Studies in bacterial anaerobiosis: the recovery of clinically important anaerobes on solid media

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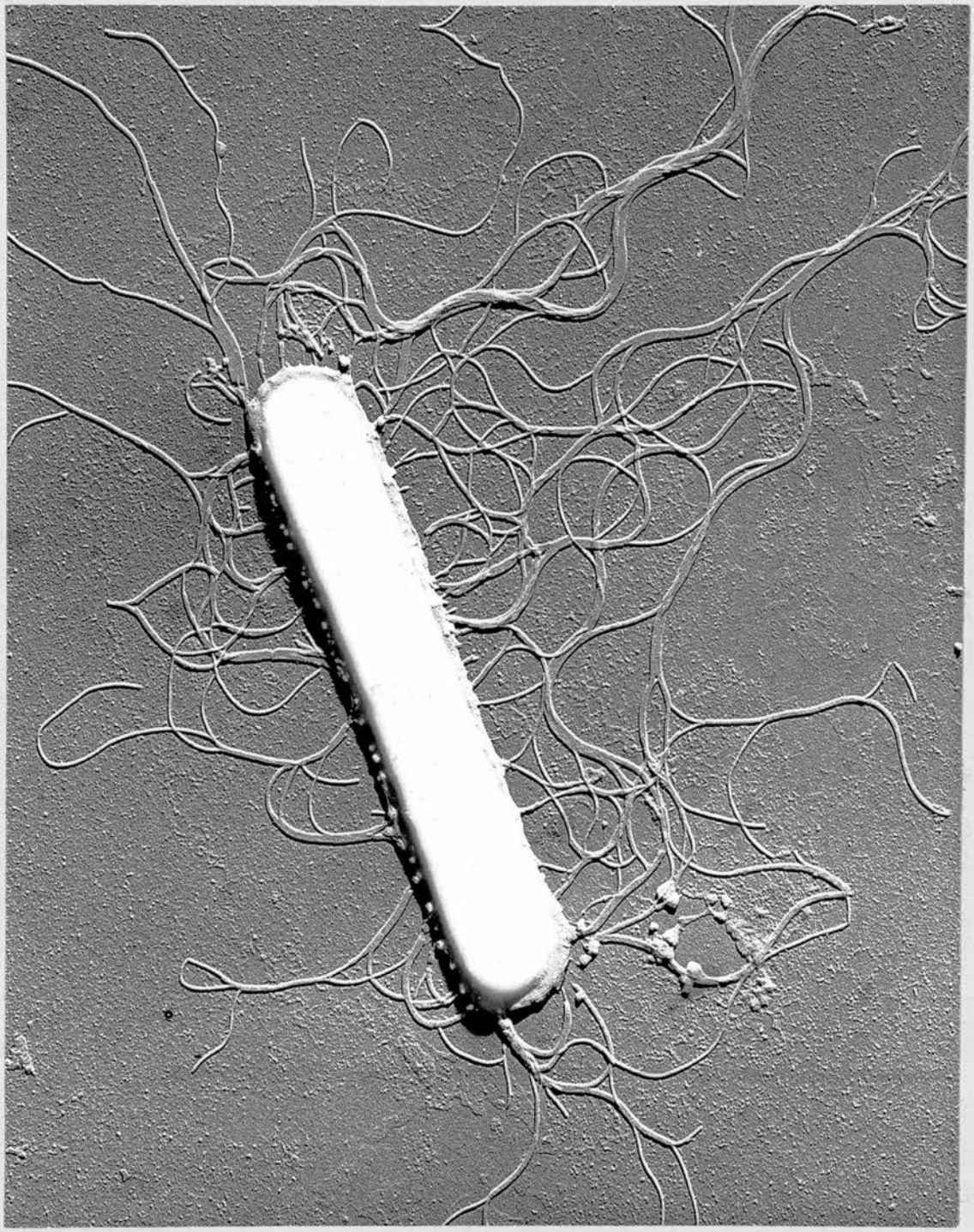

Electron micrograph of *Clostridium tetani*, with numerous peritrichous flagella. Gold-palladium shadowed preparation. x 20,000.

PREFACE

When the literature on bacterial anaerobiosis is reviewed, and the many theories on anaerobiosis are critically assessed, it appears that no single unitarian hypothesis can be developed and that the basis of anaerobiosis may vary in different organisms.

In the course of the present investigation of problems associated with the recovery of anaerobes on solid media, it was necessary to develop a suitably demanding model. The choice of Clostridium oedematiens of types B and D required, inter alia, critical reappraisal of anaerobic technique and the development of a standardised anaerobic procedure. Only when special supplements were used in conjunction with blood agar media was it possible to obtain consistently reliable surface growth of these organisms. An enumeration procedure was then developed to allow the reliable determination of surface viable counts . Then it was possible to assess the relative importance of the spore and vegetative cell components of the inocula when demanding types of Cl. oedematiens were subcultured from fluid to solid media. These studies led to the formulation of the concept of the "significantly viable particle" and its extension to other sporing anaerobes of more direct clinical importance. It became clear that vegetative cells can be the major component of the viable inoculum on solid media and the importance of these findings for the clinical bacteriologist became evident.

Similar studies with non-sporing anaerobes showed that they too can be quantitatively recovered on solid media from fluid cultures with the standard anaerobic procedure. The recovery from faecal

samples similarly processed was also investigated, and the surface viable counts obtained with conventional methods compared favourably with those of other workers who used more elaborate techniques.

It was then worthwhile to investigate the role of possible enhancing or inhibiting factors that may affect the recovery of exacting anaerobes on solid media. The use of the supplements that dramatically improved recovery of Cl. oedematiens did not enhance the growth of other anaerobes studied. Other possible enhancing factors were sought for anaerobes such as Cl. tetani and a synthetic defined solid medium developed in the course of the work may well prove useful in future studies.

The concept of "unintentional selection" by heat or selective media is introduced, and the importance of aeration during dilution, exposure of seeded plates to aerobic conditions and omission of carbon dioxide from the incubation environment in such selection was evaluated. Carbon dioxide profoundly affected the growth of several sporing and non-sporing anaerobes; these findings are assessed in relation to the increasing role of non-sporing anaerobes in the causation of infections in man. Variations in anaerobic procedure were now defined and their effects on recovery of anaerobes on solid media were assessed. The Gaspak system appears to offer a simple standardised method of achieving anaerobic conditions; it was evaluated in relation to the requirements of the diagnostic laboratory, with a range of clinically important anaerobes: in general, equally good recovery of anaerobes was obtained with the Gaspak system as with the standard anaerobic procedure.

The BTL jar was extensively used in the present studies.

Variations were noted in colony yields on plates seeded with identical inocula and incubated in different jars processed in a standard manner. This "jar variation" appeared to occur almost exclusively with Cl. oedematiens strains, but experimental design was standardised to take account of possible variables with other models, so that statistically valid data could be obtained. The standard anaerobic procedure was re-formulated to take account of the results of the present study, and to minimise errors due to variations in technique.

It appeared that vegetative cells from demanding anaerobes and from faecal samples can be quantitatively recovered on solid media, and this suggested that the use of an anaerobic cabinet would not significantly affect their recovery. A relatively simple anaerobic cabinet was developed, and although the atmosphere within the cabinet was sufficiently anaerobic to allow growth of demanding anaerobes, recovery from faecal samples was not improved by manipulation within the cabinet under anaerobic conditions, as compared with that achieved under aerobic conditions at the bench. The counts obtained in the present study were equivalent to or higher than those obtained by other workers who used anaerobic cabinets.

The gratifying improvements in the recovery of demanding anaerobes achieved by techniques developed in the present study are discussed in relation to routine clinical bacteriology.

Acknowledgements

During the present studies, I have received valuable help from many of my colleagues. I am greatly indebted to Dr J. G. Collee, who initially stimulated my interest in this subject, for his enthusiastic support and helpful guidance. My thanks are also due to Mr R. Brown and his colleagues for their technical skill and assistance. I have received invaluable help at various stages from Miss Margaret V. Hoare, Dr N. R. Belton, and Dr J. R. W. Govan. Professor J. O. Forfar kindly allowed me to use the desk computer in his department. I am grateful to Professor B. P. Marmion for his encouragement and advice and my thanks are also due to Professor G. S. Boyd for his advice on biochemical problems and to Mrs. Elizabeth Welsh for her care and patience in typing the manuscript. For much help in the preparation and photography of the figures in this thesis, I am greatly indebted to the staff of the Medical Photography Unit. It is a pleasure to acknowledge the constant assistance and helpful advice of Miss Margaret Bell and her staff at the Central Medical Library, Edinburgh, during the preparation of this work. I am a member of a team engaged in research on clinically important anaerobes and we are grateful to the Medical Research Council for their financial support (Grant G971/113/B).

I am especially indebted to my wife, without whose constant support and patient help this thesis could not have been written.

The results of some of the work incorporated in this thesis have already been reported. The relevant reference is:

COLLEE, J. G., RUTTER, J. M., and WATT, B. 1971. The significantly viable particle: a study of the subculture of an exacting sporing anaerobe. J. Med. Microbiol., 4: 271-288.

It has been a privilege to be involved in these studies and to develop concepts with my colleagues. The individual experiments recorded in this thesis were personally designed and performed or supervised by the author.

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Experimental results

SUMMARY

(1) The literature concerning bacterial anaerobiosis is reviewed, with special reference to anaerobes of clinical interest. Theories on the nature of anaerobiosis are summarised and assessed, and the possible defects inherent in a unitarian hypothesis are discussed.

(2) A general consideration of the literature on the laboratory culture of anaerobes is followed by a detailed review of (i) the use of reducing agents in culture media; (ii) cultural methods that involve the removal of oxygen; (iii) the development of the anaerobic jar; (iv) the development of oxygen-free gassing systems and anaerobic cabinets; (v) the recovery of clostridial spores; and (vi) the use of selective procedures. The lack of quantitative data on the growth and recovery of anaerobes on solid media is emphasised.

(3) The development of models of demanding anaerobes necessitated a review of the problems associated with the surface growth of Clostridium oedematiens of types B and D. The irregular growth of these organisms called for a re-appraisal of anaerobic technique, with the formulation of a standard anaerobic procedure.

(4) The enhancing effect of sterile iron-filings for Cl. oedematiens strains, previously observed in this laboratory, was confirmed by quantitative studies.

(5) The incorporation of a cysteine/dithiothreitol system into blood agar media gave consistently reliable surface growth of type-B and type-D strains of Cl. oedematiens.

(6) The development of a standardised counting procedure for exacting anaerobes allowed the rapid processing of large numbers of plates, with only minimum exposure to aerobic conditions.

(7) In a study of the subculture of Cl. oedematiens from fluid to solid media, it was shown that vegetative cells can be the predominantly viable component of an inoculum on solid media, especially in the case of young (5-hr) cultures.

(8) The concept of the "significantly viable particle"is introduced for demanding models, and extended to anaerobes of more direct clinical interest; vegetative cells of sporing anaerobes can be recovered on simple blood agar media by conventional anaerobic techniques at the bench. The need to determine the "significantly viable particles" that can be redeemed from clinical specimens is stressed.

(9) Studies with non-sporing anaerobes showed that quantitative recovery of these organisms can also be achieved with the standard anaerobic procedure.

(10) In an evaluation of the effects of possible stimulating factors on the recovery of anaerobes on simple media, it was found that the stimulatory effects of the iron-filings technique and the cysteine/dithiothreitol system for Cl. oedematiens strains are specific for that species;none of the other factors tested was shown to have an enhancing effect for any of the test organisms.

(11) A defined, synthetic solid medium is developed for Cl. tetani, and used to investigate possible enhancing factors for this model.

(12) The effects of a series of variable factors on the recovery of anaerobes on solid media are investigated quantitatively. Factors such as the use of selective procedures, and the exposure of organisms to aerobic conditions in diluents or on seeded plates, are shown to have an inhibitory effect. The use of thick plates, prolonged incubation, and the flushing of anaerobic jars before processing are shown to have an enhancing effect for certain models. The concept of "selection by intent", as opposed to "unintentional selection" is introduced.

(13) The addition of 10 per cent. carbon dioxide to the incubation environment is shown to produce a significant improvement in the recovery of many test anaerobes, especially non-sporing anaerobes. Its routine inclusion as a feature of anaerobic procedure is recommended.

(14) The Gaspak anaerobic system was evaluated with a range of test anaerobes. It performed well in comparative tests, and could only be surpassed when very exacting models were used. The Gaspak system was also evaluated in terms of the recovery of anaerobic organisms from faecal samples. Recovery was as good as that observed with the standard anaerobic procedure. The place of the Gaspak in diagnostic bacteriology is assessed in the light of these findings.

(15) Variations in recovery of Cl. oedematiens strains from plates incubated in BTL anaerobic jars were shown to be due to "jar variation". The nature of this phenomenon was investigated, and several possible contributory factors are noted. The investigation led to the development of improved design, recording and analysis

of experiments, so that statistically meaningful data can be obtained.

(16) The effect of the provision of multiple catalyst sachets in BTL jars was investigated and the recovery of Cl. oedematiens strains was shown to be significantly improved by such a procedure.

(17) The standard anaerobic procedure was re-formulated and improved in the light of the findings of the present study; the "revised anaerobic procedure" should lead to more consistent recovery of clinically important anaerobes on solid media.

(18) An anaerobic cabinet was constructed, and used in comparative studies of the recovery of test anaerobes and faecal samples on solid media. The use of a cabinet in which the gaseous environment was demonstrably anaerobic, did not improve the recovery of laboratory cultures of demanding anaerobes. Similarly, the recovery from faecal samples was equivalent to that obtained by standard methods at the bench and the viable counts obtained compare favourably with those obtained by workers sampling from a similar population.

(19) The present study has defined and evaluated several variable factors, chemical and physical, that affect the growth and recovery of anaerobes on solid media. These now merit further study with a view to the development of improved techniques and recovery media.

(20) The requirement for adequately controlled quantitative experiments is discussed, and the importance of statistically designed experiments to take account of possible variations is stressed.

(21) The present evidence indicates that quantitative recovery of anaerobes from laboratory cultures and from faecal samples can be obtained by routine anaerobic procedures; there is no evidence that the use of anaerobic cabinets is of value to the clinical bacteriologist. If this view is challenged, there is an obligation to define clinically important bacterial groups likely to occur in clinical specimens that can only be isolated by specialised procedures. It is submitted that the future development of this work should consider improvement of existing methods in addition to the further evaluation of specialised techniques.

INTRODUCTION

The clinical microbiologist is obliged to reach a definite diagnosis in a reasonable time. In the case of aerobic organisms, he is generally successful, but with some anaerobic organisms his present recovery techniques and media require further development. The recovery of anaerobes from clinical specimens is often regarded as an exacting exercise, and failures of growth on solid media are a common experience.

There is no simple answer to the question "What is an anaerobe?", as many different factors and several concepts seem to be involved. The clinical microbiologist must meanwhile apply present knowledge to everyday practical problems as intelligently and carefully as is consistent with his routine commitments. There is therefore a challenge for the research worker to link theoretical concepts with practical effectiveness in this field. It is against this background that the present state of knowledge of bacterial anaerobiosis is reviewed, and the development of anaerobic techniques is discussed, especially in relation to anaerobes of clinical interest.

The medically important anaerobes comprise a large group of organisms: Clostridium, the sporing anaerobes; the Gram-negative non-sporing anaerobes such as Veillonella and Bacteroides spp. (the author here includes the Fusiformis, Sphaerophorus and Dialister groups); other anaerobic cocci; anaerobic lactobacilli including bifidobacteria (Rogosa and Sharpe, 1959); and the anaerobic Spirochaetes and Actinomyces. Of these organisms, the sporing anaerobes and the non-sporing Gram-negative anaerobes are most important as recognised pathogens of man. The prominence of the Clostridia in this regard has

long been recognised; they are considered in detail in standard works of reference (e.g. Willis, 1969). The Gram-negative anaerobes on the other hand have come into prominence as human pathogens in recent years; they are dealt with relatively briefly in textbooks (e.g. Willis, 1964), yet their role in the causation of infections of man is being increasingly stressed (e.g. Spencer, 1971). Many of the organisms listed above are capable of causing infections in man, but in addition many may play an important part in the ecology of such habitats as the bowel (Haenel, 1961; Gorbach and Tabaqchali, 1969), and therefore justify inclusion as "medically important anaerobes".

All of the organisms mentioned above are commonly considered to be "anaerobes", but the term is loosely applied. It is therefore necessary to consider the basis of bacterial anaerobiosis in some detail.

Bacterial Anaerobiosis

It is not possible to give a description of bacterial anaerobiosis that is both brief and precise, and the difficulty involved in producing even a working definition of the term gives an indication of the complexities and confusions surrounding the subject.

It is convenient to consider bacterial anaerobiosis as being the ability of bacteria to maintain vital metabolic activity in biological systems under conditions of low oxidation-reduction potential. Bacteria are traditionally divided into (i) the obligate aerobes which require conditions of high-oxidation reduction potential - (redox potential or E_h) -: (ii) aerobes and facultative anaerobes which are organisms capable of growth within a fairly wide E_h range, and (iii) obligate anaerobes which can only grow under conditions of low E_h. Thus the E_h requirements for initiation of bacterial growth represent a fairly wide spectrum, with obligate aerobes and anaerobes at opposite extremes, and the aerobes and facultative anaerobes occupying a relatively wide band in the centre.

The present study is concerned with obligately anaerobic bacteria, usually briefly referred to as "the anaerobes". However this term, too, is open to several interpretations. Smith (1967) considered anaerobes as

" (i) Organisms growing better in the absence of air than in its presence.

(ii) Bacteria that perish even on transient contact with atmospheric oxygen.

(iii) Bacteria that are unable to initiate growth from small inocula unless the oxidation-reduction potential of the medium is low."

Of these three definitions, Smith suggests that (iii) is most

useful, as the other two are not applicable to all anaerobic organisms; he cites the example of a culture of Clostridium welchii in which, once growth has been initiated, growth will continue even if air is bubbled through the medium.

The literature concerning bacterial anaerobiosis is extensive and confusing, but theories on the nature of anaerobiosis seem to fall into three groups:

(1) Theories in which the roles of catalase and of hydrogen peroxide are important.

(2) Theories in which requirements of E_h are assumed to have the central and primary role.

(3) Theories in which the toxic effects of oxygen are considered to have the primary role.

The literature supporting or challenging each of these concepts will be considered in turn.

Theories in which requirements of oxidation-reduction potential (E_h) are assumed to have the central and primary role.

In 1929, Fildes investigated the E_h requirements for the germination and outgrowth of spores of Bacillus tetani (sic) in fluid media, using indicator dyes to measure the oxidation-reduction potential of the medium. He found that if the E_h of the medium was too positive (greater than 0.01 volt at pH 7.0), germination and subsequent outgrowth did not take place. In another paper (Fildes, 1929b), he showed that normally, germination and outgrowth of spores of B. tetani were inhibited in the subcutaneous tissues of the guinea-pig, but if the supply of oxygen to these tissues was interrupted, germination and outgrowth could take place because of the resultant fall in E_h. He suggested

that normally the E_h of the tissues was too positive to allow germination and outgrowth, but if this fell to suitable values, then germination and outgrowth could take place.

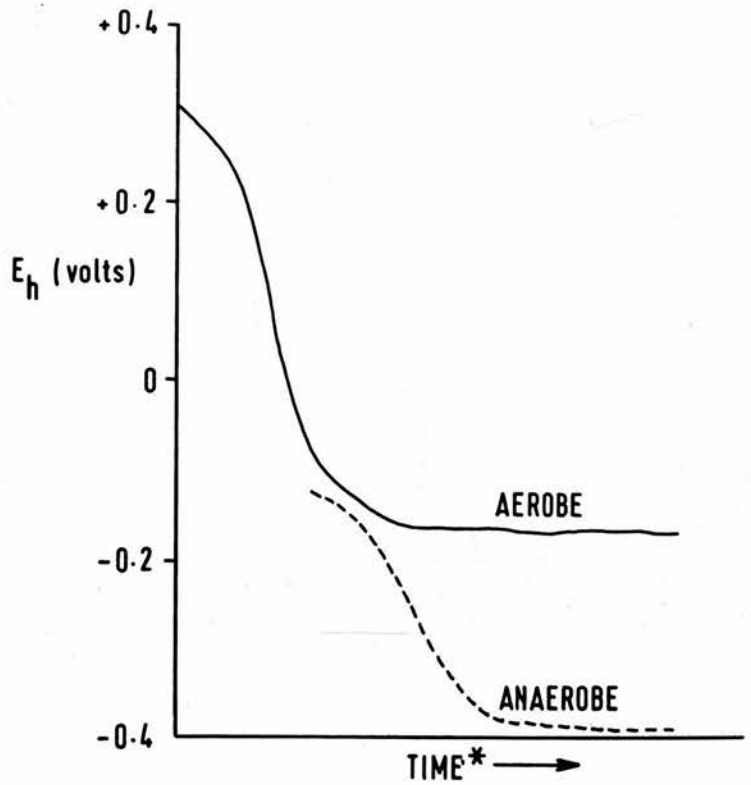
Since that time, many workers have investigated the effect of various E_h levels on the growth of anaerobes. Knight and Fildes (1930) used an electrometric technique for measurement of oxidation-reduction potentials and confirmed Fildes' finding of the inhibitory effects of high E_h values for the tetanus bacillus. These workers considered that for the growth of a given anaerobe, there was a limiting E_h value above which growth would not take place. This concept was extended to non-sporing anaerobes by Vennesland and Hanke (1940) who showed that growth of Bacteroides vulgatus in glucose broth at pH 6.6 would only occur at values below +0.150 volt. These workers suggested that oxidation-reduction potentials may play an important part in anaerobiosis by altering essential enzyme-substrate interactions. Later workers showed that several species of clostridia, in addition to Cl. tetani, would only grow in liquid media if the E_h conditions of the media were kept below certain limits, these limits being different for different organisms (Hanke and Katz, 1943; Hanke and Bailey, 1945). If the conditions were poised at E_h values that were too positive, inhibition of growth of the test organisms occurred. Hanke and Katz concluded that as the limiting oxidation-reduction potentials observed for a sporing anaerobe (Cl. sporogenes) and a non-sporing anaerobe (B. vulgatus) were independent of marked variations in oxygen tension, E_h, rather than oxygen tension, was the limiting factor for the growth of these anaerobes.

Reed and Orr (1943) investigated the optimum, rather than the

limiting E_h values for the growth of several species of clostridia in liquid media, and suggested that an E_h of approximately -0.2 volt gave optimal growth of the organism tested. They suggested that their results showed that correct conditions of E_h were an essential factor for the growth of anaerobes.

One of the main contributors to the elucidation of the role of E_h in anaerobiosis has been Hewitt. He showed that in media in which the initial conditions were kept aerobic, inoculation of the media with anaerobes did not produce growth, and the oxidation-reduction potential of the media remained at a steady positive value of approximately +0.3 volt (Hewitt, 1950). Aerobic organisms on the other hand, grew well under such conditions, and the E_h then fell to negative values. He ascribes to E_h a central role in anaerobiosis, but emphasizes the primary importance of the E_h conditions at which growth can be initiated, rather than those ultimately achieved by growth in the medium. He illustrates this diagrammatically (see Fig. [1]). From this, it is clear that the final E_h value achieved in a culture of an aerobic organism may differ little from that achieved in a culture of an anaerobe, but the E_h values required for initiation of growth differ considerably. The term "initiation of growth" as used by Hewitt has little meaning, however, unless the size of the original inoculum used is specified, as the numbers of organisms originally present will have an effect on the rate of production of a suitable micro-environment.

Jacob (1970), in a review of procedures for measurement of redox potential, shows diagrammatically that the observed E_h in cultures of anaerobes may fall to values of -0.5 volt or less. These



Adapted from Hewitt L.F. 1950. "Oxidation-reduction potentials in Bacteriology and Biochemistry", 6th. ed., Edinburgh.

* Time-course was not specifed by Hewitt.

FIGURE 1. - The typical changes in redox potential developed during the growth of a hypothetical aerobic and anaerobic bacterium.

results support the earlier work of Lepper and Martin (1930a,b) who showed that tubes of cooked-meat broth, that were able to sustain the growth of obligate anaerobes even when exposed to the air, had a strongly negative E_h value before inoculation. They found that in the depths of the medium, the E_h before inoculation was -0.2 volt, falling to -0.4 volt when growth of the anaerobes took place. From these results they concluded that growth of anaerobes could not begin unless essential requirements for negative E_h values were satisfied. In the case of cooked-meat broth cultures, they had previously ascribed the strongly reducing conditions to the autoxidation of lipids, catalysed by haematin present in the muscle of the cooked meat (Lepper and Martin, 1929).

Further evidence for the role of E_h in bacterial anaerobiosis comes from the studies of Barnes and Ingram (1956), who related the number of clostridia found in the sterno-cephalicus muscle of the horse to changes in redox potential; they showed that the number of clostridia present increased as the redox potential decreased, and that strins isolated from the muscle were E_h dependent, being inhibited by E_h values above +0.23 volt, the levels found in the normal healthy muscle.

The primary role of E_h in anaerobiosis has, however, been challenged by several workers. Dack and Burrows (1935) found, when growing non-sporing anaerobes such as Bacteroides spp., that the E_h produced was less negative than expected, and concluded that "all obligate anaerobes do not produce very negative potentials, nor are such potentials necessary to their growth processes." A careful study of their paper, however, reveals that the conditions of E_h required,

or observed, at initiation of growth of the test anaerobes, were in fact all negative; in addition, the positive drift in E_h values observed by them during culture of the test anaerobes could well be explained by the phenomenon previously described by Hewitt (1931) namely that lysis of cells in liquid cultures can produce a positive drift in E_h values.

Hanke and Bailey (1945) confirmed that the limiting E_h values for growth of clostridia are related to the conditions of pH prevailing in the medium. Grunberg-Manago (1951), using Cl. sporogenes as a test organism, showed that there was a fall in E_h values during the latter part of the lag phase of growth, while pH adjustment tended to occur later in the log phase. She concluded that the fall in E_h was caused when actively growing organisms liberated reducing substances and produced hydrogen, with a resulting pH change in the medium (i.e. that E_h is a result of, rather than an initiating factor in growth). The relationship between E_h and pH is more complex than these conclusions might suggest; the complexity is illustrated by the following equation, derived by Hewitt (1950):

$$E_h = E_o + \frac{RT}{F} \log \frac{(Oxid)}{(Red)} - \frac{RT}{F} \log \frac{K_d}{(H^+) + K_d}$$

where R = gas constant

T = absolute temperature

F = Faraday

K_d is a constant (Hewitt)

$\frac{(Oxid)}{(Red)}$ = ratio of concentrations of oxidised/reduced forms of the system under consideration.

Such work, therefore, does not invalidate the hypothesis that E_h plays, if not a primary, at least an essential role in the

initiation of anaerobiosis. Broh-Kahn and Mirsky (1938) attempted to "make" an obligate anaerobe by treating cultures of Esch. coli with KCN. This, they argued, would inhibit catalase activity, and interfere with the aerobic electron-transfer system of the bacterium, thus converting it to an obligate anaerobe. They found, however, that such KCN-treated bacteria could survive in air on suitable media. From this finding, they argued that E_h was not a primary factor in anaerobiosis. Apart from the obvious dangers of equating KCN-treated cultures of Esch. coli (an aerobe and facultative anaerobe), with an obligate anaerobe, the discovery that iron-containing electron carriers such as ferredoxins found in Esch. coli have different properties from those found in obligate anaerobes (Vetter and Knappe, 1971) suggests that there are considerable fundamental differences between the oxidation-reduction systems of Esch. coli and those of the obligate anaerobes. Thus at present, there seems little evidence on which to refute the concept of the primary role of E_h in anaerobiosis, and one must await with interest further studies in this field. The histochemical localisation of sites of oxidation-reduction activity (Vandenvinkel and Murray, 1962) may yield useful clues in studies of the obligate anaerobes.

Theories in which hydrogen peroxide and catalase are assumed to have an important role in anaerobiosis

In 1922, McLeod and Gordon investigated the factors responsible for the autolytic changes observed in cultures of pneumococci exposed to aerobic conditions for long periods of time. They showed that these changes were related to the production of an inhibitory substance that caused a green discoloration on chocolate blood agar, and suggested that this substance was hydrogen peroxide. Organisms that formed demonstrable amounts of this substance were shown by these workers to lack the ability to catalyse the breakdown of hydrogen peroxide (H_2O_2)in dilute solution. This finding complemented the earlier work of Löwenstein (1903), who noted that cultures of tetanus (sic) failed to break down hydrogen peroxide.

In a later paper, McLeod and Gordon (1923a) tested the effect of adding hydrogen peroxide to cultures of test organisms, both obligate aerobes and obligate anaerobes, and found, as might be expected, that the sensitivity of cultures of a given organism to H_2O_2 was less if the organism concerned produced a catalase enzyme than if it showed no catalase activity. Non-catalase-producing bacteria, they suggested, died in culture because of production of hydrogen peroxide with accumulation of lethal concentrations of the substance.

As the action of catalase is to "neutralise" hydrogen peroxide, as follows:

$$2H_2O_2 \rightleftharpoons 2H_2O + O_2$$

they postulated that anaerobic bacteria, when exposed to aerobic conditions, produce hydrogen peroxide from molecular oxygen; the

bacterial cell is thus exposed to increasing concentrations of hydrogen peroxide which, in the absence of catalase, prove lethal to it. Their paper implies that they consider that the sensitivity of anaerobes to oxygen is related both to sensitivity to hydrogen peroxide and inability to produce catalase. Support for the role of catalase in anaerobiosis came from the work of Callow (1923) who showed that all of 12 test aerobes showed demonstrable catalase activity whereas none of 9 test anaerobes showed any such activity.

In a later paper McLeod and Gordon (1923b) showed that they could enhance the anaerobic growth of Cl. welchii by addition of an exogenous impure catalase preparation, although the growth of Cl. tetani was only slightly enhanced. Their attempts to grow obligately anaerobic bacteria on "catalase agar" under aerobic conditions were unsuccessful. In this paper they amend the implications of their previous paper by the statement that "it would appear in any case that the sensitivity of anaerobes to H_2O_2 is not due to their inability to produce catalase." The fact that this statement is in italics in the original article makes it clear that they attached considerable importance to it, yet they have been considerably and consistently misquoted in the literature — their names are linked with a theory that both sensitivity to hydrogen peroxide and inability to produce catalase form the basis for bacterial anaerobiosis, whereas they themselves came to the conclusion that only the first part of the theory was tenable.

Support for the views of McLeod and Gordon came from the work of Avery and Morgan (1924) who found that the presence of sterile unheated plant tissues in plain broth allowed "aerobic" growth of

test anaerobes, an effect that they ascribed to decomposition of formed hydrogen peroxide by enzymes present in the plant tissues. Heating of the tissues abolished the protective effect.

Several other workers investigated the production of hydrogen peroxide by bacterial cells. Johnstone (1940) found that hydrogen peroxide was produced on aeration of anaerobically-incubated pneumococcal cultures or of autolysed cultures of obligate anaerobes. He noted that there was no correlation between the oxidation-reduction potential developed in cultures of a test organism and the ability of that organism to form peroxides. Several workers have sought to demonstrate H_2O_2 production by cultures of obligate anaerobes, usually by indirect methods. For example Hayward (1942) used the benzidine blood agar reaction of Gordon and McLeod (1940) to screen large numbers of test anaerobes, and found that many, but not all, gave a positive result by this method. Gordon et al. (1953) claimed that many clostridia gave a positive reaction (blackening of the medium) in the above test, suggestive of H_2O_2 production. No H_2O_2 production could be detected in whole cultures using direct methods, although some evidence of H_2O_2 production was obtained in a few cases if cultures were suspended in water and aerated before testing. This failure to detect H_2O_2 by direct testing of whole cultures could be explained by the finding of Mallin and Seeley (1958) that in fresh cultures of some obligately anaerobic bacteria a DPNH oxidase system was present, which could remove any peroxides formed during exposure of the culture to aerobic conditions. In ageing cultures the activity of this enzyme diminished and H_2O_2 production could be detected.

There seems little doubt that some bacteria can produce auto-

inhibitory amounts of hydrogen peroxide. For example, Gilland and Speck (1969) showed that inhibitory levels of hydrogen peroxide accumulate in cultures of lactic streptococci, while Anders, Hogg and Jago (1970) published a similar finding for group N streptococci.

Lichstein and Soule (1944) could find no detectable catalase activity in cultures of 3 different clostridia, and on the basis of a lack of this enzyme in obligate anaerobes, Holman (1955) investigated the effects of addition of exogenous catalase to cultures of various anaerobes. He claimed that addition of his catalase preparation allowed aerobic growth of these organisms, emphasizing the importance of hydrogen peroxide toxicity in anaerobiosis. More recently Dobrogosz and Stone (1962) showed that addition of exogenous catalase improved the growth of certain strains of Pediococcus pentosaceus, the effect only being observed in the case of intrinsically catalase-negative strains. However, as the pediococci tested grew equally well aerobically and anaerobically, the significance of their findings in relation to anaerobiosis is difficult to assess.

In considering the place of catalase in anaerobiosis, the present writer takes account of the fact that the presence of catalase in some aerobic organisms has been recognised for many years, and more recently Amin and Olson (1968) showed that in the case of coagulase-positive staphylococci there was a good correlation between catalase production and sensitivity to H_2O_2. That catalase can be important in microbial metabolism is underlined by findings such as those of Huddleson (1943), who showed that in cultures of Brucella, high virulence was associated with high catalase activity, and of Low, Eaton and Proctor (1968) who showed that catalase produced a noticeable

effect on the oxygen uptake of Mycoplasma species.

The possibility that H_2O_2 or catalase have a fundamental role in anaerobiosis has been challenged by several workers. Novy (1925) found that test anaerobes grew well in cultures containing pneumococci that produced hydrogen peroxide, and in some cases these "mixed" cultures grew better than if the anaerobes were incubated in pure culture, while Sherman (1926) claimed to show that propionic acid bacteria (obligate anaerobes) were copious producers of catalase.

Molland, reviewing the possible roles of H_2O_2 and catalase in anaerobiosis (Molland, 1947), criticised the indirect methods used for detection of H_2O_2 because of their lack of specificity. On the basis of his own observations he came to the conclusion that it was not possible to explain microbial sensitivity to oxygen on the basis either of catalase activity or sensitivity to H_2O_2, and that "catalase is not in any way essential to the life of bacteria". Using more direct methods of H_2O_2 detection and estimation, he showed that of many anaerobic strains tested, none was inhibited by 1:8000 H_2O_2, and none produced detectable amounts of the substance in culture. In the case of aerobic organisms the correlation between marked catalase activity and lack of sensitivity to H_2O_2 was good except in the case of Strept. agalactiae, which although catalase-negative, grew well in 0.4 per cent. H_2O_2. Molland also makes the point that members of the Pseudomonas genus are obligate aerobes, although they exhibit little if any catalase activity.

Further evidence against the role of catalase or hydrogen peroxide comes from more recent work. For example, Prevot and Thouvenot (1952) found that 6 out of 33 cultures of obligate

anaerobes showed demonstrable catalase activity, while Mateles and Zuber (1963) were unable to repeat the work of Holman in a series of experiments with purified catalase preparations. Mateles and Zuber could not produce aerobic growth of obligately anaerobic bacteria by addition of exogenous catalase, and in most cases, no enhancing effect on anaerobic growth was observed. They suggested that the dramatic effects observed by Holman could have been due to nutritional impurities in his preparation.

Whittenbury (1964) found that in the lactic acid bacteria, there was little correlation between catalase activity and requirements for aerobic or anaerobic conditions. This finding is in accord with the observation of Rogosa (1964) that although some strains of Veillonella spp. produce a catalase-like enzyme, the presence or absence of this enzyme does not determine whether a given strain is aerobic or obligately anaerobic in its requirements. Smith (1967) observed that the anaerobes Cl. sporogenes and Cl. bifermentans both possess catalase activity and that Cl. sporogenes and Bacteroides vulgatus can both be grown in air if the conditions of redox potential are suitable, yet no detectable peroxide is formed.

Thus it seems that although hydrogen peroxide may be produced under certain circumstances by bacteria, and usually produces inhibitory or lethal effects which will be diminished or removed if the organism concerned shows catalase activity, there is no correlation between the production of hydrogen peroxide, or even the lack of catalase activity, and the requirements of a given organism for anaerobic conditions. The two factors of hydrogen peroxide and catalase cannot be linked in a unitarian theory to account for the

phenomena of bacterial anaerobiosis.

The possible inhibitory effects of peroxides formed in culture media are discussed later (see pp 278-279).

Theories in which the direct toxic effects of oxygen are assumed to have the primary role

Many investigators have considered that the basis of bacterial anaerobiosis is a direct toxic action on anaerobic bacteria, rather than an indirect one that involves production of H_2O_2 or alterations in E_h values.

The toxic effects of oxygen on living systems have been known almost since the discovery of the element itself. Scheele (1882) noted that peas grown in pure oxygen could sprout normally, but no further development occurred. In 1878, Bert described the possible toxicity of increased concentrations of oxygen to living cells, and suggested that this may be due to a direct toxic action on "fermentative" reactions in the cell.

Pasteur was the first to suggest that oxygen might be toxic or inhibitory to anaerobic micro-organisms. In 1861, while examining a culture of anaerobes under a coverslip, he noted that the organisms at the periphery of the drop, and therefore nearest the air, had ceased to move, while those at the centre of the drop were still motile. This led him to investigate the possible harmful effects of air on these organisms, and in 1863, he showed that butyric fermentation could be inhibited by passing a current of air or oxygen through the fermenting liquid. The organisms responsible for this process were able to live without oxygen, and were killed by its presence. For these organisms, he proposed the name "anaerobes".

McLeod (1930) extended Pasteur's observations on anaerobic bacteria by investigating the length of exposure to air necessary to cause the death of various anaerobes, and the minimal oxygen

concentrations that would inhibit their growth. He used young cultures of sporing anaerobes in an effort to ensure the presence of a predominantly vegetative cell inoculum, and found for example that pour-plate cultures of B. tetani (sic), when exposed to the air for 6 hr, showed no growth on subsequent incubation. When broth cultures of various anaerobes were aerated, no B. tetani organisms could be recovered after 5 hr aeration, whereas appreciable numbers of B. welchii could still be recovered after this time. He also found that a partial pressure of oxygen of 4-5 mm mercury was inhibitory to the growth of B. tetani, whereas a partial pressure of oxygen of 90 mm of mercury was necessary to inhibit the growth of B. welchii completely. Over the years, many workers have commented on the sensitivity of anaerobes to exposure to aerobic conditions, and have found that the sensitivity varies in different organisms. This is well illustrated by Smith's observation that whereas exposure of a thin layer of a broth culture of Butyrivibrio to the air resulted in death of 99.99 per cent of the viable organisms, little if any decrease in viability was observed when broth cultures of Cl. welchii were exposed to similar conditions for several hours. The possibility that some anaerobes were killed by even transient exposure to oxygen has been suggested by several workers, for example Spears and Freter (1967), and has formed the basis for the use of anaerobic cabinets and oxygen-free gassing procedures (see page 45).

Several workers have attempted to quantify the oxygen sensitivity of species of anaerobes more precisely. Fredette, Planté and Roy (1967) compared the results of growing various test anaerobes in deep agar cultures in pure oxygen at pressures of 2-3 atmospheres, with those

derived from growth of the same organisms in air at normal pressures. By comparison of the zones of inhibition of growth produced under both sets of conditions, they were able to grade the anaerobic bacteria studied in a scale of oxygen sensitivity, ranging from Cl. welchii - relatively oxygen tolerant, to Ristella fragilis (sic), the most oxygen-sensitive anaerobe studied. From these results, they claimed that it was possible to express the oxygen-sensitivity of anaerobic species in mathematical terms.

Loesche (1969) studied the effects of different partial pressures of oxygen on the growth of anaerobes both on surface plates and in liquid cultures and was able to grade the anaerobes studied into three groups:

(1) Strict anaerobes showing maximum growth at partial pressures of oxygen of less than 0.5 per cent.

(2) Moderate anaerobes showing maximum growth at partial pressures of oxygen less than 3 per cent. and

(3) Micro-aerophiles showing maximum growth at pressures of oxygen greater than 3 per cent. but less than 20 per cent.

He included Cl. oedematiens type D in the "strict" group, and Bacteroides fragilis and B. necrophorus in the "moderate anaerobe" group.

In recent years, the advent of hyperbaric oxygen therapy as a means of treating anaerobic infections has led to a considerable amount of investigation into the effects of oxygen, at pressures above atmospheric, on micro-organisms. It is not possible to review this broad field adequately in the present study, and only an outline of the more important and relevant findings are given. For a more comprehensive

review of the subject, the reader is referred to the work of Ledingham (1965), to the Proceedings of a recent Conference on hyperbaric oxygenation (1969), and to a recent review by Gottlieb (1971).

In 1961, Brummelkamp, Hoogendijk and Boerema showed that oxygen at high pressures (OHP) inhibited the growth of Cl. welchii when the organism was inoculated into guinea-pigs. Their preliminary report that in a few patients studied, OHP had exerted a favourable and useful therapeutic effect in cases of Cl. welchii gas gangrene, was followed up by a report in which they showed further successes of OHP in Cl. welchii infections (Brummelkamp, Boerema and Hoogendijk, 1963).

Fredette, in 1965, examined the sensitivity of some anaerobic bacteria and their toxins to OHP, and came to the conclusion that OHP exerts a bacteriostatic effect on anaerobic bacteria, with Cl. welchii being most affected by this procedure. He found however that tetanus toxin, always assumed to be sensitive to oxygen, could withstand exposure to oxygen at 45 lb. per square inch pressure without loss of activity. In a later study (Fredette, 1967), he showed that the growth of cultures of anaerobic streptococci was inhibited by oxygen at pressures greater than 1 atmosphere, although good growth was obtained if the cultures were sealed in tubes and inaccessible to the increased oxygen concentration. Further evidence of the toxic effects of OHP on anaerobes was provided by the work of Kaye (1967), who demonstrated that oxygen at pressures of 15 or 30 lb. per square inch exerted a bactericidal effect on cultures of Cl. welchii or Cl. tetani in broth cultures, the effects being more marked in the case of young (5 hr) cultures than in the case of older (24 hr) cultures.

Several workers have also shown toxic effects of OHP on aerobic

micro-organisms. McAllister et al. (1963) showed that oxygen at 2 atmospheres exerted a bacteriostatic effect on several organisms, including Ps. pyocyanea, and their findings were confirmed by Hopkinson and Towers (1963). Caldwell (1965) found that oxygen at pressures in excess of 10 atmospheres completely suppressed the growth of test bacteria and fungi, although growth recommenced on incubation in oxygen at normal pressures. In the case of Esch. coli, oxygen at pressures of 15 atmospheres increased the doubling time in liquid cultures and caused a fall in the viable counts, as compared with controls (Stark and Orr, 1966).

The biochemical basis for oxygen toxicity, and the definition of possible target sites for the action of oxygen, has received much attention in recent years. Grunberg-Manago (1951) drew attention to the effect of oxygen on the enzymes in the Stickland reaction (1934). These effects are explained below. The reaction consists of an oxido-reduction taking place between the aminoacids alanine and glycine – one acting as hydrogen donor, the other as hydrogen acceptor:

$$CH_3 \cdot CHNH_2 \cdot COOH + H_2O \rightleftharpoons NH_3 + CH_3 \cdot CO \cdot COOH + 2H^+$$

alanine

$$CH_3 \cdot CO \cdot COOH + H_2O \rightleftharpoons CH_3 \cdot COOH + CO_2 + 2H^+$$

$$4H^+ + 2CH_2 \cdot NH_2 \cdot COOH \rightleftharpoons 2CH_3 \cdot COOH + NH_3$$

glycine

In Cl. sporogenes and possibly in other anaerobes, this process takes place as a source of energy, provided that there are physiological hydrogen acceptors available. When the organism is grown in air, oxygen

acts as the electron acceptor, and the reaction ceases. Such findings led Grunberg-Manago to criticise theories of anaerobiosis based on redox potential requirements, on the grounds that extracellular E_h values may bear no relationship to the values within the cell, which in turn may merely reflect the permeability of the cell to oxygen. She argued that intracellular oxygen itself could have a direct destructive or inhibitory effect on certain enzyme systems.

More recently, several electron carrier proteins have been discovered in clostridia and these play an important part in the electron-transport mechanisms of these organisms. In 1962, Mortenson, Valentine and Carnahan isolated an iron-containing protein, which they called ferrodoxin, from extracts of the anaerobic nitrogen-fixing bacterium Clostridium pasteurianum. This protein functioned as an electron carrier at extremely low oxidation-reduction potentials, and was a necessary co-factor in the synthesis of hydrogen from pyruvate (the phosphoroclastic reaction). It was shown to be a small acidic protein, with a molecular weight of about 6000, containing several iron-sulphide (Fe-S) groups sandwiched between the peptide backbones; the Fe-S group appears to function as the electron accepting site (Benemann and Valentine, 1971).

Ferredoxins isolated from other clostridial species, have been shown to have very similar amino-acid sequences (Matsubara et al., 1969). There have been several reports of isolation of ferredoxins from other anaerobic bacteria, and from plants, and these substances seem to be implicated as electron carriers in certain photosynthetic pathways (Togawa and Arnon, 1962; Arnon, 1965). The first reported isolation of a ferredoxin from an obligately anaerobic organism (Azotobacter

vinelandii) came in 1969 (Yoch et al., 1969). The ferredoxin from Azotobacter vinelandii differs from previously isolated ferredoxins in respect of its molecular weight (20,000 as compared with 6000 in the case of clostridial ferredoxin) and its amino-acid structure. It is unable to substitute for clostridial ferredoxins in the phosphoroclastic reaction.

Another type of electron carrier protein was isolated from Cl. pasteurianum, and this differed from ferredoxin in containing no iron or sulphide, but it had a flavin (FMN) prosthetic group; it was therefore called flavodoxin (Knight et al., 1966; Knight and Hardy, 1967). This protein could substitute for ferredoxin in several reactions, including the phosphoroclastic reaction and was formed in place of ferredoxin when cells were grown in iron-deficient medium. A similar compound has recently been isolated from Peptostreptococcus elsdenii (Beneman and Valentine, 1971).

That the ferredoxins can be inactivated by oxidation has been shown by several workers (Tagawa and Arnon, 1962; Bachhofen, Buchanan and Arnon, 1964). The latter workers found that ferredoxin could exist in two forms, oxidised ferredoxin or reduced ferredoxin, and that the activities of ferredoxin preparations were lost on exposure to aerobic conditions. Yoch and et al. (1969) showed that clostridial ferredoxin underwent fairly rapid re-oxidation in the presence of oxygen, rendering it inactive, whereas that from the aerobic organism Azotobacter vinelandii showed much slower re-oxidation in the presence of oxygen. Thus it seems that the ferredoxins and similar electron transport proteins may prove to be a target site for the toxic effects of oxygen on anaerobic bacteria.

The cellular basis for oxygen toxicity has recently been extensively reviewed (Haugaard, 1968), and the reader is referred to this work for a comprehensive review of mechanisms of oxygen toxicity in biological systems. Haugaard suggests that oxygen may exert toxic effects on biological systems in one of several ways:

(1) Oxidation of sulphydryl (-SH) groups

This process may involve compounds such as glutathione, in addition to enzyme sulphydryl groups. Haugaard considers that the inactivation of ferredoxin may occur in this way.

(2) Formation of toxic peroxides

This process could occur by means of lipid peroxide formation, or by formation of hydrogen peroxide, both acting on vital cellular components with eventual inactivation of them. The phenomenon of lipid peroxidation is not known to occur in bacteria, but it is known that hydrogen peroxide can inactivate certain enzyme groups.

(3) Formation of free radicals

Formation of free radicals such as perhydroxyl (HO) which can depolymerize DNA with inactivation of cellular function.

Menzel (1970) reviewed the roles of free radicals in systems exposed to oxygen, ozone, or ionising radiation, and considered that the formation of free radicals in these systems may account for the toxic effects observed. He emphasized that free radicals are present in small amounts in normal cellular metabolism and that only an excess is significantly harmful. Recently, McCord, Keele and Fridovich (1971) have suggested that the presence of the enzyme superoxide dismutase is essential for the survival of bacterial cells exposed to aerobic conditions. This

enzyme catalyses the conversion of the free radical superoxide (O_2^-) to hydrogen peroxide as follows:

$$O_2^- + O_2^- + 2H^+ \longrightarrow O_2 + H_2O_2$$

They showed that whereas all aerobic organisms tested showed demonstrable superoxide dismutase activity, no such activity was found in any of the anaerobic organisms tested. They postulated that cells devoid of this enzyme could only exist in conditions in which the harmful superoxide radical (an intermediate in the reduction of molecular oxygen) was not being produced, i.e. in anaerobic conditions. This hypothesis for the nature of bacterial anaerobiosis must await confirmation by further studies.

Several agents have been shown to modify the toxic effects of oxygen on biological systems; these include antioxidants (Jamieson and Van den Brenk, 1964), sulphydryl compounds (Horn and Hougaard, 1966), and the buffering agent THAM (tris-hydroxymethyl amino-methane). This latter compounds has been found to protect whole animals against such toxic effects of oxygen as oxygen-induced convulsions (Sanger et al., 1961).

It has been shown too that chelating agents such as EDYA can protect against the toxic effects of oxygen (Haugaard, 1968), and this finding may be related to the observation that whereas certain metal ions, such as cobalt and manganese, can protect whole animals against oxygen toxicity (Dickens, 1946), others such as copper or iron can markedly accentuate the inhibitory effects of oxygen on cellular metabolism (Haugaard).

The agent γ-amino butyric acid (GABA) is an intermediate compound

in the synthesis of glutamine, and has been shown to be reduced to abnormal levels in the brains of animals exposed to oxygen at high pressures (Wood and Watson, 1963). Administration of this substance appears to protect animals against some of the central nervous system effects of oxygen at high pressures (Wood et al., 1965). Other metabolites, such as succinate can exert similar protective effects on mammalian systems, perhaps by acting as an "auxiliary" electron donor to overcome toxic effects on electron transfer mechanisms (Chance et al., 1965).

There does not appear to have been adequate investigation of the possible uses of these protective substances to overcome the toxic effects of oxygen on anaerobic bacteria, although the findings of Dedic and Koch (1956) that addition of cobalt ions to liquid media allowed aerobic growth of a strain of Cl. tetani may relate to its protective role in oxygen toxicity (see above). Similarly, the protective effect of dithiothreitol on -SH groups (Cleland, 1964) may relate to oxygen toxicity. Both these findings are considered further in the present study.

There is a need to extend studies of protective agents against oxygen at high pressures to micro-organisms, to determine whether any of the above agents can minimise any contribution that the toxic effects of oxygen at normal pressures may make to the unreliability of recovery of anaerobes from clinical specimens.

There does not appear to be any one theory that explains all the phenomena of bacterial anaerobiosis; several factors may be concerned. Indeed it may be wrong to consider that the same mechanism(s) of anaerobiosis hold good for all micro-organisms. This point is

illustrated by the recent work of de Vries and Stouthamer (1969) on Bifidobacterium strains. These workers found that in 12 strains tested, differing degrees of sensitivity to oxygen were apparent, some strains being rather insensitive, while others were extremely sensitive. In two strains which showed moderate sensitivity to the effects of oxygen, production of H_2O_2 appeared to be the principal factor in anaerobiosis, inactivating a key enzymic pathway in these organisms under aerobic conditions. In the two most sensitive strains, no production of H_2O_2 could be demonstrated and the authors concluded that a requirement for low redox potential was the important factor in anaerobiosis in these strains. Thus, within a single genus, different mechanisms of anaerobiosis appear to be important in different organisms.

The differing views on the nature of the primary factors in anaerobiosis have led to different approaches to the practical problems of growth and recovery of anaerobes in the laboratory. The different methods of culturing and recovering anaerobes are reviewed below.

THE LABORATORY CULTURE OF ANAEROBES

The large volume of literature on methods for the cultivation of anaerobes suggests that no one ideal method has so far been devised that will ensure consistent and reliable growth of anaerobes in the laboratory.

The early literature abounds with reports of methods for achieving satisfactory anaerobiosis, and this field was ably reviewed by Hall (1929) who gave a comprehensive picture of early methods for the cultivation of anaerobes. A careful search of the literature suggests that with the exception of one or two omissions, mentioned below, Hall's review is a very full one.

Hall considered that available methods for the laboratory cultivation of anaerobes utilised some method of reducing the oxygen tension in the environment, coupled with some means of maintaining the oxygen tension at a low level during incubation. He showed that methods for reducing oxygen tension could involve (i) "biological reduction", as achieved for example by the presence of aerobic organisms in the immediate environment, or the use of animal or plant tissues, (ii) "physical reduction" accomplished by the boiling or pre-steaming of media, the incubation of plates under a vacuum, or the use of inert gases to displace the oxygen, and (iii) "chemical reduction" accomplished by the use of alkaline pyrogallol, iron compounds, reducing agents in the medium, or the combustion of hydrogen with any residual oxygen.

After the oxygen tension has been reduced to low levels, these low levels could be maintained by the use of seals. The seals could be mechanical (rubber, plasticine), liquid (viscous oils) or could be formed by the tightly-fitting lids of incubation chambers. Although

Hall's review is a comprehensive one, a few important contributions are omitted, notably those of Frothingham (1894) and Leclainche and Vallée (1900) who devised methods for incubating tubes of liquid media in atmospheres of hydrogen, and that of Lentz (1910) who was one of the first to utilise alkaline pyrogallol in a "double-plate" method for incubation of anaerobic plates.

Since the time of Hall's review, there has been much work in the field of anaerobic cultural methods; modification of old methods and the development of new ones. Before considering the new methods that have been developed, it is worthwhile mentioning one or two modifications of older ones that have lingered on.

The use of alkaline pyrogallol, prepared by mixing sodium hydroxide (NaOH) and pyrogallol, has appealed to many workers, and modifications of apparatus for this purpose have been proposed by Riemsdijk (1943), Ehringer (1940) and Lockhart (1953). Spray (1930-31) described a special container to hold and then to mix the NaOH pyrogallol system, the container being sealed on to the seeded surface of an agar plate. He noted that one of the disadvantages of the system was that the NaOH present absorbed carbon dioxide, and he therefore recommended the use of only a small quantity of NaOH.

Workers such as Mossel et al. (1959) and Carlquist (1959) have described methods in which the reagents are placed on the inner surfaces of the bottom halves of petri dishes, while the top halves containing the seeded agar are sealed on by suitable seals after the reagents have been mixed. However, "double-plate" methods have several disadvantages; for example it is difficult to achieve an adequate seal between the two half-plates, and there is a risk of contamination of

the seeded plate by the alkaline pyrogallol solution. To overcome these disadvantages, workers have used polyethylene bags (Mathews and Karnauchow, 1961; Müller, 1969). Mathews and Karnauchow used a Seitz filter impregnated with alkaline pyrogallol which was placed in the polyethylene bag, together with the seeded plates. The bag was then sealed and incubated. Müller used a heating system to seal the bags.

Although the alkaline pyrogallol method for removing oxygen is still used in some laboratories, it has generally fallen into disuse, perhaps because of the development of better methods, and also because of the disadvantages of carbon dioxide absorption by the sodium hydroxide and the possible production of carbon monoxide, as noted by Nicol (1929), which represents a possible hazard to the operator.

The use of plant or vegetable tissues to remove oxygen is not now used routinely, perhaps because of the "ever present source of contamination from the vegetable roots" mentioned by Carnes (1943) in his review of bacteriological methods in war-time. Similarly the use of aerobic organisms to remove oxygen has been largely abandoned. Modifications of the method of Fortner (1928) in which Serratia marcescens is used to remove oxygen, have been described by Marshall and Nordby (1942) and more recently by Paas and Grüschel (1969). The latter workers designed tightly fitting plates ("Rimseal plates"), and used a strain of Serratia marcescens to remove the oxygen from the interior of the sealed plates, the aerobe being seeded on to one third of the agar surface, the remainder being seeded with test anaerobes. Careful study of their papers shows however that when compared with other methods of achieving anaerobiosis, such as the Gaspak system (see below) their system showed one of the main disadvantages of

"biological" methods of oxygen removal, namely slowness in achieving adequate conditions of anaerobiosis.

Similarly, the use of candle jars, in which a burning candle is used to consume the oxygen present, has not been widely adopted since Hall's review. However, in 1970, Thompson and Jarvis described a compact jar, made of acrylic piping, and capable of carrying 15 petri dishes, in which removal of oxygen is accomplished by means of a lighted candle placed on top of the plates. Willis (1964) cautioned against regarding the method as an anaerobic exercise.

Modern methods for achieving anaerobiosis can be considered under several headings:-

(i) The use of reducing agents in culture media.

(ii) The removal of oxygen from individual plates or tubes.

(iii) Methods based on the anaerobic jar.

(iv) The Gaspak system.

(v) The use of anaerobic cabinets and gas flushing procedures.

The use of reducing agents in culture media

There is an extensive literature on anaerobic culture media in which reducing agents are used to lower the redox potential of the medium so as to allow initiation of growth of anaerobic organisms; only the more important and widely-used reducing agents are considered here. For further information, the reader is referred to reviews such as that given by Willis (1964).

