INDIGENOUS ANAEROBIC BACTERIA IN HEALTH AND DISEASE.

A thesis submitted by

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ABSTRACT

This thesis comprises several studies of the distribution and significance of anaerobic bacteria in the human large bowel in healthy subjects and in subjects shown to be suffering from large-bowel cancer.

Comparisons of the methods currently available for use for the isolation and cultivation of strictly anaerobic bacteria have been made. These comparisons have confirmed that an anaerobic chamber system in conjunction with pre-reduced media is a valid and convenient method for the isolation of anaerobic bacteria from faeces, especially when a large number of such samples are to be examined. In addition, an increase has been shown in the recovery of anaerobic bacteria from wound swabs seeded onto pre-reduced media in an anaerobic chamber compared with the recovery of these bacteria from the same swabs seeded on the bench onto aerobically stored media.

The use of antibiotic-disc tests for rapid identification of pure cultures of non-sporing anaerobes has been examined. Useful patterns of sensitivity were shown by Bacteroides fragilis and Bacteroides melaninogenicus. Consistent and useful sensitivity patterns were not shown by the Gram-positive anaerobes.

Using an anaerobic chamber system, non-sporing strictly anaerobic

bacteria have been isolated from human faecal samples in England, Scotland, U.S.A., India, Uganda and Japan, countries with differences in diet and incidence of large-bowel cancer. The numerical dominance of the non-sporing anaerobes in faeces was apparent. Bacteroides fragilis was the species most frequently isolated. Bifidobacterium adolescentis and Eubacterium aerofaciens were also common. Stool samples from India, Uganda, and Japan, where the diet had a high carbohydrate content and where there is a low incidence of colon carcinoma, contained a larger proportion of eubacteria and fewer bacteroides than did samples from England, Scotland and the U.S.A., where people consumed a mixed diet and where there is a high incidence of colon carcinoma.

A similar pattern has emerged on analysis of the numbers and major groups of bacteria in faeces from healthy people in three income groups within Hong Kong, where monthly income can be correlated with diet and with the incidence of large-bowel cancer.

An examination of the clostridia isolated from faeces from Hong Kong, showed that many could not be assigned to any conventional taxonomic group. Pure cultures of these clostridia and cultures of non-sporing anaerobes isolated from Hong Kong metabolized bile steroids poorly, and in the case of the clostridia, this could be partially explained by the unusual species encountered.

The bacterial flora associated with the mucosal surface of the colon of patients undergoing surgery for carcinoma of the large bowel and its relationship with the faecal flora, has been examined. Unlike

faeces, the bacteria isolated from mucosal specimens were not predominantly non-sporing anaerobes, but were mainly Gram-positive cocco-bacilli showing different degrees of oxygen tolerance. Similar bacteria were isolated from a small number of specimens of tumour tissue. Pre-operative oral administration of sulphonamide lowered the total number of bacteria which could be isolated from faeces, but did not affect the number of bacteria that could be isolated from tissue specimens.

Successful culture of large-bowel mucosa and tumour tissue *in vitro* has been achieved. Organ-cultures of normal mucosa were maintained for up to three weeks. On histological examination there was no evidence of cellular deterioration of the cultures.

Preliminary experiments indicated that cultures of mucosa and tumour tissue had antibacterial properties, but the nature of these properties was not elucidated.

When cultures of *E. coli* were added to mucosa and tumour tissue maintained *in vitro* some cells apparently adhered to the surface of the tissue. The mechanisms controlling this adherence were not elucidated.

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INTRODUCTION

Introduction and aims of the present investigation

Most of the bacteria found in the lumen of the large bowel of man are strictly anaerobic (Sanborne, 1931). These organisms comprise more than 99% of the total number of viable bacteria that can be isolated from faeces. Despite this, little is understood about the biological processes controlling their growth and many details of their distribution remain obscure.

The aim of this thesis is to examine the constitution of and mechanisms controlling, the bacterial flora of the large bowel, especially with regard to the work of other members of this laboratory which suggests that this flora may be implicated in the aetiology of cancer of the colon.

Because of the technical problems inherent in the study of oxygen-sensitive bacteria, methodology has been an important adjunct to the present study. Isolation and identification methods were examined and during the course of these investigations some of the anaerobic culture systems were compared with the methods used in a routine clinical laboratory. Many of the anaerobic bacteria encountered in studies of faeces are also important in human infections and are being increasingly realized as such.

The studies of the bacterial flora of faeces are part of a project emanating from a group of workers at St. Mary's Hospital, London, in which

the possible production of carcinogens from bile salts by faecal bacteria has been examined. Hill and his colleagues (1971) demonstrated quantitative and qualitative differences in the faecal flora of groups of people living in various parts of the world and correlated these differences with the world-wide distribution of cancer of the large-bowel. A possible determinant of these differences was diet. Part of the work described in this thesis has been an attempt to further characterize the non-sporing anaerobic bacteria isolated from the faeces of these groups of people and to extend this study to groups of people who differed in diet and risk of large-bowel cancer, but who shared a common environment and racial origin. Three income groups in Hong Kong were chosen for such a study.

Goddard (1973) showed that Clostridium paraputrificum is able to modify bile salts to produce a carcinogen-like compound and this has stimulated the examination of the clostridia in faeces from Hong Kong, the results of which are described in this thesis.

Continuing the theme of factors influencing the faecal flora, the opportunity was taken to examine the effects of pre-operative sulphonamide administration on the faecal flora at operation, of patients undergoing surgery for cancer of the large bowel. An attempt was made to relate this to any post-operative infection.

Although pre-operative regimes may affect the faecal flora, in a bowel with diminished contents such as that at operation, it is important to consider the bacterial flora intimately associated with the mucosa of the colon. This may be disturbed at operation and subsequently

give rise to post-operative infection. The close proximity of these bacteria to the mucosa may also make them important in studies of carcinogen production by bacteria in the intestine. Thus, some studies of the bacterial flora of the mucosa of the human large-bowel have been attempted.

It is hoped that the studies described in this thesis clarify our picture of the place of the anaerobic bacteria in the ecology of the large-bowel of man and that the preliminary studies of the mucosa of the colon will stimulate further investigations of the bacterial flora of this unique site.

SECTION I.

REVIEW OF THE LITERATURE.

1.1. The anaerobic bacteria.

1.1.i. Introduction.

Leeuwenhoek, in 1680, was the first to demonstrate that 'animalcules' can exist and develop without air. In 1861, during the course of work on butyric acid fermentation, Pasteur found a large sporing bacillus which failed to grow in an atmosphere of air, but grew well, with the production of butyric acid, in the absence of oxygen. He introduced the term 'aérobiès' and 'anáerobiès' to denote micro-organisms that live with or without oxygen. The occurrence of the non-spore forming anaerobic bacteria in infections was described in a series of investigations emanating from the Faculty of Medicine of Paris at the end of the nineteenth century.

Löesche (1969) recognized that anaerobes differ in their sensitivity to oxygen and four patterns were established. Strict anaerobes were species incapable of surface growth at oxygen levels greater than 0.5%. This group included various species of treponema and some bacteria usually found in the bovine rumen. Moderate anaerobes could grow in oxygen levels as high as 2-8% and could be exposed to room atmosphere for 60-90 minutes without appreciable loss of viability. Species considered as moderate anaerobes included Bacteroides fragilis, Bact. melaninogenicus, Bact. oralis, Fusobacterium nucleatum, Clostridium novyi type A and Peptostreptococcus elsdenii. A third category was formed in which were placed organisms such as Vibrio foetus and V. sputorum which grow maximally under

reduced oxygen levels, but grow poorly in the absence of oxygen. These organisms were termed Microaerophiles. Bacteria such as Clostridium histolyticum and Clostridium tertium which just grow barely on the surface of blood-agar exposed to air were termed aerotolerant anaerobes.

1.1.ii. The non-sporing anaerobes.

Whilst the taxonomy of the clostridia is, for the most part, settled, with only minor changes instigated over several years, the taxonomy of some of the non-sporing anaerobes has had a turbulent history.

Castellani and Chalmers (1919) grouped both Gram-positive and Gram-negative non-sporing anaerobic rods into a new genus which was termed Bacteroides.

In 1924, Orla-Jensen suggested that the Gram-positive non-sporulating anaerobes be placed in a separate genus Bifidobacterium. The genus name was derived from the branching forms common to these organisms. Eggerth (1935) agreed that these organisms should be separated from the Gram-negative bacteroides, but favoured the name Lactobacillus since these organisms had been shown to produce large amounts of lactic acid from glucose; however, Prévot (1938) continued to encourage the genus Bifidobacterium. In 1966, Werner, armed with D.N.A. base ratio data, showed that these organisms formed a distinct group separate from the lactobacilli and revived the genus name Bifidobacterium. The Gram-negative anaerobes remained in the genus Bacteroides.

Eubacteria (Prévot, 1938) are Gram-positive anaerobic rods which have also been isolated in fairly large numbers from the intestine. Likewise, propionibacteria (Orla-Jensen, 1909) and the anaerobic-cocci have been isolated from this environment but in much smaller numbers than the bacteroides, bifidobacteria and eubacteria. These organisms have also been isolated from the skin. Gram-positive anaerobic cocci include the genus Peptococcus and Peptostreptococcus (anaerobic streptococci) (Kluyver and Van Niel, 1936). The Gram-negative anaerobic cocci are represented by the genus Veillonella (Prévot, 1933; Rogosa, 1965).

In an attempt to find a more scientific biochemical basis for distinction of species and genera, Kluyver and Van Niel (1936) directed attention to the importance of studying the end-products of fermentation rather than the simple consideration of the production of acid. This procedure was simplified with the advent of gas-liquid-chromatography and end-product analysis is now widely applied in the study of strict anaerobes. The types and relative proportions of fatty acids and alcohols produced from glucose are specific for a particular genus or in some cases for a species. In an attempt to bring order to the chaos of non-sporing anaerobe taxonomy, Cato et al. (1970) and Holdeman and Moore (1972) published schemes for identification incorporating analysis of end-products of glucose metabolism. These are followed throughout this thesis. The major genera of non-sporing anaerobes are shown in Table 1.

TABLE 1

Major genera of non-sporing anaerobes.

	*Family	*Genus	‡Characteristics
GRAM-POSITIVE	Actinomycetaceae	Bifidobacterium	Branching (bifid) rods with spatulate ends. Acetic and Lactic acids (ratio 3 : 1) are the major end products of glucose metabolism. All strains of species in the genus Bifidobacterium make similar fermentation products.
	Propioniaceae	Eubacterium	Include bacteria previously in the genera Butyribacterium, Ramibacterium and Catanabacterium. Small rods. Produce mixtures of acids from glucose, or no major acids. Eubacterium aerofaciens produces acetic, formic and lactic acids from glucose.
		Propionibacterium	Include bacteria previously called anaerobic corynebacteria. Typically diphtheroid. Propionic and acetic acids are the major volatile acids produced from glucose. All species make similar fermentation products.
	Lactobacillaceae	Lactobacillus	Straight rods, typically in chains. Lactic acid is the sole major product from glucose. The Lactobacilli described in this thesis are anaerobes, although facultative members of this genus do exist.
		Peptostreptococcus	Cocci, often in chains, or pairs. Do not require fermentable carbohydrate.
	Micrococcaceae	Peptococcus	Cocci. Do not require fermentable carbohydrate.
GRAM-NEGATIVE	Bacteroidaceae	Bacteroides	Small rods with rounded ends, typically exhibit bipolar staining. Bacteroides fragilis produces from glucose, acetic and succinic acids with or without propionic isobutyric, butyric, isovaleric and fumaric acids.
		Fusobacterium	Includes organisms previously in the genus Sphaerophorus. Small spindle shaped rods. Butyric acid is the major product of glucose fermentation. Fusobacterium fusiforme (nucleatum) also produces small amounts of lactic, propionic and succinic acids.
	Veillonellaceae	Veillonella	Small cocci. Produce propionic and acetic acids from glucose.

* After Bergey, 1974. ‡ After Holdeman and Moore, 1972.

1.1.iii. Antibiotic susceptibility patterns for identification of non-sporing anaerobes.

In common with most schemes for bacterial identification, recommended schemes for the identification of the non-sporing anaerobes place emphasis on the results of numerous biochemical tests (Holdeman and Moore, 1972; Dowell and Hawkins, 1974; Sutter et al. 1972). Finegold, Harada and Miller, (1967) using a plate dilution method, demonstrated the use of antibacterial susceptibility patterns as aids to the characterization of anaerobic Gram-negative bacilli and Sutter and Finegold (1971) simplified and improved this method with the use of discs impregnated with high concentrations of antibiotics.

Bacteroides are often resistant to the concentrations of most antibiotics used therapeutically and to overcome this the high concentrations of antibiotics used by Sutter and Finegold are necessary. This means that whilst providing a rapid method for identification of the Gram-negative anaerobes, the results cannot be used for predicting the therapeutic effectiveness of any particular drug.

Examination of additional characteristics such as growth in bile, aesculin hydrolysis and the metabolism of tryptophan and nitrate, allows further and more reliable differentiation of all the groups.

Although the scheme appears to provide a rapid method for differentiation of Gram-negative anaerobes it has limitations. Of the 126 isolates tested by Sutter and Finegold, 43 were not identifiable and

required more conventional testing before identification could be made.

There is no comparable scheme for rapid identifications of the Gram-positive non-sporing anaerobes and the sporing anaerobes. However, studies have been carried out with therapeutic concentrations of antibiotics (Laverge et al. 1959; Miller and Finegold, 1967; Sapico et al. 1972).

A re-evaluation of the scheme for identification proposed by Sutter and Finegold (1971) has been made during the present study, together with an examination of the possibility of extending its use to include the Gram-positive non-sporing anaerobes.

1.1.iv. Methods used for the isolation of anaerobic bacteria.

Since the 'discovery' of anaerobic bacteria in 1861 by Louis Pasteur, a variety of methods for the isolation and cultivation of these organisms have been described. Although anaerobes differ in their sensitivities to oxygen (Löesch, 1969), the aim should be to provide conditions suitable for the isolation of all anaerobes. One of the first attempts at obtaining anaerobiosis was to inoculate the deeper portions of deep-agar tubes. Colonies were recovered by cutting the tube with a file near a colony and aspirating with a Pasteur pipette. The modern anaerobic jar came into existence as the McIntosh and Fildes jar in 1916,

Photograph 1. ANAEROBIC JARS.

From left to right:

Standard (Baird and Tatlock) anaerobic jar.

Vented 'Gas Pak' polycarbonate jar.

Converted Fullwood-Bland milking machine pail.

Converted beer keg.



was improved by Brewer in 1939 and further by Khairat in 1964 with the use of a palladium catalyst which was active in the cold (Heller, 1954). The most recent inovation is the 'Gas-Pak' system (Becton-Dickinson, U.K., Ltd.) which consists of a disposable carbon-dioxide/hydrogen generator (Brewer and Allgeier, 1966). Collee et al. (1972) showed this to be effective for the cultivation of even very fastidious anaerobes. For increased efficiency the 'Gas-Pak' can be combined with an evacuation technique and specially vented jars are available. During the present study, a milking machine churn, suitably converted with gas taps, has proved to be a useful type of 'anaerobic jar' for the incubation of large numbers of cultures. A number of anaerobic jars are shown in Photograph 1.

A catalyst which reacts in the cold with hydrogen and excess oxygen, forming water, is used in standard anaerobic jars. The commercially available anaerobic jars are usually supplied with a sachet of catalyst attached to the lid, but for maximum efficiency, this should be changed every time the jar is used, and reactivated by heating (Holdeman and Moore, 1972).

The use of carbon-dioxide in anaerobic jars is to be stressed. Lewis, Bedell and Rettger (1940) and Watt (1973) working with pure cultures of anaerobes have shown that addition of CO_2 increases both numbers and colony size of organisms incubated in anaerobic jars. Bacteroides have been shown to fix CO_2 and to obtain maximal amounts of succinic acid for end-product analysis by Gas-Liquid-Chromatography, CO_2 is essential. For routine incubation of plates, about 10% (v/v) is

recommended, although 30% is necessary when sugars are to be examined for end-products of metabolism. An anaerobic jar technique has been described by Collee et al. (1972).

The enzyme systems of some anaerobic bacteria are very sensitive to oxygen (Hine and Berry, 1937; Smith and Holdeman, 1968; Moore, Cato and Holdeman, 1969; Cato et al. 1970). Conventional bench techniques for isolating these organisms increase the exposure time to atmospheric oxygen and its lethal effects, especially where specimens are spread on the surface of an agar plate, and the isolation is therefore not quantitative. The loss of anaerobic bacteria may be due to a combination of atmospheric oxygen, oxygenation of media components and prolonged exposure of bacteria to oxygen dissolved in aerobically inoculated media, even when plated in an anaerobic atmosphere (Drasar, 1967; Smith and Holdeman, 1968).

In 1950, Hungate, working with the very exacting anaerobes of the bovine rumen, devised a technique by which media can be prepared, distributed, sterilized and inoculated under an atmosphere of oxygen-free gas (pre-reduced anaerobically sterilized media, P.R.A.S.). Modifications of this method have been described by Moore (1966), Latham and Sharpe (1971) and in the laboratory manuals of Sutter et al. (1972) and Moore and Holdeman (1972). The viable counts of bacteria obtained from faeces by this method are very nearly equal to the direct microscopic count. McMinn and Crawford (1970) found pre-reduced media superior to routine laboratory methods for the isolation of anaerobes from clinical specimens. A gassing apparatus is now available commercially.

Another type of system is the anaerobic glove box. Socransky, Macdonald and Sawyer (1959) described a technique involving the use of an anaerobic chamber filled with oxygen-free gas for the surface cultivation of Treponema microdentium. Properly designed and operated, an anaerobic chamber system with glove ports has two major advantages over other methods; it enables standard bacteriological techniques to be used to cultivate strict anaerobes and the media may be prepared in the conventional manner in the open laboratory and stored in the chamber for a few days before use, thereby becoming reduced. Anaerobic glove boxes have been utilized by a number of workers for the isolation of strictly anaerobic organisms and these have included both the rigid type of box (Spears and Freter, 1967; Drasar, 1967) and chambers made of flexible vinyl material (Aranki et al. 1969). Manipulations are more difficult with the rigid box and, since only very expensive cabinets are capable of withstanding evacuation, complicated deoxygenating systems are needed.

During the present study, a chamber similar to that of Aranki et al. (1969) has been used (see Section 2.8).

It has been suggested (Starr, 1974; Watt, Collee and Brown, 1974) that anaerobes often isolated from infections such as Bacteroides fragilis, B. melaninogenicus, Fusobacterium nucleatum, peptostreptococci, Clostridium perfringens and Propionibacterium acnes are 'moderate' anaerobes (Löesche, 1969) and that such stringent anaerobic methods as roll-tubes and anaerobic chambers are only really necessary for the study of 'extremely oxygen sensitive' (E.O.S.) strains isolated during normal

flora studies, such as those of faeces and of the contents of the bovine rumen.

Recent work has concentrated on the conditions of transport of clinical specimens to the laboratory for anaerobic investigation (Attebery and Finegold, 1969; Holdeman and Moore, 1972; Finegold, 1974). These methods include (1) Mini anaerobic jar (Attebery and Finegold, 1970); (2) Syringe techniques: all air is eliminated from the needle and syringe containing the specimen and the needle inserted into a sterile rubber stopper (3) Displacement technique: tubes are filled to the brim with the specimen and then capped (4) Gassed out tubes: specimen is injected into a butyl-rubber-stoppered tube which has been gassed out with O_2 free CO_2; (5) Swabs: placed into tubes of P.R.A.S. transport medium.

Techniques for the transport and storage of specimens of intestinal juice and faeces have also been devised (Drasar, Shiner and McLeod, 1969; Crowther, 1971).

Alternatives to pre-reduced media are the specially designed media which incorporate reducing agents and can be used on the bench without special equipment. Hirsch and Grinstead (1969) incorporated cysteine into media as a reducing agent and during the present study this has been used in combination with sodium formaldehyde sulphoxylate. Other specialised media include those of Moore (1968) and Watt (1972) which incorporate dithiotheritol, and Freter's medium (1969) which includes an overlay with the reducing compound palladium black. Deep tubes of

thioglycollate medium (Brewer, 1940) and Robertson's cooked meat medium with meat particles (Robertson, 1915) provide adequate reducing conditions for the cultivation of most anaerobes in liquid media.

The methods described in this thesis, for the isolation of anaerobic bacteria from faeces have been evaluated (Section 2.5) and the rates of isolation of anaerobes from wound swabs by three procedures have also been compared (Section 2.6).

1.2. Anaerobes as normal flora.

1.2.i. Introduction.

Interest in anaerobes as normal flora began early in the present century (Lewkowicz, 1901; Sanborne, 1931; Thomas and Hare, 1954) and during the last 15 years it has been established that non-sporing anaerobes are present as normal flora at various sites in the body (Rosebury, 1962; Smith and Holdeman, 1968) and are by far the most numerous of the indigenous flora of man. They are present on the mucous membranes of the upper respiratory tract, the oral cavity, the genito-urinary tract and on the skin. In these areas they outnumber the aerobes by a factor of 10 : 1. In the lower intestinal tract of man this ratio is increased to 1000 : 1 (Sanborne, 1931). Because of the numerical dominance of anaerobic bacteria in the large bowel, the

TABLE 2

Incidence of non-spore-forming anaerobes as normal flora in humans.

	Bacilli						Cocci	
	Gram-positive				Gram-negative		Gram-positive	Gram-negative
Site	Bifidobacterium	Eubacterium	Lactobacillus*	Propionibacterium	Bacteroides	Fusobacterium		
Skin	0	U	0	2	0	0	1	0
Upper respiratory tract	0	±	0	1	1	1	1	1
Mouth	1	1	1	±	2	2	2	2
Intestine	2	2	1	±	2	1	2	1
External genitalia	0	U	0	U	1	1	1	0
Urethra	0	U	±	0	1	1	±	U
Vagina	2	U	2	0	1	±	1	1

* Includes anaerobic, microaerophilic and facultative strains.

Includes nasal passages, nasopharynx, oropharynx and tonsils

0 = not found or rare; U = unknown; 1 = usually present;
2 = usually present in large numbers; ± = irregular.

From Finegold (1974).

influence they exert on the physiology and ecology of this site is of particular importance. The occurrence of non-spore-forming anaerobes as normal flora in humans is summarized in Table 2.

1.2.ii. Bacterial flora of the mouth and intestinal tract.

The mouth.

The indigenous flora of the oral cavity is complex. Many original studies concentrated on the microbial flora of the saliva, since samples were easily obtainable; Streptococcus salivarius was found to be the predominant organism. For several years now, however, it has been recognized that certain bacterial species preferentially colonize different sites in the mouth (Krasse, 1954; Van Houte, Gibbons and Banghart, 1970; Gibbons and Van Houte, 1971) and the sampling of these sites has brought to light the diverse microbial flora which is present.

Because of easy access to atmospheric oxygen, the mouth is not generally considered to be an anaerobic environment; however, anaerobic bacteria are numerous (Table 3) and improved techniques have led to the isolation of several species of anaerobe (Aranki et al. 1969). Bacteroides melaninogenicus, B. oralis, spirochaetes and anaerobic vibrios have been isolated in high numbers from the gingival crevice area, but they are found in much lower numbers on the tongue and cheek surfaces (Löesche and Gibbons, 1966). It has been suggested that B. melaninogenicus is

TABLE 3

Oral flora.

Site	Typical bacterial counts per ml	
	Aerobes	Anaerobes
Saliva	$10^7 - 10^8$	$10^8 - 10^9$
Tooth surfaces	10^6	10^6
Gingival scrapings	10^7	10^7

From Hoeprich (1970).

is involved in peridontal disease (Macdonald, Socransky and Gibbons, 1963). Infections caused by anaerobes of the mouth in cases of human bite, can be extremely virulent and cause widespread tissue damage (Finegold et al. 1972).

Bifidobacteria and fusobacteria have been isolated from saliva in counts up to 10^4/g saliva (Richardson and Jones, 1958; Drasar, Shiner and McCleod, 1969). Veillonellae are found in small numbers on all oral surfaces (Liljemark and Gibbons, 1971). Dental plaque contains a relatively high proportion of Gram-positive rods, especially Actinomyces species, and this is often accompanied by high levels of veillonellae and anaerobic Gram-negative rods (presumptive bacteroides) (Hardie and Bowden, 1973). Streptococci, to which great attention has been paid in the literature often appear to constitute a numerically small proportion of the total viable counts obtained from dental plaque. The streptococcal populations of microbial plaques developing on the surfaces of the teeth chracteristically have high proportions of Streptococcus sanguis but low proportions of Strep. salivarius (Carlsson, 1967). Bacteria from these sites may be the source of streptococci found in the saliva (Gibbons, Kapsimalis and Socransky, 1964). Possible differences in inherent abilities to attach to different surfaces may account for these observations (Van Houte et al. 1970; Gibbons and Van Houte, 1971).

It should be noted that strains of Bacteroides melaninogenicus isolated from the mouth have been shown to be biochemically different from strains isolated from other areas of the gastrointestinal tract (McFarlane, 1974).

Bacterial flora of the stomach and the small bowel.

The stomach provides an important barrier to microbial overgrowth of the small bowel (Gray and Shiner, 1967). Acid stomach contents are usually sterile (Drasar et al. 1969). The germicidal action is thought to be due to free hydrochloric acid. Immediately after a meal a count of about 10^5 bacteria per ml of gastric juice can be recorded and includes streptococci, enterobacteria, bacteroides and bifidobacteria, derived from the mouth and from the meal (Drasar et al. 1969). As the pH falls the bacterial count declines and relatively few bacteria are found when the stomach contents have fallen below about pH 3.

With the use of intubation techniques, data on the normal microbial populations of the smallintestine have accumulated (Shiner et al. 1963; Gorbach et al. 1967; Drasar et al. 1969). The upper regions, stomach, duodenum, jejunum and upper ileum have a sparse microbial flora, consisting predominantly of Gram-positive facultative organisms. In the lower small intestine, anaerobes, including bacteroides, occur and there is a richer, more permanent flora. However, the lower ileum still maintains a relatively low total concentration of organisms (10^5 - 10^8/ml) and represents a transitional zone between the sparse flora of the upper gastro-intestinal tract, and the rich flora of the large bowel (Gorbach, 1971).

Bacterial flora of the large bowel.

Distal to the ileocaecal valve a striking change in the number and types of bacteria can be seen (Gorbach, 1967). There is an increase in

anaerobic populations, bacteroides and bifidobacteria becoming the dominant micro-organisms, out-numbering the aerobic and facultative flora by 1,000 to 10,000 : 1. Data is still incomplete, due to the difficulties in sampling the contents of the intra-abdominal colon, but studies by Gorbach et al. (1967) in using an intubation technique, and by Seeliger and Werner (1963) who sampled the contents of healthy appendices obtained at operation, suggest that the flora is qualitatively similar to that of faeces, although there is controversy as to its quantitative composition. Gorbach (1971) suggests that the number of organisms per gram of colon contents is somewhat less than that normally found in faeces, i.e. 10^9 per gram versus 10^{11} per gram. Other investigators (Moore et al. 1969) have suggested counts higher than those in faeces.

The adult faecal flora.

The numerical dominance of the non-sporing anaerobes in faeces is well established (Sanborne, 1931; Eggerth and Gagnon, 1933; Lewis and Rettger, 1940). Various authors using differing bacteriological techniques generally agree that 10^{10} - 10^{11} viable bacteria can be recovered from one gram (wet-weight) faeces. However, there has been controversy concerning the dominanace of various genera. Haenel (1961) suggested that more than 50% of cultivatable bacteria in faeces were the bifidobacteria, the second largest group being bacteroides. In contrast, Zubrycki (1963) and Donaldson (1964) claimed bacteroides to be the dominant group and this view was supported by many other workers (Van-Houte and Gibbons, 1966; Gorbach, 1967; Speck, Calloway and Hadley, 1970;

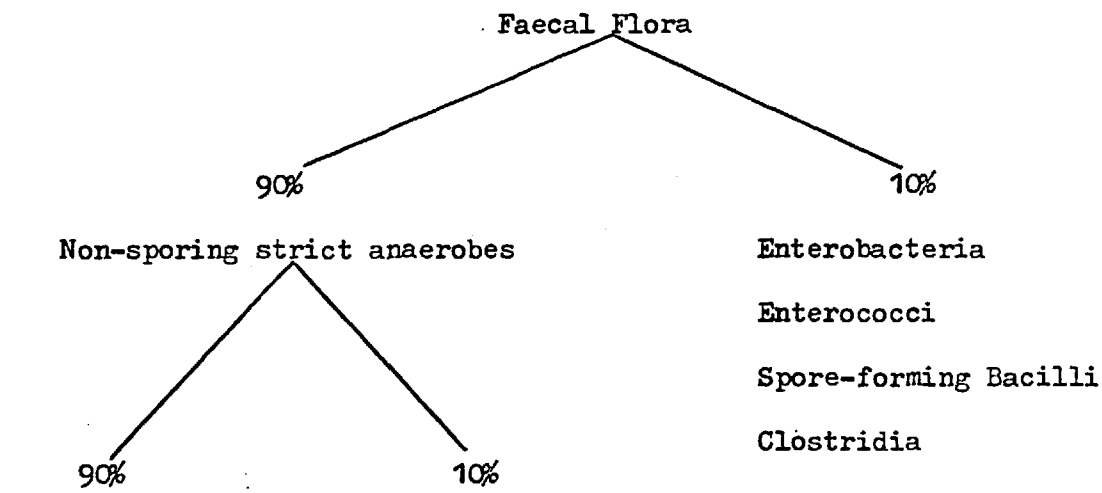
Mata, Carrillo and Villatoro, 1969).

About half the non-sporing anaerobes isolated from faeces by Moore, Cato and Holdeman (1969) were Gram-negative rods, mostly Bacteroides fragilis. Other non-sporing anaerobes isolated included various species of bifidobacteria and eubacteria. Anaerobic cocci and clostridia were isolated in relatively small numbers. These workers also showed that the faecal flora of an individual fell into one of three groups: (1) predominantly bacteroides; (2) predominantly bifidobacteria and (3) a mixture of bacteroides and bifidobacteria. A more recent study by Moore and Holdeman (1974) examined quantitatively and qualitatively the faecal flora of 20 healthy Japanese-Hawiian males. One-hundred-and-thirteen distinct types of organism were observed from a total of 1,147 isolates, most of which were non-sporing anaerobes. The commonest species were B. fragilis, Fusobacterium prausnitzii, Bifidobacterium adolescentis and Eubacterium aerofaciens. As previously demonstrated, the faecal flora of an individual could be predominantly bacteroides, bifidobacteria, or mixed, and in addition, a group was found with eubacteria predominating.

Bacteroides fragilis was the most common faecal isolate in a study by Attebery, Sutter and Finegold (1974). Other non-sporing anaerobes isolated included Eubacterium aerofaciens and Bifidobacterium adolescentis. Gossling and Slack (1974) in a study concerned only with the Gram-positive bacteria in human faeces, found 60% of isolates belonged to one of five types tentatively identified with five species - Bifidobacterium adolescentis, Eubacterium aerofaciens, E. rectale, Peptostreptococcus productus and Ruminococcus bromii. There was host idiosyncracy in the pattern of estimated counts of these five types.

TABLE 4

Main genera of bacteria in faeces.



Much of the evidence for the presence of certain types of bacteria in faeces depends on the use of suitably selective methods and nutritionally adequate media. For example, peptostreptococci not normally considered to be found in large numbers in faeces have been shown to exist at counts up to 10^9/gram wet-weight by the use of a defined medium containing urea-nitrogen in place of NH_4^+ (Varel et al. 1974).

In most studies of the faecal flora, bacterial counts are stated as logarithm_{10} colonies per gram wet-weight of faeces. However, Attebery, Sutter and Finegold (1974) consider it necessary to correct for moisture content of faeces since they have shown erratic distribution of moisture in various portions of faecal specimens. The moisture content of normal faeces is 70 - 80% (Hill et al. 1971).

A diagrammatic representation of the current idea of the normal adult human faecal flora is given in Table 4 and work published since 1969 is summarized in Table 5. The faecal flora of babies is somewhat different and is discussed later (Section 1.3.i). It will be noted that most published works refer to persons living in America or Western Europe, consuming a mixed 'western' type of diet. Diet has long been recognized as one of the determinants of the types of bacteria occurring in the intestine (Dudgeon, 1926). Hill and his colleagues (1971) showed that the faecal flora of people consuming different diets in various parts of the world, differ in their anaerobe and aerobe content (Section 1.3.i).

It is difficult to separate the effects on the faecal flora of a person's age, from those due to other factors such as diet. However, it

TABLE 5

Bacterial flora of faeces from healthy adults.

	Mata et al. 1969	Moore et al. 1969	Speck et al. 1970	Crowther 1971	Attebery et al. 1974	Gosling 1974	Varel et al. 1974	Moore & Holdeman 1974	Conn & Floch, 1970
No. of persons examined	WW 12	WW* 2	WW 6	WW 68	DW 7	WW 10	WW 3	DW 20	WW -
Total anaerobes	10.5	11.3	10.6	-	11.5	10.9	10.7	11.7	9.3
Bacteroides	10.3	10.7	-	9.8	10.8 (9-11)	-	-	10.8*1	8.9
Bifidobacteria	9.4	10.0	-	9.8	9.2 (0-11)	-	-	10.5	-
Eubacteria	-	10.30	-	-	9.3 (9-11)	-	-	10.4	-
Enterobacteria	8.7	9.7	-	7.9	10.0*2 (8-10)	-	-	9.2	6.8
Streptococci	-	-	-	-	9.5 (4-11)	-	-	8.9	5.7
Enterococci	7.9	-	-	5.8	9.5 (4-11)	-	-	8.4	-
Strep.salivarius	-	-	-	3.8	-	-	-	9.2	-
Lactobacilli	8.6	-	-	6.5	(5-10)	-	-	9.8	7.9
Clostridia	9.3	9.0	-	5.7	9.5 (4-10)	-	-	8.9	-
Veillonellae	9.2	-	-	4.2	(4-7)	-	-	-	-
Peptostrepto-cocci	10.1	9.4	-	-	(7-10)	-	9.0	10.4	-
Peptococci	-	-	-	-	(4-10)	-	-	8.9	-
Bacilli	-	-	-	3.7	(4-11)	-	-	-	-
Staphylococci	5.0	-	-	1.7	-	-	-	-	-
Fungi	4.0	-	-	1.6	(3-6)	-	-	-	-

Log_{10} no. of bacteria per gram wet weight (WW) or dry weight (DW)

All data, except Mata et al. (1969) refer to persons consuming a mixed 'western diet'.

Figures in parenthesis indicate range.

*1 Bacteroides fragilis only.

*2 E. coli.

*3 Calculated result.

seems that after an infant has been weaned, an intestinal flora is established which, within broad limits, remains stable from childhood to adult life. In old age, the numbers of bifidobacteria decrease and those of enterobacteria and clostridia increase (Haenel, 1963; Gorbach et al. 1967).

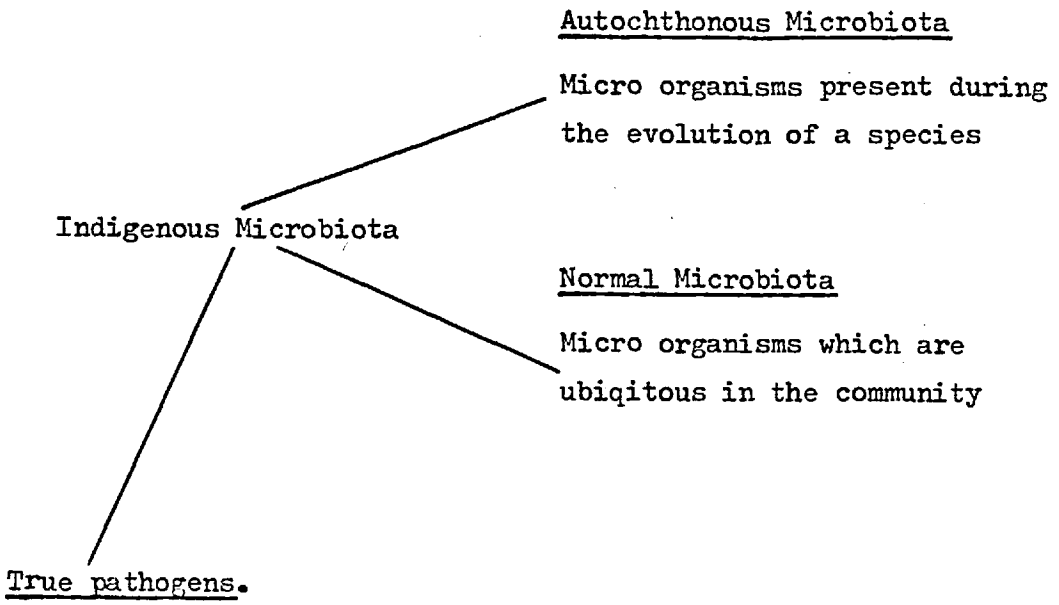
Crowther (1971) found that women in England carried significantly greater numbers of bacteroides in faeces than did men. There were no significant differences in the counts of bifidobacteria.

A large part of this thesis compares the faecal flora of people living under different conditions and consuming different diets (Section 3.5).

Mucosal surface flora.

In the mouth the bacterial flora adherent to surfaces differs from that free in the secretions (Section 1.2.ii. above). These observations have been extended to various regions of the intestine.

Dubos et al. (1965) proposed that the indigenous bacterial flora of the gastro-intestinal tract consists of two components, the 'normal' and the 'autochthonous' biotas, plus true pathogens (which may not always be present) i.e.:-



The autochthonous biota is thought to be uniform in all communities of a certain type of mammal. The normal biota is restricted to an individual community of a particular type of mammal. The concept of autochthony stimulated interest in the bacterial flora of the intestinal mucosa and on the existence of a specific mucosa-dependent flora.

Most work on this subject has been with laboratory animals, mainly rodents, and it has been demonstrated that certain microbial types in the gastro-intestinal microbiota selectively colonize various parts of the mucosal epithelia (Schaedler, Dubos and Costello, 1965). Lactobacilli, in thick layers, colonize the keratinising stratified squamous epithelium of the non-secreting portion of the stomach of all normal mice (Savage, Dubos and Schaedler, 1968; Savage, 1970), rats (Brownlee and Moss, 1961) and swine (Dubos et al. 1965). The stomach of normal laboratory mice is also populated by yeasts, which colonize the mucin on the columnar epithelium of the secreting portion of the stomach, and, although not physically attached to the underlying cells, appear to be well adapted to

to growth in the stomach mucin. The crop of chickens is populated by lactobacilli which have been shown to be actually attached to the underlying cells (Fuller and Turvey, 1971).

The acid-secreting human stomach is normally sterile (Drasar et al. 1969) and colonization of the epithelium by bacteria has not been demonstrated.

Savage (1969) noted that the villous epithelium in the ileum of rats from certain normal populations was colonized by long chains of short rods or cocci. He suggested that these bacteria may be securely attached to the ileal epithelial cells. An electron-microscope study by Hampton and Rosario (1965) demonstrated the attachment of Streptobacillus moniliformis and other unidentified organisms to villous epithelial cells in the distal ileum of normal mice. It was postulated that epithelial cells exert an influence in controlling the intestinal flora, perhaps through destruction of some species of micro-organisms.

In another study with mice (Savage et al. 1968) and rats (Savage, 1970), it was shown that the large bowels of these animals contain bacteria that associate intimately with the mucosal epithelia. These organisms are anaerobic fusiforms, and spiral shaped bacteria and spirochaetes (Savage, McAllister and Davis, 1971). They do not appear to attach directly to the epithelial cells, but colonize the mucus investing the mucosal epithelium of the caecum and colon. Light and electron microscope studies show layers of these bacteria on the epithelium.

Mucosal association of bacteria in the gastro-intestinal tract of animals appears to be selective: if removed by antibacterial drugs the micro-organisms will recolonize the same epithelium after the therapy is discontinued (Savage and Dubos, 1968; Savage, 1969). This may in turn influence the composition of the bacterial flora within the lumen of the gastro-intestinal tract.

Studies on the mucosal flora of the human intestine are few. Nelson and Mata (1970) in a light and electron-microscope study of gastro-intestinal biopsies demonstrated the presence of anaerobic streptococci embedded in the tags of mucus attached to the mucosa of the colon, especially at the tips of the villi folds.

In an electron-microscopy study, Harland and Lee (1967) demonstrated the presence of spirochaetes adherent to the surface of the epithelium of rectal biopsy specimens. These specimens came from persons with persistent diarrhoea, who could not be called 'normal' subjects. However, further studies by Gear and Dobbins (1968) and Lee et al. (1971) have indicated that these spirochaetes may be harmless commensal organisms. Intestinal spirochaetosis is confined to only a small proportion of humans (Takeuchi et al. 1974).

A summary of studies on the bacterial flora associated with various parts of the gastro-intestinal tract is given in Table 6, and the work is reviewed by Savage (1972).

The mechanisms underlying microbial association with the gastro-intestinal epithelia are not well understood. The physiology of the

TABLE 6

Indigenous micro-organisms associating with mucosal epithelia in the gastro-intestinal tracts of adult mammals.

Micro-organism	Animal	Number of micro-organisms per gram of whole bowel	Epithelium on which localised	Reference
Lactobacilli	Mouse	10^9	Non-secreting, stomach	Savage et al. 1968.
	Rat	?	Non-secreting, stomach	Brownlee and Moss, 1961.
Yeasts	Mouse	10^7-10^8	Secreting, stomach	Savage and Dubos, 1967.
	Rat	?	Secreting, stomach	Brownlee and Moss, 1961.
Anaerobic streptococci	Mouse	10^9	Non-secreting, stomach	Savage et al. 1968.
	Rat		Villous, ileum	Savage, 1969a.
	Human*	10^8-10^{10}	Mucosal, colon	Nelson and Matta, 1970.
Spirochaetes	Mouse	10^9-10^{10}	Mucosal, caecum and colon	Savage et al. 1971. Gordon and Dubos, 1970
	Human*	?	Rectum	Harland and Lee, 1967.
Spiral-shaped bacteria	Mouse	10^9	Mucosal, caecum and colon	Savage et al. 1971.
Fusiform-shaped bacteria	Mouse	10^{11}	Mucosal, caecum and colon	Savage et al. 1968, Gordon and Dubos, 1970
	Human*	?	Villous, ileum	O'Brien, 1968.

* = Patients with bowel diseases.

underlying epithelium is probably important, but this aspect has not been extensively studied. Gastro-intestinal epithelium is composed of columnar cells in most areas and of stratified squamous epithelium in portions of the stomach of certain animal species (Ham, 1965; Hummel, Richardson and Fekete, 1966). The columnar cells multiply in the crypts of Lieberkühn and migrate to the tips of the villi or the luminal surface (Leblond and Stevens, 1948): as they migrate, the activity levels of certain enzymes change, e.g. alkaline diphosphatases, disaccharidases and dipeptidases (Nordström, Dahlquist and Josefsson, 1967; Moog and Grey, 1968). These enzymes are involved in cell membrane function, and changes in membrane physiology could affect the ability of cells to accept attaching bacteria.

The role of the bacterial cell surface in attachment to epithelia has received more attention. The mechanism by which lactobacilli attach to the epithelium of the chicken crop has been studied using sophisticated histological techniques (Fuller and Brookes, 1974). Ruthenium red, an electron-dense substance with an affinity for carbohydrate, was used to demonstrate the presence of carbohydrate strands which anchor the lactobacilli to the epithelium. Other surface properties of bacterial cells probably mediate their attachment to mucosal epithelia, but little is known about them. Bacterial pili could possibly serve to secure some microbes to epithelial membranes (Swanson, Kraus and Gotschlich, 1971) and certain strains of E. coli have been shown to adhere through pili to mammalian erythrocyte membranes (Brinton, 1965). Jones and Rutter (1972) showed that pathogenic E. coli attach to small gut mucosa via the K88 antigen which is a protein.

