Aspects of Microbiology 10

Intestinal Microbiology

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Aspects of Microbiology

The American Society for Microbiology is now publishing a series of short books. We want to specify the rationale and nature of the series.

When students enroll in college science courses, they are customarily faced with large textbooks that build on secondary sources. Much of the text repeats simple material that has already been covered in high school courses, and the work in active fields is so quickly dated that the treatment in an 'inclusive' book is at best uneven. It is most discouraging that the evidence for critical inferences, the definition of the present limits of knowledge, and the excitement of scientific research are usually denied to all but the few students who go on to graduate work. Undergraduates often can only wonder what their professors are so excited about.

The disparity between science at the 'frontier' and the compilations in textbooks has led some to use collections of 'seminal papers' as a teaching aid. However, these lack continuity and clear expository prose.

The alternative that we are sponsoring is to select a number of the liveliest topics and ask active researchers who also write well to provide short books like this one. In addition to references to recent studies, each book provides a précis of the state of the field, providing the background necessary to bring students to the heart of the science. We expect these books to supplement a course, to provide additional material for undergraduate and graduate students, or to provide the complete or partial basis for all courses on microbiology, molecular biology, microbial ecology, applied microbiology, medical microbiology, etc.

With experience in providing books and scientific literature to an international audience of students and scientists, the ASM has agreed to copublish the United States editions with Van Nostrand Reinhold (UK).

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Preface

Information on and understanding of the microbiology of the alimentary tract has increased rapidly in the last 20 years and shows little sign of diminishing. There are medical and economic reasons for this.

Infectious intestinal disease is frequently manifest as diarrhoea, sometimes producing severe dehydration. Thus in developing countries, enteric viruses, enteropathogenic *Escherichia coli* and *Vibrio cholerae* are still major causes of death in adults and young alike. Even in developed countries, where standards of hygiene are relatively high, salmonellosis and *Shigella* dysentery are still prevalent. A variety of bacterial and bacterial pathogens cause diarrhoea, with differing pathogenic mechanisms, only some of which are known and understood. Thus the epidemiology, pathogenesis and prevention of intestinal diseases still present extensive fields for research and discovery.

Intestinal diseases can become epidemic if large numbers of susceptible individuals are in close contact with each other. The intensive systems of animal rearing in developed countries are therefore ideal vectors for the epidemic spread of diseases frequently occurring in young animals. Thus large financial losses can occur in agriculture.

Many components of the normal flora, however, are essentially beneficial to man and other animals. An increasing realization that the normal intestinal flora have nutritional interactions with the host has suggested the possibility of their exploitation to improve efficiency of nutrient utilization in animals. Much remains to be discovered about the metabolic contributions of the flora to the host. Animals such as ruminants obviously depend extensively on microbial activity for fulfilling their nutritional requirements. The role played by the flora in carnivores and omnivores such as man is presently largely unknown. Vitamin production by several bacteria occurs readily *in vitro* and can be demonstrated in the alimentary tract of laboratory animals. Other metabolic activities of the gut flora have been highlighted more recently because of the inadvertent detrimental effect of releasing toxic chemicals from harmless precursors which may be present in food. The reverse process of detoxification occurs but has obviously been less intensively examined.

Findings such as these have led to intensive studies into the aetiology of cancer of the large bowel and have contributed to the current vogue for diets of low fat and high fibre. The initial observations of correlations between the incidence of bowel cancer in developed and developing countries and the amount of lipid in the diet has been supported by extensive experimental evidence, suggesting a role for certain clostridia in modification of bile acids produced in response to lipid, to produce carcinogens. The importance of fibre in the diet is less well supported by experimental or other evidence.

It is not surprising that while the employment of increasingly sophisticated investigative techniques has gradually uncovered the highly complex ecology of the intestine, it has simultaneously revealed new problems and novel concepts. As a corollary a firm understanding of this complexity is necessary in attempting to study the activities of the gut flora.

