THE SEPARATION AND ESTIMATION OF MICROBIAL POPULATIONS USING BIOCHEMICAL AND BIOPHYSICAL TECHNIQUES

By


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SUMMARY

Investigation of the adsorption of micro-organisms to ion exchange resins revealed two types of interaction. In the approximate range pH 1 - 5 adsorption was not related to the ionogenic groups of the resin. At higher pH values (5 - 9) a more specific interaction occurred which was dependent upon the ionogenic groups of the resin and the ionic composition of the suspending medium.

The specific adsorption of *Staphylococcus aureus* and of yeasts to strongly basic anion exchange resin was progressively inhibited by increasing salt concentration. A large proportion of the adsorbed organisms was recovered in a viable condition by elution with salts solutions (0.2 - 0.6M).

The specific adsorption of micro-organisms to cation exchange resins did not occur from distilled water but organisms were strongly adsorbed from dilute salt suspensions (0.05M). Adsorbed organisms were eluted in a viable condition by alteration of the pH and ionic composition of the medium. Novel methods were demonstrated for the chromatographic resolution of mixtures of *Staph. aureus* and *E. coli* by differential adsorption or elution using small columns (1g) of cation exchange resin.

The mechanism by which cells adhere to ion exchange resins was investigated. Two major cell surface components, teichoic acid and lipopolysaccharide, were shown not to be involved in the adsorption of cells to anion exchange resin.
Cell surface protein was implicated in the adsorption of
Staph. aureus to both types of resin. The blockage of cell surface
cationic groups (e.g. amino) inhibited adsorption to cation exchange
resin whilst the blockage of anionic groups (e.g. carboxyl) promoted
adsorption.

The separation of micro-organisms from foods was demonstrated using
both anion and cation exchange resins. The recovery of raw meat
flora from anion exchange resin (20%) was much lower than that from
cation exchange resin (75%).

Two rapid methods for the estimation of micro-organisms in foods were
investigated. An inverse linear relationship was found between
numbers of micro-organisms and impedimetric response which permitted
the rapid estimation of micro-organisms in two foodstuffs. The rapid
estimation of micro-organisms in meat by the photometric estimation
of microbial ATP was achieved only after separation of the micro-
organisms from the meat.
TO

SUSANNE, LARA AND MICHELLE
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PART I

BACKGROUND
Ch. 1: GENERAL INTRODUCTION
The application of analytical techniques to the separation and estimation of biological materials has for several decades supported an increasing rate of progress in the biological sciences. Separation techniques such as the many forms of chromatography and electrophoresis (ion exchange and molecular sieving) in combination with estimation techniques employing for example spectrophotometric measurement, radio-assays and immunological assays, provide the biochemist with a range of sophisticated tools to aid his investigations.

Against this background of sophisticated technology, the methods currently used for the analysis of viable microbial populations have changed very little since the beginning of the century. The major reason for this apparent conservatism can be appreciated by examining the performance of these long established cultural techniques for the estimation of viable cells.

A single microbial cell has a mass of approximately $10^{-13}$ g (Strange, 1972) which is below the detection limit of all but the most sensitive analytical instruments. However, when a viable cell is placed upon a suitable growth medium in an appropriate environment it will produce a colony of cells visible to the naked eye, usually within 1 - 3 days. It is this sensitivity of detection combined with freedom from interference by non-microbial materials, low cost and the technical simplicity of cultural techniques which has ensured their continued use by generations of microbiologists.

These advantages of the traditional cultural methods are countered by the labour intensive and tedious nature of their practice and more
importantly by the lengthy period of incubation of the tests which
must elapse before a result is obtained. Delays of one to three days
may be of critical importance in medical and industrial microbiology
and in research may frequently be the rate limiting factor in the
progress of an investigation.

In recent years attempts have been made to overcome these disadvantages
of the traditional cultural techniques. One such approach has been to
reduce the labour requirement of the methods by mechanization or
miniaturization of the various stages of the cultural technique
(Thompson, et al., 1960; Fung, 1969; Sharpe and Kilsby, 1971; Kramer,
1977) and has resulted in useful devices e.g. the Spiral Plate Maker

A second approach has sought to reduce the lengthy periods which must
elapse before a result is obtained by cultural methods. Reports of
the application of sensitive analytical techniques to the estimation of
micro-organisms e.g. ATP measurement (Sharpe, Woodrow and Jackson, 1970)
Impedance measurement (Ur and Brown, 1973) Radiometry (Previte, et al.,
1974) and Micro-calorimetry (Lampi, et al., 1974) have demonstrated the
potential of such techniques for the rapid estimation of microbial
populations.

The introduction of these sensitive and rapid analytical techniques
would represent a significant advance in microbiological methodology.
All of the techniques are however less sensitive than the cultural
methods and in addition may be subject to interference from non-
microbial materials in the test sample e.g. ATP in foods (Williams, 1971).
In other areas of science these problems are commonly overcome by the
separation and concentration of the specific material to be estimated. The lack of suitable separation and concentration techniques within microbiology reflects the dominance of cultural methods of estimation in which the micro-organisms behave as self-amplifying systems and other (non-replicating) biomaterials are normally of little significance.

The development of a simple and rapid technique for the separation of original microbial populations from other bio-materials would assist the introduction of rapid analytical techniques within microbiology. It would also provide a valuable tool which might permit microbiologists to isolate and study original microbial populations rather than the progeny of these populations resulting from culture in artificial media. Recent evidence (Costerton, Geesey and Cheng, 1978) has indicated that substantial differences can exist between such populations.

The objectives of the present study were to investigate rapid methods for the estimation of microbial populations and to develop methods for the separation of micro-organisms from natural environments. The areas of investigation chosen for the study were the use of ion exchange resins for the separation of micro-organisms from foods and rapid estimation of these organisms by sensitive analytical techniques. The major question under consideration was whether an alternative and more rapid form of microbiological methodology could be found which would extend the techniques currently available in this area of science.
Ch. 2: SURVEY OF THE LITERATURE
2. SURVEY OF THE LITERATURE

The area covered by the present study includes aspects of several major fields of scientific knowledge, viz:

- The structure and physical properties of microbial cell walls
- The separation of micro-organisms from other biomaterials
- The adsorption of microbial cells to ion exchange resins
- The rapid estimation of microbial populations

These main fields of knowledge, aspects of all of which have an important bearing on the present study, are briefly surveyed below.

2.1 The structure and physical properties of microbial cell walls

The protoplasm of the microbial cell is enclosed within a semi-permeable, lipid membrane commonly termed the plasma membrane, protoplasmic membrane or cytoplasmic membrane. External to this osmotically fragile membrane is the cell wall which comprises all of the structures external to the plasma membrane with the exception of the various surface appendages i.e. flagellae, pili and fimbriae (Hodgkiss, Short and Walker, 1976). The term 'cell wall' is occasionally and wrongly used as a synonym for cell surface. The cell walls of Gram negative bacteria are between 100 and 150Å thick, those of Gram positive bacteria are between 100 and 500Å (Glauert and Thornley, 1969) and those of yeasts are of even greater thickness (Bowden and Hodgson, 1970). None of these structures is homogenous.
and it is clear that in each case only a small part of the total structure will form the surface.

The microbial cell wall has been a major topic of research in microbial biochemistry for almost a quarter of a century and the current extent of knowledge in this field is detailed in several excellent reviews (Braun and Hankke, 1974; Costerton et al, 1974; Salton and Owen, 1976; Dirienzo et al, 1978). The cell walls of Gram positive bacteria, Gram negative bacteria and of yeasts show major differences in both composition and structure. A brief summary of the main components is given here in order to permit an appreciation of the chemical structures underlying and possibly contributing to the cell surface.

The composition of bacterial cell walls

The component thought to be responsible for the mechanical strength of the bacterial cell wall is peptidoglycan which consists of glycan strands comprised of repeating units (DP > 100) of β1 - 4 N-acetyl glucosamine and β1 - 4 N-acetyl muramic acid and which are cross-linked by short peptide side chains. The composition, mode and frequency of cross-linkages may vary for different bacterial species (Schliefer and Kandler, 1972) but link the glycan strands into a three dimensional, covalently bonded network which extends around the cell (Keleman and Rogers, 1971). Bacterial peptidoglycans appear to be less densely packed in the hydrated state than are celluloses and chitins (Ou and Marquis, 1970) suggesting a more open and flexible structure than that conferred by cellulose or chitin.
Peptidoglycan is absent from some halobacteria and from protoplasts, spheroplasts and L-forms but otherwise is present in all bacteria so far studied (Glauert and Thornley, 1969). It lies immediately external to the plasma membrane and forms a small proportion, 5 - 10 per cent, of the wall of Gram negative bacteria but a much larger proportion of the wall of Gram positive bacteria, being as high as 80 - 90 per cent in *Micrococcus lysodeikticus*.

**The Gram negative cell wall**

In addition to peptidoglycan the walls of Gram negative bacteria usually contain protein, lipid, lipoprotein and lipopolysaccharide. The structure of the cell walls of Gram negative bacteria is more complex than that of Gram positive bacteria. Glauert and Thornley (1969) describe the Gram negative cell wall as a multi-layered structure bounded by an inner membrane (the plasma membrane) and an outer membrane, between which may be seen intermediate layers. The most prominent of the latter was the dense intermediate layer which contained peptidoglycan and lay adjacent to the plasma membrane. The freeze-fracture surfaces of these various layers have been demonstrated by electron microscopy (Glauert, et al., 1976).

Costerton et al., (1974), in reviewing the structure and function of the Gram negative cell wall describe both the inner and outer membranes as phospholipid bilayers and the space between them as the periplasmic space within which are located degradative enzymes. Braun (1975) demonstrated that a specific lipoprotein 120 - 140Å long and containing 57 amino acid residues is covalently linked via lysine to the peptidoglycan of several enteric bacteria in such a way that it extends towards the outer membrane. Schnaitman (1974) proposed that
the lipid component of this molecule served to anchor the outer membrane by hydrophobic interaction with the outer layer. Biophysical studies of the outer membrane (Forge and Costerton, 1973; Forge, Costerton and Kerr, 1973) have indicated that the basic continuum is formed by phospholipids and proteins and that the oligosaccharide portions of the lipopolysaccharide appear to be associated with the inner and outer surfaces. Costerton et al. (1974) have suggested that the external surface of the Gram negative cell consists of a protruding pattern of oligosaccharide moieties of the lipopolysaccharide, between which proteins and phospholipids are exposed to a greater or lesser degree. More recent investigations of the outer membranes of Gram negative organisms (Schnaitman, 1974; Rosenbusch, 1974; Hiddenach and Henning, 1975) have revealed the presence of up to five small proteins (M wt <40,000 daltons) which occur in relatively high concentrations and may be considered as integral membrane proteins. It has been demonstrated that some of these proteins have a role in the uptake of an iron complex by E. coli and also serve as receptors for bacteriophage and colicins. Other receptor proteins in the outer membrane are involved in the uptake of malto-dextrins, nucleosides and vitamins (Braun, 1978).

The Gram positive cell wall

In contrast with Gram negative bacteria, the cell walls of Gram positive bacteria do not have a layered structure (Glauert and Thornley, 1969) and, among different genera and species, show great variation in both the occurrence and quantity of components other than peptidoglycan. These frequently include large negatively charged
polymers e.g. teichoic acids (Baddiley, 1972); polysaccharides and proteins.

Glauert and Thornley (1969; 1976) have described protein structures in regular patterns which form the surface layer of the cell walls of several Gram positive bacteria. These workers have suggested that the occurrence of protein in the walls of Gram positive bacteria may be more widespread than had been realised because of the prevalence of methods of wall preparation involving digestion with proteolytic enzymes (Ghuysen, Strominger and Tipper, 1968; Nermut and Murray, 1961). This point was also made by Rittenhouse, Rodda and McFaddean (1973), who isolated immunologically cross reacting proteins from the cell walls of a range of microbial species including several Gram positive bacteria. The presence of proteins on the cell surface of certain well studied Gram positive cocci e.g. Protein A of *Staphylococcus aureus* (Forsgren and Sjöquist, 1969) and the M protein of streptococci (Fox, 1974) was established by immunological techniques. Such proteins may possibly form a very small proportion of the whole cell wall but a high proportion of the cell surface.

The yeast cell wall

The composition of the yeast cell wall differs considerably from that of bacteria. The major structural components are mannan, glucan and protein; much of this material is in the form of glycoprotein but there is no evidence for the presence of peptidoglycans similar to those present in bacterial cell walls (Lampen, 1968). Of the cell-bound external enzymes, several e.g. invertase and acid phosphatase
are mannan proteins. The cell wall protein contains a full complement of amino acids with a large proportion of glutamic and aspartic acids and is characterized by a high sulphur to protein ratio (Matile et al., 1969). Other components of the wall are lipid, phosphate and hexosamine. Hexosamine forms a few per cent of the cell wall and only one tenth or less is in the form of chitin (Bacon et al., 1966), the rest is probably a component of the glycoprotein and serves as part of the link between protein and carbohydrate (Sentandreu and Northcote, 1968).

Structure

Investigation of the gross structural organization of the yeast cell wall (Bowden and Hodgson, 1970) by histochemical techniques revealed an overall thickness of 1,400 Å comprised of an outer electron dense region of 320 Å and an inner electron dense region of 250 Å, separated by a relatively less electron dense region of 800 Å.

The chemical structure of the wall has been investigated by many workers and the model proposed by Lampen (1968) embodies many of these observations. The outer layer of the wall is constructed of large mannan molecules (M. wt. >100,000) held together by 1–6 phosphodiester bonds. This observation is supported by immunochemical studies in which mannan specific antibodies have been shown to agglutinate whole cells (Hansenclever and Mitchell, 1964; Ballou, 1970). Whether this mannan completely covers the surface is unknown (Ballou, 1976). Immediately below this surface layer are the mannan–protein enzymes and additional phosphodiester linked mannan which surrounds the enzymes and retains them within the wall.
Below this is a region containing mannan, glucan and proteins, probably as glycoprotein (Northcote, 1963) this appears to be linked to the glucan fibrils which constitute the rigid lattice that gives the cell wall its characteristic shape. In this inner stratum of the cell wall the mannan-proteins and glucan-proteins are localised, possibly linked together by disulphide bridges (Nickerson, 1963). The observation that thiols sensitize yeast cell walls to the action of snail enzyme has led to the suggestion (Bacon et al., 1966) that disulphide bonds link protein molecules in the wall to produce a lattice structure which adds to the mechanical strength of the wall. Matile et al. (1969) point out that Lampen's model is valid only in regard of the cell walls of species of Saccharomyces and that the organization of the cell wall in Candida utilis differs in having glucan rather than mannan localized at the periphery.

More recently, observations on the topography of flocculent and non-flocculent strains of Saccharomyces spp (Stewart, 1975) has shown the presence of an extensive layer of extracellular protuberances ('hairs' or fimbriae) on the surface of flocculent strains which were absent from the surface of non-flocculent strains. The removal of these structures from the surface of the flocculent yeasts rendered them non-flocculent and also changed their calcium binding pattern to that typical of non-flocculent yeasts (Stewart, et al., 1975). The authors proposed that these structures were 'glyco-peptidic' and were the sites of calcium bridging of cells during flocculation. This description of surface appendages closely resembles that of Costerton Geesey and Cheng (1978) who proposed that a mass of hair like projections, which they termed the 'glycocalyx', were involved in the attachment of bacteria to solid surfaces in nature.
The microbial cell surface

The chemical composition and structure of the microbial cell wall reveals something of the types of molecule structure which may be present at the cell surface, e.g. teichoic acids, lipopolysaccharide, protein. A more detailed picture of the chemical groups present and of the properties which they confer has been obtained from other studies.

Electrophoretic investigations

Microbial cells can be considered as colloidal particles and, if allowance is made for the fact that their surfaces are not impenetrable to ions, can be studied by electrophoresis (Haydon, 1961). The use of this technique for the study of micro-organisms has been reviewed by Richmond and Fisher (1973) and has indicated that the electrical charge of the cell surface varies with the environmental conditions. At pH values below their iso-electric points the cells behave as cations and are capable of reacting with anionic materials e.g. acid dyes (Harris, 1949) and anionic polyelectrolytes (Busch and Stumm, 1968). At pH values above their iso-electric points they behave as anions and will react with cationic materials e.g. basic dyes (Lehman, 1964), cationic polyelectrolytes (Tenney and Stumm, 1965). Neihoff and Echols (1973) investigated the effects of pH and ionic strength of the suspending medium on the electrophoretic mobility of several microbial species. An electronegative charge dominated the cell surface producing a negative mobility of similar magnitude for most of the species. Mobility varied little with pH over the range 5 – 9 but below this
range decreased sharply and for some species became positive at pH values below 3.0. Saccharomyces cerevisiae had a lower electrophoretic mobility than did the bacteria and this varied little over the range pH 2–12. Increase in ionic strength of the medium decreased the mobility of all the species examined.

The nature of the microbial cell surface, as revealed by cell electrophoresis has been investigated by several workers. Haydon (1961) found that the electrophoretic behaviour of many microorganisms was consistent with a cell surface composed of large areas of non-ionogenic material interspersed with a small number of ionogenic groups. Schott and Young (1972) found that carboxyl groups were responsible for the negative surface charge of Strep. faecalis. The charge density of the surface was reported to be one carboxyl group per (48 Å)². For E. coli the equivalent area was (38 Å)², the total surface area of this organism being 3.10⁸ Å². This data for E. coli was in agreement with the earlier results of Gittens and James (1963). Carboxyl groups were also identified as the major ionisable groups on the surface of cells of Bacillus megatherium (Douglas, 1957; Neihoff and Echols, 1973), although the low apparent pK of isolated cell walls of this organism suggested an admixture of a stronger acidic group, possibly phosphate. Results obtained for Saccharomyces cerevisiae (Eddy and Rudin, 1958; Neihoff and Echols, 1973) supported the conclusion that phosphodiester groups are present on the surface of this organism and account for the retention of negative charge (negative electrophoretic mobility) at very low pH values.
The structures with which the ionisable groups on cell surfaces are associated have been demonstrated. James and Brewer (1968a) reported that maxima in the pH-mobility curves of several strains of Staph. aureus were due to teichoic acid and that after mild periodate oxidation the maxima disappeared leaving a smooth sigmoid curve typical of a carboxyl or carboxyl-amino surface. It was also demonstrated (James and Brewer 1968b) that the presence of protein on the cell surface of strains of Staph. aureus of human origin conferred a high negative mobility. Trypsin treatment of these cells resulted in pH-mobility curves similar to those of strains isolated from animals and which possessed no surface protein. In contrast Rutter and Abbot (1978) found that trypsin treatment had little effect upon the electrophoretic mobility of Strep. salivarius or Strep. mitior but did increase the tendency of Strep. salivarius to deposit on a glass surface.

Sherbet and Lakshmi (1973) employed isoelectric focussing of microorganisms in combination with specific chemical treatments to investigate the surface groups of E. coli. They concluded that the ionisable groups occurring in the iso-electric zone were carboxyl and amino groups of the polysaccharide-phospholipid-protein complex (outer membrane) and that the iso-electric zone extended to a depth of 60 Å below the cell surface. The reactive groups of the phospholipids were not detected and the authors concluded that these lay at a depth of more than 60 Å from the surface. Heckels et al. (1976) used a similar method to demonstrate that the amino and carboxyl groups on the surface of cells of Neisseria gonorrhoeae were associated with protein.
Investigations of the nature of the charged groups on the cell surfaces of yeasts have been largely concentrated on the genus *Saccharomyces* and directed towards an understanding of the mechanism of flocculation.

Eddy and Rudin (1958) studied the electrophoretic mobility of different strains of *Saccharomyces* and noted that the component of mobility due to protein was less prominent with young cells than with old; the reverse was true for mobility attributable to phosphate. They found no simple relationship between flocculation and overall surface charge. Fisher (1975) investigated changes in surface ionogenic groups immediately prior to flocculation and found differences in the pH-mobility curves of flocculent and non-flocculent yeasts. Flocculent yeasts from both logarithmic and stationary phases of growth showed a decrease in negative charge between pH 2 and 6 indicative of the re-arrangement of charged material, probably protein, at a depth below the cell surface of 320 - 420 Å. Non-flocculent yeasts from the logarithmic phase of growth had pH-mobility curves showing negative charge over the range pH 2 - 10, indicating highly acidic surfaces. Cells harvested during the stationary phase of growth carried a positive charge at low pH values; the form of the curves was consistent with an amino-carboxyl (protein) surface. Jaytissa and Rose (1976) demonstrated the role of cell surface carboxyl groups in calcium bridge formation during flocculation, and presented evidence against the involvement of phosphate groups in this process. It should be noted however that the treatment used by Jaytissa and Rose to excise phosphodiester linkages from the cell walls was harsh i.e. exposure at 0°C to 60% (v/v) hydrofluoric acid for 3 - 5 h.
Sugano, et al. (1975) demonstrated that amino groups in the cell surface protein of sake yeast participated in the aggregation of the yeasts with cellulose.

**Ion exchange and ion binding properties**

Other physico-chemical properties of cell walls have been investigated by a number of workers.

The cation binding properties of the cell wall of *Staph. aureus* were demonstrated by Cutinelli and Galidero (1967). Approximately 14 micro-equivalents of K\(^+\) or Na\(^+\) and 85 micro-equivalents of Mg\(^{++}\) or Ca\(^{++}\) were bound per gram of cell wall (dry wt.). Ion binding capacity varied with pH. High pH levels increased the amounts of ions bound and this was more pronounced for the divalent ions. These data suggested that the cell wall behaves like a weak ion exchange resin.

Carstensen and Marquis (1968) concluded from a study of the dielectric properties of the cell wall of *Micrococcus lysodeikticus* that cell wall associated ions play a major role in the conduction of low frequency electric current by intact bacterial cells, and that in some instances the concentration of mobile counter ions in the wall exceeded the concentration in the medium by a factor of ten. Concurrent work, (Marquis, 1968), with intact cells of *Bacillus megatherium* demonstrated a physical contraction of these cells when they were transferred from water to unbuffered, non-plasmolysing sodium chloride solutions. The cell walls behaved as flexible, amphoteric polyelectrolytes and their compactness in solution varied...
with environmental pH and ionic strength. Isolated walls were most compact in low ionic strength media at a pH of about 4, a value close to the apparent iso-electric point of the cell wall peptidoglycan. These electromechanical interactions were further studied by Ou and Marquis (1970) who concluded that Gram positive bacterial cell walls have many of the properties of polyelectrolyte gels and that the expansion and contraction of the wall with changes in pH and ionic strength were due to electrostatic interactions between fixed charges on the cell wall polymers e.g. peptidoglycan and teichoic acids. Of the anionic groups in the cell wall of *Staph. aureus* more than 50% were teichoic acid phosphates and of the cationic groups nearly 50% were teichoic acid D-alanyl amino groups. In *Micrococcus lysodeikticus* nearly all of the anionic groups appeared to be peptidoglycan carboxyl groups. The electrostatic interactions of these fixed groups with each other and with mobile counter ions were said to vary with environmental pH and ionic strength, producing forces of attraction and repulsion which resulted in changes in wall volume.

Marquis, Mayzel and Carstensen (1977) determined the affinity of cell walls of a number of Gram positive bacteria for different cations. The affinity series established was $H^+ >> La^{3+} >> Cd^{2+} >> Sr^{2+} >> Ca^{2+} >> Mg^{2+} >> K^+ >> Na^+ >> Li^+$. High affinity was correlated with low mobility of bound ions in an electric field. The cation selectivity of the cell walls tested was similar to that of the commercial cation exchange resin Bio-Rex 70 - a weakly acidic resin containing carboxylic acid exchange groups on an acrylic polymer lattice and recommended for the resolution of peptides and proteins.
2.2 The separation of micro-organisms from other biomaterials

The most commonly used laboratory methods for the separation of micro-organisms from other bio-materials are centrifugation and filtration. Methods are described in most texts on practical microbiology and are particularly well treated by Thompson and Foster, (1970) for centrifugation and Mulvaney (1969) for membrane filtration. Both methods are normally employed for gross separation of micro-organisms from suspension in water, culture media or clinical fluids. The major problem encountered in the use of filtration is the blockage (clogging) of the filter. Mulvaney (1969) has suggested various sample pre-treatments which may overcome this problem when it is encountered in clinical fluids. Neither filtration nor centrifugation is very suitable for the separation of micro-organisms from homogenates of solid materials (e.g. biological tissues) however the use of density gradient centrifugation for the separation of different microbial forms has been demonstrated (Lahita and Saukkonen, 1973). Normal rod forms of E. coli were separated from filamentous forms by high speed centrifugation (25,000 X g) in a Ficoll (sucrose polymer) gradient. The method was sufficiently gentle to permit the survival of the osmotically fragile filamentous forms.

Other methods for the separation of micro-organisms from bio-materials or for the physical separation of microbial species exploit the surface properties of the micro-organisms.

Flocculation

The most common commercial process for the gross separation of micro-organisms from aqueous suspension is flocculation, forms of
which are practiced on a massive scale in the wine, brewing, water and sewage industries. A wealth of literature exists describing many aspects of flocculation (see reviews by Rainbow, 1970; Harris and Mitchell, 1973; Stewart, 1975). However, the mechanism of this phenomenon has not been fully elucidated (Jaytissa and Rose, 1976) and the application of flocculation in the fine separation of microorganisms has not been investigated. The conclusions of Busch and Stumm (1968) and of Harris and Mitchell (1973; 1975) that synthetic as well as natural polymers can form bridges between cells and that the resultant aggregation of cells is dependent upon factors such as particle size, polymer concentration, pH and ionic strength, suggest that conditions may exist in which fine separations might be achieved.

**Flotation**

Foam flotation techniques for the separation and concentration of bacteria from suspension were first demonstrated by Dogon (1941) for *Mycobacterium tuberculosis*. Boyles and Lincoln (1958) separated and concentrated bacterial spores and vegetative cells from liquid media and Gaudin, et al., (1962) demonstrated the importance of inorganic salts in the flotation of bacterial cells. Rubin et al., (1966) described a microflotation technique for the separation of bacteria and algae from suspension.

In recent years several methods previously applied in other areas of science to the separation and characterization of non-living materials have been applied to the separation of different types of microbial cells.
Counter-current distribution

Stendhal (1973) described the use of counter-current distribution for the partition of cells of *Salmonella typhimurium* which differed in their liability to phagocytosis. This technique was also used by Per Albertsson (1974) who achieved a partial separation of a yeast, an algae and a bacterium in a liquid-liquid system based on solutions of dextran and polyethylene glycol. The separated cells were viable.

Electrophoresis and iso-electric focussing

The electrophoresis of microbial cells has long been used as an analytical technique in the investigation of cell surface charge and related phenomena. (See review by Richmond and Fisher, 1973). The exploitation of surface charge for the preparative separation of microbial species was reported by Hannig (1971) who described the use of continuous free flow electrophoresis for the separation of *Pseudomonas fluorescens* from *Serratia marcescens*. Parent and mutant strains of *E. coli* could also be separated. These results were described as 'preliminary but reproducible' and it was noted that they provided 'convincing evidence for the constancy of the electrical charge of these biological particles'. The exploitation of cell charge for the separation of microbial cells was also investigated by Sherbet and Lakshmi (1973) who applied isoelectric focussing (isoelectric equilibrium analysis) to the characterization of different forms of *E. coli*; subsequently Heckels et al., (1976) applied this technique in an investigation of the influence of surface charge on the attachment of *Neisseria gonorrhoeae* to human cells. In both investigations organisms with
modified cell surfaces showed iso-electric points and elution volumes different from those observed for the original cells. Langton, Cole and Quin (1975) demonstrated the separation of cells of several species of oral micro-organisms by iso-electric focussing.

A major disadvantage of this technique in the separation of microbial species lies in the low iso-electric points of microbial cells. Harden and Harris (1953) found that the iso-electric points of 31 microbial species examined fell within the range 1.75 - 4.15. Exposure to these pH levels would be injurious to many microbial species.

2.3 The adsorption of micro-organisms to ion exchange resins

The adsorption of micro-organisms to anion exchange resin is a facet of the far broader field of adsorption of micro-organisms to solid surfaces. The extent and diversity of this broader field is indicated in a recent review by Daniels (1972) who tabulated over 70 types of solid surfaces capable of adsorbing micro-organisms. The micro-organisms involved included Protozoa, Fungi, Viruses and over 90 species of Bacteria.

Many active areas of this field such as the adsorption of micro-organisms to the roots of plants (Rovira, 1965) to animal cells (Jones, 1977) and to tooth surfaces (Gibbons and Van Houte, 1975) are of considerable practical interest. Attempts to explain the mechanisms involved (Curtiss, 1967, Marshall, 1976) are complicated by the fact that in each case two surfaces, each
with their unknowns must be considered.

For the purposes of the present study ion exchange resins offered the advantages of convenient form and defined composition and reports of the adsorption of micro-organisms to these materials have been considered.

The earliest reference to the adsorption of micro-organisms by ion exchange resins was by Barnes (1952) who observed that cells of *E. coli* were removed from suspension by anion exchange resin. The first detailed report was that of Puck and Sagik (1953) who compared the adsorption of bacteriophage to strong ion exchange resins with that of the virus to the host cell. At pH 7.0 phages T1 and T2 adsorbed to anion exchange resin from suspension in either distilled water or salt solution, but would adsorb to cation exchange resin only from salt solution. The host cells, *E. coli* also adsorbed to anion exchange resin but did not adsorb to cation exchange resin even in the presence of 200 mM sodium chloride. Subsequently Creaser and Taussig (1957) described the purification of bacteriophage using anion exchange cellulose which has since been extensively used in the purification of viruses (Klemperer and Pereira, 1959; Haruna, *et al.*, 1961; Wilson, 1962; Neurath, *et al.*, 1967).

Anion exchange cellulose has also been shown to adsorb micro-organisms from the other extreme of the spectrum of microbial size. Lanham and Godfrey (1970) demonstrated the adsorption of salivarian
trypanosomes (protozoans causing sleeping sickness in man and other mammals) to DEAE cellulose. Different species were adsorbed at different salt concentrations and retained their viability upon elution with higher salt concentrations.

Reports of the adsorption of bacteria to ion exchange resins are relatively sparse and are characterized by the broad geographical distribution of their origins and the diversity of systems studied.

The first detailed report was that of the Japanese workers Hattori and Furusaka (1959a) who adsorbed cells of *E. coli* from suspension in distilled water to the strongly basic resin Dowex 1 and observed the metabolic activity of the adsorbed cells. They noted a lower activity of adsorbed cells compared with free cells when glucose, lactose or alanine was the substrate. Later, (Hattori and Furusaka, 1959b) they noted that the adsorption of the cells raised the bulk solution pH optimum for succinate oxidation by one pH unit. Both effects were tentatively attributed to the presence of a cationic layer outside the anionic layer at the surface of the resin and sufficiently deep to cover the adsorbed cells.

Gillisen, et al., (1961) in Germany, demonstrated the adsorption of several species of bacteria including *Pseudomonas fluorescens*, *Staph. aureus*, *Bacillus subtilis* and *Bacillus anthracis* to anion exchange resin. They noted a clear contrast between the behaviour of anion and cation exchange resins, the latter showing negligible adsorption of these organisms.
The Russian worker Zvaginstev (1962) also demonstrated the adsorption of bacteria to strongly basic anion exchange resin and noted that adsorption was greater at pH 4.0 than at pH 6.0. In contrast with previous reports (see above) Zvaginstev (1962) also observed the adsorption of bacteria to cation exchange resin; not all species adsorbed and the exchangeable ion on the resin appeared to exert an influence in this system. The cells could not be removed from the resin by washing with water although some organisms e.g. Bacillus mycoides and a Sarcina sp., were eluted with 3% sodium hydroxide.

The separation of microbial species

The first investigation of the use of ion exchange resin for the separation of microbial species was reported by Kurozumi, Itoh and Shibata (1965). Mixtures of algae, yeasts and bacteria were applied to columns (30 cm long, dia. 0.85 cm) of strongly basic anion exchange resins and eluted with concentration gradients of various salts in solution. The resin bed was continuously stirred during elution. The increasing order of affinity of micro-organisms found for the resin Dowex 1 X 1 was: E. coli, Saccharomyces cerevisiae, Trigonopsis varabilis, wine yeast and Chlorella ellipsoidea. These workers monitored eluates from strongly anion exchange columns by particle counting and microscopy. They found complete or partial separations of several of the species examined but concluded that their results were '... not understandable from a simple theory of the exchange of ions on resin'.
In a later study Kurozumi (1969) demonstrated the adsorption of these same organisms to DEAE cellulose fabric but Hogg (1976) failed to demonstrate their adsorption to cation exchange resin.

The separation of bacteria on a cation exchange cellulose was reported by Zisgray et al., (1970) in the separation of donor and recipient mating types of E. coli. More than 80% of the recipient cells passed through a column of the exchanger but only 11% of the donor cells did so. This pattern of elution was not altered by changes in salt concentration or pH and the authors concluded that retention was due to the physical entanglement of filamentous appendages (pili) on the donor cells with the cellulose column rather than to ionic adsorption.