The most widely used fluid medium for the cultivation of anaerobes in use in Britain today is the cooked-meat medium described by Robertson (1915-1916) in which the reducing agents are present in the meat, as described by Lepper and Martin (1929), see page 7. In addition to the reducing conditions achieved in the depths of the medium, the meat may supply some of the nutritional demands of anaerobic bacteria, a principle made use of by some workers in the design of complex media based on protein hydrolysates (Sartory, Malgras, Touillier and Veschambre, 1951) or meat infusions (Hoffstadt, 1945; Khairat, 1964, 1966).

As long ago as 1917, Douglas, Fleming and Colebrook noted that broth to which was added a rusty nail would support the growth of some clostridial species. Since that time, several investigators have described the use of particulate iron in fluid media. Hastings and McCoy (1932) added powdered iron (reduced by hydrogen) to milk to improve the recovery rate of clostridia from heated specimens, and Spray (1936) adopted a similar method in his description of an "iron-milk" medium, in which strips of stove-pipe iron were used in place of iron powder. The use of iron in other media was described by Hayward and Miles (1943), who found for example that good growth of anaerobes could

be achieved by addition of iron strips to peptone water, and similar results could be obtained by the use of nails or iron filings. Willis (1964) noted that tin tacks or screws are more convenient as they can readily be sterilised by flaming. The use of particulate iron for cultivation of anaerobes in liquid media continues to be recommended in several textbooks (e.g. Willis 1964); the possible use of iron-filings on the surface of solid media is considered in the present study.

The use of SH-containing compounds in anaerobic culture media has been suggested by several workers; suggested compounds include alkaline sulphides (Trenkmann, 1898), cysteine (Hosoya and Kishino, 1925), thioglycollic acid (Quastel and Stephenson, 1926) and thiolactic acid (Aubertin, Aubel and Genevois, 1928). Of these, the most widely used have been cysteine, and thioglycollic acid or its derivatives.

Following the studies in which Hosoya showed that cysteine enhanced the growth of anaerobes (an observation later confirmed by Quastel and Stephenson), the use of cysteine in culture media has been extensively documented. For example, Rajagopalan (1938) used a 0.1 per cent. solution of cysteine hydrochloride to grow Cl. chauvoei, and Ryan et al. (1947) showed that similar concentrations gave better reducing conditions than sodium thioglycollate (see below) in a "defined" medium for the growth of Cl. septicum. Cysteine forms an essential component in media for clostridia such as the "reinforced clostridial medium" of Hirsch and Grinstead (1954). Cysteine has the disadvantage that it readily undergoes oxidation in air, being converted to cystine. Recently the -SH group protective reagent dithiothreitol (Cleland, 1964) has been coupled with cysteine in a solid medium for the growth

of Cl. oedematiens type B (Moore, 1968). The possible use of the cysteine/dithiothreitol system for the growth of other anaerobes is considered in the present study.

Following the report by Quastel and Stephenson that thioglycollic acid was of use as a reducing agent in cultivation of anaerobes, Brewer (1940 a,b) described a liquid medium in which 0.1 per cent. sodium thioglycollate was used in place of the less elegant thioglycollic acid. This medium allowed the "aerobic" growth of anaerobes. In addition to the thioglycollate, it contained a small percentage of agar (0.05 per cent.) to minimise the penetration of oxygen into the medium by convection, as originally described by Hitchens (1921), and methylene blue as an indicator of redox potential. Brewer claimed that this medium supported the growth of several anaerobes, including Cl. tetani and Cl. novyi, and would remain in an anaerobic condition in storage for a considerable time. McLung (1940) confirmed the value of Brewer's medium, showing that a dehydrated form of the medium, when reconstituted, compared favourably with various complex meat infusions in its ability to sustain growth of sporing anaerobes from small inocula. Since then, the efficacy of thioglycollate-containing media in the culture of anaerobes has been well documented (Vera, 1944; Schmid, 1950; Mollov, Winter and Steinberg, 1942), and their use has become widespread, notably in the U.S.A. Thioglycollate media have also been used as the basis for selective media such as that described by Shoemaker (1960), in which Kanamycin was added to a liquid thioglycollate medium to produce a selective medium for the isolation of Gram-negative anaerobic bacilli.

The general recommendation of media containing thioglycollate,

for example in media for recovery of clostridial spores, does not take account of reports of its toxicity for these spores. Although Vera did not note any toxicity in her studies, the reports of Hirsch and Grinsted regarding its possible toxicity were confirmed by Mossel and Beerens (1968), who found that sodium thioglycollate in concentrations of 0.3 - 0.5 g per litre inhibited the germination of the spores of several test clostridia, the magnitude of the effect being dependent on the presence of other constituents of the medium. Hibbert and Spencer (1970) found similar results in a range of clostridial spores tested, inhibition being noted with concentrations of 0.1 per cent. thioglycollate in some cases. Glucose appeared to reduce this inhibitory effect. The latter workers suggested that the use of sodium thioglycollate in sterility test media should be discontinued.

The use of ascorbic acid as a reducing agent has long been advocated in the cultivation of anaerobes. In 1938, Kligler and Guggenheim found that 0.2 per cent. ascorbic acid, when added to liquid media, enhanced the growth of C. welchii (sic), allowing the growth of this organism in tubes of aerobically-incubated media. Several workers have recommended its use, both as the pure substance, and even in the form of orange juice (Cianci and Palmieri, 1938). Illenyi (1939) found that addition of ascorbic acid resulted in enhanced recovery of anaerobic bacteria from teeth, and Araki (1939) obtained similar results with Cl. tetani. Ascorbic acid has been used in solid media (Cruickshank and MacDonald, 1943) and for pour plates (Tulloch, 1945). More recently, Walker et al. (1971) observed that ascorbic acid could substitute for the cysteine/dithiothreitol system in the growth of Cl. oedematiens type B. The inhibitory effects of ascorbic

acid against aerobic organisms such as M. tuberculosis (Boissevain and Spillane, 1937) or Proteus spp. (Lwoff and Morel, 1942) have not been observed in the case of anaerobic organisms (Ehrismann, 1942).

Other substances that have been suggested for use in anaerobic culture media include coconut milk (Blauvelt and Asheville, 1939) and a derivative of ascorbic acid (Prevot, 1948) called "reductone". The use of vitamin K derivatives for the cultivation of Fusiformis nigrescens (Bacteroides melaninogenicus), described by Lev (1959), and the enhancing role of cobalt ions in the growth of Cl. tetani (Dedic and Koch, 1956) and Cl. sporogenes (Faguet and Goudot, 1961), are considered further in the present study.

Methods involving the removal of oxygen

The pre-steaming or autoclaving of anaerobic media before use to render them oxygen-free has been practised for many years, and is in common use in anaerobic bacteriology. That it is effective is illustrated by the data of Fredette and Takahashi (cited by Martineau and Fredette, 1961). They found that the E_h of agar fell to values as low as -0.250 mV after being steamed for 5 min. The initial E_h value was not stated.

The use of agar deep techniques, shake cultures, or pour plate methods is also widespread, and detailed in several textbooks (Willis, 1964; Cruickshank, 1968). These methods depend on the reducing conditions in such media and various modifications have been described. For example, Miller, Garrett and Prickett (1939) described a method for obtaining shake cultures using a special tube in which a "seal" of methylene-blue agar prevented access of oxygen, and Cantor (1941) used a method in which seeded molten agar was poured through a hole in a

glass vessel, displacing the air which escaped through other holes drilled in the side of the vessel. Bladel and Greenberg (1965) adapted Cantor's method in a system in which a pouch of plastic film was completely filled with seeded molten agar, displacing the air, and then sealed. The agar was allowed to set and the filled pouch was then incubated at 37°C. These workers claimed that this method, which did not require the use of anaerobic jars, gave good growth of the two test anaerobes, Cl. welchii and Cl. botulinum. So far this method has not been applied to the culture of other anaerobes, except in combination with the alkaline pyrogallol method (see above). A similar principle has been applied by several workers using a system of two glass tubes, connected by capillary tubing. Wilson (1950) used two such tubes, connected by rubber tubing, each being equipped with airtight stoppers. One of the two tubes could be completely filled with pre-steamed liquid media, all the air being expelled into the other tube by manipulation, and anaerobic conditions were therefore produced. According to Wilson, the medium remained in a reduced condition, (as measured by a methylene blue indicator in the medium), for periods up to a month. Corona and Kane (1956) achieved growth of several anaerobes in tubes consisting of an upper portion, separated from a lower portion by a ball-bearing seal ("Hall's tubes").

Although the use of liquid or deep agar cultures has become widespread, workers such as Beerens and Castel (1958) have commented on the poorer recovery of anaerobes obtained by deep culture techniques in comparison with surface culture methods. For example these workers found that their deep culture technique gave recovery rates that were just about 25 per cent. of those obtained with surface culture methods.

Several workers have used particulate iron as a method of removing oxygen and achieving anaerobic conditions, as well as incorporating it in culture media (see p. 32). Parischa and Gosh (1941) described a method in which iron filings were either placed loose in the bottom of an anaerobic jar, or above a liquid medium in a test tube, separated from the medium by a cotton-wool plug. Parker (1955) achieved removal of oxygen by the use of iron wool, "activated" by soaking in acidified copper sulphate. He claimed that this method gave effective conditions of anaerobiosis, and showed that viable counts of Cl. butyricum obtained by his "iron wool" method were as high as those obtained by the use of a procedure involving an anaerobic jar and a heated catalyst, as described below. His method has been used by several workers since, with minor modifications (Floch, Gershengoren and Freedman, 1968; Attebury and Finegold, 1970), and on occasion has been combined with other methods for achieving anaerobiosis (Gorbach and Tabaqchali, 1969). One disadvantage of the method is that carbon dioxide has to be added separately.

Brewer (1942) described a method for removal of oxygen and growth of anaerobes, to be used in conjunction with media containing a reducing agent. In this method, a plate of seeded medium is covered with a glass vessel so designed that it forms a seal, trapping a small amount of oxygen between the medium and the dish; the oxygen is then removed by the reducing agent in the medium. Achievement of anaerobic conditions is indicated by decolorization of a methylene blue indicator in the medium.

More recently, Fabian (1965) described a chemical method for the removal of oxygen, in which a plate containing a mixture of glucose

oxidase and catalase is placed in the foot of a glass desiccator.

In the past, many workers have considered the possibility of incubating seeded plates or tubes of liquid medium in closed containers from which oxygen can be removed, usually by catalytic combination with hydrogen. This led to the development of the anaerobic jar.

The development of the anaerobic jar

In 1915, Laidlaw described a method for achieving anaerobic conditions based on the earlier work of Pfühl (1907) in which palladinised charcoal or colloidal platinum was used to catalyse the combination of hydrogen with oxygen in glass tubes containing seeded agar slopes, thus removing the oxygen present. McIntosh and Fildes (1916) used a similar principle in the design of their anaerobic jar, or "anaerobic tin". The apparatus consisted of a gas-tight container, into which the material for anaerobic incubation was placed. Hydrogen was admitted by means of a tap, and a proportion of the oxygen was displaced. The remainder reacted with the hydrogen to form water, under the influence of a catalyst consisting of asbestos wool impregnated with palladium black. The catalyst was enclosed in a wire gauze envelope suspended from the underside of the lid. The catalyst (for example Wright's capsule - Wright, 1943) had to be activated by heating it in a gas flame immediately before use. In early models, which were made of glass, an indicator of redox-potential (E_h) was placed inside the jar, and could be easily seen. If the indicator (normally alkaline methylene blue with glucose) remained colourless, this was taken to imply that satisfactory conditions of anaerobiosis had been achieved inside the jar.

In 1917, Smillie used electricity to pre-heat the catalyst, *in situ*, thus simplifying the procedure. He used platinised asbestos surrounded by an electric heating coil of wire. Brown (1921) also used an electrically heated catalyst in his anaerobic jar. This jar was based on a specimen jar, in which the palladinized asbestos and heating coil were separated from the gaseous environment inside the jar by a copper gauze screen, analagous to the system used in the design of a Davy lamp. Although the risk of explosions due to sparks igniting the hydrogen was lessened by the adoption of the "Davy lamp" principle, they still occurred. Brown (1922) records the occurrence of an explosion in one of his glass jars, and describes modifications of the heating coil so as to minimise the danger of spark formation.

At about the same time, Fildes and McIntosh (1921) described an improved form of their anaerobic jar which also incorporated an electrically-heated catalyst. This jar differed from Brown's in having two taps, each equipped with needle valves, for the introduction of hydrogen and displacement of air respectively. Their jar lacked the "Davy lamp" system of wire gauze described by Brown, and there was therefore a possible risk of explosion. This possibility led Eggerth and Gagnon (1933) to explore other methods for achieving catalysis. They found that if palladinised asbestos was finely divided, rather than compressed into pads or cylinders as in previous methods, it was capable of catalysing the combination of hydrogen with oxygen at room temperature, without the necessity for prior heating. They placed the finely-divided asbestos in a beaker, covered it with wire mesh, and put this on top of inoculated plates in their anaerobic jar; good results were obtained, although the system had the disadvantages

that first the catalytic material had to be reheated after use to recharge it, and second that hydrogen had to be admitted slowly in order to avoid the risk of explosion. In spite of these disadvantages, the system seemed to have worked well - it is surprising that the widespread use of a room-temperature catalytic system did not occur until after the report by Heller (see below).

The early anaerobic jars required displacement of oxygen from the jar by the hydrogen entering, but this method was inefficient, and resulted in an appreciable amount of residual oxygen in the jar, with the possibility of an explosive mixture occurring. Boez (1927) lessened the possible risks of explosion by prior evacuation of the jars before ingress of the hydrogen, to a negative pressure of 10-20 mm mercury. This measure not only reduced the risks of explosions occurring, but shortened the reaction time to some minutes, rather than the hours sometimes needed with older methods.

Weiss and Spaulding (1937) combined the advantages of a room-temperature catalyst with a system for evacuation of the jar before admission of hydrogen. They used glass desiccators, equipped with three-way taps, to allow admission of hydrogen, evacuation, and manometric readings. They also used a methylene blue indicator of anaerobiosis. Their claim that the system allowed satisfactory growth of anaerobes was confirmed by Miller, Garrett and Prickett (1938) who obtained better recovery rates than those achieved by the use of a deep agar shake method or by the method of Spray (1930).

Brewer (1939) described an apparatus based on the Brown anaerobe jar, in which a glass jar with a bronze lid was equipped with improved electrical connections, and a rubber tube and clamp in place of a

needle valve. The lid was secured by means of a screw clamp, and sealed by plasticine, Only a single tap was provided, as Brewer considered that prior evacuation of the jar was unnecessary. A very similar system, but employing an evacuation procedure, was described by Spaulding and Goode (1940).

The increasing use of metal jars in Britain meant that the methylene blue indicator system could no longer be seen from the outside, and no warning of a defective jar could be obtained until after incubation. Hudson (1941) proposed a modification of the original McIntosh and Fildes anaerobic tin, in which the indicator tube was held in an external side arm in continuity with the internal gaseous environment.

In spite of the work of Eggerth and Gagnon, electrically-heated catalytic systems continued to be widely used in anaerobic jars and it was not until publication of the work of Heller (1954) that room-temperature catalytic systems were more generally used. Heller described the use of catalytic pellets of alumina coated with platinum; 40 of such pellets, enclosed in a metal gauze, formed a catalytic system that could catalyse the combination of admitted hydrogen with the oxygen that remained in an evacuated anaerobic jar. These pellets had to be recharged from time to time by heating, and were liable to become "poisoned" by gases such as H_2S or SO_2, but they were cheap and reliable, and their use avoided the risks involved in the use of electrically-heated catalyst systems.

In Britain, these pellets were at first used in conjunction with jars similar to those described by McIntosh and Fildes, but in 1958 a new all-metal jar (the BTL jar) was marketed by Baird and Tatlock Ltd., and this incorporated a room-temperature catalyst sachet; the jar was

fitted with a side-arm indicator tube, a good rubber gasket between the jar and the lid, and two needle valve assemblies for admission of hydrogen and for evacuation. This type of jar has found widespread use in laboratories in Britain up to the present day, and is evaluated in the present study.

The other type of anaerobic jar in use at that time, mainly in the U.S.A., was the anaerobic jar of Brewer, described above. This has been modified by several workers; for example Evans, Carlquist and Brewer (1948) improved the jar by using a better seal between lid and jar and a better gas inlet, Richtberg (1957) suggested the use of latex rather than plasticine for the seal, and Moore et al. (1964) used an evacuation procedure in combination with an electrically-heated catalytic system in the Brewer jar. The formation of water in the jar was contained by the use of calcium chloride in a vessel at the foot of the jar (Reed and Orr, 1941). The adaptation of the Brewer jar for use with a room-temperature catalytic system was described by Khairat (1964a).

In addition to the anaerobic jars described above, various other types of containers have been used as anaerobic jars. They include stainless steel jars (Möller and Möller, 1961); pressure cookers (Gordon, 1963); desiccators (Fabian, 1965); "Pyrex" glass containers (Brewer and Allgeier, 1966); 5 mm film containers (Attebury and Finegold, 1970); and even milk churns (Schaedler, Dubos and Costello, 1965). A polycarbonate jar, used in the present study (see p.221) has recently been marketed.

The main gas used in anaerobic jars has been hydrogen. Rosenthal (1937) described an anaerobic jar in which the production of hydrogen was produced by the mixing of powdered chromium metal and

sulphuric acid, as shown in the following equations:

$$H_2SO_4 + Cr \rightleftharpoons Cr\ SO_4 + H_2 \qquad (i)$$

$$2H_2SO_4 + 4Cr\ SO_4 + O_2 \rightleftharpoons 2\ Cr_2(SO_4)_3 + 2H_2O \qquad (ii)$$

This method had the advantage (equation ii) that oxygen was absorbed, but it has the disadvantage that no carbon dioxide was produced. Carbon dioxide has generally been held to be of use in the cultivation of anaerobes (see p.266 for a discussion of this point), and Mueller and Miller (1941) accordingly modified Rosenthal's method by adding sodium carbonate to the chromium powder as a source of CO_2. The possibility that impurities in the chromium might contribute to the inhibition of growth sometimes seen with this system (Miles, 1940) was taken into account by Marshall (1960), who used pure granulated zinc and chromic sulphate, mixed with sulphuric acid, as a source of hydrogen, and calcium carbonate as a source of carbon dioxide. According to Willis (1964) this method is as effective as methods involving a palladium catalytic system.

Although the use of hydrogen in cylinders has replaced gas derived from a Kipps apparatus which is certainly inconvenient and troublesome, cylinders are cumbersome, and there is a risk of explosion. Brewer *et al.* (1955) used sodium borohydride ($NaBH_4$) for the production of hydrogen. Sodium borohydride reacts with water in the presence of cobalt chloride to form hydrogen:

$$NaBH_4 + 4H_2O \rightleftharpoons NaBO_4 + 6H_2$$

They found that 0.6 g. sodium borohydride resulted in the production of 1250 ml hydrogen in 1 hour.

A disposable system for hydrogen generation, which only required water for activation, was described by Brewer and Allgeier (1965). The original version used a mixture of magnesium metal, zinc chloride and sodium chloride to produce hydrogen:

$$Mg + Zn\,Cl_2 + 2H_2O \xrightleftharpoons{Na\,Cl} Mg\,Cl_2 + Zn\,(OH)_2 + H_2$$

but in a later paper, Brewer and Allgeier (1966) reverted to the use of the borohydride method. This was combined with citric acid and sodium bicarbonate as a source of carbon dioxide, and the whole was enclosed in a foil envelope to comprise a disposable system for the production of carbon dioxide and hydrogen marketed as the Gaspak system; it is evaluated in the present study.

Other gases used in anaerobic jars include nitrogen (Willis, 1964), alone or in combination with carbon dioxide (Morton, 1943); coal gas or "illuminating gas" (Brewer and Brown, 1938; Moore, Engwall and Moskal, 1964) or even helium (Bridges, Pepper and Chandler, 1952). The use of oxygen-free gases is considered below in relation to the development of gas-flushing procedures and anaerobic cabinets.

Some workers have used simple flushing procedures, in which inert gases are used to flush jars after prior evacuation. Even if this cycle is repeated, complete removal of oxygen is not assured and strict anaerobes may fail to grow in such jars (Willis, 1964).

The development of oxygen-free gassing systems and anaerobic cabinets

In 1946, Andreson devised a complex glass apparatus to provide an oxygen-free environment for the cultivation of anaerobes in liquid media. The apparatus was filled with nitrogen that had been rendered oxygen-free by passing it over red-hot copper. The use of oxygen-free

gases was adapted by Hungate (1950) in a technique for the cultivation of demanding cellulolytic bacteria. He seeded his culture tubes and prepared all media under an atmosphere of gas (hydrogen or nitrogen) that had been rendered oxygen-free by passing it through a chromous acid solution, or preferably over heated copper. Molten medium was dispensed into tubes, and the tubes seeded while being flushed with the oxygen-free gas. After sealing, the tubes were gently rolled until the agar had solidified, so that a thin layer of seeded agar was produced around the walls of the tube. Each tube was flushed by oxygen-free gas from a needle, and all manipulations were carried out under constant flushing by the gas. Hungate claimed that he was able to isolate very demanding anaerobes by the use of this "roll-tube" technique. Bryant and Burkey (1953) also found this technique to give good recovery of anaerobes from the bovine rumen. In 1966, Moore described methods for the isolation of fastidious anaerobes which were based on Hungate's work, in which all manipulations were performed under constant flushing by an oxygen-free gas, with all media being pre-reduced and anaerobically sterilized ("PRAS" media). Moore used a mixture of 3% hydrogen in 97% carbon dioxide in preference to the 100% hydrogen used by Bryant and Burkey; he rendered it oxygen-free by passing the gas mixture through a column containing heated reduced copper turnings.

Comparisons of the roll-tube technique with methods involving conventional anaerobic jar methods have indicated that higher recovery rates of demanding anaerobes can be achieved by the roll-tube technique, although these comparisons have usually related to organisms isolated from animals. For example, Spears and Freter (1967) found

that their roll-tube technique, involving flushing with oxygen-free CO_2 and the use of pre-reduced diluting fluids, gave higher counts than methods involving anaerobic jars. They were able to recover only 1-6 per cent. of the total microscopical cell count by the conventional method, whereas they could recover 20-34 per cent. by their roll-tube technique.

Similar results were obtained by Barnes and Burton (1970) when they compared these techniques in the recovery of anaerobic organisms from the caeca of squirrels.

Roll-tube techniques and gas flushing systems have been extensively used since the work of Hungate; for a fuller description of the details of the roll-tube technique, and recent improvements in gas flushing procedures, the reader is referred to the reviews of Barnes and Impey (1971) and Latham and Sharpe (1970).

Other workers have used oxygen-free gases in the cultivation of anaerobes by other methods. For example, Mitsuoka et al. (1969) described a "plate in bottle" method for the cultivation of fastidious anaerobes in which seeded plates of PRAS media were incubated in a glass bottle filled with oxygen-free CO_2. Any residual oxygen was removed by means of the activated iron wool method described by Parker (see above). They claimed that this method gave as good recovery rates as conventional methods.

Nordan (1970) criticised existing methods of achieving anaerobiosis on the grounds that if serial sampling of cultures was required, the environment was necessarily disturbed at each time of sampling. He therefore designed a gas flow apparatus in which liquid cultures could be incubated under a constant flow of oxygen-free gases

during sampling, without disturbance of the gaseous environment.

Similarly, certain disadvantages of the Hungate technique, notably the necessity for individual tube flushing, and the difficulty of sterilisation of the apparatus, have led to the development of methods in which oxygen-free gases are used to fill a gas-tight compartment ("anaerobic cabinet") and all manipulations, including pouring and inoculation of media are performed within this cabinet by means of gloves attached to apertures in the cabinet. Rosebury and Reynolds (1964) used a modification of the technique described by Socransky, MacDonald and Sawyer (1959) in which a vacuum-tight stainless steel glove box was used, with an air lock to permit passage of materials in and out of the cabinet. After preliminary evacuation, the cabinet was filled with a mixture of 10 per cent. hydrogen and 90 per cent nitrogen, the residual oxygen being removed by a system of room temperature catalysts (see above). They used this technique to recover anaerobic spirochaetes from human gingival scrapings.

Drasar (1967) used a "Perspex" glove box, filled with oxygen-free nitrogen containing 5 per cent. CO_2, to isolate demanding anaerobes from the human intestine. He used an electrically-heated inoculating loop, and ensured that plates of solid media were poured, dried and inoculated within the oxygen-free environment present in the cabinet, before being placed in an anaerobic jar which was removed via the gas-lock and incubated in the normal way. He claimed that recovery of anaerobes by this technique was up to 100-fold greater than that achieved by conventional techniques. Several other workers have used this technique for the isolation of demanding

anaerobes from human or animal specimens, and have claimed that the use of this method gives higher recovery rates of anaerobes than when conventional methods involving anaerobic jars are used (e.g. Lee, Gordon and Dubos, 1968; Aranki, Syed and Freter, 1968). The role of the anaerobic cabinet technique in anaerobic bacteriology is reviewed in the present study.

For full details of anaerobic cabinet techniques, the reader is referred to a recent review by Drasar and Crowther (1971).

The recovery of clostridial spores

In the design of procedures and media for the recovery and cultivation of sporing anaerobes, the clinical microbiologist must bear in mind the requirements of the spores for germination and outgrowth. For a detailed review of this complex field, the reader is referred to works such as those of Gould and Hurst (1969), and Holland, Barker and Wolf (1969); it is however necessary to consider here some of the factors involved in germination and outgrowth in relation to the methods of achieving anaerobiosis.

Many workers give conflicting reports of studies on the role of redox potential in the germination of clostridial spores. Knight and Fildes (1930) considered the effect of different E_h values on the "germination" of spores of the tetanus bacillus, noting that the time required for germination was shortened by decreasing E_h values, whereas there was an upper E_h value above which germination was totally inhibited. Although they used the word "germination" in their studies, their criteria for germination are more accurately applied to combined germination and outgrowth, as these two stages were not separated in their work.

The distinction between the E_h requirements for germination and those for outgrowth was emphasized by Hachisuka (1951) who observed that spores of Cl. tetani, when held in a complex medium under aerobic conditions (i.e. at positive E_h values) showed rapid inactivation. He suggested that this was due to germination taking place normally, the germinated spores then being inactivated by the aerobic conditions. There is little doubt that outgrowth of germinated spores requires anaerobic conditions (Shoesmith and Holland, 1968; Fujioka and Frank, 1966), but reports on the influence of E_h on germination itself seem conflicting. For example, Wynne, Mehl and Schmieding (1954) found that germination of spores of Cl. perfringens, Cl. chauvoei, Cl. botulinum type A, and putrefactive anaerobe PA 3679 could take place under aerobic conditions, whereas Hitzmann, Halvorson and Ukita (1957) found that the presence of oxygen inhibited the germination of spores of Cl. butylicum, Cl. acetobutylicum, and Cl. roseum, a finding confirmed for PA 3679 (Fujioka and Frank) and for Cl. botulinum type E (Ando and Iida, 1970).

Such discrepancies may be explained on a species-specific basis; for example Holland et al. (1969) found that although inhibition of germination by aerobic conditions was observed with spores of PA 3679 strain' h", no similar effect was observed with spores of Cl. bifermentans. The conflicting results can also be explained by possible differences in the test media used by different workers, and this is borne out by the finding of Fujioka and Frank that, in their test system, germination of PA 3679 was inhibited by aerobic conditions in an alanine-deficient medium, whereas virtually complete germination was obtained under aerobic conditions in an alanine-rich medium.

Similarly, it may be that requirements for germination differ in aerobic, as compared with anaerobic conditions, a finding reported by Shoesmith and Holland for spores of Cl. tetani.

Cultural methods for anaerobes often include addition of carbon dioxide to the gaseous environment, and there is evidence that this has a marked effect on the germination of some clostridial spores. Wynne and Foster (1948) found that carbon dioxide stimulated germination of a strain of Cl. botulinum, and Holland, Barker and Wolf (1970) showed that carbon dioxide (in the form of bicarbonate ion, HCO_3^-) increased the germination rates of the spores of three test clostridia, whereas omission of bicarbonate ion resulted in a pronounced fall in the rate and extent of germination, an observation confirmed by Ando and Iida for spores of Cl. botulinum type E.

As well as taking into account the effect of the ionic environment and the aminoacid composition of the medium on the germination of clostridial spores (Gould and Hurst, 1969), the bacteriologist attempting to recover anaerobic spores should take account of the studies of Futter and Richardson (1970a and b), who showed that cultural parameters such as temperature of incubation, time of incubation, pH, and gaseous environment markedly affect the recovery rates of clostridial spores. Their observation that spores damaged by heat seem to be more exacting in their cultural requirements may be relevant to the use of selective heating procedures.

As well as supplying suitable conditions for the germination of spores, the clinical bacteriologist must take care not to incorporate into recovery media substances that are inhibitors of germination or outgrowth; sodium thioglycollate is a well documented example (see

above) but there may be other inhibitory factors present analagous to the antisporulation factors for aerobic spore-formers described by Foster, Hardwick and Guirard (1950) and reported by them to affect clostridia. Even cysteine has been reported as inhibiting the germination of some clostridial spores (Gibbs, 1964); and the observation of Slepecky (1963) that phenethyl alcohol can inhibit germination and outgrowth of aerobic spores, might be relevant to the use of this substance as a selective agent in media for anaerobes if a similar inhibition occurs in the case of anaerobic spores.

It therefore follows that the isolation of anaerobes can be an inconsistent and unreliable exercise; in the presence of aerobic organisms it becomes much more difficult. For this reason various selective techniques have been developed to allow the growth of anaerobes and to suppress the growth of "unwanted" aerobic organisms.

Selective Procedures

Clinical specimens submitted for bacteriological examination generally contain a mixed bacterial flora, and the bacteriologist attempting to isolate anaerobic organisms from such specimens has to make use of selection procedures, which usually involve the use of heat, or of selectively inhibitory agents incorporated into culture media.

Heat has been used in the selective isolation of clostridia since the early years of this century (Hamilton, 1904), and continues to be in widespread use today (Willis, 1969; Wilson and Miles, 1964). The method depends on the fact that spores of clostridia are in general more heat resistant than the vegetative cells of aerobes or anaerobes; application of suitable amounts of heat will inactivate

all vegetative cells, but will leave the clostridial spores undamaged, and recoverable on suitable media. The disadvantages of this method are considered further in the present study.

The wide range of inhibitory agents that have been suggested as key constituents of selective media for the cultivation of anaerobes is testimony to the fact that no "ideal" selective media have so far been devised.

The earliest substances used in selective media were dyes. Thus Hall (1919, 1920) suggested the use of crystal violet or gentian violet in media, to inhibit the growth of aerobic contaminants in fluid cultures of clostridia, while Spray (1936) advocated the addition of a "tint" of crystal violet to semi-solid agar to suppress the growth of aerobic spore-forming organisms. Interest in crystal violet as an inhibitory agent waned after the discovery of antibiotics, but was revived when its use in a selective medium for clostridia was suggested by Blenden and Merilan (1961).

In 1953, Johansson suggested the addition of sodium azide to egg-yolk media for the selective isolation of lecithinase-producing anaerobes from faeces, and other media containing sodium azide have been developed by Lindsey et al. (1959), and Forget and Fredette (1962). However, the use of sodium azide as a selective agent seems limited by its toxicity; for example, Mossel et al. (1956) found that sodium azide at a concentration of 0.02 per cent. inhibited the growth of some food-poisoning strains of Clostridium welchii.

After a report by Lilly and Brewer (1953) that phenylethyl alcohol was of use in the inhibition of Proteus spp. in mixed culture, Dowell, Hill and Altemeier (1962) advocated its use in a selective medium

for the isolation of non-sporing anaerobes. More recently, the same workers discussed the possible use of phenethyl alcohol as a general selective agent for anaerobes, but warned that at the concentrations normally used (0.25 - 0.1 per cent.), it was inhibitory to other anaerobic organisms (Dowell, Hill and Altemeier, 1964).

Other substances that have been recommended include chloral hydrate (Finegold et al., 1971), tyrothricin (Sevin, Beerens and Spy, 1948), and more recently, chelating agents (Richardson, 1961). Richardson found that chelating agents such as derivatives of ethylene-diamine tetracetic acid (EDTA) can function as selective agents by altering the concentration of metallic ions in media so as to favour the growth of one organism while inhibiting the growth of another. He found that by varying the concentration of magnesium ions he could selectively isolate coagulase-positive strains of staphylococci while inhibiting the growth of coagulase-negative strains. Clearly the extension of such a concept to anaerobic bacteria seems worthy of trial.

The literature abounds with reports of the use of various antibiotics in media for the selective isolation of anaerobes; only the more important references are summarised here - for a more extensive review, the article by Finegold et al. (1971) is useful.

The finding that strains of Cl. welchii were resistant to streptomycin at concentrations inhibitory to most aerobes (Murray, Paine and Finland, 1947) led to reports of its possible use as a selective agent for clostridia (Willis, 1957; Lilly, 1958). However, Lilly discovered that whereas some strains of Esch. coli were resistant to concentrations of up to 800 µg per ml, the majority of strains of

Cl. welchii that he tested were inhibited by concentrations of greater than 100 µg per ml.

The use of neomycin as a selective agent was reported by Lowbury and Lilly (1955) who suggested a concentration of 100 µg per ml for the selective isolation of Clostridium welchii. Other selective media containing neomycin were described by Willis (1957) and Willis and Hobbs (1959), while Elston (1965) found blood agar media containing 5 mg neomycin per 100 ml, to be superior to media containing phenethyl alcohol (See above) for the selective isolation of clostridia from clinical specimens. There seems no doubt that media containing neomycin have now found general acceptance; their use is advocated in several well-known textbooks (e.g. Cruickshank, 1968; Willis, 1964).

The general acceptance of neomycin has occurred despite several adverse reports, commenting on the dangers of the use of high concentrations of neomycin. Lowbury and Lilly noted that some strains of Cl. welchii were inhibited by concentrations of neomycin greater than 100 µg per ml. In a later investigation Lilly (1958) found that although type A Cl. welchii strains could grow normally at concentrations of neomycin greater than 50 µg per ml, types B, C, D and E showed some inhibition of growth. Test strains of Cl. tetani and Cl. septicum showed some inhibition if concentrations of neomycin in excess of 25 µg per ml were used. Further studies with other clostridia showed that several organisms were inhibited by concentrations of neomycin at, or even below, that recommended by authors such as Cruickshank (Rutter, 1968; Spencer, 1969). All of these studies were performed with laboratory strains of clostridia but this effect is also observed with specimens submitted for diagnostic bacteriology: Horodniceanu and

Sasarman (1964) obtained fewer isolations of Cl. welchii from specimens of faeces or water when subcultured on to Willis and Hobbs medium than when the specimens were subcultured on to a blood medium without added antibiotics.

The use of Polymyxin was suggested by Mossel (1959); he recommended its use as a selective agent for clostridia, and his recommendations were endorsed by the later work of Angellotti et al. (1962) who sought a selective agent for the isolation of sulphite-reducing clostridia from faeces. After testing several antibiotics, these workers concluded that only Polymyxin B could inhibit non-anaerobic organisms such as faecal streptococci at concentrations that did not inhibit the growth of the clostridia. They recommended the use of media containing polymyxin with sulphadiazine, combined with iron sulphite (sulphite-polymyxin-sulphadiazine (SPS) agar.

Both kanamycin and vancomycin have been recommended as constituents of media for the selective isolation of non-sporing anaerobes (Dowell et al., 1962). The possible uses and drawbacks of such media have been extensively reviewed by Floch, Gershengoren and Freedman (1968) and Finegold, Sugihara and Sutter (1971). The latter workers review the whole field of selective agents for the isolation of anaerobes, and mention several complex media for this purpose.

Most workers recommend that antibiotics for the selective isolation of anaerobes should be incorporated in the media. Vera (1962) suggested a simpler technique, in which filter paper discs impregnated with kanamycin are placed on the surface of seeded media, and the plates are then incubated. Anaerobic organisms not inhibited by kanamycin can then be selected from areas immediately around the

disc. Such a method is simple, and commends itself to the busy clinical laboratory attempting to isolate anaerobic organisms from mixed cultures; the further development of this technique is reported by Sutter and Finegold (1971). These workers used the differing sensitivity patterns of the non-sporing anaerobes to six antibiotics as an aid to the identification of these organisms. A screening procedure could be based on this type of approach, if the antibiogram patterns can be confirmed by other workers; the variability of antibiotic sensitivity patterns is a likely drawback to this approach.

The wide variety of antibiotics used as selective agents indicates the difficulty of obtaining inhibition of aerobic organisms in mixed cultures without concomitant inhibition of anaerobic organisms. Several workers have tried combinations of inhibitory agents in an attempt to overcome this problem. For example Rogosa (1956) used basic fuchsin in combination with streptomycin in a selective medium for Veillonella species. He found that although this medium gave generally good results, it was inhibitory to some of the strains tested. Post, Allan and Reid (1967) described a medium for the selective isolation of Bacteroides organisms and related organisms from sewage, in which sodium azide, sodium desoxycholate and ethyl violet were combined in a basic medium of brain heart infusion agar.

Post and his co-workers used bile or bile salt derivatives as selective agents for intestinal anaerobes. Shimada and Finegold (1969) found that Gram-negative anaerobic bacteria show differing degrees of sensitivity to bile and desoxycholate agar, and this finding led them to suggest the possible use of these substances in

the design of selective media. In a later paper (Shimada, Sutter and Finegold, 1970) they show that the growth of most Gram-negative anaerobic organisms is inhibited by 0.1 per cent. desoxycholate solution in thioglycollate broth, and clearly the use of bile and desoxycholate as agents for the selective growth of anaerobes will require further development in order to be of practical use.

At present there does not appear to be any one ideal selective agent available for the isolation of either clostridia or of the non-sporing Gram-negative anaerobes, and Finegold et al. in their review (1971) conclude that a similar situation exists in the cases of the bifidobacteria and the lactobacilleae. The recent report of a new selective medium for Clostridium welchii in which cycloserine, at a concentration of 800 mg per ml is incorporated into 5 per cent. human blood agar (Fuzi and Csukas, 1970) seemed therefore to represent a possible advance in this field; in view of the known toxicity of neomycin for clostridia (see above), such a medium might prove useful in the selective isolation of other species of clostridia in addition to Cl. welchii. It is further evaluated in the present study.

It is clear that many of the reported studies in bacterial anaerobiosis have been based on the growth of test organisms in liquid media. When the growth of anaerobes on solid media has been studied in relation to practical problems of laboratory cultural procedures and the evaluation of new techniques, such studies have often suffered from a lack of adequate quantitation, and from the use of non-exacting models. The results of experiments designed with these pitfalls in mind may lead to a clearer understanding of the problems associated with the growth and recovery of anaerobes on solid media, and to the more rational development of improved procedures, especially for the diagnostic laboratory.

MATERIALS AND METHODS

Test strains

The following strains were used in the present study: Clostridium welchii (7 strains); Cl. oedematiens type A (4 strains); Cl. oedematiens type B (13 strains); Cl. oedematiens type D (3 strains); Cl. septicum (7 strains); Cl. tetani (5 strains); Cl. histolyticum (2 strains); Cl. bifermentans (2 strains); Cl. chauvoei (2 strains); Cl. sporogenes (1 strain); Cl. butyricum (1 strain); Anaerobic coccus, Hare's Group I, (1 strain); Bacteroides fragilis (1 strain); B. necrophorus (2 strains); B. melaninogenicus (2 strains).

Further details and a note on the source of each of the above strains are given in Appendix A; unless stated otherwise in the text, they were subcultured in cooked-meat broth (CMB).

The following organisms were subcultured in nutrient broth (NB) after isolation from clinical material and identification: 1 strain of Streptococcus pyogenes (Group A); 2 strains of Staphylococcus aureus; 1 strain of Neisseria gonorrhoeae; 1 strain of Bacillus anthracoides; 2 strains of Haemophilus influenzae; and 1 strain of Veillonella sp.

Frequent checks of the identity and purity of test strains were made, by reference to colonial morphology, Gram-staining properties and biochemical reactions. In the case of anaerobic organisms, seeded plates were incubated aerobically to detect aerobic contamination. In the case of aerobic organisms, parallel plates were incubated anaerobically.

Culture media

Cooked-meat broth (CMB) was used as a routine in the present study. It was prepared as described by Cruickshank (1968, p. 757), but the infusion broth component was made from nutrient broth (Oxoid) and the meat particles were prepared in the author's Department. Oxoid nutrient broth was used in addition for the subculture of some of the test organisms. "Pre-steamed nutrient broth" was used as a diluent in the quantitative studies; it was heated by steaming at 100°C for 30 min. to remove dissolved air, then cooled rapidly to 37°C.

Blood agar plates were prepared with Oxoid blood agar base No. 2 enriched with equine blood (10 per cent.) or human blood (16 per cent. or 33 per cent.) as specified in the text. Reinforced clostridial medium (Oxoid) was used in early studies; in later studies it was supplemented with 10 per cent. horse blood as detailed by Drasar and Crowther (1971). "Chocolated" versions of the blood media, as specified in the text, were prepared by heating the blood agar media at 80°C for 10 min. before pouring the plates.

Additions to blood agar media were prepared as sterile solutions and these were added to molten blood agar medium at a temperature of about 45°C, and plates were then poured.

Watt's modifications of Moore's medium (cysteine-dithiothreitol human blood agar, CDHBA)

CDHBA 16. The following solution was prepared immediately before use and sterilised by Millipore filtration: cysteine hydrochloride (Koch-Light) 100 mg per ml, dithiothreitol pure (Koch-Light) 9 mg per ml, in distilled water. To 100 ml of sterile molten Blood Agar Base No. 2

(Oxoid) at 45-50°C was added 20 ml human blood and 1 ml of the sterile cysteine-dithiothreitol solution. After mixing of the medium by careful inversion, plates were poured. The plates were allowed to set and their surfaces were then dried in a hot air oven at approximately 60°C for 10 min. The final concentration of ingredients in this medium was cysteine hydrochloride 82 mg per 100 ml, dithiothreitol 7.5 mg per 100 ml and human blood 16 per cent.

CDHBA 33. In a later modification, pure cysteine (Koch-Light) 50 mg per ml was used in place of cysteine hydrochloride in the initial solution, and the concentration of the dithiothreitol was reduced to 4.5 mg per ml. 2 ml of this solution was added to 66 ml of 1.5 strength molten Oxoid Blood Agar Base No. 2 at 45-50°C. 33 ml of human blood was then added, and the medium was prepared as above. The final concentration of ingredients was cysteine 100 mg per ml; dithiothreitol 9 mg per 100 ml; and human blood 33 per cent.

Cysteine-dithiothreitol equine blood agar (CDEBA)

When an outbreak of serum hepatitis occurred in Edinburgh in 1970-71, it was decided to stop the use of human blood in blood agar media. In later studies therefore, a further modification (cysteine-dithiothreitol equine blood agar, CDEBA) was prepared by substituting 10 per cent. horse blood for 33 per cent. human blood in CDHBA 33. CDEBA medium therefore contained cysteine 100 mg per 100 ml; dithiothreitol 9 mg per 100 ml; and equine blood 10 per cent. All media were used within 30 min. of being poured unless stated otherwise in the text.

Catalase-free medium

The author is indebted to Dr J. G. Collee for details of the

following medium:

4 per cent. proteose peptone (Difco)

2 per cent. yeast extract powder (Oxoid)

1.2 per cent. agar (Davis)

Make up to 1 litre with distilled water

Adjust pH to 7.4

Autoclave at 121°C for 20 min.

This medium showed no catalase activity as measured by the lack of evolution of gas produced on addition of a 0.02-ml drop of a solution of Hydrogen Peroxide solution (Analar "100 vol.") to the plate. Blood agar plates showed vigorous effervescence of gas by this test.

"109" Medium. T.C. Medium NCTC 109, Dried, ("109" medium) was supplied by Difco Laboratories, Detroit, Michigan, U.S.A. 250 ml of medium was prepared according to the manufacturer's instructions (i.e. 2.425 g dehydrated medium + 0.5 g sodium bicarbonate "Analar" were added to 250 ml distilled water) and sterilised by membrane filtration at 4°C. 4.8-ml amounts were then dispensed aseptically into stoppered glass tubes. The detailed composition of the medium is given in the text (p. 191).

"109S" Medium. A solid version of 109 medium, "solid 109" or "109S" medium was prepared as follows:-

109 medium was prepared as above, but at double strength. A solution of 1.2 per cent. Ionagar No. 3 (Oxoid) in 0.85 per cent. saline was prepared and sterilised by autoclaving. 10-ml amounts of the molten saline agar were poured into sterile plastic Petri Dishes and allowed to set. Equal volumes of double strength 109 medium and a 2.4 per cent. aqueous solution of Ionagar No. 3 were mixed at 56°C,

and 5-ml amounts of the resulting mixture were poured on to the saline agar to form "layer" plates. Thus each plate of solid medium contained 10 ml saline agar + 5 ml 109 medium in aqueous Ionagar. The plates were allowed to cool, and dried for 10 min. in a hot air oven at 60°C.

Enumeration methods

Total counts

In my initial studies, total counts were performed in a Helber bacterial counting chamber (Hawksley Ltd.) with a Thoma ruling and 0.02 mm depth; at least 400 bacteria were counted from a suitable dilution of the parent culture. Two or more observations were performed by two independent workers, in each experiment, and the results were collated. Bacteria were allowed to settle in the counting chamber for c. 5 min. before enumeration.

In the latter part of the study, the writer took account of the observations of Norris and Powell (1961) and Cook and Lund (1962) on the errors associated with the use of counting chambers of 0.02-mm depth, and a Hawksley counting chamber (B.S. 748) of 0.1-mm depth was used. Counts were made using phase-contrast microscopy, and separate counts of spores and vegetative cells were performed. All phase-contrast studies were done with a Zeiss R.A. 38 microscope fitted with a special condenser. A chain of organisms was counted as one colony-forming unit (c.f.u.). The number of spores was estimated during the total count procedure and spores were also enumerated in separate thin wet films. The author notes that the presence of fat globules from CMB cultures could be mistaken for free spores in phase-contrast preparations; to avoid errors from this source, the percentage of spores

present was checked by observations made on stained smears (see below).

In some demanding studies, young inocula were used, in which the number of spores present was expected to be small. In these studies large numbers of vegetative cells (more than 1000 and on occasion up to 5000) were counted, in order to ensure that no significant numbers of spores were present.

Viable counts

Viable counts were performed with pour-plate or spread-plate methods, as indicated. In the case of pour-plate methods, dilutions of the culture were prepared in sterile pre-steamed nutrient broth, and the mixtures were agitated gently by careful inversion or rolling to minimise aeration. An appropriate volume of each dilution was added to the cooling culture medium at about 45°C. Deep colonies appearing after anaerobic incubation were counted with a mechanical colony counter. Pour plates were prepared from Oxoid Reinforced Clostridial Medium (RCM) with 5 per cent. human blood added.

Surface (spread-plate) counts were derived from dilutions made in pre-steamed nutrient broth. 0.02-ml inocula were dropped from standard calibrated pasteur pipettes and the drops were spread with sterile glass spreaders of uniform size (see page 88). Colony counts were calculated as the mean number of colonies grown on two or more plates from a given dilution, and this figure was multiplied by the appropriate dilution factor to give the surface viable count per ml. The dilutions used to calculate the viable counts were those that gave 100-700 evenly distributed colonies per plate.

Spore studies

The spore estimates obtained by phase-contrast microscopy (see above) were checked by stained smears. Estimates of the percentage of spores present were obtained from Gram-stained smears, and from smears stained for spores by the malachite green procedure of Ashby (see Cruickshank, p. 657). Occasional checks were also made with an acid-fast staining procedure in which a 2 per cent. solution of nitric acid in absolute ethyl alcohol was used as the decolorising agent (Cruickshank, p. 657).

Statistical methods

The statistical analysis of results obtained in the present study was performed under the guidance of Miss Margaret V. Hoare. Where possible, results were expressed as "mean $\pm$ standard error of the mean (S.E.)". For simple comparisons of two treatment means, the t test was used. For more complex studies, in which a comparison of more than two treatment means was necessary, analyses of variance were used. These techniques are discussed in detail in Appendix D.

In the present study, the following convention has been adopted for levels of probability (P):

differences between means are "not significant" when $p > 0.05$;

differences between means are "significant" when $p < 0.05$;

differences between means are "highly significant" when $p < 0.01$;

differences between means are "very highly significant" when $p < 0.001$.

Electron microscopic studies

Preparation and examination of specimens was performed with the help of Dr J. R. W. Govan. The specimens were prepared as follows:

The surface growth from a plate of EBA medium was harvested in sterile distilled water, and fixed for 48 hr with 0.25 per cent. formalin* at 4°C. Following centrifugation at 3000 r.p.m. for 15 min., cells were resuspended in 1 per cent. ammonium acetate at pH 7.0.

Preparations were examined by means of a negative staining technique (Brenner and Horne, 1959) as follows:

Equal volumes of specimen and 2 per cent. phosphotungstic acid at pH 7.0 were mixed and a drop of the resultant suspension transferred to the surface of a collodion membrane supported on a copper electron microscope grid. After drainage of excess moisture with filter paper, the preparation was dried in a desiccator over anhydrous $CaCO_3$.

On occasion, a drop of specimen was allowed to dry on to the surface of the collodion membrane and shadow cast with gold-palladium alloy (60:40) at an angle of 15°.

Specimens were screened at maximum brightness in an electron microscope (Associated Electrical Industries EM6) at 50 KV.

Miscellaneous procedures

Pressure measurements. These were performed using a vacuum gauge (Proops Instruments Ltd., Tottenham Court Road, London). If small changes in pressure were being monitored, a simple mercury manometer gave satisfactory results.

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* i.e. 0.01 per cent. formaldehyde.

pH measurement. A Pye Unicam 292 pH-meter was used.

Heating. Culture samples were held in pre-heated bijou bottles totally immersed in a water-bath at the stated temperature for the stated time, as specified in the text, and were then rapidly cooled to temperatures below 50°C in running water at a temperature of 16-18°C.

Standard anaerobic procedure. The development of a standardised procedure for the setting-up of the BTL anaerobic jar (hydrogen 90 per cent., carbon dioxide 10 per cent.) is discussed in the text (see page 81). All culture media were incubated at 37°C.

Drying of plates

All plates of solid media were allowed to set and then dried in a hot-air oven at 60°C for 10 min. before use. "Well-dried" plates were dried for at least 20 min. at 60°C before use.

Antibiotic sensitivity testing. Antibiotic sensitivity testing was performed by the disc diffusion method, using discs impregnated with antibiotic solutions as detailed at page 159, and the sizes of the zones of inhibition were measured.

Animal studies. All animals were obtained from local laboratory stock. White mice (strain Balb/c) were used in toxigenicity tests for Cl. tetani strains.

Toxigenicity tests

The toxigenicity of strains of Cl. tetani was confirmed by injecting 5-8 day CMB culture supernates into unprotected mice and into mice protected by administration of 0.5 ml equine antitoxin given intraperitoneally 1 hr before injection of the culture supernate. The test material was injected into the tissues at the right of the base of

the tail. All strains tested gave evidence of classical features of tetanus when injected into unprotected mice; mice protected by antitoxin failed to develop any features of the disease.

Serological neutralisation tests

The identity of test strains of Cl. oedematiens was checked by means of procedures developed by Rutter and Collee (1969).

Anaerobic cabinet

The design and construction of the anaerobic cabinet is described on pages 248-252, together with a description of the materials used.

Fluorescent antibody studies

A smear of the test culture was fixed by gentle heat. One drop of labelled anti-Cl. oedematiens globulin (Fluorescent anti-clostridial globulin, Wellcome Research Laboratories, Beckenham, Kent) was spread evenly over the smear, and allowed to react in a moist chamber for 30 min. at room temperature. The excess reagent was removed by washing with buffered saline (0.15 M Sodium phosphate in saline, pH 7.1) and the smear was then washed for 10 min. in several changes of buffered saline. After careful drying the smear was mounted in buffered glycerin and examined under ultra-violet light.

Reagents and items of equipment

Dithiothreitol. Dithiothreitol (Cleland's reagent) was initially obtained from Koch-Light Laboratories Ltd., Colnbrook, England. In later studies, supplies were obtained from B.D.H. Biochemicals Ltd., Poole, England. The activity of the dithiothreitol was checked periodically by the method of Ellman (1959) for the estimation of reactive thiol groups. No loss of activity was observed when the

dithiothreitol was stored under dry conditions at 4°C in the dark.

Cysteine. L-cysteine hydrochloride (Koch-Light) was used in early studies (see above). In later work, pure cysteine (Koch-Light) was used in place of cysteine hydrochloride.

L-ascorbic acid. L-ascorbic acid ("Analar" grade, B.D.H.) was prepared as a stock solution containing 60 mg per ml in distilled water and sterilised by Millipore filtration.

Cobalt chloride. Cobalt chloride ("Analar" grade, B.D.H.) was prepared as a stock solution containing 1.6 mg (0.4 mg Co^{++}) per ml, and sterilised by Millipore filtration. Amounts of this stock solution were added to blood agar media and "109" medium, as specified in the text, to give final concentrations of 1, 4 and 10 µg Co^{++} per ml.

Other sulphydryl compounds. Cysteamine, penicillamine and glutathione were kindly supplied by Professor G. S. Boyd, Department of Biochemistry. 10 milli-Molar and 50 milli-Molar stock solutions of each compounds were prepared in distilled water, and sterilised by Millipore filtration. Varying amounts of the sterile stock solutions were separately tested on blood agar medium at final concentrations of 1 milli-Molar and 5 milli-Molar.

Menadione. This was supplied by Sigma (London) Chemical Company Ltd., London, England, and stored at room temperature in the dark. A stock solution containing 200 µg per ml was prepared by dissolving 20 mg in 2 ml ethyl alcohol, and diluting this to 100 ml in distilled water. The solution was sterilised by Millipore filtration, and stored at 4°C in the dark.

Catalase. Catalase ("ex beef liver lyophilised", Koch-Light) was prepared as stock solutions containing 10 mg catalase per ml and 100 mg

catalase per ml. These were sterilised by Millipore filtration and used immediately.

Cycloserine. Cycloserine powder was kindly donated by Eli Lilly & Co., Basingstoke, England. It was stored at 4°C, and stock solutions containing 80 mg cycloserine per ml and 20 mg cycloserine per ml in distilled water were prepared immediately before use and sterilised by Millipore filtration. 1 ml of the appropriate stock solution was added to 100 ml aliquots of molten blood agar medium to give final concentrations of 800 µg cycloserine per ml and 200 µg cycloserine per ml, respectively.

Neomycin. Neomycin sulphate was purchased from Upjohn of England Ltd., Crawley, England. A stock solution of 50 mg neomycin per ml was prepared in distilled water and sterilised by Millipore filtration. Varying amounts of the stock solution were added to molten blood agar medium to give final concentrations ranging from 20 to 200 µg neomycin per ml.

Ethylene-diamine tetra-acetic acid (EDTA). This was supplied ("Analar" grade) by B.D.H. Biochemicals Ltd. A stock solution containing 100 mg EDTA per ml was prepared in distilled water, and sterilised by Millipore filtration. Varying amounts of the sterile stock solution were added to molten blood agar to give final concentrations ranging from 10 mg EDTA per ml to 100 mg EDTA per 100 ml.

TRIS (hydroxymethyl) aminomethane (TRIS buffer). This was supplied by Koch-Light Laboratories Ltd. A stock solution containing 100 mg TRIS per ml was prepared in nutrient broth (Oxoid) and sterilised by Millipore filtration. Varying amounts were added to molten blood agar media to give final concentrations of TRIS ranging from 10 mg per

100 ml to 500 mg per 100 ml.

Blood. Defibrinated horse blood was supplied by Wellcome Laboratories. Outdated citrated human blood was obtained from the Blood Transfusion Department, Edinburgh Royal Infirmary; this contained disodium citrate (2 g) and dextrose (3 g) per bottle.

Anaerobic jars. Fourteen BTL (Baird and Tatlock Ltd., Chadwell Heath, Essex) anaerobic jars were used in the present study. One polycarbonate jar (Baltimore Biological Laboratories, BBL) was kindly supplied by Becton Dickinson and Co.

Catalyst sachets. Unless stated in the text, the BTL jars were each equipped with a single room-temperature catalyst sachet. Each sachet contained 20 catalytic pellets (mean of 10 sachets examined). The sachets were renewed every 2-3 months routinely, or more frequently if indicated (see text).

Gases. Cylinders of carbon dioxide gas were supplied by Distillers Company Ltd. (DCL). Cylinders of the following gases were supplied by British Oxygen Company (BOC): hydrogen; nitrogen; 10 per cent. carbon dioxide with 90 per cent. hydrogen; and 3 per cent. hydrogen with 97 per cent. nitrogen.

"Gaspak" disposable gas generator envelopes. These were supplied by Becton, Dickinson and Company.

Indicators of anaerobiosis. In the present study, external indicators of anaerobiosis were not used (see p. 264 for a discussion of this point). Reliance was placed on the prompt development of a secondary vacuum as an index of catalytic activity. In studies with the Gaspak system in conjunction with the BBL anaerobic jar, there was no means of pressure monitoring and reliance had to be placed on the

Gaspak Disposable Indicator which was placed inside the clear jar. This is discussed in the text (p. 221).

