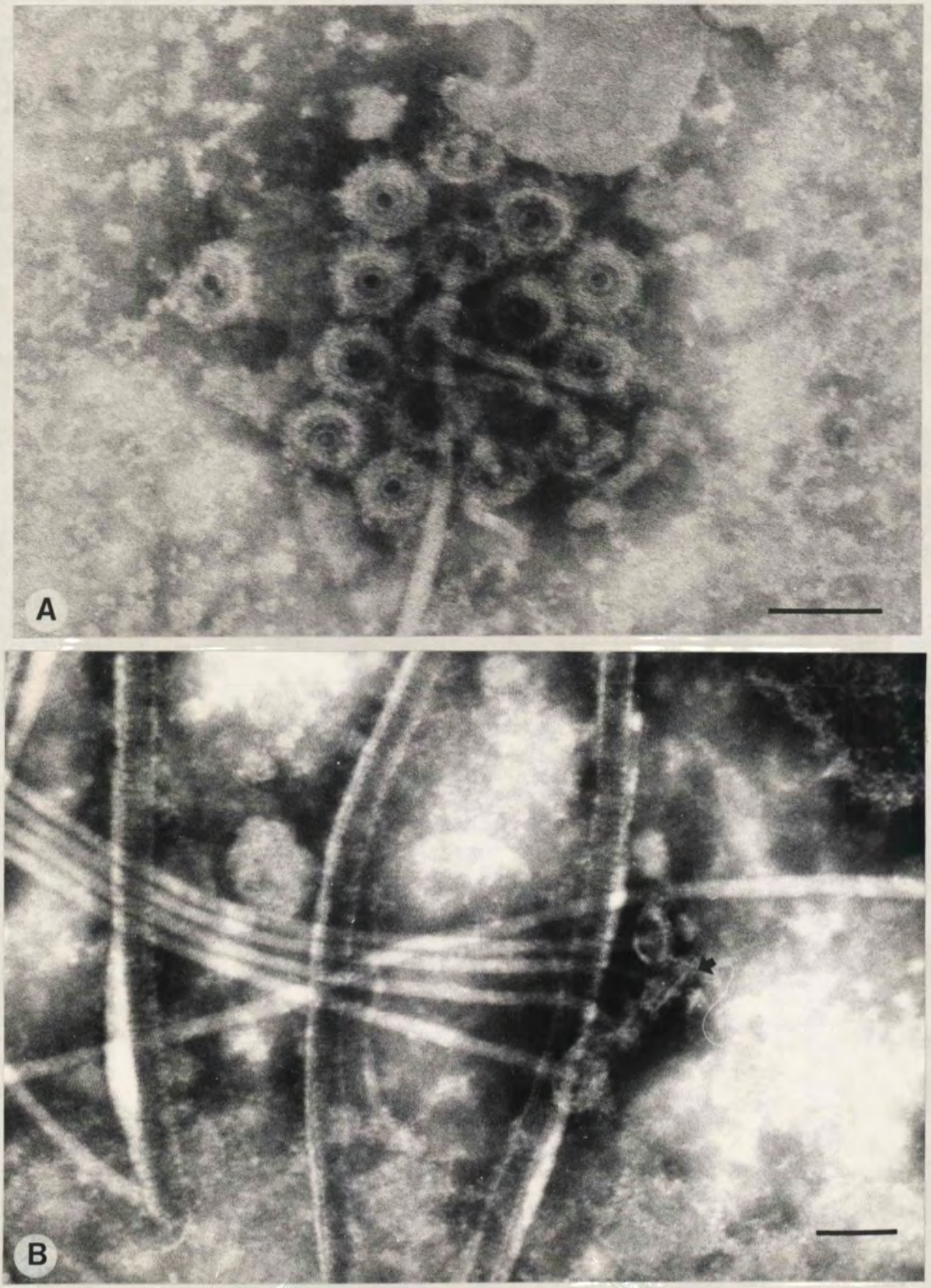

t
A
B

any point, b. there was no association between the tubules and the polar appendages (Fig.22), c. the ends of the tubules showed no specialized structures nor any internal core material (Figs.22,23), d. the tubules were of a constant diameter (28-30 nm) and did not taper at the ends, e. at higher magnification the tubules were seen to have a striated appearance; the periodicy of the bands was 9 nm (Fig.23A). Although treatment with Teepol led to the breakdown cf the sheath on the polar appendages, leaving a core 15 nm in diameter, this core remained inserted into the protoplasmic cylinder. By briefly sonicating Teepol-treated organisms for 15 s the cytoplasmic membrane was ruptured revealing components associated with the insertion of the appendages. The bases of many appendages were attached to the centre of small circular bodies (Figs.23B,24A). These circular bodies were generally found in clusters and most were not attached to appendages. The structure of these components was very similar to the Circular Membrane Rings (CMR), described by Coulton and Murray (1978), to be present in several Spirillum species; both are a series of concentric rings around a 12 nm central hole (Fig.24A). While the CMR had 7 rings, the largest of which was 90 nm diameter, the circular bodies of the TSB had only 3 rings; one of 16 to 20 nm, another of 42 to 47 nm and the external ring with a diameter of 68-72 nm. Where the circular bodies were seen in profile they appeared as two stacked discs; determination of the thickness of these discs was difficult as few were seen suitably orientated, however from Fig.29B, it was estimated that the height of the discs was 25-30 nm.

Figure 24. TEM observations of TSB isolates treated with Teepol, sonicated and negatively stained.

A. Circular bodies x 206,600, bar = 0.1 um.

B. Circular bodies in profile (arrow) x 151,800, bar = 0.1 um.


A
B

Further studies were made of the TSB ultrastructure by examining freeze-dried and freeze-fractured preparations; reports of these studies are given in the Appendix.

4.2.3 Biochemical Characteristics

Both isolates were oxidase and catalase positive. Neither was able either to ferment any of the test carbohydrates or to hydrolyze casein, starch, or tributyrin. No lipase or lecithinase activity was detected, gelatin was not liquified, indole was not produced, and nitrate was not reduced. There were no reactions in either litmus milk or cooked meat medium, and the organisms would not grow in the presence of 2% bile salts. Incubation with 1% glucose-peptone water, although not giving a positive fermentation result, yielded succinic acid and acetic acid as detectable end products. The controls, serum slopes with glucose-peptone water overlays, showed no detectable end products after 14 days of incubation.

4.3 Discussion

Although the presence, in the gastrointestinal tract, of spiral-shaped bacteria has been known of since the beginnings of microbiology (Parr, 1923), the lack of suitable culturing methods has resulted in these organisms being one of the most poorly studied components of the gut microbiota. Recently such techniques have been developed and many spiral-shaped bacteria have been isolated and

maintained *in vitro*. Using anaerobic methodology, and complex media containing rumen fluid and other additives, spirochaetes have been isolated from: the rumen (Ziolecki *et al.*, 1975; Stanton and Canale-Parola, 1979), the large bowel of dogs (Turek and Meyer, 1978), and pigs (Lemcke and Burrows, 1980), while other spiral-shaped bacteria have been isolated from the large bowels of dogs (Davis *et al.*, 1976) and mice (Roach and Tannock, 1979). These methods have been less successful for isolating organisms intimately associated with the tissue and the majority of the bacteria found in the crypts and attached to the tissue surface have not been isolated using these techniques. The methods that have been successful in isolating such populations have minimized the growth of lumen bacteria by the use of antibiotics and by using tissue scrapings (Lee and Phillips, 1978), or by utilizing high concentrations of agar to prevent swarming (Roach and Tannock, 1979). While some organisms have been isolated on media containing rumen fluid: spirochaetes from canine large bowel (Turek and Meyer, 1972), and spiral-shaped bacteria from the mouse caecum (Roach and Tannock, 1979) others have been isolated on media containing blood rather than rumen fluid: spirochaetes in man (Hovind-Hougen *et al.*, 1982) and mice (Lee and Philips, 1978) and spirillum-like bacteria in rats (Lee and Phillips, 1978). Such differences may relate to the degree of intimacy to the host; crypt organisms would be less likely to require nutrients from the intestinal lumen than would be the organisms in the mucus layers adjacent to the lumen contents. In the present study, the

organisms cultured, some appearing to be crypt bacteria, were able to grow without rumen fluid or any similar lumen derived additives. The requirement for serum or blood may indicate that some or all of the isolates growth requirements originate from the host tissue. The microaerophilic requirements of the TSB and the ileal "s" shaped isolates indicates that some sites on the surface of the tissue are more oxygenated than the highly reduced intestinal contents. Furthermore as the TSB were unable to grow anaerobically or aerobically, microaerophilic incubation was a critical factor in the successful isolation.

Clearly the major reason for the success of the isolation experiments was, as in previous studies, prevention of contamination by lumen bacteria. Washing the intestinal tissues in the changes of saline ensured that the vast majority of the lumen bacteria were removed from the tissue surface. Moreover, most of residual lumen microbes were unable to grow in the presence of the antibiotics used. The selective medium CSA69 was originally designed for the isolation of Campylobacter jejuni species using an incubation temperature of 42^{o}C. The supplement successfully inhibits the majority of gut bacteria but fortuitously many of the spiral-shaped bacteria associating with the mucosa were resistant to the combination of antibiotics it contains, and thus when incubated at 37^{o}C it is a very useful isolation medium for mucosal bacteria.

4.3 ULTRASTRUCTURE AND TAXONOMY OF THE TSB ISOLATES

The two components giving the TSB such a distinctive ultrastructure are the terminal appendages and the periplasmic fibres. Although the appearance of these components has been briefly reported previously (Erlandsen and Chase, 1972; Davis et al., 1972), prior to the present investigation little was known about their structure.

The results of the sectioning, and negative staining experiments indicate that the terminal appendages were, contrary to previous reports (Davis et al., 1972), sheathed flagella. However, the appearance of the sheath is unlike that of the bacteria with this type of flagella. While the sheath in organisms such as Bdellovibrio bacteriovorus (Burnham et al., 1970), Pseudomonas stizolobic (Fuerst and Hayward, 1969), Beneckea and Photobacterium fischeri (Allen and Bauman, 1971) and Vibrio cholerae (Das and Chatterjee, 1966), is closely associated with the flagellum core for the full length of the appendages, in the TSB the sheath is only loosely associated with the flagellum core in the region proximal to the protoplasmic cylinder.

Observation of thin sections and negatively stained preparations indicated that the insertion site of the TSB appendages resembled the insertion of flagella in Spirillum spp. more than that of other Gram-negative bacteria such as Escherichia coli (De Pamphilis and Adler, 1971). Comparisons of the TSB with bacteria possessing sheathed flagella were limited as comprehensive studies of flagella insertion have not been undertaken with these organisms.

The terminal region of the TSB had characteristics common to many polarly flagellated bacteria. The cytoplasm near the tip of the bacterium was electron-translucent and a "polar membrane" (Coulton and Murray, 1978) was found lining the cytoplasmic membrane in this region. As the components of the flagella insertion are smaller than the thickness of thin sections (70-60 nm) clearly defined detail was difficult to obtain; nevertheless a number of similarities to sections of spirilla were seen. Electron-dense material was seen in both TSB and spirilla immediately below the level of the cell wall and also in both, a large electron-dense protuberance was present adjacent to the cytoplasmic membrane inside the cytoplasm. Similarly negatively stained preparations of disrupted cells also contained common features. Specifically circular, concentric ring structures, some attached to flagella, were seen in preparations from both types of bacteria. Although the structures from the TSB were of a smaller overall diameter and contained fewer rings than the concentric membrane rings (CMR) of Spirillum serpens (Coulton and Murray, 1978), the common 12 nm control hole, similar overall appearance, and association with flagella suggest they are closely related structures.

The electron-dense protuberance seen in TSB at the attachment of appendages appeared in many sections to be U shaped. It is not known whether the membrane-like structures seen at the same site were artifactual, however as their shape and dimensions were the same as that of the protuberance it is possible they are part of the sub-

cytoplasmic membrane compartment described by Coulton and Murray (1978). Although a number of disc-like projections were seen associated with the concentric ring structures too few were observed to determine the structure of the basal complex or any similarity to those of spirilla.

The tubule-like structure of the periplasmic fibrils is apparent from observations of sections and negative stains. The striated appearance of the tubules was seen in both preparations and in each was of a similar periodicy (8-9 nm). In negative stains the tubules were very similar to tubules encasing the periplasmic fibres in a number of spirochaetes (Holt and Canale-Parola, 1968; Nauman et al, 1969; Bharier et al, 1971). Although the size, striated appearance and periodicy were similar some differences were apparent; the most obvious being the absence of a periplasmic fibre within the TSB tubules or any evidence of such a structure in any preparation of these organisms. Also the TSB were, unlike the spirochaetal tubules, readily visualized in thin sections and were more robust: they were not easily fragmented, and were never seen unravelled as were some of the spirochaete structures (Nauman et al., 1969). Further discussion on the nature of TSB ultrastructure is given in the Appendix.

Organisms similar to the rodent TSB have been found in the digestive tracts of a number of other animal species. Spiral-shaped bacteria with concentric periplasmic fibrils and flagellum-like appendages have been observed in the fundic glands of dogs and cats (Weber and Schmittdiel, 1962), the stomachs of monkeys (Takeuchi and

Sprinz, 1970), and the colonic washings of mice (Davis et al., 1973). Recently, similar organisms have also been associated with the intestinal mucosa of both cats and dogs (O'Rourke, personal communication). Although the subject of many reports, the taxonomy of this morphologically similar group of organisms has remained ambiguous. For example, when Erlandsen and Chase (1972) first observed these organisms, they suggested that the organisms "bear a close resemblance to members of the order Spirochaetales." A later report on their research, however, identifies the organism as being Spirillum species (Kessel and Shih, 1976).

The spiral-shaped organisms from the fundic glands of dogs and cats were originally classified as Spirillum species in Bergey's Manual of Determinative Bacteriology (6th ed.) (Breed et al., 1948). Subsequent editions have, however, omitted these organisms as a result of the paucity of accurate information relevant to their classification. After a detailed ultrastructural study of the organisms from the gastric mucosa of dogs and a survey of the literature, Lockard and Boler (1970) were of the opinion that the organisms were "more clearly related to the order Spirochaetales than to the genus Spirillum."

If the current edition of Bergey's Manual of Determinative Bacteriology, i.e. the eighth edition (Buchanan and Gibbons, 1974) is referred to, the rodent isolates cannot be included in any of the presently defined genera of bacteria. While the TSB possess periplasmic tubules that are very similar in structure to tubules seen in spirachaetes, the absence of flagella-like periplasmic

fibres and the different arrangement of the tubules of the protoplasmic cylinder differentiate the TSB from this type of bacteria; furthermore the presence of terminal appendage is also inconsistent with the family Spirochaetaceae.

The TSB most closely resemble organisms from the family Spirillaceae. The shape of the protoplasmic cylinder, the possession of polar flagella, being Gram-negative, microaerophilic, oxidase positive, catalase positive, and unable to ferment carbohydrates is common to both TSB and Spirillaceae spp.. Furthermore, the ultrastructure of the polar region and insertion of flagella in the TSB cells was also very similar to spirilla spp.. The occurrence of periplasmic tubules in TSB is, however, inconsistent with the definition of Spirillaceae. The morphological studies of the organisms from the cat, dog, and monkey suggest that they also do not belong to any of the known genera for the same reasons, and it is possible that they, along with the rodent isolates, belong to a new, as yet unnamed genus of bacteria.

Like the TSB few of the spiral-shaped bacteria isolated from the intestinal mucosa in this study or in others (Lee and Phillips, 1978) can be easily placed into existing genera. As these organisms have adapted to particularly specialized environments it would not be surprising if they have also diverged significantly from other, originally similar, groups of bacteria. Furthermore it can be expected that this area of taxonomy will remain confused until the majority of intestinal spiral-shaped bacteria have been cultured, studied, and genetic

similarities to each other and other bacteria are determined by methods such as: guanine-cytosine ratios, DNA homology or restriction analyses. Only then will a logical rather than arbitrary classification for these organisms be evolved.

In Chapter 3 the survey of the mucusal surface demonstrated the extent and nature of the mucosa-associated microbiota of the rat, while the perturbation experiments demonstrated how changes in host physiology influence these populations. Having isolated bacteria from the surface of the intestine it was possible to begin further investigations into the nature of the mucosal environment and its associated populations. The ability of the TSB to colonize crypts and the effect of other intestinal populations on this process is tested in Chapter 5. The following chapter describes *in vitro* experiments designed to test factors that appeared likely, from observation of the mucosal environment, to be important in crypt colonization.

CHAPTER FIVE

COLONIZATION OF EX-GERMFREE, EX-GNOTOBIOTIC, AND SPF RODENTS WITH BACTERIA ISOLATED FROM RAT AND MOUSE INTESTINAL MUCOSA

5. COLONIZATION OF EX-GERMFREE, EX-GNOTOBIOTIC, AND SPF RODENTS WITH BACTERIA ISOLATED FROM RAT AND MOUSE INTESTINAL MUCOSA.

The gut ecosystem is very difficult to study because of the variety of habitats and niches present and the diversity of the intestinal microbiota. While *in vitro* experiments are often too simplified to have relevance to the ecosystem, *in vivo* experiments can be extremely difficult to interpret. An alternate approach to the study of the ecosystem has been to experiment with animals having restricted intestinal microbiotas: such as germ-frees and gnotobiotes. When these animals are given cultures of microorganisms the ecosystems formed are more simple and therefore more easily investigated than the equivalent conventional intestinal ecosystems. Many associations between intestinal microbes and their host have been studied using animals with restricted intestinal microbiotas. Of particular interest has been the interactions between intestinal pathogens and germ-free animals; such studies also give indirect evidence for the protective role of the normal microbiota (Gordon and Pesti, 1971; Collins and Carter, 1978).

Recently mucosa-associated microbes have been introduced into rodents having restricted intestinal microbiotas; both lactobacilli spp. and the yeast *Torulopsis pintolopessi* have been shown to be capable of colonizing the surfaces of the gastrointestinal mucosa in these animals (Kotarski and Savage, 1979; Suegara *et al.*, 1979). The mode of association was similar to that seen in

conventional animals and furthermore it was often specific: mucosal colonization was not always accompanied by, and therefore a by-product of, colonization of the lumen. If such specificity is a characteristic of mucosa-associated microbes, bacteria isolated from crypts could be expected to colonize crypts in animals with restricted microbiotas: provided that the organisms can proliferate in the absence of the normal gut microbiota. Likewise, crypt colonization in these animals could help indicate whether microbes belong to the mucosa-associated microbiotas of conventional animals.

The previous chapter reported on the isolation, cultivation and characterization of bacteria isolated from the rodent intestinal mucosa. The TSB isolates were shown to have the same unusual ultrastructure as bacteria occurring in the crypts of Lieberkuhn in conventional animals, and it was postulated that they were the same organisms. Therefore the isolates were given to animals with restricted intestinal microbiota to indicate whether they were part of the mucosa-associated microbiota, i.e. if the TSB could colonize the crypts of these animals. Subsequently these studies were extended to investigate the influence of the intestinal microbiota and host factors on the ability of bacteria to colonize crypts.

5.2 INITIAL COLONIZATION EXPERIMENTS

Preliminary experiments showed that while a number of types of spiral-shaped bacteria isolated from the rodent intestinal mucosa were able to colonize crypts, the TSB isolates did so more consistently. Furthermore the TSB's

distinctive ultastructure made these organisms easier to identify in thick sections examined by LM; the method most suitable for surveying large numbers of crypts.

The animals used for these experiments had an intestinal microbiota that was less complex than the microbiota of UNSW conventionally housed animals; however as suitable holding facilities were not available, most experiments could not be conducted under strictly germ-free conditions. While the animals were bred and transported by germ-free techniques, on arrival at UNSW and during experiments, animals were housed in laminar flow cabinets, and given a sterilized diet. Consequently most of the animals used were not truely gnotobiotic; however this was not considered to be of critical importance in the experiments conducted. Accidental contamination by intestinal or faecal material was checked by monitoring faecal coliforms, and by examining uninoculated control animals housed in the same environment for mucosal colonization. No animals were found to contain coliforms, nor were any of the control animals colonized by mucosal bacteria. Consequently the results confirming the ability of organisms to colonize crypts were not compromised by lack of germ-free technique. The further experiments with TSB were comparing animals having microbiotas of different levels of complexity. It is very unlikely that gnotobiotes housed in laminar flow and free from faecal contamination could acquire a microbiota of the complexity of that found in SPF or conventional animals. Indeed microscopic observations of faecal material found that where

contamination occurred only 2-3 types of organisms were involved. As the supply of germ-free, gnotobiotic, and SPF animals was unreliable, experiments were often designed according to the availability of animals rather than to a predetermined plan. In the initial colonization experiment germ-free rats were inoculated, orogastrically, with cultures of MTSB; the RTSB had not been isolated at this time. Animals received doses of the organisms on the day they were received at the UNSW and again two days later. After 14 days the animals were killed, and then the ileal, caecal, and colonic tissues prepared for light and electron microscopy. When thick sections were examined it was found that a high proportion of the caecal crypts, and many of the colonic crypts contained TSB. This pattern of colonization contrasted with that seen in conventional animals where the heaviest colonization occurred in the small bowel. In order to investigate this difference, and to further study the relationship between the TSB's and the host animals, more extensive experiments were undertaken. In addition to the germ-free animals SPF rats, lacking mucosa-associated microbes, and gnotobiotic rats, previously associated with a Lactobacillus spp. and a Bacteroides spp., were inoculated with MTSB and compared to conventional animals. After the experiment had commenced RTSB were isolated and a similar study was conducted with these organisms: gnotobiotic and SPF rats were inoculated and compared to conventional rats. Similarly colonization experiments were also done with mice: SPF animals lacking mucosal microbes, and gnotobiotes previously associated

with a Bacteroides spp., a Lactobacillus spp., and a Fusobacterium spp., were inoculated with MTSB and compared to conventional mice. In each of the experiments colonization of the various sites was assessed using the semi-quantitative counting system described in Materials and Methods.