Micro-organisms that do not attach to epithelial cells, but colonize mucin must be highly adapted to the environment in the mucin and may be able to use the mucin as a source of carbon and energy. Germ-free animals excrete more intact intestinal mucin than do conventional animals and several species of intestinal bacteria are known to hydrolyse mucin (Hoskins and Zamcheck, 1968).

Part of this thesis describes an investigation of the mucosal flora of the human colon (Section 3.7).

1.3. Factors influencing the faecal flora.

Except where there is successful invasion by an external pathogen the gut flora is probably a stable self-regulating system. The adult faecal flora is the result of bacterial interactions, host reactions and external influences such as diet. In considering these various aspects separately, it must be remembered that the gut flora consitutes a complex ecosystem and the aspects considered below are very much inter-related.

1.3.i. Diet.

Diet has long been recognized as one of the determinants of the types of bacteria occurring in the intestine (Dudgeon, 1926). It is generally agreed that the gastrointestinal tract of the foetus is sterile

and that the host acquires its intestinal flora via the mouth. Infants born with intestinal obstruction acquire the usual colonic bacteria proximal to the site of obstruction (Bishop and Anderson, 1960). Smith and Crabb (1961) have described the development of the faecal flora in children. During the first few hours of life the intestine may become sparsely contaminated with enterococci and coliform organisms. When feeding is begun the number of bacteria in the intestine greatly increase.

In 1900, Tissier noted that a Gram-positive, non-sporing anaerobe predominated in the stools of breast-fed infants. Similarly, Gylenberg and Carlberg (1958) reported the dominance of a specific nutritional type of bifidobacterium in such stools, and Haenel (1961) showed that E. coli and Strep. faecium were also present.

Infants fed on cows' milk preparations characteristically have other bacteria, including large numbers of bacteroides, in their stools. The number of clostridia isolated is also greater than from the stools of breast-fed babies (Bullen and Willis, 1971). These differences in the faecal flora of the two groups of infants have been attributed, to some extent, to the breast milk in the diet (Willis et al. 1974). Breast milk has a high lactose content and low buffering capacity. Enterococci and coliform organisms contaminating the gut in the first few days of life ferment lactose, and the resultant pH is not adequately buffered. This provides a favourable environment for subsequent colonization of the large gut by bifidobacteria. After weaning the faecal flora becomes similar to that of the adult (Bengid, Haenel and Braun, 1968). Haenel (1958) showed that the faecal flora of breast-fed infants could be mimicked in other infants to

some extent by the feeding of lactulose. The dominant bifidobacterium in the faeces of breast-fed infants is Bifidobacterium infantis (Dehnert Group III or IV, Denhert, 1957, 1960; Bergey, 1974). Bifidobacterium bifidum is also found.

Bullen, Rogers and Leigh (1972) showed that the iron-binding protein lactoferrin, which is present in substantial amounts in human milk, but not in cows' milk, in combination with specific antibody to E. coli has a powerful inhibitory effect on the growth of this organism. This may go some way to explain the resistance of breast-fed babies to gastro-enteritis caused by enteropathogenic strains of E. coli.

Manipulation of the adult faecal flora by diet has proved difficult. The beneficial effects of a lactobacillus flora were suggested by Metchnikoff (1908) who believed that autointoxication by bacterial toxins produced in the intestine could be prevented if lactobacilli were the predominant bacteria in the gut. Various workers tried to find diets to encourage this type of flora. Their results were equivocal (Rettger and Cheplin, 1921).

More recent attempts to alter the adult faecal flora have had varying degrees of success. Moore et al. (1969) found that a change to a vegetarian diet produces no change in the predominant faecal flora. Hoffman (1964) in long term studies on a single subject showed that high protein diets did not affect the faecal flora, whereas high carbohydrate diets increased the relative numbers of bifidobacteria and high fat diets favoured the bacteroides. Speck and his colleagues (1970) fed volunteers on entirely

digestable diets containing (a) high protein, (b) high fat and (c) high carbohydrate. They found a marked stability of the normal flora and although at the beginning of the experiment there was a small fall in total faecal flora, this gradually reverted and in any case was not related to the type of diet.

Experiments by Winitz et al. (1970) with a no residue diet have indicated substantial reduction in bacterial counts and types. This effect was attributed to 'Starvation' of the bacteria. A similar study by Finegold et al. (1970) showed that extremely oxygen-sensitive anaerobes disappeared from the faeces if a completely absorbable diet was consumed, but no other consistent change occurred. The marked reduction in bacterial numbers reported by Winitz et al. was not observed by Finegold et al.

Studies of groups of people on different diets in various countries (Hill et al., 1971) have demonstrated some differences in the relative numbers of various groups of bacteria which may reflect the effect of diet although there were also other differences between the populations. The most striking differences were that people living in Britain or the U.S.A. on a mixed 'western' type of diet had more bacteroides and fewer enterococci and other aerobic bacteria than people living on largely vegetarian diets in Uganda or South India. It was postulated that the difference might be attributable to the low-fat content of the diet in Uganda and India.

Crowther (1971b) found that Sarcina ventriculi was an organism practically confined to vegetarian populations; whether this reflects

cellulose in the diet is uncertain. Filamentous fungi are also more common in the faeces of the people with largely vegetarian diets than those from people from Britain and the U.S.A., consuming 'western' diets.

1.3.ii. Bacterial interactions.

Bacterial interactions within an ecosystem may be either synergistic or competitive.

Proliferation of aerobic organisms within the large intestine is synergistic for the growth of anaerobes in providing the reducing conditions necessary. Antibiotics that have no effect on strict anaerobes *in vitro* may affect them *in vivo* indirectly by acting on the aerobes, leading to a subsequent rise in available oxygen in the gastro-intestinal tract. Streptomycin treatment of mice eliminates practically all bacteria from the gut althouth this antibiotic is not toxic for many species of anaerobe (Meynell, 1963). Direct measurements have shown that the Eh of the gut contents in germ-free animals is ~~high~~ higher (+35±22mV) than in recontaminated animals (-206±40mV) or than in the normal state (-246±24mV) (Worstmann and Bruckner-Dardoss, 1966).

Another example of synergy is the synthesis of factors essential for one organism by another, e.g. the ability of *Staph. aureus* to provide compounds similar to vitamin K for the growth of certain strains of *Bacteroides melaninogenicus* (Gibbons and Macdonald, 1960).

In a few cases the volatile fatty acids, produced by bacteroides and

Coliform organisms, act in a synergistic manner by stimulating the growth of other bacteria (Meynell, 1963; Hentges, 1969) e.g. Bacteroides succinogenes needs isobutyric and isovaleric acids for growth. More usually, however, fatty acid production acts in a competitive manner. In vitro, Lactobacillus acidophilus inhibits the growth of E. coli and Staph. aureus. The inhibitory agent is probably lactic acid (Wheater, Hirsch and Mattrick, 1952; Tramer, 1966). A Bifidobacterium (probably Bifidobacterium bifidum) inhibits the growth of E. coli and Strep. faecalis in vitro by the production of acetic and formic acids (Upton, 1929). Salmonella typhimurium is sensitive to butyric acid, which is produced by fusiform anaerobic bacteria (Meynell, 1963). Although in vitro studies may not reflect the true situation in vivo this may explain why Salmonella typhimurium fails to grow well in the alimentary canals of mice unless the alimentary microflora of such animals is disrupted (Meynell and Subbaiah, 1963). Bergeim (1940) emphasized that the antimicrobial effects of volatile fatty acids were not due to acidity alone. The toxic effects were related to the dissociated molecule and were therefore observed at pH values less than the dissociation constants of the volatile acids.

Many of the gut coliform bacteria liberate 'colicines' that are able to kill other related bacteria (Nomura, 1967). Serotypes which produce colicines appear to inhibit colonization ofthe gut by other serotypes (Branche et al. 1963). Strep. faecium and Strep. faecalis also produce similar sorts of inhibitory agents, although those of Strep. faecalis also inhibit lactobacilli, bacilli, staphylococci and clostridia (Brook, Peacher and Pierson, 1963). Recently Collee, Watt and Dewhurst (1974) have reported the production of 'colicine-like' inhibitory substances by various

strains of bacteroides. They have called these substances 'bacteroidocines' and it is possible that these may exert an influence in determining the faecal flora.

Direct competition for nutrient sources (Brock, 1966) may play a role in determining the flora of colon and faeces. Organisms capable of utilizing substrates that others cannot, will have an ecological advantage and will proliferate. Possible examples of such substrates include mucin and other intestinal secretions.

1.3.iii. Bile.

The toxic effects of bile towards some bacteria have been known for a long time (Neufeld, 1900). Early workers showed *in vitro* that bile was much more toxic towards Gram-positive than to Gram-negative bacteria (Liefson, 1935; Floch *et al.* 1970). Some Gram-negative anaerobic bacteria, notably *Bacteroides fragilis*, are stimulated by whole bile (Beerens, Castel and Abraham, 1960; Barnes and Goldberg, 1968). Bile acids are secreted in the bile in the form of conjugates with taurine or glycine and many genera of bacteria, including the bacteroides, can rapidly deconjugate them (Shimada, Bricknell and Finegold, 1969). It has been shown by Floch *et al.* (1971) that bile tolerant intestinal anaerobes may be inhibited by unconjugated bile acids such as cholic acid and deoxycholic acid. Percy-Robb and Collee (1972) showed that to some extent this is a pH associated effect, the higher the pH, the less toxic the unconjugated acid.

There is, however, little direct evidence that bile has a selective action *in vivo*. Only the sparse populations of the upper-small intestine will be influenced by whole bile, since bile is subject to enterohepatic circulation. The direct antibacterial effect of relatively large amounts of cholic acid may extend to the large bowel in patients with a resection of the terminal ileum, who have reduced bile acid absorption.

1.3.iv. Specific antibody.

The predominant immunoglobulin (Ig) in the secretions of the gastro-intestinal tract is IgA (Chodirker and Tomasi, 1963). Tomasi *et al.* (1965) reported that secretory immunoglobulins in external secretions differ in composition to serum antibodies. Secretory IgA (SIgA) has a sedimentation coefficient of 11 Svedberg units and is a dimer of two 7S IgA molecules (Serum IgA) plus a 'secretory component' and a 'J' chain linking the two molecules together. It is secreted as a dimer by the plasma cells of the sub-mucosa and the 'secretory component' is added in the mucosa before the molecule is secreted into the lumen of the gut. In the gut it forms a specific local system of immunity. Its local production has been demonstrated by immunofluorescence studies or orally administered ferritin (Crabbe *et al.* 1969 and 1970), and polio virus (Ogra *et al.* 1968).

The number of IgA-producing plasma-cells varies in different sections of the intestine, with the highest number being in the colon

(Crabbe and Heremans, 1966). There are scattered IgD and IgE containing cells in other portions of the gastrointestinal tract, especially in proximity to mucus glands (Hanson and Brandtzaeg, 1973). Few IgM producing cells have been detected and IgG, presumably of serum origin, exists primarily in an interstitial distribution in jejunal and colonic secretions.

The mode of action of IgA remains obscure. It is doubtful if it can be bactericidal as this requires complement for the lysis of bacteria and there is a school of thought that believes that IgA antibodies are unable to fix complement (South et al. 1966; Ishizaka et al. 1966). A report to the contrary suggests that IgA can accomplish bacteriolysis in the presence of complement and lysozyme (Adinolfi et al. 1966). Complement may be fixed by an alternative pathway.

It has been suggested that IgA may act as an opsonin, rendering bacteria more available for phagocytosis (Shklair, Rovelstad and Lamberts, 1969; Wernet et al. 1971). Other workers have variously suggested that IgA acts as an antitoxin (Kaur, Burrows and Furlong, 1971) or by interfering with bacterial growth (Brandtzaeg, Fjellanger and Geruldson (1968), or by masking antigenic sites on commensal bacteria (South, 1971) and dietary antigens (Beale et al. 1971). As an agglutinin, IgA, may be important in the prevention of adherence of bacterial pathogens, such as Vibrio cholerae to intestinal mucosa (Freter, 1969), or by inhibiting the actions of pathogens which adhere to the mucosa (Freter, 1970). During the present study, mucosa from the colon maintained in organ-culture has been shown to exert some antibacterial effect, but the exact nature of this is yet to be elucidated (Section 3.9.ii).

The influence of immunoglobulin on the faecal flora is not fully understood. Initiation of SIgA synthesis seems to be antigenic stimulation consequent on bacterial colonization of the gut. Germ-free animals have fewer lamina-propria immunocytes and serum IgA is about 5% of their conventionally reared litter-mates. The effects on serum IgG and IgM are less (Crabbe et al. 1969). Parkin et al. (1972) studied the jejunal flora of five patients with hypogammaglobulinaemia and could find no difference in the total numbers of organisms in comparison with a suitable control group. They concluded that IgA deficiency was having little effect on the gut flora in these cases.

Immunoglobulins, together with macrophages and local enzyme systems may prevent invasion of the mucosa and underlying tissues by micro-organisms and macromolecules (Walker and Hong, 1973). Shearman, Parkin and McClelland (1972) and Walker and Hong (1973) have reviewed the function of antibodies in the gastrointestinal tract.

1.3.v. Mechanical cleansing and antibacterial agents.

The effects of mechanical cleansing and antibacterial agents are imposed on the faecal flora by man and as such can be regarded as in a separate category from the factors considered above.

The question of desirability of 'intestinal antisepsis' prior to bowel surgery remains controversial, although it is generally agreed that

some form of mechanical cleansing facilitates operations on the colon by diminishing distension and reducing the possibility of faeces spillage. Mechanical cleansing usually consists of the administration of enemas and colon washouts together with a low residue diet (e.g. Cohn, 1968). Tyson and Spaulding (1959) claimed that mechanical preparation can reduce the flora of the remaining faeces by 99%, although this is not borne out by other workers (Bornside and Cohn, 1969) or by work with laboratory animals (Nance and Bornside, 1969).

More often, pre-operative mechanical cleansing is combined with the administration of antibacterial agents in an effort to lower the number of bacteria in the residual faeces and thus to diminish the risk of post-operative peritonitis, wound infection or breakdown of anastomoses which may result from contamination from this source. Similarly, in cases of acute myeloblastic leukaemia where patients have been treated with cytotoxic drugs, reduction of the bowel flora, combined with a 'germ-free' environment, may be desirable.

In another sphere, control of the gut flora by antibiotics may be beneficial to patients with hepatitic coma. This is thought to be due to reduction of the intestinal flora which produces ammonia. Although urease producing bacteria have been considered the major offenders, organisms producing ammonia by deamination may be equally as important (Finegold, 1970).

Various mixtures of antibacterial agents have been used in pre-operative bowel preparations and the less soluble sulphonamides (e.g. succinylsulphathiazole and phthalylsulphathiazole) were first used for this

purpose (Garlock and Seley, 1939). Such compounds owe their activity to the slow liberation of sulphathiazole in the bowel by intestinal bacteria. Aminoglycosides in combination with sulphonamide or alone have also been widely used. Neomycin, kanamycin, paramycin and framycetin, when given orally tend to eliminate the aerobic faecal flora and to reduce the Gram-positive anaerobic flora (Hewitt and Finegold, 1969; Finegold et al. 1965; Cohn, 1968). Sellwood et al. (1969) found a combination of neomycin, bacitracin and nystatin more effective than phthalysulphathiazole in lowering the numbers of bacteria in faeces and in preventing post-operative infections. Everett, Brogan and Nettleton (1969) found that addition of neomycin to a regime of pre-operative preparation caused a reduction in the number of times that various species of bacteria were isolated from rectal swabs and from gut mucosa. They failed to show that this was of benefit to the patient (i.e. in preventing post-operative infections). Bornside and Cohn (1969) looked at the effect on the faecal flora of pre-operative treatment with clindamycin and found that whilst the number of anaerobes was reduced, the aerobes were not. To overcome this, they suggested a combination of clindamycin with kanamycin. The recent reports of the incidence of pseudomembranous colitis associated with the use of clindamycin (Marks, personal communication) may now cast doubt on this suggestion.

It should be remembered that where surgical procedures are involved no amount of pre-operative antibiotic treatment can take the place of adequate aseptic technique. In addition, the rationale concerning the choice of antibacterial needs the backing of in vitro sensitivity tests, such as those of Gunn and Gould (1965), bearing in mind of course that some agents may be inactivated in vivo by the presence of faeces (Finegold, 1970).

In this thesis are reported the results of a double-blind randomized study of the effects of pre-operative mechanical bowel preparation with and without administration of phthalysulphathiazole (Section 3.6).

1.4. Anaerobes and disease.

1.4.i. The sporing anaerobes - Clostridial infections.

Many of the conditions caused by clostridia are classical infections and are fully described elsewhere, e.g. Willis (1969). Only a brief outline will be given here.

Perhaps the most widely recognized infection due to anaerobes is clostridial myonecrosis or gas-gangrene. The causative organisms include Clostridium perfringens, Clostridium histolyticum, Clostridium septicum, Clostridium novyi and Clostridium bifermentans. Swelling, pain, a sero-sanguinol exudate, gas bubbles from the wound and local myonecrosis are characteristic. Large Gram-positive rod-shaped bacteria may be recovered from the wound.

Septicaemia due to species of clostridia may occur secondary to gas gangrene and has a high fatality. Clostridial toxin causes shock and

massive haemolysis often leading to renal failure (Smith et al. 1971).

Anaerobic cellulitis is a localised clostridial infection which does not invade muscle tissue (Maclennan, 1962).

Enterocolitis or Clostridium perfringens food poisoning is a relatively common illness due to type A strains. Symptoms are mild and self limiting and organisms can be isolated from stool samples and contaminated food. A much more virulent form of clostridial food poisoning known as enteritis necroticans is associated with ingestion of food (often pork) heavily contaminated with Clostridium perfringens type C. This is a rare condition with a high mortality. Pathologically there is widespread necrosis of the bowel (primarily small bowel) (Zeissler, 1949; Murrell, 1966).

Tetanus and botulism are important conditions caused by toxins elaborated by clostridia. Tetanus results from the proliferation of Cl. tetani in deep wounds and botulism is caused by injestion of toxin produced by Cl. botulinum in food. In both cases, the toxins attack the central nervous system.

1.4.ii. Infections with non-sporing anaerobic bacteria.

As long ago as 1893, Veillon reported the recovery of an obligate anaerobic coccus from fetid pus. In 1897, Veillon and Zuber reported the

association of bacteroides-like organisms in a variety of gangrenous suppurative lesions and in 1898 these workers noted that anaerobic fusiform bacilli were the commonest organisms associated with acute appendicitis.

Little attention was paid to these organisms by clinical workers or microbiologists, until, with the advent of modern methods for the cultivation of anaerobes, interest has been revived. Bornstein reviewed the current knowledge in 1964 and more recent reviews include those of Moore, Cato and Holdeman (1969), Gorbach and Bartlett (1974) and Finegold (1974) Moore et al. (1969) claimed that 85% of clinical specimens examined in their laboratory contained non-sporing anaerobes.

The majority of anaerobic infections are of endogenous origin, arising frequently from the mucous membranes of the intestinal tract, the mouth and the female genital tract.

Bacteroides infections.

During the years following 1897 bacteroides were described as pathogens in infections at various different sites, e.g. post-partum infections (Lemierre, 1936), appendicitis (Weinberg et al. 1928), lung abscesses (Cohen, 1932) and other lesions related to ear, nose, throat, gastrointestinal (GI) tract and genito-urinary tract structures (Lemierre, 1936). The situation was reviewed by Smith and Ropes (1945) and Beigelman and Rantz (1949). Gunn summarized the literature in 1956.

Bornstein and his colleagues (1964) reviewed the records of 112 patients from whom Bacteroides species were isolated: in almost half the cases bacteroides alone were isolated and anaerobic streptococci were the partners in half of the mixed infections. There was a frequent relationship of infections to the G.I. tract and the female genital structure. Ear, nose and throat foci were rare. However, Smith and Ropes (1954) demonstrated the almost constant association of bacteroides meningitis and brain abscesses with chronic otitis media, and McVay and Sprunt (1952) showed that most bacteroides pulmonary infections are associated with putrid lung abscesses, empyema and sometimes with pneumonitis.

Dixon (1937) noted the association of malignant disease of the large bowel with post-operative bacteroides infections and Rubin and Boyd (1958) reported intra-abdominal abscesses and wound infection related to ulcerative colitis and diverticulitis. As early as 1928, Weinberg noted the association of bacteroides with normal as well as inflamed appendices and Gillespie and Guy (1956) demonstrated their presence in inflamed appendices and post-appendiceal peritonitis. Bacteroides fragilis is one of the dominant members of the normal human faecal flora and plays a major role in intra-abdominal infections. In a recent study of 501 patients with 540 intra-abdominal abscesses, Altemeier et al. (1973) found that at least 50% of the bacteria recovered were anaerobic, mostly bacteroides.

A study by Nichols, Thadepalli and Gorbach (1972) indicated the importance of bacteroides species in wound infections following abdominal trauma.

The female genital tract is a frequent site for bacteroides infections. In a study by Swensen (1974) anaerobic bacteria were isolated from 74% of female genital tract infections. Bacteroides fragilis and Bacteroides melaninogenicus were most commonly found. Convalescence from obstetric and gynaecological infections is much more protracted in patients from whom bacteroides are isolated than in patients with similar infections from whom this organism is not isolated (Finegold, 1974). Anaerobes, in particular, Bacteroides fragilis, are of particular importance in post-abortal sepsis (Rotherham and Schick, 1969).

Most reported cases of anaerobic bacteraemia have followed infections primarily in the female genital tract, gastrointestinal tract, pelvic and pharyngeal areas. In recent years, reports of bacteraemia due to anaerobes have increased. Septic thrombophlebitis is often a complication of bacteroides bacteraemia. The mortality in bacteroides bacteraemia is over 60% in patients over the age of 40, but only 6% in younger patients (Gelb and Seligman, 1970).

Infections due to anaerobic cocci.

Most known infections involving anaerobic cocci are associated with the Gram-positive peptococci and peptostreptococci (Pien et al. 1972). When isolated in pure culture the pathogenic significance can more easily be determined than in the usual situation where these organisms are isolated in mixed culture with other anaerobes or facultative organisms.

Early work was primarily focused on the association of these organisms with infections in the upper-respiratory and gastrointestinal tracts and with puerperal sepsis. There were also studies on the role of these organisms in special types of wound infection (Brewer and Meleney, 1926; Sandusky et al. 1942). The special case of progressive synergistic gangrene, an infection of wounds is interesting. An anaerobic or micro-aerophilic streptococcus is found in synergy with an aerobe, usually a staphylococcus. It is possible that the anaerobe is the pathogen, but needs the reduced conditions generated by the aerobe, for growth (Schwarz and Diekmann, 1927). Peptostreptococci have been associated with cellulitis, and myositis (Maclennan, 1943) and frequently associated with brain abscesses (Heineman and Braude, 1963). Anaerobic cocci, in general, have been associated with mouth infections, lung infections, brain abscesses, female pelvic infections, and resultant septicaemias.

Anaerobic diphtheroid infections.

There are scattered references to these bacteria as causative organisms of brain abscesses and endocarditis (Kaplan and Weinstein, 1969; Smith and Bodily, 1968), but where diphtheroids are isolated in mixed culture it is probable that they are contaminants from normal flora (Rosebury, 1962).

1.4.iii. Intestinal bacteria and the aetiology of cancer of the colon.

Cancer of the large bowel is much more common in North-West Europe and in North America than in Africa, Asia and South America (Doll, 1967 and 1969). The areas with a low incidence of the disease (e.g. Uganda, 0.6 per 100,000 population per year) have a low standard of living and high incidence areas such as England (18.2 per 100,000), Scotland (31.2 per 100,000) and the U.S.A. (28.1 per 100,000), have a high standard of living. The differences in cancer incidence are not explained merely on a racial basis: Japanese who become 'westernized' after migration to California (Buell and Dunn, 1965), or because of an increase in the standard of living in Japan (Wynder et al. 1969) have an incidence of colon cancer that is higher than that of Japanese living in the traditional style. Geographical location can also be discounted as different ethnic groups in Hawaii and South Africa (Doll, 1969) and Singapore (Lee, 1973) show different incidences of colon cancer.

Several epidemiological studies have indicated a possible relationship between colon cancer and diet. Wynder and Shigematsu (1967) showed a correlation with dietary fat and Gregor, Toman and Prusova (1969) showed a correlation with protein. A positive correlation with refined sugar in the diet and a negative correlation with 'fibre' has also been suggested (Burkitt, 1969 and 1971). Drasar and Irving (1973) showed that dietary animal protein and fat correlate more closely with the incidence of colon cancer than do other dietary components or various measures of living standards.

Inhoffen (1953) reviewed the structural similarity between steroids

and the polycyclic aromatic carcinogens and a number of bile steroids have been shown to be carcinogenic in animal studies, e.g. deoxycholic acid (Badger et al. 1940; Cook, Kennaway and Kennaway, 1940; Ghiron, 1940), bis-nor Δ^5 choleric acid (Lacassagne, Buu-Hoi and Zajdeta, 1961), apocholic acid (Lacassagne, 1966) and oestradiol (Lacassagne, 1932).

Aries et al. (1969), Hill et al. (1971) and Drasar and Hill (1972) postulated that intestinal bacteria might be able to produce carcinogens from bile steroids that are released in response to fat in the diet. Deoxycholic acid can be produced in vitro from the bile steroid, cholic acid, by several strains of intestinal bacteria (see Section 1.5). The amount of bile acid in faeces is dependent on the amount of fat in the diet (Hill, 1971). A survey conducted by Hill et al. (1971) examined the bacterial flora and steroids of faeces from people in England, Scotland and the U.S.A. where the incidence of large bowel cancer is high and of people from India, Uganda and Japan, where the incidence of this disease is low. The amount of faecal steroids (acid and neutral) was lower in the 'low-incidence-countries' than in the 'high-incidence-countries', where they were also more degraded. There were more Bacteroides spp. and fewer enterococci isolated from faeces from 'high-incidence-countries' than from 'low-incidence-countries'. Fifty percent of Bacteroides spp., 30% of Clostridium spp., and 40% of Bifidobacterium spp., isolated from the faeces of English persons could dehydroxylate bile acids. In contrast, the reaction was carried out by very few strains isolated from Ugandan faeces.

Hill (1971) postulated a pathway entailing four types of reaction by which a polycyclic aromatic type compound could be produced by gut bacteria

from faecal bile steroids (Section 1.5) and these reactions have since been shown to be carried out by gut bacteria. A key reaction is performed almost exclusively by an organism tentatively identified as Clostridium paraputrificum (see Section 1.5). Goddard (1973) showed that strains of Cl. paraputrificum capable of performing this reaction were isolated more frequently from the stools of persons from England and Scotland, than from the stools of persons from Uganda and Hong Kong. In addition Cl. paraputrificum was isolated more frequently from faecal samples from colon-cancer patients than from control groups with no large bowel carcinoma. These patients also had increased amounts of faecal steroids.

Part of the work described in this thesis is concerned with an investigation of the faecal flora and metabolic activities of faecal isolates from persons of different socio-economic class within one country (Hong Kong). The incidence of large-bowel cancer in Hong Kong correlates well with socio-economic position (Section 3.10). Future investigations must involve more detailed studies of the minor faecal bile acids, both qualitatively and quantitatively, to determine their potential as a source of carcinogen within the large bowel.

1.5. Bile acid metabolism by intestinal bacteria.

Human bile acids are substituted cholanic acids conjugated to taurine and glycine. The glycine conjugates are most common, dominating over the

TABLE 7

Demonstration of
In-vitro bile acid metabolism by intestinal bacteria

Reaction	Bacteria	Reference
Deconjugation	Clostridium perfringens	Norman and Grubb, 1955
	Strep. faecalis	
	Bacteroides sp.	Drasar, Hill and Shiner, 1966
	Cl. perfringens	Gordon and Reback, 1967
	Staph. aureus Strep. faecalis Bacteroides sp. Bifidobacterium sp. Veillonella sp. Clostridum sp.	Hill and Drasar, 1968
	Bacteroides sp. Bifidobacterium sp. Clostridium sp. Strep. faecalis	Aries and Hill, 1970
Oxidation of hydroxyl functions and dehydroxylation	Strep. faecalis Bacteroides sp. Clostridium sp. Veillonella sp.	Hill and Drasar, 1968
	Bacteroides sp. Bifidobacterium sp. Bacillus sp. Clostridium sp. Veillonella sp. Enterococci and Enterobacteria Anaerobic sarcinae	Aries, Crowther, Drasar and Hill, 1968
	E. coli Strep. faecalis Cl. perfringens Cl. sporogenes Bacteroides sp. Bifidobacterium sp. Bacteroides fragilis	Aries and Hill, 1970

taurine conjugates by a ratio of 3 : 1 (Hofmann, 1968). In addition to the conjugates of the two primary bile acids, cholic and chenodeoxycholic acid, deoxycholic acid, which results from the 7-α-dehydroxylation of cholic acid (see below), is found in normal human bile. After transit through the small intestine the bile acids are reabsorbed from the terminal ileum by an active transport mechanism (Lack and Weiner, 1961). Only about 5% of the bile acid pool is lost and passed in the faeces (Kern and Meiroff, 1970).

The faecal bile acids are deconjugated and degraded in a variety of ways. This appears to be due to bacterial action: degraded bile acids are not found in the stools of germ-free animals, but can be detected when the gastro-intestinal tracts of these animals are colonized with bacteria (Gustaffson, Midtvedt and Norman, 1968).

Many types of enzymatic reaction have been postulated to account for the production of these degraded bile acids. In the present study, only the four types of enzymatic reaction which have been postulated for the production of a polycyclic aromatic compound (Hill, 1971) have been considered. The implications of these reactions in the aetiology of cancer of the large bowel are outlined in Section 1.4.iii. These four types of reaction are discussed below. All have been demonstrated in vitro with intestinal bacteria, and a summary of previous work is outlined in Table 7.

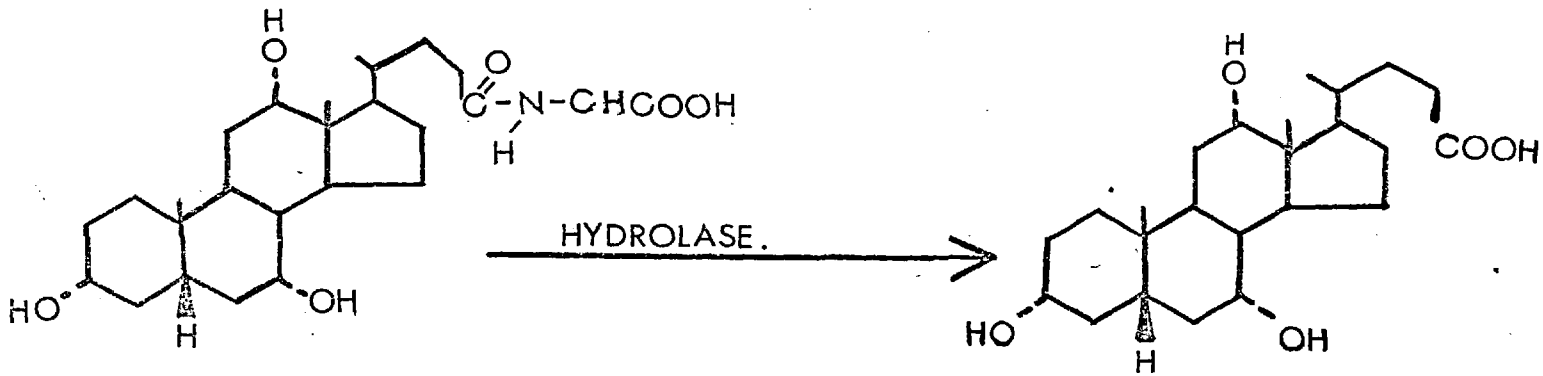
a) Deconjugation involves hydrolysis of the amide bond to release free bile acids. In-vitro reactions by intestinal bacteria are

Figure 1.

Deconjugation and dehydroxylation of bile acids.

BILE ACID METABOLISM BY INTESTINAL BACTERIA

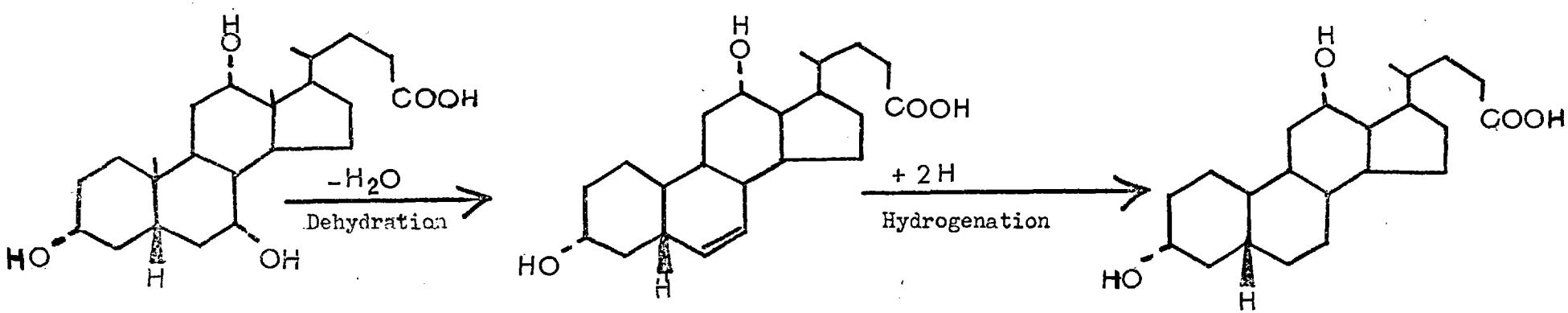
DECONJUGATION.



Glycocholic acid

Cholic acid

DEHYDROXYLATION.



Cholic acid

Deoxycholic acid

DEHYDROXYLASE.

reviewed by Shimada et al. (1969) and Lewis and Gorbach (1972).

b) Oxidation of the steroid hydroxyl functions to a keto group.

c) A dehydroxylation reaction which removes the 7-α-hydroxyl group yielding deoxycholate from cholic acid and lithocholate from chendeoxycholate.

Many of the anaerobic bacteria isolated during the present study were screened for their ability to carry out reactions (a) and (c) and examples of these reactions are shown in Figure 1.

d) Dehydrogenation of the steroid nucleus, to produce a polycyclic aromatic type of compound. There are four principal types of reaction by which double bonds may be introduced into the steroid nucleus by bacterial action (Hill, 1971):

A. Introduction of a double bond in conjugation with a keto group.

B. Oxidative methylation of the carbon 19 group linked to the introduction of double bonds to aromatise ring A.

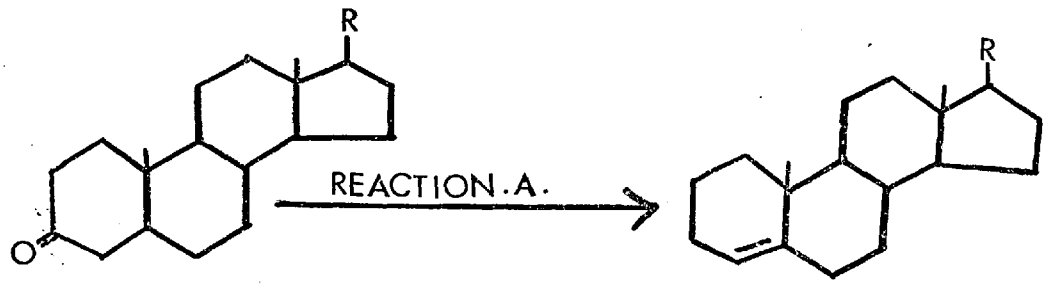
C. Double bonds introduced in association with other double bonds.

D. The introduction of a double bond by a dehydration reaction in conjugation with another double bond and keto group.

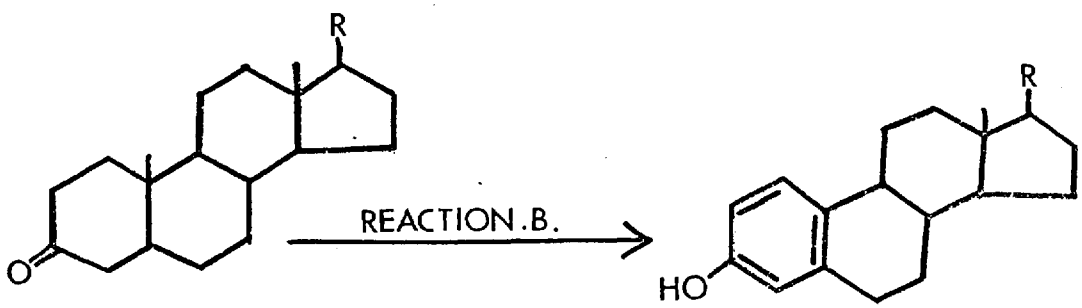
Figure 2.

Dehydrogenation of the steroid nucleus.

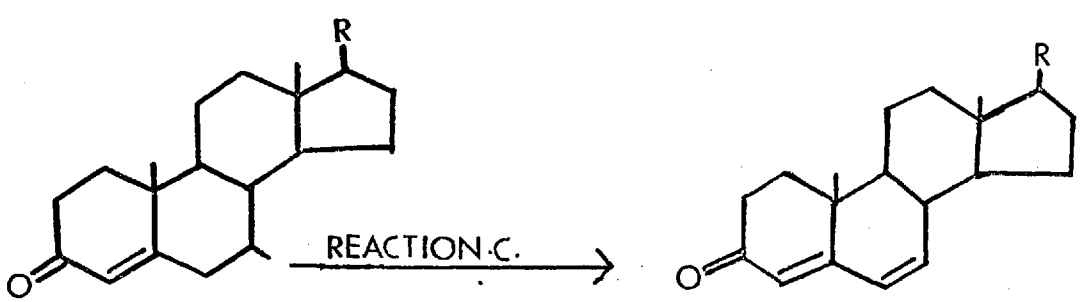
NUCLEAR DEHYDROGENATION REACTIONS.



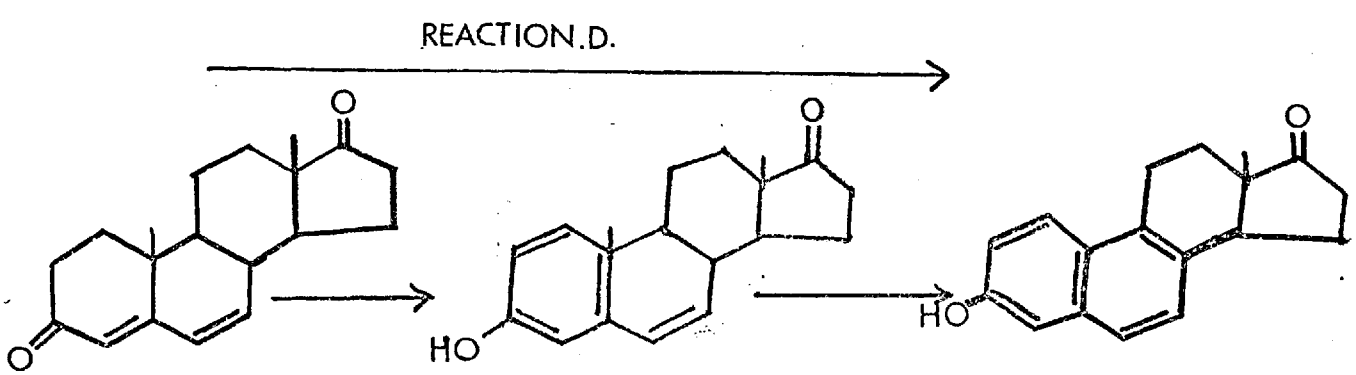
Aries et al., 1971.



Goddard + Hill, 1972.



Goddard + Hill, 1973

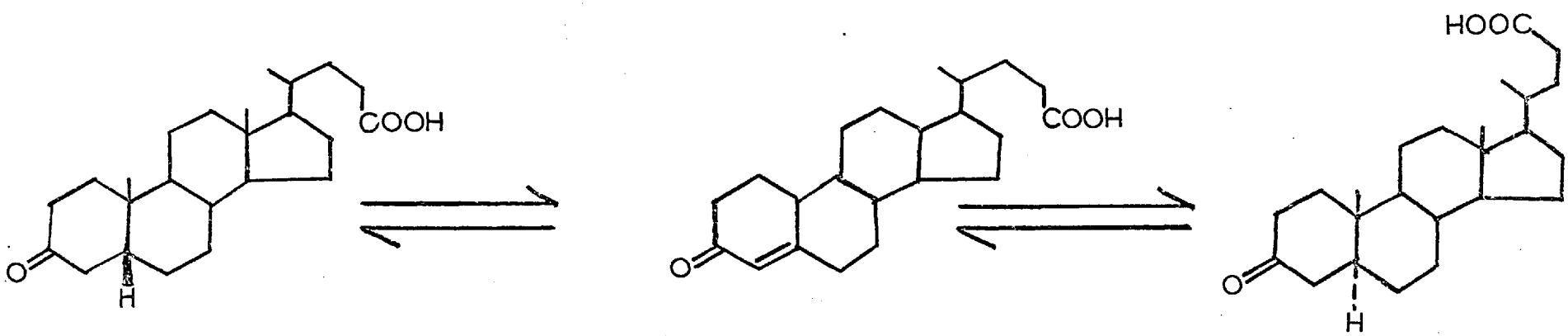


Goddard + Hill, 1973.

Figure 3.

Production of allo bile acids (Kallner, 1967).

INTERCONVERSION OF THE 5β CHOLANIC ACIDS AND THEIR 5α ANALOGUES VIA A Δ^4 INTERMEDIATE



3-oxo-5β-steroid

4-en-3-one intermediate

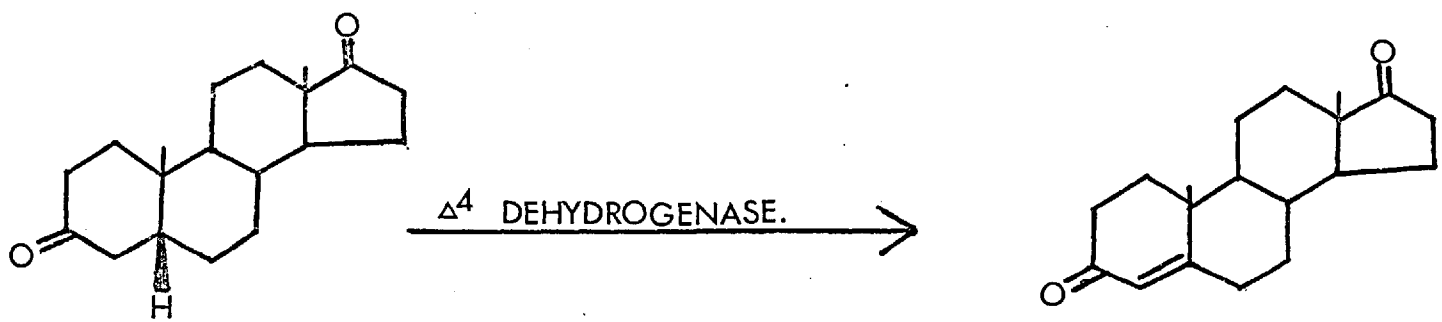
3-oxo-5α-steroid

Figure 4.

The reaction catalysed by Δ^4 dehydrogenase.