A further major study of the separation of microbial species by the use of ion exchange resins was carried out by Daniels and Kempe (1966). They were able to adsorb six bacterial species (Bacillus cereus, Bacillus subtilis, E. coli, Proteus vulgaris, Pseudomonas ovalis, and Staph. aureus) onto strongly basic anion exchange resin and to a lesser extent strongly acidic cation exchange resins. The rate and extent of binding was found to be affected by both the pH and the salt concentration of the suspending medium. Manipulation of these parameters permitted separations of microbial species by either selective adsorption or selective elution of the organisms. These workers explained their results in terms of an ion exchange process in which bacterial cells behaved as macroscopic zwitterions and were bound to either anion or cation exchange resins depending upon whether the pH of the suspending medium was, respectively, above or below the apparent iso-electric point of the cells. Elution was
accomplished either by reversing the charge on the cells (promoted by a change in pH) or by the addition of a salt e.g. potassium chloride, or by both. Elution frequently involved the lowering of the pH of the microbial suspensions to values at which the organisms would be killed e.g. to pH 3.5 or 1.5. This work was greatly enlarged but not substantially altered by Daniels (1968).

Zvyaginstev and Gusev (1971) demonstrated the differential elution from anion exchange resin of *Bacillus subtilis* and *Bacillus cereus* by use of a sodium chloride gradient in the stirred column system described by Kurozumi et al., (1965). Not all of the cells of *B. cereus* were recoverable. Eluted cells were estimated nephelometrically and microscopically; no attempt was made to determine their viability. These authors also demonstrated that esterification of the carboxyl groups in the cell walls of four species studied completely inhibited adsorption of the cells to the resin. They concluded that adsorption was attributable to the formation of electrostatic bonds between the quaternary ammonium groups on the surface of the resin and carboxyl groups on the cell surface.

More recently the Polish workers Kosinkievicz and Varonka (1975) investigated the adsorption of bacteria to a range of dextran based ion exchangers: cation exchangers Sephadex SE (strongly acidic), Sephadex SM (weakly acidic) and the anion exchanger Sephadex DEAE (basic). Cells did not adsorb to either of the cation exchangers, but the weakly acidic one was reported to have a selective affinity for the various microbial species since the components of mixtures of species emerged from columns of this exchanger at different elution
volumes. Organisms were adsorbed to anion exchange Sephadex but could not be eluted with either sodium chloride 1.0M, or Tris buffer at pH 9.0. Comparing these findings with those of Zvaginstev and Gusev (1971), the Polish workers concluded that the attachment of micro-organisms to Sephadex ion exchangers was dependent not only upon electrostatic binding but also upon gel porosity and the size and mobility of the microbial cells.

Hall et al., (1976) studied the elution of several species of Gram negative bacteria from columns of anion exchange cellulose (DEAE). Although some problems of filtration and agglutination were experienced, each of the organisms studied could be eluted in a characteristic manner. In phosphate buffer (50 mM, pH 6.0) cells of E. coli were eluted with 230 mM sodium chloride, Pseudomonas aeruginosa with 410 mM and Serratia marcescens with 395 mM sodium chloride. Cells of Salmonella typhimurium were eluted in a more complex manner; at least two and sometimes three fractions being eluted at different sodium chloride concentrations up to 1.0 M. A reproducible separation of E. coli and Salmonella typhimurium was reported, however less than half of the organisms recovered were viable. Hogg (1976) reported that in this system the pH value of the eluant, within the range pH 4 - 8, had no effect upon the binding of cells of E. coli to the resin. The possession of fimbriae (proteinaceous appendages of the cell surface) decreased the affinity of the cells for the resin. This latter observation was attributed to a lower surface charge density on the fimbriate cells although attempts to correlate adsorptive behaviour with electrophoretic mobility were unsuccessful. Attempts to adsorb a
Gram positive organism, Bacillus pumilis to this resin resulted in disruption of the columns.

An interesting report of the adsorption of streptococci to strongly acidic cation exchange resin has appeared only as an abstract of a paper to be presented to the American Society for Microbiology. Wallenstein (1975) reported that M-protein-bearing strains of streptococci were more readily adsorbed to Bio-Rad AG 5 OW than were strains not bearing this protein. Treatment of the former cells with trypsin or with homologous, but not heterologous, antiserum inhibited the adsorption of some strains. Details of this work are not available, the paper was not presented nor has the work been published in full and attempts to contact Wallenstein have been unsuccessful.

2.4 Methods for the rapid estimation of viable microbial populations in foods

Colony count techniques have provided the major methods of estimating microbial numbers since the earliest days of microbiology and still dominate this field. The success of these techniques is largely due to their sensitivity which derives from the amplifying power of microbial growth. A single bacterial cell on a suitable medium will grow to a colony detectable by the naked eye and this can be accomplished using inexpensive materials by simple practical methods.

The colony count techniques have two major disadvantages. Firstly the simple practical methods involve many manipulations e.g. serial dilutions of the sample and inoculation of the growth medium and
consequently they are labour intensive. Secondly the time required for the colony forming unit (cfu) i.e. single cell or group of cells, to grow to a visible colony is relatively long, at least 16h and frequently longer.

The search for more rapid methods of estimating microbial populations has encompassed both of these aspects. In the first case this is directed towards reducing time involved in manipulations required in colony count techniques and can be considered as 'labour saving', whilst in the second it is primarily concerned with the overall time required to obtain the estimate.

**Labour saving methods**

Several workers have achieved substantial reductions in the time required for serial dilution or inoculation of growth media by miniaturization of these procedures (Thompson, 1960; Fung, 1969).

Fung (1969) employed plastics microtitre trays and micro-loops to simplify dilution sequences. In the standard method serial tenfold dilutions were made by pipetting 1 ml of sample into 9 ml of sterile diluent. In the miniaturized system, loops calibrated to deliver 0.025 ml of sample were used in place of pipettes and wells in the plastics trays containing 0.225 ml of sterile diluent substituted for bottles of diluent. Sharpe and Kilsby (1971) developed a technique in which samples were diluted in molten nutrient agar and 0.1 ml amounts of agar cast as droplets in which the organisms could grow to visible colonies. This considerably increased the efficiency
over the standard technique although some problems were encountered in discriminating between the small colonies produced by this technique and small non-microbial particles e.g. fragments of food.

Other workers have sought to reduce the time required for standard colony counts by employing instruments to perform more efficiently the manipulations required. A major development of this approach has been in the mechanization of the loop dilution technique of Thompson (1960) for the enumeration of bacteria in milk. Posthumous et al., 1974 devised a mechanical system which was equal in accuracy to the hand operated standard colony count and less time consuming. This technique was further investigated by Fleming and O' Connor (1975) and a commercial instrument the Petri-foss (N. Foss Co. Hellerup, Denmark) has been developed which can prepare up to 300 estimates per hour compared with 20 - 30 per hour by manual methods. Gilchrist et al., (1973) developed a machine, the Spiral Plate Maker, which obviated the need for serial dilution of the sample. This machine spreads a continuously decreasing volume of the sample along a spiral track running from the centre to the periphery of a prepared agar plate, such that the volume of inoculum on any given area is known. After incubation of the plate, well separated colonies in a chosen area are counted and the concentration of organisms in the inoculum calculated. Inoculation of a plate takes 50 seconds and microbial concentrations in the inoculum of $10^3$ to $10^6$ cfu ml$^{-1}$ can be accurately determined. A comparison of estimates of microbial populations of foods obtained using the Spiral Plate Maker and four colony count techniques (Jarvis et al., 1978) showed high correlations (correlation coefficients 0.96 - 99) between all of the methods.
The methods mentioned above can reduce the time and labour required to obtain isolated microbial colonies which are the basis of estimation. The manual counting of microbial colonies is also a time consuming operation and several types of colony counter have been developed to automate this procedure. In most of the commercially available instruments a television camera is used as a scanning light detector which moves across the agar plate in a series of parallel lines. Variations in the video-signal are compared with pre-set sensitivity and size thresholds established by the operator for the type of colony to be counted. Signals exceeding the pre-set limits are registered by an electronic counting circuit and displayed. An exception to this is the Laser Bacterial Colony Counter which was designed primarily for use with spirally inoculated plates. A laser beam is used to scan the narrow spiral inoculation track produced by the Spiral Plate Maker. When the beam is interrupted a count is registered. The instrument can count all the colonies on a plate or register the area of the plate in which a pre-set number of colonies occur. In the latter case the concentration of organisms is determined from a previously constructed calibration curve.

Many factors can influence the automatic counting of colonies e.g. setting of threshold values, composition of growth medium, colonial morphology, coalescence of colonies; however it has been shown that for many purposes these instruments provide good estimates of numbers of colonies. (Schoon et al., 1970; Packard and Gin, 1974; Goss and Michaud, 1974).
Short elapsed-time techniques

**Micro-colony counting**

A major objective of research into rapid microbial methods has been to obtain estimates of viable microbial populations within a normal working day i.e. about 8 h. Some progress has been made towards this objective by the use of the microscope to count colonies before they have grown to a size detectable by the naked eye.

Postgate et al., (1961) inoculated organisms onto agar coated microscope slides which after 2 - 4 hrs incubation were viewed by phase contrast microscopy. This technique permitted estimation of both dead organisms (single cells) and viable organisms (micro-colonies) but had a lower limit of sensitivity of about $10^7$ cfu ml$^{-1}$. Greater sensitivity was achieved by Winter et al., (1971) who filtered organisms from suspension and incubated the filters on nutrient media for 4 - 6 hours. The filters were fixed and stained and micro-colonies counted using a microscope. This technique had a lower limit of sensitivity of about $10^4$ cfu ml$^{-1}$ and results correlated well with a standard colony counting technique.

A major limitation of these micro-colony counting techniques is the discomfort and fatigue induced by prolonged use of the microscope. Suhren (1975) overcame this by casting the microbial suspension in nutrient agar. After incubation the (spherical) micro-colonies were fixed with formaldehyde and the agar dissolved with acid. Micro-colonies in the suspension were enumerated using an electronic particle counter. Bowman, et al., (1967) cast inoculated nutrient agar in glass capillary tubes and monitored micro-colony development by light scattering measurements. In estimations of numbers of
micro-organisms in milk Suhren (1975) found her method to be similar in sensitivity to the microscopic method of Winter et al., (1971); however for large numbers of analyses electronic particle counting was preferred.

Estimations based upon metabolic properties

Direct estimates of microbial populations can be made by measurement of changes in the growth medium related to the physical or metabolic properties of the organisms. Such direct methods have a limited range since even sensitive analytical techniques e.g. Radiometry cannot detect populations less than $10^6$ cfu ml$^{-1}$. However, estimates of smaller populations may be obtained by measuring the time required for growth to this lower level of detection. This period, the detection time, is then related to the number of cfu originally present, and a calibration curve constructed from which the number of viable organisms originally present in a sample can be obtained.

Dye Reduction Methods

The earliest and most widespread of these techniques were the dye reduction tests (Methylene blue, resazurin) which are still used to monitor the microbiological quality of milk. Proctor and Greenlie (1939) investigated the use of resazurin test for foods other than milk and Walker et al., (1959) did so for processed poultry. These tests permitted reasonable estimates of relatively large populations ($10^7 - 10^5$ cfu) in 1 to 7 hours respectively. In general they are not used for enumeration of micro-organisms but rather as a semi-quantitative index of food quality.
More recently a range of methods based upon different aspects of microbial metabolism have been investigated.

**Acid production**

Harrison *et al.*, (1974) reported a method for the estimation of micro-organisms from beer by measurement of the time required to lower the pH value of a weakly buffered growth medium from 7.0 to 6.5. They demonstrated an inverse linear relationship between detection time and the initial concentration of micro-organisms. Low concentrations of organisms were detected; 10 cells ml⁻¹ were detected in 30h compared with 72h by the standard colony count method.

**Radiometric estimations**

Deland and Wagner (1970) described a method for the early detection of microbial growth based upon the estimation of C¹⁴O₂ produced by the metabolism of radio-labelled glucose. The sensitivity of this method was investigated by Waters (1972) who demonstrated an inverse linear relationship between detection time and the initial concentration of micro-organisms. The detection time varied with the specific activity of labelled glucose, the ratio of labelled to unlabelled glucose and the species of micro-organism. A single cell of *E. coli* could be detected in 8h and 10⁷ cfu ml⁻¹ in 1h but longer detection times were found for species of *Pseudomonas* and *Streptococcus*. Previte (1972) demonstrated the estimation of *Sal. typhimurium* and of *Staph. aureus* in foods and noted increased sensitivity with labelled glucose of higher specific activity. With
3.0 μ Curies of glucose per 36 ml of broth 10^4 cfu ml^{-1} of *Sal. typhimurium* were detected in 3h compared with 4h when only 0.5μ Curies were used. The estimation of heat shocked spores of *Clostridium botulinum* was also demonstrated. Detection times were 3 - 4h longer than those found for comparable numbers of vegetative cells. Rowley *et al.*, (1978) used the radiometric method to screen a wide range of cooked and frozen foods. Of 404 samples tested, 95 per cent were correctly classified within 6h as having microbial populations greater than, or less than, 1 X 10^5 cfu g^{-1}.

A further radiometric technique proposed by Macleod *et al.*, (1970) consisted of providing cells with ^32P labelled phosphate and measuring the uptake of the label by collecting the cells on a filter which was then placed under a thin end-window counter. Macleod *et al.*, claimed that this technique could detect 5 x 10^4 cfu in 1h. Mafart *et al.*, (1978) have described a similar technique for the rapid estimation of yeasts in beer by the uptake of C^{14} lysine which was then estimated using a scintillation spectrophotometer.

An advantage of this technique over C^{14}O_2 measurement is that all of the radioactivity taken up is measured. The result is that for the same performance only a tenth of the radioactivity is required.

**Hydrogen production**

An electrochemical method for the estimation of species of Enterobacteriaceae was devised by Wilkins *et al.*, (1974) who demonstrated a linear relationship between initial cell concentration and time to detection of molecular hydrogen produced by these species.
Detection times of 3 and 7h were found for $10^4$ and 1 cfu ml$^{-1}$ respectively when pure cultures were used. Detection times for *E. coli* in brackish estuarine waters also showed a linear relationship (Wilkins and Boykin, 1976) but were greater than those for pure cultures; 4.5h and 13h for $10^4$ and 1 cfu ml$^{-1}$ respectively.

**Calorimetric estimation**

Although micro-calorimetry has long been used to study the metabolic activity of micro-organisms (Forrest, 1972), the feasibility of differentiating between different concentrations of micro-organisms (in urine) by micro-flow calorimetry was first demonstrated by Beezer *et al.*, (1974). In a later study Beezer *et al.*, (1977) demonstrated a linear relationship between heat output and initial concentration of organisms over the range $10^5 - 10^9$ cfu ml$^{-1}$ and claimed that the lower level of sensitivity of the method was between $10^3 - 10^5$ cfu ml$^{-1}$. Previously Berridge *et al.*, (1974), who investigated the use of this technique for the rapid estimation of micro-organisms in milk, had found a lower limit of sensitivity of $5.10^5$ cfu ml$^{-1}$.

**Impedimetric estimation**

Several groups of workers have reported the estimation of viable microbial populations based upon changes in the electrical properties of the culture medium during growth. Measurements of electrical impedance are made using sensitive impedance bridges in which changes in the impedance of the sample are compared with those in a sterile reference medium. The various instruments used differ slightly in design but are based upon the same principle.
Ur and Brown (1973; 1974) used an instrument then known as the Strattometer to demonstrate an inverse linear relationship between initial number of micro-organisms and the time to obtain an impedimetric response. They claimed that as few as \(3 \times 10^2\) cfu ml\(^{-1}\) of \(E.\) coli could be detected in 2.5h. Cady (1975), using a Bactometer confirmed the inverse linear relationship between microbial concentration and detection time but found a lower sensitivity; \(10^3\) and \(10^2\) cfu ml\(^{-1}\) of \(E.\) coli were detected in 4 and 5 hours respectively. More recently Richards et al., (1978) have described a further instrument, the Malthus Conductance meter, in which only the conductivity component of impedance is measured. This modification was claimed to improve stability of the signal output. The data given indicated a sensitivity of detection similar to that obtained by Cady (1975).

The impedimetric estimation of micro-organisms in frozen vegetables was investigated by Hardy et al., (1977). Populations of micro-organisms of \(10^5\) cfu g\(^{-1}\) were detected in 5h and >90% agreement was found between impedimetric and colony count methods.

In the techniques described above changes in impedance are produced only after growth of the organisms in nutrient media to levels of about \(10^6\) cfu ml\(^{-1}\) (Richards, et al., 1978). A different impedimetric technique has been reported by Wheeler and Goldschmidt (1975) in which estimation is not dependent upon growth. The organisms are suspended in distilled water and impedance measurements made using a four electrode system at low frequency (10Hz). In this system the electrical output voltage was directly related to the concentration
of cells over the range $10^3 - 10^9$ cfu ml$^{-1}$ and estimates were obtained within minutes. It seems probable that the effect measured relates to the fixed charges and their balancing counter ions in the cell walls of the organisms as proposed by Carstensen and Marquis (1968). If this is the case then both viable and non-viable cells will be estimated. Wheeler and Goldschmidt used this technique to estimate micro-organisms filtered from urine samples. However, for solid samples prior separation of the micro-organisms would be necessary such that the organisms could be transferred to distilled water.

**Estimation methods based upon the composition or structure of micro-organisms**

The most rapid methods for the estimation of microbial populations are those made by direct estimation of a structural or metabolic component of the organisms since these estimations do not require incubation of the micro-organisms.

**Endotoxin assay**

This assay is based upon the observation by Levin and Bang (1968) that minute amounts of endotoxin from the outer membrane of Gram negative bacteria cause coagulation of a lysate of amoebocytes from the horseshoe crab (*Limulus polyphemus*). Jorgensen *et al.*, (1973) demonstrated that the time to coagulation was inversely proportional to the number of organisms present and that cell concentrations of $10^5$ ml$^{-1}$ could be estimated within an hour. Coates (1977) refined the method by measuring 'coagulation' as a change in the optical density of the lysate and enhanced the
sensitivity by including E.D.T.A. (10^{-5} M). This increased sensitivity was thought to result from the chelation of cations from the outer membrane of the bacterial cells which leads to increased concentrations of free endotoxin in solution. Coates (1977) found that for all of the Gram negative species examined 5 \times 10^2 \text{ cfu ml}^{-1} were detected in 1 hour and that this corresponded to 0.05 ng endotoxin ml^{-1}. This high sensitivity has given rise to some problems due to high background readings attributed to presence of endotoxins in water supplies (Coates personal comm.).

**ATP assay**

The bioluminescent reaction of the luciferin-luciferase system from firefly tails (McElroy, 1947) permits the photometric estimation of ATP down to picogram levels (Lyman and De Vincenzo, 1967). The use of this technique for the rapid estimation of microbial populations was by Levin et al., (1967) and by Deustachio, Johnson and Levin (1968).

Sharpe, et al., (1970) reported a 1 : 1 relationship between vegetative cells of bacteria and femtograms (10^{-15} g) of ATP. The level of ATP in bacterial spores was much lower but rapidly increased after germination and levels in yeasts were about 20 x higher than those found in vegetative bacterial cells. Attempts to estimate microbial populations in a range of foods were unsuccessful due to the high level of non-microbial ATP in the foods (Sharpe, 1970; Williams, 1972). These workers concluded that whilst ATP measurements provided good estimates of viable cell populations in pure culture the method could not be applied to food materials unless
a system for the separation of the micro-organisms from the food material could be devised. This problem has not been so apparent in clinical microbiology where the technique has been used for the estimation of levels of micro-organisms in urine (Gutekunst et al., 1976; Johnston, et al., 1976).

**Estimation of porphyrins**

Pisano et al., (1965) demonstrated that the catalytic effect of haematin on the chemiluminescent reaction of luminol (5 amino - 2.3 dihydro - l.4 amino phthalate) can be used to determine extremely small amounts of porphyrins (10\(^{-11}\) g) in cell free extracts of bacteria and yeasts. The sensitivity of the system for the estimation of 11 species of micro-organisms was studied by Oleniacz et al., (1966) who found that as few as 500 cells of *Bacillus stearothermophilus* could be detected, but that in general the lower limit of sensitivity for both bacteria and yeasts was between 10\(^3\) and 10\(^4\) cells. Strange (1973) confirmed this sensitivity but found poor reproducibility of the technique from day to day and, in the presence of sodium chloride or phosphate, extremely high blank values. Picciolo et al., (1976) demonstrated that high blank values due to metal ions could be minimized by measuring light output after 10 seconds and that those due to soluble porphyrins could be minimized by pre-reacting the sample with 0.5% H\(_2\)O\(_2\), making the reaction more specific for intact bacterial cells.

Picciolo and his co-workers (1976) also compared estimates of viable and non-viable microbial populations obtained by both the luminol chemiluminescence and luciferin-luciferase bioluminescence techniques.
When cells were killed by exposure to ethylene oxide the luminol signal was not affected whilst the luciferin-luciferase signal decreased by 99%. They concluded that the luminol method estimated all cells whilst the luciferase method measured only viable cells.

2.5 Summary

The state of our knowledge of the structure of microbial cell walls, whilst far from complete, is reasonably broad in terms of the major components and their interrelationships. In the case of Gram negative bacteria much is also known of the structure of minor components such as lipopolysaccharide and protein.

Less is known of the structure of the outer surface of the cell wall, however it is clear that this 'surface' is a three dimensional layer which may change in response to environmental conditions. A current view based upon evidence from electrophoresis and iso-electric focussing is that carboxyl and amino groups are present at the surfaces of bacterial cells and that these groups are mainly associated with proteins.

The gross separation of microbial cells from liquids are most commonly achieved by centrifugation and filtration. In other methods used or suggested for use e.g. flocculation, counter-current distribution or flotation it is probable that the surfaces of the organisms have a role, however, the mechanisms involved have not been fully elucidated.

Investigations of the use of ion exchange resins for the separation of micro-organisms have been fragmentary and in the case of cation exchange
resins, contradictory. Overall, the reports indicate effects of environmental conditions on these interactions of micro-organisms with resins. A general theory has been proposed to account for these observations (Daniels, 1968), but little confirmatory work has been carried out.

The first major advances in methods for the estimation of viable micro-organisms since the introduction of gelified media have occurred in the last decade. Miniaturization and automation of traditional colony count procedures have provided labour saving methods but, with the exception of micro-colony counting, these do not reduce the overall period required for estimation.

Novel methods based upon measurements of the metabolic activity of organisms can provide more rapid estimates than do colony counts and for higher concentrations of organisms \( (10^4 - 10^5 \text{ cfu ml}^{-1}) \) can provide results within the working day.

The most rapid methods are those based upon very sensitive techniques for the assay of cell constituents such as ATP, porphyrins or endotoxin. These methods can provide estimates within minutes. However they lack the amplification provided by microbial growth and are more susceptible to interference than are methods involving the growth of the organisms. The use of some very rapid methods for the enumeration of micro-organisms in bio-materials is currently restricted by the lack of techniques for the separation of micro-organisms from such materials.
PART II

EXPERIMENTAL
Ch. 3: GENERAL MATERIALS AND METHODS
3. GENERAL MATERIALS AND METHODS

**Micro-organisms**

The organisms mainly used in this study were *Staphylococcus aureus* ATCC 14559, *Escherichia coli* NCTC 8156 and *Saccharomyces cerevisiae* NCYC 240.

Mutant organisms with particular attributes were the gifts of other workers. *Staphylococcus aureus* strain H and the teichoic acid deficient mutant strain 52A5 were supplied by Dr. R.E. Marquis of the University of Rochester, New York. Parent and mutant strains of *Salmonella typhimurium* and *Salmonella minnesota* were supplied by Prof. O. Luderitz of the Max Planck Institut für Immunobiologie Stubeweg, Germany. Parent and outer membrane protein deficient strains of *E. coli* were supplied by Dr. I.N. Beacham of the U.C.N.W. Aberystwyth.

Other organisms were obtained from national or international collections or were isolates obtained from foods and held in the microbiology section of the Leatherhead Food R.A.

**Cultural conditions**

All of the pure cultures of micro-organisms used in this part of the study were grown in 100ml volumes of broth. The media and conditions employed are shown in Table 1.
<table>
<thead>
<tr>
<th>Micro-organism</th>
<th>Cultivation conditions</th>
<th>Recovery (Estimation) Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gram positive organisms</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>Nutrient Broth 24h. 37°C</td>
<td>Plate Count Agar 24h. 37°C</td>
</tr>
<tr>
<td><em>Lactobacillus sp.</em></td>
<td>MRS Broth 48h 30°C</td>
<td>MRS agar 72h with CO₂</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>Tryptone Soya Broth 24h 30°C</td>
<td>Tryptone Soya Agar 24 - 48h 30°C</td>
</tr>
<tr>
<td><em>Bacillus macerans</em> (NCTC 6355)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Bacillus stearothermophilus</em> (NCTC 10007)</td>
<td>Tryptone Soya Broth 24h 55°C</td>
<td>Dextrose Tryptone Agar</td>
</tr>
<tr>
<td><em>Clostridium sporogenes</em> (spores)</td>
<td>Cooked Meat Broth &gt; 72h 30°C</td>
<td>Horse Blood Agar anerobic conditions 48h 30°C</td>
</tr>
<tr>
<td><strong>Gram negative organisms</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>Nutrient Broth 24h 37°C</td>
<td>Plate Count Agar 24h 37°C</td>
</tr>
<tr>
<td><em>Salmonella sp.</em></td>
<td>Nutrient Broth 24h 37°C</td>
<td>Plate Count Agar 24h 37°C</td>
</tr>
<tr>
<td><em>Pseudomonas sp.</em></td>
<td>Nutrient Broth 24 - 48h 30°C</td>
<td>Plate Count Agar 24h 30°C</td>
</tr>
<tr>
<td><strong>Yeast</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Saccharomyces sp.</em></td>
<td>Malt Extract Broth 48h 30°C</td>
<td>Malt Extract Agar 48h 30°C</td>
</tr>
<tr>
<td><em>Kloeckera apiculata</em> (NCYC 466)</td>
<td>Malt Extract Broth 48h 30°C</td>
<td>Malt Extract Agar 48h 30°C</td>
</tr>
<tr>
<td><em>Pichia membranaefaciens</em> (NCYC 169)</td>
<td>Malt Extract Broth 48h 30°C</td>
<td>Malt Extract Agar 48h 30°C</td>
</tr>
<tr>
<td><em>Hansenula sub-pellicosa</em></td>
<td>Malt Extract Broth 48h 30°C</td>
<td>Malt Extract Agar 48h 30°C</td>
</tr>
</tbody>
</table>
Harvesting of micro-organisms

Micro-organisms were harvested from broth cultures by centrifugation. Bacterial cultures were centrifuged at 2000 \( x \cdot g \) for 20 min. Yeast cultures were centrifuged at 1,500 \( x \cdot g \) for 10 min. The precipitated cells were washed once in the appropriate diluent and then resuspended in fresh diluent before use.

Estimation of micro-organisms

Several methods were employed for the estimation of micro-organisms in suspensions.

Colony count methods

Two techniques were employed. A standard surface plating technique (Thatcher and Clark, 1968) was used in the earlier part of the work and the Spiral Plate Method (Gilchrist, et al., 1973) in later parts. The close agreement between estimates made by these two techniques has been demonstrated (Jarvis, Lach and Wood, 1977). Colony count results are expressed in colony forming units (cfu).

Optical measurements

The optical extinction of microbial cell suspensions in distilled water or buffer solutions was measured at 420 nm (Daniels, 1968) using a Uvispeck spectrophotometer (Hilger-Watts). Nephelometric measurements were made using an EEL nephelometer. In some cases a prototype nephelometer incorporating a fibre optic light guide and a photo-resistive cadmium sulphide cell was also used.
Disposable plastics cuvettes were used in optical extinction measurements and with the prototype nephelometer.

**Particle Counting**

In certain cases where organisms were rendered non-viable and/or their surfaces modified (Part II, Ch. 5) estimates of cell concentrations were made using a Coulter Counter model Zb. (Coulter Electronics Ltd.). Where necessary cell suspensions were diluted in Isoton II, a particle-free diluent (supplied by the same company). Counts were made using a 30 μm aperture, 0.05ml volumes and the following instrument settings: Aperture current $\frac{1}{2}$, Amplification $\frac{1}{2}$, lower threshold 15. The number of particles counted ranged from 20,000 to 100,000.

**Adsorbents**

**Ion exchange resins**

The resins used throughout this work were analytical grade resins (A.G.) obtained from Bio-Rad Laboratories (Richmond, California, U.S.A.). The resins used are shown below:

<table>
<thead>
<tr>
<th>Resin type</th>
<th>Polymer lattice</th>
<th>Exchange groups</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Anion exchange resins</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AG 1</td>
<td>Styrene divinylbenzene</td>
<td>$\phi$-CH$_2$ $\overset{\text{N}}{\text{N}}$(CH$_3$)$_3$ Cl$^-$</td>
</tr>
<tr>
<td>AG 3</td>
<td>Styrene divinylbenzene</td>
<td>$\phi$-CH$_2$ $\overset{\text{N}}{\text{N}}$ H (CH$_3$)$_2$ Cl$^-$</td>
</tr>
<tr>
<td>Celllex P.A.B.</td>
<td>Cellulose</td>
<td>$\overset{\text{O}}{\text{O}}$-CH$_2$ $\overset{\text{NH$_2$}}{\text{C}_6 \text{H}_4}$</td>
</tr>
</tbody>
</table>


Cation exchange resins

AG 50 W  Styrene divinylbenzene  $\text{SO}_3^2-\text{H}^+$
Bio-Rex 70  acrylic polymer  $\text{R-COO}^-\text{Na}^+$

Resins of various particle size and of different degrees of cross-linking of the polymer lattice were used. Further details are given in the appropriate chapters.

Polystyrene beads

These were obtained from Bio-Rad Laboratories as Bio-beads SX-4 (200 - 400 mesh) and consist of a styrene divinyl benzene polymer lattice without ion exchange groups.

Glass beads

Ballotini (100 - 200 mesh) and the larger glass beads 2 and 3 mm diameter were obtained from Shandon Scientific Ltd.

Adsorption of micro-organisms

Two types of system were used:

Batch systems

Two variants of the batch system were employed. In the first, the stirred batch system, 2g of the adsorbent were placed in a 50ml beaker with 40ml of the microbial suspension. The resin was agitated by a magnetic stirrer paddle operating at a speed just sufficient to prevent sedimentation of the beads. Aliquots of the suspension were removed at intervals to assess the extent of
adsorption. In this system the effect of salts on adsorption was investigated by adding a solid salt directly to the suspension.

In the second type of batch system, the *Rolled batch system*, 1 or 2g amounts of resin were placed in 30 ml straight sided bottles (Universal screw cap bottles) with 10ml of the microbial suspension. The resin was agitated by rotation of the bottles at 40 revolutions per min. on a Rolamix machine (Luckham Ltd., Burgess Hill, Sussex). After a given period the microbial suspension was removed from the resin by aspiration using a syringe fitted with a rayon cloth filter, and the resin washed with 10ml of distilled water. In this system the effect of salts was investigated by replacing the distilled water with the required salt solution and agitating (i.e. rolling the bottles) for the required period.

**Column systems**

Two types of column system were used:

The *small column system* consisted of 0.2g quantities of adsorbent contained in 3.0cm sections of rigid plastics tube (int. dia. 0.7cm). The tube was closed at one end with rayon cloth fused to the plastic after the latter had been softened with acetone. Each column was fed via silicone tubing (int. dia. 0.1cm) using a twenty channel peristaltic pump (Flow Inducer, Watson Marlowe Ltd.). Ten ml volumes of microbial suspensions were applied to the columns followed by 10ml of distilled water to remove unadsorbed organisms. Elution of adsorbed organisms was effected either by passing the eluent through the column or by dissembling the column and agitating the resin in the eluent for 10 secs. on a vortex agitator.
In the second type of column system, the **syringe column system** (Fig. 1) column holders were constructed from 2ml disposable syringes (Gillette Surgical Ltd.). A teflon tube (0.16 mm int. dia.) was passed through the plunger seal which was covered with a single layer of fine mesh cotton cloth. The syringe barrel was loaded with resin by passing an appropriate amount of resin slurry through the needle fitting. A domestic massager/vibrator (Pifco Ltd.) was used to obtain good column packing and the homogeneity of the resin bed was occasionally checked by passing blue dextran (Pharmacia Ltd.) through the column.

Microbial suspensions were applied to the columns via the needle aperture. In this system elution was effected by passing the eluent through the column. Further details are given in the appropriate experimental chapters.

**Measurement of adsorption**

Estimates of micro-organisms were made of the microbial suspensions prior to contact with the adsorbent surface and after contact. The numbers and percentage of organisms adsorbed were calculated from these data.

Similarly the percentage of organisms desorbed was calculated from the number adsorbed and that estimated in the eluant after contact with the adsorbent.
Fig. 1. The syringe column holder.
Electron Microscopy

Resins with micro-organisms adsorbed were fixed to microscope sample stubs with Durafix adhesive, frozen by immersion in liquid nitrogen and lyophilized.

Organisms from column effluents were collected by filtration on 0.2 μm pore size filters (Unipore membranes Bio-Rad Laboratories) and fixed in cacodylate buffer (0.1M, pH 7.2) containing 2% V/V glutaraldehyde.

All preparations were coated with gold-palladium alloy prior to examination with a 'Stereoscan' electron microscope (Cambridge Instruments Ltd.)

pH measurement

The pH values of microbial suspensions and reagent solutions were measured electrometrically using a Pye Unicam pH meter (Cambridge Instruments) Model 90 with an expanded scale.

Buffer solutions

All buffer solutions were prepared according to Dawson, et al., (1959) using distilled water and 'Analar' grade reagents. The pH of buffer solutions was checked electrometrically before use.