This book highlights some of the areas where greatest progress has been made in understanding the microbiology of the gut and how the establishment and use of current techniques and concepts have assisted in reaching this current understanding. We hope it will provide an introduction to the subjects for microbiologists, gastroenterologists and all students of biology and medicine.

We have been aided in this enterprise by the forbearance of our families and the support of our colleagues. We would particularly like to thank Miss Frances Bird for help with the diagrams and references.

1985

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1 Introduction and methods for investigation of the intestinal flora of man and animals

Structure and function of the intestine

The intestine is a tube extending from the lips to the anus (Figures 1 and 2). It is divided into various well defined anatomical regions whose structure and function reflect the diet and life style of particular animal species (Figure 1).

The digestive and absorptive functions are well known but, in addition to being an organ of the body, the intestine acts as a container for the most intimate portion of the chemical environment. Assimilation of food is not the only physiological function of the alimentary tract. It is also concerned with the excretion of chemical waste, the control of body metabolism and immune responses. Furthermore, the gut harbours a complex ecosystem.

The structure and indeed the particular activities of the gut differ in different sorts of mammal, but it is the similarities and the principles underlying the form and functions that are the subject of the present discussion. These represent strategies to ensure maximal utilization of food substances.

The mouth The structures and functions of the mouth have been described in another volume in this series (Marsh & Martin, 1984). In the context of intestinal function the mouth determines the physical state and degree of homogenization of the food. This affects to a considerable degree the surface area available for the action of the intestinal enzymes. Thus, the process of homogenization prepares material for digestion. In ruminant animals this process is particularly well developed and involves not only the physical disruption of plant materials but also, by use of salivary secretions, the optimization of conditions for colonization of cellulose fibrils by rumen microorganisms. Disruption of faecal pellets is probably important for those animals that practise coprophagy (consumption of faeces) as this renders available for digestion the products of bacterial attack on resistant materials. Saliva contains digestive enzymes but the extent of their overall contribution to the digestive process is uncertain. Absorption of small molecules can occur in the mouth as a consequence of partition between the aqueous oral contents and the lipid membranes of cells. This phenomenon may be crucial in the absorption of drugs and other nutrients. Local immunity is also in evidence, immunoglobulin (Ig)A in saliva being thought to control colonization of teeth with cariogenic bacteria.

The stomach Food leaving the mouth, homogenized and mixed with saliva, passes down the oesophagus and into the stomach. Here the process of digestion and absorption continues. In ruminants food may return from the stomach to the mouth for further mastication. However, this process, called rumination, is as its name implies unique to this group of animals for whom the stomach is the major digestive organ. The rumen and its associated structures may be thought of as a pre-processing plant in which bacteria degrade resistant materials, particularly cellulose, and render them available for intestinal digestion.