Clostridia isolated during the present study were screened for production of this enzyme (Section 2.10.iii)

INTRODUCTION OF A DOUBLE BOND INTO THE STEROID NUCLEUS BY INTESTINAL BACTERIA



5β-androstan-3,17-dione

4-androsten-3,17-dione

These reactions have all been demonstrated *in vitro* with intestinal bacteria and are shown in Figure 2. The reactions are carried out almost exclusively by clostridia, characterized by their lack of production of a lecithinase. The most notable organism is *Clostridium paraputrificum*, although reaction 'A' has also been demonstrated with *Clostridium indolis*, *Cl. cadaveris*, *Cl. sartagoforum* and a few strains of *Cl. perfringens*, and reaction 'D' has been demonstrated with some strains of *E. coli*.

There is some evidence that nuclear dehydrogenation of bile acids takes place in the intestine. Bile acids with the 5α-configuration (the allo-bile-acids) have been isolated from the faeces of humans (Haslewood, 1967). The bile acids synthesized by the liver have the 5β-configuration. Kallner (1967) has shown that the intestinal bacterial flora is responsible for this inversion at C 3 via an unsaturated intermediate. This reaction is shown in Figure 3.

Some of the clostridia isolated during the present study were screened for their ability to produce a C4-5 double bond in the steroid nucleus and for convenience these experiments were carried out using an androstan as a substrate. This reaction is shown in Figure 4.

SECTION II.

MATERIALS AND METHODS.

SOURCE OF MATERIALS.

2.1. Faecal samples from 'normal' persons.

Throughout the investigations described in this thesis a number of faecal samples from healthy English volunteers were required to establish the validity of the methods used for cultivation. Such stools were included as 'standards' during all experiments involving the anaerobic chamber. The samples were all obtained from laboratory staff, both men and women, none of whom were taking antibiotics or had any known gastro-intestinal disorder.

2.2. Wound swabs.

In an attempt to compare the rates of isolation of anaerobic bacteria from clinical specimens, using various isolation techniques as described in Section 2.6, swabs with plentiful material were selected from routine wound swabs sent for examination to the clinical laboratories of St. Mary's Hospital, London. The swabs were collected and transported by a porter, and in many cases several hours had lapsed between taking the swab and its arrival at the laboratory.

TABLE 8

Populations studied.

(Persons living on different diets in various countries).

Diet	Population
Normal mixed western, high fat*	England; staff and students, St. Mary's Hospital Medical School, London.
Normal mixed western, high fat	Scotland; staff and students, Edinburgh University Medical School.
Normal mixed western, high fat	U.S.A.; staff members, Center for Disease Control, Atlanta, Georgia.
Rice diet, high carbohydrate*	India; villagers, Vellore, S. India; controls in a study of tropical sprue.
Matoke (boiled mashed bananas), high carbohydrate	Uganda; villagers, Kyandondo region; parents accompanying children to Kwashiorkor Clinic.
Fruits and vegetables, high carbohydrate	Japan; villagers, Yonga; mostly farmers or housewives.

* The commonly used terms "high fat" and "high carbohydrate" applied to diets denote "rich in fat" and "rich in carbohydrate" respectively.

2.3. Dietary groups studied.

The results of these studies are presented in Section 3.5.

2.3.i. Persons living on different diets in various countries.

Stool samples were obtained from groups of people consuming a mixed western diet in England, Scotland and the U.S.A., where the incidence of large-bowel cancer is high, and from people living on high carbohydrate diets in India, Uganda and Japan, where the incidence is low. The groups contained both men and women. All were healthy individuals aged between 20 and 50. Samples from the U.S.A. came from both black and white individuals. These groups are summarized in Table 8. A more detailed description of the groups follows:-

England

The English persons were staff and students of St. Mary's Hospital, Medical School, London, aged between 20 and 40. None had any known gastrointestinal disorder, none was taking antibiotics. They all consumed normal western mixed diets.

Scotland

The Scottish persons were all staff and students at Edinburgh University Medical School and lived on normal western mixed diets.

United States

The Americans were staff members at the Center for Disease Control, Atlanta, Georgia. They all lived on normal western mixed diets.

India

The Indians were villagers living near Vellore in South India. They were healthy controls in a study of tropical sprue. Their high carbohydrate diet was a traditional one of rice and vegetables and they consumed almost no animal protein or animal fat. The diet did however contain small amounts of vegetable oils, mostly from groundnuts.

Uganda

The Ugandans were villagers from Bantu tribes of the Kyandondo region. They were mostly healthy parents accompanying their children to the M.R.C. kwashiorkor clinic in Kampala; others were hospital staff. Their diet was mainly matoke (boiled mashed bananas) supplemented with cassava, maize, beans, other vegetables and tea. This diet is characteristically high in carbohydrate and low in protein and fat and contains little animal matter (H.J.L. Burgess, 1962; A.P. Burgess, 1962; Burgess, Morton and Burgess, 1962).

Japan

The Japanese were villagers living in Sakaiminata, near Yonga. They were mostly farmers and housewives and all lived on the traditional diet containing rice, fruit and vegetables, with little meat or fat.

TABLE 9

Source of faecal samples from Hong Kong.

Group	Income per month	Population
A	>£200	Academic staff and Relatives. University of Hong Kong Hospital (UHKH)
B	£80 - £120	Technical staff and Relatives. (UHKH)
C	~~<~~£40	Laboratory assistants and Relatives. (UHKH)

2.3.ii. Persons living on different diets within one country.

The country of choice for this study was Hong Kong, where diet is very much determined by income. The population was divided into three income groups and the parameters of these groups are shown in Table 9.

Persons in high income groups consume a diet with a high meat (animal protein) content whereas the lower income groups consume a more traditional 'high carbohydrate' diet.

2.4. Colonic mucosa and faecal samples from hospital patients involved in a trial of pre-operative sulphonamide administration.

Colonic mucosa and faecal samples were obtained for determination and comparison of their normal flora and of the effect on these flora of pre-operative sulphonamide administration.

The samples were obtained during a double-blind randomized study of 20 patients, half being given pre-operative antimicrobial therapy and mechanical cleansing and the others being given placebo and mechanical cleansing. The patients were large-bowel cancer suspects and all those included in the trial had resection of the colon at operation.

The test patients received 5 g. of 'Thalazole' (Upjohn Ltd., phthalyl sulphathiazole) night and morning for three days pre-operatively,

i.e. starting at the same time as normal bowel preparation (purgation). All the patients had a bowel preparation of (1) castor oil (30 ml on the first day and the evening before the operation) and (2) a 'Veri-paque' (Winthrop Laboratories, oxyphenisatin)enema on the evening before operation. All patients received a low residue diet for three days pre-operatively and on the day prior to operation, a fluids only diet was administered.

From each patient were obtained the following specimens:-

1) Pre-operatively, 0.5 g. (wet-weight) of stool. This was the first stool passed on admission to hospital (i.e. pre-treatment).

2) At operation, (a) 0.5 g. of 'stool' (colonic contents), (b) a small piece of mucosa (dissected from the sub-mucosa). A second piece of mucosa was sometimes obtained for organ culture (see Section 2.11), (c) if available, a small piece of tumour tissue (obtained using biopsy forceps).

3) Post-operatively, 0.5 g. of the first post-operative stool passed.

The results of these studies appear in Sections 3.6 and 3.7 and organ culture of colonic mucosa is discussed in Section 3.9.

METHODS

2.5. Validation of culture techniques.

The recommendations of Crowther (Ph.D. Thesis, 1971), aimed at eliminating minor fluctuations due to variations in techniques by individuals, were observed. All manipulations of faecal specimens, including the preparation of serial dilutions and inoculation of plates were carried out by the same person using the same techniques throughout an investigation.

In addition it was thought necessary to attempt to compare the methods available for the isolation and enumeration of strictly anaerobic organisms in faeces. This was investigated in three ways:-

1) Comparison of conventional bench methods with the use of a modified Hungate technique and a flexible polyvinyl anaerobic chamber, for the isolation and enumeration of strict anaerobes in faecal samples.

2) The effect of length of time of exposure of seeded plates to the atmosphere within the anaerobic chamber and on the laboratory bench.

3) The comparison of various media recommended for the isolation and enumeration of strict anaerobes in faeces.

The results of these studies are presented in Section 3.1 and a full description of the use of the Hungate technique and anaerobic chamber is given in Section 2.8. Unless otherwise stated, incubation was at 37°C and anaerob. jars were filled with a gas mixture of 70% hydrogen and 30% carbon dioxide.

2.5.i. Comparison of methods of isolation and effect of exposure of seeded plates.

Six faecal samples were obtained from six individuals (laboratory staff). Serial dilutions of these samples were prepared in Brain-Heart Infusion broth (BHI) (Oxoid) using the Virginia Polytechnic Institute (V.P.I.) gassing apparatus as described in Section 2.8.ii. Aliquots of 0.1 ml of suitable dilutions from three of the samples were inoculated into roll-tubes which were spun until the agar hardened and incubated for three days. Each sample was inoculated in triplicate. The serial dilutions were passed into the anaerobic chamber where 0.1 ml samples were seeded onto plates of Reinforced Clostridial Medium (Oxoid) containing 10% blood (R.C.M.B.). Two sets of plates were used for each sample:-

a) plates that had been stored in an aerobic atmosphere at 4°C for three days and passed into the chamber with the serial dilutions.

b) plates that had been stored in the anaerobic chamber at room temperature (18°C) for three days prior to use.

Plates seeded with 0.1 ml of suitable dilutions from each sample were immediately placed in an anaerobic jar and the lid closed. The jar was removed from the cabinet via the air-lock, evacuated, and filled with gas mixture. Fresh palladium 'D' catalyst (Englehard Industries, Glos.) was included in the jar. Sets of plates seeded with dilutions from three specimens were left in the chamber for half an hour and for one hour before removal from the chamber, in an anaerobic jar which was refilled with gas mixture and incubated for three days.

Finally, the serial dilutions were transferred out of the chamber and 0.1 ml was seeded onto R.C.M.B. Again, two sets of plates were used for each faecal sample; those that had been stored aerobically and those that had been stored in the anaerobic chamber. All the R.C.M.B. plates used in this experiment, including those in the chamber, were poured from the same batch of medium. Seeded plates were incubated in anaerobic jars immediately after inoculation, after half an hour and after one hour. Palladium catalyst was included in the jars. The plates were examined after three days incubation. Colonies were counted with an electronic plate counter (Cooke Instruments Ltd) and all counts were made by one person.

Ordinary blood-agar plates were seeded with 0.1 ml of suitable dilutions for the enumeration of aerobic organisms in the specimens.

2.5.ii. Effectiveness of various media recommended for the isolation of strict anaerobes from faeces.

Six fresh faecal specimens were obtained from six individuals.

Ten-fold serial dilutions in B.H.I. broth were prepared in the anaerobic chamber and 0.1 ml portions of suitable dilutions were spread with a bent glass rod on the surfaces of the following media:-

1) Reinforced Clostridial Medium with 10% horse blood (R.C.M.B.).
2) Blood Agar (Oxoid).
3) Moore's Medium.
4) Freter's Medium with Palladium Black.
5) Freter's Medium without Palladium Black.

All these media had been stored in the chamber for three days prior to use. The detailed formulae of these and other media are given in the Appendix.

The seeded plates were placed into a converted milk-churn (see Section 1.1.iii), fitted with a palladium catalyst, in the chamber. This was removed via the air-lock, evacuated, filled with gas mixture and incubated for 72 hours. The sets of dilutions were removed from the chamber and 0.1 ml portions seeded onto the following media:-

6) Reinforced Clostridial Medium with 10% horse blood.
7) Blood Agar.
8) Moore's Medium.
9) Dithiothreitol Medium with 20% human blood (Watt, 1972).

Dithiothreitol medium was poured and dried immediately before use. The other media had been stored at 4°C for three days. Seeded plates were incubated for three days in a converted milk-churn fitted with a palladium catalyst. Ordinary blood agar was seeded with 0.1 ml of suitable dilutions for the enumeration of aerobic organisms in the specimens.

2.5.iii. Protective effect of picking colonies from a primary isolation plate in the anaerobic chamber.

The aim of this experiment was to assess the effect on survival of anaerobic isolates, of picking colonies from an original isolation plate on the bench and in the anaerobic chamber. Three faecal samples were obtained from three people and cultivated in triplicate in the anaerobic chamber on R.C.M.B. as described in Section 2.8.iii. After incubation for three days at 37°C, a set of isolation plates was transferred into the anaerobic chamber before being removed from their anaerobic jar. Ten colonies from each specimen were picked from plates which supported the growth of well separated colonies, into tubes of Robertson's cooked meat medium, using an electrically sterilized inoculating loop (Trotman and Drasar, 1968). The cultures were removed from the chamber for incubation. Ten colonies were also picked in conventional manner on the bench, into Robertson's cooked meat medium, from the second set of isolation plates.

The third set of isolation plates were removed from the anaerobic

jar and stored at 4°C for 24 hours before ten colonies per specimen were picked into Robertson's cooked meat medium.

All the cultures in Robertson's cooked meat medium were incubated at 37°C for three days and tested for aerobic and anaerobic growth. The number of strains of strict anaerobes isolated by each method was recorded.

2.6. Isolation of anaerobic bacteria from wound swabs.

The results of this study appear in Section 3.2.

2.6.i. Bench procedures.

The swabs were seeded onto blood-agar plates (Oxoid) which had been stored aerobically at 4°C. In each case a seeded plate was immediately placed in a standard anaerobic jar (Baird and Tatlock Ltd.), which was fitted with a palladium catalyst. The jar was evacuated, filled with hydrogen gas, and incubated at 37°C. 'Gas-Pak' anaerobic indicator (Becton, Dickinson, U.K. Ltd.) was included in the jar. A second seeded plate was left on the bench until a suitable number of plates for incubation in an anaerobic jar had accumulated. This was the usual practice in the clinical laboratories of St. Mary's Hospital. A palladium catalyst, and 'Gas-Pak' anaerobic indicator were included in the jar, which was evacuated,

filled with hydrogen-gas and incubated at 37°C. The plates were examined after 24 and 48 hours. A third plate was seeded and incubated aerobically at 37°C.

2.6.ii. Chamber procedures.

Blood-agar plates (Oxoid) were stored for three days in an anaerobic chamber prior to use; the blood became darkened indicating reduction of the media. Swabs were introduced into the chamber via the air-lock and were seeded onto the stored agar plates. The anaerobic chamber is described in Section 2.8.iii. An electrically sterilized inoculating loop (Trotman and Drasar, 1968) was used to spread the inocula on the plates. The plates were placed in an anaerobic jar inside the chamber and the lid was screwed down. On removal from the chamber, the jar was evacuated and filled with a gas mixture containing 10% carbon-dioxide and 90% hydrogen. Fresh palladium catalyst was included in all the jars. The plates were incubated at 37°C and examined on the bench after 24 and 48 hours.

2.6.iii. Isolation of anaerobes.

Colonies of suspected anaerobes were picked into Robertson's cooked meat medium and incubated at 37°C for three days. The cultures were examined for purity and for aerobic and anaerobic growth on blood-agar.

2.7. Antibiotic-disc tests for rapid identification of non-sporing anaerobes.

Sutter and Finegold (1971) suggested the use of discs impregnated with high concentrations of antibiotics for the rapid presumptive identification of Gram-negative non-sporing anaerobes. This method was used in parallel with the methods recommended in the Anaerobe Laboratory Manual (see Section 2.9) for the identification of some strains of non-sporing anaerobes isolated during the present study. The strains tested and their sources are shown in Table 10: all strains had been stored as described in Section 2.8.vi. The results of this study are presented in Section 3.3.

The culture medium for the sensitivity tests was R.C.M.B. (see Appendix). One or two drops of a three-day test culture in Robertson's cooked meat medium were spread, with a bent glass rod, over the surface of the culture plates. Antibiotic discs, no more than four per plate were then applied. The plates were incubated for three days at 37°C in Baird and Tatlock anaerobic jars filled with a gas mixture containing 70% hydrogen and 30% carbon-dioxide. Fresh palladium catalyst was included in all the jars.

Antibiotic discs

Antibiotic discs were obtained from Mast Laboratories, Liverpool. The discs used were: colistin 10μg, erythromycin 60μg, kanamycin 1000μg, neomycin 1000μg, penicillin 2u. (1.2μg), rifampicin 15μg, vancomycin 5μg.

TABLE 10

Non-sporing Anaerobes

Source of strains

FOR ANTIBIOTIC DISC TESTS

Species	Source	no. of strains tested
Bacteroides fragilis	Faeces:	
	U.S.A.	39
	India	11
	Japan	42
	Uganda	6
	Scotland	14
	Hong Kong	3
	National Collection Type Cultures 9343	1
	Wound infections	7
B. melaninogenicus	Faeces:	
	U.S.A.	3
Bifidobacterium adolescentis	Faeces:	
	U.S.A.	11
	India	4
	Japan	1
	Uganda	1
	Scotland	1
	Hong Kong	1
Bifid. bifidum	Faeces:	
	U.S.A.	3
	India	1
	Japan	3
	Scotland	3
	Hong Kong	2
Eubacterium aerofaciens	Faeces:	
	U.S.A.	3
	India	1
	Japan	1

2.8. Standard procedures used in the bacteriological examination of specimens.

2.8.i. Transport and storage of specimens.

Faecal specimens were transported and stored in a manner similar to that described by Drasar, Shiner and McLeod (1969). Fresh samples of faeces were emulsified and diluted 1 in 10 in meat infusion broth (Lab-Lemco, Oxoid) containing 10% glycerol and immediately frozen in solid carbon-dioxide (-78°C) or liquid nitrogen (-196°C) for transport to London. The samples were stored at -55°C. Experiments by Crowther (1971) showed that even fastidious anaerobic bacteria survived without significant losses when faecal specimens were stored under these conditions for up to one month.

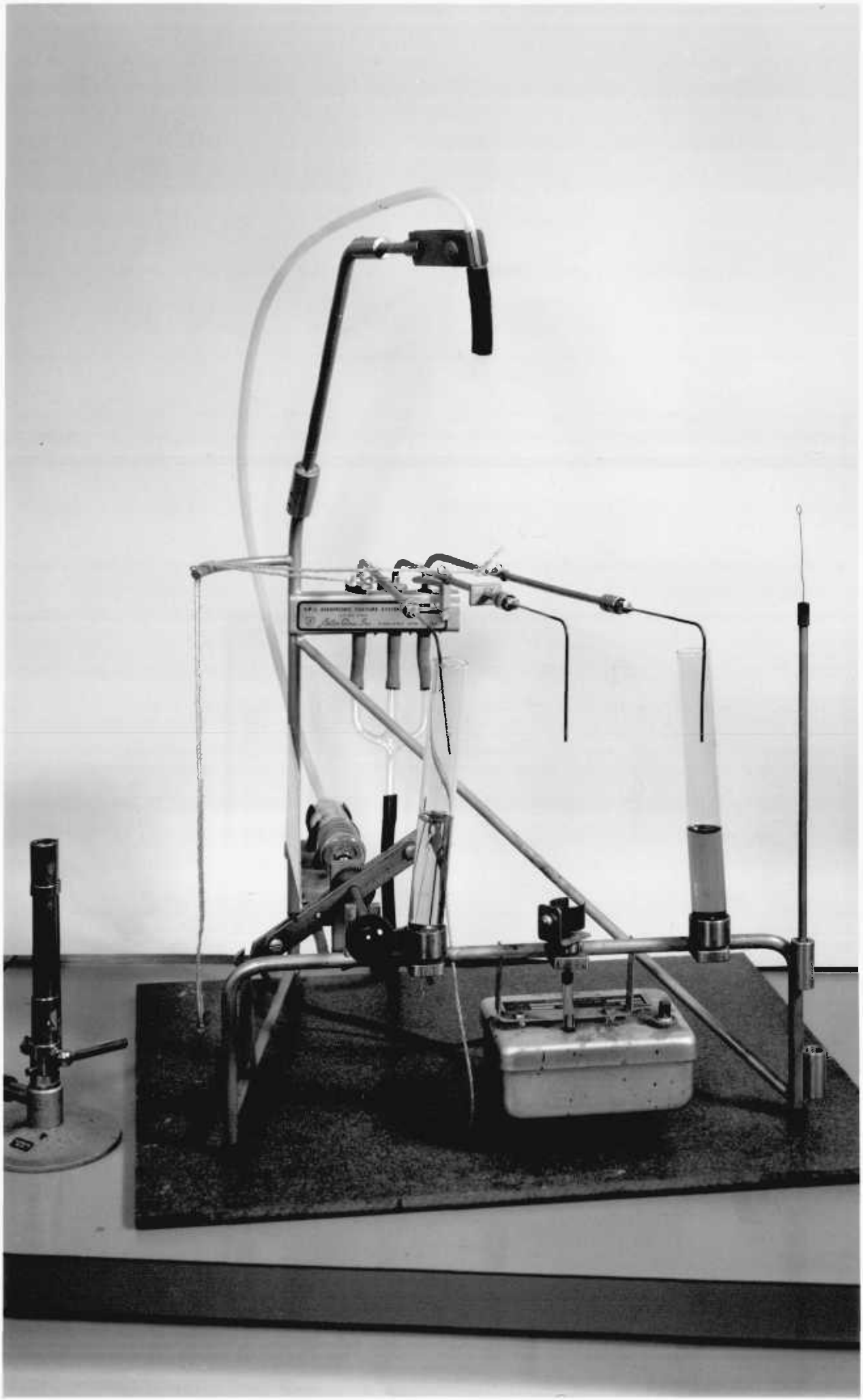
Specimens of tissues were placed in pre-weighed bijou bottles containing 4.5 ml of meat infusion transport broth. The weight of tissue was obtained by difference. All samples were immediately frozen in solid carbon-dioxide for transport to the laboratory where they were stored at -20°C prior to cultivation. Preliminary experiments showed that there were no significant losses of bacteria from tissue samples transported and stored for short periods in this manner.

None of the samples of faeces or tissue were stored for more than one month prior to cultivation.

Photograph 2. The V.P.I. Anaerobic Culture System.

The gas cannulae are treadle operated from below the bench, enabling them to be passed through the bunsen flame. The tubes of diluent shown in position beneath the gas cannulae contain rezazurin oxygenation/ reduction indicator. The liquid in the tube on the right is oxidized.

Between the two tubes can be seen the clip for holding roll tubes, attached to a small electric motor.



2.8.ii. The Modified Hungate Apparatus.

The modified Hungate apparatus, which was used for the distribution and inoculation of pre-reduced, anaerobically sterilized media (P.R.A.S.), was that designed by the staff at the Anaerobe Laboratory, Virginia Polytechnic Institute, U.S.A. It comprises treadle operated gas cannulae, an inoculater and streaker in a single compact unit. This system is commercially available as the V.P.I. Anaerobic Culture System, from Bellco-Glass Co., Inc. It is shown in Photograph 2.

Preparation of pre-reduced, anaerobically sterilized (P.R.A.S.) Media.

P.R.A.S. was used both for diluting the faecal specimen prior to inoculation and for preparing agar 'roll-tubes' for cultivation of the specimen. The preparation of diluent and culture medium was similar. Brain-Heart-Infusion broth (Oxoid) was used, with added reducing agents, salts, haemin and menadione (vitamin K). The amounts of these constituents are shown in the Appendix. The required amount of broth was reconstituted from the powdered product with distilled water in a narrow-necked conical flask, and steamed until dissolved. One rezazurin tablet (BDH Ltd.) per litre of broth (equivalent to a 0.0005% solution) was added as a reduction/oxygenation indicator. The medium was boiled on a hot plate with a magnetic stirrer (photograph 3) until the rezazurin indicator changed from pink to colourless. (At pH 6.5, rezazurin is blue in the oxidised form. It is reduced at an Eh range of +0.18 to +0.09 volts. The first reduction product, resorufin is pink and the

Photograph 3. Preparation of pre-reduced media.

A chimney fitted into the neck of the flask prevents the media from boiling over.


PYREX
1000 ml
VOSS INSTRUMENTS LTD.
INCREASE SPEED
OFF

final reduction form, dihydroresorufin is colourless). The reducing agents sodium formaldehyde sulphoxylate (final concentration 0.05% w/v) and cysteine hydrochloride (final concentration 0.03 w/v) were added after the medium had been partially reduced by boiling, since oxidized reducing agents can be toxic for some fastidious anaerobes. Special care was needed when adding sodium formaldehyde sulphoxylate, as if this compound is added to hot oxidized media, fumes of sulphur dioxide are released.

It was desirable that the final pH of the medium should be 7.0± 0.1. The medium was adjusted to pH 8.0 with 1M.NaOH and was allowed to cool under a stream of oxygen-free carbon-dioxide (British Oxygen Co., < 10 ppm O_2). The CO_2 served two purposes; to cool the medium in an anaerobic environment by excluding air, and to reduce the pH to a final value of about 7.0. Once the medium was cool, the stream of CO_2 was replaced by one of oxygen-free nitrogen (British Oxygen Co. < 10 ppm O_2) to prevent further lowering of the pH. The medium was then ready for distribution into tubes.

Preparation of dilution blanks.

Test tubes ($\frac{1}{2}$ x 6") were placed in position beneath the gas cannulae of the V.P.I. gassing apparatus (photograph 2). Aliquots of 9 ml of the pre-reduced medium were transferred by pipette to the tubes, which were continuously flushed with oxygen-free nitrogen. Care was taken that a little of the gas was taken up into the pipette before filling it with the medium, to ensure that this did not oxidize on transfer. Any media

that did turn pink, indicating oxidation, were discarded. The tubes were stoppered with butyl rubber bungs (which are impervious to oxygen), withdrawing the cannulae only as the bungs were fully inserted. Stoppered tubes were placed in a rack and press for autoclaving at 15 lb/sq" for 15 minutes. The press prevented the bungs being blown off the tubes during autoclaving and was designed specifically for the V.P.I. gassing apparatus.

Preparation of Pre-reduced agar media.

Pre-reduced Brain-Heart-Infusion broth was prepared as described above and allowed to cool. With a gas-filled pipette, 5 ml of pre-reduced medium were transferred to 'roll-tubes' (Astell Ltd.) which stood in position beneath the gas cannulae of the V.P.I. apparatus. The roll tubes contained approximately 0.1 g. agar each. The same precautions for transferring the medium and fitting the bungs were observed as for the dilution blanks. The roll tubes were likewise clamped and autoclaved at 15 lb/sq" for 15 minutes, to melt the agar. Once autoclaved and stoppered, both roll tubes and dilution blanks could be kept at room temperature for up to six months, with no deterioration.

The use of P.R.A.S. media for the isolation of anaerobic bacteria.

Faecal samples were generally provided as a one in ten dilution in transport broth and 1 ml of this was taken up into a wide-mouthed Pasteur pipette fitted to an automatic 'Cornwall' syringe. This was dispensed into the first 9 ml dilution blank, which stood in the V.P.I. gassing apparatus under a stream of oxygen-free nitrogen. Aseptic techniques

were observed throughout the operation; before opening the dilution blanks, the necks of the tubes were passed through a bunsen flame and the tubes were opened carefully, using forceps, under the gas cannulae. The bungs themselves were passed through the flame before being replaced in the tubes. In this manner a series of serial ten-fold dilutions of the original 1/10 specimen were made. The wide-mouthed Pasteur pipette and 'Cornwall' syringe were necessary only for the first transfer of faeces, ordinary sterile 1 ml pipettes sufficed for the remainder. A new pipette was used for each transfer and the cannulae were sterilized between transfers by passing through a bunsen flame. The dilutions were agitated between transfers on a vortex mixer. A total of eight dilutions of the original faecal sample were thus produced. Aliquots of 1 ml of the final three dilutions were used to inoculate the P.R.A.S. medium in roll tubes.

Prior to inoculation, the roll tubes were clamped and steamed at 100°C to melt the agar; after cooling the medium was kept in a molten state by storing the tubes in a water bath at 50°C. The tubes were opened in the V.P.I. apparatus under a stream of oxygen-free carbon-dioxide. Any tubes which turned pink were discarded. Aseptic techniques, flaming the necks of the tubes, bungs and cannulae, were observed as for the preparation of dilution blanks.

After inoculation the tubes, with the still molten medium, were spun on an 'Astell' roll-tube spinning apparatus. The spinning tubes were cooled by jets of water, and the agar solidified in a layer around the sides of the tubes. Inoculated tubes were incubated for three days at 37°C. An anaerobic jar system was not needed since the tubes had

their own anaerobic environment. Colonies of bacteria grew both on the surface of the agar and within the medium itself. The total number of organisms growing anaerobically was calculated by counting the colonies, which could be seen easily through the clear B.H.I. agar, and multiplying this count by the appropriate dilution factor.

Picking colonies for subculture from roll tubes was technically difficult, but was made easier with the use of a Pasteur pipette, the end of which was bent to form a right angle (Hungate, 1969). The roll tubes were opened under a stream of oxygen-free nitrogen in the V.P.I. gassing apparatus and the agar surrounding the required bacterial colony was stabbed with the bent Pasteur pipette. The agar and colony were drawn up into the pipette and could then be subcultured into tubes of Robertson's cooked meat medium (R.C.M.M.). Prior to use the tubes of R.C.M.M. were steamed at 100°C for ten minutes with the caps loose to remove much of the dissolved oxygen. The caps were tightened and the medium allowed to cool before inoculation. In most cases ten bacterial colonies were picked from each faecal sample. All were picked along a straight line drawn across the outside of a single roll-tube.

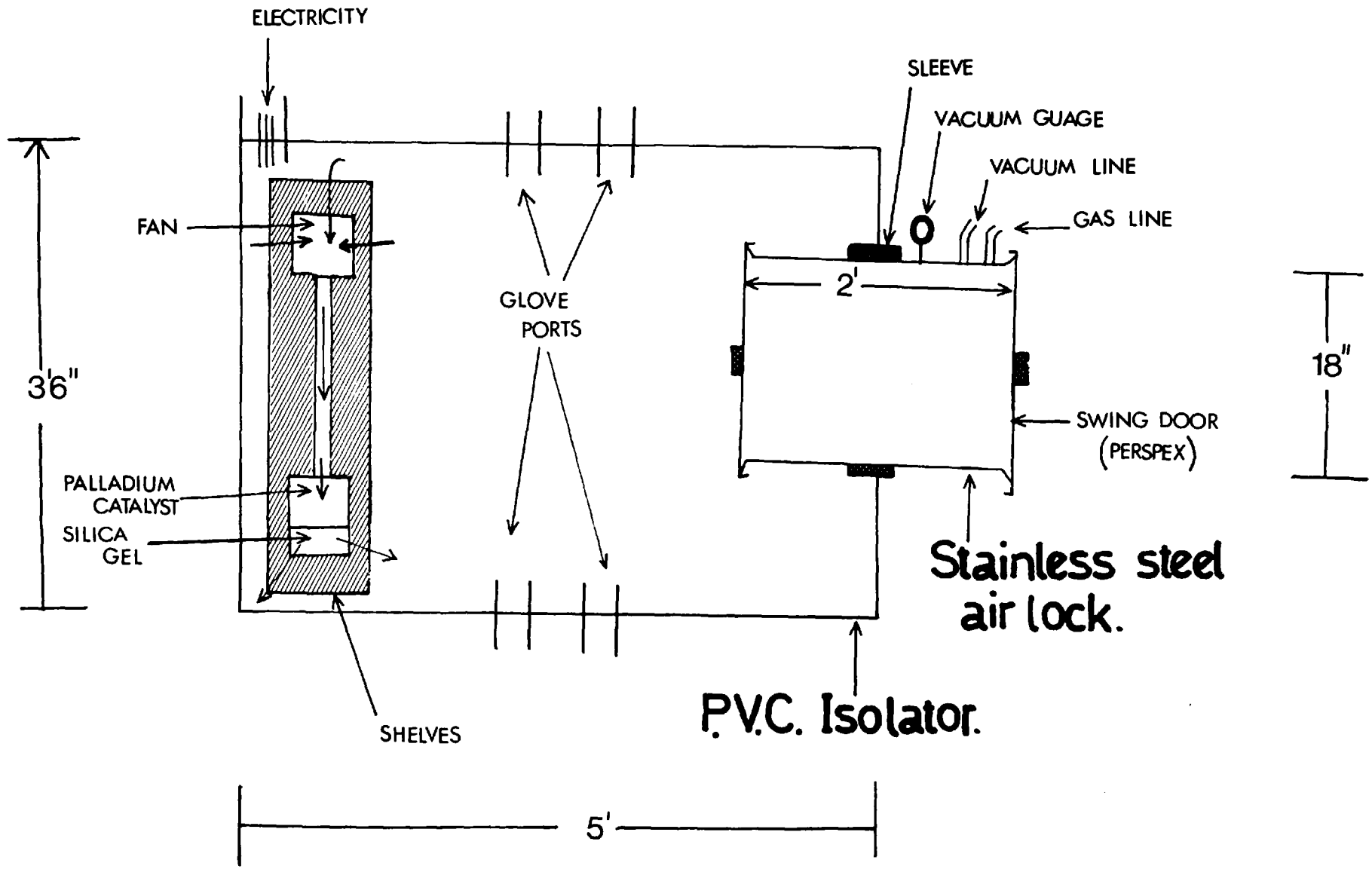
2.8.iii. The Anaerobic Chamber.

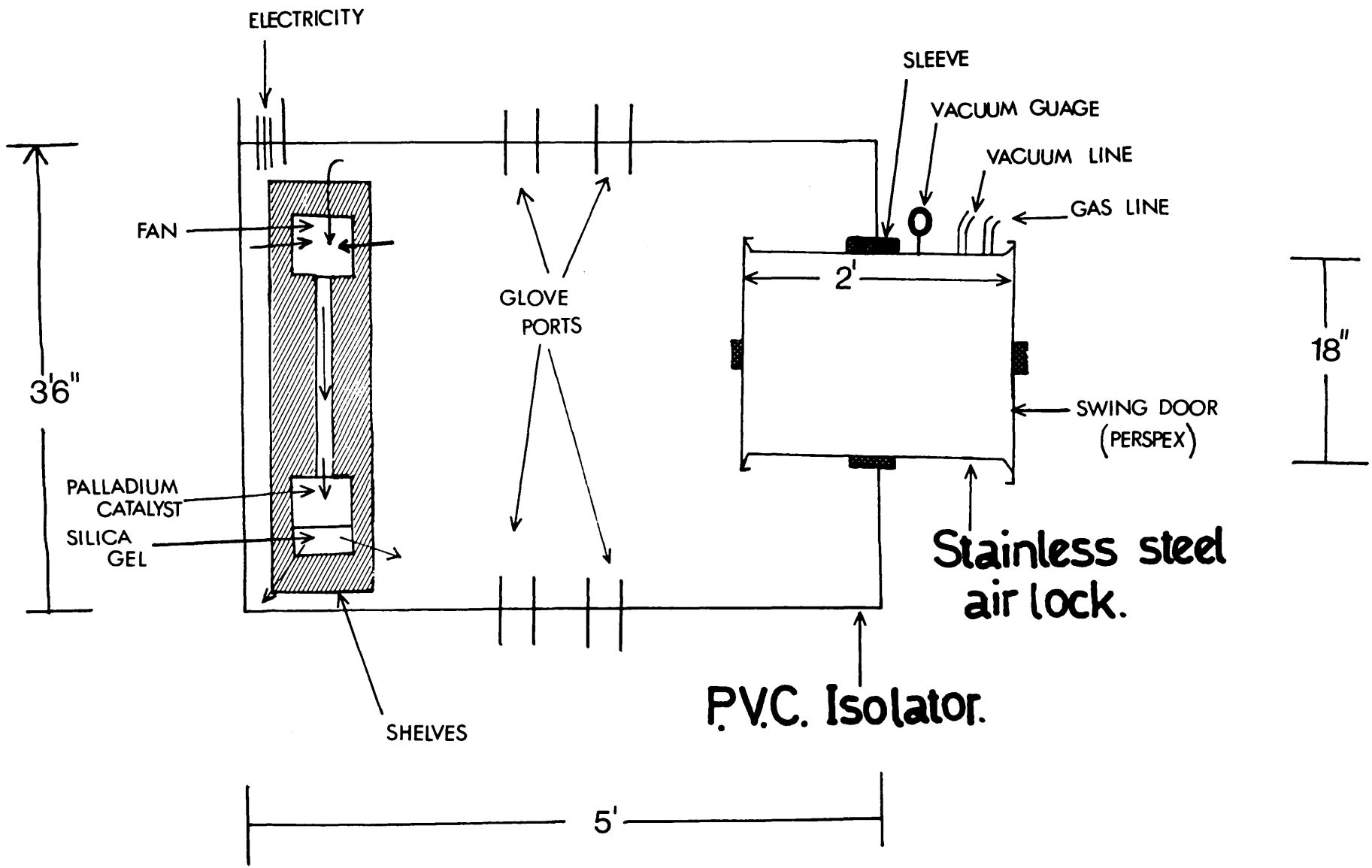
Most of the bacteria described in this thesis were isolated from material which was handled in a flexible polyvinyl chamber filled with a gas mixture of 90% nitrogen and 10% hydrogen. The chamber was similar

Figure 5.

Plan of the anaerobic chamber.

The chamber was 48" high.


ELECTRICITY
SLEEVE
VACUUM GUAGE
VACUUM LINE
GAS LINE
FAN
GLOVE PORTS
2'
3'6"
18"
SWING DOOR (PERSPEX)
PALLADIUM CATALYST
SILICA GEL
Stainless steel air lock.
P.V.C. Isolator.
SHELVES
5'


ELECTRICITY
SLEEVE
VACUUM GUAGE
VACUUM LINE
GAS LINE
FAN
GLOVE PORTS
2'
36"
18"
SWING DOOR (PERSPEX)
PALLADIUM CATALYST
SILICA GEL
Stainless steel air lock.
P.V.C. Isolator.
SHELVES
5'

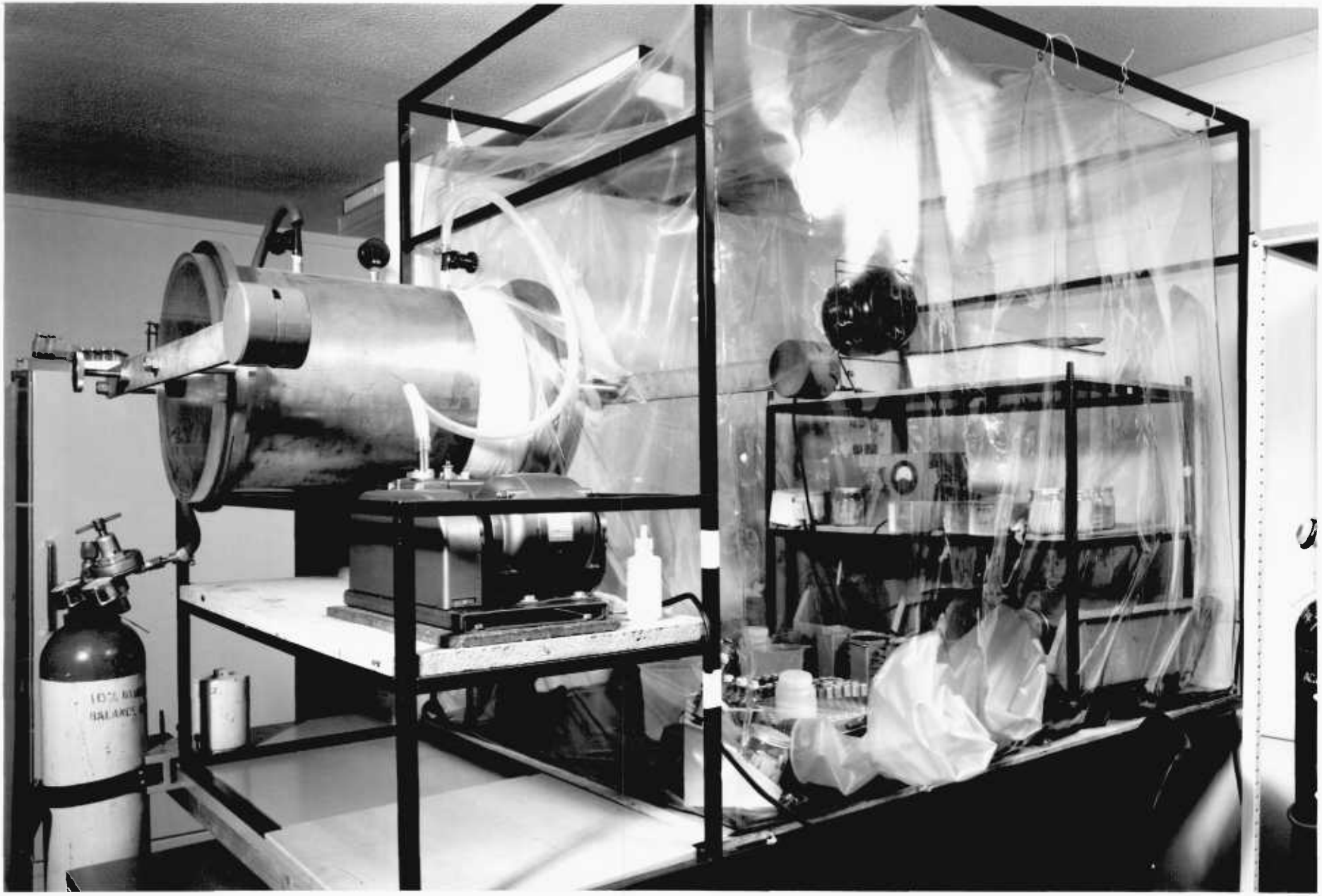
to that described by Aranki et al. (1969). The main body of the chamber was constructed from a modified polyvinyl bag, originally designed for the housing and isolation of germ-free animals (Plysu Industrial Ltd.).

The dimensions of the chamber are shown in Figure 5. A large size was chosen to enable several batches of media to be stored in it. The chamber was fitted with glove-ports in two walls, on one side the low position enabled work to be carried out whilst sitting at the bench. The higher glove ports on the opposite side were useful for transferring material in and out of the air-lock. Neoprene rubber gloves (George Angus, Cambridge) were attached to the sleeves provided. The dimension of the air-lock are also shown in Figure 5. It was constructed by Henry Hartley Ltd., London, from $\frac{1}{4}$" thick stainless steel and was fitted with 1" thick perspex swing doors with centre screw fastening operated by an arm and counterweight system. Gas inlets and exhaust, and a vacuum gauge were situated on top of the air-lock. The whole system, chamber and air-lock, was supported within a tubular steel frame. Lugs were provided on the top edges and base of the plastic chamber to enable it to be attached to the framework (photograph 4).

The chamber was provided with an aperture and sleeve into which fitted the air-lock. This joint was securely sealed with plastic tape. An inlet in one corner of the chamber was used to pass the electric flex from a 13 amp distribution board inside to an outside electricity source. The flex was sealed into the inlet with silicone-rubber moulding paste (Esbco Rubber Ltd.).

Photograph 4. The anaerobic chamber.

A vacuum pump stands next to the air-lock and the chamber is filled with gas from the cylinder (far left of picture). Inside the chamber can be seen the fan which circulates the atmosphere over sachets of palladium catalyst which lie on a grid over a tray of silica-gel.



Preparation and use of the anaerobic chamber.

A trolley with two shelves was constructed inside the chamber prior to initial inflation, and the chamber was stocked with the large pieces of equipment which would be permanently inside it. The most important of these included a 6" diameter 'Vent-Axia' fan on a stand, two large stainless-steel wire sachets (6" x 4") containing palladium catalyst (the same catalyst as used in anaerobic jars) and a tray of silical gel (self-indicating 6-20 mesh, approximately 500 g). The contents of the chamber were pushed forwards towards the air-lock and the plastic walls of the chamber were collapsed around them. The outer door of the air-lock was closed and most of the air in the chamber was removed by evacuation via the air-lock until the walls of the chamber were wrapped tightly around the contents. A gas mixture of 90% nitrogen and 10% hydrogen (a non-inflammable mixture) was introduced into the chamber until it was fully inflated with a slight positive pressure. The inside door of the air-lock was fastened and the chamber was left until the oxygen tension of the atmosphere inside was low enough to maintain rezazurin (0.0005% incorporated into Brain-Heart-Infusion agar) in the colourless state (dihydroresorufin). This was found to be a period of about 24 hours. The palladium catalysed a reaction between any residual oxygen and hydrogen in the gas mixture, with the formation of water. This was absorbed by the silica-gel, so preventing misting of the walls of the chamber. Both the silica-gel and the catalysts were changed weekly and reactivated by heating at 180°C for two hours. The gas pressure in the chamber fell slightly with time and weekly 'topping-up' was necessary. Rezazurin, incorporated into Brain-Heart-Infusion Agar monitored the state of anaerobiosis within the chamber.