Particulate iron. Crude iron filings were obtained from local laboratory stock and sterilised in a hot air oven at 160°C for 1 hr. In later experiments, particulate iron was obtained as the following B.D.H. preparations: (i) iron powder about 90 mesh; (ii) iron powder (electrolytic); and (iii) iron metal powder reduced by hydrogen.

Other particulate metals. Manganese powder and finely-powdered nickel were obtained from Messrs Hopkins and Williams Ltd., Chadwell Heath, Essex.

Antisera. The following antisera, donated to Dr J. G. Collee from Wellcome Laboratories, were used in the present study:-

Cl. tetani: experimental bovine, equine and ovine antitoxic sera.

Cl. septicum: antitoxic sera Ex 2958; Ex 3567; Ex 1825 and Ex 5655. Antitoxic sera No. Ex 3567 was found to be effective in controlling the spreading of Cl. septicum strains (see p. 95) and was used thereafter to control the spreading growth of strains of this organism.

Cl. chauvoei: Wellcome experimental Cl. chauvoei antitoxic serum No. 1664 was used in the present study.

All antisera used in the present study were applied to plates as 0.1-ml drops, which were then spread. Both undiluted sera and sera diluted 1 in 5 in saline, were used. The antitoxic potencies of these sera are not specified here; there was no correlation between any of these values and ability to inhibit spreading growth of the homologous organisms.

Saline . A solution of 0.85 per cent. NaCl in distilled water was sterilised by autoclaving.

Gelatin saline . A solution of 2 per cent. gelatin in saline was sterilised by autoclaving.

Ringer solution. This was prepared as detailed in Cruickshank (1968, p. 862) and sterilised by autoclaving.

Sodium bicarbonate. Sodium bicarbonate ("Analar" grade) was supplied by B.D.H. Biochemicals Ltd.

Phosphate buffer . This was prepared as detailed in Cruickshank (1968, p. 854).

Abbreviations. The following abbreviations are used in the text: BTL = Baird and Tatlock Ltd.; CDEBA = cysteine-dithiothreitol equine blood agar; CDEBAIF = CDEBA sprinkled with sterile iron filings; CDHBA = cysteine-dithiothreitol human blood agar; CDHBAIF = CDHBA sprinkled with sterile iron filings; cm = centimeter; CMB = cooked meat broth; EBA = equine blood agar; EBAIF = EBA sprinkled with sterile iron filings; EDTA = ethylene-diamine tetra-acetic acid; E_h = oxidation-reduction (redox) potential; g = grammes; Hg = mercury; hr = hour; l = litre; mg = milligramme; µg = microgramme; min. = minute; ml = millilitre; mm = millimetre; mV = millivolt; NB = nutrient broth; pO_2 = partial pressure of oxygen; p.p.m. = parts per million; P/S NB = pre-steamed nutrient broth; RCM = reinforced clostridial medium; r.p.m. = revolutions per minute; THAM = TRIS buffer = tris (hydroxymethyl aminomethane); V = volt.

EXPERIMENTAL OBSERVATIONS

THE DEVELOPMENT OF A DEMANDING MODEL FOR PRACTICAL STUDIES IN BACTERIAL ANAEROBIOSIS

In order to investigate some of the practical problems related to bacterial anaerobiosis, it was necessary to develop a suitable laboratory model. Such a model required to be an organism that was a strict anaerobe, preferably demanding in its requirements for anaerobiosis, and that could be grown by conventional anaerobic procedures at the bench. It appeared that Clostridium oedematiens types B and D might be suitable; both required stringent conditions of anaerobiosis, and both were difficult to culture in the laboratory. Three biotypes (A, B and C) of Cl. oedematiens were defined by Oakley, Warrack and Clarke (1947) on the basis of the distribution of soluble antigens. More recently, Cl. oedematiens (Cl. novyi) has been divided into four types (A-D) on the basis of the toxicological analysis of supernatants from broth cultures and by toxin neutralization tests on plate cultures of growing organisms (see Willis, 1969). Latterly, the sugar fermentation reactions of the organism have been used for typing (Rutter, 1968). Of the four types described, type A has been associated with infections in man, while the other types cause a variety of diseases in animals. All types require strictly anaerobic conditions for growth in the laboratory, although type-A strains are the least exacting in terms of their requirements for anaerobiosis (Willis, 1969). Types B and D are difficult to grow in the laboratory, especially if surface culture methods are used.

Failure to obtain consistently reliable surface growth of types B and D on solid media has been reported by several workers.

Albiston (1927) was unable to grow a type-B strain on solid media, and Keppie (1944) could not achieve reliable recovery of type-B strains from infected tissues; similar findings were reported by Williams (1962, 1964), who noted that frequent failures of growth of type-B and type-D strains of Cl. oedematiens occurred on solid media. Rutter summarised some of the difficulties associated with the culture of these organisms, difficulties confirmed by Willis in his observation that type-B strains of Cl. oedematiens are more difficult to grow than type-A strains, whilst "type-D strains are probably the most fastidious anaerobes known".

It was necessary to improve the surface growth of these organisms in order to develop quantitative techniques for the assessment of some of the factors involved in the growth of anaerobes on solid media.

Previous approaches to the cultivation of demanding strains of Cl. oedematiens have involved the use of subsurface culture methods to exclude oxygen, or the use of complex liquid media such as fresh brain infusion, presumably to cater for nutritional needs (Smith, 1967). Willis (1964, p. 83) considered that Cl. oedematiens is so sensitive to oxygen that death of the organism would occur unless seeded plates are immediately placed in anaerobic jars for incubation. Rutter noted that the irregular growth of type-B and type-D strains, which was in contrast to the reliable surface growth of type-A strains, occurred both with simple media and with much more complex media.

The writer soon confirmed the difficulties encountered by previous workers. Although strains of types B and D grew well in anaerobically incubated cooked meat broth (CMB) as prepared by

Cruickshank (1968, p. 757), and sparse growth could be achieved by means of "shake cultures" (i.e. cultures grown in bottled agar media seeded with 0.5-ml inocula before solidification), pour plate methods, using nutrient agar with added human blood (10-20 per cent.), gave irregular and poor results even when incubated anaerobically.

Better results were obtained if Reinforced Clostridial Medium (RCM, Oxoid) was used in the blood agar medium. Growth on the surface of seeded plates was irregular and unreliable, even when nutrient agar containing 20 per cent. of human blood was used. When colonies were present, they were small and were poorly haemolytic on blood agar media; often no growth occurred, even after 72 hr incubation. Thus, it was impossible to achieve consistently reliable growth of Cl. oedematiens of types B and D on the surface of seeded plates, a finding previously noted by Rutter (1968). In an effort to produce satisfactory surface growth, it was first necessary to determine whether defects in anaerobic technique were contributing to the inconsistent growth of these demanding anaerobes.

An appraisal of anaerobic jar procedure

The anaerobic jar in routine use in our laboratory is the BTL anaerobic jar (Baird and Tatlock, Ltd.) incorporating a catalyst active at room temperature. Although the instruction leaflet supplied with the jar recommends that 6/7 of the air should be removed by means of evacuation before the admission of hydrogen, workers such as Willis (1964, p. 10), Collee (1968), and Meynell and Meynell (1970) suggest that only 2/5 - 3/5 of the air present in the jar should be evacuated. Although the unreliable surface growth of type B and D

strains made quantitative assessments impossible at this stage, preliminary qualitative experiments suggested that more effective growth was obtained if 6/7 of the air was removed than if only 2/5 - 3/5 was removed.

Pressure changes and the demonstration of catalytic activity

Considerable pressure changes occur in a BTL jar equipped with an active room-temperature catalyst if 2/5 of the air is removed during the evacuation stage. The oxygen present in the 3/5 of the air remaining is capable of combining with the admitted hydrogen, with the formation of water and the development of a "secondary" vacuum in the jar. If 6/7 of the air is removed, there is much less oxygen available to combine with the hydrogen, and the pressure changes that develop are on a smaller scale (Table 1).

From a series of such experiments, it was found that the rapid development of a secondary vacuum in an anaerobic jar is a satisfactory indication of the activity of the room-temperature catalyst sachet.

The use of carbon dioxide in the anaerobic jar procedure

Several workers have drawn attention to the stimulatory effects of carbon dioxide on the growth of micro-organisms; in addition to its effects on the germination of spores (see p. 51), carbon dioxide has been noted to be of use in the cultivation of aerobic organisms (Rose, 1942) and its use has been advocated in the cultivation of anaerobes (e.g. Willis, 1964; Hobbs, Williams and Willis, 1971). It was decided to add 10 per cent. carbon dioxide routinely to the gaseous environment in anaerobic jars; the effect of this procedure on the growth and recovery of anaerobes on solid media is considered later (p.186).

TABLE 1

The internal pressure changes produced in a BTL anaerobic jar equipped with a new catalyst, when different procedures were used

Time after evacuation (min)	Pressure (mm Hg)* recorded after evacuation procedure**			
	A	B	C	D
0	A P	A P	A P	A P
1	- 30	- 20	- 18	- 14
2	- 60	- 30	- 24	- 18
4	- 90	- 60	- 44	- 24
6	-110	- 76	- 46	- 27
8	-120	- 88	- 52	- 28
10	-120	- 96	- 64	- 30
20	-124	-126	- 70	- 30
30	-136	-140	- 80	- 34
40	-136	-150	- 84	- 40
60	-136	-148	- 90	- 40

* pressure reading in mm Hg below atmospheric pressure (AP). All readings recorded with a simple mercury manometer at room temperature (c. 16°C).

** A = removal of 2/5 of contained air by evacuation, followed by admission of hydrogen to restore pressure to atmospheric pressure.

B = as A, with removal of 3/5 of contained air by evacuation.

C = as A, with removal of 6/7 of contained air by evacuation.

D = as C, with admission of a mixture of 10 per cent. carbon dioxide and 90 per cent. hydrogen to equilibrate to atmospheric pressure.

The standardised anaerobic jar procedure

In order to ensure that a standard procedure was followed, the following technique was introduced:

> 6/7 of the air was evacuated from the jar by means of a vacuum pump, the pressure changes being monitored by a vacuum gauge. The resultant pressure (-660 mm Hg or 100 mm Hg) was brought up to 160 mm Hg by admission of carbon dioxide from a rubber bladder, and then hydrogen was admitted, from a separate bladder, until the pressure in the jar returned to 760 mm Hg (i.e. atmospheric pressure). This ensured that the jar now contained a gaseous environment of 10 per cent. carbon dioxide + 90 per cent. hydrogen.

The jar was then attached to a simple mercury manometer, and the pressure checked after 10 min. It was found that in the presence of a normally active catalyst, a vacuum of at least 10-20 mm Hg occurred within 10 min. after admission of the hydrogen. This rapid development of a secondary vacuum is in contrast to the gradual development of a negative pressure that occurs after several hours in a jar without a catalyst (Rutter, 1968).

The BTL jar is equipped with a side arm to which is attached a methylene blue indicator of anaerobiosis. Experience showed however, that this indicator was insensitive and unreliable, failures of growth of type D strains occurring in the presence of apparently satisfactory conditions of anaerobiosis as shown by the indicator. Moreover, the time taken for the indicator to react was sometimes considerable, and leaks often developed at the rubber tubing joining

the indicator tube to the metal side-arm. For these reasons, the use of indicators was discontinued in this present study, although the problems associated with the use of anaerobic indicators merit further attention. The indicator tubes were therefore removed from all BTL jars used in the present study, and the metal side tubes carefully sealed.

The development of a secondary vacuum gave a rapid indication of catalytic activity; failure to achieve a vacuum of 20 mm Hg within 10 minutes was considered to be a requirement for immediate renewal of the catalyst. By means of this procedure, failure of a catalyst could be detected before incubation, rather than afterwards, and a new one fitted, so that any defects could be speedily remedied, and satisfactory conditions of anaerobiosis could be achieved.

The standard anaerobic procedure adopted in the laboratory is illustrated diagrammatically in Fig. 2 ; it is accompanied by frequent cleaning of the jars with spirit followed by water, by repeated checking of the jars for leaks, and by attention to the maintenance and lubrication of the needle valves. Possible faults that can arise while operating the BTL jar are considered on page 283.

A study of problems associated with the surface growth of Cl. oedematiens

The standard anaerobic technique described above allowed growth of Cl. oedematiens type A strains on blood agar media, but growth of type-B and especially type-D strains continued to be unreliable, failures of growth being frequent even after prolonged incubation. In an effort to improve the situation, many test reagents were added to human blood agar media, either mixed with the

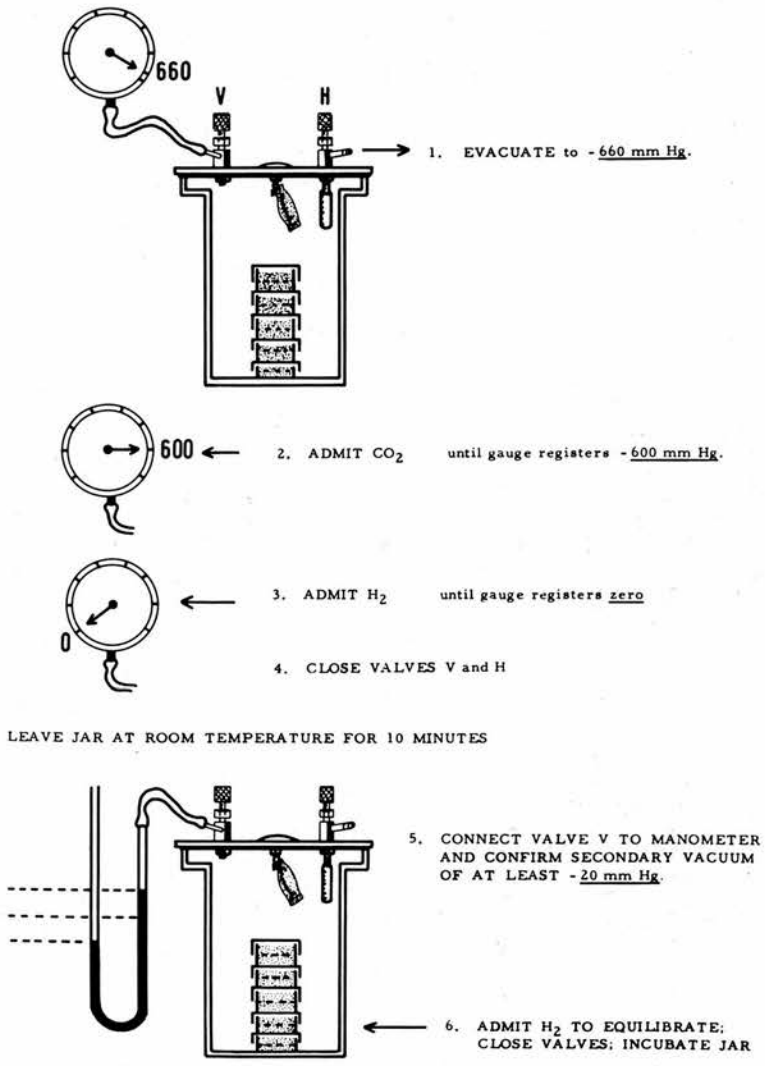


FIGURE 2. - The standard anaerobic procedure for the BTL anaerobic jar.

inoculum at the time of plating out or added to the medium after seeding with the inoculum. Of all the reagents tested, the only material giving a significant and marked effect on the surface growth of type-B and type-D strains was particulate iron.

The effect of particulate iron on the surface growth of Cl. oedematiens

Note. The following observations on the effect of particulate iron on the surface growth of the Cl. oedematiens model were originally made by Rutter (1968). It was at this point that the present author became involved in these studies, and was able to confirm Rutter's baseline observations and extend them to other models.

When heat-sterilised iron filings were sprinkled on to the surface of blood agar plates seeded with inocula derived from CMB cultures of type-B and type-D strains, subsequent anaerobic incubation yielded consistently reliable growth of these organisms (see Fig. 3). Even after 24 hr incubation, colonies were well developed, and it was notable that they developed in association with particles of iron deposited on the surface of the medium. Similar results could be obtained if iron filings, suspended in water and sterilised by autoclaving, were mixed with the inoculum in the well of a plate before streaking out with a loop. Iron filings from several sources all produced this enhancement of growth (see Materials and Methods); all were ineffective if incorporated into molten agar. However, if the inoculum was seeded into the molten agar and the sterile particulate iron was then sprinkled on to the solidified surface, enhancement of growth of colonies in the underlying medium was

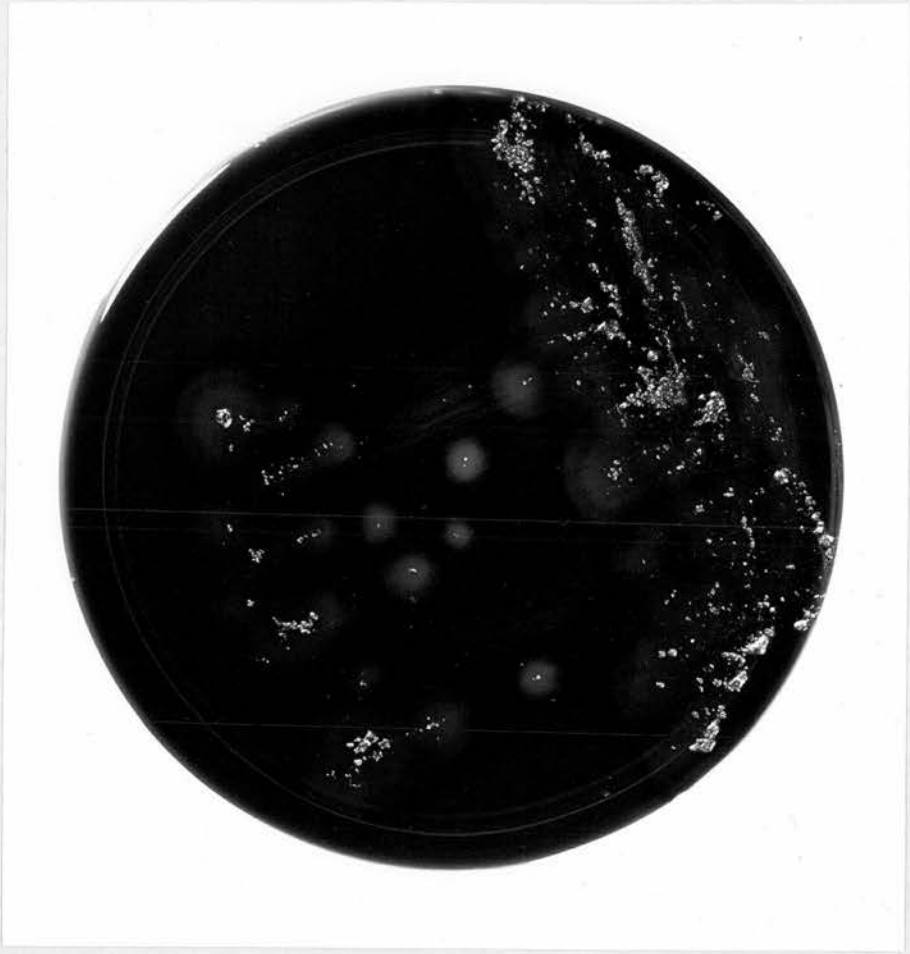


FIGURE 3. - The growth of Clostridium oedematiens type D on human blood agar sprinkled with sterile iron filings.

observed.

The stimulatory effect of iron filings on the growth of Cl. oedematiens types B and D could not be reproduced by the substitution of other sterile particulate materials. Inert materials such as carbon or crushed egg-shell gave negative results, and metallic salts, including both ferrous and ferric salts, were also ineffective. The only materials that gave even slightly positive results were palladinised asbestos and particulate nickel.

The possible use of sterile iron filings to enhance the growth of other anaerobic organisms is considered later (p. 136).

The "iron filings" technique, although ensuring reliable surface growth of type-B and type-D strains of Cl. oedematiens, had the disadvantage that accurate counting of colonies was difficult, as the individual iron filings tended to obscure the colonies. In addition, colonies tended to group around areas in which there was a high density of iron filings, often coalescing and making accurate enumeration impossible. An alternative method of producing consistently reliable growth of these organisms was then explored.

The cysteine-dithiothreitol system in the surface growth of Clostridium oedematiens types B and D

At this point in the work, Moore (1968) described a complex medium that allowed reliable surface growth of Cl. oedematiens type B. The essential components of this medium seemed to be (i) the amino-acid cysteine and (ii) dithiothreitol (DTT), a substance first described by Cleland (1964) as being a protective reagent for thiol groups. The function of the dithiothreitol (DTT) in Moore's medium appeared to be to protect the labile thiol groups of cysteine from

oxidation on exposure to aerobic conditions.

The author incorporated these two ingredients into a blood agar medium containing 10 per cent. human blood. Prototype media contained final concentrations of 10 mg cysteine and 9 mg DTT per 100 ml medium, respectively. Preliminary studies showed that such a medium greatly enhanced the surface growth of Cl. oedematiens of types B and D. The enhancing effect of the cysteine/DTT system compared with growth on simple 33) per cent. human blood agar, or 33) per cent. human blood agar supplemented with iron filings, is illustrated in Fig. 4.

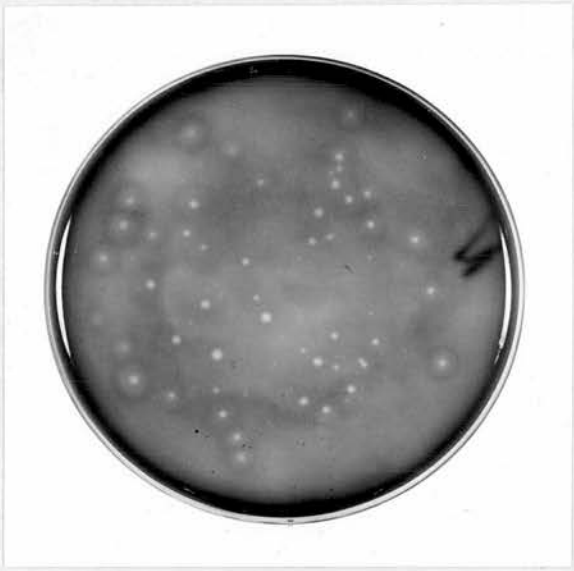
The final concentration of cysteine was increased from 10 mg per 100 ml to 100 mg per 100 ml in later work, after discussion with Dr. Moore. The influence of increasing concentrations of cysteine, up to 100 mg per 100 ml, is well illustrated in a recent report (Walker, Harris and Moore, 1971). The present author found, however, that similarly high concentrations of DTT were inhibitory to the growth of Cl. oedematiens strains.

Further development of the cysteine/DTT system led to the use of Watt's modification of Moore's medium (Cysteine-dithiothreitol human blood agar, CDEBA 16, see Materials and Methods). Later the percentage of blood in the final medium was increased to 33 per cent. (CDHBA 33), as studies suggested that an increased concentration of blood in the medium favoured the growth of Cl. oedematiens types B and D.

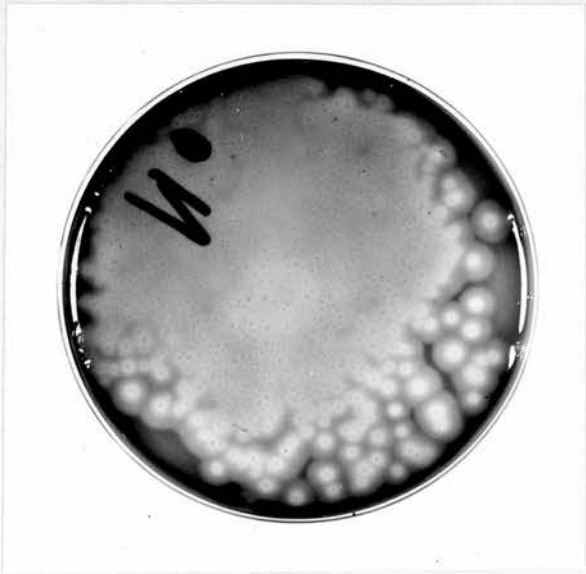
Following on the outbreak of serum hepatitis in Edinburgh in 1969-1970, the use of human blood in culture media was abandoned. Comparative qualitative studies suggested that equine blood, although

FIGURE 4. - The growth of a type-D strain of Clostridium oedematiens on solid media.

(a) Minimal growth on 33 per cent. HBA.



(b) Improved growth on 33 per cent. HBA sprinkled with sterile iron filings.



(c) Luxuriant growth on CDHBA medium.

giving slightly poorer growth than human blood, would still give good surface growth of Cl. oedematiens types B and D, when used in conjunction with the cysteine/DTT system, and a further modification of Moore's medium, cysteine-dithiothreitol equine blood agar (CDEBA 10) was therefore used in later studies. The reader is referred to the Materials and Methods section, p.61, for details of the preparation and composition of these media. Tests indicated that the use of "chocolated" media did not improve growth.

The use of media containing the cysteine/DTT system allowed remarkably reliable growth of type B and type D strains of Cl. oedematiens; development of reliable surface counting procedures with this most demanding model was now possible.

The development of a simple counting technique for demanding anaerobes

Early studies in this work, before the development of cysteine/DTT-containing media, used the pour plate method as favoured by Rutter (1968). However, preliminary studies with CDHBA 33 suggested that counts obtained by this method were appreciably lower than those obtained by surface count methods, possibly due to inactivation of heat sensitive cells by molten agar, which of necessity was at a temperature of 45°C or above at the time of addition of the inoculum. These findings are in agreement with those of earlier workers (Clark, 1967; Soestbergen and Lee, 1969) who found that surface count methods gave higher viable counts than pour-plate methods.

Roll-tube techniques such as those of Hungate (1950) or Holms (1968) were unsuitable for use with plates, and the agar-block spread

method of Chatterjee, Mukherji and Neogy (1969) was not considered suitable for viable counts of a demanding anaerobe, as it appeared to expose the organisms to aerobic conditions for unnecessarily long periods of time.

The Miles and Misra method of performing surface viable counts (Miles, Misra and Irwin, 1938) is widely used in quantitative bacteriological procedures. It has the merit of simplicity, and of economy in the use of plates, as each plate can accommodate several drops. However, pilot experiments showed that the time taken for a 0.02-ml drop to dry into a dried nutrient agar or blood agar plate was often more than 1 hr, yet spreading growth or coalescing of colonies resulted if plates were incubated in the moist environment of an anaerobic jar before the drops had dried into the plates, even if well dried agar plates (see Materials and Methods) or plates containing 4 per cent. agar were used. The Miles and Misra procedure therefore seemed unsuitable for the proposed studies, as exposure of plates seeded with a demanding organism such as Cl. oedematiens type D to aerobic conditions for periods of one hour or more might lead to inactivation of these strict anaerobes, and therefore to unrepresentative viable counts. This was confirmed in later experiments (see page 179).

The Miles and Misra technique has the additional disadvantage that plates in which the inoculum is not spread may give significantly lower counts than those in which the inoculum is spread over the surface of the plate (Snyder, 1947). Therefore, a spread plate method seemed most suitable.

Trial experiments showed that a 0.02 ml-inoculum dried in

immediately when it was spread on to a plate of freshly-poured and dried medium; the plates could be incubated anaerobically immediately after inoculation and there was no coalescing of colonies or confluent growth of normally non-spreading organisms. Spreading growth such as that observed with type-B strains of Cl. oedematiens could be controlled by one of the methods described below.

Slower "drying-in" of the inoculum was noted if (i) a larger inoculum was used, or (ii) these plates were insufficiently dried, or had been stored at 4°C. Thus, in the present studies, plates were seeded with a 0.02-ml inoculum, and freshly poured, well-dried media were used unless specified in the text.

Sterile glass spreaders, of uniform size (spreading edge 30 mm length by 3 mm diameter) were used to spread the inocula, each plate being spread by a total of 20 radial and circumferential sweeps of the spreader. A separate sterile spreader was used for each plate.

The carry-over of organisms on glass spreaders

It was appreciated that some organisms would be removed on the spreader. A series of replicate experiments were therefore performed in which a given spreader was used to spread the test inoculum on a plate of CDEBA medium, and the same spreader was then rubbed over the surface of a fresh CDEBA plate in a standard manner (20 sweeps per plate), so that the percentage carry-over to the second plate could be determined. Previous workers had reported carry-over rates of up to 4 per cent. (Soestbergen and Lee, 1969). In the present studies, the carry-over rate with the method described above was between 2 and 5 per cent. although this could rise to higher values if spreading was inadequate (see Table 2). Spreading for a longer period (i.e. with a

TABLE 2

The percentage carryover of organisms* by sterile spreaders when various spreading procedures are used

Spreading procedure	Mean percentage carryover per spreader**
5 sweeps per plate$^{\Delta}$	25.3
20 sweeps per plate	5.3
40 sweeps per plate	4.5

* The test inoculum was 0.02 ml of a $\frac{1}{5} \times 10^{-3}$ dilution of an 18-hr CMB culture of Cl. welchii strain L_2A, used to seed replicate plots of CDEBA medium.

** Expressed as the mean of five replicate experiments.

Δ All plates incubated for 18 hr after seeding.

greater number of sweeps) did not lower the carry-over significantly, and increased the time of exposure of seeded plates to the atmosphere (Table

The standard counting procedure

Freshly poured media, well dried, were each seeded with a 0.02-ml drop from a constant volume pipette. The drop was then spread in a standard manner with a sterile glass spreader of standard dimensions. The seeded plates were then immediately placed in an anaerobic jar and the jar set up by means of the standard procedure outlined above. In all experiments, counts were derived from dilutions that gave 200-700 colonies per plate (Meynell and Meynell, 1970) All plates bearing cultures of a given dilution were incubated in the same anaerobic jar.

It was found that this method allowed seeding of many plates with minimal exposure to the atmosphere. (With practised operators as many as 120 plates could be seeded and spread by 3 people within 10-15 min.).

The control of spreading growth on blood agar media

It was found that type-D strains of Cl. oedematiens gave discrete colonies on all blood agar media if well-dried plates were used. Type-B strains however gave spreading growth on blood agar media, and although media containing the cysteine/dithiothreitol system tended to reduce the amount of spreading, few discrete colonies were observed. For this reason, type-D strains were preferred to type-B strains of Cl. oedematiens in demanding quantitative studies.

From the results of several experiments, summarised in Table 3 the use of 4 per cent. agar in blood agar media was the only means of

TABLE 3

The control of spreading growth of Clostridium oedematiens type B

Test medium	Degree of inhibition of growth*	Degree of inhibition of spreading*
CDEBA ** (1.2 per cent. agar)	-	-
CDEBA (2.5 per cent. agar)	-	++
CDEBA (4 per cent. agar)	-	+++
CDEBA (1.2 per cent. agar + desoxycholate	+++ (NG)	NG
CDEBA (1.2 per cent. agar + 0.5 per cent. sodium tauroglycollate	+	-
CDEBA + 1/5000 sodium azide	+	+

* All plates seeded with 0.02-ml inocula of a 10^{-1} dilution of an overnight CMB culture of strain GRIB, and incubated for 18 hr.

** CDEBA = 10 per cent. cysteine-dithiothreitol equine blood agar (see Materials and Methods).

NG = no growth observed.

\+ = some inhibition; ++ = moderate inhibition;

+++ = marked or complete inhibition, with discrete colonies, and little or no spreading observed.

achieving sufficient inhibition of spreading of type-B strains to allow counting of individual colonies without any inhibition of growth. However, even this method occasionally failed to inhibit spreading growth satisfactorily and the number of colonies counted were therefore minimum estimates; the "true" surface viable count may have been higher.

Later experiments required the performance of surface viable counts from inocula derived from CMB cultures of Cl. tetani and Cl. septicum, and the spreading growth of these organisms posed special problems. None of the methods tried in the case of Cl. oedematiens type-B strains produced discrete growth; spreading growth was still observed when 4 per cent. agar was used in blood agar media. It was decided to determine whether the spreading growth of test strains of Cl. tetani could be inhibited by anti-tetanus antitoxic sera, as described by Willis and Williams (1970). The anti-toxin was spread on the surface of plates before inoculation, but the incorporation of anti-toxin into the medium, as suggested in a later paper (Williams and Willis, 1970) was not attempted in the present study.

The results showed that of 3 species of Cl. tetani tested, one (strain 5405) showed sufficient inhibition of spreading to produce discrete colonies, with one of the test sera (Table 4). It was decided to use strain 5405, a motile, flagellate strain (see Fig. 5) in experiments with antitoxin-spread plates, and to use a strain of Cl. tetani type VI (non-motile) without the antitoxin procedure in definitive experiments with this organism.

The use of plates spread with Cl. septicum antitoxin was then tested to see if this method could inhibit spreading growth of

TABLE 4

Results obtained when three different heterologous antitoxic sera were used to prevent spreading growth of overnight CMB cultures of Cl. tetani* on EBA medium**

Test strain	Degree of inhibition of growth	Degree of inhibition of spreading achieved with		
		Bovine antiserum	Equine antiserum	Ovine antiserum
Cl. tetani 540	-	+	+	+
Cl. tetani 5413	-	+	-	-
Cl. tetani 5405	-	+	++	+++

* Control plates of all three strains, without the addition of antitoxic sera, showed classical spreading growth with no discrete colonies.

** Plates seeded with 0,02-ml inocula of 10^{-1} dilutions of the test strains and incubated for 48 hr.

\- = No observed inhibition;

\+ = slight inhibition, few discrete colonies;

++ = moderate inhibition, with many discrete colonies, but also spreading growth;

+++ = complete inhibition, with discrete colonies, and no spreading observed.

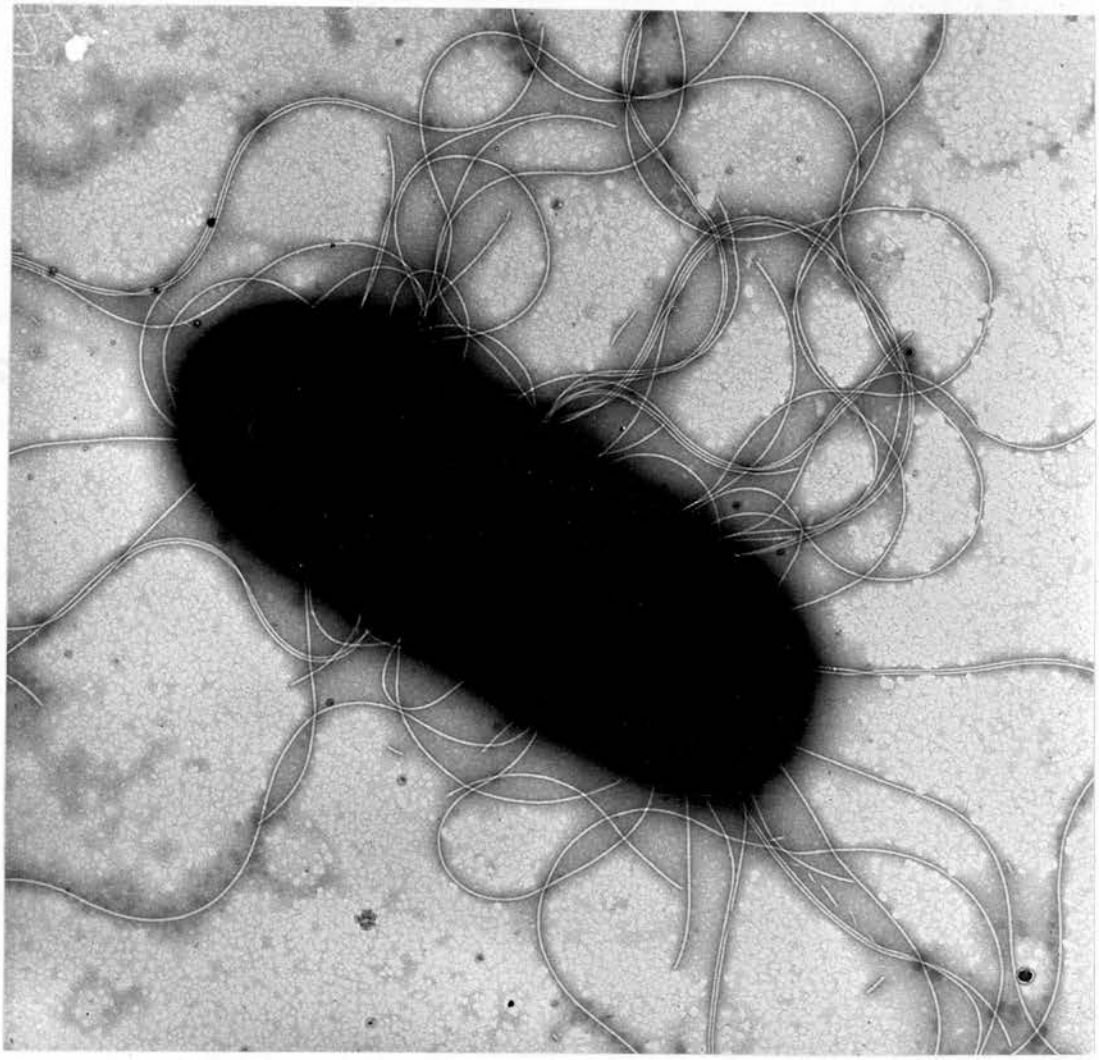


FIGURE 5. - Electron micrograph of a cell from a motile strain of Clostridium tetani, showing numerous peritrichous flagella; negatively stained with phosphotungstic acid. x 30,000.

Cl. septicum strains. Of 4 Cl. septicum strains tested, one strain (strain 101) showed satisfactory inhibition of spreading of growth with one of four test antitoxic sera (Table 5), and this combination was used in the present study.

No inhibition of spreading of Cl. chauvoei strains was observed when Cl. chauvoei strains were tested with Cl. septicum anti-toxic sera, or when Cl. septicum strains were tested with Cl. chauvoei anti-toxic sera. No inhibition of growth was observed with any of the test organisms when anti-toxic sera were used to control spreading.

The use of diluents in quantitative studies with Cl. oedematiens types B and D

In early quantitative studies with Cl. oedematiens types B and D, saline containing 20 per cent. nutrient broth was used empirically as a diluent. However, with the development of a standardised surface counting technique based on the consistently reliable growth of these organisms obtained on media containing the cysteine/DTT system, it was necessary to find a conveniently sterilised diluent that would give optimal counts of these organisms.

In contrast to the findings of Gorbach and Tabaqchali (1969), saline was found to be inferior to Oxoid nutrient broth as a diluent (Table 6), and the use of pure saline, or of related diluents such as saline/nutrient broth, was not considered in subsequent studies. For the same reason, the diluents such as that described by Bryant and Burkey (1953) containing 0.6 per cent. NaCl, were considered unsatisfactory, and it was decided to use a diluent based on nutrient broth in the present studies. It was appreciated

TABLE 5

Results obtained when four different antitoxic sera were used to prevent spreading growth of Cl. septicum* on EBA medium

Test strain	Degree of inhibition of growth	Degree of inhibition of spreading achieved with antiserum number			
		Ex2958	Ex3567	Ex1825	Rx5655
Cl. septicum 101	-	++	+++	+	++
Cl. septicum 113	-	-	-	-	-
Cl. septicum 102	-	...	-	...	...
Cl. septicum 103	-	...	-	...	...

* Control plates, without the addition of antitoxic sera, showed spreading growth with no discrete colonies.

** Plates seeded with 0.02-ml inocula of 10^{-2} dilutions of the test strains and incubated for 48 hr.

\- = no observed inhibition;

\+ = slight inhibition, few discrete colonies;

++ = moderate inhibition, some discrete colonies;

+++ = complete inhibition, with discrete colonies, and no spreading observed;

... = not done.

TABLE 6

A comparison of the observed viability of triplicate 0.02-ml samples of a 21-hr CMB culture of Cl. oedematiens type D when nutrient broth and saline were used as diluents

Diluent	Dilution	Triplicate counts, with mean in brackets, at stated dilution*
Nutrient broth (Oxoid)	10^{-1}	+++, +++, +++ (+++)**
	10^{-2}	337, 346, 390 (357.7)
NaCl 0.9 per cent. in water	10^{-1}	+++, +++, +++ (+++)**
	10^{-2}	270, 226, 235 (265.3)

* Viable counts performed by a spread-plate method on CDEBA medium; all plates seeded from a given dilution incubated for 18 hr in a single anaerobic jar.

** +++ = confluent growth.

that such a diluent was complex and not chemically defined, but there was little evidence of intra-batch variation if a large batch of nutrient broth was made up and dispensed into suitable containers. Therefore in a given series of comparative experiments, all dilutions were made from a single batch of nutrient broth. Tests showed that removal of oxygen by pre-steaming of the nutrient broth led to increased viable counts (Table 7).

The addition of cysteine/DTT solution after pre-steaming of the nutrient broth so that the diluent contained cysteine 100 mg per 100 ml and DTT 9 mg per 100 ml, was evaluated in the surface growth of Cl. oedematiens type D. There was no improvement in viable counts when the cysteine/DTT solution was added (counts were slightly lower than those obtained with pre-steamed nutrient broth alone), and therefore, unless stated otherwise in the text, Oxoid nutrient broth No. 2, presteamed for 30 min. before use, and then cooled to room temperature, was used as a diluent in this study.

TABLE 7

A comparison of the observed viability of triplicate 0.02-ml samples of a 21-hr CMB culture of Cl. oedematiens type D when nutrient broth and pre-steamed nutrient broth were used as diluents

Diluent	Dilution	Triplicate colony counts* with mean in brackets, at stated dilution
Nutrient broth (Oxoid)	10^{-1}	307, 273, 243 (274.3)
Nutrient broth (Oxoid) steamed for 30 min., and cooled to room temp.	10^{-1}	495, 391, 522 (469.3)

* Viable counts performed by a spread-plate method on CDEBA medium; all plates seeded from a given dilution incubated for 18 hr in a single anaerobic jar.

STUDIES ON THE SUBCULTURE OF CL. OEDEMATIENS

Our early difficulties encountered in the subculture of Cl. oedematiens types B and D on to solid media prompted a study to examine some of the problems involved in the laboratory subculture of exacting types of Cl. oedematiens.

It is not clear from the literature on the culture of Cl. oedematiens whether surface growth is initiated from vegetative cells or from spores. Rutter (1968) showed that smears from pathological material usually showed both spores and vegetative forms, and the author confirmed this finding for CMB cultures. It was necessary to determine whether subcultures from CMB to solid media depended on the viability of spores, or vegetative cells, or both, in the inoculum.

In preliminary studies with pour plates of RCM agar containing 5 per cent. of human blood to assist colony differentiation, the viable counts obtained on subculture of CMB cultures were considered in relation to the estimated number of spores present in the inocula. The results of a typical experiment with demanding (type B) and less demanding (type A) strains are shown in Table 8 From these results, it seemed that the viable count was related to the estimated numbers of spores present, as the viable counts increased on prolonged incubation of CMB cultures, as did the number of spores present. Even if spores alone are contributing to the viable counts, the number of spores recovered from a 4-day CMB culture of a type-B strain was considerably less than the total number of spores estimated to be present, i.e., only about 0.4 per cent. of spores of Cl. oedematiens type B proceeded to germination and outgrowth under the conditions of

TABLE 8

The total cell counts, spore counts and viable counts of samples of CMB cultures of Cl. oedematiens types A and B

Test strain and details of the culture used	Total cell count per ml	Percentage of sporing forms as judged by			Estimated number of spores per ml	Viable count* per ml after 48 hr incubation
		phase contrast	Gram's stain	Spore stain		
R32B (type B) in CMB						
at 18 hr	4.7×10^6	<1.0	<1.0	<1.0	4.7×10^4	2.8×10
at 4 days	1.5×10^7	28	29	34	4.3×10^6	1.7×10^4
GRIA (type A) in CMB						
at 18 hr	1.0×10^7	1.5	2.0	2.0	2.0×10^5	2.7×10^5
at 4 days	6.8×10^6	66	65	60	4.5×10^6	1.8×10^6

* Each viable count expressed as the mean of duplicate counts performed by a pour plate method in Reinforced Clostridial Medium with 5 per cent. human blood. All plates for each organism incubated for 48 hr in the same anaerobic jar.

the experiment, compared with the 40 per cent. recovery of spores observed in the case of a 4 day CMB culture of Cl. oedematiens type A.

Studies with Iron Filings

At this point, the iron-filings technique was being developed in relation to pour plate counts. Strains of types B and D clearly differed from type-A strains in being much more difficult to culture on solid media, and type-A strains were therefore omitted from further studies.

Experiments were performed in which total cell counts, spore estimates, and viable counts were made on samples of each sporing culture. The samples were (i) untreated, (ii) washed twice by centrifugation with saline and resuspended to the same volume, and (iii) treated as in (ii), and then heated for 20 min. at 70°C. Viable counts were performed after incubation of the plates for 24 hr and 72 hr. Results of a typical experiment with representative strains of Cl. oedematiens of types B and D are shown in Table 9.

In the case of the type-B strain, the viable counts increased slightly with prolonged incubation of the pour plates. None of the viable counts approached the total spore count, and the counts after pasteurisation indicate that the percentage of spores recovered was only 1 per cent. However, assuming that none of the spores is activated by heating at 70°C for 20 min., then the viable count after pasteurisation represents the maximum number of spores that are recoverable in the test system. On this assumption, spores could have contributed to more than 50 per cent. of the viable count of the untreated sample and 70 per cent. of the count of the washed

TABLE 9

Viable counts on treated and untreated samples of CMB cultures of a type-B and type-D strain of Cl. oedematiens with total cell counts and spore counts of the untreated samples

Test strain and details of the culture used	Treatment of sample	Total cell count per ml	Observed* percentage of spores	Estimated number of spores per ml	Time of incubation (hr)	Viable count **
Cl. oedematiens Type B incubated for 20 hr at 37°C and thereafter held on the bench at room temperature for 28 days	None	2.0×10^7	36	7.2×10^6	24	6.0×10^4
					72	1.3×10^5
	washed and resuspended in saline	...	...	...	24	4.2×10^4
					72	9.4×10^4
	washed, resuspended and heated at 70°C for 20 min	...	...	...	24	1.6×10^4
					72	7.0×10^4
Cl. oedematiens Type D incubated for 20 hr at 37°C and thereafter held on the bench at room temperature for 28 days	None	1.0×10^6	41	4.1×10^5	48	3.5×10^4
					96	4.4×10^4
	washed and resuspended in saline	6.6×10^5	38	2.5×10^5	48	3.2×10^4
					96	3.2×10^4
	washed, resuspended and heated at 70°C for 20 min	...	...	...	48	3.3×10^4
					96	3.2×10^4

* Based on the mean of separate observations with phase-contrast microscopy and stained smears.

** Each viable count expressed as the mean of duplicate counts performed by a pour plate method in Reinforced Clostridial Medium with 5 per cent. blood plus iron filings.

... = Not done.

sample, implying that a considerable proportion of vegetative cells does not grow when subcultured in pour plates.

In the case of the type-D strain, the recovery of spores from the pasteurised sample was nearly 13 per cent., and spores could have contributed to more than 70 per cent. of the viable count of the untreated sample and to 100 per cent. of that of the washed sample.

The above results indicate that when 28-day CMB cultures of Cl. oedematiens types B and D are subcultured to pour plates supplemented with sterile iron filings, spores may contribute to 50-70 per cent. or more of the viable counts, and vegetative cells may not be readily recoverable in this system.

Studies with cysteine-dithiothreitol

At this point, media containing the cysteine/dithiothreitol system were developed and, with consistently reliable surface growth of Cl. oedematiens of types B and D, it was possible to develop surface viable counting techniques. The results given in Table 10 show that appreciably higher surface viable counts of type-B strains were obtainable on the new media than on blood agar medium alone, a finding also noted for type-D strains. Although the surface viable counts obtained with the iron-filings technique were higher than those obtained with EBA medium alone, they were less than those obtained with CDEBA medium. When the two systems were combined, no cumulative effect on the surface viable count was observed with type B strains (q.v.).

Although satisfactory viable counts could be obtained with type-B strains grown on 4 per cent. agar, spreading growth still

TABLE 10

The surface viable counts obtained for duplicate 0.02-ml samples derived from a 21-hr CMB culture of Cl. oedematiens type-B when plated out on different media

Surface viable count per ml* obtained on			
EBA**	EBAIF	CDEBA	CDEBAIF
$<50^{\Delta}, <50$	$(1.0\text{-}1.1) \times 10^5$	$(6.8\text{-}6.9) \times 10^5$	$(6.3\text{-}7.0) \times 10^5$

* Viable counts expressed as duplicate counts performed by a spread-plate method, on the stated medium. All plates incubated for 18 hr in a single anaerobic jar.

** EBA = equine blood agar, containing 10 per cent. blood;
EBAIF = EBA sprinkled with iron filings;
CDEBA = EBA supplemented with cysteine and dithiothreitol;
CDEBAIF = CDEBA sprinkled with iron filings.

Δ No growth obtained from 3 replicate plates seeded with 0.02-ml undiluted inocula.

occasionally occurred, and for this reason subsequent studies were performed mainly with Cl. oedematiens type D.

The results given in Table 11 showed that when inocula derived from 24-hr CMB cultures of Cl. oedematiens type D were subcultured on to the surface of CDHBA plates, the viable counts obtained were much greater than the maximum number of spores estimated to be present. When older CMB cultures were used, the estimated number of spores present increased, but the viable count decreased to 1 per cent. of the estimated number of spores in a culture held for 6 days, and to a very small percentage indeed in the case of a 28-day culture (see Table 11) Thus it seems clear that the growth of vegetative cells on CDHBA 16 accounts for a significant component of the surface viable counts in young CMB cultures; as a culture ages, the proportion of vegetative cells that can be redeemed on CDHBA 16 is reduced. It is not clear from these results whether viability in old cultures is associated with vegetative cells or with spores; if spores are the main contributors to surface viable counts in old cultures, the proportion that proceed to successful germination and outgrowth is very small. For example, in the case of a 28-day culture (Table 11), the proportion was less than 0.0008 per cent.

If vegetative cells are being recovered on media containing the cysteine/dithiothreitol system, from inocula derived from young cultures of type-D strains, the vegetative cells should be susceptible to heat. It was decided to investigate the recovery obtained after subjecting the inocula to heating, to washing and to a combination of these procedures. The results of a representative experiment are given in Table 12).

TABLE 11

The total cell counts, spore counts and surface viable counts of 0.02-ml samples of CMB cultures of Cl. oedematiens type D

Cultural conditions of test sample	Total cell count per ml	Percentage of sporing forms as judged by			Estimated number of spores per ml	Surface viable count per ml after 20 hr incubation*
		phase contrast	Gram's stain	Spore stain		
After incubation for 24 hr	6.7×10^7	<0.2	0.2 0.1	<0.1** <0.1	6.7×10^4	3.1×10^6
After incubation for 24 hr + 6 days at room temperature	2.0×10^7	7	3 3	4 4	8.1×10^5	8.7×10^3
After incubation for 18 hr + 48 hr at room temperature	3.0×10^7	1.6	1.1	0.5	4.8×10^5	5.5×10^5
After incubation for 18 hr + 28 days at room temperature	1.0×10^8	c90Δ	...	...	c9.0 x 10^7	7.0×10^2

* Each surface viable count expressed as the mean of duplicate counts performed by a spread-plate technique on CDHBA 16 medium. All plates incubated for 18 hr in the same anaerobic jar.

** No spores seen in more than 1000 cells counted.

Δ Accurate counts were impossible in view of the large number of spores of various sizes; a minimum estimate is given.

TABLE 12

The total cell counts, spore counts, and surface viable counts of treated and untreated 0.02-ml samples of a strain of Cl. oedematiens type D cultured in CMB medium for 18 hr at 37°C

Treatment of sample	Total cell count per ml	Observed percentage of spores	Estimated number of spores per ml	Surface viable count* per ml after 48 hr incubation
Untreated CMB culture	1.5×10^7	2.4	3.6×10^5	6.6×10^3
Culture heated at 80°C for 20 min.	...	...	...	50**
Culture washed and resuspended in saline	1.5×10^7	2.4	3.6×10^5	50 - 100$^{\Delta}$
Culture washed, resuspended and heated at 80°C for 20 min.	...	...	...	$<50, <50^{\Delta\Delta}$

* Each surface viable count expressed as the mean of 3 replicate counts performed by a spread-plate method on CDHBA 16 medium. Each replicate culture was performed with the same batch of medium and all were incubated for 18 hr in a single anaerobic jar.

** Each of 3 replicate counts from inocula of 0.02 ml gave 1 colony.

Δ 3 replicate counts from inocula of 0.02 ml gave two, one and one colonies.

$\Delta\Delta$ No growth obtained from 3 replicate plates seeded with 0.02 ml undiluted inocula.

... = not done

Although the absolute recovery was low in this experiment, the viable count was significantly reduced on pasteurisation, suggesting that vegetative cells contributed the major component of the surface viable count of the untreated inoculum. A similar reduction was obtained after washing the inoculum in nutrient broth under aerobic conditions and resuspending it in saline. When the two procedures were combined, no growth was obtained even from 0.02-ml inocula of the parent culture, and there was therefore no evidence of activation of spores by the pasteurisation procedure. On the assumption that no inactivation or activation of spores occurred after heating at 80°C for 20 min, the percentage of spores recovered from the heated culture was low (0.01 per cent. of the estimated number of spores).

Although it might be considered that the pasteurisation procedure could cause inactivation of some spores, it is much less likely that significant numbers of spores were inactivated by washing, as washing did not diminish the viable count of a sporing culture of a type-D strain. That the fall in viable count produced by washing is not due to actual loss of viable cells during the washing procedure, is indicated by the unchanged total cell count after the procedure. The author appreciates that the confidence limits of such counts may be considerable, but the fact that the proportions of spores observed in samples from the washed inoculum were also unchanged implies that there was no appreciable loss of cells during the procedure.

Table 13 shows the result of an experiment in which the above procedures were performed on a culture of type-D strain in which there were very few spores. Again, there is complete inactivation of the inoculum by each of the procedures employed, suggesting that vegetative

TABLE 13

The loss of viability observed after different treatments of 0.02-ml samples of a CMB culture of a type D strain of Cl. oedematiens incubated for 18 hr at 37°C and held thereafter for 7 days at room temperature

Treatment of sample	Total cell count per ml	Observed percentage of spores	Estimated number of spores per ml	Surface viable count* per ml after 48 hr incubation
Untreated CMB culture	2.7×10^7	0.3	8.1×10^4	$>(1 \times 10^4)$**
Culture heated at 80°C for 20 min.	...	...	...	$<50^{\Delta}$
Culture washed and resuspended in saline	2.4×10^7	0.2	4.8×10^4	<50
Culture washed, resuspended and heated at 80°C for 20 min.	...	...	...	<50

* Each surface viable count expressed as the mean of 3 replicate counts performed by a spread-plate method on CDHBA 16 medium. Each replicate culture was performed with the same batch of medium, and all were incubated for 18 hr in a single anaerobic jar.

** Some confluent growth was observed; the count given is a minimal estimate.

Δ No growth obtained from 3 replicate plates seeded from 0.02 ml undiluted inocula.

... = not done.

cells were being inactivated by these procedures, and that vegetative cells were the main contributors to the surface viable counts obtained with untreated samples.

However, it was considered that more impressive proof of the contribution of vegetative cells to the surface viable counts could be obtained if the experiment was poised to ensure that the number of spores present was very small. Table 14 shows the results obtained when unheated and pasteurised samples of a 5-hr and a 21-hr-CMB culture of Cl. oedematiens type D were subcultured on (i) human blood agar (HBA) containing 33 per cent. blood; (ii) human blood sprinkled with sterile iron filings (HBAIF); (iii) Watt's cysteine/dithiothreitol blood agar (CDHHBA 33); and (iv) CDHBA 33 sprinkled with iron filings (CDHBAIF).

An 0.1-ml inoculum from a 16-hr culture of Cl. oedematiens type D was used to seed fresh anaerobic CMB medium held at 37°C. The new and parent cultures were re-incubated under anaerobic conditions for 5 hr and total cell counts and spore estimates were then performed with samples of the 5-hr and 21-hr cultures. Samples of each culture were pasteurised at 70°C and at 80°C for 20 min., and in addition samples of each culture were serially diluted in pre-steamed nutrient broth, and surface viable counts performed on the four media mentioned above.