Protein estimation

Protein estimations were made by the method of Lowry et al., (1951). Standard solutions (50–200 μg ml⁻¹) of bovine serum albumin were
prepared for each occasion and a standard curve constructed of optical extinction at 750 nm against protein concentration. The protein concentration of unknown samples was obtained by interpolation.
PART II

Ch. 4: INTERACTION OF MICRO-ORGANISMS WITH ANION EXCHANGE RESINS
4. THE INTERACTION OF MICRO-ORGANISMS WITH ANION EXCHANGE RESINS

4.1 Introduction

Reports of the adsorption of micro-organisms to anion exchange resins (Part I, 2.3) suggested that these materials might provide the basis of methods for the separation of micro-organisms.

These relatively few reports provide a rather fragmented view of the phenomenon, different systems being studied by different workers. There is little information on the mechanisms of the adsorption and recovery of micro-organisms, or on the viability of organisms recovered from the resin. In addition, the experimental systems described are of relatively large scale and therefore unsuited to the simultaneous replicate analyses commonly made in routine microbiological analysis.

The objectives of the present work were to confirm and extend previously reported work in order to obtain a better understanding and control of the interactions of micro-organisms with anion exchange resins.

4.2 Materials and Methods

Resins

Unless otherwise stated the anion exchange resin used in this section of the work was the chloride form of Bio-Rad AG1 x 8. With bacteria the 100/200 U.S. mesh size was used and for yeasts the 50/100 U.S. mesh size was used.
The method of Garrett (1965) was used. Washed cells were suspended in 0.1M NH₃ solution (to remove ester-linked alanine) and washed three times in distilled water before resuspension in buffer (pH 7) containing 0.05M sodium metaperiodate. After incubation for 30 min at 37°C the cells were washed three times in distilled water before use.

**Effect of pH on adsorption**

The pH of microbial suspensions was adjusted by dipping the tip of a teflon spatula into acid or alkali and then briefly plunging the wetted tip into a stirred suspension of the cells. Addition of acid or alkali was continued until the desired pH value was obtained. The suspensions were allowed to stand for 30 min and the pH value checked before use.

**Effect of dextran sulphate and detergents**

Prior to adsorption experiments, the resin (2g) was stirred for 1 hr in 40 ml of a 1% (w/v) solution of either dextran sulphate, M.W. 500,000 (BDH Biochemicals) sodium lauryl sulphate or Triton X-100 (Sigma Chem. Co.). The resin was allowed to settle, the solution decanted and the resin was washed three times with 40 ml volumes of distilled water.

**Treatment of cells with proteolytic enzymes**

The enzymes used were obtained from the Sigma Chem. Co., and were as follows: Trypsin (Bovine Pancreas) crystallized, Type III.
Pepsin (Hog Stomach) crystallized, and Protease (Streptomyces griseus) Type VI. Suspensions containing microbial cells (10^8 cfu ml\(^{-1}\)) and the enzyme (1.5 mg ml\(^{-1}\)) in 50 mM phosphate buffer (pH 7.5) were incubated for 90 min at 37°C. The cells were recovered by centrifugation (2000 x g for 20 min), washed twice in buffer and resuspended in distilled water.

**Ultrasonic treatment of resins and cells**

Sonication was carried out in the stirred batch system using a Soniprobe (Dawes, London) with a Type 7530A converter and 1\(\frac{1}{4}\) inch tip. For this treatment stirring of the cell-resin suspension was stopped after 5 mins and the beaker raised until the tip of the probe was just beneath the surface of the liquid. Sonication was carried out for 10 seconds after which 3 ml of the suspension was removed for optical extinction measurement. This sample was returned to the system which was stirred for 5 min before a further sample was taken.

**Removal of flagellae from E. coli**

The method of Martinez (1963) was used. The organisms were suspended in 0.01M phosphate buffer (pH 7.0) and deflagellated mechanically in a blade homogenizer (MSE Top drive) for 90 seconds. The cells were collected by centrifugation (2000 x g for 20 mins) washed twice and resuspended in distilled water.
4.3 Results

4.3.1 The adsorption of micro-organisms to anion exchange resins

4.3.1.1 Preliminary observations

The adsorption of micro-organisms to anion exchange resin was confirmed using three different methods for the detection of micro-organisms: optical extinction measurement, microbial colony counting and direct observation by electron microscopy.

When suspensions of micro-organisms in distilled water were stirred with beads of anion exchange resin the optical extinction of the suspension fell rapidly (Fig. 2) and the resin particles became agglutinated.

Passage of microbial suspensions through small columns of anion exchange resin resulted in a marked reduction in the number of colony forming units in the effluent compared with that in the original suspension. All of the organisms examined (Table 2) were adsorbed with high efficiency and, in the cases in which comparisons were made, the basicity of the resin appeared to have little effect upon the efficiency of adsorption. After each of the experiments described in Table 2, the resin columns were dismantled and the resin shaken, by hand in distilled water, to determine whether the organisms had been retained by filtration. In none of these cases was a significant proportion (>1%) of the adsorbed organisms returned to suspension.
Fig. 2. Adsorption of *Staph. aureus* in a stirred batch system.

$E_0$ = optical extinction of suspension prior to addition of resin
$E$ = .. .. .. .. after addition of resin
<table>
<thead>
<tr>
<th>Resin Type</th>
<th>Organism</th>
<th>Column Size (g)</th>
<th>No. of cfu Applied</th>
<th>% cfu Adsorbed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polystyrene matrix</td>
<td>Staph. aureus</td>
<td>1.0</td>
<td>$10^9$</td>
<td>95</td>
</tr>
<tr>
<td>strongly basic</td>
<td>Pseudomonas oleovorans</td>
<td>1.0</td>
<td>$10^8$</td>
<td>98</td>
</tr>
<tr>
<td>weakly basic</td>
<td>Pseudomonas oleovorans</td>
<td>0.5</td>
<td>$10^5$</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td>Pseudomonas oleovorans</td>
<td>0.2</td>
<td>$10^7$</td>
<td>99</td>
</tr>
<tr>
<td>Cellulose matrix</td>
<td>Lactobacillus brevis</td>
<td>1.0</td>
<td>$10^9$</td>
<td>99</td>
</tr>
<tr>
<td>weakly basic</td>
<td>Lactobacillus brevis</td>
<td>0.1</td>
<td>$10^5$</td>
<td>97</td>
</tr>
<tr>
<td></td>
<td>Micrococcus spp.</td>
<td>1.0</td>
<td>$10^8$</td>
<td>99</td>
</tr>
<tr>
<td></td>
<td>Escherichia coli</td>
<td>0.8</td>
<td>$10^7$</td>
<td>99</td>
</tr>
<tr>
<td></td>
<td>Saccharomyces rouxii</td>
<td>0.2</td>
<td>$10^5$</td>
<td>99</td>
</tr>
<tr>
<td></td>
<td>Clostridium perfringens (spores)</td>
<td>0.5</td>
<td>$10^6$</td>
<td>99</td>
</tr>
<tr>
<td>Polystyrene matrix</td>
<td>Staph. aureus</td>
<td>0.2</td>
<td>$10^9$</td>
<td>99</td>
</tr>
<tr>
<td>weakly basic</td>
<td>Pseudomonas oleovorans</td>
<td>0.2</td>
<td>$10^5$</td>
<td>99</td>
</tr>
<tr>
<td>weakly basic</td>
<td>Saccharomyces rouxii</td>
<td>0.2</td>
<td>$10^5$</td>
<td>98</td>
</tr>
</tbody>
</table>
The adherence of three different microbial forms to the strongly basic anion exchanger Dowex 1 is demonstrated in Plates I - IV. The three organisms, a yeast, a Gram positive coccus and a Gram negative rod, represent three basically different forms of cell wall structure; all adhered to the resin beads. Other material could be seen which formed a coating on the surface of the beads and this was particularly obvious in the cases of the bacteria. Close inspection of Plate III indicated that the bacteria were adsorbed to the resin surface and not to the material coating the surface of the beads.

4.3.1.2. The adsorption of bacteria to anion exchange resin in small columns

The high efficiency with which micro-organisms were adsorbed to resins indicated that very small columns of resin used in parallel for the simultaneous adsorption of organisms from multiple samples would be the most convenient practical system in which to investigate separation. A small column system was devised (see Materials and Methods, Ch. 3) and the effect of the column characteristics on the adsorption of Staphylococcus aureus to the strongly basic resin Bio Rad AG 1X8 100/200 mesh were investigated.

The effect of resin particle size

It can be seen from Fig. 3 that for a given mass of resin, adsorption was inversely related to the resin particle diameter, i.e. directly related to the surface area of the resin in the column.
Fig. 3. Effect of resin particle size on the adsorption of *Staph. aureus*. 

% cfu adsorbed

Mean particle diameter of resin beads (μm)
Plates I - IV Micro-organisms adsorbed to beads of anion exchange resin
The effect of the ionic form of the resin

The adsorption of Staph. aureus to five different ionic forms of the anion exchanger Bio Rad AG1 is shown in Table 3.

<table>
<thead>
<tr>
<th>Ionic form</th>
<th>Initial Suspension $\text{cfu ml}^{-1} \times 10^{-6}$</th>
<th>Effluent $\text{cfu ml}^{-1} \times 10^{-6}$</th>
<th>Adsorption</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloride</td>
<td>13</td>
<td>4.4</td>
<td>66</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>4.5</td>
<td>70</td>
</tr>
<tr>
<td>Formate</td>
<td>11</td>
<td>4.2</td>
<td>61</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>4.2</td>
<td>75</td>
</tr>
<tr>
<td>Hydroxyl</td>
<td>11</td>
<td>4.4</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>2.8</td>
<td>72</td>
</tr>
<tr>
<td>Nitrate</td>
<td>15</td>
<td>3.0</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>3.0</td>
<td>50</td>
</tr>
<tr>
<td>Sulphate</td>
<td>12</td>
<td>4.1</td>
<td>66</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>2.7</td>
<td>80</td>
</tr>
</tbody>
</table>

Although variation in adsorption was found between duplicates and the range of adsorption was broad, the results do not suggest major differences in the adsorptive properties of the different forms of the resin. In a further experiment little difference was found between the adsorptive properties of the chloride and hydroxyl forms of this resin for E. coli.
The adsorptive capacity of anion exchange resin for

Staphylococcus aureus

The adsorptive capacity of the resin was determined by passing a large volume (30 ml) of the microbial suspension through a small (0.1g) column of the resin.

The results (Table 4) show that in excess of a thousand million colony forming units were adsorbed to the column indicating an adsorptive capacity of in excess of ten thousand million cfu g$^{-1}$ of resin. This represents 1 mg of cells in terms of wet weight (Strange, 1972).

### TABLE 4

<table>
<thead>
<tr>
<th>No. of organisms</th>
<th>cfu</th>
</tr>
</thead>
<tbody>
<tr>
<td>Applied to 0.1g column</td>
<td>$3.9 \times 10^9$</td>
</tr>
<tr>
<td>Detected in column effluent and wash</td>
<td>$1.4 \times 10^9$</td>
</tr>
<tr>
<td>Retained on column</td>
<td>$2.5 \times 10^9$</td>
</tr>
<tr>
<td>• Adsorptive capacity of resin</td>
<td>$2.5 \times 10^{10}$ g$^{-1}$</td>
</tr>
</tbody>
</table>

The measurement of adsorption in the small column system

The precision with which estimates could be made of the adsorption of micro-organisms to small columns of anion exchange resin was tested using 18h cultures of both Staph. aureus and E. coli. In
each case eight replicate 0.2g columns were used. Ten ml of a suspension containing $10^6$ cfu ml$^{-1}$ was passed through each column at a flow rate of 0.3ml min$^{-1}$.

TABLE 5

Variation in the adsorption of micro-organisms to small columns of Bio-Rad AG1 x 8 100/200 mesh

<table>
<thead>
<tr>
<th>Organism</th>
<th>% cfu adsorbed</th>
<th>Mean*</th>
<th>Range</th>
<th>Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Staph. aureus</td>
<td></td>
<td>85</td>
<td>72-90</td>
<td>6.4</td>
</tr>
<tr>
<td>E. coli</td>
<td></td>
<td>73</td>
<td>67-78</td>
<td>2.8</td>
</tr>
</tbody>
</table>

*of eight replicates

The results (Table 5) demonstrated the variability which may be expected in estimates of adsorption in the column system. They also indicated that the adsorption of Staph. aureus in this system was slightly greater than that of E. coli, but that the adsorption of Staph. aureus was more variable.

The combined effects of some variable factors on the adsorption of Staph. aureus to small columns of anion exchange resin

The effects of three factors - quantity of resin, particle size of resin and the flow rate of the microbial suspension through the resin on the adsorption of Staph. aureus to Bio-Rad AG1 are shown
in Table 6.

**TABLE 6**

The effect of variable factors on the adsorption of 
Staph. aureus to small columns of anion exchange resin

<table>
<thead>
<tr>
<th>Flow Rate</th>
<th>Particle Size</th>
<th>Column Size</th>
<th>Initial Suspension</th>
<th>Effluent</th>
<th>Adsorption</th>
</tr>
</thead>
<tbody>
<tr>
<td>ml⁻¹ min⁻¹</td>
<td>U.S. wet mesh*</td>
<td>g</td>
<td>cfu ml⁻¹ x 10⁻⁵</td>
<td>cfu ml⁻¹ x 10⁻⁵</td>
<td>%</td>
</tr>
<tr>
<td>20/50</td>
<td>0.2</td>
<td>30</td>
<td>12</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>20/50</td>
<td>0.5</td>
<td>30</td>
<td>14</td>
<td>53</td>
<td></td>
</tr>
<tr>
<td>0.3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100/200</td>
<td>0.2</td>
<td>30</td>
<td>1.0</td>
<td>97</td>
<td></td>
</tr>
<tr>
<td>100/200</td>
<td>0.5</td>
<td>30</td>
<td>3.0</td>
<td>90</td>
<td></td>
</tr>
<tr>
<td>0.6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20/50</td>
<td>0.2</td>
<td>8.4</td>
<td>5.4</td>
<td>36</td>
<td></td>
</tr>
<tr>
<td>20/50</td>
<td>0.5</td>
<td>8.4</td>
<td>4.1</td>
<td>52</td>
<td></td>
</tr>
<tr>
<td>100/200</td>
<td>0.2</td>
<td>9.4</td>
<td>3.2</td>
<td>66</td>
<td></td>
</tr>
<tr>
<td>100/200</td>
<td>0.5</td>
<td>9.4</td>
<td>0.6</td>
<td>94</td>
<td></td>
</tr>
</tbody>
</table>

*Mesh size is inversely proportional to particle size.

All of the factors affected adsorption. The effect of resin particle size was clearly demonstrated. At both low and high flow rates adsorption increased with decrease in particle size. At the lower flow rate an effect of column size i.e. quantity of resin and resin bed depth, was apparent only as a slight decrease in adsorption to the larger columns. At the higher flow rate there
was a greater effect of column size; adsorption increased with
column size and the greatest increase was seen with 100/200 mesh
size resin.

Overall, the results showed that in this system the level of adsorption
could be controlled by manipulation of the three factors investigated.

4.3.1.3. The effect of physical conditions on the
adsorption of bacteria to anion exchange resin

The effect of the pH value of the microbial suspension on the
adsorption of Staph. aureus

The effect of pH on the adsorption of Staph. aureus strain H and of
a teichoic acid deficient mutant of this strain, (Ou, et al., 1973)
designated 52A5, to Bio-Rad AG1 x 8 100/200 mesh was investigated
in the small column system. For comparison three other types of
surface – cation exchange resin Bio-Rad AG50W x 8 100/200 mesh,
polystyrene beads, and glass beads (ballotini) all of similar
dimensions to those of the resins, were also investigated.
Adsorption was estimated by measurement of optical extinction at
420 nm and expressed as the ratio of the optical extinction of the
suspension after passage through the column (E) to that of the
initial suspension (E₀) (Daniels, 1968); a lower \( \frac{E}{E₀} \) ratio indicating
greater adsorption. The results are shown in Figs 4 and 5. The
scale denoting the absorbance ratio has been inverted for ease of
interpretation; thus the peaks of the curves denote greater adsorption
and the troughs lesser adsorption.

Each point on the graphs is the arithmetic mean of four replicate
estimates.
Fig. 4. The effect of pH on the adsorption of Staph. aureus to ion exchange resins.
Fig. 5. The effect of pH on the adsorption of *Staph. aureus* to ballotini and polystyrene beads.
An effect of pH on the adsorption of these organisms was observed for all of the surfaces. There was a general pattern of increased adsorption as the pH fell from neutrality. The maxima for the parent organism (strain H) occurred at pH values between 1 and 2 below which adsorption again decreased. This general pattern was also observed for the teichoic acid deficient mutant (strain 52A5). However, for this organism the pattern was displaced by 1 to 2 pH units towards the alkaline end of the scale. The adsorption maxima for strain 52A5 occurred at a higher pH level between pH 3 and 4, and for several of the surfaces there were indications of a second peak of adsorption at lower pH values. This was particularly well demonstrated with the glass surface for which maxima of adsorption were observed at pH 3 and pH 1.5.

The chemical removal of teichoic acid from the walls of cells of Staph. aureus H by mild metaperiodate oxidation (Garrett, 1965) resulted in a pattern of adsorption to anion exchange resin different from that exhibited by the teichoic acid deficient mutant (Strain 52A5). Metaperiodate oxidized cells (Fig. 6) showed greatest adsorption in the same pH range as the parent strain, i.e. pH 1 - 2 but adsorption exceeded that of the parent strain. At pH 5 the adsorption of oxidized cells was much greater than that of the parent strain.

In the intermediate pH range (i.e. pH 5 - 8) there was a difference in the adsorptive behaviour of the ion exchange resins. Adsorption was uniform over this range and adsorption to the anion exchanger was greater than that to the cation exchanger. In both cases the parent and mutant strains showed a similar pattern. The polystyrene and glass surfaces differed in this respect.
Fig. 6. The effect of metaperiodate oxidation of the cells on the adsorption of *Staph. aureus* to anion exchange resin.
At the alkaline end of the pH scale (pH 10 - 12) small increases in adsorption were observed for both parent and mutant strains on anion exchange resin and for the mutant strain on cation exchange resin. A similar effect was indicated for the parent organism on polystyrene beads, the other cases were not investigated.

The general pattern observed for the adsorption of Staph. aureus H to anion exchange resin at low and intermediate pH values was also observed for E. coli (Fig. 7). This organism exhibited a marked adsorption maximum close to pH 12 which was not observed with Staph. aureus.

The adsorption of E. coli to polystyrene beads over the range pH 4 to 8 paralleled, at slightly lower adsorption values, that of Staph. aureus.

The effect of salt concentration on the adsorption of Staph. aureus and E. coli

The effect upon adsorption of increasing concentrations of sodium and calcium chlorides in the suspending medium was investigated in the rolled batch system (Fig. 8). Adsorption of Staph. aureus was inhibited by each of the salts. Over the range $10^3$ to $10^6$ μM inhibition increased linearly with $\log_{10}$ salt concentration, and at the highest concentrations tested adsorption was restricted to approximately one third of that found in the absence of these salts.

Sodium chloride, at concentrations up to $10^6$ μM, had no effect upon the adsorption of E. coli.
Fig. 7. The adsorption of E. coli NCTC 8196 to anion exchange resin.
Fig. 8. The effect of salt concentration on the adsorption of Staph. aureus and E. coli to anion exchange resin.

$0' = \text{distilled water (i.e. no added salts)}$
The effect of magnesium chloride upon adsorption was studied in the stirred batch system with results (Fig. 9) similar to those found for the other salts. Concentrations of magnesium chloride increasing from 40 to 1000 mM progressively inhibited adsorption but did not completely inhibit it at 400 mM magnesium chloride. In the absence of magnesium chloride, and at the lower concentrations there was an increase in adsorption with time.

The effect of dextran sulphate and two detergents on adsorption

The effect on adsorption of pretreating the resin with a large, negatively charged molecule, dextran sulphate, was compared with the effects of similar treatment with two detergents: sodium lauryl sulphate and Triton X-100 (Octyl phenoxy polyethoxy ethanol). These effects were investigated in the stirred batch system and adsorption was monitored nephelometrically.

Pretreatment of the resin with dextran sulphate completely inhibited the adsorption of Staph. aureus to Bio-Rad AG1 x 8 over the period of the investigation (Fig. 10). The detergents had a less clear cut effect upon adsorption. Initially, resins pretreated with these detergents showed slightly greater adsorption of this organism than did the untreated resin control. With time, this effect was reversed and adsorption to the pretreated resin exceeded that to the control. This latter effect was more marked with sodium lauryl sulphate pretreatments.
Fig. 9. The effect of magnesium chloride on the adsorption of *Staph. aureus* to anion exchange resin.

*Resin omitted*
Fig. 10. The effect of pretreatment of anion exchange resin on the adsorption of *Staph. aureus*.
4.3.1.4 The effect of variable factors associated with the micro-organisms

Effect of age of culture

The adsorption of cells of Staph. aureus and E. coli to columns of Bio-Rad AG1 x 8 100/200 mesh was determined for populations cultured for 5 days at 37°C followed by 2 days at ambient temperature and harvested at intervals over seven days. The results are shown in Tables 7 and 8 in which the data for each sampling time were derived from eight simultaneous estimates.

The colony count did not vary greatly over the experimental period. The age of the culture had a relatively small but definite effect upon adsorption of both species. At 18h adsorption of Staph. aureus (85%) was greater than that of E. coli. After 3 days the adsorption of both species rose to a maximum of about 90% after which it fell to about 80% at 5 days. At seven days the adsorption of Staph. aureus fell further whilst that of E. coli rose slightly.

TABLE 7

The effect of the age of culture of Staph. aureus on adsorption to ion exchange resin

<table>
<thead>
<tr>
<th>Age of Culture</th>
<th>% Colony forming units adsorbed :</th>
<th>Mean</th>
<th>Standard deviation</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>18h</td>
<td></td>
<td>85</td>
<td>6.4</td>
<td>72-90</td>
</tr>
<tr>
<td>3 days</td>
<td></td>
<td>91</td>
<td>1.4</td>
<td>90-92</td>
</tr>
<tr>
<td>5 days</td>
<td></td>
<td>84</td>
<td>8.5</td>
<td>67-94</td>
</tr>
<tr>
<td>7 days</td>
<td></td>
<td>76</td>
<td>8.0</td>
<td>65-84</td>
</tr>
</tbody>
</table>
TABLE 8

The effect of the age of culture of E. coli on
adsorption to ion exchange resin

<table>
<thead>
<tr>
<th>Age of Culture</th>
<th>% Colony forming units adsorbed</th>
<th>Mean</th>
<th>Standard deviation</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>18h</td>
<td>73</td>
<td>2.8</td>
<td>67-78</td>
<td></td>
</tr>
<tr>
<td>3 days</td>
<td>92</td>
<td>7.0</td>
<td>77-99</td>
<td></td>
</tr>
<tr>
<td>5 days</td>
<td>79</td>
<td>9.4</td>
<td>60-90</td>
<td></td>
</tr>
<tr>
<td>7 days</td>
<td>87</td>
<td>8.5</td>
<td>66-98</td>
<td></td>
</tr>
</tbody>
</table>

The effects of variation in cell surface structures

The deletion of cell wall components

The effect upon adsorption of the deletion of teichoic acid from the
cell wall of Staph. aureus has been described (4.3.1.3). In the
intermediate pH range the parent and teichoic acid deficient strains
behaved similarly.

The effect of the deletion of cell wall components of Gram negative
bacteria was investigated in the small column system by comparing
the adsorption of Smooth (parent) and Rough (mutant) strains of two
species of Salmonellae. The mutant organisms were deficient in
parts of the lipopolysaccharide molecule of the outer cell membrane
(Fig. 11)
The lipopolysaccharide structures of Smooth and Rough strains of Salmonellae

**Sal. typhimurium**

Smooth  Lipid A-KDO-Hep-Glc-Gal-Glc N (Man-Rha-Abeq)_n
(SF 1135)

Rough  Lipid A-KDO-Hep-Glc-Gal-GlcN
(SF 1591)

**Sal. minnesota**

Smooth  Lipid A-KDO-Hep-Glc-Gal-GlcN (Gal N)_n
(SF 1167)

Rough  Lipid A-KDO
(SF 1111)

KDO  2-keto-3 deoxy octonate
Hep  Heptose (L-Glycero-D Manno heptose)
Glc  Glucose
Gal  Galactose
GlcN  Glucosamine
GalN  Galactosamine
Man  Mannose
Rha  Rhamnose
Abeq  Abequose

( )_n  = repeating unit
(SF )  = Luderitz Strain No.
TABLE 9
The adsorption of Smooth and Rough strains of Salmonellae to anion exchange resin

<table>
<thead>
<tr>
<th></th>
<th>Smooth (parent) strain</th>
<th>Rough (mutant) strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sal. minnesota</td>
<td>61 ± 8.3</td>
<td>80 ± 2.1</td>
</tr>
<tr>
<td>Sal. typhimurium</td>
<td>82 ± 2.3</td>
<td>93 ± 2.1</td>
</tr>
</tbody>
</table>

* Of 4 replicate estimates

The adsorption of strains of Sal. typhimurium was greater than that of Sal. minnesota. The Rough strains of each species showed greater adsorption than their Smooth counterparts and this difference was greater for Sal. minnesota strains.

Overall the results indicate that deletion of the polysaccharide part of the lipopolysaccharide promotes adsorption to anion exchange resin but that the effect is a relatively minor one.

The effect of flagellae on the adsorption of E. coli

When the adsorption of mechanically de-flagellated cells of E. coli was compared with that of complete cells from the same culture little difference was observed. In replicate estimates made in the small column system a mean value of 98.5% (range 97-99%) was obtained for the adsorption of complete cells compared with 95% (range 93-98) for the de-flagellated cells.
The effect of treatment with proteolytic enzymes on the adsorption of *Staph. aureus*

The typical effect of the treatment of cells of *Staph. aureus* with proteolytic enzymes on adsorption to anion exchange resin in the rolled batch system is shown in Table 10.

**TABLE 10**

The effect of treatment with proteolytic enzymes on the adsorption of *Staph. aureus*

<table>
<thead>
<tr>
<th>Enzyme treatment</th>
<th>% cfu adsorbed</th>
<th>mean*</th>
<th>range*</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>69</td>
<td>60-81</td>
<td></td>
</tr>
<tr>
<td>Pepsin</td>
<td>68</td>
<td>62-78</td>
<td></td>
</tr>
<tr>
<td>Trypsin</td>
<td>69</td>
<td>60-77</td>
<td></td>
</tr>
<tr>
<td>Pronase (non specific peptidase)</td>
<td>48</td>
<td>44-56</td>
<td></td>
</tr>
</tbody>
</table>

* of three replicate experiments

Only treatment with Pronase inhibited adsorption. At the pH used for the various treatments (7.5) Pepsin would not be expected to exhibit proteolytic activity and was included as a reagent control. Trypsin which might be expected to exhibit activity of this pH had no effect upon adsorption.

The effect of Pronase was not a major one, the adsorption of the treated cells was about two thirds of that of the untreated cells;
however the effect was consistently found in three experiments carried out on different occasions.

4.3.2 The recovery of bacteria from anion exchange resin

The adsorption of bacteria to anion exchange resin appeared to be an extensive and simply controlled phenomenon which offered the basis of a method for the separation of micro-organisms. In analytical applications of such separations the viability of the organisms recovered would be an important consideration. In the work reported here estimates of numbers of micro-organisms recovered from anion exchange resin (AG1 100/200 mesh) were made by colony count techniques and relate only to viable organisms.

4.3.2.1 Preliminary Observations

A series of preliminary experiments was carried out using the small-column system to examine the effect of 0.3M sodium citrate (Kurozumi, et al., 1965) on the recovery of bacteria from the resin. Typical results are shown in Table 11. Simple elution of the resin column was shown to be less efficient in the recovery of bacteria than was agitation of the resin in the eluant.
### Table 11

The recovery of bacteria from anion exchange resin by elution with sodium citrate

<table>
<thead>
<tr>
<th>Micro-organism</th>
<th>No. adsorbed to column (cfu)</th>
<th>% Organisms recovered (cfu)</th>
<th>without agitation</th>
<th>with agitation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Staph. aureus</td>
<td>$10^5$</td>
<td>16</td>
<td>37$^\dagger$</td>
<td></td>
</tr>
<tr>
<td>Staph. aureus</td>
<td>$10^7$</td>
<td>20</td>
<td>40$^\dagger$</td>
<td></td>
</tr>
<tr>
<td>Pseudomonas oleovorans</td>
<td>$10^5$</td>
<td>13</td>
<td>46$^*$</td>
<td></td>
</tr>
</tbody>
</table>

$^\dagger$ column agitated by vibrator during elution

$^*$ Resin removed and shaken by hand in the eluant

These results indicated that agitation promoted the recovery of organisms but recovery was still incomplete.

In further experiments more intensive agitation using ultrasonic vibration decreased rather than increased recovery.

### 4.3.2.2 The effect of salt concentration and pH value of the eluant on the recovery of Staph. aureus and E. coli

The effects of the eluants on the recovery of relatively low numbers of organisms ($<10^7$ per gram of resin) were investigated in the rolled batch system, in which mechanical agitation was applied during both the adsorption and recovery of the organisms.
The effect of phosphate buffer

The effect of the molarity and the pH value of phosphate buffer
(Sørensons) on the recovery of Staph. aureus and E. coli from anion
exchange resin are shown in Figs 12 and 13 respectively.

Greatest recovery of Staph. aureus (about 45%) was obtained at
concentrations of 0.2 and 0.3M indicating an optimum value between
these points. There was no significant recovery of E. coli at any
of the buffer concentrations tested.

The effect of the pH value of 0.25M buffer on the recovery of
Staph. aureus is shown in Fig. 13. Maximal recovery (105%) was
obtained at pH 6.5 and was only slightly lower (90%) at pH 6.0 and
7.0. Outside this range recovery fell more sharply to about 60% at
pH 5.5 and at pH 8.0. This pattern of recovery with pH was
consistently found although the maximal recovery varied between 80
and 110%.

There was no significant recovery of E. coli under any of the
conditions tested.

The effect of various eluants on the recovery of Staph. aureus

The effects of three different eluants on the recovery of Staph. aureus
are shown in Fig. 14. Two of the eluants were used at pH 7.0. The
third, sodium citrate, was at pH 8.3.

These results, with those for phosphate buffer (Fig. 12), demonstrate
that there are optimal concentrations of these salts for the recovery
Fig. 12. The effect at pH 7.0 of the concentration of phosphate buffer on the recovery of *Staph. aureus* and *E. coli* from anion exchange resin.
Fig. 13. The effect of the pH of 0.25M phosphate buffer on the recovery of *Staph. aureus* and *E. coli* from anion exchange resin.
Fig. 14. The effect of eluant concentration on the recovery of *Staph. aureus* from anion exchange resin.
of the organisms but do not precisely define the optima. Calcium chloride, phosphate buffer and sodium citrate produced greater recoveries at concentrations below 0.4M than did sodium chloride which exhibited greatest effect at about 0.6M. At higher concentrations of these salts recovery was inhibited and this contrasted with the effect of salts on the adsorption of this organism to the resin (see Fig. 8). Concentrations of calcium chloride required for a given recovery were lower than those of phosphate buffer or sodium citrate. Phosphate buffer was similarly more efficient in recovering this organism than was sodium citrate.

Sodium chloride exhibited an optimal concentration clearly different from those of the other three salts.

The effect of various eluants on the recovery of E. coli

In contrast to their effect upon the recovery of Staph. aureus, the eluants described above had little effect on the recovery of E. coli. None gave recoveries greater than 15% and only in the case of calcium chloride was the pattern of recovery with eluant concentration similar to that found for Staph. aureus. At 0.2M calcium chloride the maximum recovery of E. coli was 9%.

The effect of sodium citrate - citric acid buffer at pH 7.0 and concentrations up to 0.5M was also investigated. Maximum recovery was obtained with 0.3M buffer and was 18%.

When micro-organisms in the eluants were estimated by particle counting similar low recoveries of E. coli were obtained and when
the resin, after elution, was incubated on agar plates growth of \textit{E. coli} was observed around the resin particles. These observations indicate that this organism was retained on the resin and suggest that the cells were still viable.

4.3.3 The adsorption of yeasts to anion exchange resins

In preliminary experiments (see 4.3.1.1) it was shown that yeasts could be adsorbed to anion exchange resin in the same way as bacteria. In view of the differences between these microbial groups e.g. in size and cell wall structure, further studies were made of the adsorption of yeasts. Throughout this work the resin used was Bio-Rad AG1 x 8, 50/100 mesh, except where otherwise stated.

4.3.3.1 The effects of some variable factors associated with the small column system

When yeasts, suspended in distilled water were passed through columns of anion exchange resin several factors were found to influence the extent of adsorption of \textit{Saccharomyces cerevisiae}; the particle size of the resin, the flow rate of the suspension through the resin and the mass of resin.

The effect of changing the resin particle size (Fig. 15) was to increase adsorption with decrease in particle size i.e. increase in surface area.

The effect of the mass of the resin bed is shown in Table 12. When the flow rate was held at 0.3ml min$^{-1}$ a column of 0.1g did not
Fig. 15. The effect of resin particle size on the adsorption of *Saccharomyces cerevisiae.*
completely adsorb all of the cells from a suspension containing $10^6$ cfu ml$^{-1}$ but columns of 0.2g and greater did so. The plastics column holder and the associated tubing did not adsorb this organism.