Isolation of non-sporing anaerobes from faeces.

Serial ten-fold dilutions of faecal specimens were prepared in Brain-Heart-Infusion broth (BHI) broth (Oxoid) which contained 0.05% (w/v) cysteine hydrochloride and 0.03% sodium formaldehyde sulphoxylate as reducing agents. The diluent was heated to 100°C before introduction into the cabinet where it was dispensed and allowed to cool in the anaerobic environment. Using a pre-set, constant volume pipette, 0.1 ml of appropriate dilutions were dispensed and were spread with glass spreaders on the surface of plates of Reinforced Clostrial Medium (Oxoid) containing 10% defibrinated horse blood (R.C.M.B., see Appendix). In order to maintain the media in a reduced state the plates were stored in the anaerobic chamber for three days prior to use. Seeded plates were packed into anaerobic jars containing palladium catalyst and on removal from the cabinet the jars were re-evacuated and filled with a gas mixture containing 30% carbon-dioxide and 70% hydrogen and incubated at 37°C for three days. When a large number of plates were to be incubated, converted milking machine churns (Fullwood-Bland) were used instead of conventional anaerobic jars (photograph 1).

Ten colonies per faecal sample were picked on the open bench from the isolation plates and subcultured into Robertson's cooked meat medium. After incubation for three days the cultures were checked for purity and aerobic growth.

Isolation of bacteria from colonic mucosa biopsies.

Tissue specimens were passed into the anaerobic chamber and allowed to thaw. The samples were washed (by vigorous agitation on a vortex mixer) in two changes of 4.5 ml of pre-reduced Brain-Heart-Infusion (BHI) broth and mascerated, to release associated bacteria, by 'stomaching' for three minutes in 4.5 ml of pre-reduced BHI broth in a 'Colworth Stomacher 80'. The mascerate was diluted 1/10 and 1/100 times and 0.1 ml of the dilutions, plus the original mascerate were seeded onto R.C.M.B. The plates were packed into an anaerobic jar containing a palladium catalyst, and the jar was removed from the chamber, evacuated and filled with a gas-mixture containing 30% carbon-dioxide and 70% hydrogen. After three days incubation at 37°C the plates were removed for enumeration of the number of bacterial colonies which grew under anaerobic conditions.

The original and diluted portions of the mascerate were also seeded on the open bench onto the following media:-

Blood agar (Oxoid) - for enumeration of the total number of bacteria growing aerobically, and for streptococci.

MaConkey's agar (Oxoid) - for the enumeration of enterobacteria and enterococci.

Rogosa's medium - for enumeration of lactobacilli.

Willis and Hobbs medium - for the enumeration of clostridia, both before and after heat-shock of specimens at 70°C for ten minutes.

Details of these media are given in the Appendix.

2.8.iv. Isolation of sporing anaerobes from faeces.

The sporing anaerobes, members of the genera Clostridium and Sarcina were isolated on media manipulated on the open bench. However, freshly poured or anaerobically stored media was used, and care was taken to ensure that seeded plates were incubated in an anaerobic atmosphere as soon as possible.

Isolation of clostridia (not producing lecithinase) and sarcinae.

The serial dilutions of faeces, prepared in the anaerobic chamber for the isolation of non-sporing anaerobes, were removed from the chamber via the air-lock. 0.5 ml of dilutions 10^{-2} to 10^{-4} were transferred to 3 x $\frac{1}{2}$" tubes spaced well apart in a rack. The tubes were immersed in a water bath at 70°C for ten minutes which killed vegetative cells and stimulated the germination of heat resistant spores by the 'heat-shock' effect (Freame and Fitzpatrick, 1971; Hobbs, Williams and Willis, 1971).

After heat shock, 0.1 ml of these dilutions was spread on the surface of plates of Willis and Hobbs egg-yolk-medium (Willis and Hobbs, 1959). The culture plates were incubated for three days at 37°C in an anaerobic jar or converted milk-churn containing palladium catalyst and an atmosphere of 70% hydrogen and 30% carbon-dioxide. Colonies of

organisms not producing lecithinase were easily recognized for counting and isolation by the lack of an opaque halo in the medium surrounding the colony. Ten colonies per specimen were picked into tubes of Robertson's cooked meat medium from one of the isolation plates.

No special effort was made to isolate sarcinae during the present investigation, but on further analysis it appeared that some of the colonies isolated on Willis and Hobbs medium after heat-shock, were sarcinae.

Some of the Clostridium spp. which show lecithinase activity (e.g. Cl. perfringens) form spores only occasionally. Colonies of these organisms were counted on Willis and Hobbs agar seeded with 0.1 ml from the portions of the dilutions which had not been subjected to heat-shocking. Colonies of organisms producing lecithinase were surrounded by a halo of opaque medium which enabled them to be counted against a background of colonies which did not produce this effect.

2.8.v. Isolation of lactobacilli, veillonellae and aerobic organisms from faeces.

Although particular attention was paid to the isolation of some of the non-sporing anaerobes and clostridia from the large intestine in this study, other organisms were not ignored. Various selective and non-selective media were used to count total aerobic organisms, lactobacilli and veilleonellae.

TABLE 11

Isolation of bacteria from faeces

Isolation medium*	Dilutions plated out	Incubation	Bacteria investigated	Notes
Reinforced Clostridial Medium (R.C.M.)	10^{-5} to 10^{-7}	3 : AN	Total count of anaerobic bacteria	Stored in anaerobic chamber for 3 days prior to inoculation
Willis & Hobbs agar	10^{-1} to 10^{4}	3 : AN	Clostridia (Lecithinase producers).	Stored anaerobically
Willis & Hobbs agar (after heating at 70°C /10 mins).	10^{-1} to 10^{-4}	3 : AN	Clostridia, Lecithinase producers and non-producers.	" "
Rogosa's Lactobacilli Medium	10^{-1} to 10^{-5}	3 : AN	Lactobacilli	50% CO_2 in incubation gas
Blood Agar	10^{-2} to 10^{-6}	1 : A	Total aerobes Total Streptococci	
MacConkey Agar	10^{-1} to 10^{-5}	1 : A	Enterococci Enterobacteria	
Rogosa's Veillonellae Medium	10^{-1} to 10^{-2}	3 : A	Veillonellae	

* Detailed formulae given in Appendix.

AN = Anaerobic incubation at 37°C.

A = Aerobic incubation at 37°C.

Prefix indicates number of days incubation.

Media for the isolation and enumeration of bacteria from faeces and the conditions and length of incubation are shown in Table 11.

2.8.vi. Preservation of cultures.

When necessary for convenience, pure cultures of non-sporing anaerobes and clostridia were stored at -50°C as three day cultures in Robertson's cooked meat medium to which glycerol had been added to a final concentration of 10% (v/v). When required the cultures were thawed and immediately subcultured into fresh Robertson's meat medium.

Enterobacteria were stored as 24 hour cultures on Dorset-egg slopes at +4°C and other aerobic bacteria were stored at -50°C in Robertson's cooked meat medium as described above.

2.9. Identification criteria.

2.9.i. Identification of anaerobic isolates.

The isolates were placed into various broad groups on the basis of their appearance in a Gram-stained preparation (Preston and Morrell, 1962) and of the end-products of glucose metabolism detected by gas-liquid chromatography. They were assigned to species on the basis of their biochemical reactions.

TABLE 12

Differentiation of species of the major faecal anaerobes.

Test	*Bacteroides fragilis*	*Bacteroides melaninogenicus*	*Bifidobacterium adolescentis*	*Bifidobacterium bifidum*	*Bifidobacterium eriksonii*	*Eubacterium aerofaciens*
Gelatinase	-	+	w^-	$-^w$	$-^w$	w^-
Indole production	v	v	-	-	-	-
Acid from:						
Glucose	+	v	+	+	+	+
Lactose	+	v	+	+	+	$+^-$
Sucrose	+	v	+	-	+	$+^-$
Maltose	+	v	+	-	+	+
Fructose	+	v	+	+	+	+
Mannose	+	v	$+^-$	$-^w$	+	+
Mannitol	-	-	v	-	+	v
Arabinose	v	v	+	-	-	v
Xylose	+	w	+	$-^w$	+	$-^+$
Aesculin hydrolysis	+	v	+	$-^w$	v	$+^-$
Nitrate reduction	$-^+$	-	-	-	-	$-^+$

\+ = Positive reaction for 90-100% of strains.

\- = Negative reaction for 90-100% of strains.

w = Weak.

v = Variable among strains.

Superscripts indicate reactions of occasional strains.

After Cato *et al.* (1970).

In the present study, the genus definitions and species characteristics of the non-sporing anaerobes, published in the Anaerobe Laboratory Manual of Cato et al. (1970) were employed; they are briefly recapitulated in Tables 1 and 12.

The sporing anaerobes, Clostridium spp., were identified according to the scheme suggested in a later edition (1972) of the Anaerobe Laboratory Manual of which Holdeman and Moore are the editors; the basis of this scheme is outlined in Table 13. Inhibition of lecithinase by specific antitoxin on egg-yolk medium was used as a confirmatory test for the detection of Clostridium perfringens isolated from wounds (Nagler, 1939). The analytical methods to detect the volatile fatty acids produced from glucose are described in Section 2.10.i. Details of the biochemical tests are given in the Appendix.

2.9.ii. Identification of aerobic isolates.

After incubation, bacterial colonies on blood and MacConkey agar plates were counted. Gram-stained smears were made of each colony type. Various broad groups were defined on the following grounds:-

Enterobacteria: Gram-negative, catalase-positive rods, growing on MacConkey's agar, not producing diffusable pigment.

Streptococci: Enterococci: Gram-positive, catalase-negative cocci forming intense red colonies on MacConkey's agar.
'Viridans' streptococci: Gram-positive, catalase-negative cocci growing aerobically on blood agar.

Table 13.

Characteristics of various groups of clostridia.

Modified from Holdeman and Moore (1972).

ecithinase produced

- C. barati
- C. bifermentans
- C. ghone I
- C. haemolyticum I
- C. limosum
- C. oedematiens (novyi)A,B
- C. perenne
- C. welchii (perfringens)*
- C. sardiniensis N
- C. sardellii*

KEY

I = indole producer
N = nitrare reducer
X = xylose fermentor
* = common in faeces

Lecithinase not produced

Proteolytic (gelatin, milk or meat)

Acetic Major Volatile acid	Butyric sole acid or Major acid together with acetic	Complex acid pattern
C. histolyticum*	C. lentoputrescens* I	C. botulinum
	C. chauvoei N	C. cadaveris* I
		C. hastiforme
		C. litus-eburense
		C. magenoti I
		C. putrificum
		C. sporogenes*
		C. subteminale
		C. tetani I

Saccharolytic (Lactose, maltose and sucrose)

Acetic Major Volatile acid	Butyric sole acid or Major acid with acetic	Complex acid pattern
C. indolis* INx	C. acetobutylicum x	
C. oroticum Nx	C. bejerinkii	
C. ramosum*	C. botulinum	
C. sphenoides	C. butyricum x	
	C. fallax	
	C. paraputrificum*	
	C. pseudotetanicum	
	C. rectum*N	
	C. rubrum x	
	C. sartagoforum	
	C. tertium*N	

Proteolytic and Saccharolytic

Acetic Major Volatile acid	Butyric acid or Major acid with acetic	Complex acid pattern
	C. aurantibutyricum x	C. difficile*x
	C. felsineum x	C. oceanicum
	C. plagarum	
	C. septicum*N	

Weakly reactive both prolyosis, saccharolysis

Acetic Major Volatile acid	Butyric sole acid or Major acid with acetic	Complex a pattern
C. aminovalericum	C. barkeri	C.irregularis
C. cellobioparum x	C. carnis	C.malenominat
C. glycolicum x	C. cochlearium	C.scatologene
C. purtrifaciens	C. pasteurianum	C.sticklandii
	C. sporosphaeroides	
	C. themosaccharolyticum (45°C)	
	C. tyrobutyricum	

No volatile acids formed - C. inulinum

Staphylococci and Micrococci:

Gram-positive, catalase-positive cocci. No attempt was made to subdivide this group.

Bacillus spp.: Gram-positive, catalase-positive aerobic rods forming spores resistant to heating at 70°C for ten minutes.

2.10. Biochemical Methods.

2.10.i. Analysis of volatile acids by gas-liquid chromatography.

The volatile products of glucose metabolism (ethanol and the low molecular weight fatty acids) were detected using gas-liquid chromatography. Two types of gas-chromatograph were used. During the initial stages of this work samples were prepared as described by Cato et al. (1970) and analyses were performed on an isothermal '104' series gas chromatograph fitted with a flame-ionisation detector (Pye Instruments). Later samples were prepared for head-space analysis in an F.40 Multifract gas chromatograph (Perkin-Elmer).

Preparation of sample and chromatographic conditions.

Series '104' gas-chromatograph.

A 4 ml volume of a three day culture in 1% glucose broth (prepared as described in the Appendix) was acidified with 0.5 ml of 6M aqeuous sulphuric acid and extracted with 4 ml diethyl ether. After centrifugation to break the emulsion, the ether layer was pipetted off and dried over magnesium sulphate. Reference solutions of volatile fatty acids (1mM/100 ml aqeuous solution) were prepared, acidified and extracted with ether as above. Controls were of uninoculated glucose broth. Pyruvic, lactic and succinic acids are unstable at the operating temperature of the gas chromatograph. These acids were detected by prior preparation of their methyl esters which are stable and readily separate on the column. Freshly prepared diazomethane (de Boer and Backer, 1954) was used as the methylating agent, two to three drops being added to a portion of the ether extract. Ether extracts of reference solutions (1mM/100 ml aqeuous solution) were also methylated in this way.

Analyses were performed on a Pye '104' gas chromatograph, fitted with a flame-ionisation detector and a five foot glass column packed with 'Diatomite C' that had been treated with phosphoric acid and impregnated with 10% polyethylene glycol. The operating temperature was 125°C and the carrier-gas was oxygen-free nitrogen passing through at 30 ml per minute. A 1- µl aliquot of the sample for analysis was injected onto the column.

F.40 Multifract gas-chromatograph.

A 4 ml portion of a three day culture in 1% glucose broth (see Appendix) was acidified with 0.1 ml of concentrated sulphuric acid (final pH<2). The acidified cultures were placed in the water bath of a Perkin-Elmer F.40 Multifract automatic gas chromatograph for head space analysis. The temperature of the water bath was 95°C. The gas chromatograph was fitted with a flame ionisation detector and a 2M stainless-steel column, 0.25" diameter, packed with 'Chromosorb W' which had been treated with 2% phosphoric acid and coated with 15% 'Carbowax 20M'. The carrier gas and other conditions were similar to those for the Pye '104' gas chromatograph, but the operating temperature was slightly higher (130°C).

2.10.ii. Chemical analysis of faeces.

The chemical components of faeces shown in Tables 14 and 52 estimated quantitatively by Dr. M.J. Hill by methods described by Hill and Aries (1971). The moisture content of faeces was estimated by finding the loss in weight after drying overnight in a vacuum freeze drier (Edwards (High vacuum) Ltd.).

TABLE 14

Faecal steroid composition of 26 English subjects.

(mg. per g. dry-weight).

Neutral steroids	
Cholesterol	3.32
Coprostanol	6.77
Coprostanone	0.72
Acid steroids	
Trisubstituted	0.48
Disubstituted	2.49
Mono and unsubstituted	3.08

From Hill and Aries (1971).

2.10.iii. Steroid metabolism by intestinal bacteria.

Deconjugation and dehydroxylation of bile-acids.

Non-sporing anaerobes isolated from faeces from Hong Kong were tested for their ability to deconjugate bile acids and to carry out the 7-dehydroxylation reaction (Section 1.5). The procedures for both these tests were similar. Unless otherwise specified all cultures were incubated in an anaerobic jar fitted with a palladium catalyst and filled with a gas mixture containing 30% carbon-dioxide and 70% hydrogen.

Deconjugation.

Todd-Hewitt broth (Oxoid) distributed in 50 ml volumes in screw-capped bottles was inoculated with a Pasteur pipette full of a three day test culture in Robertson's cooked meat medium. After incubation the cells were harvested by centrifugation at 2,500 g for 30 minutes at 10°C. The supernatant was discarded and the pellet resuspended in 0.5 ml phosphate-buffered saline. Conjugated bile salt was added to a final concentration of 0.5% and the cultures were transferred to 3 x $\frac{1}{2}$ inch tubes for overnight incubation. 10 µl aliquots of these cultures were spotted onto commercially prepared thin-layer-chromatography (TLC) plates (Polygram Sil.G 0.25 mm. thick), activated at 100°C for ten minutes. Controls were 10 µl of 0.1% conjugated and unconjugated bile salts in chloroform. The spots were separated by ascending chromatography in solvent s_1 (see Appendix), allowed to air-dry and separated steroids located by spraying the plates with 10% phosphomolybdic acid in ethanol

and heating at 100°C for about a minute. Products of bacterial metabolism appeared as blue spots which could be identified by comparing the Rf values with those of the reference compounds.

Dehydroxylation.

The procedure was similar to that for deconjugation, but cholic acid in concentrations of 0.01% and 0.1% respectively was used as enzyme inducer in the original Todd-Hewitt broth and as substrate. Controls were 10 µl of cholic acid and deoxycholic acid in chloroform.

Dehydrogenation of steroid nucleus.

Clostridia isolated from faeces from Hong Kong were tested for their ability to introduce a double bond, in conjugation with a keto group into the steroid nucleus (see Section 1.5). Fifty ml of Todd-Hewitt broth were prepared in 100 ml bottles and to each was added 5 mg of 5β-androstan-3-17-dione as inducer. It was convenient to add the steroid as 1 ml of a 0.005% (w/v) ethereal solution. These broths were inoculated from test strains grown for three days at 37°C in Robertson's cooked meat medium. The broths were incubated anaerobically for three days. A further 1 ml of the saturated steroid was added and the cultures were shaken at 37°C for three days on a 'New Brunswick' shaker (arbitrary setting 3.5). Steroids were extracted using an equal volume (50 ml) of chloroform/methanol (2 : 1) and 50 ml chloroform only. The extracts were evaporated to dryness, the residue dissolved in 1 ml of chloroform and 5 µl were spotted onto silica-gel T.L.C. plates (Polygram G.U.V./254, 0.25 mm. thick). The

steroids were separated in solvent V_1 (see Appendix) and the plates were examined under ultra-violet light (254 nM) for the production of 4-androsten 3-17-dione and other unsaturated steroids. Authentic samples of this compound were used for reference. Anisaldehyde-spray (see Appendix) could be used to further identify the products.

2.11. Organ culture of colonic mucosa.

2.11.i. Transport and preparation of the specimens.

Pieces of mucosa obtained at operation were immediately placed in small amounts of Waymouth's tissue-culture medium (see Appendix) in screw-capped pots. There was enough medium to wet the specimen, but not to submerge it. In this manner, the specimens were transported to the laboratory. The interval between removal of the specimen from the patient and cultivation was usually less than one hour and never greater than two hours. In the laboratory, the specimens were washed with three changes of Waymouth's medium, using a Pasteur pipette to force the medium over the surface of the mucosa. Using a rocking motion and a very sharp scalpel the initial ragged edges were removed and the specimens were cut into sections about 2 - 3 mm square with De Wecker's scissors. Throughout the procedure the specimen was handled as little as possible, and then only with fine watchmakers' forceps. The sections of tissue were placed,

mucosa-side upwards, on small squares of moistened sterile filter paper (Millipore, R.A. 0.2 μ) on metal grids in culture chambers similar to those described below. A botanical section-spatula was used to transfer the specimens. Tumour specimens were treated in a similar manner although it was often difficult to orientate the specimen on the filter paper.

During initial cultivation, the transport and washing medium contained antibiotics, but subsequently it was found that these could be omitted without adverse effect.

All manipulations in the laboratory were carried out in a 'Hepaire' laminar flow cabinet (Hepaire Manufacturing Ltd.).

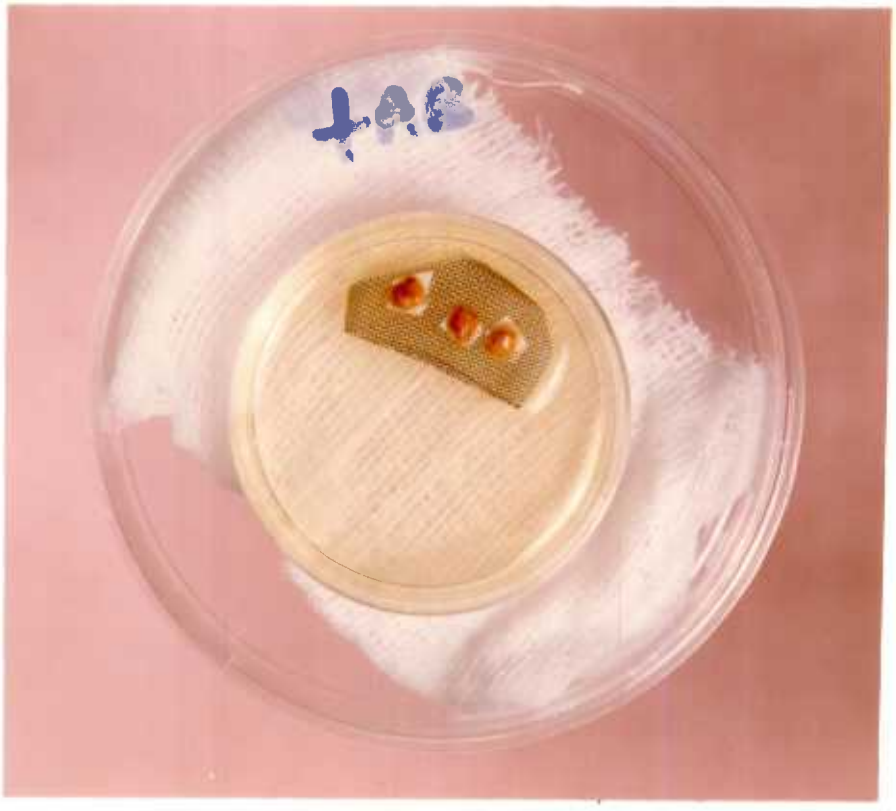
2.11.ii. The culture chambers.

A culture chamber is shown in photograph 5. Each chamber consisted of a 4" plastic petre-dish, lined with lint which had been soaked in 10 ml of double-distilled water. On this stood a small plastic crystallising dish containing Waymouth's tissue-culture medium. The mucosal specimen, on a piece of filter paper, was supported on a grid tressel (stainless-steel, 50 mesh squares/inch). The specimen was not submerged in the culture medium, the level of which had been adjusted so that it just touched the underside of the grid.

Photograph 5.

Culture chamber with specimens of mucosa. The level of Waymouth's medium in the small dish just touches the underside of the grid tressel and wets the filter paper supporting the cultures.

ORGAN - CULTURE OF LARGE BOWEL MUCOSA



Culture chamber with specimen.

The specimen in this photograph had been maintained for five days.

2.11.iii. Maintenance of cultures.

The culture chambers were covered with a lid and placed in plastic lunch boxes which were gassed with carbon-dioxide (final concentration, approximately 5%) and sealed with plastic tape. Cultures were incubated at 37°C. The medium was changed after 24 hours and then every 48 hours, for as long as the culture was maintained. When changing the medium, all manipulations were carried out in the laminar flow cabinet and fresh medium was warmed to 37°C before addition to the culture.

2.12. Attachment of E. coli to colonic organ-culture.

2.12.i. Preparation of bacterial culture.

A strain of E. coli (EB_{11}), which had been stored on a Dorset-egg slope, was subcultured onto blood-agar and incubated at 37°C for 24 hours. A well isolated colony was selected from this plate, subcultured into 50 ml of Todd-Hewitt broth and shaken on a 'Brunswick' shaker (arbitrary setting 3.5) for five hours at 37°C. The bacteria were harvested by centrifugation at 2,000 g for half an hour and the pellet was washed twice with 20 ml of Waymouth's medium. The final pellet was resuspended in 20 ml of Waymouth's medium and the number of viable bacteria per millilitre were estimated by the method of Miles and Misra (1938).

2.12.ii. Addition of bacterial culture to mucosa.

With the use of an automatic fixed volume pipette a 10 µl aliquot of the prepared bacterial suspension was dropped onto the surface of a 72 hour mucosal culture. After half an hour, the organ culture was washed twice with 5 ml of Waymouth's medium (without antibiotics), forced over the surface of the tissue with a Pasteur pipette. Associated bacteria were released by 'stomaching' the washed tissue in 4.5 ml of Waymouth's medium in a 'Colworth Stomacher 80'. The number of viable bacteria in this mascerate and in the medium in which the tissue had been cultured, were estimated by the method of Miles and Misra (1938).

SECTION III

RESULTS AND DISCUSSION

3.1. Validation of culture techniques.

3.1.i. Comparison of methods of isolation and effect of exposure of seeded plates.

The recovery of anaerobic bacteria from the faeces of six subjects when samples were manipulated in various ways are compared in Table 15. These experiments involved the use of large numbers of plates and in order to keep to the time schedule only one plate per dilution was seeded. Colony counts were made on plates where the number of colonies could be accurately assessed, i.e. those with between 30 and 200 colonies. A pre-set constant volume pipette had been used for the preparation of all dilutions and for seeding the plates (0.1 ml inoculum).

In all cases colony counts of aerobic bacteria were 100 to 1,000-fold lower than counts on anaerobically incubated plates. Thus, aerobic bacteria in these samples were fewer than 1% of the total and colony counts on anaerobic plates were taken to represent the 'total anaerobes'.

Higher colony counts were obtained on plates that had been seeded in the anaerobic chamber than on plates that had been seeded on the open bench and incubated without delay. However, this advantage was only maintained when anaerobically stored (pre-reduced) agar plates were used. There was no advantage when the plates had been stored in an aerobic environment.

TABLE 15

Recovery of faecal anaerobes using various methods.

(Counts expressed as $\log_{10}$ bacteria/gram wet-weight of faeces)

Specimen	1.		2.		3.		4.		5.		6.		Mean ± SD	
Storage of Media	$-O_2$	$+O_2$	$-O_2$	$+O_2$	$-O_2$	$+O_2$	$-O_2$	$+O_2$	$-O_2$	$+O_2$	$-O_2$	$+O_2$	$-O_2$	$+O_2$
Chamber. Immediate	10.07	9.92	9.97	9.77	10.69	10.34	9.87	9.78	10.10	9.12	9.83	9.87	10.09±0.31	9.80±0.39
Chamber. ½ hour	ND	ND	ND	ND	ND	ND	9.95	9.89	10.15	9.15	ND	9.85	10.05±0.14	9.63±0.42
Chamber. 1 hour	ND	ND	ND	ND	ND	ND	10.03	9.97	10.16	9.16	9.84	9.86	10.01±0.16	9.67±0.45
Bench. Immediate	9.94	9.92	9.87	9.87	10.11	10.16	9.66	ND	9.68	9.84	9.60	9.56	9.81±0.20	9.87±0.21
Bench. ½ hour	9.75	9.82	9.65	9.62	9.83	9.83	9.67	9.85	9.61	9.71	9.30	9.69	9.62±0.18	9.75±0.09
Bench. 1 hour	9.81	9.82	9.61	9.52	ND	ND	9.41	9.66	9.69	9.75	9.30	9.62	9.56±0.21	9.67±0.12
P.R.A.S.	ND		ND		ND		9.27 ±0.13		10.05 ±0.01		9.64 ±0.07		9.65±0.39	

ND = Not done. SD = Standard deviation.

The results obtained using the Hungate method (P.R.A.S.) were not consistent: in two cases the mean count was lower (up to half a logarithm) than the count obtained using pre-reduced media in the anaerobic chamber. In the third case the count was similar to that obtained using the chamber.

When seeded plates were left on the bench for half an hour, colony counts of total anaerobes were lower than those on plates which had been incubated without delay. The fall in count continued, though less markedly, if the plates were left for a further half an hour. There was no loss of recovery of anaerobes when plates were left in the chamber for up to one hour. The differences between the mean counts of total anaerobes obtained when the plates were left in the chamber for half and one hour and the mean counts when the plates were left on the bench for those periods, were probably significant ($p < 0.05$ calculated from Student's 't' test). Further experiments showed that when plates seeded in the anaerobic chamber on pre-reduced media were removed and exposed to the atmosphere of the laboratory, the count of total anaerobes began to fall during the first ten minutes (Figure 6, solid lines, samples A and B). Examination of a further five faecal samples from five individuals indicated that the overall rate at which the total anaerobe count fell was similar for all these specimens (Figure 6, dotted lines, samples C to G) and resembled the rate of fall of samples A and B. The fall was statistically significant in three cases and in one case (G) the fall was highly significant (p 0.001). The percentages by which the total anaerobe count fell for these five samples are shown in Table 16.

Figure 6.

Effect of time of exposure of seeded plates to room atmosphere on the recovery of bacteria from faeces: the count of total anaerobes per gram of faeces fell significantly for specimens E, F and G.

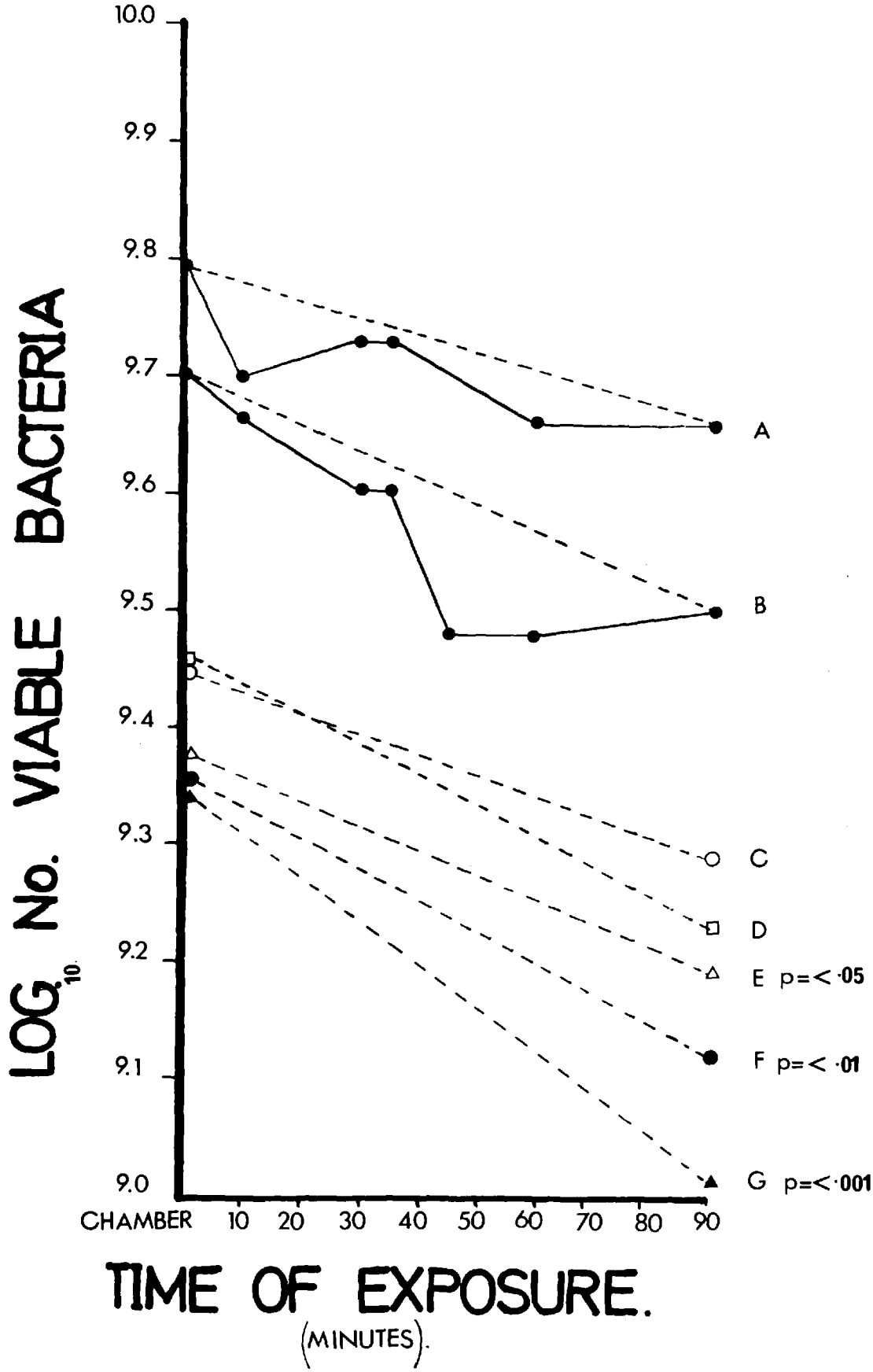

10.0
9.9
9.8
9.7
9.6
9.5
9.4
9.3
9.2
9.1
9.0
LOG10 No. VIABLE BACTERIA
CHAMBER 10 20 30 40 50 60 70 80 90
TIME OF EXPOSURE.
(MINUTES).
A
B
C
D
E p=<·05
F p=<·01
G p=<·001

TABLE 16

Effect on total anaerobe count of exposure of seeded plates to laboratory atmosphere.

Specimen	Percentage by which total anaerobe count fell after exposure for 90 minutes.
C	31
D	36
E	38
F	44
G	55

3.1.ii. The effect of various media recommended for the isolation of strict anaerobes from faeces.

A comparison of total anaerobe counts obtained on various media is shown in Table 17. The counts quoted are not mean values obtained from dilutions plated in triplicate but are derived from counting the number of colonies on a single plate seeded with a dilution which yielded between 30 and 200 colonies.

Assessment of results.

For each of the specimens, the media were arranged in descending order of merit according to the total anaerobe count obtained, each medium could therefore be assigned a rank number. Where an identical count was obtained from two media, the rank was shared and the number of the next best medium was correspondingly ammended. The rank numbers of each medium obtained for each of the six specimens were totalled to give a final score value. The lower the final score, the more efficient, overall, the medium. Those media consistently high in the order of merit had low total score values.

The media were then arranged in a final order of merit, one to eight, according to the total score values (Table 18). Pre-reduced Reinforced Clostridial Medium with blood (RCMB) used in conjunction with an anaerobic chamber appeared to be superior to the other media tested and a separate experiment was carried out to compare its performance with that of dithiothreitol medium, which was designed to be seeded on the

TABLE 17

Total anaerobe counts obtained from six faecal specimens seeded on various media.

	Medium							
	R.C.M.B.		Moore's		Blood Agar		Freter's Palladium	Freter's without Palladium
Sample	Bench	Chamber	Bench	Chamber	Bench	Chamber	Black	Black
H.	9.93	9.91	9.69	10.00	9.91	9.69	9.91	9.78
I.	9.81	10.11	9.82	9.99	9.93	10.04	10.20	10.07
J.	10.09	10.30	9.99	10.30	10.05	10.18	10.05	10.30
K.	9.91	9.85	9.88	9.81	9.75	9.89	9.90	9.96
L.	10.03	10.04	10.05	9.95	10.05	10.03	9.95	9.97
M.	9.92	10.06	9.91	9.95	9.94	9.92	10.23	9.92

Counts expressed as $\log_{10}$ per gram wet-weight faeces.

TABLE 18

Media for the enumeration of total anaerobes in faeces:

Descending order of merit.

Position	Medium	Score
1	Reinforced Clostridial Agar Anaerobic chamber	18
2	Freter's Medium with Palladium black Anaerobic chamber	21
3	Blood Agar - Anaerobic chamber	22
4	Freter's Medium without Palladium black - Anaerobic chamber	24
5	Moore's Medium - Anaerobic chamber	25
6	Blood Agar - Bench	29
7	Reinforced Clostridial Agar - Bench	33
8	Moore's Medium - Bench	37

bench. The results of this comparison are shown in Table 19.

3.1.iii. The effect on survival of isolates of picking colonies in the anaerobic chamber and on the bench.

Of the ten colonies picked under various conditions, the number which were strict anaerobes are shown in Table 20. There was no difference between the numbers of anaerobes isolated by picking colonies in the chamber and on the bench. Very few anaerobes survived on a plate kept under aerobic conditions for 24 hours. All those that did were found on further examination to be strains of Bacteroides fragilis.

Discussion.

The need for specialised equipment for the isolation of strictly anaerobic bacteria has been supported and contended by various workers (Drasar, 1967; Watt, Collee and Brown, 1974). If efficient isolation of these bacteria could be achieved using simple bench techniques many questions could be answered more quickly and research in this field would undoubtedly benefit.

The results of the present study indicate no statistical advantage

TABLE 19

Comparative recovery of total anaerobes from six faecal specimens using Reinforced clostridial agar in an anaerobic chamber and Dithiothreitol Medium on the bench

	Log_{10} Total anaerobes/g.wet weight faeces	
Sample Number	Reinforced Clostridial Agar (chamber)	Dithiothreitol Medium (bench)
1	9.91	9.78
2	10.30	9.83
3	10.04	10.20
4	9.66	9.67
5	9.60	9.79
6	9.77	9.59
Overall	9.88 ± 0.26	9.81 ± 0.21

TABLE 20

Effect of picking colonies under various conditions on the subsequent success of the cultures.

	Number of anaerobic cultures obtained when colonies picked under the stated conditions		
Specimen	Chamber	Bench	Bench after 24h. @ 4°C
1	8/10	7/10	5/10
2	10/10	10/10	2/10
3	10/10	10/10	3/10

gained from the use of an anaerobic chamber compared with rapid bench techniques for the isolation of strict anaerobes from faeces. However, the lowering of the total anaerobe count observed when seeded plates are exposed to the laboratory atmosphere for longer periods is not observed when such plates are left in an anaerobic chamber. Thus, used in conjunction with suitably pre-reduced media, the anaerobic chamber is advantageous when the cultivation of a large number of specimens is involved and immediate incubation in an anaerobic jar is not a practical proposition. This is of special advantage in a study such as described in part of this thesis - the examination of a large number of faecal specimens from different sources. The use of such a chamber for the isolation of strict anaerobes from clinical specimens is also described in this thesis (Section 2.6).

The full potential of the anaerobic chamber is not realised unless pre-reduced mediaare used. Thus, some of the advantages afforded by the flexible type of chamber lie in the fact that it can maintain an anaerobic environment in which plates can be stored, pre-reduced, ready for use and manipulation of specimens and seeded plates within this environment by one operator can proceed at a pace akin to that of more conventional aerobic bacteriology.

The flexible type of anaerobic chamber has several advantages over its rigid counterpart. Flexible vinyl film is low in cost and easy to work with and shape and, since it is optically clear, it allows good vision across the walls of the chamber. Flexible chambers are also much more comfortable to work in than are their rigid counterparts. Since there is a positive pressure of gas inside, any small leaks are

outwards and produce no change in the oxygen tension inside the chamber. Large leaks are usually obvious, but for small leaks Jones and Denver (1974) demonstrated the use of the inert gas freon (CCL_2F_2) added to the atmosphere of an anaerobic chamber as an aid to the location of leaks. Escaping freon was detected by passing the probe of a halogen leak detector specific for freon '12' over the outside surface of the chamber.

During the present study, the isolation of anaerobes from faeces was not improved numerically by the use of the Hungate technique with pre-reduced anaerobically sterilized media (P.R.A.S.). It should be remembered, however, that in this study not only were the two methods being compared, but the media also differed. The Brain-Heart-Infusion agar used as P.R.A.S. contained no blood, but was supplemented with haemin. Since bacterial colonies grow both in the body of the medium and on the surface, media for enumeration of these colonies must be clear and this automatically precludes any media containing red blood cells. The Hungate technique is technically difficult and tedious, even with the improvements of the Bellco-V.P.I. anaerobic culture system. Media in roll-tubes must be kept in a molten condition prior to inoculation and spinning, but must not exceed a temperature above which delicate bacteria would be killed. Roll-tubes containing small amounts of media fall in temperature quickly, and the process thus becomes a compromise between starting with fairly cool media ($50^{\circ}C$) and working quickly before it becomes solid. It is also technically awkward to pick for subculture subsurface colonies.

Consistently high numbers of strict anaerobes were isolated from

faeces during the present study on plates of pre-reduced Reinforced Clostridial Medium containing 10% horse blood, used in an anaerobic chamber. Of the other media tested, ordinary blood agar, pre-reduced and used in the anaerobic chamber, gave results which were as good as those obtained using more complex, often expensive (cf. palladium black), specially designed media. As a medium for use on the bench, ordinary blood agar also appeared to be markedly better than the more complex R.C.M.B. and Moore's medium. It may be that the complex media, designed for use in an anaerobic environment, produce more complex inhibitory agents when allowed to oxidize. All the bench media tested gave overall a poorer performance than the pre-reduced media. Dithiothreitol medium used on the bench gave results that were no better than those obtained using R.C.M.B. in the anaerobic chamber. This medium had the added disadvantage of having to be poured fresh each day. Human blood is recommended for use in this medium, but it was noted that colony size was markedly reduced on this and indeed any medium, to which such blood had been added. This may be an effect of the citrate usually added in blood-banks to prevent clotting, or could be due to natural inhibitors such as immunoglobulin.

Methods of isolation of strictly anaerobic bacteria from various sources have received much attention, but the problem of manipulation of isolated strains must not be forgotten. The results of the present study indicate that no advantage is gained by attempting to pick colonies from a plate within the anaerobic chamber. A few hardy strains of Bact. fragilis appear to be able to survive on an initial isolation plate in an aerobic environment for up to 24 hours. Tally, Stewart, Sutter and

Rosenblatt (1975) studied the oxygen tolerance and sensitivity of 57 freshly isolated anaerobic bacteria from clinical specimens and found that all the isolates tolerated eight hours or more of exposure to oxygen in room air. They concluded that although strictly anaerobic procedures may be necessary for the initial isolation of anaerobes, subsequent handling may safely be carried out on the open laboratory bench.

The present study, endorses this theory to some extent, for faecal isolates, although many isolates did not survive prolonged exposure (24 hours) to room atmosphere. In addition, the present study dealt with mixed populations of bacteria on a single isolation plate. Further investigations of the survival times of pure cultures of faecal isolates in atmospheric oxygen are necessary.

3.2. The isolation of anaerobic bacteria from wound swabs.

3.2.i. Selection of swabs for comparison.

Of 97 swabs cultured by the three different procedures, 21 of those processed in the anaerobic chamber, 24 of those examined on the bench with immediate incubation, and 26 of those incubated after delay, yielded no bacterial growth. Within those three groups, overgrowth by spreading organisms made analysis of growth impossible in twelve, six and four cases respectively. The number of swabs remaining for comparison in each group is shown in Table 21. In no instance were anaerobes

TABLE 21

Selection of swabs for comparison.

	Cabinet	Bench	
		Immediate Incubation	Delayed Incubation
Total no. of swabs examined	97	97	97
Number recorded as no bacterial growth	21	24	26
Number overgrown	12	6	4
Total remaining	64	67	67
Number of patients	50	49	48

isolated from a swab that was retained in the results from one method and discarded in another.

3.2.ii. Comparative recovery of anaerobic organisms.