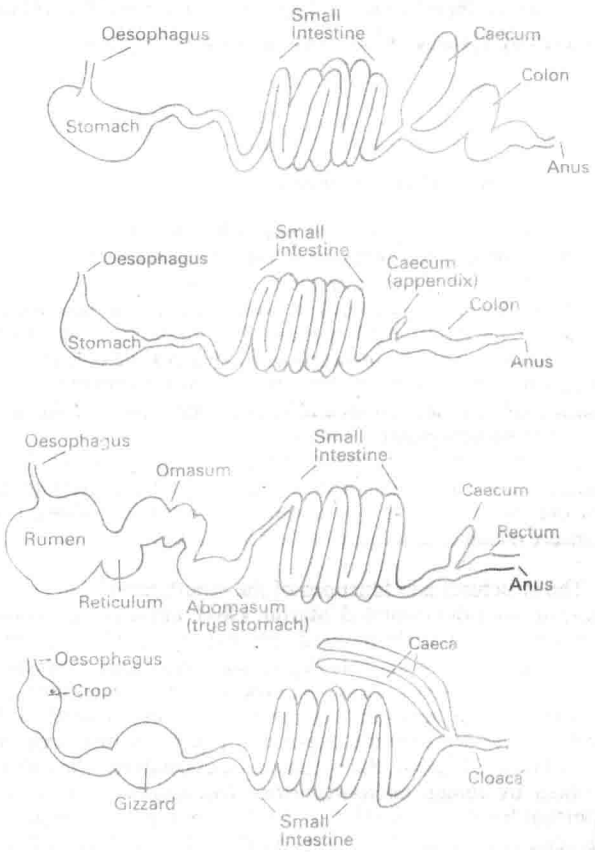

Fig. 1 Intestinal structure of different dietary types of animal. (a) monogastic herbivore, e.g. horse, rabbit, rat, pig. (b) Carnivore or omnivore, e.g. cat, dog, man. (c) Ruminant, e.g. cow, sheep. (d) Granivorous bird, e.g. chicken, turkey.

In most animals the stomach is not so central to the digestive process and functions as a reservoir regulating the entry of food into the small intestine. The stomach empties fastest when full, although the rate of emptying is modified by the nature of the food with which it is filled. Thus, lipids cause delay in gastric emptying. While in the stomach food undergoes preparation for entry into the small intestine. Important aspects of this treatment include further homogenization by the movements of the stomach, adjustment of osmotic pressure by various secretions and reduction of the bacterial load by the action of gastric hydrochloric acid. The pH of the stomach may be as low as 2.

The digestion and absorption started in the mouth continue in the stomach. Gastric enzymes include acid endopeptidases, gelatinase and a lipase. Intrinsic factors produced in the stomach potentiate the absorption of vitamin B_{12}. Activity

of gastric enzymes is related to the low pH of the stomach and the endopeptidases are inactivated when they enter the duodenum. The relative importance of gastric digestion is difficult to assess since disturbances in emptying and osmotic regulation produced by gastric surgery are usually compensated by the activity of the small intestine.

Absorption from the stomach again relates to the acid conditions. Thus, molecules whose ionization is suppressed by the low pH are absorbed. This is of importance for the absorption of some drugs, e.g. salicylate and ethanol. Nevertheless, in the stomach considerably more secretion than absorption occurs and food entering the small intestine has an increased water content and is osmotically better balanced.

The small intestine Most digestion and absorption occurs in the small intestine and its structures and activities tend to maximize efficiency in these respects. The volume of material entering the intestine is very large. Perhaps surprisingly, ingested material, i.e. food and drink, contributes only a minor component. Other contributions include the secretions of the gastric mucosa, the intestinal mucosa, the pancreas, and the gallbladder. It has been estimated for man that some 7 litres of liquid enter the duodenum each day, perhaps only 2 litres being contributed by the diet. This swamping of dietary substances ensures a constant intestinal environment and thus enables the digestive enzymes to operate under controlled conditions.

Carbohydrate, protein and fat are all digested and absorbed from the small intestine (Figure 2). Pancreatic juice is a major source of enzymes and includes the buffering capacity needed to neutralize gastric acid and optimize the conditions of enzyme action. Pancreatic enzymes include trypsin, chymotrypsin carboxipeptidases, an amylase, various lipases, ribonuclease, deoxyribonuclease, collaganase and elastase. The products of the action of these enzymes are then available for further digestion and absorption. The digestion of fats involves not only the pancreatic enzymes but also the bile acids secreted by the liver. These solubilize the fats and products of fat digestion and facilitate their absorption. Disaccharidases occur at the mucosal surface and these complete the hydrolysis of carbohydrates.

Specific mechanisms exist to potentiate the absorption of some amino acids and some monosaccharides, e.g. glucose. Bile acids are recirculated and vitamin B_{12} is absorbed by specific transport systems. The role of bile acids in the absorption of fat has already been mentioned. Thus, particular mechanisms exist for various essential substances but not all materials are absorbed like these. Partition between the mucosa and intestinal contents and passive diffusion make an important contribution. The structure of the small intestine maximizes the area available for absorption to occur. The surface of the mucosa is convoluted and folded. From these folds and convolutions spring the finger-like villi, the surface of which are covered with the absorptive cells. The surface of these cells, which face into the intestinal lumen, are themselves covered with microscopic protuberances, the microvilli. The small intestine is the main organ of digestion and absorption.