Table 14 indicates that in the case of the young (5-hr) CMB culture, the viable counts obtained on all four media exceeded the spore estimate of $< 9.0 \times 10^3$ per ml; the viable count on CDHBAIF (4.7×10^5 organisms per ml) was at least 50 times greater than the number of spores estimated to be present. Although appreciable growth was obtained on HBA plates, the colonies were small; better growth

TABLE 14

The surface viable counts determined for heated and unheated samples of 5 hr and 21 hr CMB cultures of Cl. oedematiens type D with recovery on different media

Period of incubation (hr)	Treatment of sample	Surface viable count per ml* estimated from 0.02-ml samples grown on**			
		HBA$^{\Delta}$	HBAIF	CDHBA	CDHBAIF
5$^{\Delta\Delta}$	None	$(6.5\text{-}6.6)\times10^4$	$(1.3\text{-}1.4)\times10^5$	$(2.6\text{-}3.0)\times10^5$	$(4.2\text{-}5.3)\times10^5$
	Heated at 70°C for 20 min.	<10, <10	<10, 50	<10, <10	<10, <10
	Heated at 80°C for 20 min.	<10, <10	<10, 20	<10, <10	<10, <10
21 $^{X}_{X}$	None	$>(5\times10^4)^{\S}$	$(3.7\text{-}4.3)\times10^6$	$(3.6\text{-}3.9)\times10^6$	$(4.6\text{-}5.7)\times10^6$
	Heated at 70°C for 20 min.	<10, <10	$(1.6\text{-}2.2)\times10^3$	$(2.8\text{-}2.9)\times10^2$	$(1.08\text{-}1.12)\times10^3$
	Heated at 80°C for 20 min.	<10, <10	$(7.3\text{-}7.8)\times10^2$	90, 120	$(4.9\text{-}7.1)\times10^2$

* Surface viable counts of duplicate samples, performed by a spread-plate technique on the stated medium. All plates seeded from samples similarly treated were incubated for 18 hr in the same anaerobic jar.

** HBA = human blood agar containing 33 per cent. blood; HBAIF = HBA sprinkled with iron filings; CDHBA = HBA supplemented with cysteine and dithiothreitol; CDHBAIF = CDHBA sprinkled with iron filings.

Δ Tiny colonies were observed on this medium.

$\Delta\Delta$ The total cell count was 3.6×10^6 per ml; the spore estimate was $<9.0\times10^3$ per ml.

$^{X}_{X}$ The total cell count was 8.0×10^6 per ml; the spore estimate was 4.3×10^5 per ml.

§ Minimum count based on profuse growth of c. 1000 discrete colonies from 0.02 ml of undiluted sample. 10-fold dilutions yielded no growth when cultured on HBA medium.

was obtained with HBAIF, much better growth was obtained with CDHBA, and the highest surface viable counts were obtained in the case of CDHBAIF. Nevertheless, the fact that the surface viable counts obtained on HBA plates exceeded the total number of spores present was clear evidence that vegetative cells could be successfully subcultured on occasions on a fresh, but relatively simple medium. The average recovery of vegetative cells on these media was 1.8, 3.9, 7.8, and 13.3 per cent. respectively. After pasteurisation at 70°C for 20 min., only one colony was recovered from 0.1 ml of the undiluted sample when subcultured on a CDHBAIF plate, and only five colonies when subcultured on an HBAIF plate. After pasteurisation at 80°C for 20 min., two colonies were subcultured on an HBAIF plate. Thus, if heat-resistant spores were being recovered, they were few in number, and their recovery was enhanced by the presence of iron filings.

In the case of the 21-hr culture, different results were obtained (see Table 14). Growth was obtained on HBA from undiluted samples only, no growth being obtained from diluted samples. Counts obtained on HBAIF, CDHBA and CDHBAIF all exceeded the total number of spores estimated to be present (4.3×10^5 per ml) by about tenfold; in the case of CDHBAIF, the best count obtained (5.2×10^6 per ml) was remarkably near the total cell count (8.0×10^6 per ml). Spore recovery after pasteurisation was poor, and obviously HBA alone failed to redeem any spores. The percentage recovery on HBAIF, CDHBA and CDHBAIF after heating of the inoculum at 70°C was 0.4, 0.07 and 0.3 per cent., respectively, whereas that obtained after heating at 80°C was 0.2, 0.02 and 0.1 per cent. respectively, suggesting that some inactivation of spores occurred on heating to 80°C. Even the

best recovery of spores (obtained on HBAIF after heating to 70°C), was less than 0.5 per cent., and it appears that more than 99 per cent. of the spores in this model are dormant, or non-viable in the above test system. It is clear that the cysteine/dithiothreitol system has an inhibitory effect on spore recovery when compared with HBAIF, and when the stimulatory effect of the iron filings is added (in the case of CDHBAIF medium), the recovery was still less than that on HBAIF medium.

The combination of the iron-filings technique with the cysteine/dithiothreitol system gave greater recovery from unheated samples with each culture, than when the cysteine/dithiothreitol system was used alone, although a cumulative effect was not observed in the case of type-B strains.

From the above results, it is clear that early studies, with pour-plate techniques and sporing cultures, gave low recovery rates on solid media, possibly due to inactivation of vegetative cells by heat. These studies, weighted against recovery of vegetative cells, suggested that spores were the main contributors to the surface viable counts. It was not until the development of (i) the iron filings technique, and (ii) the use of the cysteine/dithiothreitol system in conjunction with a spread plate counting method, that it was demonstrably possible to achieve quantitative recovery of vegetative cells of type-D strains on solid media.

The results show that it is possible to recover vegetative cells of Cl. oedematiens type-D, a demanding anaerobe, on solid media, using a standard anaerobic technique. In the case of young (5-hr) cultures it is possible to recover from a given inoculum of spores

and vegetative cells many more organisms than can be accounted for by spores alone. If the "significantly viable particle" is considered as that component of a CMB culture of a sporing anaerobe (either spore or vegetative cell), from which colonies on solid media are primarily derived, it is clear that vegetative cells are the significantly viable particles when young CMB cultures of type-D strains are subcultured on solid media. The marked decrease in the surface viable counts produced by heating or washing under anaerobic conditions is further evidence that vegetative cells contribute appreciably to surface viable counts derived from fluid inocula.

It was now necessary to determine whether the concept of the significantly viable particle could be applied to clostridia of more direct clinical importance than Cl. oedematiens type D.

THE ROLE OF SPORES AND VEGETATIVE CELLS IN THE RECOVERY OF SOME MEDICALLY IMPORTANT CLOSTRIDIA ON SOLID MEDIA

The nine test strains of clostridia used for this study were representative strains of Cl. oedematiens type B; Cl. oedematiens type A; Cl. tetani (non-motile) type VI; Cl. septicum (2 strains); β-haemolytic, non-heat-resistant Cl. welchii, type A; non-haemolytic heat-resistant Cl. welchii type A; Cl. histolyticum and Cl. bifermentans.

All strains were readily cultivated in cooked-meat broth (CMB) and when subcultured on to the surface of equine blood agar (EBA) or cysteine-dithiothreitol blood agar (CDEBA), a consistent growth of easily visible colonies was achieved after overnight incubation. Surface viable counts could therefore be readily performed. The spreading growth of Cl. septicum was avoided by the use of plates spread before inoculation with anti-toxin (see p. 92) All strains were regularly checked for purity (see Materials and Methods).

Studies with Cl. welchii

The results given in Table 15 show that in the case of a "classical" (β-haemolytic non-heat-resistant) strain of Cl. welchii, appreciably more vegetative cells could be recovered than the number of spores present: 5×10^7 organisms per ml were obtained from a young (5-hr) CMB culture in which there was less than 6.6×10^4 spores per ml.

Similar results were obtained in the case of a non-haemolytic, heat-resistant strain of Cl. welchii (Table 16); $(5\text{-}6) \times 10^7$ cells per ml were recovered from an inoculum that was estimated to contain 6.6×10^7 cells per ml and less than 6.7×10^4 spores per ml. In

TABLE 15

The surface viable counts determined for duplicate 0.02-ml samples of a 5-hr CMB culture of Cl. welchii (β-haemolytic, non heat-resistant strain)* before and after pasteurisation, with recovery on different media

Treatment of sample	Surface viable count per ml** estimated from culture grown on			
	EBA$^{\Delta}$	EBAIF	CDEBA	CDEBAIF
nil	$(6.5\text{-}6.7)\times10^7$	$(4.9\text{-}6.1)\times10^7$	$(5.9\text{-}6.0)\times10^7$	$(4.9\text{-}5.4)\times10^7$
heated at 70°C for 20 min	$<10, <10^{\Delta\Delta}$	$<10, <10$	$<10, <10$	$<10, <10$
heated at 80°C for 20 min	$<10, <10$	$<10, <10$	$<10, <10$	$<10, <10$

* The total cell count was 1.1×10^8 per ml; the spore estimate was $<(6.6 \times 10^4)$ per ml. (No spores seen in over 1700 cells counted)

** Surface viable counts of duplicate samples, performed by a spread-plate technique on the stated medium. All plates seeded from samples similarly treated were incubated for 18 hr in the same anaerobic jar.

Δ EBA = 10 per cent. equine blood agar; EBAIF = EBA sprinkled with sterile iron filings; CDEBA = EBA supplemented with cysteine and dithiothreitol; CDEBAIF = CDEBA sprinkled with sterile iron filings.

$\Delta\Delta$ No growth obtained from 0.1-ml samples of undiluted inoculum.

TABLE 16

The surface viable counts determined for duplicate 0.02-ml samples of a 5-hr CMB culture of Cl. welchii (non-haemolytic, heat-resistant strain)* before and after pasteurisation, with recovery on different media

Treatment of sample	Surface viable count per ml** estimated from culture grown on			
	EBA$^{\Delta}$	EBAIF	CDEBA	CDEBAIF
nil	$(6.1\text{-}6.4)\times10^7$	$(5.9\text{-}6.9)\times10^7$	$(5.3\text{-}5.9)\times10^7$	$(5.9\text{-}6.0)\times10^7$
heated at 70°C for 20 min.	$<10, <10^{\Delta\Delta}$	10-70	<10, 10	30-40
heated at 80°C for 20 min.	<10, 10	10-30	10-20	20-70

* The total cell count was 6.6×10^7 per ml; the spore estimate was $<6.6 \times 10^4$ per ml. (No spores seen in over 1000 cells counted)

** Surface viable counts of duplicate samples, performed by a spread-plate technique on the stated medium. All plates seeded from samples similarly treated were incubated for 18 hr in the same anaerobic jar.

Δ EBA = 10 per cent. equine blood agar; EBAIF = EBA sprinkled with sterile iron filings; CDEBA = EBA supplemented with cysteine and dithiothreitol; CDEBAIF = CDEBA sprinkled with sterile iron filings.

$\Delta\Delta$ No growth obtained from 0.1-ml samples of undiluted inoculum.

both cases, there was a marked diminution in the surface viable count after mild pasteurisation of the sample. Although this could be due either to inactivation of vegetative cells or of immature heat-sensitive spores, a similar finding was observed in the case of older (21-hr) CMB cultures of both heat-sensitive and heat-resistant strains, suggesting that the inactivation of vegetative cells was responsible for the diminution in viable counts observed after heating.

Studies with Cl. septicum

In the case of the Cl. septicum strains, the spreading surface growth rendered accurate colony counting difficult until it was controlled by a suitably selected Cl. septicum antitoxin (see page 93). It was then possible to show that in the case of Cl. septicum also, considerably more organisms could be recovered than could be accounted for by the number of spores estimated to be in the inoculum. The results of a representative experiment with a 5-hr culture (Table 17) show that c. 6×10^7 organisms per ml were recovered from inocula containing $<6.7 \times 10^4$ spores per ml.

Studies with Cl. tetani

In the case of Cl. tetani, quantitative studies were conveniently performed with a non-motile type VI strain; CMB cultures of this strain contained few spores, although occasional sporing forms could be demonstrated (Fig. 6). Recovery of vegetative cells in both young and old cultures greatly exceeded the total numbers of spores present. From the results of a typical experiment given in Table 18, it is clear that at least 2×10^5 organisms per ml could be recovered from inocula containing less than 6.6×10^3 spores per ml. There was

TABLE 17

The surface viable counts determined for duplicate 0.02-ml samples of a 5-hr CMB culture of Cl. septicum (strain 101)* before and after pasteurisation, with recovery on different media

Treatment of sample	Surface viable count per ml** estimated from cultures grown on			
	EBA$^{\Delta}$	EBAIF	CDEBA	CDEBAIF
nil	$(6.5\text{-}6.7)\times10^7$	$(4.9\text{-}6.1)\times10^7$	$(5.9\text{-}6.0)\times10^7$	$(4.9\text{-}5.4)\times10^7$
heated at 70°C for 20 min.	$<10, <10^{\Delta\Delta}$	$<10, <10$	$<10, <10$	$<10, <10$
heated at 80°C for 20 min.	$<10, <10$	$<10, <10$	$<10, <10$	$<10, <10$

* The total cell count was 1.1×10^8 per ml; the spore estimate was $<(6.6\times10^4)$ per ml (No spores seen in over 1700 cells counted).

** Surface viable counts of duplicate samples, performed by a spread-plate technique on the stated medium. All plates seeded from samples similarly treated were incubated for 18 hr in the same anaerobic jar

Δ EBA = 10 per cent. equine blood agar; EBAIF = EBA sprinkled with iron filings; CDEBA = EBA supplemented with cysteine and dithiothreitol; CDEBAIF = CDEBA sprinkled with iron filings.

$\Delta\Delta$ No growth obtained from 0.1 ml of an undiluted sample.

TABLE 18

The surface viable counts determined for duplicate 0.02-ml samples of a 5-hr CMB culture of Cl. tetani type VI* before and after pasteurisation, with recovery on different media

Treatment of sample	Surface viable count per ml** estimated from culture grown on			
	EBA$^{\Delta}$	EBAIF	CDEBA$^{\Delta\Delta}$	CDEBAIF
nil	$(2.8\text{-}3.0)\times10^5$	$(1.8\text{-}2.2)\times10^5$	$(3.2\text{-}4.2)\times10^5$	$(2.1\text{-}2.2)\times10^5$
heated at 70°C for 20 min.	$<10, <10^{\text{XX}}$	$<10, <10$	$<10, <10$	$<10, <10$
heated at 80°C for 20 min.	$<10, <10$	$<10, <10$	$<10, <10$	$<10, <10$

* The total cell count was 4.6×10^7 per ml; the spore estimate was $< (6.6 \times 10^3)$ per ml. (No spores seen in over 7000 cells counted).

** Surface viable counts of duplicate samples, performed by a spread-plate technique on the stated medium. All plates seeded from samples similarly treated were incubated for 48 hr in the same anaerobic jar.

Δ EBA = 10 per cent. equine blood agar; EBAIF = EBA sprinkled with iron filings; CDEBA = EBA supplemented with cysteine and dithiothreitol; CDEBAIF = CDEBA sprinkled with iron filings.

$\Delta\Delta$ Colonies on this medium were slightly more haemolytic than on the other media.

XX No growth obtained from 0.1 ml of an undiluted sample.

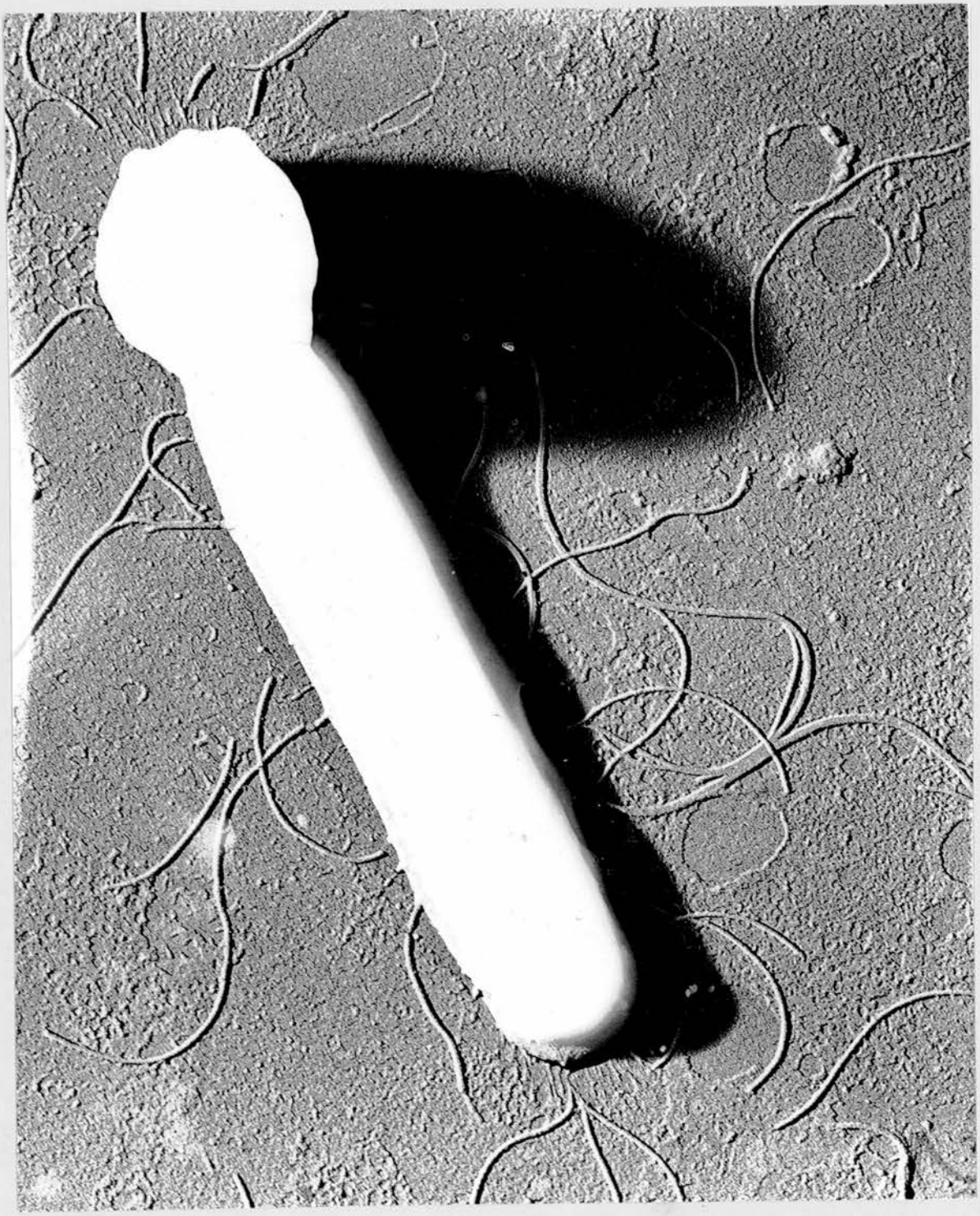


FIGURE 6. - Electron micrograph of Clostridium tetani, showing a terminal spore and several flagella. Gold-palladium shadowed preparation. x 36,000.

a dramatic fall in the surface viable count after pasteurisation, suggestive of inactivation of vegetative cells or of heat-sensitive spores.

Although the numbers of viable organisms recovered exceeded the total number of spores present, this was still only a small proportion (0.5 per cent.) of the total cell count and it is presumed that the inocula were derived from a log-phase culture in which a high degree of viability was to be expected. This indicated that recovery of Cl. tetani in the test system was far from the theoretical maximum and the challenge is taken up later in studies with Cl. tetani type VI as a model.

Studies with Cl. oedematiens types A and B

The results of representative experiments with Cl. oedematiens strains of types A and B are shown in Tables 19 and 20 respectively. It was possible to recover considerably more organisms from inocula of these organisms than the estimated number of spores present and to prove that vegetative cells are the significantly viable particles in these models. For example, from a 5-hr CMB culture of a type-A strain (Table 19), it was possible to recover c. 1.0×10^5 organisms per ml from inocula that contained 7.9×10^3 spores per ml.

Studies with Cl. histolyticum and Cl. bifermentans

The recovery of Cl. histolyticum was compared in parallel on various media, under aerobic and anaerobic conditions, and the results of representative experiments (Tables 21 and 22) show that more organisms were recovered on the surface of seeded plates than could be accounted for by spores alone; this was true for both aerobically and anaerobically incubated plates; in fact, marginally better counts

TABLE 19

The surface viable counts determined for duplicate 0.02-ml samples of a 5-hr CMB culture of Cl. oedematiens type A (strain GRIA)* before and after pasteurisation, with recovery on different media

Treatment of sample	Surface viable count per ml** estimated from culture grown on			
	EBA$^{\Delta}$	EBAIF	CDEBA	CDEBAIF
nil	$(4.8\text{-}5.1)\times10^{5}$	$(1.7\text{-}2.1)\times10^{5}$	$(1.0\text{-}1.2)\times10^{6}$	$(1.0\text{-}1.4)\times10^{5}$
heated at 70°C for 20 min.	$(1.20\text{-}1.12)\times10^{4}$	$(6.6\text{-}9.8)\times10^{3}$	$(1.1\text{-}1.4)\times10^{4}$	$(6.9\text{-}9.5)\times10^{3}$
heated at 80°C for 20 min.	$(7.5\text{-}8.2)\times10^{3}$	$(6.0\text{-}6.9)\times10^{3}$	$(6.7\text{-}8.3)\times10^{3}$	$(7.1\text{-}8.3)\times10^{3}$

* The total cell count was 2.9×10^{6} per ml; the spore estimate was 7.9×10^{3} per ml.

** Surface viable counts of duplicate samples, performed by a spread-plate technique on the stated medium. All plates seeded from samples similarly treated were incubated for 18 hr in the same anaerobic jar.

Δ EBA = 10 per cent. equine blood agar; EBAIF = EBA sprinkled with iron filings; CDEBA = EBA supplemented with cysteine and dithiothreitol; CDEBAIF = CDEBA sprinkled with iron filings.

TABLE 20

The surface viable counts determined for duplicate 0.02-ml samples of a 5-hr CMB culture of Cl. oedematiens type B (strain GRIB)* before and after pasteurisation, with recovery on different media

Treatment of sample	Surface viable count per ml** estimated from culture grown on			
	EBA$^{\Delta}$	EBAIF	CDEBA	CDEBAIF
nil	50, 50$^{\Delta\Delta}$	5×10^2 §	$(2.5\text{-}3.1)\times10^4$ §	$(3.0\text{-}5.0)\times10^4$ §
heated at 70°C for 20 min.	<10, <10^{XX}	<10, <10	<10, <10	<10, <10
heated at 80°C for 20 min.	<10, <10	<10, <10	<10, <10	<10, <10

* The total cell count was 1.5×10^6 per ml; the spore estimate was (5.21×10^3) per ml; (no spores seen in over 300 cells counted).

** Surface viable counts of duplicate samples, performed by a spread-plate technique on the stated medium. All plates seeded from samples similarly treated were incubated for 18 hr in the same anaerobic jar.

Δ EBA = 10 per cent. equine blood agar; EBAIF = EBA sprinkled with sterile iron filings; CDEBA = EBA supplemented with cysteine and dithiothreitol; CDEBAIF = CDEBA sprinkled with sterile iron filings. All media contained 4 per cent. agar to inhibit spreading growth.

$\Delta\Delta$ No growth from 0.02 ml of undiluted inoculum.

§ Spreading growth rendered accurate colony counting difficult; minimum estimates are given.

XX No growth from 0.1 ml of undiluted inoculum.

TABLE 21

The surface viable counts determined for duplicate 0.02-ml samples of a 21-hr CMB culture of Cl. histolyticum*, before and after pasteurisation, with recovery on different media and anaerobic incubation of the plates

Treatment of sample	Surface viable count per ml** estimated from cultures grown on			
	EBA$^{\Delta}$	EBAIF	CDEBA	CDEBAIF
nil	$(6.0\text{-}6.5)\times10^{8}$	$(7.0\text{-}7.3)\times10^{8}$	$(5.0\text{-}7.6)\times10^{8}$	$(3.5\text{-}6.2)\times10^{8}$
heated at 70°C for 20 min.	$(3.3\text{-}3.7)\times10^{7}$	$(2.0\text{-}2.2)\times10^{7}$	$(3.1\text{-}3.9)\times10^{7}$	$(2.2\text{-}2.3)\times10^{7}$
heated at 80°C for 20 min.	$(2.6\text{-}2.8)\times10^{7}$	$(2.1\text{-}2.2)\times10^{7}$	$(2.6\text{-}3.0)\times10^{7}$	$(2.0\text{-}2.3)\times10^{7}$

* The total cell count was 5.3×10^{8} per ml; the spore estimate was 8.8×10^{7} per ml.

** Surface viable counts of duplicate samples, performed by a spread plate technique on the stated medium. All plates seeded from samples similarly treated were incubated for 18 hr in the same anaerobic jar.

Δ EBA = 10 per cent. equine blood agar; EBAIF = EBA sprinkled with sterile iron filings; CDEBA = EBA supplemented with cysteine and dithiothreitol; CDEBAIF = CDEBA sprinkled with sterile iron filings.

TABLE 22

The surface viable counts determined for duplicate 0.02-ml samples of a 21-hr CMB culture of Cl. histolyticum*, before and after pasteurisation, with recovery on different media and aerobic incubation of the plates

Treatment of sample	Surface viable count per ml** estimated from cultures grown on			
	EBA$^{\Delta}$	EBAIF	CDEBA	CDEBAIF
nil	$(8.6\text{-}9.4)\times10^{8}$		$(8.3\text{-}8.4)\times10^{8}$	
heated at 70°C for 20 min.	$(4.3\text{-}4.5)\times10^{7}$	Colonies too small to count and presence of IF made counting unreliable	$(4.3\text{-}4.7)\times10^{8}$	Colonies too small to count and presence of IF made counting unreliable
heated at 80°C for 20 min.	$(2.9\text{-}3.1)\times10^{7}$		$(2.8\text{-}3.3)\times10^{7}$	

* The total cell count was 5.3×10^{8} per ml; the spore estimate was 8.8×10^{7} per ml.

** Surface viable counts of duplicate samples, performed by a spread-plate technique on the stated medium. All plates seeded from samples similarly treated were incubated for 18 hr in the same anaerobic jar.

Δ EBA = 10 per cent. equine blood agar; EBAIF = EBA sprinkled with sterile iron filings; CDEBA = EBA supplemented with cysteine and dithiothreitol; CDEBAIF = CDEBA sprinkled with iron filings.

were obtained when the media were incubated aerobically (Table 22). The results of a typical experiment with a 5-hr culture of Cl. bifermentans (Table 23) show that more than 1 x 10^8 organisms per ml were recovered from inocula estimated to contain c. 1.8 x 10^7 spores per ml.

The results of this series of experiments show clearly that for all the organisms tested, it is possible to recover considerably more organisms on the surface of seeded plates than the estimated numbers of spores present in the inocula; this was most clearly demonstrated when the proportion of spores was poised at low levels by the use of young (5-hr) CMB cultures. Thus, it is possible to recover quantitatively vegetative cells of medically important anaerobes by traditional anaerobic techniques; this is true not only for non-demanding organisms such as Cl. welchii, but also for more demanding ones such as Cl. oedematiens of types A and B and Cl. tetani. The concept of the "significantly viable particle", introduced with the Cl. oedematiens type-D model, can therefore be extended usefully to other anaerobes of more direct clinical importance: vegetative cells can contribute proportionately and absolutely more to the surface viable counts than the spores present in the inoculum.

The fact that the standard anaerobic technique enabled quantitative recovery of vegetative cells on solid media encouraged the writer to determine the recovery rates of other anaerobes on solid media.

TABLE 23

The surface viable counts determined for duplicate 0.02-ml samples of a 5-hr CMB culture of Cl. bifermentans (strain 506)* before and after pasteurisation, with recovery on different media

Treatment of sample	Surface viable count per ml** estimated from cultures grown on			
	EBA$^{\Delta}$	EBAIF	CDEBA	CDEBAIF
nil	$(1.45\text{-}1.48)\times10^{8}$	4.2×10^{7} $\Delta\Delta$	$(1.1\text{-}1.2)\times10^{8}$	$(1.0\text{-}1.1)\times10^{8}$
heated at 70°C for 20 min.	$(0.9\text{-}1.2)\times10^{6}$	8.85×10^{5}	$(1.1\text{-}1.2)\times10^{6}$	$(0.9\text{-}1.0)\times10^{6}$
heated at 80°C for 20 min.	$(5.6\text{-}5.8)\times10^{5}$	5.45×10^{5}	$(4.2\text{-}5.2)\times10^{5}$	$(3.3\text{-}3.7)\times10^{5}$

* The total cell count was 2.6×10^{8} per ml; the spore estimate was 1.8×10^{7} per ml.

** Surface viable counts of duplicate samples, performed by a spread-plate technique on the stated medium. All plates seeded from samples similarly treated were incubated for 18 hr in the same anaerobic jar.

Δ EBA = 10 per cent. equine blood agar; EBAIF = EBA sprinkled with sterile iron filings; CDEBA = EBA supplemented with cysteine and dithiothreitol; CDEBAIF = CDEBA sprinkled with sterile iron filings.

$\Delta\Delta$ Spreading growth made accurate colony counting difficult; the figures given are minimum estimates.

STUDIES WITH NON-SPORING ANAEROBES

The recovery of Bacteroides organisms from CMB cultures

The recovery rate of young (15 hr) and older (23 hr) CMB cultures was estimated by determination of the surface viable counts on plates of equine blood agar (EBA) medium, and this was compared with the total cell count determined by phase-contrast microscopy. Each total cell count is the mean of 2 - 4 replicate observations made by two observers. The results of a representative experiment are shown in Table 24, in which representative strains of Bacteroides fragilis and B. necrophorus were used.

The results demonstrate that, especially in the case of older cultures, a large proportion of the organisms present can be recovered on simple blood agar medium, by standard anaerobic techniques. Better recovery was obtained with the B. fragilis strain than with B. necrophorus strain. It should be noted that in all these experiments, 10 per cent. carbon dioxide was added to the anaerobic environment. The effects of incubation in atmospheres with and without carbon dioxide are considered later (page 186).

The recovery of anaerobic organisms from human faeces

It has been claimed by workers such as Drasar (1967) that quantitative recovery of obligate anaerobes from intestinal samples can only be achieved by techniques involving the use of anaerobic cabinets or gas flushing procedures. The evidence given above that vegetative cells of demanding sporing anaerobes can be recovered by routine anaerobic techniques encouraged the author to investigate the recovery of anaerobic organisms from human faeces, using the standard anaerobic

TABLE 24

The recovery from dilutions of 5-hr and 23-hr CMB cultures of two Bacteroides strains on EBA plates, and the total cell counts of the inocula

Culture	Organism	Dilution	Mean surface viable count per ml ± S.E.*	Total cell count per ml
5-hr CMB	*Bacteroides fragilis* NCTC 9343	10^{-4}	$(4.5 \pm 0.14) \times 10^8$	1.07×10^9
23-hr CMB		10^{-5}	$(2.9 \pm 0.09) \times 10^9$	3.2×10^9
5-hr CMB	*Bacteroides necrophorus* NCTC 7155	$\frac{1}{2} \times 10^{-3}$	$(1.6 \pm 0.04) \times 10^8$	1.9×10^8
23-hr CMB		$\frac{1}{2} \times 10^{-3}$	$(6.6 \pm 0.25) \times 10^7$	3.4×10^8

* Each count is expressed as the mean ± S.E. (standard error of the mean) of ten replicate plates, each seeded with 0.02-ml inocula at the stated dilution.

procedure already described.

In these experiments, a sample (c. 1 g) of freshly passed faeces was added to a conical flask containing 100 ml of sterile pre-steamed nutrient broth and a magnetic stirring rod. The flask and contents were weighed before and after the addition of the faecal sample, and the weight of faeces was accurately determined. The flask and contents were placed in an anaerobic jar, which was promptly evacuated, filled with a 10 per cent. CO_2 and 90 per cent. hydrogen mixture, held in the cold room on a magnetic stirrer and the flask was stirred at 4°C for 5 hr. Pilot experiments had shown that satisfactory stirring occurred through the metal floor of the anaerobic jar. After stirring for 5 hr, the flask and contents were removed from the jar, and tenfold dilutions of the faecal suspension were made in pre-steamed nutrient broth at room temperature. Replicate 0.02-ml volumes of the dilutions were used to seed plates of freshly-poured equine blood agar (EBA) medium. Seeded plates were incubated aerobically and anaerobically at 37°C for 18 hr. It was found that the aerobic organisms present in the samples could be "diluted out", i.e., in dilutions that yielded 100-1000 colonies per anaerobic plate, there were only 1 - 10 aerobes respectively. The counts on anaerobically incubated plates seeded from dilutions giving 100-1000 colonies per plate were therefore assumed to represent the "total anaerobic" viable counts; however, aerobic checks were always performed on all dilutions from serially diluted samples. The results of a typical experiment, summarised in Table 25, show that viable counts of more than 1×10^{10} organisms per g can be achieved.

Similar results were obtained when attempts were made to isolate maximum numbers of Bacteroides-like organisms from faeces in

TABLE 25

The recovery of anaerobic organisms from dilutions of a sample of human faeces

Weight of sample	Dilution*	No. of colonies subcultured from 4 x 0.02 ml of faecal suspension at stated dilution on EBA medium				Mean total anaerobic count per g of faeces
1.79g	10^{-5}	127	142	125	125	3.6×10^{10}

* The dilution refers to the dilution of the faecal suspension; the initial dilution (1.79 g in 100 ml) is taken into account in determining the total anaerobic count.

experiments in which BTL jars equipped with "Gaspak" anaerobic systems were used.

A fresh faecal sample was emulsified in transport broth (See Materials and Methods) by a magnetic stirrer. Parallel serial dilutions were made in pre-steamed nutrient broth and transport medium that had both been held for some hours before use under anaerobic conditions. Tenfold dilutions were made in an atmosphere constantly flushed with CO_2. With continuous CO_2 flushing, 10^{-4}, 10^{-5} and 10^{-6} dilutions of each suspension were prepared, and 0.02-ml drops from each dilution were used to seed blood agar plates that had been anaerobically stored. Three plates were seeded from each dilution, and each dilution series was performed in duplicate, so that a total of 36 plates were prepared. As soon as each batch of nine plates was ready, the plates were placed in a jar, a Gaspak envelope added and activated by addition of water, the jar closed, and incubated for 48 hr. The colonies were then counted; Gram-stained smears showed that the predominant organisms on plates used for enumeration were Gram-negative bacteria morphologically typical of Bacteroides, and these were not identified further; organisms typical of lactobacilli were also present.

The results obtained with the special transport medium (1.7×10^{10} Bacteroides-like organisms per g of faeces) did not differ appreciably from those obtained with the nutrient broth diluent (1.9×10^{10} Bacteroides-like organisms per g of faeces). Both counts however fall within the range of counts said to be obtainable only by specialised anaerobic techniques (Drasar, 1967). An experiment was then devised in which the recovery of anaerobic organisms from a fresh sample of faeces

was compared with the total cell count of the sample as estimated by phase-contrast microscopy. The author sought to avoid the possibility of particulate matter contributing to falsely high counts, by examining several stained smears from the dilutions of the faecal sample that were used for phase contrast enumerations; at these dilutions, there was no evidence of inert particulate matter in Gram-stained smears, and the phase-contrast counts were therefore assumed to be reasonably accurate. The results of the experiment are shown in Table 26. It is clear that although the recovery of organisms is as high as recorded by other workers who used more elaborate techniques, the numbers of organisms recovered are a small percentage of the total cell count determined by phase-contrast microscopy.

The recovery of Bacteroides-like organisms was also determined in studies with a faecal suspension that had been held deep frozen for varying periods of time. No appreciable diminution in the surface viable counts was noted (see Table 27) and it appears that deep-freeze storage of faecal samples at -30°C does not significantly affect the recovery of anaerobes, under the test conditions and during the period of time tested in this series.

Thus quantitative recovery of both sporing and non-sporing anaerobes could be obtained on solid media with the standard anaerobic procedure. The sub-optimal recovery observed with some species, however, prompted a study of possible factors that would stimulate the growth of anaerobes on solid media.

TABLE 26

The recovery of anaerobic organisms from dilutions of a sample of human faeces and the total cell count of the inoculum

Weight of sample	Dilution*	No. of colonies subcultured from 3 x 0.02 ml of faecal suspension at stated dilution on EBA medium			Mean total anaerobic count per g of faeces	Total cell count per g of faeces
0.92 g	10^{-5}	48	60	59	3.0×10^{10}	7.2×10^{12}

* The dilution refers to the dilution of the faecal suspension; the initial dilution (0.92 g in 100 ml) is taken into account in determining the total anaerobic count.

TABLE 27

The recovery of anaerobic organisms from dilutions of fresh and frozen samples of a suspension of human faeces

Treatment of sample	Dilution*	Mean viable count per ml ± S.E.**
Fresh	10^{-5}	2.3×10^{10} △
Kept at -30°C for 24 hr	$\frac{1}{5} \times 10^{4}$	$(2.0 \pm 0.09) \times 10^{10}$
Kept at -30°C for 48 hr	$\frac{1}{5} \times 10^{4}$	$(1.8 \pm 0.06) \times 10^{10}$
Kept at -30°C for 72 hr	$\frac{1}{5} \times 10^{4}$	$(2.2 \pm 0.06) \times 10^{10}$
Kept at -30°C for 14 days	$\frac{1}{5} \times 10^{4}$	$(1.6 \pm 0.03) \times 10^{10}$

* The dilution given is the dilution of the faecal suspension. The original sample of faeces was diluted 1/20 to provide the faecal suspension.

** Each count is expressed as the mean ± S.E. (standard error of the mean) of ten replicate plates, each seeded with 0.02 ml of the faecal sample, at the stated dilution.

△ Expressed as the mean of triplicate plates.

A STUDY OF FACTORS THAT MAY STIMULATE THE GROWTH OF ANAEROBES ON SOLID MEDIA

As noted above (page 133), almost complete recovery of many anaerobes on solid media could be achieved with the use of simple blood agar media in conjunction with careful anaerobic technique. However, in the case of some organisms, notably Cl. tetani type VI and Bacteroides sp., although very good recovery rates could be obtained on occasions, this success was inconsistent and recovery was often poor. In the case of Cl. oedematiens of types B and D, (the most demanding models) the cysteine/dithiothreitol system gave greatly improved recovery of these organisms on solid media, but the recovery was sometimes only 10-20 per cent. of the total cell count, even when young (5-hr) cultures were used. Several possible enhancing factors were therefore investigated to determine whether the efficiency of recovery of these organisms on solid media could be improved.

The influence of the iron filings technique and the cysteine/dithiothreitol system on the growth of various anaerobic bacteria

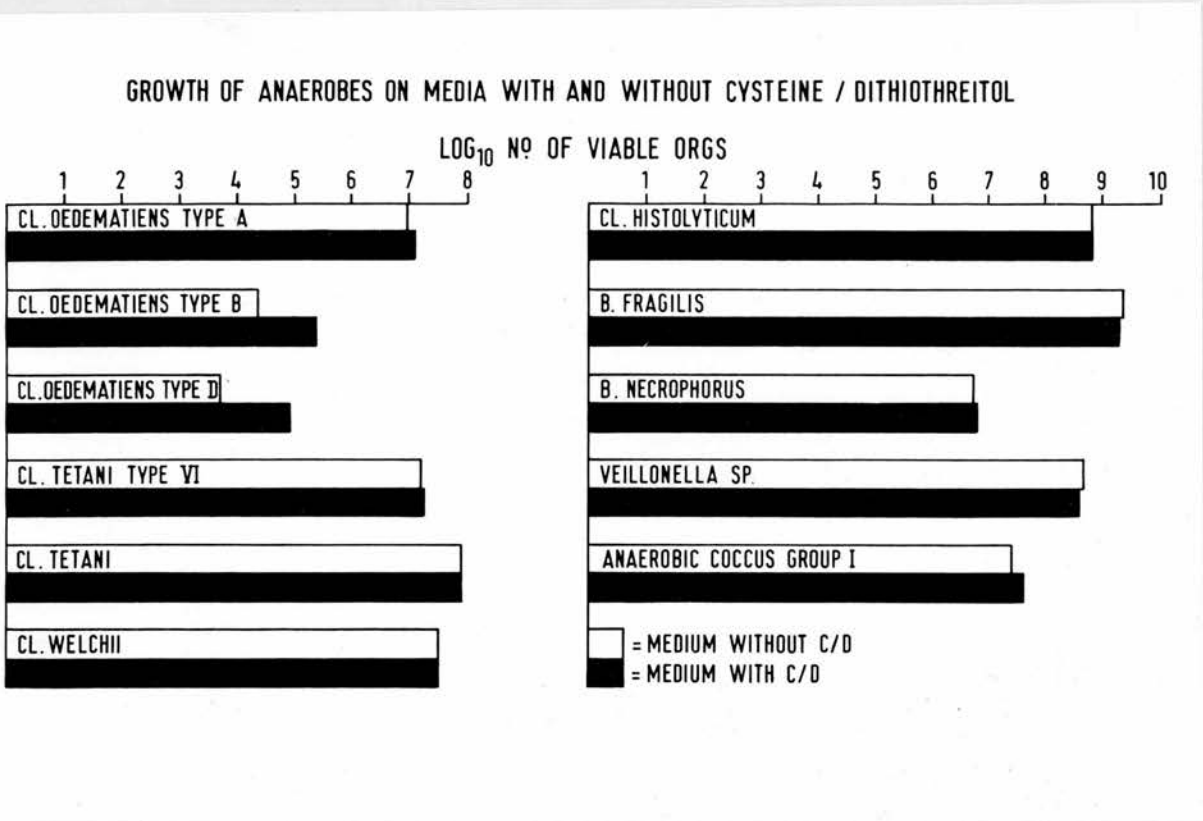
The recovery of the following clostridia was compared on four media used in parallel; non-haemolytic heat-resistant Cl. welchii type A; beta-haemolytic non-heat resistant Cl. welchii type A; Cl. tetani type VI; Cl. septicum (2 strains); Cl. bifermentans; Cl. histolyticum and Cl. oedematiens of types A, B and D. The four media used were (i) 10 per cent. equine blood agar (EBA); (ii) EBA with sterile iron filings sprinkled on the surface of seeded plates (EBAIF); (iii) 10 per cent. equine blood agar with cysteine-dithiothreitol (CDEBA); and (iv) CDEBA with iron filings (CDEBAIF). With the exception of the test

strains of Cl. oedematiens of types A, B and D, the other test clostridia showed no qualitative or quantitative enhancement of growth when the cysteine/dithiothreitol system or the iron filings technique were used separately or in combination. In the case of type-B and type-D strains of Cl. oedematiens, enhancement of growth was achieved by both systems, although the combination of the two systems did not appear to have any cumulative effect. The cysteine/dithiothreitol system was also effective in enhancing the growth of these organisms when 0.1 ml of a suspension containing 9 mg dithiothreitol and 100 mg cysteine per ml was spread on to the surface of freshly-poured EBA plates prior to seeding, but the spreading growth that occurred as a consequence of the increased moisture content of the plates rendered colony counting difficult. In the case of type-A strains, a minimal enhancement of surface growth was observed with the cysteine/dithiothreitol system, whereas iron filings produced a slightly inhibitory effect. The relevant data have already been summarised in Table 19.

In an extended study to compare the growth of a wider range of anaerobic organisms on EBA medium with and without the cysteine/dithiothreitol system, the results summarised in Fig. 7 indicate that, apart from the Cl. oedematiens group, the only other organism to show even a minimal enhancement of growth was an anaerobic coccus. Several aerobic organisms were also tested; no enhancement of growth was observed with CDEBA medium in comparison with that obtained on EBA.

The cysteine/dithiothreitol system, and the iron-filings technique seem therefore to be specific enhancing factors for organisms of the Cl. oedematiens group; under the test conditions, neither of these procedures seems to offer a useful basis for a general purpose

FIGURE 7


GROWTH OF ANAEROBES ON MEDIA WITH AND WITHOUT CYSTEINE / DITHIOTHREITOL
LOG10 Nº OF VIABLE ORGS
1 2 3 4 5 6 7 8
CL. OEDEMATIENS TYPE A
CL. OEDEMATIENS TYPE B
CL. OEDEMATIENS TYPE D
CL. TETANI TYPE VI
CL. TETANI
CL. WELCHII
1 2 3 4 5 6 7 8 9 10
CL. HISTOLYTICUM
B. FRAGILIS
B. NECROPHORUS
VEILLONELLA SP.
ANAEROBIC COCCUS GROUP I
= MEDIUM WITHOUT C/D
= MEDIUM WITH C/D

anaerobic culture medium. Several other substances were therefore tested to determine whether their addition to EBA medium had similar enhancing effects on the surface growth of a series of test anaerobes. The substance included ethylene diaminetetra-acetic acid (EDTA), catalase, Tris (hydroxymethyl) aminomethane, several sulphydryl compounds, menadione and cobalt.

Studies with ethylene diaminetetra-acetic acid (EDTA)

Preliminary qualitative studies showed that addition of EDTA to blood agar media did not enhance the growth of any of a range of test anaerobes. The growth of Cl. oedematiens type D, Cl. tetani type VI, and B. fragilis (NCTC 9343) was then compared in a series of quantitative studies. Varying amounts of a solution of EDTA in distilled water (100 mg per ml), and sterilised by Millipore filtration, were added to molten blood agar. Plates were then poured and dried with final concentrations of 0, 10, 20, 50 and 100 mg EDTA per 100 ml of medium. Tenfold dilutions of overnight CMB cultures of the test organisms were used to seed three replicate plates of each medium with 0.02-ml inocula. Duplicate plates of each medium were incubated anaerobically, and parallel plates incubated aerobically.

In the case of Cl. tetani and B. fragilis, no enhancement of growth was observed on any of the anaerobically incubated plates, and some inhibition of growth was observed with the plates that contained 100 mg EDTA per ml. No growth of Cl. oedematiens type D was observed on any of the anaerobically incubated media containing EDTA, although control plates of CDEBA plates, seeded from the same inocula and incubated in the same anaerobic jar, gave good growth of colonies

typical of Cl. oedematiens type D. No growth was observed on any of the aerobically incubated plates. Further studies with type-D and type-B strains of Cl. oedematiens confirmed that addition of EDTA to EBA medium (in concentrations up to 1 g per 100 ml) failed to improve the irregular growth of these organisms on this medium, and at 1 g per 100 ml, positively inhibited growth of the test strains.

Studies with catalase

Addition of catalase (ex bovine liver, lyophilised" from Koch-Light) to blood agar media did not affect the anaerobic growth of the test anaerobes. No growth occurred on seeded plates with added catalase when these were incubated aerobically. As blood agar media possess intrinsic catalase activity, it was decided to develop a medium in which the amount of intrinsic catalase activity was minimal. The author is indebted to Dr. J. G. Collee for developing a medium that showed no catalase activity, as measured by the evolution of gas produced on addition of a 0.02-ml drop of a ("100 Vol.") Hydrogen Peroxide Solution (Analar) to the plate. The details of this "catalase-free" medium are given in the Material and Methods section.

Growth of Cl. oedematiens types B and D was very poor on this medium, and the addition of catalase to the medium in concentrations ranging from 10 to 100 mg per ml of medium, failed to improve the growth of these organisms; failures of growth sometimes occurred, although seeded control plates of CDHBA 16 medium incubated in the same anaerobic jar gave confluent growth. Addition of cysteine and dithiothreitol to the catalase-free medium to give final concentrations of 100 mg per 100 ml and 9 mg per 100 ml respectively greatly enhanced

the growth of type-B and type-D strains of Cl. oedematiens. Addition of catalase to this medium did not further improve the surface growth of these organisms even if concentrations of catalase up to 100 mg per ml were used. This lack of enhancement was observed both when catalase was incorporated into the medium, and when discs impregnated with catalase solution were placed on the surface of seeded plates.

Cl. tetani type VI showed only poor growth on catalase-free medium, but addition of catalase to the medium failed to produce any enhancement of growth even when concentrations as high as 100 mg per 100 ml were used.

In the case of B. fragilis and B. necrophorus strains, growth was adequate, but not luxuriant, on catalase-free media alone. Plates seeded from inocula derived from CMB cultures of B. fragilis required incubation under anaerobic conditions for 48 hr before easily visible colonies appeared. Preliminary studies suggested that addition of catalase to the medium at a final concentration of 10 mg per 100 ml medium produced some enhancement of growth, although the effect was not progressively marked with increasing concentrations of catalase. It was therefore decided to compare the recovery of B. fragilis and and B. necrophorus on catalase-free medium with and without the addition of catalase (10 mg per 100 ml medium) in quantitative studies. The results of a typical experiment are shown in Table 28. In the case of B. fragilis, the colonies on catalase-free medium were too small to count after 24 hr incubation, compared with easily visible colonies on medium with added catalase. After 48 hr incubation, although there was still a difference in the size of the colonies, those on the catalase-free medium could be counted, and results showed

TABLE 28

The recovery from dilutions of overnight CMB cultures of two Bacteroides strains on catalase-free medium with and without the addition of catalase

Test strain	Dilution	Mean colony count ± S.E.* of replicate samples grown on		t-value for difference of means on 18 d.f.
		Catalase-free medium	Catalase-free medium + 10 mg catalase per ml	
B. necrophorus (NCTC 7155)	10^{-4}	150.1 ± 18.2	157.7 ± 9.9	0.49 ** (not significant)
B. fragilis (NCTC 9343)	$\frac{1}{2} \times 10^{-5}$	237.5 ± 7.2	206.6 ± 14.6	1.90 ** (not significant)

* Each count is expressed as the mean ± S.E. (standard error of the mean) of ten replicate plates, each seeded with 0.02 ml of the test sample, at the stated dilution.

** See Materials and Methods for explanation of convention adopted.

that there was no significant difference in the numbers of organisms recovered on the two types of media.

In the case of B. necrophorus, colonies on catalase-free medium after 24 hr incubation, were easily visible, but smaller than those on the medium with added catalase. There was no significant difference in the recovery rates on the two media (see Table 28).

Aerobically incubated plates, seeded from CMB cultures of all of the above organisms failed to show any growth, even when catalase in a concentration of 100 mg per 100 ml was added to the plates.

Thus, although the added catalase appeared to produce some effect in terms of luxuriance of growth, there was no quantitative evidence of improved recovery. This is considered in the Discussion.

Studies with TRIS (hydroxymethyl) amino-methane (TRIS buffer)

As TRIS (THAM) buffer has been shown to protect animals against some of the effects of oxygen toxicity (see p. 25) it was decided to determine whether addition of this substance to culture media enhanced the growth of test anaerobes, or allowed growth to proceed under aerobic conditions. Solutions of TRIS buffer of varying strengths were prepared in distilled water and sterilised by Millipore filtration. 5-ml amounts of these solutions were added to molten EBA medium at a temperature of c. 45°C, and plates were poured. The final concentrations of TRIS in the plates were 0, 10, 100, 500, 1000 and 2500 mg per 100 ml medium respectively.

Qualitative studies failed to show any enhancement of growth of the test anaerobes (Cl. oedematiens types B and D, Cl. tetani type VI, and B. fragilis) and concentrations of TRIS of 1000 mg per 100 ml

or more were inhibitory. Quantitative studies, in which recovery was compared from dilutions of the test strains on blood agar plates containing 0, 10, 20, 50 and 100 mg per 100 ml, confirmed that no enhancement of growth occurred on TRIS-containing media. Growth of the Cl. oedematiens strains on EBA medium alone was irregular and was not improved by the addition of TRIS, even in concentrations of 500 mg per ml.

No growth of any of the test strains occurred when seeded plates containing TRIS buffer in concentrations up to 100 mg per 100 ml were incubated aerobically.

Studies with sulphydryl (-SH) compounds

In view of the specific enhancing effect of the cysteine/dithiothreitol system for organisms of the Cl. oedematiens group, and as both of these compounds have reactive sulphydryl groups, other sulphydryl compounds were tested to determine whether they could substitute for the cysteine/dithiothreitol system in the growth of Cl. oedematiens. The growth of Cl. oedematiens type D was compared on blood agar media, and on blood agar media with various supplements. Three sulphydryl compounds, cysteamine, penicillamine and glutathione, were kindly supplied by Professor G. S. Boyd, and stock solutions of these compounds containing 10 millimoles (mmol) per ml and 50 mmol per ml were prepared in distilled water and sterilised by Millipore filtration. 1-ml amounts of the stock solutions were added to 100-ml aliquots of EBA medium, to give media containing the sulphydryl compounds at final concentrations of 1 milliMolar (mM) and 5 mM respectively. Growth was also assessed in parallel on CDEBA media. Duplicate

plates of each medium were seeded from dilutions of an overnight CMB culture of Cl. oedematiens type D. The media used, and the results, are given in Table 29. All media containing a stated sulphydryl compound were prepared on a single day, so that no direct comparison between the viable counts on media containing different sulphydryl compounds is possible.

It can be seen that although addition of the test compounds to EBA medium at concentrations of 1 mM enhanced the surface growth of the organism compared with the growth on EBA medium alone, better results were obtained if the -SH compound was used at a concentration of 5 mM. A combination of cysteine (100 mg per ml) and any of the three compounds (5 mM) gave surface viable counts similar to those obtained on cysteine/dithiothreitol medium. In the case of glutathione, slightly higher counts were obtained when cysteine was combined with glutathione than when it was combined with dithiothreitol.

Similar comparative studies were then performed with inocula derived from CMB cultures of the following test organisms: Cl. tetani type VI NCTC 9567; Cl. oedematiens type D NCTC 9692; Bacteroides fragilis NCTC 9343; and an anaerobic coccus (Hare's Group I) NCTC 9801. The growth of the inocula of the test organisms was compared on the media shown in Table 29. No enhancement of the growth of the test strains was observed when the sulphydryl compounds were added to EBA medium and the lack of enhancement of growth on media containing cysteine and dithiothreitol with these organisms was confirmed.

Plates of media containing the stated sulphydryl compounds at concentrations of 1 mM and 5 mM did not show any growth when seeded with the test strains and incubated aerobically at 37°C.

TABLE 29

The growth of Cl. oedematiens type D on blood agar medium with and without the addition of sulphydryl compounds to the medium

Sulphydryl compound	Surface viable count per ml* estimated from colonies grown on medium**					
	A	B	C	D	E	F
Cysteamine$^{\Delta}$	$(5.5\text{-}7.0)\times10^{5}$	$(2.3\text{-}2.5)\times10^{6}$	$(2.0\text{-}4.9)\times10^{5}$	$(2.1\text{-}2.6)\times10^{6}$	$(2.4\text{-}2.9)\times10^{6}$	$(2.6\text{-}5.0)\times10^{5}$
Glutathione	$(1.5\text{-}3.0)\times10^{4}$	$(7.3\text{-}8.3)\times10^{5}$	$(0.6\text{-}1.7)\times10^{5}$	$(1.0\text{-}1.5)\times10^{4}$	$(1.1\text{-}1.4)\times10^{6}$	$(6.3\text{-}7.0)\times10^{5}\Delta\Delta$
Penicillamine	$(1.2\text{-}1.3)\times10^{5}$	$(3.3\text{-}3.4)\times10^{6}$	$(4.9\text{-}9.1)\times10^{5}$	$(1.61\text{-}1.62)\times10^{6}$	$(3.4\text{-}3.7)\times10^{6}$	$(1.5\text{-}2.8)\times10^{6}$

* For each test series A-F the recovery is expressed as estimated numbers per ml of the standard inoculum, which was 0.02 ml of a 10^{-2} dilution of a 23-hr CMB culture in each case. The counts were done in duplicate and the ranges are given.

** The test media were: A = EBA; B = EBA + cysteine 100 mg per 100 ml and dithiothreitol 9 mg per 100 ml; C = EBA + the stated sulphydryl compound (1 milli-Molar); D = EBA+ the stated sulphydryl compound (5 milli-Molar); E = EBA + cysteine 100 mg per 100 ml + the stated sulphydryl compound (1 milli-Molar); F = EBA + cysteine 100 mg per 100 ml + the stated sulphydryl compound (5 milli-Molar).

Δ All seeded plates of a given sulphydryl compound were incubated in a single anaerobic jar for 18 hr.

$\Delta\Delta$ Spreading growth rendered accurate colony counting difficult; the figures given are therefore minimum estimates.

Studies with menadione

Menadione has been recommended as a growth-enhancing factor for some strains of Bacteroides melaninogenicum on solid media (Bowden and Hardie, 1971). In addition many other organisms, including anaerobes, have been shown to metabolise this substance (Gibbons and Engle, 1964). It was therefore decided to determine whether addition of menadione to blood agar media affected the growth of a range of test anaerobes. The test organisms were Cl. septicum (1 strain); Cl. welchii, β-haemolytic, non heat-resistant (2 strains); Cl. welchii, non-haemolytic, heat resistant (2 strains); Cl. tetani, type VI (1 strain); Cl. oedematiens, type B (1 strain); Cl. oedematiens, type D (1 strain); one strain of an anaerobic coccus (Hare's group 1); B. fragilis (1 strain); B. necrophorus (1 strain); and B. melaninogenicum (2 strains). Duplicate plates of EBA medium, and of EBA medium with added menadione (5 μg per ml, see Materials and Methods) were seeded from dilutions of CMB cultures of the test organisms, and the colonies counted after 24 hr and 48 hr incubation. The results of these studies showed no obvious enhancement with any of the organisms; in the case of the anaerobic coccus, some inhibition was noted on plates containing added menadione.

The recovery of each of the Bacteroides strains was then compared in a series of statistically-designed experiments in which larger numbers of plates of each medium were used. No significant enhancement occurred with any of the three strains, including the two strains of B. melanogenicum. The author takes note of the fact that only certain strains of this organism are reported to require

menadione as a growth factor.

Studies with cobalt

Cobalt has been claimed by some workers to enhance the growth of Cl. tetani (Dedic and Koch, 1956) and Cl. sporogenes (Faguet and Goudot, 1961). The possible growth enhancing effect of the addition of cobalt (as cobalt nitrate - see Materials and Methods) to blood agar medium was tested with Cl. septicum lab. no. 114; Cl. oedematiens type B, strain GR1B; Cl. oedematiens type D, strain GR1D; Cl. welchii lab. no. L2A; Cl. welchii, Hobbs type 1; Cl. welchii, Hobbs type IV; Cl. tetani, type VI; B. fragilis; B. necrophorus; B. melaninogenicum and an Anaerobic coccus, Hare's Group I.