When the data from these experiments was examined it was found that the differences between the various types of animals resulted from the pattern of colonization: the comparative colonization of the ileum, caecum and colon, rather than the total amount of colonization per animal. Consequently statistical analysis of this data was needed to be able to test for interactions between the type of animal used and the site sampled. Although a large proportion of crypts in conventional animals contain bacteria, very few contain TSB, similarly a majority of crypts in the inoculated animals were not colonized; as a result the data does not follow a normal distribution. While the data was suitable for non-parametric analysis only parametric tests are able to allow for interactions. For this reason while statistical comparisons were made of transformed data (log(x+1)), using Two Factor Analysis of Variance with Replication, and Newman-Keul multiple range tests (Zar, 1974), the analyses were used only as indicators of differences rather than proofs.

5.3. COLONIZATION OF RATS WITH TSB ISOLATES

5.3.1 RTSB

A high proportion of the caecal crypts of SPF (71%) and ex-gnotobiotic (80%) rats, inoculated with RTSB,

Table 8. Semi-quantitative assessment of crypt colonization by TSB in conventional rats, and in ex-gnotobiotic and SPF rats given cultures of the isolate RTSB.

Animal Group	Conventional			Ex-Gnotobiotic			SPF		
Site	Ileum	Caecum	Colon	Ileum	Caecum	Colon	Ileum	Caecum	Colon
% of crypts in group[a] colonized	70	8	9	22	80	10	24	71	0
Semi-quantitative scores[b]									
Animal number									
1	22[c]	1	5	1	40	0	5	17	0
2	21	0	0	18	63	2	5	33	0
3	20	0	5	1	34	3	6	22	0
4	36	0	0	4	63	1	4	20	0
5	27	8	0	10	44	4	4	20	0
Group average scores,	25.2	1.6	2.0	6.8	48.8	2.0	5.0	22.4	0.0
± standard deviation	5.9	3.0	2.7	7.2	13.4	1.6	0.7	7.1	0.0

Significant differences at 1% level[d]

Between groups, Ileum : Conventional>Gnotobiote and SPF
Caecum: Gnotobiote and SPF>Conventional
Colon : None

Between sites, Conventional: Ileum>caecum and colon
Gnotobiotic : Caecum>ileum and colon
SPF : Caecum>ileum>colon

a 100 crypts per site per group (5 animals, 20 crypts each)
b for details see Materials and Methods.
c Total score for 20 crypts assessed in each animal.
d Data transformed (logx+1) and compared using Two-Factor Analysis of Variance with replication and Newman-Keul multiple range test; see Materials and Methods for details.

contained these organisms compared to only 8% of the caecal crypts in conventional rats. The difference was further demonstrated when the numbers of organisms within the crypts were quantitated (Table 8): both types of inoculated animals have significantly higher scores than the conventional animals at this site, with the ex-gnotobiotes generally greater than the SPF's. Each of the inoculated rats had ileal crypts containing TSB however both the proportion of crypts and the quantitative counts at this site were considerably less than in conventional animals. Unlike the other sites there appeared to be little difference in the association of TSB with the colonic crypts of the three groups of animals.

5.3.2. MTSB

The pattern of colonization in the rats inoculated with MTSB was similar to that of animals given RTSB however there was greater variability between these groups. While a high proportion of caecal crypts contained TSB in each inoculated group: ex-germ-frees 100%, ex-gnotobiotes 93% and SPFs 77%, the numbers of organisms in the crypts varied. The quantitative counts (Table 9), for the ex-germ-frees were the highest and considerably greater than those of the SPF animals; the ex-gnotobiotes were at an intermediate level, and each was significantly greater than the conventional rats. Colonization of colonic crypts followed a similar pattern: greatest in ex-germ-frees, less in ex-gnotobiotes and SPFs. The quantitative counts of the ex-germ-free animals were significantly greater than those of conventional rats; the

Table 9. Semi-quantitative assessment of crypt colonization by TSB in ex-germfree, ex-gnotobiotic, and SPF rats inoculated with cultures of the isolate MTSB in comparison to conventional rats.

Animal Group	Ex-germfree			Ex-Gnotobiotic			SPF			Conventional		
Site	Ileum	Caecum	Colon	Ileum	Caecum	Colon	Ileum	Caecum	Colon	Ileum	Caecum	Colon
% of crypts in group[a] colonized	1	100	52	7	93	6	36	77	0	70	8	9
Semi-quantitative scores[b]												
Animal number												
1	1[c]	70	21	0	55	1	20	29	0	22	1	5
2	0	72	9	3	56	0	1	38	0	21	0	0
3	0	72	11	4	53	7	2	35	0	20	0	5
4	0	74	14	0	51	0	8	31	0	36	0	0
5	0	64	8	0	48	0	8	34	0	27	8	0
Average scores[d]	0.2	70.4	12.6	1.4	52.6	1.6	7.8	33.4	0.0	25.2	1.6	2.0
± standard deviation	0.45	3.8	5.2	1.9	3.2	3.0	7.6	3.5	0.0	5.9	3.0	2.7

Significant differences at 1% level[e]

Between groups, Ileum : Conventional>SPF>Ex-gnotobiotic and Ex-germ-free
Caecum: Ex-germ-free, Ex-gnotobiotic and SPF>Conventional
Colon : Ex-germ-free>Ex-gnotobiotic, SPF and Conventional

Between sites, Ex-germ-free : Caecum>Colon>Ileum
Ex-gnotobiotic: Caecum>Ileum and Colon
SPF : Caecum>Ileum and Colon
Conventional : Ileum>Caecum and colon

a. 100 crypts per site, per group:5 animals, 20 crypts each.

b. For details see Materials and Methods.

c. Total score for 20 crypts assessed in each animal.

d. Average for the 5 animals in each group.

e. Data transformed (logx+1) and compared using Two-Factor Analysis of Variance with Replication and Newman-Keul multiple range test; see Materials and Methods for further details.

other groups were similar to these animals at this site. An interesting observation was that in SPF rats given either isolate no TSB were ever seen in colonic crypts. Colonization of the ileal crypts was the reverse of that occurring in the large bowel: very few organisms in the crypts of ex-germ-free or ex-gnotobiotes, significantly higher number in the crypts of SPF animals but still much less than in the conventional rats.

5.4 COLONIZATION OF MICE WITH TSB ISOLATES

The site of highest crypt occupancy by TSB in conventional mice was, as in rats, in the ileum: 63% of crypts contained these organisms. However, in contrast to conventional rats a high proportion of the large bowel crypts also contained TSB: 38% in the caecum and 42% in the colon. Nevertheless the quantitative counts of the ileum were significantly higher than those of the large bowel (Table 10). The pattern of colonization in ex-gnotobiotes and SPF animals given MTSB was similar to that seen in the inoculated rats; a high proportion of caecal crypts were colonized: ex-gnotobiotes 98% and SPFs 71%, and the quantitative counts were significantly higher than in conventional mice. Furthermore in contrast to the conventional animals no TSB were found in any of the ileal crypts. A difference in comparison to the inoculated rats was the increased numbers of TSB in the colonic crypts; counts in the ex-gnotobiotes were significantly higher than the conventional mice while the SPF crypts, unlike those of rats, contained high numbers of TSB. The mode of association of the TSB isolated with the host tissue in

Table 10. Semi-quantitative assessment of crypt colonization by TSB in conventional mice, and in ex-gnotobiotic and SPF mice innoculated with cultures of the isolate MTSB.

Animal Group	Conventional			Ex-Gnotobiotic			SPF		
Site	Ileum	Caecum	Colon	Ileum	Caecum	Colon	Ileum	Caecum	Colon
% of crypts in group[a] colonized	63	38	42	0	100	81	0	71	54
Semi-quantitative scores[b]									
Animal									
1	18c	17	9	0	69	23	0	26	9
2	10	21	12	0	66	38	0	34	26
3	36	6	10	0	75	28	0	42	17
4	39	25	17	0	74	25	0	37	20
5	38	15	12	0	77	41	0	31	19
Average scores[d]	28.2	16.8	12.0	0.0	72.2	31.0	0.0	34.0	18.2
± standard deviation	13.3	7.1	3.1	0.0	4.5	8.0	0.0	6.0	6.1

Significant differences at 1% level[e]

Between groups, Ileum : Conventional, SPF and Ex-gnotobiotic
Caecum: Ex-gnotobiotic SPF Conventional

Between sites, Conventional :Ileum>Colon
Ex-gnotobiotic: Caecum>Colon>Ileum

a. 100 crypts per site, per group:5 animals, 20 crypts each.
b. For details see Materials and Methods.
c. Total score for 20 crypts assessed in each animal.
d. Average for the 5 animals in each group.
e. Data transformed (logx+1) and compared using Two-Factor Analysis of Variance with Replication and Newman-Keul multiple range test; see Materials and Methods for further details.

both rats and mice was similar to that occurring in normal animals: the organisms were found in the lumens of the crypts and not attached to the surface (Fig.25). Furthermore the orientation of the organisms was also similar to conventional animals: in the heavily colonized crypts in the ileum or caecum organisms were found principally at the base and towards the middle, with organism occasionally being expelled from the openings into the intestinal lumen. The organisms in the crypts were often orientated, like the bacteria in normal animals, parallel to the crypt axis (Fig.25A) .

A feature of the colonization of inoculated animals was the frequent occurrence of TSB in the cells lining the crypts; such tissue invasion was not seen in either control or conventional animals. The organisms were found free in the cytoplasm and the cells were often degenerate with numerous vacuoles, swollen mitochondria, and diffuse cytoplasm (Fig.26). A possible entry site could be goblet cells as the organisms were frequently seen within mucus granules (Fig.27)

The experiments with rats and mice demonstrated two major trends; firstly a large difference between the colonization of the inoculated and conventional animals and secondly less dramatic differences amongst the inoculated animals: in each case the large bowel crypts of ex-germ-free and ex-gnotobiotic animals contained, on average, higher numbers of TSB than those of SPF animals.

Figure 25. Crypt colonization in gnotobiotic rodents after oral inoculation of TSB cultures.

A. Mouse caecal crypt colonized by MTSB. LM x 2,200, bar = 10 um.

B. Rat caecal crypt colonized by RTSB. An organism (arrow) can be seen within a goblet cell. TEM x 8,100, bar = 2 um.

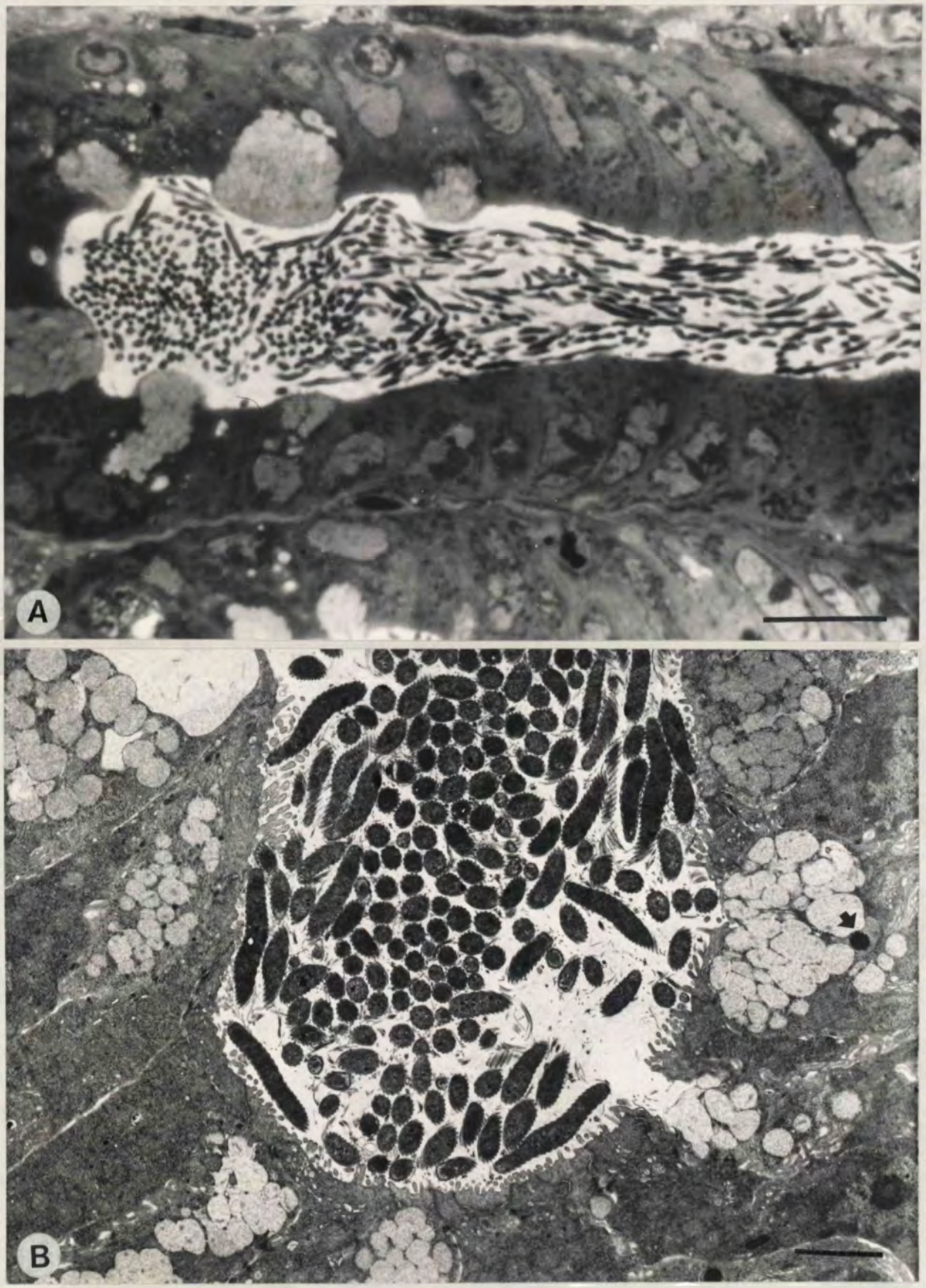

A
B

Figure 26. Gnotobiotic rat caecal tissue after oral inoculation of RTSB cultures. TSB (T) are present within a degenerate epithelial cell x 7,000, bar = 2 um.

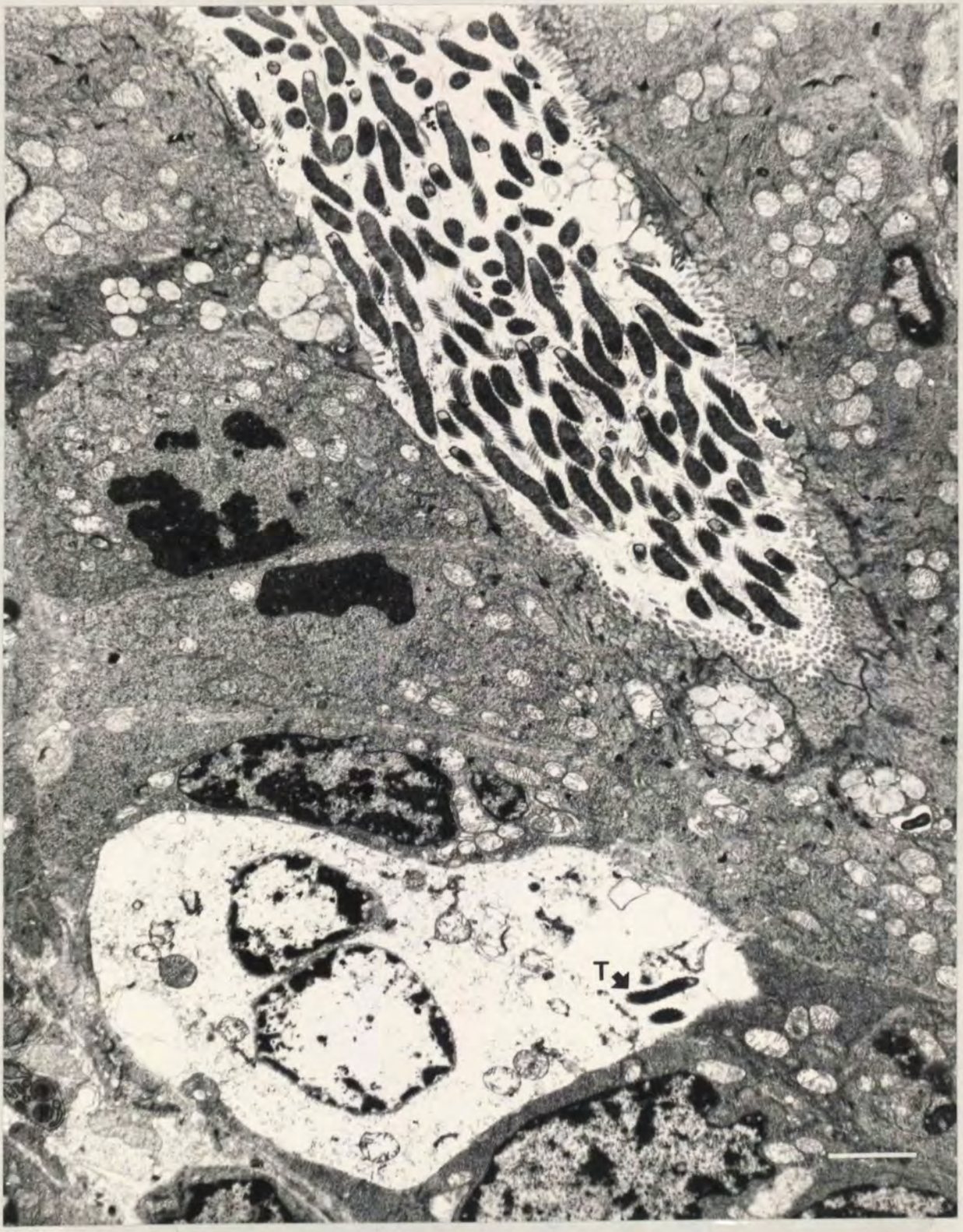
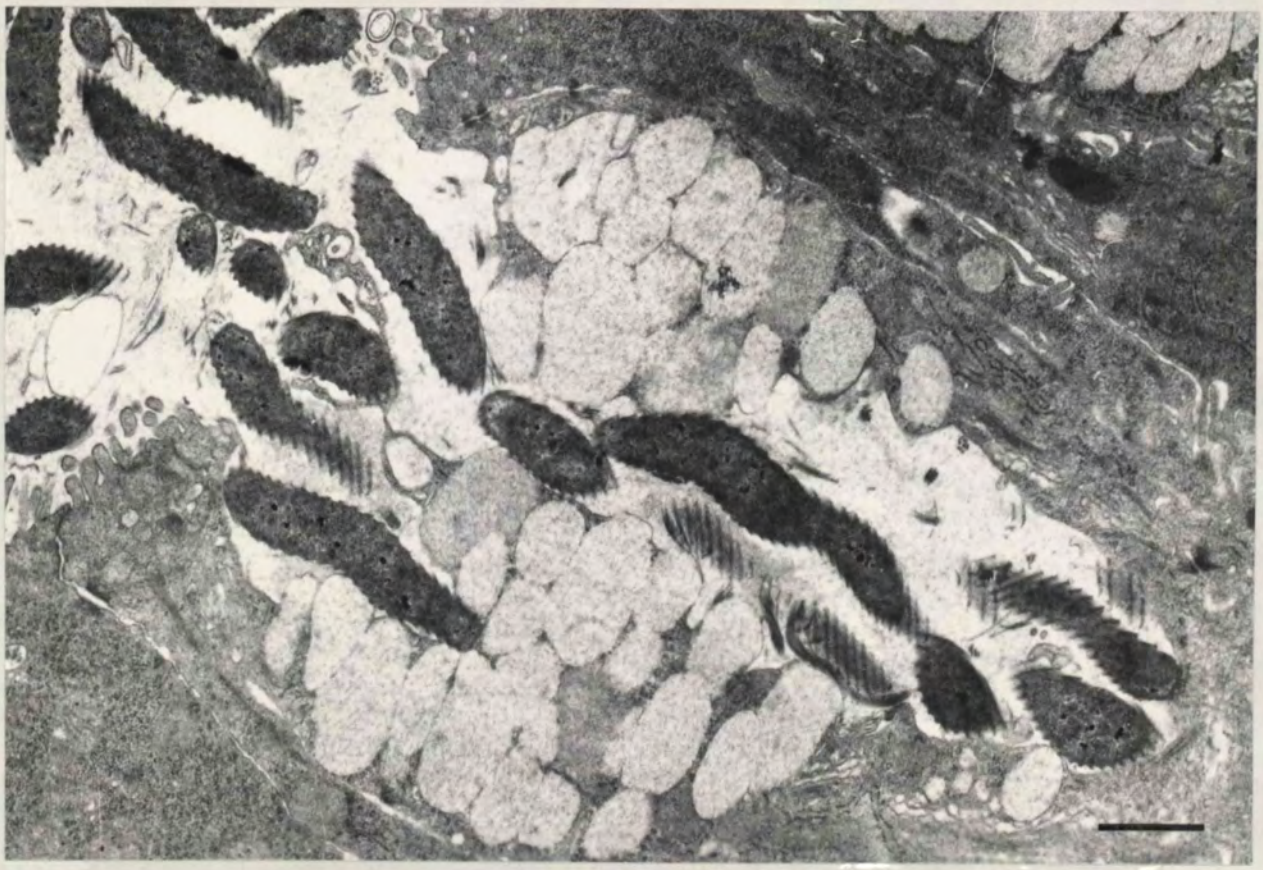

T

Figure 27. Gnotobiotic rat caecal tissue after oral inoculation of RTSB cultures. TSB are present deep within a mucus granule in a goblet cell x 14,800, bar = 1 um.