The large intestine The residue and debris left after digestion and absorption in the small intestine pass into the large intestine to await elimination from the body. This part of the intestine contains the richest and most complex part of the intestinal flora. Some authorities have regarded it as a septic swamp. Some animals use this hectic activity in their caecum to digest cellulose. Products of

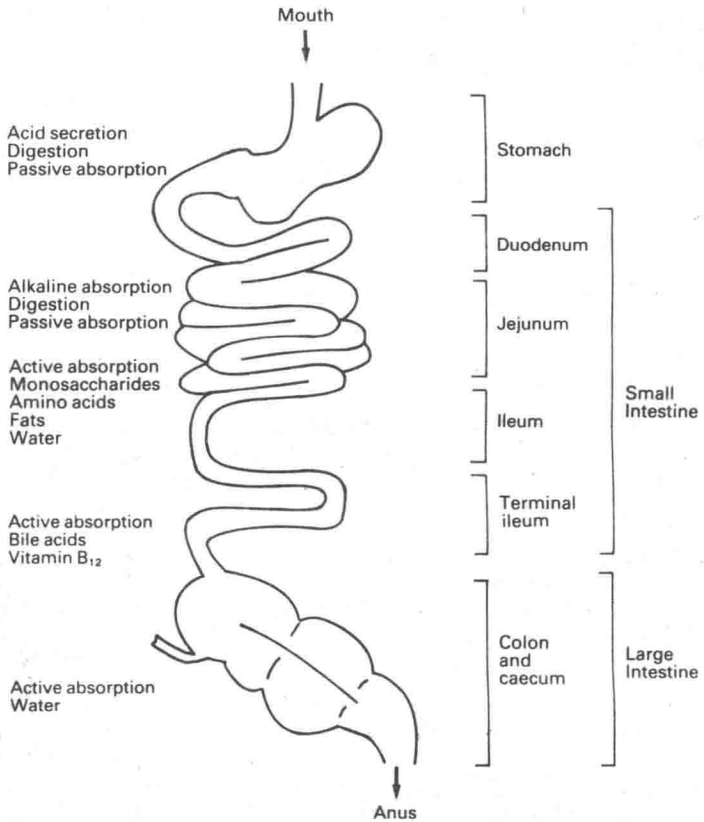

Fig. 2 Outline of intestinal structure and function.

cellulose digestion may be absorbed directly or faeces may be eaten and the food passed again through the intestine. In this way many useful products of bacterial action may be conserved.

Water is absorbed from the intestinal contents as faecalization occurs and this is the most obvious absorptive activity of the large gut, but it should be realized that, even in the absence of a specific mechanism, absorption can occur by simply diffusion. Bacterial products and other materials are absorbed from the large gut but their importance in many animals is unclear.

The intestinal flora

The intestine of man and other animals harbours a complex mixed culture of bacteria. This intestinal flora develops as a result of the influence of intestinal physiology on the interactions between those bacteria from the environment that

contaminate the body. The initial inoculum is usually derived from the mother at the time of birth. The climax flora is different in different animal species and alters as the host ages. Many factors, including diet, have been suggested as influencing the flora, but direct evidence is difficult to obtain. Further, although the intestinal flora is usefully considered as a whole, the structural and functional identity of the different regions of the intestine result in distinct ecologies.

In the study of both human and animal medicine it is important to realize the limitations of the techniques used since the quality of the results obtained reflect the methods used to obtain them. Many of the methodological and conceptual problems involved in the study of the human gut flora have been discussed by Drasar & Hill (1974) and these also apply to methods used for studying the alimentary tract of animals (Clarke, 1977). Although some of the problems peculiar to animal work have been mentioned by the latter author the advantages of using animals and of comparative studies in general have not been dealt with.

Conceptual approaches Those bacteria that grow in the intestine are regarded as the indigenous flora and this can be regarded as having two major components, the normal flora and the autochthonous flora.

The composition of the normal flora is variable and differs from population to population.