Tenfold dilutions were made from overnight CMB cultures of the test organisms, and dilutions were chosen to give 100-700 colonies per plate. Freshly poured plates of EBA medium, and of EBA medium containing 4 μg Co^{++} per ml were prepared, and duplicate plates of each medium were seeded with 0.02-ml inocula from the dilution of the test organism, and spread in a standard manner. The procedure was repeated for all the test organisms; all plates seeded from a given test organism were incubated in the same anaerobic jar. After 48 hr incubation at 37°C, the plates were examined, and the colonies counted.

There was no qualitative or quantitative enhancement of growth of any of the test organisms on media containing added cobalt; in the case of the Cl. welchii strains, some inhibition of growth was observed. The absence of any enhancing effect in the case of the Cl. tetani strain prompted the author to compare the growth of

Cl. tetani type VI on EBA medium with and without the addition of 4 mg Co^{++} per ml, in a statistically-designed experiment. Twenty plates of each medium were prepared, and seeded from a 10^{-4} dilution of an overnight CMB culture of the test organism. The plates were seeded so that a plate of each medium was sown alternately. The plates were spread in a similar order, and distributed randomly between 4 anaerobic jars so that each jar contained 5 plates of each medium. The distribution of the plates within a given jar was also randomised. The jars were then incubated anaerobically for 48 hr. It was clear from the results of this experiment that there was no increase in the size or numbers of colonies on plates containing EBA medium alone. The mean colony count $\pm$ standard error of the mean derived from 20 plates of EBA medium was 194.8 $\pm$ 8.9; that from plates of EBA medium with added cobalt was 190.5 $\pm$ 9.7. The author was increasingly aware that there was no substance(s) that would allow the general enhancement of growth of all anaerobes. This led to a search for specific enhancing factors (see above) that could be added to blood agar media. However, variations between batches of media resulted in such studies being performed with a shifting base-line; therefore it seemed worthwhile to attempt to control the base-line, so that variables could be defined for future study. Cl. tetani was chosen as a model that had both general biological interest as an example of a strict anaerobe, and clinical importance.

A STUDY OF THE GROWTH OF CL. TETANI ON A DEFINED MEDIUM

The results of earlier experiments showed that recovery of Cl. tetani on blood agar media was on occasion sub-optimal. Even if plates of adequate thickness were incubated for 72 hr (see p.178), recovery on the surface of solid media from young (5 hr) CMB cultures was occasionally appreciably less than the number of cells in the inoculum, as estimated by phase-contrast microscopy, and addition of several possible enhancing factors to blood agar media did not improve the number or size of colonies of Cl. tetani recovered. However, variation between batches of a complex, non-defined medium such as EBA could lead to variations in recovery of anaerobes. It was therefore decided to attempt to grow Cl. tetani on a defined medium to avoid batch variation and to allow accurate comparisons of the recovery of this organism on basal medium with various additions.

Although the growth of Cl. tetani in defined synthetic or semi-synthetic liquid media has been described by several workers (e.g., Feeney, Mueller and Miller, 1943; Kaufman and Humphries, 1958), the present author could find no references to the growth of this organism on a defined solid medium. As it was not the purpose of the present study to investigate the growth requirements of the organism (see Discussion), it seemed reasonable to use an existing defined, synthetic medium, to which could be added a "pure" agar such as Ionagar, and which would allow minimal growth of Cl.tetani. Following a report by Nakamuru, Cook and Cross (1968) that Clostridium perfringens could be grown in NCTC 109 medium (Grand Island Biological Company, Grand Island, New York) growth and serial passage of a strain of Cl. tetani

was attempted on this medium. (Note: the author appreciates that this medium is far from minimal, i.e., it supplies several nutrients not known to be essential for the growth of Cl. tetani). The composition of this medium is shown in Table 30. Pilot studies showed that little or no growth of Cl. tetani strains occurred in this medium unless the cysteine and ascorbic acid was supplemented, and therefore to each tube of medium was added 0.1 ml of solution containing 50 mg cysteine per ml, and 0.1 ml of a solution containing 50 mg ascorbic acid per ml in distilled water. The final medium is hereafter referred to as "109" medium.

Duplicate tubes prepared as above were each seeded with 0.1 ml of an overnight CMB culture of Cl. tetani type VI (NCTC 9569), and incubated anaerobically for 48 hr. The tubes were examined at 24 and 48 hr, and Gram stained smears prepared. After 48 hr, 0.1-ml inocula from each tube were used to seed quadruplicate plates of EBA medium; duplicate plates were incubated aerobically and anaerobically.

Visible turbidity was observed after 24 and 48 hr, and anaerobically incubated EBA plates showed colonies typical of a non-motile strain of Cl. tetani. Gram stained smears revealed organisms morphologically typical of Cl. tetani. Similar results were obtained in replicate experiments but in order to ensure that growth of Cl. tetani in 109 medium was not merely due to carry-over of nutrients from CMB cultures, it was necessary to passage the organism serially in the 109 medium. Accordingly, 0.1-ml inocula from tubes of 109 medium, treated as in the above experiment, were used to seed fresh tubes of 109 medium which had been held anaerobically at 16-18°C for 24 hr. These latter tubes were incubated anaerobically for 48 hr at

TABLE 30

The formulation of NCTC 109 medium

	mg per litre
L-alanine	31.5
L-α-amino butyric acid	5.5
L-arginine	31.2
L-asparagine	8.0
L-aspartic acid	9.9
L-cysteine HCl-H_2O	259.9
L-cysteine	10.5
L-glutamic acid	8.3
glycine	13.5
L-histidine HCL.H_2O	26.7
L-hydroxyproline	4.1
L-isoleucine	18.0
L-leucine	20.4
L-lysine	38.4
L-methionine	4.4
L-ornithine	7.4
L-phenylalanine	16.5
L-proline	6.1
L-serine	10.8
L-taurine	4.2
L-threonine	18.9
L-tryptophan	17.5
L-tyrosine	16.4
L-valine	25.0

	mg per litre
p-aminobenzoic acid	0.13
ascorbic acid	49.9
D-biotin	0.03
calciferol	0.3
D-calcium pantothenate	0.03
choline chloride	1.3
cocarboxylase	1.0
folic acid	0.03
i-inositol	0.13
menadione	0.03
Nicotinamide	0.06
nicotinic acid	0.06
pyridoxal HCl	0.06
pyridoxine HCl	0.06
riboflavin	0.03
DL-α-tocopherol phosphate (Na_2)	0.03
thiamine HCl	0.03
vitamin A	0.25
vitamin B_{12}	10.0
coenzyme A	2.5
deoxyadenosine	10.0
deoxycytidine HCl	10.0
deoxyguanosine	10.0
diphosphopyridine nucleotide tetrahydrate (DPN.$4H_2O$)	7.0

	mg per litre
flavin adenine dinucleotide (FAD)	1.0
glucose	1000.0
D-glucosamine	3.2
D-glucuronolactone	1.8
L-glutathione	10.1
5-methylcystosine	0.1
phenol red	17.0
sodium acetate · $3H_2O$	83.0
sodium glucuronate	1.8
thymidine	10.0
triphosphopyridine nucleotide monosodium salt (TPN)	1.0
Tween 80	12.5
uridine-5'-triphosphate tetrasodium tetrahydrate (UTP)	1.0
($CaCl_2$ (anhydrous)	200.0
(K Cl	400.0
($MgSO_4 \cdot 7H_2O$	200.0
(NaCl	6800.0
($NaH_2PO_4.H_2O$	1140.0
($NaHCO_3$	2200.0

pH 7.2

37°C, after which time the process was repeated. A total of 12 serial passages were performed; Gram stained smears were prepared and EBA plates seeded and incubated both aerobically and anaerobically from each subculture. After the 12th subculture, 0.1-ml inocula were used to seed plates of "solid 109 medium" (see below), which were anaerobically incubated for 48 hr at 37°C. Gram stained smears revealed colonies morphologically typical of Cl. tetani. A representative colony was subcultured onto EBA medium and into tubes of CMB. After incubation for 48 hr, 0.25-ml amounts of the filtrate from a representative CMB culture were injected into unprotected mice, and into mice protected with 0.5 ml equine antitoxin (see Materials and Methods); the death (from tetanus) of the unprotected mice only, showed that toxigenic Cl. tetani was being recovered. Thus liquid 109 medium supported the growth and serial passage of a strain of Cl. tetani; it was necessary to confirm that the organism would grow on a solid version of the same medium ("solid 109" or "109 S" medium). For details of the preparation of this medium in the form of layer plates see Materials and Methods.

Preliminary tests showed that even if stored anaerobically for 24 hr before seeding, 109 S medium would not support the growth of the test strain of Cl. tetani. However, the addition of 0.1 ml of an aqueous solution of cysteine (50 mg per ml) to the NCTC 109 medium before mixing with the Ionagar (see Materials and Methods), to give a final concentration in the upper layer of the layer plate of 1 mg per ml, supported the growth of Cl. tetani. Cultural and toxicological checks (see above) confirmed that toxigenic Cl. tetani could be recovered on this solid medium, both directly from CMB cultures, and

from serially-passaged cultures in fluid 109 medium. Later studies showed that four other stock strains of Cl. tetani (NCTC 540; NCTC 5404; NCTC 5405 and NCTC 5413), could all be recovered on 109 S medium from CMB cultures. In all cases, 48 hr anaerobic incubation was required for the appearance of visible colonies, but even after prolonged incubation, these were too small for accurate colony counting. Thus it seemed that this complex but defined solid medium, although unnecessarily rich in its supply of nutrients (see above), allowed minimal growth of Cl. tetani strains.

A series of possible enhancing factors were then tested to determine whether their addition to "basal 109 S medium" (i.e., without added cysteine) or to "109 S medium", improved the recovery of five test strains of Cl. tetani from overnight CMB cultures. Growth was assessed qualitatively after 48 hr incubation. The factors were added as 0.1 ml amounts of membrane-filtered aqueous solutions to the NCTC 109 medium (see above), and their final concentration was estimated as the concentration in the NCTC/aqueous agar layer in the layer plates. The results of a large series of replicate experiments are summarised in Table 31.

It is clear that addition of cysteine to basal solid 109 medium is necessary for the surface growth of these organisms, and that higher concentrations increase the surface growth; this contrasts with the absence of enhancement noted with the cysteine/dithiothreitol system. On the other hand, none of the other factors tested, when added to the basal medium, allowed growth of the test strains. In later studies (not shown in the Table), the effects of adding these factors, singly and in combination to 109 S medium (i.e. with added

TABLE 31

The effect of various additions to a basal defined solid medium on the recovery of Cl. tetani strains from overnight CMB cultures

Medium	Observed growth of strain no.				
	NCTC 9569	NCTC 540	NCTC 5404	NCTC 5405	NCTC 5413
Basal medium* (= Medium A).	NG**	NG	NG	NG	NG
Medium A + added cysteine (1 mg per ml).	++	++	++	++	++
Medium A + added cysteine (5 mg per ml).	+++	+++	+++	+++	+++
Medium A + ascorbic acid (5 mg per ml).	NG	NG	NG	NG	NG
Medium A + cobalt ($4\mu g$ Co^{++} per ml).	NG	NG	NG	NG	NG
Medium A + cobalt ($10\mu g$ Co^{++} per ml).	NG	NG	NG	NG	NG
Medium A + dithiothreitol (0.1 mg per ml).	NG	NG	NG	NG	NG
Medium A + cysteine (1 mg per ml) + dithiothreitol (0.1 mg per ml).	++	++	++	++	++
Medium A + menadione ($5\mu g$ per ml).	NG	NG	NG	NG	NG

* The preparation of this medium is described in the text.

** NG = no growth after incubation for 48 hr.

++, +++ = increasing degrees of growth as assessed visually after 48 hr incubation.

cysteine) was determined; no improvement in surface growth (as estimated qualitatively) was observed with any combination, as compared with growth on solid 109 medium alone. The failure of cobalt, as cobalt nitrate, to produce any enhancement was especially striking.

VARIABLE FACTORS THAT MAY AFFECT THE RECOVERY OF ANAEROBES ON SOLID MEDIA

Selection by heat

The precipitate fall in the surface viable counts of various anaerobes after heating samples of young fluid cultures at 70°C and 80°C has already been noted. Vegetative cells can be the significantly viable particles in the case of many sporing anaerobes, and the use of selective heating procedures, even if no spores are inactivated, will remove the significantly viable component of the inoculum for solid media. If spores are also inactivated by such procedures, the surface viable counts obtained from heated samples may be very low (see p. 111).

The use of heat as a selective procedure may be inadvertent; pour-plate methods expose organisms to temperatures at or above 45°C and this may inactivate vegetative cells. This is clearly demonstrable with Cl. oedematiens type D. An overnight CMB culture of Cl. oedematiens type-D, was mixed with an old (28-day) CMB culture of the same organism, so that a CMB culture containing about 3 per cent. spores was obtained. 1-ml aliquots were transferred into pre-heated bijou bottles and held for 20 min. at varying temperatures in thermostatically-controlled water baths. After exposure to the test temperature for 20 min., 0.02-ml drops from each sample were used to seed replicate plates of CDEBA medium. The plates were incubated overnight at 37°C in BTL jars set up according to the standard anaerobic procedure. The results of a typical experiment are shown in Table 32. It can be seen that there is a very marked diminution in

TABLE 32

The recovery of a 10^{-1} dilution of an 18-hr CMB culture* of Cl. oedematiens type D on CDEBA medium after exposure of samples to various temperatures for 20 min.

Jar	Colony counts** of replicate 0.02-ml inocula after treatment for 20 min. at					
	Room temp. (18.5°C)	40°C	50°C	60°C	70°C	80°C
H2	>1000, >1000$^{\Delta}$	12, 18	13, 22	15, 18	15, 20	6, 14
H1	>1000, >1000$^{\Delta}$	16, 11	10, 13	12, 10	16, 18	10, 7

* The total cell count was 1.5×10^{7} per ml; the spore estimate was 4.9×10^{5} per ml.

** Surface viable counts of duplicate samples, performed by a spread-plate technique on CDEBA medium. All plates incubated for 18 hr.

Δ Confluent growth rendered accurate counting impossible; the figures given are minimum estimates.

the surface viable counts at 40°C, i.e. below the temperature at which agar solidifies.

The use of selective agents in culture media

Neomycin

The inhibitory effects of selective media containing neomycin have been reviewed above (see page 55). Although there are considerable data for many anaerobes, the unreliability of surface growth of strains of Cl. oedematiens of types B and D has rendered quantitative data on the inhibitory effects of neomycin difficult to obtain. The reliable growth obtained with the cysteine/dithiothreitol system permitted studies on the growth of strains of Cl. oedematiens on media containing varying amounts of neomycin.

It was necessary to check qualitatively the sensitivity patterns of strains of Cl. oedematiens to neomycin, and it was decided to combine this with sensitivity testing of all the available Cl. oedematiens strains to a range of antibiotics. The results are given in Table 33.

Apart from one type-B strain, all of the strains tested were resistant to neomycin as shown by lack of a zone of inhibition round the neomycin (10 mg) disc. Qualitative studies, in which varying concentrations of neomycin were incorporated into CDEBA medium, showed that some inhibition of growth was observed when concentrations of 70 μg per ml or more were used. It was therefore necessary to compare quantitatively the surface growth of Cl. oedematiens on media containing varying concentrations of neomycin. To this end, plates of CDEBA 16 medium containing 0, 25, 70 and 200 μg neomycin per ml were prepared.

TABLE 33

The sensitivity of Cl. oedematiens strains to eight antibiotics determined by disc diffusion tests on CDEBA medium

Organism	Strain	Result of test with the stated antibiotic*							
		TS(25)**	S(10)	CY(75)	PG(4)	Ne(10)	T(25)	E(5)	Amp(10)
Cl. oedematiens type A	1A	R	R	S(4)△	S(3.7)	R	S(3.3)	S(3.1)	S(4.5)
	2A	R	R	R	S(3.4)	R	S(3.8)	S(3.2)	S(4.0)
	3A	R	R	S(3)	RR(1.6)	R	S(3.0)	S(2.5)	S(3.2)
	4A	R	R	S(3.4)	S(3.8)	R	S(3.4)	S(3.4)	S(3.8)
Cl. oedematiens type B	1B	R	R	S(3.6)	S(2.8)	R	S(3.6)	S(2.5)	S(3.0)
	2B	R	R	S(3.6)	S(3.6)	S(3.0)	S(3.8)	S(3.7)	S(5.2)
	3B	R	R	S(4.1)	S(3.0)	R	S(3.9)	S(3.1)	S(4.0)
	7B	R	R	S(3.6)	S(2.0)	R	S(3.6)	S(3.0)	R
	8B	R	R	S(3.6)	S(2.5)	R	S(3.6)	S(3.1)	S(3.6)
	9B	R	R	R	S(2.6)	R	S(3.1)	S(2.5)	S(2.5)
	10B	S(2)	R	R	S(2.5)	R	S(3.0)	S(2.2)	S(2.5)
	11B	R	R	R	S(2.3)	R	S(3.1)	S(2.4)	S(3.3)
	12B	R	R	S(4.0)	S(2.7)	R	S(3.7)	S(3.4)	S(3.2)
	13B	R	R	S(4.1)	R	R	S(4.3)	S(3.7)	S(4.8)
Cl. oedematiens type D	1D	R	R	S(3.6)	S(2.5)	RR(1.0)	S(3.9)	S(2.9)	S(3.1)
	2D	R	R	S(3.9)	S(3.4)	RR(1.6)	S(3.5)	S(3.6)	S(3.5)
	3D	R	R	S(3.4)	S(3.0)	R	S(3.9)	S(3.3)	S(3.0)

* TS = sulphamethoxazole/Trimethoprim, S = Sulphonamide, CY = cycloserine, PG = Penicillin, Ne = Neomycin, T = Tetracycline, E = Erythromycin, Amp = Ampicillin.

** Figures in parentheses are amount of antibiotic expressed as mg per disc.

△ Figures in parentheses = zone of inhibition in cm (mean of two replicate plates).

R = resistant (zone of inhibition <1 cm); RR = relatively resistant (zone of inhibition <2 cm); S = sensitive (zone of inhibition 2 cm or more).

Undiluted and diluted samples of overnight CMB cultures of each of the test organisms were used to seed duplicate plates of each medium with an inoculum of 0.02 ml. All plates seeded with a given organism were incubated in the same anaerobic jar for 24 hr at 37°C. The results summarised in Table 34 show that at concentrations of neomycin of 70 µg per ml or less, there is little inhibitory effect on the growth of the test strains, but at a concentration of 200 µg per ml there is more marked inhibition, especially in the case of type-D strains.

As a concentration of neomycin of 70 µg per ml in blood agar media did not appear to inhibit the growth of Cl. oedematiens strains to a significant extent, it was decided to determine whether the recovery of non-clostridial organisms from clinical material was inhibited by neomycin-containing media. The recovery of "total anaerobes" (see p.130) from samples of faeces was compared on blood agar containing varying concentrations of neomycin. Preliminary qualitative studies suggested that inhibition of growth occurred if concentrations of neomycin in excess of 70 µg per ml were used. Quantitative studies were therefore performed, in which the recovery of anaerobic organisms from faeces was compared on blood agar media with and without 70 µg neomycin per ml (see Materials and Methods). The results of a representative experiment are shown in Table 35. It can be seen that there was no significant difference between the recovery rates on the two media.

Cycloserine

Recently, Fuzi and Csukas (1969) described a selective medium for Cl. welchii, in which cycloserine (800 mg per ml) was added to

TABLE 34

The recovery of dilutions of overnight CMB cultures of Cl. oedematiens strains on CDEBA medium containing varying concentrations of neomycin

Organism	Strain	Dilution	Surface viable counts per ml* derived from duplicate samples on medium**			
			A	B	C	D
Cl. oedematiens type A	GR1A	10^{-2}	$(6.7\text{-}7.1)\times10^{7}$	$(6.2\text{-}6.5)\times10^{7}$	$(5.9\text{-}7.3)\times10^{7}$	$(5.1\text{-}5.6)\times10^{7}$
	GR2A	10^{-2}	$(1.6\text{-}1.7)\times10^{7}$	$(1.7\text{-}1.9)\times10^{7}$	$(1.9\text{-}2.2)\times10^{7}$	$(0.9\text{-}1.7)^{\Delta}\times10^{7}$
	GR4A	10^{-2}	$(2.1\text{-}2.2)\times10^{7}$	$(1.4\text{-}1.9)\times10^{7}$	$(1.8\text{-}2.3)\times10^{7}$	$(1.3\text{-}1.5)\times10^{7}$
Cl. oedematiens type B	GR1B	10^{-1}	$(1.9\text{-}2.0)\times10^{5}$	$(1.0\text{-}1.1)\times10^{5}$	$(1.0\text{-}1.2)\times10^{5}$	$(7.9\text{-}8.3)\times10^{4}$
	GR2B	10^{-1}	$(1.1\text{-}1.4)\times10^{3}$	$(4.0\text{-}6.0)\times10^{3}$	$(5.0)\times10^{4}$ $^{\Delta}$ ^{3}	$(5.0\text{-}9.0)\times10^{2}$
	GR3B	undiluted	$(4.0\text{-}4.5)\times10^{5}$	$(2.3\text{-}3.0)\times10^{5}$	$(2.5\text{-}3.5)\times10^{5}$	$(6.5\text{-}9.8)\times10^{2}$
	GR13B	10^{-1}	$(3.8\text{-}4.1)\times10^{5}$	$(4.1\text{-}4.2)\times10^{5}$	$(1.0\text{-}1.4)\times10^{5}$	$(0.9\text{-}1.6)\times10^{5}$
Cl. oedematiens type D	GR1D	$\frac{1}{2}\times10^{-2}$	$(2.0\text{-}2.2)\times10^{6}$	$(0.5\text{-}1.4)\times10^{6}$	$(5.0\times10^{5})^{\Delta}$	$(7.1\text{-}1.1)\times10^{5}$
	GR2D	undiluted	$(5.7\text{-}8.8)\times10^{3}$	$(6.1\text{-}6.8)\times10^{3}$	$(3.7\text{-}5.6)\times10^{3}$	$(5.4\text{-}7.5)\times10^{3}$
	W3D	undiluted	$(7.9\text{-}8.3)\times10^{3}$	$(4.0\text{-}4.2)\times10^{3}$	$(2.0\text{-}2.1)\times10^{3}$	$(4.0\text{-}7.0)\times10^{2}$

* Surface viable counts of duplicate samples, performed by a spread-plate technique on CDEBA medium. All plates were seeded with 0.02-ml inocula of the test dilution; plates seeded with samples of a given organism were incubated in a single anaerobic jar for 18 hr.

** A = CDEBA; B = CDEBA + 25 μg neomycin per ml; C = CDEBA + 70 μg neomycin per ml; D = CDEBA + 200 μg neomycin per ml.

Δ Spreading growth rendered accurate counting difficult; a minimum estimate is given.

TABLE 35

The recovery of anaerobic organisms from dilutions of a suspension of human faeces on blood agar media with and without neomycin

Medium *	Mean colony count ± S.E.**	t-value for difference of means on 38 d.f.
EBA	232.0 ± 9.2	
EBA + Neomycin (70 µg per ml)	224.0 ± 9.0	0.72 (not significant)△

* EBA = 10 per cent. equine blood agar.

** Each count is expressed as the mean ± S.E. (standard error of the mean) of ten replicate plates of the stated medium.

△ See Materials and Methods for explanation of convention adopted.

5 per cent. human blood agar. The possible use of cycloserine as a selective agent for Cl. welchii strains and for other anaerobes was evaluated in the present study.

The sensitivity of 25 strains of Cl. welchii was determined by disc diffusion method; all were resistant in tests with discs containing 75 mg cycloserine per disc. Qualitative tests with media containing varying concentrations of cycloserine, showed that some inhibition of growth was observed at concentrations of 800 mg cycloserine per ml in blood agar media. Table 36 shows the results of an experiment in which six representative strains of Cl. welchii were grown on EBA medium containing varying amounts of cycloserine. The pH of the media in this experiment was not adjusted, and the plates had final pH values of 6.5-6.9. Five of the six strains tested showed considerable inhibition of growth on EBA medium containing 800 mg cycloserine per ml, but very little when concentrations of 200 mg per ml were used. In their study, Fuzi and Csukas had used media at a pH of 7.4; as the activity of cycloserine is increased by a pH of <7 (Dr. B. I. Davies, personal communication) it was decided to repeat the above study with media buffered to a pH of 7.6 with phosphate buffer before autoclaving. The final pH of the media was pH 7.4-7.5. The results are shown in Table 37, and it is clear that at this higher pH value, at which the activity of cycloserine is presumably diminished (see above), there was little or no inhibition of the test strains of Cl. welchii. It was then decided to investigate the growth of a wider range of anaerobes on cycloserine-containing media.

The results with some other test anaerobes, summarised in Table 38 , show that a proportion failed to grow on media containing 800 mg

TABLE 36

The recovery from dilutions of overnight CMB cultures of six strains of Cl. welchii on media with and without addition of cycloserine, without buffering of the media

Strain	Surface viable counts per ml* derived from duplicate samples grown on		
	10 per cent. EBA	10 per cent. EBA + 200 mg cycloserine per ml**	10 per cent. EBA + 800 mg cycloserine per ml
Hobbs type I	$(6.6\text{-}7.6) \times 10^7$	$(4.9\text{-}5.9) \times 10^7$	$<50, <50^{\Delta}$
Hobbs type II	$(5.0\text{-}5.3) \times 10^7$	$(4.7\text{-}5.7) \times 10^7$	$(4.3\text{-}4.5) \times 10^6$
Hobbs type III	$(5.50\text{-}5.52) \times 10^7$	$(3.8\text{-}4.1) \times 10^7$	$(2.6\text{-}3.7) \times 10^7$
NCTC 8588	$(4.8\text{-}6.5) \times 10^7$	$(4.7\text{-}4.9) \times 10^7$	$(4.0\text{-}5.2) \times 10^7$
L2A	$(9.6\text{-}10.1) \times 10^7$	$(7.6\text{-}9.9) \times 10^7$	$1.5 \times 10^6, <5 \times 10^5$ $\Delta\Delta$
Lab. no. Cl	$(9.6\text{-}10.1) \times 10^7$	$(7.6\text{-}9.9) \times 10^7$	$1.5 \times 10^6, <5 \times 10^5$

* Surface viable counts of duplicate samples, performed by a spread-plate technique on the stated medium, each plate being seeded with 0.02-ml inocula of the test dilution. All plates seeded from samples of a given organism were incubated in the same anaerobic jar for 18 hr.

** Colonies on cycloserine-containing media were smaller than those on EBA medium alone.

Δ No growth from 0.02-ml of undiluted inoculum.

$\Delta\Delta$ No growth from 0.02-ml inocula at 10^{-4} dilution.

TABLE 37

The recovery from dilutions of overnight CMB cultures of six strains of Cl. welchii on media with and without addition of cycloserine, at controlled pH*

Strain	Surface viable counts per ml** derived from duplicate samples grown on		
	10 per cent. EBA	10 per cent. EBA + 200 mg cycloserine per ml	10 per cent. EBA + 800 mg cycloserine per ml
Hobbs type I	$(7.1\text{-}7.2) \times 10^7$	$(5.2\text{-}5.5) \times 10^7$	$(4.4\text{-}4.7) \times 10^7$
Hobbs type II	$(8.5\text{-}9.8) \times 10^7$	$(6.8\text{-}7.8) \times 10^7$	$(4.9\text{-}6.2) \times 10^7$
Hobbs type III	$(0.7\text{-}1.0) \times 10^8$	$(0.7\text{-}1.0) \times 10^8$	$(0.8\text{-}1.0) \times 10^8$
NCTC 8588	$(1.0\text{-}1.3) \times 10^8$	$(1.2\text{-}1.3) \times 10^8$	$(1.0\text{-}1.1) \times 10^8$
L2A	$(0.9\text{-}1.1) \times 10^8$	$(8.7\text{-}9.5) \times 10^7$	$(8.4\text{-}9.0) \times 10^7$
Lab. no. Cl	$(9.2\text{-}9.9) \times 10^7$	$(7.1\text{-}8.7) \times 10^7$	$(0.9\text{-}1.0) \times 10^7$

* The media were buffered with 0.2M phosphate buffer at pH 7.6 before autoclaving. The final pH of the media ranged from 7.4 to 7.5.

** Surface viable counts of duplicate samples, performed by a spread-plate technique on the stated medium, each plate being seeded with 0.02-ml inocula of the test dilution. All plates seeded from samples of a given organism were incubated in a single anaerobic jar for 18 hr.

TABLE 38

The recovery from dilutions of overnight CMB cultures of test anaerobes on media with and without the addition of cycloserine at controlled pH*

Organism	Surface viable counts per ml** derived from duplicate samples grown on		
	10 per cent. EBA	10 per cent. EBA + 200 mg cycloserine per ml	10 per cent. EBA + 800 mg cycloserine per ml
B. fragilis (NCTC 9343)	$(3.2\text{-}3.9)\times10^9$	$(4.1\text{-}4.5)\times10^9$	$(3.3\text{-}4.0)\times10^9$
B. necrophorus (NCTC 7155)	9.0×10^7	$(8.8\text{-}9.6)\times10^7$	$(7.1\text{-}8.0)\times10^7$
Cl. bifermentans (lab. no. B4)	$(1.1\text{-}1.5)\times10^8$	$(1.0\text{-}1.3)\times10^8$	$(8.1\text{-}8.4)\times10^7$
Cl. histolyticum	$(5.1\text{-}5.4)\times10^8$	<50, $<50^{\Delta}$	<50, <50
Cl. chauvoei (lab. no. CC1)	$(8.4\text{-}9.2)\times10^6$	$(3.7\text{-}5.6)\times10^6$	<50, <50
Anaerobic coccus (Group 1)	$(6.3\text{-}6.5)\times10^7$	<50, <50	<50, <50
Cl. septicum (lab. no. 110)	$>5\times10^4$	$>5\times10^4$	$(0.9\text{-}3.5)\times10^2$
Cl. septicum (lab. no. 111)	$(1.0\text{-}1.7)\times10^6$	$(1.1\text{-}1.2)\times10^6$	$(1.0\text{-}2.0)\times10^5$
Cl. septicum (lab. no. 114)	$(8.4\text{-}8.6)\times10^6$	$(6.0\text{-}6.5)\times10^6$	<50, $50^{\Delta\Delta}$

* The media were buffered with 0.2M phosphate buffer at pH 7.6 before autoclaving. The final pH of the media ranged from 7.4 to 7.5.

** Surface viable counts of duplicate samples performed by a spread-plate technique on the stated medium, each plate being seeded with 0.02-ml inocula of the test dilution. All plates seeded from samples of a given organism were incubated in the same anaerobic jar for 18 hr.

Δ No growth from 0.02-ml of undiluted inoculum.

$\Delta\Delta$ One colony from 0.02-ml of undiluted inoculum.

cycloserine per ml, and some were inhibited by only 200 mg cycloserine per ml.

In addition, in tests of nine Cl. oedematiens strains on CDEBA media containing varying concentrations of cycloserine, all failed to grow at concentrations of 200 mg per ml or more. Thus, although cycloserine may be useful as a selective agent for Cl. welchii, inhibition of some strains will be observed if the pH of the medium is not carefully controlled. Its use as a general-purpose selective agent in anaerobic culture media cannot be recommended, in view of the observed inhibition of several anaerobic species.

The effect of variations in procedure

The effect of several variable factors in anaerobic procedure on the recovery of test anaerobes on solid media was investigated. The factors studied included aerobic agitation of dilutions of CMB cultures; the mode of storage of solid media; the thickness of plates of solid media; the exposure of seeded plates to aerobic conditions; the presence or absence of carbon dioxide in the BTL anaerobic jar; the flushing of anaerobic jars before processing; and the prolonged incubation of plates in anaerobic jars.

Note: The studies reported in this section were complex, and considerable detail was required in the setting down of the results; a simplified summary of the important findings is therefore given on p. 200.

The effect of aerobic agitation

When a bacterial suspension is serially diluted during the normal counting procedure, it is essential to ensure adequate mixing

between the dilution steps and this involves a considerable amount of agitation and some aeration. The effect of such agitation on the viable bacterial content of dilutions of a CMB culture of Cl. oedematiens type D was therefore studied; samples were shaken for periods up to 4 hr under aerobic conditions.

A ten-fold dilution of an overnight CMB culture was made in pre-steamed nutrient broth, and 20-ml aliquots were added to sterile 100-ml conical flasks. Triplicate plates were seeded with 0.02-ml drops from the parent dilution, spread in the standard manner, and held anaerobically at room temperature. These gave the time "0" (unshaken) viable counts. The flasks were then placed in an empty shaking water-bath at 18.5°C and shaken at 135 oscillations per min. This rate was determined in preliminary studies; it gave vigorous shaking without loss of fluid and the rate was kept constant in all subsequent experiments. After a given flask had been agitated for the allotted time, triplicate plates were each seeded with a 0.02-ml sample of the shaken suspension. The plates were then spread and held anaerobically at room temperature until the end of the experiment, when all the plates were placed in a single anaerobic jar which was then processed according to the standard anaerobic procedure.

Initial studies showed that shaking of a given dilution for 20 min. produced an appreciable fall in the surface viable count. If diluents other than pre-steamed nutrient broth were used, even greater inactivation was produced after aerobic agitation. Table 39 shows the result of an experiment in which the degrees of inactivation were compared when the suspending agent was pre-steamed nutrient broth, saline or gelatin/saline. In this experiment, the plates were

TABLE 39

The effect of aerobic agitation of dilutions of an overnight CMB culture of Cl. oedematiens type D for 20 min. at room temperature on the surface viable counts derived from these dilutions, and the influence of different diluents

Test strain	Diluent*	Dilution	Mean surface viable count, with ranges in parentheses, derived from triplicate CDEBA plates each seeded with 0.02 ml of the stated dilution	
			before agitation	after agitation for 20 min.
Cl. oedematiens type D, strain GRID	P/S NB	10^{-2}	6.8×10^5 ($(4.5\text{-}8.2) \times 10^5$)	7.3×10^4 ($(5.0\text{-}9.0) \times 10^4$)
	Saline	10^{-2}	7.5×10^5 ($(6.8\text{-}8.6) \times 10^5$)	$< 5 \times 10^3$ **
	Gelatin/saline	10^{-2}	1.4×10^5 ($(1.1\text{-}1.5) \times 10^5$)	1.7×10^4 ($(1.5\text{-}1.9) \times 10^4$)

* P/S NB = pre-steamed nutrient broth; saline = 0.09 per cent. NaCl in water; gelatin/saline = 2 per cent. gelatin in saline.

** No growth from 0.02-ml inocula at the stated dilution.

Room temperature = 18.5-20°C.

distributed between two anaerobic jars, so that representative plates seeded from each sample were present in both jars. It is clear that a greater inactivation occurred in the case of dilutions made with saline, or gelatin/saline, than when the diluent was pre-steamed nutrient broth. An inhibitory effect of gelatin/saline is also obvious in the preparation of the original dilution, the recovery from the "before agitation" sample being less than that from samples prepared from the other two diluents.

The sensitivity of Cl. oedematiens type D to aerobic agitation was well demonstrated in an experiment in which a dilution of a culture of a type-D strain was agitated aerobically in parallel with a dilution, prepared with the same diluent, of a culture of an aerobe, Pseudomonas aeruginosa. The results of this experiment are shown in Table [40]. In the case of Cl. oedematiens type D a dramatic fall in the surface viable counts was demonstrated after agitation for 20 min.; there was even an appreciable decrease when a test dilution was left on the bench for 20 min. at room temperature without agitation. Comparative tests with Ps. aeruginosa showed no such effect. The author appreciates that the lack of any observed effect in the case of Ps. aeruginosa could be due in part at least to the greater dilution of the parent culture that was necessary to produce countable colonies on solid media, and the proportionately greater amount of pre-steamed nutrient broth that was therefore present.

It could be argued that the decrease in the recovery of Cl. oedematiens after aerobic agitation was due to loss of organisms during the agitation procedure, although the consistent recovery observed in the case of Ps. pyocyanea suggests that no loss of organisms occurred,

TABLE 40

The effect of aerobic agitation of dilutions* of cultures of Ps. aeruginosa and Cl. oedematiens type D for 20 min. at room temperature on the surface viable counts derived from these dilutions

Test culture	Dilution	Mean surface viable count, with ranges in parentheses, derived from triplicate plate cultures, each seeded with 0.02 ml of the stated dilution		
		Before agitation	After agitation for 20 min.	After exposure to aerobic conditions for 20 min. without agitation
Overnight CMB-culture of Cl. oedematiens type D, strain GR2D	10^{-1}**	2.5×10^5 ($2.2 - 2.8) \times 10^5$	$<1 \times 10^3$ Δ	2.2×10^4 ($1.9 - 2.7) \times 10^4$
Overnight NB culture of Ps. aeruginosa	10^{-5}	5.9×10^8 ($5.2 - 7.2) \times 10^8$	6.2×10^8 ($5.5 - 6.8) \times 10^8$	5.7×10^8 ($4.4 - 6.6) \times 10^8$

* All dilutions were performed in pre-steamed nutrient broth.

** All plates seeded from this dilution were incubated in a single anaerobic jar, and incubated for 18 hr.

Δ Surface viable count derived from triplicate plate cultures yielding 1, 2, and 2 colonies respectively.

Room temperature = 19°C.

and careful pilot studies repeated several times, had shown that no loss of fluid occurred, even if flasks were agitated for 60 min. Nevertheless, in all subsequent experiments, total cell counts were performed on samples before and after the agitation procedure. In order to detect any alteration in recovery rates due merely to the breaking up of the chain forms sometimes seen in cultures of Cl. oedematiens, two types of microscopical counts were performed:

(i) Total cell count in which individual organisms were counted irrespective of whether or not they occurred singly, or as components of a chain.

(ii) Colony-forming unit (c.f.u.) count in which a chain was counted as a c.f.u. irrespective of its length.

Experiments in which microscopical counts were performed confirmed that the fall in surface viable counts occurring after aerobic agitation was not due to loss of cells during the agitation procedure.

Experiments were performed in which dilutions of suspensions of other organisms (Cl. tetani type VI; Bacteroides fragilis; Anaerobic coccus) were subjected to similar procedures. The results showed that Cl. oedematiens type D was very much more sensitive to aerobic agitation than any of the other test organisms. The results of an experiment in which the effect of aerobic agitation for up to 60 min. on the recovery from dilutions derived from cultures of B. fragilis and Cl. oedematiens was compared, are shown in Table 41. It is clear that whereas no effect was observed in the case of B. fragilis, a considerable decrease in the surface viable count was observed with Cl. oedematiens type D, although the test strain used on this occasion, GR1D, seemed less sensitive to

TABLE 41

The effect of aerobic agitation of dilutions* of overnight CMB cultures of Cl. oedematiens type D and B. fragilis for varying periods of time at room temperature on the surface viable counts derived from these dilutions

Test culture	Dilution	Mean surface viable count, with ranges in parentheses, derived from triplicate plates, each seeded with 0.02 ml of the stated dilution**			
		Before agitation	After agitation for 20 min.	After agitation for 60 min.	After exposure to aerobic conditions for 60 min., without agitation
Cl. oedematiens△ type-D strain GR1D	10^{-1}	2.0×10^5 ($1.8-2.1) \times 10^5$	1.1×10^5 ($1.0-1.2) \times 10^5$	1.8×10^4 ($1.6-1.9) \times 10^4$	4.4×10^4 ($3.9-4.9) \times 10^4$
B. fragilis△△ NCTC 9343	10^{-5}	2.2×10^9 ($2.1-2.3) \times 10^9$	2.4×10^9 ($2.3-2.5) \times 10^9$	2.3×10^9 ($2.1-2.4) \times 10^9$	2.4×10^9 ($2.2-2.6) \times 10^9$

* All dilutions were performed in pre-steamed nutrient broth.

** All plates seeded from a given dilution were incubated in a single anaerobic jar.

△ Total cell count before agitation = 2.0×10^7 organisms per ml; total cell count after agitation for 60 min. = 2.3×10^7 organisms per ml. Total c.f.u. count before agitation = 1.3×10^7 organisms per ml; total c.f.u. count after agitation for 60 min. = 1.5×10^7 organisms per ml;

△△ Total cell count before agitation = 1.9×10^{11} organisms per ml; total cell count after agitation for 60 min. = 1.7×10^{11} organisms per ml.
Only single organisms were seen in phase contrast preparations of this organism.

Room temperature = 18-19.5°C.

aerobic agitation than the strain of GR2D (see Table 40). This is considered further in the Discussion.

The effect of the mode of storage of solid media

Unless stated otherwise in the text, all experimental work was performed on freshly-poured plates of solid media, which were seeded, spread in a standard manner, and incubated anaerobically within 30 min. of being dried. As the clinical bacteriologist cannot always ensure that a supply of freshly-poured plates is available, and may have to place reliance on stored plates for the isolation of anaerobes, it was deemed necessary to investigate the effect of storage of plates of solid media on the recovery of a demanding anaerobe.

Plates of CDEBA medium were freshly prepared on three successive days. Plates prepared on the first and second days were stored aerobically (i.e. on the bench) and anaerobically (in anaerobic jars processed in the standard manner) at room temperature, until the start of the experiment. On the third day, freshly poured plates of CDEBA medium, together with the stored plates, were seeded from tenfold dilutions of an overnight CMB culture of Cl. oedematiens type D. Duplicate plates of each medium were seeded with 0.02-ml inocula from a given dilution. All the plates seeded from a given dilution were then incubated in a single anaerobic jar for 24 hr at 37°C. The anaerobic jars were each equipped with three catalyst sachets. The results of this experiment are shown in Table 42; note that the 10^{-1} dilution gave colonies within a suitable range for counting.

From these results, it is clear that aerobic storage, even for 24 hr leads to very poor recovery of these organisms. Storage of plates under anaerobic conditions gave much better recovery; in this

TABLE 42

The influence of storage of plates for 24 and 48 hr under aerobic or anaerobic conditions on the recovery of Cl. oedematiens type D from an overnight CMB culture

Test organism	Dilution	Surface viable counts per ml derived from duplicate plates of CDEBA medium*				
		Freshly-poured plates	Plates stored aerobically at RT for 24 hr	Plates stored anaerobically** at RT for 24 hr	Plates stored aerobically at RT for 48 hr	Plates stored anaerobically at RT for 48 hr
Cl. oedematiens type D strain GR1D	10^{-1}	$(2.8\text{-}3.5)\times10^{5}$	$<5\times10^{2}$, $<5\times10^{2}$ Δ	$(4.3\text{-}4.5)\times10^{5}$	$<5\times10^{2}$, $<5\times10^{2}$	$(6.2\text{-}7.5)\times10^{5}$

* All plates were incubated in a single anaerobic jar.

** All "anaerobically-stored" plates were placed in anaerobic jars, processed by the standard anaerobic procedure, and left on the bench at room temperature (RT) until the start of the experiment.

Δ No growth from 0.02 ml of a 10^{-1} dilution of the parent CMB culture.

Room temperature = 19.5°C.

experiment the recovery was better than that achieved with freshly poured media. When these experiments were repeated, using statistically designed experiments to take account of "jar variation" (see p. 224) the recovery on aerobically stored plates was shown to be significantly poorer than on freshly-poured, or anaerobically stored plates. No statistically significant difference was observed, however, between the recovery on freshly-poured plates, and that on anaerobically stored plates. Anaerobically-stored plates had the disadvantage that the considerable humidity often observed on the surface of the plates gave rise to spreading growth which rendered accurate counting of surface colonies difficult.

The recovery of other test anaerobes on fresh and stored EBA media was then compared. Cl. tetani type VI; B. fragilis NCTC 9343; B. necrophorus NCTC 7155; and an Anaerobic coccus, Group I, were used as test organisms. The recovery from overnight CMB cultures of these organisms was compared on fresh plates, and on plates stored aerobically or anaerobically at room temperature for up to 48 hr. From the results of these experiments it was clear that recovery was somewhat poorer on plates stored aerobically, but that freshly poured plates gave equivalent, or on occasion slightly better recovery than if anaerobically stored plates were used. Storage of plates at 4°C was compared with storage at room temperature; plates stored at room temperature gave as good recovery as those stored at 4°C, without the excessive moisture often present in the plates stored at the lower temperature. The results of such an experiment in which Cl. tetani type VI was used as the test organism are shown in Table 43.

Thus it was clear that aerobic storage of plates led to

TABLE 43

The influence of storage of plates for 24 hr under aerobic or anaerobic conditions, at 4°C and at room temperature on the recovery of Cl. tetani type VI from an overnight CMB culture

Test organism	Dilution	Mean surface viable count per ml, with range in parentheses, derived from culture grown on triplicate plates of EBA medium*				
		Freshly-poured plates	Plates stored aerobically at RT for 24 hr	Plates stored anaerobically** at RT for 24 hr	Plates stored aerobically at 4°C for 24 hr	Plates stored anaerobically at 4°C for 24 hr
Cl. tetani type VI NCTC 9569	10^{-3}	4.7×10^7 ($(4.6\text{-}4.8)\times10^7$)	3.6×10^7 ($(3.3\text{-}3.8)\times10^7$)	4.3×10^7 ($(3.9\text{-}4.7)\times10^7$)	3.7×10^7 ($(3.6\text{-}3.7)\times10^7$)	4.2×10^7 ($(4.1\text{-}4.5)\times10^7$)

* All plates incubated in a single anaerobic jar.

** All "anaerobically-stored" plates were placed in anaerobic jars, processed by the standard anaerobic procedure, and left on the bench at room temperature (RT) or in the cold-room at 4°C, until the start of the experiment.

Room temperature = 19°C.

decreased recovery of laboratory cultures of anaerobes. In a series of later experiments the recovery of "Total anaerobes" (see p. 130) from faecal samples was compared on fresh EBA medium, and EBA medium stored under varying conditions; no significant difference was observed between the recovery on freshly-poured or anaerobically-stored plates, although on occasion slightly lower recovery was observed on plates stored aerobically at room temperature.

The effect of the thickness of plates (volume of medium)

In all of the experimental work hitherto reported, plates containing 10-12 ml solid medium per plate were used. In view of the finding of Domey et al. (1969) that the recovery of an aerobic organism could be affected by variations in the amount of solid medium in a plate, it was decided to compare the recovery of some anaerobic organisms on plates containing differing amounts of medium.

The recovery from overnight CMB cultures of Cl. tetani type VI, Cl. oedematiens type D and B. fragilis, and from a faecal suspension, was compared on plates of EBA medium (CDEBA medium in the case of Cl. oedematiens type D) containing 8, 15 or 20 ml medium per plate. Suitable dilutions of the CMB cultures or of the faecal suspension were prepared in pre-steamed nutrient broth, and 0.02-ml inocula were used to seed 10 plates of each medium. The order of seeding of the plate was randomised according to a pre-arranged schedule and the plates were distributed randomly between three anaerobic jars; the position of the plates within the jars was also randomised. The jars were incubated anaerobically for 48 hr.

The results of several replicate experiments showed that there was no difference in the recovery rates with different thicknesses of

media in the case of Cl. oedematiens type D, B. fragilis, or the faecal suspension, but that the recovery of Cl. tetani was significantly diminished on plates containing 8 ml solid medium per plate as compared with recovery on plates containing 15 or 20 ml medium per plate. In later experiments, the recovery of this organism was compared on plates containing 8, 12, 15, and 20 ml medium, using a statistically-designed experiment analogous to that described in Appendix B. The results of this experiment, when analysed statistically (see p.345 in Appendix E) showed that while recovery on plates containing 8 ml medium per plate was significantly poorer than on plates containing 12, 15 or 20 ml per plate, there was no significant difference between these latter media in terms of their recovery of Cl. tetani. Thus the use of plates containing 12 ml of medium would appear to be satisfactory.

The effect of exposure of seeded plates to aerobic conditions

In the clinical laboratory, plates seeded with anaerobic organisms may be left for varying periods on the bench, thus exposing them to aerobic conditions. It was therefore decided to investigate the recovery of a series of anaerobic organisms from seeded plates before and after exposure to aerobic conditions for varying periods of time. All experiments were performed at room temperature (18-20^{o}C).

In initial qualitative studies, replicate plates were seeded with 0.02 ml of an overnight CMB culture of the following organisms: Cl. tetani (5 strains); Cl. oedematiens type D; Cl. oedematiens type B; Cl. welchii (2 strains); B. fragilis and B. necrophorus. The seeded plates were left on the bench for various periods before incubation. It was clear that in the case of the Cl. oedematiens strain,

little if any growth was observed if seeded plates were exposed to aerobic conditions for more than 2-3 hr, whereas there was little detectable difference in the growth of other organisms if seeded plates were incubated immediately or left for up to 24 hr on the bench. These results were observed in studies in which plates exposed for differing periods were put up in different anaerobic jars, and also when all plates seeded from a given organism were incubated in the same jar, the jar being opened and reprocessed after the addition of each seeded plate.

A series of quantitative studies was then done in which the recovery of various anaerobes from seeded plates was compared after exposure of the plates to aerobic conditions for various periods of time. In the case of Cl. oedematiens type D, plates were seeded from dilutions of overnight CMB cultures and randomly allocated to several anaerobic jars. The jars were then processed at various time intervals from 30 min. to 24 hr so that all the plates in a given jar were exposed to aerobic conditions for a constant time. The distribution of plates within a given jar was randomised. All jars were equipped with 3 catalysts and incubated for 48 hr at 37°C. Because of the possible occurrence of "jar variation", in several experiments a freshly seeded plate was included in each jar as a control.

Results of repeated experiments showed that poor recovery was obtained if seeded plates were left on the bench under aerobic conditions for more than 1-2 hr. Table 44 shows results of an experiment that compared the recovery from seeded plates incubated immediately with that from plates held aerobically or anaerobically at 20°C on the bench for 90 min. Two jars were used for each exposure time.

TABLE 44

The effect of aerobic and anaerobic exposure of seeded plates at room temperature on subsequent recovery of Cl. oedematiens type D*

Jar	Treatment of seeded plates**	Mean colony count ± S.E. △
C 1	Processed immediately	239.0 ± 11.1
C 5	Processed immediately	246.6 ± 9.8
C 2	Held anaerobically at room temp. before processing	166.9 ± 11.3
C 7	Held anaerobically at room temp. before processing	111.7 ± 17.1
C 3	Held anaerobically at room temp. before processing	<1 △△
C10	Held anaerobically at room temp. before processing	5.2 ± 2.4

* Plates were seeded with a $\frac{1}{2} \times 10^{-2}$ dilution of an overnight CMB culture of strain GRID.

** 20 plates of CDEBA medium were incubated anaerobically at 37°C immediately; 20 plates of the same medium were exposed to aerobic conditions for 90 min. before anaerobic incubation at 37°C; and 20 plates of the medium were exposed to anaerobic conditions for 90 min. before anaerobic incubation at 37°C.

△ Each count is expressed as the mean ± S.E. (standard error of the mean) of ten replicate plates of CDEBA medium, each seeded with 0.02-ml inocula and incubated for 18 hr.

△△ One colony on each of two plates; other plates gave no growth.

Little or no recovery was observed after exposure of seeded plates to aerobic conditions for 90 min.; even anaerobic storage at room temperature led to a decreased recovery. One such set of results should not be regarded as conclusive, as "jar variation" (p.224) could account for these findings, but freshly seeded control plates gave good growth in jars showing low counts and this suggests that the low recovery was not due to factors associated with the jars. Similar results were obtained from experiments in which jars contained both freshly seeded plates and plates left exposed to aerobic conditions for varying periods of time, the plates being added to the jar, and the jar then being re-processed and re-incubated, after each sampling time. Thus, this demanding anaerobe is inactivated by exposure to aerobic conditions for relatively short periods of time.

The recovery of Cl. tetani type VI and B. fragilis was then determined in similar experiments with plates stored anaerobically for 24 hr and with aerobically-stored plates. The results of two typical experiments for Cl. tetani (Table 45) show that little or no reduction in recovery from seeded plates is observed unless these are exposed to aerobic conditions for 24 hr; even then, there is still appreciable recovery of the organism. In similar experiments with B. fragilis strains, little or no evidence of a reduction in the recovery was observed, even after exposure of seeded plates to aerobic conditions for 24 hr.

It was then decided to determine the recovery of "total anaerobes" from a faecal sample that had been held at -30°C, in experiments similar to those above. Previous studies had shown that no significant decrease in viability occurred in faecal samples held

TABLE 45

The recovery of Cl. tetani type VI from dilutions of overnight CMB cultures on anaerobically incubated plates after exposure of the seeded plates to aerobic conditions at room temperature for varying periods of time

Time of exposure of seeded plates	Dilution of inoculum on Medium A	Mean colony counts ± S.E.* derived from replicate plates of Medium A	Dilution of inoculum on Medium B	Mean colony counts ± S.E.* derived from replicate plates of Medium B
0	$^1/3 \times 10^{-3}$	609.7 ± 15.0	10^{-4}	154.8 ± 14.3
30 min.		588.1 ± 15.5		170.9 ± 10.7
1 hr		624.4 ± 13.1		208.1 ± 7.8
2 hr		577.6 ± 21.6		127.2 ± 6.6
4 hr		595.7 ± 30.1		122.2 ± 7.4
7 hr		511.7 ± 29.9		92.5 ± 7.9
24 hr		194.1 ± 7.4		46.2 ± 2.6

* Each colony count is expressed as the mean ± standard error of the mean /(S.E.) derived from 10 replicate plates of each medium.

** A = EBA medium held anaerobically at room temperature for 24 hr before use; B = EBA medium held aerobically at 4°C for 24 hr before use.

Medium A and Medium B were seeded from dilutions of different parent cultures.

at -30°C for several days (p.135). The experiments were performed using both aerobically and anaerobically-stored media, and the results, summarised in Table 46 , demonstrate that there is a gradual fall in the recovery, but that even after 24 hr exposure, it is possible to recover a considerable proportion of the original colony counts. Although it was clear that vegetative cells of a non-sporing anaerobe could survive aerobic exposure for 24 hr, little difference was observed between aerobically and anaerobically-stored plates in terms of the rate of decrease of the colony counts with time. In fact, in the example given, there was a greater percentage fall-off in colony counts after 24 hr aerobic exposure in the case of the anaerobically-stored plates, although this was not a consistent feature when replicate experiments were performed.

The ability to recover appreciable numbers of Cl. tetani organisms from seeded plates after 24 hr exposure to aerobic conditions (see above) might be explained on the basis of the survival of spores of assumed resistance to aerobic conditions. However, the present study has already shown that it is possible to recover vegetative cells of Cl. tetani on solid media using techniques involving transient exposure of seeded plates to aerobic conditions, and it seemed possible that vegetative cells could account for at least some of the persisting inoculum in the above experiments. To test this possibility, an experiment was designed in which 20 plates of freshly poured EBA medium, and 20 plates of EBA medium stored anaerobically at 16-18°C for 24 hr were randomly seeded from a dilution of an overnight CMB culture of Cl. tetani type VI. The plates were randomly assigned to 4 anaerobic jars so that each jar contained 5 plates of each medium.

TABLE 46

The recovery of "total anaerobes" from dilutions of faecal suspensions* on plates incubated anaerobically after exposure of the seeded plates to aerobic conditions at room temperature for varying periods of time

Time of exposure of seeded plates	Dilution of inoculum	Mean colony counts ± S.E.** derived from replicate plates of	
		Medium A$^{\Delta}$	Medium B$^{\Delta\Delta}$
0		513.5 ± 36.9	423.7 ± 7.4
30 min.		503.5 ± 33.6	354.7 ± 10.1
1 hr		455.8 ± 22.3	377.6 ± 10.6
2 hr	$\frac{1}{4} \times 10^{-4}$	432.2 ± 19.0	390.5 ± 20.3
4 hr		387.4 ± 12.1	343.0 ± 7.7
7 hr		401.8 ± 12.4	337.8 ± 10.0
24 hr		249.1 ± 12.4	309.8 ± 17.8

* Faecal samples were diluted 1 g in 20 ml to give the faecal suspensions.

** Each colony count is expressed as the mean ± S.E. (standard error of the mean) derived from 10 replicate plates of each medium, each seeded with 0.02-ml inocula from the test dilutions.

Δ Medium A = EBA medium held anaerobically at room temperature for 24 hr before use; Medium B = EBA medium held aerobically at 4°C for 24 hr before use.

Medium A and Medium B were seeded from dilutions of different parent suspensions.

The order of the plates within the jars was also randomised. Two of the jars were set up immediately using the standard procedure, and the plates for the other two jars were left for 24 hr on the bench at room temperature (18°C). After 24 hr the plates were placed in the other two jars, and these were set up in the standard manner. All the jars were incubated for 48 hr at 37°C. Total cell counts and spore counts were performed on the parent culture. The results (Table 47) show that good recovery was obtained from plates incubated immediately, and that even after 24 hr, it is possible to recover far more organisms than can be accounted for by spores alone. Thus vegetative cells of Cl. tetani can still be recovered from plates seeded from a CMB culture, after exposure of the seeded plates to aerobic conditions for 24 hr.