5.5 INOCULATION OF GERM-FREE RATS WITH RTSB AND TISSUE HOMOGENATES

At this time a small number of germ-free rats and a germ-free isolator became available and an experiment was designed to test if the differences between conventional animals and inoculated animals kept under laminar flow would also be seen in rats mono-associated with the TSB. Also tested was: whether increasing the complexity of the intestinal microbiota altered the numbers of TSB in the crypts.

Nine germ-free rats housed in a germ-free isolator were each given 1.0 ml oral inoculations of RTSB culture with a further 5.0 ml spread on food, bedding, and the fur of the animals; this was done twice four days apart. After 14 days six rats were removed from the isolator; three were killed and prepared for examination, and the other three were given orogastric inoculations of homogenized conventional rat intestine and then housed with conventional animals. The three animals remaining in the isolator were given oral inoculations of asceptically prepared homogenized SPF rat intestine and left in the isolators. Two weeks later the animals were removed from the isolators and along with the conventionalized rats, killed and prepared for evaluation.

As a result of the smaller numbers of animals used and variability, possibly due to the less efficient inoculation technique, the results of this experiment are not as easily interpreted as the previous colonization experiments. Nevertheless it appeared that the rats

mono-associated with RTSB had a pattern of colony similar to that of ex-germ-free and ex-gnotobiotic rats given TSB and maintained under laminar flow (Table 11). Furthermore as in the previous experiments the site of heaviest colonization in all the inoculated animals was the caecum. The introduction of a SPF microbiota, or a conventional microbiota containing mucosa-associated microbes into the mono-associated animal did not give clear-cut results. The inoculations of tissue homogenates appeared to influence colonization of the ileal crypts as both these groups of animals had significantly higher counts at this site compared to the mono-associated animals, and thus resembled conventional animals.

The inoculation of homogenized SPF tissue had no noticeable effects on the TSB colonization of the caecal or colonic crypts. The effects of conventional tissue homogenate were less clear: while two of the three animals had high caecal counts the other had a low count. Unfortunately as one of the mono-associated animals also had a low count it is uncertain whether the conventionalized animals' TSB population was reduced by the tissue inoculation or whether it was originally low.

5.6. INFLUENCE OF DIFFERENT INTESTINAL MICROBIOTAS ON TSB COLONIZATION

In order to clarify the effects of introducing a conventional intestinal microbiota on established populations of TSB a more extensive experiment was completed using SPF animals kept under laminar flow conditions. A larger number of animals were used and a

Table 11. Semi-quantitative assessment of crypt colonization by TSB in germ-free rats given: cultures of the isolate RTSB, RTSB and tissue homogenates from SPF or conventional animals, and conventional rats.

Animal Group	Conventional			Germ-free+RTSB			Germ-free+RTSB+SPF Homogenate			Germ-free+RTSB+Convent. Homogenate		
Site	Ileum	Caecum	Colon	Ileum	Caecum	Colon	Ileum	Caecum	Colon	Ileum	Caecum	Colon
% of crypts in group[a] colonized	63	13	3	0	72	7	47	90	10	52	65	35
Semi-quantitative scores[b]												
Animal												
1	20c	0	2	0	68	0	23	37	6	30	46	4
2	18	6	0	0	15	0	9	52	0	12	38	11
3	12	3	0	0	40	4	8	31	2	7	8	8
Average scores[d]	16.7	3.0	0.7	0.0	41.0	1.3	13.3	40.0	2.7	16.3	30.7	7.7
± standard deviation	4.1	3.0	1.2	0.0	26.5	2.3	8.4	10.8	3.1	12.1	20.0	3.5

Significant differences at 1% level[e]

Between groups, Ileum : Conventional, Germ-free+RTSB+SPF and Germ-free+RTSB+Con.>Germ-free+RTSB
Caecum: Each inoculated group.Conventional
Colon : None

Between sites, Conventional : Ileum>Colon
Germ-free+RTSB : Caecum>Ileum and Colon
Germ-free+RTSB+SPF : Caecum>Colon
Germ-free+RTSB+Conv.: None

a. 100 crypts per site, per group:5 animals, 20 crypts each.
b. For details see Materials and Methods.
c. Total score for 20 crypts assessed in each animal.
d. Average for the 5 animals in each group.
e. Data transformed (logx+1) and compared using Two-Factor Analysis of Variance with Replication and Newman-Keul multiple range test; see Materials and Methods for further details.

period longer than used in the previous experiment was allowed for the establishment of the microbiota. Fifteen SPF rats were separated into three groups and two groups given orogastric inoculation of RTSB cultures. After two weeks one of the inoculated groups of rats were taken from the laminar flow, killed and the tissue prepared for examination. The remaining RTSB associated SPF animals and the uninoculated SPF animals were taken from the laminar flow and given orogastric doses of homogenized conventional rat intestine, and then housed with conventional animals for 4 weeks before sacrifice.

The inoculations of homogenized conventional rat intestine resulted in normal numbers of bacteria being present in the ileal and caecal crypts (Table 12). In contrast the colonic crypts contained significantly fewer bacteria than the conventional animals and this may be explained by the absence of the thin, spiral-shaped bacteria attaching to the crypts' cells (Chapter 3). Despite this normalization process the patterns of colonization by the TSB in both the groups inoculated with RTSB and homogenized tissue and the group given just homogenized tissue were different to that found in conventional animals (Table 13). In neither group were the numbers of organisms in the ileal crypts similar to that of conventional rats. The colonization of the caecal crypts in animals just given homogenized tissue was similar to the numbers in conventional animals, however those given RTSB before the tissue homogenate had, as a group, elevated numbers of TSB. Two of this latter group did, however,

Table 12. Semi-quantitative assessment of crypt colonization by microorganisms in conventional rats, SPF rats inoculated with cultures of RTSB and then given homogenized conventional rat intestine, and SPF rats given homogenized conventional rat intestine.

Animal Group	SPF+RTSB+Conv.homog.			SPF+Convent.homog.			Conventional		
Site	Ileum	Caecum	Colon	Ileum	Caecum	Colon	Ileum	Caecum	Colon
% of crypts in group[a] colonized	97	84	50	99	90	53	85	94	84
Semi-quantitative scores[b]									
Animal									
1	35	37	14	38	44	16	24	53	13
2	23	48	15	38	61	2	27	57	35
3	43	13	10	30	38	10	24	40	29
4	34	49	6	44	25	16	43	60	33
5	28	34	12	36	66	13	34	39	31
Average scores[d]	32.6	36.2	11.4	37.2	46.8	11.4	30.4	49.8	28.2
± standard deviation	7.6	14.5	3.6	5.0	16.8	5.8	8.1	9.7	8.8

Significant differences at 1% level[e]

Between groups, Ileum : None
Caecum: None
Colon : Conventional>SPF+RTSB+Conv.homog. and SPF+Conv.homog.

Between sites, SPF+RTSB+Conv.homog.: Ileum and Caecum>Colon
SPF+Conv.homog. : Ileum and Caecum>Colon
Conventional : None.

a. 100 crypts per site, per group:5 animals, 20 crypts each.
b. For details see Materials and Methods.
c. Total score for 20 crypts assessed in each animal.
d. Average for the 5 animals in each group.
e. Data compared using Two-Factor Analysis of Variance with Replication and Newman-Keul multiple range test; see Materials and Methods for further details.

Table 13. Semi-quantitative assessment of crypt colonization by TSB in SPF rats and SPF rats colonized with cultures of RTSB, given homogenized conventional rat intestine compared to, SPF rats colonized with cultures of RTSB, and Conventional rats

Animal Group	SPF+RTSB+Conv.homog.			SPF+Convent.homog.			SPF+RTSB			Conventional		
Site	Ileum	Caecum	Colon	Ileum	Caecum	Colon	Ileum	Caecum	Colon	Ileum	Caecum	Colon
% of crypts in group[a] colonized	9	53	1	4	9	0	21	74	0	70	8	9
Semi-quantitative scores[b]												
Animal number												
1	1	8	1	0	3	0	3	32	0	22	1	5
2	4	28	0	2	2	0	4	21	0	21	0	0
3	0	6	0	0	3	0	6	22	0	20	0	5
4	2	20	0	2	2	0	5	20	0	36	0	0
5	2	16	0	0	0	0	3	29	0	27	8	0
Average scores[d]	1.8	15.6	0.2	0.8	2.0	0.0	4.2	24.8	0.0	25.2	1.6	2.0
± standard deviation	1.5	9.0	0.4	1.1	1.2	0.0	1.3	5.4	0.0	5.0	3.0	2.7

Significant differences at 1% level[e]

Between groups, Ileum : Conventional>others, SPF+RTSB+Conv.homo. and SPF+RTSB>SPF+Conv.homog.
Caecum: SPF+RTSB and SPF+RTSB+Conv.homog.>SPF+Conv.homog. and Conventional
Colon : None.

Between sites, SPF+RTSB+Conv.homog: Caecum>Ileum and Colon
SPF+Conv.homog. : None
SPF+RTSB : Caecum>Ileum>Colon
Conventional : See previously (Table 8)

a. 100 crypts per site, per group:5 animals, 20 crypts each.
b. For details see Materials and Methods.
c. Total score for 20 crypts assessed in each animal.
d. Average for the 5 animals in each group.
e. Data transformed (logx+1) and compared using Two-Factor Analysis of Variance with Replication and Newman-Keul multiple range test; see Materials and Methods for further details.

have lower counts suggesting the normal microbiota may have reduced the numbers of TSB present. The TSB were seen in only 1 colonic crypt from the 3 groups of SPF derived animals and this is consistent with the results obtained in the previous colonization experiments.

5.7 THE EFFECT OF ANIMAL AGE ON THE ABILITY OF TSB TO COLONIZE INTESTINAL CRYPTS

The ability of TSB to colonize intestinal crypts in infant rats and mice was compared to the equivalent adult animals in a series of experiments. Infant animals were inoculated with the cultures in the first week of life and kept with their mothers for the length of the experiment. Three different experiments were undertaken: in the first unweaned infant ex-germ-free rats were given cultures of MTSB (RTSB had not been cultured at this time), and examined two weeks later. In the second experiment infant ex-gnotobiotic rats were given RTSB; half the litter were killed and examined two weeks later while the remainder were left until weaned (a further two weeks), before examination. In the third experiment unweaned infant ex-gnotobiotic mice were given cultures of MTSB or RTSB and examined two weeks later.

In all three experiments the pattern of colonization in the young animals generally resembled that of the adult animals, with some exceptions (Tables 14,15). While a similar proportion of caecal crypts contained TSB in both young and old animals the quantitative scores were usually lower in the infants, especially in mice. Colonization of

Table 14. Semi-quantitative assessment of crypt colonization by TSB in ex-gnotobiotic rats given cultures of the isolate RTSB; weaned and unweaned infants compared to adults.

Animal Group	Unweaned Infants			Weaned Infants			Adults		
Site	Ileum	Caecum	Colon	Ileum	Caecum	Colon	Ileum	Caecum	Colon
% of crypts in group[a] colonized	32	77	77	21	85	72	22	80	10
Semi-quantitative scores[b]									
Animal number									
1	4	24	25	2	43	19	1	40	0
2	8	21	32	5	49	34	18	63	2
3	2	31	34	10	34	16	1	34	3
4	13	38	29	0	29	42	4	63	1
5	8	29	23	9	23	29	10	44	4
Average scores[d]	7.0	28.6	28.6	5.2	35.6	28.0	6.8	48.8	2.0
± standard deviation	4.2	6.6	4.6	4.3	10.5	10.7	7.2	13.4	1.6

Significant differences at 1% level[e]

Between groups, Ileum : None
Caecum: None
Colon : Weaned and Unweaned Infants>Adults.

Between sites, Unweaned Infants: Caecum and Colon>Ileum
Weaned Infants : Caecum and Colon>Ileum
Adults : See previously (Table 9).

a. 100 crypts per site, per group:5 animals, 20 crypts each.
b. For details see Materials and Methods.
c. Total score for 20 crypts assessed in each animal.
d. Average for the 5 animals in each group.
e. Data transformed (logx+1) and compared using Two-Factor Analysis of Variance with Replication and Newman-Keul multiple range test; see Materials and Methods for further details.

Table 15. Semi-quantitative assessmentof crypt colonization by TSB in ex-germ-free rats given cultures of the isolate MTSB; unweaned infants compared to adults.

Animal Group	Unweaned infants			Adults		
Site	Ileum	Caecum	Colon	Ileum	Caecum	Colon
% of crypts in group[a] colonized	54	100	19	1	100	52
Semi-quantitative scores[b]						
Animal						
1	19	68	6	1	70	21
2	4	69	3	0	72	9
3	20	71	2	0	72	11
4	19	69	4	0	74	14
5	25	66	4	0	64	8
Average scores[d]	17.4	68.6	3.8	0.2	70.4	12.6
± standard deviation	7.9	1.8	1.5	0.45	3.8	5.2

Significant differences at 1% level[e]

Between groups, Ileum : Unweaned infants>Adults
Caecum: None
Colon : Adults>Unweaned infants

Between sites, Unweaned infants: Caecum>Ileum>Colon
Adults : See previously (Table 9)

a. 100 crypts per site, per group:5 animals, 20 crypts each.
b. For details see Materials and Methods.
c. Total score for 20 crypts assessed in each animal.
d. Average for the 5 animals in each group.
e. Data transformed (logx+1) and compared using Two-Factor Analysis of Variance with Replication and Newman-Keul multiple range test; see Materials and Methods for further details.

other sites in mice was not significantly different, however several differences were seen in rats: infant rats given MTSB had significantly higher numbers of TSB in the ileal, and lower numbers in the colonic crypts. In contrast both groups of animals given RTSB: weaned and unweaned, had much greater numbers of these organisms in the colonic crypts. Weaning did not appear to have an obvious effect on the numbers of organisms in crypts at any site.

5.8 TISSUE INVASION BY THE TSB ISOLATES

As previously noted a feature of the colonization of ex-germ-free, ex-gnotobiotic, and SPF animals by TSB was the frequent observation of these organisms in the cells surrounding the crypts. Such tissue invasion was seen in a proportion of each group of inoculated animals, but not in conventional controls. TSB of bacteria with a similar appearance have been reported previously in two papers describing the appearance of ileal tissue of immunologically compromised rodents: irradiated mice (Quastler and Hampton, 1962), and mice treated with nitrogen mustard (Hampton, 1967). In these papers the bacteria were seen to have passed from the intestinal crypts into the tissue cells. In order to test whether immunological competence would influence the occurrence of TSB in the crypt cells two types of compromised hosts were given cultures of TSB: thymus free (nude) Balb c SPF mice or ex-gnotobiotic and SPF mice given sub-lethal doses of γ-radiation.

The nude mice were given doses of MTSB, left for two weeks, and then examined. Ex-gnotobiotic and SPF mice were exposed to 560 rads γ-radiation from a cobalt source and with unirradiated controls transferred to UNSW. All animals were given an orogastric dose of MTSB on arrival and again two days later; animals were then kept under laminar flow until sacrifice seven days post-inoculation. In addition to preparing tissues for microscopic examination, samples of spleen, liver and mesenteric lymph nodes were removed, homogenised and plated on CSA69 and Horse Blood agar.

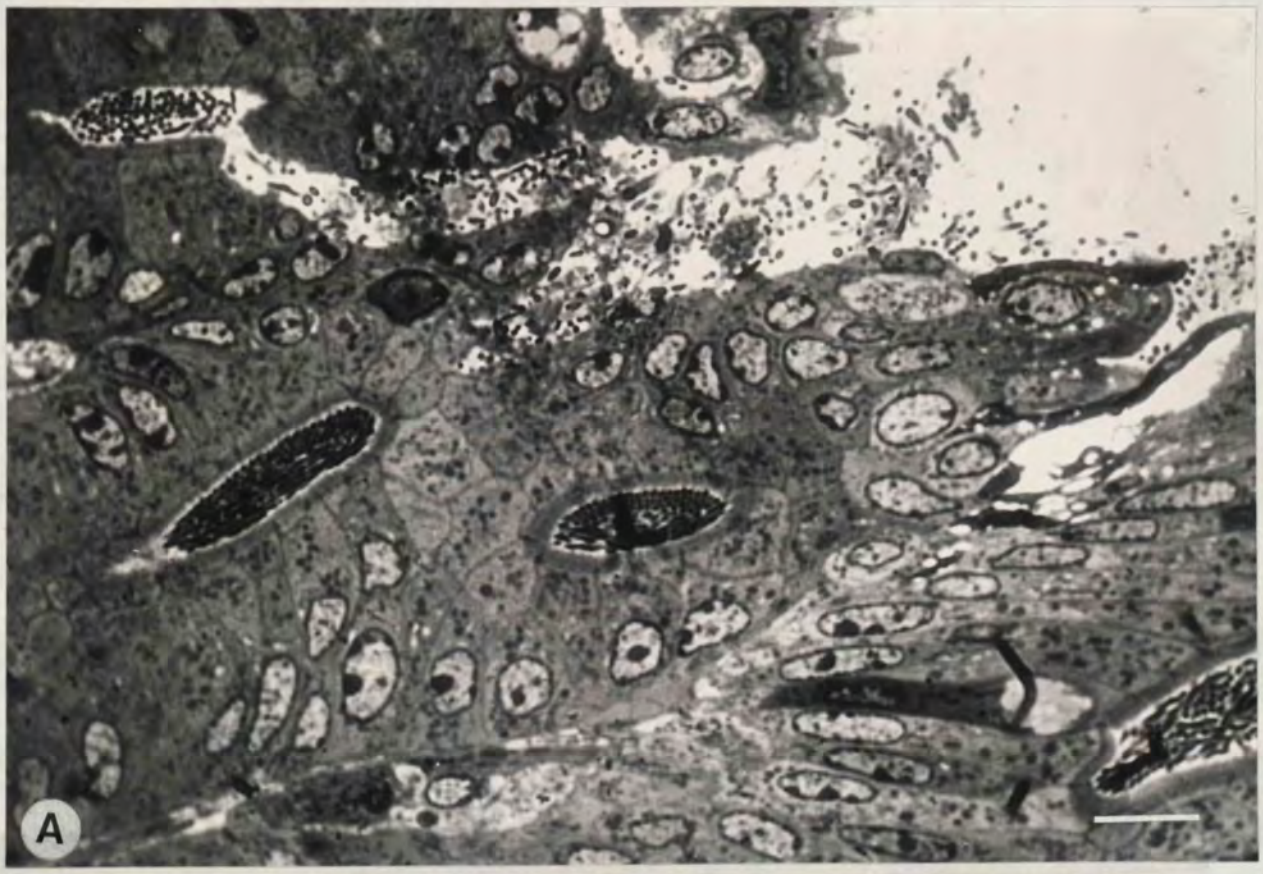
As quantitation of tissue invasion was not practicable comparisons between the various groups was subjective. Nevertheless while tissue invasion was seen in each group there appeared to be higher numbers of organisms in the cells of the irradiated animals. Crypt colonization in all groups was similar to the other animals with restricted microbiotas, having greater numbers of organisms present in the caecal crypts. Tissue invasion appeared to be more common in the irradiated ex-gnotobiotes compared to the irradiated SPFs however, due to the subjective nature of assessment, this difference is probably not significant. While the appearance of the bacteria in the tissue was similar in each group it was noted that TSB and fusiforms were occasionally seen in areas of disrupted surface tissue in irradiated animals (Fig.28).