Anaerobic bacteria were isolated from 42.5% of the swabs processed in the anaerobic chamber, from 13.5% of those processed on the bench and immediately incubated and from 4.5% of the swabs processed routinely (Table 22). For all the methods used, bacteroides organisms were the most common isolates. Anaerobic cocci and clostridia were also isolated. The recovery rates of all anaerobic bacteria, including the spore-forming clostridia were improved by the use of the anaerobic chamber with pre-reduced media; the recovery of the bacteroides was most noticeably improved. There was a less dramatic improvement with the rapid bench procedure compared with the delayed bench procedure.

3.2.iii. Distribution of anaerobes isolated by cabinet procedures.

The 64 wounds swabs that were processed in the anaerobic chamber included 33 swabs from abdominal and rectal sources, 13 of which yielded facultative organisms only and 20 of which yielded anaerobic organisms (Table 23). In five cases only anaerobes were isolated. A single species of anaerobe was recovered in pure culture from three swabs, two or more species of anaerobes were isolated from two swabs. A single strain of anaerobe and one or more facultative bacteria were recovered

TABLE 22

Numbers of isolates of various species.

		Bench	
	Cabinet	Immediate Incubation	Delayed Incubation
Bacteroides species	22	5	2
Anaerobic cocci	7	3	1
Clostridia	5	1	1
Total Anaerobes	34	9	4
Number of swabs	64	67	67
Percentage from which anaerobes isolated	42.5	13.5	4.5
No. of patients from whom anaerobes isolated	23	9	3

TABLE 23

Distribution of anaerobes in 64 wound swabs.

(Numbers of swabs in each category)

Organisms	TYPE OF SPECIMEN (SOURCE OF WOUND SWAB)						
		Other					
	Abdominal and rectal Total	Total	Skin/Bone	Superficial Abscesses	Respiratory	Muscle	Renal
Specimens with:							
Facultatives only	13	24	10	7	4	2	1
Anaerobes	20	7	4	1	1	-	1
Anaerobes only	5	2	1	1	-	-	-
1 species	3	2	1	1	-	-	-
2 species	2	-	-	-	-	-	-
Anaerobes and Facultatives	15	5	3	-	1	-	1
1 species	11	4	3	-	-	-	-
2 species	4	1	-	-	1	-	-

from four others. Thirty-one swabs from other sites were also examined, these included burns, osteochondritis, abscesses, and swabs from respiratory and renal operation wounds. Twenty-four of these yielded facultatives only. Anaerobes were isolated from only seven, which included two swabs from which a single species of anaerobe was isolated in pure culture. Single strains of anaerobic bacteria in mixed culture with facultative organisms were recovered from four swabs, and one swab yielded facultative organisms with two species of anaerobe.

3.2.iv. Nature of anaerobic isolates.

Of the anaerobes isolated by the anaerobic chamber method, 65% from abdominal swabs and 62.5% from other sources were bacteroides (Table24). Fewer anaerobic cocci (19%) were isolated from abdominal sources than from other sources (25%). Fifteen percent of the abdominal isolates were clostridia, as were 12.5% of isolates from other sources.

The majority of the bacteroides isolated were identified as Bacteroides fragilis (Table 25), Bacteroides malaninogenicus was isolated twice, in both instances from swabs of small bowel operation wounds. The anaerobic cocci isolates were all Gram-positive organisms of the genera Peptococcus and Peptostreptococcus. The peptococci were Peptococcus asaccharolyticus and Peptococcus magnus. The anaerobic streptococci were identified as Peptostreptococcus intermedius. The clostridia were all Clostridium perfringens.

TABLE 24

Anaerobes isolated from wound swabs on pre-reduced media.

	Abdominal and Rectal Source	Other Sources
No. of swabs with Anaerobes	20	7
No. of Anaerobes isolated	26	8
% Bacteroides	65	62.5
% Anaerobic Cocci	19	25
% Clostridia	15	12.5

TABLE 25

Species of anaerobic bacteria isolated from wound swabs.

Organism	Number of Isolates
Bacteroides fragilis	20
Bacteroides melaninogenicus	2
Peptococcus asaccharolyticus	2
Peptococcus magnus	2
Peptostreptococcus intermedius	2
Clostridium welchii	5
Total	34

Discussion.

The clinical significance of anaerobes in wound infections cannot be assessed from the data obtained in this study. No follow-up was made of the patients and there was no assurance that the swabs were taken in a way that avoided contamination with normal flora. Indeed the high rate of recovery of anaerobes from abdominal and rectal sources might be accounted for by such contamination. This seems less likely in the seven cases where only anaerobes and no facultative organisms were recovered. It is interesting to note here that anaerobes were isolated more frequently together with facultative organisms than on their own. Macdonald, Gibbons and Socransky (1960) and Moore and Gross (1968) have shown cases where anaerobic bacteria are pathogenic only where they exist in a synergistic situation with other bacteria.

The results of the present study indicate that the use of pre-reduced (P.R.) media in an anaerobic chamber improves the recovery of anaerobic bacteria from wound swabs, and that immediate incubation of plates seeded on the bench is preferable to methods which involve delayed anaerobic incubation of seeded plates.

No direct comparison can be made between the results obtained by cabinet and bench procedures since the carbon dioxide content of the anaerobic jars differed in these two situations. However, the results of a similar study by Vargo et al. (1971) in which CO_2 was included in all anaerobic jars, were comparable with those obtained in this study (Table 26). Lewis, Bedell and Rettger (1940) and Watt (1973), working

with pure cultures, have shown that adding CO_2 increases both numbers and colony size of organisms incubated in anaerobic jars. The inclusion of CO_2 in the gas mixture in anaerobic jars is to be recommended, although its effect on the recovery of anaerobes in the clinical situation may not be as dramatic as with pure cultures. Even with CO_2 in the anaerobic jar, efficient isolation of anaerobes from routine clinical specimens can only be achieved with the use of pre-reduced media.

McMinn and Crawford (1972) found that pre-reduced liquid media were superior to solid media incubated in anaerobic jars for the isolation of anaerobes from clinical specimens known to contain anaerobes.

In contrast, Rosenblatt, Fallon and Finegold (1973) showed that anaerobes could be isolated from clinical specimens just as effectively on the bench with anaerobic jar procedures as on pre-reduced solid media in an anaerobic chamber (Table 26). However, in the latter study, the specimens were specially collected under anaerobic conditions, and speedily transported to the laboratory. Similarly, Dowell (1972) using pre-reduced solid media in an anaerobic chamber, found no improvement in the recovery of anaerobes from clinical specimens which had been submitted under anaerobic conditions.

In the present study the clinical specimens had been through the normal hospital transport system, and in many cases, several hours had elapsed between taking and cultivating the swabs. Throughout transportation the swabs were in an aerobic atmosphere. It is probable that many more organisms would have been recorded had the specimens been submitted

TABLE 26

Isolation of anaerobes from clinical specimens under various conditions.

No. of Specimens	McMinn and Crawford (1970)		Rosenblatt et al. (1973)			Vargo et al. (1971)		This study	
	38 specimens		23 specimens			145 specimens		64 specimens	
	Bench	Prereduced Anaerobically Sterilized Media	Bench	Cabinet	Prereduced Anaerobically Sterilized	Bench	Cabinet	Bench	Cabinet
Anaerobic Transportation	-	-	+	+	+	-	+	-	-
+ CO^2	+	+	+	+	+	+	+	-	+
% Positive	8.2	100	100	100	100	18	41	13.5	42.5

under anaerobic conditions, and it is hoped that, in future, hospital laboratories in Britain will make efforts in this direction.

It is encouraging that anaerobic bacteria were isolated without employing specialised complicated media. Ordinary blood agar was used for all three procedures in this study.

In order to have a constant supply of pre-reduced media, a method of storing media in a reduced state is required. In addition, to try to ensure that every culture for anaerobic incubation is put into an anaerobic jar immediately it is seeded would require extra technician time and a great many jars and so a method of keeping seeded plates in an anaerobic environment is also required. The anaerobic chamber meets both these requirements. It is appreciated that many people feel that the chamber and air-lock system, although a useful tool for research, is too cumbersome for the ordinary clinical laboratory. However, the increased rate of isolation of anaerobes should outweigh any disadvantage. Martin (1971) has described a cupboard and jar both capable of being flushed with oxygen-free gas, and this type of apparatus may also prove useful to the routine laboratory. Pre-reduced, anaerobically sterilized media are now being marketed commercially in the United States and if this service is extended to Britain, it would be a further possibility for routine use.

3.3. Antibiotic disc tests for rapid identification of non-sporing anaerobes.

Strains were designated 'resistant' to an antibiotic when there was no zone of inhibition around the disc. Strains of 'intermediate' resistance had zone diameters less than 10 mm. The discs were 6 mm in diameter. There were no outstanding differences in the sensitivity patterns of strains of the same species that had been isolated from faeces of varying geographical origins.

3.3.i. Bacteroides fragilis.

The antibiotic susceptibility patterns obtained for Bacteroides fragilis were comparable with those obtained by Sutter and Finegold (1971) and are shown in Table 27.

All strains of Bacteroides fragilis tested were found to be resistant to a 5 µg disc of vancomycin.

3.3.ii. Bacteroides melaninogenicus.

Only three strains of Bacteroides melaninogenicus were tested and these differed in sensitivity to colistin and neomycin from twelve strains tested by Sutter and Finegold (1971) (see Discussion). All three strains

TABLE 27

Susceptibility patterns of Bacteroides fragilis.

		Sutter and Finegold 1971 (100 strains)	This study (123 strains)
Colistin 100 µg	R	100	94
	S	--	2.5
	Intermediate	--	3.5
Erythromycin 60 µg	R	--	1
	S	100	98
	Intermediate	--	1
Kanamycin 1000 µg	R	100	96
	S	--	1
	Intermediate		3
Neomycin 1000 µg	R	78	83
	S	20	15
	Intermediate	2	2
Penicillin 2u (1.2 µg)	R	93	78
	S	7	19
	Intermediate	--	3
Rifampicin 15 µg	R	-	2.5
	S	100	97.5
	Intermediate	--	--

Figures expressed as percentages Disc diameter = 6 mm

R = No zone of inhibition

S = Diameter of inhibition zone $>$ 10 mm

Intermediate = diameter of inhibition zone $<$ 10 mm

were resistant to a 5 µg disc of vancomycin.

3.3.iii. Bifidobacteria.

The antibiotic susceptibility patterns of 19 strains of Bifidobacterium adolescentis and twelve strains of Bifidobacterium bifidum were smaller and are summarized in Table 28. These organisms were in general much less resistant to the test antibiotics than were the Gram-negative anaerobic bacteria. In addition, all strains tested were sensitive to a 5 µg disc of vancomycin.

5.3.iv. Eubacteria.

Five strains of Eubacterium aerofacines were tested and no clear pattern of antibiotic sensitivity emerged (Table 29). However, all strains were sensitive to the 5 µg disc of vancomycin.

Discussion.

The need for a rapid method for the presumptive identification of non-sporing anaerobic bacteria, especially those isolated from clinical material, is becoming increasingly important. Gram-negative anaerobic bacteria, in particular, Bacteroides fragilis and Bacteroides melaninogenicus, are frequently isolated from clinical specimens (Stokes, 1958;

TABLE 28

Susceptibility patterns

of Gram-positive non-sporing anaerobes.

Antibiotic		Bifidobacterium adolescentis (19 strains) Percentage Resistant	Percentage Sensitive	Bifidobacterium bifidum (12 strains) Percentage Resistant	Percentage Sensitive	Percentage Intermediate
Colistin	10 µg	95	5	92	8	-
Erythromycin	60 µg	-	100	-	100	-
Kanamycin	1000 µg	32	68	17	75	8
Neomycin	1000 µg	16	84	17	75	8
Penicillin	2 µ (1.2 µg)	10	90	-	100	-
Rifampicin	15 µg	-	100	-	100	-

Moore, Cato and Holdeman, 1969) and a means of identification that could be carried out during primary isolation would be advantageous.

The antibacterial susceptibility patterns of strains of Bacteroides fragilis tested during this study were similar to those obtained by Sutter and Finegold (1971). However, fewer isolates were found to be resistant to a disc containing two units (1.2 µg) of penicillin. Strains used in the present study had recently been isolated from the intestine. Those tested by Sutter and Finegold included stock strains which would have been subjected to repeated subculture. Fievez (1963) noted that the Gram-negative anaerobe Sphaerophorus (Fusobacterium) necrophorus was more sensitive to penicillin on primary isolation than after several subcultures. It may be that a similar phenomenon occurs with bacteroides. Most of the clinical isolates tested in the present study were resistant to 2 u of penicillin.

Only three strains of Bacteroides melaninogenicus were available for study and their sensitivity to colistin and neomycin differed from the twelve strains studied by Sutter and Finegold. The most striking difference was sensitivity to neomycin. All the strains of Bact. melaninogenicus studied by Sutter and Finegold were sensitive to 100 µg neomycin; in contrast, the three strains in the present study were resistant to this concentration. Garrod (1955) in a plate dilution study found Bact. melaninogenicus was resistant to streptomycin. In this study and in the study by Sutter and Finegold Bact. melaninogenicus was found to be resistant to the related antibiotic, kanamycin. Martin, Gardner and Washington (1972) using an agar dilution technique found Bact. melaninogenicus to be sensitive to rifampicin and erythromycin.

TABLE 29

Antibiotic susceptibility patterns of five strains of Eubacterium aerofaciens.

Strain		Individual Sensitivities A	B	C	D	E
Colistin	100 µg	R	S	R	S	R
Erythromycin	60 µg	S	S	S	R	S
Kanamycin	1000 µg	S	S	S	S	R
Neomycin	1000 µg	S	S	S	S	R
Penicillin	2 µ (1.2 µg)	R	S	S	S	S
Rifampicin	15 µg	S	S	S	R	S

R = No zone of inhibition

S = Diameter of inhibition zone $>$ 10 mm

Intermediate = Diameter of inhibition zone $<$ 10 mm

Most strains were sensitive to penicillin G, and resistant to kanamycin. These trends are also shown in the disc sensitivity tests of Sutter and Finegold and in the present investigation.

Susceptibility to a 5 µg disc of vancomycin as recommended by Sutter et al. (1972), proved to be a useful 'back-up' for a Gram reaction. All the Gram-negative anaerobes in this study were resistant to such a disc. All the Gram-positive organisms were sensitive.

There is no solid evidence for pathogenicity of the Gram-positive non-sporing anaerobic bacteria, although they are often isolated from clinical material, in mixed culture with facultative organisms or with Gram-negative anaerobes (Smith and Holdeman, 1968; Moore et al., 1969). However, these bacteria also form a major part of the intestinal microflora of man (Gossling and Slack, 1974). There were few differences in antibiotic sensitivity between the two species of bifidobacteria tested in the present study. Both Bifidobacterium adolescentis and Bifid. bifidum were sensitive to the disc of penicillin and erythromycin. More strains of Bifid. adolescentis were resistant to the kanamycin disc than were the strains of Bifid. bifidum. These results are similar to those of a plate dilution sensitivity test reported by Miller and Finegold (1967). Miller and Finegold found that Bifid. adolescentis was more sensitive to neomycin than Bifid. bifidum. Laverge et al. (1959) found the sensitivity of Bifid. bifidum to erythromycin and neomycin to be similar to that found by Miller and Finegold.

Courtieu et al. (1960) reported a single strain of bifidobacteria to be sensitive to colistin. However, Miller and Finegold (1967) found

bifidobacteria to be resistant to the related antibiotic polymixin B and in the present study most strains of Bifid. adolescentis and Bifid. bifidum were resistant to colistin.

Eubacterium aerofaciens was more sensitive to the test antibiotics than were the bifidobacteria. Martin, Gardner and Washington (1972) using an agar dilution technique found that in general, bifidobacteria (unidentified species) were more sensitive than eubacteria to erythromycin, penicillin and rifampicin. In the present study using antibiotic discs, eubacteria and bifidobacteria showed similar sensitivity patterns to these antibiotics.

The results of the present study indicate that antibiotic disc susceptibility patterns as proposed by Sutter and Finegold (1971) provide a reliable method for the rapid presumptive identification of Bacteroides fragilis. Identification of Bacteroides melaninogenicus can also be achieved provided the system is backed up by other simple tests, such as those recommended by Sutter and Finegold. This method would be useful for the initial presumptive identification of these two species of anaerobe, particularly when isolated from clinical specimens. However, identification on an initial isolation plate is not achieved and the time taken to isolate and purify a culture is not eliminated.

The sensitivity of an isolate to the high concentrations of antibiotics used in this study would give no indication of the sensitivity of this strain to concentrations of antibiotics obtainable 'in vivo'. For this purpose a separate sensitivity plate would have to be prepared.

Using the antibiotics recommended for the identification of Gram-negative anaerobes, no distinguishing pattern of antibiotic sensitivity suitable for identification of Gram-positive anaerobes emerged. It may be that different antibiotics, or different concentrations of antibiotics are needed to provide a similar rapid preliminary identification of these organisms and an investigation in this direction is desirable.

As with all tests for antibiotic susceptibility the possibility of acquired resistance, even to the high concentrations of antibiotics used in this study, should be remembered in interpretation of results.

3.4. Survival of cultures of non-sporing anaerobes during storage.

The strains isolated during the geographical surveys had been stored at -50°C in Robertson's cooked meat medium with 10% glycerol for between six months and two years. More than 80% of the organisms were recovered for identification.

Data for the survival during storage of organisms isolated from stools from the U.S.A., Japan and Hong Kong is presented in Table 30.

TABLE 30

Survival of non-sporing anaerobes at -50°C in Robertson's cooked meat medium with 10% glycerol.

Country of origin	Length of storage	Percentage of cultures remaining viable
U.S.A.	1 year	81.5
Japan	1 year	96.0
Hong Kong	6 months	95.6
Hong Kong	2 years	81.3

3.5. The faecal flora.

3.5.i. The non-sporing anaerobes in faeces from six countries.

A total of 224 strains of non-sporing anaerobes were isolated from the faeces of 34 people on a mixed western diet in the U.S.A., England and Scotland; 215 strains were isolated from the faeces of 45 people on carbohydrate-rich diets in Uganda, India and Japan.

Identification of the isolates.

All the isolates could be assigned to genera. The predominant groups of non-sporing strict anaerobes in faeces were found to be similar for all the populations studied; they fell into three main genera: Bacteroides, Bifidobacterium and Eubacterium. Minor components of the anaerobic flora consisted of fusobacteria, propionibacteria and lactobacilli. Although similar groups of organisms were isolated from all the faecal samples, with bacteroides the most common isolates, there were differences in the distribution and relative proportions of these organisms. Faeces from 'western-diet' countries (England, U.S.A. and Scotland) appeared to have a larger proportion of Gram-negative organisms (bacteroides and fusobacteria), than did those of people from countries with 'high-carbohydrate' diets (India, Uganda and Japan). The consequently larger proportion of Gram-positive organisms found in faeces from these countries seemed to be due mainly to the

TABLE 31

Percentage frequency of genera of non-sporing anaerobes among isolates from faeces of populations consuming different diets.

Category of diet* and populations sampled	Number of isolates identified	Percentage frequency among isolates from the stated populations of genus					
		Bacteroides	Fusobacterium	Bifidobacterium	Eubacterium	Propionibacterium	Lactobacillus
"Mixed-Western"; U.S.A, England, Scotland.	224	61.6	0.9	25.0	6.7	3.1	0
"High-carbohydrate"; Uganda, India, Japan.	215	37.7	1.4	27.0	23.2	5.1	1.4

* See Table 8.

contribution of the eubacteria (Table 31): this difference in the proportion of eubacteria was statistically significant ($p < 0.001$, obtained by evaluation of normal deviate of two proportions; England, 1975). Propionibacteria were found in both of the population groups studied. Strictly anaerobic lactobacilli, however, were found only in faeces from Uganda and Japan and in numbers too small to draw any conclusions.

Sub-division of major genera.

More than 80% of the isolates could be assigned to species, and the same major species of non-sporing anaerobes were found in faeces from all the dietary groups.

Almost all the bacteroides organisms were identified as Bact. fragilis (Table 32). Two-thirds of the bifidobacteria were identified as Bifid. adolescentis; Bifid. bifidum and Bifid. eriksonii were also common. However, 8% of bifidobacteria from 'western-diet' countries and 14% from 'high-carbohydrate' countries, could not be assigned to any species. More than 80% of the eubacteria could be shown to be E. aerofaciens.

TABLE 32

Major species of non-sporing anaerobes found in faeces.

	USA, ENGLAND AND SCOTLAND	UGANDA, INDIA AND JAPAN
No. of strains tested	224	215
% of strains identified at species level	88.5	85
No. of Bacteroides tested	138	81
% found to be *Bact. fragilis*	92	98
No. of Bifidobacteria tested	56	58
% found to be:		
Bifid. adolescentis	59	63.5
Bifid. bifidum	23	16
Bifid eriksonii	10	6.5
No. of Eubacteria tested	15	50
% found to be *E. aerofaciens*	86	82

TABLE 33

Minor species of non-sporing anaerobes isolated from faeces in the present study.

Species	Number of isolates	Source
Bacteroides melaninogenicus	5	USA and India
Bact. hypermegas	1	India
Bact. ruminicola	1	Japan
Bact. trichoides	1	Japan
Bact. oralis	1	Japan
Fusobacterium fusiforme (nucleatum)	3	Japan
F. plauti	2	Japan
F. abscendens	1	Japan
Eubacterium lentum	9	Scotland and India
E. tortousum	4	Japan
E. limosum	4	Japan
E. ventriosum	1	Japan
E. contortum	2	Japan
Propionibacterium acnes	11	Scotland, USA and India
P. avidum	9	Scotland, USA, India and Japan
P. jensenii	5	India and Japan
P. freundreichii	3	USA and Japan
P. theonii	1	Japan
Lactobacillus catenaforme	4	Japan and Uganda
L. crispatus	1	Japan

Other species of non-sporing anaerobes.

A variety of other species of non-sporing strict anaerobes were isolated in small numbers and identified (Table 33). The isolation of Bact. melaninogenicus from human faeces is not unusual, but other more unexpected bacteroides species were also found (see Discussion).

3.5.ii. The faecal flora of persons from Hong Kong.

Cultural counts.

Table 34 shows the geometric means, standard deviations and ranges of the viable counts for the various bacterial groups investigated. In general, the number of anaerobes outnumber the aerobes 100 to 1000-fold, anaerobes numbering 10^9 to 10^{10} bacteria per gram (wet-weight) of faeces and aerobes 10^6 to 10^7 per gram.

Bacteroides, bifidobacteria and eubacteria were not enumerated on selective media and counts of these bacteria were estimated as a proportion of the total anaerobe count, using the ratios of those groups of bacteria to each other obtained by picking and identifying colonies of organisms from an R.C.M.B. plate.

TABLE 34

Cultural counts of various groups of bacteria in the faeces of persons from Hong Kong.

Bacterial Group	Income Group		
	A	B	C
Total anaerobes	9.85± 0.43 (8-10)	9.85± 0.45 (9-10)	9.61± 0.36 (9-10)
Bacteroides*	9.70± 0.42 (8-10)	9.62± 0.49 (8-10)	9.33± 0.41 (8-9)
Bifidobacteria*	9.05± 0.63 (7-10)	9.30± 0.44 (8-10)	9.04± 0.32 (8-9)
Eubacteria*	8.65± 0.46 (7-9)	8.83± 0.45 (8-9)	8.83± 0.36 (8-9)
Lecithinase - producing clostridia	4.24± 1.95 (4-6)	4.76± 1.22 (3-6)	5.73± 1.77 (3-7)
Clostridia not producing lecithinase	5.90± 0.70 (5-6)	5.27± 1.03 (5-6)	5.56± 0.68 (5-6)
Veillonellae	4.07± 1.08 (2-5)	4.14± 0.93 (3-5)	4.07± 1.20 (3-6)
Lactobacilli	5.43± 1.89 (3-8)	4.15± 1.19 (4-8)	6.11± 1.96 (4-8)
Total aerobes	7.16± 1.25 (5-8)	7.03± 0.69 (5-9)	7.48± 0.68 (6-8)
Enterococci	5.41± 1.80 (2-7)	4.89± 1.87 (4-8)	5.61± 1.34 (4-8)
Enterobacteria	7.17± 1.24 (4-8)	7.23± 1.07 (5-8)	7.09± 1.18 (5-8)

Counts expressed as $\log_{10}$ bacteria per gram wet-weight of faeces.

± standard deviation. Figures in parenthesis indicate range.

* estimated count (see text).

Identification of the isolates.

The non-sporing anaerobes.

A total of 96 strains of non-sporing anaerobes were isolated from the faeces of 15 people in income group A; 146 strains were isolated from the faeces of 20 people in income group B and 114 strains were isolated from the faeces of 19 people in income group C.

All the isolates were assigned to genera, but were not identified further. Bacteroides, bifidobacteria and eubacteria were the most common genera isolated; lactobacilli and propionibacteria were isolated in small numbers. Similar organisms were isolated from all three income groups, but faeces from persons in the high income group A appeared to have larger proportions of bacteroides than did those of persons in the lower income groups (groups B and C). These differences were statistically significant ($p < 0.05$). Persons in income group B had a greater proportion of bifidobacteria than persons in group A. Persons in group C also had a greater proportion of bifidobacteria than group A and in addition, more eubacteria than both groups A and B (Table 35); these differences in the proportions of bifidobacteria (B : A) and eubacteria (C : B) being statistically significant ($p < 0.01$ and $p < 0.05$, respectively). Propionibacteria were isolated from the faeces of persons in groups B and C only. Strictly anaerobic lactobacilli were isolated from all three income groups.

TABLE 35

Percentage frequency of genera of non-sporing anaerobes among isolates from faeces of persons in Hong Kong.

Income Group	No. of Persons	No. of isolates identified	Percentage frequency among isolates from the stated populations of genus				
			Bacteroides	Bifidobacteria	Eubacteria	Lactobacilli	Propionibacteria
A	15	96	72	14.5	9.4	4.2	-
B	20	146	60.3	28.8	9.6	0.7	0.7
C	19	114	57	23.7	17.0	1.7	0.9
ALL	54	356	63.1	22.3	11.9	2.2	0.5

The clostridia

Only clostridia that did not produce a lecithinase on Willis and Hobbs agar (Willis and Hobbs, 1958) were examined, since various species of such clostridia have been shown to produce desaturated steroids from bile salts and as such are probably implicated in the aetiology of large-bowel cancer (see Section 1.4.ii).

Ninety-two strains from 26 people in the three income groups were examined and the identities of these isolates are shown in Table 36. In cases where strains were similar to type strains (Holdeman and Moore, 1972) but differed in one or two sugar reactions, they are included in that group and named in quotes. A difference in the reaction with one sugar was permissible where the reactions for other sugars of that biochemical group (e.g. other disaccharides) were normal.

Strains that did not correspond to any conventional species were grouped on the basis of fatty acid production, i.e. strains that produced butyric or acetic acid as the major products of glucose metabolism and strains that produced a variety of acids.

Unidentified butyric acid-producing organisms were isolated from all persons studied in income group A and accounted for a third of the isolates in this group. In all, more than two-thirds of the isolates could not be assigned to any recognized species. Species corresponding to a group of clostridia designated Clostrium 'A' and Clostridium 'D', reported as isolates from the faeces of Japanese-Hawiians by Moore and Holdeman (1974), were also isolated.

TABLE 36

Clostridia (non-lecithinase producers) isolated from the faeces of persons from Hong Kong.

Income group	No. of persons	No. of strains	Identity of isolates	No. of isolates	No. of individuals from whom isolated
A	15	15	'Cl. innocuum'	1	1
			Cl. 'A' (Moore)	1	1
			Cl. rectum	1	1
			Cl. ramosum	1	1
			unidentified butyric acid producers	5	5
			unidentified acetic acid producers	4	3
			unidentified mixed acid producers	2	2
B	14	59	'Cl. paraputrificum'	7	3
			Cl. paraputrificum	1	1
			Cl. rectum	4	4
			Cl. beijerinckii	1	1
			'Cl. felsineum'	5	4
			'Cl. innocuum'	1	1
			Cl. innocuum	3	2
			Cl. inulinum	5	4
			Cl. 'A' (Moore)	1	1
			Cl. ramosum	2	2
			Cl. sphenoides	2	2
			Cl. chauvoei	1	1
			Cl. pasteuraneum	1	1
			unidentified butyric acid producers	18	11
			unidentified acetic acid producers	2	2
			unidentified mixed acid producers	5	2
C	7	18	Cl. rectum	2	1
			Cl. beijerinckii	2	1
			'Cl. innocuum'	1	1
			Cl. aurantibutyricum	1	1
			Cl. 'D' (Moore)	1	1
			Cl. sphenoides	1	1
			unidentified butyric acid producers	4	3
			unidentified acetic acid producers	2	2
			unidentified mixed acid producers	4	2

Strains in quotes are similar to the type strain, but not identical.

Forty percent of the isolates from persons in income group B were unidentifiable and 70% of these produced butyric acid as their main end product. A strain resembling Cl. paraputrificum was isolated from half of the people in this group and Cl. rectum and Cl. inulinum were isolated from one third of the people. Clostridium 'A' (Moore) was again isolated.

Fifty-five percent of the isolates from persons in group C did not fall into any conventional group. The majority of these bacteria produced butyric acid, or mixed fatty acids from glucose. Cl. auranti-butyricum was isolated from one person in this group. Moore's Clostridium 'D' was also isolated from one person.

Discussion.

The non-sporing anaerobes.

The results of the present study are in agreement with numerous other studies on this topic in showing that non-sporing strict anaerobes numerically dominate the bacterial flora of human faeces. The anaerobe most frequently isolated was Bacteroides fragilis, a species shown to be the most numerous in human faeces by several other workers (Moore et al. 1969; Moore and Holdeman, 1974; Attebery et al., 1974). Moore and Holdeman found Bact. fragilis sub-species vulgatus to be the dominant sub-species in faeces from Japanese-Hawiian males. However, a study of

DNA homologies has cast doubt on the value of sub-grouping Bact. fragilis (Johnson, 1973) and no attempt was made during the present study to further differentiate Bact. fragilis.

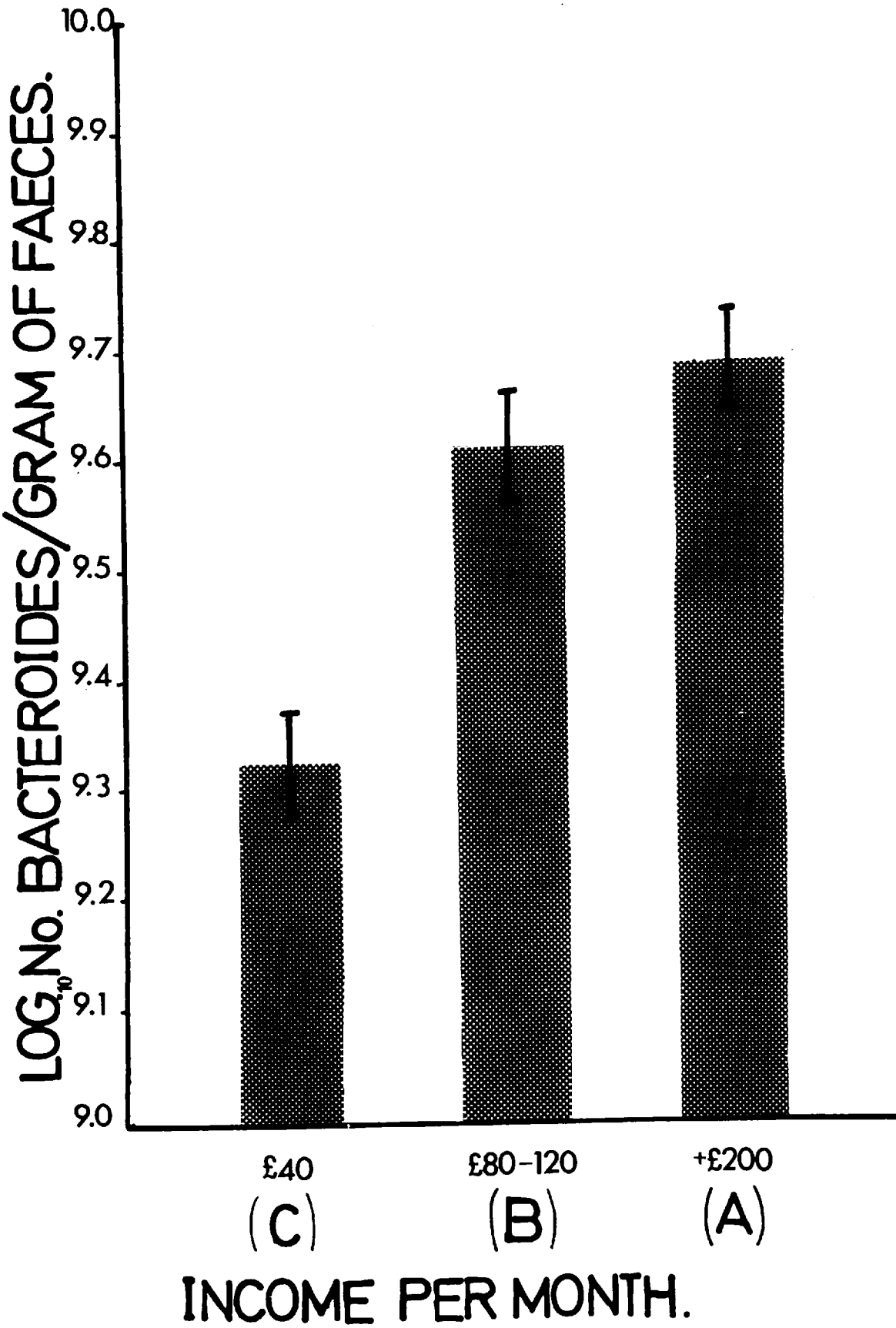
Bifidobacteria and eubacteria, in particular Bifidobacterium adolescentis and Eubacterium aerofaciens were also common isolates and these results are in agreement with those of Attebery et al. (1974), Gossling and Slack (1974) and Moore and Holdeman (1974). The isolates of Bifid. bifidum and Bifid. eriksonii in small numbers conforms to the patterns observed by Moore and Holdeman (1974).

Several unexpected species of non-sporing anaerobe were isolated during the present study. Bact. hypermegas is common in the intestine of poultry and Bact. ruminicola in the bovine rumen. Bact. trichoides is common in the intestine of pigs. Bact. oralis is frequently isolated from the human mouth and is similar to Bact. fragilis. Fusobacterium fusiforme is also found in the mouth and organisms of these species that were recovered from faeces were probably passed down the intestinal tract with food. All the species of eubacteria isolated are common intestinal organisms (Holdeman and Moore, 1972). It is possible that Propionibacterium acnes and P. avidum were contaminants derived from the skin of the peri-anal area, skin being the more common habitat of these organisms. P. jensenii, P. freundreichii and P. theonii have not in the past been thought of as predominantly intestinal organisms, more usually being isolated from dairy products. Their isolation from faeces is interesting; some were isolated from persons whose diet would not have included much dairy produce. It is probable that the strictly anaerobic Lactobacillus catenaforme is truly a human intestinal organism. L. crispatus is

Figure 7.

The relationship between the numbers of bacteroides in faeces and monthly income in Hong Kong.

Black bars indicate one standard deviation on either side of the mean values.


10.0
9.9
9.8
9.7
9.6
9.5
9.4
9.3
9.2
9.1
9.0
LOG10 No. BACTEROIDES/GRAM OF FAECES.
£40
£80-120
+£200
(C)
(B)
(A)
INCOME PER MONTH.

common in the gastrointestinal tract of cows and pigs.

Studies by Moore and Holdeman (1972) with 1,057 isolates from six faecal specimens have demonstrated the presence of 86 species of anaerobe and undoubtedly many more would be found if a greater number of isolates could be characterised. The main interest in the present study was the comparison of dietary groups rather than the exhaustive study of individuals.

The present study demonstrates the relative predominance of eubacteria in the faeces of people on a carbohydrate-rich diet in India, Uganda and Japan and persons in low income groups in Hong Kong. Conversely, faecal samples from persons from the U.S.A., England and Scotland and a high income group in Hong Kong, had significantly larger proportions of Bacteroides spp. The relationship between the numbers of bacteroides in faeces and income group in Hong Kong is shown in Figure 7. Income reflects dietary habits in Hong Kong. The diet of persons in high income groups contains some foods similar to those of western countries such as England and the U.S.A. Persons in low income groups consume only traditional chinese meals. In addition the diet of persons in high income groups is richer in animal protein whilst persons in low income groups consume more carbohydrate.

The populations compared in the present study differed in many respects other than diet so to assign a controlling role to this factor is probably not justified. Indeed, consideration of the data from individuals indicates that the difference between the groups might be accounted for on the basis of the different dominant organisms in various individuals (Table 37). Individuals with four types of flora were found,

TABLE 37

An analysis of the predominant bacterial groups in faeces samples from individuals of various origins.

Number of Individuals	Origin	Percentage of individuals in whom organisms of the stated group predominate			
		Bacteroides	Bifidobacteria	Eubacteria	Mixed
21	Japan	38	19	9.5	33.5
24	U.S.A.	46	21	-	33
	Hong Kong:				
12	Income group A	67	8	8	17
13	Income group B	46	31	7.5	15.5
9	Income group C	44.5	22.0	22.0	11.5

i.e. (1) predominantly bacteroides (2) predominantly bifidobacteria (3) a mixture of bacteroides and bifidobacteria (4) predominantly eubacteria. This supports the results of a study by Moore and Holdeman (1974) on the faecal flora of Japanese-Hawiians which on analysis indicate that these individuals can also be assigned to type, although this was not mentioned by them. Moore et al. (1969) described individuals of types (1) to (3) and showed that the flora was very little influenced by dietary manipulation and the dominant bacterial species in the intestine of an individual tended to remain constant for long periods. It is interesting that all three income groups in Hong Kong contained individuals with eubacteria dominant in the faecal flora. The mechanisms by which a particular bacterial species is selected for dominance in an individual are unknown. Moore and Holdeman (1974) point out that several species of bacteria which are numerous in faeces have only weak proteolytic activity and specific carbohydrates are essential for the growth of many. Thus, factors such as the type and amount of complex carbohydrate in the diet, or in mucin may exert a controlling influence on the composition of the flora. The effects of initial feeding regime (breast feeding versus bottle feeding) and weanling diets must also be considered. Long-term studies on the effects of dietary changes in adults (in, for instance, Japanese migrants to the U.S.A.) may be necessary before the role of this factor can be assessed. Presumably host factors are also very important and diet may act indirectly through its influence on the host rather than directly on the flora.

The clostridia.

Only 50% of the isolates could be assigned to any recognized species and about 25% of these were only tentative identifications. This is not unexpected since the criteria applied for identification were those evolved from studies of strains indigenous in Europe and the United States. The unidentifiable strains were not randomly distributed, however, and fell into three fairly homogeneous groups on the basis of analysis of the fatty acids produced from glucose. All three income groups contained strains which did not fall into any recognized species. Persons in income groups C and B had a larger proportion of these bacteria than persons from group A. This may reflect the variations in diet consumed by people in these groups. Many of the strains were assigned to species which have not previously been described as faecal organisms. In general, the more unexpected species were isolated from the faeces of persons in the lower income groups (Table 38). However, the isolation from faeces of the species which were common to all income groups is not unusual, likewise, Cl. ramosum, which was isolated only from persons in income groups A and B, is common infaeces. The isolation of Moore's Clostridium 'A' and Clostridium 'D' is interesting since these two strains were isolated in small numbers by Moore and Holdeman (1974) during a study of the faecal flora of Japanese-Hawiian males, some of whom consumed an oriental or oriental/western diet. The diet of the person(s) from whom Moore's strains were isolated is not known.

Most of the strains confined to income groups B, C and B and C were unusual, with the exception of Cl. paraputrificum (group B only).

TABLE 38

Usual sources of certain strains of clostridia isolated from Hong Kong.

Strain	Source	Reference
1. Strains common to all three income groups		
'Cl. innocuum'	Pathogenic material	Bergey, 1974
		Smith and Holdeman, 1968
	normal faecal flora	Holdeman and Moore, 1972
	Rodent faecal flora	
Cl. rectum	Plant material and horse faeces	Bergey, 1974
Clostridium 'A' or 'D'	Faeces of Japanese-Hawaiins	Moore and Holdeman, 1974
2. Strains common to Income groups A and B only		
Cl. ramosum	Human and animal infections and faeces	Bergey, 1974
	Faeces of man	Attebery and Finegold, 1974
	Faeces of man and swine	Holdeman and Moore, 1972
3. Strains common to Income groups B and C only		
Cl. sphenoides	Human wounds	Bergey, 1974
Cl. beijerinkii	Human infections and soil	Holdeman and Moore, 1972
4. Strains confined to Income group B only		
'Cl. paraputrificum'	Human faeces and soil	Bergey, 1974
	Human infections	Holdeman and Moore, 1972
	Human faeces	Attebery and Finegold, 1974
Cl. felsineum	Soil and water	Holdeman and Moore, 1972
Cl. chauvoei	Bovine infections, bovine and canine intestines	Bergey, 1974
	Animal infections, rumen and faecal flora	Holdeman and Moore, 1972
	Animal intestines	Smith and Holdeman, 1968
Strains confined to Income group C only		
Cl. aurantibutyricum	Plant	Bergey, 1974
		Holdeman and Moore, 1972

This species is particularly interesting since it has been shown to be capable of desaturating the steroid nucleus and the faeces of persons with carcinoma of the large bowel contain larger numbers of these bacteria than do faeces from normal persons (see Section 1.4.iii). Seven of the eight strains isolated during the present study were atypical and were unable to metabolize steroids (Section 3.8).

3.6. The effect of pre-operative sulphonamide versus no antibacterial on the faecal flora at operation and afterwards.

Tables 39, 40 and 41 (pre-operative, operative, post-operative) show the faecal flora of the 20 patients studied together with the geometric means and standard deviations of the bacterial groups investigated. The mean values include results from those individuals for whom counts of certain groups of bacteria could not be assigned since they were below the limits of detection of the method. In these cases an arbitrary value equal to a count of one colony on a plate at the lowest dilution tested, was assigned. One patient (F) received pre-operative ampicillin and these results are therefore not included. The flora of colonic mucosa from this patient is included in the results of Section 3.7. Figure 8 is a graphical representation of the faecal flora before, at operation and after operation, for the patients in each group from whom the complete series of samples was obtained.

TABLE 39

Pre-operative faecal flora of the test patients (Counts expressed as $\log_{10}$ bacteria/gram wet-weight faeces).

Patient	Total Anaerobes	Total Aerobes	Entero-bacteria	Entero-cocci	Total Streptococci	Lactobacilli	Clostridia producing Lecithinase	Clostridia not producing Lecithinase
ANTIMICROBIAL GROUP								
A	9.26	7.49	5.0	7.38	7.46	5.65	3.0	4.84
B	8.89	7.76	7.45	6.28	6.90	3.0	4.04	6.83
C	9.92	9.57	9.36	8.0	9.18	8.0	7.52	5.0
D	9.40	7.97	6.11	7.69	7.95	4.70	5.30	6.70
E	9.10	8.65	8.60	7.10	8.64	3.0	3.0	5.36
F	Patient received Ampicillin, not included							
G	9.00	8.46	8.23	7.0	5.0	5.66	4.26	4.54
H	9.38	8.72	8.18	7.0	8.51	6.04	-	4.91
I	9.60	9.18	7.39	8.51	9.18	3.70	7.48	5.0
J	9.72	8.23	7.90	5.0	8.10	4.0	4.84	4.0
Overall	9.36± 0.34	8.45±0.67	7.58±1.32	7.10±1.02	7.88±1.32	4.86±1.64	4.93±1.77	5.24±0.94
NO ANTIMICROBIAL								
K	8.90	6.0	6.0	6.0	6.0	6.40	4.95	3.95
L	8.54	6.70	6.90	6.0	6.48	5.0	3.0	3.90
M	7.48	5.0	5.0	5.0	5.0	4.0	3.0	4.76
N	9.04	6.18	5.60	5.30	6.00	3.20	4.00	7.00
O	10.04	9.38	9.20	7.85	8.70	3.95	5.18	6.30
P	9.58	6.97	8.02	5.0	5.0	4.0	3.0	4.0
Q	9.63	7.62	7.28	6.0	9.0	4.90	3.0	5.43
R	9.00	7.32	-	7.0	6.98	5.0	3.0	5.60
S	7.0	5.0	5.0	5.0	5.0	3.0	4.0	4.0
T	7.90	6.30	-	6.60	-	-	4.34	4.80
Overall	8.71±0.98	6.65±1.30	6.63±1.50	5.98±0.96	6.46±1.52	4.38±1.05	3.75±0.87	4.97±1.09

(± = standard deviation).