The intestinal flora may be regarded as either the most intimate portion of the environment or as an organ of the body. Consideration of the flora as an organ, at least in a metabolic sense, is again highlighted by studies of cellulose digestion in the rumen; however, studies of ammonia metabolism, drugs and food additives by the bacteria in the intestine emphasize the practical importance of such metabolic considerations. It should be noted that if the flora is considered as an organ it is potentially the most metabolically adaptable and rapidly renewable portion of the body.

Though consideration of the evolutionary development of the gut flora and its metabolic activity provide important insights, one must remember that the bacteria growing in the intestines are an ecological unit. Study of the intestinal ecosystems may be performed at several levels. It has been stated that the ultimate aim of microbial ecology is to explain the ecosystem in terms of the biochemical properties of purified enzymes, explaining in this way the microbial interactions and the modifications to the environment. Five categories of experimental and conceptual complexity can usefully be distinguished:

(i) The study of microorganisms in the natural environment. Studies at this level are usually limited to enumeration of bacteria and measurement of the results of complex metabolic processes. One outcome of such studies may be the alteration or distinction of parts of the ecosystem.

(ii) The study of isolated strains of bacteria in the laboratory. Many such studies are directed towards examination of the ability of isolated strains to perform metabolic transformations known to occur in the intestine. Analysis of the chemical composition of isolated strains may be used as background data during attempts to estimate microbial biomass by chemical analysis of intestinal samples.

(iii) The study of physiological aspects of the environment. The intestinal ecosystem is complicated by the impacts of the physiology of the host. Thus, though natural homeostasis of the intestine acts to stabilize the

environment, changes in host physiology, diet or immune status can cause short- or long-term environmental variations. Animal experiments may be regarded as attempts to elucidate the human intestinal environment; for example, physiological mechanisms only postulated to occur in man may be demonstrated in animals.

(iv) The study of cell suspensions enables the conditions under which ecologically significant reactions and transformations occur to be defined more closely. Suspensions may be prepared from samples of intestinal contents or isolated bacterial strains; faecal homogenates have been commonly investigated.

(v) The study of purified enzymes. In theory at least an understanding of bacterial metabolism should provide a biochemical explanation for interactions between bacterial species and with their host. The genetic potential of particular bacterial strains may also be of crucial importance. It should be remarked that the bacteria chosen for study by biochemists and geneticists are seldom ecologically important.

Following the characterization or description phase in studying a microbial ecosystem in man or other animals the ecological or pathogenic relationships of interest can be transferred to animals under controlled conditions or to *in vivo* model systems so that many sources of variation can be controlled or excluded. Extrapolation to models is always fraught with problems relating to the host and tissue specificity of microbial activity. The current debate on animal experimentation has some bearings on this and wherever possible attempts should be made to develop *in vitro* models where the microbial characteristic to be tested has been well characterized *in vivo*. It must be emphasized, however, that the use of inappropriate or untested models could produce erroneous results and should be avoided at all costs. Nevertheless, some *in vitro* tests are already well established. *E. coli* enterotoxin can be assayed using monolayers of adrenal and Chinese hamster ovary cells. In this case, however, the activities of enterotoxin had been well studied *in vivo* and in tissue culture cells. Other micro-ecological characteristics such as adhesion to intestinal epithelial cells can also be reproduced using isolated intestinal cells *in vitro*. But here the use of tissue cultures can be misleading since, for example, many non-enteropathogenic strains of *E. coli* in addition to enteropathogenic strains show adhesive properties in these systems.

If a particular ecological relationship is to be studied in depth the molecular and genetic basis of the various interactions must be studied. While some of the steps involved can only be carried out *in vitro* the work can still use microbial cells which have been cultured *in vivo* to ensure full expression of the important microbial characteristics concerned. This is particularly true for pathogens (Smith, 1968). Similarly, the genetic basis of virulence can be identified by mapping the relevant loci in the case of chromosomally located alleles or by plasmid transfer accompanied by testing the resultant manipulated strains for virulence in the correct animal (Chapter 3) or in a suitable model. In all cases it is the animal in which the ecological and pathological relationships exist and any departure from the system can lead to erroneous assumptions and suppositions and eventually misleading results.