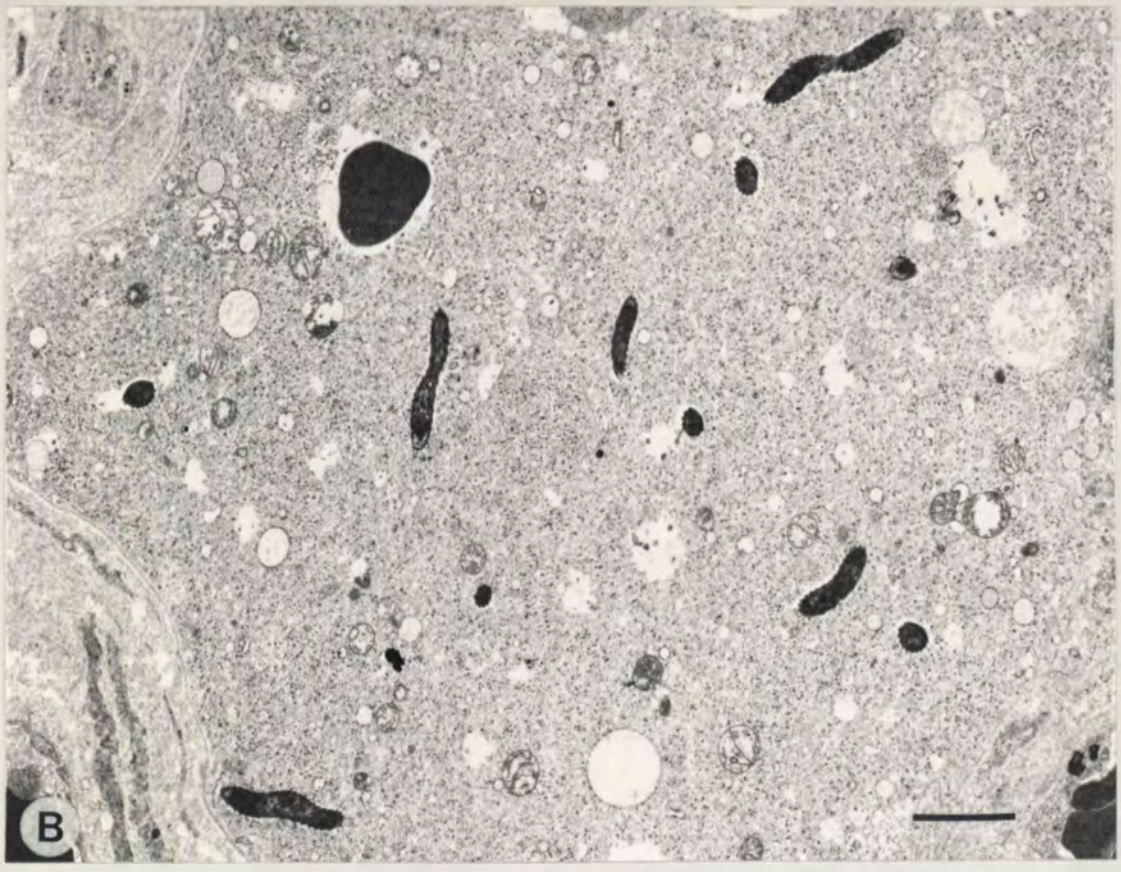
The TSB were seen predominantly in epithelial cells and only occasionally in the laminar propria, furthermore invasion only occurred in the large bowel and never in the

Figure 28. Tissue invasion by bacteria in irradiated mice.

A. The disrupted caecal tissue surface of an SPF mouse colonized with bacteria. LM x 1,420, bar = 10 um.

B. Disrupted caecal tissue in a gnotobiotic mouse containing numerous TSB. TEM x 6,700, bar = 2 um.


A


B

ileum. All cultures of liver, spleen and MLN were negative for TSB although isolated lactobacilli colonies were seen in the MLN of some irradiated animals.

5.9 COMPARISON OF RAT AND MOUSE ISOLATES

There were no major differences in the colonization by MTSB and RTSB isolates. In experiments where the equivalent animal groups were given the same isolates, most sites were colonized similarly although some differences occurred. In the animals with restricted microbiotas the large bowel crypts of rats and mice given the host isolate generally contained higher numbers of organisms than those in animals given the non-host isolate. An exception to this trend was in the caecal crypts of SPF rats where higher numbers of MTSB compared to RTSB were found.

5.10 COLONIZATION EXPERIMENTS WITH OTHER BACTERIAL CULTURES

A number of different types of bacteria other than TSB were given to animals with restricted microbiotas and included: á Proteus spp., a campylobacter-like organism isolated from rat small bowel mucosa, and a spiral-shaped bacterium isolated from rat colonic mucosa following $MgSO_4$ induced diarrhoea.

5.10.1 Proteus mirabilis

Cultures of these highly motile bacteria were given to SPF rats to test the specificity of crypt colonization. Although the organisms established, and remained in the faeces of animals up to the time of sacrifice two weeks post-inoculation, no surface or crypt colonization was seen.

5.10.2 Campylobacter-like Isolates

Cultures of small spiral-shaped bacteria isolated from rat ileum were able to establish in animals with restricted microbiotas and to colonize intestinal crypts. The pattern of colonization was essentially the same as that seen in animals given TSB: heaviest colonization in the caecum (Fig.29A), some in the colon and none in the ileum. Colonization was more variable than that seen in the TSB experiments with some animals being well populated and others containing none or few organisms. Because of this variability and the difficulty in observing these organisms in thick sections the factors influencing colonization were not further investigated.

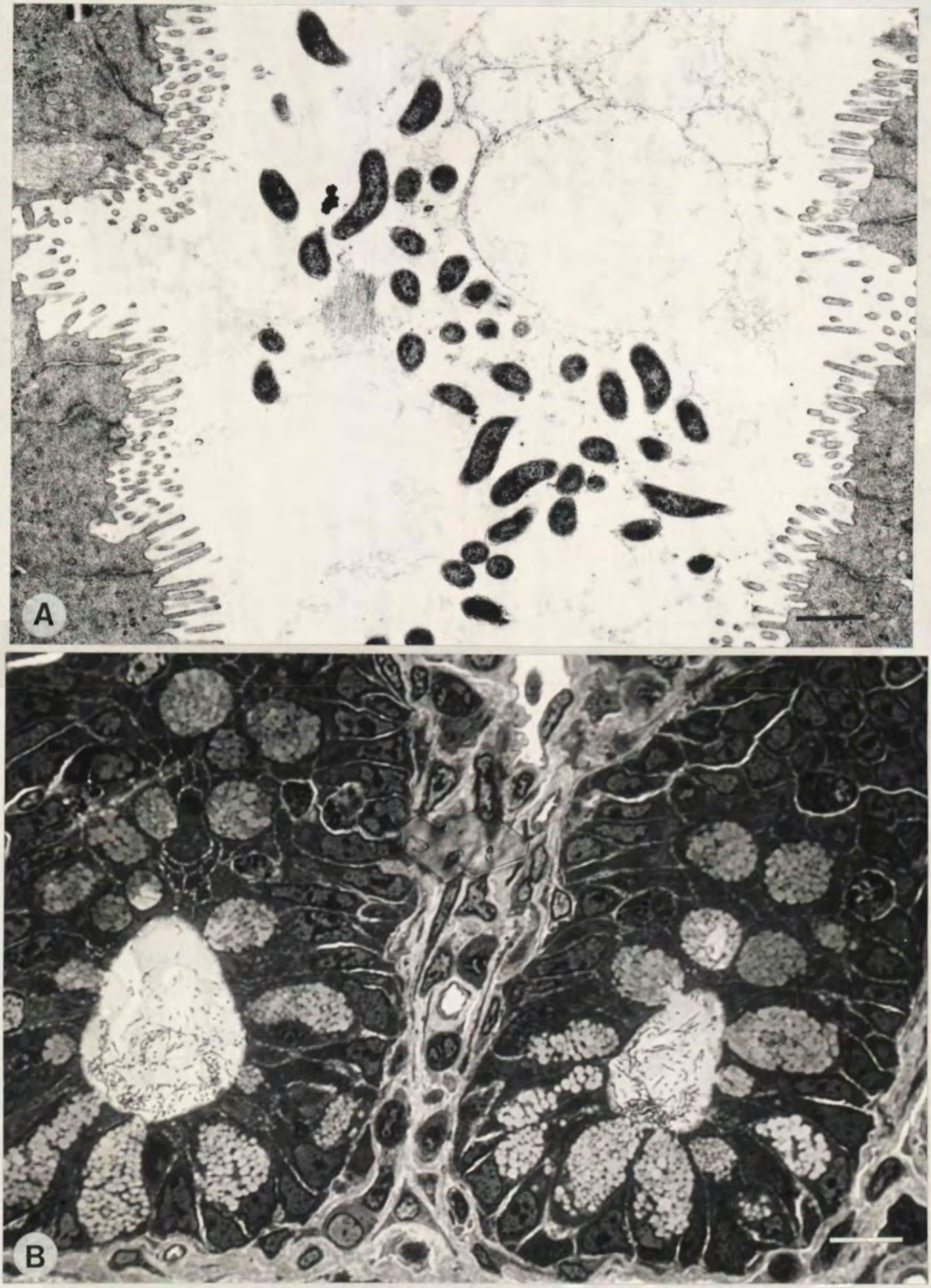
5.10.3 Thin Spiral-shaped Bacteria

Thin spiral-shaped bacteria isolated from the colonic mucosa of $MgSO_4$-treated animals were similar in appearance to organisms colonizing the surface in these animals, and also in SPF rats given a conventional microbiota; the morphology of these organisms was also similar to "s" shaped bacteria isolated from normal rat colon. An attempt was made to reproduce surface colonization by giving SPF animals cultures of the thin spiral-shaped isolates. When the animals were examined two weeks post-inoculation the organisms were found to be capable of associating with the mucosa: the organisms were found in many caecal crypts (Fig.29B), but none were seen attached to the mucosa in the colon or caecum.

Figure 29. Colonization of gnotobiotic rats with cultures of mucosal isolates.

A. A caecal crypt containing campylobacter-like bacteria. TEM x 11,600, bar = 1 um.

B. Caecal crypts containing thin spiral-shaped bacteria. LM x 1,300, bar = 10 um.


A
B

5.11 DISCUSSION

A suggestion made in Chapter Three was that the spiral and curved morphology common to all the bacteria found in the intestinal crypts of rats was an adaption to the special conditions of this environment. Conversely, the absence of other organisms was postulated to be due to an inability to exist in those conditions. Findings in this chapter support this hypothesis. The SPF rats used in the current investigation, although possessing a complex and varied luminal microbiota, had no microorganisms present in the intestinal crypts. While the lumen organisms are adapted for survival in the gut none could colonize the nearby crypt habitats. Proteus mirabilis was also able to colonize the lumen but not capable of penetrating into the crypts. Only in SPF mice were any microbes seen in the crypts and the organisms involved, fusiform-shaped bacteria often present in the upper portions of the crypts, were also seen at the same site in conventional mice and thus are probably part of the normal mucosa-associated microbiota.

In contrast with the inability of almost all of the lumen microbes to colonize crypts, each of the three spiral-shaped bacteria isolated from the mucosa, TSB, campylobacter-like bacteria and the thin "s" shaped bacteria isolated from the colon, when inoculated into animals with restricted microbiotas were able to colonize these sites. A possible explanation for the presence of these organisms in the crypts was that each has the ability

to physically enter the habitat. This ability would give the organisms the same ecological advantage that attachment does for microbes elsewhere in the tract (Lee, 1980). Studies with gastric lactobacilli and the yeast Torulopsis pintolopesii have shown, however, that the ability to attach to mucosal cells is by itself insufficient to enable colonization of the mucosa in vivo; nutritional and environmental factors are also vitally important (Suegara et al., 1979). Similarly, being able to penetrate the crypt environment would be only one of many adaptions needed for the organisms to colonize.

The ability of the TSB isolates to colonize intestinal crypts and their distinctive ultrastructure is strong evidence that these organisms are the same as the TSB seen in the crypts of conventional animals. Likewise the colonization of crypts by the other mucosal isolates may show that they also occupy this site in normal animals. Alternately, these organisms may inhabit habitats in the gut that have similar environmental parameters to the crypts, such as the mucus layers on the mucosal surface and, in the absence of competing organisms, are able to colonize this site.

The colonization experiments demonstrated that the numbers and types of microorganisms present in the gastrointestinal tracts of animals had a large influence on the ability of the TSB to colonize intestinal crypts. This is most clearly demonstrated by the differences between the conventional and inoculated animals (Table 16). Ex-gnoto-

Table 16. Summary of Semi-quantitative assessments of TSB colonization in rats and mice[a].

Tissue	Ileum			Caecum			Colon		
Animal Type	Rats		Mice	Rats		Mice	Rats		Mice
Isolate	RTSB	MTSB	MTSB	RTSB	MTSB	MTSB	RTSB	MTSB	MTSB
Animal Group									
Conventional	b++c	NDd	++	+	ND	+	±	ND	+
SPF	±	+	-	++	++	++	-	-	+
Ex-gnotobiotic	+	±	-	+++	+++	++++	±	±	++
Ex-germ-free	-	±	ND	+++	++++	ND	±	+	ND

a. Further details see Figs.8, 9 and 10.

b. Representation of average semi-quantitative counts for 5 animals.

c. Symbol range of average scores

-	0
±	0-5
+	6-20
++	21-35
+++	36-50
++++	51+

d. Not done.

biotic ex-germ-free and SPF animals all had low numbers of TSB in the ileal crypts and high numbers in the caecum, when compared to conventional animals. Differences amongst the inoculated animals also suggested the influence of different microbiotas on TSB colonization of crypts: the ex-germ-free and ex-gnotobiotic animals were less like conventional animals than the SPF animals that had a more diversified microbiota.

These results are similar to previous studies of microorganisms associating with the mucosa in rodents: Watanabe et al. (1977) found that the numbers of lactobacilli in the gastrointestinal tract were higher in mono-associated rats than in conventional animals, especially in the large bowel. Zachar and Savage (1979) found that while Listeria monocytogenes was unable to persist in SPF mice the organisms were able to proliferate in the intestines of germ-free mice or mice associated with a Bacteroides spp. and a Clostridium spp.. Moreover the presence of these organisms diminished the ability of the Listeria to colonize the mucus layers on the surface of the mucosa.

From the results of the colonization experiments using TSB and also other spiral-shaped bacteria, it could be inferred that the caecum was the site most favourable for crypt colonization. This was also true of conventional animals as the caecal crypts contained higher numbers of bacteria than those of the ileum or colon. The most obvious explanation for lower numbers of TSB in the caeca

of conventional animals is competition from other crypt bacteria: specifically the spirochaetes and thin spirilla which are found at this site. When these competing populations are absent, as in the SPF, ex-gnotobiotic or ex-germ-free animals, the introduced organism are able to occupy the crypt habitat fully.

The influence of the intestinal biota on ileal colonization by the TSB is less obvious than in the caecum. If the semi-quantitative results for the ileum are compared there appears to be a correlation between the "normality" of the intestinal biota and the ability of the TSB to colonize the ileum: 0.6% of these crypts in ex-germfree rats contained TSB compared to, 14.5% in ex-gnotobiotic, 40% in SPF and 70% in conventional rats. Nevertheless, quantitative counts for all the inoculated groups, included those given homogenized intestine, were condiderably less than those of conventional animals. An explanation for this phenomenon may be that other microorganisms in the intestine create conditions suitable for TSB colonization, e.g. changes in surface chemistry (Abrams et al., 1963), production of growth factors (Gordon and Pesti, 1971) or alterations in redox potentials more suitable for microaerophilic growth (Celesk et al., 1976). Alternatively, the presence of normal bacterial populations may affect the intestine itself and hence alter crypt conditions, e.g. by increasing peristalsis (Savage, 1972), affecting cell turnover (Abrams et al., 1963), altering the structure of the ileal mucosa (Gordon and Bruckner-Kardos, 1961) or

changing the fluid balance of the mucosa (Savage and Dubos, 1968).

Experiments where ex-germ-free or SPF rats, having established crypt populations of TSB, were given tissue homogenates from conventional animals were undertaken to help determine how other microorganisms influence TSB colonization. However, the introduction of a more complex microbiota, including mucosa-associated microbes, did not, as expected, result in the animals being colonized by TSB in a manner similar to that occurring in conventional animals. The presence of other caecal populations appeared to depress numbers of TSB in the caecal crypts of some animals but not in others. Similarly introduction of a complex microbiota did not result in normal numbers of TSB being present in the ileal crypts. A possible explanation for the equivocal results was that feeding animals homogenized intestine did not lead to the establishment of a normal conventional microbiota. Koopman et al. (1982) found that increasing the number of doses of a SPF microbiota to germ-free animals led to the intestinal ecosystem becoming more like that of SPF animals, nevertheless even after four doses many environmental parameters remained different.

The presence of other intestinal microbes has been known to be necessary for colonization by intestinal organisms (Koopman et al., 1982) and could also be necessary for TSB to colonize normally. A related explanation could be that suitable conditions for TSB

growth occur only as a result of a succession of populations, such as occurs in newborn animals (Savage et al., 1968). Such successions could also be expected to occur in the similar intestinal environments of the animals with restricted microbiotas. The inability of organisms to establish in germ-free animals was explained by Koopman et al. (1982) to be caused by the absence of a true succession of populations creating suitable conditions. Although in the present experiment the composition of the lumenal biota was not analysed, the mucosa-associated microbiota of the SPF animals given homogenated conventional tissue was found to be different to that of conventional animals: by the absence of the attached spiral-shaped bacteria in the colon and, more relevantly to the present experiments, by the low numbers of TSB in the ileal crypts. The most important time in the succession that occurs prior to the establishment of the climax community is weaning, when the initial populations of lactobacilli and facultive anaerobes are substantially replaced by strict anaerobes. Although many of these members of the climax community are unable to establish in either conventional (Savage et al., 1968) or gnotobiotic animals (Ducluzeau et al., 1974) prior to weaning, spiral-shaped bacteria have been seen in conventional animals within the first week of life (Davis et al., 1973). Colonization studies showed that TSB were able to establish in the intestinal tract of pre-weaned rats and mice; furthermore weaning appeared to have little effect on colonization. Interestingly of all the animals having

restricted microbiota, only one group contained ileal populations of TSB comparable to that of conventional animals: germ-free infant rats given TSB cultures. A possible explanation of this result is that the sequence of colonization is important, and that for TSB to establish normally the organisms must be introduced into the animal early in life, before any climax communities have established. Further studies with infant animals and, in particular, with animals lacking other mucosal populations will be needed to better understand how these organisms colonize.

An important feature of the colonization experiments was the occurrence ofthe TSB within the cells surrounding the crypts. The passage of members of the normal intestinal microbiota through the mucosal barrier occurs at a low rate in normal animals. Organisms such as Lactobacilli spp. can be found in the mesenteric lymph nodes, liver and spleen of conventional and SPF animals in the absence of any pathogenic consequences (Hale and Hill, 1973). Irradiation leads to a large increase in translocation although the mechanism appears the same (Bazin, 1981). The tissue invasion involving the TSB followed a different pattern as it was characterized by organisms being present in the epithelial cells rather than in the mesenteric lymph nodes, liver or spleen. This type of invasion is similar to that of Shigella flexneri which are found in highest numbers in cells adjacent to the lumen with diminishing numbers deeper in the tissue, few in the

lamina propria, and rarely in the bloodstream or other tissues (Takeuchi, 1971); however the TSB infiltration did not cause the same disruptions to the tissue as did the pathogen. From the observations made in the experiments it was unclear how the organisms penetrated the tissue, although the observation of the TSB in the cytoplasm near the surface of goblet cells suggests these may be a portal of entry.

The occurrence of intracellular TSB in inoculated but not in normal animals may be due to the very high numbers of these organisms in the large bowel. Alternatively tissue invasion may occur at a higher frequency in the large bowel compared to the ileum. Another reason may be that animals with restricted microbiotas had deficient immune systems. Further experimentation needs to be undertaken to ascertain whether one or a combination of these reasons or other factors are involved; however, the studies with athymic and irradiated mice did not show immunological factors to be important. Although athymic mice are characterized by deficient cellular immunity and reduced production of antibodies, particularly IgA, tissue invasions appeared to occur at the same rate as in SPF animals and similarly TSB were not found in other sites apart from the intestine. In contrast, irradiation did seem to increase invasion in both gnotobiotic and SPF mice. Sub-lethal irradiation causes many changes to the host animal, two of which are pertinent to host defence: alterations to the immune system and changes in the nature of the intestinal mucosa. Irradiation before exposure to an antigen, as in the

present study, results in a marked depression of antibody production against these antigens: TSB antigens (Anderson and Warner, 1976). Irradiation also changes the mucosa by inhibiting mitosis at the base of the crypts, and thus disrupting the cell cycle (Maisin, 1966, cited by Bazin, 1981), by causing shrinkage of epithelial cells and creation of intercellular spaces (Bazin et al., 1971), and also by causing other physiological changes such as decreasing mucus production (Bazin, 1981). As irradiation renders the host immune status similar to that of athymic mice it could be expected that if this was a critical factor, infiltration in both groups would occur at similar rates. Similarly previous experiments where mice were irradiated with 3000 rads X radiation or given nitrogen mustard, produced TSB invasion one to four days post-treatment; a period not normally associated with bacterial translocation (Bazin, 1981) and more likely to be related to tissue integrity than immune status. However, before such conclusions can be drawn, more accurate assessments of the levels of invasion need to be made, e.g. by immunofluorescence, autoradiography or radioactive labelling of the TSB.

The colonization experiments demonstrated that organisms isolated from the intestinal mucosa, in particular the TSB, were able to colonize intestinal crypts in animals lacking any existing mucosal microbiota. The final results chapter will examine in detail the TSB isolates with respect to parameters thought to be important for survival in this micro-habitat.

CHAPTER SIX

IN VITRO STUDIES OF BACTERIA, ISOLATED FROM RODENT INTESTINAL MUCOSA

6. IN VITRO STUDIES OF BACTERIA, ISOLATED FROM RODENT INTESTINAL MUCOSA

The limited variety of microorganisms found within intestinal crypts is indicative of a very selective environment. While many of the abilities needed to colonize these habitats would be shared by other intestinal bacteria some would be directly related to the nature of the crypt environment. Having isolated bacteria that were able to inhabit intestinal crypts: the TSB isolates, it was possible to begin examining what factors may be important in the colonization of these sites. Three parameters: the ability to move in viscous mucus secretions, sensitivity to oxygen and carbon dioxide (CO_2) and the ability to degrade mucin, were chosen as being of possible significance, and the response of the TSB isolates to each tested in separate experiments.