### TABLE 12

**Effect of column mass on the adsorption of***

*Saccharomyces cerevisiae*

<table>
<thead>
<tr>
<th>Mass of column (g)</th>
<th>Percentage of organisms adsorbed (cfu)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0$^+$</td>
<td>0</td>
</tr>
<tr>
<td>0.1</td>
<td>72</td>
</tr>
<tr>
<td>0.2</td>
<td>100</td>
</tr>
<tr>
<td>0.3</td>
<td>100</td>
</tr>
<tr>
<td>0.4</td>
<td>100</td>
</tr>
</tbody>
</table>

$^+$ Empty column holder

The effect of the flow rate upon adsorption (Table 13) was also found to be important in this system. At a flow rate of 0.6 ml min$^{-1}$ adsorption to a column of mass 0.4g was incomplete and at a flow rate of 1.5 ml min$^{-1}$ was greatly reduced.
TABLE 13

The effect of the flow rate of the microbial suspension on the adsorption of Saccharomyces cerevisiae

<table>
<thead>
<tr>
<th>Flow Rate (ml min⁻¹)</th>
<th>Percentage of organisms adsorbed (cfu)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.3</td>
<td>100</td>
</tr>
<tr>
<td>0.6</td>
<td>95</td>
</tr>
<tr>
<td>1.5</td>
<td>20</td>
</tr>
</tbody>
</table>

The adsorption of seven species of yeasts in the small column system (Table 14) showed that all of the species of Saccharomyces tested had a high affinity for the resin as also did Pichia membranefaciens. Cells of the other two species tested exhibited a slightly lower affinity for this resin.

TABLE 14

The adsorption of seven species of yeasts to anion exchange resin

<table>
<thead>
<tr>
<th>Species</th>
<th>Percentage of organisms adsorbed (cfu)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saccharomyces cerevisiae</td>
<td>99</td>
</tr>
<tr>
<td>Saccharomyces bayanus</td>
<td>98</td>
</tr>
<tr>
<td>Saccharomyces carlsbergiensis</td>
<td>99</td>
</tr>
<tr>
<td>Saccharomyces spp</td>
<td>97</td>
</tr>
<tr>
<td>Pichia membranefaciens</td>
<td>98</td>
</tr>
<tr>
<td>Kloechera apiculata</td>
<td>85</td>
</tr>
<tr>
<td>Hansenula subpelliculosa</td>
<td>82</td>
</tr>
</tbody>
</table>
4.3.3.2 The effect of pH and salt concentration of the microbial suspension

The effect of magnesium chloride on the adsorption of *Saccharomyces cerevisiae* to anion exchange resin was investigated in both stirred batch and small column systems. In the batch system (Fig. 16) adsorption varied with both time and magnesium chloride concentration. Initially (1 min) organisms were rapidly adsorbed from distilled water and from solutions of magnesium chloride; adsorption was inversely related to the concentration of magnesium chloride. After 5 min some organisms were desorbed from resin suspended in water and the lower concentrations of magnesium chloride, but adsorption continued at the higher concentrations. An inverse relationship between adsorption and magnesium chloride concentration persisted over the whole experimental period.

The effect of magnesium chloride on the adsorption of this organism to small columns of the resin was to progressively inhibit adsorption (Fig. 17). This effect was first evident at a concentration of 0.075M magnesium chloride and increased with increase in salt concentration; at 0.3M magnesium chloride adsorption was inhibited by about 40% of the value obtained in distilled water.

In further studies using the stirred batch system it was shown that, when the cells and the resin were exposed to solutions of magnesium chloride (1.0M) prior to mixing in distilled water, adsorption was not inhibited. In contrast, prior exposure of the resin to solutions (1.0% w/v) of dextran sulphate completely inhibited the adsorption of the organisms from distilled water.
Fig. 16. The effect of magnesium chloride on the adsorption of *Sacch. cerevisiae* in a batch system.
Concentration of magnesium chloride (molar)

% cfu adsorbed

Fig. 17. The effect of magnesium chloride on the adsorption of *Sacch. cerevisiae* in a column system.
The pH value of the microbial suspension had a marked effect upon the adsorption of \textit{Saccharomyces cerevisiae} in the stirred batch system (Fig. 18). Maximum adsorption occurred at pH 3.0 and fell steeply as the pH was lowered. At pH 1.0 adsorption was not observed. With increase in the pH value of the suspension adsorption increased to a maximum at pH 3.0 then fell steadily. Substantial adsorption was evident over the range pH 5 - 8 but at pH 12.0 adsorption was only slightly greater than that observed at pH 1.0.

4.3.4 The recovery of yeasts from anion exchange resin

4.3.4.1 The effect of sonication

The adsorption of cells of \textit{Sacch. cerevisiae} from suspension in water and their subsequent recovery by sonication of the resin suspension was demonstrated in a batch system (Fig. 19). The adsorbed organisms were returned to suspension by sonication at the lowest energy level. At successively higher energy levels the optical extinction of the suspension of resin plus organisms paralleled, at a slightly higher value, that of the original suspension of organisms.

These results indicated that the organisms were desorbed at the lower energy level, and that at the higher energy levels of sonication the treatment caused an increase in the concentration of particles in suspension. This was most probably due to the splitting of clumps of yeast cells.

4.3.4.2 The recovery of several species of yeast by elution with magnesium chloride

When solutions of magnesium chloride were used to elute yeasts from
Fig. 18. The effect of pH on the adsorption of *Sacch. cerevisiae*. 
Optical density (420 nm)

Fig. 19. The effect of ultrasonic vibration on the adhesion of *Sac. cerevisiae.*
Fig. 20. The recovery of yeasts from anion exchange resin by elution with magnesium chloride solutions.
anion exchange resin by agitation in the rolled batch system. Three patterns of recovery were observed, but in no case was complete recovery obtained. The first pattern (Fig. 20a) was shown by three species. These organisms were not desorbed by water but were desorbed to a substantial extent (60 - 80%) by concentrations of magnesium chloride of between 0.1 and 0.5M. The second pattern (Fig. 20b) was similar to the first but the level of desorption was lower, (generally 30 - 50%). The maximum desorptive effect of magnesium chloride occurred at or below a concentration of 0.1M and at higher concentrations the effect was decreased. In the third pattern of desorption (Fig. 20c) the organisms were desorbed to a substantial extent by water, and magnesium chloride effected further desorption in only one of the two cases.

The pattern of desorption did not appear to be related to the genus of the organism; different species of Saccharomyces demonstrated different patterns of desorption.

4.4 Discussion

The results amply confirm the reports of previous workers (Part I 2.3) that a wide range of microbial species can be adsorbed to ion exchange resins. In particular the adsorptive capacity of anion exchange resin for Staph. aureus was of the same order (10^10 cells g^-1) as that found by Daniels (1968) for Bacillus subtilis and the general effects of pH and salt concentration were in some respects similar to those previously described (Zvaginstev, 1962; Daniels, 1968).

The small column system developed for this investigation provides a simple and convenient system in which the adsorption of bacteria and
yeasts can be controlled by choice of resin particle size, column size and flow rate. The variation in estimates of adsorption (Table 5) was small enough to permit reasonably precise measurement of the factors affecting adsorption and it is probable that the colony count technique provides a more sensitive method than those used by Zvaginstev, (1962) or Daniels (1968) (direct cell count and optical extinction respectively). In addition, the use of colony counts provides information on the viability of organisms recovered from the resin. This is an important aspect of cell-resin interactions which has received little attention from previous workers.

One objective of the present study was to determine whether microorganisms could be adsorbed to anion exchange resins and recovered in a viable condition. The results show that this can be accomplished for the Gram positive bacterium Staph. aureus and for yeasts but that whilst the adsorption of the Gram negative organism E. coli is easily accomplished the recovery of this organism is less straightforward.

A further objective of this work was to gain some knowledge of the mechanism of the cell-resin interactions. A mechanism had been suggested by Daniels (1968) who considered that microbial cells behaved as very large zwitterions which, at pH values above their isoelectric points, adsorbed to anion exchangers and at pH values below these points adsorbed to cation exchangers. Microbial cells do exhibit iso-electric points and these are usually low, e.g. pH 1.5 - 3.5 (Harden and Harris, 1949); therefore Daniel's observations that microbial cells adsorbed to anion exchange resin at higher pH values and were desorbed at lower values apparently supported his theory.
It is clearly shown however, by the effects of pH on the adsorption of *Staph. aureus* to ionogenic and non-ionogenic surfaces (Figs. 4 and 5), that the major interaction between micro-organisms and resin particles at low pH is largely independent of the charge on the adsorbent surface. In all cases *Staph. aureus* showed a peak of adsorption close to its iso-electric point of pH 2.0 (Harden & Harris, 1953) and the teichoic acid deficient mutant which has fewer negatively charged cell wall groups exhibited a similar peak at a slightly higher pH value. These data suggest that this major effect is due to iso-electric point precipitation and that many observations upon which Daniels formulated his theory were attributable to a general effect of iso-electric point precipitation rather than a specific interaction between charged groups on the resin surface and those on the cell surface.

A more specific interaction between anion exchange resin and *Staph. aureus* does occur in the intermediate pH range (pH 5 - 9). This was not observed with any of the other surfaces (Figs. 4 & 5) and appears to be due to the positive charge on the resin surface. This view is supported by the effect of dextran sulphate on the interaction (Fig. 10). Pre-treatment of the resin with this strongly negatively charged polymer completely inhibited the adsorption of both bacteria and yeasts.

It is this specific interaction of anion exchange resin with micro-organisms which is of greatest interest in the separation of micro-organisms from other bio-materials because it occurs within a pH range which the organisms can withstand. The more general, iso-electric point interaction is likely to be less useful since the
low pH values involved would kill, or seriously impair the functions of the majority of microbial species.

**Primae facie** the mechanism of the more specific interaction may be attributed to an electrostatic attraction between the positively charged groups on the resin and negatively charged groups on the microbial surface. All of the organisms investigated possess an overall negative charge in this pH range (Neihoff and Echols, 1973).

The location of these negative charges has not been demonstrated but several cell surface structures viz. flagellae, lipopolysaccharide and teichoic acid appear not to be involved. This latter is perhaps surprising in view of the report of Ou and Marquis, (1970) that the phosphate groups of teichoic acids account for 50% of the anionic groups in the cell wall of *Staph. aureus*.

The fact that all three groups of micro-organisms investigated, Gram positive bacteria, Gram negative bacteria and yeasts adsorbed to the resin may suggest a common factor responsible for adsorption. However the cell wall structures of these groups are different and none of the major structural components viz. peptidoglycan, teichoic acid, lipopolysaccharide or mannan phosphate is common to all three.

Protein is a minor cell wall component which has been reported to lie close to the cell surfaces of all three groups of organisms and the carboxyl groups associated with proteins have been cited as determinants of the electrophoretic and iso-electric focussing behaviour of bacteria (Part I, 2.1). In Gram negative groups
Salmonella the polysaccharide chains of the lipopolysaccharide are extant from the protein rich outer surface (Costerton, et al., 1974) and the deletion of parts of these chains might be expected to increase adsorption due to protein. The greater adsorption of Rough strains of Salmonellae (Table 9) might therefore suggest that the adsorption of these micro-organisms to anion exchange resin is due to protein components of the cell surface and the effect of protease on the adsorption of Staph. aureus (Table 10) supports this view.

The effect of salt concentration on the adsorption of Staph. aureus and of yeasts can be broadly explained in terms of the suppression of electrostatic attractive forces, however the identical effects of equi-molar concentrations of sodium and calcium chloride (Fig. 8) indicate that ionic strength is not the only factor involved. Furthermore, the effect of salt concentration on the recovery (i.e., desorption) of this organism does not parallel that on adsorption. The occurrence of optimal salt concentrations for recovery (Fig. 14) contrasts with the uniform effect of salt concentration on adsorption (Fig. 8).

It is possible that the adhesive interaction of the organisms with the resin introduces constraints which override the normal relationship between ionic concentration and electrostatic forces of attraction between freely suspended particles. If this was the case then the optimal concentrations of the different salts in the recovery of Staph. aureus from the resin might be explained in terms
of salt induced changes in the conformation of the cell wall and surface. Salt induced changes in the cell wall volume of *Staph. aureus* have been described by Ou and Marquis (1970) who found that with increasing concentration of sodium chloride the walls contracted to a minimum volume at about 0.25M, above which they re-expanded. This salt concentration for maximum contraction of the cell wall is lower than the optimum sodium chloride concentration for recovery of *Staph. aureus* from the resin; however this might be explained by the different states of the cell walls in the free and adsorbed conditions.

The interactions of yeasts with anion exchange resin are sufficiently similar to those of *Staph. aureus* to support a similar electrostatic mechanism of adsorption. This is not the case for cells of *E. coli*.

The adsorption of *E. coli* from solutions of high ionic strength (Fig. 8) and the failure to recover the organisms from the resin indicate very strong binding and also suggest that adhesion is not due to electrostatic attraction. It appears more probable that electrodynamic forces are involved in the adhesion of this organism to the resin and this view is further considered in the General Discussion (Part 10).

4.5 **Summary**

The specific adsorption of both Gram positive and Gram negative bacteria and of yeasts to anion exchange resins has been demonstrated in two simple practical systems.

Major factors which affect adsorption of *Staphylococcus aureus* and yeasts are the pH value and the salt concentration of the microbial
suspension. The particle size of the resin and the period of contact of the suspension with the resin also influenced adsorption.

The mechanism of adsorption of micro-organisms to anion exchange resin appeared to involve electrostatic attraction between the positively charged groups of the resin and negatively charged groups on the surfaces of the microbial cells. Teichoic acids of the staphylococcal cell wall, lipopolysaccharides of the cell surface of salmonellae or the flagellae of *E. coli* did not appear to be directly involved in adsorption. Evidence was obtained which indicated that cell surface protein was involved in adsorption.

The recovery of *Staph. aureus* from the resin was achieved by elution with solutions of salts and was promoted by agitation of the resin. A substantial proportion of the adsorbed organisms were recovered in a viable state.

The interactions of *E. coli* with anion exchange resin differed from those shown by *Staph. aureus* and yeasts. The independence from salt concentration of the interactions of *E. coli* with this resin suggests that the mechanism of the interactions differ from that of the other organisms.
Ch. 5: THE INTERACTION OF MICRO-ORGANISMS WITH CATION EXCHANGE RESINS
5. THE INTERACTION OF MICRO-ORGANISMS WITH CATION EXCHANGE RESINS

5.1 Introduction

Whilst microbial cells bear an overall negative surface charge at pH values greater than about 3.5 (Harden and Harris, 1953), positively charged groups are also present at the cell surface and are potentially available for interaction with negative groups on an adsorbent surface.

There have been diverse reports of the adsorption of micro-organisms to cation exchange resins. Zvaginstev (1962) and Zisgray, et al., (1970), found that micro-organisms were adsorbed whilst other workers (Gillisenn et al., 1961; Hogg, 1976) concluded that the adsorption of micro-organisms to these resins was negligible. In the present study (Part 4.3.1.3) adsorption of Staph. aureus to a strongly basic cation exchanger was found to be negligible over the range pH 4 - 10.

In earlier work Puck and Sagik (1953) demonstrated the adsorption of bacteriophage to cation exchange resin from solutions of salts but not from distilled water. It seemed possible that this effect of salt concentration on adsorption might also occur with free living micro-organisms and that it could explain the previous contradictory reports of the adsorption of micro-organisms to cation exchange resin.

The objectives of the investigation of the cation exchange systems were to gain knowledge of the interaction of organisms with these systems and to determine the potential of cation exchange resins for the separation of micro-organisms.
5.2 Materials and Methods

Resin and Buffers

The resins used throughout this work were Bio-Rex 70, 100-200 U.S. wet mesh size and Bio-Rad AG.50 W 100/200. Prior to use the resin was washed with 0.5M sodium hydroxide, approximately 10 bed volumes, followed by two washes with distilled water each of 10 bed volumes.

Citric acid-sodium citrate buffers were made from equi-molar solutions of the two components, which had been filter sterilised; pH values were determined electrometrically. Disodium hydrogen phosphate-citric acid buffers were prepared in a similar way from equi-molar solutions. Except where otherwise stated all buffer solutions were used at a concentration of 50 mM with respect to total salt levels.

Adsorption of Micro-organisms to Ion Exchange Resins

Except where otherwise stated the resin columns were prepared for the adsorption of micro-organisms by equilibration with 100 bed volumes of the appropriate buffer solution. The organisms for each experiment were also suspended in buffer of the same composition.

Five ml of a suspension of organisms was run through each column at a flow rate of 0.3 - 0.5 ml min$^{-1}$ followed by 5 ml of the sterile buffer to wash out any unadsorbed organisms. The percentage adsorption was calculated from the total number of organisms added and the total number recovered in the column effluent, as determined by total cell counts or colony counts.
Separation of Staph aureus and Escherichia coli by use of ion exchange resin

Two methods were used to effect the separation of a mixed culture of St. aureus and E. coli. The first method used was selective adsorption in which the culture was separated by selectively adsorbing one of the two organisms. The second was the selective elution of the separate species after adsorption of all the organisms to the resin column.

Selective adsorption

Citric acid - sodium citrate buffer at pH 5.9 was used with 1 g resin columns. Ten millilitres of a mixture of Staph. aureus (10^5 cfu ml^-1) and E. coli (10^6 cfu ml^-1) were run on to the column followed by 5 x 5 ml volumes of sterile buffer: 3 x 5 ml of sterile distilled water were then used to elute adsorbed organisms from the column. All of the column effluent was collected in 5 ml fractions. Each fraction was plated on to MacConkey No. 3 agar (Oxoid) and Tellurite Polymixin Egg Yolk agar (BBL Ltd). All plates were incubated at 37°C for 24 hours.

Selective elution

Citric acid - sodium citrate buffer at pH 4.7 was used with 1 g resin columns. Ten millilitres of a mixture containing Staph. aureus (10^5 cfu ml^-1) and E. coli (10^5 cfu ml^-1) were run on to the column followed by 3 x 5 ml of sterile buffer to wash out unadsorbed organisms: 3 x 5 ml 50 mM sodium citrate was used to elute E. coli followed by 3 x 5 ml sterile distilled water to elute Staph. aureus. 5 ml fractions were collected and all were analysed by viable count as above.
Effect of pH on the Adsorption of Food-associated Micro-organisms

In this series of experiments the effect of pH was assessed using phosphate: citric acid buffer over the range pH 4.0 to 8.0 at intervals of 0.5 units. Two gram columns were used with a flow rate of 0.3 ml min$^{-1}$.

Chemical modification of cell surfaces

Cells were prepared for the following treatments by washing three times in distilled water and were resuspended in distilled water or the appropriate buffer to give a final concentration of about $10^{10}$ cfu ml$^{-1}$.

After the treatments the cells were washed twice in 50mM buffer and resuspended in buffer.

Modification of carboxyl groups by reaction with methylamine in the presence of carbodiimide (Heckels, et al., 1976)

Five ml of a suspension of cells in 0.8% sodium chloride solution was added to 5 ml of a solution containing 1-ethyl-3-(dimethyl-amino propyl) carbodiimide, 0.5g; methylamine hydrochloride, 0.6g; Mg Cl$_2$ H$_2$O, 5.0mg and adjusted to pH 4.5 with 1M HCl. The reaction mixture was stirred at 25$^\circ$C for 4h and at intervals the pH was re-adjusted to 4.5 by the addition of 0.1N NaOH.

Esterification of carboxyl groups (Zvyaginstev and Gusev, 1971)

Cells were washed in methanol and then suspended in 0.1N HCl in
methanol for 24h at ambient temperature (10 - 15°C).

Reaction of anionic groups with safranine
(Fraenkel-Conrat and Cooper, 1944)

Two ml of a suspension of cells in phosphate buffer (0.2M; pH 11.5) were added to 4 ml of an 0.2% (w/v) solution of safranine. The mixture was shaken in a test tube at ambient temperature for 24h.

Reaction of carboxyl groups with Alcian Blue
(Everson-Pearce, 1968)

Cells were suspended in a solution containing 0.2M MgCl\textsubscript{2} and 0.2% (w/v) Alcian Blue (T. Gurr Ltd.) and held at ambient temperature for 24h.

Reaction of amino groups with Dinitrofluorobenzene
(Ghuysen and Strominger, 1963)

0.2 ml of 100mM dinitrofluorobenzene was added to 2ml of a suspension of cells in 1% (w/v) sodium borate and the mixture heated at 60\textdegree C for 30 min.

Reaction of basic groups with Orange G
(Fraenkel-Conrat and Cooper, 1944)

The procedure used was identical with that shown above for safranine except for the dye and the buffer. Phosphate-citrate buffer (0.2M; pH 2.2) was used with Orange G.
The reaction of amino groups with formaldehyde
(Sherbet and Lakshmi, 1973)

A 1.5% (w/v) solution of formaldehyde was prepared by dissolving solid paraldehyde in 0.146M sodium chloride solution at 65°C. The solution was cooled and the pH adjusted to 7.5 using 0.146M HCl. Cells were suspended in the solution for 8 weeks at ambient temperature.

Reaction of amino groups with citraconic anhydride

The cells were suspended in phosphate buffer (0.1M; pH 8.0) containing 1.0% w/v of citraconic anhydride and the mixture held at ambient temperature for 2h. During this period the pH was maintained at 8.0 by addition of 1.0M NaOH.

Treatment with proteolytic enzyme

Cells were suspended in phosphate–citric acid buffer (0.05M; pH7.5) containing 1.5 mg ml$^{-1}$ of a non-specific protease. (Sigma Chem. Co. 'Pronase' type IV; 3 - 5 units mg$^{-1}$). The suspension was incubated at 37°C for 30 mins.

5.3 Results

5.3.1 The adsorption of bacteria to strongly acidic cation exchange resin

The effect of pH on the adsorption of *Staph. aureus* from suspension in distilled water to the strongly acidic cation exchange resin Bio–Rad AG 50 W is described in Section 4.3.1.3. At pH values
between 4.0 and 10.0 virtually no adsorption was observed.

When salts were introduced into this system and the pH maintained at 5.5 the results shown in Fig. 21 were obtained. In distilled water adsorption was low (10%) but adsorption from 0.05M phosphate-citrate buffer was high (80 - 90%) and from 0.1 M buffer exceeded 90%. Further increases in buffer concentration up to 0.50M produced little further increase in adsorption.

When each of the above suspensions was made to 0.50M with respect to sodium chloride the effect was to increase greatly adsorption relative to that from distilled water and decrease slightly that from the buffered solutions. Further increases in sodium chloride concentration up to 3.0M had little effect upon adsorption.

5.3.2 The adsorption of bacteria to weakly basic cation exchange resin

In the investigation of cation exchangers it was considered that weakly acidic resins, which have a charge density similar to that of the bacterial cell wall, might be less likely to damage labile components of the microbial surface. Marquis et al., (1978) reported that the cation selectivity of bacterial cell walls was similar to that of the carboxyl resin Bio-Rex-70 and therefore this resin was selected for the adsorption studies.

5.3.2.1 The adsorptive capacity of Bio-Rex 70 for Staph. aureus.

When suspensions (10^6 cfu ml^{-1}) of Staph. aureus in 50 mM phosphate citrate buffer were passed through resin columns of different sizes
Fig. 21. The effect of salt concentration on the adsorption of *Staph. aureus* to strongly acidic cation exchange resin (at pH 5.5).
it was found that the percentage of organisms adsorbed increased with column size for columns of less than 1g. Columns of 1g and greater adsorbed about 90 per cent of the organisms.

The adsorptive capacity of this resin was determined by passing a concentrated suspension of the organism \(10^8\text{ cfu ml}^{-1}\) through a 1g column until minimum adsorption was taking place (Fig. 23).

It was found that the adsorption of \textit{Staph. aureus} to the column decreased as the number of cells already adsorbed increased. When the number of cells adsorbed reached about \(5 \times 10^9\) little further adsorption occurred, indicating that the adsorptive capacity of the resin was close to this value.

5.3.2.2 The effect of pH value and salt concentration of the microbial suspension on the adsorption of food associated microbial species

The effect of salt concentration

The effect of the concentration of phosphate-citrate buffer (pH 5.5) on the adsorption of \textit{Staph. aureus} is shown in Fig. 24.

Adsortion from distilled water was low (0-20\%\) but increased to about 70\% when the cells were suspended in 10mM buffer, and exceeded 90\% from 50 and 100 mM buffers. When the concentration of the buffer was further raised to 500 mM a slight decrease in adsorption was observed. When the buffer solutions were made to 0.5M with sodium chloride the result was similar to that found with strongly acidic
Fig. 22. Effect of column size on adsorption of *Staph. aureus* to Bio-Rex 70 at pH 5.5.
Fig. 23. Adsorptive capacity of Bio-Rex 70 for *Staphylococcus aureus* at pH 5.5.
Fig. 24. The effect of salt concentration on the adsorption of *Staph. aureus* to weakly acidic cation exchange resin (*Bio-Rex 70*).
A similar effect of salt concentration upon adsorption was demonstrated for E. coli (Table 15).

**TABLE 15**

The effect of salt concentration on the adsorption of E. coli to Bio-Rex 70

<table>
<thead>
<tr>
<th>Suspending medium</th>
<th>% Adsorption at pH:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4.5</td>
</tr>
<tr>
<td>Distilled Water</td>
<td>22</td>
</tr>
<tr>
<td>Phosphate-citrate buffer 50mM</td>
<td>67</td>
</tr>
</tbody>
</table>

The effect of salt was seen at both pH levels. At the lower pH appreciable adsorption occurred from distilled water (cf. Part 4.3.1.3) whilst at the higher pH adsorption was more dependent upon the presence of salts.

The effect of pH

There was a marked effect of pH of 50 mM phosphate-citrate buffer on the adsorption of all the species studied and differences in the patterns of adsorption for Gram positive and Gram negative species of bacteria were observed.

The Gram positive species, Staph. aureus and Bacillus stearothermophilus (Fig. 25) showed higher adsorption over the range pH 4.5 - 5.5 than over the range pH 5.5 - 7.5.
The Gram negative species (Fig. 26) including *E. coli* (Fig. 25) all showed a marked decrease in adsorption within the range pH 4.0 - 5.5 followed by an increase in adsorption over the range pH 5.0 - 6.0. The pattern of adsorption of *Salmonella typhimurium* was similar to that of the other Gram negative species but appeared in comparison to be displaced by 0.5 pH units towards the acidic end of the scale. This organism was also the only one of the six species studied which did not show an increase in adsorption within the range pH 7.5 - 9.0.

5.3.3 The effect of chemical modification of the microbial cell surface on the adsorption of micro-organisms

5.3.3.1 The effect of the modification of cell surface anionic and cationic groups of *Staph. aureus*

The effects of a range of cell surface modification procedures on the adsorption of *Staph. aureus* to Bio-Rex 70 at pH 5.5 are shown in Table 16. The effects of the various treatments are expressed as percentage inhibition or enhancement of adsorption in comparison with the adsorption of untreated controls which were made simultaneously.
Fig. 25. Effect of pH on the adsorption of three species of microorganisms to Bio-Rex 70.
Fig. 26. Effect of pH on the adsorption of three Gram negative species of micro-organisms to Bio-Rex 70.
TABLE 16
The effect on adsorption of the modification of cell surface groups of *Staph. aureus*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Groups affected</th>
<th>% Inhibition or enhancement of adsorption</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Anionic Groups</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methanolysis (Zvaginstev, 1972)</td>
<td>carboxyl</td>
<td>+70</td>
</tr>
<tr>
<td>Alcian Blue (Everson-Pearce, 1968)</td>
<td>carboxyl</td>
<td>+53</td>
</tr>
<tr>
<td>Safranine (Fraenkel-Conrat, 1944)</td>
<td>carboxyl, hydroxyl, phosphatidyl, sulphydryl</td>
<td>+58</td>
</tr>
<tr>
<td>Carbodiimide (Heckels et al., 1976)</td>
<td>carboxyl</td>
<td>-56</td>
</tr>
<tr>
<td><strong>Cationic groups</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dinitrofluorobenzene (Ghuysen &amp; Strominger, 1963)</td>
<td>amino</td>
<td>-34</td>
</tr>
<tr>
<td>Orange G (Fraenkel-Conrat, 1944)</td>
<td>amino, guanidyl, imidazole</td>
<td>-52</td>
</tr>
<tr>
<td>Citraconic anhydride</td>
<td>amino</td>
<td>-36</td>
</tr>
<tr>
<td>Formaldehyde (Sherbet &amp; Lakshmi, 1973)</td>
<td>amino</td>
<td>-93 *</td>
</tr>
<tr>
<td>Pronase</td>
<td>peptide bonds (non-specific)</td>
<td>-59</td>
</tr>
<tr>
<td>Teichoic acid deficient mutant of <em>Staph. aureus</em> (Chatterjee, 1969)</td>
<td>Teichoic acid in cell wall</td>
<td>0</td>
</tr>
</tbody>
</table>

* Contrast with Fig 27 p.124
The results show that, generally, treatments which attack anionic groups promote adsorption whilst treatments which attack cationic groups inhibit adsorption. The carbodiimide treatment was an exception to this pattern. However, the microscopical appearance of carbodiimide treated cells together with a fall in the cell count of the suspension during the treatment, suggested that this treatment caused far greater cell damage than did the other treatments.

Deficiency of teichoic acid in the cell wall did not affect adsorption but treatment with the proteolytic enzyme, Pronase, considerably reduced adsorption.

Overall, these results indicate that adsorption is due to the positive charges on cationic groups associated with cell wall proteins.

5.3.3.2 The effect of formaldehyde treatment on the pH-adsorption spectra of micro-organisms

The adsorptive behaviour of all of the microbial species examined was modified by the formaldehyde treatment previously demonstrated by Sherbet and Lakshmi (1973) to block amino groups on the microbial cell surface.

The results obtained with the three bacterial species (Figs. 27 to 29) showed a relatively small inhibitory effect of formaldehyde treatment on the adsorption of cells over the range pH 4.5 to 6.5; the greatest difference was observed for *Bacillus stearothermophilus* (Fig. 29).

A greater inhibitory effect was observed within the range pH 6.5 to 8.5. Complete inhibition of the adsorption of formaldehyde treated cells of *Bacillus stearothermophilus* occurred at pH 6.5 and for
Fig. 27. The effect of formaldehyde treatment on the adsorption of *Staph. aureus* to Bio-Rex 70.
Fig. 28. The effect of formaldehyde treatment on the adsorption of *E. coli* to Bio-Rex 70.
Fig. 29. The effect of formaldehyde treatment on the adsorption of *Bacillus stearothermophilus* to *Bio-Rex 70*.
those of the other two species at pH 7.5. Complete inhibition of all three species was maintained at pH 8.5. Within this range the untreated cells generally exhibited greater adsorption than did the formaldehyde treated cells and in all cases some reversal of the trend of decreasing adsorption with increasing pH was observed.

Cells of the yeast, **Saccharomyces cerevisiae** were investigated over a more limited range of pH (Fig. 30) but showed a general trend of decreasing adsorption with increasing pH similar to that observed for the bacteria. At the two highest pH levels studied the adsorption of the untreated cells was greater than that of the formaldehyde treated cells.

Overall the results indicated that the chemical groups affected by formaldehyde treatment of the cells have a relatively minor role in adsorption at pH values below about 5.0 but assume a greater role in adsorption as the pH rises.

### 5.3.4 The adsorption of cell-surface-protein deficient mutants of E. coli

The adsorptive behaviour was examined for two species of **E. coli** known to lack major protein components of the outer cell membrane.

In the first experiment (Fig. 31) the adsorptive behaviour of a mutant strain deficient in outer membrane protein 1 was compared with that of the parent organism. Little difference was observed in adsorption of the two strains at pH values between 4.0 and 6.0.
Fig. 30. The effect of formaldehyde treatment on the adsorption of *Sacch. cerevisiae* to Bio-Rex 70.
Fig. 31. The adsorption of parent and outer membrane protein 1 deficient mutant strains of *E. coli* to Bio-Rex 70.
At pH values between 6.0 and 7.5 the parent strain adsorbed but the mutant strain did not. The pattern of adsorption of the parent strain was similar to that previously observed for *E. coli* (Fig. 25 & 28) in that there were two peaks of adsorption separated by a trough in the region pH 5.5 - 6.0. However the patterns were not identical; the parent and mutant organisms exhibited much higher adsorption at pH 5.0.

*E. coli* strain K-12 which is deficient in outer membrane protein 2 (DiRienzo, et al., 1978) was also examined in this system and showed behaviour identical with the other two organisms between pH 4.0 and 6.0. Over the range 6.0 to 7.5 this strain showed only slight adsorption, intermediate between that of the mutant and parent organisms shown in Fig. 31.

5.3.5 The separation of specific bacterial forms by the use of cation exchange resin

The separation of a mixed culture of *E. coli* and *Staph. aureus* by selective adsorption

When a mixed suspension of *E. coli* and *Staph. aureus* in 50 mM buffer (pH 5.9) was applied to the resin column and eluted with the same buffer (Fig. 32), only cells of *Staph. aureus* were adsorbed. Cells of *E. coli* were washed through the column by this buffer. The adsorbed cells of *Staph. aureus* were subsequently eluted with distilled water.
Fig. 32. Separation of *Staph. aureus* and *E. coli* by differential adsorption to Bio-Rex 70.
The extent of the separation achieved is shown in Table 17.