The effect of addition of carbon dioxide to the gas in anaerobic jars

The gas mixture employed in the laboratory at the time for anaerobic work was hydrogen 90 per cent. with carbon dioxide 10 per cent. The CO_2 was included because of the impression that it improved the growth of some demanding anaerobes. It was decided to determine in formal studies whether the presence of carbon dioxide in the gaseous environment affected the growth of the anaerobic organisms in which we were interested.

The presence or absence of carbon dioxide did not appear to effect the growth of Cl. oedematiens type D as judged by qualitative results; good growth occurred in the absence of carbon dioxide. However the "jar variation" phenomenon (p .224) made quantitative confirmation of these findings difficult, and it was therefore decided to check the quantitative growth of other anaerobes of more direct

TABLE 47

The recovery of Cl. tetani from plates seeded from a dilution of an overnight CMB culture* before and after exposure of the plates to aerobic conditions at room temperature for 24 hr

Strain	Dilution	Treatment of seeded plates	Mean surface viable count per ml ± S.E.** on	
			Medium A$^{\Delta}$	Medium B
Cl.tetani type VI NCTC 9569	10^{-4}	Incubated immediately	$(8.2\pm0.7)\times10^{7}$	$(8.5\pm0.7)\times10^{7}$
	10^{-4}	Exposed to aerobic conditions for 24 hr at room temp. before incubation	$(4.9\pm0.3)\times10^{7}$	$(6.0\pm0.4)\times10^{7}$

* The total cell count was 1.4×10^{8} organisms per ml; the spore estimate was $<1.4 \times 10^{5}$ organisms per ml (no spores seen in over 1000 cells counted).

** Each count is expressed as the mean ± S.E. (standard error of the mean) of ten replicate plates, each seeded with 0.02 ml inocula at the stated dilution, and incubated for 48 hr.

Δ Medium A = freshly poured EBA medium; medium B = EBA medium stored anaerobically at 18°C for 24 hr before use.

clinical importance in environments with and without carbon dioxide, with the counting techniques described on p.90. Some representative results are given in Tables 48 to 51.

It is clear that, although addition of carbon dioxide has little effect on the growth of Cl. welchii, it has a marked stimulatory effect on the two test Bacteroides strains, and on Cl. tetani. In no case was the presence of CO_2 in a concentration of 10 per cent. inhibitory to the test organism, although none of the organisms could be cultured in an atmosphere of pure carbon dioxide.

The enhancing effect of CO_2 on Bacteroides strains prompted the author to investigate the effect of CO_2 on the recovery of "total anaerobes" from faecal samples, in which organisms morphologically resembling Bacteroides are predominant (p.132). From several such experiments, it was clear that without the addition of 10 per cent. CO_2, a significant proportion of the total anaerobes failed to be recovered in solid media, i.e. that omission of CO_2 from the anaerobic environment was a powerful selective procedure. The results of a typical experiment are given in Table 51.

The author takes account of the work of Futter and Richardson (1970b), who showed that the presence of carbon dioxide in the anaerobic jar decreased the recovery note of clostridial spores. In the present study, no attempt was made to investigate the effect of carbon dioxide on spore preparations, but only on mixed spore/vegetative cultures, in which the number of spores present was usually small. With young (5-hr) CMB cultures of two strains of Cl. welchii, in which the proportion of spores present was much less than 0.5 per cent., the absence of an inhibitory effect when 10 per cent. carbon dioxide was included in the

TABLE 48

The recovery of two strains of Cl. welchii from diluted samples of 24-hr CMB cultures grown on EBA medium in BTL jars with and without CO_2 in the gaseous environment

Culture	Jar	Gaseous environment	Dilution	Mean colony count ± S.E.*	t-value for difference of means on 38 d.f.
24-hr CMB culture of Cl. welchii Hobbs type 1	H 1 C 9 C10 C 7	90 per cent. H_2 + 10 per cent. CO_2 100 per cent. H_2	$\frac{1}{2} \times 10^{-3}$	573.0±28.9 604.7±21.6	0.86 (not significant)**
24-hr CMB culture of Cl. welchii strain L2A	C 9 H 1 C10 C 7	90 per cent. H_2 + 10 per cent. CO_2 100 per cent. H_2	$\frac{1}{4} \times 10^{-3}$	507.1±13.0 521.6±15.4	0.67 (not significant)**

* Each colony count is expressed as the mean ± S.E. (standard error of the mean) derived from 20 replicate plates of EBA medium, each seeded with 0.02 ml of the stated dilution and incubated in two anaerobic jars for 18 hr.

** See Materials and Methods for explanation of convention adopted.

TABLE 49

The recovery of Bacteroides strains from diluted samples of overnight CMB cultures grown on EBA medium in BTL jars with and without CO_2 in the gaseous environment

Strain	Jar	Gaseous atmosphere	Mean colony count ± S.E.*	t-value for difference of means on 18 d.f.
B. necrophorus NCTC 7155	C 5	10 per cent. CO_2 + 90 per cent. H_2	739.6 ± 21.2	33.6 (very highly significant)**
	C 7	100 per cent. H_2	17.8 ± 3.5	
B. fragilis NCTC 9343	C 3	10 per cent. CO_2 + 90 per cent. H_2	1153 ± 32.4	6.7 (very highly significant)**
	C 4	100 per cent. H_2	873 ± 26.4	

* Each colony count is expressed as the mean ± S.E. (standard error of the mean) derived from 10 replicate plates each seeded with 0.02 ml of $\frac{1}{2} \times 10^{-3}$ dilution of the test organism and incubated for 21 hr.

** See Materials and Methods for explanation of convention adopted.

TABLE 50

The recovery of Cl. tetani from dilutions of CMB cultures grown on EBA medium in BTL jars with and without CO_2 in the gaseous environment

Culture	Jar	Gaseous atmosphere	Mean colony count ± S.E.*	t-value for difference of means on 18 d.f.
18-hr CMB culture of Cl. tetani type VI, NCTC 9569	C 7	90% H_2:10% CO_2	1014.0 ± 27.8	3.06 (highly significant) **
	C 5	100% H_2	893.0 ± 28.1	

* Each colony count is expressed as the mean ± S.E. (standard error of the mean) derived from 10 replicate plates, each seeded with 0.02 ml of ½ x 10^{-3} dilution of the test organism, and incubated for 48 hr.

** See Materials and Methods for explanation of convention adopted.

TABLE 51

The recovery of anaerobic organisms from dilutions of a faecal sample cultured on EBA medium in BTL jars with and without CO_2 in the gaseous environment

Jar	Gaseous environment	Mean colony count ± S.E.*	t-value for difference of means on 38 d.f.
C 9 C 8	90 per cent. Hydrogen + 10 per cent. CO_2	483 ± 7.2	34.35 (very highly significant)**
C 6 H 1	100 per cent. Hydrogen	149.1 ± 6.6	

* Each colony count is expressed as the mean ± S.E. (standard error of the mean) of 20 replicate plates of EBA medium seeded with 0.02-ml inocula of a $\frac{1}{2} \times 10^{-6}$ dilution of a faecal sample, and incubated for 72 hr.

** See Materials and Methods for explanation of convention adopted.

gaseous environment was confirmed (Table 52).

Thus the addition of 10 per cent. carbon dioxide to the gaseous environment in anaerobic jars as a routine part of the standardised anaerobic procedure seemed worthwhile.

The effect of flushing of anaerobic jars before incubation

In the standard anaerobic procedure already described, the jars were evacuated, filled with a mixture of 10 per cent. CO_2 in 90 per cent. hydrogen and then incubated. It was decided to investigate the effect of flushing anaerobic jars 1, 2 or 3 times with the 10 per cent. CO_2/90 per cent. hydrogen mixture before incubation, on the recovery of Cl. tetani and Cl. oedematiens type D. Preliminary studies showed that although the recovery of Cl. tetani type VI on EBA medium was not affected by flushing, such a procedure did increase the recovery of Cl. oedematiens type-D strains two or three-fold. In view of the jar variation phenomenon observed with the type-D model (see p .224) it was decided to compare bacterial recovery from faecal samples in anaerobic jars processed with and without additional flushing with the carbon dioxide/hydrogen mixture. As the results of a series of preliminary experiments showed that additional flushing did increase the recovery from faecal samples, this was further investigated in the following statistically-designed study.

A sample of a faecal suspension held at -30°C was thawed at 37°C, and a 1/5 x 10^{-4} dilution of the suspension was made in pre-steamed nutrient broth. 0.02-ml inocula were used to seed 60 plates, which were distributed randomly between six anaerobic jars according to a pre-arranged schedule. The plates were then arranged randomly within the jars, according to the schedule. The jars were then

TABLE 52

The recovery of two strains of Cl. welchii from diluted samples of 5-hr. CMB cultures grown on EBA medium in BTL anaerobic jars with and without CO_2 in the gaseous environment

Culture	Jar	Gaseous environment	Dilution	Mean colony count ± S.E.*	t-value for difference of means on 38 d.f.
5-hr CMB culture** of Cl. welchii Hobbs type 1.	H 3 C 1	90 per cent. H_2 + 10 per cent. CO_2	$\frac{1}{4} \times 10^{-3}$	437.0±15.6	1.06 (not significant)△
	C 8 C 2	100 per cent. H_2		461.9±17.4	
5-hr CMB culture△△ of Cl. welchii strain L2A	H 3 C 1	90 per cent. H_2 + 10 per cent. CO_2	$\frac{1}{4} \times 10^{-3}$	695.0±18.6	1.95 △ (not significant)
	C 8 C 2	100 per cent. H_2		632.4±26.4.	

* Each colony count is expressed as the mean ± S.E. (standard error of the mean) derived from 20 replicate plates, each seeded with 0.02 ml of the stated dilution and incubated in two anaerobic jars for 18 hr.

** The total cell count was 1.6×10^8 per ml; the spore estimate was 3.2×10^5 per ml (no spores seen in over 500 cells counted).

△ See Materials and Methods for explanation of convention adopted.

△△ The total cell count was 1.7×10^8 per ml; the spore estimate was 3.4×10^5 per ml (no spores seen in over 500 cells counted).

processed as follows:-

(i) 2 jars were processed according to the standard procedure;

(ii) 2 jars were evacuated to -660 mm Hg, filled with 10 per cent. CO_2 in 90 per cent. H_2, and then processed as in (i);

(iii) 2 jars were flushed as in (ii), flushed again, and then processed as in (i).

The order of processing of the jars was as far as possible random, and the jars were incubated at 37°C for 48 hr. The results of this experiment, together with the order of processing of the jars, are shown in Table 53 . It is clear that flushing of the jars before processing increases the recovery of "total anaerobes" from faeces, although repetition of the flushing did not give higher recovery than if a single flushing step was used.

From these results, it seemed worthwhile to include a flushing step in the procedure for BTL anaerobic jars. A modified procedure is described on p .246.

The effect of prolonged incubation of plates

It is customary to incubate plates seeded with anaerobes for 48 hr or longer; Willis (1969, p. 161) recommends incubation of plates seeded with Cl. oedematiens type D for four days in order to obtain adequate growth. The use of the cysteine/dithiothreitol system, however, yielded large, easily visible colonies of demanding strains of Cl. oedematiens after only 18 hr incubation at 37°C. Although it was clear from several experiments that quantitative recovery of type-D and type-B strains could be achieved on blood agar media (see page 113), it was deemed necessary to determine whether incubation for periods in excess of 18 hr would lead to increased

TABLE 53

The effect of flushing BTL jars with a mixture of hydrogen and carbon dioxide before processing on the recovery of anaerobic organisms from a suspension of human faeces

Jar*	Dilution	Procedure**	Mean colony count ± S.E.△
A E	10^{-6}	Standard procedure	141.7 ± 5.0
C D	10^{-6}	Flushed once, then processed by the standard procedure	369.7 ± 13.2
B F	10^{-6}	Flushed twice, then processed by the standard procedure	323.0 ± 10.7

* The jars were processed in the order A, B, C, D, E, F.

** The standard procedure is detailed on p. . The flushing consisted of evacuating the jar to - 660 mm Hg, then filling with a mixture of 90 per cent. hydrogen and 10 per cent. carbon dioxide, after which the jar was processed in the normal way, i.e. by evacuation, followed by filling with the hydrogen carbon dioxide mixture etc.

△ Each count is expressed as the mean ± S.E. (standard error of the mean) of 20 replicate plates of EBA medium, each seeded with 0.02 ml of the stated dilution, and incubated in two anaerobic jars for 72 hr.

recovery of these organisms. BTL anaerobic jars containing plates seeded with type-D strains were incubated for 24, 48, 72 and 96 hr, and the recovery rates compared. These experiments were repeated several times, in jars equipped with three catalysts, to minimise the "jar variation" phenomenon (see p.224), and were repeated for other strains of Cl. oedematiens. From the results, it was clear that incubation of seeded plates for periods in excess of 18 hr resulted in only a marginal increase in recovery, of 5 per cent. or less. Similar experiments with Cl. tetani, however, showed that incubation for 48-72 hr, resulted in increased recovery as compared with that from plates incubated for only 18 hr. This finding was investigated further, in the following experiment:-

20 freshly-poured plates of EBA medium were each seeded with 0.02 ml of a 10^{-3} dilution in pre-steamed nutrient broth of an overnight CMB culture of Cl. tetani type VI (NCTC 9569). The plates were randomly distributed between two anaerobic jars, according to a pre-arranged schedule, and the position of the plates within each jar was also randomised. Both jars were processed according to the standard anaerobic procedure, one being incubated for 18 hr at 37°C, the other for 66 hr at 37°C. Total counts and spore estimates were performed for the parent culture. The results are given in Table 54. It is clear that prolonged incubation results in a considerable increase in the surface viable count (more than two-fold), which could not be accounted for by germination of spores alone, as these formed only a small proportion of the inoculum. These findings were confirmed in later studies, which also showed that little increase in recovery (5 per cent.) occurred if incubation was continued after 48 hr.

TABLE 54

The influence of prolonged incubation on the recovery of Cl. tetani NCTC 9569 from an overnight CMB culture*

Jar	Dilution	Time of incubation (hr)	Mean surface viable count ± S.E.**
C 9	10^{-3}	18	$(2.03 \pm 0.15) \times 10^{6}$
C10	10^{-3}	66	$(5.96 \pm 0.22) \times 10^{6}$

* The total cell count was 6.05×10^{6} per ml; the spore estimate was $< 3.90 \times 10^{3}$ per ml (no spores seen in over 1500 cells counted).

** Counts expressed as mean ± S.E. (standard error of the mean) derived from ten replicate plates of EBA media, each seeded with 0.02-ml inocula at the stated dilution.

Careful examination of plates after 24 hr and 48 hr incubation showed that the increase in recovery was due to the growth to a visible size of colonies that were so small after 24 hr as to be almost invisible, even with a hand lens. A similar phenomenon occurred with faecal samples; many tiny colonies seen with difficulty after 24 hr could be easily seen and accurately counted after 48 hr or 72 hr incubation. It therefore seems reasonable to incubate plates seeded with demanding organisms, or seeded from a clinical specimen, for 48 hr; organisms such as Cl. welchii show almost complete recovery after overnight incubation (see Table 16).

A summary of the effects of the above variable factors on the recovery of organisms on solid media is given overleaf (Table 55).

TABLE 55

A summary of the effects of various factors, separately tested, on the recovery of test organisms from solid media*

Factor tested	Observed effect with the stated test inoculum					
	Cl. oedematiens type D	Cl. tetani type VI	Anaerobic coccus	Ps. pyocyanea	Bacteroides spp.	"Total anaerobes" in faecal suspension
Aerobic agitation of suspension in diluents	I +++	I +	I +	-	-	. . .
Pre-flushing with H_2/CO_2 before the standard anaerobic procedure	E ++	-	. . .	. . .	-	E ++
Omission of CO_2 from standard anaerobic procedure	±	I +	. . .	. . .	I +++	I ++
Exposure of seeded plates to aerobic condition before anaerobic incubation	I +++	I +	. . .	. . .	I +	I +
Anaerobic storage of plates before seeding**	E ±	-	I ±	. . .	-	I ±
Aerobic storage of plates before seeding **	I ++	I ++	I ++	. . .	I ++	I ++
Effect of special thick agar△	-	E +	. . .	. . .	-	-
Prolonged incubation (48-72 hr)	E ±	E ++	. . .	. . .	E ±	E ±

* See text for details of these studies. I = inhibition of growth. E = enhancement of growth.

+, ++, +++, = increasing degrees of observed effect. ± = doubtful effect. - = no effect observed.

. . . = not done.

** In comparison with recovery on normal freshly-poured plates.

△ In comparison with plates of normal thickness (see text).

AN ASSESSMENT OF THE GASPAK SYSTEM IN THE LABORATORY CULTURE OF ANAEROBIC BACTERIA

The recent introduction of a self-contained disposable source of hydrogen and carbon dioxide for the anaerobic jar (marketed as the "Gaspak" by Becton Dickinson U.K. Ltd., York House, Empire Way, Wembley, Middlesex), removes the need for cylinders of compressed gases, pumps and metering equipment. The development of the Gaspak system has been reviewed above (see p.44).

The production of gas by this system is simply accomplished by introducing a 10-ml volume of water into a disposable aluminium-foil pack (Fig. 8) within the jar containing the plates to be incubated; the jar is then closed and incubated without further attention. Thus the system is quick and easy to operate, and if found to be acceptable, could provide a standardised, consistent procedure for anaerobic jars in diagnostic laboratories, especially in those laboratories that lack special facilities for anaerobic work. A limited report on the use of the Gaspak system has already appeared (Williams, 1970). It was decided to compare the recovery of a wide range of anaerobes by the Gaspak system, with that achieved by the standard procedure already described. Unless specified in the text, all studies were performed with the Gaspak system being used in conjunction with BTL jars. Fourteen BTL jars were used in the present study; all were used in conjunction with the room-temperature catalyst sachets supplied by BTL, and each jar was equipped with a single catalyst sachet.

The standard procedure developed for use with the BTL jar is described on page 81. The Gaspak anaerobic procedure is as follows:

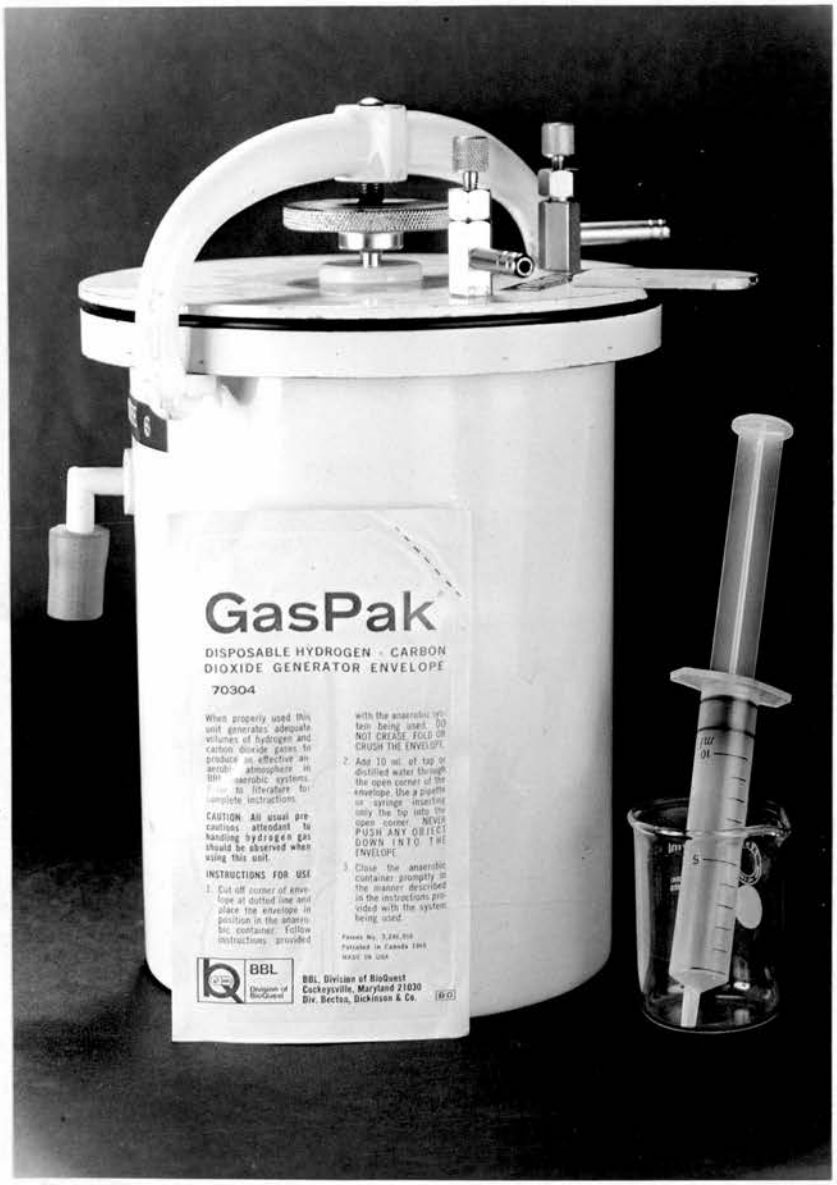


FIGURE 8. - The Gaspak envelope and the BTL anaerobic jar. The side arm of the jar is sealed.

The Gaspak foil envelope is opened by peeling back the corner to the dotted line and 10 ml of water is then added by means of a syringe, the nozzle being inserted a little way into the envelope before placing the Gaspak upright and unfolded into the jar. The lid of the jar is then immediately put on, and screwed down finger-tight. If a BTL jar is used, both inlets must be closed. The jar is then incubated in the normal way. The organisms used in this study are listed in Table 56.

Pressure changes

The pressure changes occurring within a BTL anaerobic jar containing an activated Gaspak envelope and equipped with a new catalyst sachet were monitored with a mercury manometer. In the absence of a catalyst, the pressure may rise steeply to about 30 cm Hg within the first 10 min. When an active catalyst is present, the increase in pressure caused by the evolution of gas is countered by the combination of the hydrogen with the oxygen present, and an initial rise in pressure is followed by a precipitate fall. The results of a typical experiment are summarised in Fig. 9.

Fig.10 shows the results of a similar experiment in which the gas was collected over concentrated KOH solution so that the carbon dioxide was absorbed, and it is obvious that a substantial volume of hydrogen remains on completion of the reaction. It should be noted, however, that the above studies were performed with empty jars, which therefore contained maximum amounts of air.

The volume of gas produced by the Gaspak

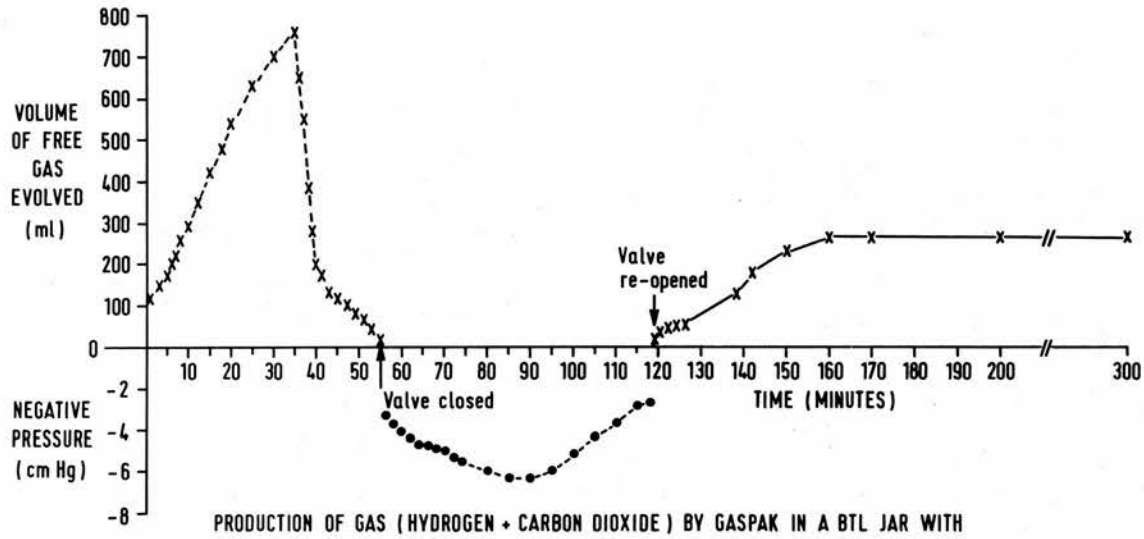
The mean volume of 10 BTL jars examined was 3110 ml, with a range of 3060-3160 ml. The total volume of gas released from a Gaspak

TABLE 56

Test organisms used in comparative studies of the Gaspak and standard procedures for the BTL anaerobic jar

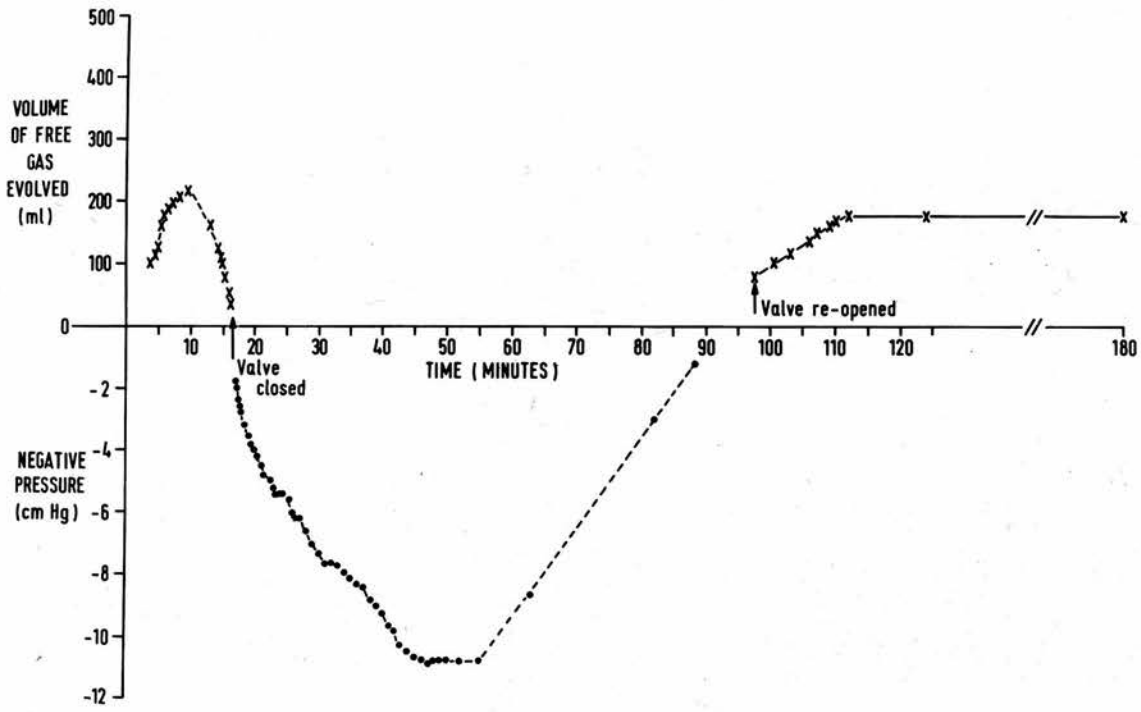
Bacteroides fragilis	NCTC 9343
B. necrophorus	NCTC 10575
Clostridium tetani	NCTC 5405
Cl. tetani, type VI	NCTC 9567
Cl. sporogenes	Dr. Nancy Hayward's strain 60
Cl. chauvoei	Wellcome No. CN3600
Cl. histolyticum	NCTC 503
Cl. welchii (*Cl. perfringens*)	Type A, lab. No. L2A
Cl. bifermentans	NCTC 506
Cl. butyricum	NCTC 7423
Cl. septicum	Lab. No. 117
Cl. oedematiens (*Cl. novyi*), Type A	NCTC 538
Cl. oedematiens, Type B	Glaxo No. 474E.
Cl. oedematiens, Type D	(NCTC 9692 (NCTC 8350 (NCTC 8145
Anaerobic coccus, Group 1	NCTC 9801

FIGURE 9



PRODUCTION OF GAS (HYDROGEN + CARBON DIOXIDE) BY GASPAK IN A BTL JAR WITH CATALYST PRESENT AND THE NEGATIVE PRESSURE PHASE OBSERVED (WATER TEMPERATURE 16·5°C; AIR TEMPERATURE 20°C)

FIGURE 10



PRODUCTION OF FREE HYDROGEN BY GASPAK IN A BTL JAR WITH CATALYST PRESENT AND THE NEGATIVE PRESSURE PHASE OBSERVED (GAS EVOLUTION MEASURED OVER KOH TO ABSORB CO_2; WATER TEMPERATURE 15·5 °C ; AIR TEMPERATURE 22 °C)

contained in a BTL jar was about 1600-1900 ml, this volume being evolved over the course of 5-6 hr at room temperature (18.5-20°C). The results of a typical experiment with a jar devoid of a catalyst are shown in Fig. 11. The rate of evolution of gas from the Gaspak sachets was very variable, especially in the early stages, and the total volume produced also varied. The results obtained with 5 Gaspaks, tested on different days, are given in Table 57.

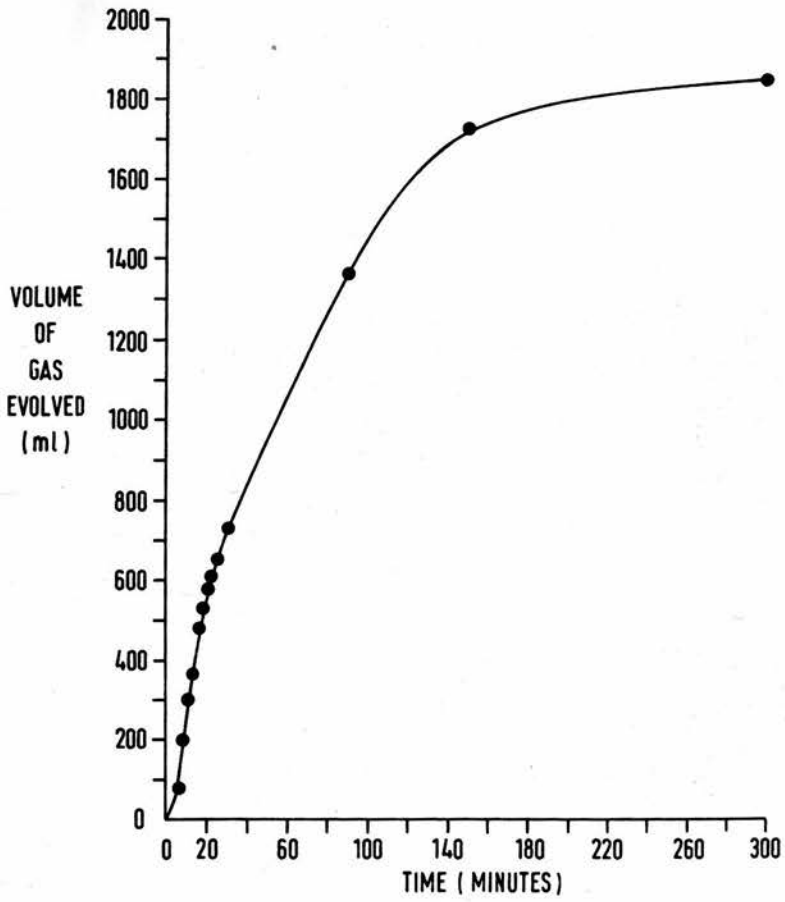
The culture of laboratory stock strains

Studies with various test clostridia

The recovery of Cl. welchii type A was compared in the standard and Gaspak procedures, after varying intervals of incubation. Comparable results were obtained with the two procedures; although plates incubated with the standard procedure showed slightly more growth after 3½ hr incubation, no perceptible difference could be seen after 6 hr incubation, or thereafter. The viable counts obtained by both procedures were equivalent, at 1.4×10^7 organisms per ml.

Cultures of other test clostridia were then set up with the Gaspak system and with the standard system, plates being seeded with a loopful of a 20 hr CMB culture of the test organism, and the growth after overnight incubation at 37°C was compared on the basis of naked-eye appearances only. The organisms tested are listed in Table 56. In the case of Cl. bifermentans, Cl. chauvoei, Cl. histolyticum, Cl. butyricum and Cl. sporogenes, there was no obvious difference between the results obtained with the standard and Gaspak procedures. When similar experiments were performed with a more demanding anaerobe, Cl. oedematiens type-A, comparable results were usually obtained,

FIGURE 11



PRODUCTION OF HYDROGEN BY GASPAK IN BTL JAR WITH NO CATALYST PRESENT (ROOM TEMPERATURE 18 °C ; WATER TEMPERATURE 10-12°C)

TABLE 57

Cumulative volumes of gas produced from five Gaspaks separately tested in BTL anaerobic jars* by a water displacement method

Time	Volume of gas (Cm^3) evolved				
	Test 1 (18°C)**	Test 2 (17.5°C)	Test 3 (17.5°C)	Test 4 (16.5°C)	Test 5 (16.5°C)
5 min.	360	30	20	0	340
10 min.	530	100	120	30	420
15 min.	680	120	260	140	450
20 min.	780	180	358	310	490
30 min.	960	240	630	565	550
1 hr	1120	410	920	1200	660
2 hr	1800	590	1250	1810	...
3 hr	1860	790	1470	1875	1280
4 hr	1865	1210	...	1875	1510
5 hr	1865	1470	1920	1875	1720
6 hr	1865	1850	1935	...	...

* Tests 1, 2 and 3 were performed in the same anaerobic jar; tests 4 and 5 were performed in another anaerobic jar.

** Figures in parentheses = temperature of water used in water displacement method.

... = not done.

although the standard procedure occasionally gave better naked-eye evidence of growth. It was therefore decided to compare the two systems in a series of quantitative experiments with the most exacting clostridia available, i.e. strains of Cl. tetani and Cl. oedematiens type-D.

Studies with Cl. tetani

In qualitative studies, comparable results were obtained with cultures grown on blood agar plates with the two systems.

Initial quantitative studies with a non-motile type VI strain showed that comparable surface viable counts could be obtained in the two systems. An overnight CMB culture of a type VI strain was diluted 10^{-2}, 10^{-3} and 10^{-4}, and 0.02-ml drops were used to seed blood agar plates, which were then spread by the method already described (page 90). Four plates were used for each dilution, and random pairs were set up in the standard and in the Gaspak system. The Gaspak procedure yielded viable counts of (1.3-3.1) x 10^7 per ml, and the standard procedure gave slightly better results of (1.9-3.9) x 10^7 per ml.

In a further study, a motile strain of Cl. tetani (NCTC 5405) was used. Inocula derived from tenfold dilutions of an overnight CMB culture were used to seed blood agar plates, spreading growth being inhibited by the use of tetanus antitoxic serum (Wellcome), according to the method of Willis and Williams, 1970 (see p.92). Slightly higher viable counts could be obtained with the standard procedure than with the Gaspak system; the results of a representative experiment shown in Table 58 indicate that a mean surface viable count of 5.7 x 10^7 per ml was obtained with the standard procedure, whereas a mean count

TABLE 58

A comparative test of the Gaspak and standard anaerobic systems with a motile strain of Cl. tetani NCTC 5405

Anaerobic system	No. of colonies subcultured from 3 x 0.02 ml of a 10^{-4} dilution of an 18-hr CMB culture on EBA medium*			Estimated total viable count per ml
Standard procedure	119,	99,	125	5.7×10^7
Gaspak system	78,	110,	110	5.0×10^7

* All plates incubated for 48 hr.

of 5.0×10^7 per ml was obtained with the Gaspak system. It was notable that under the relatively moist conditions in the Gaspak system, confluent growth of colonies tended to occur.

The above studies were performed with small numbers of plates and it was therefore decided to compare the growth of cultures on large numbers of plates in the Gaspak and standard systems, using a type VI strain. Four plates were seeded and spread, and randomly distributed between four anaerobic jars, 3 equipped with the Gaspak system (G) and 3 set up according to the standard procedure (S). The jars were processed in pairs, e.g. S1 and G1 were set up simultaneously, then S2 and G2. After initial treatment, all 4 jars were left on the bench for 10 min. and then those in the "S" series received more hydrogen to equilibrate. Jars S1 and G1 were incubated together, and so on. All jars were incubated at 37°C; jars S1 and G1 were incubated for 24 hr, jars S2 and G2 were incubated for 72 hr. The results of this experiment showed that there was no significant difference between the recovery in jars processed by the standard anaerobic procedure and that in jars processed with the Gaspak system.

Studies with Cl. oedematiens type-D and type-B strains

Qualitative studies with these two organisms gave variable results when human blood agar medium was used, and no reliable comparison could be drawn between the two systems. The following quantitative studies were therefore performed with media containing the cysteine/dithiothreitol system. Preliminary results suggest that superior results were obtained with the standard procedure (Table 59), but there was a considerable degree of variation. The following experiment was therefore performed:

TABLE 59

A comparative test of the Gaspak and standard anaerobic systems with two strains of Cl. oedematiens type D

System*	Organism	Jar	No. of colonies subcultured from 3 x 0.02 ml of a 10^{-1} dilution of an 18-hr CMB culture on CDEBA medium			Estimated total viable count per ml
S	Cl. oedematiens	H 3	149	113	150	6.9×10^4
G	Type D NCTC 9692	C 5	36**	36**	36**	1.8×10^4
S	Cl. oedematiens	H 3	467	412	254	1.9×10^5
G	Type D NCTC 8350	C 5	29	3	10	7.0×10^3

* G = Gaspak; S = standard procedure

** Some confluent growth occurred, and these numbers are therefore minimum estimates.

An 8-day CMB culture of Cl. oedematiens type D (strain NCTC 8145) was diluted 10^{-1} in pre-steamed nutrient broth, and a 0.02-ml drop of the undiluted or diluted culture was spread on each of a series of plates of CDHBA medium. Twelve plates were spread with the undiluted sample, and another twelve plates were spread from the diluted sample. Six of each were randomly assigned to a jar set up with the standard or Gaspak procedure. On the following day, the results were recorded, and the experiment repeated with the same anaerobic jars subjected to the alternative procedure. The results are given in Table 60; only the data obtained with the undiluted sample are shown, as the diluted samples gave colony counts considerably below the acceptable range for colony counting.

The results indicate variables in this work that cannot be defined. For example, the counts obtained on day 1 are generally superior to those on day 2. The reasons for these variations are not clear; it may be that variations in batches of media are responsible for this effect. However, the results clearly demonstrate that comparable recovery of this demanding anaerobe can be achieved with the Gaspak and standard systems, and that on occasion, the counts obtained with the Gaspak system may even exceed those obtained with the standard anaerobic technique.

It was then decided to design an experiment in which a large number of jars was used. Twelve BTL anaerobic jars were serviced and fitted with new catalyst sachets that had been used once and proved active. Each jar was equipped with one catalyst sachet. An overnight

TABLE 60

The results of replicate surface viable counts obtained from plates seeded with a type-D strain of Cl. oedematiens grown in different anaerobic systems on two occasions

Day	Anaerobic jar	Anaerobic system	Mean colony count ± S.E.*
1	A	Gaspak	192.6 ± 6.23**
	B	Standard	106.0 ± 5.0
2	B	Gaspak	77.3 ± 1.8
	A	Standard	79.7 ± 5.9

* Each colony count expressed as the mean ± S.E. (standard error of the mean) of six replicate plates of CDHBA 33 medium, each seeded with 0.02 ml of a 10^{-1} dilution of an 18 hr CMB culture of strain GRID.

** The author appreciates that statistical interpretation of data based on small numbers may be misleading; the figures for standard deviations are here derived to illustrate the variables involved in the test system.

CMB culture of Cl. oedematiens type D (strain NCTC 8350) was diluted 10^{-1} and 10^{-2} in pre-steamed nutrient broth. Thirty-six plates of freshly-poured CDHBA 33 medium (see Materials and Methods) were then seeded with 0.02-ml drops of each dilution, the drops being immediately spread with sterile glass spreaders as described on page 90. The plates were randomised and three plates for each dilution were immediately placed in each of the 12 anaerobic jars. Six of the jars (S1-S6) were put up by the standard procedure and six (G1-G6) received a Gaspak envelope. The jars were processed in pairs, S1 and G1 being set up simultaneously, then S2 and G2, and so on. The total time taken to seed the plates, place them in the jars, and process the jars was less than 10 min. This speed was achieved by training 4 technical colleagues to work as a team. After the initial treatment, all 12 jars were left on the bench for 10 min; the S series than received more hydrogen to equilibrate. Jars S1 and G1 were incubated together, then jars S2 and G2, and so on. All jars were incubated at 37°C for 42 hr, care being taken to incubate paired jars (i.e. S1 and G1) on the same shelf of the incubator, so that any differences due to temperature variations within the incubator were minimised. The results of this experiment are given in Table 61.

The most striking feature of these results is the large degree of variation that occurred between the six jars processed by the standard system, with viable counts ranging from 3.1×10^4 to more than 2.5×10^5. This "jar variation" is much less marked in the case of the Gaspak system, counts ranging from (2.9×10^4) - (3.5×10^4). If the minimum figure obtained with jar S6 is taken, the mean value for the standard series is 1.1×10^5, whereas the mean for the Gaspak

TABLE 61

A comparative test of the Gaspak and standard anaerobic systems: the results of replicate viable counts obtained on plates of a solid medium* with a type-D strain of Cl. oedematiens, incubated in two two parallel series of six anaerobic jars

System used	Jar	Number of colonies subcultured from 3 x 0.02 ml of a 10^{-1} dilution of an 18-hr CMB culture			Mean total viable count per ml
Gaspak	G 1	62	60	54	2.9×10^4
	G 2	66	70	71	3.5×10^4
	G 3	44	61	90	3.3×10^4
	G 4	74	53	61	3.1×10^4
	G 5	50	57	67	2.9×10^4
	G 6	67	72	73	3.5×10^4
Standard	S 1	72	112	143	5.5×10^4
	S 2	60	63	64	3.1×10^4
	S 3	201	145	208	9.2×10^4
	S 4	208	169	164	9.0×10^4
	S 5	386	295	210	1.5×10^5
	S 6	>500	>500	>500	$>2.5 \times 10^5$

* CDHBA 33 medium was used throughout; all plates incubated for 18 hr.

series is 3.2×10^4. Alternatively, the medians are ($9.0 - 9.2 \times 10^4$) and ($3.1 - 3.3 \times 10^4$).

It is clear that comparisons between the recovery rates for type-D strains obtained with the Gaspak and standard systems are difficult to interpret, because of the variables observed in the test system, especially with the "jar variation" factor now apparent in Table 61 . With the type-D model, the standard procedure was superior, and on occasion markedly so. However, in other experiments, the Gaspak was consistently successful in redeeming large numbers of exacting test strains.

The "jar variation" phenomenon clearly required further investigation and the results of relevant studies are reported on page 224.

Studies with other stock laboratory cultures

The recovery of other organisms was then compared using the Gaspak and standard procedures. The recovery of two Bacteroides species was similar in the two systems; the results of a typical experiment (Table 62) show that the surface viable counts achieved by the two systems were very similar.

In a later experiment, the surface viable counts of these two strains of Bacteroides were compared when the Gaspak system was used in conjunction with a BBL polycarbonate jar. The results for B. fragilis (NCTC 9343) and B. necrophorus (NCTC 10575) respectively were: 8.4×10^9 and 3.0×10^8 by the standard procedure, and 6.6×10^9 and 3.3×10^8 by the Gaspak system.

Similar results were obtained with other test organisms, including a beta-haemolytic streptococcus and an anaerobic coccus

TABLE 62

Comparative tests of the standard and Gaspak anaerobic systems with dilutions of overnight CMB cultures of Bacteroides species

Date	System*	Organism	Jar	No. of colonies subcultured from 3 x 0.02 ml of CMB culture at stated dilution on EBA medium**			Dilution used	Mean total viable count per ml
8.4.70	S	B. fragilis NCTC 9343	C 8	62	76	60	10^{-6}	3.3×10^9
	G		H 3	76	80	70		3.8×10^9
8.4.70	S	B. necrophorus NCTC 10575	C 5	28	16	21	10^{-4}	1.1×10^7
	G		C 7	38	26	30		1.6×10^7

* S = Standard procedure, G = Gaspak anaerobic system.

** Plates incubated for 48 hr.

group I; little or no difference was observed in the performance of the two systems.

Studies with freshly isolated obligate anaerobes

It may be argued that freshly isolated strains of anaerobic bacteria are more exacting in terms of their requirements for anaerobiosis than laboratory stock strains such as those used in the present study. It was therefore decided to compare the isolation of Bacteroides species from faeces (in which those organisms are present in large numbers) with the Gaspak and standard procedures.

A fresh specimen of faeces was collected in a pre-weighed sterile bijou bottle and a subsequently defined amount (0.6 g) was immediately added to 50 ml cool pre-steamed nutrient broth in a 100-ml conical flask equipped with a magnetic stirrer; an overlay of sterile liquid paraffin prevented the access of oxygen. The faeces was then suspended in the diluent by stirring at room temperature for 2 hr. Thereafter, a sample of the faecal suspension was serially diluted (up to 10^{-7}) in pre-steamed broth at 37°C under anaerobic conditions.

0.1-ml volumes from each dilution were used to spread plates of well-dried equine blood agar (EBA). Four plates were seeded from each dilution, and paired plates were promptly allocated to the standard and Gaspak procedures. The results recorded in Table 63 show the colony counts obtained after anaerobic incubation at 37°C for 20 hr and for 44 hr with the two systems.

Gram stained smears from the dilution used for enumeration (10^{-4}) showed Gram-negative bacteria morphologically typical of Bacteroides species. The colonial appearances were also typical of those seen

TABLE 63

The surface viable counts of anaerobes grown on solid media from dilutions of a faecal suspension incubated anaerobically by the standard and Gaspak procedures

Time of incubation (hr)	Total anaerobic viable counts per g* of faeces with the stated anaerobic system	
	Standard	Gaspak
20	2.0×10^8	1.0×10^9
44	1.0×10^{10}	1.3×10^{10}

* expressed as the mean from four replicate plates of EBA medium each seeded with 0.02 ml of a 10^{-5} dilution of the faecal suspension, and incubated for 72 hr.

with laboratory stock cultures of these organisms, and as they proved to be anaerobic, further identification was not attempted. The results suggest that after a short period of incubation, the viable counts obtained with the Gaspak system (1.0×10^9 per g) were appreciably higher than those obtained with the standard system (2.0×10^8 per g), whereas on incubation for 44 hr there was little difference between the counts. Similar experiments confirmed that there was little difference in the recovery of organisms from faeces with the two systems; the higher recovery observed in this experiment after 20 hr incubation was not observed in subsequent experiments. It was decided to compare the recovery of organisms from faeces using several anaerobic jars, so that the results might be analysed statistically.

A frozen sample of faecal suspension was thawed at 37°C and tenfold dilutions made in cool pre-steamed nutrient broth. From previous experiments with aliquots of the same suspension, it had been shown that there was no decrease in the surface viable counts on freezing at -30°C. A dilution was chosen that would yield 200-500 colonies per plate, and 0.02-ml drops were used to seed 60 plates. The plates were randomly allocated to six anaerobic jars, three equipped with a Gaspak (G1 - G3) and three processed by the standard procedure (S1 - S3); the position of the plates within a given jar was also randomly determined (see p. 235). Jars S1 and G1 were then processed together, followed by jars S2 and G2 and then jars S3 and G3. The jars were left on the bench for 10 min., when hydrogen was added to the "S" series jars to equilibrate the pressure, and all the jars were then incubated anaerobically at 37°C for 18 hr. Each jar was

equipped with 3 catalysts (see p. 246).

The results are given in Table 64, and it is clear by inspection that there is little difference between the colony counts achieved in each pair of jars; this was confirmed by statistical analysis of the results obtained with paired jars. Similarly the mean colony count for all the jars containing Gaspaks (307.9 $\pm$ 7.2) was very similar to that obtained with all the standard jars (312.6 $\pm$ 8.3).

Class studies

As the Gaspak system seemed convenient for practical classwork when inexperienced operators were involved, a study was set up in which a class of students, unused to anaerobic techniques, investigated 10 samples of human faeces from different individuals. About 0.5 g of the freshly passed specimen was added to a bijou bottle containing a known volume of transport medium (see Materials and Methods), with instructions that the sample should be processed within 12 hr if held at 4°C and within 2 hr if unrefrigerated. The samples were all labelled with the name, age and sex of the donor. On receipt in the laboratory, all samples were immediately frozen by immersion in solid CO_2 chips, and held thus until the start of the experiment. Working in groups of 2 or 3, and supervised by demonstrators with special experience in this work, serial dilutions of the thawed faecal suspensions were prepared in transport medium (from undiluted to 10^{-8} by hundred-fold dilutions). Then 0.02-ml amounts of the dilution were spread on blood agar plates, and on plates of blood agar medium containing 70 µg neomycin sulphate per ml. The

TABLE 64

The mean colony counts observed from a $^{1}/_{5} \times 10^{-6}$ dilution of a faecal sample incubated anaerobically by the Gaspak and standard procedures

Order of evacuation*	Jar	System	Mean colony count ± S.E. **	t-value for difference of means on 18 d.f.
1st	S 1	Standard	319.3 ± 17.3	0.56
	G 1	Gaspak	331.4 ± 12.9	(not significant)Δ
2nd	S 2	Standard	279.6 ± 10.4	1.2
	G 2	Gaspak	296.2 ± 9.1	(not significant)
3rd	S 3	Standard	332.3 ± 14.9	1.14
	G 3	Gaspak	312.8 ± 8.3	(not significant)

* The jars were processed in pairs, i.e. S 1 and G 1 together, followed by S 2 and G 2 etc.

** Each colony count is expressed as the mean ± S.E. (standard error of the mean) of ten replicate plates, each seeded with 0.02 ml of the stated dilution, and incubated for 24 hr.

Δ See Materials and Methods for explanation of convention adopted.

plates were incubated immediately after being seeded in BTL anaerobic jars equipped with Gaspaks, and the colonies were counted after incubation for 48 hr at 37°C. The numbers of colonies ranged from 60 to 200 per plate, and Gram-stained smears from representative plates confirmed that the predominant organisms isolated resembled Bacteroides species. The total viable counts per g of faeces were as follows: 2.8×10^9; 1.2×10^9; 3.2×10^9; 1.2×10^9; 4.4×10^8; 6.1×10^7; 2.9×10^9; 1.2×10^9; 9.5×10^9; 4.1×10^9; and 3.2×10^9. The viable counts therefore ranged from (6.1×10^7) to (9.5×10^9). All the students found the system easy to operate, and the counts obtained compare favourably with the counts obtained by the author in the present study, e.g. Table 63.

The Gaspak anaerobic indicator, and the BBL jar

The Gaspak foil envelope can be used in a specially designed clear polycarbonate jar, in conjunction with the improved anaerobic indicator described by Brewer, Allgeier and McLaughlin (1966).

The author has had little experience with the anaerobic indicator, as the standard procedure developed in the present work relies on the development of a secondary vacuum as an index of catalytic activity. The indicator system supplied is intended for use inside a clear jar, and is therefore of no use in the case of the metal BTL jars. In the BBL/polycarbonate jar it seemed to work satisfactorily, but the development of the indicator effect was delayed and slow compared with the prompt development of a secondary vacuum observed in the case of the BTL jar with the standard procedure.

The polycarbonate jar is expensive, costing about £35 in

Britain, compared with the BTL jar at £22. It worked well in tests, but only one jar was available and reliable comparative studies could not be performed. As mentioned above, the polycarbonate jar is designed for use with a specially-designed indicator system. Preliminary tests with the Gaspak system suggested that it might be possible to develop a monitoring system of catalytic activity based on pressure changes within the jar, similar to that employed with the BTL jar. As the pressures developed are greater, a simple mercury manometer would not be suitable for this purpose; preliminary results suggested that a rubber balloon attached to one of the outlets of the BTL jar might provide a suitable indication of pressure changes signifying an active catalyst. The lack of outlets in the case of the BBL polycarbonate jar would obviously be a disadvantage for such a system.

The humidity associated with the use of the Gaspak system is considerably greater than that developed with the standard procedure, as water is added to the foil pack. This is a problem in the case of spreading organisms and may frustrate attempts to determine accurate colony counts, or to produce discrete colonies from clinical specimens. Williams (1970), reporting the results of a small comparative trial, noted that the humidity associated with the Gaspak system tended to shorten the life of the catalyst sachets.

The results obtained show that the Gaspak can perform consistently and reliably, achieving comparable recoveries of clinically important anaerobes with those achieved by the standard procedure. Only when a very demanding organism was used did the Gaspak system show poorer recovery, on occasion, than that achieved with the

standard procedures; the variables associated with the exacting model however, made accurate quantitative comparisons difficult.

STUDIES WITH THE BTL ANAEROBIC JAR

Jar variation

Results of studies in which several jars were used at one time (Table 61), made it clear that, in the case of the Cl. oedematiens type-D model, there was on occasion a marked degree of variation between jars containing plates seeded with identical inocula. This variation - the "jar variation"-phenomenon seemed to affect all the plates in a given jar, and seemed only to occur with Cl. oedematiens type-D strains. To confirm that jar variation really occurred, the following experiment was designed.

> Twelve BTL anaerobic jars were selected, of which 5 were unused ("new") and the remaining were in current use ("old"). Each jar was equipped with a room temperature catalyst sachet. Forty-eight plates of freshly-poured CDEBA 10 medium were seeded from a 10^{-2} dilution of an overnight CMB culture of Cl. oedematiens type-D, each plate being seeded with 0.02 ml and spread with a sterile spreader. The plates were then distributed without a formal randomisation schedule between the 12 anaerobic jars, each jar receiving 4 plates. The jars were then evacuated and processed by the standard anaerobic procedure in the order shown, and incubated at 37°C for 21 hr. The results of the colony counts are given in Table 65.

The wide range of variation observed between jars contrasts with that observed between the plates in a given jar, and this suggests that the variable factor(s) involved may affect the whole jar. Jar C1 gave no growth in spite of the development of a satisfactory secondary

TABLE 65

The mean colony counts observed on replicate plates each seeded from a dilution of an overnight CMB culture of Cl. oedematiens type-D, when the plates were incubated in 12 BTL anaerobic jars

Order of evacuation	Jar	Condition* of jar	No. of colonies subcultured from 4 x 0.02 ml of CMB culture at 10^{-2} dilution				No. of colonies per jar	Mean colony count per plate	Secondary vacuum developed after 10 min. at room temp. (mm Hg below atmospheric pressure)
1	C3	Used	11	17	17	10	55	13.8	-72
2	H1	New	21	33	31	30	115	28.8	-60
3	H2	New	131	102	152	172	557	139.3	-70
4	C8	Used	3	3	8	2	16	4.0	-64
5	C1	Used	0	0	0	0	0	0	-57
6	H4	New	34	43	34	23	134	33.5	-48
7	C4	Used	34	16	16	19	85	21.3	-51
8	C1.4	Used	111	85	148	76	420	105.0	-24
9	H3	New	71	92	57	63	283	70.8	-32
10	C5	Used	61	44	71	61	237	59.3	-52
11	C6	Used	6	4	2	5	17	4.3	-32
12	T6	New	53	96	91	81	321**	80.3**	-22

* Used = jar in current use; New = unused BTL jar, used for first time in this experiment.

** No secondary vacuum was observed in this jar after 10 min. and the catalyst was therefore replaced and the jar reprocessed.

All jars were processed according to the standard anaerobic procedure, and incubated in a single incubator at 37°C for 18 hr.

vacuum; on the other hand, jar C6 which was last to be processed, and which had to be re-processed because of an initially faulty catalyst, gave reasonably high counts. There seemed to be little association between the age of the jar and the numbers of colonies developed, although the least successful of the "new" jars had a higher mean colony count per plate than some of the "old" jars. Similarly, there was no correlation between the extent of the secondary vacuum developed after 10 min., and the colony counts observed; the jar showing the smallest secondary vacuum (jar C1. 4, with a secondary vacuum of - 24 mm Hg) gave high colony counts, whereas the jar showing the highest secondary vacuum (jar C 3, with a secondary vacuum of - 72 mm Hg) gave low colony counts.

It was decided to investigate the surface viable counts obtained from plates incubated in 10 BTL anaerobic jars, on three separate occasions, each jar to receive twelve plates, all seeded with identical inocula. On day 1, 120 plates of freshly poured CDEBA 10 medium were each seeded with 0.02 ml of a 10^{-1} dilution of an overnight CMB culture of Cl. oedematiens type-D. After the plates had been spread, twelve plates were randomly assigned to each jar, and the jars processed in the order shown. Each jar was equipped with a single room-temperature catalyst sachet, and all the jars were incubated at 37°C for 18 hr. On day 2, the procedure was repeated with freshly poured media and a fresh overnight parent CMB culture. The same 10 anaerobic jars were used, with the same catalyst sachets as on day 1, and the jars were processed in the same order. On day 3, the same procedure was followed except that a 10^{-1} dilution of a 51-hr CMB culture was used as inoculum. Total counts and spore counts were

performed on samples from the inocula used each day. The results are given in Table 66.