6.1.1. Motility of Mucosal Isolates in Solutions of varying Viscosities.

An important facet of tissue association is that the organisms are contained within the mucus layers also associated with the tissue surface. While the mucus on the surfaces in contact with the intestinal lumen form generally unstirred layers (Allen, 1981), the mucus produced by the numerous goblet cells lining the intestinal crypts is not static: with other crypt secretions the mucus flows from the base of the crypt to the opening and out into the intestinal lumen. Even if this flow is

spasmodic, the organisms would need to be able to move more rapidly than the outflow of secretions to remain in the habitat. It was therefore postulated that the TSB isolates should be able to move efficiently in solutions of high viscosity. This was tested by monitoring the motility of the isolates in solutions of varying viscosity.

The major components giving intestinal mucus its rheological properties are long, flexible, negatively charged glycoproteins (Clamp et al., 1978). Interactions between these large asymmetric molecules leads to the formation of viscous solutions and gel formation, even at low concentrations (Clamp et al., 1978). In contrast other mucins, such as pig gastric mucin, form viscous solutions by the interaction of globular polymeric glycoproteins. Berg and Turner (1979) have shown that the ability of microorganisms to move in viscous environments is dependent on the structural characteristics of the viscous agent. The ideal solution to test for the motility of organisms in mucus would, therefore, be various concentrations of rat or mouse intestinal mucin; however it was not possible to obtain sufficient quantities of these glycoproteins for viscosity experiments. Solutions of commercially available hog gastric mucin were not considered suitable because, as previously stated, the structures formed by these glycoproteins in solution are quite different to those formed by intestinal mucins. Previously a number of

different substances have been used to test the effect of viscosity on bacterial motility: methyl cellulose (Schneider and Doetsch, 1974; Strength et al., 1976; Greenberg and Canale-Parola, 1977a), polyvinylpyrrolidone (Schneider and Doetsch, 1974; Greenberg and Canale-Parola, 1977a,1977b) and Ficoll (Berg and Turner, 1979). For the present investigation solutions of methyl cellulose were chosen, as these more closely resembled mucin glycoproteins: a low concentration of the very long methyl cellulose molecules in solution gives a gel-like medium.

The response of the TSB to different viscosities was tested using the rat isolate; the mouse isolate was also tested at several viscosities and was found to behave similarly. Also tested were other mucosal isolates, campylobacter-like bacteria grown from ileal scrapings, and for comparison a non-mucosal peritrichously flagellated bacterium: Serratia marcescens. For the viscosity experiments the bacteria were grown on overlaid slopes, as described in Chapter 2. Liquid overlays were poured off the slopes, combined and centrifuged at 3,000 g. The supernatant was removed and the centrifuge tube inverted to drain any free liquid. Test solutions were prepared by combining appropriate amounts of either 1%, 2% or 4% stock solutions of methyl cellulose with the broth supernatant. The broth component was necessary as the microaerophilic bacteria quickly lost motility in solutions with Adlers motility buffer, or phosphate

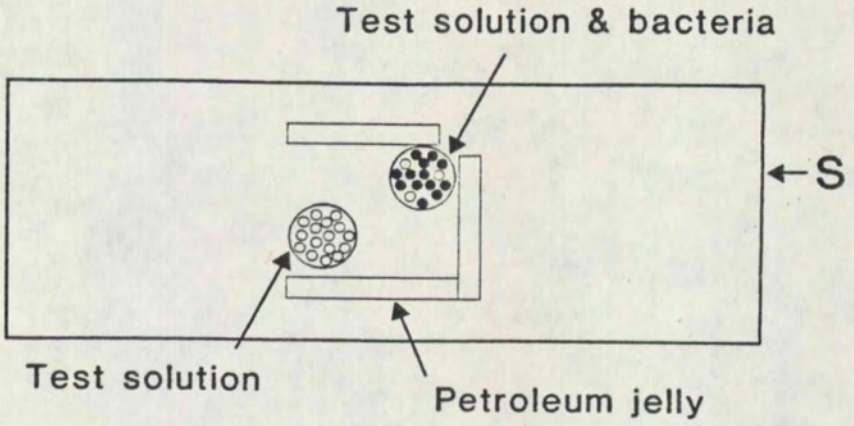
buffered saline as the solvent. After the methyl cellulose and broth had been mixed using a vortex agitator, 0.5 ml was used to resuspend the bacterial pellet and the remainder kept for viscometry measurements. The motility of the organisms was quantitated by a modification of methods described by Schneider and Doetsch (1974), using video tape recording of preparations observed by dark field microscopy. A thin film of solution containing bacteria, suitable for microscopic observation, was prepared as follows. Lines of petroleum jelly were drawn on the surface of a clean microscope slide, in a partly formed square grid (Fig.30A). A small drop of test solution containing bacteria was placed at the open corner of the square whilst another drop of test solution without bacteria was placed at the open side of the grid (Fig.30A). A clean coverslip was then placed on the vaseline grid, covered with an absorbent tissue and lightly pressed to create a film of test solution between the coverslip and slide; if pressed too firmly extensive streaming occurred. The slide was examined with a Ziess microscope using a 16x objective. Only areas adjacent to the open side of the coverslip were examined (Fig.30B), and slides with obvious streaming were discarded. One side of the coverslip was left unsealed to permit diffusion of oxygen. The use of two drops of solution was to minimize the amount of debris and non-motile bacteria in the field of view, as these interfered with motility and observation of bacteria. Only organisms capable of

Figure 30. Preparation of a microscope slide (S) for observation of bacterial motility in different viscous solutions.

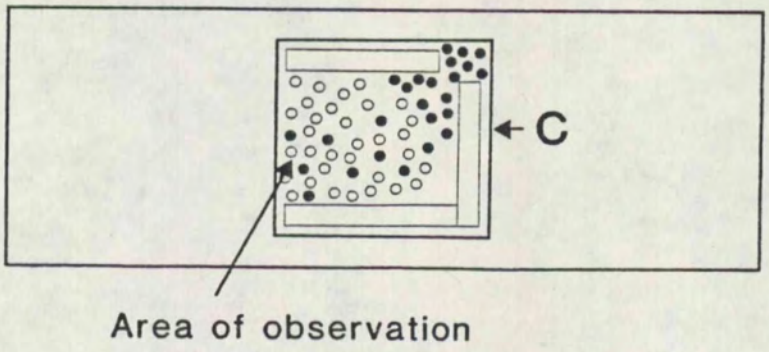
A. Two drops of viscous test solution, one containing bacteria, were placed at opposite sides of an open grid of petroleum jelly.

B. A coverslip (C) was then placed on the grid and microscope observations made in the area indicated.

A.


Test solution & bacteria
S
Test solution
Petroleum jelly

B.


C
Area of observation

moving through the bacteria-free test solution to the open side of the grid, were examined. After a suitable field had been chosen, the motility of the bacteria were recorded on a video tape recorder using a television camera mounted on the microscope. The tape was replayed on a monitor and the paths of individual bacteria traced onto a transparent sheet of plastic, overlaying the monitor screen. These paths were measured with a calibrated planimeter, the tape replayed, and the time taken to traverse the path measured with a 1/10 sec stopwatch. As previously (Schneider and Doetsch, 1974), the 10 greatest velocities were used to calculate the average velocity. The experiment was conducted in a constant temperature room set at 21°C. The viscosities of the test solutions were measured with an Ostwald-type viscometer in a water bath kept at 21°C. The viscometers were calibrated with ethylene glycol at various temperatures.

The results of the viscosity experiments are displayed in Fig.31. The response of the Serratia marcescens to increasing viscosities was similar to that seen by Schneider and Doetsch (1974): the velocity of the organisms increased until it reached a maximum at a viscosity of 4-5 centipoise (cp) and then gradually diminished with further increases in methyl cellulose concentration. The TSB isolates moved at much greater velocities than the Serratia over the range of viscosities tested. The response to increasing viscosities was similar

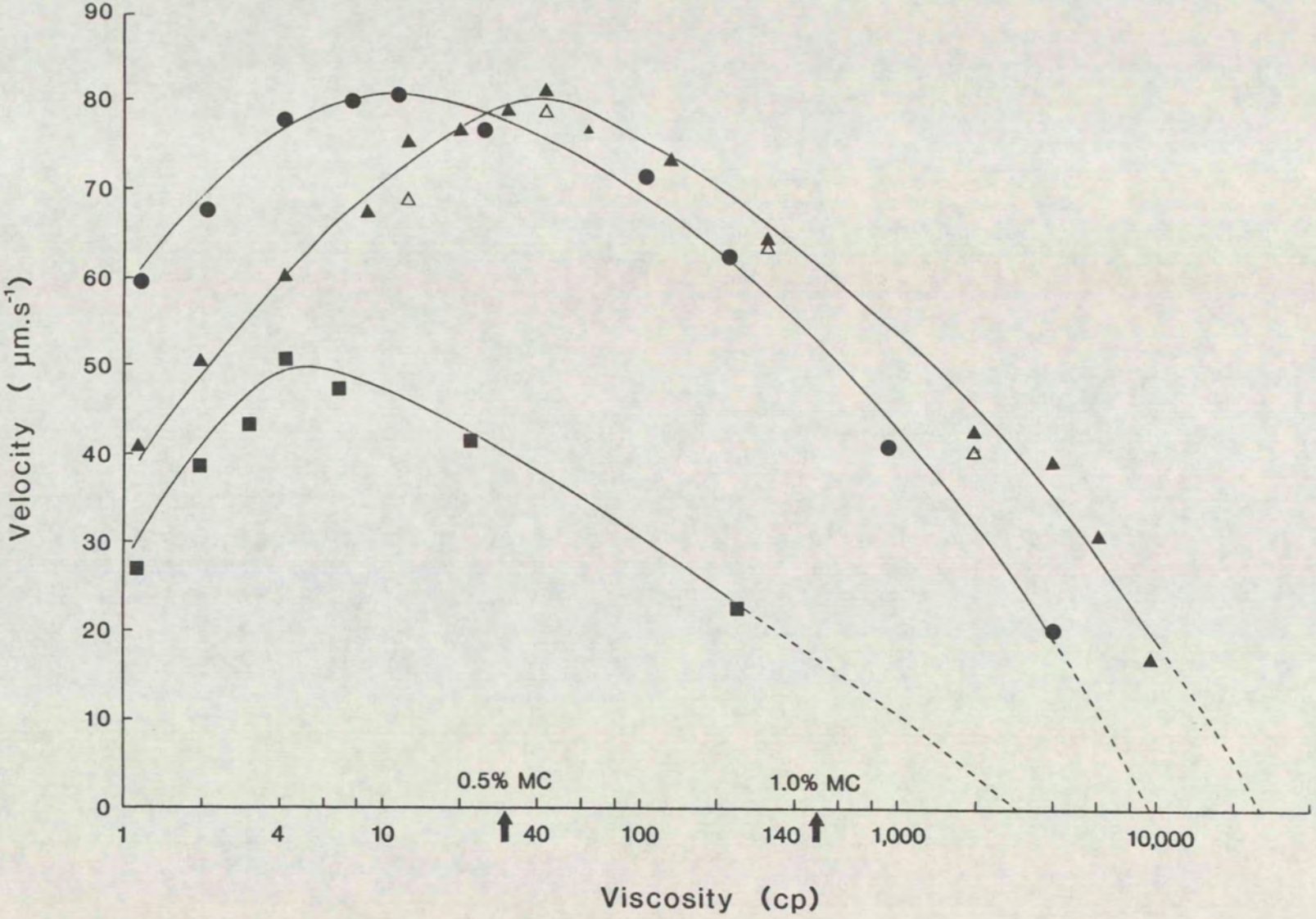
Figure 31. The effects of viscosity on bacterial motility.

RTSB isolates ▲

MTSB isolates △

Campylobacter-like bacteria ●

Serratia marcescens ■


Velocity (µm.s-1)
90
80
70
60
50
40
30
20
10
0
0.5% MC
1.0% MC
1
4
10
40
100
140
1,000
10,000
Viscosity (cp)

to that of the Serratia with velocities initially increasing to a maximum and then slowly decreasing; however the optimum viscosity for velocity was considerably higher: around 40 cp. The organisms were still highly motile at high viscosities of methyl cellulose; at 1000 cp the TSB had a higher velocity than the Serratia sp. at 4-5 cp. While the results were obtained with the RTSB, tests with the MTSB using four different viscosities indicated that the response of these two organisms were very similar.

In addition to the effects on the velocity of bacteria other changes in the motility of the organisms were noted as the viscosity increased. At low viscosities, such as 1-2 cp, the organisms were usually seen moving in short runs with many changes of direction and pauses. These short paths were often curved rather than straight. As the viscosity increased, the paths of the organisms became straighter, and the runs became longer with fewer changes of direction and pauses. Although not quantitated, there appeared also to be an increase in the proportion of organisms translocating as the optimum velocity was reached. At very high viscosities the nature of motility was the same as that seen at the optimum concentration except for the diminished velocity. In these preparations the flagellum-like appendages occasionally could be seen as single rotating helical bundles. Also the corkscrew-like motility of the TSB was apparent, and there appeared to be very little slip as the organism moved through the medium. The other mucosa-associated isolates, the campylobacter-like bacteria, like

the TSB, had an optimum viscosity for motility (11 cp) above that of the Serratia sp. and other flagellated bacteria (Schneider and Doetsch, 1974); however this viscosity was still considerably below that of the TSB. Nevertheless the campylobacter-like bacteria were still able to move at high velocities at viscosities over 100cp.

6.1.2. Oxygen and CO_2 Requirements of the TSB Isolates.

It was shown in Chapter 4 that the TSB, unlike the vast majority of intestinal microorganisms, were unable to grow anaerobically. The organisms were isolated using atmospheres suitable for the growth of campylobacter: reduced partial pressures of oxygen with added CO_2 (Smibert, 1978). An investigation was undertaken to determine more precisely the gaseous requirements of the TSB: whether these organisms were truely microaerophilic or whether, like other bacteria (Austrian and Collins, 1966) the TSB only required elevated CO_2 levels for growth. Furthermore such information would also indicate the conditions that are likely to be present in the crypt habitats.

The growth of TSB under various gaseous conditions was tested by methods similar to those used to assess Campylobacter spp. (Kiggins and Plastridge, 1956; Flecher and Plastridge, 1964). A partial vacuum was drawn in Brewer gas jars and replaced with measured amounts of oxygen, nitrogen, or CO_2.

As in the previous studies with campylobacter it was necessary to grow the organisms on solid media as the diffusion of oxygen and CO_2 into any liquid media would make interpretation of such results very difficult. The organisms were grown on two types of media, Lysed Horse Blood agar (LHBA) and serum agar, and each was slanted in 25 ml bottles to give standard surfaces. Slopes were inoculated with 0.01 ml of a three day biphasic broth culture spread evenly over the entire surface, excluding the lower 1 cm. After 48 hrs incubation at 37°C the slopes were removed from the gas jars and the cells washed from the agar surface with two 2.5 ml washes of formalized phosphate buffered saline. The washes were combined and the turbidity measured in a spectrophotometer at 650 nm. The growth of the RTSB and MTSB isolates on LHBA in various gas mixtures is shown in Figs.32A and 32B. Neither isolate grew in anaerobic gas jars while both grew well in atmospheres containing 15% or less oxygen with 5% or 10% CO_2. With 5% CO_2 growth diminished at oxygen concentrations greater than 15% with negligible growth in atmospheric oxygen levels: 21%. In contrast, with 10% CO_2 better growth was obtained with higher oxygen levels, even at 21% oxygen. Between the range of 1% to 13% there was slightly better growth with increased oxygen while greatest growth was obtained at 15% oxygen, in either 5% or 10% CO_2. Both isolates grew very poorly without added CO_2: i.e. less than 0.03% CO_2, and the organisms only grew in the range of 7% to 15% oxygen.

Figure 32. Growth of TSB isolates in various gas mixtures when incubated on LHBA; average of two experiments ± standard deviation.

a. MTSB

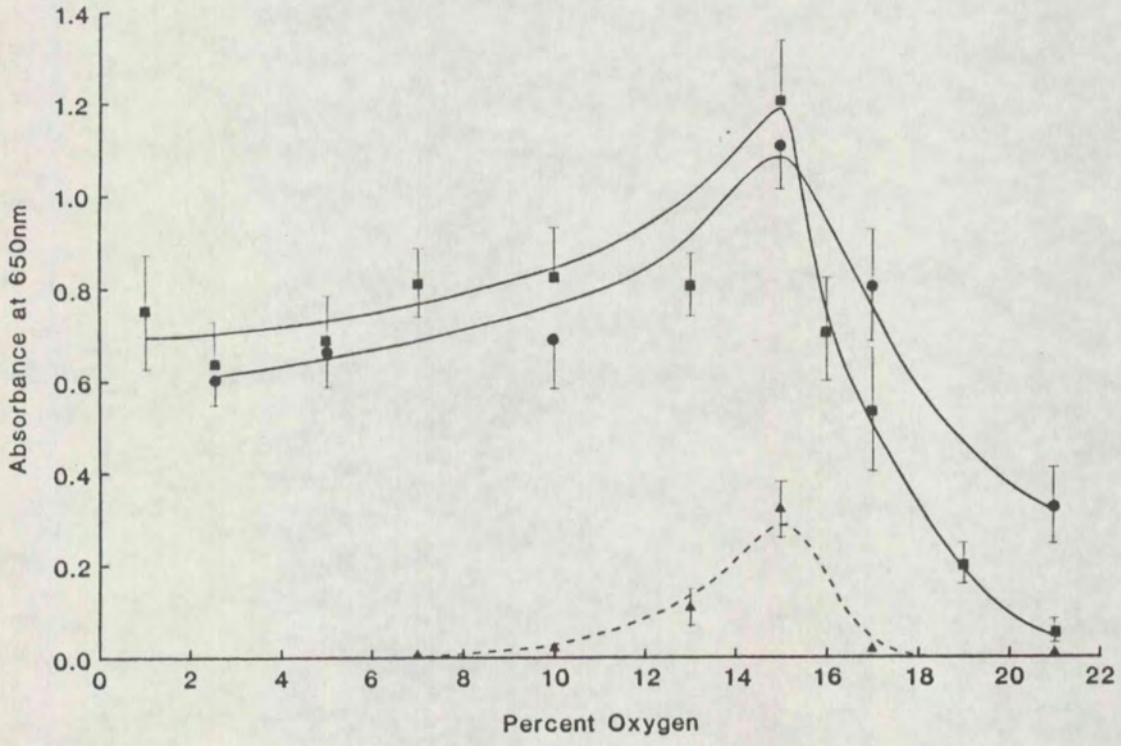
b. RTSB

0.03% CO_2 ▲

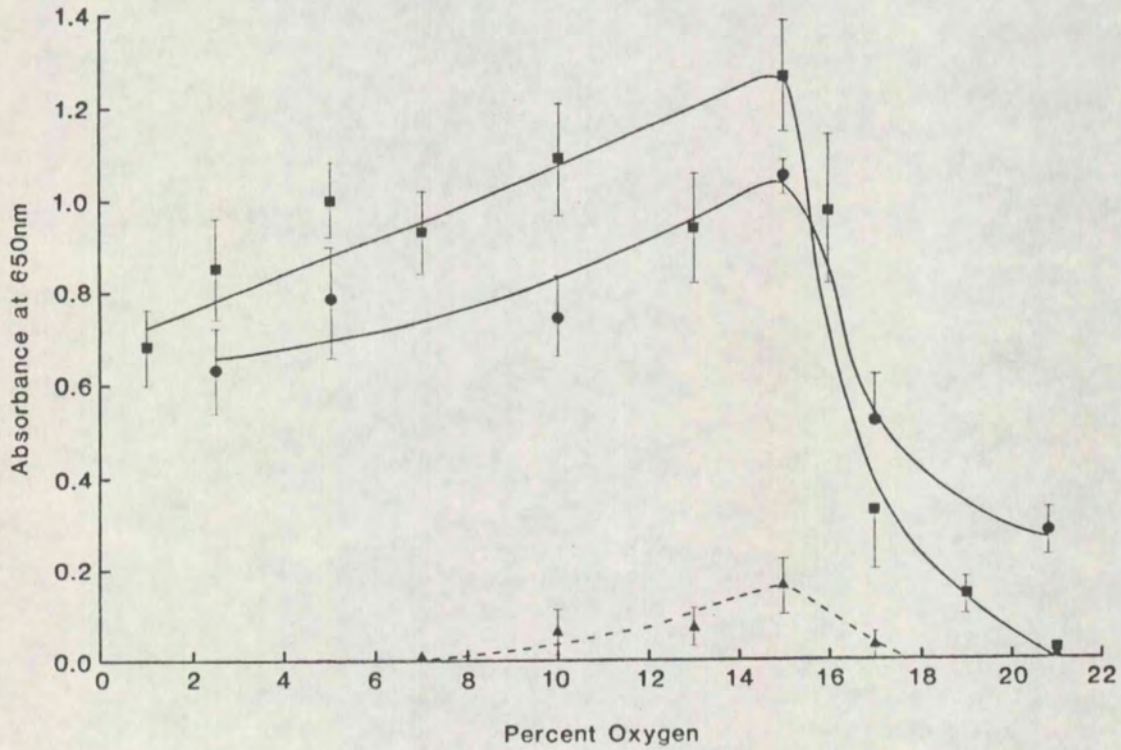
5% CO_2 ■

10% CO_2 ●

a)


Absorbance at 650nm
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
2
4
6
8
10
12
14
16
18
20
22
Percent Oxygen

b)


Absorbance at 650nm
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
2
4
6
8
10
12
14
16
18
20
22
Percent Oxygen

Although the isolates grew less vigorously on serum agar compared to LHBA, the response to the various atmospheres was similar (Fig.33); with the main differences being that the isolates did not show a preference for atmospheres with 15% oxygen, and those with 5% CO_2 were unable to grow in oxygen concentrations of greater than 16%. Growth without added CO_2 was negligible.