**TABLE 17**

*The separation of E. coli and Staph. aureus by selective adsorption to cation exchange resin*

<table>
<thead>
<tr>
<th>Microbial composition</th>
<th>Percentage of:</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E. coli</td>
<td>Staph. aureus</td>
</tr>
<tr>
<td>Initial suspension</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td>Initial effluent</td>
<td>99.96</td>
<td>0.04</td>
</tr>
<tr>
<td>Fractions 8 and 9</td>
<td>4.0</td>
<td>96</td>
</tr>
</tbody>
</table>

A considerable enrichment of *E. coli* was evident in the initial effluent from the column compared with the mixture applied to the column. Enrichment of *Staph. aureus* was evident in fractions 8 and 9 i.e. distilled water. Overall, 87% of the cfu of *E. coli* and 97% of the cfu of *Staph. aureus* present in the original mixture were recovered in a viable state after the separation process.

**The separation of a mixed suspension of E. coli and Staph. aureus by selective elution**

When a mixed suspension of the two organisms in 50 mM buffer at pH 4.7 was passed through the column both types of cell adsorbed (Fig. 33). Elution with sodium citrate (50mM) released large numbers of cells of *E. coli* which were collected in fractions 7 and 8. Subsequent elution with distilled water released large numbers of cells.
Fig. 33. Separation of *Staph. aureus* and *E. coli* by differential elution from Bio-Rex 70.
of Staph. aureus which were collected in fractions 9 and 10. In both cases considerable enrichment of one cell type was achieved (Table 18).

**TABLE 18**

The separation of E. coli and Staph. aureus by selective elution from cation exchange resin

<table>
<thead>
<tr>
<th>Microbial composition of:</th>
<th>Percentage of:</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E. coli</td>
<td>Staph. aureus</td>
</tr>
<tr>
<td>Initial suspension</td>
<td>55</td>
<td>45</td>
</tr>
<tr>
<td>Fractions 7 and 8</td>
<td>96</td>
<td>4</td>
</tr>
<tr>
<td>Fractions 9 and 10</td>
<td>8</td>
<td>92</td>
</tr>
</tbody>
</table>

Overall, 94% of the cfu of E. coli and 120% of those of Staph. aureus present in the original mixture were recovered in a viable state after the separation process.

5.4 **Discussion**

Two types of adsorptive interaction can be demonstrated between micro-organisms and cation exchange resins. The first type occurs in suspensions of low ionic strength at pH values in the approximate range pH 1 - 5. This interaction appears to be, except at very low pH i.e. below 1.5, largely independent of the charge on the resin since similar interactions occur with anion exchange resin, glass beads and polystyrene beads (See Part 4.3.1.3).
The second type of interaction occurs at intermediate pH values i.e. pH 5 - 8 in suspensions containing dissolved salts and within this range adsorption may vary with pH (Figs 25 and 26).

The extent to which adsorption is dependent upon the pH and the salt concentration of the suspension has not been demonstrated previously. These effects can satisfactorily explain the contradictory reports of earlier workers such as Gillisen et al., (1961) and Zisgray, et al., (1970) who found that bacteria did not adsorb to cation exchange resin and of Zvaginstev, (1962) and Wallenstein (1975) who reported that such adsorption did occur. The two former workers used suspensions of micro-organisms in water and EDTA solutions respectively. In contrast, Wallenstein (1975) suspended the organisms in 2M sodium chloride. Zvaginstev suspended the organisms in water but noted that adsorption was increased by saturation of the resin and of the microbial cells by cations.

The effect of salt concentration on adsorption (Figs. 22 and 25) is an apparently striking observation since at neutral pH the micro-organisms are negatively charged and would not be expected to adsorb to the negatively charged resin.

Reversible adsorptive interactions between negatively charged particles may be explained in terms of the DLVO theory of colloid stability (Derjaguin and Landau, 1941; Verwey and Overbeek, 1948) as an adhesive bond formed when the electrostatic forces of repulsion are balanced by the Van der Waal forces of attraction. It is possible that even low concentrations of salts might suppress the electrostatic forces of repulsion sufficiently to permit this type
of interaction (Marshall, 1976). However, such interactions are weak and occur slowly (Sherbet, 1978) whilst the sharpness of the adsorption and elution peaks observed in the present work (Figs. 32 and 33) suggest strong attractions and repulsions more usually associated with purely electrostatic interactions.

There is some basis for a purely electrostatic attraction between the cells and the resin because whilst the cells bear an overall negative surface charge they also possess surface cationic groups (Haydon, 1961; Gittens & James, 1963; Heckels, 1976), and in the present study such groups have been shown to be involved in adsorption (Table 16). Even so, to propose an electrostatic mechanism of attraction between two overall negatively charged surfaces appears to present a paradox.

A closely analogous situation was encountered by Snoeren (1976) who observed that increases in ionic strength up to 0.2M promoted aggregation between a large negatively charged polysaccharide, K-carrageenan and a smaller negatively charged protein, K-casein. This effect was attributed to the asymmetric distribution of charge on the protein molecule. K-casein contains 165 amino acid residues but examination of the primary structure shows that the negatively charged residues are located in the terminal portions of the chain, leaving an extensive positively charged region between residues 20 to 115. This accumulation of positive charge was absent in the other casein fractions, αS1 and β-caseins, neither of which aggregate with K-carrageenan.
Snoeren suggested that the overall negative charge of K-casein was suppressed by salt and that an electrostatic attraction ensued between the clusters of positively charged residues on the protein and the negatively charged groups of the polysaccharide.

The major charged groups on the surface of micro-organisms are associated with proteins. This has been demonstrated for several classes of micro-organisms: Gram positive bacteria e.g., staphylococci (James and Brewer, 1968a) and streptococci (Rutter and Abbot, 1978); Gram negative bacteria; E. coli (Sherbet and Lakshmi, 1973) Neisseria gonorrhoeae (Heckels et al., 1976); and yeasts (Eddy and Rudkin, 1958). The involvement of cell surface protein in the salt dependent adsorption of micro-organisms to cation exchange resin has been noted by Wallenstein (1975) and confirmed in the present study. Consequently whilst the dimensions of the cell-resin system are orders of magnitude greater than those of the protein-polysaccharide system of Snoeren (1976) the elements are analogous; a large negatively charged particle and a smaller proteinaceous particle. This analogy is further supported by the report of Neihoff and Echols (1978) that the amino groups in the microbial surface are normally shielded by anionic groups which are in excess and lie closer to the surface. Thus both K-casein and microbial cells may show localised areas of potentially positively charged groups whilst possessing an overall negative charge.

The results of the present work (Table 16, Fig. 31) indicate that protein is involved in adsorption and that the balance of positively and negatively charged sites influences the adsorption (Table 16). The positively charged sites create attractive forces;
loss of attraction after reaction with formaldehyde (Figs. 27 - 30), indicates that these are amino groups.

The decrease in overall adsorption and the increasing influence of amino groups with increasing pH probably reflects the overall charge of the microbial cells which for *E. coli* show increasing negative charge with increasing pH up to a maximum at pH 4.5 - 5.5 (Neihoff and Echols, 1973). It is in this pH range that adsorption of the Gram negative cells falls sharply, presumably due to electrostatic repulsion. Above this pH there is no further increase in the overall negative charge but there is potential for the creation of positively charged sites.

The most important basic groups that associate with protons to create positively charged sites on proteins around neutral pH are the imidazolyl groups of histidine which ionize in the range 6.4 - 7.4 and chain terminating α-amino groups which ionize in the range pH 7.5 - 8.0 (Morris, 1973). It is within these ranges of pH values that reaction of bacterial cells with formaldehyde (Figs. 27 - 29) and the deletion of cell surface protein (Fig. 31) showed their greatest effect upon adsorption.

It is possible therefore to explain the observed results by a mechanism in which overall negatively charged particles are attracted due to positively charged sites on protein on one of the particles. The creation of these positive sites is dependent upon the pH and ionic composition of the suspending medium and most probably upon their position relative to other chemical groups in the
cell surface. However the association or dissociation of chemical
groups may not be the only means of creating or revealing positive
sites; other phenomena may also contribute.

The cell walls of Gram positive bacteria have been shown to possess
many of the properties of polyelectrolyte gels (Marquis, 1968;
Ou and Marquis, 1970). In particular the walls can expand and
contract in response to changes in pH value and ionic concentration
of the suspending medium. These volume changes are attributed to
electrostatic interactions between fixed charges on the major cell
wall polymers; peptidoglycan and teichoic acid. However it is
probable from these and other considerations of the conformational
stability of macromolecules (Von Hippel and Schleich, 1969) that
minor wall polymers e.g. surface proteins may also change their
conformations in response to changes in their solvent environment.
In their cell electrophoretic studies James and Brewer (1968a) found
that for some strains of Staph. aureus isolated from humans the cell
surface changed with fall in pH to reveal protein which did not
have surface expression of higher pH levels.

If such conformational changes of surface macromolecules expose
additional positively charged groups at the surface then even though
the overall charge within the electrokinetic zone remains negative
there can still be revealed at the surface sufficient positive groups
to promote an electrostatic adsorption of the cells to the negative
resin. If the surface of the adsorbed cell was returned to its
normal conformation electrostatic repulsion would result and rapid
desorption of the cells such as that observed for E. coli and Staph.
aureus (Figs. 32 and 33) would be expected.
At a more practical level the separations of *E. coli* and *Staph. aureus* achieved by the use of cation exchange resin indicate the potential of these resins in microbiological methodology. The separation system is simple, inexpensive and easily controlled by manipulation of pH and salt concentration. All of these factors are desirable; however the most significant factor is that the organisms remain viable after the separation process. This is an essential requirement of any method for the separation of micro-organisms prior to analysis since the great majority of analytical microbiological methods depend upon the growth of the organisms.

5.5. **Summary**

The adsorption of Gram positive and Gram negative bacteria and of yeasts to cation exchange resin has been demonstrated in a simple practical system.

Two types of adsorption were demonstrated. The first type occurred at low pH values (<5) and was independent of salt concentration. The second type occurred at pH values above 5 and was dependent upon the presence of salts.

The second type of interaction was further investigated. The adsorption of different microbial species varied with pH within the range pH 4 – 8 and Gram negative bacteria were separated from Gram positive bacteria by differential adsorption from buffer at pH 5.9. Separation of these species was also achieved by differential elution. In both cases the recovered organisms were viable.
Within the pH range 4 - 8 the adsorption of bacteria to the resin was inhibited by the blockage of cationic cell surface groups and was promoted by the blockage of anionic groups.

Evidence was obtained to suggest the involvement of protein in the adhesion of micro-organisms to the resin and a theory was proposed to account for the electrostatic adsorption of overall negatively charged micro-organisms to negatively charged resin particles.
Ch. 6: THE INTERACTION OF MICRO-ORGANISMS WITH A
GLASS SURFACE UNDER THE INFLUENCE OF AN
ELECTRICAL FIELD
6. THE INTERACTION OF MICRO-ORGANISMS WITH A GLASS SURFACE UNDER THE INFLUENCE OF AN ELECTRICAL FIELD

6.1 Introduction

The adsorption of micro-organisms to solid surfaces under the influence of an electrical field was reported by a group of Russian workers (Gvozdyak et al., 1974). These workers found that a wide range of micro-organisms could be retained on 'granular filter materials' e.g. soil particles, glass, silica gel and ion exchange resins, if these materials were placed in an electrical field and a suspension of organisms was passed through the stationary material. They also reported that the adsorbed organisms were returned to suspension when the field was switched off.

This phenomenon was of interest in the present study, firstly because it provided a separate system for the study of the adsorption of micro-organisms to solid surfaces, and secondly because it offered a possible alternative to the passive adsorption previously investigated for the separation of micro-organisms.

An apparatus was constructed (Fig. 34; Plates V & VI) similar to that described by Gvozdyak, et al., (1974) except for the substitution of platinum or stainless steel electrodes for the manganese oxide coated titanium electrodes used in their original apparatus. A series of experiments was carried out to substantiate the original observations and investigate further the potential application of this phenomenon in the separation of micro-organisms from foods.
6.2 Materials and Methods

Electrical Adsorption Cell

The cell used in this work is shown in Fig. 34. Plates V & VI show the cell in the dissembled and assembled forms respectively. The cell was constructed of perspex and consisted of three basic parts: two end plates and a centre section. Each end plate consisted of a block of perspex into which a recess of 25 mm diameter and 7 mm depth was machined. The electrodes which were of platinum foil were mounted in the recesses and channels were provided in the upper and lower edges of the recesses to permit the flow of coolant past the electrodes. The centre section of the cell consisted of a ring of perspex of int. diameter 25 mm, ext. diameter 28 mm and thickness 10 mm. Diametrically opposed holes were provided to permit the passage of the samples. The end plates were provided with holes through which studs were passed to clamp the parts together.

The cell was assembled by placing a disc of Visking dialysis membrane of diameter 38 mm into each of the end plates. The centre section was placed on top of one end plate and filled with packing material, which in this series of experiments consisted of acid washed glass beads of 2 mm diameter. The second end plate was placed on top and the cell components were clamped together. The assembled cell consisted of three chambers separated by dialysis membranes. The outer chambers contained the electrodes and permitted the passage of coolant whilst the centre chamber contained the packing material and was the site of adsorption. The void volume of the cell was 1 ml.
Plate V  Component Parts

Plate VI  Assembled Cell

Plates V and VI  The electrical adsorption cell
Fig. 34. Electrical adsorption cell.
Experimental test system

The system comprised a number of standard items of laboratory equipment connected to and controlled by a purpose built timer unit.

Two peristaltic pumps supplied the sample and the wash/elution fluid respectively to the adsorption cell at 1 ml min$^{-1}$. A standard electrophoresis power pack (Vokam, Shandon Southern Ltd.) produced the D.C. supply for the electric field. An integrated circuit timer connected in series with the power supply output provided the switched D.C. source for elution. The frequency of this switched D.C. supply was adjustable.

During the sample application and wash periods the electric field was maintained at the required D.C. voltage. During elution a switched D.C. field of the same voltage was applied at a frequency of 2 c.p.s.

The coolant for the cell was continuously supplied by gravity from two 2½ litre reservoirs situated about 1 m above the cell. After passing through the cell the coolant was run to waste, care being taken to avoid contact of the two streams.

Experimental Method

In all experiments carried out using the electrical adsorption cell, with the exception of those in which protein solutions or minced beef homogenates were used, the following experimental method was employed.
Ten ml of sample was run through the cell with the required voltage applied and the cell was then washed with a 10 ml volume of distilled water. Organisms were eluted in a further 5 ml volume of eluant and using a slowly cycled D.C. field. The initial column effluent, the wash and the concentrated eluant were collected and examined for viable micro-organisms and for optical extinction at 420 nm. In some experiments in which buffer solutions were used all the liquids were chilled to about 2°C before passage through the cell. pH measurements were made on the various samples. In all of the work carried out the electrolyte used in the cooling system was the same as that used to suspend the micro-organisms.

Minced beef

Minced beef homogenate was prepared by adding 90 ml of distilled water to 10 g of minced beef and stomaching for 30 secs. The homogenate was centrifuged in an MSE minor centrifuge by taking the centrifuge up to maximum speed (= R.C.P. of 2000) and then allowing it to run down. The supernatant solution was passed through the electrical adsorption cell.

6.3 Results

6.3.1 The effect of the applied potential on the adsorption and recovery of micro-organisms

The effect of the applied potential

In all of the experiments carried out with suspensions of micro-organisms in distilled water significant adsorption to glass beads
occurred only under the influence of an electrical field. The adsorption of *E. coli* to glass beads increased with the magnitude of the applied field over the range of potential investigated. When, after adsorption of the organisms, distilled water was substituted for the microbial suspension and the direct voltage maintained at 350 volts some organisms were observed in the effluent. The effect of switching i.e. repeatedly reversing the polarity of the field, at a frequency of 2 cycles per second (c.p.s.) was rapidly to desorb the organisms from the beads, effecting a marked concentration of the organisms in the effluent compared with that in the original suspension (Fig. 35).

A similar effect of increasing adsorption with increasing potential was observed for other microbial species but variations in the extent of adsorption were observed between the different organisms. (Fig. 36). This was particularly evident with the parent and teichoic acid deficient strains of *Staph. aureus*. The mutant strain was less strongly adsorbed than the parent strain. The yeast *Sacch. cerevisiae* showed an overall increase in adsorption with increasing electrical potential but the results were erratic possibly due to aggregation of the organisms of this strain, which showed a tendency to flocculate in distilled water.

All of the species investigated, including *Salmonella typhimurium* and *Sacch. carlsbergiensis*, were adsorbed to glass beads under the influence of an electrical field and desorbed when the field polarity was switched but in no case was desorption observed when the field was merely removed. The effect on the desorption of
Effluent during adsorption (Constant potential)

Effluent during desorption (Alternating potential)

Fig. 35. The effect of electrical field potential on the interaction of E. coli with glass beads.
Fig. 36. The effect of applied potential on the adsorption of micro-organisms to glass beads.
Staph. aureus of removing the electrical field (Fig. 37) was to increase very slightly the concentration of organisms in the effluent but this effect was negligible in comparison with that produced by switching the field.

The recovery of viable organisms

The combined effects on the viability of cells of adsorption under the influence of an electrical field of 350V and desorption in a switched field are shown in Table 19.

<table>
<thead>
<tr>
<th>Species</th>
<th>Concentration of cfu in initial suspension (cfu ml⁻¹)</th>
<th>% cfu adsorbed</th>
<th>% cfu desorbed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Staph. aureus</td>
<td>10⁷</td>
<td>93</td>
<td>114</td>
</tr>
<tr>
<td>E. coli</td>
<td>10⁷</td>
<td>89</td>
<td>64</td>
</tr>
<tr>
<td>Sacch. carlsbergiens</td>
<td>10⁴</td>
<td>97</td>
<td>81</td>
</tr>
<tr>
<td>Sacch. cerevisiae</td>
<td>10⁴</td>
<td>96</td>
<td>77</td>
</tr>
</tbody>
</table>

In all of the cases substantial adsorption occurred. Only in the case of Staph. aureus was recovery of adsorbed organisms complete and it seems probable that this result was due to the splitting of
Fig. 37. The effect of electrical field potential on the interaction of Staphylococcus aureus strain H with glass beads.
clumps of cells leading to an increase in the number of colony forming units. The incomplete recovery of adsorbed cells of the other species indicates that a fraction of these cells was not desorbed, or that the cells were killed. However, the levels of recovery observed indicated that the majority of the cells of all four species survived both the adsorption and desorption processes.

6.3.2 The effect of the adsorbent on the efficiency of adsorption

It was observed, that under comparable conditions, the substitution within the adsorption cell of glass beads by silica gel resulted in more efficient adsorption of micro-organisms.

When experiments were performed without an adsorbent in the adsorption cell organisms were retained less efficiently than when an adsorbent was present. In the absence of an adsorbent several effects were observed by eye. When a turbid suspension of Sacch. cerevisiae was passed through the apparatus the cells accumulated on the dialysis membrane adjacent to the anode where they appeared as a white precipitate. A single reversal of the electrical field resulted in the 'explosive' repulsion of the precipitate from the membrane and an immediate increase in turbidity within the cell and in the effluent. This was followed by the accumulation of a precipitate at the new anode.

In an experiment in which ion exchange resin (Bio-Rad AG1) was used as the adsorbent very high currents were encountered, the adsorbent bed rapidly darkened in colour and the apparatus became very hot.
This investigation was abandoned.

6.3.3 Effect of the composition of the suspending medium on the operation of the adsorption cell

Electrolyte concentration

In the experiments on the adsorption of micro-organisms from distilled water, low currents (1 - 2 mA) were observed across the cell. It was also observed that the pH value of the effluent tended to fall during adsorption and rise during desorption. When attempts were made to minimize the pH changes by suspending the organisms in buffer solution (McIlvaines) of low molarity (1 mM) the following effects were observed. The efficiency of adsorption of the micro-organisms decreased compared with that from distilled water. There was a marked increase in the current across the cell; from 2mA to 40mA at 350V and the cell became warm. When 5mM buffer was employed it was not possible to maintain a potential of 350V due to excessive heating within the cell.

These effects were confirmed when a solution (0.09% w/v) of sodium chloride was passed through the cell under a potential of 200V. A current in excess of 80mA was produced and the following pH values were observed for the various fractions: initial solution, 7.6; initial effluent, 2.1; wash, 2.8; effluent during switching of field 8.2. A very marked heating effect occurred and no attempt was made to study the adsorption of micro-organisms in this system.
### TABLE 20

The effect of bovine serum albumin in the electrical adsorption cell

<table>
<thead>
<tr>
<th>Stage</th>
<th>Suspending fluid</th>
<th>Applied voltage</th>
<th>pH value</th>
<th>Protein/mg ml⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td></td>
<td></td>
<td>5.9</td>
<td>8.0</td>
</tr>
<tr>
<td>Effluent</td>
<td>Distilled water</td>
<td>350V</td>
<td>4.9</td>
<td>7.4</td>
</tr>
<tr>
<td>Wash</td>
<td></td>
<td></td>
<td>5.4</td>
<td>2.8</td>
</tr>
<tr>
<td>Eluant</td>
<td></td>
<td></td>
<td>8.2</td>
<td>0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Stage</th>
<th>pH value of suspension</th>
<th>Protein/mg ml⁻¹</th>
<th>Log₁₀ Total viable count (c.f.u. ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>6.1</td>
<td>9</td>
<td>8.04</td>
</tr>
<tr>
<td>Effluent</td>
<td>4.8</td>
<td>7.5</td>
<td>7.74</td>
</tr>
<tr>
<td>Wash</td>
<td>4.7</td>
<td>2.5</td>
<td>7.31</td>
</tr>
<tr>
<td>Eluant</td>
<td>8.4</td>
<td>0</td>
<td>6.07</td>
</tr>
</tbody>
</table>
The effect of protein and of meat on the adsorption of micro-organisms

There was no adsorption of bovine serum albumin from dilute solutions passed through the cell, however the pH value of the solutions was lowered (Table 20). When Staph. aureus was suspended in the protein solution there was relatively little adsorption of this organism (Table 21).

When minced beef supernatant was passed through the cell several effects were observed. This red, cloudy preparation was decolourised and clarified and a white precipitate formed on the beads. The pH of the effluent fell from 6.5 to 3.5 and the microbial count fell by five orders of magnitude.

6.4. Discussion

The results obtained here confirm the observations of Gvodzyak, et al., (1974) that micro-organisms adsorb to solid surfaces under the influence of electrical fields and that the extent of adsorption is proportional to the magnitude of the applied voltage. The observation that adsorbed organisms were returned to suspension when the electrical field was switched off was not confirmed since the adsorbed cells were removed only when the field was alternated.

Overall it appears that the adsorption of micro-organisms in this system is initiated by electrokinetic forces exerted on the organisms but that the adhesive forces between the cells and the adsorbent are independent of the electrical field. These observations can be explained in terms of the DVLO theory of colloid stability.
(Derjaguin and Landau, 1941; Verwey and Overbeek, 1948).

The micro-organisms and adsorbents (glass and silica) bear overall negative surface charges and the electrostatic forces of repulsion normally prevent a sufficiently close approach for any effect of the shorter range attractive forces which exist between particles to occur. The electromotive force exerted on the organisms by the electrical field provides sufficient kinetic energy to overcome the electrostatic repulsion and permit the organisms to approach the adsorbent surface more closely. At this closer range the attractive forces, e.g. the London dispersive force, are effective and balance the electrostatic repulsive forces; the resultant adhesion can be independent of the electrical field.

However, the observation that the parent strain of \textit{Staph. aureus} was more readily adsorbed than was the teichoic acid deficient mutant does not fit this theory. The teichoic acid deficient mutant which bears fewer negative groups (Ou & Marquis, 1970) would be expected to experience lower forces of electrostatic repulsion than the parent organism and therefore greater adsorption. The observation that the reverse is true indicates an overall electrostatic attraction. It seems more probable that the phenomena observed are due to induced positive electrical charges on the glass beads and are wholly electrostatic in nature. The retention of the organisms after the field has been switched off would then be explained by retention of the induced charge on the beads when surrounded by a solvent of low conductivity i.e. distilled water.

Whichever mechanism is involved in adsorption and adhesion the mechanism of desorption seems clear. The reversal of the polarity of
the field reverses the direction of the electromotive force on the adsorbed micro-organisms which then reinforces the electrostatic repulsion between the particles and the micro-organisms are desorbed, move towards the new anode but are intercepted by other particles of adsorbent to which they adhere. In the intervening period the hydrodynamic forces exerted by the fluid stream displace the organisms towards the electrical cell exit and, upon continuous reversal of the field, eventually wash the organisms from the cell.

The differences in the levels of adsorption of the different microbial species under identical conditions (Fig. 36) are probably related to the electrical charge characteristics of the cell surfaces and to the mass and shape of the microbial cells. These properties will affect the electrokinetic energy of the cells as they approach the adsorbent surface and will also influence the magnitude of the electrostatic repulsion between the particles. The difference in the level of adsorption of the parent and mutant strains of Staph. aureus (Fig. 36) lends support to this view.

The processes of adsorption and desorption appeared to have little effect upon the viability of the micro-organisms and this type of system may be usefully applied to the collection and concentration of micro-organisms from water. The application of this 'electrically assisted' adsorption to the separation of micro-organisms from food homogenates appears less promising. The adsorption of organisms will be severely limited by the presence of salts, proteins and other small charged particles and the concomitant effects of low pH values and heating within the apparatus may kill or seriously impair the organisms.
In a broader context, the experimental system may be of practical value in the study of the forces involved in the adhesion of microbial cells to solid surfaces. Since the mass and cell surface charge density of many microbial cells are known (Heckels, 1978) it would be possible from measurements to be made in this system to calculate the kinetic energy of microbial particles approaching adsorbent surfaces. This would give estimates of the electrostatic forces of repulsion between cells and adsorbents. Conversely, the forces of adhesion might be estimated from the applied voltage required to desorb the organisms. Such measurements would be practically simple and could be made for a broad variety of surfaces and of organisms. In addition the use of cells with specifically modified surface groups would provide data on the quantitative contribution of specific surface groups to the adhesion of organisms to solid surfaces.

6.5 Summary

The adsorption of micro-organisms to solid surfaces under the influence of an electrical field has been demonstrated. The level of adsorption of micro-organisms was directly related to the applied potential and also varied with the microbial species.

Adsorbed micro-organisms were not recovered when the electrical field was switched off but were recovered when the polarity of field was alternated at low frequency.

A mechanism was proposed which could account for these phenomena in terms of electrostatic forces.
This 'electrically assisted' adsorption system permitted the collection and concentration of viable bacteria and yeasts from distilled water. The presence of small electrically charged particles e.g. salt ions and protein molecules in the microbial suspension decreased the efficiency of adsorption of the micro-organisms and gave rise to pH changes and heating effects within the apparatus. These effects would severely limit the use of this type of adsorption for the separation of micro-organisms from foods.

The simplicity of the experimental system and the ability to adsorb and desorb micro-organisms by the controlled application of electromotive forces suggest that this type of adsorption might be of value in the investigation of the forces involved in adhesion of micro-organisms and other colloidal particles.
PART II

Ch. 7: THE SEPARATION OF MICRO-ORGANISMS FROM FOODS
7. THE SEPARATION OF MICRO-ORGANISMS FROM FOODS

7.1 Introduction

An important practical aspect of the study of the interactions of micro-organisms with ion exchange resins was the potential use of such systems for the recovery of micro-organisms from natural environments, particularly foods.

This aspect of the work was investigated using both anion and cation exchangers and the food systems chosen were largely meats. It was felt that the separation of micro-organisms from such complex systems would adequately demonstrate the potential of this approach.

In preliminary investigations it became obvious that crude homogenates of meats were unsuitable for presentation to columns of ion exchange resin. The larger meat particles tended to block the columns and it was necessary to devise a preliminary separation stage to remove these particles from the homogenates.

7.2 Materials and Methods

Centrifugation of food homogenates. Centrifugation was carried out in 50ml polypropylene tubes using an MSE Minor bench centrifuge (MSE Instruments Ltd., Crawley). The instrument was calibrated by measuring the speed of revolution developed after various periods at maximum setting. Speed of revolution was monitored using a tachometer and the relative centrifugal force (R.C.F.) calculated from the following equation:

\[ RCF = 1.118 \times R \times N^2 \]
where \( R \) = radius of rotation (cm) and \( N \) = speed of rotation in revolutions per minute.

The relative centrifugal forces developed during short periods of centrifugation are shown in Fig. 38.

**Dry weight measurements.** Duplicate 10ml amounts of the homogenate were pipetted into pre-weighed, shallow dishes constructed from aluminium foil. The samples were dried, at 95°C, to constant weight. The average weight was used.

**Preparation of food samples for application to anion exchange resin**

Solid foods were homogenized (Stomacher, A.J. Seward) in distilled water, normally 10g in 90ml, and centrifuged. The supernatant solution was used. Liquid foods were diluted with deionized water. Ginger ale was thoroughly agitated before use to remove dissolved carbon dioxide.

Jars (400g) of the two types of baby food were inoculated with 1.0ml of a 24h culture of *Bacillus stearothermophilus* and incubated for three days at 55°C. Twenty grams of the food was homogenized in 80ml of distilled water. Slices of ham (40g) were spread with 0.2ml of a 24hr culture of *Staph. aureus* and incubated for 24hr at 37°C. Minced pork was inoculated with a suspension of *Clostridium sporogenes* spores in distilled water.

**Application of food homogenates to anion exchange resin.** Supernatant solutions of solid foods derived by centrifugation of homogenates at 2000 xg for 30 secs (unless otherwise stated) and dilutions of liquid foods were used. Ten ml of the solution was applied to Bio-Rad
Fig. 38. The relative centrifugal force exerted during periods of centrifugation.
AG1 x 8 (100/200 mesh) in the small column system. The resin was then washed with 10ml of sterile deionised water. Where required, micro-organisms were recovered by agitation of the resin in 10ml of the chosen eluant.

Separation of micro-organisms from raw beef using cation exchange resin

Ten grams of minced beef were homogenised in 90ml of phosphate-citric acid buffer (0.05M, pH 5.8) for 30 seconds and the homogenate centrifuged at 2000 x g for 30 seconds. Five ml of the supernatant solution was applied to Bio-Rex 70 cation exchange resin in the syringe column system followed by 2 x 5 ml of sterile buffer. The protein adsorbed to the column was then eluted with 5ml of 0.25M sodium phosphate followed by 2 x 5 ml 0.25M phosphate – citric acid buffer pH 6.5. The residual salts were eluted with 3 x 5 ml of distilled water; 5 ml fractions were collected throughout. Total viable counts at 30°C on plate count agar after 24 hours, protein estimation by the method of Lowry et al., 1951) and pH measurements were carried out on all fractions. The sample used for subsequent rapid estimation experiments comprised the first two 5ml fractions from the column.

7.3 Results

7.3.1 The centrifugal separation of gross particles from food homogenates

The physical composition of food homogenates varies with the type of food and the homogenization procedure used but in all meat homogenates some particles remain of sufficient size to cause blockages in columns of resin beads. Efforts to overcome this problem by filtering the homogenates through paper or cloth resulted in the loss of a
substantial proportion (>50%) of the microbial population and this proportion varied widely with the sample.

The use of centrifugation for the separation of gross food particles from bacteria in these homogenates was more successful. When homogenates of meats in distilled water were centrifuged for short periods at 2000 x g a large proportion of the food was removed from suspension whilst the greater part of the microbial population remained in the supernatant solution.

The typical effect of centrifugation is shown for minced beef in Fig. 39. The greater part of the meat ( > 80%) was precipitated from suspension during the period required for the centrifuge to attain a force of 2000 x g (30 secs) after which no further precipitation occurred. During this initial period the concentration of micro-organisms in suspension remained constant or increased slightly. As the period of centrifugation was increased from about 2 mins the microbial concentration decreased. A similar effect was demonstrated for a processed meat product; pork luncheon meat. The results obtained for a range of meats and meat products are shown in Table 22.

**TABLE 22**

<table>
<thead>
<tr>
<th></th>
<th>Period of centrifugation*</th>
<th>Supernatant soln.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(secs)</td>
<td>% dry wt.</td>
</tr>
<tr>
<td>Minced beef</td>
<td>30</td>
<td>6</td>
</tr>
<tr>
<td>Pork</td>
<td>30</td>
<td>8</td>
</tr>
<tr>
<td>Bacon</td>
<td>15</td>
<td>11</td>
</tr>
<tr>
<td>Beefburger</td>
<td>15</td>
<td>10</td>
</tr>
<tr>
<td>Ham</td>
<td>15</td>
<td>10</td>
</tr>
</tbody>
</table>

*Holding period at 2000 x g
Fig. 39. The centrifugal separation of microorganisms from minced beef.
In all cases, and particularly with beefburgers, fat accumulated at the surface of the centrifuged suspension but provided no obstacle to the sampling of the supernatant liquid beneath.

Overall these results indicate that this method is suitable for the preliminary separation of micro-organisms from meats prior to the application of the organisms to resin columns.

7.3.2 The separation of micro-organisms from foods by adsorption to anion exchange resin

7.3.2.1 Adsorption of micro-organisms from a range of foods

In order to assess the applicability of the resin systems evolved in work with pure cultures to more practical situations, the adsorption of micro-organisms from a range of foods was investigated.

When homogenates of foods in distilled water were passed through small columns of Bio-Rad AG1 x 8 100/200 anion exchange resin the percentage of the total viable organisms adsorbed varied with the type of food (Table 23).

Generally, adsorption of micro-organisms comprising the natural flora of raw meats was high (75-98%) as was the adsorption of Staph. aureus grown in ham. However only 50% of spores of Clostridium sporogenes inoculated into minced beef became adsorbed to the resin.
For the two frozen products lower levels of adsorption were observed and in the case of frozen beans was the lowest observed for any of the products examined.