Obtaining samples of intestinal contents

Studies on the human intestine and, indeed, the intestine of animals are complicated by the problems of obtaining samples of intestinal contents. Most of the intestinal tract is normally inaccessible for examination. Many studies of the intestinal flora are by necessity studies of the bacteria found in faeces. In some cases this is inevitable but it must be remembered that faeces are probably a final decadent phase of the flora and accurately represents only the flora of the rectum.

Gastric, small intestinal and colonic contents are difficult to obtain and are probably much affected by the procedures used in collection. Animals can be killed and their intestinal contents examined after death, but this procedure cannot be applied to man and is undesirable in animal studies. Metabolic studies are particularly difficult.

Several workers have obtained specimens from patients undergoing abdominal surgery. Various methods of sampling the intestine have been used. Buffered saline introduced with a syringe and needle has been used to wash out a length of intestine isolated between clamps. The intestine has been opened and the contents sampled. Excised appendices have also been examined.

Samples of intestinal contents can be obtained through a peroral or nasal tube. Intubation can be used to obtain samples from normal people and the period for which samples can be obtained can be extended so that subjects can be studied while performing normal functions such as eating. However, the technique has several disadvantages, apart from discomfort to the subject; the tube is likely to stimulate the intestine, causing gastric and biliary secretion and thus changing the intestinal contents.

Open-ended tubes have been widely used but it has been suggested that contamination introduced during the swallowing of the tube may influence the results. Special sampling capsules and other forms of tube closure have been devised to overcome this problem but their usefulness has been questioned. Various types of biopsy tube have been devised in order to obtain samples of the intestinal wall for histological studies in the diagnosis of disease and parts of such samples may occasionally be available for bacteriological studies.

Free capsules that can be swallowed by the subject, opened and closed by radio and collected in the faeces have sometimes been used. Samples are, however, incubated during their passage through the gut.

In studying the *in vivo* microecology of the alimentary tract the main advantage of animals over man is in taking samples. In the subsequent steps of processing the samples and culture and identification of the microorganisms isolated the same limitations are involved. Portions of the gut can be removed immediately following slaughter, while the animal is under anaesthesia or even from a conscious animal by using surgically inserted sampling tubes or fistulae. For some studies, particularly those involving the nutrition of the ruminant, the construction of fistulae has become a matter of routine surgery and the fistula can remain in place for several years providing the animal is mature at the time of the operation. They can also be fitted to the colon and caecum of other large animals. Their use for studying the physiology of germ-free animals, however, is complicated by excess mucus production at the site of the fistula. Cannulae can be constructed which loop outside the body and thus can facilitate collection of digesta and measurement of flow rates. However, some disturbances in the animal's physiology usually occur and sheep under this technique seldom survive for more than one year.

Samples must be processed as soon as possible after removal, preferably immediately. This is because following death massive growth of many intestinal bacteria occurs which may obliterate small quantitative differences which are sought. Invasion of the intestinal wall may also occur. Consequently little useful information can be gained from *post mortem* examination of animals that have been dead for more than 1 or 2 hours. As with human samples, if storage is to be carried out at all this should be done at −70°C and transport media should never be used in experimental work. Deterioration in cellular structure of the host tissues also begins immediately *post mortem* and for microscopy immediate fixation is required to minimize this. A further complication arises in culturing strict anaerobes. Any exposure to oxygen may reduce the viability of these organisms and consequently samples should be transferred immediately to and processed under an anaerobic atmosphere.

Even if samples are taken immediately, quality can vary according to the method of sacrifice used. The use of ether is known to affect some biochemical activities, particularly glycolysis, of intestinal epithelial cells while the physical methods of killing may produce some detachment of epithelial cells. This latter effect may have some significance for the quantitation of microorganisms whose normal ecological niche is the mucosal layer. The use of barbitone compounds has been found to be generally satisfactory for sacrifice since shock is minimal while death is nevertheless very quick.