Although slight differences were seen in the responses of the RTSB and MTSB isolates they were not considered significant.

6.1.3. In vitro Test for Degradations of Purified Rat Small Bowel Mucin by TSB Isolates.

Intestinal mucus is, as previously stated, of importance to surface-associated microbes because of its rheological characteristics. Another potentially important role for this substance is as a nutritional source for the intestinal microbiota. While the breakdown of mucin by lumen microbes is well established (Hoskins, 1981), it is not known if the organisms most closely associated with the mucus producing cells: the mucosa-associated microbes, are also able to metabolise these substances. Having isolated crypt dwelling bacteria, i.e. the TSB, it was possible to test for the ability of such organisms to degrade mucin.

The isolates were inoculated onto serum slopes and incubated for 48 h and then overlaid with 5 ml of test solution: a 0.5% w/v solution of purified rat small bowel mucin in tryptone water. Other solutions tested were

Figure 33. Growth of TSB isolates in various gas mixtures when incubated on serum agar: average of two experiments ± standard deviation.

a. MTSB

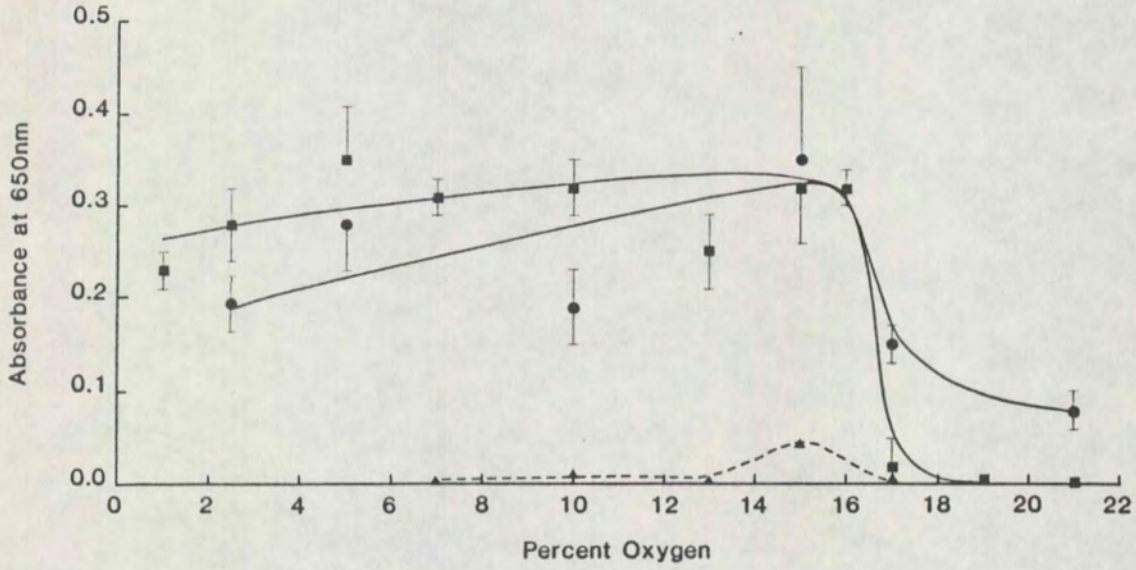
b. RTSB

0.03% CO_2 ▲

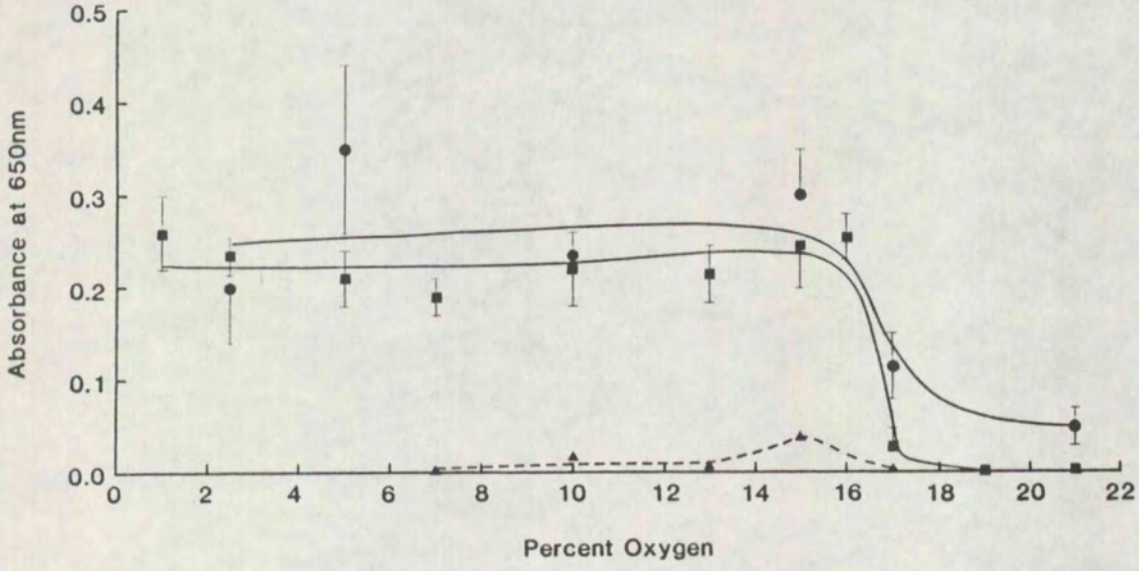
5% CO_2 ■

10% CO_2 ●

a)


Absorbance at 650nm
0.5
0.4
0.3
0.2
0.1
0.0
0
2
4
6
8
10
12
14
16
18
20
22
Percent Oxygen

b)


Absorbance at 650nm
0.5
0.4
0.3
0.2
0.1
0.0
0
2
4
6
8
10
12
14
16
18
20
22
Percent Oxygen

tryptone water overlaying inoculated slopes and also mucin solutions overlaying uninoculated slopes. As positive controls slopes overlaid with the mucin solution were inoculated with 0.1 g of conventional rat faeces. The solutions containing rat faeces were incubated for a further 3 days while all other solutions were incubated for a further 7 days; then the broths were collected, centrifuged at 3000 g and the supernatant analysed for the levels of bound sialic acid using the method of Jourdian et al. (1971), and also for methyl pentoses using the method of Dische and Shettles (1948).

The results of the mucin degradation experiment are seen on Table 16. While incubation with conventional rat faeces led to the removal of over 99% of bound sialic acids and 98% of methyl pentoses from solutions containing mucin, incubation with the TSB isolates appeared not to alter the levels of these terminal mucin oligosaccharide residues: the results for the isolates were within the variations seen in duplicate samples of the control mucin solution.

6.1.4. Discussion

The lactobacilli and yeast present on the gastric mucosa and the segmented, filamentous-bacteria found on the ileal villi have overcome adverse physical environments by evolving mechanisms to attach to host tissues (Lee, 1980). Nevertheless experiments with isolates of the lactobacilli and the yeast Torulopsis pintolopesii, have indicated that attachment is only one of many abilities needed to colonize these habitats (Tannock et al., 1982 ; Suegara et al., 1979).

Table 16. Changes in methyl pentose and bound sialic acid concentrations in solutions containing purified rat small bowel mucin and inoculated with either RTSB, MTSB or conventional rat faeces.

Test solution	Methyl Pentoses µg/ml ± standard deviation[b]	Sialic Acids µg/ml ± standard deviation
Tryptone Water (TW)	18.8 ± 0.4	24.0 ± 6.1
TW + RTSB[c]	59.8 ± 6.9	51.9 ± 7.2
TW + MTSB[c]	45.6 ± 0.42	34.0 ± 5.3
TW + 0.5% Mucin	171.1 ± 10.5	271.9 ± 14.7
TW + 0.5% Mucin + RTSB[c]	179.0 ± 8.9	263.2 ± 9.1
TW + 0.5% Mucin + MTSB[c]	169.2 ± 2.5	264.5 ± 13.9
TW + 0.5% Mucin + Rat faeces	4.1 ± 0.9	2.5 ± 2.1

a. Overlaying a serum slope

b. Duplicate samples.

c. TSB isolates grown on slopes prior to addition of other solution components.

Similarly colonization of intestinal crypts is also likely to be reliant on an ability to physically remain within these habitats and also on the suitability of the organisms' biochemical and physiological makeup to conditions occurring at these sites. Accordingly, the experiments reported in this chapter have sought to begin determining what physical and metabolic abilities enable the TSB to colonize crypts.

6.1.4.1 Effect of viscosity on the motility of mucosal isolates

Before considering the results of the viscosity experiments it is necessary to consider the research done previously in this field. Furthermore an important aspect to consider when comparing these reports is the viscous agent used in experiments. Berg and Turner (1979) have demonstrated that the microscopic viscosity is of more importance to bacterial motility than the macroscopic viscosity. As a consequence agents with different molecular shapes and sizes, while having similar macroscopic viscosities, can present very different viscous properties to the bacteria. Thus while even low viscosities of a spherical molecule such as Ficoll can dampen the movement of E.coli flagella, it needs considerably higher viscosities of methyl cellulose to achieve the same effect. Many of the reports investigating the effects of viscosity on bacteria use polyvinylpyrrolidone (PVP), and this compound, like Ficoll, has a much more pronounced effect on motility than methyl cellulose at equivalent macroscopic viscosities

(Greenberg and Canale-Parola, 1977a; Schneider and Doetsch, 1974). An example of the differences that can occur in results obtained by using methyl cellulose and PVP can be seen in results of experiments determining the minimum inhibitory viscosities (MIV): the viscosity at which motility ceases, of Spirochaeta aurantia M1 (Greenberg and Canale-Parola, 1977a). In an experiment conducted using both viscous agents the MIV was 300 cp with PVP while the methyl cellulose results extrapolate to give a MIV of approximately 20,000 cp.

Schneider and Doetsch (1974) investigated the ability of a variety of flagellated bacteria: Bacillus megaterium, Escherichia coli, Pseudomonas aeruginosa, Sarcina urea, Serratia marcescens, Spirillum serpens, and Thiospirillum jenense, to translate in viscous solutions of PVP or methyl cellulose. The response of these organisms was similar in each case: as the viscosity was raised from that of water (1 cp) the velocity of the cells increased to a maximum at viscosities of 2-5 cp. Further increases in viscosity lead to decreases in the organism's velocity. These experiments confirmed the observations of Shoesmith (1960) that the optimum viscosity for flagellated bacteria was slightly above that of water. It was also found that polarly flagellated organisms, though often having higher maximum velocities, were more affected by viscosity increases than peritrichously flagellated organisms. Greenberg and Canale-Parola (1977a) extended these observations by increasing the viscosity until

organisms lost all movement. They found that the minimum inhibitory viscosity (MIV) of Pseudomonas aeruginosa, Spirillum serpens and Escherichia coli, were all about 60cp in PVP.

While these studies have demonstrated that most bacteria are not suited to move in solutions of high viscosity, a number of organisms have been shown to have the ability to move efficiently in such solutions. Spirochaetes have been shown to be well suited to movement in viscous environments. Increased viscosity results in both a higher percentage of leptospires translating, and increases in these organisms' velocity (Cox and Twigg, 1974; Kaiser and Doetsch, 1975). In methyl cellulose solutions the optimum viscosity, for both maximum velocity and also for the maximum percentage of cells moving, was well above 300 cp. Furthermore Petrino and Doetsch (1978) demonstrated that Leptospira interrogans preferentially moved towards a viscosity gradient of PVP and thus exhibited "viscotaxis".

In addition to spirochaetes a number of flagellated bacteria have been found that appear to be adapted to viscous environments. Greenberg and Canale-Parola (1977b) found that two bacteria; Spirillum gracile and a rod-shaped bacterium, PFR-3, each had MIVs of 1000 cp in PVP, though neither had elevated optimal viscosities (2 and 6.5 cp respectively). Both organisms were also able to migrate through agar gels, that would have had very high macroscopic viscosities. Also able to move in very

viscous solutions was the rod-shaped bacterium Aquaspirillum fasciculus (Strength et al., 1976). These organisms were found to move with maximum velocity at a viscosity of 203 cp in methyl cellulose, moreover the motility was relatively unaffected at a viscosity of 633 cp. These organisms, which were isolated from freshwater sediments, moved at a comparatively low speed: a maximum of 13.5 um/sec. A much less dramatic adaption to viscosity has been seen in Rhizobium spp.: strains with complex flagella were shown to be less affected by increases in viscosity; however this was only in the range of 5-20 cp in methyl cellulose (Gotz et al., 1982).

The response of bacteria to viscous solutions cannot be fully explained, as the nature of bacterial motility in both spirochaetes and flagellated bacteria has yet to be fully elucidated. Nevertheless explanations for the abilities of some organisms to move in viscous solutions have been proposed. Each of the spirochaetes have small cross-sectional areas, an important factor when considering the lattice-like structures created by some of the viscous agents. Berg and Turner (1979) have proposed that spirochaetes move through the solvents of a viscous solution and corkscrew through or around the lattice-like solute. In viscous solutions with no propensity to form lattices, such as formed by the branched spherical molecules of Ficoll, Leptospira interrogans lacked the efficient motility seen in methyl cellulose.

Another factor common to these spirochaetes is tight cell coiling. Greenberg and Canale-Parola (1977a) found that while mutants with loose, or little cell coiling, could move adequately in low viscosity solutions they lacked the ability of the tightly coiled isolates to move efficiently in higher viscosities. The importance of cell coiling may be related to the corkscrew-like motility of the spirochaetes. Such motion is only effective if slip along the helical axis is reduced to a minimum. Jahn and Landman (1965) demonstrated that the higher the number of coils in Cristispira sp. the lower the translational slip, and efficiencies of near to 100% (or no slip) were recorded. Such efficiency is achieved by having a small area in cross-section, pushing through the medium, with a very large surface area, (the coils) providing forward motion.

Unlike the spirochaetes there is no common cell shape that characterizes the ability of flagellated bacteria to move in viscous solutions: both rod-shaped and spiral-shaped organisms were able to move in viscous solutions and organisms of both types were also unable to move in such solutions. Consequently explanations for their behaviour relate more to the nature of their flagellum/flagella. As previously noted most flagellated organisms move faster at viscosities slightly above 1 cp; one such organism, Thiospirillum jenense has a large flagellum bundle which is visible under the light microscope (Schneider and Doetsch, 1974). It was observed

that the conformation of this bundle changed according to the viscosity of the surrounding solution. From these observations and from theoretical considerations Schneider and Doetsch (1974) postulated that the ability of bacteria to move faster at viscosities higher than 1 cp was due to changes in the conformation of their flagella. Optimum efficiency of a flagellum is obtained when its wavelength is 2π times its amplitude; wavelengths longer or shorter are less efficient and thus give less propulsive power. If this is so, the stiffness of the flagella will influence efficiency in different viscosities; a stiffer flagellum will not dampen as much as one with a less rigid structure. Schneider and Doetsch (1974) suggested that the cumulative stiffness of flagellum bundles explained the different responses of the polarly flagellated organisms and those with peritrichous flagella. Similarly the rigidity of the complex flagella found in Rhizobium isolates could explain their enhanced ability to move in viscous solutions when compared to isolates with normal flagella.

In the present investigation the response of Serratia marcescens was similar to that gained by Schneider and Doetsch (1974), except that the organisms moved more rapidly; this may have been due to differences in the viscous agents or to differences in the isolates. The TSB, like leptospires and Aquaspirillum facile, moved optimumly at a viscosity (40 cp) considerably above that of most flagellated bacteria (2-5 cp). The response of

the TSB to increased viscosity was like that of Spirochaeta sp., Spirillum gracile or the rod-shaped bacterium PFR-3 isolated by Greenberg and Canale-Parola (1977b) rather than of the leptospires; initially as viscosity increased so did motility, before reaching a maximum, and then gradually declining. The MIV of the TSB was not reached, however by extrapolation it was found to be 20,000 cp in methyl cellulose. This is probably similar to the MIV of 300-1000 cp in PVP obtained for other bacteria, as the MIV of Spirochaeta aurantia M1 was 300 cp in PVP and 20,000 cp in methyl cellulose.

The other mucosal isolate, the campylobacter-like organism, responded to the changes in viscosity in a similar manner to the TSB, however these organisms had an optimal viscosity that was more similar to that of the Serratia sp. than to the other mucosal isolate. Nevertheless, the high velocity, in comparison to the Serratia sp., of the campylobacter-like bacteria meant that in above optimum viscosities these organisms were able to move 2-2½ times as rapidly as the peritrichously flagellated organisms. However, if the results are extrapolated the Serratia sp. motility does appear to decrease at a slower rate than the campylobacter-like bacteria at very high viscosities and this may be due to the presence of multiple flagella, compared to the bipolar single flagella of the campylobacter-like organisms. The viscosity studies were all conducted at 21^{o}C as the means of controlling the stage temperature was not available,

however it could be expected that the velocities of mucosal isolates would be considerably higher at the 37°C temperature of the mucosal environment (Schneider and Doetsch, 1977).

Although experiments were not undertaken to determine why the TSB had an elevated optimum viscosity for motility, several explanations can be proposed on the basis of the ultrastructural studies and similarities to both spirochaetes and flagellated bacteria.

It is clear from the ultrastructural studies that the terminal appendages are sheathed flagella and that spirochaete-like periplasma fibres are not present: thus propulsion is due solely to the work of the appendages. Consequently the characteristics of the TSB motility in viscous solution is most likely to be due to changes in the conformation of the flagella and thus related to their "stiffness". Furthermore the optimum shape of the flagella, with the wavelength equal to 2π times the amplitude, could be expected to be seen at a viscosity of 40 cp in methyl cellulose. The stiffness of the flagella could be due to the nature of the flagella core or due to the sheath membrane. Interestingly another organism known to move efficiently in mucus, Vibrio cholerae also has a sheathed flagellum. It would be interesting to test the ability of these vibrios to move in viscous solutions.

The periplasmic tubules and the associated structures in the cell wall create prominent ridges on the

surface of the TSB, and these could be expected to influence the hydrodynamic properties of the bacteria. The ridges could promote auger-like motion by the TSB, particularly if the organisms were moving through lattice-like viscous solutions. The ridges could minimise translational slip, as increased cell coiling does in spirochaetes.

The surfaces of the intestinal mucosa are bathed in mucus secretions originating from the host cells. The presence of mucin glycoproteins in various amounts gives mucus its characteristic rheological properties of viscosity and gel formation. There are several ecological advantages to the TSB, and other mucosa-associated bacteria, endowed with the ability to move in solutions of high viscosity. Active motility is necessary if bacteria are to effectively penetrate the mucus layers on the tissue surface and to initially colonize this habitat. Furthermore having a high optimum viscosity for motility means that these organisms would move through the mucus more rapidly and thus be less likely to be flushed into the lumen from the intestinal crypts.

6.1.4.2 Gaseous requirements of the TSB isolates

The requirements of the spiral-shaped organisms for oxygen and carbon dioxide were very similar to those reported for Campylobacter jejuni strains (Smibert, 1978) which can also be found in the intestine. The isolates grew optimally in reduced partial pressures of oxygen and

increased partial pressures of carbon dioxide. The toxicity of oxygen to the isolates was dependent on the carbon dioxide concentration. Growth in atmospheric concentrations of oxygen, ie. 21%, occurred only if the concentration of carbon dioxide was increased substantially from the level in air, 0.03%, to higher concentrations, e.g. 5 to 10%.

At low concentrations of oxygen, CO_2 was needed to give normal growth, while at high concentrations of oxygen over 15-16%, high CO_2 concentrations (10%) appeared to diminish the toxic effects of oxygen.

It is likely that the TSB's inability to grow aerobically is caused, as with campylobacter spp. (Smibert, 1978) and other microaerophiles such as Spirillum volutans (Padgett et al., 1982), by an acute sensitivity to toxic forms of oxygen such as hydrogen peroxide or superoxide radicals. Such sensitivity can be due to an absence of enzymes necessary to detoxify these compounds (Blakemore et al., 1979) or due to an inability of enzyme systems to act against them exogenously (Hoffman et al., 1979). In order to confirm the influence of toxic forms of oxygen on the TSB, experiments need to be undertaken to test the effects of adding catalase and a superoxide dimutase to growth media, on aerotolerance. Nevertheless, intolerance to toxic oxygen compounds would explain the rapid decline in growth above 15% oxygen; as this could be the threshold at which the TSB are unable to remove these substances. If so, the presence of high

concentrations of CO_2 either enhances the ability of TSB to tolerate or remove the toxic compounds, or the presence of this gas inhibits the formation of hydrogen peroxide and/or superoxide radicals in the medium. The poor growth of the isolates on serum agar may be due to nutritional factors or it could be due to the absence of detoxifying agents such as catalase and superoxide dimutase known to be present in whole blood (Fridovich, 1974).

6.1.4.3 Mucin degradation by the TSB.

Bacterial degradation of mucin in the intestinal lumen has been shown to be caused by only a small sub-population of the intestinal microbiota (Hoskins, 1981). Moreover, the process of degradation requires the presence of a number of bacterial types, each with different, specific, glycosidases, acting in a step-wise breakdown of the mucin oligosaccharides (Hoskins, 1981). The glycosidases are extracellular and thus it is probable that non-mucin degrading bacteria benefit from their activity.