**TABLE 23**

The adsorption of micro-organisms onto anion exchange resin from food homogenates

<table>
<thead>
<tr>
<th>Food homogenate</th>
<th>No. of cfu applied to resin column</th>
<th>Column size g</th>
<th>% cfu Adsorbed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naturally contaminated foods</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacon</td>
<td>$10^5$</td>
<td>0.5</td>
<td>90 : 75</td>
</tr>
<tr>
<td>Minced Beef</td>
<td>$10^6$</td>
<td>0.5</td>
<td>96 : 94*</td>
</tr>
<tr>
<td>Pork</td>
<td>$10^4$</td>
<td>0.2</td>
<td>98 : 92</td>
</tr>
<tr>
<td>Fish fingers</td>
<td>$10^3$</td>
<td>0.2</td>
<td>67 : 56</td>
</tr>
<tr>
<td>Frozen beans</td>
<td>$10^4$</td>
<td>0.2</td>
<td>39 : 27</td>
</tr>
<tr>
<td>Milk**</td>
<td>$10^4$</td>
<td>0.2</td>
<td>70 : 63</td>
</tr>
<tr>
<td>Ginger Ale**</td>
<td>$10^3$</td>
<td>0.2</td>
<td>90</td>
</tr>
<tr>
<td>Artificially contaminated foods</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ham (Staph. aureus)</td>
<td>$10^6$</td>
<td>0.2</td>
<td>100</td>
</tr>
<tr>
<td>Chocolate Baby Food</td>
<td>$10^7$</td>
<td>0.2</td>
<td>47</td>
</tr>
<tr>
<td>Minced Pork (Cl. sporogenes spores)</td>
<td>$10^3$</td>
<td>0.2</td>
<td>48</td>
</tr>
</tbody>
</table>

: = Simultaneous duplicate estimates

* In a further 168 estimations an average value of 89% was obtained

**1 in 10 dilution
The adsorption of the micro-organisms to the resin was in several cases confirmed by electron-microscopic examination of the resin bed. The surface of resin particles removed from the top of the resin bed before and after the passage of a homogenate of bacon are shown in Plates VII and VIII respectively.

The degree of separation of micro-organisms from the food indicated by this result may be appreciated from the fact that at the contamination level of this bacon \(10^5 \text{ cfu g}^{-1}\) the microbial mass would form less than one millionth of the mass of the bacon.

Overall these results demonstrate the separation of micro-organisms from food homogenates and that in this system the proportion of the population adsorbed varies with the food. This variation may reflect qualitative variations in the microbial flora and/or inappropriate experimental conditions for optimal adsorption from the particular food. One of the experimental conditions, the dilution of the homogenate, was examined.

The effect of the dilution of the food homogenate on the adsorption of micro-organisms

In several of the food systems examined low adsorption of micro-organisms to the resin was observed from the supernatant solution of the standard (1 in 10) homogenate, but with increasing dilution of the homogenate adsorption increased markedly.

The effect of dilution with distilled water on the adsorption of Bacillus stearothermophilus (a thermophilic spoilage organism) from vegetable and lamb baby food is shown in Fig. 40. The concentration
Plates VII and VIII The adsorption of micro-organisms from meat to anion exchange resin.
Fig. 40. The effect of dilution of a baby food on the adsorption of *B. stearothermophilus*.
of organisms in the original food was $10^6$ cfu g$^{-1}$. Adsorption did not occur from the 1 in 10 homogenate but at a dilution of 1 in 20 about 40% of the organisms were adsorbed. At a dilution of 1 in 50 adsorption rose to 70% and at 1 in 100 was almost complete. A similar effect was shown for this organism in a 'chocolate pudding' baby food.

An effect of increasing adsorption with increasing dilution was also observed for a beverage, ginger ale, naturally contaminated with low levels ($10^4$ cfu ml$^{-1}$) of a *Saccharomyces* sp.

The results of experiments performed on different occasions (Fig. 41) show that in this case organisms were adsorbed from the neat beverage but that the level of adsorption increased markedly with dilution and at a dilution of 1 in 5 over 90% of the organisms were adsorbed.

A similar effect was observed with pasteurized milk. Less than 10% of the viable microbial population was adsorbed from neat milk whilst at dilutions of 1 in 10 and 1 in 100 70% and 99% respectively of the population became adsorbed to the resin.

### 7.3.2.2 The recovery of micro-organisms from minced beef

Homogenized minced beef was selected as a suitably complex food with which to examine the potential of the anion exchange resin system for the adsorption and recovery of micro-organisms from food.

The supernatant solution from minced beef homogenate was applied to replicate columns of Bio-Rad AG1 x 8. The various fractions from the separation were analysed for viable organisms (cfu ml$^{-1}$) and food content (dry wt.).
The typical separation achieved is shown in Table 24. Virtually all of the food mass was lost in the first three stages of the separation procedure whilst almost 90% of the micro-organisms were retained on the resin columns. Good agreement was found for the three replicate columns.

Extraction of the columns with 0.3M sodium citrate recovered less than 20% of the initial flora. However, these organisms were recovered in clear suspension and could be concentrated by centrifugation to produce suspensions containing levels of micro-organisms of the same order of magnitude as those found in the original homogenate. In the recovery of organisms from the resin, agreement between the replicate analyses was generally less good than that found for adsorption.

The recovery of yeasts from ginger ale by adsorption to anion exchange resin

The use of anion exchange resin for the recovery of low concentrations of Saccharomyces spp. \(3 \times 10^2\) cfu ml\(^{-1}\) from a 1 in 5 dilution of ginger ale is demonstrated in Table 25. In this experiment the effect of the concentration of magnesium chloride was also investigated. At low concentrations of organisms viable counts are more variable; however the results show that a large proportion of the organisms were recovered in a viable condition. Desorption was of pattern III type (See Section 4.3.4.2) i.e. it was independent of magnesium chloride concentration.
TABLE 24
The separation of micro-organisms from minced beef by adsorption to anion exchange resin

<table>
<thead>
<tr>
<th>Fraction</th>
<th>No. of organisms</th>
<th>Dry wt.</th>
<th>% total cfu adsorbed</th>
<th>% total Dry wt.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cfu 10ml⁻¹ x 10⁻⁶</td>
<td>g 10ml⁻¹</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Homogenate</td>
<td>45</td>
<td>0.3058</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Supernatant solution</td>
<td>52</td>
<td>0.0528</td>
<td>-</td>
<td>81</td>
</tr>
<tr>
<td>Column effluents</td>
<td>3.1</td>
<td>0.0458</td>
<td>94</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>4.4</td>
<td>0.0458</td>
<td>91</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>5.4</td>
<td>0.0434</td>
<td>90</td>
<td>14</td>
</tr>
<tr>
<td>Column washes</td>
<td>2.6</td>
<td>0.0158</td>
<td>89</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>1.9</td>
<td>0.0146</td>
<td>89</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>0.8</td>
<td>0.0152</td>
<td>88</td>
<td>5</td>
</tr>
<tr>
<td>Column extracts</td>
<td>8.8</td>
<td></td>
<td></td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>8.2</td>
<td></td>
<td></td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>3.7</td>
<td></td>
<td></td>
<td>7</td>
</tr>
</tbody>
</table>

% total cfu recovered*

*based on supernatant solution
The recovery of low numbers of *Saccharomyces* spp. from ginger ale by adsorption to anion exchange resin

<table>
<thead>
<tr>
<th>Column</th>
<th>No. of cfu in 10ml suspension</th>
<th>% adsorbed to column*</th>
<th>Eluent MgCl₂(M)</th>
<th>No. of cfu desorbed in 10ml eluant</th>
<th>% cfu recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>289</td>
<td>94</td>
<td>0.00</td>
<td>260</td>
<td>90</td>
</tr>
<tr>
<td>2</td>
<td>289</td>
<td>0.00</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>289</td>
<td>93</td>
<td>0.01</td>
<td>200</td>
<td>69</td>
</tr>
<tr>
<td>4</td>
<td>289</td>
<td>0.05</td>
<td>290</td>
<td>&gt;100</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>289</td>
<td>97</td>
<td>0.10</td>
<td>250</td>
<td>78</td>
</tr>
<tr>
<td>6</td>
<td>289</td>
<td>0.20</td>
<td>370</td>
<td>&gt;100</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>289</td>
<td>100</td>
<td>0.30</td>
<td>270</td>
<td>93</td>
</tr>
<tr>
<td>8</td>
<td>289</td>
<td>0.40</td>
<td>200</td>
<td>69</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>289</td>
<td>88</td>
<td>0.50</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>289</td>
<td>1.00</td>
<td>270</td>
<td>93</td>
<td></td>
</tr>
</tbody>
</table>

* calculated from pooled effluents of pairs of columns

- not estimated
Experiment 1

Dilution Factor - final volume in which 1 vol of Ginger Ale was present

Fig. 41. The effect of dilution on the adsorption of a Saccharomyces spp. from Ginger Ale.
The separation of micro-organisms from minced beef by the use of cation exchange resin

The results of the previous section (7.3.2.) demonstrated that micro-organisms could be separated from minced beef by adsorption to anion exchange resin but the recovery of the organisms from the resin was far from complete. Since the major objective of the separation was to recover the organisms, it was considered that the use of cation exchange resin might give an equally good separation in which the food material became adsorbed and the organisms did not. Thus, the problem encountered in the recovery of the organisms from anion exchange resin might be avoided.

On the basis of results obtained with pure cultures (Chapter 5) a pH value was selected at which Gram negative organisms (which form the major part of the microbial flora of raw meat) would be least likely to be adsorbed to the resin. When the supernatant solution from an homogenate of minced beef in citrate phosphate buffer (pH 5.8) was applied to columns of the cation exchange resin Bio Rex-70 a considerable separation was achieved by elution with the citrate-phosphate buffer (Fig. 42). The major proportion of the meat protein was adsorbed to the column whilst the majority of the organisms were eluted in the first two fractions. It should be noted that in Fig. 42 the scale on which the concentration of micro-organisms is recorded is a logarithmic one whilst that for protein is arithmetic.

When elution with citrate-phosphate buffer was continued, little further recovery of either organisms or protein was achieved. The adsorbed protein was recovered from the resin by elution with salt solutions of higher ionic concentration and pH value but in neither case were appreciable levels of organisms recovered.
Fig. 42. Separation of micro-organisms from beef steak.
Typical results for the viable count and protein levels determined in column effluent fractions during a separation experiment are shown in Table 26. The first two fractions from the ion exchange column (effluent and Wash 1) contained 81% of the organisms from the sample with only 13% of the protein from that sample. Overall only 84% of the organisms were recovered whilst the amount of protein recovered slightly exceeded that estimated in the initial sample.

**TABLE 26**

The separation of micro-organisms from beef steak

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total viable count (cfu ml⁻¹)</th>
<th>Protein (µg ml⁻¹)</th>
<th>% Recovery of:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial sample</td>
<td>5.36 x 10⁵</td>
<td>2180</td>
<td>-</td>
</tr>
<tr>
<td>Effluent</td>
<td>3.55 x 10⁵</td>
<td>196</td>
<td>66 )</td>
</tr>
<tr>
<td>Wash 1</td>
<td>8.1 x 10⁴</td>
<td>90</td>
<td>15 ) 81*</td>
</tr>
<tr>
<td>Wash 2</td>
<td>1.09 x 10⁴</td>
<td>20</td>
<td>2</td>
</tr>
<tr>
<td>0.25M Na₂HPO₄</td>
<td>7.5 x 10³</td>
<td>1400</td>
<td>1</td>
</tr>
<tr>
<td>0.25M Buffer</td>
<td>10³</td>
<td>500</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>Distilled water</td>
<td>&lt; 10³</td>
<td>64</td>
<td>&lt; 1</td>
</tr>
<tr>
<td></td>
<td>&lt; 10³</td>
<td>10</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>Total recovered</td>
<td></td>
<td>84</td>
<td>104</td>
</tr>
</tbody>
</table>

*Organisms in effluent and wash 1 = 81% of initial
Organisms in effluent and wash as % of total organisms recovered = 96%
A summary of results obtained using this separation system is presented in Table 27. The data for the treated samples was obtained in each case from analysis of the first two fractions from the column.

**TABLE 27**

Summary of the results obtained for separation of micro-organisms from raw meat using a cation exchange resin.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Initial suspension</th>
<th>Column effluent</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total viable count</td>
<td>Protein</td>
</tr>
<tr>
<td></td>
<td>cfu ml$^{-1}$</td>
<td>µg ml$^{-1}$</td>
</tr>
<tr>
<td>Steak</td>
<td>$5.3 \times 10^5$</td>
<td>2180</td>
</tr>
<tr>
<td>Minced Beef</td>
<td>$7.8 \times 10^6$</td>
<td>2540</td>
</tr>
<tr>
<td>Minced Beef</td>
<td>$1.36 \times 10^6$</td>
<td>3000</td>
</tr>
<tr>
<td>Minced Beef</td>
<td>$8.0 \times 10^4$</td>
<td>4000</td>
</tr>
<tr>
<td>Minced Beef</td>
<td>$6.1 \times 10^4$</td>
<td>3000</td>
</tr>
<tr>
<td>Minced Beef</td>
<td>$3.5 \times 10^5$</td>
<td>2900</td>
</tr>
<tr>
<td>Minced Beef</td>
<td>$3.0 \times 10^6$</td>
<td>3500</td>
</tr>
</tbody>
</table>

**Average recovery**

72  
14.5

The recovery of organisms varied from about 50 to 100% whilst the level of protein recovered was much less variable with limits of 12 – 16%.

In further experiments this pattern of results was continued.
The separation achieved in the cation exchange system was further demonstrated by electron microscopic examination of the initial suspension and of the column effluent.

Plate IX shows the material retained when the initial sample (supernatant solution of minced beef homogenate) was passed through a membrane filter (pore size 0.22μ). The shapes of micro-organisms can be seen but these and the filter surface are overlaid by non-cellular material which completely occludes the pores of the filter.

When part of the initial sample was passed through cation exchange resin and the initial effluent fractions applied to the filter, the extraneous material was absent (Plate X). The micro-organisms are clearly seen, as are the pores of the filter. These effects are shown in greater detail in Plates XI and XII.

These results demonstrate the efficiency of the separation on cation exchange resin and also indicate that filtration offers a further stage of separation. The electron micrographs indicate that the soluble protein which passes through the resin column with the organisms may be removed by simple filtration leaving only the organisms on the filter. The recovery of these organisms from filters has not been investigated. However two preliminary experiments with pure cultures of Staph. aureus and E. coli have suggested that recovery from these polycarbonate filters is superior to that from cellulose based filters.
IX Micro-organisms filtered from supernatant of minced beef homogenate
x 5,700

X Micro-organisms filtered from supernatant solution of minced beef homogenate after passage through cation exchange resin
x 6,000

Plates IX - XII The separation of micro-organisms from meat by the use of cation exchange resin
XI Micro-organisms filtered from supernatant solution of minced beef homogenate
x 20,000

XII Micro-organisms filtered from supernatant solution of minced beef homogenate after passage through cation exchange resin
x 24,000
The centrifugation of food homogenates to remove coarse particles provides a considerable separation of micro-organisms from foods and is an essential preliminary step in the application of foods to columns of ion exchange resins. The slight but definite increases in the microbial concentration of the supernatant solutions after short periods of centrifugation (Fig. 39) might be explained by the dissociation of clumps of micro-organisms during centrifugation but, as the shear forces exerted during centrifugation are most probably lower than those exerted during the prior homogenization, this is unlikely. The increases in colony count observed for meats were relatively small and the cause was not pursued. It is possible, however, that this phenomenon occurs in other suspensions of biomaterials and might provide for more accurate estimates of their microbial populations.

The separation of micro-organisms from food by the use of anion or cation exchange resins was also considerable. In both cases viable organisms representing only 1 ppm of the original food mass were recovered in clear suspensions.

The effect of the dilution of foods on the adsorption of Gram positive micro-organisms to anion exchange resin may reflect the level of salts in these foods (cf Fig. 8, Ch. 4) but this has not been investigated and in view of the composition of the foods it could be argued that sugars e.g. lactose and sucrose may be responsible.

The low recovery of fresh meat microflora (largely Gram negative bacteria) from anion exchange resin might be exploited for the removal of these organisms from meat preparations but is a disadvantage in the use of this system for the fine separation of micro-organisms. This low
recovery parallels that obtained for the gram negative bacterium E. coli from this resin (Part 4.3.2.2) and a possible explanation of this phenomenon is discussed in Part 10.

The incomplete recovery of micro-organisms from columns of cation exchange resin, to which the organisms do not themselves adsorb, might be explained by entrapment of the organisms by the adsorbed meat protein. This is not, however, supported by microbial counts of the subsequently eluted protein fractions. If the missing organisms are not dead or retained on the resin, the most likely explanation of the incomplete recovery is that they are present in the column effluent but are not detected. This could arise if passage through the column caused aggregation of some organisms such that two or more cells or colony forming units merged to form a single larger colony forming unit.

The flocculation of micro-organisms by natural polymers including proteins, nucleic acids and polysaccharides has been described by Harris and Mitchell (1973) and it is possible that such an effect occurs during passage through the resin column. Although in the present work effluent fractions were agitated on a vortex stirrer prior to sampling, further precautions e.g. addition of surfactants may reveal higher recoveries of organisms in this system.

The use of the cation system separation in combination with filtration provides an excellent separation of micro-organisms from raw meat and permits the estimation of micro-organisms in the meat by the assay of microbial ATP (See Ch. 9). A further application might be found in the detection of food pathogens by the fluorescent antibody technique.
In these techniques food debris may obscure the microscopic observation of the stained organisms and can also give rise to high levels of non-specific fluorescence which are rarely encountered with preparations of pure cultures of the organisms (Goldman, 1968).

The broader significance of the results reported here is that they demonstrate the separation of viable micro-organisms from complex mixtures of bio-materials by rapid and simple techniques. This may provide biologists with a novel opportunity to analyse the composition of natural microbial populations and compare them with the traditional observations made on pure (i.e. artificial) cultures of the organisms.

### 7.5 Summary

A considerable separation of micro-organisms from food was achieved by centrifuging food homogenates for short periods.

The adsorption of micro-organisms from food homogenates onto anion exchange resins has been demonstrated.

The separation of micro-organisms from meat has been demonstrated by two methods employing columns of ion exchange resins.

When anion exchange resin was used the organisms became adsorbed to the columns whilst the food material passed through. In several cases the level of adsorption was dependent upon the dilution of the food material.
When cation exchange resin was used much of the meat protein was adsorbed whilst the organisms passed through with a small proportion of soluble protein. In this system the greater part of the micro-flora of beef was consistently recovered in a viable condition.
PART II

Ch. 8: THE RAPID ESTIMATION OF MICROBIAL POPULATIONS

BY IMPEDANCE MEASUREMENT
8. THE RAPID ESTIMATION OF MICRO-ORGANISMS BY MEASUREMENTS OF ELEcTRICAL IMPEDANCE

8.1 Introduction

Many of the novel techniques suggested for the rapid estimation of microbial populations are at an early stage of development. One of the most interesting of the instrument based, short elapsed time techniques (Part I, 2.4), impedimetric estimation, is based upon changes in the electrical properties of growth media which are brought about by the metabolic activities of the micro-organisms.

The initial reports of this technique have indicated that estimates of viable populations of micro-organisms may be obtained within the working day. In addition the technique is reported to be practically simple and offers a high sample capacity.

These early investigations have involved the use of different instruments and, except in one case (Hardy et al., 1977), have been carried out using pure cultures of micro-organisms. The objective of the present investigation was to confirm reports of the performance of the impedimetric technique and further investigate the use of this technique for the rapid estimation of micro-organisms in foods.

8.2 Materials and Methods

Culture media. Conventional microbiological media were used throughout. Liquid culture media for impedimetric measurements were: PPL0 Broth (Difco; PPL0), Brain Heart Infusion Broth (Difco; BHI), Tryptone Soya Broth (BBL) + 0.1% yeast extract (TSBY) and 10 g l⁻¹
Brain Heart Infusion Broth + 0.5% dextrose (BHIG). Viable counting procedures were carried out on Plate Count Agar, (Oxoid), Nutrient Agar (Oxoid) and Milk Agar (Oxoid), the medium used depending upon the type of sample being tested.

Food Samples. Retail food samples were obtained locally in Leatherhead. Other samples were obtained and analysed during trials carried out in food manufacturing plants to compare the various methods of estimation under industrial conditions.

Sample preparation. Pure cultures were prepared by incubating 10 ml of the appropriate inoculated culture medium for 18h at 37°C. Dilutions of cultures were prepared in the same medium for introduction directly into the test systems.

Frozen vegetables were prepared by stomaching 50g of sample with 50 ml of BHIG for 30 seconds. The resultant puree was used to inoculate the test systems.

Meat samples were prepared by stomaching 10 g of meat in 90 ml of TSBY for 30 seconds. The supernatant liquid from this was used as the inoculum.

Colony counting methods

In all studies the Spiral Plate technique (S.P.M.) (Gilchrist et al., 1973; Jarvis, Lach and Wood, 1977) was used.

Pure cultures were plated on to Nutrient Agar (Oxoid) and incubated for 24h at 37°C. Food samples were examined using Plate Count Agar
Incubation was for 24h and 48h at 30°C and 35°C.

A number of conventional viable counting methods were used concurrently by the factory laboratories in the production trials to examine the same, or duplicate, samples of the foods under test. During a factory trial samples of frozen peas were examined in the factory laboratory by: the Roll tube technique (Harrigan and McCance, 1966) with incubation for 48h at 30°C; the Pour Plate technique (Thatcher & Clark, 1968) with incubation for 48h at 30°C and the Colworth Droplette technique (Sharpe and Kilsby, 1971) incubated for 24h and 48h at 30°C.

**Measurement of Impedance**

Three different instruments were used to measure changes in the electrical impedance of microbial cultures. Two were first generation commercial instruments whilst the third, the Malthus Conductance meter, was a laboratory prototype kindly supplied by Dr. G. Hobbs of the Torry Research Station, Aberdeen. All of these instruments are impedance bridges but differ in cell geometry, electrode composition and sample capacity. A short description of each of the instruments used is given below.

**Bactobridge.** This is a commercial instrument (supplied by T.E.M. Sales Ltd., Crawley, U.K.) available in two models with capacities of 3 and 6 simultaneous tests or channels. Each channel consists of an independent bridge circuit in which the sample and reference cells form two arms of the bridge, the circuits are energized by a 10 KHz supply. Measuring cells are of precision glass capillary tubing 30 mm x 2 mm int. dia. giving a capacity of 0.1 ml. Electrical contacts are provided by means of gold electrodes deposited on the glass at each end of the cell.
The ends of the cells are sealed with soft rubber plugs designed to allow injection of sample by syringe. A matched pair of cells is required for each measurement, one test cell and one reference cell for sterile medium.

A thermostatically controlled cell and syringe holder was used to ease cell filling and prewarm the cells and solutions.

Each measuring circuit has its own thermostatically controlled 37°C incubator unit into which a pair of cells is inserted to carry out the impedance measurements. The individual channels are each provided with a pair of interacting controls which must be manually set to give a starting value of 0 volts bridge output. Changes in the impedance of the sample, compared with the sterile reference solution, are displayed on a chart recorder in terms of mV bridge output. Three sensitivity settings are provided but use of all but the lowest requires considerable skill in use of the instrument.

Measuring cells were autoclaved without the rubber seals in place to allow steam penetration; after cooling the rubber sealing plugs were aseptically inserted into each end of the cells. The sealed cells were then placed in the cell holder and a 25 gauge syringe needle inserted through each rubber seal. Inoculum/sterile medium was then injected using a 2 ml syringe attached to one needle. The second needle allowed displaced air to escape; some care was required to ensure that no air bubbles were trapped inside the cell since these would interfere with the measurements. Removal of the two syringe needles rendered the cells ready for insertion into the incubation units in the instrument. A three channel instrument was employed in the present study.
Bactometer. This is a commercial instrument (supplied by Bactomatic Inc., Palo Alto California, USA) available with capacities of 8, 32, 120 (and multiples thereof) simultaneous tests. A 32 channel instrument was employed here. One self-balancing bridge circuit is employed and sequentially switched to each of the measuring channels. Two measurement frequencies are available 400 Hz and 2K Hz. The latter frequency was used throughout the present study. Measuring cells consist of sterile, disposable plastics modules with in-built stainless steel electrodes. Each module contains 8 reference and 8 test wells. The instrument capacity is 4 modules giving a total capacity of 32 simultaneous tests. Modules are supplied in sterile packages with the sample/reference wells sealed with adhesive tape. Filling of the wells requires removal of the adhesive tape followed by addition of the samples and reference solutions to the module. The wells are resealed using fresh, sterile tape supplied in the module package. Sample capacity is 0.5 - 2 ml per well.

Incubation of the modules is carried out in an integral, thermostatically controlled air incubator which contains the electrical connections into which the modules are plugged. Incubation temperature can be varied between 45°C and ambient + 6°C.

For the 32 channel model used, changes in the impedance of the sample are displayed on a chart recorder in terms of percentage change of initial impedance. A number of sensitivity settings are provided by means of a thumbwheel switch.
After removal of the sealing tape 1 ml of inoculum was added to each test well and 1 ml of sterile medium added to each reference well. All wells were resealed using the tape strips provided in the module package. Where possible modules were filled with sterile medium and incubated overnight as a test for any contamination introduced during the filling procedure.

**Malthus conductance meter.** This instrument, supplied by M.A.F.F. Torry Research Station, Aberdeen, is not available commercially at the present time and was tested in prototype form. Capacity is at present five channels although a 128 channel instrument is under commercial development. Each channel consists of a separate bridge circuit in which two of the arms are composed of the sample and reference cells. A 10 kHz supply is used to energize the circuits which employs phase sensitive demodulation to measure purely the resistive component of the impedance.

Sample and reference cells are constructed from pyrex test tubes (12mm x 150mm) with implanted platinum wire electrodes at 50 mm spacing. Glass side arms are used to insulate the electrical connections from each other and their surroundings. Each cell has a capacity of 10 ml and is autoclavable and reusable.

Filling and inoculation of the cells is by conventional microbiological techniques. A water bath set at $37^\circ \pm 0.01^\circ C$ is used as the incubation system in this instrument to give precise temperature control.
A single control is provided for each bridge circuit to enable the output to be adjusted to zero volts. Changes in the impedance of the sample are displayed on paper charts in terms of \(\mu\)siemens change in conductance.

Sterile culture medium was used as a reference solution throughout.

**Impedimetric detection criteria**

The output signals from the three impedance instruments were displayed on paper charts. Some difficulty was experienced in comparing these since for each instrument different units of measurement were used. For clarity of presentation, all three instruments have been considered to measure "impedance". The detection criteria were arbitrarily determined as the point at which a given accelerating change of impedance had occurred. These points were determined by eye, the criteria being as follows:

- **Bactobridge**: 10 chart units of bridge output (mV)
- **Bactometer**: 0.8% impedance change
- **Malthus**: 10\(\mu\) siemens change in conductance.

Response ('detection') times were calculated as the time from inoculation to the detection point.

**Analysis of results**

A logarithmic transformation was used to permit statistical analysis of colony count data.

The results obtained for pure cultures of micro-organisms were subjected to regression analysis of colony count \(\log_{10} \text{cfu ml}^{-1}\).
and detection time to produce relationships which could be expressed graphically.

The results obtained for food samples were subjected to computer analyses of correlation and regression in order to elucidate relationship between colony count \((\log_{10} \text{ cfu g}^{-1})\) and detection time.

A convenient method of representing the agreement between viable count results and impedimetric detection times was found to be the use of percentage agreement between results obtained by both methods about an assumed specification level. This analysis was carried out using the results expressed in graphical form. All of the data analysed was obtained with the Bactometer 32 because of the much greater capacity of this instrument.

**Calculation of percentage agreement between impedimetric and colony count methods**

The impedimetric detection time for a specified level of organisms was obtained from the graph of colony count against time to impedimetric detection. Assuming a specific level of organisms, the equivalent time for impedimetric detection was defined as the detection time corresponding to the point of intersection of this specified level of organisms with the calculated regression line. For each specified level of organisms a grid was constructed about the intersection point. The percentage agreement values were calculated by determining the number of samples for which results were in agreement by both methods, e.g. for a level of \(10^4 \text{ cfu g}^{-1}\) in frozen peas (Fig. 51) a cut-off time of 7.1h was established and agreement was decided as follows:
Results in agreement

a) Samples having detection times shorter than cut-off time with counts greater than the specified level i.e. time <7.1h, count > 10^4 cfu g^{-1}.

b) Samples having detection times longer than the cut-off time with counts lower than the specified level, i.e. time >7.1h, count < 10^4 cfu g^{-1}.

Results not in agreement

c) False positive: samples having detection times shorter than the cut-off time with counts lower than the specified level, i.e. time <7.1h, count < 10^4 cfu g^{-1}.

d) False negative: samples having detection times longer than the cut-off time with counts greater than the specified level i.e. time >7.1h count > 10^4 cfu g^{-1}.

The impedimetric response of fractions separated from raw meat

The separation procedure used was based on the cation exchange system described in Ch. 7 except that after application of the supernatant solution to the column, the column was allowed to drain in order to avoid dilution of the sample with buffer.

Aliquots of the homogenate, the supernatant solution and the column effluent were diluted 1:1 with double strength BHI and 1ml amounts placed in cells in the Bactometer 32.

1 ml volumes of the column effluent were filtered through 0.22 µm polycarbonate filters and the filters placed in Bactometer 32 cells.
containing 0.5ml of double strength BH1G and 0.5ml of citrate phosphate buffer (50mM; pH 5.8). Impedance measurements were made at 30°C.

8.3 Results

8.3.1 Impedimetric response of pure cultures of bacteria

The impedimetric response of pure cultures of E. coli in the Malthus conductance meter is shown in Fig. 43. Typically, the curve was level for a period of time inversely proportional to the number of organisms initially present; after this period impedance decreased exponentially (represented in Fig. 43 as an increase in conductance). The corresponding response for this organism in the Bactometer 32 also showed a similar lag period followed by a sharp decrease in impedance.

Relationship between colony count and impedimetric response of bacteria in pure culture

Typical relationships between colony count and time to detection of impedimetric response in the Bactometer 32 are shown for E. coli (Fig. 44), Staph. aureus (Fig. 45) and Bacillus subtilis (Fig. 46). Over the range of microbial concentrations tested an inverse linear relationship existed between log_{10} colony count and detection time. In the cases of E. coli and Staph. aureus the slopes of the lines were identical and indicated a population doubling time of 27 minutes whilst for B. subtilis the slope was less steep and indicated a population doubling time of about 36 mins.

With inoculum levels of E. coli greater than 10^7 cfu ml^{-1} the inverse linear relationship was not seen (Fig. 47). Extrapolation of the
Fig. 43. 'Impedimetric' response of E.coli in the Malthus conductance meter.
Slope: 1.5 h/log cycle
Population doubling time: 27 min

**Fig. 4.4.** Relationship between initial colony count and impedimetric detection time for *E. coli* in Brain Heart Infusion broth at 37°C.
Fig. 45. Relationship between initial viable count and detection time for *St. aureus* in Brain Heart Infusion broth at 37°C.
Slope: 2h/log cycle
Population doubling time: 36.1 min

Fig. 46. Relationship between initial colony count and detection time for *B. subtilis* in PPLO broth at 37°C.
Fig. 47. The effect of large inocula of *E. coli* on impedimetric detection time.
relationship obtained with lower inoculum levels indicated that instantaneous detection would result from an inoculum of $1 \times 10^8$ cfu ml$^{-1}$; observed detection times for $2.5 \times 10^8$ and $2.5 \times 10^9$ cfu ml$^{-1}$ were 10 min and 2 min respectively.

**Comparison of three impedimetric detection systems**

The response of the three impedance measuring instruments to *E. coli* growing in PPLO broth is shown in Fig. 48. It is clear that the slopes of the curves are similar and that the response times of the instruments differ by less than one hour for any specified inoculum level. The Malthus conductance meter gave the most rapid response, the response of the Bactometer 32 was about 45 mins slower than this and the Bactobridge response fell between that of the other two instruments.