It is evident that large variations occur between the mean number of colonies per jar on a given day. Although differences in the total cell counts, spore counts and age of cultures used preclude quantitative comparisons of the colony counts observed on different days, it is nevertheless clear that a "good" jar (i.e. one giving a high mean colony count per plate) on one occasion, may be a "poor" jar on another occasion (i.e. give a low mean colony count per plate). For example, Jar C8, although giving comparatively high mean plate colony counts on days 1 and 2, performed badly on day 3 compared with most of the other jars.

Thus it seemed that the "jar variation" phenomenon was not a consistent feature of a given jar, but seemed to affect different jars to a different extent on different days. The possible causes of jar variation that were considered are listed in Table 67; it was decided to investigate these systematically.

The possible presence of variable factors associated with the processing of plates of solid media

It was found very difficult to design experiments in which all but one of the possible variables was kept constant; however, several could be dismissed. Checks on the size of inoculum used showed that the volume used was constant at 0.02 ml, and the possible volumetric errors involved were very small compared with the size of the variations observed. Similarly, all experiments on a given day were performed with plates from the same batch of medium, which in turn was derived from a "parent" concentrate; the latter was diluted, and agar

TABLE 66

The colony counts obtained from replicate plates seeded from CMB cultures of Cl. oedematiens type-D on 3 separate occasions; a fresh parent culture being used each day

Order of evacuation	Jar	DAY 1*		DAY 2**		DAY 3Δ	
		Total no. of colonies per jarΔΔ	Mean no. of colonies per plate	Total no. of colonies per jar	Mean no. of colonies per plate	Total no. of colonies per jar	Mean no. of colonies per plate
1	C 1	38	3.2	2208	184.0	66	5.5
2	C 2	196	16.3	8943	745.3	44	3.7
3	C 3	80	6.7	1958	163.2	89	7.4
4	C 4	13	1.1	437	36.4	98	8.2
5	C 5	300	25.0	6283	523.6	307	25.6
6	C 6	189	15.8	2801	233.4	74	6.2
7	C 7	2388	199.0	5472	456.0	657	54.8
8	C 8	1917	159.8	9046	753.1	68	5.7
9	C 9	149	12.4	2886	240.5	219	18.3
10	C10	1873	156.1	5153	429.4	235	19.6

* The total cell count was 1.8×10^7 per ml; the spore estimate was 7.8×10^4 per ml.

** The total cell count was 2.0×10^7 per ml; the spore estimate was 1.6×10^5 per ml.

Δ The total cell count was 8.4×10^6 per ml; the spore estimate was 5.5×10^5 per ml.

ΔΔ Each jar contained 12 replicate plates. Each plate was seeded with 0.02 ml of a 10^{-1} dilution of the parent overnight CMB culture, and incubated for 18 hr.

TABLE 67

Some possible factors contributing to the "jar variation" phenomenon

1. The inoculum

   Differing times of exposure of organisms in diluted samples to the possible toxic effects of the diluent.

   Effect of exposure of organisms in diluent to aerobic conditions.

   Exposure of seeded plates to aerobic conditions for varying periods of time.

   Variations in size of a standard inoculum (i.e. experimental error).

2. The medium

   Variation between batches of media.

   Toxic substances randomly dispersed in the media.

3. Factors associated with the BTL anaerobic jar

   The order of evacuation of the anaerobic jars.

   The position of plates within a given jar.

   The state of the catalyst in a given jar.

   The position of the catalyst in a given jar.

   Variations in anaerobic technique:
   - amount of carbon dioxide admitted to the gaseous environment.
   - degree of evacuation.

   Possible toxic substances in the jars.

   Variation in the temperature of incubation.

   Variation in the length of time of incubation.

added, for each experiment. The above factors would in any case be unlikely to operate in a constant manner for a given jar, but rather to have a more scattered effect. Similarly, any inhibitory substances present would be unlikely to affect all the plates in one anaerobic jar, and none in another, unless the material of the jar carried the inhibitor.

The standard anaerobic technique was performed with great care and attention to detail in all experiments; some variation may have occurred, but all experiments were performed under careful supervision, with the same team of workers on each occasion. All jars were put in the same incubator, and thermographic records were kept; the temperature remained constant at 37°C ± 0.5°C. Similarly, although some variations in temperature may have occurred at different positions within the incubator, it was noted that "jar variation" occurred between jars incubated on the same shelf of the incubator. All jars in a given experiment were removed from the incubator after the same period of time.

It seemed that one variable factor in the experiments was the length of time of exposure in the diluent. All experiments were performed on diluted samples of CMB cultures, and it followed that plates seeded early in a series would be exposed to the environment in the diluent for less time than those seeded later. The following experiment was designed to investigate this factor.

100 plates of freshly poured CDEBA 10 medium were allocated to 10 piles of 10. Each plate received a 0.02-ml volume of a 10^{-1} dilution of an overnight CMB culture of Cl. oedematiens type-D. Groups of 20 plates were seeded and spread at time "0", and thereafter at 10, 20,

40 and 60 minutes. The dilution used to seed the plates was mixed by inversion at each sampling time, and each group of 20 seeded plates was subdivided between 2 BTL anaerobic jars, which were immediately processed by the standard procedure. Each jar was equipped with a single room temperature catalyst sachet. Thus the inoculum used to seed the plates was held in the diluent for varying periods of time. The results are shown in Table 68 . It is clear that there is a fall in the observed viable counts with increasing time in the diluent, although such findings should be interpreted with care in view of the "jar variation" phenomenon.

Similar experiments were designed to determine whether exposure of seeded plates to aerobic conditions resulted in decreased recovery. The results of these studies, reported on p.179 , indicated that in the case of Cl. oedematiens type-D strains, inactivation of vegetative cells did occur when seeded plates were exposed to aerobic conditions, even for short periods of time.

The possible presence of inhibitory factors in the environment of the jar

It was considered possible that inhibitory substances might be present in the gaseous environment of jars and that these might affect the growth of organisms in the jars to a variable extent. Possible factors considered included mercury (from the manometer); hydrogen sulphide produced by the colonies, or by other cultures incubated in the same jar; water vapour; suspensions of metallic salts (notably copper sulphate) from the inside surfaces of the jar; and the effects of incubating seeded plates in the presence of solid or liquid cultures of other anaerobic organisms. Careful and prolonged testing

TABLE 68

The recovery of Cl. oedematiens type-D on solid media after varying times of explosure in a diluent

Order of evacuation	Jar	Time in diluent (min)	Total no. of colonies per jar	Mean colony count per plate ± S.E.*
1	C 1	0	4965	496.5 ± 29.6
2	C 2		8095	809.5 ± 33.4
3	C 3	10	960	96.0 ± 9.4
4	C 4		1338	133.8 ± 9.0
5	C 5	20	706	70.6 ± 5.6
6	C 6		562	56.2 ± 5.8
7	C 7	40	120	12.0 ± 1.9
8	C 8		65	6.5 ± 1.4
9	C 9	60	NG**	—
10	C10		29	2.9 ± 0.6

* Each colony count expressed as the mean ± S.E. (standard error of the mean) of ten replicate plates of CDEBA medium, each seeded with 0.02 ml of a 10^{-1} dilution of an overnight CMB culture of strain GRID, and incubated for 18 hr.

** NG = no growth.

failed to reveal any variable effects on colony counts due to the above factors; for example, when H_2S (produced by the reaction of ferrous sulphide with hydrochloric acid) was admitted to the test jars in concentrations up to 1 per cent., no effect was observed on the recovery of Cl. oedematiens type-D strains, as compared with that in control jars. In other studies, dishes of copper sulphate were placed in jars and incubated with the plates, or strips of paper soaked with a solution of copper sulphate were placed around the internal surface of the test jars, which were then filled with plates and incubated anaerobically for 48 hr. No consistent effect on the recovery of type D strains was observed in jars containing copper sulphate, as compared with control jars. The increased recovery, observed on one occasion in one jar so equipped, could not be repeated in any of several replicate experiments, and was considered to be due to some other factor in the test jar. Colony counts did not seem to be affected by the growth of other organisms in the same anaerobic jar. Although large amounts of water vapour led to some spreading growth, no effect on the magnitude of the colony counts could be ascertained. The author bears in mind that these studies were difficult to control, and conclusions drawn are only on the basis of semi-quantitative data; nevertheless, no clear-cut variables were found.

Variables associated with catalyst sachets

In addition to the factors already investigated, possible variations in the performance of the catalyst were tested in a series of experiments. However, the presence of the "jar variation" phenomenon made any quantitative studies impossible to analyse. It

seemed that "old" catalysts (i.e. catalysts already used several times, but still showing satisfactory activity as judged by the prompt production of a secondary vacuum, performed less well than "new" catalysts (previously unused); in other words greater mean plate counts were obtained with jars equipped with a "new" catalyst than in those equipped with an "old" one. Pilot studies carried out to determine whether the presence of multiple catalysts in the BTL jars increased the surface viable counts obtained indicated that there was some correlation between the counts obtained and the numbers of catalysts present, although it was impossible to ensure that observed differences between jars equipped with differing numbers of catalysts were not due to the "jar variation" phenomenon.

Although accurate quantitative studies were not carried out, preliminary results suggested that in addition to the variables mentioned above, the position of plates within a given jar should be considered as a possible variable factor, and taken account of in experimental design.

At this point in the work, it became necessary to seek statistical advice, in order to control some of the variables involved, so that a fuller investigation of the "jar variation" phenomenon could be carried out. The author is greatly indebted to Miss M. V. Hoare for her co-operation in the design of experiments and the analysis of results.

The influence of the number of catalyst sachets in BTL jars on the surface growth of Cl. oedematiens type D

In this experiment, six BTL anaerobic jars were used on three separate occasions. Each jar was equipped with 1, 3 or 5 new catalyst sachets on Day 1, and these were re-distributed on Day 2 and Day 3, so that the 6 jars were equipped as shown in Table 69; i.e. during the experiment, each jar had all the possible permutations of numbers of catalysts. On Day 1, 60 plates of CDEBA medium were poured, dried and labelled 1-60. Plates 1-30 were seeded from a 10^{-2} dilution of an overnight culture of a type-D strain, each plate being seeded and spread in the order 1-30, the same dropping pipette being used throughout. The plates were then randomly allocated to one of three anaerobic jars by means of random number tables. The random distribution of plates within a given jar was then performed, again by means of random number tables, and the jars were then processed in a predetermined random order. The process was then repeated for plates 31-60. All jars were incubated for 18 hr at 37°C.

On Days 2 and 3, the process was repeated, a 10^{-2} dilution of a fresh overnight CMB culture of the same type-D strain being used to seed the plates on each occasion. Although fresh plates were prepared on each day (a possible source of variation), all the medium was prepared from a concentrate, which was diluted for use on each day, and a supplement of cysteine and dithiothreitol was freshly prepared on each of the 3 days. Thus as far as possible, the likely variables were controlled, so that differences between jars due to varying numbers of catalysts could be investigated by statistical analysis of the results. The full experimental design for this

TABLE 69

Experimental design:
The distribution of catalyst sachets between six BTL anaerobic jars on three separate occasions

	No. of catalysts in stated jar on		
Jar	Day 1	Day 2	Day 3
C 2	3	1	5
C 4	1	5	3
C 6	5	3	1
C 7	1	3	5
C 9	5	1	3
C10	3	5	1

experiment is given in Appendix B.

In order to record the results satisfactorily, so as to check that the experimental design had been followed, a special record sheet (shown in Appendix C) was constructed, and this allowed recording of each individual plate colony count as well as the number of the plate (i.e. the order in which it was inoculated and spread) and its position within a given anaerobic jar. The full results are given in Appendix E , a shortened version in Table 70.

The data indicate that higher colony counts are obtained in jars equipped with 3 or 5 catalysts, than in those equipped with only one catalyst. Inspection of the data also suggests that although there is a considerable "day-to-day" variation in the colony counts, there is little evidence of the "jar variation" phenomenon in this experiment. The results were then analysed statistically, by an Analysis of variance (Appendix E), to determine (i) whether the number of catalysts had a significant effect on the colony counts (Analysis A), and (ii) whether the number of catalysts affected the variability of colony counts within a given jar (Analysis B).

In Analysis A, the amount of variability due to different days, different jars, and to differing numbers of catalysts was measured. As the data are of Poisson type, the variance was stabilized by taking the square roots of the original colony counts. The detailed analysis is given in Appendix E , and an explanation of the statistical methods employed is given in Appendix D ; a summary of the results is given in Table 70 . The results show that although very large differences are observed between the colony counts on different days, the differences between jars are not significant in this

TABLE 70

The mean colony counts per jar, in BTL anaerobic jars equipped with varying numbers of catalyst sachets, on three occasions, using Cl. oedematiens type-D as the test organism

Jar	Mean colony count* per jar on		
	DAY 1	DAY 2	DAY 3
C 2	384.3 (3)**	1046.2 (1)	289.7△ (5)
C 4	170.0 (1)	1340.9 (5)	252.5 (3)
C 6	391.6 (5)	1083.8 (3)	123.1 (1)
C 7	313.3 (1)	1001.0 (3)	270.9△ (5)
C 9	328.8 (5)	784.3△(1)	286.1 (3)
C10	348.0 (3)	1126.5 (5)	94.1 (1)

* Colony counts expressed as the mean of ten replicate plates of CDEBA medium each seeded with 0.02 ml of a 10^{-2} dilution of an overnight CMB culture of strain GRID, and incubated for 18 hr. A fresh parent CMB culture was used on each day.

** Figures in parentheses refer to number of catalysts in the jar on the stated day.

△ One plate in the jar showed spreading growth, and figure given is therefore the mean of nine plate counts.

experiment. The difference between catalysts is statistically significant ($P < 0.01$): in other words, the presence of 3 or 5 catalysts in an anaerobic jar does produce higher counts than if only one catalyst is present.

In Analysis B, the effect of the number of catalysts on the variability of colony counts within a given jar was investigated. In this Analysis, the variances of the square roots of the original counts within each jar have been calculated, and the logarithm of each taken to stabilize the variance. It is clear that there was no significant effect of the number of catalysts within a jar on the variability of the plates within that jar, i.e. given that the averages of the counts for different numbers of catalysts are different, the relative variability of the counts on the plates is not significantly influenced by the number of catalysts present.

The results show that higher counts are obtained if anaerobic jars are equipped with 3 or more catalyst sachets; they also show the need for careful experimental design when quantitative studies are performed with this model, and the unreliability of comparing quantitative data on different occasions is evident. It seemed that by randomising the distribution of plates between and within jars, useful, statistically valid comparisons could be made. The absence of significant jar variation in this experiment was puzzling, although it may have been excluded by the experimental design.

A series of similar experiments was then performed to determine whether the presence of more than one catalyst increased the counts of the test organism, or affected the variability of plates seeded with the test organism, within a given jar. Although exhaustive

tests with less sophisticated experimental design had failed to reveal any "jar variation" phenomena except in the case of organisms of the Cl. oedematiens group, it was possible from these more elaborate experiments to show whether any significant degree of "between jar" variation existed. The test organisms used were: Cl. tetani type VI (NCTC 9569); B. fragilis (NCTC 9343); Cl. oedematiens type A (GR1A); and an Anaerobic coccus Group I (NCTC 9801). In addition, the influence of multiple catalysts on the recovery of anaerobic organisms from faecal samples was also investigated in a similar experiment. Each experiment was designed as shown for Cl. oedematiens type-D (appendix A) with different randomisation schedules for each organism, and the test jars were equipped with new catalysts for each 3-day experiment. The results were subjected to analyses of variance similar to those for Cl. oedematiens type-D. The results and analyses are given in Appendix E, and they are discussed in the following section.

The influence of multiple catalyst sachets in BTL anaerobic jars on the recovery of test anaerobes

Analysis of the results from the above experiments showed that for Cl. tetani type VI, B. fragilis, and the anaerobic coccus, no statistically significant differences were observed in the colony counts obtained in jars with differing numbers of catalysts; i.e. increased numbers of catalysts in a given anaerobic jar did not significantly affect the magnitude of the colony counts in that jar. Similarly the numbers of catalysts present in a jar did not significantly affect the variability of the plate counts within the jar.

In the case of the type-A strain of Cl. oedematiens, the results summarised in Table 71 when analysed (p. 335) show that the number of catalysts significantly affects the magnitude of the colony counts ($p < 0.01$), although the average colony counts obtained in jars equipped with 3 catalysts, while significantly higher than those obtained from jars equipped with 1 catalyst, were slightly higher than in those with 5 catalysts. Thus, while having 3 or more catalysts in an anaerobic jar increased the recovery of this organism, there is no evidence that the recovery is further increased by more than 3 catalysts per jar.

The "B" analysis shows that the influence of number of catalysts on the variability of plates within a given jar is not quite significant at the 5 per cent. level, although this again may be due to the greater variability observed between plates in jars equipped with 3 catalysts.

With all the test organisms, the "between jar" variation was not significant at the 5 per cent. level, and this is consistent with the results of preliminary tests. Since these experiments were performed, many hundreds of anaerobic jars have been processed with different test organisms; with the exception of type-D and type-B strains of Cl. oedematiens, no evidence of jar variation has been found.

The influence of multiple catalyst sachets in BTL anaerobic jars on the recovery of anaerobic organisms from faeces

The recovery of anaerobes from faeces was investigated using the experimental design shown in Appendix B, but with different

TABLE 71

The mean colony counts per jar, in BTL anaerobic jars equipped with varying numbers of catalyst sachets, on three occasions using Cl. oedematiens type A as the test organism

Jar	Mean colony count* per jar on		
	DAY 1	DAY 2	DAY 3
C 4	177.3 (1)**	189.3 (5)	275.5$^{\Delta}$ (3)
C 2	220.8 (5)	221.6 (3)	234.4$^{\Delta\Delta}$(1)
C10	220.1 (3)	172.9 (1)	267.9 (5)
C 9	188.8 (5)	192.8 (1)	243.4$^{\Delta}$ (3)
C 6	169.8 (1)	187.9 (3)	235.6$^{\Delta}$ (5)
C 8	221.5 (3)	226.8 (5)	274.0$^{\Delta}$ (1)

* Colony counts expressed as the mean of ten replicate plates of CDEBA medium, each seeded with 0.02 ml of a 10^{-2} dilution of the parent overnight CMB culture. A fresh parent culture was used on each day.

** Figures in parentheses refer to number of catalysts in the jar on the stated day.

Δ One plate in the jar showed spreading growth; the figure given is therefore the mean of nine plate counts.

$\Delta\Delta$ Two plates showed spreading growth; the figure given is therefore the mean of eight plate counts.

The seeded plates were incubated for 18 hr.

randomisation schedules. All jars were equipped with new catalyst sachets before the beginning of the experiment. In order to ensure consistency of the inoculum, aliquots of a freeze-dried faecal suspension were used, as it had already been shown that no appreciable diminution in the total anaerobic counts occurred on holding at -30°C (see Table 27). A parallel series of plates, incubated aerobically, showed that at the dilution used, aerobic organisms contributed to less than 1 per cent. of the colony counts and the colony counts were therefore taken as an index of the total anaerobic counts. The results and analysis are shown in full in Appendix E, and the results are summarised in Table 72. There was no significant difference between the colony counts in jars with different numbers of catalysts. Similarly, the number of catalysts in a given jar did not have any significant influence on the variability of the plates within that jar. No evidence of significant jar variation was observed.

These negative findings are important as a basis for further quantitative studies with faecal samples; the BTL anaerobic jar can be used with confidence for such studies, provided that the precautions outlined on page 283 are taken.

Measures to reduce the variables in anaerobic procedure

Although there was no evidence to suggest that appreciable difference in the proportions of carbon dioxide and hydrogen in different jars occurred when the standard anaerobic procedure was used, the separate addition of these two gases to the anaerobic jars was a possible source of variation. It was therefore decided to use a single cylinder containing a mixture of 90 per cent. hydrogen and 10 per cent. carbon dioxide (British Oxygen Company Ltd.), so that the gaseous

TABLE 72

The recovery of anaerobic organisms from deep-frozen aliquots of a sample suspension of human faeces by culture of dilutions on plates in BTL anaerobic jars equipped with varying numbers of catalyst sachets, on 3 occasions

Jar	Mean colony count* per jar on		
	DAY 1	DAY 2	DAY 3
C 2	469.1$^{\Delta}$ (3)	419.2 (1)	175.6 (5)
C10	417.6 (1)	368.2$^{\Delta}$(5)	193.0 (3)
C 5	408.7$^{\Delta}$ (5)	357.0 (3)	421.7 (1)
C 9	568.1 (5)	344.1 (1)	378.0 (3)
C 3	478.9$^{\Delta\Delta}$(3)	392.8$^{\Delta}$(5)	401.9 (1)
C 1	482.8$^{\Delta}$ (1)	378.0 (3)	432.4 (5)

* Colony counts expressed as the mean of ten replicate plates of CDEBA medium each seeded with 0.02 ml of a 10^{-4} dilution of the parent aliquot and incubated for 72 hr. A fresh aliquot was used on each day.

** Figures in parentheses refer to number of catalysts in the jar on the stated day.

Δ One plate in the jar showed spreading growth, and figure given is therefore the mean of nine plate counts.

$\Delta\Delta$ Two plates showed spreading growth and figure given is therefore the mean of eight plate counts.

environment in anaerobic jars would be standardised.

All jars were regularly cleaned and checked for leaks, and all needle valves were regularly lubricated.

A REVISED ANAEROBIC PROCEDURE

The studies described above indicated that the standard procedure outlined on page 81 could be improved in several ways:

(i) By the provision of a 90 per cent. hydrogen/10 per cent. carbon dioxide mixture in a single cylinder, to avoid variations in the relative proportions of the two gases in anaerobic jars.

(ii) By the inclusion of a flushing stage in the procedure.

(iii) By fitting all anaerobic jars with multiple catalyst sachets.

Such improvements required a reassessment of the degree of the secondary vacuum (p. 79) that would be acceptable as an index of catalytic activity, as a decreased amount of residual oxygen would remain after a flushing procedure, and this could be expected to lead to the development of a smaller secondary vacuum, even in the presence of a fully functional catalyst. This was confirmed in a series of studies in which six jars, each filled with 10 plates, were processed according to the standard procedure, but with the addition of a flushing stage as detailed on page 175. The secondary vacuums developed after 5, 10 and 15 min. at room temperature on the bench were determined with a simple mercury manometer; each jar was equipped with a single unused catalyst sachet.

The mean negative pressures observed were: - 5 mm Hg after 5 min; - 9 mm Hg after 10 min; and -15 mm Hg after 15 min. The experiments were then repeated on this occasion with each jar equipped with three unused catalyst sachets. The mean negative pressures observed were: - 12 mm Hg after 5 min; - 17 mm Hg after 10 min;

and - 20 mm Hg after 15 min (pressures expressed as mm Hg below atmospheric pressure). Thus it was decided that the development of a secondary vacuum of at least 15 mm Hg after 10 min would be adopted as a satisfactory index of catalytic activity in jars fitted with three catalyst sachets and processed with an additional flushing stage.

The revised anaerobic jar procedure formulated for the BTL anaerobic jar is as follows:-

(1) All jars should be equipped with three functional catalyst sachets.

(2) Connect inlet H (closed) to pump and V (open) to gauge.

(3) Start pump and slowly open inlet H.

(4) Evacuate to -660 mm Hg (25 inches Hg).

(5) Close inlet H; disconnect pump line; stop pump.

(6) Connect a bladder filled with a mixture of 90 per cent. hydrogen and 10 per cent. carbon dioxide to inlet H, and run in until gauge registers zero.

(7) Repeat steps (2) to (5).

(8) Close both inlets; leave jar on bench.

After 10 min: check with manometer that a secondary vacuum of at least 20 mm Hg has been produced. Admit more of the H_2:CO_2 mixture to equilibrate the pressure.

(9) Close both inlets. Disconnect bladder. Check that gas cylinder is closed.

(10) Incubate jar.

STUDIES WITH AN ANAEROBIC CABINET

The development of an improved anaerobic procedure, and the evident success of recovering demanding anaerobes quantitatively on solid media, led to a series of studies in which the recovery of demanding anaerobes from pure cultures and from faecal samples was investigated when all manipulations were performed in an anaerobic cabinet, and the results compared with those obtained with the conventional anaerobic techniques already described.

The design and construction of an anaerobic cabinet

In the latter part of this study, funds became available to allow the construction of an anaerobic cabinet. After preliminary discussions with Dr B. S. Drasar, it was decided to construct a cabinet that would combine simplicity and ease of operation with the provision of an anaerobic environment.

The basic cabinet consisted of a glove box (Model IIIc, Lintott Engineering, Horsham, Sussex), constructed from 10 s.w.g. steel, and fitted with a posting port at one side. The apparatus is shown in Figures 12 to 16 . There was a large transparent viewing panel (of 6 mm Perspex sheeting) at the front of the cabinet, underneath which were two glove ports. "Neoprene" gloves were attached to the ports. When the gloves were not in use, the ports could be closed by gas-tight seals. The posting port was connected to the cabinet by a transfer port, and both had seals so that the port could function as an air-lock between the cabinet and the external environment. This air-lock was fitted with a needle valve to allow evacuation and flushing (see below).

FIGURE 12. - Side view, showing posting port and modified BTL jar.

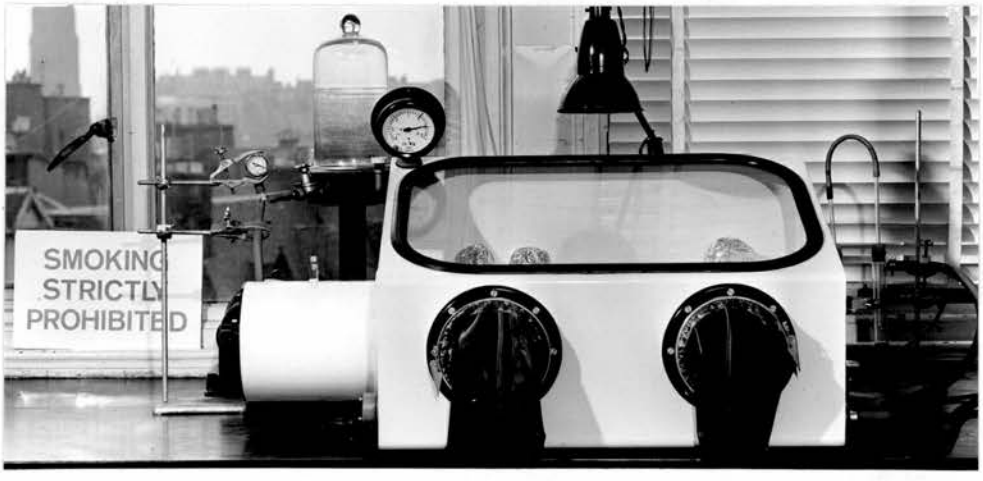


FIGURE 13. - Front view of cabinet.

FIGURE 14. - The cabinet seen from above. Tins containing spreaders and pipettes are suspended from the gas inlet pipe within easy reach.

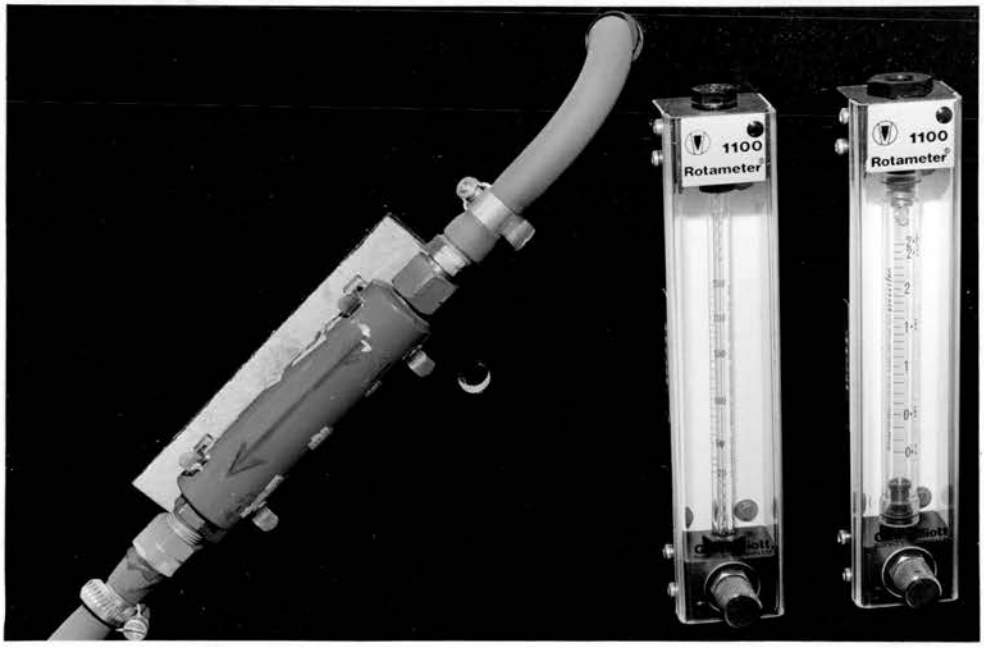


FIGURE 15. - Close up view of catalytic purifier and flowmeters.

FIGURE 16. - The anaerobic cabinet in operation (Mr. R. Brown).

The dimensions of the base of the cabinet were 60.5 cm x 45 cm, and the maximum height of the cabinet was 45 cm. The volume of the cabinet was 110 litres, and that of the airlock 5.7 litres.

A mixture of 3 per cent. hydrogen in 97 per cent. nitrogen (British Oxygen Company, Special Gases Division) was allowed to mix with carbon dioxide (Distillers Company), and the final mixture was admitted to the cabinet through a nozzle in the wall, after being rendered oxygen-free by passage through a D5/50 catalytic purifier (Engelhard Industries Ltd., Gloucestershire) as suggested by Latham and Sharpe (1971). The inner part of the nozzle was continuous with a length of 4.5 mm bore copper tubing, perforated at intervals, and extending along two sides of the cabinet, so that there was adequate mixing of the gases in the cabinet. The flow of gases was monitored by calibrated flowmeters (Rotameter Manufacturing Co. Ltd., Purley Way, Croydon), and the pressure inside the cabinet was monitored by a sensitive water manometer fitted to the cabinet wall. The exhaust or outlet gases passed through a nozzle to an adjustable pressure release valve, and were then filtered through cotton-wool plugs before reaching the outside environment. All gas connections were made with special 5/16 gas-tight tubing ("Saffire" hose No. 153478, British Oxygen Company) and all joints were sealed with Araldite, in addition to being secured by jubilee clips.

The setting-up and operation of the cabinet

The cabinet had been tested by the manufacturers for leaks (certificate no. RAL/PR/5679). In the laboratory, it was thoroughly cleansed with soap and water and dried. A large polythene bag was

inflated in the cabinet with the nitrogen/hydrogen mixture, so that the bag filled the cabinet and airlock, and displaced most of the air from the cabinet. The outer airlock seal was then closed and the bag was collapsed in the cabinet by manipulation with the gloves; the cabinet was then flushed continuously with the nitrogen/hydrogen mixture for 72 hr at a rate of 0.4 - 0.5 litres per min. After this time, catalyst sachets were cautiously introduced through the airlock (see below) and distributed on the floor of the cabinet in glass Petri dishes, the flow of gas being maintained for a further 24 hr. The gas flow was then stopped, and the system checked for leaks over periods of several hours; no leaks were detected, and the cabinet was then considered ready for use. During the use of the cabinet, eight to ten catalyst sachets were always present in the cabinet, and a further three were placed in the airlock. The sachets were replaced every 2-3 weeks.

During an experiment, the cabinet was flushed continuously with the hydrogen/nitrogen mixture (0.3 - 0.4 l per min.) and carbon dioxide (30 - 40 ml per min.); i.e. a carbon dioxide concentration of 10 per cent. At all other times, the flow rates were reduced to 50 - 100 ml per min. and 5 - 10 ml per min. respectively. The pressure release system was arranged so that the cabinet was maintained at a constant positive pressure of 2.5 cm Wg, irrespective of the gas flow rates through the system.

The procedure developed for passage of materials through the airlock was as follows:-

After careful closure of the inner and outer seals, the airlock was evacuated to - 200 mm Hg then filled with the nitrogen/

hydrogen mixture until the pressure returned to atmospheric. This process was repeated a further four times, but on the last occasion a mixture of 90 per cent. hydrogen and 10 per cent. carbon dioxide was used to equilibrate the pressure, to ensure that there was an excess of hydrogen present. The airlock was then left for at least 1 hr, hydrogen being admitted from time to time to counteract the secondary vacuum produced by the catalytic combination of the hydrogen in the airlock with any residual oxygen. After 1 hr, the airlock could be opened and materials transferred into the cabinet.

A BTL anaerobic jar was modified (Dr J. G. Collee) so that it would fit into the airlock; the jar can be seen in Figure 12. Spreaders and pipettes were as described previously, but the spreaders were carefully heated to remove any sharp edges that could cut the Neoprene gloves. Spreaders and pipettes were stored in the cabinet in foil-covered tins clipped to the copper gas entry pipe; they could be easily removed for re-sterilisation when required.

The size of the cabinet precluded the pouring of plates within it, and all media and diluents were therefore stored anaerobically for at least 24 hr before use - media and diluents were normally stored in the cabinet, the darkening of the media attesting to the reducing environment. On occasion, this storage was preceded by storage for 48 hr in BTL anaerobic jars under anaerobic conditions on the bench, to remove most of the dissolved oxygen and minimise the amount liberated from plates in the cabinet.

As mentioned above, careful testing revealed no leakages; the cabinet could maintain a positive or negative pressure satisfactorily for long periods of time. In early experiments, some moisture was

produced from the catalysts, but once the airlock procedure was standardised, no evidence of moisture formation was seen, and this indicated that no significant leakage of oxygen was occurring.

Tests to determine the concentration of oxygen in the cabinet by means of an electrometric method indicated that the level was <1 per cent., but as a suitable instrument for monitoring levels of oxygen below 1 per cent. was not available, it was decided to use biological indicators of anaerobiosis. Plates were seeded inside the cabinet from overnight CMB cultures of Cl. tetani type VI and Cl. oedematiens type D and, still within the cabinet, the plates were then placed in the modified BTL jar described above; on this occasion, however the normal catalyst sachets had been removed and the jar contained the atmosphere of the cabinet - i.e. the jar was incubated at 37°C for 72 hours without further processing. Good growth of Cl. tetani (checked by toxigenicity tests in mice) was obtained on EBA and CDEBA plates, and growth of Cl. oedematiens type D (checked by an immunofluorescence method, see Materials and Methods) was also obtained on both media. Thus the atmosphere within the cabinet was sufficiently anaerobic to support the growth of very demanding anaerobes on simple blood agar media.

Experimental Results

Preliminary studies revealed that the recovery of demanding anaerobes such as Cl. tetani and Cl. oedematiens type D from CMB cultures was not qualitatively improved when dilutions were made and plates were seeded inside the cabinet. However, it was necessary to check these findings quantitatively in the following series of

experiments:

Dilutions of an overnight CMB culture of the test organism were made inside the cabinet, and anaerobically-stored plates of EBA medium (or CDEBA medium in the case of Cl. oedematiens type D) were seeded with 0.02-ml inocula from a dilution known to give 100-700 colonies per plate. Nine plates were seeded, placed in the modified BTL jar (see above) and the jar, together with the dilutions was passed through the airlock to the exterior. The dilutions prepared in the cabinet were then used to seed another set of anaerobically stored plates at the bench. In addition, a similar series of plates was seeded at the bench from replicate dilutions prepared under aerobic conditions. To minimise errors due to dilution, the pre-set constant volume pipette used for the preparation of dilutions in the cabinet was passed out through the airlock and used for the preparation of dilutions under aerobic conditions at the bench. All plates seeded at the bench were then randomly distributed between four BTL anaerobic jars. These jars, with the jar from the cabinet, were then processed according to the procedure described on page 246 and incubated at 37°C for 18 hr (Cl. oedematiens type D) or 48 hr (Cl. tetani).

The results (Tables 73 and 74), show that no increase in recovery was obtained when the anaerobic cabinet was used for the preparation of dilutions; even when both preparation of dilutions and seeding of plates were performed within the cabinet, the recovery was not improved in comparison with that obtained by standard anaerobic procedures at the bench. In the case of Cl. oedematiens type D, the most exacting model, complete recovery was obtained when

TABLE 73

The effect of different modes of processing of the inoculum and plates on the recovery of Cl. oedematiens type D, strain GR1D, on EBA medium from an overnight CMB culture

Dilution	Mean surface viable count per ml ± S.E. derived from samples processed according to mode**		
	A	B	C
10^{-2}	$(6.6\pm0.1)\times10^{6}$	$(6.7\pm0.1)\times10^{6}$	$(6.7\pm0.2)\times 10^{6}$

* The total cell count was 6.0×10^{6} per ml; the spore count was $<6.0 \times 10^{4}$ per ml.

** A - dilutions prepared and plates processed within the anaerobic cabinet. Surface viable count expressed as mean ± S.E. (standard error of the mean) derived from nine replicate plates, each seeded with 0.02-ml inocula of the test dilution.

B - dilutions prepared within the cabinet were used to seed plates under aerobic conditions at the bench. Surface viable count expressed as mean ± S.E. (standard error of the mean) derived from 10 replicate plates, seeded as in A.

C - dilutions prepared, and plates processed under aerobic conditions at bench. Surface viable count expressed as mean ± S.E. (standard error of the mean) derived from six replicate plates seeded as in A.

All manipulations were performed at room temperature (18.5°C).

TABLE 74

The effect of different modes of processing of the inoculum and plates on the recovery of Cl. tetani type VI on EBA medium from an overnight CMB culture

Dilution	Mean colony count ± S.E. derived from samples processed according to mode		
	A*	B**	C△
$1/5 \times 10^{-3}$	179.3 ± 7.9	190.6 ± 5.4	183.0 ± 13.1

* A - dilutions prepared, and plates processed within the anaerobic cabinet. Colony count expressed as mean ± S.E. (standard error of the mean) of counts from nine replicate plates, each seeded with 0.02-ml inocula of the stated dilution.

** B - dilutions made within the cabinet were used to seed plates under aerobic conditions at the bench. Colony count expressed as the mean ± S.E. (standard error of the mean) of seven replicate plates, each seeded as in A.

△ C - dilutions were prepared, and plates processed under aerobic conditions at the bench. Colony count expressed as the mean ± S.E. (standard error of the mean) of seven replicate plates, each seeded as in A.

All manipulations were performed at room temperature (19.0°C).

all manipulations were performed under "aerobic conditions" at the bench (see Table 73).

Thus there was no evidence that the use of an anaerobic cabinet increased the recovery of demanding anaerobes on solid media. It could be argued, however, that pure cultures of stock anaerobes are not representative of fresh isolates; faecal samples were therefore used to give wild strains of strict anaerobes. Comparative studies were now done with these samples to assess the importance of an entirely anaerobic manipulation in their recovery. Recovery of strict anaerobes from human faecal samples on plates seeded inside the cabinet was compared with that from plates seeded under normal aerobic conditions at the bench. Over the course of some weeks, 12 freshly-passed samples of faeces were obtained from volunteers, and each sample was processed as follows:

A weighed portion was added to pre-steamed nutrient broth in a sterile beaker on the bench to give a dilution of 1 g of faeces in 100 ml broth, but the faeces was not homogenised at this stage. The beaker and contents were then sealed in the airlock, and after completion of the flushing procedure (see above), were admitted into the cabinet. Homogenisation was then achieved with a glass rod, and 10^{-4} and 10^{-5} dilutions of the suspension were made in pre-steamed nutrient broth. 0.02-ml inocula were seeded on to five plates from the 10^{-5} dilution and four from the 10^{-4} dilution. The plates (of Reinforced Clostridial Medium + 10 per cent. horse blood, as described by Drasar and Crowther, 1971) had been stored in the cabinet for at least 24 hr before use. The plates were placed in the modified BTL jar, which was then closed and passed, together with the

dilutions, through the airlock to the exterior. The dilutions were then used to seed a further series of plates of the same medium, which were placed in a BTL jar, and both jars were then processed according to the procedure described on page 246, and incubated at 37°C for 72 hr. Duplicate plates of EBA medium were seeded with 0.02-ml inocula from the 10^{-4} and 10^{-5} dilutions, and incubated aerobically for 48 hr.

From all samples, the counts of colonies on aerobically incubated plates were 1 per cent. or less of the counts on anaerobically incubated plates, and the counts from the anaerobic plates were therefore taken to represent the "total anaerobic counts" (page 130). The majority of organisms on anaerobic plates were morphologically typical of Bacteroides spp., although some organisms typical of lactobacilli were also seen.

The results given in Table 75 show that identical recovery is obtained, whether the specimens were processed in the cabinet or by standard anaerobic procedures at the bench. The results obtained compare favourably with those obtained in a previous survey, performed in Edinburgh in 1970 (B. S. Drasar, unpublished details), in which several of the volunteers in the present study participated. The results obtained on that occasion were derived from samples processed entirely within an anaerobic cabinet (Drasar and Crowther, 1971); the mean total anaerobic count (± S.E.) obtained from 15 samples was $(2.1 \pm 0.2) \times 10^{10}$ organisms per ml. Thus the counts obtained were in fact slightly lower than the counts in the present study.

In a further series of experiments, the recovery from faecal

TABLE 75

The comparative recovery from a series of 12 faecal samples processed within an anaerobic cabinet and on the bench

Dilution	Mean surface viable counts of total anaerobes ± S.E.* derived from samples processed	
	Within the anaerobic** cabinet	On the bench△
10^{-7}	$(2.4 \pm 0.4) \times 10^{10}$	$(2.4 \pm 0.3) \times 10^{10}$

* Expressed as the mean ± S.E. (standard error of the mean) of the results of experiments with 12 different samples of human faeces.

** Dilutions were prepared, and plates were processed within the anaerobic cabinet.

△ Plates were seeded under aerobic conditions at the bench from dilutions made within the cabinet.

All manipulations were performed at room temperature (18.8°C).

samples was compared on:

(i) plates seeded inside the cabinet, with dilutions also made inside the cabinet.

(ii) plates seeded under aerobic conditions at the bench from the dilutions prepared in (i).

(iii) plates seeded on the bench from dilutions of the faecal suspension prepared outside the cabinet.

To minimise dilution errors, the same pre-set constant-volume pipette used for the preparation of the dilutions in (i) was passed out of the cabinet via the airlock, and used for the preparation of the dilutions in (iii).

The results (Table 76) again demonstrate that no significant increase in recovery is obtained when the anaerobic cabinet is used for the preparation of dilutions or the seeding of plates; equally good recovery is obtained by conventional anaerobic procedures at the bench.

These experiments have demonstrated no benefit in terms of quantitative recovery of anaerobes when an anaerobic cabinet with a demonstrably anaerobic environment is used in place of the careful techniques developed for use at the bench in the present study.

TABLE 76

The effect of different modes of processing of inocula and plates on the recovery of total anaerobes from faecal samples

Dilution	Mean surface viable counts of total anaerobes ± S.E.* derived from samples processed according to mode**		
	A	B	C
10^{-7}	$(2.6 \pm 0.7) \times 10^{7}$	$(2.4 \pm 0.5) \times 10^{10}$	$(2.5 \pm 0.7) \times 10^{10}$

* Expressed as the mean ± S.E. (standard error of the mean) of the results of six experiments, with six different samples of human faeces.

** A - Dilutions prepared, and plates processed within the anaerobic cabinet.

B - Dilutions made within the cabinet were used to seed plates under aerobic conditions at the bench.

C - Dilutions were prepared, and plates processed under aerobic conditions at the bench.

All manipulations performed at room temperature (18.5°C).

DISCUSSION

Bacterial anaerobiosis has long presented a formidable challenge to microbiologists; difficult to understand and beset with practical problems, it remains an enigma. Although many of the associated phenomena have been described, and some practical methods of achieving anaerobiosis in the laboratory have been developed, there is still much to be learned. Even to formulate a satisfactory definition is difficult, as new observations may necessitate the revision of traditional concepts. For example, a recent study (O'Brien and Morris, 1971) suggests that in the case of Clostridium acetobutylicum, an obligate anaerobe, growth can be sustained even if the E_h of the culture is maintained at high (positive) levels, provided that the concentration of oxygen is kept low. These authors refer to the possibility raised by earlier workers (Wimpenny, 1969; Morris, 1970) that the inhibitory effects of oxygen on strict anaerobes may relate to the inability of these organisms to maintain sufficient concentrations of primary electron donors such as NADPH; they suggest that such donors would be consumed in the "energetically unrewarding task of detoxifying oxygen".

Theories of anaerobiosis that ascribe a central role to the direct or indirect toxicity of oxygen must also take account of the fact that some anaerobes can be shown to consume oxygen (Mallin and Seeley, 1958; Bullen, Cushnie and Stoner, 1966). Bullen and his colleagues considered that oxygen consumption by anaerobes in infected tissues would create an extremely reduced local environment and that this might be an important factor in the pathogenesis of fatal infections caused by Cl. welchii. The therapeutic use of

hyperbaric oxygen may reverse this process, and restore the balance of oxygen in the tissues. Whilst it is tempting to assume that oxygen exerts specific toxic effects on anaerobic bacteria, it must be borne in mind that a wide range of aerobic organisms can also be poisoned by hyperbaric oxygen (see Gottlieb, 1971). Thus, Bullen's theories concern the host-parasite association, whereas the direct toxic effects that may occur with hyperbaric oxygen require to be explained in relation to aerobes and anaerobes at the cell level. Any theory that ascribes a primary toxic role to atmospheric oxygen in bacterial anaerobiosis must therefore take account of the range of antibacterial effects associated with oxygen at various pressures.

Thus the postulated mechanisms associated with, or responsible for bacterial anaerobiosis increase in number; their constant modification in the light of new knowledge indicates that no one unitarian hypothesis will be sufficient, but rather that different mechanisms of anaerobiosis will relate to different models, as suggested by the work of de Vries and Stouthamer (1969).

Many of the reported studies relate to the growth of anaerobes in liquid media, under carefully defined conditions but often with poorly quantitated data. Although much of importance has come from these studies, comparatively little work has been done on the factors that affect growth of anaerobes on solid media; for the clinical bacteriologist attempting to isolate anaerobes from clinical specimens, the recovery of anaerobes on solid media remains a demanding exercise. The present study was designed to take particular account of possible factors affecting the growth of anaerobes on solid media, and to produce quantitative as well as qualitative evidence.

It was decided initially to use Cl. oedematiens, especially types B and D, as models. The recovery of these demanding organisms seemed to offer a challenge that needed to be taken up, especially in the case of Cl. oedematiens type D strains, "probably the most fastidious anaerobes known" (Willis, 1969, p. 161). It was hoped that the development of improved methods for the recovery of these organisms on solid media would elucidate the problems involved, and lead to better methods for the recovery of other anaerobes.

Early studies confirmed the work of Rutter (1968), who noted that although type-A strains of Cl. oedematiens grew readily on the surface of solid media, growth of type-B and type-D strains was often poor. The use of pour plate methods did not markedly improve the reliability of surface growth of these organisms; although good growth was achieved in liquid media containing meat particles, the poor surface growth observed on blood agar suggested that the successful surface growth of type-B and type-D strains might require not only the provision of adequate nutritional factors, but also a suitably reducing environment. The anaerobic procedure in use at the time was therefore reviewed.

It became clear that details of practical procedure, and the correct maintenance of equipment, were of importance in the growth of these demanding organisms. As a result of these studies, a standard anaerobic procedure for use with the BTL anaerobic jar was formulated. One of the features of this standard procedure was the reliance placed on the prompt development of a secondary vacuum as an index of catalytic activity.

Since the development of the anaerobic jar, it has been

customary to include in each jar at the time of incubation some indicator of redox potential (E_h) to serve as a check that satisfactory conditions of anaerobiosis have been achieved. Several authors have suggested the use of biological indicators of anaerobiosis, such as Cl. tetani (Willis, 1964) or Cl. sporogenes (Wiel-Korstange and Winkler, 1970), but this method has the two disadvantages that

(i) the results are not known until after the test plates have been incubated - too late for timely correction of a fault in the anaerobic system, and

(ii) if an exacting organism is used, failure of growth of the indicator organism may be due to factors other than faulty anaerobic procedure. Nevertheless, faulty anaerobic technique may be overlooked, and correct maintenance of equipment neglected, unless a demanding test anaerobe is cultured routinely on solid media.

Indicator solutions such as that recommended by Fildes and McIntosh (1921) and modified by Parker (1955) can be used to monitor the establishment of anaerobic conditions in an anaerobic jar. They are based on the principle that an alkaline solution of methylene blue with glucose, colourless in a reduced state, becomes coloured blue when exposed to oxidising conditions. Such solutions can be used inside glass anaerobic jars, or attached to the side-arms of metal ones, but their preparation often involves the mixing of stock solutions. To overcome this disadvantage, Ulrich and Larsen (1948) designed a single stage anaerobic indicator, but their solution appeared to deteriorate rapidly on storage (Parker, 1955), and may be toxic because of the presence of sodium arsenate. Stokes (1960)

cited details of Lucas' semi-solid indicator, in which methylene blue, borax, thioglycollic acid and phenol red are incorporated into semi-solid agar. The complete indicator can be dispensed into ampoules, which are then sealed until required, when they can be opened and connected by means of rubber tubing to the side-arm of an anaerobic jar such as the BTL anaerobic jar. Details of the use of E_h indicators are given by Hewitt (1950) and more recently by Drollette (1970).

Some of the difficulties associated with the use of external indicators were considered by Rutter (1968); for example the external semi-solid indicator supplied with the BTL jar was unreliable in operation, so that Rutter resorted to the use of an internal indicator. Whilst an internal indicator has the obvious disadvantage that it cannot be seen during incubation, the use of an external indicator solution has the disadvantage that the rubber tube connecting the indicator to the side arm is a common source of leaks. Moreover, it is questionable whether the E_h range of the commonly used indicators, which are based on methylene blue, is sufficient to determine whether conditions are sufficiently reduced for the growth of demanding anaerobes such as Cl. oedematiens. Further information is required on this point.

An alternative index of catalytic activity was developed in the present study and this obviated the need for an indicator of anaerobiosis. The determination of a secondary vacuum with a simple mercury manometer allowed the prompt detection and replacement of faulty catalyst sachets; moreover, the metal side-arms of BTL anaerobic jars could then be permanently closed to prevent leakage.

At this stage, the addition of 10 per cent. carbon dioxide to

the gaseous environment of anaerobic jars was included empirically in the standard anaerobic procedure on the basis of early qualitative studies. Later quantitative studies showed that the addition of carbon dioxide to anaerobic jars increased the recovery both of stock strains of medically-important anaerobes and of anaerobes from faecal specimens; the inhibitory effect of carbon dioxide on the recovery of Cl. welchii from spore suspensions noted by Futter and Richardson (1970b) was not observed with CMB cultures of these organisms. The pronounced enhancing effect of carbon dioxide for Gram-negative non sporing anaerobes observed in the present study is of especial importance when related to the increasing significance of these organisms in the causation of serious infections of man (Spencer, 1971; Felner and Dowell, 1971). The addition of carbon dioxide to the anaerobic environment facilitates the isolation of these organisms on solid media, and this should therefore be part of routine anaerobic procedure; the assured provision of carbon dioxide by the Gaspak system is an advantage in this regard.

In spite of the use of a standard anaerobic procedure, surface growth of demanding strains of Cl. oedematiens continued to be irregular, and failures of growth still occurred. The use of iron-filings, sprinkled on to seeded plates, had been noted in preliminary observations by Rutter to enhance the growth of type-B and type-D strains. These findings were confirmed by the present author; growth of colonies of type-B and type-D strains occurred in 48 hr, instead of the 4 days recommended by some authorities (e.g. Willis, 1969). The mechanism of this effect was not clear; the effect could not be reproduced with other particulate metals, nor with inert particles

such as carbon or crushed egg-shell. Similarly, addition of iron salts to the surface of seeded plates did not produce this effect; only iron filings, sprinkled on to the surface or mixed in with the inoculum and "plated out" with it, gave the enhancing effect. It seems reasonable to suggest that the effect observed with iron filings is related to the production of adequately reduced micro-environments on the surface of the medium, but elucidation of this point awaits further study. Later studies failed to show any enhancing effect when the iron-filings technique was used with the other organisms, suggesting that the effect was specifically associated with organisms of the Cl. oedematiens group.

The iron-filings technique was imprecise, however, and the presence of iron filings on the surface of plates rendered accurate colony counting difficult, so that it seemed worthwhile to search for some other method of enhancing the surface growth of demanding strains of Cl. oedematiens. The cysteine/dithiothreitol system described by Moore (1968) offered an alternative. Moore had noted the enhancing effect of this system on Cl. oedematiens type-B strains, when the cysteine and dithiothreitol were incorporated into a complex basal medium, but the present author found that incorporation of these substances into simple blood agar media gave equally good and dramatic results. Not only was the enhancing effect on the surface growth of type-B strains confirmed, but consistently reliable surface growth on media containing the cysteine/dithiothreitol system was observed with type-D strains. Large, easily visible colonies, showing good haemolysis, could be obtained after overnight incubation, and it seemed possible that the cysteine/dithiothreitol system could now be extended

to enhance the growth of other anaerobes on solid media.

However, the results of later studies with a range of aerobic and anaerobic organisms showed that with the exception of the Cl. oedematiens group, the addition of cysteine and dithiothreitol to blood agar medium had little or no effect on the surface growth of the test organisms. Thus it appears that (i) the cysteine/dithiothreitol system is a specific requirement for organisms of the Cl. oedematiens group, or (ii) the system functions as a special reducing system, and these organisms are more demanding in terms of their requirements for anaerobiosis than the other test organisms. This latter explanation can only be valid if the enhancing effect of the cysteine/dithiothreitol system can be shown to be due to its reducing action. Although cysteine is well known as a reducing agent, and dithiothreitol has also been shown to be effective in this respect (Mead, 1969), it was found that other -SH compounds could substitute for dithiothreitol in the cysteine/dithiothreitol system, including penicillamine, which is a poor reducing agent. Thus it may be that organisms of the Cl. oedematiens group have a specific requirement for -SH compounds. As cysteine undergoes rapid oxidation under aerobic conditions, cysteine alone may not be sufficient to meet these requirements. Other -SH compounds may stabilise the cysteine in a manner analagous to that described by Cleland (1964) for dithiothreitol. / Szent-Györgi, Együd and McLaughlin (1967) noted that cysteine was effective in reversing the inhibition of bacterial growth produced by certain keto-aldehydes analogous to methylglyoxal. It may be, as Moore suggested, that cysteine is a specific requirement to prevent this inhibition, and that its presence in the -SH form is essential.

As dithiothreitol and penicillamine are both chelating agents, an alternative explanation of their action might be that they remove toxic metallic ions from the environment. However, another chelating agent (EDTA) used in the present study had no enhancing effect on the growth of Cl. oedematiens type-D and this suggests that this action of dithiothreitol was not primarily responsible for its enhancing effect.

It appears, therefore, that as both the iron-filings technique and the cysteine/dithiothreitol system are specific enhancing factors for organisms of the Cl. oedematiens group, their use cannot be extended on the present evidence to the isolation of other anaerobes; inhibition of growth may even occur in some cases.

It now became possible for the first time to perform reliable surface viable counts with Cl. oedematiens types B and D. In preliminary work, pour-plate techniques had been the only means of obtaining quantitative data but although such techniques have been shown to be more precise than surface methods (Soestbergen and Lee, 1969) they introduced the risk of possible inactivation of heat-sensitive cells by the molten agar (the heat sensitivity of vegetative cells of type-D strains was well shown in the present studies). In addition, such methods were not directly relevant to studies of growth on the surface of solid media. The surface counting method of Miles, Misra and Irwin (1938) was found to be unsuitable as it required a considerable period of time for the drops to dry into plates, even if the plates were well dried. The counting procedure that we finally developed utilised a spreading technique, with sterile glass spreaders of uniform dimensions, and involved the rapid processing of seeded plates that were promptly transferred to anaerobic jars, thus

minimising exposure of the plates to aerobic conditions. It should be noted that the speed of the spread-plate procedure can be readily achieved with practice, and a minimum of training is required. The use of this procedure could well be extended to studies with other demanding anaerobes.

The choice of diluent, and the method of making dilutions, was shown to be of great importance in the case of these demanding anaerobes. Saline was found to be rather inhibitory, whereas pre-steamed nutrient broth appeared to have less toxic effects in the present studies. Careful mixing of dilutions is essential, but care must be taken to avoid oxygenation by vigorous shaking. This is well shown by studies in which dilutions were shaken aerobically for a constant period at a constant rate - a dramatic fall-off in the surface viable counts could be demonstrated in a short period of time in the case of a demanding anaerobe. From the results of these studies it is clear that pre-steamed nutrient broth is superior to gelatin or gelatin/saline as a diluent in dilution procedures. Such procedures should be done as rapidly as possible with minimum aerobic agitation, consistent with adequate mixing of the bacterial suspension.

Thus, in quantitative work with anaerobes, the spreading procedure can give reliable results when coupled with careful dilution technique. The spreading growth of many anaerobes can be minimised by the use of well-dried plates, or by the use of plates with an increased agar content. In the case of Cl. tetani, the efficacy of antitoxic sera as described by Willis and Williams (1970) was confirmed and its use extended to Cl. septicum. In the present study, the results obtained with Cl. tetani were not as good as those obtained

by Willis and Williams in their elegant studies; this may relate to the potency of the antitoxic sera used or to differences in technique. Nevertheless, although different sera varied in their ability to control spreading growth, the results were sufficiently encouraging to warrant a fuller investigation with other clostridia.