TABLE 40

Faecal flora of the test patients at operation (Counts expressed as $\log_{10}$ bacteria/gram wet-weight faeces).

Patient	Total Anaerobes	Total Aerobes	Entero-bacteria	Entero-cocci	Total Streptococci	Lactobacilli	Clostridia producing Lecithinase	Clostridia not producing Lecithinase
ANTIMICROBIAL GROUP								
A	8.90	8.32	8.62	6.0	8.0	5.0	3.0	3.0
B	9.15	6.73	6.50	5.0	6.20	-	3.0	4.00
C	7.0	7.10	5.00	5.30	7.10	4.0	3.0	3.0
D	7.0	6.97	7.04	5.0	5.0	4.0	6.26	4.0
E	7.0	6.53	6.63	5.0	-	5.0	3.0	3.0
F	Patient received Ampicillin			not included				
G	8.26	7.28	5.60	5.0	7.28	4.0	6.26	4.0
H	7.86	8.59	8.15	5.0	5.0	4.0	3.0	4.0
I	7.85	7.78	6.41	6.18	7.78	4.0	4.82	4.98
J	8.47	7.93	7.57	5.47	7.88	4.0	3.47	4.0
Overall	7.49±0.82	7.47±0.71	6.84±1.16	5.32±0.47	6.78±1.24	4.25±0.46	3.98±1.42	3.78±0.66
NO ANTIMICROBIAL								
K	10.15	9.40	9.46	6.0	6.0	7.64	3.0	5.86
L	9.26	7.70	8.96	5.0	-	5.0	3.0	3.0
M	8.32	6.56	5.00	5.0	6.15	3.48	4.0	4.0
N	8.18	7.11	7.25	5.0	-	5.0	3.0	3.0
O	7.80	5.0	5.0	5.0	5.0	5.0	7.0	3.0
P	7.0	7.0	7.0	5.0	5.0	4.0	3.0	4.0
Q	9.00	7.38	7.77	6.59	7.38	4.0	3.0	5.79
R	8.96	7.80	7.80	7.80	5.0	5.0	3.0	4.63
S	8.30	8.50	8.12	5.0	-	5.0	3.0	3.0
T	8.75	6.53	7.96	5.0	-	5.0	3.0	3.0
Overall	8.57±0.86	7.32±1.21	7.45±1.48	5.56±1.00	5.76±0.96	4.89±1.07	3.50±1.27	3.84±1.13

(± = standard deviation).

TABLE 41

Post-operative faecal flora of the test patients (Counts expressed as $\log_{10}$ bacteria/gram wet-weight faeces).

Patient	Total Anaerobes	Total Aerobes	Entero-bacteria	Entero-cocci	Total Streptococci	Lactobacilli	Clostridia producing Lecithinase	Clostridia not producing Lecithinase
ANTIBACTERIAL GROUP								
A	9.15	8.94	6.96	9.00	8.94	6.25	5.18	4.00
B	-	7.61	6.98	7.51	7.34	4.00	3.0	4.0
C	8.65	8.11	7.18	7.63	8.04	2.00	6.08	4.08
D	8.11	7.28	7.20	5.00	7.26	-	4.51	5.61
E	9.54	7.43	8.11	3.00	3.00	6.00	6.00	4.64
F	Patient received Ampicillin not included							
G	7.00	7.08	5.00	6.70	7.04	4.00	3.00	4.08
H	-	8.53	8.50	5.00	5.00	4.00	3.00	6.65
I								
J	9.94	7.93	6.04	7.91	7.88	4.00	3.00	3.95
Overall	8.54±0.98	7.86±0.64	6.99±1.10	6.47±1.98	6.81±1.90	4.32±1.43	4.22±1.39	4.63±0.99
NO ANTIBACTERIAL								
K	10.70	8.79	8.96	6.00	6.00	7.08	-	6.54
L	9.36	6.18	5.49	4.20	6.00	2.00	3.08	4.74
M	9.28	8.08	7.57	6.43	8.00	4.00	6.07	4.00
N	9.64	8.61	8.30	7.48	8.04	2.30	2.48	6.49
O								
P								
Q	9.43	8.32	8.34	5.00	6.00	4.00	3.00	6.08
R	10.00	9.00	9.00	7.64	9.00	4.00	3.78	4.00
S	-	7.89	7.86	5.00	5.00	3.00	3.00	4.46
T	8.18	8.00	8.53	5.00	78.00	5.00	5.11	5.08
Overall	9.51±0.70	8.17±0.88	8.00±1.13	5.84±1.26	7.00±1.42	39.2±1.62	3.79±1.32	4.99±0.99

(± = standard deviation).

Discussion.

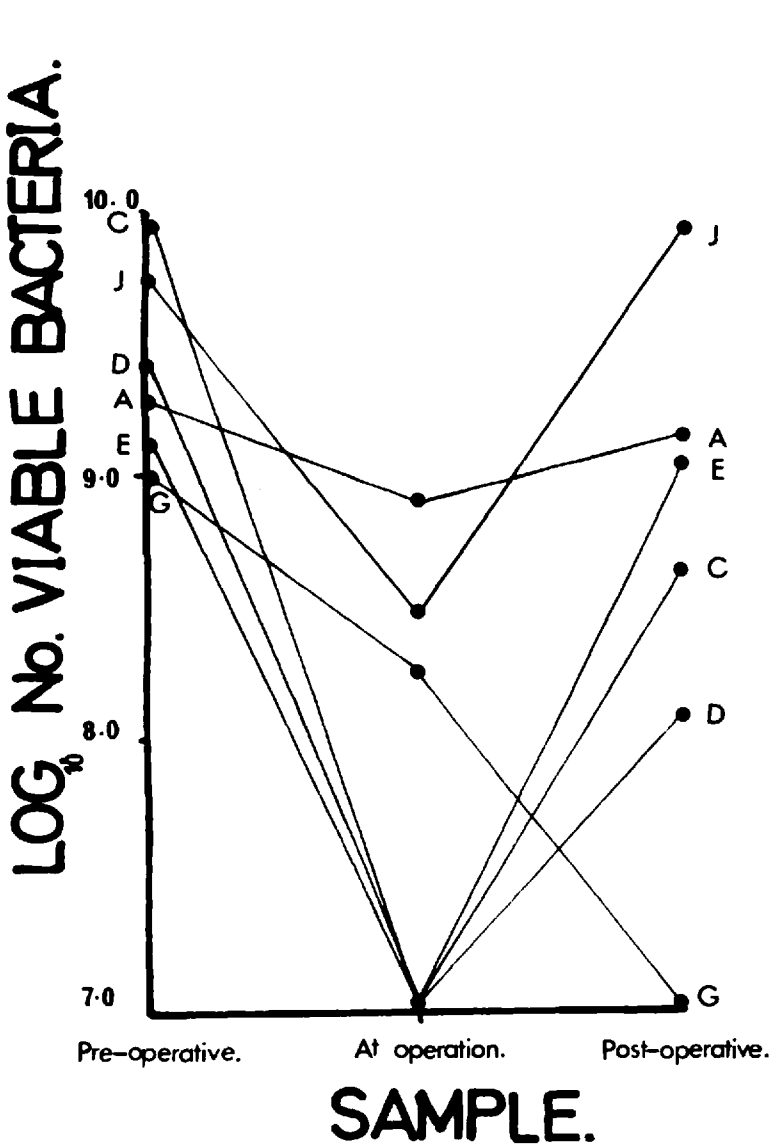
It is particularly noticeable that for many of the test patients the margin by which the total number of anaerobic organisms recovered per gram of pre-operation faeces predominated over the total number of aerobic organisms was not as marked as has been found in other studies of the normal faecal flora (e.g. Moore et al. 1969). However, the population studied was by no means 'normal' since these were all patients undergoing surgery for carcinoma of the large bowel and may have had associated conditions, such as diarrhoea, which could tend to lower the number of anaerobic bacteria per gram of faeces.

It also appears that the two groups of patients were not entirely similar. Overall, the counts of total aerobes per gram of pre-operative faeces were significantly lower ($p < 0.01$) for the group of people not treated with antibacterial. The mean count of total anaerobes was also lower for this group, but the difference was not statistically significant. However, since this was a double-blind randomized study there was no way that these differences could have been forseen and avoided.

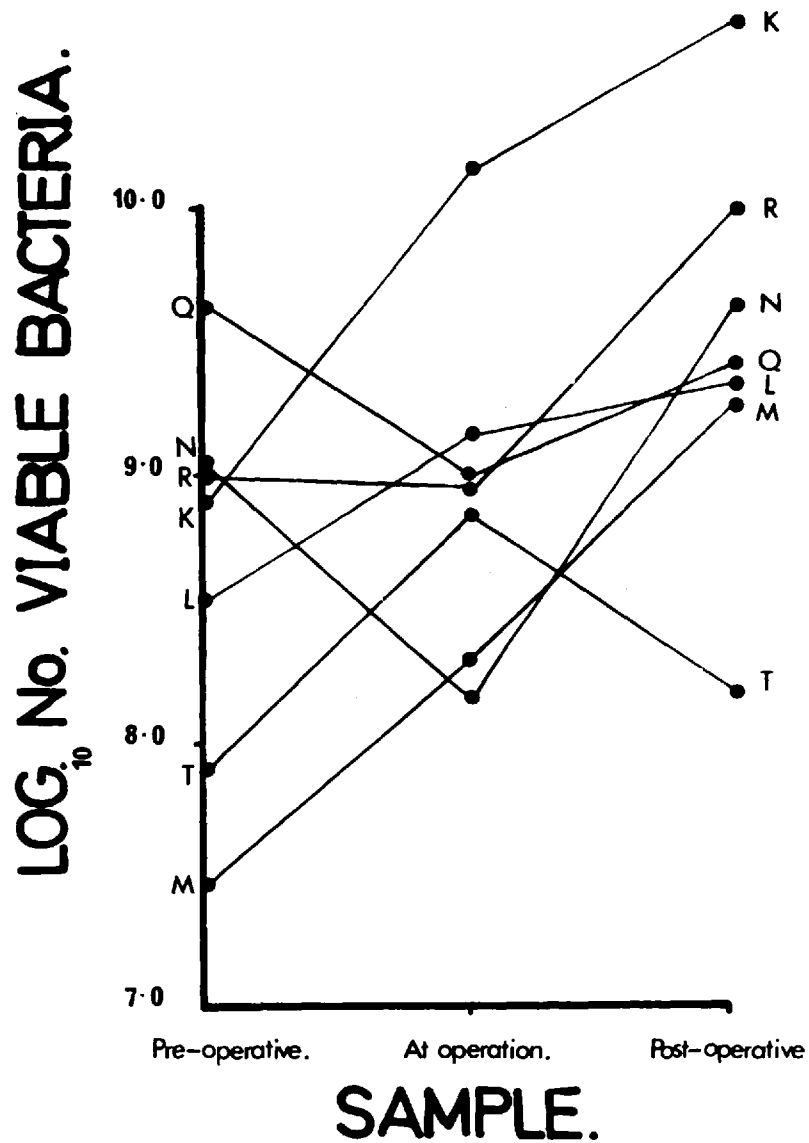
At operation, the bacterial counts per gram of faeces were reduced in many of the patients who had taken sulphonamide (Tables 39 and 40). In all but one patient, where the count remained similar to that pre-operatively, there was a decrease in the total number of anaerobes. The counts were lowered 10 to 1000-fold. Likewise the count of total aerobes showed a general decrease: in some patients the fall was 100-fold. In one patient, (A), the total aerobe count was raised to 10-fold its pre-operative level. In this case, however, the overall picture was

Figure 8.

Total anaerobe counts obtained from the faeces of some of the patients during the study of the effects of administration of pre-operative sulphonamide on the faecal flora. The letters refer to individual patients (see Tables 39, 40 and 41).



GROUP TAKING ANTIBACTERIAL.



GROUP NOT TAKING ANTIBACTERIAL.

of little change since the total anaerobe count fell.

A different picture emerged for the group of patients who had not taken any antibacterial. Changes in the counts of total anaerobes were diverse; half of the patients in this group had reduced anaerobe counts (10 to 100-fold) whilst half had counts raised by similar margins. In six patients the aerobe count remained at the pre-operative level and in only one patient was the count reduced, admittedly by a large margin. This patient (O) also had a decreased number of anaerobes.

Overall, for the group of patients who had pre-operative sulphonamide the mean count of total anaerobes and total aerobes was significantly lower ($p < 0.001$ and $p < 0.01$ respectively) in the stool at operation, than pre-operatively.

The mean total anaerobe and aerobe counts of the operation stools had not been altered significantly ($p > 0.05$) for the group of patients who had no pre-operative sulphonamide.

Thus, it appears that pre-operative sulphonamide has caused a lowering of the total number of viable bacteria which can be cultivated from faeces at operation.

The normal pre-operative regime, experienced by both groups of patients, disturbs the flora, but the outcome is variable. The value of the pre-operative regime cannot be assessed on the basis of these bacteriological results. However, although the effect may not be to lower the number of viable bacteria per gram of faeces, observations at

Figure 9.

The relationship between the total number of anaerobes in faeces obtained pre-operatively and in those obtained at operation. No trend can be seen.

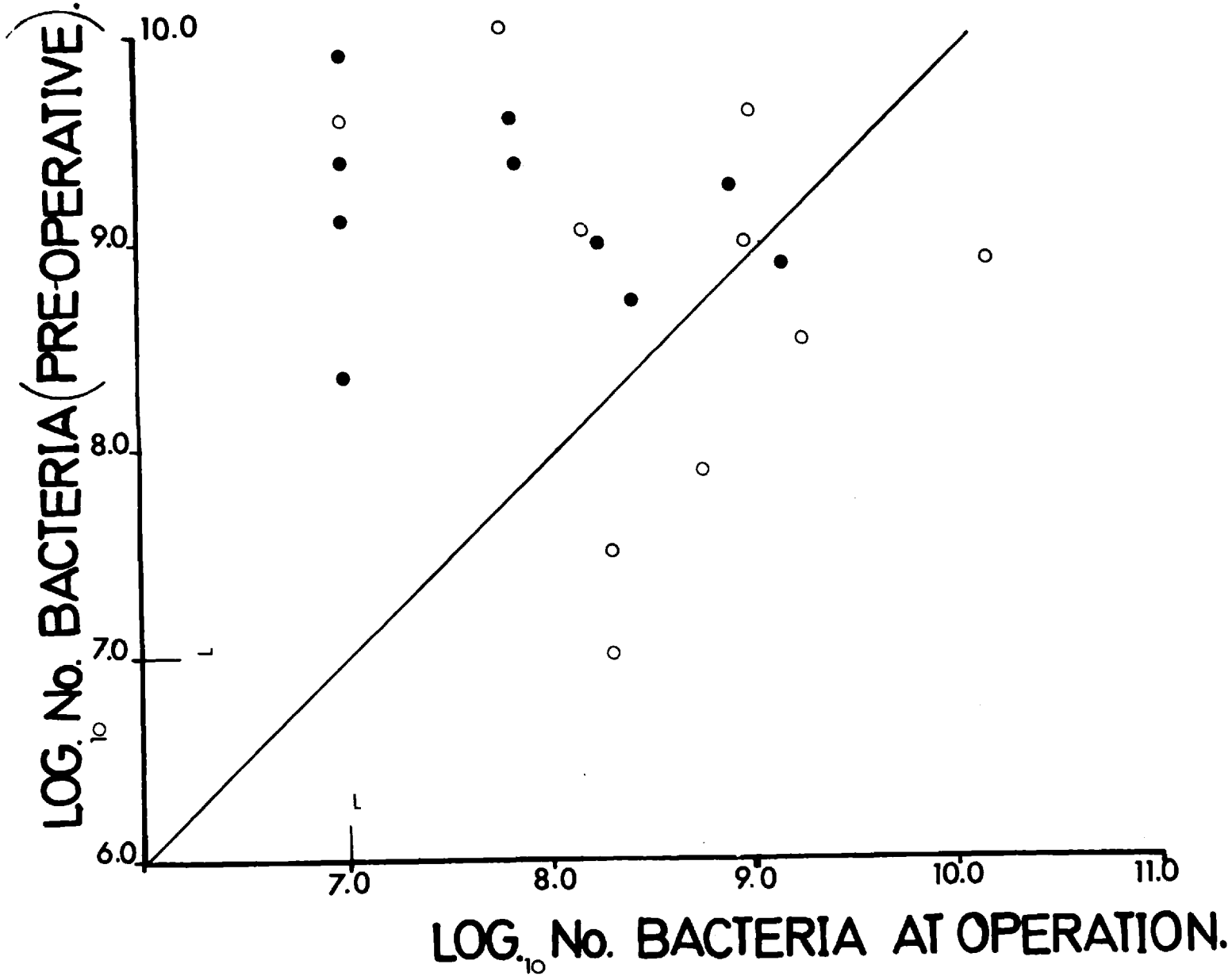
Counts expressed as $\log_{10}$ number viable bacteria per gram (wet weight) faeces. Each spot represents a single patient:

Black spots .....Patients receiving sulphonamide

Open spots .....Patients not receiving sulphonamide

L- shows the limits of detection of bacteria imposed by the method.

The line represents a theoretical 1 : 1 relationship.


LOG. No. BACTERIA (PRE-OPERATIVE).
10.0
9.0
8.0
7.0
6.0
LOG. No. BACTERIA AT OPERATION.
7.0
8.0
9.0
10.0
11.0

operation showed a decrease in the total contents of the colon, with only small areas containing visible amounts of faeces. Thus the total numbers of bacteria in the colon available as a reservoir of possible infection, have been reduced.

Regardless of treatment, the pre-operative and operative anaerobic flora does not appear to be numerically related (Figure 9). Whatever the treatment, the possible reduction, or in some cases, increase, in total number of anaerobes in the faeces at operation cannot be predicted from the pre-operative faecal flora. However, a similar diagram (Figure 10) shows the relationship between the numbers of anaerobic bacteria per gram of pre-operative and post-operative flora. This is interesting since it has already been noted that the number of anaerobes in the pre-operative faeces was in many cases abnormally low. These results also illustrate the rapid return of the numbers of bacteria to pre-operative levels after operation and the cessation of sulphonamide administration. This may be in part due to the bacteriostatic action of the drug. If the drug were bactericidal it could take several days (as with the development of intestinal flora in babies) for the intestinal tract to be recolonized and for stable ecological relationships between groups of bacteria to develop.

Although the results of the present investigation demonstrate that pre-operative sulphonamide causes a decrease in the numbers of viable bacteria in the faeces at operation, no evidence was gained to suggest that this had any influence on the outcome of the operations. There were no significant post-operative wound infections. Leakage of the anastomosis made at operation occurred in three patients in the group

Figure 10.

The relationship between the total number of anaerobes in faeces obtained before and after operation.

Within broad limits, the pre-operative anaerobe count is similar to the post-operative count.

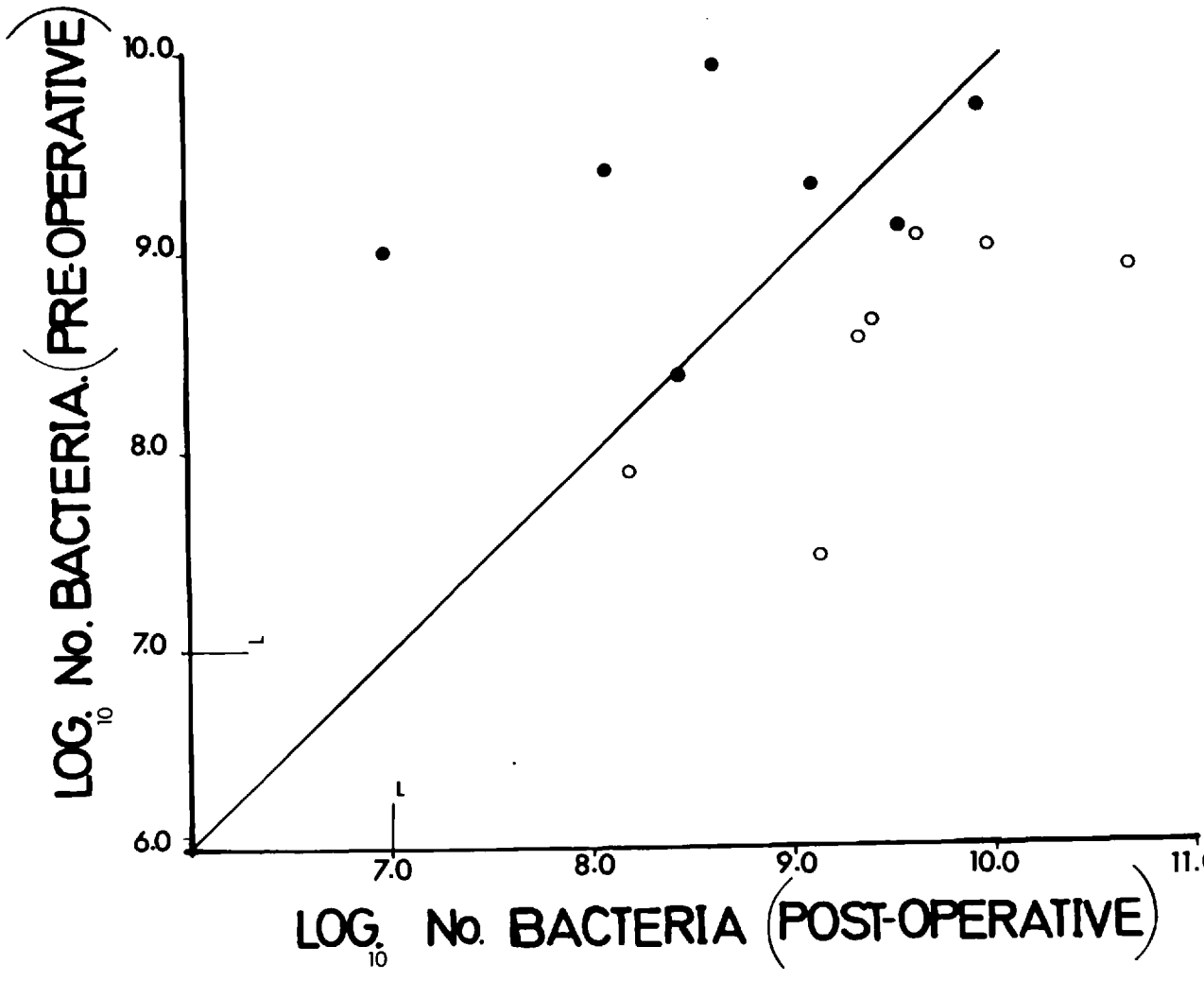
Each spot represents a single patient

Black spots ..... Patients receiving sulphonamide

Open spots ..... Patients not receiving sulphonamide

L- shows the limits of detection imposed by the method.

The line represents a theoretical 1.: 1 relationship.


LOG. No. BACTERIA. (PRE-OPERATIVE)
10.0
9.0
8.0
7.0
6.0
L
L
7.0
8.0
9.0
10.0
11.0
LOG. No. BACTERIA (POST-OPERATIVE)

who had received sulphonamide and in one patient in the group who had not. In all these patients the number of anaerobes in the faeces at operation had been reduced and in all but one, the number of aerobes had also decreased. In future, it may be necessary to conduct a study to determine specifically which, if any, of the faecal organisms are associated with breakdown of anastomoses or post-operative infections and in this light, suitably modify pre-operative antibacterial regimes.

3.7. The bacterial flora of the colonic mucosa.

3.7.i. The bacterial flora of colonic mucosa and tumour tissue.

The various groups of bacteria that were isolated and enumerated from the colonic mucosa are shown in the lower portion of Table 42. The tissue was obtained at operation from ten patients who had not received any antibacterial agent, but who had normal pre-operative bowel preparation. Similarly, the flora of samples of tumour tissue from six of these patients is shown in Table 43. The geometric means of the numbers of bacteria per gram of tissue and the standard deviations of these means, are also shown. Not all the groups of bacteria investigated were isolated from every sample. Where this is the case, it is indicated by a dash.

Ten well separated colonies were picked at random from one of the

TABLE 42

Mucosal flora of test patients at operation (Counts expressed as $\log_{10}$ bacteria per gram wet-weight of tissue)

Patient	Total no. bacteria growing Anaerobically	Total no. bacteria growing Aerobically	Entero-bacteria	Entero-cocci	Streptococci	Non Lecithin-ase producing Clostridia	Lactobacilli
ANTIBACTERIAL							
A	5.72	5.80	5.54	-	5.83	-	-
B	5.88	4.25	4.18	-	3.81	6.44	-
C	6.08	4.32	3.79	-	-	-	-
D	5.91	5.23	5.15	-	4.23	3.56	-
E	5.32	5.15	5.28	-	5.15	-	-
F	5.23 (5.20)	5.15 (5.41)	4.65	4.93	5.15	-	4.00
G	6.05	5.78	3.37	5.13	4.54	-	-
H	5.99	5.83	5.43	-	-	-	-
I	5.91	5.86	5.95	5.20	5.86	2.95	-
J	4.80	3.92	3.00	3.60	3.91	-	-
Overall	5.69±0.43	5.13±0.73	4.63±1.00	4.72±0.75	4.77±0.76	4.32±1.86	4.00
NO ANTIBACTERIAL							
K	4.79	4.36	4.29	-	-	-	-
L	4.52	4.41	4.32	-	-	-	-
M	5.18	5.15	5.08	-	-	-	-
N	6.32 (5.10)	6.53 (4.10)	6.52	-	-	-	-
O	5.70	5.38	5.29	-	-	3.36	2.89
P	5.18	3.43	-	3.43	-	-	-
Q	6.50	5.52	5.62	3.87	3.99	-	-
R	6.00	5.58	5.58	-	-	-	-
S	6.58	5.34	5.34	-	-	-	-
T	4.86	4.57	4.43	-	-	-	-
Overall	5.56±0.76	5.01±0.87	5.16±0.73	3.65±0.31	3.99	3.36	2.89

(± = standard deviation).

TABLE 43

Tumour flora of some patients at operation.

(Counts expressed as $\log_{10}$ bacteria per gram wet-weight of tissue)

Patient	Total No. bacteria growing Anaerobically	Total No. bacteria growing Aerobically	Entero-bacteria	Entero-cocci	Streptococci	Non Lecithinase producing Clostridia	Lactobacilli
ANTIBACTERIAL							
A	6.15	4.88	4.95	-	-	-	-
D	4.25	4.08	3.83	-	-	-	-
E	5.79	5.20	3.43	4.97	5.20	-	-
G	6.00	6.00	6.00	-	-	-	-
Overall	5.54± .88	5.04± .79	4.55± 1.16				
NO ANTIBACTERIAL							
M	6.80	5.23	3.26	-	5.23	-	-
O	7.00	6.46	6.58	-	-	-	-
P	5.43	5.20	4.30	-	-	4.11	-
Q	4.99	3.73	3.95	-	-	4.36	-
R	6.25	6.00	6.23	-	-	-	-
S	5.18	5.04	4.95	-	-	-	-
Overall	5.94± .86	5.28± .94	4.88± 1.31			3.75± .53	

(± = standard deviation)

plates which had been incubated anaerobically, and subcultured into tubes of Robertson's cooked meat medium. Many of these isolates were found to be Gram-positive cocci with unusual characteristics. These are shown in Table 44. The isolates varied in their sensitivities to oxygen. Some grew well aerobically, others grew only anaerobically and a few appeared to be microaerophils. Although much less frequently encountered, several similar strains were isolated from faecal specimens from these patients (pre-operative, operative and post-operative).

3.7.ii. The effect of pre-operative sulphonamide versus no antimicrobial.

The types and numbers of bacteria isolated from the mucosa of ten patients who had received sulphonamide prior to operation were compared with those isolated from ten patients who had no pre-operative antibacterial agent. These results are shown in Table 42. The mean numbers of bacteria per gram of tissue and the standard deviations of these means are also shown. Both groups of patients had received normal mechanical pre-operative bowel preparation. A sample of mucosa was obtained pre-operatively from one patient in each group and the results of their cultivation are included in parenthesis for comparison. Samples of tumour tissue were obtained from four patients who had received pre-operative sulphonamide and from six control subjects. The bacteriology of these samples is shown in Table 43.

TABLE 44

Characteristics of Gram-positive bacteria isolated from colon mucosa.

All groups.

Gram-positive cocco bacilli, sometimes forming chains. Some produce a distinct pungent foul odour in liquid media containing amino acids. Aerobes, microaerophiles or strict anaerobes. Anaerobes may become aerotolerant on subculture. All fermentative. All ferment galactose, maltose, glucose and lactose. Do not ferment melzitose or arabinose. Do not produce indole from tryptophan or reduce nitrate. Not resistant to KCN. Produce lactic, acetic ± propionic and butyric acids from glucose.

Aerobic isolates only.

Group 1.

Mostly catalase positive.
Oxidase positive.
Resistant to Tween 80 and 20.
Most grown in 6%, but not 20%, NaCl.
Most ferment melibiose, rhamnose, mannitol, raffinose and xylose.
Some hydrolyse urea.
Some hydrolyse gelatin.
Most hydrolyse aesculin.
Some hydrolyse starch.

Group 2.

Catalase negative.
Oxidase negative.
Sensitive to Tween 80 and 20.
Sensitive to NaCl (6% and 20%).
Ferment melibiose, rhamnose, mannitol, raffinose and xylose.
Urea hydrolysed.
Gelatin not hydrolysed.
Aesculin hydrolysed.
Starch not hydrolysed.

Tests as described by Cowan and Steel (1965).

3.7.iii. The relationship between the mucosal and faecal bacterial flora.

For an accurate quantative assessment of the mucosal flora it is useful to express counts of bacteria in relation to the surface area of the specimen. A formula for the calculation of surface area from weight of tissue is available (Drasar, personal communication). During the present study, however, the mucosal flora was compared quantitatively with that of faeces, and for continuity all counts were expressed per gram wet-weight of tissue.

The total number of bacteria per gram of mucosa which grew on an anaerobically incubated isolation plate are shown in comparison with the total number of anaerobes isolated per gram of faeces obtained at operation (Figures 11 and 12). Results for two groups (pre-treatment with sulphonamide and no sulphonamide) are given. One pre-operative mucosal sample was obtained from each group and the results are shown in comparison with a pre-operative faecal sample. A question-mark indicates that the number of bacteria per gram of faeces could not be ascertained since they were below the limits of detection imposed by the method.

Discussion.

Even taking into account the low proportion of anaerobic bacteria in the faeces of the test patients at operation, the bacterial flora of both mucosa and tumour differ noticeably from that of faeces in that

Figure 11.

Comparison of numbers of bacteria from mucosa and faeces which grew on an anaerobically incubated isolation plate.

Light shading ..... mucosa

Dark shading ..... faeces

Each bar represents one patient.

A question mark indicates that the number of bacteria per gram of faeces could not be ascertained.

This group of patients had received sulphonamide.

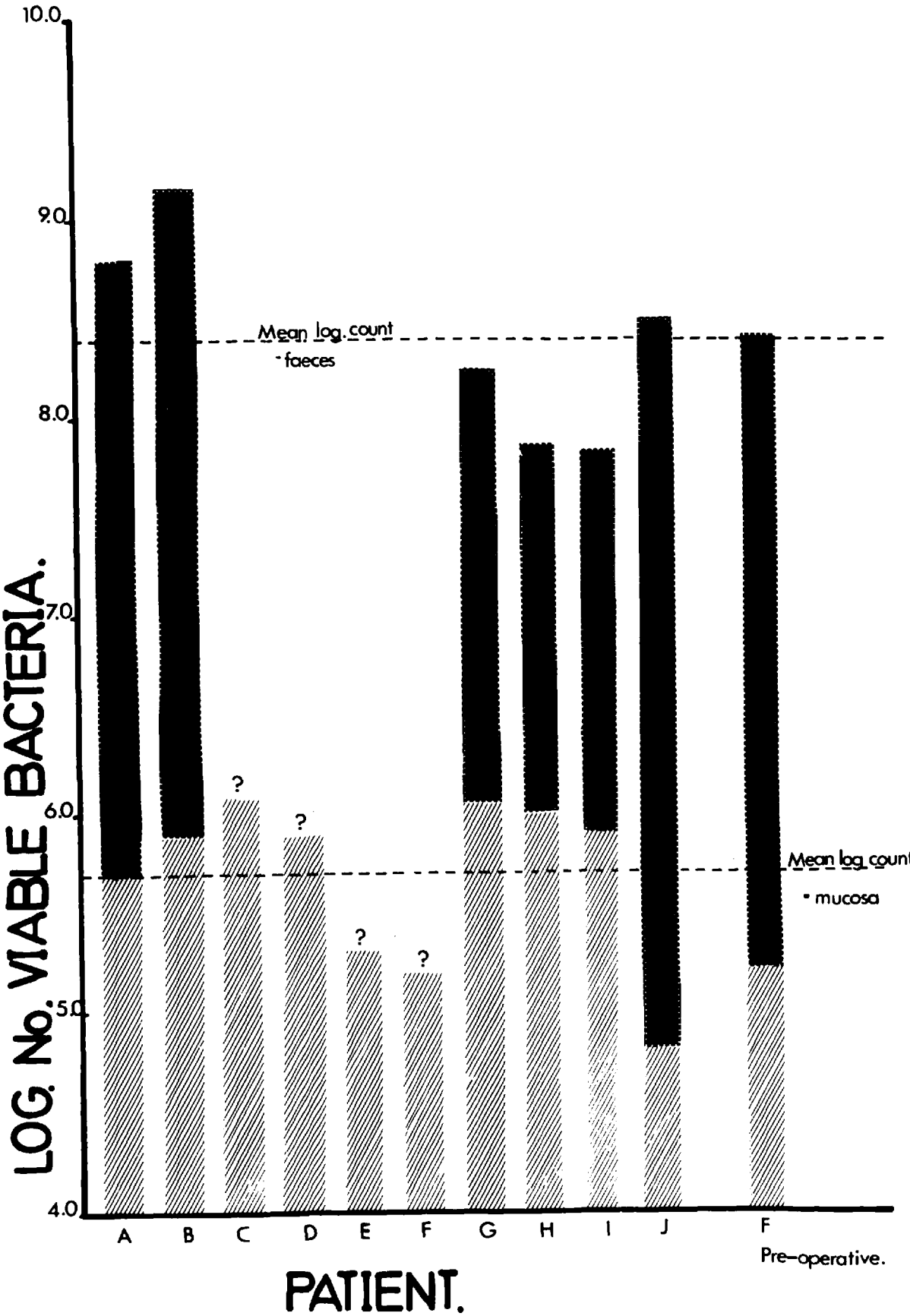

10.0
9.0
8.0
7.0
6.0
5.0
4.0
LOG. No. VIABLE BACTERIA.
Mean log. count
- faeces
Mean log. count
• mucosa
?
?
?
?
A
B
C
D
E
F
G
H
I
J
F
Pre-operative.
PATIENT.

Figure 12.

Comparison of the numbers of bacteria from mucosa and faeces which grew on an anaerobically incubated isolation plate.

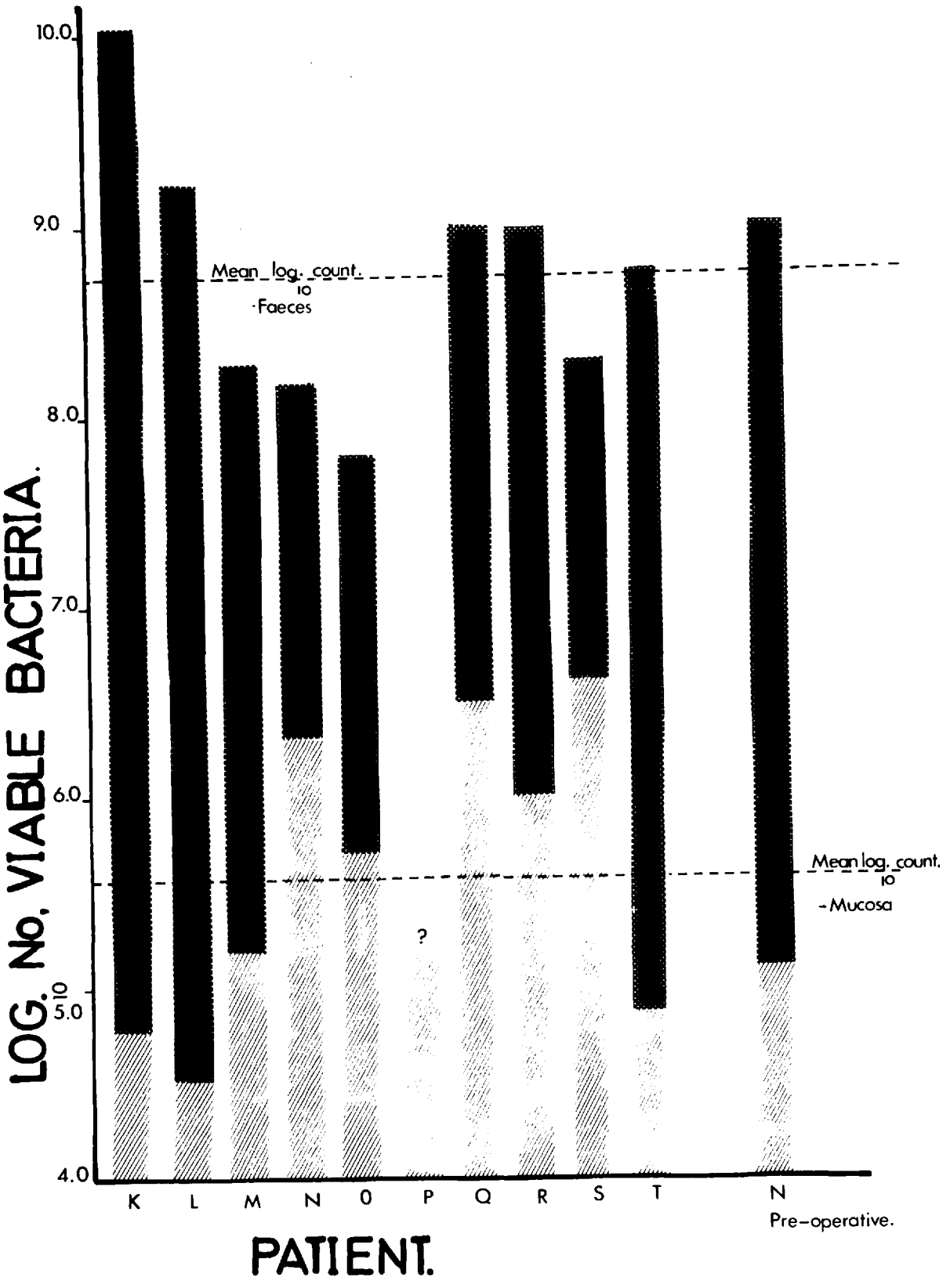
Light shading ..... mucosa

Dark shading ..... faeces

Each bar represents one patient.

A question mark indicates that the number of bacteria per gram of faeces could not be ascertained.

This group of patients had not received sulphonamide.


10.0
9.0
8.0
7.0
6.0
5.0
4.0
LOG. No. VIABLE BACTERIA.
Mean log. count. (10)
-Faeces
Mean log. count. (10)
-Mucosa
?
K
L
M
N
O
P
Q
R
S
T
N
Pre-operative.
PATIENT.

anaerobes do not predominate. In many cases, the total number of colonies growing on anaerobically incubated plates was equal to, or only slightly less than, the number of colonies which grew on aerobically incubated plates. This ratio of anaerobes to aerobes was slightly greater with the tumour specimens. Although only a small number of tumour samples were examined, the numbers and groups of bacteria isolated were similar to those of mucosal samples. Thus, it appears that the bacterial flora of colonic mucosa is primarily facultative. Enterobacteria were isolated from all the mucosal and tumour specimens from the patients in this group. Enterococci and other streptococci were also frequently isolated, but non-lecithinase producing clostridia and lactobacilli were isolated from only a few specimens. Nelson and Mata (1970) reported the isolation from mucosal specimens of large numbers of anaerobic streptococci ($10^{7/g}$), together with microaerophilic streptococci, enterobacteria, bacteroides, bifidobacteria, clostridia and enterococci. During the present study the numbers of bacteria per gram of mucosal specimen were found to be slightly lower than those reported by Nelson and Mata. This may be a reflection of differences between the site from which the biopsies were taken or of the different populations studied.

Nelson and Mata do not give any definitions for their "anaerobic streptococci" and these may well be similar to the large number of Gram-positive cocci isolated during the present study. Many of these bacteria were able to decarboxylate several amino acids forming primary and secondary amines (Johnson, personal communication). Since some secondary amines are known carcinogens, they too, could be implicated in the aetiology of cancer of the colon. Any metabolites produced by those

bacteria 'in situ' would be likely to exert an influence on the mucosa, since they appear to be closely associated with this tissue (they remain after two vigorous washings). Furthermore, many faecal bacteria utilise simple nitrogen sources in preference to proteins and amino acids (Bryant, 1974) and the bacteria isolated from mucosa may contribute towards the determination of the bacterial flora of faeces by production of such necessary metabolites. A more detailed study of the biochemistry and ecology of these bacteria is necessary, including electron-microscope and other studies to determine the mechanisms by which they are associated with the mucosa.

There were no outstanding differences between the number and types of bacteria isolated from mucosal or tumour specimens from patients who had been treated with pre-operative sulphonamide and those who had not. It appears therefore that pre-operative sulphonamide was not affecting the bacterial flora of the mucosa. The penetration of sulphonamide from faeces into the folds of mucosa would, in any case, be uncertain.

There appeared to be no correlation in either group of patients between the numbers of anaerobic and facultatively anaerobic bacteria isolated from faeces at operation and those isolated from mucosal specimens. This may be a reflection of the unique flora of the mucosa which, in many aspects of its composition does not resemble the faecal flora, and of the close association of this flora with the tissue, which would leave it unaffected by rigorous mechanical bowel preparations.

3.8. Steroid metabolism by pure strains of anaerobic bacteria.

3.8.i. Deconjugation.

A small number of non-sporing anaerobes isolated from the faeces of persons from Hong Kong, U.S.A., Japan, India and Scotland and a strain of Bacteroides fragilis from the National Collection of Type Cultures (N.C.T.C. 9343) were tested for possession of cholanoyl hydrolase (which deconjugates bile salts releasing free bile acid). The strains are shown in Table 45. All the strains produce this enzyme, but the non-sporing anaerobes from the U.S.A., the strains of Bacteroides fragilis from Japan and N.C.T.C. and two of the four strains of B. fragilis from Hong Kong were especially active. The latter strains were isolated from persons from income group A.

TABLE 45.

Country of Origin	No. of Strains	Species
Hong Kong	4	Bacteroides fragilis
U.S.A.	2	B. fragilis
	1	Bifidobacterium adolescentis
Japan	1	B. fragilis
India	1	Propionibacterium acnes
Scotland	1	Eubacterium lentum
N.C.T.C. (9343)	1	B. fragilis

TABLE 46

Source of strains tested for 7-α-dehydroxylase activity.