8.3.2 The impedimetric estimation of micro-organisms in foods

8.3.2.1 The estimation of micro-organisms in retail samples

**Frozen peas and beans.** The relationship between colony count and impedimetric response for 36 samples of frozen vegetables is shown in Fig. 49. The detection times used were the mean values from quadruplicate, simultaneous estimations. The distribution of the data points about the calculated regression line was wider than that found for pure cultures. Percentage agreement between the two methods at specified levels of colony count are shown in Table 28. At $10^5$ cfu g$^{-1}$ there was 97% agreement and a detection time of 5.5h; at $10^4$ cfu g$^{-1}$ there was 89% agreement and a detection time of 6.8h.
Fig.48. The response given by \textit{E.coli} in three impedance measuring instruments.
Fig. 49. Relationship between detection time and bacterial colony count for 36 samples of retail frozen vegetables
TABLE 28

Percentage agreement between colony count and time to impedimetric detection for 36 samples of frozen vegetables

<table>
<thead>
<tr>
<th>Colony count (cfu g⁻¹)</th>
<th>Detection time (hours)</th>
<th>Impedimetric classification</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>correct</td>
</tr>
<tr>
<td>10^6</td>
<td>4.2</td>
<td>100</td>
</tr>
<tr>
<td>10^5</td>
<td>5.5</td>
<td>97</td>
</tr>
<tr>
<td>10^4</td>
<td>6.8</td>
<td>89</td>
</tr>
</tbody>
</table>

Meat Products. The relationship between colony count and impedimetric response for 24 samples of retail meat products including minced beef, sausages, beefburgers and stewing steak is shown in Fig. 50 as the distribution of data points about the calculated regression line. The percentage agreement between the two methods about specified levels of colony count (Table 29) was good and levels as low as 10^4 cfu g⁻¹ were estimated by the impedimetric method in 7.8h. Higher levels than this were estimated well within the working day.
Fig. 50. Relationship between colony count and detection time for 24 miscellaneous retail raw meat products.
### TABLE 29

Percentage agreement between colony count and time to
impedimetric detection for 24 samples of retail meat products

<table>
<thead>
<tr>
<th>Colony count (cfu g⁻¹)</th>
<th>Detection time (hours)</th>
<th>Impedimetric classification</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>correct</td>
</tr>
<tr>
<td>$10^7$</td>
<td>2.7</td>
<td>98</td>
</tr>
<tr>
<td>$10^6$</td>
<td>4.5</td>
<td>96</td>
</tr>
<tr>
<td>$10^5$</td>
<td>6.2</td>
<td>100</td>
</tr>
<tr>
<td>$10^4$</td>
<td>7.8</td>
<td>92</td>
</tr>
</tbody>
</table>

8.3.2.2 The estimation of micro-organisms in frozen peas

The data in Table 3C shows the mean, and range of colony count ($\log_{10}$ cfu g⁻¹) values and the impedimetric detection time for the 117 samples tested. No gross differences occurred in mean colony counts obtained by the different counting methods.
When the impedimetric response (detection times) of the samples was compared with colony count carried out by eight different methods (Table 31), correlation coefficients ranged from -0.53 to -0.90 for all methods, and from -0.86 to -0.90 for colony counts determined by the Spiral Plate Method. The correlations between impedimetric measurements and colony count methods were at least as good as between different colony count methods.
## TABLE 31

Correlation coefficients between estimation methods used on 117 samples of peas

<table>
<thead>
<tr>
<th>Method</th>
<th>Coefficient</th>
<th>Temperature 1</th>
<th>Temperature 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bactometer</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>- 0.876</td>
<td>SPM* 24h @ 30°C</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- 0.863</td>
<td>SPM 48h @ 30°C</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- 0.906</td>
<td>SPM 24h @ 35°C</td>
<td>SPM 48h @ 35°C</td>
</tr>
<tr>
<td></td>
<td>- 0.888</td>
<td>SPM 24h @ 35°C</td>
<td>SPM 48h @ 35°C</td>
</tr>
<tr>
<td></td>
<td>- 0.735</td>
<td></td>
<td>Roll tube</td>
</tr>
<tr>
<td></td>
<td>- 0.629</td>
<td>SPM 24h @ 35°C</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- 0.682</td>
<td>SPM 24h @ 35°C</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- 0.532</td>
<td>SPM 24h @ 35°C</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Pour plate</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Droplette 24h</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Droplette 48h</td>
</tr>
</tbody>
</table>

*SPM – Spiral Plate Method
Fig. 51 shows the regression line through the data point for the impedimetric estimation and the SPM counts with plates incubated for 48h at 30°C. The intersection points relating colony count to impedimetric detection time have been superimposed to demonstrate the way in which agreement between the methods was calculated. At colony count levels of $10^4$ and $10^5$ cfu g$^{-1}$ the corresponding impedimetric 'cut-off' times were 7.2 and 5.2h respectively. The percentage agreement between the two methods about these levels is shown in Tables 32 and 33.

The agreement between the two methods at the $10^5$ cfu g$^{-1}$ level was very high (98%); however, it can be seen from Fig. 51 that few points fell above this level. A more critical test of the agreement was obtained at the $10^4$ cfu g$^{-1}$ level where agreement was still very good (91%). Overall, the agreement between impedimetric and colony count methods was very similar to that between the different colony count methods.
Fig. 5.1. The relationship between colony count and detection time for 117 samples of frozen peas.
### TABLE 32

Percentage agreement values at a level of $10^5$ organisms g$^{-1}$ between methods used for estimation of micro-organisms in peas

<table>
<thead>
<tr>
<th>Method</th>
<th>Time</th>
<th>Temp</th>
<th>Percentage Agreement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bactometer</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SPM</td>
<td>24h</td>
<td>30°C</td>
<td>94</td>
</tr>
<tr>
<td>SPM</td>
<td>48h</td>
<td>30°C</td>
<td>98 99</td>
</tr>
<tr>
<td>SPM</td>
<td>24h</td>
<td>35°C</td>
<td>98 98 99</td>
</tr>
<tr>
<td>SPM</td>
<td>48h</td>
<td>35°C</td>
<td>98 98 99 100</td>
</tr>
<tr>
<td>Roll tube</td>
<td></td>
<td></td>
<td>94 98 98 99</td>
</tr>
<tr>
<td>Pour plate</td>
<td></td>
<td></td>
<td>93 98 97 100 100 99</td>
</tr>
<tr>
<td>Droplette 24h</td>
<td></td>
<td></td>
<td>100 98 97 100 99 100</td>
</tr>
<tr>
<td>Droplette 48h</td>
<td></td>
<td></td>
<td>94 98 97 99 98 99 99</td>
</tr>
</tbody>
</table>

### TABLE 33

Percentage agreement values between methods used for estimation of micro-organisms in peas at a level of $10^4$ organisms g$^{-1}$

<table>
<thead>
<tr>
<th>Method</th>
<th>Time</th>
<th>Temp</th>
<th>Percentage Agreement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bactometer</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SPM</td>
<td>24h</td>
<td>30°C</td>
<td>93</td>
</tr>
<tr>
<td>SPM</td>
<td>48h</td>
<td>30°C</td>
<td>91 97</td>
</tr>
<tr>
<td>SPM</td>
<td>24h</td>
<td>35°C</td>
<td>93 88 88</td>
</tr>
<tr>
<td>SPM</td>
<td>48h</td>
<td>35°C</td>
<td>81 90 93 99</td>
</tr>
<tr>
<td>Roll tube</td>
<td></td>
<td></td>
<td>90 83 84 92 94</td>
</tr>
<tr>
<td>Pour plate</td>
<td></td>
<td></td>
<td>86 90 90 89 88 91</td>
</tr>
<tr>
<td>Droplette 24h</td>
<td></td>
<td></td>
<td>88 81 73 86 87 89 88</td>
</tr>
<tr>
<td>Droplette 48h</td>
<td></td>
<td></td>
<td>88 88 88 94 87 92 90 89</td>
</tr>
</tbody>
</table>

SPM - Spiral Plate Method
Simultaneous estimates were made by both colony count and impedimetric techniques on 32 replicate sub-samples from a homogenate of frozen peas. The mean impedimetric detection time was 6.7h with range 5.94 - 7.45h and standard deviation 0.23h. In order to compare these estimates with those obtained by colony count techniques the detection times were converted into colony count values by use of the curve, obtained for industrial samples (Fig. 51), relating the two types of measurement. The resultant values and those obtained by a single expert analyst using three colony count techniques are shown in Table 34.

The range and confidence limits for colony count estimates on peas by a single operator were narrower than those obtained by the impedimetric method or by a colony count method when two operators were employed.
### TABLE 34

Intra-sample variation in estimates of the microbial population of peas by colony count and impedimetric methods

<table>
<thead>
<tr>
<th>Method</th>
<th>No. of replicates</th>
<th>Transformed colony count ($\log^{10}$ cfu g$^{-1}$)</th>
<th>mean</th>
<th>range</th>
<th>S.D</th>
<th>95% C.L.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>*Spiral Plate</td>
<td></td>
<td></td>
<td>4.35</td>
<td>4.27 - 4.47</td>
<td>0.06</td>
<td>± 0.12</td>
</tr>
<tr>
<td>*Spread Plate</td>
<td>32</td>
<td></td>
<td>4.45</td>
<td>4.23 - 4.67</td>
<td>0.10</td>
<td>± 0.20</td>
</tr>
<tr>
<td>*Pour Plate</td>
<td></td>
<td></td>
<td>4.51</td>
<td>4.38 - 4.58</td>
<td>0.06</td>
<td>± 0.12</td>
</tr>
<tr>
<td>*Impedance</td>
<td></td>
<td></td>
<td>4.35</td>
<td>4.05 - 4.51</td>
<td>0.13</td>
<td>± 0.26</td>
</tr>
<tr>
<td>(Bactometer)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>^Data of Hall</td>
<td>79</td>
<td></td>
<td>4.27</td>
<td>3.96 - 4.58</td>
<td>0.16</td>
<td>± 0.31</td>
</tr>
</tbody>
</table>

† Data cited as colony counts transformed from detection times.

*All analyses carried out by a single operator

Hall L.P. (1977) results include estimates by 2 operators

S.D. – standard deviation

C.L. – confidence limits

8.3.3 The impedimetric response of micro-organisms separated from meat

The results shown above demonstrate that the impedimetric technique can provide useful estimates of numbers of viable micro-organisms in foods if calibrated against the colony count technique. However the results give no indication of the effect of food constituents on the impedimetric response of the food flora.

In order to determine the effect of meat constituents on the response of the meat microflora, micro-organisms were separated from meat (minced beef) and the impedimetric responses of the various fractions monitored separately.
Fig. 52. The impedimetric response of fractions obtained during separation of microorganisms from meat
A typical result is shown in Fig. 52. In this example the final concentration of organisms in each Bactometer well was the same; 4.5 x 10^6 cfu ml^-1. The filtrate was sterile. The impedimetric response of the separated micro-organisms occurred earlier but was considerably greater than that of either the meat homogenate or the column effluent. The filtrate showed least decrease in impedance. Typically all of the fractions except for the filtrate showed regular decreases in impedance with time which plotted as smooth curves but occasionally the homogenate fractions produced more undulating curves as the incubation period exceeded 5 - 6 hr.

8.4 Discussion

The results obtained for the estimation of populations of pure cultures generally confirm the initial reports of this technique (Ur and Brown, 1973; Cady et al., 1974). The inverse linear relationship between colony count and impedimetric detection time provides a good basis for the estimation of colony forming units over the approximate range 10^2-10^7 ml^-1. Above this range the relationship is non linear, probably asymptotic (Fig. 47) and the sensitivity of the method will be lower for these higher concentrations of micro-organisms.

The sensitivity of detection of micro-organisms is in broad agreement with that reported by Ur and Brown (1973). Differences in sensitivity of the three instruments in the estimation of E. coli were small (Fig. 48). The Bactobridge and the Malthus detected impedimetric changes about one hour earlier than did the Bactometer, however the Bactobridge was erratic compared with the other two instruments. The greater sensitivity of the
Malthus conductance meter may be attributable to the incorporation in this instrument of a phase sensitive demodulation system designed to eliminate the capacitive component of impedance and measure only the conductive component (Richards, et al., 1978). These authors have reported that only the conductive component of impedance is relevant in the estimation of micro-organisms and that capacitive changes tend to obscure the measurements.

The relationship between colony count and impedimetric detection time for pure cultures of micro-organisms (Figs. 44 - 46) provide a basis for rapid estimation in these systems, however, natural microbial populations are rarely pure cultures. In natural populations, the different species present may exhibit different growth rates and produce different metabolic end products. They may also interact with one another e.g. by antagonism or synergism. These effects might be expected to modify the relationship between colony count and impedimetric response found for pure cultures. In addition, other components from a natural environment might modify the impedimetric response. It has been shown by Lawrence and Moores (1972) that enzymic activities can be followed conductimetrically, and by Ur and Brown (1973) that the buffering capacity of the medium can depress the impedimetric response of micro-organisms.

In view of the above, the results obtained for the impedimetric estimation of micro-organisms in foods compare well with colony count estimates. The relationships between impedimetric detection times and colony count of food products, as judged by correlation analyses and the fit of the data points about the calculated regression line, are generally good. They are also in agreement with the only other
published data; those of Hardy et al., (1977) for frozen peas. The variation in impedimetric estimates of micro-organisms in frozen peas was slightly greater than that found for the colony count methods (Table 34) but this difference is unlikely to be of practical significance. This, and the generally high level of agreement between the methods suggests that impedimetric estimates can provide substantially the same information as do colony count estimates but more rapidly and with considerably less labour.

The impedimetric response of organisms separated from meat was more rapid than was that of the meat homogenates (Fig. 52). In terms of detection times (time to produce a decrease of 0.8% in the impedance of the sample) this did not appear to offer an advantage commensurate with the additional manipulation required for the separation. The greater magnitude of the impedimetric response of the separated organisms compared with that of the other fractions which contain organisms suggests that constituents of the food affect either the growth of the organisms or the impedance of the medium. It appears unlikely that constituents of the meat should inhibit organisms which have grown in the meat and a more likely explanation is that the impedimetric response of the organisms is suppressed by the buffering capacity of the meat constituents.

Overall it appears that impedimetric techniques for the estimation of micro-organisms are a valuable addition to the inventory of techniques available to the biologist. The main advantages of the technique are short elapsed time, practical simplicity and the capacity to perform simultaneous multiple analyses. This combination of advantages represents a significant advance in methodology which may have far
reaching effects upon the practice of Microbiology. A second generation of instruments has recently been developed which permit the simultaneous analysis of up to 120 samples and incorporate data handling systems. The introduction of such powerful techniques may be anticipated to produce a considerable impact in a field which, for several decades, has been dominated by manual colony count techniques.
8.5 Summary

An inverse linear relationship between the impedimetric response and the concentration of micro-organisms was demonstrated for three species of organisms in pure culture.

The impedimetric response of *E. coli* was observed in three different impedimetric detection systems. Differences in the sensitivity of the systems were found, but the pattern of the responses was similar.

Impedimetric and colony count methods were compared for the estimation of micro-organisms in commercial food samples. Good agreement was found between the methods.

The impedimetric method produced results more rapidly than did the traditional method and in many cases provided results within the working day.

The impedimetric response of micro-organisms may be decreased by constituents of meat. This effect is probably attributable to the buffering capacity of the meat constituents.
Ch. 9:  THE RAPID ESTIMATION OF MICRO–ORGANISMS BY
THE BIOLUMINESCENCE ASSAY FOR ATP
9. THE RAPID ESTIMATION OF MICRO-ORGANISMS BY THE BIOLUMINESCENCE ASSAY FOR ADENOSINE TRIPHOSPHATE (ATP)

9.1 Introduction

McElroy (1947) first recognized that the light emission from firefly lantern extract could be induced by the addition of ATP and that the light emitted was proportional to the amount of ATP added. The chemistry of the reaction is shown below (McElroy and Deluca, 1974; E indicates luciferase; \( LH_2 \), luciferin).

\[
E + LH_2 + ATP \xrightarrow{Mg^{++}} E.LH_2AMP + PP
\]

\[
E.LH_2AMP + O_2 \rightarrow E + Oxyluciferin + CO_2 + AMP + \text{light}
\]

This observation formed the basis of a method sufficiently sensitive to estimate the ATP content of relatively small populations of micro-organisms (Chapelle and Levin, 1968). Such estimations may be made far more rapidly than can microbial colony counts and this method has been successfully used as a rapid means of estimating micro-organisms in urine (Alexander et al., 1976).

The use of this method for the rapid estimation of micro-organisms in foods was investigated by Sharpe et al., (1970) and by Williams (1971) who found that in most cases the total amount of ATP in foods exceeded microbial ATP in the microbial flora by several orders of magnitude. Sharpe, et al., (1970) concluded that the method was unlikely to be of use for the estimation of micro-organisms in foods unless a suitable preliminary separation of the micro-organisms from the food components could be made.
The objective of the work reported here was to determine whether the
cation exchange system developed for the separation of micro-organisms
from raw meat (Ch. 7) could overcome the problems posed by non-microbial
ATP in the rapid estimation of micro-organisms in minced beef.

9.2 Materials and Methods

Reagents

Luciferase. Chromatographically purified luciferase was obtained from
the Sigma Chemical Co. (Catalogue No. L.5256). The lyophilized
powder was rehydrated by mixing for 30 min at 0°C in 0.5M glycylglycine
buffer (pH 7.8) to give a stock solution containing 5 units ml⁻¹ of
activity, this was stored at -25°C. Before use the stock solution was
diluted to give an activity of 0.5 units ml⁻¹ (1 unit will hydrolyse
10⁻⁹ moles of ATP min⁻¹ when 6 x 10⁻⁴ moles of ATP and 1 x 10⁻⁴ moles of
D-luciferin are present).

Luciferin. Synthetic D-luciferin (Calbiochem Cat. No. 438477) was
dissolved in 0.1 ml glycylglycine buffer (pH 7.8) to give a
concentration of 1 mg ml⁻¹. This solution was then diluted with equal
parts of distilled water to produce a stock solution which was stored
at -25°C until required.

Standard ATP solutions

Standard vials containing 1 mg of ATP were obtained from Calbiochem
The contents of the vials were dissolved in distilled
water and standard solutions were freshly prepared in the assay buffer
prior to each experiment.
Assay buffer. The buffer used was 20mM boric acid-sodium borate at pH 7.4.

In later parts of the work a commercial reagent, ATP monitoring kit (L.K.B. Ltd. Croydon) was used. This single reagent contained both luciferin and luciferase and provided a very stable light output.

Extraction of ATP from micro-organisms

ATP was extracted from microbial cells by boiling for 2 mins in assay buffer containing 0.1% Triton x-100. After cooling the samples were assayed directly by the procedure described below or, if the assay was delayed the samples were held at -25°C.

Assay procedures

The assay reagents were added to scintillation vials in the amounts shown below:

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Assay buffer</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>Luciferase</td>
<td>0.1 ml</td>
</tr>
<tr>
<td>Luciferin</td>
<td>0.2 ml</td>
</tr>
<tr>
<td>Sample</td>
<td>1.0 ml</td>
</tr>
</tbody>
</table>

The first three components listed were mixed and the background luminescence measured. The sample was then added and luminescence measured once more.

The instrument used was a liquid scintillation counter, the Coru/matic 25 (Tracerlab Hersham, Surrey).

In all cases the non-coincidence counting mode was used.
Standard curves were prepared for each experiment using ATP concentrations in the range $10^{-8}$ to $10^{-12}$ M.

When the LKB monitoring reagent was used the assay procedure was modified. 0.2 ml of the reagent was added to 0.7 ml of buffer and placed in small scintillation vials to which 0.1 ml of the sample was then added. Measurements were made using a Luminometer 1250 (L.K.B. Ltd., Croydon).

Assay of ATP in micro-organisms separated from meat

Micro-organisms were separated from raw meat by the cation exchange system previously described (Part II, Ch. 7). The column effluent was passed through a microbial filter (Unipore, 0.2 μm pore size. Bio-Rad Laboratories). The filters were washed in situ with 1 ml of the assay buffer before removal from the filter holder for extraction.

Assay of ATP in cells from pure cultures of micro-organisms

Broth cultures of micro-organisms were harvested by centrifugation and resuspended in the assay buffer. Serial, tenfold dilutions of this suspension were prepared in the assay buffer.

9.3 Results

Estimation of ATP

A typical calibration curve for the estimation of ATP is shown in Fig. 53. The curve shown was produced by use of the LKB monitoring reagent. A closely similar response was obtained when separate sources of luciferin and luciferase were used.
**Fig. 5.3.** A typical calibration curve for the photometric estimation of ATP.
A linear relationship between light emission and the amount of ATP present was found over the range $10^{-8}$ to $10^{-12}$ g of ATP. The limit of detection in both of the estimation systems was close to $10^{-12}$ g of ATP.

The estimation of ATP in standard solutions was not affected by the procedure used to extract ATP from microbial cells.

Estimation of the ATP content of micro-organisms from pure cultures

Determinations of the ATP content of microbial cultures were made on three successive tenfold dilutions of each of the cultures. The mean and range values for the ATP content of single colony forming units were calculated from these data. Typical values obtained for four organisms are shown in Table 35.

<table>
<thead>
<tr>
<th>microbial species</th>
<th>femtograms ATP cfu$^{-1}$</th>
<th>mean*</th>
<th>range*</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli</td>
<td>0.24</td>
<td>0.21</td>
<td>0.27</td>
</tr>
<tr>
<td>Pseud. fluorescens</td>
<td>0.31</td>
<td>0.27</td>
<td>0.41</td>
</tr>
<tr>
<td>Staph. aureus</td>
<td>2.4</td>
<td>2.0</td>
<td>2.8</td>
</tr>
<tr>
<td>Sacch. cerevisiae</td>
<td>100</td>
<td>60</td>
<td>150</td>
</tr>
</tbody>
</table>

*of six estimates
Colony count g⁻¹ based upon ATP estimation

\[ \text{Colony count g}^{-1} \text{ based upon ATP estimation} \]

\[ \log_{10} \text{Colony count g}^{-1} \]

\[ \log_{10} \text{Actual colony count (cfu g}^{-1}) \]

* Assuming that 1 cfu contains \( 1 \times 10^{-16} \) g of ATP

Fig. 54. Relationship between actual colony counts of meat and rapid estimates of colony count based upon estimation of ATP.

The straight line is not a regression line.
The ATP content of the yeast was approximately two orders of magnitude greater than that found for any of the bacterial species. The values found for the two Gram negative species were considerably lower than those found for the Gram positive organism, \textit{Staph. aureus}.

The estimation of micro-organisms separated from meat

The results obtained for replicate estimates of microbial ATP in samples of raw beef are shown in Fig. 54. These data are expressed as \(\text{cfu g}^{-1}\) of meat, calculated on the basis of an ATP content of \(10^{-16} \text{g ATP cfu}^{-1}\), and plotted against the actual number of \(\text{cfu g}^{-1}\) in the meat samples as determined by colony count.

A linear relationship was found between values of colony count obtained by the two methods over the range \(10^5 - 10^8 \text{ cfu g}^{-1}\) of meat.

Estimates of the ATP content of meat homogenates and supernatant solutions from homogenates were in every case several orders of magnitude greater than the values obtained for microbial ATP after separation of the micro-organisms from the food. The ratio of total ATP to microbial ATP in the samples ranged between 100 and 10,000.

9.4 Discussion

The assay procedure used to determine the ATP content of microbial cells was relatively simple since the time available for this work precluded detailed investigation of the conditions. Several factors
which may affect such estimates e.g. the efficiency of extraction of ATP from the cells (Lundin and Thore, 1975) and the stress of manipulative procedures (Chapman and Atkinson, 1977) were not investigated. The estimates of ATP content of colony forming units of the various microbial species (Table 35) are, however, in agreement with previous reports (Chapelle and Levin, 1968; Thore et al., 1975 and Hysert et al., 1976.

Estimates of the ATP content of meat homogenates confirmed previous reports of massive interference from non-microbial ATP which precluded the rapid estimation of micro-organisms in meat by the estimation of microbial ATP (Sharpe et al., 1970; Williams, 1971). The relationship found between estimates of cfu in meat made by ATP estimation and those made by colony count indicate that in the separation of micro-organisms from meat the micro-organisms are also separated from non-microbial ATP.

It seems probable that in this separation the role of the resin column is to remove proteins, cells and other components of meat which normally prevent the filtration of the supernatant solution of the meat homogenate (See Plates VIII -- XII, Ch. 7). The micro-organisms are then separated from other materials, including ATP in the filtration stage. It may therefore be possible to avoid this filtration stage by enzymic degradation of non-microbial ATP in the column effluent. This procedure has been demonstrated by Johnston et al., (1976) who used potato apyrase to remove non-microbial ATP from urine, and might be more convenient than filtration.
Whilst the relationship between microbial ATP and colony count in meat (Fig. 54) provides an empirical basis for the rapid assessment of colony count, the value of 0.1 fg of ATP cfu$^{-1}$ appears low in comparison with the values determined for bacteria in pure culture. The microbial flora of raw meat is normally composed of gram negative rods which might be expected to have ATP contents similar to those obtained for E. coli and Pseud. fluorescens (Table 35).

This difference can be partially explained by the incomplete recovery of micro-organisms from meat. The value of 0.1 fg ATP cfu was obtained by dividing the value for microbial ATP separated from the meat by the total number of cfu g$^{-1}$ in the meat, in practice only an average of 70% of the cfu were recovered from the meat (see Ch. 7).

It is also possible that the stresses exerted on the organisms during extraction from their normal environment may result in lower ATP content (Chapman and Atkinson, 1977). There is the further possibility that this is a true value for micro-organisms growing in raw meat.

Further investigations to assess the effect of the separation procedure on the ATP content of pure cultures of meat spoilage organisms would help to resolve this question.

Overall the results of this brief investigation indicate that it is possible, by the use of a combination of separation and estimation techniques, to obtain very rapid estimates of numbers of micro-organisms in meat. These estimates which can be made within 30 min compared with periods of 1 to 3 days by conventional techniques have obvious advantage in the microbiological quality control of meat.
broader context the results raise the prospect of the analysis of
natural populations of micro-organisms since once separation has
been achieved not only ATP but other constituents of the cells might
also be analysed.

9.5 Summary

The values determined for ATP content of cells of four microbial species
were in agreement with previously reported values.

The separation of micro-organisms from non-microbial ATP in meat was
achieved by the passage of the sample through a cation exchanger followed
by collection of the organisms on filters.

A linear relationship was found between the ATP content of microbial
populations separated from meat and the colony count of the original
sample over the range $10^5$ - $10^8$ cfu g$^{-1}$ of meat.

The method of separation overcomes the problems previously encountered
in the rapid estimation of micro-organisms in meat by the measurement
of microbial ATP.
Ch. 10: GENERAL DISCUSSION
10. GENERAL DISCUSSION

The broad objective of the work described here was to investigate the application of physical and chemical techniques to the analysis of microbial populations.

This work, though a single study, is comprised of two major facets: the development and evaluation of practical methods and the consideration of the mechanism underlying the interaction of microorganisms with solid ionogenic surfaces. Whilst these facets are intimately related they can be more clearly discussed if considered separately.

The development of methods

Adequate methods for the gross separation of micro-organisms from other biomaterials in aqueous suspension are well established. Procedures such as filtration, flocculation and centrifugation are applied on both the laboratory and industrial process scales. These procedures have necessarily received much attention since in some processes e.g. sewage treatment and water purification, they are of vital importance to the maintenance of large human communities. In other processes e.g. microbial fermentations, they are an essential stage in the production of materials of vast economic importance.

By contrast, methods for the fine separation of micro-organisms are less well developed. Techniques investigated for the fine separation of specific microbial forms have included foam flotation (Dogon, 1941)
continuous flow electrophoresis (Hannig, 1971) and counter-current distribution (Stendhal, 1973). Each of these techniques demonstrates that differences in the surface properties of micro-organisms can provide a basis for the separation of different microbial species. None of the methods have been further developed, possibly because they are too complex or unwieldy to provide the simultaneous replicate analyses frequently required in microbiological laboratories.

The fine separation of biological materials by adsorption to solid surfaces has been widely exploited in the biological sciences and offers considerable advantages for the simple and convenient manipulation of micro-organisms. Whilst this potential was realised for the separation of viruses and in one case protozoans, investigations with bacteria and yeasts have been less successful. Previous investigations of the use of ion exchange resins for the separation of bacteria and yeasts (see Ch. 2) have been almost exclusively concerned with the use of anion exchange resins for the separation of microbial species, the major objective being the demonstration of such separations. The mechanism of the interaction and several practical considerations important in the development of useful separation techniques received less attention.

The foremost of these practical considerations was the viability of the micro-organisms recovered from the resins. Estimation methods such as particle counting (Kurozumi, et al., 1965), optical absorbance (Daniels, 1968) or microscopy (Zvaginstev and Gusev, 1971) do not discriminate between viable and non-viable cells.
This distinction is of great importance in microbiological analyses, the majority of which depend upon the metabolic properties of the organisms. Very acid conditions such as those used by Daniels (1968) to recover organisms from anion exchange resin (pH 1.5) or adsorb them to cation exchange resin (pH 1.0) would be lethal to the majority of micro-organisms.

Other practical considerations of cell-resin interactions which have previously received little attention are the separation of micro-organisms from non-microbial materials and the scale and capacity of the experimental systems in relation to the analytical applications of the phenomenon.

A major contribution of the present study lies in the demonstration that Gram positive bacteria, Gram negative bacteria and yeasts can be adsorbed to ion exchange resins and recovered in a viable form by the use of simple rapid systems.

Previous reports of the adsorption of micro-organisms to cation exchange resins were contradictory (Ch. 2.3) and may have discouraged work with these systems. In the current study the elucidation of conditions for the adsorption and recovery of micro-organisms from the cation exchanger Bio-Rex 70 resolves these contradictions. In addition, the separation of micro-organisms from minced beef (Ch. 7) and the separation of Staph. aureus and E. coli (Ch. 5) clearly demonstrate the potential of cation exchange resin for the manipulation of micro-organisms.

The development of such separations has several methodological implications. The separation of micro-organisms from minced beef
permits the rapid estimation of the meat flora by measurement of microbial ATP (Ch. 9). Without prior separation of the organisms such estimates could not be made (Sharpe, et al., 1970; Williams, 1971). Other very rapid estimates of microbial populations based upon sensitive methods for the detection of cell constituents e.g. porphyrins (Oleniacz, et al., 1966) or endotoxin (Jorgensen, et al., 1973) are also susceptible to interference by extracellular material. The application of these methods would also be extended by prior separation of microorganisms from the interfering materials.

The degree of separation of micro-organisms from visible food particles (Ch. 7, Plates IX to XII) suggests that such procedures would resolve many of the problems encountered in the visual discrimination of micro-organisms from small food particles during total microscopic count and micro-colony count estimates of micro-organisms in foods. A similar consideration might apply in the identification of microorganisms by fluorescent antibody techniques in which food particles give rise to non-specific fluorescence (Goldman, 1968).

The separations of Staph. aureus and E. coli were achieved using a single type of resin under specific conditions. Other adsorbents and conditions might provide equally effective systems for the separation of specific microbial forms. However, the number of possible combinations of resins and experimental conditions would argue against a broad empirical investigation and a knowledge of the mechanism of the interactions may indicate the more productive areas for investigation.

The broader development of this type of separation of microbial species would have considerable advantages in the estimation of specific groups
of micro-organisms in natural populations. Conventional estimates of such organisms are made by inoculating the whole population onto culture media selective for the specific group of interest. Methods for the physical separation of microbial populations from foods and their resolution into the component groups might provide an alternative to the selective media which at present are virtually the only practicable means for the isolation of specific groups of micro-organisms.

A broader advantage of the rapid isolation and resolution of natural microbial populations is that such methods would permit biologists to compare organisms in their natural and cultured states. Our current knowledge of microbial composition and metabolism is largely derived from micro-organisms isolated and grown in model systems i.e. culture media. The opportunity to compare such organisms with their natural counterparts would be of considerable interest to biologists and might provide a more accurate assessment of the activities and roles of micro-organisms in nature.

Whilst this study has been largely concerned with isolation and estimation of microbial populations there is a further consideration which is not concerned with analysis.

The immobilisation of organisms on a physical support is the basic requirement in one form of industrial fermentation. In these processes organisms are held stationary by a solid matrix and the substrate passed through the microbial mass or 'reactor'. Methods suggested for the immobilisation of the micro-organisms (Nishida, et al., 1979) include permanent fixation using glutaradehyde or physical entrapment
in gums or polyacrylamide gels. These processes require care in the preparation of the 'microbial reactor' and are for most practical purposes irreversible. The use of ion exchange resins as supports might simplify the preparation of such 'microbial reactors' and would have the advantage of reversible attachment which might permit the removal and replacement of the microbial catalyst whilst the support remains in situ. It is possible however that attachment to an ionogenic surface may modify the metabolic activity of micro-organisms (Hattori and Furusaka, 1959).

The mechanism of the interaction of micro-organisms with ion exchange resins

A theory to explain the adsorption of micro-organisms to ion exchange resins was proposed by Daniels (1968). In this, the cells were said to behave as 'macroscopic zwitterions' which at pH values above their iso-electric points would adsorb to anion exchange resin and at pH values below their iso-electric points would adsorb to cation exchange resins.

This theory has not been challenged and this view of adsorption has been taken by other workers. Marshall (1976) has proposed that since most surfaces in nature are negatively charged it is unlikely that electrostatic phenomena are directly involved in the attraction and adhesion of micro-organisms to such surfaces.

In the work leading to the proposal of his theory, Daniels (1968) did not include non-ionogenic control surfaces e.g. polystyrene beads (Fig. 5) and so did not distinguish between adsorption due to ionogenic groups and those related to other properties of the resin surface.
Thus, the observation that microbial cells at pH values below their iso-electric points adsorb to cation exchange resins (Fig. 4) is also true of polystyrene beads (Fig. 5) which do not possess ionogenic groups. A further difficulty in the interpretation of such results obtained at very low pH values is that the surface of the microbial cell may undergo irreversible changes in structure (Neihoff and Echols, 1973). These considerations indicate that the observations of Daniels (1968) are open to a broader interpretation and his theory might be further considered.

It is possible that the theory proposed by Daniels (1968) could explain the interaction of micro-organisms with anion exchange resin (Ch. 4); however this simple theory cannot accommodate the adsorption of negatively charged cells to cation exchange resin (Ch. 5). An explanation of this phenomenon must be sought elsewhere.

The adsorptive interaction of negatively charged biological particles, including microbial cells, to negatively charged surfaces has been explained by Curtiss (1967) in terms of the DVLO theory of colloid stability. A reversible adhesive bond is formed by a balance of attractive forces (London dispersion forces) and distractive forces (electrostatic repulsive forces).