The situation for bacteria within intestinal crypts would be quite different to that of lumen microbes with respect to mucin degradation. It is unlikely that significant amounts of glycosidases or released mucin carbohydrates would diffuse into the depths of the crypts against the outflowing secretions. Consequently the crypt bacteria could not rely on the subpopulation of mucin degrading bacteria in the lumen and because of the limited diversity in the crypts a higher proportion of organisms

could be expected to have the enzyme systems needed for degradation. Moreover as the TSB isolates were able to colonize crypts in germ-free rats in the absence of other bacteria, the organisms would by necessity, have the enzymes needed to remove the terminal oligosaccharide residues; that is, if mucin is a major nutritional source for these organisms.

The results of the *in vitro* experiments gave no evidence of mucin degradation by the TSB isolates. While the intestinal lumen microbiota present in rat faeces were able to remove a very high proportion of the terminal mucin sugars: the bound sialic acids and also methyl pentoses, from mucin solutions, incubation of the same solutions with TSB caused no significant reductions. The TSB may be able to metabolize mucin at a very slow rate, below the accuracy of the present experiments; nevertheless it is unlikely, from these results, that the organisms do rely on mucin as a major nutritional source.

The aim of the studies reported in this chapter have been to begin investigating what characteristics enable the TSB to associate with the intestinal mucosa in rodents. Experiments have demonstrated that these bacteria are able to move efficiently in solutions of high viscosity and thus appear well adapted for the mucus environment adjacent to the tissue surface. Requirements, by the TSB, for oxygen and CO_2 suggest that these gases are readily available in areas near the mucosa and

moreover may give the microaerophiles a selective advantage over other anaerobic microorganisms at these sites.

The inability of the TSB to degrade purified rat small bowel mucin indicates that these organisms rely on the catabolism of other compounds for a source of energy and carbon; it is probable that these compounds arise from the host tissue. Clearly, if the relationship between the host and the TSB or other mucosa-associated bacteria, is to be better understood a high priority must be given to studying these organisms' nutritional, biochemical and physiological characteristics.

CHAPTER SEVEN

GENERAL DISCUSSION

7. GENERAL DISCUSSION

Although the microbiology of the gastrointestinal tract has been studied extensively over the past 100 years the understanding of how this ecosystem functions remains remarkably poor (Freter, 1983c). While some of this lack of progress can be attributed to the way research has been undertaken (Freter, 1983c), the main reason for this situation is probably the great complexity of the microbiota. Most research has concentrated on the micro-organisms colonizing the lumen of the tract, a site of great species diversity: for example up to 400 types of bacteria have been identified from human faeces (Holdeman *et al.*, 1976). Despite many advances in techniques for the isolation, cultivation, and study of fastidious gut bacteria, and also the application of ecology theory for analysing data, the interpretation of results from such ecosystems remains extremely difficult. The present investigation concentrated on a component of the intestinal ecosystem: the mucosa and its mucus layers, as the apparent lack of microbial diversity reported to occur in these habitats implied that it was a simpler, and thus more easily studied system.

The investigation has sought to integrate information from a number of sources including: *in vivo* observations of conventional animals, *in vivo* experiments with both conventional animals and those with restricted intestinal microbiotas, and also *in vitro* experiments with mucosal isolates. The *in vivo* studies were of tissue sections as these specimens showed the various microbial

populations *in situ*; moreover existing culture techniques and also those developed during the study were inadequate for accurately identifying or monitoring mucosal populations. Three sites were chosen for *in situ* studies; one each in the ileum, caecum, and colon. The areas were selected as being representative of each region of the gut on the basis of a survey of the mucosa-associated microbiota obtained from examining tissue scrapings (Leach, W, M.Sc. Thesis, 1977); however the results obtained are not intended to be complete descriptions of the mucosa-associated microbiota throughout the tract.

An important insight into the understanding of the nature of the gut ecosystem was made by Dubos *et al*. (1965) when these authors indicated that various regions of the tract had characteristic, and different microbiotas. This concept was extended when Savage *et al*. (1968) showed that some populations of microbes preferentially colonized the tissue surface. Subsequently it has been recognized that a great variety of different microhabitats exist throughout the tract. Each has its own characteristic set of physical and chemical parameters; moreover it could be expected that each should also possess populations of microbes adapted to survive and multiply in these conditions, i.e. to fill the niches associated with the microhabitats. The physical and chemical parameters in some microhabitats, such as those in the lumen of the stomach, are too extreme for a normal microbiota to establish; others, such as those in the lumen of the large bowel,are able to support a great diversity of microorganisms (Savage, 1977). In the

present study the low species diversity seen in the microhabitats intimately associated with the host tissue surface, such as in the crypts or on the microvillus border of the intestine, suggests that there are environmental extremes at these sites that select for only the most adapted species (Alexander, 1971). From the observations made of the normal and perturbed mucosal ecosystem in situ, and also from the results of experiments with the TSB isolates it is possible to speculate on the nature of the crypt environment, and thus the reason for the lack of species diversity.

The mucosal habitats are physically small when compared to the intestinal lumen, as they consist only of the regions between the tissue microvilli and the outer boundaries of the mucus layers. Microorganisms colonizing these sites are likely to be influenced by factors originating from both the tissue and the lumen of the tract, and also by the nature of the mucus gel. The relative importance of these components probably depends on the locality of the microhabitat: crypt bacteria are more likely to be affected by tissue related factors than those factors originating from the intestinal lumen, whereas the reverse could be expected for the fusiform-shaped bacteria colonizing the mucus layers interfacing with the large bowel lumen. The characteristics of the mucus layers varies in different regions of the tract: in the crypt the mucus is a physically dynamic substrate, while the mucus layers in the surface of the large bowel in contact with the lumen are relatively static. The composition of the

mucus also varies with different proportions of sulphated, non-sulphated acid, and neutral mucosubstances being found throughout the gut (Sheahan and Jervis, 1976; Sakata and Engelhardt, 1981).

Physical conditions are very important in determining the composition of the microbiota in different regions of the gastrointestinal tract; for example the rapid flow of material through the stomach and small bowel, and the long periods when these sites are free of digesta makes them adverse environments for most microbes (Luckey, 1974). As noted previously the lactobacilli and yeast in the stomach and the filamentous procaryotes in the ileum overcome these difficult conditions by attaching to the mucosal surface (Lee, 1980). The present study has shown that two crypt populations, the crescent-shaped bacteria in the ileum, and the thin, spiral-shaped bacteria in the colon also attach to the tissue surface; this indicates that these organisms are also overcoming the movement of fluids. Moreover the presence of unattached populations at the same sites suggests that these organisms have other mechanisms for countering the physical extremes of these environments. From observations of the interactions between mucosa-associated microorganisms and the mucus components of intestinal scrapings and also from the orientation of these organisms in tissue sections it was suggested that the unattached crypt bacteria needed to be highly motile in mucus to remain within the crypts. The results of other experiments were consistent with this hypothesis. When animals were given $MgSO_4$ diarrhoea, the

unattached lumen bacteria were removed by the increased fluid flow, while the attached organisms remained. The ability of the TSB isolates to move optimally in solutions with elevated viscosities correlated with an apparently preferential motility in mucus exhibited by the crypt microorganisms. Such an ability is consistent with the need to move against the outflow of viscous mucus secretions.

A characteristic of many mucosa-associated micro-organisms is rapid motility and this ability is likely to be important if these organisms are to remain near the tissue surface (Stanton and Savage, 1983). Nevertheless to be of optimum value the organisms would need to have the motility directed towards the tissue. A probable means of orientation is chemotaxis: for the organisms to detect a gradient of an attractant chemical originating from the tissue surface (Freter et al., 1981). Chemotactic responses have been demonstrated with both intestinal pathogens such as Vibrio cholerae (Freter et al., 1981) and with autochthonous intestinal bacteria (Stanton and Savage, 1983).

The concentration gradient of mucin from the tissue towards the lumen could provide a means, other than chemotactic, of attracting microorganisms to the mucosa. Petrino and Doetsch (1978) demonstrated that leptospires preferentially moved towards an increasing viscosity gradient and thus exhibited "viscotaxis". As the TSB had an elevated optimal viscosity for motility it is possible that the higher viscosities associated with the mucus on

the surface and streaming from the crypts could form a viscous gradient. Viscotaxis could explain the ability of organisms such as the TSB to colonize a high proportion of intestinal crypts when these organisms were not present elsewhere in the tract. Unfortunately accurate measurements or estimates of crypt viscosities have yet to be obtained. Forstner et al. (1973) found that the reduced viscosity (η sp/c) of mucin glycoprotein isolated from rat small bowel was approximately 8 dl/g in water at concentrations above 0.1 g/dl (0.1%). Given mucin concentrations of 1-10% in intestinal mucus,this (Allen, 1981) suggests viscosities of 8-80 cp; however it is probable that native mucins are more viscous. The ability of the TSB to move relatively rapidly even in high viscosities of methyl cellulose (the RTSB isolates retained 50% of maximum velocity at a viscosity of 3000 cp), could therefore be expected to be of advantage in the crypt environment.

Another important characteristic of the gastrointestinal ecosystem is its anaerobiosis: the low redox potential, especially in the large bowel has resulted in the autochthonous microorganisms adapting to the absence of oxygen. As anaerobic growth has been universal amongst all the indigenous populations previously cultured from the gut it has been used as one of the criteria for defining autochthonous populations (Savage, 1977).

Experiments with TSB isolates suggest that this definition may be inaccurate. While the present studies did not prove that the TSB were autochthonous the presence of these organisms in all of the University of NSW conven-

tional rodents sampled and the occurrence of similar organisms in other colonies (Davis et al., 1973; Erlandsen and Chase, 1972) indicates they could belong to this component of the microbiota. Even if the TSB, like other mucosa-associated organisms such as the yeast Torulopsis pintolopesii is not truly autochthonous (Savage, 1972) the ability of these obligate microaerophiles to colonize the mucosa shows that oxygen can be obtained by microorganisms in these habitats. Savage (1980) has suggested that intestinal pathogens such as Vibrio cholerae could grow aerobically in the bowel by stimulating the excretion of oxygen-rich fluid; the TSB and other indigenous microbes may be able to utilize the oxygen contained in normal secretions. If oxygen is obtainable by microorganisms in the gut there is no reason why organisms adapted to utilize this resource, and living in habitats near the mucosa, but unable to proliferate in the highly reduced intestinal lumen, can not be regarded as being autochthonous.

An interesting aspect of crypt colonization was the ability of both obligatory microaerophilic and obligatory anaerobic bacteria to colonize the same intestinal sites immediately adjacent to oxygenated eucaryotic cells. For example large numbers of anaerobic spiral-shaped bacteria were found in the caecal crypts of conventional rodents, while equally large populations of TSB colonized the same sites in both gnotobiotic and SPF animals. It is unlikely that colonization by the different types of bacteria represents overall differences in intestinal redox potential: while the redox in gnotobiotes would be high,

that occurring with the diversified SPF microbiota could be expected to be equivalent to that of conventional animals (Celesk et al., 1976). Furthermore the frequent occurrence of both types of bacteria in the crypts of conventional animals indicates other factors are involved.

Although the oxygen content of intestinal crypts is unknown, some insights into its interaction with the crypt bacteria can be made from observation of an analogous environment: the peridontal pockets in the oral cavity. By using a very small oxygen tension (pO_2) electrode, Loesche et al. (1983) were able to measure the pO_2 of peridontal pockets and correlated these measurements with assessments of the microbiota present at the same sites. The pO_2 of pockets was found to be considerably lower than that occurring in blood: 13.3 mm Hg compared to 155 m Hg for arterial blood and 20-40 mm Hg for venous blood. However the pO_2 was higher in the pockets than at the openings to the oral cavity, and it was suggested that oxygen was defusing into the sites from the surrounding non-secreting epithelial cells. Various amounts of oxygen were detected in pockets and it was found that those with low oxygen content had a predominantly anaerobic microbiota, while those with higher oxygen pO_2 contained more microaerophiles and oxygen tolerant anaerobes such as Bacteroides intermedius. Nevertheless all three types of organisms were found in pockets with both high and low oxygen concentrations. Bleeding increased the proportion of microaerophiles and oxygen-tolerant anaerobes and was correlated to pockets with higher than average oxygen contents.

An interesting observation was that strictly anaerobic organisms appeared to be much less oxygen sensitive *in vivo* than *in vitro*. For example organisms identified as *Treponema denticola*, were unable to grow at 4 mm Hg (0.5%) O_2 *in vitro* but were found in pockets with oxygen levels of 27 mm Hg (3.6%) O_2. Loesche *et al*. (1983) suggested that the difference was because of removal of toxic oxygen compounds (hydrogen peroxide and superoxide radicals) from the environment by the action of eucaryotic cell catalases, peroxidases and superoxide dimutases.

The cohabitation of crypts by anaerobes and microaerophiles is analogous to the colonization of peridontal pockets and accordingly the explanations offered by Loesche *et al*. (1983) have relevance to intestinal microbiology. That is, that the ability of both types of bacteria to co-exist near oxygenated eucaryotic cells may be due to the anaerobes having an inherent tolerance to oxygen or due to host enzymes removing toxic oxygen compounds from the crypts, or a combination of both.

Another factor likely to be of great relevance in the ability of the TSB to colonize the intestinal crypts is their nutritional characteristics. The crypt habitat of the TSB is surrounded by host cells and bathed with host secretions. Consequently it is likely that the crypt populations rely on host products for nutrition; the requirement by the TSB isolates of serum or blood in media may be indicative of these needs. Mucins seemed to be a likely source of energy, carbon and nitrogen for crypt bacteria, however experiments with the TSB isolates showed

that these glycoproteins were not degraded. It was also demonstrated that these organisms were unable to ferment carbohydrates so that the most likely energy and carbon sources for the TSB appeared to be host proteins or B-oxidation of fatty acids. Obviously if the relationship between the host and the crypt bacteria is to be understood the nutritional requirements of the TSB will need to be more fully studied.

The study demonstrated two interesting aspects of the relationship between the mucosal microbiota and the host. Firstly it was shown in experiments with the TSB that mucosa-associated microorganisms were able, in some experimental situations, to invade the nearby mucosal tissue. An interesting feature of this phenomenon was that the TSB, although occurring frequently within epithelial cells, were not seen in the mesenteric lymph nodes, liver or spleen. This implies that the organisms were eliminated at the site of invasion: the mucosal cells, and probably by macrophages. Although TSB were not seen within such cells, macrophages containing other spiral-shaped crypt bacteria were observed in animals given cascara.

Another aspect of tissue associations was the ability of "S"-shaped bacteria to colonize the colonic mucosa after perturbation. These apparently allochthonous organisms were able to remain on the tissue surface for long periods and unlike organisms such as V.cholerae (McNabb, 1981), did not appear to initiate an immune response sufficient to prevent them from attaching to the mucosal surface.

Previously it has been demonstrated that some members of the lumen microbiota avoid stimulating the host immune system by sharing antigens with the mucosal tissue, and thus being recognized as "self" (Foo and Lee, 1972, 1974; Berg and Savage, 1972). Preliminary experiments with mucosa-associated fusiform-shaped bacteria indicate that some members of the intestinal microbiota are able to stimulate immune responses (A. Lee, personal communication). Clearly an interesting avenue of research will be detailed investigations of the immunogenicity of organisms such as the TSB and, if they can be cultured, attached populations such as the filamentous bacteria in the ileum, the thin, spiral-shaped bacteria from the colonic crypts and the "S" shaped bacteria seen on the colonic mucosa.

An important aspect of the gastrointestinal ecosystem is the interactions occurring between members of the microbiota; the study of these relationships will become increasingly important as more is learnt about the individual microorganisms. Little direct information on microbial interactions was obtained in the current investigation, nevertheless some inferences could be made from *in situ* observations. The ability of several different types of microorganisms to colonize the caecal crypts of rats suggests that competition is a likely relationship (Alexander, 1971). Evidence for competition was that: while the TSB, campylobacter-like isolates, and thin, spiral-shaped isolates were able to heavily colonize the caecal crypts of gnotobiotic or SPF rodents, few could be found in the crypts of conventional animals: these were

colonized by spirochaetes and spirilla-like bacteria. Competition requires there to be a limiting resource/ resources that is more efficiently utilized by the predominant populations (Alexander, 1971), the spirochaetes and spirilla-like bacteria; in vitro studies may be able to determine the identity of this/these component of the crypt ecosystem.

Another possible relationship between crypt bacteria is symbiosis. For example commensalism could occur if the microaerophilic TSB utilize oxygen in the caecal crypts and by doing so create more suitable conditions for the strictly anaerobic spirochaetes and spirilla-like bacteria. This process could be especially important in the succession of populations and establishment of the climax community in growing animals: oxygen utilizing bacteria are usually found to be precursors to the establishment of the anaerobic components of the lumen microbiota (Savage et al., 1968). Mutualism could occur if components of the crypt microbiota possessed enzymes able to degrade the host's mucin and thus make carbohydrates, amino acid or peptides available for microorganisms such as the TSB, unable to attack these glycoproteins.

Interactions between crypt bacteria and the microorganisms colonizing the lumen of the tract were indicated by the results of colonization experiments with germ-free, gnotobiotic and SPF rodents. Increasing the complexity of the lumen biota appeared to cause reductions in the numbers of TSB colonizing the caecal crypts. Alternatively in the absence of a conventional intestinal biota

the TSB were unable to colonize ileal crypts to the same level as in normal animals. Possible reasons for these results have been discussed previously, however the precise mechanisms remain unclear. Nevertheless the observations demonstrate that microorganisms do not need to be present in the same habitat to exert influence on other microbial populations; this process makes the interpretion of *in situ* studies of the gut particularly difficult.

An important component of this thesis was the investigation of the effects of intestinal perturbation on the composition of the mucosal microbiota. As was expected the changes in the host's physiology, caused by the purgatives, altered both the number of bacteria found in the intestinal crypts and also the number of mucosal bacteria to be found in faeces. By removing normal mucosal populations $MgSO_4$ treatment resulted in habitats and niches being vacated and accordingly a number of allochthonous populations were found to be present on the mucosal surface. While this phenomenon could be predicted by ecology theory the ability of the "S" shaped bacteria to remain on the surface of the colonic mucosa was unexpected and thus relevant to an understanding of gastrointestinal ecology. If perturbation can, as suggestd by the $MgSO_4$ experiments, lead to long term changes in the mucosal microbiota this implies that similar sequences of colonization could occur with other microorganisms including pathogens. There are a number of diseases where perturbation or differences in the intestinal ecosystem appear to be a prerequisite to infection, e.g. pseudo

membranous colitis (Bartlett et al., 1978), and necrotic enteritis (Egerton and Walker, 1978). In others, altered reactivity to antigens in the intestinal tract may be the result e.g. inflammatory bowel disease (Beeken, 1980). Unrelated physical disturbances in the intestine may be an as yet unsuspected, but important, predisposing factor in some of these diseases.

The major reason for the lack of information on the mucosa-associated microbiota has been an inability to isolate and cultivate the individual microorganisms (Lee, 1980). The successful isolation of mucosa-associated bacteria, in particular the TSB, reported in this thesis is therefore a very important step in the investigation of this ecosystem. Clearly by continuing and broadening the in vitro studies of these organisms' physiological, nutritional, and ultrastructural characteristics, the understanding of interactions with the host and other microorganisms will be enhanced. Similarly demonstration, for the first time, of crypt colonization in germ-free, gnotobiotic, and SPF animals by mucosal isolates is also important as it provides the opportunity for the in vivo study of the mucosa-associated microbiota and its interaction with its host. The information gained from these in vitro and in vivo experiments have value not only for studies of the normal microbiota but also for the information gained on the ecology of the vital interface between the host and its microorganisms: the gastro-intestinal mucosa. A detailed knowledge of microbial ecology is now recognised as being necessary if the

aetiology of intestinal infection diseases are to be fully understood (Cheng *et al.*, 1981). Moreover recent observations have indicated that interactions with the mucus layers near the mucosa, such as those occurring with the TSB, are important in colonization of the intestine by some pathogens. Lee *et al.* (1983) have demonstrated that one such organism *Campylobacter jejuni* is, like the TSB, able to colonize intestinal crypts in rodents and exhibits a preference for motility in viscous secretions. These authors postulated that the ability to remain in the mucus blanket, near the mucosal surface, could be an important component of the disease process. Interaction with mucus has also been reported to occur with a similar spiral-shaped bacterium that has been implicated in gastritis and duodenal ulcers (Marshall and Warren, 1983). These organisms have been found to be present in 55 of 70 patients with gastritis, 13 of 14 with duodenal ulcers but in only 2 of 31 normal biopsies. The organisms were found below the surface of the mucus blanket and the authors suggested that this feature of colonization enabled the organisms to exist in the hostile, low pH gastric environment. Penetration of the mucus layers can also be postulated to be important for organism such as *Vibria cholerae*, enterotoxigenic *Escherichia coli*, *Salmonellae* spp. and *Shigellae* spp. that require close proximity to the mucosa to exert pathogenic effects. For example, while attachment has been implicated in the pathogenesis of cholera, *in vivo* and *in vitro* studies with *V.cholerae* (G.N. Cooper, personal communication) indicate that entry of, and

multiplication in the mucus blanket may also be a necessary component of the disease aetiology. Studies of mucosa-associated microorganisms, such as the TSB isolates, that have adapted to the mucosal environment may therefore provide important insights into the factors enabling intestinal pathogens to colonize the mucosal surface.