Chemical analyses of the sample may be required, particularly for nutritional studies. Some of these, particularly volatile fatty acid analysis, may require immediate processing while less volatile and stable compounds should resist storage at −70°C.

From this discussion it will be clear that the problems of obtaining intestinal samples are far from solved. The difficulties are greatest for samples from the large intestine; the disturbance resulting from a long tube passed from above or by sampling from below means that few satisfactory samples have been obtained from this area. This is unfortunate in that the caecum and colon are undoubtedly the areas of greatest microbial activity. Some studies of intestinal metabolism attempt to overcome these problems by comparing the metabolism of drugs and food additives in groups of antibiotic treated and conventional animals or occasionally in man.

Direct examination of intestinal samples

Little systematic consideration has been given to a direct examination of intestinal samples or the search for alternative methods for detection of the effects of intestinal bacteria. This is unfortunate because it is known that not all intestinal organisms are grown by the best methods currently available. Thus, the numbers of bacteria isolated from the duodenum and jejunum of normal people (not more than 10^4 ml^{-1}) fall well below the direct microscopic count (at least 10^5 ml^{-1}).

Examination of ecosystems by light or electron microscopy alone is of limited value. The presence of certain morphological types can be detected but accurate identification is impossible unless immunofluorescence is used or duplicate samples are cultured in the ways already described. Transmission and scanning electron microscopy have been used extensively to study adhesion of bacteria to various epithelial niches in the gut. The quality of the information, particularly in

regard to the preservation and visualization of the fine fibrillar material connecting bacteria with each other and with the host, depends on the use of relatively mild fixatives and suitable stains.

Quantitation of bacteria present in intestinal samples

Estimates of microbial number These often rely on culture techniques but microscopic examination of samples is a most useful means of evaluating the efficacy of other systems. The combination of microscopy with specific stains or fluorescent antibody enables particular organisms to be detected in mixed populations. Some organisms such as spirochetes can be recognized because of their unique morphology but most rods and cocci remain anonymous.

The mass of bacteria can be estimated chemically. This approach has been little used for studies on intestinal samples because of the large amounts of organic material from other sources that are present. To be of value, analysis must concentrate on uniquely bacterial components or rely on an initial purification to separate bacteria from other cells and food debris. Such a procedure, based on the use of filtration and centrifugation, has been used for examination of human faecal material and may well be applicable to other samples.

Two methods can be used to quantitate the microorganisms present in the sample: the total microscopic count and the viable count of the numerically most important groups of microorganisms, normally made from serial dilutions of the homogenized sample. For aerobes and facultative anaerobes homogenization presents little problem. Simple shaking of the sample and a diluent such as saline or nutrient broth may suffice for some samples of intestinal contents. Others including samples of epithelia with associated bacteria may require more vigorous maceration with a pestle and mortar. For counting strict anaerobes a diluent such as Reinforced Clostridial Medium (RCM) should be chosen containing reducing substances such as cysteine or sodium thioglycollate and a small amount of agar in solution to reduce mixing caused by convection.

Both methods for counting have inherent inaccuracies in addition to those arising from the dilution series (Meynell & Meynell, 1970). The total viable count made on a non-selective solid medium estimates the number of colony forming units (c.f.u.) per gram or other unit. Since each colony can arise from one cell or a clump or chain or cells it is important to count microscopically in the same way. Comparison of the results from using the two methods normally produces the finding that the total viable count is less than the total microscopic count. In the past this was attributed to a large proportion of dead organisms in the sample. Although this may be true for highly inimical sites such as the adult stomach, in other sites, particularly those predominated by strict anaerobes, it is more likely to be due to an inability to culture the majority of the organisms present.

Estimate of microbial activity This is aided by the element of amplification inherent in such microbiological assays. Thus, the estimation of faecal β-glucuronidase of bacterial origin has been suggested as a more sensitive indicator of the influence of diet on the flora than the counting of bacterial species. Similarly, a faecal incubation system has been used to study the generation of ammonia from non-urea sources. The incubation of faeces with ^{14}C-labelled cyclamate to examine its degradation is a similar approach.