The development of standard counting procedures allowed an investigation of the problems related to the subculture of demanding strains of Cl. oedematiens. The work of Rutter (1968) with the Cl. oedematiens type-D model indicated that growth of this organism on solid media was primarily derived from spores present in the inoculum. However, these studies used old stationary phase cultures, coupled with aerobic washing of the centrifuged deposit; these factors, together with the use of a pour-plate technique involving exposure of the inoculum to temperatures of 40-45°C operated against the survival of vegetative cells. It seems that his experimental design was heavily weighted against disproof of the hypothesis that growth on solid media is primarily derived from spores. It soon became clear that from overnight CMB cultures it was possible to recover on occasion more organisms on the surface of plates than the number of spores present in the inoculum, suggesting that vegetative cells were also contributing to the surface viable counts. The fall in the viable counts produced by heating and by washing in air was further evidence that vegetative cells were being recovered on solid media; although heating procedures may inactivate heat-sensitive spores in addition to vegetative cells, the fall in the viable count after washing could be expected to be due to inactivation of vegetative cells alone.

When young (5-hr) cultures were used to poise the numbers of

spores present in the inoculum at low levels, the surface viable counts derived from such inocula consistently exceeded the estimated numbers of spores present, sometimes by a hundred-fold. The studies included many attempts to ensure that under-estimation of spore numbers did not occur; the estimates given were based on a colony-forming unit counting procedure that gave maximum rather than minimum indices of spore proportions. Although the author accepts that free spores might occasionally escape detection, the examination of stained smears in parallel with the phase-contrast preparations should have avoided this.

One consistent finding in the present study was the low proportion of spores of type-D strains that proceeded to successful germination and outgrowth; on occasion, more than 99 per cent. of the apparent spore population failed to produce surface colonies. If colony production from spores is as inefficient as these findings suggest, then there must be a very considerable contribution from vegetative cells to the surface viable counts that were obtained, especially in the case of young cultures. The finding that in older CMB cultures the surface viable counts decrease as the proportion of spores present increases implies either that (i) spores are the primary component of the viable count and become progressively more dormant and difficult to activate, or (ii) that vegetative cells are the primary component, and die in ageing CMB cultures.

The above findings make it clear that spores were not the primary component of the surface viable counts, especially in the case of young cultures. If the "<u>significantly viable particle</u>" is defined as that component of a CMB culture of a sporing anaerobe (either spore

or vegetative cell) from which colonies on solid media are primarily derived, it is clear that in the case of Cl. oedematiens type D, vegetative cells are the significantly viable particles when young CMB cultures are subcultured on to solid media. In other words, it is possible to redeem vegetative cells of this strict anaerobe by careful anaerobic technique, without the use of anaerobic cabinets, or of gas-flushing procedures. Although the vegetative cells are vulnerable to such procedures as aerobic washing, they can obviously survive transient exposure to aerobic conditions; the spore is not the only insurance involved in the subculture of this organism on to solid media. It seems clear that the past difficulties associated with the surface growth of this organism related both to its low rate of spore germination, and to the vulnerability of its vegetative cells.

Although Cl. oedematiens served the author well as a model of a demanding anaerobe, it was the purpose of the present study to relate findings from this model to anaerobes of more direct clinical interest. Accordingly, the recovery of medically important clostridia on surface media was investigated. Again, it was possible to show that vegetative cells could be the significantly viable particles for all the organisms tested, especially when young cultures were used; in other words, not only is it possible to recover vegetative cells quantitatively, in addition to spores, from laboratory cultures of clostridia, but vegetative cells may contribute proportionately and absolutely more to the surface viable counts than the spores that are present. For the clinical bacteriologist, the message is clear: with careful anaerobic technique, vegetative cells of medically important anaerobes can be redeemed on simple blood agar media. Reliance should

not, and need not, be placed on the germination and outgrowth of spores to provide surface colonies on solid media. The findings are of particular significance in relation to the observations of Smith and McIver (1969), who found that vegetative forms of Cl. tetani could develop from a spore inoculum, and persist for up to nine days in the muscles of experimentally-infected guinea pigs, and that virtually no heat-resistant spores seemed to be present in such lesions. The work of Garcia and McKay (1969), who showed that vegetative forms of Cl. septicum could persist in the soil, may relate to wound infection in man in cases where the wound is contaminated with soil, and there is a need to extend these studies to "in-vivo" models.

When my studies showed that vegetative cells of sporing anaerobes could be quantitatively recovered on blood agar media, it was necessary to determine the recovery of non-sporing anaerobes on similar media. It was soon clear that a high proportion of Bacteroides organisms could be recovered, especially in the case of older cultures, provided that carbon dioxide was added to the gaseous environment in the BTL anaerobic jar (q.v.) As laboratory strains of Bacteroides could be considered to be less demanding in terms of their anaerobic requirements than wild strains, the recovery of anaerobes from specimens of faeces was studied. The viable counts of "total anaerobes" obtained exceeded 1×10^{10}, and most of the organisms present were morphologically typical of Bacteroides although organisms typical of lactobacilli were also present. Similar counts were obtained when the Gaspak system was used, and the use of a special transport medium did not appreciably alter the counts. The counts obtained in these studies fell within the range of counts said to be obtainable only by the use of techniques

such as the anaerobic cabinet (Hill et al., 1971). However, a large proportion of organisms present, as determined by phase-contrast microscopy, were not recovered on the surface plates, suggesting either that the majority of these organisms were non-viable, or that they could only be recovered with specialised techniques. The present study attempted to ensure, by parallel examination of stained smears, that all particles counted were in fact micro-organisms, rather than inert particles, but specialised staining techniques and exhaustive microscopy will be required to elucidate this point. There does seem to be a "non-recoverable" component. It seems unlikely that there are no non-viable organisms in faecal samples. As the special procedures employed in the study have not defined any group of organisms that cannot be recovered in significant numbers at the bench, we cannot define the non-recoverable component and we doubt whether others have yet defined it.

At a time when there is much interest in the quantitative recovery of anaerobes from the gastro-intestinal tract of man, it is important to avoid false standards. The relationship between the number of bacterial cells present in faecal samples, and the numbers that can be recovered, is clearly important in the assessment of cultural techniques. Many of the techniques used have been derived or adapted from studies on the microbiology of the rumen; workers in this field appear to accept that a significant proportion of visible cells cannot be recovered even by the use of specialised techniques (Hobson, 1969). In the case of human faeces, the literature is conflicting, and often suffers from a lack of description of the methods used and a concomitant lack of statistically valid data.

The reported studies must be considered in the context of the calculations of Luckey (1965), that a count of 10^{12} organisms per gram of faeces implies that 100 per cent. of the volume consists of bacterial cells. Thus the microscopical counts of 10^{11} organisms per gram observed by workers such as Moore, Cato and Holdemann (1969) seem acceptable, whereas higher total counts, such as those obtained in the present study, may reflect inadvertent counting of particulate debris. The situation in relation to the viable counts obtained from faecal samples is also confused; not only do the counts vary in different geographical locations (Hill et al., 1971) but in different samples from the same location. For example, although Drasar (1967) originally obtained total anaerobic viable counts of up to 10^{11} organisms per gram wet weight of faeces in a small series of normal adults, a later study based on larger numbers but in the same geographical area, gave counts of around 10^{10} organisms per gram (Drasar and Shiner, 1969). Thus it is difficult to find a suitable "standard" for comparative studies: details of methodology and quantitative techniques are often lacking. It seemed to the author that meaningful comparisons can best be made with counts obtained by other workers sampling from a similar population, with well-documented methodology. This is discussed on pages 289 - 290.

Although quantitative recovery of vegetative cells of demanding anaerobes could be achieved on simple blood agar, the occasionally incomplete recovery of some organisms such as Cl. tetani led to an investigation of several possible factors that might have improved the recovery in a manner analogous to that of the cysteine/dithiothreitol system for Cl. oedematiens. Apart from the stimulatory

effect of carbon dioxide already mentioned, none of the other factors tested had any enhancing effect on the growth of the test anaerobes. The author was able to confirm, for example, the negative results obtained by Mateles and Zuber in their attempts to enhance the growth of anaerobes by addition of catalase (Mateles and Zuber, 1964). Similarly, factors such as TRIS (hydroxymethyl) aminomethane, and EDTA, which might have been expected to have an enhancing effect (see Introduction) gave negative results on testing. An appreciation of the possible occurrence of batch variation in media such as blood agar gave impetus to the search for a defined solid medium with which these factors could be more competently investigated. The development of such a medium for Cl. tetani allowed qualitative studies that showed the lack of enhancing effect of any of the factors tested for this model. The finding that addition of cobalt to blood agar media and to the defined medium had no effect on the qualitative or quantitative recovery of Cl. tetani conflicts with the claims made for this substance by Dedic and Koch (1956). There seems no justification for the addition of cobalt salts to culture media for Cl. tetani.

The defined medium developed was not a minimal medium for Cl. tetani, yet only tiny colonies were obtained even in the presence of reducing substances such as cysteine, and the addition of dithiothreitol did not improve the size of these colonies, nor the density of growth as gauged by macroscopic appearances. This raises the intriguing difference between essentials for growth - already studied by such workers as Feeney, Mueller and Miller (1943); Mueller and Miller (1942), and Kaufman and Humphries (1958) - and stimulating factors that are of practical importance. The defined medium will be

a useful tool in studies on these stimulating factors.

The clinical microbiologist is often obliged to attempt to isolate anaerobes from a clinical specimen containing a very mixed bacterial population, and it is necessary to resort to procedures such as heat, or to selective media. Such intentional selective procedures should be clearly distinguished from the unintentional selection that may occur as a result of delay in transit to the laboratory, with consequent exposure to aerobic and drying conditions, or as a result of poor anaerobic technique. Clearly such factors may selectively inhibit the growth of vegetative cells of clinically important anaerobes. Similarly, although standard textbooks clearly advise that attempts to isolate sporing anaerobes should be made by culturing unheated as well as heated samples (Wilson and Miles, 1964; Cruickshank, 1968; Willis, 1969), there is a danger that the use of heat as a selective procedure may encourage the clinical microbiologist to place undue reliance on recovery from spores - sometimes an inefficient process - and therefore to select out an often small proportion of the potentially viable inoculum.

The antibiotic-containing selective media are often useful, but their use should be based on careful surveys of the antibiotic sensitivities of the relevant species. Too little is known of the effects of selective agents on the recovery of anaerobes from clinical specimens, as opposed to laboratory cultures, and careful quantitative studies may reveal disadvantages associated with selective media; caution should prevail before new selective media are adopted for routine use. Media may contain unsuspected selective agents: for example, antisporulation factors may be present (Foster et al., 1950),

as may be toxic peroxides (Proom et al., 1950; Barry et al., 1956). Clearly the occurrence of these agents in media may jeopardise further the quantitative recovery of anaerobes from clinical material.

In addition to the factors mentioned above, the present study has shown that recovery of anaerobes on solid media may be positively or negatively affected by other factors. On the one hand, recovery may be enhanced in certain models by the addition of carbon dioxide to the anaerobic environment, by the use of thick plates, and by flushing of anaerobic jars before incubation. On the other hand, recovery may be inhibited by such factors as storage of plates under aerobic conditions, by the use of selective procedures, or by exposure of seeded plates to aerobic conditions. This latter effect is variable; although rapid inactivation of vegetative cells of Cl. oedematiens type D was observed under these circumstances, the cells of Cl. tetani appeared more resistant. Cl. tetani is classically considered to be a strict anaerobe, with cells of implied sensitivity to oxygen, but the present study has shown that quantitative recovery of these cells can be achieved after exposure of the organisms on seeded plates to aerobic conditions for considerable periods. Analogous results were also obtained in the case of Bacteroides strains, both from stock culture, and from "wild" strains in faecal samples.

Thus exposure of anaerobes on seeded plates to aerobic conditions demonstrates a spectrum of sensitivity; in some organisms, notably Cl. tetani and Bacteroides species, the vegetative cells appear to be protected by the presence of the solid media, whereas other organisms are rapidly inactivated. It is a paradox that the

vegetative cells of a sporing anaerobe (Cl. oedematiens type D) are most sensitive to these conditions, and that the spores do not compensate for this because they are recovered very inefficiently on solid media. Clearly, there is a need to investigate the degree of inactivation that is produced when anaerobic organisms are exposed in wound swabs to aerobic and drying conditions. Such studies could encourage improved methods of sampling (see Ellner and Ellner, 1966), and may lead to more effective transport of specimens to the laboratory.

From the results of the present study, it seems reasonable to suggest that where the identity of the inoculum is unknown, as in the case of clinical specimens, plates should be rapidly processed and incubated, as a routine. The present study has shown however that such a recommendation requires to be based on quantitative data, rather than empirical assumptions.

These studies have yielded data that are based on quantitative techniques and the analyses have been made with strict statistical control. There is a clear need for studies to be extended to other models, based on the results and techniques of the present study, so that a fuller knowledge can be obtained of the factors involved in the recovery of other organisms on solid media.

Even if promptly incubated, the success of recovery of anaerobes from seeded plates is dependent on the conditions of incubation. Temperature is important, and the present study has shown that the time of incubation should be sufficiently long for maximal recovery. However, anaerobic technique is the most important determining factor in the recovery of anaerobes from seeded plates. Poor anaerobic technique can only be revealed when demanding models are used;

Cl. oedematiens type D is a severe test of anaerobic procedure, and its consistent surface growth is a more valid indicator of satisfactory anaerobic technique than repeated isolations of Cl. welchii.

The use of a standard anaerobic procedure in the present studies was of great value, but results suggested that it could be improved, notably by the inclusion of a flushing stage, and by attention to possible variable factors such as the number of catalyst sachets (see below) and the provision of carbon dioxide. The revised anaerobic technique eventually developed should lead to more consistent conditions of anaerobiosis being achieved in the laboratory, and therefore to the recovery of strict anaerobes on solid media being regarded as an everyday event, rather than an unusual achievement.

The Gaspak disposable anaerobic system offers a method of cultivation of anaerobes that avoids the use of cylinders of compressed gases or cumbersome hydrogen-generating systems, and its use in routine diagnostic laboratories could lead to more consistent results. The recovery of anaerobes obtained by the Gaspak system was compared with that obtained with the standard anaerobic procedure developed in the early stages of the present study. In this study, the term "Gaspak" denoted the Gaspak system, which was usually used in conjunction with a BTL jar; the Gaspak polycarbonate jar was used in only a few experiments.

The results obtained when a fairly wide range of anaerobes of clinical importance were tested, showed that the Gaspak system performed reliably, and consistently gave as good recovery of the test organisms as was obtained by means of the regular procedure. Only when Cl. oedematiens type-D strains were used could the Gaspak

of anaerobes
system be faulted on occasion. Recovery/from faecal samples was equally good with either system.

When Gaspak sachets were used in conjunction with BTL jars, the pressure changes that occurred were such that it was not possible to monitor them on a simple mercury manometer, and no index of catalytic activity was therefore available. This is especially important in the case of the Gaspak, where the pressures developed are considerable, and in the presence of a faulty, non-functional catalyst severely stress the BTL jar. The use of a rubber balloon attached to one of the outlets of the BTL jar seems to offer a simple means of recording such pressure changes, but the development of such a procedure would have to take account both of the permeability of a rubber balloon to oxygen, and the fact that the needle valves of the BTL may leak at the valve stems when they are not tightly closed. The valves should therefore not be left open with the balloon in situ.

The Gaspak polycarbonate jar was only used in a few experiments, and in these it performed satisfactorily. As it was transparent, plates could be inspected, and the colour changes in the Gaspak indicator observed, without the dangers associated with glass jars. The polycarbonate jar was however expensive (its price was £35 in Britain, compared with £22 for the BTL jar), and the absence of outlet taps was a disadvantage if monitoring of pressures was desired.

Each Gaspak costs about 21 pence in Britain, and although the capital expenditure is minimal, the cost of setting up several jars daily in this way would certainly be significantly more than the cost involved in the standard procedure. Such financial considerations

should however be set against the findings that the Gaspak system worked well, was reliable in operation, and could be used by staff unskilled in anaerobic technique. It may well have a place in routine service laboratories, especially if facilities for anaerobic procedures and experience in anaerobic bacteriology are limited.

The BTL anaerobic jar was extensively used in the present study, and although not inexpensive, it performed reliably and safely, provided that it was used with care and attention to detail. For example, regular cleaning of the jars is recommended, and regular lubrication of the valves with a suitable grease is a necessary maintenance too often omitted.

Several potential disadvantages were observed when the BTL jars were in use; for example the rubber at the side-arm tubes commonly leaked and we permanently closed off the side-arms of all BTL jars in the laboratory. The rubber "O" ring seals leaked unless they were carefully fitted and free of dirt particles. Leakage through the screw-holes in the lid was observed on one or two occasions. Leakage through the valve stems of partly open valves during monitoring of pressures could occur, especially if the valves were inadequately greased, and this could allow air into jars or hydrogen out, and might vitiate the success of the procedure of Futter and Richardson (1971) in which a bladder of hydrogen is attached to an open valve after processing of the jar, to allow ingress of the gas. We have found that regular checking is necessary; the ability to maintain a vacuum, and the testing of jars at increased pressures under water, are both essential measures of jar efficiency that are all too often neglected.

In the course of studies in which large numbers of BTL jars

were being used at one time, it became clear that differences had existed between the performance of BTL jars processed in a standard manner, especially when Cl. oedematiens type D was used as a model. On occasion, all the plates in a given jar had many more colonies per plate than those in other jars, although all the jars showed satisfactory catalytic activity as measured by the production of a secondary vacuum. This "jar variation" phenomenon seemed to be associated with the jars themselves rather than with specific plates. It was not related to a specific jar, but occurred irregularly; in other words, a jar that performed well on one occasion did not necessarily do so on a subsequent occasion. The phenomenon was clearly related to the culture of exacting strains of Cl. oedematiens; it could have serious implications if it occurs with other anaerobes. This has not been the case with a wide range of organisms, although jar variation has been observed on a single occasion with Cl. tetani.

Preliminary studies with a series of possible causative factors gave inconclusive results, although one factor that seemed important in the case of Cl. oedematiens at least was the number and age of the catalyst sachets present in a given jar. Although each sachet contained the same number of catalytic pellets (see Materials and Methods), carefully designed statistical studies confirmed that recovery of organisms of the Cl. oedematiens group on solid media was improved if more than one catalyst sachet was used in each jar; little advantage was gained if more than three were used. There is little mention of the use of multiple catalyst sachets in the literature on anaerobic procedure: Futter and Richardson, while criticising the rate at which a single catalyst sachet catalyses the

combination of hydrogen and oxygen, did not investigate the effects of multiple catalysts on the rate of catalysis. Similarly, although the use of two catalyst sachets per jar has been described (e.g. Moore, 1968; Walker, Harris and Moore, 1971), there have been no reports of adequately designed studies to test the effect of multiple catalysts on the recovery of anaerobes. In the present study, all catalyst sachets were fixed to the lid of the anaerobic jar: further studies are needed to determine whether the positioning of one or more catalyst sachets on the floor of the jar enhances anaerobic growth. Such studies could usefully be extended to test the recovery of other anaerobes using different numbers of catalyst sachets. It could be argued that the use of catalytic pellets (Engelhardt) placed on a tray in the jar is more practicable and economical than the use of multiple catalyst sachets; although this point will require elucidation, it seems that in the absence of suitable methods for measuring the efficiency of such a system, an empirical replacement routine would have to be introduced. Such a routine would have to strike a balance between waste due to rapid replacement (the pellets cost £5 per 200 g) and inefficiency due to excessive "in use" service. The secondary vacuum method on the other hand, is a rapid and easy measurement of the function of catalyst sachets.

The nature of the jar variation encountered in studies with type-D and type-B strains of Cl. oedematiens remains obscure, in spite of extensive investigation. No inhibitory factor in the environment of the jars was found and no discernible difference in technique between the processing of different jars has been noted. Great care was taken to ensure that all possible variables in technique were

standardised. Several possible variables were discovered, including exposure of organisms in the diluent, exposure of seeded plates to aerobic conditions, and the number of catalysts in a given jar. Experiments with spore suspensions and with young cultures of Cl. oedematiens containing very few spores have shown that the "jar-variation" phenomenon is associated with vegetative cell growth, rather than with germination of spores. The fact that no evidence of a statistically significant degree of jar variation was observed in statistically designed experiments with two types of Cl. oedematiens indicates that randomisation of plates may help to control this factor. However, in the author's opinion, until the nature of the jar-variation phenomenon has been elucidated, comparative quantitative studies with Cl. oedematiens should not be undertaken without stringent precautions in the design and analysis of experiments (see below). In the case of other anaerobes, although little evidence of the jar-variation phenomenon has been observed, certain precautions should also be employed when initial comparative studies are being performed; failure to demonstrate jar-variation should not lead any investigator to assume that it will not occur. The author is aware that jar-variation is an artificial phenomenon, which could perhaps be abolished by the use of anaerobic jars of different size and construction. Nevertheless it seemed worthwhile to try to elucidate the problem and plan experiments on the basis of the existence of jar variation; as the BTL anaerobic jar is a piece of apparatus that is in widespread use, allowing for jar-variation in experimental design is easier than re-equipping diagnostic laboratories with new, large, anaerobic jars which may be too large for existing

incubators.

The following points should be incorporated into the design and analysis of quantitative experiments involving Cl. oedematiens type D:

(1) All jars should be equipped with at least three functional catalysts per jar.

(2) All plates should be allocated randomly to anaerobic jars, according to a pre-arranged randomisation schedule.

(3) All plates should be randomly distributed within a given jar, according to a randomisation schedule.

Note: if a number of media are being compared, then the order of seeding of the plates should also be random, and the number of plates of each type of medium should be equalised in each jar.

(4) The jars should be processed in a random order.

(5) In critical experiments, at least four and preferably six "replicate" jars should be used.

(6) In comparisons between jars processed in different ways, the experiment should be repeated on three successive occasions. The same dilution should be used on each occasion, so that errors due to dilution are standardised.

(7) All jars should be incubated for equal times at 37°C.

All data should be recorded so that the distribution of every plate in terms of the anaerobic jar used, and the position in the jar are noted. The form illustrated in Appendix C is a convenient method of achieving this. The analysis of experiments must assume that surface counts derived from different jars do not belong to a single

statistical population, and therefore an analysis of variance must be performed.

It is not permissible, given the degree of "between-days" variation observed in the present studies, to compare the counts obtained on a given day with those obtained on another day, except as part of a statistically designed experiment. Similarly, if the recovery of a test anaerobe on two media, A and B, is to be compared, it is not permissible to put all plates of medium A into one anaerobic jar, and all plates of medium B into another anaerobic jar, as any observed differences may merely be due to jar variation.

In the design and analysis of quantitative experiments with other test anaerobes, there is less need for the elaborate precautions detailed above. However, as a routine, the author randomises the distribution of plates between and within anaerobic jars. At least two, and preferably four or six jars are used for every experiment, and are processed in a random manner. All jars are incubated for equal times at 37°C. If several media are being compared, equal numbers of plates of each medium are placed in each jar. The plates are seeded in a random order. In the case of Cl. oedematiens strains, or if "unknown" organisms are used, each jar is equipped with three catalyst sachets, otherwise only one functional catalyst sachet is placed in each jar.

The results are recorded as explained above. If only two variables are being compared, then provided that there is no jar variation evident on inspection of the results, the author is advised (Miss M. V. Hoare) that it is acceptable in the first instance at least to assume that the jars belong to a single population, and to

compare the colony counts from variable A with those from Variable B, using the t test; the more exact analysis of variance can be reserved for special cases. If more than two variables are being tested, a two-way analysis of variance, similar to those detailed on p.329, is necessary.

Note: in the present study, all data on which statistical comparisons have been performed have been derived from experiments designed as shown above. In the case of experiments involving comparisons of the processing of BTL anaerobic jars, the results of "t" tests have been included only as a guide; their validity would be endangered if a significant degree of jar variation were to exist.

The planning and execution of such experiments is obviously time-consuming and tedious, and they have been reserved for situations in which the results of simpler, pilot experiments have proved unsatisfactory or equivocal. Similarly, such complex experiments are only necessary where comparisons are made; if absolute recovery is being measured, such as in the "significantly viable particle" experiments, they are unnecessary.

The development of anaerobic cabinets and of gas flushing procedures is outlined on pp.45-49. Such procedures are expensive, and their use in routine clinical laboratories can be justified only if they significantly improve the recovery of clinically important anaerobes. Although several workers have claimed that the use of specialised procedures are essential for the growth and recovery of exacting anaerobes, their studies have lacked adequate comparative data; for example, Aranki et al. (1969) claimed that their simplified

glove box procedure recovered more organisms from human gingival flora than could be obtained by conventional anaerobic jar techniques, yet no details of their results with the latter technique are given in the text. In the present study, it was clear that vegetative cells of demanding anaerobes could be quantitatively recovered by conventional anaerobic procedures, and this suggests that more specialised techniques might be unnecessary when standard procedures are carefully performed. It was necessary to construct an anaerobic cabinet to investigate this point in a series of quantitative studies. Although the atmosphere within the cabinet was shown to be sufficiently anaerobic to support the growth of the most demanding models, quantitative recovery was not improved by the use of the cabinet for dilution procedures and for the seeding of plates. This finding applied both to laboratory cultures and faecal samples, and confirmed the impression that equally good recovery would be obtained by the careful techniques developed in the present study. It could be argued that these findings merely represent "equally poor" rather than "equally good" recovery by the two systems, i.e. that both methods were failing to recover a large proportion of the potentially viable inoculum. However, complete recovery of the most demanding model was obtained both with and without the use of the anaerobic cabinet; similarly, the counts obtained from faecal samples compared favourably with those of other workers. Thus, although improved recovery of some minor component of faecal flora in the anaerobic cabinet would not have been detected in the present study, the overall recovery was not improved, even when manipulations were performed in a demonstrably anaerobic environment. It was shown earlier in this

work that complete recovery with non-demanding models of clinically important anaerobes can be achieved by conventional techniques; now there is the additional evidence that this can also be achieved with demanding models. All of these results combine to indicate that there is no evidence to support the use of anaerobic cabinets in the diagnostic laboratory; while anaerobic cabinets may have a place in the recovery of anaerobes from other species not investigated in the present study, it appears that the clinical microbiologist can achieve as good results by careful use of conventional techniques, without the costs and possible dangers (e.g. of explosive gases) associated with specialised procedures.

The present study has defined some of the factors associated with the growth of anaerobes on solid media, and their recovery from fluid cultures. The definition of these factors must be continued and extended to new areas,especially in relation to practical medical microbiology. It is suggested that much remains to be learned by the application and improvement of existing techniques in the light of carefully quantitated baseline studies.

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APPENDIX A

The test strains used in the present study

Note: Many of these strains have been subcultured several times in the laboratory; their current laboratory numbers are probably more relevant than the original source references, but where possible, both references are given.

Anaerobic coccus (Group 1)

NCTC strain 9801 (lab. no. GP1)

Bacteroides fragilis

NCTC strain 9343

B. melaninogenicus (syn. B. melaninogenicum)

NCTC strain 9336; NCTC strain 9337

B. necrophorus

NCTC strain 7155; NCTC strain 10575

Clostridium bifermentans

1 strain received from Professor C. L. Oakley (lab. no. B4)

Strain no. 506

Cl. butyricum

NCTC strain 7423

Cl. chauvoei

2 strains from the Wellcome collection: CC1 and CN3600

Cl. histolyticum

NCTC strain 503

Cl. oedematiens type A

1 strain from the Glaxo collection, No. S277/64 (B) - lab. no. GR2A.

NCTC strains 538, 6737 and 6735 - lab. nos. GR1A, GR3A and GR4A

Cl. oedematiens type B

Strains from the Glaxo collection:

474E (lab. no. GR1B); S281/64 (lab. no. GR2B); 475E (lab. no. GR3B); 513E (GR7B); S277/64(A) (lab. no. GR8B); S77/64(C) (lab. no. GR9B); S284/64 (GR10B); S286/64 (GR11B); S291/64 (GR12B); S292/64 (GR13B).

1 strain provided by Dr J. A. A. Watt (see Rutter, 1968) - lab. no. R3/2.

Cl. oedematiens type D

NCTC strains 8450 (lab. no. GR1D); 8145 (lab. no. GR2D); and 9692 (lab. no. W3D)

Cl. septicum

Wellcome strain CN3204 (lab. no. 103)

1 strain from the Veterinary School, University of Edinburgh, no. 688/52 (lab. no. 102)

1 strain from the Royal Veterinary College, London University, no. 41 (lab. no. 111)

NCTC strains 547 (lab. no. 101); 282 (lab. no. 113); and 286 (lab. no. 114)

Laboratory strain 117

Cl. sporogenes

Laboratory strain 60

Cl. tetani

NCTC strains 540; 5404; 5405; 5413 and a type VI strain no. 9569 (lab. no. TVI)

Cl. welchii type A

2 classical strains from Professor C. L. Oakley (lab. nos. L2A and L3A)

1 classical strain, laboratory no. 8588.

1 classical laboratory strain (Cl) was isolated from a leg wound in Edinburgh

(All of the classical strains were β-haemolytic on horse BA, and non-heat-resistant).

Typical food poisoning strains (non-haemolytic, heat-resistant) included NCTC strains 8359 (lab. no. Hobbs I); 8238 (Hobbs II); 8239 (Hobbs III) and laboratory strain 8588.

Strain F9191, an atypical food-poisoning strain, was obtained by Dr. Collee from the Food Hygiene Laboratory, Central Public Health Laboratory, Colindale Avenue, London.

APPENDIX B

Experimental design for a "multiple catalyst" experiment

DAY 1

Spread measured inoculum by standard procedure on plates 1-30, then allocate to BTL anaerobic jars as follows:

Jar	Plate number									
C 2	2	6	9	10	15	16	21	24	25	30
C 4	3	4	8	12	13	17	20	22	27	28
C 6	1	5	7	11	14	18	19	23	26	29

Then distribute plates <u>within</u> each anaerobic jar as follows:

Order of evacuation	Jar	Position of plate in jar Top Plate → Bottom plate									
1	C 6	29	1	23	7	14	26	18	11	5	19
2	C 4	13	22	27	28	8	12	4	20	17	3
3	C 2	15	2	16	24	30	25	10	6	21	9

The jars are then processed in the order shown above.

Spread inoculum on plates 31-60, then allocate to BTL anaerobic jars as follows:

Jar	Plate number									
C 7	31	36	38	41	43	46	49	53	55	59
C 9	32	35	39	42	45	47	51	54	57	58
C10	33	34	37	40	44	48	50	52	56	60

Then distribute plates <u>within</u> each anaerobic jar as follows:

Order of evacuation	Jar	Position of plate in jar Top plate → Bottom plate									
4	C 9	51	39	57	58	47	35	54	42	45	32
5	C 7	59	38	36	55	53	46	43	31	41	49
6	C10	50	60	48	52	33	34	40	44	56	37

The jars are then processed in the order shown above.

DAY 2

Spread inoculum on plates 1-30, then allocate to BTL anaerobic jars as follows:

Jar	Plate number									
C 2	2	6	8	10	13	17	19	22	27	28
C 4	3	4	9	12	14	18	20	24	26	29
C 6	1	5	7	11	15	16	21	23	25	30

Then distribute plates within each anaerobic jar as follows:

Order of evacuation	Jar	Position of plate in jar Top plate → Bottom plate									
1	C 6	1	7	11	30	5	23	15	21	16	25
2	C 2	13	6	10	19	8	2	28	17	27	22
3	C 4	14	3	24	20	26	18	12	9	4	29

The jars are then processed in the order shown above.

Spread inoculum on plates 31-60, then allocate to BTL anaerobic jars as follows:

Jar	Plate number									
C 7	33	34	37	41	43	47	49	53	55	58
C 9	31	36	38	40	44	48	51	54	56	60
C10	32	35	39	42	45	46	50	52	57	59

Then distribute plates within each anaerobic jar as follows:

Order of evacuation	Jar	Position of plate in jar Top plate → Bottom plate									
4	C 7	37	33	55	47	49	34	41	58	43	53
5	C10	50	32	46	52	35	57	39	42	45	59
6	C 9	44	56	60	31	36	40	54	48	38	51

The jars are then processed in the order shown above.

DAY 3

Spread inoculum on plates 1-30, then allocate to BTL anaerobic jars as follows:

Jar	Plate number									
C 2	3	4	8	10	13	17	20	24	26	29
C 4	1	6	9	12	15	18	21	22	27	28
C 6	2	5	7	11	14	16	19	23	25	30

Then distribute plates within each anaerobic jar as follows:

Order of evacuation	Jar	Position of plate in jar: Top plate → Bottom plate									
1	C 4	18	27	9	12	15	1	21	22	6	28
2	C 2	3	24	4	17	20	26	13	8	10	29
3	C 6	19	11	30	23	7	16	5	2	14	25

The jars are then processed in the order shown above.

Spread inoculum on plates 31-60, then allocate to BTL anaerobic jars as follows:

Jar	Plate number									
C 7	31	36	38	42	45	47	51	54	56	58
C 9	33	34	37	41	43	46	49	53	55	59
C10	32	35	39	40	44	48	50	52	57	60

Then distribute plates within each anaerobic jar as follows:

Order of evacuation	Jar	Position of plate in jar: Top plate → Bottom plate									
4	C 9	33	37	43	46	34	53	49	55	59	41
5	C10	40	35	32	57	48	44	52	39	60	50
6	C 7	56	45	54	31	36	58	47	42	38	51

The jars are then processed in the order shown above.

APPENDIX C

A laboratory record sheet for results of quantitative experiments with anaerobic jars

Order of Evacuation	Jar Experiment No.	Jar Code No.	No. of catalysts	POSITION OF PLATES IN JAR											
				TOP - - - - - - - - - - - - - - - BOTTOM										Plates down side of jar△	
				*											
				**											

* Order of inoculation of plate recorded here.

** Colony counts recorded here.

△ Not used in statistically designed experiments.

APPENDIX D

Statistical methods

Statistical Methods

The following methods and the analysis of results, have been performed under the guidance of Miss Margaret V. Hoare of the Department of Statistics, University of Edinburgh:

1. Comparison of two treatment means.

In the present study, it has been necessary to compare the effect on the recovery of anaerobes of two treatments A or B. These treatments may involve different media, or different processing of anaerobic jars. The design of such experiments is detailed on page If $x_{11}, x_{12}, x_{13}, \ldots, x_{1n_A}$ are the n_A observations resulting from treatment A, then the sample mean is given by

$$\bar{x}_A = \frac{1}{n_A} \sum_{i=1}^{n_A} x_{1i} \quad .$$

Similarly, if $x_{21}, x_{22}, x_{23}, \ldots, x_{2n_B}$ are the n_B observations resulting from treatment B , then the sample mean is given by:

$$\bar{x}_B = \frac{1}{n_B} \sum_{i=1}^{n_B} x_{2i} \quad .$$

The sample of treatment A is assumed to come from a distribution with mean μ_A and variance δ^2_A , and similarly that from treatment B is assumed to come from a distribution with mean μ_B and variance δ^2_B . If δ^2_A and δ^2_B were known and if the distributions were normal, the hypothesis that $\mu_A = \mu_B$ could be tested by using normal distribution tables. However in the present experiments, it was necessary to draw reasonable conclusions about treatment A and treatment B in general, from a limited number of observations, and without knowledge of δ^2_A or δ^2_B . An estimate of δ^2_A can be

obtained by

$$S^2_A = \frac{1}{n_A - 1} \sum_{i=1}^{n_A} (x_{1i} - \bar{x}_A)^2$$

and of δ^2_B by

$$S^2_B = \frac{1}{n_B - 1} \sum_{i=1}^{n_B} (x_{1i} - \bar{x}_A)^2$$

where S^2_A and S^2_B are the variances of the samples of the two treatments A and B.

If S^2_A and S^2_B are approximately equal, then let $\delta^2_A = \delta^2_B$ = a common value, say δ^2. The estimate for δ^2 can be obtained from $S^2 = \dfrac{(n_A - 1)\, S^2_A + (n_B - 1)\, S^2_B}{n_A + n_B - 2}$.

To compare μ_A and μ_B, it is necessary to consider $\bar{x}_A - \bar{x}_B$. The standard error of $\bar{x}_A - \bar{x}_B$ is estimated by

$$SE(\bar{x}_A - \bar{x}_B) = \sqrt{S^2 \left(\frac{1}{n_A} + \frac{1}{n_B}\right)}$$

To test the null hypothesis that $\mu_A = \mu_B$, one takes $t = \dfrac{\bar{x}_A - \bar{x}_B}{SE(\bar{x}_A - \bar{x}_B)}$ which follows the t distribution on $n_A + n_B - 2$ degrees of freedom. t-tables are then consulted to determine whether the observed value of t exceeds the tabulated one, i.e. whether μ_A is significantly different from μ_B.

2. Comparison of more than two treatment means.

The t test is useful for comparing the means of two samples (see above) but for larger numbers of samples, a one-way analysis of variance is a generalisation that is appropriate for any number of groups.

Suppose that there are K different treatment groups, with the

i^{th} treatment giving n_i observations, and denote the j^{th} observation in the i^{th} group by x_{ij} where $i = 1,\ldots,K$ and $j = 1,\ldots,n_i$.

The further notation can be considered as follows:

Group	1,	2, ...,i, ...,K	All groups combined
Number of observations	n_1,	$n_2,\ldots,n_i,\ldots,n_k$	$N = \sum_{i=1}^{K} n_i$
Mean of x	$\bar{x}_1$,	$\bar{x}_2,\ldots,\bar{x}_i,\ldots,\bar{x}_k$	$\bar{x} = T/N$
Sum of x	T_1,	$T_2,\ldots,T_i,\ldots,T_k$	$T = \sum_{i=1}^{K} T_i$
Sum of x^2	S_1,	$S_2,\ldots,S_i,\ldots,S_k$	$S = \sum_{i=1}^{K} S_i$

The deviation of any observation from the grand mean, $\bar{x}$, can be expressed as

$$x_{ij} - \bar{x} = (x_{ij} - \bar{x}_i) - (\bar{x}_i - \bar{x}) \quad .$$

If this expression is squared and summed for all N observations it can be shown that the following relationship holds:

$$\sum_{i,j} (x_{ij} - \bar{x})^2 = \sum_{i,j} (x_{ij} - \bar{x}_i)^2 + \sum_{i,j} (\bar{x}_i - \bar{x})^2 \quad .$$

In other words, the total sum of squares of deviations from the grand mean consists of two parts: the sum of squares of each reading from its own treatment mean, and the sum of the squares of the deviations of each treatment group mean about the grand mean. Thus:

Total sum of squares (S.Sq) = within groups S.Sq + between groups S.Sq . It is possible to perform an F test for differences between treatment means as follows:

Computation can be more conveniently performed using totals rather

than individual observations (i.e. if $S = \sum S_i$ and $T = \sum T_i$ etc. then $T_i = \sum_j x_{ij}$, and $S_i = \sum_j x^2_{ij}$). Note also that $S = \sum_i S_i = \sum_{i,j} x^2_{ij}$.

$$\text{Thus} \quad \sum_{i,j} (x_{ij} - \bar{x}_i)^2 = \sum_{i,j} x^2_{ij} - \sum_i \left(\frac{(\Sigma_j x)^2}{n_i} \right)$$

$$= S - \sum_i \frac{T_i^2}{n_i} .$$

These formulae can be used to compile an analysis of variance table as follows:

	Degrees of Freedom	Sum of squares	Mean square = sum of squares / degrees of freedom
Between groups	K - 1	$\sum (T_i^2 / n_i) - T^2/N$	
Within groups	N - K	$S - \sum (T_i^2 / n_i)$	
Total:	N - 1	$S - T^2/N$	

To compare the treatment groups, it is necessary to consider the null hypothesis that all means are equal to some common value μ, with variance δ^2. The appropriate test is expressed by the ratio:

$$\frac{\text{Between groups mean square}}{\text{Within groups mean square}} .$$

If the null hypothesis is true, both numerator and denominator are independent estimates of δ^2, and the ratio should approximate to unity. If there are real differences between the means of the treatment groups, however, the "between groups mean square" will be inflated, resulting in a ratio of > 1. The ratio of the mean squares follows the F-distribution, and the test is completed by checking from tables whether the observed ratio is significantly larger than the tabulated

value, i.e., whether the differences between the means are larger than would be expected by chance. In such a case, the conclusion is that there are real differences between the means of different treatments.

A similar reasoning can also be used when more than one variable is being observed; e.g., in the present study, variable factors such as anaerobic jars, number of catalysts and days. The design of these experiments is detailed on page 287. Computation of the results of such experiments was complex, and the analyses were conveniently handled on an electronic desk computer (Olivetti Programma 101) and on an IBM360/50 computer (Edinburgh Regional Computer Centre).

Note: For a fuller discussion of the principles of analyses of variance, the reader is referred to Armitage (1971)*.

Levels of probability.

Both t and F tables exist for varying levels of probability (p) . For example, a given ratio would be significant ($p < 0{\cdot}05$) if the observed result occurred by chance in less than 5 per cent. of repeated experiments when the means are in fact equal. In the present study, the following convention has been adopted:

differences between means are "not significant" when $p > 0{\cdot}05$;

differences between means are "significant" when $p < 0{\cdot}05$;

differences between means are "highly significant" when $p < 0{\cdot}01$;

and differences between means are "very highly significant" when $p < 0{\cdot}001$.

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*ARMITAGE, P. 1971. Statistical methods in medical research, 1st ed., Oxford and Edinburgh.

APPENDIX E

Analyses of variance

Effect of varying numbers of catalyst sachets on recovery of Cl. oedematiens type D

"A" data: averages of square roots of original colony counts

Jar	DAY		
	1	2	3
C 2	19.56 (3)*	32.24 (1)	16.82 (5)
C 4	13.00 (1)	36.59 (5)	15.85 (3)
C 6	19.73 (5)	32.87 (3)	11.07 (1)
C 7	17.65 (1)	31.55 (3)	16.44 (5)
C 9	18.12 (5)	27.98 (1)	16.88 (3)
C10	18.60 (3)	33.50 (5)	9.68 (1)

Average for numbers of catalysts:- 1 catalyst = 18.60
3 catalysts= 22.55
5 catalysts= 23.53
Standard error = 0.79

Analysis of variance

Factor	Degrees of freedom	Sums of squares	Mean square	Variance ratio (F)	Significance**
Day	2	1101.192	550.596	>100	very highly significant
Jar	5	9.773	1.955	0.52	not significant
Catalyst	2	81.679	40.839	10.90	highly significant
Residual	8	29.986	3.748		
Total	17	1222.630			

* Figures in parentheses represent numbers of catalyst sachets in the stated jar.

** See Appendix D for explanation of convention adopted.

Effect of varying numbers of catalyst sachets on recovery of Cl. oedematiens type D

"B" data: logarithm of variance of square root of count on each plate

Jar	DAYS		
	1	2	3
C 2	0.6312 (3)*	1.9907 (1)	0.4892 (5)
C 4	0.0388 (1)	0.9278 (5)	0.4422 (3)
C 6	0.8652 (5)	0.8659 (3)	-0.5682 (1)
C 7	0.6972 (1)	1.8390 (3)	-0.3394 (5)
C 9	-0.3846 (5)	0.4279 (1)	0.1654 (3)
C10	-0.8665 (3)	1.5819 (5)	-1.0607 (1)

Averages for numbers of catalysts: 1 catalyst = 0.2543
3 catalysts = 0.8017
5 catalysts = 0.5234
Standard error = 0.2336

Analysis of variance

Factor	Degrees of freedom	Sums of squares	Mean square	Variance ratio (F)	Significance**
Day	2	6.077	3.039	9.41	highly significant
Jar	5	1.615	0.323	0.99	not significant
Catalyst	2	0.899	0.450	1.37	not significant
Residual	8	2.620	0.328		
Total	17	11.211			

* Figures in parentheses represent number of catalyst sachets in the stated jar.

** See Appendix D for explanation of convention adopted.

Effect of varying numbers of catalyst sachets on the recovery of Clostridium oedematiens type-A

"A" data: averages of square roots of original colony counts

JARS	DAYS		
	1	2	3
C 4	13.29 (1)*	13.74 (5)	16.56 (3)
C 2	14.74 (5)	14.82 (3)	15.19 (1)
C10	14.64 (3)	13.12 (1)	16.32 (5)
C 9	13.61 (5)	14.01 (1)	16.47 (3)
C 6	12.98 (1)	13.83 (3)	15.58 (5)
C 8	14.74 (3)	14.98 (5)	15.33 (1)

Average for numbers of catalysts: 1 catalyst = 13.99
3 catalysts = 15.18
5 catalysts = 14.83
Standard error = 0.18

Analysis of variance

Factor	Degrees of freedom	Sums of squares	Mean square	Variance ratio (F)	Significance**
Days	2	13.941	6.970	36.70	very highly significant
Jars	5	1.473	0.295	1.53	not significant
Catalysts	2	4.471	2.235	11.48	highly significant
Residual	8	1.557	0.195		
Total	17	21.442			

* Figures in parentheses represent number of catalyst sachets in the stated jar.

** See Appendix D for explanation of convention adopted.

Effect of varying numbers of catalyst sachets on the recovery of Clostridium oedematiens type A

"B" data: logarithm of variance of square root of colony count on each plate

JAR	DAYS		
	1	2	3
C 4	-0.3253 (1)	-0.5076 (5)	0.4005 (3)
C 2	1.2835 (5)	0.8025 (3)	1.4184 (1)
C10	1.8512 (3)	-0.2024 (1)	0.5375 (5)
C 9	1.3615 (5)	0.5525 (1)	1.1230 (3)
C 6	0.2772 (1)	0.4387 (3)	-0.2576 (5)
C 8	1.5689 (3)	0.9732 (5)	-0.3706 (1)

Averages for numbers of catalysts: 1 catalyst = 0.225
3 catalysts = 1.031
5 catalysts = 0.565
Standard error = 0.204

Analysis of variance

Factor	Degrees of freedom	Sums of squares	Mean square	Variance ratio (F)	Significance**
Days	2	1.463	0.732	2.92	not significant
Jars	5	3.835	0.767	3.04	not significant
Catalyst	2	1.964	0.982	3.9	not significant
Residual	8	1.993	0.249		
Total	17	9.255			

* Figures in parentheses represent number of catalyst sachets in the stated jar.

** See Appendix D for explanation of convention adopted.

Effect of varying numbers of catalyst sachets on the recovery of Anaerobic coccus group I

"A" data: average of square roots of original colony counts

JAR	DAYS		
	1	2	3
C 3	17.75 (3)*	12.63 (1)	16.75 (5)
C 9	17.65 (5)	13.52 (3)	17.59 (1)
C 7	17.13 (1)	13.42 (5)	16.91 (3)
C 1	16.24 (5)	12.91 (1)	17.12 (3)
C 6	16.46 (1)	13.35 (3)	16.15 (5)
C 4	15.75 (3)	13.99 (5)	14.12 (1)

Averages for numbers of catalysts: 1 catalyst = 15.14
3 catalysts = 15.73
5 catalysts = 15.70
Standard error = 0.356

Analysis of Variance

Factor	Degrees of freedom	Sums of squares	Mean square	Variance ratio (F)	Significance**
Days	2	44.866	22.433	29.52	very highly significant
Jars	5	4.578	0.916	1.21	not significant
Catalysts	2	1.330	0.665	0.87	not significant
Residual	8	6.081	0.760		
Total	17	56.855			

* Figures in parentheses represent number of catalyst sachets in the stated jar.

** See Appendix D for explanation of convention adopted.

Effect of varying numbers of catalyst sachets on the recovery of an Anaerobic coccus Group I

"B" data: logarithm of variance of square root of colony count on

JAR	DAYS		
	1	2	3
C 3	0.7176 (3)*	1.5049 (1)	0.6058 (5)
C 9	1.4493 (5)	0.7445 (3)	-0.6546 (1)
C 7	1.8140 (1)	0.3825 (5)	0.8512 (3)
C 1	1.1455 (5)	1.3114 (1)	0.9730 (3)
C 6	1.6440 (1)	1.5594 (3)	0.4466 (5)
C 4	1.5151 (3)	1.2465 (5)	0.3083 (1)

Averages for numbers of catalysts: 1 catalyst = 0.988
3 catalysts = 1.060
5 catalysts = 0.879
Standard error = 0.230

Analysis of Variance

Factor	Degrees of freedom	Sums of squares	Mean square	Variance ratio (F)	Significance**
Days	2	2.960	1.480	4.67	Significant
Jars	5	0.915	0.183	0.58	not significant
Catalysts	2	0.099	0.050	0.16	not significant
Residual	8	2.533	0.317		
Total	17	6.507			

* Figures in parentheses represent number of catalyst sachets in the stated jar.

** See Appendix D for explanation of convention adopted.

Effect of varying numbers of catalyst sachets on the recovery of Cl. tetani type VI

"A" data: average of square roots of original colony counts

JAR	DAYS		
	1	2	3
C 7	15.06 (3)*	13.83 (1)	13.80 (5)
C 2	15.39 (1)	13.28 (5)	14.07 (3)
C 1	15.60 (5)	13.37 (3)	13.58 (1)
C 8	15.22 (1)	14.18 (3)	13.65 (5)
C 4	15.26 (5)	14.08 (1)	14.34 (3)
C 6	14.68 (3)	14.04 (5)	13.76 (1)

Average for number of catalysts: 1 catalyst = 14.31
3 catalysts = 14.28
5 catalysts = 14.27
Standard error = 0.18

Analysis of variance

Factor	Degrees of freedom	Sums of squares	Mean square	Variance Ratio (F)	Significance**
Day	2	7.248	3.624	19.48	very highly significant
Catalyst	2	0.004	0.002	0.34	not significant
Jar	5	0.314	0.063	0.01	not significant
Residual	7	1.304	0.186		
Total	16	8.870			

* Figures in parenthesis represent number of catalyst sachets in the stated jar.

** See Appendix D for explanation of convention adopted.

Effect of varying numbers of catalyst sachets on the recovery of Cl. tetani type VI

"B" data: logarithm of varience of square root of count on each plate

JAR	DAYS		
	1	2	3
C 7	-0.6777 (3)*	0.0136 (1)	-1.7608 (5)
C 2	-0.2843 (1)	0.2473 (5)	-0.6356 (3)
C 1	0.2391 (5)	-1.7004 (3)	-0.4604 (1)
C 8	-0.4318 (1)	-0.4840 (3)	-0.8331 (5)
C 4	-0.5086 (5)	-0.8025 (1)	-0.3274 (3)
C 6	-0.0117 (3)	-0.5937 (5)	-0.4001 (1)

Average for number of catalysts: 1 catalyst = -0.4017
3 catalysts = -0.6395
5 catalysts = -0.5350
Standard error = 0.28838

Analysis of variance

Factor	Degrees of freedom	Sums of squares	Mean square	Variance ratio (F)	Significance**
Days	2	0.755	0.377	0.76	not significant
Catalysts	2	0.143	0.071	0.14	not significant
Jar	5	0.576	0.115	0.23	not significant
Residual	8	3.493	0.499		
Total	17	4.967			

* Figures in parentheses represent number of catalyst sachets in the stated jar.

** See Appendix D for explanation of convention adopted.

Effect of varying numbers of catalyst sachets on the recovery of Bacteroides fragilis

"A" data: averages of square roots of original colony counts

JAR	DAYS		
	1	2	3
C 7	16.54 (3)*	17.10 (1)	18.80 (5)
C 2	16.08 (1)	17.52 (5)	18.67 (3)
C 1	16.41 (5)	17.66 (3)	18.10 (1)
C 8	16.08 (1)	17.15 (3)	18.36 (5)
C 4	15.83 (5)	17.65 (1)	18.20 (3)
C 6	15.95 (3)	18.18 (5)	18.97 (1)

Averages for numbers of catalysts:
1 catalyst = 17.33
3 catalysts = 17.36
5 catalysts = 17.52
Standard error = 0.15

Analysis of Variance

Factor	Degrees of freedom	Sums of squares	Mean square	Variance ratio (F)	Significance**
Days	2	16.969	8.484	60.17	very highly significant
Jars	5	0.501	0.100	0.71	not significant
Catalysts	2	0.118	0.059	0.42	not significant
Residual	8	1.125	0.141		
Total	17	18.714			

* Figures in parentheses represent number of catalyst sachets in the stated jar.

** See Appendix D for explanation of convention adopted.

Effect of varying numbers of catalyst sachets on the recovery of *Bacteroides fragilis*

"B" data: logarithm of variance of square root of colony count on each plate

JAR	DAYS		
	1	2	3
C 7	-0.8907 (3)*	-0.9286 (1)	-0.6237 (5)
C 2	-0.8094 (1)	0.1227 (5)	-0.8836 (3)
C 1	-1.2613 (5)	-0.1436 (3)	0.7739 (1)
C 8	0.1412 (1)	-0.0743 (3)	-0.1322 (5)
C 4	-0.7580 (5)	-0.3209 (1)	-1.8418 (3)
C 6	-0.1496 (3)	-1.2046 (5)	-0.5921 (1)

Average for numbers of catalysts: 1 catalyst = -0.289
3 catalysts = -0.664
5 catalysts = -0.643
Standard error = 0.287

Analysis of Variance

Factor	Degrees of freedom	Sums of squares	Mean square	Variance ratio (F)	Significance**
Days	2	0.119	0.059	0.12	not significant
Jars	5	1.957	0.391	0.79	not significant
Catalysts	2	0.532	0.266	0.54	not significant
Residual	8	3.941	0.493		
Total	17	6.548			

* Figures in parentheses represent number of catalyst sachets in the stated jar.

** See Appendix D for explanation of convention adopted.

Effect of varying numbers of catalyst sachets on the recovery of total anaerobes from a sample of human faeces

"A" data: average of square roots of original colony counts

JAR	DAYS		
	1	2	3
C 2	21.57 (5)*	19.09 (3)	12.62 (1)
C10	20.38 (3)	18.87 (1)	13.88 (5)
C 5	20.17 (1)	20.45 (5)	20.50 (3)
C 9	23.78 (3)	19.42 (5)	20.78 (1)
C 1	21.91 (5)	18.52 (1)	20.02 (3)
C 3	21.85 (1)	19.79 (3)	19.42 (5)

Average for number of catalysts:
1 catalyst = 18.80
3 catalysts = 20.60
5 catalysts = 19.44
Standard error = 0.824

Analysis of Variance

Factor	Degrees of freedom	Sums of squares	Mean square	Variance ratio (F)	Significance**
Days	2	42.607	21.304	5.23	significant
Jars	5	34.145	6.829	1.68	not significant
Catalysts	2	9.906	4.953	1.22	not significant
Residual	8	32.594	4.074		
Total	17	119.252			

* Figures in parentheses represent number of catalyst sachets in the stated jar.

** See Appendix D for explanation of convention adopted.

Effect of varying numbers of catalyst sachets on the recovery of total anaerobes from a sample of human faeces

"B" data: logarithm of variance of square roots of colony counts on each plate

JAR	DAYS		
	1	2	3
C 2	1.4837 (5)*	1.4390 (3)	-0.4574 (1)
C10	0.8521 (3)	-0.1476 (1)	-1.4114 (5)
C 5	0.6953 (1)	0.1112 (5)	0.5950 (3)
C 9	0.9853 (3)	0.0290 (5)	-0.1250 (1)
C 1	1.0666 (5)	0.3015 (1)	0.0578 (3)
C 3	1.0159 (1)	-0.0681 (3)	-0.0681 (5)

Averages for numbers of catalysts:
1 catalyst = 0.214
3 catalysts = 0.644
5 catalysts = 0.202
Standard error = 0.192

Analysis of Variance

Factor	Degrees of freedom	Sums of squares	Mean square	Variance Ratio (F)	Significance**
Days	2	4.751	2.375	10.70	highly significant
Jars	5	1.803	0.361	1.64	not significant
Catalysts	2	0.760	0.380	1.71	not significant
Residual	8	1.775	0.221		
Total	17	9.089			

* Figures in parentheses represent number of catalyst sachets in the stated jar.

** See Appendix D for explanation of convention adopted.

The effect of varying thicknesses of solid media on the recovery of Cl. tetani type VI

Averages of original colony counts

JAR	Media*			
	A	B	C	D
1	142.0	196.5	235.5	203.5
2	135.0	213.0	236.0	229.5
3	168.0	203.0	236.5	178.0
4	130.5	189.0	226.5	203.5
5	168.5	181.0	146.0	193.5
6	160.0	211.5	176.0	237.5

Analysis of Variance

Factor	Degrees of freedom	Sums of squares	Mean square	Variance ratio (F)	Significance**
Media	3	27638.409	9212.803	6.958	highly significant△
Jars	5	4664.780	932.956	0.705	not significant
Media x Jars	15	19864.091	1324.273	0.92	not significant
Residual	24	34464.000	1436.000		
Total	47	86631.280			

* A = 8 ml medium per plate; B = 12 ml medium per plate; C = 15 ml medium per plate; D = 20 ml medium per plate.

** See Appendix D for explanation of convention adopted.

△ Medium A gave significantly lower recovery than that obtained from other three media.