1. Ultrastructural Studies of the TSB isolates

The interesting ultrastructure of the TSB revealed by sectioning and negative staining experiments in Chapter 4 was further investigated by undertaking freeze-fracture and freeze-drying studies.

2. Freeze-dried preparations of the RTSB isolates

Examinations of preparations of RTSB isolates freeze-dried as described in Chapter 2 confirmed the arrangement of the periplasmic tubules seen in negative stains: concentrically coiled around the length of the organism (Fig. 34). Some preparations showed organisms apparently dividing by binary fusion (Fig.35A). Occasionally the cell walls of organisms were etched to reveal the underlying periplasmic tubules. As in other preparations the tubules were striated with a band width of 8-9 mm (Fig.35B).

3. Freeze-fracture preparations of the RTSB isolates

Freeze-fracture preparations of RTSB isolates fixed for 15 min in 1% gluteraldehyde and suspended in 15% glycerol were prepared as described in Chapter 2. From examination of all the fractured preparations it was determined, using the guidelines of Sjostrand (1979), that the major fracture planes were intramembrane fractures of the cell wall and also of the cytoplasmic membrane. Convex fractures were the most easily interpreted, however all structures seen in these specimens were also seen in reciprocal concave fractures (Figs.36,37).

Fractures through the cell wall were characterized by the presence of concentric ridges, nevertheless these

Figure 34. Freeze dried preparation of RTSB.

A. x 14,300, bar = 1 um.

B. x 19,700, bar = 1 um.

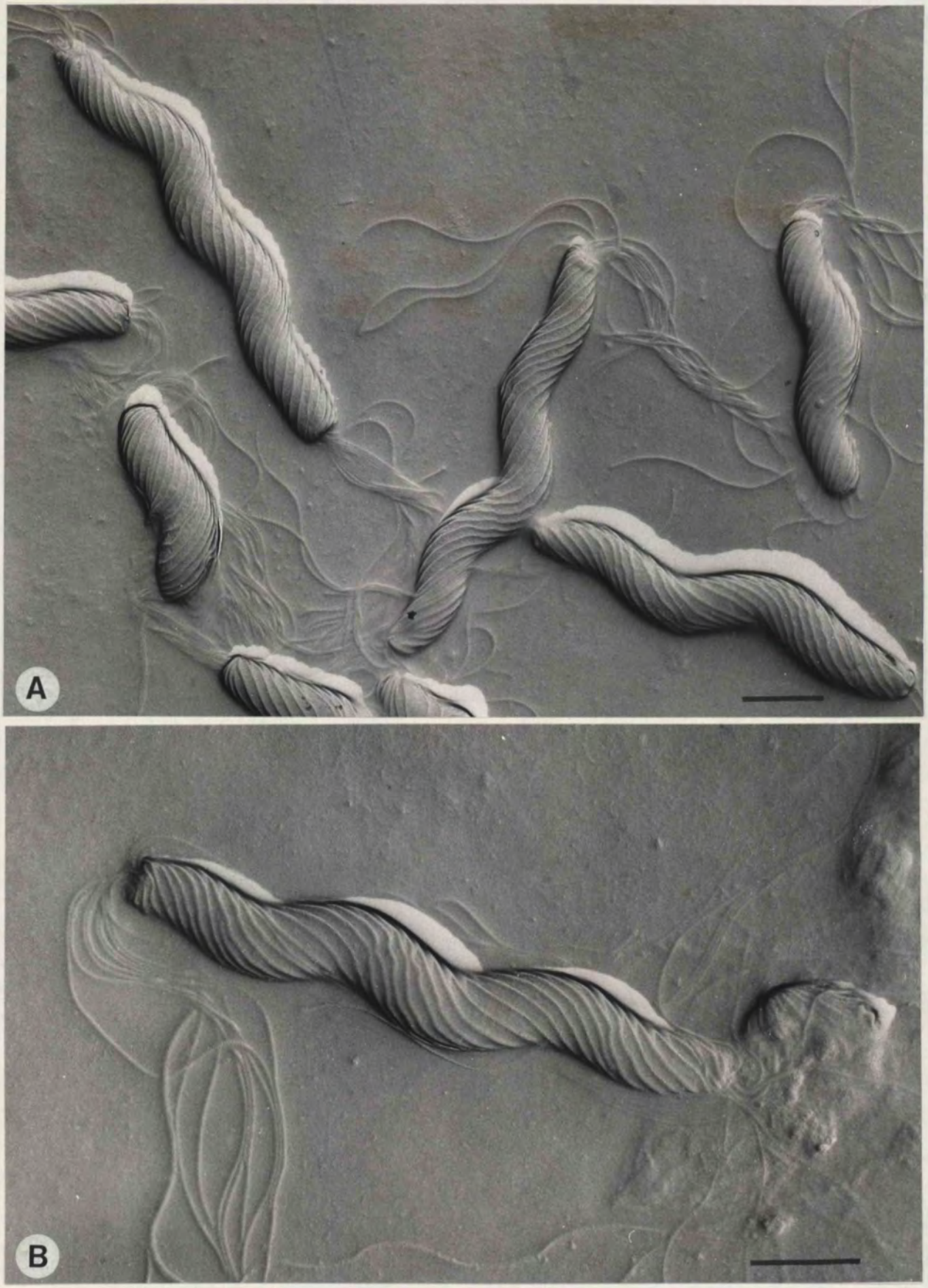

A
B

Figure 35. Freeze dried preparation of RTSB.

A. Division by binary fission x 19,700, bar = 1 um.

B. A spherical body etched to reveal striated periplasmic tubules (T) x 77,250, bar = 0.2 um.

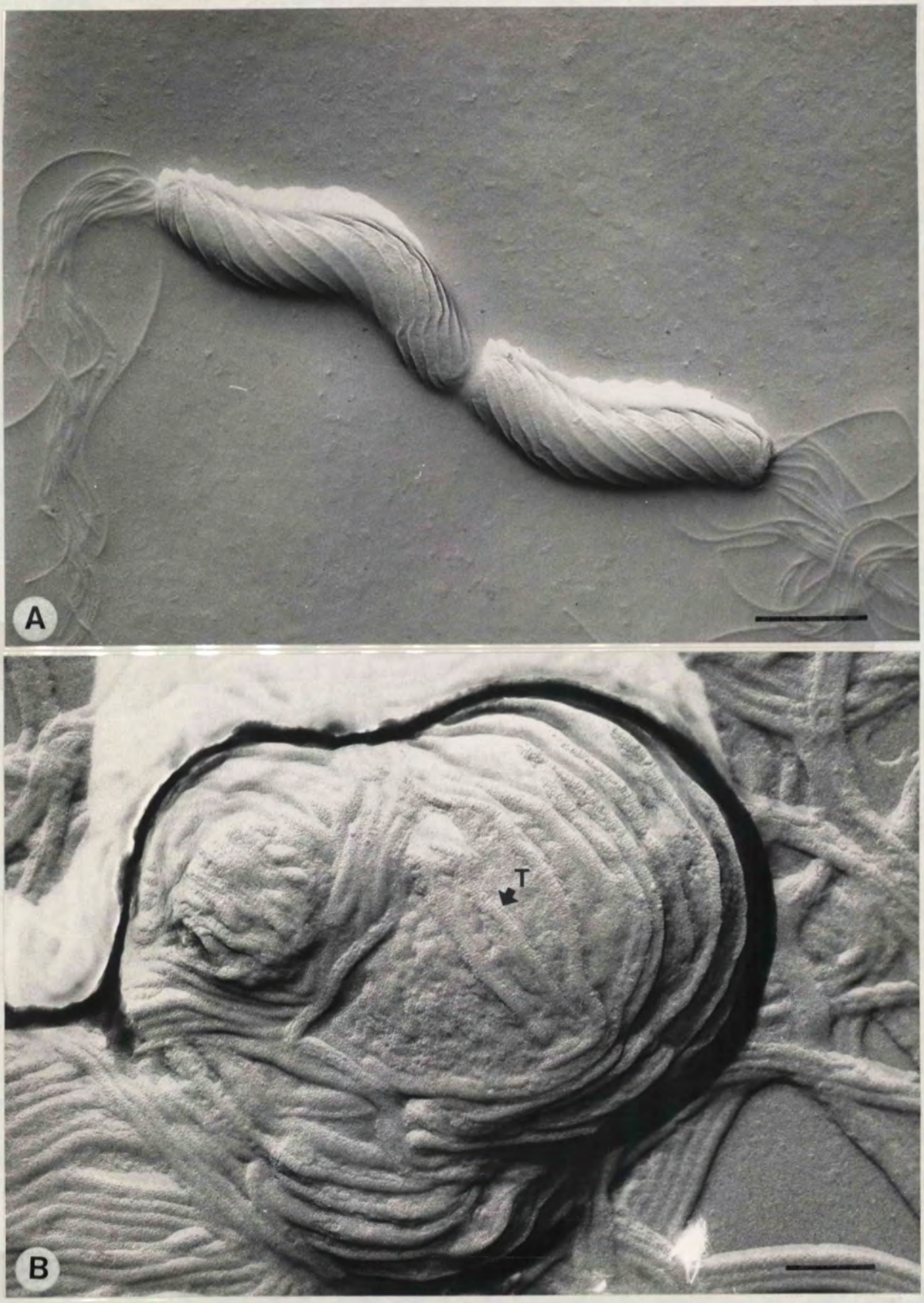

A
B
T

Figure 36. Freeze fracture preparations of RTSB. Large arrows indicate direction of shadow.

A. Convex (X) and concave (V) fracture faces of bacteria showing the ridged nature of the cell wall x 38,100, bar = 0.5 um.

B. Concave fractures, one of the polar region (p) x 50,500, bar = 0.5 um.

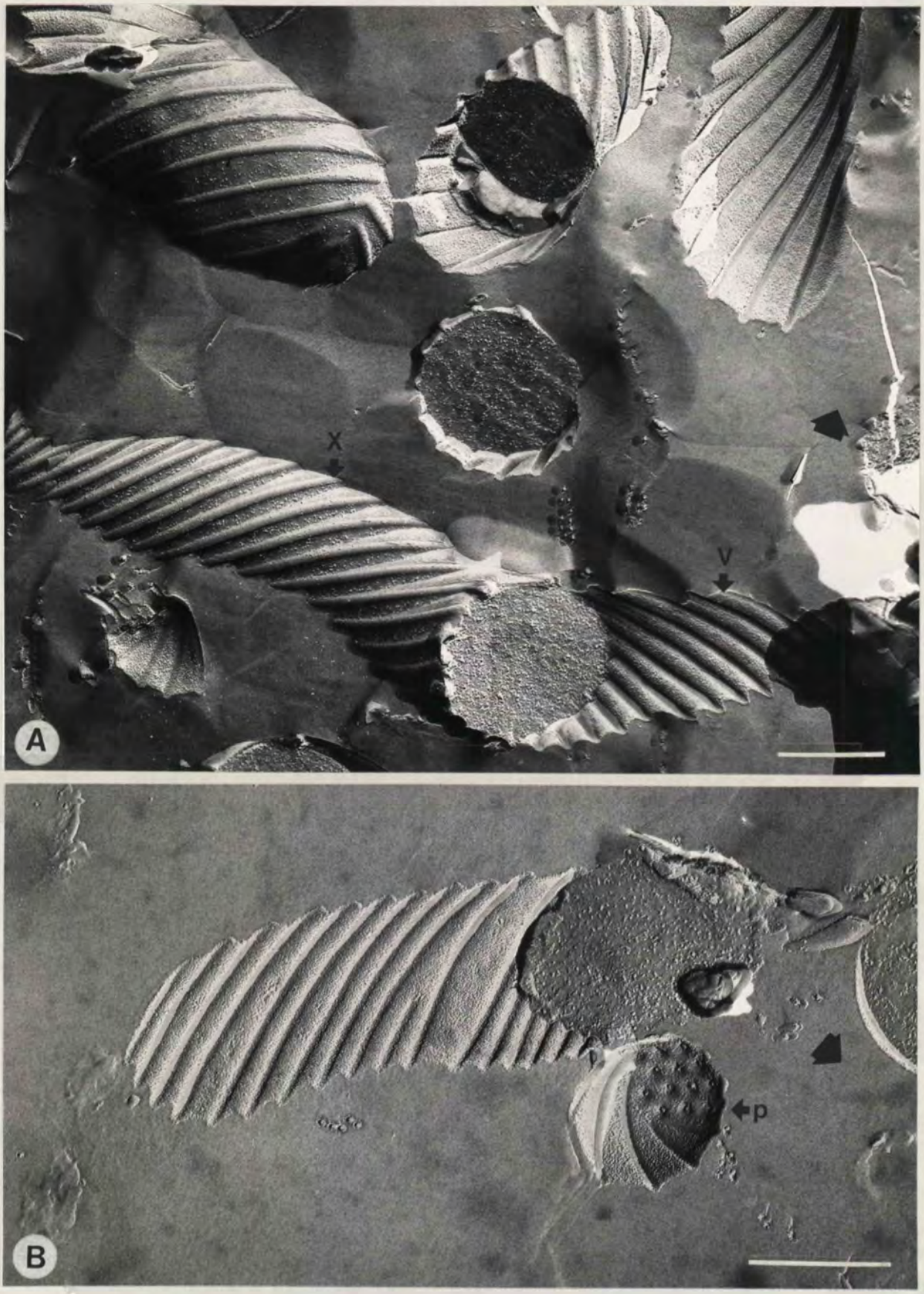

X
V
A
p
B

Figure 37. Freeze fracture preparation of RTSB. Large arrow indicates direction of shadow. The polar region can be seen in both convex (x) and concave (v) fractures. Tube-like projections (arrows) can be seen at both sites x 41,900, bar = 0.5 um.


X
V

preparations gave little information on the underlying periplasmic tubules as they were always covered with part of the cell wall, even in angled cleavages (Fig. 36). However, observation of many preparations did indicate that the arrangement of the periplasmic tubule was not constant: the spacing, distribution and termination of the ridges was variable (Figs.36,37).

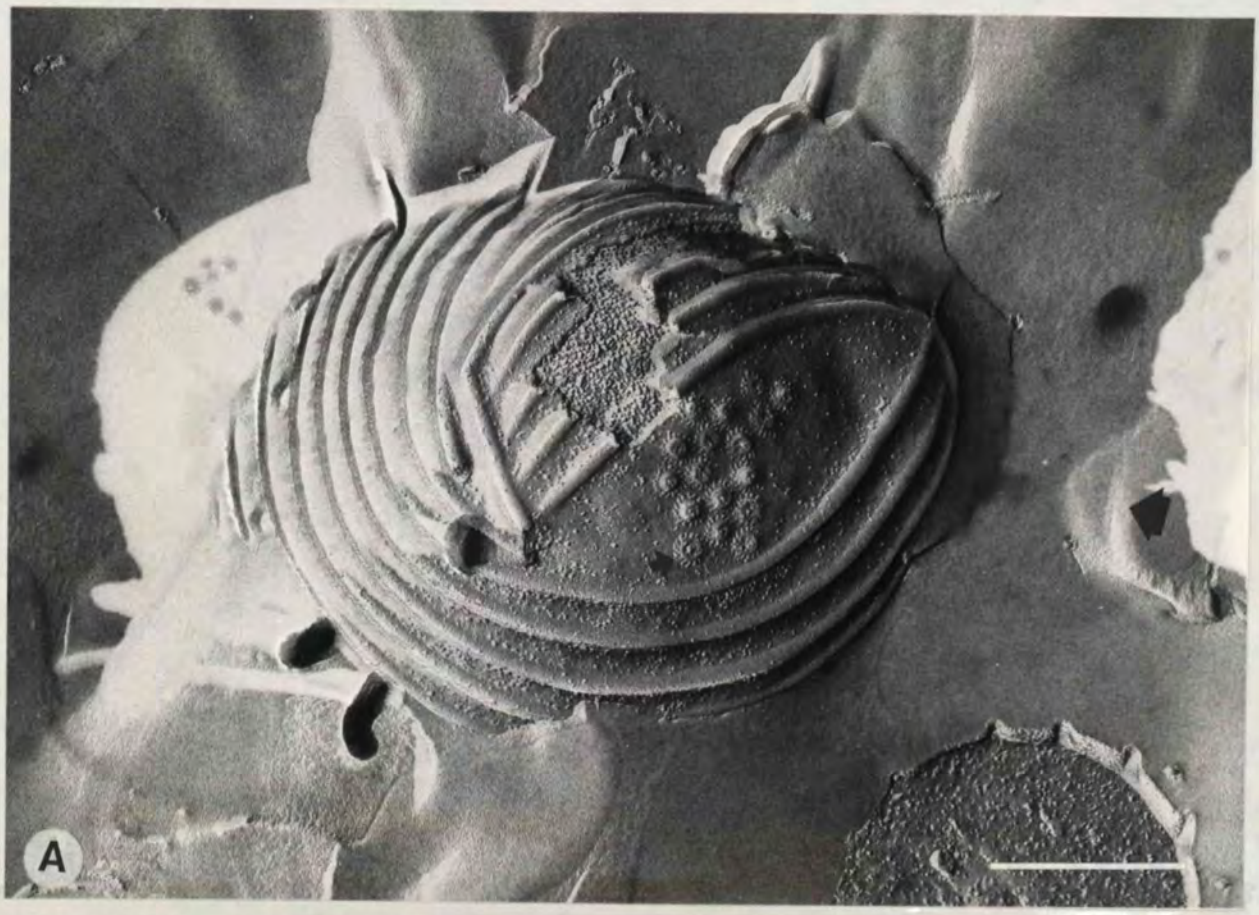
Fracture through the polar region of the cells revealed interesting components associated with insertion of the polar appendages. Characteristically this region was free of ridges and was the site of variable numbers (average of 13-15) of circular complexes (Figs.37,38). Where the appendages were cleaved longitudinally they entered the protoplasmic cylinder and appeared as tapering stalks; thick at the base and diminishing in width further from the cell (Fig.37).

These structures were assumed to be the polar flagella cleaved through the middle of the sheath membrane. At the base of these components were circular arrays of particles (Fig. 37). The distribution of these particles was seen even more clearly in cross-cleavages of the polar region (Fig.38). Appendages were cleaved near the base and appeared as a central circular component surrounded by 17-20 oval-shaped particles, each approximately 15x7 nm in size and in a ring with a diameter approximately 90 nm. The central body was of variable shape; some cross-cleavages gave this structure a dome-like appearance. In others, where the fracture was further from the

Figure 38. Freeze fracture preparations of the polar region of the RTSB. Large arrows indicate direction of shadow.

A. Circular projections, surrounded by small particles with a Catherine-wheel-like orientation (arrow) on the fracture face of a spherical body x 51,700, bar = 0.5 um.

B. The particles surrounding the circular projections have a less regular orientation in this fracture x 122,300, bar = 0.1 um.


A


B

protoplasmic cylinder, the results were more pointed structures (Fig.38). If stereo-pairs of such fractures are examined these structures project from the surface. Many of the conical bodies had a central spike or core.

The arrangement of the particles was radial around the central body; in many fractures the particles were offset by 30-50^{o} giving the complex a Catherinewheel-like appearance (Fig.38A). The complexes were well spaced on spherical bodies (Fig. 38A), but on organisms with a normal morphology they were closely associated, often with the particles of adjacent complexes in contact (Fig.38B).

4. Discussion

In Chapter 4 it was noted that the TSB had both similarities and differences with members of the family Spirillaceae; the freeze fracture studies gave similar findings. Preparations fractured at the terminal region were unlike those of spirilla as they were cleavages of the cell wall rather than, as in the spirilla, of the cytoplasmic membrane. Despite this, a common feature of fractures from both organisms were rings of intramembrane particles surrounding the core of the flagella. The intra-cell wall particles seen in TSB were, in comparison to the intra-cytoplasmic membrane particles of spirilla, larger (15x7 nm compared to 5x5 nm), and forming a larger ring array (85-90 nm diameter compared to 50-55 nm).

If the information on the nature of the terminal flagella gained by examining sections, negative stains, and freeze fractures is compared, a number of correlations

occur. The dark band underlying the cell wall in sections, is of the same diameter (70 nm) as the circular rings seen in the negative stains, while the central hole is the same width (15 nm), as the appendages in section. Similarly the core at the base of the array of particles seen in freeze fractures is of the same width as the base of the appendages in sections: i.e. where the membrane begins curving outwards. The ring of particles seen in the fracture preparations had a diameter similar to those of the circular ring structures, and would lay immediately above these structures in the cell wall.

Coulton and Murray (1978) suggested that the most likely role for the CMR in spirilla was to stabilize and reinforce the cell wall membrane against the force of the spinning flagella. Such a role could also be applicable to the TSB, especially as the cell wall of these organisms does not enclose the basal complex as it does in unsheathed bacteria such as S.serpens. Similarly the bearing-like function attributed to the interaction between the L and P discs on the cell wall membrane, could not apply with sheathed flagella. It is possible that the circular ring structures in TSB could act to form a stable seat for the flagella, the same as that formed by the cell wall membrane in other gram-negative organisms, or the peptidoglycan in gram-positive bacteria.

The positioning of the circular ring structures suggests that only the outer ring could be in direct contact with the cell wall membrane. Interestingly if the

intramembrane particles seen in freeze fractures are positioned in the membrane they reside immediately adjacent to the outer rim of the circular ring structures. From this observation a possible function for the particles can be made: as a means by which the circular ring structures are connected to the cell wall membrane. The Catherine-wheel appearance of some arrangements of particles could be due to torque-induced twisting of the circular ring structures.

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