Income group	No. of persons	No. of strains tested	Bacteroides	Bifidobacteria	Eubacteria	Clostridia
A	8	35	14	9	2	10
B	11	58	42	14	2	0
C	10	48	27	12	8	1
Total	29	141	83	35	12	11

3.8.ii. Dehydroxylation.

One-hundred-and-forty-one strains of non-sporing anaerobes isolated from the faeces of 29 persons from Hong Kong were tested for 7α-dehydroxylase activity. The identities of the strains and the income groups of the persons from whom they were isolated are summarized in Table 46. Several strains of bacteroides and a few strains of bifidobacteria had 7α-dehydroxylase; this enzyme was not demonstrated with the strains of clostridia and eubacteria tested (Table 47). The greatest proportion of bacteroides showing 7α-dehydroxylase activity originated from persons in income group B, whilst the largest proportion of bifidobacteria with the enzyme came from persons in income group A. None of the strains of bifidobacteria tested from persons in group C showed any dehydroxylase activity.

3.8.iii. Dehydrogenation of the steroid nucleus.

The 92 strains of lecithinase-negative clostridia which had been isolated from the faeces of persons from Hong Kong were tested for their ability to dehydrogenate the steroid nucleus. The 'identities' of these strains and the income groups from which they originate are shown in Table 36 and discussed in Section 3,5,ii. Only four strains were able to dehydrogenate the steroid nucleus and all had been isolated from persons in income group B. One of these strains was identified as Clostridium paraputrificum. The remaining three strains were clostridia that could not be assigned to any definite species and of these, two

TABLE 47

7α-dehydroxylase production by pure strains of anaerobic bacteria.

	Income Group							
	A		B		C		All Groups	
	No. strains tested	% strains producing enzyme	No. strains tested	% strains producing enzyme	No. strains tested	% strains producing enzyme	No. strains tested	% strains producing enzyme
Bacteroides	14	7.0	42	14.0	27	7.5	83	11
Bifidobacteria	9	11.0	14	7.0	12	0	35	6
Eubacteria	2	0	2	0	8	0	12	0
Clostridia	10	0	0	-	1	0	11	0
Total	35	6.0	58	12.0	48	4	141	8

strains were butyric acid producers and one produced a complex mixture of fatty acids from glucose. The strain of Cl. paraputrificum and one of the butyric acid producing strains were isolated from the same person.

Seven strains of Cl. paraputrificum isolated from the stools of bottle-fed infants by Dr. T. Willis (Public Health Laboratory Service, Luton) were tested in parallel with the Hong Kong isolates and were shown to be able to dehydrogenate the steroid nucleus.

Discussion.

The results of the present study demonstrate the ubiquitous nature of cholanoyl hydrolase in anaerobes isolated from faeces from varying geographical sources. It is unfortunate that only a small number of strains were tested and that they were mainly Bacteroides fragilis; however, the results of this limited survey are in agreement with those of Hill et al. (1971) who, during a study of isolates from faeces from various countries, found little difference in the proportions of strains with the enzyme.

The possession of 7-α-dehydroxylase by several strains of Bacteroides spp. and Bifidobacterium spp. was again in agreement with the findings of Hill et al. (1971). However, these workers also showed that some strains of clostridia were able to dehydroxylate. None of the strains of clostridia tested during the present study were able to carry out this

reaction. Hill et al. (1971) noted a geographical variation in the proportion of strains with 7-α-dehydroxylase; very few Indian strains possessed the enzyme, compared with 40 - 50% of the anaerobic bacteria isolated from American and Scottish people. In the present study only 8% of the strains from Hong Kong could dehydroxylate, a proportion similar to that shown by Indian strains. Most of the strains with 7-α-dehydroxylase activity came from persons in income group B. The proportion of positive strains isolated from groups A and C were similar. This may, in part, be a reflection of the large numbers of strains tested which originated from persons from income group B and its true significance cannot be assessed.

More bifidobacteria than bacteroides from group A were capable of dehydroxylation; however, the situation was reversed amongst the isolates tested from the other two income groups and overall it appeared that a greater proportion of bacteroides than bifidobacteria possessed the 7-α-dehydroxylase. Amongst strains of bacteroides and bifidobacteria isolated from persons in England, Scotland and the U.S.A., Hill et al. (1971) found that dehydroxylase was distributed evenly. Isolates from Ugandan faeces showing 7-α-dehydroxylase activity, were almost always Bacteroides spp.

Relatively few of the clostridia isolated from faeces from Hong Kong produced Δ^4 -dehydrogenase. Many of the species were somewhat unusual as faecal isolates and this may be a contributing factor. Goddard (1973) showed that this dehydrogenation was carried out almost solely by strains of Cl. paraputrificum. The seven strains of Cl. paraputrificum from babies tested during the present study were positive

for this reaction and confirm previous work with this species. The one strain definitely classified as Cl. paraputrificum, isolated from faeces from Hong Kong, was also shown to possess Δ^4-dehydrogenase, but similar, although not identical, strains did not. The few other strains found to produce dehydrogenase were unidentifiable and did not resemble Cl. paraputrificum or any other species previously shown to possess the enzyme. Goddard (1973) found several unidentifiable faecal clostridia capable of dehydrogenating the steroid nucleus. He also noted a geographical variation in the distribution of dehydrogenating clostridia in faeces, with low numbers being found in faeces from Uganda and high numbers in faeces from England and Scotland. A possible relationship between the finding of these bacteria in faeces and the incidence of carcinoma of the large bowel was observed by Goddard (1973) and Drasar et al. (1975). The implications of this relationship for certain groups in Hong Kong will be discussed later (Section 3.10).

Since only four positive strains were found during the present study, and of these, two came from a single individual, the significance of the income group of the persons from whom the strains were isolated cannot be assessed.

Future work in this field must involve more detailed studies of the role of environmental factors in the ecology of Cl. paraputrificum and other dehydrogenating clostridia in faeces.

3.9. Organ culture of colonic mucosa.

3.9.i. Survival of the cultures.

Pieces of mucosa survived in culture in antibiotic-free medium for up to three weeks. The tissue remained intact and retained its original colour. Tumour tissue was more friable and could be kept no longer than four or five days before disintegration.

Some of the cultures of mucosa were fixed in glutaraldehyde, embedded in 'Araldite' and sectioned and examined in an electron-microscope by Dr. M. Shiner at the Central Middlesex Hospital. The ultrastructural appearance of cells of mucosa, which had been maintained in culture for up to 14 days, was well preserved. Older cultures and cultures of tumour tissue were not examined.

3.9.ii. Attachment of E. coli to mucosa and tumour tissue in organ culture.

Table 48 shows the recovery of E. coli from organ cultures of mucosa and tumour inoculated with about 10^7 organisms. No other aerobic bacteria were recovered from the organ culture. In addition, about 10% of the inoculum was recovered from the filter paper which supported the tissue in culture. Although the washings from the organ culture were not examined, it would seem that the number of bacteria in

TABLE 48

Recovery of E. coli from organ cultures.

Patient	Specimen	Size of inoculum (Log_{10})	No. of bacteria recovered (Log_{10})
S	Mucosa	7.23	4.89
S	Mucosa	7.23	4.40
T	Mucosa	7.68	4.61
T	Mucosa	7.68	5.28
S	Tumour	7.23	4.63

such samples represented only a small proportion of the inoculum, since the bulk of this inoculum was recovered from the organ-culture medium. However, if the culture medium was sampled 24 hours later, after storage at $4^{o}C$, no bacteria could be recovered. If, in turn, a sample of this medium was inoculated into a Todd-Hewitt broth, prolific growth resulted. This phenomenon was observed with the medium from both mucosa and tumour organ cultures. The same strain of E. coli used for these experiments grew well when about 10^{7} organisms were inoculated into fresh Waymouth's medium (Table 49).

Discussion.

The survival of pieces of mucosa in organ-culture is encouraging. Few workers have attempted to culture pieces of large bowel from adults because of the inherent problem of contamination of such tissue. Warren and Kaufman (1956) and Watson, Quigley and Bolt (1966) maintained adult colonic tissue for short periods, but contamination of the culture with bacteria from colonic contents was an obstacle to long-term maintenance. Attempts to culture pieces of small bowel have been successful (Mitchell, Mitchell and Peters, 1974) but the tissue has only been maintained for a day or two before disintegration. Chlopkow (1930) claimed to maintain rabbit ileum and duodenum in culture for 13-14 days, but the viability of the cultures after that time is uncertain. Small bowel tissue has a higher rate of cell turnover than large-bowel tissue

TABLE 49

Recovery of E. coli (EB_{11}) from organ culture medium

Culture	Recovery of EB_{11}
*MCM + EB_{11} @ t = 10 minutes (culture A)	+++
MCM + EB_{11} @ t = 24 hours (culture B)	-
1ml 'B' into 50ml Todd-Hewitt broth	+++
EB_{11} in Weymouths medium	++
EB_{11} in Todd-Hewitt broth	+++

*MCM = Mucosal culture medium.

(Lipkin, 1973) and this may account for its early breakdown in culture. The practice of keeping the culture on a grid, effectively on the surface of the culture medium, may also have contributed in some way to the survival of the tissue. Organ cultures are usually maintained in roll-tubes and thus continually flushed with the culture medium. Cultures of large-bowel tissue from rabbits does not maintain well if the medium is allowed to flood over the culture (De Vries, personal communication).

The results obtained from incubation of the organ culture medium indicate that this medium is bacteriostatic after it has been used to maintain either mucosa or tumour tissue. The mechanism by which this is achieved is not apparent. It may be a non-specific inhibition, e.g. by mucin or by cationic proteins, or it may be specific, e.g. residual locally produced immunoglobulin. Similar systems may prevent invasion by bacteria of the colon tissue '<u>in situ</u>'. Whatever the mechanism, it enables tissue from a site which is potentially contaminated with bacteria, to be maintained in culture without the use of antibiotics in the medium. Bernik, Donellan and Hirsch (1968) maintained adult human colonic mucosa in culture for 8-12 weeks. As in the present study, they found that bacterial contamination of the cultures was not a problem, even when antibiotics were not included in the medium, if the biopsies were washed well first. They concluded that natural protective antibacterial mechanisms of the mucosa may be able to dispose of a limited number of residual organisms '<u>in vitro</u>'. Thus, diligence during initial washing of the specimen may be all important for the subsequent success of the culture.

<u>E. coli</u> appeared to adhere readily to pieces of tissue in organ

culture, with no preference for either mucosa or tumour cultures. The cultures were about 4 sq. mm. in area and weighed about 0.5 g. The number of bacteria adhering was equivalent, therefore, to a mucosal population of about 10^5 to 10^6 bacteria/g tissue, which is similar to the numbers of bacteria which have been isolated from mucosal specimens during the present study.

If specific sites are involved in bacterial attachment it might be concluded the the number of sites available in the cultured tissues were equivalent to those found *in vivo*.

Bacteria, other than *E. coli* used as the inoculum, were not isolated from the organ cultures, which again suggests activity by a locally produced antibacterial agent in removing the normal mucosal flora.

The present study is only a small beginning and a great deal of future work will be necessary to establish whether strains of bacteria prominent in the large bowel (e.g. bacteroides) and those isolated from mucosal specimens can be shown to attach to pieces of mucosa in culture and to establish the mechanisms by which this is achieved.

3.10. Discussion on the role of intestinal bacteria in the aetiology of cancer of the large bowel.

Aries *et al.* (1969) postulated that intestinal bacteria producing carcinogens or co-carcinogens from bile acids may be involved in the

aetiology of colon cancer. Since then, the reaction necessary for the colonic flora to produce a carcinogen of the polycyclic aromatic type has been demonstrated (Goddard, 1973). It should be emphasized that production of such a compound is not the prerogative of one single strain or genus of bacteria since several preliminary steps are also involved and carcinogen formation would thus be the resultant of interactions between various groups of bacteria. Therefore, in relating the faecal flora of a population to the incidence of large-bowel cancer within that population, caution must be exercised. Any one facet of the results should not be over emphasized and a balanced view of the situation as a whole should be maintained.

During the present study, examination of the faecal flora has shown variations between groups of people who differ in many aspects, including incidence of large-bowel carcinoma (Table 50). Fewer bacteroides were isolated from the faeces of persons from Uganda and India, where the incidence of colon cancer is low, than from the faeces of persons from the U.S.A., England, Scotland and Hong Kong where the incidence is high. Correspondingly there was an inverse relationship between the numbers of eubacteria isolated and the colon cancer incidence rate. This trend was also seen between groups of people in different socio-economic groups in Hong Kong. The incidence of colon cancer in Hong Kong is positvely correlated with income (Table 51)*. Income in turn reflects life-style and thus diet.

Animal fat and protein in dietary intake have been positively correlated with the incidence of large bowel carcinoma (Drasar and Irving, 1973). The populations examined during the present study differed in

TABLE 50

Incidence of cancer of the colon.

(standardized for age)

Country	Incidence of colon cancer annual rates per 100,000 persons aged 35 - 64		
	Men	Women	Mean for Men and Women
England (South Metropolitan)	15.8	20.5	18.2
Scotland (Edinburgh)	28.2	34.1	31.2
U.S.A. (Atlanta) (white)	26.6	29.6	28.1
U.S.A. (Atlanta) (black)	25.6	31.9	28.8
India (Bombay)	6.6	4.8	5.7
Uganda (Kyadondo)	0.0	1.2	0.6
Japan (Miyagi)	5.0	4.8	4.9
Hong Kong (overall population)	14.5	13.6	14.0

From Doll (1969).

TABLE 51

Cancer incidence rates for Hong Kong.

(standardized for age)

Cancer mortality rates per 100,000 by site, sex and average monthly income per person (aged 35 - 64).

Site	Sex	INCOME <£16	£16 - £33	+ £33
Colon	Male	7.4	11.5	13.6
	Female	7.7	9.4	No figures
Rectum	Male	4.6	6.5	13.6
	Female	3.3	6.5	No figures
Colon-Rectum	Male	12.0	18.0	27.3
	Female	10.9	15.8	No figures

McLennan - personal communication.

the amounts of these commodities consumed in the diet. People from Uganda and India and from the low income groups in Hong Kong, consumed a diet which was high in carbohydrate, but low in animal fat and protein. People from England, Scotland, the U.S.A. and the high income groups in Hong Kong consumed a diet which contained fewer carbohydrates but more animal fat and protein.

Dietary fat stimulates the secretion of bile. An indication of bacterial breakdown of bile steroids in the colon can be assessed from an examination of the concentrations of some faecal steroids and Hill et al. (1971) noted that steroids were present in much lower concentrations in faeces from Ugandans and Indians than in the faeces of British and Americans. It was suggested that people living in Britain and North America have an intestinal bacterial flora that is more active at degrading both cholesterol and bile salts. A similar situation has been shown with the faeces of persons from various income groups in Hong Kong (Table 52, Hill, personal communication).

Hill et al. (1971) showed that a high percentage of strains of non-sporing anaerobes isolated from the faeces of persons living in areas where large-bowel cancer incidence is high were capable of 7-α-dehydroxylating bile steroids; however, this trend was not found during the present study of various income groups in Hong Kong and, overall, the number of isolates capable of performing this reaction was low; the concentrations of mono and unsubstituted acid steroids in the faeces was also correspondingly low (Table 52). Similarly, steroid metabolism by strains of clostridia isolated from faeces from Hong Kong was low. Goddard (1973) in a geographical survey, showed a positive correlation between the proportion of strains of faecal clostridia showing Δ^4-dehydrogenase

TABLE 57

Steroid concentrations (mg per g. dry weight) in faeces from three income groups in Hong Kong.

		Income Group A	B	C
No. of samples examined		16	20	12
(a)	Total steroids	10.05	7.72	5.66
(b)	Neutral steroids	5.42	4.71	3.53
(c)	Cholestrol	1.85	2.73	2.11
(d)	Coprostanol	3.24	2.29	1.21
(e)	Coprostanone	0.33	0.22	0.21
%	(d + e)/b	71	53	39
(f)	Acid steroids	4.63	3.01	2.13
(g)	Trisubstituted	0.68	0.73	0.43
(h)	Disubstituted, Total	2.54	1.50	1.30
	dihydroxyl	1.43	1.15	1.09
(i)	Mono + unsubstituted	1.42	0.80	0.46
%	i/f	30.67	26.6	21.10
	Ratio i/g	2.08	1.10	1.06

Hill - personal communication.

activity and colon carcinoma. This variation was not due, as with the 7-α-dehydroxylase, to the proportion of certain strains performing the reaction, but to the variation in numbers of non-lecithinase producing organism Clostridium paraputrificum, the major contributor to this type of desaturation. The lecithinase negative clostridia isolated from Hong Kong were somewhat unusual and nuclear desaturation of steroids by these bacteria was rare. Only one true strain of Cl. paraputrificum was isolated and this did possess Δ^4-dehydrogenase, however, due to small numbers it was impossible to try and correlate carriage of Cl. paraputrificum or any similar strains with socio-economic class.

The bacteriological and biochemical studies of faeces has yielded the following information:-

a) Differences in the proportions of non-sporing anaerobes isolated from the faeces of groups with different rates of incidence of carcinoma of the colon.

b) Differences in the faecal steroid concentrations in these groups.

c) The number of isolates of non-sporing anaerobes and clostridia, from faeces from Hong Kong, able to metabolize steroids was too small to show differences between groups within that one country.

d) Only very small numbers of Cl. paraputrificum and other clostridia capable of desaturating the steroid nucleus were isolated from faeces from Hong Kong, and many of the isolates

were species not generally considered to be faecal organisms.

The low rate of metabolism of steroids by Hong Kong isolates may indicate the possible importance of additional mechanisms involved in the aetiology of large-bowel cancer in that country. Metabolites produced by the colonic mucosal flora may be important as carcinogens or co-carcinogens, and in Hong Kong they may assume an increased importance.

*The income groups shown in Table 51 do not directly correspond to the income groups examined during the present study. They merely indicate the trend towards a higher incidence of colon carcinoma associated with increased income.

CONCLUSIONS.

Conclusions.

Information gathered about the numbers of viable bacteria isolated from a specimen ultimately relies on the assumption that one viable bacterial cell gives rise to one macroscopic colony. Specimens studied during the present investigation yielded a mixed bacterial population and it should be remembered that the inhibition of one species by another on an agar plate may possibly have caused some species to be under-represented.

The bacteriological examination of large bowel specimens (faeces and tissue) has been facilitated by the advent of the anaerobic chamber and the flexible vinyl type described in this thesis is cheap and easy to use and maintain. The present study has also demonstrated the potential of such a chamber for the isolation of anaerobic bacteria from wound swabs and routine clinical laboratories could benefit by the adoption of a similar system or of some other means of preparing pre-reduced media and storing seeded plates in an anaerobic environment. Isolated strains of anaerobic bacteria may be handled on the open bench, at least for short periods and with the development of rapid techniques for identification, our knowledge of the biochemistry, pathogenicity and taxonomic inter-relationships of these bacteria will greatly increase.

The anaerobic bacteria are abundant and important members of the indigenous flora of mans' large bowel and may influence the host in a variety of ways. They also represent a large pool of potential pathogens which may take the opportunity to renounce their symbiotic status and

become parasitic, e.g. in wound infections and peritonitis following bowel surgery. Under other circumstances, some of these bacteria may elaborate carcinogen-like compounds from bile steroids, again to the possible detriment of their host. The factors which determine the composition of the bacterial flora of the large bowel thus assume an equivalent degree of importance.

The present study has demonstrated differences in the faecal flora of groups of people in various parts of the world and has tentatively ascribed these differences to the type of diet. The high percentage ~~properties~~ of bacteroides isolated from the faeces of persons whose diets contained large amounts of animal protein may reflect the association of this dietary constituent with animal fat (Drasar and Irving, 1973). Bile is secreted into the intestine in response to fat consumed. The growth of some species of bacteroides is stimulated *in vitro* by the presence of bile and it is possible that this could account for the situation noted *in vivo*. However, the studies described in this thesis have dealt only with the cumulative results from the examination of faeces from groups of people and much work remains to assess the factors determining the faecal flora of individuals, especially with regard to carriage of bacteria such as *Clostridium paraputrificum*.

In addition, the present study has demonstrated that it is not only the flora of faeces which must be taken into account when looking at the ecology of the large bowel. The bacterial flora associated with the mucosa appears very different from the anaerobe dominated faeces and a great many questions about the exact nature of these bacteria, the factors which control their distribution and their biochemical activities,

especially with regard to the possible production of carcinogens, remain to be answered. A small beginning has been made using organ culture, and the adherence of *E. coli* to colonic mucosa has been demonstrated. Future studies could further exploit organ culture of mucosa to examine the adherence of other bacteria, especially those that have been isolated from tissue specimens, and to determine the factors involved in adherence (biochemical and physical). In addition light may fall on the nature of the antibacterial properties exhibited by mucosa maintained in culture. The factors determining the relationship between the mucosal flora and the faecal flora also await investigation.

Organ cultures of colonic mucosa may be a useful tool for future studies of the mechanism of carcinogenesis and for the testing of bacterial metabolites.

It seems that the large bowel of man represents a highly developed ecological system and a great many of the factors controlling its development and maintenance remain to be elucidated.

APPENDIX.

Biochemical tests.

Liquid media for biochemical tests were distributed in bijou bottles, and inoculated with 2 drops of actively growing culture from Robertson's cooked meat medium. The tops of the bottles were loosened before incubation in an anaerobic jar containing an atmosphere of 30% CO_2 and 70% hydrogen.

Fermentation tests

The fermentation tests were modifications of those described by Cato et al. (1970). 0.5 g. aesculin or 1.0 g. of other test carbohydrates were added to a 100 ml. volume of basal medium containing 2.0 g. peptone, 1.0 g. yeast extract, 0.05 g. cysteine hydrochloride, 0.3 g. sodium formaldehyde sulphoxylate, 0.001 g. $CaCl_2$, 0.001 g. $MgSO_4$, 0.005 g. KH_2PO_4, 0.005 g. K_2HPO_4, 0.05 g. $NaHCO_3$, and 0.01 g. NaCl. The pH of the media was adjusted to 7.1± 0.1 before autoclaving at 115°C for 20 minutes. Cultures were incubated anaerobically at 37°C for three days. The final pH of the various test media was estimated by the addition of two drops of bromocresol purple indicator (B.D.H.) and noting the colour change.

Aesculin hydrolysis

Two to three drops of a 1% solution of ferric chloride were added to the culture in aesculin medium. A positive test was denoted by the development of a black colour.

Gelatin liquefraction

The test cultures were inoculated into Robertson's cooked meat medium, to which were added charcoal gelatin discs (Oxoid). The appearance of free charcoal particles in the medium, after 14 days incubation at 37°C indicated liquefraction of the gelatin.

Nitrate reduction and indole production

Nitrate, nitrite and indole were estimated by conventional procedures (Cowan and Steel, 1965) in cultures grown anaerobically for 72 hours in indole-nitrate medium (Baltimore Biological Laboratories, U.S.A.).

Growth in bile medium

The test cultures were inoculated into thioglycollate broth (Oxoid) which contained 20% ox-bile (Oxoid) and 0.1% deoxycholic acid. Stimulation or inhibition of growth was determined by comparison with a control of the same culture in thioglycollate broth. These tests were incubated aerobically.

Glycerol Transport Broth.

Lab. Lemco	10 g.
Glycerol	100 ml.
Distilled water	900 ml.

pH adjusted to 7.3± 0.1.

Dispensed in 4.5 ml amounts in bijou bottles.

Sterilized by autoclaving at 10 lb/sq" for 20 minutes.

Brain-Heart-Infusion Broth

(B.H.I.)

As diluent - anaerobic chamber.

Brain-Heart Infusion (Oxoid)	37 g.
Yeast extract (Oxoid)	5.0 g.
Cysteine Hcl.	0.5 g.
Sodium formaldehyde sulphoxylate	0.3 g.
Distilled water	1000 ml.

pH adjusted to 7.3± 0.1.

Dispensed in 100 ml volumes in bottles to give minimum air gap over surface of liquid.

Sterilized by autoclaving at 10 lb/sq" for 20 minutes.

In roll tubes.

Basic medium as above, supplemented with haemin/menadione solution (10 ml/1000 ml medium), added after autoclaving and cooling to 50-55°C.

Haemin/menadione solution.

MENADIONE STOCK SOLUTION: 100 mg menadione in 20 ml 95% ethanol. Sterilized by filtration.

HAEMIN STOCK SOLUTION: 50 mg haemin dissolved in 1 ml of 1M NaOH. Made to 100 ml with distilled water. Sterilized by autoclaving at 15 lb/sq" for 15 minutes.

FINAL SOLUTION: 1 ml sterile menadione stock solution mixed with 100 ml haemin stock solution.

All solutions stored at +4°C.

Todd-Hewitt Broth.

Todd-Hewitt Broth (Oxoid)	36.4 g.
Cysteine Hydrochloride	0.5 g.
Sodium formaldehyde sulphoxylate	0.3 g.
Distilled water	1000 ml.

pH adjusted to 7.3± 0.1

Sterilized by autoclaving at 10 lb/sq " for 20 minutes.

Reinforced Clostridial Medium with blood.

(R.C.M.B.).

Reinforced Clostridial agar (Oxoid)	52.5 g.
Glucose	5.0 g.
Liver digest ('Panmede', Paines and Byrne, Greenford).	10.0 g.
Cysteine HCl.	0.5 g.
Sodium formaldehyde sulphoxylate	0.3 g.
Distilled water	1000 ml.

pH adjusted to 6.8± 0.1.

Dispensed in 500 ml amounts.

Sterilized by autoclaving at 10 lb/sq" for 20 minutes.

Cooled to 50-55°C and 50 ml sterile, defibrinated horse blood added.

Dispensed in Petri dishes.

Willis and Hobbs (1958) medium - modified.

Basal Medium

Agar (New Zealand)	9.0 g.
Lactose	9.6 g.
Neutral Red 1% solution	4.0 ml.
Brain Heart Infusion (Difco)	37.0 g.
Cysteine - HCl	1.0 g.
Distilled water	900 ml.

Agar, lactose, neutral red and BHI dissolved by steaming.

Dispensed in 450 ml amounts.

Sterilized by autoclaving at 15 lb/sq" for 25 minutes.

Cooled to 50-55°C and 50 ml of egg yolk suspension added.

Dispensed into Petr dishes.

Egg Yolk Suspension

Hen's eggs broken under aseptic conditions.

Separated yolks emulsified in an equal volume of 0.9% sodium chloride solution.

Rogosa's medium for lactobacilli.

(Rogosa et al. 1951).

Tryptone (Oxoid)	10 g.
Yeast extract (Oxoid)	5 g.
KH_2Po_4	6 g.
Ammonium citrate	2 g.
Salts solution*	5 ml.
Glucose	20 g.
Sodium acetate (Hydrate)	25 g.
Sodium acetate (anhydrous)	15 g.
Acetic acid	1.32 ml.
Agar	15 g.
Distilled water	1000 ml.

pH adjusted to 5.4± 0.1.

Dispensed in 500 ml amounts.

Sterilized by autoclaving at 10 lb/sq" for 20 minutes.

Dispensed in Petr dishes.

*Salts solution.

$CaCl_2$ (anhydrous)	0.2 g.
$MgSo_4$	0.2 g.
K_2HPo_4	1.0 g.
KH_2Po_4	10.0 g.
$NaHCO_3$	2.0 g.
NaCl	

$CaCl_2$ and $MgSo_4$ mixed in 300 ml distilled water undissolved. 500 ml water added and while swirling, remaining salts slowly added. When all salts dissolved, 200 ml distilled water added. Stored at +4°C.

Rogosa's Medium for Veillonellae

(Rogosa, 1956).

Tryptone (Oxoid)	5 g.
Yeast extract (Oxoid)	3 g.
Sodium thioglycollate	0.75 g.
Tween 80	1.0 g.
Sodium lactate (50% solution)	25 ml.
Basic fuchsen (1% solution)	0.2 ml.
Agar (New Zealand)	20 g.
Water	1000 ml.

All reagents, except agar, dissolved by steaming. pH adjusted to 7.5± 0.1. Agar added and medium sterilized by autoclaving at 15 lb/sq" for 15 minutes.

Before pouring vancomycin added to a concentration of 7.5 ug/ml.

Once poured, plates of this medium should be used within one week.

Watt's Medium.

(Watt, 1972).

Blood Agar Base no. 2 (Oxoid)	2.5 g.
Distilled water	100 ml.

Sterilized by autoclaving at 15 lb/sq" for 20 minutes.
After cooling to 50°C, 20 ml human blood (outdated, citrated), or 10 ml of defibrinated horse blood, added plus 1 ml of cysteine-dithiothreitol solution.

Cysteine-dithiothreitol solution.

Cysteine hydrochloride	100 mg/ml.
Dithiothreitol	9 mg/ml.
Distilled water	

Prepared immediately before use and sterilized by filtration.

Moore's Medium.

Neopeptone (Difco)	10 g.
Yeast extract (Difco)	5 g.
Liver extract ('Panmede', Paines and Byrne, Greenford)	5 g.
Glucose	10 g.

The above constituents were dissolved in 400 ml distilled water and 100 ml of salts solution (see below) were added. The pH was adjusted to 7.6± 0.1. Agar (Difco), dissolved in 500 ml distilled water by autoclaving at 10 lb/sq" for 10 minutes, was added whilst still hot. The medium was dispensed in suitable amounts and sterilized by autoclaving at 10 lb/sq" for 20 minutes. This basal medium was stored at room temperature, and melted when required.

Before use, liquefied portions of the basal medium were cooled to 50°C and defibrinated horse blood (10% v/v) and dithiothreitol supplement (0.75% v/v) were added.

Salts solution:

$MgSo_4 7H_2O$	40 g/l.
$MnSo_4\ 4H_2O$	2 g/l.
$FeCl_3$ (anhydrous)	0.4 g/l.
HCl (concentrated)	0.5 ml/l.

Dithiothreitol Supplement:

cysteine hydrochloride	12 mg/ml.
glutamine	6 mg/ml.
Dithiothreitol	12 mg/ml.

Made immediately prior to use and sterilized by filtration.

Moore, 1968.

Freter's Medium (modified)

(Aranki et al. 1969)

Trypticase Soy Broth (Baltimore Biological Laboratories, U.S.A.)	30 g.
Cysteine hydrochloride	0.5 g.
Sodium carbonate	0.42 g.
Palladium chloride	1 g.
Agar (Difco)	20 g.
Distilled water	1000 ml.

Sterilized by autoclaving at 15 lb/sq" for 20 minutes. Haemin/menadione solution (see preparation of B.H.I.) and defined supplement (20 ml) added as sterile solutions after cooling. For medium without palladium black, omit palladium chloride.

Defined supplement

Supplement A.

Co Carboxylase	0.001 g.
L-glutamine	0.5 g.
Dextrose	20.0 g.
Distilled water	100 ml.

Supplement B.

Ferric nitrate	0.5% solution

Sterilized by filtration and stored at -20°C until required.

For use, 10 ml of A mixed with 1 ml of B.

Waymouth's medium for organ culture.

Waymouth's medium (Wellcome)	7 g.
Foetal calf serum	50 ml.
Distilled water	475 ml.
Hydrocortisone	1 mg/ml.
Ascorbic acid	300 mg/ml.
Ferrous sulphate	0.45 mg/ml.

Antibiotics (if included)

Penicillin G	50 units/ml.
Streptomycin	50 µg/ml.
Amphotericin B	5 µg/ml.

Sterilized by filtration, then 25 ml of a sterile 4.4% solution of sodium bicarbonate added.

Chromatography solvents and developing sprays.

Solvents.

S_1 : Benzene/Dioxan/Acetic acid (75 : 20 : 2)

V_1 : Benzene/Dioxan/Acetic acid (100 : 10 : 1)

Sprays.

Phosphomolybdic acid

Phosphomolybdic acid (10%) in ethanol.

Anisaldehyde reagent

Anisaldehyde (B.D.H.)	0.5 ml.
glacial acetic acid	50 ml.
concentrated sulphuric acid	1 ml.

Suppliers of Equipment.

Anaerobic Jars	Baird and Tatlock (London) Ltd., P.O. Box 1 Freshwater Road, Romford, RM1 1HA.
Standard	Tel: 01-590-7700
Large	Becton Dickinson UK Ltd., York House, Empire Way, Wembley, Middx.
Milking machine pails (for conversion)	Fullwood and Bland Ltd., 35 Bevenden Street, London, N.1. Tel: 01-253-1721
Anaerobic Chambers (Flexible plastic)	Plysu Industrial Ltd., Woburn Sands, Bletchley, Bucks.
	Vickers Ltd., Priestly Road, Basingstoke, Hampshire.
Stainless steel air-locks suitable for flexible plastic isolators	Lintott Engineering Ltd., Foundry Lane, Horsham, Surrey.
Cold Catalyst	'D' Catalyst Code 4586, Engelhard Industries, Valley Road, Cinderford, Glos., GL14 2PB.
Rubber moulding paste	'Silescol' SR300, Esko (Rubber) Ltd., 14-18 Great Portland Street, London, W1N 5AB
Fan for Chamber	'Vent Axia', 339 Finchley Road, London, N.W.3.
V.P.I. Anaerobic Culture System: Gassing stand, clamps.	Bellco Glass Inc., Vineland, New Jersey, U.S.A.
Roll-tube spinner	Astell Laboratory Service Co., 172 Brownhill Road, London, S.E.6.
Oxygen Free Gas for P.R.A.S.	B.O.C. Special Gasses Division, Deer Park Road, London, S.W.19.
Culture System	Air Products Special Products Dept., Weston Road, Crewe, Cheshire, CW1 1DF.
Gas Chromatography	Pye Unicaim Ltd., York Street, Cambridge, CB1 2PX.

Gloves for Chamber (Neoprene-rubber)	Dunlop Ltd., Dunlop House, Ryder Street, London, S.W.1.
Laminar flow cabinet	Hepaire Manufacturing Ltd., Robell Way, Water Lane Industrial Estate, Storrington, Sussex.
Electronic colony counter	Scientifica and Cook Electronics, 78 Bollo Bridge Road, London, W.3.
Brunswick shaking apparatus	New Brunswick Scientific, New Brunswick, N.J., U.S.A.
Stomacher	'Colworth Stomacher 80', A. J. Seward, U.A.C. House, Blackfriars Road, London, SE1 9UG.

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The relation between diet and the gut microflora in man

By B. S. DRASAR, J. S. CROWTHER, P. GODDARD, GABRIELLE HAWKSWORTH, M. J. HILL, SUSAN PEACH and R. E. O. WILLIAMS, *Department of Bacteriology*, and A. RENWICK, *Department of Biochemistry, St Mary's Hospital Medical School, London W2 1PG*

The chemical nature of the diet has long been considered to exert a controlling influence on the bacterial flora of the intestine (Rettger & Cheplin, 1921; Dugeon, 1926). The type of diet and frequency of feeding influences the distribution of bacteria within the small intestine of both man and animals (Smith, 1965; Drasar, Shiner & McCleod, 1969). But, in adult man, attempts to change the colonic flora by dietary manipulation have produced equivocal results, at least in so far as alterations can be detected by culture of faecal specimens (Sanborn, 1931*a*,*b*; Haenel, Müller-Benthow & Scheunert, 1957).

In this paper we describe differences in the faecal flora of groups of people living on very different diets and the results of some studies of individuals whose diet was varied. Because of the variations in bacteriological technique and to facilitate direct comparisons detailed results are presented only for our own studies. The methods used have been described in detail (Drasar, 1967).

Differences between the faecal flora of various dietary groups

As part of a larger study on the aetiology of cancer of the colon the faecal flora of people living in Uganda, India, and Japan was compared with that of people living in England, Scotland, and the USA. Although there are many other differences between these groups the principal dietary variations were in the amount of carbohydrate, fats, and animal proteins consumed (Hill, Crowther, Drasar, Hawksworth, Aries & Williams, 1971). The Ugandan diet consisted principally of matoke (boiled, mashed banana) whereas the Indians and Japanese consumed a rice diet. Compared with these people the western diet was rich in fat and animal protein. The people living on the high-carbohydrate diet had significantly ($P<0{\cdot}001$) fewer bacteroides and more enterococci in their faeces than did those on a Western diet (Table 1); aerobic bacteria were also more prominent.

Study of the bacteria isolated from these groups revealed other differences. Eubacteria occurred more often in specimens from people on a high-carbohydrate diet and there was also greater variation in the aerobic bacteria isolated from these specimens (Table 2).

Table 1. *The faecal flora of normal people belonging to various dietary groups (mean log_{10} viable count/g faeces). From the results of Hill* et al. (1971) *and Aries, Crowther, Drasar, Hill & Ellis* (1971)

Diet	Mixed Western				Wholly or primarily vegetarian			
Country of origin	USA	Scotland	England	Uganda	Rice India	Matoke Uganda	Rice Japan	Vegan* England
Enterobacteria	7·4	7·6	7·9	7·4	7·9	8·0	9·4	7·0
Enterococci	5·9	5·3	5·8	5·3	7·3	7·0	8·1	4·8
Lactobacilli	6·5	7·7	6·5	5·3	7·6	7·2	7·4	7·4
Clostridia	5·4	5·6	5·7	4·7	5·7	5·1	5·6	5·4
Bacteroides	9·7	9·8	9·8	9·8	9·2	8·2	9·4	9·7
Gram-positive, non-sporing anaerobes	10·0	9·9	9·8	9·5	9·6	9·3	9·7	9·6

*Subject living on strictly vegetarian diet.

Table 2. *Comparisons of the prominence of certain bacterial species within some groups of faecal bacteria*

Group	Species	Percentage of group identified as species designated: England, Scotland and USA	Uganda, India and Japan
Enterobacteria	*Escherichia coli*	92	81
Enterococci	*Streptococcus faecalis*	65	38
	Streptococcus faecium	30	45
Bacteroides	*Bacteroides fragilis*	92	98
Gram-postive anaerobes	*Bifidobacterium bifidium*	13	10·5
	Bifidobacterium adolscentis	35	28
	Bifidobacterium eriksonii	6	4·5
	Eubacterium biforme	16	33·5

The influence of vegetarianism on the intestinal flora was studied in an attempt to elucidate the differences between the developed and under-developed countries. The high-carbohydrate diets eaten in Uganda, India, and Japan are basically vegetarian. However, when a group of people living on a balanced but strictly vegetarian diet were studied, their flora was similar to that of people eating a normal Western diet (Table 1). *Sarcina ventricula* was the only bacterial species found solely in the faeces of vegetarian groups (Crowther, 1971).

Dietary alteration of the flora

Although differences are demonstrable between groups of people living on different diets, attempts to change the composition of the flora by variations in diet have, in general, proved unsuccessful. A change to a vegetarian diet produces no demonstrable change in the predominant faecal flora (Moore, Cato & Holdeman, 1969). One study on the faeces of astronauts (Gall, 1965) showed changes in the flora on a synthetic diet; however, a further study demonstrated no such effect (Cordaro, Sellers, Ball & Schmidt, 1966). On the other hand, cellulose (Haenel, Gassman, Gratte & Müller-Benthow, 1964) and lactulose (Haenel, Feldham, Müller-Benthow

& Ruttloff, 1958) apparently produced slight changes. In one long-term study in which one subject was fed successively for periods of some weeks on high-protein, high-carbohydrate and high-fat diets, the protein diet did not affect the flora whereas the high-carbohydrate diet increased the relative numbers of the bifidobacteria, and the high-fat diet favoured the bacteroides (Hoffmann, 1964).

Winitz, Adams, Seedman, Davies, Jayko & Hamilton (1970) reported the disappearance of most of the faecal flora (total count as low as 10^3/g faeces) when volunteers were given a 'no-residue diet'. Our studies did not support this finding, the total bacterial numbers remaining unchanged. A similar study reported by Attebery, Sutter & Finegold (1972) showed that 'extremely oxygen-sensitive anaerobes' disappeared from faeces if a completely absorbable diet was consumed but that no other consistent change occurred.

Changes in the metabolic activity of the flora

Although changes in the bacterial species and genera comprising the flora are difficult to demonstrate, changes in the metabolic activity of the flora due to changes in dietary compounds do occur. This is best illustrated by reference to our studies on cyclamate metabolism (Drasar, Renwick & Williams, 1972). The giving of a dose of 3 g cyclamate daily for 10 d was accompanied by the urinary excretion of cyclohexylamine in one of our volunteers. In this subject but not in the two others studied the flora had changed in its ability to metabolize cyclamate; initially the flora had not metabolized the cyclamate but after exposure the flora converted the cyclamate to cyclohexylamine (Table 3).

Table 3. *The change in the ability of faecal flora to metabolize cyclamate by feeding a cyclamate diet in one human subject*

Dietary conditions	Percentage conversion of cyclamate to cyclohexylamine by faecal flora
Cyclamate-free diet	0·06
Cyclamate diet (3 g/d) for 10 d	74·9
Cyclamate-free diet for 6 d after cyclamate diet for 18 d	0·14

Physiological considerations

There is little information concerning the composition of the intestinal fluid entering the large intestine. Intestinal secretions and cells together with dietary residues probably comprise the greater part of this material. The difficulty in changing the flora of individuals by changes in diet probably reflects the efficiency of intestinal absorption. However, the studies on cyclamate demonstrate that, if dietary compounds reach the flora, changes do occur.

Furthermore, studies on bile salt metabolism have demonstrated that diet can control intestinal secretions and therefore the composition of the fluid entering the colon (Hill, 1971). In this context it should be noted that the faeces of the Ugandans,

Indians, and Japanese studied contained much lower concentrations of bile salts than do those of Western people (Hill *et al.* 1971). The influence of diet may be indirect.

The problem of demonstrating changes in bacterial flora may be partly explained by the nature of bacteriological procedures for bacterial identification. These have been developed to distinguish bacterial groups in the laboratory, not biological types in an ecological situation. Thus many of the substrates whose utilization is important in bacterial identification may be without significance in the bio-economics of bacterial growth in the intestine.

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THE NON-SPORING ANAEROBIC BACTERIA IN HUMAN FAECES

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DIET has long been assumed to be one of the major determinants of the faecal flora (Dudgeon, 1926). Studies with experimental animals provide support for this hypothesis (Smith, 1965); however, studies with human faeces have provided less satisfactory evidence (Moore, Cato and Holdeman 1969).

In a study of faecal specimens from different dietary groups in various parts of the world, we demonstrated differences in the numbers of bacteroides organisms present in faeces (Hill *et al.*, 1971). In the present paper we report on the more detailed classification of the non-sporing anaerobes isolated in that study and during subsequent extensions of the study.

MATERIALS AND METHODS

Source of faeces. Stool samples were obtained from groups of people consuming a mixed western diet, in England, Scotland and the USA, and from people living on diets with a high carbohydrate content in India, Uganda and Japan. The characteristics of these groups are shown in table I. The groups contained both men and women. All were healthy individuals, mostly aged between 20 and 50. Samples from the USA came from both black and white persons.

Transport and storage of specimens. Fresh samples of faeces were diluted 1 in 10 in 10% glycerol broth and immediately frozen in solid carbon dioxide or in liquid nitrogen for transport to London (Crowther, 1971). The samples were stored at −50°C.

Isolation of non-sporing anaerobic bacteria. Isolation plates were seeded in an anaerobic cabinet. During the initial stages of this investigation a rigid Perspex cabinet filled with oxygen-free nitrogen (N_2) containing 5% carbon dioxide (CO_2) was employed (Drasar, 1967). Later studies were carried out in a flexible polyvinyl cabinet filled with 5% CO_2 in a mixture of 90% N_2 and 10% hydrogen (H_2) (Aranki *et al.*, 1969). Ten-fold dilutions of the specimens were prepared in Brain-Heart-Infusion (BHI) broth (Oxoid) containing 0·05% (w/v) cysteine hydrochloride and 0·03% sodium formaldehyde sulphoxylate as reducing agents. The diluent was heated to 100°C before being introduced into the cabinet, where it was dispensed and allowed to cool in the anaerobic environment.