This interaction is relatively weak and only slowly reversible (Heckels, 1978), a description which does not correspond with the interactions observed between microbial cells and cation exchange resin (Ch. 5). Furthermore, the observation that the blockage of cell surface cationic (e.g. amino) groups inhibits the adsorption of Staph. aureus to cation exchange resin (Table 16) indicates
that the attractive forces are electrostatic ones rather than Van der Waal forces. This suggests a largely electrostatic mechanism of adhesion.

An electrostatic mechanism for the adhesion of overall negatively charged particles (as determined by electrophoresis) has been proposed by Snoeren (1976) to explain the salt dependent aggregation of K-carrageenan with K-casein (See Ch. 5.4). This mechanism could also explain the interactions of micro-organisms with cation exchange resin, and in particular the effect of salt in these systems. In this context it is interesting to compare the structure of K-casein (Snoeren, 1976) with a recent description of the spatial arrangement of charged groups on the microbial surface (Neihoff and Echols, 1978). Both may show localised areas of positive charge whilst possessing an overall negative charge. It appears that in both cases salts may alter the arrangement of the various charges to permit the positive charges to participate in electrostatic bonding with other particles whilst the organism or K casein molecule remains electrophoretically negative.

It is known that at certain concentrations salts can cause electro-mechanical contractions in the cell walls of micro-organisms (Marquis, 1968) and it is possible that these contractions could result in changes in the proportions of oppositely charged groups at the cell surface. These interactions occurred at sodium chloride concentrations of approx. 0.2M (Marquis, 1968) and may be more relevant to the recovery of Staph. aureus from anion exchange resin (Fig. 14) than in the adsorption of micro-organisms to cation exchange resins.
It is possible therefore to explain the reversible interactions of micro-organisms with ion exchange resins in terms of a balance of electrostatic forces which is primarily determined by the nature of the microbial surface. These forces cannot be predicted from the overall charge of the cell as determined by electrophoresis but appear to result from the inter-relationships between charged groups on the cell surface under the influence of environmental conditions.

The irreversible adsorption of E. coli to strongly basic anion exchange resin (Ch. 4) is difficult to explain in terms of purely electrostatic forces and furthermore contrasts with the observation of Hogg (1976) that this organism could be recovered from columns of DEAE cellulose by elution with sodium chloride solutions.

This difference in behaviour might be explained by the particularly high surface charge density of E. coli (Haydon, 1961; Neihoff and Echols, 1973) which in combination with strongly basic resin will result in a very strong electrostatic attraction. Such forces might overcome the balancing electrostatic repulsive forces and permit a very close approach of the organism to the resin particle. At distances of <10^6 A, the DVLO theory predicts that Van der Waals forces will predominate and irreversible adhesive bonds will be formed. It is possible that the forces of attraction between E. coli and DEAE resin are insufficient to produce such a close approach and that a reversible adhesion occurs by a balance of electrostatic forces. This explanation of these interactions could be tested by devising treatments to alter the surface charge density of the cells or the resin.
Whilst there is evidence that largely electrostatic forces are involved in the interaction of micro-organisms with ion exchange resins, the particular cell surface structures involved are less clearly defined. Peripheral structures such as the lipopolysaccharide of salmonellae and the flagellae of *E. coli* do not appear to be involved and this directs attention to the main body of the cell envelope.

Investigations of the cell wall structure of bacteria have until relatively recently emphasized the major components such as teichoic acids, lipopolysaccharides and peptidoglycan. It appears unlikely that these are specifically involved in the adsorption of micro-organisms to resins. The involvement of the first two could not be demonstrated in the present study and the deep location of peptidoglycan within cell walls argues against cell surface expression. A further consideration is that none of these structures are found in the cell walls of yeasts which also interact with ion exchange resins.

Of the minor components of microbial cell walls, protein appears the most probable source of both anionic and cationic groups for interaction with resins. The presence of protein in yeast cell walls has long been established, as has the presence of protein on the cell surface of certain Gram positive bacteria e.g. staphylococci and streptococci. In much of the earlier work on bacterial cell walls the use of proteolytic enzymes in the preparation of wall samples tended to obscure the presence of proteins. Later, the presence of protein was reported in cell walls of a wide range of bacteria (*Rittenhouse, et al.*, 1973) and more recent work on the structure and function of cell envelope proteins (See Review by *Di-Rienzo et al.*, 1978) has demonstrated that the outer membrane proteins
of Gram negative bacteria are ideally situated for interaction with other particles.

Wallenstein (1975) demonstrated the role of the 'M' protein of streptococci in their adsorption to ion exchange resin. In the present study the involvement of *E. coli* outer membrane protein 1. (Fig. 31) demonstrates a similar role for a specific protein of a Gram negative organism. More general evidence of the involvement of cell surface proteins is that of the effect of procedures for the modification of proteins on the adsorption of *Staph. aureus* to cation exchange resin (Table 16), and the effect of proteolytic enzymes on the adsorption of this organism to both cation and anion exchange resins (Tables 10 and 16).

On balance, the cell surface proteins are considered to be the most likely structural components responsible for the electrostatic forces involved in the interactions of micro-organisms with ion exchange resins. However, the evidence for this is relatively sparse and none relates to yeasts. It would therefore be premature to state categorically that proteins were the determinants of these interactions.

The purpose of this study was to explore the application of biochemical and biophysical techniques to the analysis of microbial populations. The work demonstrates the use of these techniques in both qualitative and quantitative analysis and reveals new approaches to the physical manipulation of microbial populations.
The effects demonstrated may have immediate applications in fields as diverse as microbial ecology, industrial fermentation and the analysis of foods and other biomaterials. In the longer term the broader significance is that this type of methodology may release microbiologists from the constraints of model systems and permit the study of natural microbial populations.


donor and recipient bacteria by column chromatography.

J. Bact., 103, 302 - 4.

ZVGAGINTEV, D.G. (1962). Some regularities of adsorption of micro-

ZVAGINSTEV, D.G. and GUSEV, V.S. (1971). Concentration and separation
of bacteria on Dowex anionite. Mikrobiologiya, 40, 123 - 6.
The Rapid Estimation of Colony Count Levels in Foods by Impedance Measurements.
By V. H. LACH, J. M. WOOD and B. JARVIS (Leatherhead Food R.A., Randalls Road, Leatherhead, KT22 7RY).

Several workers have investigated the use of impedance measurements for the rapid estimation of microbial populations (Cady, P. 1977, Food Product Development 80–85). The basis of the impedimetric estimation of micro-organisms is the measurement of small changes in the electrical properties of nutrient media effected by the growth of micro-organisms. The estimation of microbial numbers is dependent upon the relationship between initial numbers of microbial cells and the period of incubation required to produce a given impedance change. Previous studies have demonstrated this relationship both for pure cultures (Ur, A. & Brown, D. F. J. 1974, Biomedical Engineering 18–20) and for frozen vegetables (Hardy et al. 1977, Applied and Environmental Microbiology 34, 14–17). A preliminary report on our investigations has also been published (Wood et al. 1977, Journal of Applied Bacteriology 43, xiv). The relationship between impedance and colony count levels has been further investigated for a number of food products examined in manufacturing plants. An inverse linear relationship was found between log_{10} colony count and impedimetric detection time which permitted the estimation of microbial populations in selected food products within the working day. Impedimetric detection times of 4–7 h corresponded with total viable counts of 10^6–10^4 colony forming units (c.f.u.)/g, respectively in frozen peas. Similarly, total viable counts on fresh meats and meat products of 10^8–10^5 c.f.u./g corresponded with impedimetric detection times of 2–8 h respectively. Correlation coefficients between impedimetric detection times and colony counts for these two product types were 0.86 and 0.83 respectively.

The use of impedance measurement for the estimation of microbial numbers has been studied by several workers (Cady, P. Food Product Development April 1977, 80–85). The instruments available for impedance measurement differ in both design and capacity. However, all are basically impedance bridges which measure changes in electrical properties resulting from growth of micro-organisms in nutrient media. Two instruments, the ‘Bactometer 32’ (Bactomatic Inc., California) and the ‘Malthus’ (Torry Research Station, Aberdeen) have been evaluated for the estimation of food associated micro-organisms such as *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis*, *Lactobacillus casei* and *Streptococcus faecalis*. For both instruments, linear relationships were found between initial numbers of microbial cells and impedance change with time. This permitted the construction of calibration curves relating numbers of colony forming units (c.f.u.) in the inoculum with time-to-detection by impedance change. For the species examined, populations of $10^6$–$10^7$ c.f.u. ml$^{-1}$ were detected in 3–5 h and populations of $10^5$–$10^4$ c.f.u. ml$^{-1}$ in 5–7 h. Preliminary assessments of these instruments for monitoring levels of micro-organisms in foods were reported.
Evaluation of the Spiral Plate Maker for the Enumeration of Micro-organisms in Foods

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Received 7 March 1977 and accepted 13 April 1977

A statistically based comparison of five methods of enumerating bacteria in foods has been undertaken to assess the potential application of the Spiral Plate Maker (Gilchrist et al. 1973) which is claimed to reduce very significantly the costs involved in the quantitative estimation of viable micro-organisms in foods. The performance of the Spiral Plate Maker was compared with that of three conventional methods (pour plate, surface spread plate and drop count) for the examination of four types of food by four different operators. Analysis of variance showed that there were no differences between the methods at the 5% level although some isolated interactions occurred. Regression and correlation coefficients between the various methods were all highly significant and the results obtained by the spiral plate method were within the limits of error for traditional quantitative methods. The spiral plate method, in many cases, can replace advantageously any of the other methods for the quantitative estimation of viable micro-organisms in foods. Labour requirements for the enumeration of micro-organisms by the spiral plate method were only 31% of that required for one conventional method. In addition considerable savings in materials were achieved.

Traditional methods for the estimation of viable micro-organisms, e.g. the pour plate or surface plate methods, are both labour intensive and costly in materials. A major part of the effort employed at the bench lies in the preparation of dilutions from the sample and the inoculation of the various dilutions on to or into the culture medium. A lesser but significant amount of effort is involved in the preparation of sterile diluent and of culture media prior to the actual estimation. A further disadvantage of traditional methods lies in the repetitive nature of the work and it is not surprising that microbiologists have long sought for a means to mechanize viable count techniques.

One of the more interesting approaches to this problem was that of Trotman (1971) who devised a system by which an inoculum could be spread mechanically on the surface of an agar plate. This device could replace the technique of hand streaking to obtain isolated colonies but the application of the inoculum to the agar surface was not carried out quantitatively and therefore did not permit the estimation of the number of colony forming units in the inoculum. Trotman’s device is most suitable for the isolation of pure cultures, a task which forms a large part of the work of medical microbiology laboratories.

A device more suited to the quantitative work which occupies much of the efforts of food microbiologists was described by Gilchrist et al. (1973). This device deposits a small, standard volume of inoculum on the surface of a rotating agar plate via a hollow stylus. The stylus moves from the centre to the edge of the rotating plate producing a spiral track; simultaneously, the amount of inoculum delivered to the surface is continually decreased and the quantity of inoculum deposited on any given area of the plate is known. After incubation any area of the plate with discrete colonies can be used
for the calculation of the concentration of micro-organisms in the inoculum. This system permits the estimation of microbial concentrations over a range of about three orders of magnitude, e.g. $6 \times 10^2 - 6 \times 10^5$ or $10^3 - 10^6$ c.f.u. ml$^{-1}$, without recourse to serial dilution of the sample and using only a single agar plate for each sample. For counts in excess of these values, prior dilution of the sample is necessary.

The Spiral Plate Maker has an obvious potential for reducing labour and material costs incurred in carrying out viable counts, but is of value only if it can produce results which compare in accuracy and reliability with those obtained by the conventional viable count methods. In a preliminary evaluation of the Spiral Plate Maker in this laboratory aerobic and anaerobic counts on a variety of food and other samples were compared with drop counts. In every instance counts correlated well and differed by no more than $\pm 0.5$ log cycle. Further comparisons have been made in the United States (Donnelly et al. 1976; Gilchrist et al. 1976). The objective of the work reported here was to evaluate more thoroughly the potential of the spiral plate method for the enumeration of viable micro-organisms in foods.

**Materials and Methods**

**Apparatus**

The Spiral Plate Maker* (Plate 1) was obtained from J. E. Campbell, 1618 Dell Terrace, Cincinnati, Ohio, U.S.A. (present supplier: Spiral Systems Marketing, 1200 Quince Orchard Boulevard, Gaithersburg, Maryland 20760, U.S.A.). A schematic diagram (Fig. 1) illustrates the operating mechanism. Standard 9 cm diameter Petri dishes (Sterilin Ltd.) were used throughout the work.

**Culture medium**

Plate Count Agar (Oxoid) was used for total viable counts of bacteria. Plates were poured on a carefully levelled surface and allowed to set completely before handling. Prepared plates were dried overnight at 37 °C with the lids in place. For the pour plate method the agar was dispensed in 200 ml amounts in screw-capped bottles and was carefully tempered to $45 \pm 1$ °C before use.

**Sample preparation**

Food samples were prepared by stomaching 10 g of food with 90 ml of $\frac{1}{2}$ strength Ringer's solution containing 0-1% (w/v) peptone (RP) for 30 s. In experiments in which four operators were testing the same sample, the above quantities were quadrupled and the resulting homogenate was divided into four equal portions. Serial decimal dilutions of the homogenates were prepared in RP solution to $10^{-7}$.

When conventional plating methods were used, all dilutions were plated. Only the 1/10 homogenate and the 1/100 dilution were plated on the Spiral Plate Maker. The 1/100 dilution was necessary only for those samples expected to have a very high count i.e. $> 10^9$ colony forming units (c.f.u.) g$^{-1}$. After inoculation plates were incubated aerobically at 30 °C for 3 d.

* Now available from Don Whitley Scientific, Shipley, Yorks, U.K.
PLATE 1. The Spiral Plate Maker.
Experimental design

The investigation was designed as a multifactorial experiment to compare results obtained by several operators, using various food samples and estimation methods. Four operators tested five samples of each of four types of foodstuffs by five plating methods. Three of the operators were experienced microbiologists but the fourth was relatively inexperienced. The food types used were: sausages, minced beef, coleslaw, and pasteurized cream. All samples were purchased from local retail outlets. The plating methods examined were: (1) pour plate method (Thatcher & Clark 1968); (2) 0.1 ml surface spread plate method (Thatcher & Clark 1968); (3) drop count (Miles & Misra 1938); (4) spiral plate method (Gilchrist et al. 1973); (5) spiral plates from (4) recounted by a single operator. The last method was included to assess the extent to which spiral plate results would be influenced by selection of the countable areas on the plates, since, unlike conventional methods, only a proportion of the colonies on each plate are counted. The areas selected for counting are thus subject to a certain amount of interpretation by the operator. The transformed colony counts (as log_{10} c.f.u. g^{-1}) were subjected to computer analysis of variance and regression. Correlation coefficients between the results were also determined.

Results

Analysis of variance on the methods of estimating micro-organisms in foods

Table 1 shows the ranges and overall mean colony counts obtained by each method when five samples of each of four food products were analysed by four operators. Analysis of variance of the three factors (method, operator and sample type) with
Table 1

Comparison of total viable counts on four food products determined by five methods

<table>
<thead>
<tr>
<th>Food product</th>
<th>No. samples</th>
<th>pour plate</th>
<th>surface spread</th>
<th>drop count</th>
<th>spiral plate</th>
<th>recounted spiral plate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sausage</td>
<td>5</td>
<td>7-26</td>
<td>7-32</td>
<td>7-19</td>
<td>7-30</td>
<td>7-27</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(6-03-8-96)</td>
<td>(5-88-9-17)</td>
<td>(5-83-8-86)</td>
<td>(6-28-9-25)</td>
<td>(6-32-8-58)</td>
</tr>
<tr>
<td>Minced beef</td>
<td>5</td>
<td>7-55</td>
<td>7-62</td>
<td>7-54</td>
<td>7-45</td>
<td>7-48</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(7-18-8-06)</td>
<td>(7-17-8-23)</td>
<td>(7-03-8-11)</td>
<td>(6-86-8-26)</td>
<td>(7-23-7-76)</td>
</tr>
<tr>
<td>Cream</td>
<td>5</td>
<td>5-95</td>
<td>6-01</td>
<td>6-00</td>
<td>6-08</td>
<td>6-14</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(4-00-7-66)</td>
<td>(3-90-7-83)</td>
<td>(4-11-7-53)</td>
<td>(4-18-7-51)</td>
<td>(4-00-7-45)</td>
</tr>
<tr>
<td>Coleslaw</td>
<td>5</td>
<td>3-86</td>
<td>3-91</td>
<td>3-85</td>
<td>4-05</td>
<td>4-02</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(3-13-4-49)</td>
<td>(3-19-4-71)</td>
<td>(3-15-4-73)</td>
<td>(3-45-4-76)</td>
<td>(3-45-4-76)</td>
</tr>
</tbody>
</table>
| * Each mean value is the average for five samples examined in parallel by each of four operators.

replication of tests showed no statistically significant differences between the methods at the 5% level of probability (Table 2). Neither were there any significant differences between operators nor interactions. The differences in mean $\log_{10}$ colony counts were well within the arbitrary limits of ±0.5 log cycle. Further analysis of variance for particular food types showed some operator × method interactions, but these were of low significance.

Determination of the variances between operators for particular foods showed only low magnitude differences. For example, with minced beef the operator variances ($S^2$) were 0-0024 (pour plate), 0-0172 (surface spread), 0-0118 (drop count), 0-0088 (spiral plate method) and < 0-002 (spiral plates recounted by a single operator). Similar variances were seen for the other foods. Although such variances should be interpreted with caution, the operator errors in the spiral plating and counting operation were no worse than those found using conventional methods.

Table 2

Analysis of variance of $\log_{10}$ bacterial colony counts on four foods analysed by four operators using five methods, with replication of samples

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Degrees of freedom</th>
<th>Corrected sum squares</th>
<th>Mean square</th>
<th>$F$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Method (M)</td>
<td>4</td>
<td>0-454</td>
<td>0-114</td>
<td>0-133</td>
</tr>
<tr>
<td>Operator (O)</td>
<td>3</td>
<td>0-840</td>
<td>0-280</td>
<td>0-326</td>
</tr>
<tr>
<td>Sample Type (S)</td>
<td>3</td>
<td>805-652</td>
<td>268-551</td>
<td>312-632*</td>
</tr>
<tr>
<td>M × O</td>
<td>12</td>
<td>1-593</td>
<td>0-133</td>
<td>0-155</td>
</tr>
<tr>
<td>M × S</td>
<td>12</td>
<td>1-256</td>
<td>0-105</td>
<td>1-170</td>
</tr>
<tr>
<td>O × S</td>
<td>9</td>
<td>0-631</td>
<td>0-070</td>
<td>0-081</td>
</tr>
<tr>
<td>M × O × S</td>
<td>36</td>
<td>0-990</td>
<td>0-028</td>
<td>0-033</td>
</tr>
<tr>
<td>Residual</td>
<td>320</td>
<td>274-965</td>
<td>0-859</td>
<td>—</td>
</tr>
<tr>
<td>Total</td>
<td>399</td>
<td>1086-381</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

* Significant at $P < 0-01$.
No symbol, not significant ($P > 0-05$).
Regression analysis between methods

The results of each method were analysed by regression against the results obtained on all the other methods. The regression and correlation coefficients between all methods together with the standard error of the regression coefficient are presented in Table 3.

**Table 3**

<table>
<thead>
<tr>
<th>Method 1</th>
<th>Method 2</th>
<th>Regression coefficient $B$</th>
<th>Intercept</th>
<th>S.E. $B$</th>
<th>Multiple correlation coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pour Spread</td>
<td>0.988</td>
<td>0.030</td>
<td>0.014</td>
<td>0.99</td>
<td></td>
</tr>
<tr>
<td>Pour Drop</td>
<td>1.001</td>
<td>0.010</td>
<td>0.018</td>
<td>0.99</td>
<td></td>
</tr>
<tr>
<td>Pour Spiral</td>
<td>1.050</td>
<td>-0.366</td>
<td>0.023</td>
<td>0.98</td>
<td></td>
</tr>
<tr>
<td>Pour Spiral recount</td>
<td>1.056</td>
<td>-0.415</td>
<td>0.020</td>
<td>0.99</td>
<td></td>
</tr>
<tr>
<td>Spread Drop</td>
<td>1.010</td>
<td>-0.001</td>
<td>0.014</td>
<td>0.99</td>
<td></td>
</tr>
<tr>
<td>Spread Spiral</td>
<td>1.055</td>
<td>-0.355</td>
<td>0.023</td>
<td>0.98</td>
<td></td>
</tr>
<tr>
<td>Spread Spiral recount</td>
<td>1.059</td>
<td>-0.393</td>
<td>0.021</td>
<td>0.99</td>
<td></td>
</tr>
<tr>
<td>Drop Spiral</td>
<td>1.034</td>
<td>-0.285</td>
<td>0.024</td>
<td>0.98</td>
<td></td>
</tr>
<tr>
<td>Drop Spiral recount</td>
<td>1.037</td>
<td>-0.316</td>
<td>0.023</td>
<td>0.98</td>
<td></td>
</tr>
<tr>
<td>Spiral Spiral recount</td>
<td>0.988</td>
<td>0.063</td>
<td>0.018</td>
<td>0.99</td>
<td></td>
</tr>
</tbody>
</table>

* Data from Table 1; n = 80.

Fig. 2. Regression lines for microbiological enumeration methods. Regression of data from pour plate ($Y$) on: ▲, surface spread ($Y = 0.988X + 0.030$); ■, Miles & Misra method ($Y = 1.001X + 0.010$); ○, spiral plate method ($Y = 1.050X - 0.355$); ▼, spiral plate method (recounted) ($Y = 1.056X - 0.415$).
For all the methods tested the regression lines fit almost ideally to the expected line (Fig. 2). A small discrepancy occurs at low levels of count for the spiral plate data compared with pour plate data. The distribution of the results obtained by the pour plate and the spiral plate methods about the calculated regression line is illustrated in Fig. 3.

**Comparison of the labour and materials requirements for the spiral plate method and the Miles & Misra method**

To carry out 100 colony counts by the spiral plate method required only 31% of the labour necessary for the drop count method which is a method used routinely in this laboratory (Table 4). The greatest difference in labour requirements was at the bench

<table>
<thead>
<tr>
<th>Method</th>
<th>Operator time (h)</th>
<th>Support labour*</th>
<th>Bench labour†</th>
<th>Total</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drop count</td>
<td>6.5</td>
<td>16.5</td>
<td>23.1</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Spiral plate</td>
<td>2.2</td>
<td>5.0</td>
<td>7.2</td>
<td>31</td>
<td></td>
</tr>
</tbody>
</table>

* Preparation of culture media, diluents, sterile apparatus, etc.
† Preparation of homogenates and dilutions; inoculation of media; transfer to and from incubators; counting colonies; disposal of used apparatus and materials.
although the simplicity of the spiral plate method was also reflected in a reduction in support services. In addition to these savings in labour there was also a considerable saving of materials i.e. media, Petri dishes and pipettes.

Discussion

The objective of this work was to determine whether the Spiral Plate Maker could advantageously replace methods currently used for the estimation of viable micro-organisms in foods. It has already been shown (Gilchrist et al. 1973) that the spiral plate and pour plate methods can produce comparable results for pure cultures of several micro-organisms and for inoculated milk and cream samples. More recently it has been demonstrated that the spiral plate method could be substituted for the pour plate method in the analysis of raw milk (Donnelly et al. 1976). The results of the present study indicate that for the samples examined the Spiral Plate Maker could replace any of the other methods tested without producing results which are different for the purposes of practical microbiology. The basic statistical analysis showed no significant differences between the results produced by the different methods.

Our results confirm and extend those reported by other workers who have compared the spiral plate method with conventional methods (Gilchrist et al. 1973, 1976; Donnelly et al. 1976) but are not in agreement with the findings of Ruosch (1976). Ruosch, who also compared the spiral plate method with conventional methods for the enumeration of viable micro-organisms in minced meat and in sausage emulsion, found that the spiral plate method produced results statistically different from those produced by the pour plate and drop count methods. It is not possible at present to determine the reason for this difference.

The regression analysis indicates that at low levels of contamination (e.g. $10^3$–$10^4$ c.f.u. g$^{-1}$) slightly higher counts would be expected from the spiral plate method than from the pour plate method. For instance a count of $10^4$ c.f.u. g$^{-1}$ by spiral plate would be expected to be equivalent to a count of $6.8 \times 10^3$ c.f.u. g$^{-1}$. Such differences are well within normally accepted tolerances for microbiological colony enumeration methods. However, it should be noted that in the present study the initial sample preparation was common to all methods and operations. Individual sample preparation might be expected to increase the variances observed. The choice by the different operators of suitable areas of the spiral plate on which to perform the colony count did not give rise to statistically significant differences between results, although the operator variance was lower for the recounted plates. This implies that plates prepared by several operators give consistent counts for individual samples.

The spiral plate method has many advantages which recommend its use in a routine laboratory. The machine is small and robust; it requires very little operator training and, once trained, operators can inoculate 50–60 plates/h which is close to the maximum capacity of a single machine. It appears quite feasible for a single operator to double this rate by the synchronous use of two machines as was suggested by the originators. A problem which might arise in the use of the spiral plate method is that the efficiency of the method may give rise to very large numbers of plates for manual counting. To alleviate such a situation a laser-based automatic colony counter has been developed specifically for use with spiral plates (Model 500 Bacterial Colony Counter, Spiral Systems Marketing, 1200, Quince Orchard Boulevard, Gaithersburg, Maryland, 20760,
This has been evaluated also by us (Lach & Wood, unpublished data) and has not produced results significantly different from those obtained by manual counting of the spiral plates.

The only problem encountered in the use of the Spiral Plate Maker in the present study was the occasional blockage of the stylus by large suspended food particles. This was simply overcome by inserting a syringe into a convenient fitting on the inoculum delivery system and flushing liquid through the stylus tube. An anticipated problem of damage to the surface of soft agars, e.g. Barnes' Thallous Acetate Tetrazolium Glucose Agar or Malt Extract Agar at low pH, did not occur on any of a wide range of selective media tested in preliminary studies, although it was found that uneven or very dry plates gave rise to poor inoculation patterns.

The limits of estimation using the spiral plate method are $10^3$–$10^6$ c.f.u. ml$^{-1}$ suspension (Gilchrist et al. 1973) with a 9 cm Petri plate. The upper limit can obviously be extended by dilution of the sample, but the lower limit is fixed by the amount of inoculum which can be applied to the medium. By increasing the inoculum size and/or plate dimensions the range can be extended to $5 \times 10^2$–$5 \times 10^6$ c.f.u. ml$^{-1}$. The sensitivity of this method will be greater for liquid foods, e.g. milk, than for solid foods which must be homogenized in a diluent to produce a representative inoculum.

The spiral plate method has considerable potential for the estimation of microorganisms in foods and, in our opinion, the claims made by its originators are justified. The benefits in cost savings and convenience to be gained will obviously vary according to the operation of individual laboratories but in our experience cost savings are highly significant. It is difficult, therefore, to understand the findings of Ruosch (1976) who reported that the drop count method was less costly than the spiral plate method for the enumeration of microorganisms in minced beef and sausage emulsion.

In our opinion the introduction of the Spiral Plate Maker represents a major step forward in the mechanization of microbiological methodology which is long overdue. In this context it is pertinent to note that the spiral plate method has now been accepted as a U.S. Official Method by the Association of Official Analytical Chemists (Anon. 1977).

The authors would like to thank all of the members of the Microbiology Section of the Leatherhead Food R.A. who contributed in various ways to this study. We are indebted to Dr J. E. Campbell for providing much unpublished information.

References

TROTMAN, R. E. 1971 The automatic spreading of bacterial cultures over a solid agar plate. Journal of Applied Bacteriology 34, 615–616.
Food Group Symposium
Short Paper Reading Meeting

The following are summaries of papers presented at the "Short Paper Reading Meeting" organised by the Food Group in conjunction with the Institute of Food Science and Technology. It was held at the University of Nottingham, Sutton Bonnington, on 17 March 1976. The papers so published are entirely the responsibility of the authors and in no way reflect the views of the Editorial Board of the Journal of the Science of Food and Agriculture.

The Separation of Microorganisms from Foods

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In recent years sensitive analytical instruments, e.g. the luminescence biometer, the Coulter counter and impedance bridges, have been used to give a rapid estimate of the numbers of microorganisms in model systems, e.g. broth culture. Whilst these instruments are useful in model systems their use in the more applied branches of microbiology has been retarded by the inability of the instruments to discriminate between microorganisms and non-microbial materials in real samples, e.g. foods. The separation of microorganisms from such materials would permit applied microbiologists to benefit from these advances in instrumentation.

In preliminary investigations of possible separation systems it was found that cells of Staphylococcus aureus could be adsorbed on to the surface of beads of anion exchange resin. The adsorptive capacity of the resin was found to be $10^{10}$ cells per g. This indicated that even very small columns (0.2 g) of resin would have the capacity to adsorb all of the organisms normally encountered in 1 g of a food sample.

When suspensions of food-associated microorganisms were passed through columns of anion exchange resin it was found that a wide range of organisms including Gram positive and Gram negative bacteria, bacterial spore forms and yeast were adsorbed from suspension with high efficiency. This was demonstrated by both viable count and optical extinction measurements of the initial suspension and the column effluent. The attachment of the organisms to the resin beads was confirmed by scanning electron microscopy and the organisms were not removed from the surface when the resin column was disassembled and shaken in water. Profuse growth was observed when the particles of resin were incubated on nutrient media.

The major factors influencing the adsorption of microorganisms to anion exchange resin were the mesh size of the resin particles, the contact time of the microbial suspension with the resin, the pH and the electrolyte concentration of the suspending medium. Variable factors associated with the microorganisms, e.g. species, age of culture and composition of the cell envelope, had a lesser effect upon adsorption.

When food homogenates were prepared in distilled water (1:10) using the Colworth "Stomacher" (A. J. Seward, Norwich) and passed through resin columns adsorption of total viable flora varied from 50 to 100% depending upon the type of food. In certain cases adsorption could be increased by decreasing the pH value or by increasing the dilution of the homogenate.

When foods containing large particles were passed through the columns the particles were often retained on the resin bed. Investigations showed that these particles could be removed from suspension by very short periods of centrifugation (2000 g 30 s) following which the viable count of the supernatant solution remained similar to, and often slightly exceeded, that of the original homogenate.

Using centrifugation in conjunction with adsorption to resin it was found that 90% of the total flora from a sample of minced beef was adsorbed to the resin whilst 99% of the dry weight of the samples was accounted for in the precipitate from centrifugation and in the column effluent. With a starch based food 75% of the total flora was adsorbed with only 6% of the dry weight of the food. In this latter case it was found that the organisms could be desorbed by shaking the resin in Ringer's solution.
In recent years the application of sensitive analytical instruments e.g. the Luminescence biometer, the Coulter Counter and Impedance bridges to the estimation of microbial populations has been demonstrated. Much of this proving work has been carried out with pure cultures of micro-organisms. In many cases problems have not been encountered until real systems i.e. foods have been examined when it has frequently been the case that food components interfere with the estimation of micro-organisms.

Conventional methods of enumerating micro-organisms e.g. colony counts accept a wide range of samples of vastly different composition including whole ranges of food products and it appears that this is less likely to be so for the more sophisticated techniques now proposed as rapid methods.

The obvious solution to this type of problem is to separate the microorganisms from the interfering components of food prior to estimation. Currently there are no simple and practical methods of doing this. Whilst we have a wealth of methods for the isolation of microbial species relatively little work has been done on the recovery of origional microbial populations from large amounts of non microbial materials.

A rapid and practically simple system of separation was required and differential adsorption on ion exchange resin was selected for investigation.

Preliminary investigations showed that a wide variety of food associated microbial species could be adsorbed from aqueous suspension onto the surface of beads of anion exchange resin and that these resins had a large adsorptive capacity of bacteria \((10^{10} \text{ cfu g}^{-1})\). It was also demonstrated that organisms could be adsorbed from homogenates of a wide range of foods. The major factors influencing the adsorption of micro-organisms were the mesh size of the resin particles, the contact time of the microbial suspension with the resin, the pH and electrolyte concentration of the suspending medium. Variable factors associated with the micro-organisms e.g. species, age of culture, and composition of the cell envelope had a lesser effect on adsorption.

In experiments with minced beef it was demonstrated that, after prior separation of large particles by low speed centrifugation 90% of the total flora could be adsorbed to a small column of anion exchange resin whilst 99% of the dry wt of the sample could be accounted for either in the centrifugation pellet or in the column effluent.

Recovery of micro-organisms from resin columns was achieved by elution with salt solutions and varied with the concentration and pH of the eluant.

A cation exchange resin system for the separation of micro-organisms from minced beef has also been devised. This is the converse of the anion exchange system, i.e. the food material is adsorbed and the organisms pass through the column, and obviates the need for elution and simplifies the separation.

With the cation exchange system substantial separation of micro-organisms from minced beef can be achieved. The separation has been demonstrated by both chemical analysis and electron microscopy and the effect of the separation on the impedometric estimation of micro-organisms in minced beef has been investigated.

Summary of a paper presented to the Microbiology and Enzyme Technology Group of the Society of Chemical Industry at a Symposium on Rapid Methods in Microbiology 6th October 1977.
THE SEPARATION OF MICRO-ORGANISMS FROM FOODS

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The results demonstrate that a considerable degree of separation of organisms from food may be attained using relatively simple apparatus. It seems probable that the efficiency of separation might be improved but this may not be necessary. Our current work is directed towards desorption of the organisms from resin prior to estimations by instrumental techniques mentioned above.