0·1-ml samples of appropriate dilutions were spread on the surface of plates of Reinforced Clostridial Medium (Oxoid) with 1% glucose (w/v), 1% liver digest (Panmede, Paines and Byrne, Greenford, England) and 10% defibrinated horse blood. In order to maintain the media in a reduced state, the plates were poured and dried in the Perspex cabinet, or stored for 3 days in the flexible cabinet.

Seeded plates were packed into anaerobic jars and on removal from the cabinet the jars were re-evacuated and filled with a gas mixture containing 30% CO_2 and 70% H_2 and incubated at 37°C for 3 days. Cold " D " catalyst was used in the anaerobic jars (Englhard Industries, Cindeford, Gloucestershire).

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Preservation of cultures. Ten colonies were picked from the isolation plates and sub-cultured into Robertson's cooked meat medium. After incubation for 3 days, the cultures were checked for purity and aerobic growth. Glycerol was then added to a final concentration of 10% (v/v) and the stock cultures were stored at −50°C.

Identification of isolates

The isolates were placed into various broad groups on the basis of their appearance in a Gram-stained preparation (Preston and Morrell, 1962) and on the detection of the end-products of glucose metabolism by gas-liquid chromatography. They were assigned to species on the basis of their biochemical reactions.

TABLE I

Populations studied

Diet	Population	Number of samples	Number of isolates
Normal mixed western, high fat*	England; staff and students, St Mary's Hospital Medical School, London	6	30
Normal mixed western, high fat*	Scotland; staff and students, Edinburgh University Medical School	16	28
Normal mixed western, high fat*	USA; staff members, Center for Disease Control, Atlanta, Georgia	33	116
Rice diet, high carbohydrate*	India; villagers, Vellore, S. India; controls in a study of tropical sprue	17	37
Matoke (boiled mashed bananas), high carbohydrate	Uganda; villagers, Kyandondo region; parents accompanying children to Kwashiorkor Clinic	9	10
Fruits and vegetables, high carbohydrate	Japan; villagers, Yonga; mostly farmers or housewives	31	168

* The commonly used terms "high fat" and "high carbohydrate" applied to diets denote "rich in fat" and "rich in carbohydrate" respectively.

Gas-liquid chromatography

Preparation of the sample. A 4-ml volume of a 3-day culture in 1% glucose broth (Cato *et al.*, 1970) was acidified with 0·5 ml of 50% aqueous sulphuric acid and extracted with 4 ml of diethyl ether. After centrifugation to break the emulsion, the ether layer was pipetted off and dried over magnesium sulphate. Reference solutions were prepared as described by Cato *et al.* (1970). Controls were of uninoculated glucose broth. Pyruvic, lactic and succinic acids are unstable at the operating temperature of the gas chromatograph. These acids were detected by prior preparation of their methyl esters which are stable and readily separate on the column. Freshly prepared diazomethane (de Boer and Backer, 1954) was used as the methylating agent, 2–3 drops being added to a portion of the extract.

Chromatographic conditions. Analyses were performed on a Pye 104 gas chromatograph, fitted with a flame-ionisation detector and a 5-ft glass column packed with "Diatomite C" that had been treated with phosphoric acid and impregnated with 10% polyethylene glycol. The operating temperature was 125°C and the carrier gas was oxygen-free N_2 passing through at 30 ml per min. A 1-μl aliquot of the sample for analysis was injected on to the column.

Biochemical tests

Liquid media for biochemical tests were distributed in 5-ml screw-capped bottles, and seeded with two drops of actively growing culture from Robertson's cooked meat

medium. The tops of the bottles were loosened before incubation in an anaerobic jar containing an atmosphere of 30% CO_2 and 70% H_2.

Fermentation tests. The fermentation tests were modifications of those described by Cato *et al.* (1970); 0·5 g aesculin or 1·0 g of other test carbohydrates were added to a 100-ml volume of basal medium containing 2·0 g peptone, 1·0 g yeast extract, 0·05 g cysteine hydrochloride, 0·03 g sodium formaldehyde sulphoxylate, 0·001 g $CaCl_2$, 0·001 g $MgSO_4$, 0·005 g KH_2PO_4, 0·005 g K_2HPO_4, 0·05 g $NaHCO_3$, and 0·01 g NaCl. The *p*H of the media was adjusted to 7·1±0·1 before autoclaving at 115°C for 20 min. Cultures were incubated anaerobically at 37°C for 3 days. The final *p*H of the media was estimated by the addition of two drops of bromocresol purple indicator (BDH) and noting the colour change.

Test for aesculin hydrolysis. Two or three drops of a 1% solution of ferric chloride were added to a 3-day culture in aesculin medium. A positive test was denoted by the development of a black colour.

Test for gelatin liquefaction. The test cultures were seeded into Robertson's cooked meat media, to which were added charcoal gelatin disks (Oxoid). The appearance of free charcoal particles in the medium, after incubation for 14 days at 37°C indicated liquefaction of the gelatin.

Tests for nitrate reduction and indole production. Nitrate, nitrite and indole were estimated by conventional procedures (Cowan and Steel, 1965) in cultures grown anaerobically for 72 hours in indole-nitrate medium (Baltimore Biological Laboratories, USA).

Growth in bile medium. The test cultures were seeded into Thioglycollate Broth (Oxoid) supplemented with 20% ox-bile (Oxoid) and 0·1% deoxycholic acid. Stimulation or inhibition of growth was determined by comparison with a control of the same culture in thioglycollate broth. These tests were incubated aerobically.

RESULTS

Survival of the isolates

The isolates had been stored at −50°C in Robertson's cooked meat medium with 10% glycerol for between 9 months and a year; 81·5% of organisms isolated from USA specimens and 96% of Japanese isolates were recovered after storage for 1 year.

Identity of the isolates

A total of 196 strains of non-sporing anaerobes were isolated from the faeces of 34 people on a mixed western diet in the USA, England and Scotland; 215 strains were isolated from the faeces of 45 people on carbohydrate-rich diets in Uganda, India and Japan.

In the present study the genus definitions and species characteristics published in the Anaerobe Laboratory Manual of Holdeman and Moore (1972) were employed; they are briefly recapitulated in tables II and III.

Genera of non-sporing anaerobes

All the isolates could be assigned to genera. The predominant groups of non-sporing strict anaerobes in faeces were found to be similar for all the populations studied; they fell into three main genera: *Bacteroides*, *Bifidobacterium* and *Eubacterium*. Minor components of the anaerobic flora consisted of fusobacteria, propionibacteria and lactobacilli. Although similar groups of organisms were isolated from all the faecal samples, with bacteroides

TABLE II

Genera of non-sporing anaerobes cited in this paper (modified from Cato et al., 1970)

BACTEROIDES

Small Gram-negative rods, with rounded ends, typically exhibit bipolar staining. *Bact. fragilis* produces from glucose, acetic and succinic acids with or without propionic, isobutyric, butyric, isovaleric and fumaric acids.

FUSOBACTERIUM

Small, Gram-negative spindle-shaped rods. Butyric acid is the major product of glucose fermentation.

F. fusiforme (nucleatum)
Also produces small amounts of lactic, propionic and succinic acids.

BIFIDOBACTERIUM

Gram-positive, branching (bifid) rods with spatulate ends. Acetic and lactic acids (ratio 1+:1) are the major end-products of glucose metabolism. All strains of species in the genus *Bifidobacterium* make similar fermentation products.

EUBACTERIUM

Small Gram-positive rods. Produce mixtures of acids from glucose, or no major acids.
E. aerofaciens produces acetic, formic and lactic acids from glucose.

PROPIONIBACTERIUM

Gram-positive rods, typically diphtheroid. Propionic and acetic acids are the major volatile acids produced from glucose. All species of propionibacteria make similar fermentation products.

LACTOBACILLUS

Straight Gram-positive rods, typically in chains. Lactic acid is the sole major product from glucose.

TABLE III

Differentiation of species of the major faecal anaerobes (after Cato et al., 1970)

Test	Reactions of the stated species*					
	Bacteroides fragilis	*Bacteroides melaninogenicus*	*Bifido-bacterium adolescentis*	*Bifido-bacterium bifidum*	*Bifido-bacterium eriksonii*	*Eubacterium aerofaciens*
Gelatinase	−	+	w^-	$-^w$	$-^w$	w^-
Indole production	v	v	−	−	−	−
Acid from:						
glucose	+	v	+	+	+	+
lactose	+	v	+	+	+	$+^-$
sucrose	+	v	+	−	+	$+^-$
maltose	+	v	+	−	+	+
fructose	+	v	+	+	+	+
mannose	+	v	$+^-$	$-^w$	+	+
mannitol	−	−	v	−	+	v
arabinose	v	v	+	−	−	v
xylose	+	w	+	$-^w$	+	$-^+$
Aesculin hydrolysis	+	v	+	$-^w$	v	$+^-$
Nitrate reduction	$-^+$	−	−	−	−	$-^+$

* + = positive reaction for 90–100% of strains; − = negative reaction for 90–100% of strains; w = weak reaction; v = variable among strains; superscripts indicate reactions of occasional strains.

TABLE IV

Percentage frequency of genera of non-sporing anaerobes among isolates from faeces of populations consuming different diets

Category of diet* and populations sampled	Number of isolates identified	Percentage frequency among isolates from the stated populations of genus					
		Bacteroides	*Fusobacterium*	*Bifidobacterium*	*Eubacterium*	*Propionibacterium*	*Lactobacillus*
"Mixed-western"; USA, England, Scotland	224	61·6	0·9	25·0	6·7	3·1	0
"High-carbohydrate"; Uganda, India, Japan	215	37·7	1·4	27·0	23·2	5·1	1·4

* See table I.

the most common isolates, there were differences in the distribution and relative proportions of these organisms. Faeces from " western-diet " countries (England, USA and Scotland) appeared to have a larger proportion of Gram-negative organisms (*Bacteroides* spp. and fusobacteria), than did those from people from countries with " high-carbohydrate diets " (India, Uganda and Japan). The consequently larger proportion of Gram-positive organisms found in faeces from these countries seemed to be due mainly to the contribution of the eubacteria (table IV); this increase in the proportion of eubacteria was statistically significant ($p = <0{\cdot}001$). Propionibacteria were found in both of the population groups studied. Strictly anaerobic lactobacilli, however, were found only in faeces from Uganda and Japan.

TABLE V

Major species of non-sporing anaerobes found in faeces

	Percentage number of isolates classified as stated and country of origin of the samples	
	USA, England or Scotland	Uganda, India or Japan
Organisms identified at species level	88·5	85
Bacteroides spp. found to be *Bact. fragilis*	92	98
Bifidobacterium spp. found to be:		
Bi. adolescentis	59	63·5
Bi. bifidum	23	16
Bi. eriksonii	10·0	6·5
Eubacterium spp. found to be *E. aerofaciens*	86	82

Sub-division of major genera

More than 80% of the isolates could be assigned to species, and the same major species of non-sporing anaerobes were found in faeces from all the dietary groups.

Almost all the bacteroides organisms were identified as *Bact. fragilis* (table V). Two-thirds of the bifidobacteria were identified as *Bi. adolescentis*; *Bi. bifidum* and *Bi. eriksonii* were also common. However, 8% of bifidobacteria from " western-diet " countries and 14% from " high-carbohydrate-diet " countries, could not be assigned to any species. More than 80% of the eubacteria could be shown to be *E. aerofaciens.*

Minor species of non-sporing anaerobes

A variety of minor species of non-sporing strict anaerobes were isolated and identified (table VI). The isolation of *Bact. melaninogenicus* from human faeces is not unusual, but other, more unexpected, bacteroides species were also found (see *Discussion*).

TABLE VI

Minor species of non-sporing anaerobes isolated from faeces in the present study

Species	Number of times isolated	Source
Bacteroides melaninogenicus	5	USA and India
Bact. hypermegas	1	India
Bact. ruminicola	1	Japan
Bact. trichoides	1	Japan
Bact. oralis	1	Japan
Fusobacterium fusiforme (nucleatum)	3	Japan
F. plauti	2	Japan
F. abscendens	1	Japan
Eubacterium lentum	9	Scotland and India
E. tortuosum	4	Japan
E. limosum	4	Japan
E. ventriosum	1	Japan
E. contortum	2	Japan
Propionibacterium acnes	11	Scotland, USA, India
P. avidum	9	England, USA, India and Japan
P. jensenii	5	India and Japan
P. freundreichii	3	USA and Japan
P. theonii	1	Japan
Lactobacillus catenaforme	4	Japan and Uganda
L. crispatus	1	Japan

DISCUSSION

The complete characterisation of the bacterial flora of the intestine is not yet possible. Many previous studies of the faecal flora have concentrated upon the aerobic component of the flora. While the numerical dominance of the non-sporing anaerobes in faeces is well established (Eggerth and Gagnon, 1933) little is known about the factors controlling them.

In the present study several unexpected species of anaerobe were isolated. *Bact. hypermegas* is common in the intestine of poultry, and *Bact. ruminicola* in the bovine rumen. *Bact. trichoides* is common in the intestine of pigs. *Bact. oralis* is frequently isolated from the human mouth and is similar to *Bact. fragilis*. *Fusobacterium fusiforme* is also found in the mouth, and organisms of this species that we recovered from faeces were probably passed down the intestinal tract with food. All the species of eubacteria isolated are common intestinal organisms (Holdeman and Moore, 1972). It is possible that *Propionibacterium acnes* and *P. avidum* were contaminants derived from the skin of the perianal area, skin being the more common habitat of these organisms. *P. jensenii*, *P. freundreichii* and *P. theonii* have not in the past been thought of as predominantly intestinal organisms, more usually being isolated from dairy products. Their isolation from faeces is interesting; some were isolated in the present study from persons whose diet would not have included much dairy produce. It is probable that the strictly anaerobic

Lactobacillus catenaforme is truly a human intestinal organism. *L. crispatus* is common in the gastro-intestinal tract of cows and pigs.

Studies by Moore and Holdeman (1972) with 1057 isolates from six faecal specimens have demonstrated the presence of 86 species of anaerobe and undoubtedly many more would be found if a greater number of isolates could be characterised. Our main interest in the present study was in the comparison of dietary groups rather than the exhaustive study of individuals. The study demonstrates the predominance of eubacteria in people on a carbohydrate-rich diet. However, the groups studied differ in many respects other than diet, so to assign a controlling role to this factor is probably not justified. Indeed,

TABLE VII

An analysis of the predominant bacterial groups in faeces sampled from subjects on different diets in Japan and the USA

Number of individuals	Diet*	Country of origin	Percentage occurrence of organisms of the stated group as the predominant component of the faecal flora			
			Bacteroides spp.	Bifido-bacteria	Eubacteria	Mixed
21	" High carbohydrate "	Japan	38	19	9·5	33·5
24	" Mixed western "	USA	46	21	...	33

* See table I.

consideration of the data from individuals in this study indicates that the difference between the groups might be accounted for on the basis of the different dominant organisms in various individuals (table VII). This contention is supported by the finding of Moore *et al.* (1969) that the flora was very little influenced by dietary manipulation and the dominant bacterial species in the intestine of an individual tended to remain constant for long periods. The mechanisms by which a particular bacterial species is selected for dominance in an individual are unknown, but presumably host factors are very important and diet may act indirectly through its influence on the host rather than directly on the flora.

SUMMARY

Non-sporing strictly anaerobic bacteria were isolated from human faecal samples from England, Scotland, USA, India, Uganda and Japan. Strains were assigned to genera on the basis of the Gram reaction and on the type of fatty acid produced from glucose. Analysis of these acids was by gas-liquid chromatography. The isolates were further classified on the basis of various biochemical reactions.

Bacteroides fragilis was the non-sporing anaerobe most frequently isolated from human faeces. *Bifidobacterium adolescentis* and *Eubacterium aerofaciens*

were also frequently isolated. Stool samples from India, Uganda and Japan contained a larger proportion of eubacteria and fewer *Bacteroides* spp. than did the samples from England, Scotland and the USA.

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The isolation of anaerobic bacteria from wound swabs

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The isolation of anaerobic bacteria from wound swabs

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SYNOPSIS The isolation of anaerobic bacteria from routine wound swabs by three procedures was evaluated.

Recovery of anaerobic organisms was doubled by immediate incubation of seeded plates, and the recovery could be further dramatically improved by the use of prereduced media, in conjunction with an anaerobic chamber.

Recommendations for the treatment of swabs and cultures for anaerobic investigation are made.

As long ago as 1898, Veillon and Zuber noted that anaerobic fusiform bacilli were the commonest organisms associated with acute appendicitis but little attention was paid to these organisms, and since there was no reliable method of cultivating them, their significance in clinical infections was largely ignored.

With the advent of the modern anaerobic jar (McIntosh and Fildes, 1916; Brewer, 1939) and subsequent improvements (Khairat, 1964), more anaerobes have been recognized from clinical specimens. Using anaerobic jar systems, Stokes (1958) demonstrated the isolation of 496 anaerobes from 4737 positive cultures in her clinical laboratory over several years, and Gillespie and Guy (1956) isolated 117 strains of anaerobic bacteria from 111 clinical specimens.

Hungate (1950) studying oxygen-sensitive rumen bacteria devised a technique based on the production of oxygen-free culture media. Moore (1966) extended this method of production and the use of prereduced anaerobically sterilized media. McMinn and Crawford (1970) found prereduced media superior to routine laboratory methods for the isolation of anaerobes from clinical specimens.

Several workers have used prereduced media in anaerobic chambers for the study of normal flora (Spears and Freter, 1967; Drasar, 1967; Arank, Syed, Kenney, and Freter, 1969).

More recent work has concentrated on the conditions of transport of clinical specimens to the laboratory for anaerobic investigation (Attebery and Finegold, 1969). Specialized media for the isolation of anaerobes on the bench have also been devised (eg, Watt, 1972).

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In the present investigation, the isolation of anaerobes from routine wound swabs on simple solid media was examined by three methods in parallel: (1) the use of prereduced media in an anaerobic chamber; (2) anaerobic jar techniques specially performed; and (3) routine clinical laboratory techniques.

Materials and Methods

SOURCE OF SWABS

Swabs with plentiful material were selected from routine wound swabs sent for examination to the clinical laboratories of St Mary's Hospital. They had travelled from the wards by the hospital's usual collection and transport system.

BACTERIOLOGICAL PROCEDURES

Bench procedures

The swabs were seeded onto blood agar plates (Oxoid Ltd, London, SE1) which had been stored aerobically at 4°C. In each case a seeded plate was immediately placed in a standard anaerobe jar (Baird & Tatlock Ltd, Chadwell Heath, Essex), which was fitted with a palladium catalyst. The jar was evacuated, filled with hydrogen gas, and incubated at 37°C. Gas-Pak anaerobic indicator (Becton, Dickinson, UK Ltd, Wembley, Middlesex) was included in the jar. A second seeded plate was left on the bench until a suitable number of plates for incubation in an anaerobic jar had accumulated. This was the usual practice in the clinical laboratories of St Mary's Hospital. A palladium catalyst

and Gas-Pak anaerobic indicator were included in the jar, which was evacuated, filled with hydrogen gas, and incubated at 37°C. The plates were examined after 24 and 48 hours. A third plate was seeded and incubated aerobically at 37°C.

Cabinet procedures

A flexible polyvinyl anaerobic chamber, similar to that described by Arank *et al* (1969), was employed. It was fitted with a stainless steel air lock and filled with a 90% nitrogen, 10% hydrogen gas mixture. The atmosphere was circulated by a fan over granules of palladium D catalyst (Engelhard Industries, Cinderford, Glos). The catalyst was changed weekly and was reactivated by heating at 180°C for one hour. A tray of silica gel was included in the chamber to absorb any moisture produced by the action of the catalyst.

Blood agar plates (Oxoid Ltd) were stored for three days in the chamber before use; the blood became darkened indicating reduction of the media. Swabs were introduced into the chamber via the air lock and were seeded onto the stored blood-agar plates. An electrically sterilized inoculating loop (Trotman and Drasar, 1968) was used to spread the inocula on the plates. The plates were placed in an anaerobe jar inside the chamber and the lid was screwed down. On removal from the chamber, the jar was evacuated and filled with a gas mixture containing 10% CO_2 and 90% H_2. Fresh palladium catalyst was included in all the jars. The plates were incubated at 37°C and examined on the bench after 24 and 48 hours.

Isolation of anaerobes

Colonies of suspected anaerobes were picked into Robertson's cooked meat medium (Southern Group Laboratories) and incubated at 37°C for three days. The cultures were examined for purity and for aerobic and anaerobic growth on blood-agar medium.

Identification of anaerobes

The isolates were assigned to genera on the basis of their appearance in a Gram-stained preparation (Preston and Morrell, 1962), and on the end products of glucose fermentation as detected by gas-liquid chromatography. Species were assigned on the basis of biochemical tests (Holdeman and Moore, 1972). Inhibition of lecithinase by specific antitoxin on egg yolk medium was used as a confirmatory test for the detection of *Clostridium welchii* (Nagler, 1939).

Gas-liquid chromatography

A 4-ml portion of a three-day culture in 1% glucose broth (Holdeman and Moore, 1972) was acidified with 0·1 ml of concentrated sulphuric acid (final pH < 2). The acidified cultures were placed in the water bath of a Perkin-Elmer F.40, multifract automatic gas chromatograph for head-space analysis. The temperature of the water-bath was 95°C. The gas chromatograph was fitted with a flame-ionization detector and a 2m stainless steel column packed with Diatomite C which had been treated with phosphoric acid and impregnated with 3% polyethylene glycol. The operating temperature was 125°C and the carrier gas was oxygen-free nitrogen passing through at 30 ml/min.

Results

SELECTION OF SWABS FOR COMPARISON

Of 97 swabs cultured by the three different procedures, 21 of those processed in the anaerobic chamber, 24 of those examined on the bench with immediate incubation, and 26 of those incubated after delay yielded no bacterial growth. Within those three groups, overgrowth by spreading organisms made analysis of growth impossible in 12, six, and four cases respectively. The number of swabs remaining for comparison in each group is shown in table I. All swabs that yielded anaerobes by one or more methods are included.

	Cabinet	*Bench*	
		Immediate Incubation	*Delayed Incubation*
Total no. of swabs examined	97	97	97
No. recorded as no bacterial growth	21	24	26
No. overgrown	12	6	4
Total remaining	64	67	67
No. of patients	50	49	48

Table I *Selection of swabs for comparison*

COMPARATIVE RECOVERY OF ANAEROBIC ORGANISMS

Anaerobes were isolated from 42·5% of the swabs processed in the anaerobic chamber, from 13·5% of those processed on the bench and immediately incubated, and from 4·5% of the swabs processed routinely (table II). For all the methods used, bacteroides organisms were the most common isolates. Anaerobic cocci and clostridia were also isolated (table III). The recovery rates for all anaerobic organisms, including the spore-forming clostridia, were improved by the use of the anaerobic chamber with prereduced media; the recovery of bacteroides was most noticeably improved. There was a less dramatic improvement with the rapid bench procedure.

	Cabinet	*Bench*	
		Immediate Incubation	*Delayed Incubation*
Number of swabs	64	67	67
Swabs (%) yielding isolates of anaerobes	42·5	13·5	4·5

Table II *Recovery of anaerobes from wound swabs*

Organism	*Cabinet*	*Bench*	
		Immediate Incubation	*Delayed Incubation*
Bacteroides species	22	5	2
Anaerobic cocci	7	3	1
Clostridia	5	1	1
Total anaerobes	34	9	4
No. of patients from whom anaerobes isolated	23	9	3

Table III *Numbers of isolates of various species*

DISTRIBUTION OF ANAEROBES ISOLATED BY CABINET PROCEDURES

The 64 wound swabs that were processed in the anaerobic chamber included 33 swabs from abdominal and ischio-rectal sources, 13 of which yielded facultative organisms only and 20 of which yielded anaerobic organisms (table IV). In five cases only anaerobes were isolated. A single species of anaerobe was recovered in pure culture from three swabs and multiple species were isolated from two swabs. A single anaerobe and one or more facultative organisms were recovered from four others. Thirty-one wound swabs from other sites were also examined: these included burns, abscesses, and swabs from respiratory and renal operation wounds. Twenty-four of these yielded facultatives only. Anaerobes were isolated from only seven, which included two swabs from which a single anaerobe was isolated in pure culture. Single strains of anaerobic bacteria in mixed culture with facultative organisms were recovered from four swabs, and one swab yielded facultative organisms with two species of anaerobe.

NATURE OF ANAEROBIC ISOLATES

Of the organisms isolated in the anaerobic chamber, 65% of isolates from abdominal swabs and 62·5% of isolates from other sources were bacteroides (table V). Fewer anaerobic cocci (19%) were isolated from abdominal sources than from other sources (25%). Fifteen per cent of the abdominal isolates were clostridia, as were 12·5% of isolates from other sources.

The majority of the bacteroides isolated were identified as *Bacteroides fragilis* (table VI). *Bacteroides melaninogenicus* was isolated twice, in both instances from swabs of small bowel operation wounds. The anaerobic cocci isolates were all Gram-positive organisms of the genera *Peptococcus* and *Peptostreptococcus*. The peptococci were *Peptococcus asaccharotyticus* and *Peptococcus magnus*. The anaerobic streptococci were identified as *Peptostreptococcus intermedius*. The clostridia were all *Clostridium welchii*.

	Abdominal and Rectal Source	*Other Sources*
No. of swabs with anaerobes	20	7
No. of anaerobes isolated	26	8
% Bacteroides	65	62·5
% Anaerobic cocci	19	25
% Clostridia	15	12·5

Table V *Anaerobes isolated from wound swabs on prereduced media*

Organism	*No. of Isolates*
Bacteroides fragilis	20
Bacteroides melaninogenicus	2
Peptococcus asaccharolyticus	2
Peptococcus magnus	2
Peptostreptococcus intermedius	2
Clostridium welchii	5
Total	34

Table VI *Identity of anaerobic isolates*

Organism	*Type of Specimen (Source of Wound Swab)*						
	Total Abdominal and Rectal	*Other*					
		Total	*Skin/Bone*	*Superficial Abscesses*	*Respiratory*	*Muscle*	*Renal*
Facultatives only	13	24	10	7	4	2	1
Anaerobes	20	7	4	1	1	—	1
Anaerobes only	5	2	1	1	—	—	—
One species	3	2	1	1	—	—	—
Two species	2	—	—	—	—	—	—
Anaerobes and facultatives	15	5	3	—	1	—	1
One species	11	4	3	—	—	—	1
Two species	4	1	—	—	1	—	—

Table IV *Distribution of anaerobes in 64 wound swabs*[1]

[1]Figures represent actual numbers of specimens.

Discussion

The clinical significance of anaerobes in wound infections cannot be assessed from the data obtained in this study. No patients were followed up and there was no assurance that the swabs were taken in a way that avoided contamination with normal flora. Indeed the high rate of recovery of anaerobes from abdominal and rectal sources might be accounted for by such contamination. This seems less likely in the seven cases where only anaerobes and no facultative organisms were recovered. It is interesting to note here that anaerobes were isolated more frequently together with facultative organisms than on their own. Macdonald, Gibbons, and Socransky (1960) and Moore and Gross (1968) have shown cases where anaerobic bacteria are pathogenic only where they exist in a synergistic situation with other bacteria.

The results of the present study indicate that immediate incubation of plates seeded on the bench is preferable to methods which involve delayed anaerobic incubation of seeded plates. No direct comparison can be made between the results obtained by cabinet and bench procedures since the CO_2 content of the anaerobic jars differed in these two situations. However, the results of a similar study by Vargo, Michaelson, Spaudling, Vitagliano, Swanson, and Forsch (1971) in which CO_2 was included in all anaerobic jars, were comparable with those obtained in this study (table VII). Lewis, Bedell, and Rettger (1940) and Watt (1973), working with pure cultures, have shown that adding CO_2 increases both numbers and colony size of organisms incubated in anaerobic jars. The inclusion of CO_2 in the gas mixture in anaerobic jars is to be recommended, although its effect on the recovery of anaerobes in the clinical situation may not be as dramatic as with pure cultures. Even with CO_2 in the anaerobic jar, efficient isolation of anaerobes from routine clinical specimens can only be achieved with the use of prereduced media.

McMinn and Crawford (1972) found that prereduced liquid media were superior to solid media incubated in anaerobic jars for the isolation of anaerobes from clinical specimens known to contain anaerobes.

In contrast, Rosenblatt, Fallon, and Finegold (1973) showed that anaerobes could be isolated from clinical specimens just as effectively on the bench with anaerobic jar procedures as on prereduced solid media in an anaerobic chamber (table VII). However, in the latter study, the specimens were specially collected under anaerobic conditions, and speedily transported to the laboratory. Similarly, Dowell (1972), using prereduced solid media in an anaerobic chamber, found no improvement in the recovery of anaerobes from clinical specimens which had been submitted under anaerobic conditions.

In the present study the clinical specimens had been through the normal hospital transport system, and in many cases, several hours had elapsed between taking and cultivating the swabs. Throughout transportation the swabs were in an aerobic atmosphere. It is probable that many more organisms would have been recorded had the specimens been speedily submitted under anaerobic conditions, and it is hoped that, in future, efforts will be made to transport clinical specimens in anaerobic conditions.

It is encouraging that anaerobic organisms were isolated without employing specialized complicated media. Ordinary blood agar was used for all three procedures in this study.

Especially where it is not possible for specimens to be collected and transported anaerobically, methods are required for storing media in a reduced state and of keeping seeded plates in an anaerobic environment. To try to ensure that every culture for anaerobic incubation is put into an anaerobic jar immediately it is seeded would require extra technician time and a great many anaerobic jars. It is appreciated that many people feel that the anaerobic

No. of Specimens	*McMinn and Crawford (1970)*		*Rosenblatt* et al *(1973)*			*Vargo* et al *(1971)*		*This Study*	
	38 Specimens		*23 Specimens*			*145 Specimens*		*64 Specimens*	
	Bench	*Prereduced Anaerobically Sterilized*	*Bench*	*Cabinet*	*Prereduced Anaerobically Sterilized*	*Bench*	*Cabinet*	*Bench*	*Cabinet*
Anaerobic transportation	−	−	+	+	+	−	+	−	−
$+CO^2$	+	+	+	+	+	+	+	−	+
% Positive	8·2	100	100	100	100	18	41	13·5	42·5

Table VII *Isolation of anaerobes from clinical specimens under various conditions*

chamber and air lock system, although a useful tool for research, is too cumbersome for the ordinary clinical laboratory. However, the increased rate of isolation of anaerobes should outweigh any disadvantages. Martin (1971) has described a cupboard and jar both capable of being flushed with oxygen-free gas, and this type of apparatus may also prove useful in the routine laboratory. Prereduced, anaerobically sterilized media are now being marketed commercially in the United States, and if this service is extended to Britain it would be a further possibility for routine use.

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Antibiotic-disc tests for rapid identification of non-sporing anaerobes

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Antibiotic-disc tests for rapid identification of non-sporing anaerobes

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SYNOPSIS The sensitivity patterns to a number of antibiotic-impregnated discs of 126 strains of Gram-negative anaerobes and 36 strains of Gram-positive non-sporing anaerobes from various sources have been examined. The sensitivity patterns shown by *Bacteroides fragilis* and *Bacteroides melaninogenicus* were useful for the rapid identification of these organisms. Consistent and useful sensitivity patterns were not shown by the Gram-positive anaerobes.

Improvements in techniques for the isolation and cultivation of anaerobic organisms have led to an increase in the frequency of isolation of non-sporing anaerobes from clinical specimens (Moore, Cato, and Holdeman, 1969; McMinn and Crawford, 1970). These bacteria are also found in very large numbers in the human gastrointestinal tract (Sanborn, 1931; Haenel, 1970; Moore and Holdeman, 1974).

Identification of these organisms based on morphological and biochemical characteristics is tedious and time consuming; however, Finegold, Harada, and Miller (1967) have demonstrated the use of antibacterial susceptibility patterns as aids to the characterization of anaerobic Gram-negative bacilli, and Sutter and Finegold (1971) simplified and improved this method with the use of discs impregnated with high concentrations of antibiotics for rapid presumptive identification.

In this paper are presented the results obtained from the examination of a collection of non-sporing anaerobes using the special antibiotic-impregnated discs suggested by Sutter and Finegold (1971) as a guide to identification. It should be noted that the concentrations of antibiotics used for identification were in most cases too high for the results to be an indication of therapy.

Materials and Methods

Most of the bacteria in this study were isolated during a large survey of the faecal flora of people consuming different diets in various parts of the world (Peach, Fernandez, Johnson, and Drasar, 1974). The wound isolates came from routine wound swabs sent to the clinical laboratories of St Mary's Hospital, London (Peach and Hayek, 1974). A strain of *Bacteroides fragilis* (NCTC 9343) was obtained from the National Collection of Type Cultures, Colindale, London. Strains were identified

Species	*Source*	*No. of Strains*
B. fragilis	Faeces:	
	USA	39
	India	11
	Japan	42
	Uganda	6
	Scotland	14
	Hong Kong	3
	National Collection	
	Type Cultures 9343	1
	Wound infections	7
B. melaninogenicus	Faeces:	
	USA	3
Bifid. adolescentis	Faeces:	
	USA	11
	India	4
	Japan	1
	Uganda	1
	Scotland	1
	Hong Kong	1
Bifid. bifidum	Faeces:	
	USA	3
	India	1
	Japan	3
	Scotland	3
	Hong Kong	2
Eubacterium aerofaciens	Faeces:	
	USA	3
	India	1
	Japan	1

Table I *Non-sporing anaerobes: source of strain*

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by the morphological and biochemical tests described by Holdeman and Moore (1972).

One hundred and twenty three strains of *B. fragilis* and three of *B. melaninogenicus* were examined (table I). Nineteen strains of *Bifidobacterium adolescentis*, 12 strains of *Bifid. bifidum*, and five strains of *Eubacterium aerofaciens* were also examined.

CONDITIONS OF CULTURE

Test strains were maintained by subculture in Robertson's cooked meat medium (Southern Group Laboratories Ltd). The culture medium for the sensitivity tests was Reinforced Clostridial Agar (Oxoid) containing 1% glucose, 1% liver digest (Panmede, Paines and Byrne, Greenford, England), and 10% defibrinated horse blood. One or two drops of a three-day culture in Robertson's cooked meat medium were spread with a bent glass rod over the surface of the culture plates. Antibiotic discs, no more than four per plate, were then applied. The plates were incubated for three days at 37°C in a Baird and Tatlock anaerobe jar filled with a gas mixture containing 10% CO_2 and 90% hydrogen. Fresh Palladium D Catalyst (Englehard Industries, Cinderford, Glos.) was included in all the jars.

ANTIBIOTIC DISCS

Antibiotic discs were obtained from Mast Laboratories, Liverpool. The discs used were: colistin 10 μg, erythromycin 60 μg, kanamycin 1000 μg, neomycin 1000 μg, penicillin 2 u (1·2 μg), rifampicin 15 μg, and vancomycin 5 μg.

Results

Strains were designated 'resistant' to an antibiotic when there was no zone of inhibition around the disc. Strains of 'intermediate' resistance had zone diameters less than 10 mm and 'susceptible' strains had zone diameters greater than 10 mm. The discs were 6 mm in diameter. There were no outstanding differences in the sensitivity patterns of strains of the same species that had been isolated from faeces of varying geographical origins.

	Sutter and Finegold (1971) (100 strains)	*This study (123 strains)*
Colistin	100 μg	
	R 100	94
	S —	2·5
	Intermediate —	3·5
Erythromycin	60 μg	
	R —	1
	S 100	98
	Intermediate —	1
Kanamycin	1000 μg	
	R 100	96
	S —	1
	Intermediate —	3
Neomycin	1000 μg	
	R 78	83
	S 20	15
	Intermediate 2	2
Penicillin	2 units (1·2 μg)	
	R 93	78
	S 7	19
	Intermediate —	3
Rifampicin	15 μg	
	R 100	97·5
	S —	2·5
	Intermediate —	—

Table II *Susceptibility patterns of* B. fragilis

Figures expressed as percentages; disc diameter 6 mm; R = no zone of inhibition; S = diameter of inhibition zone > 10 mm; Intermediate = diameter of inhibition zone < 10 mm.

BACTEROIDES FRAGILIS

The antibiotic susceptibility patterns obtained for *B. fragilis* were comparable with those obtained by Sutter and Finegold (1971) and are shown in table II.

All strains of *B. fragilis* tested were found to be resistant to a 5 μg disc of vancomycin.

BACTEROIDES MELANINOGENICUS

Only three strains of *B. melaninogenicus* were tested and these differed in sensitivity to colistin and neomycin from 12 strains tested by Sutter and Finegold (1971). All three strains were resistant to a 5 μg disc of vancomycin.

Antibiotic		Bifid. adolescentis (*19 strains*) *Percentage*		Bifid. bifidum (*12 strains*) *Percentage*		
		Resistant	*Sensitive*	*Resistant*	*Sensitive*	*Intermediate*
Colistin	10 μg	95	5	92	8	—
Erythromycin	60 μg	—	100	—	100	—
Kanamycin	1000 μg	32	68	17	75	8
Neomycin	1000 μg	16	84	17	75	8
Penicillin	2 u (1·2 μg)	10	90	—	100	—
Rifampicin	15 μg	—	100	—	100	—

Table III *Susceptibility patterns*

BIFIDOBACTERIA

The antibiotic susceptibility patterns of 19 strains of *Bifid. adolescentis* and 12 strains of *Bifid. bifidum* were similar and are summarized in table III. These organisms were in general much less resistant to the test antibiotics than were the Gram-negative anaerobic bacteria. In addition, all strains tested were sensitive to a 5 μg disc of vancomycin.

EUBACTERIA

Five strains of *Eubacterium aerofaciens* were tested and no clear pattern of antibiotic sensitivity emerged (table IV). There was a marked variation between strains. However, all strains were sensitive to the 5 μg disc of vancomycin.

Strain		*Individual Sensitivities*				
		A	*B*	*C*	*D*	*E*
Colistin	100 μg	R	S	R	S	R
Erythromycin	60 μg	S	S	S	R	S
Kanamycin	1000 μg	S	S	S	S	R
Neomycin	1000 μg	S	S	S	S	R
Penicillin	2 u (1·2 μg)	R	S	S	S	S
Rifampicin	15 μg	S	S	S	R	S

Table IV *Antibiotic susceptibility patterns of five strains of* Eubacterium aerofaciens

R = no zone of inhibition; S = diameter of inhibition zone > 10 mm; Intermediate = diameter of inhibition zone < 10 mm.

Discussion

The need for a rapid method for the presumptive identification of non-sporing anaerobic bacteria, especially those isolated from clinical material, is becoming increasingly important. Gram-negative anaerobic bacteria, in particular *B. fragilis* and *B. melaninogenicus*, are frequently isolated from clinical specimens (Stokes, 1958; Moore *et al*, 1969) and a means of identification during primary isolation would be advantageous.

The antibacterial susceptibility patterns of strains of *B. fragilis* tested during this study were similar to those obtained by Sutter and Finegold (1971). However, fewer isolates were found to be resistant to a disc containing 2 units (1·2 μg) of penicillin. Strains used in the present study had been recently isolated from the intestine. Those tested by Sutter and Finegold included stock strains and clinical isolates. Fievez (1963) noted that the Gram-negative anaerobe *Sphaerophorus necrophorus* was more sensitive to penicillin on primary isolation than after several subcultures. It may be that a similar phenomenon occurs with *Bacteroides*. Most of the clinical isolates tested in the present study were resistant to 2 units of penicillin.

Only three strains of *B. melaninogenicus* were available for study and their sensitivity to colistin and neomycin differed from that of the 12 strains studied by Sutter and Finegold. The most striking difference was sensitivity to neomycin. All the strains of *B. melaninogenicus* studied by Sutter and Finegold were sensitive to 100 μg neomycin; in contrast, the three strains in the present study were resistant to this concentration. Garrod (1955), in a plate dilution study, found *B. melaninogenicus* was resistant to streptomycin. In this study and in the study by Sutter and Finegold *B. melaninogenicus* was found to be resistant to kanamycin. Martin, Gardner, and Washington (1972), using an agar dilution technique, found *B. melaninogenicus* to be sensitive to rifampicin and erythromycin; most strains were sensitive to penicillin G and resistant to kanamycin. These trends are also shown in the disc sensitivity tests of Sutter and Finegold and in the present investigation.

Susceptibility to a 5 μg disc of vancomycin as recommended by Sutter, Attebery, Rosenblatt, Bricknell, and Finegold (1972) proved to be a useful 'back-up' for a Gram-reaction. All the Gram-negative anaerobes in this study were resistant to such a disc. All the Gram-positive organisms were sensitive.

There is no solid evidence for pathogenicity of the Gram-positive non-sporing anaerobic bacteria, although they are often isolated from clinical material, in mixed culture with facultative organisms or with Gram-negative anaerobes (Smith and Holdeman, 1968; Moore *et al*, 1969). However, these bacteria also form a major part of the intestinal microflora of man (Peach *et al*, 1974). There were few differences in antibiotic sensitivity between the two species of bifidobacteria tested in the present study. Both *Bifid. adolescentis* and *Bifid. bifidum* were sensitive to the discs of penicillin and erythromycin. More strains of *Bifid. adolescentis* were resistant to the kanamycin disc than were the strains of *Bifid. bifidum*. These results are similar to those of a plate dilution sensitivity test reported by Miller and Finegold (1967). Miller and Finegold found that *Bifid. adolescentis* was more sensitive to neomycin than *Bifid. bifidum*. Lavergne, Burdin, Schmitt, and Manciaux (1959) found the sensitivity of *Bifid. bifidum* to erythromycin and neomycin to be similar to that found by Miller and Finegold.

Courtieu, Monnier, de Lajudie, and Guillermet (1960) reported a single strain of bifidobacteria to be sensitive to colistin. However, Miller and Finegold (1967) found bifidobacteria to be resistant to the related antibiotic polymyxin B and in the present study most strains of *Bifid. adolescentis* and *Bifid. bifidum* were resistant to colistin.

Eubacterium aerofaciens was more sensitive to the test antibiotics than were the bifidobacteria. Martin *et al* (1972), using an agar dilution technique, found that, in general, bifidobacteria (unidentified species) were more sensitive than eubacteria to erythromycin, penicillin, and rifampicin. In the present study, using antibiotic discs, eubacteria and bifidobacteria showed similar sensitivity patterns to these antibiotics.

The results of the present study indicate that antibiotic disc susceptibility patterns as proposed by Sutter and Finegold (1971) provide a reliable method for the rapid presumptive identification of *B. fragilis*. Identification of *B. melaninogenicus* can also be achieved provided the system is backed up by other simple tests, such as those recommended by Sutter and Finegold (1971). This method would be useful for the initial presumptive identification of these two species of anaerobe, particularly those isolated from clinical specimens. However, identification on an initial isolation plate is not achieved and the time taken to isolate and purify a culture is not eliminated.

The sensitivity of an isolate to the high concentrations of antibiotics used in this study would give no indication of the sensitivity of this strain to concentrations of antibiotics obtainable *in vivo*. In this light a separate sensitivity plate would have to be prepared.

Using the same antibiotics recommended for the identification of Gram-negative anaerobes, no distinguishing pattern of antibiotic sensitivity suitable for identification of Gram-positive anaerobes emerged. It may be that different antibiotics, or different concentrations of antibiotic, are needed to provide a similar rapid preliminary identification of these organisms, and an investigation in this direction is desirable.

As with all tests for antibiotic susceptibility the possibility of acquired resistance, even to the high concentrations of antibiotics used in this study, should be remembered in interpretations of results.

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